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Insulin Resistance, Ethnicity and Cardiovascular Risk

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Thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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Abstract

Cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality. The literature supports a series of established risk factors for CVD: age, gender, family history of CVD, ethnicity (un-modifiable); and high blood pressure, blood cholesterol, TGs, LDL, diabetes, pre-diabetes, obesity, smoking, physical inactivity, stress and unhealthy diet (modifiable). High blood pressure (hypertension) shares many of these risk factors. However, much of the variance/risk in both conditions cannot be explained. This has led to a search for novel risk factors, including insulin resistance and subclinical inflammation, the significance of which at present are controversial, particularly in relation to hypertension. There are also ethnic differences in the incidence, prevalence, risk factors and progression of cardiovascular disease. In some populations CVD occurs at an earlier age and progresses more rapidly.

In this thesis I worked on two datasets in relation to hypertension, cardiovascular disease and their risk factors: (i) the RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study (chapters 2, 3, 5 and 6); and (ii) routinely-collected national data in Scotland via the SDRN (Scottish Diabetes Research Network) and SCI-Diabetes (chapter 2 and 7). Work on data from the RISC cohort focused on the relation between clamp-measured insulin sensitivity (its unique feature), inflammatory markers and hypertension; the SDRN work addressed ethnic differences in relation to diabetes and CVD.

The first study (Chapter 3) examined the importance of insulin sensitivity/resistance in the development of hypertension and change in blood pressure over three years of follow-up in the healthy European (EU) RISC population. Systolic BP (SBP) was higher at baseline in insulin resistant (IR) women. There was no difference in BP in relation to IR in men. After adjustment for age, BMI, baseline BP and other covariates, low insulin sensitivity (M/I) predicted a longitudinal rise in SBP in women but not men, and SBP over time did not increase in insulin sensitive women.

The second study (Chapter 4) was a systematic review of the relationships between two markers of low grade inflammation (IL-6 and CRP) and BP/hypertension, considering the roles of adiposity and insulin resistance.

The systematic review showed evidence of considerable variation in the relationships amongst low grade inflammation, adiposity, insulin resistance and the development of hypertension. There appeared to be a positive association in the literature between CRP and DBP in younger individuals, although none of the studies were adjusted for insulin sensitivity determined by clamp technique. This association was further explored using RISC study data in Chapter 5 with stratification by sex and adjusting for clamp-derived insulin sensitivity.

The third study (Chapter 5) examined the relationship of inflammatory markers with the development of hypertension and change in blood pressure over three years in the same healthy European population and whether any relationship was independent of clamp-measured insulin sensitivity (IS). High sensitivity C reactive protein (hsCRP) predicted prospective change in diastolic BP independent of insulin sensitivity and BMI whereas IL-6 had no relation with BP (both systolic and diastolic) or the incidence of hypertension.

The fourth study (Chapter 6) evaluated all available predictors of BP rise over time (both systolic and diastolic) in a healthy EU population; moreover the significance of different predictors was examined within subgroups defined by age and sex. This analysis showed that baseline BP was the principal determinant of follow-up BP in all age and sex groups. Obesity was the second most important predictor (BMI in adults aged 30-44 years; percent change in BMI in middle age people aged 45-60 years). Lifestyle factors influenced BP via their effect on BMI. People who maintained their BMI during the three year follow-up did not exhibit a rise in BP (whether systolic or diastolic). Other important predictors identified in this analysis were insulin sensitivity in middle aged women and hsCRP in adult men.

The fifth study (chapter 7) evaluated the role of ethnicity in the development of cardiovascular disease in people with type 2 diabetes living in Scotland. Over a follow-up of seven years, Pakistani people had increased risk of CVD and Chinese people had decreased risk of CVD as compared to White population. Pakistanis had an increased risk of CVD at a younger age independent of other conventional risk factors.

In summary, insulin sensitivity and inflammation influence blood pressure, but their role is not generalised across different age and sex groups. BMI and change in BMI are important predictors of follow-up BP in adults and middle age healthy people, supporting a role for maintenance of BMI in preserving cardiovascular health. In addition to the known ethnic differences in the development of diabetes, I identified ethnic differences in the development of CVD.

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Dedication

I dedicate this work to my beloved parents who greatly encouraged me to do PhD even when they were ill and needed my company and services.

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Author's Declaration

This thesis is submitted in fulfilment of the requirement for the degree of Doctor of Philosophy at the University of Glasgow. Unless stated otherwise, the work is that of the author. Parts of the research work included in this thesis have been published or submitted with co-authors.

Definitions/Abbreviations

Akt	Protein kinase B
Ang-II	Angiotensin II
BMI	body mass index
CHD	coronary heart disease
CI	confidence interval
CRP	C Reactive protein
CV	Cardiovascular
CVD	cardiovascular disease
DM	Diabetes Mellitus
EU	European
ET-1	Endothelin-1
ERK1/2	extracellular-signal-regulated kinases
F	female
HR	hazard ratio
ICD	International Classification of Diseases
IL-6	Interleukin-6
IS	Insulin sensitivity
IR	Insulin resistance
HOMA	Homoeostasis model assessment
M	male
MFG-E8	milk fat globule epidermal growth factor-8
MR	Mineralocorticoid receptor
mtDNA	mitochondrial DNA
NE	Norepinephrine
NHS	National Health Service
NO	Nitric Oxide
OR	odds ratio
PDK- 1	phosphoinositide-dependent kinase 1
PI3 K	phosphatidylinositol kinase
PVAT	perivascular adipose tissue
RAAS	Renin Angiotensin Aldosterone System
ROS	reactive oxygen species
RR	relative risk

SA	South Asian
SES	Socioeconomic status
SD	standard deviation
SDRN	Scottish Diabetes Research Network
SIMD	Scottish Index of Multiple Deprivation
SMR	Scottish Morbidity Record
SNS	Sympathetic nervous system
T2DM	Type 2 diabetes mellitus
TNF-α	Tumour Necrosis Factor-α
ψm	Mitochondrial membrane potential
UK	United Kingdom
VSMC	Vascular smooth muscle cells
WC	waist circumference
WHO	World Health Organization
WHR	waist-to-hip ratio

List of Publications

Chapter 3

Petrie JR, Malik MO, Balkau B, Perry CG, Hojlund K, Pataky Z, et al. Euglycaemic Clamp Insulin Sensitivity and Longitudinal Systolic Blood Pressure: Role of Sex. Hypertension 2013;62(2):404-9.

Chapter 5

Malik MO, Govan L, Petrie JR, Ghouri N, Leese G, Fischbacher C, Colhoun H, Philip S, Wild S, McCrimmon R, Sattar N, Lindsay RS; Scottish Diabetes Research Network (SDRN) Epidemiology group. Ethnicity and risk of cardiovascular disease (CVD): 4.8 year follow-up of patients with Type 2 diabetes living in Scotland. Diabetologia 2015; 58(4):716-25.

Chapter 5

Malik MO. Ethnicity and risk of cardiovascular disease (CVD): 4.8 year follow-up of patients with Type 2 diabetes living in Scotland. Diabetic Medicine 2014; 31(S1): 76-77 (Abstract)

Abstracts and Presentations

- Hs-CRP and blood pressure in men and women: the RISC study (EGIR Annual Meeting 2012, Lyon, France)

- Ethnicity and risk of cardiovascular disease (CVD): 4.8 year follow-up of patients with Type 2 diabetes in Scotland (DREAM Symposium 2013, Glasgow)

- Ethnicity and risk of cardiovascular disease (CVD): 4.8 year follow-up of patients with Type 2 diabetes in Scotland (Diabetes UK Professional Conference 2014, Liverpool)

- Role of maintaining weight in cardiovascular disease risk (3 Minute thesis competition (University of Glasgow)

- Predictors of carotid intima media thickness (cIMT) progression in the RISC cohort at baseline and three years: role of NT-proBNP (EGIR meeting 15-17 May 2014, Glasgow).

1 Introduction

In this thesis I present work evaluating the relationships between cardiovascular disease (including hypertension) and novel risk factors including insulin resistance, inflammation, weight change and ethnicity. Special emphasis is given to the influence of age and sex on these associations in order to examine the hypothesis that these relationships differ in men and women and through different periods of life. The introduction comprises four Parts. In the first, I discuss the epidemiology of cardiovascular disease and its major risk factors focusing on hypertension, obesity and diabetes. As vascular (macro- and microvascular) dysfunction is an early step in the initiation of cardiovascular disease, its physiology and different mechanisms of vascular dysfunction are discussed in the second Part. In the third Part I discuss novel risk factors including insulin resistance and inflammation. The final Part covers the influence of age, sex and ethnicity on cardiovascular risk.

Part 1

1.1 Cardiovascular disease- epidemiology

Non-communicable diseases (NCD) were responsible for two-thirds of all deaths globally in 2011, a 7 % increase from 2000 (60% in 2000) (1). The four main NCDs are cardiovascular disease (CVD), cancer, diabetes and chronic lung disease, with CVD causing most deaths (2). CVD includes coronary heart disease (heart attacks), cerebrovascular disease (stroke), peripheral arterial/vascular disease, rheumatic heart disease, congenital heart disease and heart failure.

About 30% of all global deaths in 2008 were from CVD alone, approximating 17.3 million (2). Within deaths due to CVD, an estimated 7.3 million were due to coronary heart disease and 6.2 million were due to stroke (3). It is projected that the number of people dying from CVD (mainly heart disease and stroke) will increase to reach 23.3 million by 2030 (2;4). CVD is projected to remain the single leading cause of death in future (4).

1.1.1 Disease load in middle and low income countries

About 80% of CVD deaths occur in low and middle-income countries (1) and occur almost equally in men and women (2). People living in these countries have a greater exposure to risk factors; in addition, they have poorer access to prevention programmes than people in high-income countries. Early detection services are fewer and they have less access to effective and equitable health care services. As a result many people die at a younger (productive) age: this places a heavy burden on the economies of low and middle income countries. Among NCD, CVD and diabetes are estimated to reduce Gross Domestic Product (GDP) by around 6.8% in these countries (2).

In certain low and middle income countries, current health expenditure on CVD alone accounts for 20% of total health expenditure. It is projected that over the period 2011-2025 the cumulative lost output in low- and middle-income countries associated with non-communicable diseases will be US$ 7.28 trillion (3.76 trillion for CVD alone) (5). The current annual loss due to NCD in low to middle income countries is approximately US$ 500 billion and CVD including hypertension accounts for nearly half of the cost (1).

1.1.2 Hypertension- epidemiology

High blood pressure or hypertension (HTN) already affects one billion people worldwide and is a major risk factor for heart attacks and strokes (6). About 9.4 million deaths each year (around 16.5% of all deaths) can be attributed to hypertension (7). Deaths due to hypertension mainly include deaths due to strokes (51%) and deaths due to coronary heart disease (45%) (6).

In 2008 the global prevalence of hypertension in adults (aged 25 and over) was approximately 40%. It affected around 600 million people in 1980 rising to 1 billion in 2008 (2).The prevalence of HTN is highest in the African region at 46% of adults aged over 25 years and lowest at 35% in the Americas. Global prevalence of HTN is low (35%) in high-income countries, while high (40%) in low and middle income countries (2). Due to the weak health systems in those countries, many cases remain undiagnosed and untreated or have uncontrolled HTN.

1.1.3 Obesity- epidemiology

According to WHO, in 2014, more than 1.9 billion people (18 years and older) were overweight [Body mass index (BMI) between 25-30] and among these over 600 million were obese (BMI 30 or more) (8). Of the global adult population, 39% (38% men and 40% women) were overweight and 13 % (11% men and 15% women) were obese in 2014. The prevalence of obesity has more than doubled between 1980 and 2014. Moreover, 42 million children under the age of 5 are now overweight or obese (8).

Obesity was once considered a problem of high income countries but is now also on the rise in low and middle income countries, particularly due to urbanisation. Urbanisation leads to decrease in physical activity and increase in BMI and upper body adiposity. Moreover in developing countries, the rate of increase of childhood overweight and obesity has been more than 30% higher than that of developed countries (8). The most likely cause of this may be poor nutrition in the pre-natal, infant and toddler periods. Many children are exposed to food which is low in quality: high-fat, high-sugar, high-salt, energy-dense, and micronutrient-poor foods (8).

Worldwide, overweight and obesity are thought to be responsible for at least 2.8 million deaths each year as well as an estimated 35.8 million (2.3%) of global disability-adjusted life years (DALY) (9). The prevalence of overweight and obesity is highest in the WHO regions of the Americas (62% for overweight in both sexes, and 26% for obesity) and lowest in South East Asia (14% overweight in both sexes and 3% for obesity). Women are more likely to be obese than men in all the WHO regions,, especially in Africa, the Eastern Mediterranean and South East Asia [where prevalence of obesity in women is double that of men] (9). Obesity is associated with insulin resistance and associated adverse metabolic effects on blood pressure and lipids.

1.1.4 Diabetes- epidemiology (incidence, current load, projected load)

347 million people worldwide have diabetes (10). According to the WHO prediction, it will be the 7th leading cause of death in 2030 (11). In 2004, an estimated 3.4 million people died from complications related to diabetes

mellitus (12). More than 80% of deaths due to diabetes or its consequences occur in low- and middle-income countries (4).

Part 2

1.2 Anatomy and physiology of blood vessels

Blood vessels consist of layers (also called tunicae) from inner to outer; intima, media and adventitia (externa). The arterial intima consists of a single layer of endothelial cells (ECs) that is in contact with blood and underlying layer of smooth muscle cells. The internal elastic lamina (membrane) separates intima from media. The capillaries only contain single layer of EC. The medial layer consists of concentric layers of elastic lamina interspersed with smooth muscle cells and collagen (13). The smooth muscle cells within the vessel wall are called vascular smooth muscle cells (VSMC). The adventitial or outermost layer is rich in collagen, fibroblasts, dendritic cells, mast cells, macrophages, lymphocytes and adipocytes. The adventitia also contains nerve endings and microvessels. Healthy large arteries have a high elastin: collagen ratio. The extracellular matrix (ECM) is an important factor modulating vascular stiffness. Other important factors regulating vascular stiffness come from ECs, VSMC and adventitia (e.g. cytokines and inflammatory signals) (13). Small and large vessels are discussed in Section 1.3. ECs are directly in contact with circulating blood and have a crucial role in regulating vascular physiology in states of health by releasing substances with potent anti-thrombotic properties (see below Section 1.2.1). They are particularly vulnerable to damage by molecules in the blood and can sense and respond to metabolic alterations either directly or by transmitting reactive signals to nearby cells, such as VSMC (13).

1.2.1 Endothelial function and dysfunction:

The vascular endothelium is a single layer of endothelial cells forming an interface between circulating blood and the vessel wall. The endothelium is continuously exposed to shear forces generated by blood flow and has an essential role in the maintenance of vascular integrity. Laminar blood flow stimulates the release of endothelial-derived nitric oxide (NO) which in turn stimulates anti-atherosclerotic and anti-thrombotic pathways (14;15). NO is

released in response to shear stress and is the major vasodilator and pro-angiogenic factor. It regulates local vascular tone and blood pressure as well as stimulating the release of vascular endothelial growth factor (which plays a key role in angiogenesis and vascular remodelling). In addition NO also have anti-inflammatory, anti-atherogenic and antithrombotic properties which can be demonstrated by its ability to inhibit endothelial leukocyte adhesion, platelet aggregation and smooth muscle cell proliferation and migration (16;17).

Endothelial function can be evaluated by measuring NO dependent vasodilation when stimulated by pharmacological (after acetylcholine perfusion) or mechanical (flow-mediated vasodilation) mechanisms (18). Loss of NO dependent vasodilation is a feature of endothelial dysfunction (17). Endothelial dysfunction (ED) may be the earliest vascular manifestation of macro and microvascular dysfunction (18;19). Loss of endothelial derived NO leads to ECs apoptosis; combined with reduced angiogenesis, the result can be microvascular rarefaction (20-22). Rarefaction is abnormally low spatial density of microvessels (see below Sections 1.3.3.1 and 1.3.7.4). Likewise impaired endothelium-dependent coronary vasoreactivity (characterized by vasoconstrictor response to acetylcholine infusion- normally vasodilator response) appears to be an independent predictor of atherosclerotic disease progression and cardiovascular events (19).

Different cardiovascular risk factors cumulatively generate a pro-oxidative environment which leads to endothelial dysfunction. Reactive oxygen species switch the endothelial anti-inflammatory/ NO donor response into a pro-inflammatory response, with hydrogen peroxide accumulation, abnormal redox signalling (23) and decreased NO bioavailability. Endothelial dysfunction is also associated with leukocyte adhesion and accumulation in the intima of the vascular wall (17). These mechanisms related to endothelial dysfunction will be discussed in more detail below in the sections of obesity, insulin resistance, inflammation and immunity (Section 1.7.4).

1.2.2 Mitochondria and endothelial function

This thesis does not contain data on mitochondrial function or structure but, as these subcellular organelles play an important role in determining endothelial

and vascular function, their role will be considered here in relation to the other phenotypes discussed (endothelial dysfunction, obesity, diabetes, hypertension, atherosclerosis). Mitochondria are considered to be the major intracellular source of reactive oxygen species (ROS) and ROS is involved in the pathology of endothelial dysfunction. Mitochondrion-mediated endothelial dysfunction has been linked to a variety of disease states, including hypertension, diabetes mellitus, atherosclerosis, coronary artery disease, and hypercholesterolemia (24).

The mitochondrial content of ECs is small in comparison to other body cells with higher energy requirements (e.g. muscles) (25) and ECs obtain most of their energy from the anaerobic glycolytic metabolism of glucose (25). The main function of the mitochondria in ECs is unlikely to be energy production but rather as signalling organelles which orchestrate cellular function and homeostasis (26). Mitochondria are an important Ca2+ buffering system within ECs and work with the endoplasmic reticulum to maintain cellular Ca2+ homeostasis (25). Internal metabolic disturbances (e.g. hypertension, diabetes) are associated with mitochondrial damage, which in turn produce excessive ROS and accelerate EC senescence, death and dysfunction. As EC serve as the first barrier of the vascular system, dysfunction contributes to the development of nearly all vascular diseases including essential hypertension, pulmonary hypertension, and atherosclerosis (27).

1.2.2.1 Mitochondrial content and dynamics in endothelial cells

The mitochondrial content of any cell is very important for its cellular functions and is critically regulated. The mitochondrial content in turn depends on the balance between mitochondrial biogenesis and mitophagy (28). Mitophagy is the selective degradation of defective mitochondria by the process of autophagy (self-eating). Whenever there is damage to mitochondria, it is followed by a mitochondrial fusion and fission process, which then generate functionally normal (healthy) and damaged mitochondria. Healthy mitochondria re-enter fusion cycles and perform physiological functions in cell. The damaged daughter mitochondriae still produce mitochondrial ROS and are depolarized and undergo mitophagy (28). If this normal mitochondrial life cycle (biogenesis and mitophagy) is disturbed, there are detrimental consequences on cellular

bioenergetics which contribute to endothelial dysfunction and the pathogenesis of cardiovascular diseases (29). Ageing is associated with inhibition of mitochondrial biogenesis in vascular ECs. Moreover, impaired mitophagy is one of the contributing factors in the pathogenesis of vascular diseases including diabetes mellitus, atherosclerosis, and hypertensive heart diseases (25). The Mitochondria are involved in cellular necrosis but EC necrosis remains elusive (24). Mitochondrial damage has been shown to be involved in endothelial dysfunction as improvement of mitochondrial dysfunction prevents endothelial dysfunction (30) (see section 1.7.5.1) and evidence that endothelial dysfunction leads to mitochondrial dysfunction is still lacking.

1.2.2.2 Mitochondrial reactive oxygen species (ROS) in endothelial cells

Mitochondria are an important source of cellular ROS but also serve as an important ROS buffering system. Mitochondria can sense toxic signals such as infectious agents or cholesterol crystals and generate mitochondrial ROS. Risk factors including ageing, hypercholesterolemia, hyperglycaemia, smoking, infections and hypoxia alter mitochondrial membrane potential (ψm) which then triggers excess mitochondrial ROS production (25). In healthy humans, cellular mitochondrial ROS production is tightly regulated: manganese superoxide dismutase (MnSOD) in mitochondria is rapidly inducible and buffers the ROS in the mitochondria matrix by converting superoxide to H_2O_2 (25). CuZnSOD is another superoxide dismutase which buffers superoxide in the intermembranous space, cytoplasm and extracellular space. H_2O_2 thus produced is metabolised locally by antioxidant enzymes such as catalase and peroxidases (28).

Under physiological conditions, concentrations of mitochondrial ROS are low. However, these are critical signalling molecules for normal cellular metabolism (31). Mitochondrial ROS are involved in the body’s response to hypoxia, autophagy, immunity, differentiation, and longevity (31). However, if mitochondrial ROS production is significantly increased and exceeds the buffering capacity of MnSOD, free ROS causes oxidative damage and cellular dysfunction. Free superoxide anions in the matrix are highly reactive and can damage mitochondrial DNA, lipids, and proteins. Mitochondrial ROS can also damage some complexes of the electron transport chain, further exacerbating mitochondrial ROS production and setting up a positive feedback loop that

contributes to the severity of endothelial dysfunction and the pathogenesis of vascular diseases (31).

In health and in the early stages of vascular diseases, cellular repair mechanisms may replace damaged mitochondria or their components and maintain normal mitochondrial function. However, if these quality control and repair mechanisms are impaired (e.g. due to ageing or diabetes), dysfunctional mitochondria may be retained and continue producing excess ROS and further exacerbate vascular diseases (32;33). It has been reported that many vascular diseases are accompanied by elevated mitochondrial ROS levels, but the underlying pathological mechanisms are complex (34). One of the mechanisms by which mitochondrial ROS participates in endothelial dysfunction and vascular diseases is by uncoupling the endothelial NO synthase (eNOS) which results in a subsequent decrease in the production of NO. Another mechanism is that $O_2−$ reacts with NO to form peroxynitrite (ONOO−), which together with ROS production leads to mitochondrial dysfunction. Reaction with O_2- decreases available NO, resulting in eNOS uncoupling. This further decreases NO production, further promoting pre-existing oxidative stress, and exacerbating endothelial dysfunction and vascular diseases (35). In addition increased angiotensin II (secondary to activation of renin-angiotensin system in hypertensive patients) activates endothelial NADPH oxidase leading to oxidative stress and ROS generation which combines with NO to form peroxynitrite (36) further enhancing the destructive process.

1.2.2.3 Mitochondrial regulation of endothelial senescence, apoptosis, and mitophagy

Mitochondrial dysfunction, reduced mitochondrial mass, somatic mtDNA mutations and respiratory (electron transport) chain dysfunction are strongly associated with EC senescence (37;38). Damaged mitochondria produce excessive superoxide and H_2O_2, which are major determinants of telomere length shortening and associated telomere dependent senescence (39). Moreover, in senescent ECs, the mitochondrial antioxidant- MnSOD is significantly down regulated; further impairing ROS buffering capacity of mitochondria (40;41). Similarly in cell culture models of senescence in ECs, mitochondrial dynamics are impaired along with loss of ψm. In contrast,

improved mitochondrial fitness, as manifested by higher ψm, increased ATP production, and decreased damage to mtDNA was associated with prolonged lifespan of cultured ECs (42).

1.3 Macro- and microvascular alterations

Hypertension and diabetes are a complex chronic systemic disorder exhibiting both functional and structural alterations in macrovascular and microvascular circulation.

1.3.1 Macrovascular disease

Macrovascular disease is a disease of any large blood vessels in the body, including aorta, coronary arteries and sizable arteries in the brain and the limbs. It is associated with development of cerebrovascular disease (stroke, transient ischemic attacks), coronary artery disease (myocardial infarction, angina), and peripheral vascular disease. The key features of macrovascular disease are arterial stiffening, disturbed wave reflection and altered central to peripheral pulse pressure amplification (16;43;44).

In health, the heart pumps blood into the aorta which stretches to accommodate the blood and reduce or “damp” the pulsatility of ventricular ejection. The elastic recoil of the aorta then pumps blood back into the peripheral circulation as a steady (continuous) flow and limits the pulsatile strain imposed to the peripheral microcirculation. The physiological ageing process and other metabolic insults (hyperglycaemia, dyslipidaemia) are associated with structural and functional changes of the aortic wall leading to aortic stiffness. Structural changes include increase in collagen and elastin content and alterations of elastin fibres resulting in increased arterial wall thickness. It is also associated with inflammation, VSMC alterations (hypertrophy, phenotype modulation) and increased endothelial permeability, along with diffusion of macromolecules within the arterial wall (45). Elevated wall tensile stress (in hypertension) also causes smooth muscle cells to undergo hypertrophy and change from the physiological contractile phenotype to a secretory and proliferating phenotype. This structural modification in large arteries maintains the wall tensile stress

roughly at its baseline values but is possible only at the expense of increased arterial wall thickness, leading to increased vascular stiffness (46).

Increased aortic stiffness leads to increased pulse wave velocity. The central pressure waveform is composed by an early systolic peak and a subsequent peak in diastole due to the return of the reflected wave coming from the peripheral vessels. Due to aortic stiffness and increased pulse wave velocity there is amplification of the primary wave in systole rather than diastole leading to an increase of central systolic and pulse pressures which in turn is closely related to target organ damage.

1.3.2 Arterial stiffness

Arterial stiffness collectively accounts for distensibility, elasticity and compliance of the arterial vascular system. It describes the reduced capability of an artery to expand and contract in response to pressure changes. Arterial stiffness is a very important independent (beyond classical cardiovascular risk factors) risk factor for the progression of cardiovascular and chronic kidney disease (CKD) and an independent predictor of cardiovascular events (47;48). Arterial stiffness increases with ageing, however, the process is accelerated in the presence of obesity and diabetes and occurs at earlier ages if these conditions coexist (48;49). Age-related stiffness is associated with intimal thickening with marked increase in intimal to medial thickness ratio (50). Within the medial layer there is fragmentation and depletion of arterial elastin coupled with deposition of matrix metallo-proteins and collagen (51;52). The dominance of collagen content is associated with increased non-enzymatic cross-linkages between collagen structures (53). VSMC in the medial layer also undergo intrinsic stiffness with ageing (49;54;55) and hypertension (49;54;55).

Arterial stiffness is also associated with insulin resistance and activation of the renin-angiotensin aldosterone system (RAAS) in obesity (56;57), even in obese children (58). It is present in conditions such as hyperglycaemia of diabetes even in the absence of insulin resistance (56;59). It is also seen in pre hypertensive subjects and in normotensive subjects predisposed to develop hypertension (60). Obese individuals are likely to have aortic stiffness independent of BP. Both obesity and arterial stiffness are also independent factors for left ventricular

diastolic dysfunction (heart failure with preserved ejection fraction) which is in turn related to CVD (57;61;62). Similarly insulin resistance is associated with diastolic dysfunction (obesity cardiomyopathy), independent of hypertension or heart disease (63), explaining the fact that arterial stiffness alone even without hypertension is a cardiovascular risk factor

1.3.2.1 Aortic stiffness as a predictor of incident hypertension and CVD

Liao et al. demonstrated in the Atherosclerosis Risk in Communities (ARIC) Study that arterial stiffness and elasticity were independent predictors of incident hypertension in normotensive participants over a 6-year mean follow-up (64). They also showed that, each standard deviation decrease in elasticity was associated with a 15% increase in developing hypertension (64). Later, in the Baltimore Longitudinal Study of Ageing, carotid-femoral pulse wave velocity was demonstrated as an independent predictor of longitudinal increase in SBP and the development of hypertension in individuals followed up for more than 4 years (65). More recently Kaess et al. have suggested that aortic stiffness is a precursor of future altered systolic haemodynamic load and incident hypertension (66).

Aside from the effect of hypertension, the main factors causing aortic wall alterations include age-related modifications, epigenetic factors, nutritional habits and pathological processes associated with diabetes, metabolic syndrome and chronic renal insufficiency (67). Ageing is a major predictor of aortic stiffness and a determinant of pulse wave velocity progression in both hypertensive and normotensive individuals (68). It is also associated with decrease and fragmentation of elastin fibres, increase in collagen content, diffuse intimal thickening, VSMC hyperplasia, increased extracellular matrix and luminal enlargement of the aorta (69;70). In addition, the activated RAAS effects arterial wall remodelling via direct inflammatory and pro-fibrotic actions of Ang II and aldosterone on vascular cells. Moreover, genome wide association studies have demonstrated that the genes related to the RAAS were also associated with arterial stiffness (71). In summary, physiological age-related modifications of the aortic wall likely account for the higher prevalence of isolated systolic hypertension (ISH) among older adults (72). The measurement of aortic stiffness improves cardiovascular disease risk prediction and may avoid patients being

mistakenly classified as at low or moderate risk, when they actually have a high aortic stiffness placing them within a higher-risk group (47;73). A recent individual participant meta-analysis showed that consideration of arterial stiffness improves model fit and reclassifies risk for future cardiovascular events in models that include standard vascular risk factors (74).

1.3.2.2 Measurement of arterial stiffness

In clinical settings the evaluation of arterial stiffness is accomplished by: 1) measurement of arterial compliance and distensibility by ultrasound, 2) measuring the velocity of the pressure wave travelling between two arterial segments (pulse wave velocity or PWV) and 3) measuring the augmentation pressure divided by blood pressure (augmentation index) (48;51). PWV is closely associated with arterial wall stiffness whereas augmentation index is related to arterial wall stiffness and also wave reflection. Moreover, augmentation index is also related to coronary artery flow (51). The wave reflection is dependent on peripheral resistance and is also affected by heart rate variation (48;51) (also see Section1.3.7). The carotid-femoral pulse wave velocity (the gold standard non-invasive measurement for aortic stiffness) is also used to determine subclinical target organ damage and risk assessment in hypertensive patients (75). In addition augmentation index *In vitro* measurement of tissue and cell stiffness is done by use of atomic force microscopy and complimented by confocal imaging (49).

1.3.3 Microvascular disease

Microvascular disease (micro-angiopathy) associated with diabetes affects arteries, arterioles, venules and capillaries resulting in diabetic retinopathy, nephropathy and neuropathy. The key features of microvascular disease include altered wall-to-lumen ratio of larger arterioles, vasomotor tone abnormalities and network rarefaction, decreased vasodilation reserve, disturbed tissue perfusion and susceptibility to ischaemia (16;76).

Arterioles or resistance arterioles are one of the most important components of the microcirculatory network which supplies the micronutrients and removes by-products from the tissues. The main physiological inherent response mechanism

is by a myogenic (smooth muscle) reduction in lumen diameter (77). This smooth muscle contraction controls tissue perfusion and organ function via three main mechanisms: 1) protecting capillaries from damaging blood pressure increase, 2) regulating the nutritive role of the vascular network within tissues in response to variations in demand, and 3) determining local and systemic peripheral vascular resistance (78). Any alteration in resistance arterioles may have consequences for tissue perfusion, metabolism, susceptibility to ischaemia, or blood pressure (78;79). The skin capillaries are considered a surrogate marker of systemic microvascular function and resistance and reflect microvascular network in different vascular beds (79-81).

1.3.3.1 Microvascular alterations in hypertensive patients

In resistance arterioles there is no remodelling in the absence of an increase in wall tensile stress. However, increase in blood pressure induces local vasoconstriction by increasing myogenic tone and thus maintaining the arteriolar wall tensile stress at normal or even lower values to protect downstream capillaries (82;83). Chronic high blood pressure is associated with structural and functional alteration of micro vessels. In the beginning, increased myogenic tone and arteriolar vasoconstriction promote functional rarefaction (increased number of non-perfused micro-vessels) which progresses to structural rarefaction (anatomical disappearance of non-perfused vessels) if the pathology persists (77;83). Serne et al. evaluated the relative contribution of functional and structural rarefaction in never treated patients with essential hypertension and normotensive controls. They showed that at the most, 62% of rarefaction was explained by structural defects, with at least 38% explained by functional defects (84). Loss of shear stress in non-perfused micro-vessels leads to decreased NO production, promoting ECs apoptosis and structural rarefaction (see above Section 1.2.1)(20-22). This micro-vascular damage is a predictor of long term cardiovascular events. Interestingly, the Framingham score for cardiovascular risk in hypertensive patients, whether treated or not, appears to be negatively correlated to skin capillary density (85).

Structural changes are characterized by a decrease in lumen and an increase in media-to-lumen ratio and are an independent (i.e. independent of blood pressure itself) predictor of cardiovascular events in hypertensive patients

(86;87). They may also have prognostic significance: reduction of media-to-lumen ratio in larger subcutaneous arterioles has been used to evaluate the effectiveness of antihypertensive treatment over and above blood pressure reduction (88).

1.3.4 Association between macro and microvascular alterations

Macro and microvascular alterations are inter-correlated. Aortic stiffening leads to increased pulse wave velocity and premature reflected waves with enhanced central pulse and elevated systolic pressure. This leads to change of steady continuous flow towards a more pulsatile flow in peripheral vessels resulting in peripheral tissue microcirculatory damage (89). Similarly aortic stiffness is associated with increased peripheral vascular resistance (90). Conversely, increased peripheral resistance leads to chronic elevated blood pressure (91), a major determinant of aortic stiffness. Similarly in hypertensive patients, increased media-to-lumen ratio of subcutaneous arterioles is positively correlated with carotid-femoral pulse wave velocity independently of age and mean blood pressure (92).

The capillaries are the smallest blood vessels and constitute the major part of the vascular structure: if spread out on a horizontal surface, the endothelial surface area (500-700 square meters) is almost the size of a football field (93); explaining a larger effect on BP by slight alteration of micro vessels. Microcirculatory functional or structural alteration possibly enhances the phenomenon of reflected pressure waves and in turn contributes to an increase in central systolic and pulse pressure (92). Alterations in the adventitial microvascular network in the wall of conduit artery may result in a mismatch between supply and metabolic demand, further accentuating large artery disease (94). It has also been suggested that microcirculatory alterations may precede larger artery dysfunction and atherosclerosis and represent an indirect marker of large vessel dysfunction (95). Structural rarefaction and remodelling of the micro vessels explains the long-term elevation of systemic vascular resistance and enhanced wave reflection, both of which contribute to aortic wall stiffness (90;92;96;97). This “vicious circle” accounts for the inter correlation between macro and microvascular alterations leading to difficult blood pressure control and antihypertensive treatment resistance (see Figure 1.1)

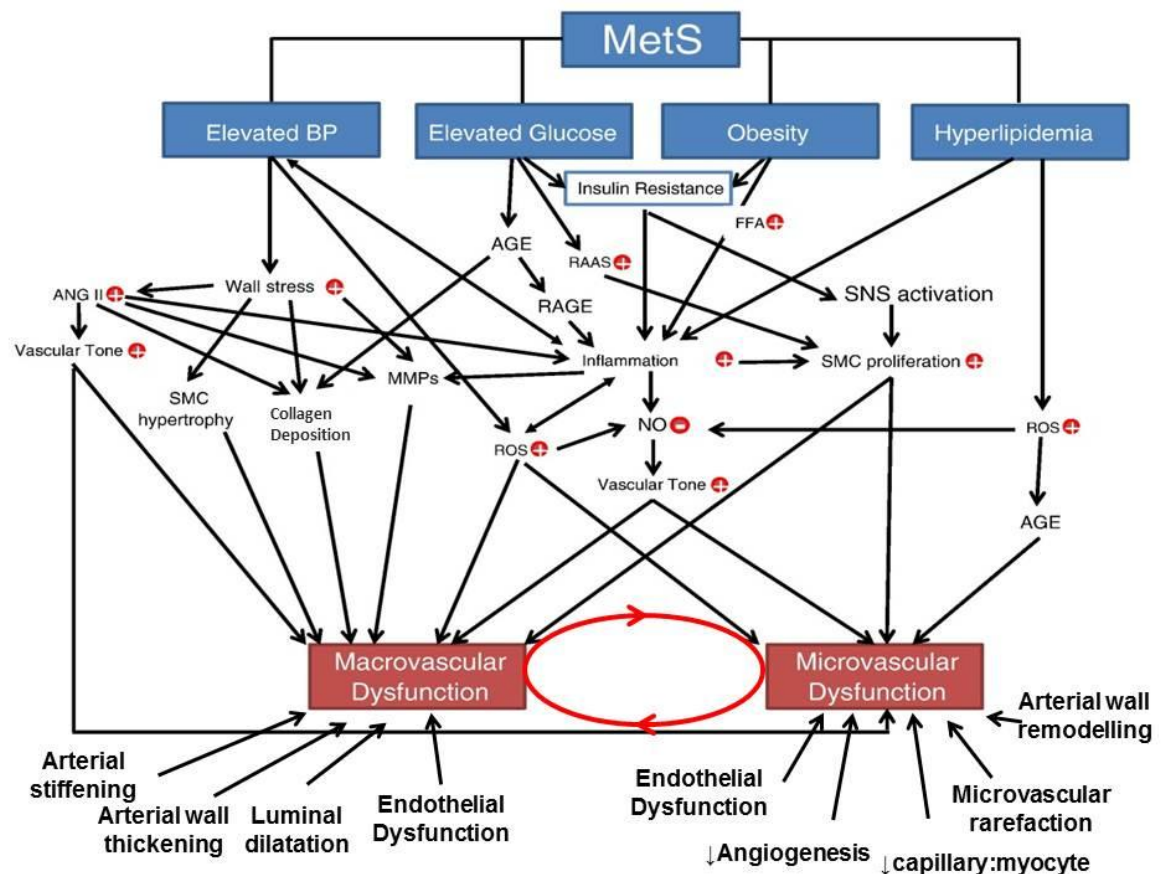


Figure 1.1 Cyclical association between macro and microvascular dysfunction and its association with obesity and raised BP.

1.3.5 Possible mechanism in relation to hypertension

Aortic stiffness and large vessel disease are related to hypertension in epidemiological studies. Nonetheless, the relationship is complex and unclear in terms of cause and effect i.e. whether aortic stiffness causes HTN, high blood pressure leads to aortic stiffness, or the relationship is bidirectional. The reason for this uncertainty may be that multiple factors are potentially implicated in the pathophysiology of hypertension, including genetic, epigenetic (environmental, nutritional) and metabolic factors as well as ageing. On the other hand, both observational and experimental evidence suggest that alteration in the microvascular circulation precedes and even predicts incident hypertension (77;78). A plausible mechanism may be that a cumulative metabolic burden and chronic oxidative stress lead to chronic endothelial injury and activation which promotes structural and functional alterations in the microvascular circulation (16), with subsequent effects on macrovasculature.

1.3.6 Hypertension associated structural changes in vasculature

Hypertension leads to increased pressure on the vessel wall (tensile stress) (see Section 1.3.1) which is the most important determinant of vascular adaptive remodelling. Remodelling depends on the original vessel structure and its location within the vascular tree. It may vary from myogenic constriction to structural changes such as medial hypertrophy (and increased wall-to-lumen ratio). In large arteries (including the aorta and others conduit arteries), there is an increase in wall thickness to maintain wall tensile stress constant as it is exposed to increased pressure (43). Larger arterioles (100–300 μm) undergo a combination of growth (leading to wall hypertrophy) and myogenic tone (lumen reduction) (44). Small resistance arterioles (<100 μm) undergo inward remodelling without growth. The inherent myogenic response reduces vascular lumen and normalizes wall tensile stress (76). These control mechanisms protect fragile capillaries from excessive pressure but in chronic hypertensive disease are associated with a reduction in mean blood flow which may result in a mismatch with local tissue metabolic demands.

Thus, structural and functional alterations in both macro and microvascular circulations appear to be common precursors leading to target organ damage in hypertensive patients (16).

1.3.6.1 Vascular remodelling – Mechanism

ECs sense any type of vascular injury (eg. pulmonary or arterial hypertension, hyperlipidaemia) and transmit signals to the medial VSMCs which promote the inflammatory response. This is followed by accumulation of VSMC in the intimal layer forming a “neointima” - this is associated with an increase in the width of the tunica media leading to increased vascular resistance and therefore raised BP. The VSMC phenotype is adaptable to the environment and may change from contractile to a synthetic state and may also migrate in response to environmental stimuli. In addition, VSMC progenitor cells from the circulation or the adventitia can be recruited to the neointima, where they adopt a VSMC phenotype. Moreover, fibroblasts and vascular stem cells from the adventitia can migrate into neointima and differentiate into VSMC (98).

Atherosclerosis is also associated with vascular remodelling (by activating ECs and VSMC) but is additionally accompanied by endothelial accumulation of modified lipoproteins which accentuate the inflammatory response, especially characterised by recruitment of monocytes and macrophages (98). CVD events due to hypertension or atherosclerosis are usually associated with injury to the large arteries of the brain, heart or kidney. Structural and functional changes of the arterial wall media (hypertrophy, ECM, calcium deposits) and of the vascular endothelium (imbalance of vasodilation and vasoconstriction) (see above Section 1.3.2) combined with increased vascular tone ultimately lead to a reduction of lumen diameter, elasticity and increased stiffness of vessels. This change in elasticity is responsible for an increase in aortic systolic pressure and a relative decrease in aortic diastolic pressure, leading to increased afterload on the heart, causing ventricular hypertrophy (99;100). Arterial remodelling also includes arterial calcification which is discussed below (Section 1.7.8) Hypertension-associated brain vascular remodelling is also related to cognitive decline and dementia (in addition to stroke); lowering BP reduces the risk of stroke-related cognitive decline or dementia (101). Tzourio C et al. recently reviewed the evidence, that hypertension has a stronger impact on the brain especially in middle age, and BP in middle age (not in old age) is a risk factor for dementia (102). The benefits of controlling BP in patients with history of stroke or transient ischemic attack were evaluated in more than 6000 patients in the Perindopril Protection Against Recurrent Stroke Study (PROGRESS) trial. It clearly showed that the risk of post-stroke dementia was decreased by one third and the risk of post-stroke severe cognitive decline was almost halved by lowering BP with angiotensin converting enzyme inhibitors (ACE) or diuretics (101). Similarly the Framingham Heart Study also showed that cognitive function and visuomotor skills are related to midlife arterial BP (103).

1.3.7 Prehypertension and hypertension

The JNC-7 guidelines defined prehypertensive individuals as those having systolic BP: 120-139 mm Hg or diastolic BP 80-89 mm Hg and advised health-promoting lifestyle modifications in prehypertension to prevent the progressive rise in blood pressure and CVD (104). It is acknowledged that more recent JNC-8 guidelines do not define hypertension and prehypertension but instead define thresholds for pharmacologic treatment (105). Prehypertension is associated

with increased stiffness of large to middle-sized arteries which is then associated with hypertension and increased risk of CVD. Tomiyama et al. compared arterial stiffness (measured by brachial-ankle PWV) in 1349 Prehypertensive and 984 normotensive people, reporting arterial stiffness to be increased in prehypertensives. This was the case even after adjustment for potentially confounding variables, including age, sex and mean BP (106). Similarly a review of seven longitudinal studies showed that measures of arterial stiffness are independent risk factors for the development of hypertension (106). In another prospective study, 777 middle-aged Japanese men with prehypertension were successfully followed up for 3 years for the development of hypertension (107). Despite evidence of "tracking" (i.e. subjects with higher BP at the start of the follow-up period also had higher BP at the end of the follow-up period), higher brachial-ankle PWV values at baseline were independently associated with the risk of new onset of hypertension even after adjustment for major confounders and baseline BP(107).

Apart from prehypertensives, Najjar et al. (65) demonstrated that higher carotid-femoral PWV (arterial stiffness) was also an independent risk factor for new-onset hypertension in normotensive subjects. Increased arterial stiffness has also been reported in hypertensive children (108). Tomiyama et al. evaluated change in arterial stiffness in normotensive and prehypertensive people over a follow up period of 5-6 years (109): they found that change in brachial-ankle PWV during the study period was higher in prehypertensive subjects (n=550) than in those with persistent normal blood pressure (n= 612) (109).

Thus, prehypertension is a risk factor for arterial stiffness, while increased arterial stiffness contributes to elevation of BP. Some more mechanistic detail is given below.

1.3.7.1 Mechanism of increased arterial stiffness contribution to the development of hypertension

The medial layer of the aorta is enriched with elastic fibres which are responsible for its elasticity. With each cardiac contraction, the systolic pressure of blood is dampened by the aorta due to its elasticity. This cushioning effect of

the aorta attenuates the pressure wave (energy) as it is propagated to the peripheral organs (110-113). Increased arterial stiffness reduces this "cushioning" effect and exaggerates the propagated pressure energy wave to peripheral vessels causing microvascular damage, especially in blood-flow-rich organs, such as the brain and kidney (110-113). Tomiyama et al. demonstrated in a middle aged cohort, that over six years of follow up, increased stiffness of large arteries as measured by brachial-ankle PWV was an independent risk factor for progression of the renal function impairment (estimated glomerular filtration rate- eGFR). In addition to this renal dysfunction, increased arterial stiffness also predicted increased peripheral vascular resistance and increase in BP (114). Thus, arterial stiffness related microvascular alteration increases peripheral vascular resistance, which may lead to development of hypertension (see Section 1.3.3).

1.3.7.2 Mechanism of prehypertension contribution to arterial stiffness

The changes associated with increased tensile stress on the vascular wall include, VSMC hypertrophy, fatigue and degradation of elastic fibres, increase in the collagen content and increase in inflammation (115;116). In turn, these changes induce medial layer hypertrophy along with neointima formation in the arterial wall. Cumulatively, all these changes decrease elasticity and/or increase arterial stiffness (see Section 1.3.6) (115;116). The increase in BP in prehypertension also augments the age-associated increase in arterial stiffness (109), and is also associated with increased arterial stiffness in old age (109).

Antihypertensive medications reduce arterial stiffness along with reduction in BP; especially, drugs blocking the RAAS. The TROPHY study was a landmark trial showing the importance of controlling BP in prehypertension range. It demonstrated that a two year treatment of prehypertension with Candesartan (an angiotensin receptor blocker-ARB) reduced the risk of development of hypertension over an additional two years (117). Another provocative finding from TROPHY trial was that the rate of development of hypertension was 13.6% in candesartan group and 40.4% in placebo group after 2 years. Candesartan treatment was stopped after two years, and at the end of four years, the rate of incident hypertension was less in Candesartan group (53.2% vs placebo 63%) (117). This can be taken to show the importance of controlling or maintaining BP

in prehypertension range as it has the potential to rapidly progress to hypertension.

As mentioned earlier (Section 1.3.7.1), elevated BP and arterial stiffness may aggravate each other, establishing a positive feedback loop; conversely, improving one abnormality may prove beneficial for the other (Figure 1.1).

1.3.7.3 Barker hypothesis

Low birth weight is a recognized risk factor for the development of hypertension and also CVD (118;119). Low birth weight is associated with structural and functional changes in the vasculature, which are then implicated in the development of CVD in adult life. Low birth weight is associated with reduced renal mass, and some studies have also shown its association with a reduced capillary network in peripheral organs (118;119). Both reduced renal mass and reduced capillary network may act to elevate BP. Mori et al. also showed increased aortic stiffness in new-born infants that were born small for gestational age (120). In addition low birth weight is also associated with raised fasting plasma cortisol concentration in adult life and suggests involvement of hypothalamic-pituitary-adrenal axis as the link between low birth weight and raised BP in adult life (121). In summary both hypertension and increased arterial stiffness are more likely to occur in low birth weight infants.

Another possible mechanism in relation to birth weight is the changes in microcirculation. The primary evidence comes from the work of Barker et al. who found that BP and the risk of hypertension among middle aged (approximately 50 years) men and women was predicted by a combination of their birth weight and placental weight (122). The highest BP levels were found among people who had been small babies with large placentas, and they suggested that reduced blood flow in the trunk of a foetus that is small in relation to its placenta could lead to reduced microcirculatory growth (122). Another proposition is that a primary deficit in the development of the microcirculation could have led to impaired growth of the foetus. The reduced microcirculatory growth in a foetus due to any mechanism may predispose the person to the development hypertension in later life.

Similarly examination of men and women in different UK populations which had low growth rates up to the age of one year; were associated with increased prevalence of known risk factors for CVD, including BP, blood glucose, insulin, fibrinogen, factor VII, apolipoprotein B; along with increased death rates from CVD (123).

1.3.7.4 A "vicious cycle" in hypertension

The microvascular abnormalities have been shown to both result from and contribute to hypertension. A "vicious cycle" may exist in which the microcirculation maintains or even exaggerates an initial increase in BP. Delano et al. described the pressure changes from central to peripheral circulation and have indicated that as much as 70% to 90% of the systemic pressure is delivered to the microcirculation in many skeletal muscles (124). Moreover Pries et al (125) showed that almost all the contribution in decreasing intravascular pressure before delivery to peripheral tissues was by micro vessels with a diameter of 100µm or less. An increase in BP might raise microvascular resistance which may lead to a further elevation of BP. Pries and colleagues (125;126) used computer simulation techniques to study the long term effects of increased BP and blood flow on the resistance and structural adaptation of microvascular circulation. They showed that a small increase in pressure can lead to larger structural increases in pressure and flow resistance by a mechanism involving the tendency of vessels to reduce their luminal diameter in response to increased intraluminal pressure (125;126).

On the other hand, microvascular abnormalities might initiate the pathogenic sequence in primary hypertension by increasing peripheral vascular resistance. Increased peripheral resistance to blood flow raises central pressure in the aorta and large arteries, ultimately increasing vascular stiffness in large vessels as they are exposed to higher pressure. From this perspective primary hypertension may be seen as a developmental abnormality of the microcirculation. Microvascular rarefaction reduces the vessel surface area available for oxygen delivery and also increases the diffusional distance between vessels and their target cells. If there is progression in rarefaction, it will result in tissue ischaemia which may be responsible for much of the end organ damage associated with hypertension (77).

Part 3

1.4 Blood pressure/hypertension

The heart pumps oxygenated blood to all parts of the body in blood vessels. High blood pressure (BP) is a condition of increased arterial tone. Under these conditions, the heart has to perform increased work leading to hypertrophy and ischaemia, infarction and ultimately heart failure (6).

Hypertension is one of the important factors causing atherosclerosis (narrowing of the blood vessels). Hypertension is responsible for multiple target organ damage through atherosclerotic macro- and micro-angiopathy. Atherosclerosis results in decrease blood supply to target organs and is responsible for ischaemic stroke, myocardial infarction, peripheral vascular disease, and cardiac failure (127). It has been recorded that 60% of all stroke patients have a past medical history of arterial hypertension (128;129).

Blood vessels may develop aneurysms and weak spots due to high pressure, making them more likely to block or rupture. Hypertension can also lead to kidney failure, blindness, rupture of blood vessels (in other areas) and cognitive impairment (6)

1.4.1 Normal BP and hypertension

Blood pressure, measured in millimetres of mercury (mm Hg), is recorded as the systolic blood pressure (SBP) [maximum pressure during contraction of the left ventricle (systole)] and diastolic blood pressure (DBP) [minimum pressure recorded in blood vessels during ventricular relaxation (diastole)]. According to the US Eighth Joint National Committee (JNC 8) evidence-based guideline for the management of high blood pressure in adults (age <60 years), normal adult BP is defined as a systolic blood pressure of <140 mm Hg and a diastolic blood pressure of <90 mm Hg (105).

Hypertension is defined as a SBP equal to or above 140 mm Hg and/or diastolic blood pressure equal to or above 90 mm Hg (105). Normal levels of both systolic and diastolic blood pressure are crucial for the efficient function of vital organs such as the heart, brain and kidneys and to prevent them from damage (6).

1.4.1.1 Physiology

The product of normal cardiac output (CO) and total peripheral resistance (TPR) determines the arterial BP. These two principal determinants are influenced by many physiological and pathological determinants. CO depends primarily on heart rate and stroke volume. The systemic vascular resistance is influenced by multiple vasoactive mechanisms under the control of local, regional, and systemic neural, humoral and renal factors (130).

1.4.1.2 Pathophysiology

Chronic increases in arterial BP may result from combinations of inappropriate levels of CO and TPR. In hypertension an abnormal factor perturbs either one or both of CO and TPR and appropriate compensatory mechanisms that could normalize the changes in TPR and/or CO to return BP to normal range are ineffective.

A high CO and a normal or low TPR normally occurs in early phase diabetes mellitus, patients needing dialysis and hyperdynamic or hyperadrenergic hypertension usually seen in youth. A high TPR and low or normal CO mostly occur in accelerated or malignant hypertension or hypertension in the elderly. A high CO and a high TPR pattern is normally present in renovascular hypertension (6;130).

1.4.2 Factors influencing blood pressure/hypertension

There are many factors influencing BP and the development of hypertension. The following is a brief description of the factors influencing BP but their detailed discussion in relation to hypertension, endothelial dysfunction and macro and microvascular dysfunction is explained in the relevant sections (see Sections 1.2 and 1.3).

1.4.2.1 Role of genes

Multiple genes along with involvement of multiple environmental factors determine susceptibility to develop primary HTN (131). The role of genes in the development of hypertension varies according to race and population and the variance explained may be as low as 15–20% to as high as 65-70% (132). It has

also been observed that a child with a family history of hypertension (hypertension in one or both parents, and/or a sibling with HTN), has up to four times increased chance of developing HTN as an adult (133;134). Gene-environment interaction is such that the same genotypic susceptibility to HTN may manifest as normal BP (normotensive) in one environment, and hypertensive in another (135). Genetic factors may play a role in the development of HTN early in life but when it develops before the age of 40 years it is important to exclude secondary causes such as kidney disease, endocrine disease and vascular malformations (6).

1.4.2.2 Role of environment

Genetic variance is presumed to have a weak effect on BP but may produce substantial hypertension in the presence of the necessary environmental conditions (136). Key environmental factors include geographical area, diet, physical activity, psychosocial stress, socioeconomic status, alcohol intake, smoking, obesity and other life style factors (135). Obesity, inflammation and insulin resistance are important factors related to HTN and will be discussed in detail.

1.4.2.3 Age

HTN is uncommon in children and young adults but its prevalence increases with age, approaching 65% at age 65 years and 75% at 75 years(135). The risk of HTN increases with age is attributable at least in part to stiffening of blood vessels (see Section 1.8). Progression of arterial stiffness can be slowed by healthy living (physical activity, smoking), including healthy eating and reducing salt intake in the diet (6).

1.4.2.4 Weight/BMI

Obesity is one of the most important risk factor for phenotypic expression of HTN. There is a strong positive correlation between body fat and BP with obesity and HTN frequently co-existing (137). Weight gain and especially obesity is a consistent predictor of subsequent development of hypertension (138).

Various mechanisms by which obesity plays a role in the development of HTN (135) are shown in Table 1.1 but will be discussed in detail in Section 1.7.

Table 1.1 Mechanisms by which obesity promotes development of hypertension

Mechanism
1. Activation of sympathetic nerve activity leading to renal sodium retention.
2. Hyperleptinemia: also stimulates sympathetic nervous system (SNS)
3. Hyperinsulinaemia: also stimulates SNS
4. Increased Angiotensin II (Ang II)
5. Increased Aldosterone: salt and fluid retention, also stimulates Ang II
6. Perirenal Fat: fat surrounding the kidneys raises intra-renal pressure

1.4.2.5 Autonomic Nervous System (ANS)

Numerous studies have documented that in essential hypertension, the Sympathetic Nervous system (SNS) is hyperactive particularly in patients who are young or borderline hypertensive (139). Moreover, many people newly diagnosed with hypertension have increased plasma nor-epinephrine (NE) levels with increased heart rate. The effects of SNS stimulation are peripheral vasoconstriction, release of NE from the adrenals, an increase in heart rate and an increase in systemic BP. Other effects of increased SNS stimulation are myocardial hypertrophy, vascular smooth muscle hypertrophy leading to vascular stiffness and reduced arterial distensibility and compliance (140). The renal SNS directly stimulates sodium reabsorption and renin release from the juxtaglomerular apparatus which ultimately leads to activation of the renin angiotensin system (140;141).

Autonomic Nervous System and vascular disease

Cardiovascular risk factors like diabetes, hypertension, hyperlipidaemia and smoking impair endothelial function from the luminal side of the vessel (142) whereas the autonomic nervous system (ANS) is considered to affect the endothelial function from outside of vessel. ANS innervation and control of vascular structure and tone, are complex as both the sympathetic (adrenergic) and the parasympathetic (cholinergic) nervous systems innervate blood vessel walls and regulate wall tension (143;144). The sympathetic nerve fibres are

found in the tunica media whereas cholinergic nerve endings are found both in the muscular and endothelial layers. Endothelial muscarinic (M) 3 receptors are coupled to the formation of NO and cause vasodilation. However, the M3 and M2 receptors on smooth muscle cause contraction when formation of NO is blocked. Imbalance of ANS affects vascular function and structure and hence becomes a cardiovascular risk factor (145). Increased sympathetic activity increases peripheral vasoconstriction and reduces venous capacitance and renal sodium and water excretion; all leading to sustained increase in blood pressure (146), and may contribute to the pathologic process of hypertension. Similarly ANS dysfunction has been associated with the development of diabetes in healthy adults and also increases the risk of atherosclerosis progression (147). Obesity is also associated with central stimulation of the SNS by reactive oxygen species. The levels of oxidative stress markers within the brain are raised in obesity and may be a cause of increased sympathetic tone leading to hypertension in high fat fed animals (148).

Endothelial function and ANS

Gamboa, et al. investigated the endothelial NO and ANS derived NO relationship in normotensive subjects and found that endothelial NO is the most potent metabolic determinant of BP. Endothelial NO was responsible for tonically restraining BP by approximately 30mmHg in normotensive state (149), However the impairment of endothelial NO availability was insufficient to raise BP and the ANS pathway was considered to be critical for the early development of hypertension (150).

Some studies ascribe a physiologically relevant role for neuronal nitric oxide synthase (nNOS), as NO produced is involved in smooth muscle cell relaxation (144), hence playing a role in the modulation of systemic arterial pressure (151). Moreover ANS denervation alters normal endothelial function in animal studies (152). Similarly in hypertensive patients, ANS modulation by alpha 2 adrenoreceptor agonists has been shown to improve endothelial dysfunction (153). These animal and human findings suggest a contribution of sympathetic nervous tone on the maintenance of basal vascular function. When sympathetic activity is exaggerated, it modifies the normal endothelial function by increasing immune-reactivity of ECs and also promotes the uptake of low-density

lipoprotein cholesterol (LDL) by ECs (154;155). Hijmering, et al. also demonstrated that vascular flow-mediated dilation responses were impaired by sympathetic stimulation (via an alpha-adrenergic mechanism) (156) Similarly anxiety has been shown to be associated with endothelial dysfunction (157). Ghiadoni, et al. also reported that acute mental stress induced transient (lasting up to 4 hours) endothelial dysfunction, accompanied by increase in heart rate, blood pressure and salivary cortisol (158).

It should be noted that vascular function may also modulate ANS, although the mechanisms by which NO modulates neuronal activity are still unclear. It has been shown that NO alters neuronal responses to excitatory amino acids (159). Similarly within the central nervous system NO acts as a sympatho-inhibitory substance (160). Animal studies have shown that after induction of diabetes in rats, there was reduction in nerve blood flow causing endoneurial hypoxia (161) and it has been proposed that diabetes induced endothelial dysfunction and reduced NO may be responsible for this reduction in blood flow.

The inter-related mechanisms are complex: some factors (e.g. NO, reactive oxygen species (ROS), endothelin, and the RAAS) appear to influence both ANS and vascular function. ROS and Ang II affect both systems, perhaps explaining the basal physiological interrelationship between vascular function and the ANS. Similarly, inflammation also explains some of the interactions between endothelial function and the ANS. Parasympathetic stimulation reduces the inflammatory response, whereas, sympathetic activation increases the production of inflammatory cells (162). In addition, inflammation also impairs endothelial function (163).

1.4.2.6 The renin angiotensin aldosterone system (RAAS)

The renin angiotensin aldosterone system is one of the most important mechanisms in the regulation of blood volume and pressure. Angiotensinogen is a protein substrate produced by the liver which is cleaved by renin (a proteolytic enzyme released from kidney) to form Angiotensin I. Angiotensin I does not have any vascular effects, but in the presence of angiotensin converting enzymes (ACEs), is converted to Angiotensin II (Ang II). Most angiotensin I is converted to Ang II during its passage through the pulmonary circulation (164).

Ang II is a potently vaso-active compound and acts through multiple mechanisms in different organs. Increases in Ang II lead to stimulation of vascular smooth muscle contraction and hypertrophy, increased cardiac contractility, stimulation of the SNS (in both central and peripheral nervous system), increased thirst and vasopressin release (also stimulation of aldosterone synthesis). In the kidneys, Ang II causes vasoconstriction, a decrease in blood flow, and an increase in vascular resistance. It also increases sodium re-absorption, both directly and by increasing aldosterone secretion. Ang II also increases the generation of reactive oxygen species in the vasculature and aggravates the atherosclerotic process (165). The detailed role of RAAS in altering vascular system is discussed in Section 1.7.4.7

1.4.2.7 The kidneys and primary hypertension

One of the main steps in development of hypertension is impairment of renal sodium excretion. A genetic reduction in the number of nephrons may be the initiating event but over time hyper-filtration and increased glomerular pressure limit capacity to excrete salt (166). Moreover with ageing, the degree to which BP is sensitive to a dietary sodium load increases, such that, by 70 years of age almost all patients with hypertension are salt sensitive (167;168). Salt sensitivity can be assessed formally as described by Sharma using the change in BP in response to a low salt diet (169). Those who are sensitive to salt are more likely to have high BP than those who are resistant to salt (167).

1.4.2.8 Nephron number and blood pressure

In healthy humans the nephron number at birth ranges from 250 000 per kidney to as high as 1,800,000 (170). It has been shown that kidney size and nephron number per kidney at birth are closely related to birth weight (see Section 1.3.7.3) which is in turn associated with increased risk for developing adult metabolic syndrome and obesity (171;172). The number of nephrons is directly correlated with birth weight and inversely correlated with age, blood pressure in adult life, glomerulosclerosis and cortical fibrosis (171;173). In this regard, it can be hypothesized that individuals endowed at birth with a greater number of nephrons are more resistant to the deleterious effects of obesity and other factors than those with a smaller number.

1.4.2.9 Mitochondrial dysfunction in hypertension

Hypertension is strongly associated with oxidative stress, endothelial dysfunction, and increased vascular resistance as a consequence of elevated levels of ROS and nitrogen species. As mitochondrial dysfunction precedes endothelial dysfunction (see Section 1.2.2 and Figure 1.7), it might have a role in hypertension. Jin et al showed in a Korean population that age-dependent polymorphisms in the mitochondria shaping gene, OPA1 correlated with blood pressure and hypertension (174). Similarly Wang et al. showed that mitochondrial dysfunction caused by mitochondrial tRNAlle 4263A>G mutation was involved in hypertension (175). Puddu et al. also proposed that increased mROS generation in situations of metabolic disturbance, might trigger endothelial dysfunction, possibly contributing to the development of hypertension (176). Similarly nicotinamide adenine dinucleotide phosphate-oxidase 2 (NADPH oxidase 2 or Nox2) and Ang II elevates mROS production and endothelial dysfunction (177). In contrast, Nox2 depletion in gp91phox knockout mice inhibits Ang-II-induced cellular mROS and attenuates hypertension (178). The mitochondria specific antioxidant enzymes (eg. thioredoxin 2) also attenuate Ang-II-induced hypertension (179). Moreover, transgenic mice overexpressing mitochondria MnSOD also attenuates Ang-II induced hypertension (180). The eNOS and associated NO production are crucial for endothelial function, but direct association of mitochondria with eNOS uncoupling (observed in hypertension) remains elusive.

1.4.2.10 Hypertension and other cardiovascular risk factors

The association of hypertension with insulin resistance, inflammation and obesity is explained in detail in the relevant sections (Sections 1.5, 1.6 and 1.7).

1.4.3 Renal pathway for the development of hypertension (impaired natriuresis)

In the normal kidneys norepinephrine (NE) and Ang II have sodium retaining properties. The increase in BP due to these or any other agents results in a physiological pressure natriuresis with sodium loss and a return to a normal or low blood volume state (181). Similarly, increased salt intake (sodium loading) is likely to increase renal blood flow (182) but brisk and exaggerated natriuresis

will normalise BP (183). Another important process is renal autoregulation. With increase in BP, the afferent arteriole and interlobular artery vasoconstrict; secondary to a myogenic reflex and by tubulo-glomerular feedback. This prevents the transmission of high pressure distally to the sensitive structures in the glomerulus and peri-tubular capillaries (135).

In the early phases of hypertension, the increase in BP may be in the prehypertension range and intermittent. At this stage variability may be higher than in normotensive individuals. During this phase hypertension is salt resistant and the pressure natriuresis system is intact (181). Intermittent activation of the SNS and RAAS eventually results in permanent injury which leads to impairment of salt excretion. The vasoconstriction of afferent arterioles (renal autoregulation) in response to Ang II and NE gradually leads to the development of pre-glomerular arteriolopathy; arteriolosclerosis of the afferent arterioles is the classic renal biopsy finding in HTN patients (184).

Permanent or irreversible injury to the kidneys occurs via two main mechanisms.

The development of arteriosclerosis and the deposition of extracellular matrix within the arterioles decrease compliance and they lose the autoregulatory response (185;186). As a consequence increase in pressure is transmitted distally and leads to glomerular damage (sclerosis) and tubulointerstitial injury.

Renal injury by ischaemia is caused by pre-glomerular arteriopathy. With increased BP and arteriosclerosis of the afferent arteriole, the arteriolar lumen becomes progressively smaller and finally collapses, leading to distal glomerular and peritubular ischaemia. Ischaemia leads to infiltration of T cells, macrophages and other inflammatory cells, increase in reactive oxidants and local inflammatory markers further increasing renal vasoconstriction (187;188). This leads to a reduction in sodium filtration (by reducing cortical filtration coefficient and glomerular filtration rate) and increased renin and Ang II, which further increase tubular reabsorption of sodium, leading to increased BP. As a consequence, blood volume and pressure increase, increasing renal perfusion pressure. Sodium handling return towards normal, but all at the expense of a shift in pressure natriuresis and an increase in systemic BP (189).

In summary, HTN may shift from a salt resistant, renin-dependent type to a salt-sensitive, volume-dependent type in time (135). This is consistent with ageing (190) and obesity (191), in which there is a progressive increase in salt sensitivity.

Explained above is the renal pathway for the development of hypertension. Hypertension is a dynamic condition and involves many other risk factors including, ageing, autonomic nervous system, obesity, diabetes, vascular stiffness and calcification, RAAS, insulin resistance and immunity. The role of each factor in the development of hypertension will be explained in the relevant sections and at the end a unifying mechanism correlating all the risk factors will be explained.

1.5 Insulin resistance

Insulin resistance (IR) is a state in which the body has an impaired response to the normal actions of the hormone, including transport of circulating glucose into cells. This contributes to the development of hyperglycaemia and type 2 diabetes (T2DM). ß-cells in the pancreas subsequently increase their production of insulin, further contributing to hyperinsulinaemia (192).

1.5.1 Explanation

Insulin is an anabolic hormone produced by the ß-cells of the pancreas which has many functions in the regulation of carbohydrate, lipid and protein metabolism. As well as regulating glucose transport into muscle, insulin decreases hepatic glucose production. When this function is impaired in insulin resistant states, excess glucose is produced contributing to hyperglycaemia. In fat cells, insulin increases uptake of circulating lipoproteins from blood and decreases hydrolysis of stored triglycerides. Insulin resistance thus causes reduced uptake and increased mobilization of lipids, leading to elevated concentrations of free fatty acids in blood. Glucose transported into the cells is used to generate energy and excess is stored as glycogen in liver and muscle cells, and as triglycerides in fat cells. In summary, reduced muscle glucose uptake, increased hepatic glucose production, and elevated blood fatty-acid concentration all contribute to elevated blood glucose levels (hyperglycaemia) (193).

When hyperglycaemia is sensed by ß-cells, additional insulin is secreted causing hyperinsulinaemia. Hyperglycaemia and hyperinsulinaemia are major components of the metabolic syndrome (194). When the pancreas does not produce sufficient insulin to compensate for the high blood glucose and insulin resistance, blood glucose concentrations increase further, leading to T2DM (193).

1.5.2 Mechanism of diabetes

Insulin sensitivity is determined by genetic and lifestyle factors, including those associated with obesity. Increased insulin resistance, whether genetic or environmental, causes hyperinsulinaemia (192;195), the latter expedites the development of fatty liver and ectopic fat deposition (195). As glucose utilization decreases in muscle, it is redirected to the liver where it is stored as fat. This creates hepatic insulin resistance and triggers a fatty liver vicious cycle (195).

During positive energy balance fat will accumulate in the liver and this accumulation is promoted by insulin. An individual with a degree of muscle insulin resistance, if prone to more energy intake; will accumulate liver fat more readily than others (195). As liver fat increases it leads to: 1) hepatic insulin resistance, 2) increased hepatic glucose production, as the liver becomes less sensitive to suppression by insulin, 3) a rise in plasma glucose; further increasing hyperinsulinaemia, and 4) additional hepatic fat deposition; exacerbated by raised plasma glucose and portal hyperinsulinaemia. The increase in insulin and glucose leading to fatty liver will from a positive feedback loop (195).

Increased fat in the liver will increase secretion of triacylglycerols (TGs) from liver into blood; these accumulate in ectopic sites including pancreatic islets. The ectopic fat and raised plasma glucose attenuates ß-cell insulin secretion in response to ingested glucose; further raising plasma glucose. These processes further impair insulin release from ß-cells and promote cell death, ultimately leading to clinical diabetes (195). Early intervention reversing energy balance (by low calorie diet or bariatric surgery) may prevent ß-cell death and reverse fatty liver.

In conclusion, insulin resistance is a complex phenotype and involves cross-talk between tissues including muscle, liver and pancreas. Ectopic fat deposition in the liver underlies the defect of hepatic insulin resistance and fat deposition in islets forms the basis of β-cell dysfunction. All of these effects can be reversed early in the course of diabetes by hypoenergetic (low calorie diet) feeding conditions (195).

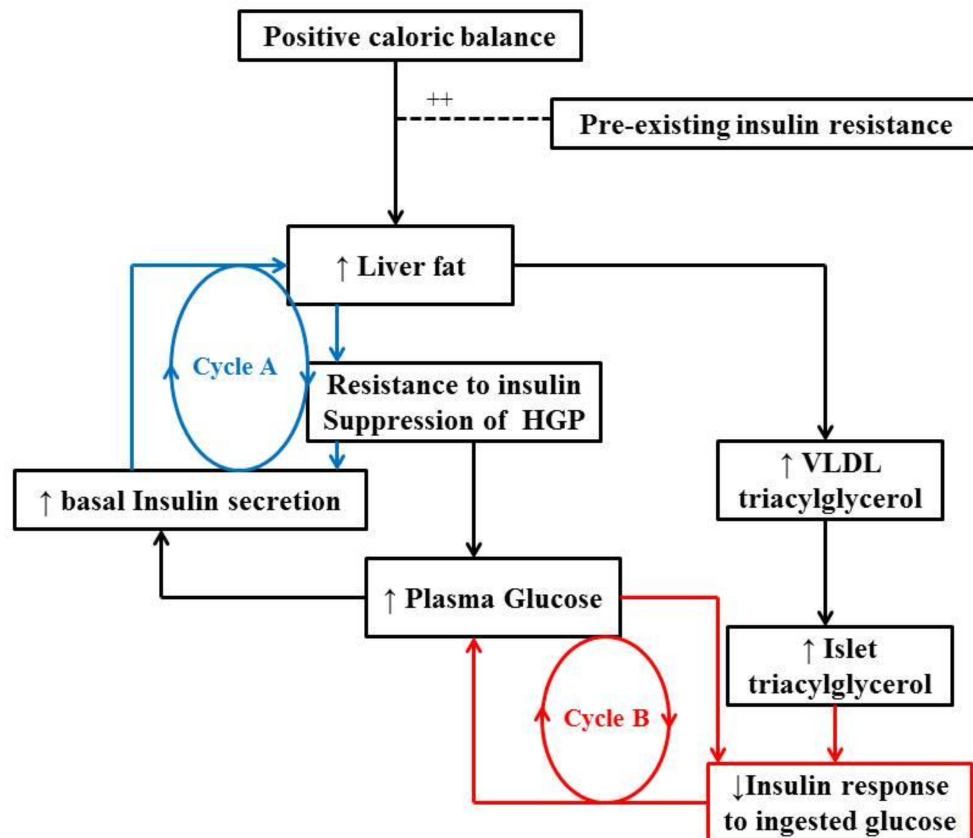


Figure 1.2 The twin vicious cycles of type 2 diabetes.
Redrawn with permission from Taylor R, Diabetologia 2008 (195)
HGP= Hepatic glucose production, VLDL= Very low density lipoprotein

1.5.3 Relation with hypertension

Hyperinsulinaemia and underlying insulin resistance have been associated with essential hypertension independently of weight or body mass index (196) but their exact role in pathophysiology remains unclear. Allerman Y et al demonstrated in 1993 that Insulin resistance and hyperinsulinaemia exist in normotensive, first-degree relatives of patients with essential hypertension (197), suggesting that the link may be causal.

However, the relationships between obesity and both insulin resistance and HTN complicates the analysis. The association between IR and HTN is not strong if compared to the relation between IR and dyslipidaemia, as only about 50% of hypertensive subjects are insulin-resistant (198).

The role of endothelial dysfunction in the link between HTN and IR further opens another avenue, that defective vasodilation actually produces insulin resistance. Normal blood flow may be required in some tissues for optimal glucose uptake and defective arteriolar vasodilatation is a characteristic of insulin resistant individuals. For example, the Heart Outcomes Prevention Evaluation (HOPE) Study showed that treatment with the angiotensin-converting enzyme inhibitor ramipril was associated with a reduced incidence of diabetes; one interpretation of this finding was that inhibiting vasoconstriction improved muscle blood flow, enhancing insulin sensitivity and thereby improving tissue glucose uptake(199).

It is well know that hyperinsulinaemia increases renal sodium and water retention (200), but it is still not clear how often volume-dependent hypertension is present in IR individuals and people with T2DM. The sympathetic nervous system is overactive in obese and IR individuals (201), but it is not known if this is the primary event or the result of IR.

The Insulin Resistance Atherosclerosis Study (IRAS) also showed that, insulin resistance was significantly associated with hypertension in non-Hispanic whites (NHW) and Hispanics (H), but not African Americans (AA). It also showed that neither insulin resistance nor hyperinsulinaemia was related to HTN or BP in patients with T2DM (202).

In conclusion, the relation between insulin resistance and hypertension is still controversial as it varies by ethnicity and disease. Moreover it is still under debate whether impaired vasodilatation in association with HTN is a cause or an effect of insulin resistance.

1.5.4 Effect of insulin/ insulin resistance on vasculature

Insulin resistance and hyperinsulinaemia are known to play major roles in the pathophysiology of obesity and diabetes but beyond these classic targets also

affect cardiovascular tissue; contributing to hypertension and atherosclerosis. Abnormalities in vasodilation and blood flow have been suggested as a mechanism linking hypertension and insulin resistance. In the 1990s, Baron et al identified a vasodilator effect of insulin in human skeletal muscle (203) which is impaired in insulin-resistant states including obesity and T2DM (204). Within the vessels, insulin has an important role in vasodilation but several factors affect vascular reactivity independent of insulin including catecholamines, glucagon-like protein-1 (GLP-1) and RAAS. Attenuation of the vasodilator action of insulin may be more relevant to the pathophysiology of hypertension than to that of diabetes (205). It has also been suggested that resistance to the vascular effects of insulin may contribute to the pathogenesis of CVD (206) and may play a role in increased rates of CVD in T2DM (207).

The vasodilatory effects of insulin are mediated at least in part by endothelial release of nitric oxide; moreover, blocking nitric oxide production can induce insulin resistance *in vivo* by preventing insulin-mediated vasodilation in skeletal muscle and thereby reducing glucose uptake (208). Thus, insulin may enhance its own delivery and that of its substrates to relevant capillary beds. In addition, endothelial dysfunction, characterized by reduced nitric oxide production and exaggerated release of endothelin, is also a feature of insulin resistant states (209).

Normal insulin signalling in endothelial cells is thought to involve activation of phosphoinositide 3-kinase (PI3k), insulin receptor substrate 1 (IRS-1) and mitogen-activated protein kinases (MAPK) pathways; endothelial insulin sensitivity is a balance between the vasculoprotective PI3K pathway and the proatherogenic MAPK pathway (210). Insulin induces release of NO (through IRS-1/PI3K pathway), which decreases vascular tone, VSMC proliferation, adhesion of inflammatory cells and platelet aggregation to EC (211). Blockade of the PI3K pathway induces insulin resistance in cultured ECs and results in blunted production of NO with increased expression of pro-atherosclerotic molecules (212). The PI3K pathway shares common signalling elements with those utilized by insulin to upregulate glucose transport in metabolic target tissues including muscle (211). Moreover, insulin regulates production of prostaglandins and endothelium derived hyperpolarizing factors (211). The MAPK pathway mainly acts via endothelin (211), and results in mitogenic and proatherosclerotic

responses in the vascular wall (210). The excess of endothelin and its actions appears to contribute to insulin resistance in human obesity (211). ET-1 also causes vasoconstriction, increase oxidative stress, and promote cell growth and mitogenesis in VSMCs (213). Spontaneously hypertensive rats exhibiting vascular insulin resistance also showed decreased insulin-stimulated NO production and enhanced ET-1 secretion (214).

Clark et al explained that insulin (via NO release) regulates capillary recruitment and perfusion by affecting pre-capillary resistance vessels (215) and insulin resistance is associated with impaired capillary recruitment and altered skeletal muscle perfusion. Vincent et al. demonstrated in rats that the onset of the acute vascular action of insulin precedes the induction of glucose uptake (216), consistent with the notion that the vascular effects of insulin are primary and do not arise as a consequence of changes in cellular metabolism. In keeping with suggestion, abnormal vasoreactivity has been demonstrated in human studies comparing lean and obese subjects. After a mixed meal, lean humans exhibited increased brachial blood flow and forearm microvascular recruitment, whereas obese subjects have a blunted response despite hyperinsulinaemic conditions (217;218). This blunted response was related to insulin resistance and associated endothelial dysfunction (211). Both obesity and T2DM are associated with abnormal vasoreactivity (204). Hyperinsulinaemia has also shown its contribution in vascular stiffness. In lean and insulin sensitive individuals, insulin reduces central arterial stiffness before it exerts its slow vasodilatory effect on peripheral small vessels. In contrast, in obese individuals, its effect on arterial stiffness is severely blunted and this attenuation correlates with the degree of obesity (219). It is thought that post receptor abnormalities causing resistance to insulin-mediated glucose uptake in metabolic tissues also cause resistance to insulin-mediated vasodilation in vascular tissues (see below Section 1.5.4.1)

In summary, insulin stimulates vasodilation in insulin sensitive states, but may promote vasoconstriction and vascular proliferation in insulin resistant states (211)

1.5.4.1 Actions of insulin in skeletal muscle vasculature

Insulin serves two main functions in the skeletal muscle: 1) vasodilation and capillary recruitment; 2) glucose uptake (220). The skeletal muscles receives 0.03–0.04 mL/min of blood flow per gram of tissue in resting (Non-exercising) conditions (221), but exercise initiation increases blood flow up to 100-fold (222). The capillary recruitment in the skeletal muscle during resting and exercising state is determined by vasomotor changes in the terminal arterioles (221). During exercise oxygen is depleted and it initiates an ascending vasodilation response which extends from the contracting skeletal muscle arterioles to the proximal large arteries, resulting in increased blood flow (223). This ascending vasodilation response also depends on an intact and normal functioning endothelium. Capillary recruitment is crucial for the normal metabolic effects of insulin in skeletal muscles, (224) and clinical conditions characterized by insulin resistance such as obesity and T2DM demonstrate impaired capillary recruitment (225). Insulin increases the blood flow and in this way also regulates its own delivery to the tissues (221). However, the tissue extraction of insulin declines to approximately 7% at higher insulin concentration (~250 pmol/l), indicating that delivery alone may not be a critical rate-determining step (221)

In lean, insulin-sensitive humans insulin increases whole-limb blood flow in a dose dependent manner and is also associated with increased skeletal muscle glucose uptake (226). The effects of increase in blood flow are largely dependent on increased NO production (224), as it is blunted by eNOS inhibition (208). The decreased blood flow due to eNOS inhibition of NO, also results in impaired insulin-mediated skeletal muscle glucose uptake (203). The capillary recruitment increases delivery of insulin to the skeletal muscle along with increasing the endothelial surface available for nutrient exchange (227). The insulin-mediated increases in microvascular blood flow and microvascular recruitment precedes the increase in total-limb blood flow caused by insulin (228). In contrast, bradykinin (a vasodilator) infusion increased limb blood flow but did not increase insulin-mediated glucose uptake by skeletal muscle (229) suggesting that only increasing flow alone does not increase glucose disposal at the same time (205). Therefore, insulin is essential for both capillary recruitment and glucose disposal, although without capillary recruitment glucose

disposal will not increase as peripheral insulin delivery depends on capillary recruitment.

1.5.4.2 Vascular insulin resistance: evidence from transgenic models

Endothelial insulin receptor (IR) knock out (KO) leads to impaired eNOS and ET1 expression (230). Similarly utilizing a transgenic mouse with endothelium overexpression of a dominant negative mutant human IR containing a mutation in the tyrosine kinase domain (ESMIRO) demonstrated decreased vasodilation in response to insulin and acetylcholine with blunting of insulin-induced eNOS phosphorylation (231). However, ESMIRO mice exhibited preserved whole body insulin sensitivity and were normotensive (231). This study suggested that haemodynamic factors may play a relatively small role in the pathogenesis of insulin resistance and several human studies also support this interpretation (205). In ESMIRO mice, the changes above were also accompanied by increased ROS production in vessels from the Nox2 isoform of NADPH oxidase. Furthermore inhibition of Nox2 under insulin-resistant conditions led to improvement in Ach-mediated vasodilation and decreased ROS production (232). The IR KO in mice also accelerated atherosclerosis (233)

1.5.4.3 Lipotoxicity and insulin resistance

Lipotoxicity is a common finding in both obesity and T2DM and its deleterious effects have been studied in both humans and animals. Intravenous infusion of lipids and heparin in rodents decreased muscle glucose uptake and blunted insulin-mediated microvascular recruitment in skeletal muscle (234). Similarly, lipid infusion in healthy humans increased insulin resistance and decreased forearm microvascular recruitment (235). Increased plasma lipids increase intracellular production of diacylglycerol (DAG) and ceramides (236) which then activates protein kinase C (PKC) (236). PKC is known to inhibit the vascular effects of insulin (237). Tabit et al. also showed that PKC expression was markedly increased in ECs of T2DM patients (238) and that a PKC inhibitor restored eNOS activation by insulin (238). In insulin-resistant Zucker fatty rats, PKC activation was associated with decreased Akt-dependent eNOS activation (237) and treatment with a PKC inhibitor (ruboxistaurin) restored the insulin-induced eNOS activation (237).

Lipotoxicity also induces inflammation within the vascular cells. Jang et al. showed that vascular cultured ECs exposure to palmitate results in activation of pro-inflammatory molecules through activation of Toll-like receptor 2 (TLR2). TLR2 knockdown ameliorated these inflammatory effects and also protected mice from whole body and endothelium insulin resistance in spite of high fat feeding (239).

1.6 Inflammation

Inflammation is a non-specific immune response that occurs in reaction to any type of bodily injury. Inflammation can be classified as either acute or chronic.

Acute inflammation is the initial response of the body to harmful stimuli (infection, tissue injury, neoplastic growth, or immunological disorders) and is characterized by increased movement of plasma and leukocytes (especially neutrophils and macrophages) from the blood into the injured tissues to limit injury or aid healing (240;241). Acute inflammation is also characterised as acute phase reaction (APR), which is beneficial in restoring disturbed physiological homeostasis (240). There is also induction of acute phase proteins (like C-reactive protein- CRP) which are mostly synthesized in the liver. Their production is stimulated by cytokines including interleukin 6 (IL-6) and tumour necrosis factor (TNF-α) (242;243). Other metabolic processes accompanying the APR include increased hepatic glycogenesis and glucose synthesis, reduced glucose uptake in muscles, increased insulin secretion and insulin resistance (244).

Acute inflammation is a self-limiting process but in some disorders the inflammatory process becomes continuous and chronic inflammation develops. Prolonged or chronic inflammation is characterized by simultaneous destruction and healing of tissue via the inflammatory process. It leads to replacement of the type of cells present at the site of inflammation. It is characterised by infiltration of T lymphocytes and plasma cells. Macrophages play a central role in chronic inflammation and contribute to the final step of fibrosis leading to loss of tissue (241). Research in recent years has implicated inflammation as a key pathogenic mechanism in the initiation and progress of many cardiovascular

risk factors (including physiological ageing) and diseases including obesity, diabetes, atherosclerosis and coronary heart disease (245).

1.6.1 Inflammation related vascular changes in ageing and CV risk

Chronic inflammation is strongly associated with arterial ageing and occurs in the absence of any microorganisms and with little or no white blood cell infiltration (246). Phenotypic shifts in arterial ECs and VSMCs during the ageing process, promote pathogenic inflammation (247-250). Most of the changes in the vessel wall during ageing are also associated with inflammation including; endothelial disruption, enhanced VSMC migration and proliferation and matrix calcification/amyloidosis/glycation (246). Vascular inflammation is also related to the pathogenesis of hypertension and atherosclerosis. Age-associated arterial pro-inflammation is to some extent modifiable and may have the potential to ameliorate or retard age-associated arterial diseases. The transcription and activity of ACE1 and chymase (both increasing Ang II production) increases with age (251). This leads to increased Ang II in older arteries (30 months old rats) (252) and is also associated with up regulation of AT1 receptor expression in old coronary arteries (246;252).

Ageing is also associated with increased aldosterone/mineralocorticoid receptor (MR) signalling and increased sensitivity of MR to aldosterone thus increasing MR activity (252;253), promoting a pro-inflammatory phenotype via an extracellular signal-regulated kinase 1/2/mitogen-activated protein kinase/epidermal growth factor receptor (ERK/MAPK/EGFR)-dependent pathway (253). Moreover aldosterone mediated increase in the expression of EGFR in VSMCs also reinforces the inflammatory effects (253).

In contrast, the key defence system of antioxidant enzymes protecting against the cytotoxic effects of oxidative stress is downregulated with age. This is due to the inactivation of transcription factors of detoxifying and antioxidant genes by ROS in the vasculature of older animals (254). Similarly levels of the antioxidant enzymes like glutathione are reduced in old age as compared with young animals (254).

The relationship between inflammation and endothelial dysfunction explained above is biologically plausible but does not confirm causality. Chronic low grade inflammation promotes cellular and biochemical changes in vessel wall which favour endothelial dysfunction (246). Low grade inflammation also decreases basal NO production promoting increased expression of cell-surface adhesion molecules for leucocytes and platelets, promoting interaction between these cells and the vascular endothelium, and inducing pro-coagulant activity (255). In addition TNF-α (a pro-inflammatory factor) has been shown to reduce the half-life of the mRNA encoding endothelial NO synthase (256). Cumulatively these changes may cause endothelial dysfunction and increase the likelihood of vasospasm, thrombosis and vessel occlusion (255).

1.6.1.1 Inflammation and phenotypic shift of vascular cells in ageing

Low grade chronic inflammation has been suggested as the key to most of the age-related alterations in arterial structure and function such as diffuse intima-medial thickening, increased stiffening and VSMC migration, proliferation and senescence (246). Many characteristics of vascular ageing like endothelial dysfunction, oxidative stress and increased apoptosis can be reproduced by recombinant TNF-α and chronic infusion of Ang II (246;252). These pro-inflammatory molecules increases activity of pro-inflammatory molecules, for example, MMP-2, MCP-1, TGF- β1, NADPH oxidase and calpain-1, affecting the arterial wall cells and matrix and leading to adverse arterial restructuring (246;252). Continuous ACE inhibition, AT1 blockade and/or inhibition of MMPs from an early age delays the progression of age-associated aortic remodelling in animal models, by markedly inhibiting the pro-inflammatory molecules (246).

Age-related vascular changes involve inflammation as an intermediary step. The phenotypic shift in different vascular cells will be discussed in relation to both ageing and inflammation in the following section.

Endothelial cells

Cellular senescence is a condition in which the cell is metabolically active but loses the ability to proliferate. With each cell division the telomere length is shortened until a critical length is exceeded, at which cell signalling is triggered

for the arrest of cell proliferation and start of senescence and apoptosis (257). Telomere dysfunction and endothelial senescence are related to increased ROS, decreased NO, and increased production of pro-inflammatory molecules (258). The senescent endothelial cells impact negatively on neighbouring cells; further enhancing endothelial dysfunction (258).

In vitro, the number of cellular replications is correlated with a decrease in NO synthase and an increase in the number of monocytes adhering to the ECs (257). Ageing also increases the sensitivity of the endothelium to apoptotic stimuli. Oxidized LDL also increase the inflammatory activity more than three times in old cells as compared to young cells (259).

ECs are in direct contact with the blood and carry the components of the pro-inflammatory burden that originates within the circulation. The Ang II, MCP-1, and MFG-E8 inflammatory load is increased in ECs isolated from the vessels of older animals (246;258). This pro-inflammatory state enhances ROS generation, which damages endothelial mitochondrial DNA and also interferes with the mitochondrial life cycle (246-250). All of these mechanisms initiate, and also promote EC senescence and apoptosis (247-250). MMPs break down the ECM ultimately damaging basement membrane and old enlarged ECs are likely to detach from the damaged basement membrane (247-250). The disrupted basement membrane is more likely to recruit and also concentrate the inflammatory factors such as Ang II and MFGE8, which form a local inflammatory focus that disturbs EC (247;248). The pro-inflammation and associated cellular and micro environmental changes lead to endothelial dysfunction and are also the perpetrators of enhanced permeability, infiltration, pro-thrombosis or coagulation within the vessel wall (247;248).

Telomerase transfection (introducing nucleic acids into cells) which stabilises the expression of telomerase in EC, induces a younger EC phenotype with an increase of NO synthase and higher NO activity (257). Ageing and endothelial dysfunction are also associated with a reduction of vascular expression of Sirtuin (SIRT), with lower SIRT1 lead to a reduction in the capacity for vascular repair in the elderly. SIRT1 is a key sensor system for regulating EC survival, proliferation and senescence and may possess beneficial effects against ageing-related diseases (260).

Vascular smooth muscle cells (VSMC)

Old VSMCs (i.e. isolated from older animals) lose their contractile function and instead become stiffened and develop heterogeneous phenotypes within the arterial wall. In addition to change in phenotype VSMC also have changes in other characteristics such as pro-inflammatory secretion, senescence, proliferation, migration, and ECM deposition (247-250).

Senescence and secretion

In arteries from older animals, both proliferative and senescent VSMC subsets coexist. When old VSMC enter an irreversible growth arrest, it is known as cellular senescence (246). Ang II is known to play a role in VSMC senescence through induction of stress induced premature senescence (SIPS) or telomere shortening (261;262). Both SIPS and progressive telomere shortening consequently leads to activation of the DNA damage machinery and p53 enzymes (261-263). Ageing changes the VSMC phenotype from contractile to secretory and VSMC derived from arteries of old non-human primates show increased expression of the age-associated arterial secretory phenotype (AAASP) (247;264). The AAASP in old cells is associated with increased secretion of IL-1ß, IL-6, MCP-1, and TNF-α (264). Similar to the AAASP of old untreated cells, young VSMCs when treated with Ang II, also secrete a large amount of pro-inflammatory factors, including MFG-E8 (247;265). The AAASP likely delivers signals to the neighbouring VSMC (in a paracrine/juxtacrine manner), enhancing the phenotypic shift with ageing (246).

Proliferation

VSMC proliferation increases with age and has been proposed to be due to imbalance of calcium homeostasis or platelet derived growth factor (PDGF) gene over-expression (266). Moreover, old cultured VSMCs have an increased replication rate compared to young cells (267). Old cultured VSMCs have a greater percentage of cells in the S and G2/M phases, and a lower percentage in the G0/G1 phase of the cellular life cycle, compared to young cells (267). MFG-E8 increases in vessels with age and by Ang II, and triggers phosphorylation of ERK1/2 which enhances proliferation signalling in young cultured VSMCs. In

contrast MFG-E8 silencing, or the blockade of ERK1/2 phosphorylation in young cells reduces inflammation and decelerates the cell cycle S phase, conferring a reduction in proliferative capacity (267). Oxidative stress and sympathetic activity increases with age and both might play an important role in modification and proliferation of the muscle (258;268).

Migration/invasion

The migration/invasion of medial VSMC, into the arterial intima is a key cellular event in age-associated diffuse intimal thickening. Old VSMC cease to interact normally with the ECM due to changes in ECM composition or due to change in the expression of integrins (269). In addition, the capacity of invasion of VSMC increases many fold with ageing and has also been demonstrated in cultured old VSMC, via increased activation of MMP-2/-9 (247-250;270). Similarly exposure of cultured young VSMCs to Ang II, MFG-E8, calpain-1, PDGF-bb, or MCP-1 enhances invasive capacity to levels observed in untreated old cells (246;271;272). MFG-E8 silencing RNA considerably reduces the expression of MCP-1, PDGF, and the PDGF receptor and also reduces VSMC invasion capacity (265;267). Collectively MFGE8 inhibition can be used in future as a target to reduce proliferation and invasion of VSMC.

1.6.1.2 Changes in the vascular extracellular matrix (ECM) with ageing

The ECM is a complex mixture of structural proteins and glycoproteins, including collagens, elastins, fibronectins, and proteoglycans. Its main function is to provide and maintain the structural framework which is essential for the functional properties of the vessel wall. The maintenance of three dimensional organization of the ECM contents especially elastin, collagens, proteoglycans and structural glycoproteins are essential for optimal vascular functions (273). In healthy (uninjured) vessels some proteases are constitutively expressed but their activity is controlled by inhibitors and balance is maintained. This balance is lost due to ageing and other vascular pathologies and there is induction of matrix metalloproteinase gene expression, activation of zymogens and secretion of enzymes by inflammatory cells (273). VSMCs have the ability to respond to these injurious stimuli and synthesize ECM (including collagen types I, II and III) and protease inhibitors but the three dimensional organization of the newly

synthesized ECM is never functionally optimal (273-275). In old VSMC, MMP-2-activated TGF-b1 signalling is involved in the increased collagen I, II, and III production (274;275). In contrast some pathological conditions overcome the VSMC response and the quantity of ECM decreases (273).

1.6.2 Role of Inflammation in hypertension

Elevated blood pressure is known to be pro-inflammatory and prothrombotic. There is evidence of upregulation of local and systemic inflammatory mediators including cytokines, tissue factor (TF), components of the renin-angiotensin system (RAS), endothelial adhesion molecules and chemokines (276-278). Attica et al demonstrated that prehypertensive subjects had 31% higher CRP levels than normotensive controls (279), and the same was observed in the Third National Health and Nutrition Examination Survey (NHANES III) (280). In the Framingham offspring study serum CRP levels were higher in non-hypertensive children of hypertensive parents compared with offspring of parents without hypertension (281).

The association of chronic low grade inflammation with HTN is widely documented in experimental and clinical results and inflammatory activation is implicated in the development of the cardiovascular consequences of HTN. However, it is remains unclear whether inflammation is a pathogenetic inducer of HTN or whether HTN precedes the inflammatory events of atherosclerosis (282) .

1.6.3 Role of inflammation in atherosclerosis and cardiovascular disease

Inflammation has been clearly linked with atherosclerosis over the past 2 decades (283;284). Inflammation is involved in initiation, growth and rupture of atherosclerotic plaque regardless of the initial stimulus (285;286). The established risk factors for promoting atherosclerosis are cigarette smoking, hypertension, atherogenic lipoproteins and hyperglycaemia. These give rise to a variety of stimuli that elicit secretion of leukocyte soluble adhesion molecules, which facilitate the attachment of monocytes to ECs as well as chemotactic factors which facilitate migration of monocytes into the subintimal space. These monocytes are transformed into macrophages which take up lipoproteins to form

fatty streaks. Additional injurious stimuli may continue the attraction and accumulation of macrophages, activated T cells and mast cells within the growing atherosclerotic lesion. Oxidized low-density lipoproteins (LDL) and many other factors contribute to loss of smooth muscle cells through apoptosis in the atherosclerotic plaque cap. Activated macrophages also secrete metalloproteinases and other connective tissue enzymes which may break down collagen, weakening the cap and making it prone to rupture. The disruption of the atherosclerotic plaque exposes the plaque core to arterial blood and induces thrombosis. In summary, nearly all the steps in atherogenesis are believed to involve cytokines, other bioactive molecules, and cells that are characteristic of inflammation (287).

The arterial ageing process is connected with hypertension and atherosclerosis at the molecular and cellular levels because all the three process exhibit the same structural and functional characteristics (246). The similarities between ageing, hypertension and atherosclerosis in relation to inflammation are shown in Table 1.2 and the common pathway is shown in Figure 1.3.

Table 1.2 Molecular and cellular remodelling in Ageing, hypertension, atherosclerosis and Angiotensin II signalling

		Ageing >56	HTN	Ather	Ang II signalling
Inflammatory molecules	Local Ang II	↑	↑	↑	↑
	MMPs	↑	↑	↑	↑
	Calpain-1	↑	↑	↑	↑
	MCP-1/CCR2	↑	↑	↑	↑
	TGF-β1	↑	↑	↑	↑
	NADPH oxidase	↑	↑	?	↑
	NO bioavailability	↓	↓	↓	↓
	TNF-α	↑	↑	↑	↑
	ICAM	↑	↑	↑	↑
	MFG-E8	↑	↑	↑	↑
	PDGF	↑	↑	↑	↑
Cellular matrix structure and function	EC dysfunction	↑	↑	↑	↑
	Diffuse IMT	↑	↑	↑	↑
	Stiffness	↑	↑	↑	↑
	Matrix	↑	↑	↑	↑
	Calcification	↑	↑	↑	↑
	FN/Collagen	↑	↑	?	↑
	VSMC migration	↑	↑	↑	↑
	VSMC proliferation	↑	↑	↑	↑
	Hypertension prevalence	↑	↑	?	↑
	Atherosclerosis prevalence	↑	?	↑	↑

Symbols and abbreviations: ↑= increase, ↓= decrease, Ang II= Angiotensin II, Ather= atherosclerosis, CCR2= C-C chemokine receptor type 2, EC= endothelial cell, FN= fibronectin, HTN= hypertension, ICAM= intercellular adhesion molecule; IMT= intima-media thickening, MCP-1= monocyte chemo-attractant protein-1, MFG-E8= milk fat globule epidermal growth factor-8, MMPs= matrix metalloproteases, NO= nitric oxide, PDGF= platelet-derived growth factor, TGF-b1= transforming growth factor b1, TNF-α = tumor necrosis factor α, VSMC= vascular smooth muscle cell.
Adapted with permission from Wang M 2014 (246)

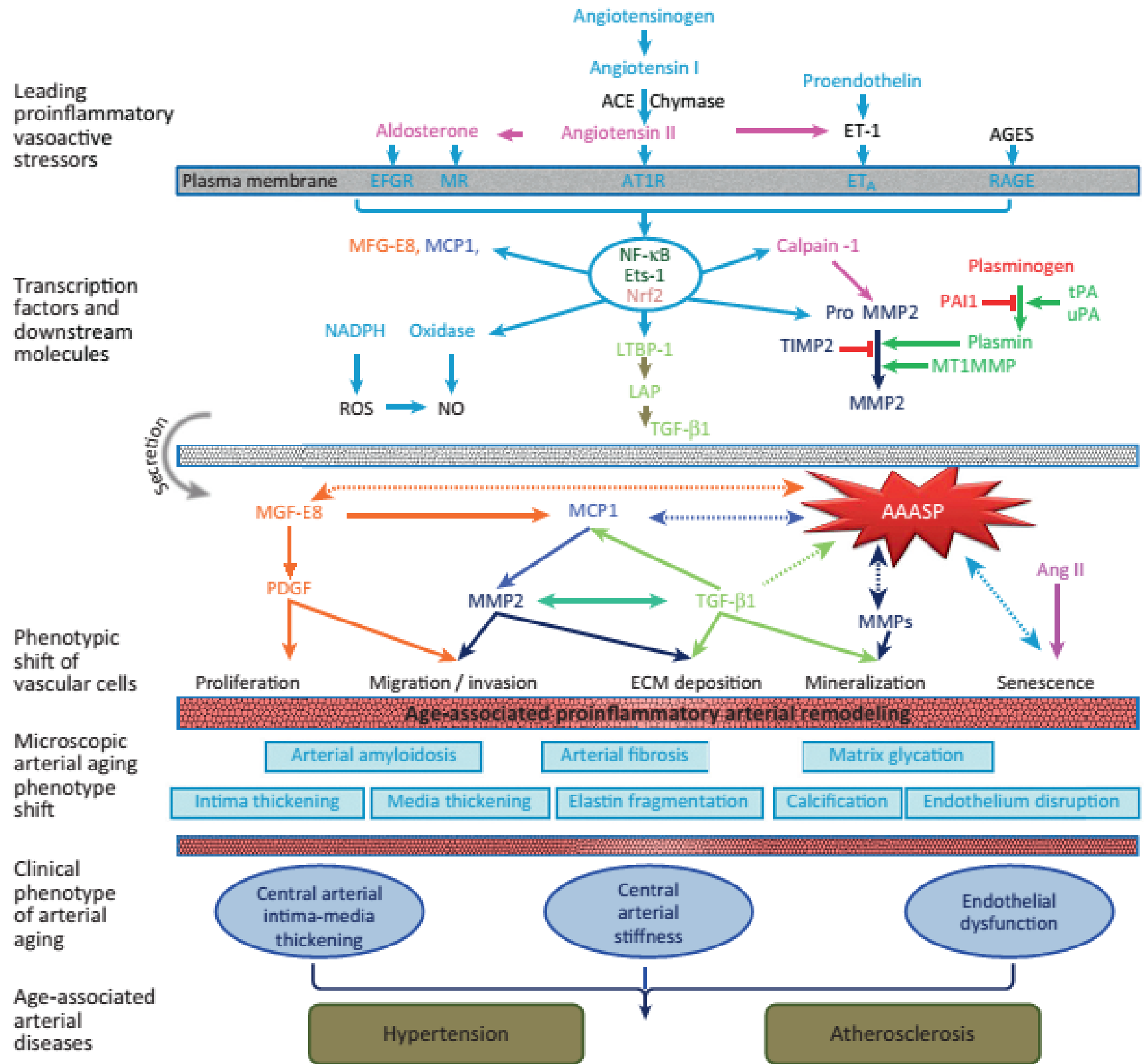


Figure 1.3 Age associated pro-inflammatory arterial remodelling and development of hypertension and atherosclerosis.
Reproduced with permission from Wang et al. 2014 (246).
Abbreviations: AAASP= age-associated arterial secretory phenotype, ACE = angiotensin converting enzyme, Ang II= angiotensin II, AT1R= angiotensin II type 1 receptor, AGE= advanced glycation end products, ECM= extracellular matrix, ET-1= endothelin-1, ETA= endothelin-1 receptor A, Ets-1= v-ets erythroblastosis virus E26 oncogene homolog 1, LAP= latency-associated peptide, LTBP-1= latent transforming growth factor (TGF)-binding protein-1 (LTBP-1), MCP-1= monocyte chemo attractant protein-1, MFG-E8= milk fat globule epidermal growth factor-8, MMP= matrix metalloprotease, MR= aldosterone/mineralocorticoid receptor, NF- kB= nuclear factor k light-chain-enhancer of activated B cells, Nrf-2= NF-E2-related factor 2, NO= nitric oxide, PAI= plasminogen activator inhibitor, PDGF= platelet-derived growth factor, RAGE= receptor for AGE, ROS= reactive oxygen species, TGF- b1= transforming growth factor b1, t-PA/u-PA= tissue-type/plasminogen-type plasminogen activators and VMSC= vascular smooth muscle cell

1.6.4 Hypertension and immune system

This thesis does not contain direct measurements of the immune system in relation to blood pressure and hypertension. However, it contains two chapters which focus on biomarkers of inflammation. The role of immunity in relation to hypertension is therefore briefly described below.

The role of the immune-inflammatory component in the pathogenesis of hypertension is underappreciated. Innate and adaptive immune cell infiltration is continuously found in the kidney, vessel wall and perivascular adipose tissue (PVAT), along with the more conventional inflammation and ROS in hypertensives (288-290). T effector lymphocytes appear to play a key role in the development of hypertension (289) and also exaggerate the inflammatory response by their interaction with innate cells (288). In contrast, T regulatory lymphocytes (Tregs) limit the innate and adaptive immune responses and neutralise the elevation of BP and associated kidney and vascular damage (288).

1.6.4.1 T-lymphocyte subsets

T lymphocytes are characterised by the presence of the T-cell receptor (TCR) complex containing two TCR chains (α and ß), a CD3 co-receptor and a ζ-chain accessory molecule. During development in the lymphoid tissues, CD3+ T lymphocytes mature into the active forms: CD4+ or CD8+ cells. These immunocompetent T cells require two signals for activation: 1) recognition of an antigenic peptide (via TCR) presented by antigen-presenting cells (APC) via their major histocompatibility complex (MHC) class II molecules, 2) generation of a co-stimulatory signal, that is the interaction between B7 ligands (CD80 and CD86) on APC with the T-cell co-receptor CD28 (291). Upon activation, the naive CD4+ T helper (Th) cells differentiate into Th1, Th2 and Th17 effector cells, each producing its own panel of cytokines which then mediate separate functions (292). Th1 cells secrete interferon (IFN)-γ, interleukin 2(IL-2), and tumor necrosis factor (TNF)-ß and play roles in cell-mediated defence against intracellular microorganisms as well as involvement in hypertension. Th2 cells produce IL-4, IL-5, IL-10, and IL-13, which assist in B-cell activation and also suppress cell-mediated immunity. Th17 cells secrete IL-17 and IL-22 and participate in defence against extracellular bacteria and fungi. CD4+ cells can also differentiate into T regulatory lymphocytes (Tregs) which regulate and can suppress innate and adaptive responses to autoantigens, alloantigens, tumor antigens, and infectious agents (292). Tregs have an important role in the maintenance of immunologic self-tolerance, immune homeostasis and anti-inflammatory effects by producing IL-10 (292-294). The body response in production of Th1, Th17 or Th2 and Treg depend on the stimulus, environment and other cytokines. For example Treg and Th17 are derived from the same

precursor but their quantity within the cells depends upon the amount of IL-6 (295). CD8+ T effectors differentiate into cytotoxic (Tc) cells that secrete perforin, granzyme B, IFN-γ, and TNF-α and may play a role in hypertension (293).

1.6.4.2 T-effector lymphocytes in hypertension

Guzik et al. exposed C57BL/6 mice lacking recombination activating gene-1 (Rag1−/−), which are deficient in mature T and B cells (296), to Ang II infusion or desoxycorticosterone acetate (DOCA)-salt hypertension. These mice exhibited protection from the development of hypertension and vascular oxidative stress. Moreover, adoptive transfer of T cells (but not of B cells) in these mice restored the hypertensive phenotype. This restoration of hypertension was also dependent upon Ang II type 1a receptors (AT1aR), suggesting that T-cell AT1aR activation and NADPH oxidase-dependent ROS formation also play important roles in hypertension (296). The Rag1−/− mice also showed blunted adventitial collagen deposition and aortic stiffening in response to Ang II; this was restored on adoptive transfer of T-cells (297). Interestingly, this vascular remodelling was not achieved through adoptive transfer of CD4+ or CD8+ T-cells alone, indicating that a combination of both CD4+ and CD8+ T-cells was required (297). In contrast, immunosuppressive therapy has been shown to prevent BP elevation in experimental models of hypertension. For example, mycophenolate mofetil, a compound which depletes B and T cells, protects against hypertension and development of renal disease. Activated T cells increase TNF-α and treatment with the TNF-α antagonist, etanercept prevents Ang II-induced BP and increased in ROS (298). Crowley et al. used severe combined immunodeficiency (SCID) mice which also lack lymphocyte immune responses (299). On Ang II infusion these mice did not develop hypertension, cardiac hypertrophy or renal injury. These protective effects may be due to enhanced production of NO, prostaglandin E2 and prostacyclin via stimulation of eNOS and COX-2 dependent pathways (299). Senchenkova et al. studied Ang II induced arteriolar thrombosis in cremaster arterioles of different mice models. The thrombosis response was found to be greater in wild-type mice compared with Rag1−/−, CD4+ T-cell- or Nox2 (gp91phox)-deficient (Cybb−/−) mice, whereas CD8+ T-cell-deficient mice exhibited an intermediate phenotype (300). Moreover, adoptive transfer of wild-type or Cybb−/− T cells into Rag1−/− restored the pro-thrombic effects of Ang II.

This suggests greater contribution of CD4+ T cells compared to CD8+ T lymphocytes (300). The respective roles and amount of contribution by $CD4^+$ and $CD8^+$ T lymphocytes in hypertension are still under investigation and relevance in humans may be demonstrated by studies in pre-eclampsia (301;302). Recently Youn et al. (303) showed that T-lymphocytes from patients with hypertension had increased immunosenescent pro-inflammatory cytotoxic CD8 + T-cells, which were associated with loss of CD28 and the presence of CD57. The loss of CD28 occurs in ageing associated hypertension. These T-cells also secreted the pro-inflammatory and BP increasing factors: perforins, granzyme B, IFN-γ and TNFα. Moreover, circulating levels of C-X-C chemokine receptor type 3 chemokines were also found to be higher in hypertensive patients (303), suggesting a role of T-lymphocyte-dependent inflammation in human hypertension.

1.6.4.3 Alteration of Th1/Th2 balance in hypertension

Ang II infusion is associated with a shift of T cells balance towards a pro-inflammatory state with an increase in Th1 and decrease in Th2-mediated responses (304;305). As outlined above, the Th1 response is characterised by increased production of IFN-γ (304;305), while increase in IL-4 production is a hallmark of the Th2-mediated response (304). The effects of Ang II can be blocked by AT1aR antagonists independently of haemodynamic responses to Ang II (304). Lozovoy et al. (306) recently compared Th1/Th2 ratio in patients with SLE (active and non-active) with controls. They showed that Th1/Th2 ratio exhibited by IL-12/IL-4 ratio, IL-12/IL-10 ratio, IFN-γ/IL-10 and IFN-γ/IL-4 ratio was raised in patients with active SLE compared to non-active SLE or controls. Patients with a higher Th1/Th2 ratio had a higher probability of developing hypertension (306). However this may not apply directly to people with connective tissue diseases.

1.6.4.4 Role of Th17 cells in hypertension

Th17 cells mainly secrete IL-17 which, as outlined above, are associated with Ang II induced hypertension, inflammation and vascular dysfunction. Madhur et al. (307) demonstrated that IL17a KO mice (IL-17a−/−) receiving chronic infusion of Ang II exhibited a blunted BP response, preserved vascular function, decreased ROS production, and decreased aortic T-cell infiltration. Moreover,

these IL-17a−/− mice were protected against aortic collagen deposition and vascular stiffening (297;307). Nguyen et al. also showed that giving IL-17 infusion to C57BL/6 mice significantly increased SBP and decreased aortic NO-dependent relaxation (308). Similarly Amador et al. showed that treatment of DOCA-salt hypertensive rats with an anti-IL-17 antibody reduced arterial hypertension, expression of profibrotic and pro-inflammatory mediators as well as collagen deposits in the heart and kidney (309). These effects of IL-17 are thought to be due to activation of RhoA/Rho-kinase and also lead to endothelial dysfunction (309). Reduced uterine perfusion pressure (RUPP) rats (a model of pre-eclampsia) have also been shown to exhibit lower levels of Tregs and higher levels of Th17 cells (302). The adoptive transfer of CD4+ T cells from pregnant RUPP rats, into normal pregnant rats induced a significant increase in BP and inflammatory markers. Furthermore administration of IL-17 soluble receptor C in RUPP rats reduced circulating Th17 cells along with decrease in ROS and hypertension (301).

1.6.4.5 Role of the co-stimulation in activation of T effector lymphocytes

Hypertension is associated with activation of T effector lymphocytes suggesting a role of APCs in antigen presentation. Ang II induced hypertension is also associated with increased expression of activated (CD86+) dendritic cells in secondary lymphatic tissues. Vinh et al. demonstrated that preventing T-cell co-stimulation either pharmacologically, using a CTLA4-Ig (which blocks CD28 interactions with B7 ligands), or by genetic deletion of B7 ligands in mice prevented Ang II induced hypertension, inflammation, ROS increase and T cell activation (310). The CTLA4-Ig also reversed the Ang II and DOCA-salt induced hypertension (310).

1.6.4.6 Role of the central nervous system (CNS)

The CNS may influence the pathophysiology of hypertension by modulating innate and adaptive immune responses. Ang II administration into the lateral cerebral ventricles was associated with increased expression of pro-inflammatory splenic cytokines, such as IL-1β and IL-6 (311). Furthermore these responses were abolished by splenic sympathetic denervation, suggesting involvement of ANS in this peripheral response. Similarly, lesions anterior and

ventral to the third ventricle in mice are known to disrupt signals from the subfornical organ to the hypothalamus (312). This intervention prevents most forms of experimental hypertension as well as blunting T-cell activation and vascular infiltration of leucocytes (312). Cre/Lox-mediated deletion of extracellular SOD in the circumventricular organs of mice increases oxidative stress and elevated sympathetic outflow. This leads to a slight increase in baseline BP and exaggerates hypertension induced by low dose Ang II (313). Similarly T-cell activation was increased in SOD3 KO mice infused with a low dose of Ang II (289). In contrast, intracerebroventricular injections of an adenovirus encoding for cytoplasmic SOD reported a blunting of Ang II-induced hypertension in C57Bl/6 mice (314).

1.6.4.7 T regulatory (Treg) lymphocytes in hypertension

The Treg lymphocytes are involved in regulating innate and passive immunity. Barhoumi et al. and showed that adoptive transfer of Tregs in C57Bl/6 mice blunted Ang II-induced hypertension, endothelial dysfunction, circulating pro-inflammatory cytokines, vascular oxidative stress and stiffness, aortic macrophage and T-cell infiltration (315;316). Similarly Kasal et al. suggested protective effects of Tregs adoptive transfer in a model of aldosterone-induced hypertension (317). Kavakan et al. further showed that the preventive effects of adoptive transfer of Tregs on Ang II induced cardiac hypertrophy and fibrosis, TNF-α expression and immune cell infiltration were independent of BP lowering (318). Matrougui et al. also reported similar findings i.e. reduction in BP elevation, macrophage activation and infiltration into coronary arterioles and the heart, local TNF-α release, and coronary arteriolar endothelial dysfunction when C57Bl/6 mice received intraperitoneal injections of Tregs (316).

Most Treg effects are mediated by production of IL-10 which decreases inflammation and oxidative stress in the development of hypertension. The protective role of IL10 is illustrated by exacerbation of Ang II-induced endothelial dysfunction and hypertension in IL 10 KO mice (IL 10−/−) (319). In addition, transfer of Tregs (isolated from control mice) into hypertensive IL 10−/− mice reduced SBP and NADPH oxidase activity along with improvement of endothelium-dependent relaxation in resistance arteries (320).

In conclusion, T effector and T regulator subsets of lymphocytes play an opposite role in hypertension. Moerover, any stimulus causing CNS stimulation can increase sympathetic outflow resulting in mild BP elevation (in prehypertension range). This modest pressure elevation brings about an inflammatory response, likely by generating neoantigens that activate T cells (289). This inflammatory response also leads to entry of effector-like T cells into the perivascular fat and the kidney; cause tissue injury (289) and formation of damage-associated molecular patterns (DAMPs) (288;321). Activation of innate APCs by DAMPs, or by pathogen-associated molecular patterns (PAMPs) generated in response to low-grade infection (321), and direct stimulation by CNS, may be the cause of activation of CD4+ and CD8+cells which differentiate towards pro-inflammatory Th1/Th17 phenotypes (304). The Th1 and Th17 effector lymphocytes produce pro-inflammatory mediators, including ROS, IFN-γ, TNF-α, and IL-17, to promote low-grade inflammation, contributing to the progression of hypertension (296;304;305;307;308). Tregs on the other hand, counteract progression of hypertension by suppressing innate and adaptive immune responses, perhaps by secreting IL-10 (315-320;322).

1.7 Obesity

Obesity or “adiposity” is the excessive deposition of fat in adipose and other tissues of the body. The main cause of adiposity is imbalance between energy intake and output, i.e. calorie intake exceeds calorie expenditure with the excess stored as “fats”. Adipocytes are present throughout the body: their main function is to store energy in the form of fat (neutral triglycerides), along with providing insulation and mechanical support. When needed, fats provide the energy for most body organs, including liver, muscle and heart. Adipose tissue not merely works as an energy store but is also an important component of metabolic control as an endocrine organ. Due to its size, adipose tissue can be considered one of the largest endocrine organs. Obesity is associated with an increase in both adipocytes number and size (323).

1.7.1 Normal adipose tissue function

Normal adipose tissue contains mature adipocytes. Other cell types present include pericytes, ECs, monocytes, macrophages, pluripotent stem cells

(including preadipocytes) and fibroblasts. When food is scarce or energy requirement increases, lipid reserves are released for providing energy. Adipocytes contain enzymes “lipases” which breakdown stored triglycerides into glycerol and free fatty acids (FFA) and release them into the blood, from where FFAs are transported to organs including liver and muscle for oxidation and generation of energy. Glycerol and FFAs can be re-esterified in adipocytes so their level is closely regulated. Adipocytes also respond to hormonal (insulin) and sympathetic stimulation. As an endocrine organ, adipose tissue secretes several hormones and cytokines commonly called adipokines or adipocytokines. These adipokines have effects on multiple biological systems, including energy homeostasis (lipid and carbohydrate metabolism, thermogenesis, appetite), the immune system, blood pressure, angiogenesis and reproductive function (323). The role of adipose tissue in glucose homeostasis has been demonstrated in adipose specific GLUT4 knockout mice in which disruption of insulin-stimulated glucose uptake caused peripheral insulin resistance and glucose intolerance even without alteration in adipose tissue mass (324). In non-obese physiology, cytokines make surplus fuel readily available for use by activated immune cells during infection and/or inflammation. The cytokines, adiponectin and leptin promote insulin-stimulated lipogenesis leading to triglyceride accumulation and adipose expansibility. They also have insulin sensitising actions and promote fuel oxidation in muscle and protect non-adipose tissue from accumulating lipids (325). Leptin is involved in regulation of appetite, resting metabolism and fertility (326). Other important adipokines are TNF-α and IL-6 in inflammation, plasminogen activator inhibitor-1 in coagulation (325). Adipose tissue also produces angiotensinogen (AGT) and angiotensin-converting enzyme (ACE) under physiological conditions (327).

1.7.2 Obesity and adipose tissue dysfunction

With positive energy balance, extra energy is stored in adipose tissue with development of new adipocytes and growth of mature adipocytes. The genetic profile of an individual determines the storage capacity of adipose tissue; when the storage capacity fails to cope with excessive demand then adipose tissue becomes dysfunctional. Adipose tissue dysfunction is associated with overweight and obesity and also alters adipokine production with increased production of

leptin, TNF-α and IL-6 but decreased adiponectin. It is characterized by hypertrophied adipocytes and infiltration by macrophages (325).

In obesity, chronic increase in pro-inflammatory adipokines impairs whole body energy balance with loss of the normal response to fluctuations in nutritional status (328). Increased secretion of TNF-α impairs insulin sensitivity, inhibits adipocyte differentiation, promotes lipid mobilization in mature adipocytes and alters production of other adipokines (329). This limits adipose tissue lipid storage capacity and contributes to obesity associated hyperlipidemia and lipotoxicity in other organs such as muscles, liver and ß-cells (329). Reduction in leptin and adiponectin and their decreased activity contributes to impaired adipose tissue expansion while accumulating lipids in non-adipose tissue (323). Decrease in adiponectin also adds to insulin resistance as adiponectin receptors on liver and muscle cells mediate ß-oxidation of fatty acids, glucose uptake, gluconeogenesis and peroxisome proliferator activated receptor-γ activation (325;330).

1.7.3 Obesity related hypertension

The Framingham heart study implicated obesity as a contributory factor in 60%-70% of essential hypertension (331). Obese individuals have a 3.5-fold increase in the likelihood of developing hypertension (332). Increased risk starts from a young age and even obesity acquired during childhood is a predictor of hypertension in adulthood (333;334). Severely obese but normotensive children were also reported to have dilation (without arterial wall hypertrophy) and increased stiffness of the carotid artery (335). Similarly Rocchini et al. demonstrated decreased maximal blood flow and increased structural vascular resistance in the fore arm of obese adolescents but these changes were partially reversed with weight loss (336). Wildman et al. evaluated the age-related association between obesity and arterial PWV. They clearly showed that being overweight or obese even in young age, around 30, is significantly associated with increased arterial PWV. However this association of PWV with obesity appeared to plateau after 60 years of age with obesity not being associated with any further increases in vascular stiffness in elderly patients (337). This may suggest a reduced input of obesity on PWV with age, but could also suggest a “ceiling” effect due to limitations of the technique.

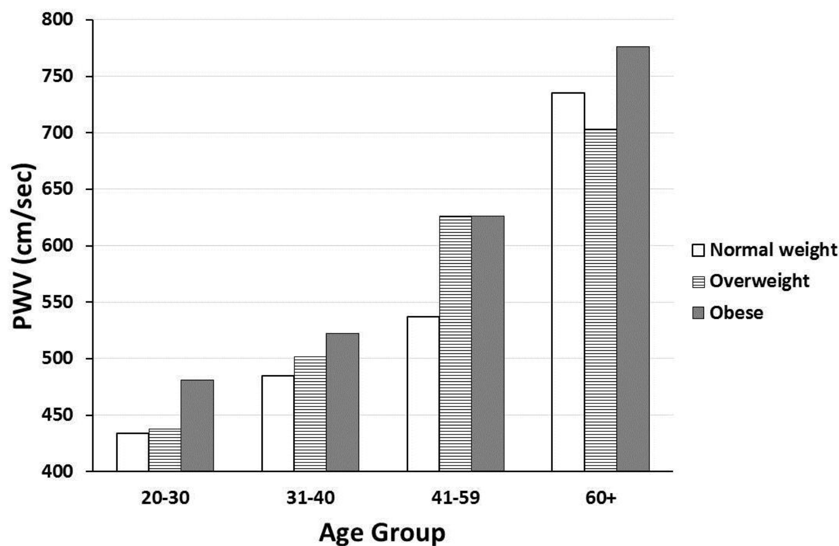


Figure 1.4 Mean PWV by BMI categories.
Modified from Wildman et al. 2003) (337)
Normal weight (BMI <25), overweight (25> BMI <30) and obese (BMI> 30) subjects. Mean values were adjusted for age, sex, SBP and race.

Gosmanov et al. showed the effects of fat load (both oral and intravenous) in normotensive but obese individuals (338). They suggested that both bolus oral ingestion and/or the intravenous infusion of fat resulted in a significant rise in SBP, attenuated endothelial function (assessed by flow mediated dilatation), increased oxidative stress and also activated the sympathetic nervous system (338). Moreover 10 weeks of high fat diet in obesity-prone rats was associated with hypertrophy of the aorta, accompanied by elevated plasma renin activity, glomerulosclerosis and the development of hypertension (339). Similarly high salt diet in obese rats (diet induced) accelerated the development of hypertension along with a significant increase in superoxide levels within aortic rings (340). These studies show that obese people are highly prone to development of hypertension and any additional load of fat diet, salt intake or other stress may exhibit the clinical picture of hypertension.

1.7.4 Mechanism of obesity related disorders (inflammation, insulin resistance and hypertension)

The cellular mechanisms linking obesity to disorders like insulin resistance, inflammation, and hypertension are explained below.

1.7.4.1 Normal intracellular Signalling

Insulin stimulates the uptake of glucose in skeletal muscles and adipose tissue through stimulation of phosphatidylinositol (PI3) kinase dependent signalling pathways. This pathway involves the insulin receptor, insulin receptor substrate 1 (IRS-1), PI3- kinase, phosphoinositide-dependent kinase 1 (PDK- 1), and protein kinase B (Akt) (341). The vasodilator action of insulin is classically thought to operate via PI3-kinase dependent stimulation of Akt which directly increases endothelial NO synthase (eNOS) activity, leading to increase in NO production (341;342) (Figure 1.5). Insulin also has vasoconstrictor effects which are mainly mediated by the vasoconstrictor peptide endothelin-1 (ET-1) (341). ET-1 is produced within the vascular endothelium through stimulation of the intracellular MAP-kinase signalling pathway (343). Thus insulin has both vasodilator and vasoconstrictor effects within the endothelium which counterbalance each other. In normal homeostasis state, the net result is either neutral or vasodilatation.

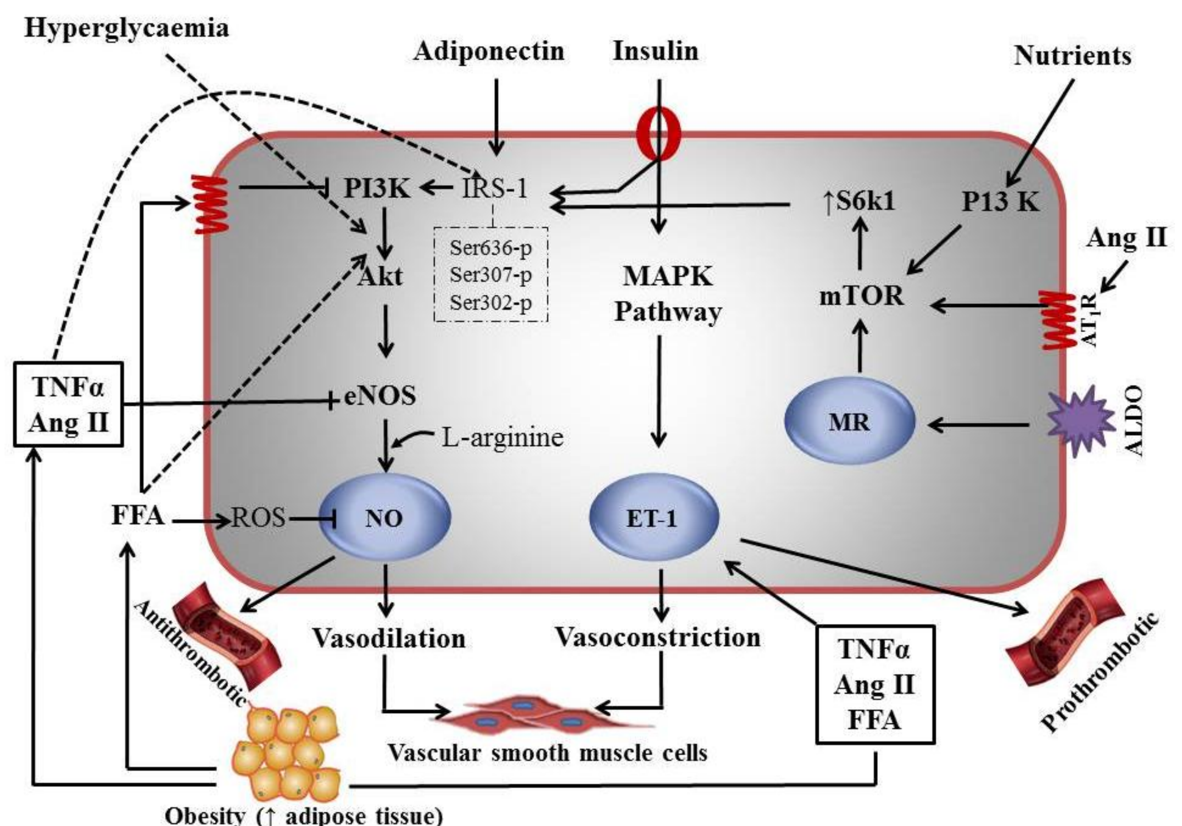


Figure 1.5 Mechanisms of insulin-mediated nitric oxide and endothelin 1 production in endothelial cells.
Modified from Jonk et al. 2007 (344).
ALDO= aldosterone, Ang II= angiotensin II, PI3K= phosphoinositide 3-kinase, S6k1= Ribosomal protein S6 kinase beta-1, MAPK= Mitogen-activated protein kinases, mTOR= mammalian target of rapamycin, MR= mineralocorticoid receptor, ET-1= endothelin 1, Akt=

Protein kinase B, eNOS= endothelila nitric oxide synthase, TNF-α= Tumour necrosis factor alpha, IRS1= Insulin receptor substrate 1, Ser= serine

1.7.4.2 Possible mechanisms for obesity associated micro vascular dysfunction

Elevation of circulating free fatty acid levels in obesity (secondary to insulin resistance and increased lipolysis) induces serine phosphorylation of IRS-1 which interferes with the normal insulin-receptor mediated phosphorylation of IRS-1, thus impairing activation of PI3-kinase (345). Obesity thus disturbs normal intracellular signalling through multiple mechanisms resulting in obesity associated microvascular dysfunction. For example, there is increased production of reactive oxygen species (ROS) which decreases the bioavailability of NO via reduced NO production and direct inactivation of NO by superoxide (O_2^-) (346;347) (see Figures 1.5 and 1.6). Obesity also leads to reduced expression and activity of eNOS in muscle and kidney (348-352), resulting in blunted NO production. Lastly, the intracellular insulin signalling transduction pathway is also impaired (353). In contrast the insulin-mediated vasoconstrictor pathway remains intact or only selectively impaired in obesity. Thus, there is an imbalance between NO and ET-1 production, shifting the vascular reaction from vasodilatation towards vasoconstriction (344). As a consequence of these cellular defects insulin-mediated endothelium-derived vasodilatation is blunted in obesity.

These effects have been demonstrated in human studies in which obese, hypertensive individuals exhibited insulin-induced vasoconstriction and increased ET-1 dependent vasoconstrictor tone as well as decreased NO-dependent vasodilator tone (354;355). This microvascular dysfunction may contribute to obesity-associated insulin resistance and hypertension. Changes in vascular stiffness, calcification, mitochondrial function, cytokine and inflammatory system activation are explained in detail in the relevant sections (see Sections 1.2, 1.3 and 1.6).

As well as an increased production of free fatty acids (FFA) (356), obese individuals also exhibit increased circulating levels of leptin, resistin, TNF-α, IL-6 and angiotensinogen (357-359). It has been shown that FFA and TNF-α elevation impair insulin sensitivity and increase blood pressure (360). Along with that the

production of adiponectin, an anti-inflammatory adipokine, is reduced (361). All these are discussed as follows

1.7.4.3 Free fatty acids (FFA)

Fatty acids exposure in humans leads to endothelial dysfunction exhibited as reduction in endothelium dependent vasodilatation (362;363). De Jongh et al demonstrated the effect of FFA elevation (by intravenous lipid plus heparin infusion) in lean subjects. It resulted in impairment of basal and insulin-induced skin capillary recruitment and endothelium-dependent vasodilatation along with reduced glucose uptake (360). It was also shown that lowering FFA in obese women leads to improvement in basal and insulin mediated skin capillary recruitment and also increased glucose uptake (360).

The mechanisms by which circulating FFAs impair basal and insulin-mediated effects on micro vascular function are not completely understood. However it has been shown that elevation of FFA interferes with insulin-induced activation of PI3-kinase in human muscle (345;353;364) and in cultured cells (365;366) (Figure 1.5). In addition FFA elevation increases ROS production (367) and increases the release of vasoconstrictor ET-1, both of which cause endothelial dysfunction (356).

1.7.4.4 Tumour necrosis factor-α (TNF-α)

Obesity increases the release of TNF-α which then impairs the balance between endothelial-derived vasodilator and vasoconstrictor substances. It decreases expression of eNOS (352;368), increases expression of ET-1(73) and inhibits IRS-1 phosphorylation (359;369) in human ECs. In vascular smooth muscle cells and also endothelium it directly activates NADPH oxidase and increase ROS production (370;371) (Figure 1.5). Besides these direct effects, TNF-α also stimulates lipolysis and increases FFA in plasma. *In vivo* rat studies show that acute administration of TNF-α inhibits insulin mediated increase in femoral blood flow and muscle capillary recruitment thus potentially contributing to insulin resistance (372). In humans, weight loss has been shown to result in a significant improvement of endothelial function and was closely correlated with a reduction in TNF-α (373).

1.7.4.5 Leptin

The hormone leptin is released from adipocytes; its concentration rises with increasing percentage of body fat (358). Increased leptin levels in obesity have been shown to increase ROS production in endothelial cells (358). More detail of leptin related vascular dysfunction is discussed in detail in Section: 1.7.6

1.7.4.6 Adiponectin

Adiponectin is also released from adipocytes; its concentration is inversely related to body fat (361). Adiponectin activates tyrosine phosphorylation of IRS-1 and other molecules in the insulin signalling cascade, enhancing glucose uptake and endothelium-dependent relaxation (374). The role of adiponectin in obesity is explained in detail in the Section: 1.7.6

1.7.4.7 The renin-angiotensin-aldosterone system (RAAS)

The involvement of the kidneys in obesity-related hypertension is highlighted by three main factors, including increased activity of the renal SNS, activation of the RAAS and deposition of intrarenal fat causing physical compression of the kidneys and ECM modifications (375). In addition to the conventional circulating RAAS, RAAS components have been detected in tissues such as heart, brain, kidney, vasculature, immune cells and adipose tissue (376-381). Obesity causes increased activation of the RAAS, both systemically and within adipose tissue; the latter can generate Ang II (342;382).

Most of the effects of RAAS occur via production and activation of Ang II which signals through G protein coupled membrane-bound type 1 and type 2 receptors (AT1R and AT2R) (383). At the cellular level Ang II stimulates phosphorylation of IRS-1 (211), which interferes with the normal insulin-dependent activation of PI3-kinase, resulting in decreased glucose uptake and NO synthesis (211). Ang II is a well-known stimulant of reactive oxygen species (ROS) production which decreases cellular level of NO (384-387) and this ROS production was reduced by ARB (388;389). Ang II also stimulates the production of ET-1 in the endothelium (390;391) and causes the release of other inflammatory cytokines like TNF-α (392;393).

In normal weight healthy people, infusion of Ang II causes a redirection of blood flow between different vascular beds. This redistribution, increases total muscle blood flow and capillary recruitment, which as a result increases insulin induced glucose uptake (394;395). In obese people, it has been suggested that the RAAS seems to have opposite effects and decreases insulin induced glucose uptake, and also contributes to obesity associated hypertension (396;397). In animal models Ang II induced hypertension was associated with endothelial dysfunction (384) and chronic administration of Ang II caused insulin resistance in muscle and adipose tissue (385;386); conversely blocking the RAAS decreased insulin resistance in muscle of diabetic mice (389).

In humans it has been shown that Ang II subtype 1 (AT1) receptor blockers (ARB) and angiotensin-converting enzyme (ACE) inhibitors decrease the risk of new-onset diabetes mellitus in hypertensive patients by about 25% (398) and enhance blood flow in peripheral tissues such as skeletal muscle (399;400). A study in humans demonstrated that a FFA induced impairment in the endothelial function was completely prevented by a single dose of either an ARB or an ACE, which suggests RAS involvement in FFA induced endothelial dysfunction (363). In contrast to these effects, a reduction in body weight reduced RAS activity in both plasma and adipose tissue and was associated with a decrease in BP (397;401).

Obesity and RAAS modulation of skeletal muscle microvasculature

The RAAS has been ascribed both beneficial and deleterious effects but in overweight, obesity and T2DM there is inappropriate activation of the RAAS, which plays an important role in the modulation of the skeletal muscle vasculature by promoting fibrosis, remodelling, proliferation, migration, and hypertrophy (206;211;402-404).

Within the vessel wall, AT1R activation increases oxidative stress and promotes vasoconstriction and remodelling (405). In the EC, AT1R activation leads to increased activity of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, enhanced production of ROS, and uncoupling of eNOS (383). eNOS uncoupling in turn decreases bioavailable NO (403). Moreover, in EC Ang II also interferes with insulin-stimulated eNOS activation (via decreased Ser1177

phosphorylation) and consequently diminishes NO production via the mammalian target of rapamycin/p70S6 kinase 1 pathway (mTOR/S6K1) (406). AT1R signalling has been shown to impair endothelial-mediated vasorelaxation (211;220), insulin sensitivity and glucose uptake in cultured myotubules (407). The increase in ROS due to ATR1 signalling promotes the serine phosphorylation of IRS and reduces insulin metabolic signalling. Impaired insulin signalling in turn attenuates activation of eNOS, increases destruction of NO, and increases intracellular calcium and calcium sensitization in VSMC (206;211;402)

In contrast, AT2R activation antagonises the deleterious effects of AT1R signalling by causing vasodilation (408). The vasodilation by AT2R signalling results from activation of the bradykinin and NO system (409). In insulin resistance and diabetes this AT2R mediated dilatation is reduced due to increased ROS. However, treatment with an AT1R blocker in hypertensive diabetic persons for 1 year resulted in increased AT2R expression and enhanced vasodilatory response (410). Similarly, weight loss due to caloric restriction and exercise in overweight and obese adults also improved endothelial function, NO availability, and vascular dilatation (411;412).

ATR1 blockers (irbesartan) were also associated with improvement in endothelial function, inflammation, oxidative stress and flow mediated dilatation in people with metabolic syndrome (407). Similarly AT1R blockade with losartan resulted in increased blood flow and glucose extraction and these effects were abolished by the NO inhibitor NG-nitro- l-arginine methyl ester (l-NAME). In contrast, AT2R blockade decreased microvascular blood flow by 80%, along with a decrease in glucose extraction. (413). AT2R antagonism was also associated with development of whole body insulin resistance and attenuation of microvasculature recruitment. All of these ATR2 blockade related changes were paralleled by a decrease in plasma NO and skeletal muscle eNOS activation (414).

Plasma aldosterone levels are also elevated in the setting of IR and obesity (404) and impair insulin sensitivity in healthy humans (415). Aldosterone reduces IRS-1 levels in VSMCs (416) and also increases the proteasomal degradation of IRS-1 by a ROS mediated mechanism in vessels (417) leading to insulin resistance (through PI3K–Akt pathway). In contrast MR blockade and use of antioxidants and Src

inhibition reduces vascular dysfunction (418). Tissue effects of aldosterone are also important and are discussed below

Role of tissue RAAS in the development of endothelial dysfunction and arterial stiffness

Inappropriate activation of RAAS is now acknowledged as an important determinant of endothelial dysfunction, arterial stiffness, and progression to CVD and CKD (56;59;376;419;420). The role and significance of local RAAS is not completely understood but increased expression of RAAS has been detected in vascular tissues in animal models of obesity (421;422), and its expression in vascular tissue was modified by insulin (421;423) which favours its role in modulating endothelial dysfunction and arterial stiffness. In obesity and diabetes there is evidence suggesting inappropriate activation of RAAS which is associated with immune and inflammatory responses (424-426). There is increased secretion of cytokines by dysfunctional visceral adipocytes, leading to activation of the vascular RAAS (56;424;425). In addition, there is increased oxidative stress and a decrease in the level of interleukin (IL)10 which cause increased expression of Ang II type 1 (AT1) receptor and impaired function of T regulatory cells (424;425;427). Therefore, inappropriate activation of RAAS causes cytokine imbalance, which in turn activates vascular RAAS resulting in a feed forward loop of RAAS activation in obesity and diabetes (424;425;428).

Visceral adiposity (429) and waist to hip ratio (430) correlate directly with plasma aldosterone levels . Increased aldosterone in obesity raises ROS which further stimulate MR receptors which produce more ROS, i.e. a further feed forward cycle (431). Moreover, Aldosterone also decreases endothelial glucose 6 phosphate dehydrogenase (G6PD) activity, a main source of intracellular NADPH. NADPH in turn functions to limit ROS activity (432). Aldosterone also increases the expression of TNF-α from macrophages which further contribute to increase in ROS. In keeping with these mechanisms, blockade of the MR receptor with eplerenone leads to a reduction of ROS and increased levels of adiponectin in obese and diabetic mice (433)

In premenopausal women oestrogen has a cardioprotective effect but this effect is lost in the setting of obesity and diabetes (434;435). Oestrogen modulates

both Ang II signalling and inflammatory responses by suppressing actions of Ang II by inhibiting the expression of AT1 (436;437). However, this protective effect of oestrogen is lost in obese premenopausal women suggesting the loss of inhibition of AT1 expression (438)

Cellular and molecular mechanisms underlying vascular RAAS-mediated endothelial dysfunction, and arterial stiffness in physiological ageing, and pathological obesity and diabetes are not well understood. It has been demonstrated that Ang II and aldosterone increase serine phosphorylation of IRS-1 and impair insulin signalling (63;406;423) but the role of mammalian target of rapamycin (mTOR)/S6 kinase (S6K) mediated IRS-1 serine phosphorylation in ECs are not well characterized. Kim et al. recently showed that Ang II treatment activated tissue RAAS which increased serine phosphorylation of IRS-1 and decreased phosphorylation of eNOS (406). Moreover, rapamycin, an inhibitor of (mTOR) activation attenuated the effects of Ang II on IRS-1 and eNOS and lead to NO-dependent arteriole vasodilation (406).

The precise role of aldosterone in endothelial function is not directly and fully elucidated, but blocking the mineralocorticoid receptor (MR) improves endothelial function (63;439-442) and reduces inflammation and vascular stiffness (439;441-443). Aldosterone levels are correlated with BMI and insulin resistance in normotensive subjects (444) and primary hyperaldosteronism is associated with insulin resistance (445). However, spironolactone (a MR blocker) raises HbA1c, Ang II and cortisol (in spite of decreasing BP) in people with T2DM and hypertension (446). Spironolactone also did not improve endothelial function in people with T2DM (446). Aldosterone has shown to increase epithelial Na + channel expression on the ECs surface which is correlated with ECs stiffness (447). The EC stiffness is associated with a reduction in NO release (447) and so aldosterone may have some role in vascular stiffness. In addition aldosterone has been shown to contribute significantly to target organ injury that include atherosclerosis, myocardial hypertrophy, fibrosis, heart failure, and kidney disease (448).

Increased sodium reabsorption is a major contributor in development of hypertension and both AngII and aldosterone have a direct action on kidneys to

increase sodium reabsorption. These results suggest the role of tissue RAAS in vascular endothelial functions and are shown in Figures 1.5 and 1.6.

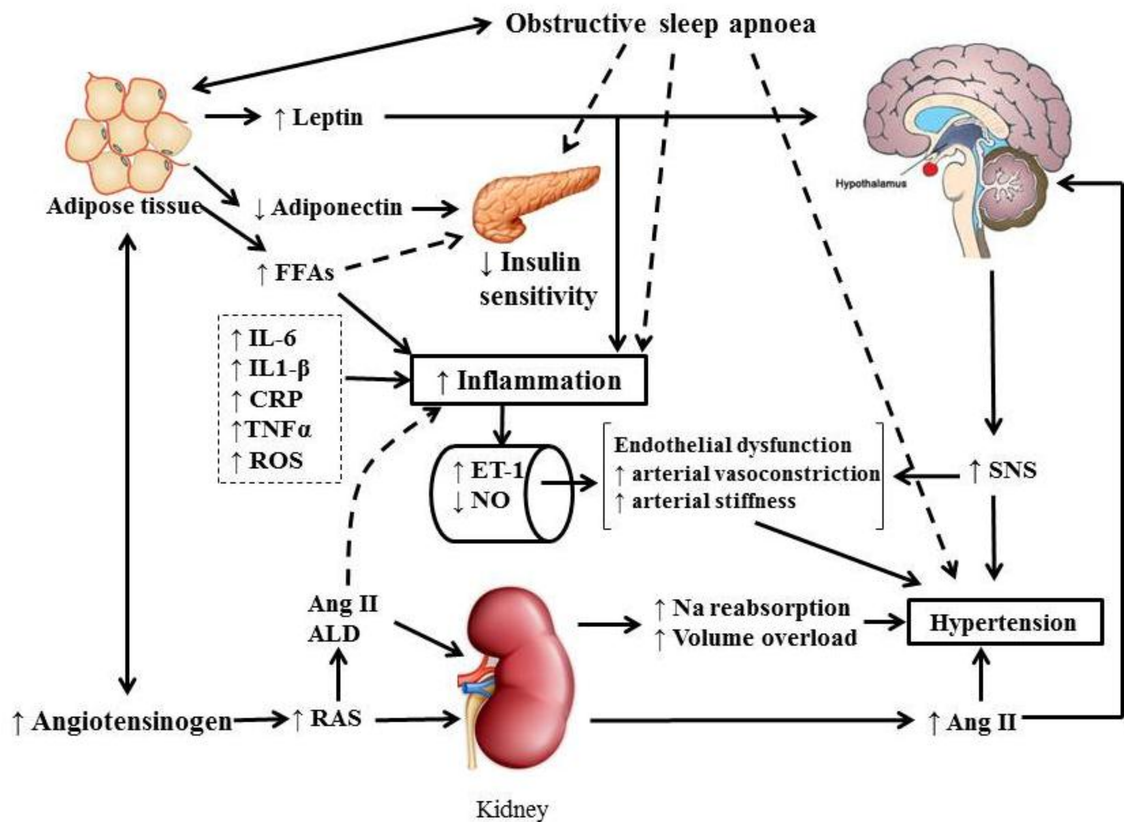


Figure 1.6 Mechanism of obesity induced hypertension,
Redrawn with permission from Kotsis et al. 2010 (449).
Mechanisms involved in the pathogenesis of obesity-induced hypertension. Ang II, angiotensin II; ALD, aldosterone; IL-6, interleukin-6; IL-1β, interleukin-1β; TNFα, tumour necrosis factor-α; CRP, C-reactive protein; ROS, reactive oxygen species; FFAs, free-fatty acids; NO, nitric oxide; ET-1, endothelin-1; RAS, renin–angiotensin system; SNS, sympathetic nervous system.

1.7.5 Mitochondrial dysfunction in obesity and T2DM

Skeletal muscle metabolism and mitochondrial function are also impaired in obesity. Skeletal muscle from obese people exhibits increased fatty acid uptake, lipid accumulation and oxidative stress (450;451). Fatty acids are degraded within cells to diacyglycerol and ceramide, which are associated with impaired insulin sensitivity in this tissue (452;453). One of the contributing factors in lipid accumulation and oxidative stress is the reduction of fatty acid (FA) oxidation in obesity and T2DM (454;455). However, later studies have demonstrated that FA oxidation is either moderately increased or not different compared to lean controls in both rodent and human studies (456;457). The differences in mitochondrial oxidation in different studies may be due to the differences in

cellular mitochondrial content (456;458). Studies comparing skeletal muscle mitochondrial content of obese and T2DM vs lean individuals show it to be reduced in both obese and T2DM patients (459) i.e. reduced mitochondrial mass may be responsible for mitochondrial dysfunction. Similarly levels of mitochondrial proteins and their genes are also reduced in skeletal muscle in obesity and T2DM (460;461).

Apart from reduction in oxidation, FA oxidation is incomplete in obesity (462), and also after having a high fat diet in obese rodents and humans (462-464). Incomplete FA oxidation is associated with accumulation of by-products of metabolism, namely acylcarnitines and other short chain fatty acids which are proposed to cause mitochondrial dysfunction and insulin resistance (463;464). Moreover the activity of ETC in mitochondria is also reduced in obese individuals with T2DM as compared to lean controls (459).

Type II glycolytic fibres have a reduced capacity to oxidise fat (465) and to counter oxidative stress (466), and possibly contribute to increased oxidative stress. In the skeletal muscles of individuals with diabetes, type IIx glycolytic fibre expression is higher (467). Similarly the weight gain response to overfeeding is associated with type IIa fibre expression (468). Fibre type expression may play an important role in skeletal muscle function and weight loss success in obesity.

Mitochondrial morphology is also changed in obesity and T2DM. Higher rates of mitochondrial fission are implicated in the development of diabetic neuropathy (469;470). In fasting and stress conditions mitochondria are elongated, whereas obesity and high fed state is associated with shorter and rounder mitochondria. In addition increases in mitochondrial fission proteins dynamin-related protein 1 and fission protein 1 have been observed in the skeletal muscle of ob/ob and high-fat fed mice, and palmitate-treated C2C12 cells (471). Smaller, rounded mitochondria and a fragmented mitochondrial network are associated with a reduction of the fusion protein, mitofusin-1 in skeletal muscle of obese rodents and humans (472). In addition, lower levels of the fusion proteins mitofusin 1 and optic atrophy 1 have also been observed in the individuals with T2DM (473).

Lower mitochondrial capacity in obesity and T2DM is not irreversible as mitochondrial capacity can be restored. Due to the hypothesis that mitochondrial dysfunction could be secondary to excess lipid accumulation in skeletal muscle, weight loss was initially tested as a strategy but with negative results (474). Toledo et al. compared the relative contribution of weight loss versus weight loss combined with exercise training. They showed that both groups experienced a comparable degree of weight and fat mass loss along with improvement in insulin sensitivity. However, improvement in mitochondrial content and ETC activity was only observed in the combined training group (475). Others have also shown improvement of mitochondrial content and activity with exercise training in insulin-resistant subjects with and without T2DM (476;477). These effects of exercise are not triggered by amelioration of the insulin resistant state or a reduction in intra myocellular lipid content, but are more likely due to an increase in contractile activity induced by exercise (474).

1.7.5.1 Mitochondrial dysfunction in atherosclerosis

Atherosclerosis begins with the recruitment of inflammatory cells to the intima and endothelial dysfunction is frequently involved in atherosclerosis (see Section 1.2.2). Elevation of endothelial mitochondrial ROS (mROS) initially leads to endothelial dysfunction and apoptosis and later enhanced inflammation – a dominant feature of atherosclerosis. Moreover EC are more sensitive to ROS as compared to VSMC (33). The increase in mROS is in response to many atherosclerosis inducers, including hypertension, hyperglycemia, ox-LDL and TG. For example, exposure of ECs to free fatty acids, levels of which are upregulated in patients with metabolic syndrome, increases mROS (478;479).

In samples from human atherosclerotic plaque , mitochondrial DNA damage is increased, probably because of proximity to the electron transport chain and the relative lack of mtDNA repair mechanisms (32). The resulting mitochondrial mutations may lead to increased production of ROS and may initiate a cycle of positive feedback. Increased DNA damage and failure of DNA repair cause defects in cell proliferation, apoptosis, and mitochondrial dysfunction which concomitantly lead to ketosis, hyperlipidemia, and increased fat storage further promoting atherosclerosis and the metabolic syndrome. Recently Yu et al.

showed that mitochondrial DNA damage can promote atherosclerosis independently of ROS, through its effects on VSMC and monocytes and is associated with higher risk plaques in human (34).

In summary, mitochondrial dysfunction is involved in atherosclerosis by impairing endothelial function but its independent role in atherosclerosis still needs to be evaluated.

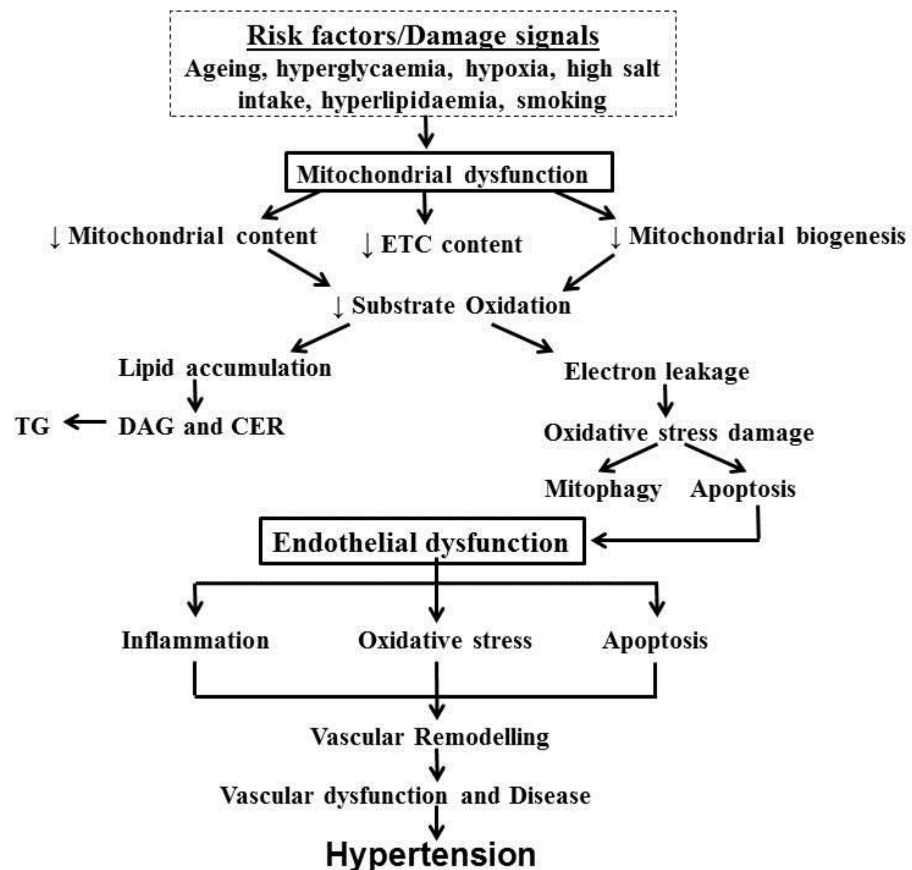


Figure 1.7 Proposed relationship between mitochondrial dysfunction, endothelial dysfunction and hypertension. Adapted from Tang et al 2014 (24).

1.7.6 Role of perivascular adipose tissue in relation to obesity related hypertension

Most of the arteries and veins with an internal diameter >100 µm are invested with a layer of perivascular adipose tissue (PVAT). Comprising adipocytes, inflammatory cells, and stem cells it is mostly found in the coronary arteries, aorta, and the micro vascular beds of the mesentery, muscle, and kidney (480). On stimulation, PVAT release a single or a combination of factors (acting in a paracrine and vasocrine fashion) and this release may depend on the stimulus

applied, the vascular bed examined, and the phenotypic state of the fat. Vasocrine signalling is said to occur when cytokine accesses the nutritive vascular tree to inhibit insulin-mediated capillary recruitment (480). Healthy PVAT exerts an anti-contractile effect on adjacent small vessels when it is stimulated (481). This anti-contractile effect operates by both endothelium dependent and independent mechanisms (482) but the exact mechanism is still under study. PVAT secretes a number of molecules and the ones with vasorelaxant properties include, adiponectin, angiotensin 1–7 (Ang1–7), nitric oxide (NO), leptin, and palmitic acid methyl ester (PAME).

Adiponectin is one of the most abundant adipokines and has significant vasorelaxant effect on small arteries. It can reverse endothelial dysfunction in diet-induced obese rats via the 5’-adenosine monophosphate activated protein kinase (AMPK)-eNOS pathway (483). Human studies have shown its levels to be low in hypertension, and antihypertensive therapy increases adiponectin levels (484). The adiponectin released from PVAT serves as an adipose tissue derived relaxant factor and modulates the tone of the adjacent vessel (485). Greenstein et al. demonstrated in humans that blocking the adiponectin receptor type-1 abolishes PVAT anti-contractile effect on adjacent small arteries from healthy human tissue (486).

Angiotensin 1–7 is also secreted from PVAT and exert anti-contractile effects. It stimulates the release of endothelial NO, thus activating calcium dependent potassium channels (43) in arteries and voltage dependent potassium channels in veins (487). Similarly Ang 1–7 receptor antagonists have been shown to attenuate PVAT anti contractile function (488). Ang 1–7 has also been shown to affect AT2 and Mas receptors and can decrease the nerve stimulated overflow of noradrenaline (489), and this property can be further explored for therapeutic use as obesity also increase SNS outflow.

Leptin is secreted from white adipose tissue and its plasma levels are increased in obesity. It acts centrally on the hypothalamus to reduce appetite and also increases SNS activity (490). Locally, in healthy conditions, it has a direct endothelial NO dependent vasorelaxant effect. It is proposed to play a major role in pathophysiology of obesity related hypertension as it has been shown that leptin deficient ob/ob mice remain normotensive in spite of developing severe

obesity (491). An acute rise in leptin does not significantly affect BP despite SNS activation and may be due to its stimulation of endothelial NO (492). However, chronic infusion of leptin for 7 days (similar to chronically raised leptin in obesity) lead to increase in BP and hearty rate, possibly through decreases NO bioavailability (493). So in obesity, the vasopressor effects of leptin become more apparent.

PVAT damage is characterised as a disturbance in the normal metabolic and vasoactive function of adipocytes surrounding the blood vessels. In obesity, the anti-contractile function of PVAT is either attenuated or completely lost. The most likely factors responsible for the loss of anti-contractile effect include; oxidative stress, inflammation, adipokine dysregulation and increased SNS action. Sympathomimetic stimulation of ß3-adrenoreceptors on adipocytes leads to the activation of PKG and increases the bioavailability of adiponectin ultimately reducing vascular tone. Obesity as well as metabolic syndrome is associated with a loss of PVAT mediated anti-contractile function. In these conditions, fat cells undergo hypertrophy and there is clear evidence of local inflammation (486), and also reduction in bioavailability of adiponectin (486). Greenstein et al. have shown that incubation of healthy PVAT with TNF-α and IL-6 leads to significant attenuation of PVAT anti-contractile function, similar to that observed in obese people (486).

Obesity is associated with increased macrophage recruitment in adipose tissue. These macrophages secrete a number of inflammatory cytokines including TNF-α, IL-6, and also produce free radicals such as the superoxide anion. Moreover, different adipose tissue depots have unique inflammatory profiles. In comparison with subcutaneous and visceral fat, PVAT from murine aortic arch expresses lower levels of adipocyte associated genes. However, two weeks of high-fat feeding up regulates pro-inflammatory genes (494). Furthermore, visceral adipose tissue as compared to subcutaneous fat exhibits a higher inflammatory profile with a higher macrophage content (495). This may explain why hypertension is more strongly related to central obesity than to BMI (496).

Grant (497) has suggested a phylogenetic basis for this on the basis that humans did not historically have access to abundant availability of food. So development of peripheral insulin resistance with decreased glucose utilization and deposition

of fat in existing fat cells helped to preserve energy stores in anticipation of periods of starvation. At the time of food shortage, this fat was used. Yudkin et al (480) proposed that obesity-induced production of pro-inflammatory cytokines by PVAT might cause low-grade inflammation which then impairs normal functioning of vessels. This impaired vascular function may be the link between arterial function and insulin resistance and make type II diabetes mellitus a vascular disease. The vasoconstriction will decrease glucose uptake into skeletal muscle and will be followed by insulin resistance.

1.7.6.1 Bariatric surgery and hypertension

Bariatric surgery is growing in popularity as a method for weight loss in comparison to diet control and exercise. A systematic review evaluated data of 16,867 patients, 49% of whom had hypertension before the operation. 34 months follow-up showed that hypertension had either improved or completely resolved in 68% of cases (498). Similarly another review (of 18 studies) evaluating bariatric surgery outcomes, demonstrated an increase in serum adiponectin levels by nearly 70% in patients after gastric bypass, and by 36% post gastric banding procedures. Moreover the greatest increase in adiponectin was achieved after loss of at least 35% of the original body weight (499). There was a strong correlation between percentage increase in adiponectin levels and percentage decrease in BMI (499).

In contrast weight loss by liposuction did not increase adiponectin or improve insulin resistance (500). This difference in metabolic results between bariatric surgery and liposuction is likely due to the differing qualities of adipose tissue depots, with visceral fat exhibiting a more inflammatory profile as compared with subcutaneous fat (501).

Bariatric surgery has also been shown to improve the inflammatory profile of obese individuals as it significantly decreases the expression of IL-6 and TNF-α mRNA in subcutaneous adipose tissue. In addition to increasing adiponectin levels, it also increases expression of adiponectin receptors (502;503). Aghamohammadzadeh et al. recently evaluated change in PVAT structure and function before and six months after bariatric surgery. Before intervention, gluteal artery PVAT had evidence of adipocyte hypertrophy and inflammation

with increased cell adhesion molecules (CAMs) and a complete loss of PVAT-mediated anti-contractile function (504). Six months after surgery, there was a significant decrease in BMI, insulin resistance, and BP. Although patients remained morbidly obese, adipocyte hypertrophy had been completely reversed along with the disappearance of CAMs and inflammation. PVAT anti-contractile activity was restored and adiponectin bioavailability was also improved (504). The significant degree of weight loss, improvements in adipokine expression, decrease in inflammation, increase in insulin sensitivity and resolution or improvement in diabetes status (505), makes bariatric surgery an invaluable procedure for obese people although in individual cases the risks need to be considered in the context of the potential benefits.

1.7.6.2 PVAT changes in obesity

In white adipose tissue of lean and healthy animals, macrophages constitute 10% to 15% of stromal cells and express markers that link them with the phenotype of alternatively activated macrophages. The latter are critical for maintaining insulin sensitivity in adipocytes, through the production of IL-10. In obesity, Ly6chi monocytes are recruited, which increases macrophage content to 46% to 60% and induce the CAM inflammatory phenotype that promotes insulin resistance (506;507). In obesity, adipocytes also hypertrophy and release chemokines, such as CCL2, CCCL5, and CCL8; further exacerbating the process (506). Studies of human PVAT from obese individuals and adipocytes from animal models of obesity and diabetes mellitus show evidence of adipocyte hypertrophy and low grade inflammation (508).

The central role of inflammation in loss of PVAT anti-contractile function was shown by Withers et al. in mouse models (509). In mice deficient of macrophage CD11b-diptheria toxin receptor, there was no loss of PVAT mediated anti-contractile activity when pro-inflammatory stimuli, such as hypoxia, were applied to small arteries surrounded by PVAT (509).

Norepinephrine constricts the small arteries and the anti-contractile activity of PVAT is lost (486;510). However, this constriction can be fully restored *in vitro* using a combination of catalase and dismutase giving an indication of inflammation and ROS production in PVAT. It was also associated with reduced

NO bioavailability and uncoupling of NO synthase (511). Likewise, antagonism of TNF-α using preincubation with infliximab in the organ bath or aldosterone antagonism by spironolactone and eplerenone can restore normal PVAT anti-contractile function (486;509). Aldosterone antagonists also restore the hypoxia induced loss of PVAT function suggesting a role of oxidative stress (509).

1.7.7 Role of chemokines in hypertension

Chemokines are low molecular weight proteins of the cytokine family which participate in the inflammatory reactions within the vascular wall. They are called chemoattractant due to the ability to activate and control leukocyte (monocytes and macrophages) migration. They also play an important role in the development of endothelial dysfunction and hypertension. Other vascular functions of chemokines include: VSMC proliferation, angiogenesis, hematopoiesis, embryogenesis, organogenesis, maturation of dendritic cells, tumour growth, tumour metastasis, autoimmune and inflammatory processes, promotion of cancer cell growth and increased severity of hypertension complications such as atherosclerosis, hypertensive heart disease and hypertensive nephrosclerosis (17;512-515).

Chemokines control inflammation in the vascular walls and have a role in hypertension as inhibition of inflammation and oxidative stress results in a decrease in blood pressure (516). The chemokines playing some role in the pathogenesis of hypertension include monocyte chemoattractant protein-1 (MCP-1, CCL2), interferon inducible protein (IP-10, CXCL10) interleukin-8 (IL-8; CXCL8), Gro-α (growth-related oncogene), CXCL1/RaNTeS (CCL5)/CCR5 and fractalkine (CX3CL1)/CX3CR1.

1.7.7.1 Chemokines and endothelial dysfunction

Chemokines disturb the normal vascular homeostasis both by increasing inflammation and oxidative stress and by impairing the protective factors. They are involved in migration and adhesion of mononuclear leukocytes, increasing inflammation and ROS in the vascular wall and increasing ET1 and plasminogen activation inhibitor 1 (PaI-1) in ECs (517;518). All of these factors lead to endothelial dysfunction and development of hypertension. Chemokines also

increase the proliferation of VSMC and so involved in the pathogenesis of hypertension, atherosclerosis and cardiovascular disease (512;513;515).

1.7.8 Vascular calcification

Vascular calcification is characterised by the extracellular deposition of calcium in the vascular wall. It is a complex biological process that is based on a continuous balance between promoting and inhibiting factors (519;520). Cell types potentially involved in calcium deposition with the vessel wall include VSMCs (521), interstitial valve cells (522), circulating osteoprogenitor cells (523) and mesenchymal pluripotent cells (524).

1.7.8.1 Types of vascular calcification

Two main types of extracellular vascular calcification are recognized, intimal and medial (122). Intimal calcification is primarily associated with atherosclerosis and appears as punctate and disorganized mineral deposition in the intima. Intimal calcification forms an important part of atherosclerotic plaques, which mainly constitute VSMCs, lipids, macrophages, connective tissue, and necrotic debris (525).

Coronary artery calcification is very important in the development of CVD and predominantly affects the intima (526). A major complication of atherosclerosis is plaque rupture followed by serious sequelae including myocardial infarction (MI) and stroke. Although the role of plaque rupture is certain, the direct contribution of calcification to plaque rupture is still unclear as recent studies suggest that the distribution of calcification, rather than its mere presence, may predispose to plaque rupture. It has been found that diffuse and speckled micro calcium deposits (spotty calcification) are associated with greater risk of plaque rupture (527;528). Hypertension is an independent risk factor for the development of atherosclerosis and is also associated with acute plaque rupture; by increasing the pulsatile mechanical stress on plaques (529).

Medial calcification is predominantly associated with ageing, diabetes mellitus, hypertension and uraemia, and morphologically appears as organized mineral deposition along the elastic lamellae of media. Medial calcification and hypertension are very closely linked and are proposed to potentiate each other

as BP stress on vascular walls promotes calcification (530). Medial calcification decreases vascular elasticity and increases stiffness which accelerates pulse wave velocity and contributes to increases in BP (531;532). Patients with resistant hypertension also have exaggerated arterial stiffness and calcification (533). Apart from other factors arterial calcification is independently associated with arterial stiffness. This association between arterial calcification and arterial stiffness is mainly due to medial calcification as intimal calcification (atherosclerosis) has only a modest association with arterial stiffness (534).

Vascular calcification is quantified by non-contrast computed tomography (CT). It is a sensitive method of measuring total vessel calcium content but is not specific as it does not distinguish between intimal and medial mineralization (529).

1.7.8.2 Calcification and VSMCs

Vascular calcification is now recognized as an active and regulated process and has similarities with developmental osteogenesis. VSMCs are the chief modulators and orchestrator of vascular calcification. In response to stress stimuli or any damage signals (such as hyperphosphatemia, oxidative stress and inflammation) VSMCs change their phenotype (as a repair mechanism) and transforms to an osteogenic/calcifying phenotype and are then called calcifying vascular cells (CVCs) (535;536). This VSMC modification is also accompanied by increase in inflammatory cytokines and oxidized lipids along with mineral imbalance (530). Moreover, VSMC phenotypic transformation is also accompanied by the secretion of micro vesicles (MVs) which are integral to calcification process (537). Osteogenic VSMCs have the capacity to secrete an osteoid-like matrix which can calcify (529). The release of MVs by VSMC is one of the earliest events in calcification. Researchers have identified two populations of MVs: 1) relatively large apoptotic bodies (200–800 nm) derived from dying cells, and 2) smaller MVs (50–150 nm) released by living VSMCs, particularly in response to calcium stress; both furnishing nucleation site for crystals (537).

Potent inhibitors of calcification, such as matrix GLA protein (MGP) and inorganic pyrophosphate (PPi), are locally produced (by vascular cells) and expressed in the arterial wall and prevent mineralization of elastic lamellae

(538) and also prevent differentiation of VSMC into chondrogenic cells. It is plausible that the differentiation of vascular cells into a chondro/osteoblast-like phenotype is accompanied by reduced production of calcification inhibitors by these modified cells themselves or the 'adjacent' vascular elements; as calcification inhibitors are expressed differentially in calcified arterial walls as compared to healthy artery walls (539). In the absence of calcification inhibitors, MVs form the nidus for nucleation of mineral calcium, by providing a micro-environment which raises the calcium:phosphate product above the threshold for precipitation (537;540). In non-stress state, these inhibitors are loaded into MVs and inhibit calcification, but are differentially expressed in calcified MVs; favouring calcification (537). Under conditions of acute stress, VSMC release MVs as an initial adaptive response to prevent cell death by removing excess calcium, which is bound by the inhibitors in the MVs. These are then deposited in the ECM. However, in the presence of prolonged stress (e.g. mineral imbalance) this adaptive mechanism becomes overwhelmed and calcification ensues (537).

Extracellular space calcium is elevated in people with chronic renal failure and in atherosclerosis plaques (at sites of cell death and necrosis), however, in hypertension there is intracellular calcium overload (541). Mechanistically calcium promotes the loss of inhibitors such as MGP from MVs, and also exposes the calcium binding protein annexin A6 together with phosphatidylserine (PSer) on the surface of MVs; forming a complex. This complex is highly efficient at nucleating hydroxyapatite, thus enabling MVs to seed extracellular matrix calcification in the vessel wall (541;542). *In vitro* studies have suggested that calcium channel blockers may prevent calcification by preventing MVs from mineralizing (543). Similarly animal models of medial calcification have demonstrated that various anti-hypertensive therapies (diuretics, calcium channel blockers, ARBs and endothelin receptor antagonists) can reduce pulse wave velocity and slow or prevent medial calcification (544-547) but their efficacy in humans still needs further work. Both calcification and hypertension are related to each other and may influence each other as shown in Figure 1.8 and explained below.

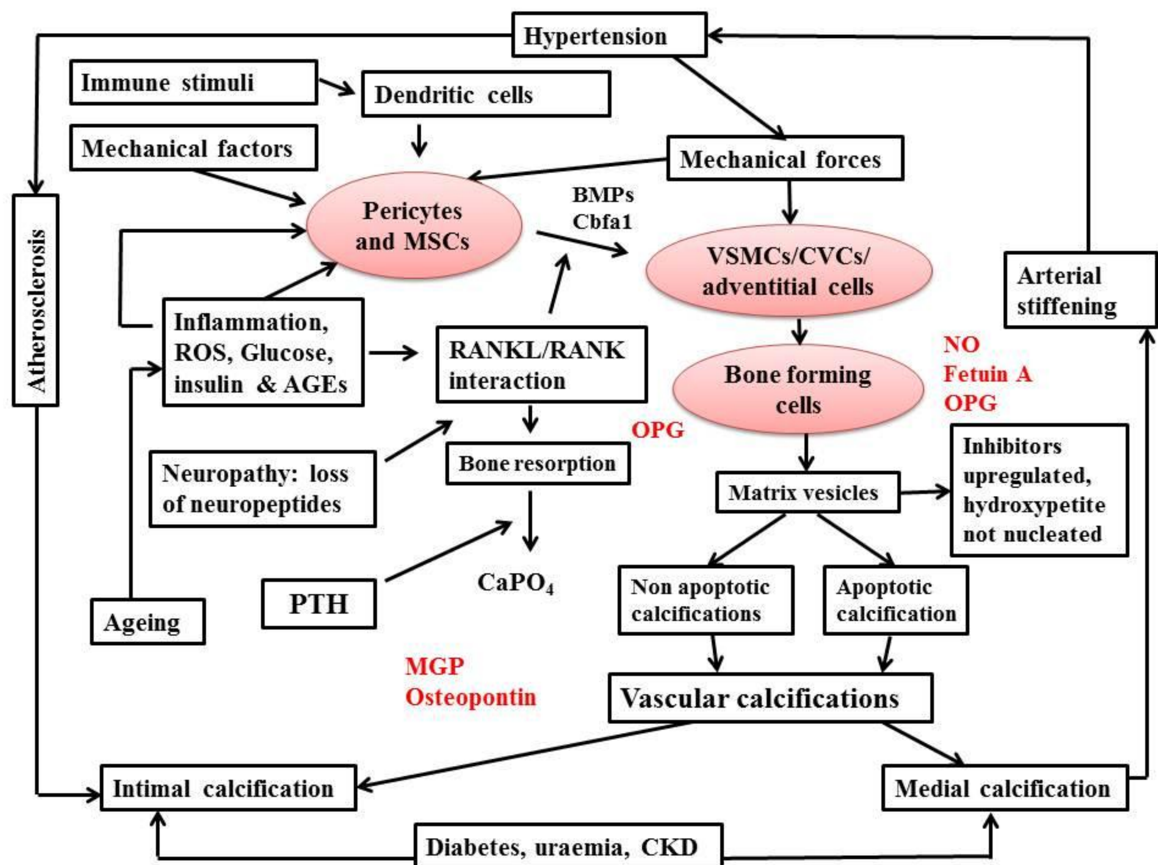


Figure 1.8 The cyclical association of vascular calcification, arterial stiffness, atherosclerosis and hypertension.
Redrawn and modified with permission from Jeffcoate et al. 2009 (548)
Red coloured compounds are calcification inhibitors. MSC= mesenchymal stem cells, VSMC= vascular smooth muscle cells, CVC= calcifying vascular cells, MV= micro-vesicles, OPG= osteoprotegerin, NO=Nitric oxide , BMPs= Bone morphogenetic protein, Cbfa1= Transcription factor core-binding protein, ROS= reactive oxygen species, RANKL/RANK= Receptor activator for nuclear factor κB/ Receptor activator for nuclear factor κB ligand, PTH= parathyroid hormone, CaPO4= calcium phosphate.

1.7.8.3 Hypertensive remodelling causing calcification

Hypertension is associated with remodelling of the arterial wall mainly characterised by changes in composition and quantity of ECM, along with proliferation/differentiation of VSMC (321;549;550). Elastic fibres are an important constituent of aorta and large and medium sized arteries. They are composed of an elastin core which is surrounded by fibrillin rich microfibrils (551). The elastic properties of large conduit arteries are determined by the presence as well as special arrangement of elastic fibres; organized in concentric rings of fenestrated lamellae intercalated with aligned VSMCs (116). However, increased pressure on elastic fibres (e.g. hypertension) can induce quantitative and qualitative changes in the elastic fibre organization. Increased BP increases elastin production and deposition in vascular wall (552). Increased

pressure causes fatigue and damage to elastic fibres, leading to fragmentation and generation of elastin degradation products (EDPs) (116). EDPs are endowed with chemotactic activity and also have proliferative and migratory effects on VSMCs (553). Hypertension is also associated with increased deposition of several ECM components, including collagen, fibronectin and proteoglycans by vascular cells especially VSMC (116;552). Adijiang et al showed the effect of high BP in calcification by exposing Dahl salt-sensitive hypertensive rats to a uremic (indoxyl sulfate) toxin; and this accelerated vascular calcification (554). In contrast exposure of normotensive rats to the same uremic toxin did not induce calcium deposition (554). It is suggested that hypertension-associated changes in ECM and vascular cells might create an environment which is prone to calcium deposition, and calcification is accelerated in the presence of noxious or pro-calcification mediators.

EDPs are represented as a preferential site for hydroxyapatite crystal nucleation (555); they also induce phenotypic transition of VSMC toward an osteoblast-like profile and amplify phosphate driven calcium deposition (556-558). EDPs also promote the release of metalloproteinases (MMPs), through interaction with specific receptors (556;557). During early hypertension MMP activation helps to limit the pulse pressure rise by increasing vascular compliance (559), however, MMP accumulation amplifies ECM damage, including elastin degradation and elastin calcification. Moreover, blockade of MMPs prevents the calcification of elastin (560-562).

Elastic fibres are composed of several microfibrillar molecules including the latent transforming growth factor-ß (TGFß) binding proteins and these contribute to formation of TGFß large latent complex (LLC) (563). LLC interacts with ECM components and in response to any insult (including MMP), converts from latent TGFß to active TGFß (563). During hypertensive remodelling active TGFß is known to drive the synthetic and proliferative response of vascular cells (564). TGFB has also been shown to be involved in the osteogenic differentiation of VSMC in synergy with other procalcific mediators, including EDPs (556;565).

VSMCs also produce type I collagen during hypertensive remodelling (552). Type 1 collagen represents an ideal matrix for apatite crystal nucleation and propagation. *In vitro* studies have shown that it is produced by calcifying VSMC,

and on exogenous administration it facilitates matrix calcification along with collagen and fibronectin (566;567). Increased type I collagen production has been also documented in senescent VSMCs (568). Moreover, it provides the matrix for MV driven calcification (569) and also acts as modulator of VSMC differentiation into osteoblast-like cells (567;570). Type 1 collagen gene deletion leads to significant reduction in calcium deposition (568). Similarly its receptor gene [discoidin domain receptor-1 (DDR1)] deletion was also associated with significant reduction in vascular calcification (570). VSMCs deficient for DDR1 show reduced ability of osteogenic differentiation and also express higher level of the calcification inhibitors such as ENPP1 (570). Arterial proteoglycan content (including chondroitin sulfate, biglycan and decorin) increases during hypertension (571) and they are also associated with increased vascular calcification (565;572).

In summary, hypertensive remodelling of the large arteries is characterised by changes in vascular cells and ECM composition that might create a favourable environment for the initiation and propagation of calcium deposition.

1.7.8.4 Aortic calcification causing systolic hypertension

Ageing is associated with progressive increase in stiffening of aorta and other large arterial conduits. The structural modifications of the vascular wall in ageing are similar to hypertension in several ways. Ageing of the large arteries is characterized by progressive collagen accumulation along with fracture and disorganisation of elastic lamellae (111). The net effect of the imbalance of collagen and elastin in ageing is a progressive reduction in vascular elasticity and compliance. This change in vascular elasticity and compliance explains the age associated rise in SBP, the fall in DBP and the acceleration of the pulse wave velocity (PWV) as observed in the elderly. Moreover, increased stiffness may result in returning of the aortic reflected wave during the systolic period, further increasing the left ventricular load, favouring cardiac hypertrophy and susceptibility to MI (111;532).

Vascular calcification is associated with arterial rigidity which in turn is responsible for the mechanical abnormalities and cardiac consequences associated with vascular stiffening (532). As mentioned above, vascular

calcification can be both intimal and medial, and both types increase vascular rigidity. However, the relative contribution of each in arterial stiffening is still unknown as the available CT imaging technique does not differentiate between the two types.

Through the experimental generation of medial calcification (elastocalcinosis) in thoracic and abdominal aorta in rats was associated with accelerated PWV, increased SBP and augmented PP (531;545;547;573). These changes were similar to those observed in isolated systolic hypertension (ISH), and were also accompanied by a significant increase in left ventricular mass (573).

In human studies, vascular calcification and associated vascular stiffness has mostly been shown in patients with chronic kidney disease (CKD). Population studies clearly showed that the amount of aortic calcification in CKD patients was positively correlated with PWV (574;575). It has also been shown that both PWV and the extent of arterial calcium deposition are predictive of future CVD mortality (576;577). The Twins UK cohort of middle aged women with normal kidney functions also showed that aortic calcification was significantly correlated with carotid femoral PWV. This positive correlation remained significant even after adjustment for age, mean arterial pressure (MAP), glucose, heart rate and menopausal status (578). More recently, Sekikawa et al. also confirmed these findings in a multi-ethnic cohort of 906 middle-aged men without history of any CVD. They showed that carotid femoral PWV was positively and significantly correlated with the amount of calcium deposits in the aorta observed between the aortic arch and the iliac bifurcation. This correlation was also independent of the effect of age, BMI, MAP, smoking, diabetes and medications (579).

The association between hypertension and aortic calcification had been reported previously (580;581). Recently McEniery et al. (533) confirmed that increased aortic calcium deposition was accompanied by higher aortic PWV in a group of healthy individuals. This link was independent of age and MAP and mainly observed between PWV and the calcification of the abdominal aortic tract. Along with aortic PWV, peripheral PP was also positively associated with aortic calcium deposition in any vascular site (abdominal, ascending and descending aorta). Considering all the factors together in a multivariate analysis, they found that

the presence of aortic calcification was independently associated only with age, aortic PWV and calcium phosphate product. When they compared normotensive people with those with ISH, they found that hypertensive individuals exhibited higher aortic PWV along with increased calcification of abdominal and descending thoracic aorta. Moreover, patients with resistant ISH had an even higher amount of aortic calcification (533). More recently Jensky et al. (582) investigated the association of individual BP parameters (SBP, DBP and PP) with the extent of calcification in different vascular segments. Calcification in all the large and medium sized arteries (except for the iliac and subclavian arteries) was significantly associated with SBP and PP, with the latter showing an even stronger association (582). They also found that in older people (<60 years), differences in arterial calcification between hypertensive and normotensive individuals were more pronounced (582). As the studies examining direct relationship (533;582) were cross sectional and not longitudinal, causal relationship between aortic calcification and BP cannot be confirmed. However, findings from these studies strongly suggest a significant association between calcification and ISH, SBP and PP. Moreover, exhibition of higher aortic calcium accumulation in patients with resistant ISH (533) also underscores the possibility of a contribution in the pathophysiology.

In humans, three monogenic diseases are characterized by extensive and premature onset of arterial calcification. These conditions are 1) Generalized arterial calcification of infancy (GACI), associated with mutations in the ENPP1 gene (583), 2) Pseudoxanthoma elasticum (PXE), which results from mutations in the ABCC6 gene (584) and 3) arterial calcification and distal joint calcification (ACDC), which is caused by CD73 deficiency (mutations in the NT5E gene) (585). Patients affected by GACI and PXE exhibit diffuse calcific deposits in the medial layer of large arteries, whereas calcification in patients with ACDC is mainly restricted to lower limbs vessels. Both GACI and PXE are characterized by severe increase in arterial BP and development of renovascular hypertension (586).

In summary, *in vitro*, animal and human data supports the role of vascular calcification in the generation of arterial stiffness and subsequent increase in SBP and PP but more work is needed to confirm the independent causal association.

1.8 Ageing, vascular changes and CV risk

Ageing is often considered to be a progressive deterioration of biological functions and structure after the organism has attained its maximal reproductive competence (587). In particular vascular ageing is associated with both structural and functional changes taking place in endothelium, VSMC and the vascular ECM and characteristic alterations are increased arterial stiffness, dilation of central elastic arteries and endothelial dysfunction (588). Vascular ageing is closely associated with cardiovascular disease (589). However it can also be argued that ageing is mainly determined by the number of diseases by which it is accompanied(590).

The following are important changes taking place within vessel wall.

1.8.1 Ageing associated changes in vascular cells

Ageing related changes in vascular cells exhibit the same characteristics as in low grade chronic inflammation and often involve inflammation as an intermediary step. Many characteristics of vascular ageing like endothelial dysfunction, oxidative stress and increased apoptosis can be reproduced by recombinant TNF-α and chronic infusion of Ang II; both of these induce inflammation within the vascular wall. The detailed functional and structural changes in different vascular cells and matrix observed in association with inflammation are explained in Section 1.6.1.1

1.8.2 The enhancement of oxidative stress

Reactive oxygen and nitrogen species are essential signalling molecules involved in maintaining vascular homeostasis, but are also important contributors to the ageing process (591). Age-dependent increase in ROS disturbs the nitric oxide (NO) signalling and associated functions. ROS also alters and activates prostaglandin metabolism, and promotes oxidative posttranslational protein modifications which in turn interfere with vascular and cell signalling pathways leading to vascular dysfunction. In the initial stages compensatory mechanisms are activated to cope with this age-induced oxidative stress, but become counterproductive with time. This results in irreversible oxidative modifications of vascular structures (591;592). It has been suggested that in ageing there is a

reduction in the number of mitochondria and an increase in the generation of dysfunctional proteins, which leads to an increase in superoxide production (258). Mitochondrial oxidative stress and mitochondrial damage and biogenesis are also thought to play a central role in cardiac and vascular ageing (37).

1.8.3 The reduction of NO bioavailability

NO is synthesized from l-arginine through the action of NO synthase (NOS) (258). Reduction in NO levels in the vessel may be due to 1) a deficiency in NOS substrates and cofactors; 2) increase in endogenous eNOS inhibitors; 3) decreased activity or expression of eNOS; and 4) augmented NO scavenging due to oxidative stress (589). ROS such as superoxide anions either degrade NO or quench it by forming peroxynitrite ($ONOO^-$). With age there is increased expression and enhanced activity (due to S nitrosylation) of the enzyme arginase which degrades l-arginine; the substrate for the formation of NO (258).

Both human and animal studies show that NO production decreases with age (593) and as NO is one of the most important signalling molecules in our body, its loss marks the beginning of many disease processes. Moreover clinical studies also provide evidence that decreased or insufficient NO is associated with all major cardiovascular risk factors, such as hyperlipidaemia, diabetes, hypertension (593).

1.8.4 Imbalance in the production of vasoconstrictor/ vasodilator factors and vascular response

During ageing there is a decrease in the NO or the endothelium derived hyperpolarizing factor (EDHF) induced vasodilation but an increase in vasoconstriction induced by cyclooxygenase products, such as thromboxane A2 (TXA2) (594). These may also be involved in reduction of the vasodilator response to agonists in resistance and capacitance arteries. Ageing is also associated with increased plasma concentrations of ET-1 and endothelin converting enzyme-1 (ECE-1) mRNA(258). There is also evidence of increased vascular expression of Ang II and ACE with ageing and it is a known fact that Ang II is a potent inducer of endothelial dysfunction and vascular oxidative stress (258). Ang II acting through AT1 also increases oxidant damage to mitochondria and affects mitochondrial function. In contrast, inhibition of Ang II activity by

targeted disruption of the Ang II type 1A receptor (AT1A) in mice was associated with prolongation of life span (595;596). Age-related decline in EDHF (a vasodilator) can be linked to an up-regulation of RAAS, since chronic ACE inhibition or AT1 receptor blockade, recovered EDHF-mediated responses in arteries from old rats (597).

Taking together; decrease in NO, increased ROS, and lower levels of antioxidants impair the vasodilatory response (258). The formation and accumulation of advanced glycation end-products (AGEs) with age and hyperglycaemia also induces fibrosis and remodelling in VSMC further accentuating the dysfunction, and impairing vasodilation (598).

1.8.5 Impaired angiogenesis

Angiogenesis is an essential adaptive response to physiological stress and is also an endogenous repair mechanism after vascular injury. In old age both impaired angiogenesis and endothelial dysfunction are present and likely contribute to the increased prevalence of cardiovascular diseases in the elderly (599).

1.8.6 Arterial stiffness during ageing

Arterial stiffness describes the reduced capability of an artery to expand and contract in response to pressure changes. It increases with ageing, however, the process is accelerated in the presence of obesity and diabetes and occurs at earlier ages if these conditions coexist. This is explained above in the context of aortic and arterial stiffness; Section 1.3.2

1.8.7 Ageing, insulin resistance, hypertension and diabetes

Arterial endothelium-dependent relaxation is diminished during ageing in both normotensive and hypertensive rats (600;601). This reduced response can be due to: 1) an impairment of either the generation (synthesis or release) of relaxant factors; 2) an impairment of the cellular response to them during ageing; 3) both 1 and 2. During the late stages of ageing in SHR, there is reduction in NO production, NO bioavailability (due to ROS) and also decreased VSMC response to NO (258).

Insulin resistance is associated with both ageing and vascular malfunction. The prevalence of T2DM increases with age: almost 20% of people over age 65 have diabetes (602). Moreover insulin resistance is the most important predictor and cause of T2DM. In addition there are twice as many hypertensive patients among type 2 diabetics subjects (603). Both ageing and insulin resistance interact with each other to increase sympathetic tone and may alter vascular responses to insulin. Insulin within physiological limits causes vasodilation in young adults but may cause vasoconstriction in healthy elderly individuals (604). This may further potentiate insulin resistance in the elderly (604). Another proposed mechanism of insulin induced vasoconstriction is through production and release of ET-1, which may contribute to hypertension (258).

From another perspective, hypertension also leads to insulin resistance by a number of mechanisms including stimulation of AT1 receptor, which then interferes with the insulin signalling pathways and also decreases NO production (258). In addition, patients with essential hypertension are more prone to develop diabetes as compared to normotensive subjects. This tendency may be due to decreased ability of insulin to promote relaxation and glucose transport in vascular and skeletal muscle tissue.

1.8.8 Effect of drugs and lifestyle on vascular remodelling with ageing

Amongst the many properties of NO (see Section 1.2.1), it has been shown to activate telomerase in ECs, delaying senescence (257;258). Any strategies to diagnose and treat NO insufficiency may therefore be considered as potential therapies to prevent ECs senescence associated with ageing.

To date no genetic or molecular solutions exist to slow the progression or reverse cardiovascular ageing but exercise has been shown to decreases the progression of arterial stiffness and improves endothelial function in skeletal muscle (605) promoting vasodilatation (268). Exercise also lowers BP and heart rate, thereby reducing the vascular shear stress and slowing vascular and myocardial remodelling (268). In terms of drug treatment, ACEi or AT1-blockers and calcium channel blockers have also been shown to reduce pulse wave reflection and improve endothelial function (258). Wray et al. have shown acute

reversal of endothelial dysfunction in the elderly after oral administration of an antioxidant cocktail (vitamin C + vitamin E + lipoic acid) (606); this requires further evaluation and for long term effects.

- In women, oestrogen replacement therapy in older (> 60 years) postmenopausal women is not currently thought to be associated with cardiovascular benefit (607). The proposed reason is the "Timing Hypothesis," which states that oestrogen mediated vascular benefits occur only before the detrimental effects of ageing are established i.e. it may require to be commenced prior to the age of 60 years (607). Similarly lipid-lowering and aspirin therapy have not been conclusively shown to significantly reduce CVD in older women (608). However, the Danish Osteoporosis Study (DOPS), which is the only prospective longitudinal randomized trial conducted specifically in women less than 60 years of age (average age = 50), showed that hormone replacement therapy started early in postmenopausal women significantly reduced the risk of the combined endpoint of mortality, myocardial infarction, or heart failure without increasing risk of breast cancer or stroke (609).

1.8.8.1 Anti-inflammatory drugs

Ageing is a state of low grade chronic inflammation and anti-inflammatory and antioxidant agents have been shown to have useful effects in the elderly. Aspirin has a potent antioxidant effect, diminishing lipoperoxidation levels, reducing ROS and inhibiting NOX in ECs (258;610;611). Salsalate, which is closely related to aspirin, has been shown to decrease blood glucose, C peptide, insulin clearance, free fatty acids, NF-κB activity and triglycerides (610;612). Despite these effects, anti-inflammatory drugs increase BP, probably through inhibition of prostaglandin synthesis (610;613). Inhibition of prostaglandin action might also cause salt and water retention. Despite the apparently beneficial properties of aspirin and salsalate in small studies, they can only be recommended for use in patients at high risk owing to adverse effects including GI haemorrhage (611;614)

The following table shows mechanistic similarities between arterial ageing, hypertension and atherosclerosis. Ageing is not a disease but is of course highly associated with many disease processes.

1.9 Unifying mechanism for the development of hypertension

The following figure indicates the inter-related mechanisms contributing to the development of hypertension. Genetic predisposition may act through increasing renin, insulin resistance, hyperinsulinaemia and an enhanced sympathetic response. These result in a shift of the renal function curve towards sodium and water retention. The increase in BP in turn also leads to increasing vascular stiffness, calcification, generation of ROS, inflammation, stimulation of RAAS and immune mechanisms. It also accentuates impairments of vascular function associated with ageing, obesity, diabetes, CKD and insulin resistance. In conclusion, these processes are self-perpetuating and may amplify each other in their deleterious effects on the vasculature. On the other hand treating one condition may also lead to improvement in others.

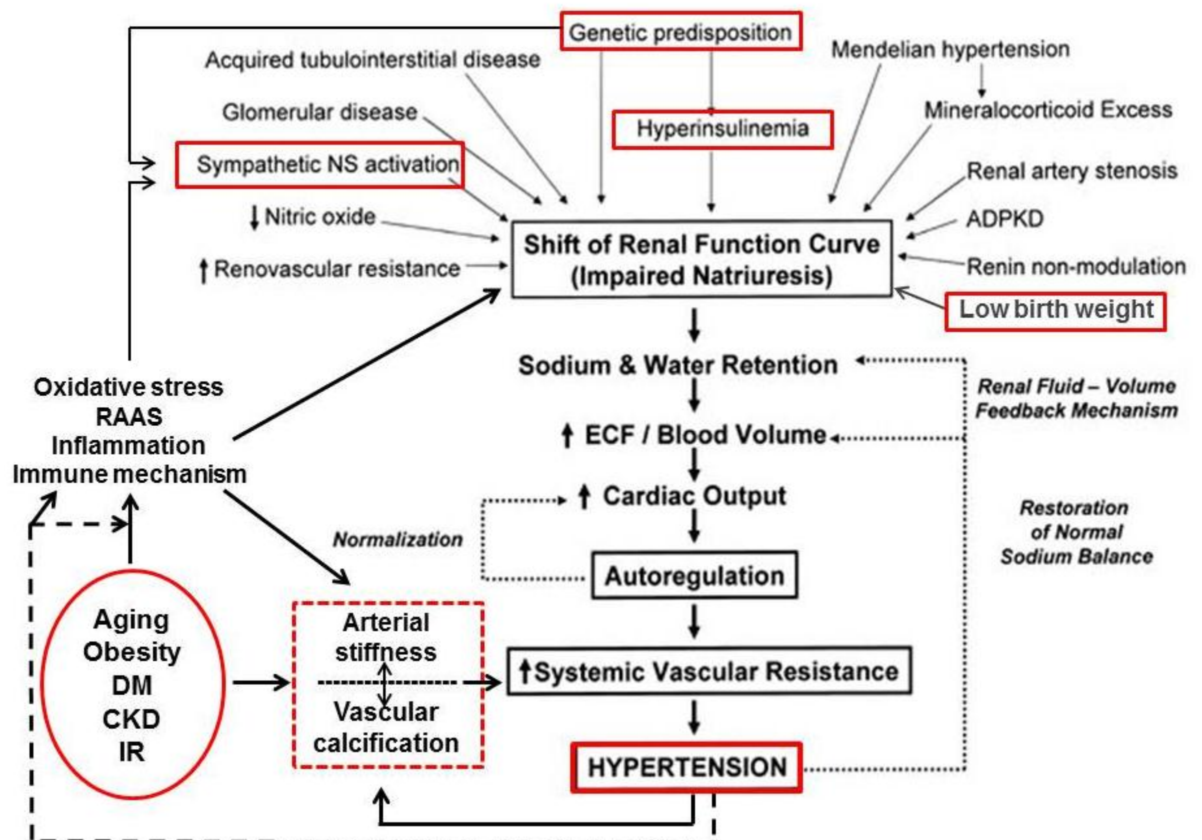


Figure 1.9 Contribution of different mechanisms in the development of hypertension

1.10 Diabetes mellitus

Diabetes is a chronic disease that occurs either when ß-cells in pancreas do not produce enough insulin or when the body cannot effectively use the insulin it produces. Hyperglycaemia (raised blood sugar) is a common outcome of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially blood vessels, heart and nerves (12).

Type 1 diabetes

Type 1 diabetes (previously known as insulin dependent, juvenile or childhood onset diabetes) is characterized by deficient insulin production and requires daily administration of insulin. A series of functional defects in the ß-cells, immune system, bone marrow and thymus collectively contribute to the pathophysiology of type 1 diabetes (615). However, it is not preventable with current knowledge.

Type 2 diabetes

Type 2 diabetes (formerly called non-insulin dependent or adult onset) is associated with insulin resistance (particularly hepatic) as well as with ß-cell dysfunction (see Section 1.5)(616). T2DM comprises 90% of people with diabetes around the world (12).

1.10.1 Mechanism of CVD in diabetes

Type 2 diabetes is associated with an increased risk of premature mortality from vascular causes (617). It is estimated that people with T2DM have double the risk for an incident vascular event compared to people without T2DM (618). Diabetes leads to elevation of many cardiovascular (CV) risk factors including hyperglycaemia, insulin resistance or deficiency, free fatty acidaemia, sympathetic stimulation, hypertension, hyperlipidaemia, and inflammation. Hyperglycaemia is also an important factor and produces tissue damage via a number of pathways, including the aldose reduction pathway, advanced glycation end product (AGE) pathway, reactive oxygen intermediate pathway, and protein kinase (PKC) pathway (619). In spite of the close association between diabetes and the development of CVD, intensive management of

glycaemic control is only of limited benefit in decreasing CVD risk. In contrast, control of other risk factors, such as hypertension and hypercholesterolemia has marked benefits in terms of reducing rates of CVD (620). The reason that glucose-lowering is less effective in type 2 than in type 1 for reducing CVD may actually be because lipids and BP are just as important hallmarks of the condition as glucose, even though we diagnose it using glucose levels.

Diabetes is associated with both vascular and autonomic nervous system (ANS) dysfunction. Both these mechanisms generally co-exist in the setting of diabetes, and also progress simultaneously. The possible interrelationship between vascular and autonomic dysfunction may also impact on the pathological process of organ damage in diabetes. Meyer et al. studied the relationship between ANS and vascular function and compared T2DM patients with controls (621). Patients with T2DM had arterial dysfunction with increased PWV, carotid intima media thickness (cIMT), and reduced systemic arterial compliance. Vascular dysfunction correlated with hyperinsulinaemia and autonomic neuropathy as assessed by heart rate variability during breathing and postural manoeuvres (621). Similarly hypertension has been implicated as a strong risk factor for distal polyneuropathy observed in T2DM (622). Moreover treatment with ACE inhibitors has been shown to be associated with an improvement of nerve conduction velocity in distal symmetrical polyneuropathy (623). The Atherosclerosis Risk In Communities (ARIC) study also demonstrated an independent association of impaired cardiac autonomic control with the development of ischemic heart disease among individuals with diabetes (624).

In diabetes, insulin resistance within the cardiovascular system is associated with chronic low-grade inflammation, increased oxidative stress, lipotoxicity, and activation of the RAAS (402). These conditions promote serine phosphorylation of different insulin signalling molecules such as IRS-1 and the impairment of the normal tyrosine phosphorylation cascade (625), thus impairing insulin metabolic signalling.

1.10.1.1 Endothelial dysfunction in diabetes

Endothelial dysfunction is considered one of the important mechanisms in CV complications and is impaired from the onset of diabetes. It is still unclear

whether endothelial dysfunction is primarily caused by diabetes or other factors (626). Proposed mechanisms for diabetes related endothelial dysfunction are as follows:

1. Hyperglycaemia leads to increased intracellular glucose concentration within ECs causing structural changes in ECs in the form of increased deposition of collagen and fibronectin. It also decreases endothelial proliferation, NO production and increased apoptosis (627;628).

2. Hyperglycaemia alters EC function indirectly by the alteration of growth and vascular factors in other cells (629)

3. Other associated metabolic alterations (dyslipidaemia, hypertension and inflammation) also cause endothelial dysfunction (630).

1.10.1.2 Diabetes induced mitochondrial dysfunction and vascular disease

Diabetes-associated hyperglycaemia affects mitochondria in ECs; mitochondrial dysfunction plays a central role in endothelial dysfunction in T2DM (631). Hyperglycemia induced mitochondrial dysfunction cause vascular dysfunction through at least three pathways: mROS production, apoptosis and damage memory.

Mitochondrial dysfunction in T2DM is evident from lower mitochondrial O_2 consumption, ψm, GSH/GSSG ratio, and higher mROS production (632). Hyperglycaemia increases ROS production by the mitochondrial electron transport chain causing vascular damage (633;634), whereas activation of AMPK reduces hyperglycaemia-induced mitochondrial ROS production and promotes mitochondrial biogenesis in ECs (635). Recently, Li et al. also showed that endothelium-selective activation of AMPK prevents diabetes-induced impairment in vascular function and favours reendothelialization (636).

Hyperglycaemia induces EC apoptosis, and in addition to elevated mROS, mitochondrial membrane depolarization is also implicated in hyperglycaemia-induced apoptosis of human aortic ECs (637).

The existence of persistent vascular damage and poorer CVD outcomes in people with diabetes despite apparently good glycaemic control can be regarded as a paradox. However, the average level of glycaemic control since the time of diagnosis is much more important than the current level of control: the concept of glycaemic memory can be demonstrated and appears to contribute to prevention of CVD over long durations of follow-up (638-640). The mitochondrial ROS-driven hyperglycaemic stress is remembered in the vasculature even after glucose normalization and promotes vascular dysfunction. The mitochondrial adaptor protein p66Shc has a critical role in the hyperglycaemic memory. When EC from human aorta and aortas of diabetic mice were exposed to high glucose, the activation of p66Shc by protein kinase C ß II (PKCßII) persisted even after achievement of normoglycaemia. Persistent p66Shc up regulation and mitochondrial translocation are associated with continued ROS production, reduced NO bioavailability, and EC apoptosis. After achievement of normoglycaemia, *in vitro* and *in vivo* gene silencing of p66Shc, blunted ROS production, restored endothelium dependent dilatation and attenuated apoptosis (641).

In summary, hyperglycaemia upregulates mROS production, impairs ROS buffering system, damages mitochondrial DNA, alters mitochondrial membrane potential and finally impairs the balance between anti-apoptotic and pro-apoptotic pathways; all leading to endothelial and vascular dysfunction.

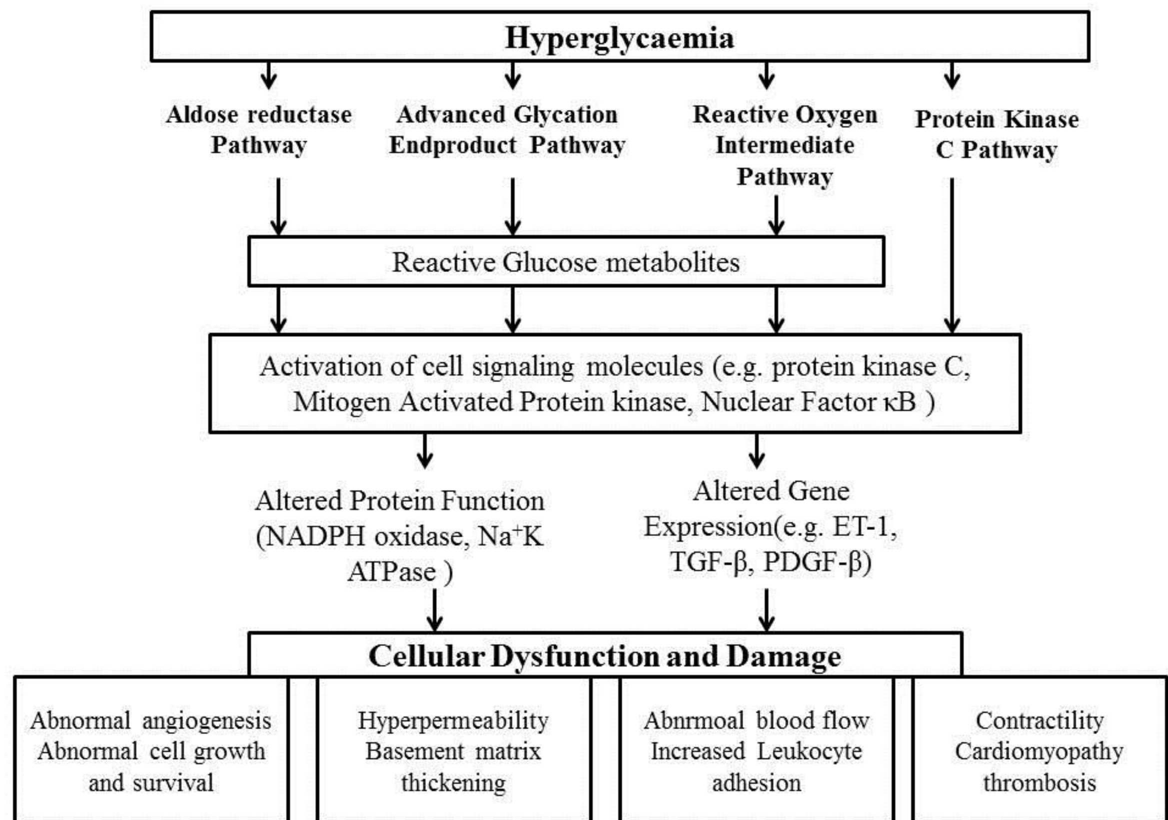


Figure 1.10 The toxins and signalling pathways contributing to hyperglycaemia's adverse effects for complications (642)
Redrawn with permission from Scott JA, Annals of the New York Academy of Sciences, 2004

Part 4

1.11 Age-related changes in body composition

Cardiovascular risk factors are positively correlated with obesity, abdominal obesity and visceral adipose tissue and negatively with fat free mass or lean mass (643;644). During the normal process of ageing there is a change in body composition; increasing fat mass and decreasing fat free mass or lean body mass (645-648). Kyle et al. has demonstrated in a population of 5225 healthy white people that fat free mass in men peaked at 35-44 years whereas in females this occurred at 45 to 54 years (decreasing in both genders afterwards). Fat mass remained stable until the age of 44 in females and 54 in males and then increased with age in both genders (649). Thus weight in healthy young people is due to both fat mass and fat free mass, but fat mass dominates in the older population (649). Similarly Sylvia et al. have shown in an Austrian population of 513 women and 412 men between age range of 19- 92 years (mean ± SD 51.7 ±

15.2) that in both sexes there is an increase in fat mass and decrease in lean body mass with increasing age (648). The steep increase/decrease is within the age range of 40-49 years in women and between 50-59 years in men (648). In relation to additional adiposity measures, Nassis and Geladas reported in 441 healthy Greek women that BMI, fat mass, waist to hip ratio and skin fold thickness, all started increasing from the age of 50 (steep increase)(650). Similarly Jackson et al. reported in 7265 American men that fat free mass increased until the age of 47 years and then declined afterwards, whereas fat mass increased from 20 to 96 years. They also reported that even if BMI remains the same with age, the proportions of fat mass and fat free mass change over time (647). Ito et al. reported differences in increasing fat mass in relation to age. In 2411 Japanese people, they showed that LBM remained constant until the fifth decade and then decreased in both sexes. For fat mass there was a linear increase in females while in males there was a curvilinear pattern with peak in 40-50 years (646). Moreover, Kuk et al also demonstrated that WC and VAT are age dependent in 483 white American and Canadian population (651). Table 1.7 and Figure 1.12 shows the age-related changes in fat mass and fat free mass in males and females in different ethnic population.

Table 1.3 Age-related differences in body composition and cardiovascular risk factors

Author	N	Male/ female	Ethnicity	Age range	Fat mass Increase	FFM peak age
cross sectional						
Kyle UG et al. 2001(649)	5225	2735/ 2490	White (Western European)	15-98	Progressed between 15-98 years	M: 35-44, F: 45-54
Sylvia 2010 (648)	925	412/ 513	Austrian	19-92	Gradual ↑ in both but slope ↑ in F: 40-49 and M: 50-59	M: 50-59, F: 40-49
Chittawatanarat K et al. 2011 (645)	2324	1000/ 1324	Thai	18-60+	M & F: 55 -60	M: 50, F: 40
Nassi GP 2003 (650)	441	441 women	Greece	18-69	F: 50 (peak)	Remain unchanged
Henche SA et al. 2007 (652)	1113	397/ 716	Spain	0-80	M: ↑ from 50 F: ↑ from 36	M: 40-45 F: 40
Ito H et al. 2001 (646)	2411	625/ 1786	Japanese	20-79	M: peak 40-50 F: linear ↑ through life	M & F: 40
Larsson I et al. 2004 (653)	1135	524/ 611	Swedish	37-61	M & F: Linear ↑ in both	M: 51 F: 46
Longitudinal						
Jackson AS 2012 (647)	7265	7265 men	USA	20-96	↑ from 20- 80	M: 47

N= no of people, FFM= fat free mass, F= female, M= male

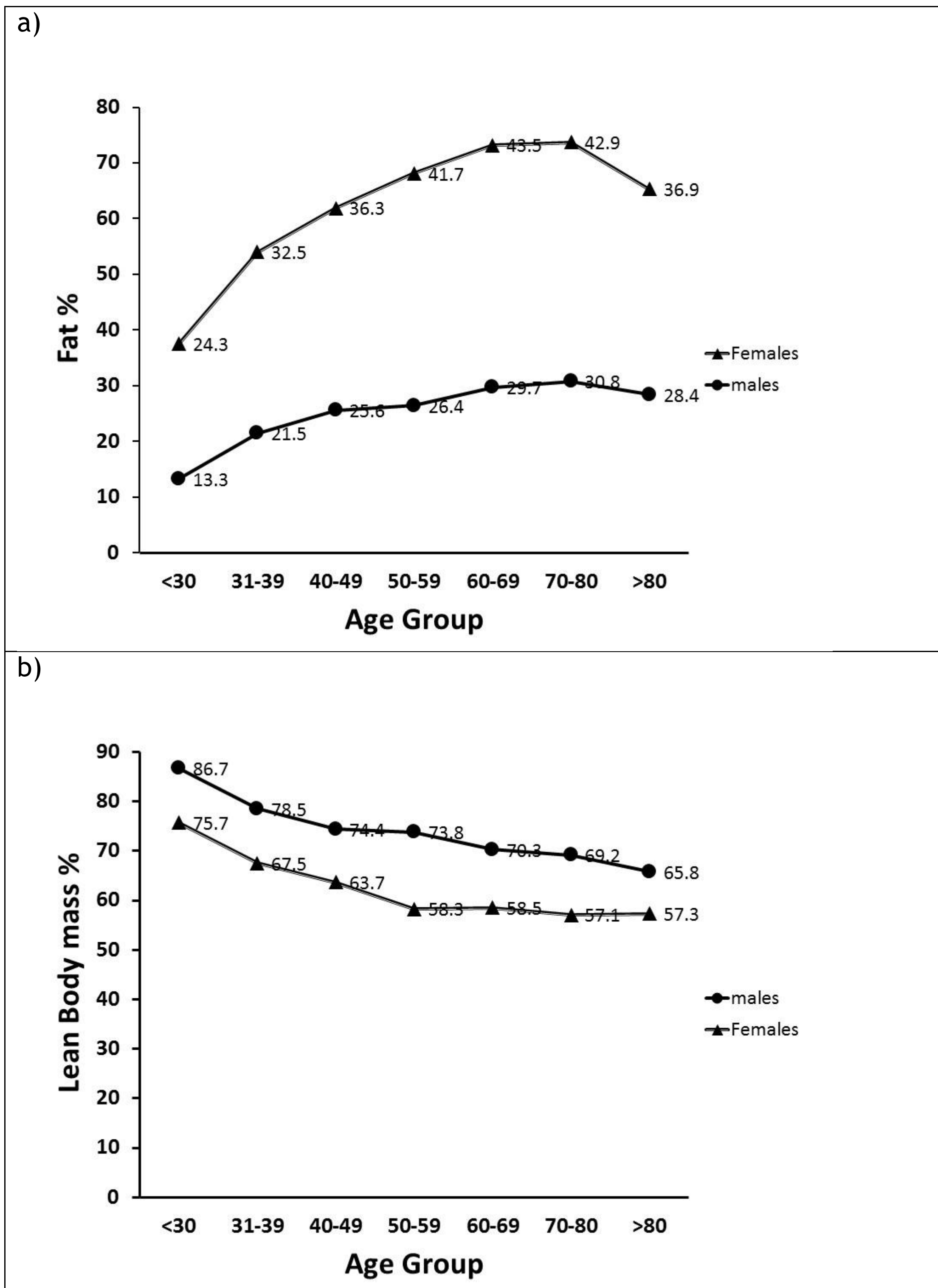


Figure 1.11 Changes in fat and fat free mass with age
Adapted from Sylvia 2010 (648).

1.11.1 Age, body composition and inflammatory markers

The concentration of inflammatory markers also changes (increases) with age. In addition to increasing adiposity with age, ageing is also associated with redistribution in the pattern of obesity; decrease in subcutaneous adipose tissue (SAT) and increase in visceral adipose tissue (VAT) (651). Cartier et al. reported

that in a French- Canadian male cohort VAT increased with age along with increases in inflammatory markers CRP, IL-6 and TNF-α. This may have been related to increases in VAT and not age as differences in inflammatory markers between young and middle age (< and > 40 years) were eliminated after adjustment for VAT (654).

1.11.2 Age, body composition and autonomic nervous system (ANS)

With increasing age there are alterations in ANS responses which may be associated with changes in body composition and change in distribution of fat. Christou et al. demonstrated that ANS responses like baroreflex sensitivity and heart rate variability were significantly different in younger and older men. The univariate effect of age was abolished after adjustment for total and abdominal body fat and abdominal-to-peripheral body fat distribution(655). This effect of adiposity, especially VAT on ANS was also observed in young men (656) and young women (657). Obesity causes depression of parasympathetic nervous system activity manifested as a decrease in heart rate variability (HRV); this is accompanied by over-reaction of sympathetic nervous system on exercise (handgrip) causing raised BP (657).

1.11.3 Sex-related differences in body composition and cardiovascular risk factors

Henche et al. compared 113 Spanish males and females from age 0-80, and showed that all measures of adiposity (fat mass, LBM, trunk/legs fat mass) are different between the sexes from ages 10 to 75 years (652). Similar differences were also reported in fat mass, fat free mass, waist circumference and centralized fat distribution in 1135 Swedish males and females (653). In addition Kuk et al. demonstrated that for the same WC females have more adipose tissue, although with increasing WC this difference diminishes (651). Sex differences will also be explained below (Section 1.12)

1.11.4 Menopausal effects on body composition and cardiovascular risk factors

In a study of 316 Chinese women aged 40-59 years, Chen et al. showed no effect of different menstrual status (pre or post menopause) on BMI, body weight and waist circumference(658). In contrast, Diebert et al. reported higher BMI, fat mass, WC, SBP, TG and glucose in postmenopausal women. However, this was without adjustment for age (659). Douchi et al. also demonstrated that ageing made a more significant contribution than menopause to changes in body composition, especially truncal obesity, in 642 Japanese women (aged 20-53) (660). In keeping with these findings, Aloia et al. additionally showed the effect of HRT in a randomised control trial over three years in 118 American women, reporting that fat mass increased and lean body mass decreased after menopause but HRT had no positive effect (661). Moreover, Douchi et al also demonstrated in a study of 365 pre and 201 postmenopausal Japanese women (aged 20-70 yrs) that lean mass and bone mineral density (BMD) were inversely correlated with age and that fat mass and truncal obesity were positively correlated with age (662).

From twin study data, Schousboe et al. conclude the that adult adiposity phenotype is highly heritable; however, with advancing age, the importance of environmental factors increases while the genetic influence decreases (663).

1.12 Sex differences

Sex differences in lean muscle mass, visceral adiposity, insulin sensitivity, the impact of ageing, menopausal transition, and altered susceptibility to free fatty acid are well described in humans (664). Similarly the influence of sex on the clinical expression and pathophysiology of obesity and other cardiovascular risk factors and disease has been well studied but will be reviewed briefly here

1.12.1 Sex differences in prevalence of dysglycaemia

As discussed above, in healthy people, the fasting blood glucose concentration depends on basal secretion of insulin and insulin sensitivity of the liver, limiting hepatic glucose output. Similarly after taking a meal or ingestion of a carbohydrate load there is surge of insulin secretion from pancreatic β-cells

which enhances glucose uptake in insulin sensitive liver and muscle. Impaired fasting glucose (IFG) mainly depends on the basal secretion of insulin while impaired glucose tolerance (IGT) is associated with peripheral insulin resistance at the skeletal muscle level, where most postprandial glucose disposal occurs. IGT is considerably more prevalent than IFG (665).

IFG is 1.5-3 times more common in men than in women in nearly all age groups, but up to 7–8 times higher in older men (50–70 years). However, the prevalence of IGT is higher in women albeit with minor ethnic differences (665).

1.12.2 Sex differences in body fat distribution, adipose size and function

Around half a century ago when describing adipose tissue accrual in the upper body, men were described as having android obesity (trunk and abdomen) and women gynoid obesity (hips and thighs) (666). Men have twice as much visceral adipose tissue (VAT) as premenopausal women, (667) even though women have higher total body fat, BMI, and abdominal subcutaneous adipose tissue (SAT) (668). In men VAT is linked to total body fat and increases with weight gain while in women VAT is less associated with total adiposity (668). Similarly for the same waist circumference, men have more VAT than women (651). It has been demonstrated that when obese people lose weight, men lose relatively more VAT then women even if both have similar weight loss (669). Therefore, men have potentially greater improvements in metabolic profile than women, even with similar levels of weight loss.

Obese women accumulate more fat in the gluteofemoral region than their leaner counterparts, but this is not the case in men (670). During weight gain, lower body adipose tissue tends to expand via adipocyte hyperplasia in women but via adipocyte hypertrophy in men. Moreover, lower-body SAT adipocytes of women tend to be larger than men but the sex differences in abdominal SAT adipocyte size are less marked (670;671). Irrespective of obesity level, adipocyte size is an important determinant of adipocyte function and metabolic activity (adipokine secretion), and larger adipocytes have higher basal and stimulated rates of lipolysis. Larger adipocytes predominantly secrete pro-inflammatory adipokines (672).

Leptin expression is greater in subcutaneous than in visceral adipocytes and as women have more subcutaneous fat than men, so leptin signalling and effects are different in males and females (673), also explained in Section 1.7.6

As well as being released by the ovaries, oestrogen is produced through peripheral aromatization in fat and other tissues in both sexes. It plays a role in cell proliferation, differentiation, and homeostasis (674;675). Oestrogen has multiple effects in different body systems. Some important functions of oestrogen include:

(a) Anorexigenic action (decrease in appetite) via the central nervous system.

(b) Increased glucose transport in skeletal muscles.

(c) Prevention of visceral fat accumulation

(d) Decreased lipogenic activity of lipoprotein lipase in adipose tissue, and

(e) Anti apoptotic effects on pancreatic ß-cells (674).

After the menopause there is alteration in partitioning of fat with a preferential increase in visceral adiposity (676). Due to these metabolic, hormonal and fat partitioning changes, many mechanisms may contribute to the development of HTN including endothelial dysfunction, activation of the RAS, activation of the sympathetic nervous systems, oxidative stress, and increased pro inflammatory mediators (664). However, for incidence of CVD, a recent meta-analysis of five prospective cohorts (showing sex stratified results), did not show any sex related difference in incidence of CVD in people with metabolic syndrome (664).

1.13 Ethnicity and diabetes

South Asians have differences in cardiovascular (CV) risk factors and diabetes as compared to Europeans and other ethnicities (677). In the 2011 UK (NICE) guidelines, the reason for the fourfold or even higher risk of diabetes in South Asians as compared to Europeans is attributed to weight, diet and physical activity (678). However, epidemiological findings are different; Bangladeshis in the UK have lower weight (and BMI) than White, Indians or Pakistanis yet have

the highest rate of type 2 diabetes (679;680). Similarly, consumption of fruit and vegetables by Bangladeshi people is similar to the general population (as is fat consumption) yet there is a higher prevalence of diabetes (680;681). South Asians have low muscle mass (682), especially in buttocks and legs (683). This phenotype is insulin resistant and is characterized by reduced total body capacity for oxidation and this insulin resistance is not associated with lipid content of the muscle (683;684). In South Asians excess energy (from higher caloric intake) in the form of fat is stored in the truncal and deep subcutaneous tissues which are highly active and lead to derangements in metabolic profile (685). Excess fat is also stored in ectopic fat depots intra abdominally, and in the liver, pancreas and around the heart (686). In Europeans excess fat (energy from caloric intake) is principally deposited in superficial subcutaneous compartments, especially those in lower limbs. Fat in these superficial subcutaneous compartments is either neutral or even protective in relation to cardiovascular and metabolic outcomes (687-690). Several theories and mechanisms are proposed for the ethnic differences in early development or higher prevalence of diabetes and are summarised below.

1.13.1 Birth and early life

At birth, South Asians babies are smaller in size than European babies but even then have more fat mass (and lower muscle mass) than Europeans (691). Smaller babies have fewer β-cells in pancreatic islets (692). This may be a consequence of intrauterine programming (Barker hypothesis- Section 1.3.7.3)

1.13.2 Childhood and early adulthood

Size at birth is not a problem in itself but in later life the mismatch between phenotype at birth and later phenotype is manifested as a higher level of subcutaneous truncal fat (693). In adult life small babies have comparatively large, metabolically active adipocytes (686;694).

South Asian children in UK, along with their parents have low physical activity compared to Europeans (695). As leisure time activity or exercise is determined by cultural factors and resistant to change (696), a sedentary lifestyle is passing between generations.

1.13.3 Metabolic changes in later adulthood and middle and old age

Nair demonstrated that mitochondria in South Asians have different oxidative phosphorylation capacity, and convert more energy from calories ingested into ATP (and thereafter) with less heat generated (697;698). In addition, the energy requirement of South Asians is less, due to lower lean mass and more adipose tissue given that adipose tissue is metabolically less active than muscles. The combination of all of these factors, i.e. efficient mitochondria, less lean mass, lower metabolic rate, truncal and liver fat deposition and insulin resistance render South Asians more prone to the adverse effects of excess energy intake (677). These effects (of excess energy intake) therefore occur in South Asians at a lower BMI compared to Europeans. It has also been demonstrated clinically that, to achieve the metabolic profile of a European origin person with a BMI of approximately 30 kg/m2, a South Asian person needs a BMI as low as 22 kg/m2 (699-701). The recent International Diabetes Federation (IDF) has defined central obesity according to ethnicity keeping in regard the ethnic distribution of fat. For SA women, the cut-off for waist circumference is the same as for EU women (≥80 cm), but for SA men the cut point is lower than for their EU counterparts (≥90 cm versus ≥94 cm) (702).

Clinically overt diabetes develops at the time of ß-cell failure in the pancreas. There are also many postulated mechanisms for early ß-cell failure in South Asians, for example: a low volume of pancreatic ß-cells islets at birth (due to genetic or developmental reason); and a high rate of ß-cell apoptosis (due to genetically low resistance to insult or ectopic fat and other metabolic stresses). Increased workload on these cells due to insulin resistance may lead to early ß-cell exhaustion (195) (see Section 1.5.2 above).

1.13.4 Ethnicity and CVD

1.13.4.1 CVD in South Asians

Coronary heart disease (CHD) mortality is 90% greater in SA than in EU individuals and this increase is not explained by CV risk factors like smoking, hypertension, hypercholesterolaemia and features of the insulin resistance syndrome (703). CHD risk is high in young Asian men (704;705) and sex-related

differences in CHD risk are also less marked in Asians (705;706). The Study of Health Assessment and Risk Evaluation (SHARE) also reported that SA had higher prevalence of carotid atherosclerosis and associated CVD compared to EU and Chinese individuals (707). In the INTERHEART study, it was demonstrated that at the time of their first heart attack SA were younger (median age 52 years) than the overall population (median age 58 years), or EU population (median age 62 years) (708).

1.13.4.2 Risk Factors in South Asians; conventional and novel

South Asians (SA) have a less favourable metabolic profile than Europeans (EU) as has been shown in the Southall study (709). It showed that among men with similar BMI (mean BMI: SA 25.7 kg/m2; EU 25.9 kg/m2), SA compared with EU men had higher diabetes prevalence (20% versus 5%), fasting insulin levels (9.8 mU/L versus 7.2 mU/L), insulin levels after glucose load (41 mU/L versus 19 mU/L), systolic blood pressure (126mmHg versus 121 mmHg), waist/hip ratio (0.98 versus 0.94) and triglyceride (TG) levels (fasting: 1.73 mmol/L versus 1.48 mmol/L) and lower high-density lipoprotein-cholesterol (HDL-C) levels (1.16 mmol/L versus 1.25 mmol/L). Furthermore, the prevalence of metabolic syndrome was higher in SA (46.3 %) than EU (18.8 %) according to WHO definition (710). SA develop diabetes at a younger age (46 years versus 57 years) and at lower BMI (28.7 kg/m2 versus 29.9 kg/m2) than EU (711). In addition it has also been shown that total caloric intake is higher in immigrant SA (712).

1.13.4.3 Physical activity

Fischbacher et al reported in a systematic review of 17 UK studies, that SA had a substantially lower level of physical activity and fitness than the local white population. The difference was even greater in women for all types of activities: sports, cycling and heavy manual work (713).

1.13.4.4 Vascular factors

Plasminogen activator inhibitor 1 (PAI-1)

Plasminogen activator inhibitor 1 (PAI-1) is a prothrombotic factor released by the endothelium and also adipose tissue. Its physiological role is inhibition of

fibrinolysis (degradation of blood clots). SA have been shown to have increased levels of prothrombotic factors including PAI-1 and fibrinogen compared with Europeans (707;714). PAI-1 is an independent predictor of diabetes and also plays a role in the development of vascular disease in people with insulin resistance (715).

Homocysteine

Homocysteine is linked with development of endothelial dysfunction and atherosclerosis and its concentration is higher in SA men compared with EU (716).

Lipoprotein (a)

Lipoprotein (a) is a genetically determined factor: a meta-analysis of 67 prospective studies show a clear association of lipoprotein (a) with increased risk of CHD (717). SA show increased circulating lipoprotein (a) levels compared to Europeans (712;718).

Adiponectin

As discussed in Sections 1.7.1, 1.7.2, 1.7.4.6 and 1.7.6, adiponectin has beneficial effects in glucose and lipid metabolism and also protect against vascular and metabolic dysfunction via anti-inflammatory pathways (719;720). People with central obesity have low adiponectin levels (719) and its levels are also reduced in type 2 diabetes (720). SA have significantly low levels of adiponectin compared to EU matched for age and BMI (721).

CRP

SA have higher CRP levels compared to EU and CRP levels are more closely associated with visceral adiposity and insulin resistance. The difference was even higher in women (722;723).

1.13.4.5 Early development of risk factors

Yajnik et al reported that SA babies were small but had more central obesity measured by subscapular skinfold thickness and this subscapular skinfold was better preserved than triceps skinfold thickness further augmenting the central adiposity tendency present at birth (691). Similarly cord blood leptin concentration, a reflection of percent body fat mass, was higher in SA babies even when adjusted for the difference in weight (691). Circulating Insulin concentration was also higher at birth in SA babies demonstrating an insulin resistant phenotype at birth (691). Similarly, immigrant SA children (around 10 years of age) were more insulin resistant than EU children (724). Din JN et al demonstrated increased pulse wave velocity (PWV) in healthy young SA men. PWV is an index of arterial stiffness and a marker of cardiovascular events (725).

1.13.4.6 Role of immigration

Immigration may also have an effect and is associated with atherosclerosis (726) and CVD (727). This may be related to dietary changes towards high calorie and refined diets which are very common in immigrants (728).

1.13.4.7 CVD in Other Ethnic Groups

African/ Caribbeans

It was reported in the Southall study that African/ Caribbean people had higher BP (128mmHg versus 121 mmHg) and greater prevalence of diabetes (15% compared with 5% in Europeans). However, they had healthier (less atherogenic) lipid profile than Eu, HDL-C (e.g. 1.37 mmol/L versus 1.25 mmol/L, respectively) and triglyceride levels lower (1.09 mmol/L versus 1.48 mmol/L) (709). CHD mortality was almost similar in African-American and White men (224 versus 236 per 100,000), but stroke mortality was much greater in African American men (89 versus 62 per 100,000) and women (76 versus 58 per 100,000) compared with white (729).

Chinese

People of Chinese ethnicity have a favourable risk factor profile with lower rates of smoking and obesity, and lower levels of total and LDL cholesterol, but have higher BP levels (730). It has been reported that Chinese men have a lower prevalence of CHD (4.9%) as compared to EU men (16.6%) (730), but the incidence of stroke is higher in the Chinese as compared to EU population (731).

Conclusion

These data suggest that there is a higher prevalence of metabolic derangements, diabetes, higher CV risk factors and cardiovascular disease among South Asians. Unhealthy CV risk factors are present at birth and are exacerbated more easily with obesity or other factors and there is also a culturally-determined trend towards physical inactivity and higher sedentary behaviour. The results from the INTERHEART study showed that smoking, hypertension, diabetes, dyslipidaemia and obesity accounted for 80% of the population risk of CHD in all ethnic groups and across all geographical regions. It also showed that 80% of the risk of CHD can be reduced by being physically active, consuming fruits and vegetables, moderate amount of alcohol intake and decreasing and/or stopping smoking (708). It is suggested that within population risk is determined mainly by environmental factors, while between-population risk (ethnic difference in risk) is determined by a larger genetic element (732). So within populations, ethnicity-specific guidelines on primary and secondary prevention of CVD may be an appropriate solution.

1.13.5 Is the ethnic difference in CVD explained by diabetes?

It is well known that type 2 diabetes is one of the most important risk factors in relation to CVD (733). In many large prospective studies it has been shown that there is two to threefold increase risk of CVD in people with diabetes (734-736). Ethnic variation in prevalence of diabetes (736) and poor CVD outcomes in some ethnic groups has also been well documented (708;734). Ethnic variation in glucose control in people with diabetes (734) has been described in the literature but after the diagnosis of diabetes management of blood glucose (although the main pathology determining diabetes) is not a potent method of

reducing CVD risk (640;737). Tanika NK et al demonstrated in a systematic review of five trials indicating that intensive glucose control mildly reduced the CVD risk (relative risk [RR], 0.90 [95% CI, 0.83 to 0.98) but did not reduce the risk for cardiovascular death or all-cause mortality (although increasing the risk of severe hypoglycaemia) (738). Overall the available literature is controversial regarding the relation of ethnic variation in diabetes and related glucose control with cardiovascular disease outcomes. Further evaluation of risk of CVD is needed in different ethnicities irrespective of diabetes, glucose control or duration of diabetes.

1.14 Summary of the introduction

Cardiovascular disease is the leading cause of death worldwide. Its prevalence is decreasing in developed or high income countries potentially as a result of better health facilities, prevention programmes and screening. However it is on the rise in the low and middle income countries in which the majority of the world's population resides. Many cardiovascular risk factors have been studied for decades but the absolute risk of a particular individual for developing CVD cannot be predicted with accuracy. This has led to a search for novel risk factors and further study of the mechanisms of diseases in an effort to optimise prevention and management strategies.

Hypertension is a well-known risk factor for coronary heart disease and also cerebrovascular disease. The association of insulin resistance and inflammation with the development of hypertension remains controversial despite a large number of studies. Although many animal studies suggest a causal association of inflammation with hypertension, few human epidemiological studies have supported this finding. It remains uncertain whether inflammation is an inducer of high BP or is merely a step in the pathological process towards developing hypertension – similar questions apply for insulin resistance. Similarly, it is still debated whether impaired vasodilatation associated with hypertension, is a cause or an effect of insulin resistance.

When considering ethnicity, the relative contribution of cardiovascular risk factors in the development of CVD is different in different ethnic groups. South Asians develop diabetes and CVD early while others (Chinese and African

Caribbeans) have a greater incidence of stroke as compared to CHD. The effect of intense glucose control in reducing the incidence of CVD is limited.

In this thesis, I have therefore evaluated CV risk factors in a healthy population (RISC) and a diabetic (SDRN) population to examine their inter-relationships. In particular, I focused on whether the various risk factors behave differently in different groups defined by age, sex and ethnicity.

1.15 Aims and Objectives

In this thesis I aimed to evaluate the associations amongst novel cardiovascular risk markers (insulin resistance, inflammation) and traditional cardiovascular risk factors (hypertension, obesity) as well as evaluating the independent role of ethnicity in relation to cardiovascular complications in T2DM.

There were five specific objectives:

1. To evaluate insulin sensitivity as an independent longitudinal predictor of BP rise in healthy adults in a healthy European population.

   Hypothesis: insulin resistance is an independent predictor of BP rise and/ or incident hypertension

2. To conduct a systematic review of the relationships between two markers of low grade inflammation (IL-6 and CRP) and BP/hypertension, considering the roles of adiposity and insulin resistance.

   Hypothesis: low grade inflammation is associated with BP and incident hypertension independently of adiposity and insulin resistance

3. To evaluate low grade inflammation as an independent predictor of BP rise in a healthy European population well-characterised for insulin sensitivity.

   Hypothesis: low grade inflammation is associated with BP rise and/ or incident hypertension independently of adiposity and insulin resistance.

4. To determine the main predictors of change over time in systolic and diastolic BP in different age and sex groups in healthy adults.

   Hypothesis: Predictors of systolic and diastolic BP change over time differ according to age, sex and body composition.

5. To evaluate the role of ethnicity in the development of cardiovascular disease in relation to the control and duration of diabetes.

   Hypothesis: Ethnicity is an independent risk factor for cardiovascular disease

2 Methods

Chapter 2

Basis for measurements of the cardiovascular risk factors

The methodological background including measurement of obesity, insulin resistance, inflammation and diabetes are explained and critically appraised, along with potential alternative measurement options. This section includes detailed aspects of the measurement of body composition, insulin resistance and inflammatory markers in relation to the RISC cohort and the SDRN cohort. The relationship of these measures with cardiovascular risk is also summarised. The cohort specific methods will be discussed in the relevant section below.

2.1 Measurement of obesity

The most common measurements used for measuring adiposity or body fat mass are indirect and include body mass index (BMI), waist circumference (WC), and waist-to-hip ratio (WHR). More complex ways of measuring adiposity are laborious and costly and so less commonly used for population studies: including skin fold thickness, bioelectrical impedance, underwater weighing (hydrostatic weighing), dual-energy x-ray absorptiometry (DXA), magnetic resonance imaging (MRI), computed tomography (CT), whole body air displacement plethysmography (ADP) and isotope dilution. Some of the measures are briefly discussed below:

2.1.1 Body mass index

BMI is the most widely used measure of obesity and is used for screening of individual and also population studies (due to cost effectiveness). It is calculated by a simple formula; weight in kilograms (kg) divided by the square of individual height in meters (kg/m^2). Standardized cut-off points have been developed by the World Health Organization (WHO) and are used by various national and international organisations (739) (Table 1.3). The National Health Service (NHS), in the United Kingdom also recommends using the same WHO BMI cut-offs to identify those needing interventions.

Table 2.1 Body Mass Index category for adults

Classification	BMI range (kg/m^2)
Underweight	<18.5
Normal-weight	18.5 to 24.9
Overweight	25 to 29.9
Obese	≥30
Class I	30 to 34.9
Class II	35 to 39.9
Class III	≥40

South Asian populations are more prone towards obesity related diseases and so additional intermediate cut-off points are suggested in this population group for labelling risk; 23.0 kg/m^2 and 27.5 kg/m^2 for increased risk and higher risk respectively (740).

The main advantage of BMI is that only height and weight is required for calculation and the same cut-off can be applied to all ages and both genders. In children and adolescents, BMI calculation method is same as for adults but age and sex specific percentiles are used to determine cut-offs. BMI has been used for decades and across the globe and so provides opportunities to compare it over time and between different populations. BMI within the normal limits is ideal and both underweight and overweight to obese have increased morbidity and mortality (741). Despite its usefulness, BMI is based on simple weight measurement and does not differentiate between fat and lean mass (742). BMI only categorises individuals as normal, overweight or obese and this can be misleading particularly in the case of athletes and body builders who have a high lean body mass. Similarly women and old people may have same BMI but are likely to have more body fat as compared to men and young people. However, misclassified individuals are relatively uncommon at a population level as BMI correlates well with the direct measure of body fat at population level (743) and so is still used extensively in research and at population level.

2.1.2 Waist circumference (WC)

WC is the most widely used measurement of central adiposity as it is strongly correlated with central or abdominal fat mass (744). It is a very easy and cheap method and can be easily assessed with a normal inelastic measuring tape. It is

measured between the two bony landmarks i.e. mid-way between the lower border of last palpable rib and the upper border of the iliac crest in standing position and at the end of a gentle expiration (745). The WHO has recommended sex-specific thresholds for WC which have been adopted by many national and international organizations for community and clinical settings (Table 1.4).

Table 2.2 Waist circumference categories

Classification	Men	Women
Normal-weight	<94 cm	<80 cm
Overweight	94 cm to 102 cm	80-88 cm
Obese	>102 cm	>88 cm

For Chinese and South Asian adults, lower thresholds of <90 cm and <80 cm are recommended for men and women respectively (746). Several studies have shown that BMI is not a good estimate for central adiposity and is the reason why WC has gained a considerable attention as a complementary or alternative anthropometric measure to BMI (747). WC use in children is limited due to non-availability of recommended cut-offs. In addition its use is challenging in some populations as it requires physical contact and also lifting the shirt of the participants. Training of staff is also required to ensure accurate measurements between the two bones and at the end of expiration; both can change the results. WC is widely used but not as commonly as BMI, particularly in population studies.

2.1.3 Waist-to-hip ratio (WHR)

WHR is the second most widely used measure of central adiposity after WC, and is shown to be significantly correlated with abdominal fat (745). WHR is an indirect estimate of abdominal and hip fat mass; the latter being representative of subcutaneous (less morbid) fat. In contrast to WC it requires two measurements; waist and hip circumference. Hip circumference is measured at the widest part of the buttocks (at the level of the greater trochanter) using a stretch resistant measuring tape. Both measurements should be done in a relaxed standing position with feet together, and at the end of gentle expiration. Two measurements should be taken and in the case of one

centimetre difference, the mean should be calculated. The WHO recommended sex-specific thresholds of WHR are given below (Table 1.5).

Table 2.3 Waist-to-hip-ratio categories

Classification	Men	Women
Normal-weight	<0.94	<0.80
Overweight	0.94 to 0.99	0.80-0.84
Obese	≥1	≥0.85

South Asians and other ethnic groups have different BMI cut-offs, similarly the cut-offs for threshold of high risk WHR for the South Asian ethnic groups is <0.90 for men and <0.80 for women (748). As compared to BMI, central obesity is a stronger predictor of adverse outcomes (749). Recent studies showed that WHR was more strongly associated with CVD than BMI and WC (750;751). Although WC is strongly associated with diabetes (752), WHR is a stronger predictor of myocardial infarction compared to other measures (753). Recently Huxley et al. compared BMI, WC and waist hip ratio as predictors of CV risk (diabetes, hypertension and dyslipidaemia). They showed in both men and women that measures of central obesity were superior to BMI as discriminators of cardiovascular risk factors, although the differences were small and unlikely to be of clinical relevance (754). Moreover South Asian (SA) have higher prevalence of type 2 diabetes and CVD at lower BMI. Moreover, SA have lower BMI's at a given percentage of body fat compared with Europeans (754). WHR has certain limitations, 1) the need for two measurements thereby increasing the chance of measurement error, 2) expression of the result as a ratio which may be more difficult to interpret, 3) the need for physical contact and training. In addition fewer studies are available for ethnic groups other than White and so is difficult to derive ethnic-specific cut-offs. Moreover, due to less literature reference percentiles for use in children is still not recommended by WHO, although different population specific percentiles are available (755).

2.1.4 Direct and other measures of adiposity

2.1.4.1 Skin fold thickness

A special calliper can be used to measure subcutaneous body fat. The skin fold thickness is measured by pinching the skin at a number of predefined points on

the body, such as upper arm, trunk, and thighs (756). The recorded measurements are then compared with age-sex-specific charts.

2.1.4.2 Underwater Weighing

Underwater weighing is the gold standard method of directly measuring body fat. Participants are weighed in air and then weighed again after they are submerged in a specialized tank in a laboratory setting. This procedure is based on Archimedes Principle; where density of different body components (fat mass, lean mass) is compared with the density of water. Individuals with high fat mass weigh less inside the water compared to those with a high lean mass. In addition residual lung volume is also calculated in maximal inhalation and about 100 cc of air is estimated as air trapped in the intestines (757;758). All these values are then put in the following equation to calculate body density

$$\text{Density of body} = \frac{\text{density of water} * \text{weight of body}}{(\text{Weight of body} - \text{weight of immersed body}) - \text{density of water} * (\text{residual lung vol} + 100\text{cc})}$$

2.1.4.3 Whole body air displacement plethysmography (ADP)

Air displacement plethysmography (ADP) measures body composition using the same principles as underwater weighing except with air instead of water. Subjects are asked to remove clothes and enter a sealed chamber that measures their body volume through the displacement of air in the chamber. The body volume is combined with body mass to determine body density. The ADP uses known equations to estimates the percentage of body fat and lean body mass by using the previously calculated body volume, mass and density. ADP is a very fast, simple and non-invasive technique and does not uses x-rays and so is preferred over DEXA and underwater weighing where available. In addition the acceptance of ADP is also high (100%) as compared to underwater weighing (69%), despite both giving almost identical results (759;760). ADP measurements are extremely reproducible, making them ideal for monitoring pharmaceutical therapy, nutritional or exercise intervention, or sports training. However ADP was not a feasible option for use in the RISC study due to the cost of the equipment for each centre in a multicentre study.

2.1.4.4 Bioelectrical impedance or bioimpedance

Bioelectrical Impedance Analysis (BIA) using a variety of proprietary devices is one of the most reliable and accessible methods of screening body fat and composition in clinical settings. As such it was measured in participants of the RISC study in all study centres using the Tanita bioimpedance balance (a machine was purchased for each centre) (see Chapter 2, Section 2.1.6). In BIA, a person's height, age, gender and weight and other physical characteristics such as body type, physical activity level, ethnicity, etc. are first entered. Subjects are asked to have only a light meal at least one hour before and to void their bladder before the start of the measurements. More sophisticated methods are also available: in these, the person is asked to lie down and electrodes are attached to various parts of the body. If in the standing position, the measurements are done after a period of at least 10 min standing upright to minimize potential errors from acute shifts in fluid distribution. Weight is calculated and recorded by the balance. A small and safe electric signal (usually a 50 kHz, 500 µA current) is passed thorough the body and the impedance or resistance to the signal is recorded as it travels through the water that is found in body tissues. Every measurement is taken in duplicate and averaged unless the difference in two measurements was greater than 10 Ohms. The basis of BIA is that the greater the amount of water in a person's body, the easier it is for the current to pass through it. The more muscle a person has, the more water their body contains and so offers less resistance or impedance. In contrast, more fat a person has; less water is present, hence resistance is higher. BIA is a safe method and the electric signals passed cannot be felt at all either by an adult or a child (761;762).

Along with the body fat and body fat% BIA can be used for other measures including; body water %, muscle mass, physique rating, daily caloric intake (DCI), basal metabolic rate (BMR), metabolic age, visceral fat and bone mass (761-763). BIA has many advantages including being non-invasive, relatively inexpensive, portable, whilst not involving exposure to ionizing radiation and low between-observer variation. BIA can be done in healthy people or in chronic disease which have the validated BIA equations available. However, its use is not recommended in people at extremes of BMI ranges and in subjects with abnormal hydration (762).

BIA is also the preferred method for monitoring changes in body composition (fat mass and fat free mass) with time if they have normal hydration and BMI between 16-34 kg/m2. Studies suggest that BIA gives good results in individuals with stable water and electrolyte balance as abnormal hydration and electrolytes facilitate the flow of electric current. It is also complex to develop algorithms and equations in these abnormal hydration conditions (761;762). It has been suggested by some studies to use segmental or multi-frequency BIA or bioelectrical impedance spectroscopy in conditions of abnormal hydration, but these techniques require further research (761;762). Abnormal hydration conditions include: oedema, ascites, patients undergoing dialysis, kidney disease, liver pathology, cardiac disease, large volume intravenous fluids, diuretic therapy, post major surgery, patient in intensive care and pregnancy. Hypothyroid patients due to increased skin thickness and patients with any orthopaedic implants may also register inaccurate results and should not undergo BIA.

2.1.4.5 Imaging Methods

These are the ideal methods and their main advantage is accuracy and detailed body composition. Dual-energy x-ray absorptiometry (DEXA) uses low dose x-ray to record fat distribution in the body. It also calculates bone mineral density and muscle mass. The DEXA technique is based on the attenuation properties of bone, lean and fat tissues at two different x-rays energies. It measures directly the lean and fat mass and bone mineral content. The bone mass added to lean mass constitutes the fat free mass. DEXA usually takes 5 minutes to complete (764;765). Isotope dilution is another method where participants drink isotope-labelled water and then their body fat is calculated by analysing for isotope levels. Computerized tomography (CT) and magnetic resonance imaging (MRI) are the two other frequently used measures and can directly measure body fat mass in different parts of the body. Some of the limitations of imaging methods include: price, use of ionizing radiation, time, and suitability for serial measurements; these factors influence choice of method for large population studies. However they are mostly used in clinical settings or for validating other methods of body composition measurement. In general no agreed cut-offs are available for body fat percentage (BF%) but the routine values in men are; normal weight (<18%), overweight (18-25%) and obese (>25%). Similarly in

women the BF% values are; normal weight <25%, overweight 25-32% and obese >32%.

2.1.5 Comparison of imaging techniques and BIA

A lot of work has been done to compare BIA with imaging techniques with the main advantage that BIA is inexpensive and easy to use. It has been shown that BIA is significantly correlated with imaging techniques but it slightly over or underestimates different components of body composition when using different apparatus, subjects or body conditions (763;766;767).

Wang et al recently compared four and two limb BIA with DEXA and MRI for measuring body composition. They showed that both four and two limb BIA were significantly correlated (r= 0.7- 0.9) to both DEXA and MRI. The BIA underestimated body fat percentage, whereas the measurements for fat free mass were in close limits to DEXA and MRI (768). Similarly Pateyjohns et al. (769) compared BIA using three different methods (and three different machines) against DXA. Validity of BIA against DEXA was assessed using linear regression and limits of agreement analysis. BIA methods showed good correlation but poor agreement with DEXA in overweight and obese men (769) and in Hispanic diabetic people (770). In contrast Lloret et al demonstrated that BIA overestimated fat mass and fat mass % as compared to DEXA (771).

Boneva-Asiova et al. compared BIA and DEXA measurements in different BMI and sex categories. They showed that both measurements were highly correlated. However, in lean participants BIA tended to give lower values for fat mass and fat percentage, while higher values for fat free mas as compared to DEXA. This trend was reversed with higher BMI (>35). In addition the correlations between BIA and DEXA tended to decrease with increasing BMI. Moreover the agreement between the two methods was better in men as compared to women (763). The segmental BIA tends to underestimate muscle mass in men and to overestimate in women; with reverse for fat mass (767).

In summary, BMI is the anthropometric measure of choice for most large population studies in spite of being a surrogate marker body fat as it measures weight and height. There is a chance that BMI might give misleading results with

increasing age (772), different ethnic groups (773), sportsmen and women (774), and weight loss with enhancing physical activity (775). It is suggested that combining BMI with WC or WHR might produces more informative results (643). The next inexpensive and easy option is the BIA, which can also be used for following up or monitoring prognosis. DEXA or MRI should be used in clinical or research settings due to their high cost and expertise required.

2.2 Measurement of Insulin Sensitivity/Resistance

Insulin sensitivity can be estimated by several biological measurements. It ranges from a single fasting blood sample for simple indices like HOMA or QUICKI, to a research setting for performing the hyperinsulinaemic- euglycaemic clamp test. The choice of method depends on the study requirement, available resources and choice of information (either global, muscle or liver insulin sensitivity) (776).

Table 2.4 Methods of measuring insulin resistance

Directly measuring insulin sensitivity tests	**Simple surrogate indices of insulin sensitivity**	
	Indices derived from OGTT values	**Indices derived from fasting values**
Hyperinsulinaemic euglycaemic glucose clamp (777) Insulin suppression test (778) Minimal model analysis of frequently sampled intravenous glucose tolerance test (779)	ISI Belfiore (780) ISI Cederholm (781) ISI Gutt (782) ISI Matsuda (783) ISI Stumvoll (784) OGIS (785) SIisOGTT (786) Insulin (120 min) Glucose (120 min)	Homoeostasis model assessment (HOMA) (787) Quantitative insulin-sensitivity check index (QUICKI) (788) Revised QUICKI (789) FIRI (790) I_0/G_0 (791) G_0/I_0 (792)

BW, body weight; Imean, mean insulin during OGTT; Gmean, mean glucose during OGTT, G0=fasting glucose, I0= Fasting insulin, ISI= insulin sensitivity index

2.2.1 Hyperinsulinaemic–euglycaemic glucose clamp

The hyperinsulinaemic–euglycaemic glucose clamp (HEC) is widely accepted as the gold standard procedure for the assessment of insulin sensitivity. It comprises: 1) a constant intravenous infusion of insulin to create a state of exogenous hyperinsulinaemia, and 2) a variable glucose infusion to maintain euglycaemic (777). For blood glucose measurement, arterialized blood is obtained by either arterial catheterization or via retrograde cannulation of a wrist vein warmed with a heating pad; warming opens up of arteriovenous anastomoses as is required for blood glucose measurement. Although HEC performed using venous, 'arterialized' venous, or capillary euglycaemia appear to be almost equally useful for the determination of insulin sensitivity (793). Glucose levels are maintained at 80–90 mg/dL (or 4.5–5.00 mmol/L) by monitoring the glucose level every 5 or 10 min at the bedside. Euglycaemia is maintained by adjusting the infusion rate of a 20% dextrose solution. The clamp test lasts for two to three hours.

The constant insulin infusion produces a plateau of insulin concentration sufficiently above fasting levels to suppress hepatic glucose production (HGP), which increases glucose disposal in skeletal muscle and adipose tissue. As the HGP is suppressed, the rate of glucose infusion approximates the rate of glucose uptake into peripheral tissues, or insulin sensitivity: the more sensitive the tissues the more glucose is required and vice versa. The glucose infusion rate (GIR) during the last 30 min of the clamp adjusted for a space correction is known as "M" or glucose metabolised. This can be adjusted for the achieved level of plasma insulin concentration at steady state (M/I). M is expressed in mg/kg body weight/min. As most of the glucose uptake occurs in muscle and only a small proportion in adipose tissue, expressing M in mg/kg/min could overestimate insulin resistance in obese subjects (as they have more fat mass) (794). M can therefore also be normalized for lean body mass (mg/kgLBM/min) in order to give a value for insulin sensitivity independent of body mass index (BMI).

The validity of the HEC clamp depends on the complete suppression of HGP by insulin. If HGP is not suppressed, glucose will be released from liver and M underestimates GIR. Suppression of HGP is achieved with an insulin infusion rate

of 40–60 mU/m2/min in non-obese non-diabetic subjects (777). In some situations (overweight, obesity or type 2 diabetes) where HGP is not totally inhibited by such an infusion rate, a higher rate of insulin infusion (≥80 mU/m2/min) is used to ensure HGP suppression (795).

Although the HEC clamp test is the reference method and generates valuable information it has some disadvantages: (1) it requires two intravenous catheters and calibrated pumps; (2) online glucose-level determination; (3) requires trained staff; and (4) time-consuming (lasts 2-3 hours), thereby precluding widespread use in large cohorts (794).

2.2.1.1 Potential errors in insulin sensitivity estimation by HEC

During HEC, the aim of insulin infusion is to completely suppress HGP which enable an accurate estimation of peripheral insulin sensitivity. If a disproportionately low dose of insulin is administered, it leads to incomplete suppression of HGP and will lead to the underestimation of insulin sensitivity. Likewise, overweight and diabetic individuals need higher insulin doses to suppress hepatic glucose production (777;796), and if the same dose of insulin is used for healthy, normal weight, obese and type 2 diabetic individuals, then for some people insulin sensitivity will be underestimated.

Another assumption of HEC is a steady state glucose infusion but it has been noted that in some studies that the amount of infused glucose continues to rise even at the end of the examination (797;798), indicating that the same participant may exhibit better insulin sensitivity during a longer duration HEC compared to a shorter duration clamp. It has also been noted that within the same individual glucose infusion rate is higher at the second examination than at the first (797). One possible explanation is that attending the second examination may be associated with lower stress than the first attendance, and we know that stress leads to release of corticosteroid hormones which decrease insulin sensitivity.

2.2.2 Insulin suppression test

The insulin suppression test (IST) is another direct method of measuring insulin sensitivity. After an overnight fast, somatostatin (250 μg/h) is infused

(intravenously) to suppress the endogenous production of insulin. Simultaneously, glucose (6 mg/kg body weight/min) and insulin (50 mU/min) are infused over first 150 min of the test at a constant rate. For the first two and half hours, glucose and insulin are determined every 30 minutes and for the last 30 minutes (150 to 180 min) glucose and insulin are evaluated every 10 minutes. The tissue insulin sensitivity is estimated by the steady-state plasma glucose (SSPG) concentration obtained during the last 30 min of infusion and the higher the SSPG concentration, the more insulin-resistant the individual is (778).
As described with HEC, the IST is difficult to apply in large epidemiological studies as it needs more time and labour. Moreover, in insulin-sensitive subjects there is a risk of hypoglycaemia and in people with type 2 diabetes, IST can provoke glycosuria. Glycosuria in turn can lead to underestimation of insulin resistance by SSPG (778).

2.2.3 Minimal model analysis of frequently sampled intravenous glucose tolerance test (FSIVGTT)

This is an indirect measurement of insulin sensitivity and is based on glucose and insulin values obtained during a frequently sampled intravenous glucose tolerance test. It uses a mathematical model that uses both insulin and glucose values. The minimal model uses the dynamics of increasing insulin and decreasing glucose concentration to obtain two different indices; 1) SiMM (insulin sensitivity index) and 2) SgMM (glucose effectiveness index) (779)
The SiMM index provides information on peripheral and liver insulin sensitivity. It shows the link between insulin level and disappearance of glucose from plasma. In contrast, the SgMM provides information on the effects of glucose on its own disappearance independent of any insulin variation. The minimal model also gives values of early and late phase insulin secretion (779).

The minimal model is a complex test with duration of three to four hours, requiring frequent blood sampling and also specific software is needed for analysis (779). Regarding the efficacy, the ‘standard’ minimal model test is less reliable in people who have major insulin resistance and/or impaired insulin secretion, such as people having T2DM. In these people a ‘modified’ minimal model test is used which requires exogenous insulin infusion (799).
The methods described above are complex, time consuming, costly, need

specialist staff and are not easily performed in large populations, thus surrogate indices have been developed for assessment of insulin sensitivity/resistance and are used in studies containing large cohorts.

2.2.4 Surrogate indicators of insulin sensitivity/resistance

2.2.4.1 Indices derived from OGTT values

The oral glucose tolerance test (OGTT) is widely used in clinical settings for the diagnosis of impaired glucose tolerance (IGT) and T2DM. A fasting blood sample is taken for blood glucose and insulin. The individual is then given an oral glucose load (75 g) at time 0 min and then blood samples are taken every 30 minutes up to two hours. For the clinical diagnosis of IGT and T2DM, fasting and two hour post load glucose values are sufficient. However, for the assessment of insulin sensitivity or secretion, additional samples for both plasma insulin and glucose obtained every 30 min following an oral glucose load (75 g) are required (783). Surrogate indices derived from the OGTT use plasma glucose and insulin values during the OGTT, into mathematical equations for evaluating insulin sensitivity. Some of the indices additionally use other parameters like weight, BMI and glucose volume of distribution (780;783;784;800).

Some of the indices are described in detail below along with their mathematical formula; most of them are also validated against HEC.

The Matsuda index

This was first described in 1999 by Matsuda and DeFronzo in subjects with a wide range of glucose tolerance from normal glucose tolerance (NGT) to T2DM. The following formula was used:

Whole body insulin sensitivity (IS) = 10,000/square root of [fasting glucose X fasting insulin] X [mean glucose X mean insulin during OGTT]) (783)

The Matsuda index was positively correlated with HEC ($r = 0.73$, $P < 0.001$). The basis of this index was that fasting glucose and insulin values mainly reflect hepatic insulin sensitivity, whereas mean OGTT values reflect insulin sensitivity in peripheral skeletal muscle (783). The same group has recently modified the

Matsuda index and have proposed different formulas for more specific evaluation of hepatic and muscle insulin resistance (801).

Hepatic insulin sensitivity is equal to the product of total area under curve (AUC) for glucose and insulin during the first 30 min of the OGTT

IS = (glucose(0-30) [AUC] X insulin(0-30) [AUC]) (801).

It is strongly positively correlated with hepatic insulin resistance index (fasting plasma insulin X basal endogenous glucose production) ($r = 0.64$, $P < 0.0001$).

The formula for muscle insulin sensitivity is the rate of decay of plasma glucose concentration from its peak value to the lowest value during the OGTT divided by the mean plasma insulin concentration

IS = (dG/dt divided by MPI) (801)

Where dG/dt is the rate of change in plasma glucose from its peak to its nadir and MPI is mean plasma insulin concentration during OGTT. It is also strongly correlated with muscle insulin sensitivity measured with HEC ($P = 0.78$, $P < 0.0001$).

The Stumvoll index

This was first described by Stumvoll and colleagues in 2000. Its equation was developed from a multiple linear regression model evaluating the effect of different demographic and OGTT parameters.

Stumvoll ISI = $0.226 - 0.0032 \times BMI - 0.0000645 \times I_{120} - 0.00375 \times G_{90}$ (784)

Where I_{120} = plasma insulin at 120 min, G_{90} = blood glucose at 90 min

It is highly correlated with metabolic clearance of glucose ($r = 0.80$, $P <0.001$) and insulin sensitivity index ($r = 0.79$, $P <0.001$) (784).

The Belfiore index

Belfiore et al. proposed a formula in 1998, which had very high correlation (r= 0.99, P= <0.01) with HEC. In contrast to clamp performed under artificially induced steady state (sustained hyperinsulinaemia, suppression of FFA, etc.), this index was obtained under physiological conditions with hormonal and metabolic variables unmodified and is more convenient in clinical setting to assess whole body insulin sensitivity. It uses the area under the curve (AUC) of insulin and glucose during a two hour OGTT (780).

Belfiore ISI = 2/[(AUC insulin + AUC glucose) + 1] (780)

(AUC glucose = $0.25 \times G_0 + 0.5 \times G_{30} + 0.5 \times G_{60} + 0.5 \times G_{90} + 0.25 \times G_{120}$)

(AUC insulin = $0.25 \times I_0 + 0.5 \times I_{30} + 0.5 \times I_{60} + 0.5 \times I_{90} + 0.25 \times I_{120}$)

Where $_0$, $_{30}$, $_{60}$, $_{90}$ and $_{120}$ are blood glucose and insulin concentration at fasting (0 min), 30, 60, 90 and 120 minutes of OGTT.

The OGIS index

The oral glucose insulin sensitivity (OGIS) index was developed by Mari et al. in 2001. It is complex in comparison to other OGTT derived indices as it requires the use of two primary formulas that have to be incorporated into a third one. Furthermore, the final calculation requires the incorporation of six parameters; weight, height, oral glucose dose, blood glucose (at 0, 90 and 120 min), blood insulin (at 0 and 90 min) and body surface area (BSA). From these six parameters glucose dose per m^2 BSA and two other calculations are done. Finally glucose dose per m^2 BSA and the two calculations are entered into the final formula to calculate OGIS. These calculations also vary depending on the duration of the OGTT (2 or 3 h) and the units used to express glycaemia (mg/dL or mmol/L). However, OGIS can be easily calculated using a calculator available through the website (http://webmet.pd.cnr.it/ogis/), or excel sheet downloaded through the website which contains all the calculations and formulas (785). OGIS is well-correlated with M as measured by HEC as shown by Otten et al. in a meta-analysis of 7 studies (r= 0.70, p= <0.001) (802).

2.2.5 Indices derived from fasting values

Fasting can be thought of as a steady state of the body in which blood glucose is tightly maintained between normal values as a result of the effect of insulin on HGP, which equals whole body glucose disposal. In healthy conditions blood glucose will remain almost constant and plasma insulin levels will vary according to the degree of insulin resistance of the body. Under fasting conditions, glucose utilization is mainly cerebral which is non-insulin dependent. It is a very simple and cost effective way to determine insulin sensitivity, but it mainly represents hepatic insulin sensitivity. Peripheral insulin resistance and hepatic insulin resistance are closely related to each other and this may be the reason why fasting indices have been validated against the HEC, which more specifically measures muscle insulin sensitivity. The clamp technique can also be used to measure hepatic insulin sensitivity but then requires labelled glucose, which makes the test more complicated and costly (776). Another difference from the clamp technique is the use of arterialized blood for blood glucose estimation in clamp techniques whereas arterialized sampling is never done in clinical practice for calculation of fasting indices. However, the insulin resistance values obtained from arterialized or venous blood are roughly comparable (793).

Many surrogate indices are based on fasting measures and among them most common and widely used are homoeostasis model assessment (HOMA) and quantitative insulin-sensitivity check index (QUICKI).

2.2.5.1 Homoeostasis model assessment (HOMA)

Homoeostasis model assessment (HOMA) has been widely used as an estimate of insulin sensitivity in cross-sectional, longitudinal and prospective studies over the last 3 decades (803). It is one of most widely used surrogate measures of insulin sensitivity and is based on simple fasting measurements of insulin and glucose.

$$\text{HOMA} = \frac{[\text{fasting insulin } (\mu U/mL)] \times [\text{fasting glucose } (mmol/L)]}{22.5} \quad (787)$$

The denominator effectively standardizes insulin and glucose to a normal fasting levels [insulin 5μU/mL ; glucose 4.5 mmol/L](787). HOMA demonstrates

reasonable correlations (Pearson r = 0.6-0.7) with the HEC clamp results in several populations and has proved useful in large epidemiological studies. Additionally, an index of ß-cell function (HOMA-B) can also be derived which reflects insulin secretion (787). The HOMA software has been recently updated and is currently called HOMA-%S; is available on the Oxford University website (www.dtu.ox.ac.uk/homacalculator/index.php). HOMA-%S allows the estimation of insulin sensitivity (%S) and steady-state ß-cell function (%B) as a percentage of a normal reference population.

Due to the pulsatile secretion of insulin it is recommended to take a mean of three samples for computation of HOMA (787) but a single sample also provides similar results in large datasets (804). Nevertheless, for one individual or a small sample, the use of the mean insulin concentration from three samples is preferable as the use of a single sample results in intra subject coefficients of variation that are higher than when three samples are used (804).

As healthy subjects maintain fasting glucose with little variability, HOMA performance is weaker in clinically healthy populations. As fasting glucose and insulin concentrations depend on many physiological processes other than insulin sensitivity (glucose absorption, ß-cell function, insulin clearance), direct measures like HEC clamp are the preferred technique if feasible (787).

2.2.5.2 Quantitative insulin sensitivity check index (QUICKI)

This is based on the same principal as HOMA but is inverse logarithm of the fasting glucose and insulin values

$QUICKI = 1/(\log G_0 + \log I_0)$ (788)

Where G_0 and I_0 are fasting glucose and insulin respectively.
The effectiveness of QUICKI in estimating insulin sensitivity was evaluated in a heterogeneous population of non-obese, obese and type 2 diabetic people. In a recent meta- analysis QUICKI showed good correlation with HEC (Pooled r= 0.61, $p < 0.05$) (802). The fasting insulin distribution is not linear and log transformation improves its linear correlation with HEC. Similarly the log transformation of HOMA also improves its linear correlation with HEC (776).

Fasting sample derived indices show weaker correlation in healthy individuals. This may be due to the fact that in healthy people fasting glucose and insulin values are maintained within narrow ranges, despite having different levels of insulin sensitivity. It further suggests that small, but clinically relevant, variations in insulin sensitivity may be overlooked by simple fasting indices and needs direct measures like HEC to detect these changes (776). To address this issue new indices have been developed and some of them have included other body parameters like BMI and Lipids.

2.2.5.3 Revised QUICKI

Perseghin et al. (789) modified the QUICKI formula by adding the log of the fasting value of non-esterified fatty acids (NEFA).

Revised QUICKI = $1/(\log G_0 + \log I_0 + \log \text{NEFA})$ (789)

The principle behind addition of NEFA was that insulin inhibits lipolysis at lower levels as compared to the levels required to effect glucose metabolism. Thus, fasting NEFA concentrations can reflect insulin resistance (by estimating the anti-lipolytic effect of insulin) earlier than do fasting glucose values. This improved its correlation with HEC and in a recent meta- analysis comparing correlations of OGTT and fasting based indices with HEC, the revised QUICKI had almost same correlations as the OGTT based measures (802). In addition the correlation with HEC were high ($r= 0.68$, $p= <0.05$) in healthy subjects as well as T2DM (802). Despite improving the fasting based index, revised QUICKI added NEFA to the formula and so required an additional biochemical measure, in addition to glucose and insulin. The NEFA concentrations may also be affected by dietary interventions and weight loss (802).

2.2.5.4 The Disse index

This index was developed by Disse et al. in 2008 by using multiple forward regression analysis. They found that fasting insulin, NEFA and the HDL cholesterol/total cholesterol ratio explained 53% of the variation of insulin sensitivity and were included in the Disse index (805)

Disse index = 12 × [2.5 × (HDL-cholesterol (mmol/L)/total cholesterol (mmol/L)) - NEFA (mmol/lL)] – Insulin (IU/mlmL) (805)

The Disse index was highly correlated with HEC (r = 0.79, P < 0.001) (805) and can be used in healthy, overweight, obese and T2DM people (776).

2.2.6 Rationale behind surrogate measurement of insulin sensitivity

2.2.6.1 Basis for surrogate measurement of insulin sensitivity

The two most important components of insulin sensitivity are glucose clearance in peripheral (mainly muscles) tissues (i.e. peripheral insulin sensitivity) and insulin mediated suppression of hepatic glucose production (i.e. hepatic insulin sensitivity) (802). The HEC mainly measures muscle insulin sensitivity, fasting indices mainly measure hepatic insulin sensitivity and the OGTT-based indices measure both types of insulin sensitivity (783;801). The reason behind moderate to high correlations (r>0.5) of fasting surrogate measures with the HEC is that, in most people, hepatic insulin sensitivity is closely related to peripheral insulin sensitivity (801). OGTT based surrogate markers are based on changes in insulin and glucose during the OGTT and incorporate both peripheral and hepatic insulin sensitivity. During the first hour of OGTT, changes in hepatic glucose production are dominant, while peripheral glucose uptake is best measured during the second hour (801).

Measurement of insulin resistance is very complex when considering whole body glucose metabolism. The surrogate markers are based on blood levels of glucose, insulin and/or non- esterified fatty acids (NEFA), which in turn are influenced by following important biological processes: dietary glucose absorption, renal glucose loss, insulin clearance, lipolysis, lipids re- esterification, hepatic glucose production, insulin secretion rate, ß-cell glucose sensitivity, muscle cells or peripheral insulin sensitivity and muscle cells activity or glucose utilization. Each of these components influences circulating glucose and insulin concentrations and at any moment of blood sampling; the blood glucose and insulin level are the product of all of these components.

Resistance to insulin-mediated peripheral glucose disposal or insulin resistance (measured by HEC) is just one component of glucose metabolism and refers mainly to muscle cells. Hepatic insulin sensitivity or hepatic glucose production is largely suppressed during the clamp procedure and so the term insulin sensitivity in this context refers mainly to muscles glucose disposal. The measurement of interest when measuring insulin sensitivity is only muscle cells insulin sensitivity and not the combined effect of all measurements and thus surrogate markers can only provide an approximation of a true value.

Although OGTT derived indices take at least 2-3 hours, they have the additional advantage of evaluating other parameters apart from insulin sensitivity, such as glucose tolerance and insulin secretion.

The HEC is considered the gold standard for measuring peripheral insulin sensitivity as it is not influenced by changing glucose and insulin levels (and the factors influencing blood glucose and insulin), the hepatic insulin extraction or clearance, ß-cells insulin secretion and feedback mechanism between glucose and insulin (777).

2.2.6.2 Correlations between surrogate markers and HEC in relation to blood glucose status

Surrogate measures of insulin sensitivity exhibit weaker correlations with the HEC in healthy normal weight individuals as compared to people having insulin resistance (776). This finding is further supported by a recent meta-analysis showing that surrogate measures like Matsuda, Stumvoll MCR, Stumvoll ISI and revised QUICKI were more strongly correlated with HEC in individuals with IGT than in those with NGT or type 2 diabetic patients (802). However OGIS, QUICKI and HOMA showed almost equal correlations in individuals with NGT, IGT and type 2 diabetes (802).

The strength of the correlations between HEC and surrogate measures in individuals with different levels of insulin sensitivity (NGT, IGT and T2DM) depends on the insulin dose used during the clamp (806). Lower insulin doses show strong correlations for healthy NGT people, and higher insulin doses show higher correlation for insulin resistant individuals (802). The studies of fasting surrogate measures showing higher correlations with HEC in which low dose

insulin is used, may be due to the fact that it may be measuring hepatic insulin sensitivity instead of peripheral glucose uptake (807) as low dose insulin does not suppress HGP. In OGTT based surrogate measures insulin levels around 120 min exhibit a strong correlation with HEC in a healthy population, but not in a population with diabetes as it is strongly influenced by islet dysfunction (802).

2.2.6.3 Other potential errors associated with surrogate measures

In a healthy individual fasting glucose is tightly regulated by other factors apart from insulin sensitivity, such as islet cells function and insulin release and HGP. So there will be minimal variation in fasting glucose in healthy subjects with various degrees of insulin sensitivity. In addition insulin levels at any point are also regulated by ß-cell insulin secretion and insulin clearance by liver; in addition to insulin sensitivity (802). On the other hand measuring insulin concentration is one of the most important components of many surrogate indices but there are several sources of error in insulin measurement. Some insulin assays show cross reactivity with pro-insulin and partially processed proinsulin products: this can be a source of error when using some radioimmunoassys. Newer and more specific assays have reduced this cross reactivity (808). Another issue with inulin assays is that many show more variability at low insulin levels and may be a possible cause of lower correlation coefficients in healthy individuals vs type 2 diabetic patients (806). The OGTT based surrogate markers are also influenced by inter individual variability of gastric emptying, glucose absorption, insulin secretion and incretin hormones (802).

Closing the discussion, surrogate markers have an importance in large scale studies and can be used in place of HEC when it is desired to check insulin sensitivity at one point in time. However, surrogate markers are less reliable for analysing change in insulin sensitivity in response to metabolic factors or the relationship of insulin sensitivity with other risk factors (inflammation, BP, hypertension etc). The main reason why surrogate markers are not used in mechanistic and physiological studies is that their validity is dependent upon intact function of other biological processes; normal pancreatic ßcell function, normal liver glucose and fat metabolism and normal insulin clearance.

For measuring insulin sensitivity surrogate markers depend upon detecting insulin and glucose concentration. The glucose concentration can be low or high depending on many factors specially β-cell insulin secretion. In insulin resistant states, if β-cells are functioning normally they will secrete extra insulin to compensate for insulin resistance leading to 'compensatory hyperinsulinaemia'. This is true for obese insulin resistant people having normal blood glucose. On the other side some people have weak or more prone β-cells of pancreas and develop dysfunction with slight metabolic stress and may show abnormal plasma glucose in insulin sensitive or mild insulin resistant individual. In HEC, physiological feedback between glucose and insulin concentrations is disrupted and HEC is not influenced by β-cell insulin secretion capacity and insulin clearance etc (802).

In a recent meta- analysis comparing correlations of all surrogate markers with HEC, Otten et al found that the OGTT-based surrogate measures (Stumvoll metabolic clearance rate r=0.70, OGIS r=0.70, the Matsuda index r=0.67, the Stumvoll insulin sensitivity index r=0.67) had highest correlation with HEC. The non-OGTT surrogate measure which exhibited the highest correlation coefficient with HEC was 'revised QUICKI' r=0.68) (802). It was further concluded that surrogate indices derived from fasting measurements, are valid measures of insulin resistance, and that OGTT with multiple sampling is not necessary for estimating insulin sensitivity in both clinical and epidemiological studies (802).

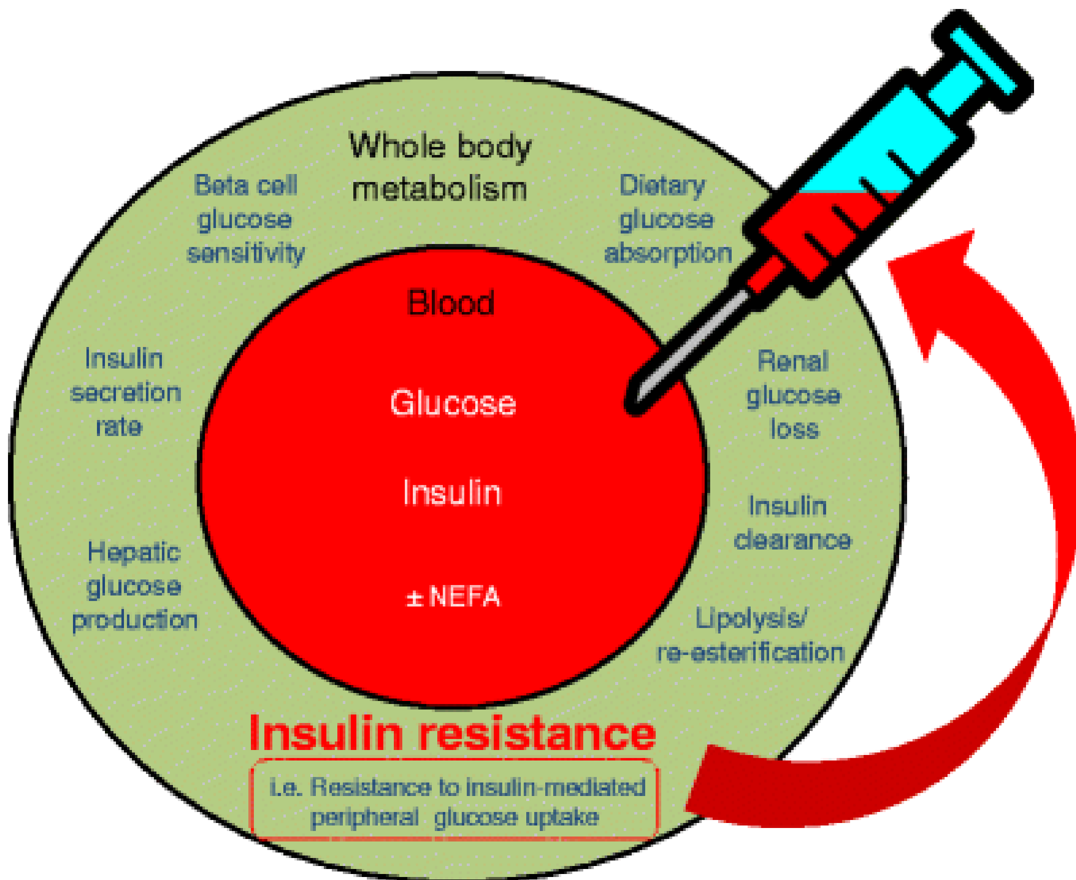


Figure 2.1 Basis of surrogate markers of insulin sensitivity and hyperinsulinaemic euglycaemic clamp
Reproduced with permission from Petrie JR 2014 (809).
Whole body glucose metabolism, blood glucose, insulin levels and peripheral insulin sensitivity in relation to measurement of insulin sensitivity.

2.2.7 Comparison of surrogate measures with HEC in relation to other disease factors

Literature shows that HOMA-IR and other surrogate measures are significantly correlated to Insulin sensitivity determined by the HEC (Pearson r values 0.6-0.7) (802;810). When examining the relation of other factors (inflammation, blood pressure, hypertension and different drugs) with insulin sensitivity, surrogate estimates of insulin sensitivity can lead to conclusions that are totally different from those based on the results of HEC.

In an observational prospective cohort, Arnlov et al showed that women had lower BMI, lower fasting insulin and glucose and lower HOMA-IR as compared to men. However, insulin sensitivity (determined by ISI index) was same in both genders (811). In keeping with this, in a randomised trial of the effect of salsalate compared with placebo on insulin sensitivity, Goldfine et al showed

that there was no improvement in insulin sensitivity (determined by HEC) by salsalates (610). An additional finding in their results was the decrease in fasting plasma glucose and insulin concentration by salsalates, which determine HOMA-IR. Although HOMA-IR was not reported in the results but it is clear from the formula (fasting glucose × fasting insulin/22.5) that it had decreased. In conventional terms a decrease in HOMA-IR would be interpreted as improvement of insulin sensitivity, but direct measurement of insulin sensitivity by HEC showed that there was no change after salsalate administration (610). HOMA-IR relies on fasting insulin levels for determination and any drug or mechanism influencing insulin's pancreatic β-cell secretion or clearance will directly affect HOMA-IR and will influence the relation, e.g. salsalates have been reported to decrease insulin clearance.

In keeping with the argument Pisprasert et al demonstrated in their study of normo- glycaemic people that African Americans were more insulin resistant than European Americans as assessed by a number of surrogate estimates of insulin action. Surrogate estimates used by them ranged from ones based on fasting plasma glucose and insulin concentrations (HOMA-IR and QUICKI) to ones using insulin and glucose concentrations resulting from an OGTT (Matsuda index and Stumvoll index). However, in the same study no difference in insulin sensitivity could be found in the two ethnic groups when a hyperinsulinemic euglycaemic clamp was performed (810). They also showed that race and gender affected the relationship of surrogate markers with HEC as in African Americans males HOMA-IR was not related to HEC but Matsuda index and SIISOGTT were significantly correlated. These findings further support that HEC is the gold standard, and surrogate markers may be used with caution.

2.3 Measurement of markers of Inflammation

Acute phase reactants; interleukin-6 (IL-6) and C-reactive protein (CRP) are increased during acute and chronic inflammation and are the most studied and widely used markers of inflammation.

2.3.1 C-reactive protein (CRP)

C-reactive protein (CRP) can be measured in blood and has been used as an indicator of acute inflammation for decades. Macrophages and adipose cells secrete factors (interleukin-6) which cause synthesis of CRP in the liver. In physiological states, CRP binds to phosphocholine expressed on the surface of dead or dying cells and bacteria in order to activate the complement system. This facilitates phagocytosis by macrophages and ultimately clears the body of necrotic and apoptotic cells (812).

More recently, inflammatory markers have received attention for their ability to predict CVD risk (287). Among these, CRP is one of the more powerful with a recent meta-analysis showing that for every 1-standard deviation (SD) increase in CRP, vascular risk (adjusted for age and sex) increases by more than 60% (813). CRP is stable in plasma or whole blood at 4 and 21 degrees C for at least three days and for several years at -80°C. Moreover, it is stable after five freeze-thaw cycles and is therefore a stable marker of inflammation (814).

A review of the literature indicates that CRP levels in blood are influenced by a number of environmental and lifestyle factors including age, gender, cholesterol level, body mass index, blood pressure, insulin resistance, smoking and sleep deprivation(815). There is also genetic variation in CRP levels (816). Several single-nucleotide polymorphisms (SNPs) in the CRP gene have been shown to directly influence steady state CRP levels in blood and are inherited independent of the above risk factors (816;817) with effects across the lifespan. Earlier studies showed conflicting result with regard to the relationship between serum CRP and SNPs with cardiovascular risk (816;817) but more recent larger studies have been consistent in showing no association between serum CRP as determined by Mendelian randomization and CVD (especially hypertension) (818-820), casting some doubt on the causality of the association.

In animal studies it has been reported that chronic elevation of CRP is associated with a greater risk of hypertension. Vongpatanasin et al reported that Ang II leads to exaggerated blood pressure elevation in CRP transgenic mice, and this response was reversed by a nitric oxide (NO) donor, indicating a role for NO deficiency in the process (821). Schwartz et al also showed in mice that CRP

downregulates endothelial NO synthase (eNOS) and attenuates re endothelialization (822). Other *in vitro* and animal studies also show inhibition of (eNOS) and impaired endothelial vasoreactivity following CRP administration (823). In rats, delivery of adeno- associated virus overexpression of CRP (AAV-hCRP) increased BP and impaired vasoreactivity (824;825), as well as increasing oxidative stress, expression of angiotensin 1 receptors and endothelin-1. The same genetic manipulation also decreased expression of eNOS and impaired endothelium dependent vaso-relaxation (824;825). These experimental observations suggest that CRP may have physiologically-relevant biological activity at least in animals.

However, there are many discrepancies between human and animal studies in relation to CRP. For example, statins have been shown to decrease BP without lowering CRP in mice (825). However in humans, statins lower CRP (826) but do not affect BP (827). Nevertheless, some epidemiological studies support a relationship between high levels of CRP and hypertension (827).

So even if animal studies show a causal association between CRP and the development of HTN (824;825), the evidence of a causal association in humans is not strong (818-820;828). It remains uncertain whether CRP might increase BP directly or via some other mechanism (e.g. obesity, insulin resistance) or whether both are affected by some other feature of the metabolic syndrome (829). CRP is unlikely to be on the causal pathway in relation to development of hypertension, but rather a risk marker for chronic low grade inflammation.

2.3.2 Interleukin-6 (IL-6)

IL-6 is a cytokine released from a number of cells ranging from adipocytes, skeletal muscle cells, monocytes, lymphocytes etc. IL-6 acts both as a pro-inflammatory and anti-inflammatory cytokine. The main anti-inflammatory effects are the release of IL-1ra, IL-10 and sTNF-R (830). The pro-inflammatory effects of IL-6 are through expansion and activation of T cells, differentiation of B cells, and the induction of acute-phase reactants by hepatocytes (831). Serum samples of IL-6 can be stored at -20°C for several years and are not significantly altered by repeated freeze-thaw cycles (832); it is therefore a stable marker of inflammation.

In disease states, IL-6 is released in response to tumour necrosis alpha (TNF-α) and has pro-inflammatory actions. It is raised to chronically high levels which have pro-inflammatory and damaging effects (833). However, it can also be released physiologically during exercise (in a TNF-α- independent manner) which can have protective and anti-inflammatory effects (830). Skeletal muscle-derived IL-6 is beneficial in modulating glucose and fatty acid metabolism during exercise. It stimulates adipose tissue lipolysis, fat utilization and has positive effects on pancreatic β-cells function, contributing to improved glycaemia following exercise (833;834).

Healthy adipose tissue is populated with 5–10% macrophages but this macrophage infiltration increases up to 60% in obesity (507). Activated macrophages release TNF-α and IL-6 resulting in insulin resistance (835). Obesity leads to activation of inflammatory pathways in all insulin target tissues, including fat, liver and muscle, signifying a role for inflammation in driving the pathogenesis of systemic insulin resistance (831). Proposed mechanisms leading to inflammation in obesity include oxidative stress, lipotoxicity, glucotoxicity, endoplasmic reticulum stress, hypoxia, amyloid and lipid deposition (831).

There is therefore considerable evidence that IL6 secretion promotes insulin resistance and that its concentration is elevated in obesity and type 2 diabetes mellitus (836). Moreover, in a study in a non-diabetic Caucasian population, Succurro et al found that increased IL6 levels were related to an increased risk of developing insulin resistance (837). However, there are also studies which question this finding. When muscle cells are treated with IL-6 *in vitro* there was increased glucose uptake and translocation of glucose transporter GLUT4 - an insulin-sensitising action (830). Carey et al also found that IL-6 was not elevated in lean subjects with insulin resistance and suggested that fat mass was the proximal cause for raised IL6 in T2DM (836). In keeping with this suggestion, an epidemiological study suggested that IL-6 lost its association with insulin resistance after adjustment for BMI and waist-to-hip ratio; however, the majority of participants were men so the results cannot be generalized (838). Contradicting these results, Andreozzi et al found a negative correlation between IL-6 and clamp-derived insulin stimulated glucose disposal (M). The correlation remained significant even after adjustment for age, sex, BMI and

free fatty acids (839). In another study, IL-6 also correlated negatively with the Insulin sensitivity index (840). Given these uncertainties, further investigation is required in well-characterised human cohorts.

2.4 Measurement of diabetes

2.4.1 Blood glucose

Blood glucose is the traditional method used for diagnosis of diabetes. It is also used for monitoring of diabetes. WHO criteria for the diagnosis of diabetes is fasting plasma glucose of ≥ 7.0 mmol/L (126mg/dl) or a venous plasma glucose 2 hour after ingestion of 75 gram oral glucose load of ≥ 11.1 mmol/L (200 mg/dl) (841).

2.4.2 HbA1c

From 2011, WHO also recommended use of HbA1c as a diagnostic test for diabetes. An HbA1c of 48 mmol/mol (6.5%) is the cut off for diagnosing diabetes. However, a value of less than 48 mmol/mol (6.5%) does not exclude diabetes diagnosed using blood glucose tests (fasting or two hour post prandial) (842).

HbA1c is used both as a screening and diagnostic test for T2DM and for monitoring of both types 1 and 2 diabetes. Advantages of using HbA1c over plasma glucose levels are as follows: 1). No requirement for fasting; 2) longer term glycaemia information compared to plasma glucose; 3) standardized and reliable laboratory methods; (843).

2.5 Relationship between Insulin Sensitivity and Cardiovascular disease- RISC study

The RISC study (Relationship between Insulin Sensitivity and Cardiovascular disease) is a large euglycaemic clamp-based prospective study of the association between insulin resistance and cardiovascular risk (844). There are 21 RISC centres, 19 of which are recruiting centres (two are data management, analysis and modelling) across 14 countries in Europe (Austria, Denmark, Finland, France, Germany, Greece, The Netherlands, Ireland, Italy, Sweden, Spain, Switzerland, United Kingdom and Serbia).

2.5.1 Objectives

The three principal objectives of the RISC study were:

1. To establish whether insulin resistance predicts deterioration of CVD risk markers: diabetes, obesity, hypertension, dyslipidemia, atherosclerosis and CVD.

2. To determine genetic and environmental contributions to insulin resistance and CVD.

3. To develop a method based on mathematical modelling to identify insulin resistant subjects in clinical practice

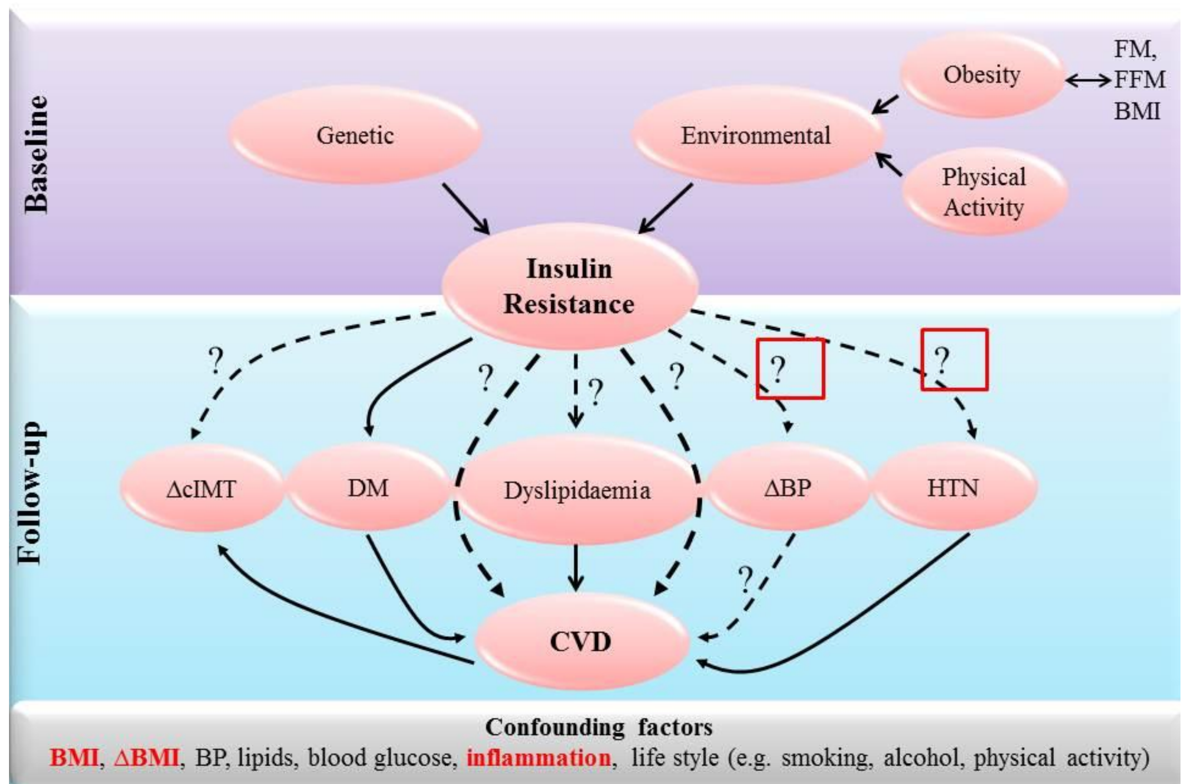


Figure 2.2 Schema of the main Objectives of the RISC study
At baseline the main objective was to examine the associations amongst genes, environmental factors and insulin resistance (IR). At 3 year follow-up the key objective was to analyse the longitudinal relationship between IR and cardiovascular risk factors (e.g. hypertension, diabetes, atherosclerosis, dyslipidaemia) and markers (carotid IMT) taking into account potential confounding factors. The 10 year follow-up objective is to test the hypothesis that IR is related to CVD. Solid lines= established relationship; dashed lines = hypothesized relationship. The red boxes are the objectives of Chapters 3, 5 and 6. Bold and red are the major potentially confounding factors considered in the Results chapters.

FM= fat mass, FFM= fat free mass, DM= diabetes mellitus, HTN= hypertension, cIMT= carotid intima media thickness, CVD= cardiovascular disease

2.5.2 Ethical considerations

The protocol was approved by local ethics committees at each recruiting centre before the study commenced. Volunteers were invited from the local population and were given detailed written information as well as an oral explanation of the study and protocols. Written consent was obtained from each participant and separate consent was obtained for the genetic analyses. All clinical assessments were conducted according to the principles of the Declaration of Helsinki.

2.5.3 Protocol and methodology

RISC is a prospective (3- and 10-year follow up), observational, cohort study of healthy people. Participants were recruited from the local population, according

to specific inclusion criteria (Table 2.1). Initially 1563 people were screened based on the inclusion criteria and underwent physical examination, local blood screening and an oral glucose tolerance test. Of these, 1384 participants met the eligibility criteria and underwent a hyperinsulinaemic euglycaemic clamp procedure (see Section 2.2.1) to measure insulin resistance. 32 individuals were then excluded following quality control checks. The final cohort consists of 1352 individuals whose baseline clamp studies were considered suitable for analysis. Baseline examinations began in June 2002 and continued through spring 2004.

Table 2.5 Inclusion and exclusion Criteria for the RISC (Relationship between Insulin Sensitivity and Cardiovascular disease) study (844)

Inclusion criteria
clinically healthy aged between 30 and 60 years available for follow-up in the next 10 years
Initial exclusion criteria
treatment for obesity, hypertension, lipid disorders, diabetes pregnancy cardiovascular disease weight change 5 kg or more in last month steroid treatment chronic lung disease cancer (in last 5 years) kidney failure, kidney dialysis or transplant recent major surgery seizure disorder or epilepsy inability to give informed consent
Exclusion criteria after clinical examinations
systolic/diastolic blood pressure ≥140/90 mmHg or treatment fasting plasma glucose ≥7.0 mmol/l (126 mg/dl) or treatment 2h plasma glucose ≥11.1 mmol/l (200 mg/dl) or treatment total cholesterol ≥7.8 mmol/l (300 mg/dl) or treatment triglycerides ≥4.6 mmol/l (400 mg/dl) or treatment ECG abnormalities acute myocardial ischaemia injury or pericarditis poor ultrasound imaging of carotid artery

2.5.4 Lifestyle and medical history questionnaire

Information was collected on lifestyle and medical history using questionnaires. Information about personal medical history and family history of CVD, stroke, hypertension and diabetes in first-degree relatives, as well as information on body shape of family members, smoking and alcohol habits, and physical activity was recorded. Study nurses collected information about smoking habits via a standard Case Report Form. The modified versions of the Rose questionnaire for

angina (845) and the Edinburgh claudication questionnaire (846) were used for exclusion. The questionnaires were prepared in English and translated into 11 other languages. All questionnaires were independently back-translated into English to ensure accuracy.

2.5.5 Physical examinations

Height: Measured with a standard clinic ruler (stadiometer). Participants were without shoes with head in the Frankfort (horizontal) plane and feet/ankles were together.

Waist circumference: Measured on bare skin (not over clothes), at the smallest point between costal margins and iliac crests.

Hip circumference: Measured at level of the greater trochanters. If greater trochanters were not palpable then measurement of the largest gluteal circumference was taken.

Thigh circumference: Circumference around the right thigh, just below the gluteal fold. Participant stands with both feet (slightly apart) flat on the floor.

See Section 2.1 for detailed appraisal of body mass index, waist circumference, waist hip ratio and other measures of obesity.

2.5.6 Body composition:

Body weight, BMI, percent body fat and fat-free mass were evaluated by the TANITA bioimpedance balance (Tanita International Division, UK). Participants were weighed in the fasting state, dressed in light clothes, with empty bladder and bare feet (no lotion, cream, powder etc.) (847). Digital print-out was obtained of body weight, impedance, total body water, fat mass and fat-free mass (for detailed appraisal see Introduction Sections 1.11.4 and 1.11.5)

2.5.7 Biological samples

Standardised procedures for the drawing, centrifugation, freezing, transport and storage of blood, plasma, serum and urine samples were observed to ensure

quality. Central laboratories were asked to report and mark samples, if they were not received in good condition. Collected blood was separated into plasma and serum, aliquoted and stored at -20°C for glucose, and -80°C for lipids and NEFA. Serum aliquots were stored at -80°C for insulin and C-peptide; urine samples are stored at -20°C. All samples were transported on dry ice at pre-arranged intervals to central laboratories.

Fasting blood samples of glucose were taken for exclusion criteria (Table 2.1) before and during a 75 g oral glucose tolerance test (OGTT). Samples were also taken for central analysis of glucose, insulin and C-peptide (see Section 2.1.12). Extra aliquots of serum and plasma from the OGTT and clamp were stored for future analyses of inflammatory markers, haemostatic factors and other research questions.

2.5.8 Euglycaemic hyperinsulinaemic clamp and related data

The clamp main procedure and basis are explained in Section 2.2.1

Insulin sensitivity was measured using a standardised hyperinsulinemic euglycaemic clamp technique following central training of site staff (844). Target plasma glucose concentration was between 4.5 and 5.5 mmol/l; insulin was infused at a rate of 240 $pmol \cdot min^{-1} \cdot m^{-2}$. Serum glucose was measured at 5 to 10 min intervals to ensure it remained within 0.8mmol/l (±15%) of target glucose concentration.

Data from each clamp study was assessed for quality control criteria on receipt in the project office (sent by fax following clamp). When clamp quality was considered, not only the CV is important but also the time course of the glucose and glucose infusion data. To ensure safety and consistent quality, feedback was provided for each clamp. Data from each clamp is stored in graphic form on the EGIR website (www.EGIR.org).

For quality control the acceptable glucose concentration was within 0.8mmol/l (15%) of target glucose level (4.5-5.5 mmol.l).

- Values >15% above target level were provisionally acceptable:

- If a blood glucose value was above the mean by < 1.5 mmol/l

- and – at time 120 minutes blood glucose was < 5.5 mmol/l
- and – this was not due to stress but due to an error of infusion rate

- Values >15% below target level were acceptable if:

- the blood glucose value was >3.5 mmol/l (hormonal response)
- The plasma glucose at 120 min was between 4.5 and 5.5 mmol/l

Recruiting centres were alerted about non-acceptability and advised to repeat the clamp if possible. If repetition was not possible, the data were 'flagged' for later analysis with one flag graded "minor deviation" and two flags "serious deviation". The former signified that only one 'expert' had reported a problem with the clamp' while the latter meant that two had found problems. 180 clamps were flagged: 117 flag 1, 63 flag 2.

Upon receipt of clamp data, automatic flags were generated by a mathematical quality control procedure with the following criteria: hypoglycaemia (less than 3.5 and 3.0), hyperglycaemia (more than 120% of fasting), failed achievement of target glucose and glucose variability greater than 15%. All flags were confirmed by visual inspection of the graphs. During interim analysis it was observed that the hypo- and hyper-glycaemias did not really affect the clamp data (in terms of systematic trends in means or in correlations) therefore it was decided to elevate the threshold for the "flagged" clamp studies. Clamps were examined by two experts independently blinded to clinical data. Ultimately, clamps were flagged only when both experts considered that the procedure was affected by serious problems of quality.

Mean glucose infusion rate: The steady-state period (for calculation of insulin sensitivity) was between 80 to 120 min (G80 and G120) and expressed in mg.min-1.kg(LBM)-1. Less than 40 individuals had missing local values for G120 or G80; these values were interpolated from adjacent values.

The glucose infusion rate (M value) was expressed in mg.min-1.lbm (kg)-1, with lean body mass (lbm) measured using a TANITA bioimpedance balance. Insulin sensitivity was expressed as M/I (μmol·min-1·kgffm-1·nM-1) i.e. the M value divided by the achieved steady state insulin concentration. As the distribution of this variable (M/I) was skewed, a logarithmic transformation was undertaken prior to use in regression and other analyses using parametric statistical tests [Log (M/I)].

2.5.9 Measures of physical activity

Accelerometry has been used in many cohorts and trials as a measure of physical activity (848). In the RISC study, physical activity was measured objectively by a small single-axis accelerometer (Actigraph, AM7164-2.2; Computer Science and Applications, Pensacola, Florida, USA). The Actigraph is a small (43 g) single-channel recording accelerometer capable of continuous data collection for up to 22 days. The acceleration signal was digitized with 10 samples per second and registered as counts over 1-min intervals (849). Participants were asked to wear the device for up to 8 days on a belt in the small of the back, from waking to bedtime except during water-based activities. Participants actually wore the accelerometer for an average of 5.7 days (median 6 days) (849).

2.5.9.1 Comments

Data was analysed for participants with at least 3 days of data, including days when the device was worn for more than 10 h. It was assumed that the device was not worn if there were 60 consecutive min with no counts. Accelerometer data were processed with custom software developed for this project using SAS version 9 for cleaning for outlying values. Data were also checked for spurious recording: high counts >20,000 counts/min or repeated counts (850). The following are the major summary measures provided by the software:

Total activity: average number of counts per minute when accelerometer was worn

Intensity of activity: on days when accelerometer was worn, participants were classified as having

1) Some vigorous activity (>5,724 counts/min for at least 10 consecutive min),

2) Some moderate activity (1,952–5,724 counts/min for at least 10 consecutive min), or

3) Neither moderate nor vigorous activity on any day (851)

percent time sedentary: <100 counts/min when accelerometer worn (852)

percent time in light activity: not inactive nor in moderate or vigorous activity.

2.5.10 Carotid artery intima media thickness (cIMT)

Ultrasonographic measurement of cIMT has been evaluated in many large trials as a reliable measurement of measuring subclinical and clinical atherosclerosis (853;854). Carotid arteries were investigated by high-resolution ultrasonography. Image acquisition and IMT measurement were made according to the Atherosclerosis Risk in Communities (ARIC) study protocol (855). Carotid images were obtained in each centre, with the participant supine with neck slightly extended and head rotated contra laterally to the side. Longitudinal B-mode image was obtained of the distal 10 mm of right and left common carotid arteries, carotid bifurcation, and internal carotid artery from anterior, lateral and posterior angles. Whole imaging procedure was recorded on super VHS videotape. IMT measurement was performed in a centralized reading centre (Pisa) by a single reader blinded to clinical data, using a high resolution video recorder (Panasonic AG-MD830) coupled with the computer-driven image analysis system MIP (Medical Image Processing; Institute of Clinical Physiology, CNR, Pisa, Italy) (856). IMT was measured by bow compasses in digitized zoomed diastolic frames of each carotid segment at 5 different points and the average was calculated for each segment. For statistical analysis IMT in all 12 carotid segments were averaged (mean IMT).

Sonographers attended a training course, following which they sent five ultrasound scans for accreditation and quality control to the reading centre before undertaking actual RISC recordings. Different machines were used in each centre, but final cIMT was calculated by a single reader (blinded to clinical

data). Moreover the same machine was used for baseline and three year follow-up scans at each centres. Equipment for IMT ultrasound in 19 centres: Toshiba Power vision, Siemens Elegra, Acuson Sequoia, ATL / Philips HDI 5000, Esaote MEGAS, Acuson Aspen 128 XP, Aplio, Toshiba, 5000 HDI, Toshiba SSA 270, Philips Sonos 4500, Agilent Sonos 5500, with 7.5 MHz linear transducer.

2.5.10.1 Power calculations

The primary endpoint was progression of atherosclerosis (see page ….) as measured by the change in cIMT. The secondary endpoints were the change in CVD risk factors (e.g. blood pressure, lipids, glucose metabolism and body composition).

From published studies (857;858), this change is at least 0.01 mm over 1 year, with a standard deviation of 0.06 mm. Over the initial follow-up of 3 years, a mean change of at least 0.03 mm and standard deviation below 0.11 mm was estimated. To detect a difference of 0.03 mm in the mean cIMT between the insulin resistant subjects (in the lower 20% of the insulin sensitivity distribution) and the remainder of the population, with an alpha error = 0.05 and power=0.80; 1500 subjects will be adequate for a two-sided test and 1200 subjects for a one-sided test (in the case of drop outs). The secondary endpoint (BP, lipids, glucose metabolism and body composition) requires fewer subjects than 200.

2.5.11 Lipids and NEFA

Non-esterified fatty acids (NEFA) analysis was carried out using Randox enzymatic kit - Cat. No. FA115. The analyser used was Hitachi Modular P unit and CV was less than 5%.

Total cholesterol was measured by Roche Cholesterol Method for Modular systems which uses enzymatic colourimetric test and CV was less than 2%.

LDL cholesterol calculated by the Friedwald formula (859)

HDL cholesterol was analysed by Roche HDL 2nd Gen Method for Modular systems which uses homogeneous enzymatic colourimetric test and CV was less than 2%.

Triglycerides were measured by Roche Triglyceride Method For Modular systems which uses enzymatic colourimetric test and CV was less than 2%.

Expected values according to NCEP: Normal range: less than 2.3 mmol/l (N.B. If the free glycerol is taken into account, then 0.11 mmol/l must be subtracted from the TG value obtained.)

2.5.12 Glucose, plasma insulin, C-peptide and pro-insulin

Glucose was measured by Cobas Integra, Roche which uses Glucose Oxidase Technique and CV was less than 2%.

Coefficient of variation

Control	Mean mmol/l	Within assay variation % CV	Between assay variation % CV	Total variation % CV
1	5.38	1.8	2.1	2.9

Insulin, proinsulin and C-peptide were measured by Auto DELFIA Insulin kit, Wallac Oy, Turku, Finland which uses two-sited, time-resolved fluoroimmunoassay using monoclonal antibodies.

Normal range: insulin 12-77 pmol/l, proinsulin 2-23 pmol/l, C-peptide 130-760 pmol/l.

Sensitivity: insulin 1-2, proinsulin 0.3, C-peptide 5 pmol/l.

Oral glucose tolerance test (OGTT) was carried out according to a standardised protocol with 75g glucose monohydrate solution.

2.5.13 Plasma hsCRP

Levels of hsCRP were quantified by commercially available monoclonal antibodies (R&D Systems, Abingdon, UK) on a clinically validated automated platform: c311 (Roche, Burgess Hill, UK). In short, wells were coated with 0.1µg anti-CRP antibody (MAB17071, R&D Systems) in 100 µl 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) overnight at 4¤C. Residual protein-binding sites were blocked with 40 mmol/l phosphate buffer, 5% Tween20 and 25 µM EDTA for 1 h at room temperature and washed in PBS

containing 0.05% Tween 20 (PBS/Tw). Recombinant human CRP (1707-CR, R&D Systems) in the range from 25 to 0.19 ng/ml was used as standard and samples were diluted 100 fold in PBS/Tw containing 0.1% BSA and incubated overnight at 4¤C. Bound CRP was determined by incubation with 25 ng biotinylated anti-CRP antibody (BAM17072, R&D System) in 100 µl PBS/Tw for 2h at room temperature. The wells were washed and subsequently incubated with 10 ng Eu3+-labelled streptavidin (Perkin Elmer, Life Sciences, Turku, Finland) in 100 µl PBS/Tw containing 25 µM EDTA for 1 h at room temperature. After wash, bound europium was detected by the addition of 200 µl of enhancement solution (Perkin Elmer), 5 min of vigorous shaking and reading the time resolved fluorescence on a DELFIA fluorometer (Victor3, Perkin Elmer). The limit of detection was 0.15 mg/l. The intra- and inter-assay variations (%CV) were below 5 and 10%, respectively

2.5.14 Interleukin- 6

IL-6 was measured by human IL-6 Quantikine high sensitivity commercial ELISA (R&D systems, Oxon, UK). Briefly, a microplate pre-coated with capture antibody is provided. Plasma was added and IL-6 present is bound by the immobilized antibody. Unbound materials are washed away. A second Alkaline Phosphatase (AP)-labelled antibody (detection antibody) is added which binds to the captured IL-6 and any unbound detection antibody is washed away. NADPH substrate solution is added and a rose colour develops. Plates are NOT washed after this step. Amplifier solution is added and the rose colour deepens to a red colour, in proportion to the amount of IL-6 present in the sample. Stop solution is added (colour remains red) and the absorbance of the colour at 490 nm is measured. The intra- and inter-assay variations (%CV) were below 10%.

2.5.15 Current Medication

Participants were requested to bring prescriptions and containers for prescribed and not prescribed (vitamins, homeopathy etc) drugs. For all medicines, generic and commercial name and strength were recorded as well as the reason for the medication. Medication for diabetes, obesity, hypertension, dyslipidaemia was the exclusion criteria.

The generic name of the drug was obtained for each medicine and was entered into the WHO website http://www.whocc.no/atcddd/. When more than one code was available, the commercial name of the drugs as well as its route of administration was also noted.

All prescriptions of oestrogen monotherapy, a combination of oestrogen and progestogen or progestogen alone were considered to be on hormone replacement therapy.

2.5.16 Menopause

Women were classified as postmenopausal if their last menstrual period was more than 12 months prior to baseline measurements.

2.5.17 Blood Pressure

Outer garments were removed to expose left or non-dominant arm. Participants were seated and rested for at least 5 min before recording. No exertion, physical exercise, eating, smoking or exposure to cold for at least 30 min before recording was confirmed from each participant. Arm should rest comfortably on table, elbow level with the heart and upper arm at an angle of about 40 degrees to the trunk. Blood pressure was measured in triplicate following five minutes' rest at each visit by OMRON 705CP (Omron Healthcare GmbH, Hamburg, Germany) using a standard protocol: the median of these readings was used in this analysis for both baseline and follow-up examinations.

Hypertension: Median systolic BP (SBP) ≥ 140 mmHg and/or median diastolic BP (DBP) ≥ 90 mmHg at follow-up was taken to indicate hypertension (104;860). Participants who had been started on antihypertensive treatment in routine care (n=40) were classified as hypertensive.

2.5.17.1 Modification of BP by treatment of hypertension at follow-up

The method of Cui et al was used to estimate numerical BP values for individuals who had been commenced on antihypertensive medication between baseline and follow-up.(861),(862). For SBP, 10 mmHG was added to the value at follow-up and for DBP; 5 mmHG was added.

2.5.18 Follow up

Annual telephone follow-up by study nurses recorded changes in addresses, medication or medical treatment and also schedule examinations for follow-up. After three years selected examinations were repeated: progression to diabetes was assessed by OGTT; obesity was measured by standard criteria of BMI; blood pressure for development of hypertension and standard anthropometric measures. Ankle: brachial pressure index was measured for progression of peripheral artery disease. Blood examinations were done for lipids, renal and liver markers. The lifestyle questionnaire was repeated to record changes in medical status, smoking and alcohol habits and prescribed and non-prescribed medication. Hospital records with a diagnosis of CVD were reviewed.

2.5.19 Data analysis and management

Study documentation can be accessed from the EGIR website (password protected), where recruitment information is updated weekly. Data were entered into the program Epi Info 2002 (Centres for Disease Control, Atlanta, Georgia, USA) at the recruiting centres and sent by e-mail as an Excel file to the coordinating office or via the website data transfer system. Data are maintained centrally on a computer with restricted access and with back-up.

The participant’s name and address is kept only at the recruiting centre and identification documents of the participant are stored separately from the study data. Data are identifiable only by a 9-digit code and the coordinating centre only receives the coded information. Identification codes are used only as long as necessary to maintain confidentiality.

Local laboratory normal ranges were collected for each of the 19 recruiting centres. Laboratory data for each centre was compared to local range and any out of range data had to be accompanied by an explanation of clinical significance before the data were accepted into the database.

2.5.20 Distinguishing features of RISC study

The main strengths of the RISC Study are:

(i) A large sample of healthy Europeans,

(ii) Gold-standard methodology for measurement of insulin sensitivity and cIMT,

(iii) State-of-the-art measurement of physical activity,

(iv) Availability of DNA samples,

(v) Centralised laboratory assays,

(vi) Centralised continuous quality control of data.

2.5.21 Follow-up and Inclusion in Study/Analysis

Of the 1352 healthy individuals followed after baseline examination, 1073 (587 women and 486 men) had complete data at three year follow up after exclusion of those who had developed diabetes and/or symptomatic cardiovascular disease (n=21).

2.6 Scottish Diabetes Research Network dataset- SDRN study

2.6.1 Introduction

Insulin resistance, blood pressure, inflammation and hypertension are all cardiovascular risk factors (863) and it will be demonstrated in chapters 3, 4, 5 and 6 that insulin resistance and inflammation have independent relation with BP. BMI and change in BMI are also related to all other CV risk factors. So the first four results chapters show that cardiovascular risk factors are interrelated to each other. As the RISC population was healthy at baseline with no chronic condition, there were only three people who developed CVD and 21 people who developed diabetes in three years. This means that we could not examine the relationship of baseline factors to incident CVD in this dataset.

In addition to the risk factors studied in RISC dataset, ethnicity and diabetes are also very important CV risk factors. Moreover it has been shown that ethnicity and diabetes are also very closely linked to insulin resistance, blood pressure, anthropometric measures and lifestyle factors. To study the independent relation of these outcomes with cardiovascular disease I was fortunate to have access to another dataset from the Scottish Diabetes Research Network (SDRN) epidemiology group. This is one of the largest population based dataset available

internationally for non-intrusive anonymised epidemiological research in diabetes.

2.6.2 Methods

In 2014 the population of Scotland was 5,254,800 of whom 258,570 were recorded as having a diagnosis of diabetes (864). Population-based data are available for people with diagnosed diabetes in Scotland in the Scottish Care Information-Diabetes (SCI-Diabetes) dataset (previously known as SCI-DC), an electronic patient record of National Health Service (NHS) Scotland patients with diabetes. For the purpose of this study an extract of SCI-Diabetes data until 31/12/2011 were linked to Scottish Morbidity Records (SMR01) and National Records of Scotland for mortality provided by the National Records of Scotland. Data were also linked to The Scottish Index of Multiple Deprivation (SIMD) - 2012 data set. All of these are explained in detail below.

2.6.2.1 Scottish Care Information – Diabetes (SCI- Diabetes)

SCI – Diabetes is one of the world's best electronic patient record systems (SCI-Diabetes) for people having diabetes. Scottish Diabetes framework was launched in 2001 to shape diabetes care in Scotland. It was identified that information technology system is the best way to manage an integrated diabetes care and Scottish Care Information – Diabetes Collaboration (SCI-DC) Project was started. From 2002 SCI- DC has been successfully operating. In 2011 SCI- diabetes (Phase III development of SCI-DC) was started in Western Isles and is now implemented in all health boards of Scotland (864).

SCI- Diabetes is an integrated diabetes system which is not only an electronic database of patients with diabetes but also has clinical and speciality modules for paediatrics, Podiatry, diabetes specialist nursing and dietetics. (See http://www.sci-diabetes.scot.nhs.uk/ for more information) (864).

Key Features and Benefits of SCI- Diabetes (865):

SCI- Diabetes features and benefits; modified from the SCI-DC website (865).

A single shared electronic record	Available regardless of geographical boundaries and patient location
Cross boundary support	Patients record available to all providing treatment
Real time data entry	Data entered will be available immediately Can be used to determine cost of treatment Shows strategic department usage for service audit
Full patient contact record and record of care	Can be used to determine cost of contact
Reduced Health Board cost to support service	No clinical system to host or technically support Current estimated cost of the systems is £4 per patient, this is forecast to be reduced to £2 a patient by the end of the current project Training of Clinical staff will be easier and more cost effective with a single system Less duplication Greater confidence in data being held Facility to flag erroneous data Reduction of potential data transcription errors through reduced interface requirements
Greater range of information held in a fully integrated diabetes patient record	A single point of data entry across primary and secondary care providers reducing data duplication and transfer (SCI-DC Back-Population)
Improved functionality through convergence of existing SCI-DC systems onto a common and sustainable technical platform	Reduction in data interface and transfer; Additional data held (Ulcer Management, Dietetics, etc) No need for paper trail between Primary and Secondary care and other specialists.
Register will continue to underpin national programmes and surveys	Diabetes Retinal Screening Programme Scottish Diabetes Survey (Scotland has an international reputation for having some of the best data of diabetes anywhere in the world. SCI-DC has allowed us to demonstrate year on year improvements in the quality of diabetes care) Scottish Foot Framework support SIGN Clinical guidelines support

All of the above contribute in giving SCI- Diabetes an international reputation, as one of the best data base of diabetes anywhere in the world.

2.6.2.2 The Scottish Index of Multiple Deprivation (SIMD)

SIMD is the Scottish Government's official tool for identifying deprivation and aspects of deprivation in all areas of Scotland. It divides Scotland into 6505 small areas called datazones, each containing 350 households or 800 people on average. It considers several different aspects of deprivation like employment, current income, other financial resources, health, education, skills and training, physical environment, social relations and social capita, geographic access to services, crime and housing. It combines them into a single index and provides a relative ranking for each datazone from 1 (most deprived) to 6,505 (least deprived). It gives an overall picture in addition to individual aspects of deprivation for each area. By identifying small areas where there are concentrations of multiple deprivations, the SIMD can be used to target policies and resources at the places with greatest need (866).

Quintiles of the index are defined at a national level, and Q1 and Q5 were used to identify the most affluent and most deprived quintiles, respectively. An area-based measure of Socio Economic Status (SES) quintile was assigned to individual people with diabetes on the basis of where they live by using the Scottish Index of Multiple Deprivation (SIMD) 2012. Hence the SES quintiles are:

1 = Most Affluent

2 = Affluent

3 = Middle

4 = Deprived

5 = Most deprived

(See http://www.scotland.gov.uk/Topics/Statistics/SIMD/ for more information) (866)

2.6.2.3 Scottish Morbidity Records (SMR01)

SMR01 is an episode-based patient record relating to all inpatients and day cases discharged from non-obstetric and non-psychiatric specialties, also excluding geriatric long stay. Data collected include patient identifiable and demographic

details, episode management details and general clinical information (867). Currently diagnoses are recorded using the International Classification of Diseases-10 (ICD-10) classification and operations are recorded using the OPCS Classification of Interventions and Procedures version 4 (OPCS-4). The Information Services Division (ISD) Scotland, Data Quality Assurance (DQA) team assesses the accuracy rate and quality of SMR01. For quality assessment, a sample of episodes is extracted from the national database for the hospitals; which are to be assessed. DQA staff compares data from the sample against information held in medical records for the relevant episode. For ischemic heart disease the accuracy, sensitivity and completeness of SMR01 in the 2012 report were 97.3%, 94.8% and 97.4% respectively (868). For cerebrovascular disease the accuracy, sensitivity and completeness were 94.5%, 98.9% and 100% respectively (868). The detailed methodology of quality assessment can be found at http://www.isdscotland.org/Products-and-Services/Data-Quality/Methodology/.

Inpatients: are patients who occupy an available staffed bed in a hospital and remains there overnight; OR - at admission, is expected to remain overnight but is discharged earlier. Discharges include transfers-out and deaths. Haemodialysis patients are excluded from this category.

Day Case: is a patient who makes a planned attendance to a specialty for clinical care and is seen by a doctor or dentist or nurse and requires the use of a bed or trolley in lieu of a bed. The patient is not expected to, and does not, remain overnight. Many of these patients require anaesthesia.

From 1980 to March 1996 ICD9 classification was used, but from April 1996 onwards ICD10 classification is used in SMR01 (867).

2.6.2.4 Variables included in SCI-DC

ID, date of admission, year of admission, date of discharge, year of discharge, continuous in patient stay, marital status, ethnic group, health board of treatment, speciality, speciality area code, clinical facility start date, clinical facility end date, patient category, admission date, waiting list type, admission type, admission reason, admission/transfer from, discharge date, discharge type, discharge/transfer to, diagnosis 1, diagnosis 2, diagnosis 3, diagnosis 4,

diagnosis 5, diagnosis 6, main operation A, main operation B, main operation date, other operation 1A, other operation 1B, date of other operation 1, other operation 3-A, other operation 3-B, date of other operation 3, inpatient day case marker, old speciality code, old type of admission code, waiting time code, SE urban rural code 2004, SIMD Score, SIMD Scotland quintile, SIMD Scotland decile, SIMD health board quintile, SIMD health board decile, SIMD top 15% marker, SIMD bottom 15% marker, carstairs 2001 score, carstairs 2001 Scotland quintile, carstairs 2001 Scotland decile, carstairs 2001 health board quintile, carstairs 2001 health board decile, carstairs 1991 score, carstairs 1991 quintile, carstairs 1991 decile, carstairs 1991 category, electoral ward, UK Parliamentary constituency, Scottish Parliamentary constituency, local government district, council area, age in Years, age in months, days waiting, length of stay, body mass index, systolic blood pressure, diastolic blood pressure, renal failure, HbA1c, high density lipoprotein, low density lipoprotein, total cholesterol, triglycerides, smoking history, year of diabetes diagnosis, socio economic status, anti-diabetic drug group.

2.6.2.5 International Classification of Diseases (ICD)

ICD is the international standard for defining and reporting disease, disorders, injuries and other health conditions. It allows the countries to compare and share health information using a common language. Information recording is very comprehensive and covers all conditions and classifications and is easy to retrieve, analyse and share. ICD is used as a diagnostic classification standard for all clinical and research purposes and is used for mortality and morbidity statistics, injuries, symptoms, reasons for encounter, factors that influence health status, external causes of disease and also incidence and prevalence of disease (869).

ICD is used as a tool in epidemiology, health management, clinical settings, reimbursement and resource allocation, policy making and insurance. WHO was entrusted with ICD in 1948 and ICD-10 has been used since 1990. ICD-11 development is currently in progress and will be completed in 2015. ICD-10 is available in the six official languages of WHO (English, Russian, Chinese, Spanish, French and Arabic) as well as in 36 other languages. ICD-10 Version: 2010 (International Statistical Classification of Diseases and Related Health Problems

10th Revision) is available online (http://apps.who.int/classifications/icd10/browse/2010/en) (869).

2.6.2.6 Mortality

For mortality the SCI-Diabetes extract was linked to National Records of Scotland for mortality provided by the General Register Office for Scotland for getting date and cause of death (both 1a and 1b on death certificate). Data were also matched with SMR01.

2.6.2.7 Type of diabetes

The type of diabetes was based on the type of diabetes assigned by the clinician. In case of any coding errors it was further refined by an algorithm using age at diagnosis and use and timing of treatment with oral hypoglycaemic agents and insulin (870). Diabetes was wrongly diagnosed in 365 people (0.1% of total), and so the diagnosis has been clinically revised and taken off from the register and were removed from analysis

2.6.2.8 Ethnicity

Ethnicity information was obtained from SCI-Diabetes based on the fact that people with diabetes are asked to identify their ethnic group from a standard list used in the 2001 Census in Scotland (734). Census Form of Scotland used for defining ethnicity is attached as Appendix C. The following classification was used for this analysis.

White: Includes Scottish, English, Welsh, Northern Irish, British, Irish, Gypsy Traveller, Polish and any other white

Multiple: Any mixed background or multiple ethnic

Indian: Indian, Indian Scottish or Indian British.

Other Asian: Other Asian, Asian Scottish or Asian British, Bangladeshi, Bangladeshi Scottish or Bangladeshi British

Pakistani: Pakistani, Pakistani Scottish or Pakistani British

Chinese: Chinese, Chinese Scottish or Chinese British

African Caribbean: African, Caribbean, Black, Other African Caribbean or Black,

Other Ethnic: Arabs

2.6.2.9 Data storage and Ethical Approval:

A research database containing anonymous data was used for analysis. Data were stored in the Department of Biostatistics, University of Glasgow and used for analysis in the Cardiovascular Research Centre, through a secure virtual private network (VPN). Approval for the linkage and analysis was obtained from SCI-Diabetes steering committee, the Scottish multicentre research ethics committee, the Privacy Advisory Committee of NHS - National Services Scotland (NSS), and the Caldicott guardians of all 14 Health Boards in Scotland; PAC Approval – 33/11 and MREC-Reference: 11/AL/0225.

2.6.2.10 Data Clean up

Implausible values were removed from the data at SCI- Diabetes research database. Following were the coding and boundaries used for cleaning the data. Note that the bounds were kept deliberately very broad- seeking only to remove impossible values.

Date of Birth: 1900 < value < 31st December 2011

Sex: 0 = unknown; 1 = male; 2 = female; 9 = not specified

Ethnic groups: 0 = scottish; 1 = carribean; 2 = african; 3 = indian; 4 = pakistani; 5 = bangladeshi; 6 = chinese; 29 = unknown; 30 = any other ethnic origin; 31 = irish; 32 = other british; 33 = any other white background; 34 = any other black background; 35 = any other asian background; 36 = any other mixed background; 40 = not known; 50 = not disclosed

Date of diabetes diagnosis: 1900 < value < 31st December 2011

Fasting Venous Plasma Glucose at Diagnosis (mmol/L): bounds: 0.5 <value <100

2Hour Venous Plasma Glucose at Diagnosis (mmol/L): bounds: 0.5 <value <100

Random Venous Plasma Glucose at Diagnosis (mmol/L): bounds: 0.5 <value <100

Type of Diabetes: 0 = not diabetic; 1 = type 1; 2 = type 2; 3 = impaired glucose tolerance; 4 = impaired fasting tolerance; 5 = gestational; 6 = maturity onset diabetes of the young; 7 = stress event; 8 = other diabetes mellitus; 9 = type unknown; 10 = diabetes resolved

Patient Weight (Kg): bounds: 40 < value < 300

Patient HEIGHT (meter): bounds: 1 < value < 3

Body Mass Index (BMI): bounds: 14 < value < 75

Patient Smoking Status: 0 = unknown; 1 = current; 2 = ex; 3 = never

Systolic Blood Pressure (mmHg): bounds: 80 < value < 400

Diastolic Blood Pressure (mmHg): bounds: 40 < value < 300

Serum Creatinine (μmol/l): bounds: 40 < value < 1999

Serum Total Cholesterol (mmol/L): bounds: 2 < value < 50

Serum HDL Cholesterol (mmol/L): bounds: 0.5 < value < 5

Triglycerides (mmol/L): bounds: 0.5 < value < 100

Glycated Haemoglobin (HbA1c) (%): bounds: 4 < value < 30

Blood Glucose (mmol/L): bounds: 3 < value < 100

Myocardial Infarctions: bounds: value < 15

Stroke: bounds: value < 15

2.6.2.11 Preparation of data for analysis

The following steps were applied to clean data before analysis

Sex: Unknown and not specified were removed. Females were coded= 0 and males = 1.

Ethnic groups: Ethnic groups were merged and the detail is written in the Section; Ethnicity

BMI: BMI values were removed from the analysis if they were <14 (n= 1) and ≥ 52 (n=89).

Blood Pressure: BP values were not considered for five people whose DBP was more than SBP. For SBP; values less than 80mmHG and values more than 260mmHg were removed (n=11). For DBP; values less than 40mmHg and more than 140mmHg were removed (n= 8). Both the SBP and DBP show trend of terminal digit preference (also shown in histogram of Figure 2.1)

Total cholesterol: total cholesterol levels were not considered if it were more than 30µmol/L (n= 29 excluded).

LDL-c: LDL values more than 7mmol/L were removed from analysis (n=10 excluded).

Triglycerides: TG values more than 30 mmol/L were excluded from the analysis (n= 47 excluded)

HbA1c: values more than 20% were excluded (n= 5)

Creatinine: values more than 500µmol/l were excluded from analysis (n= 49 excluded)

Duration of diabetes: People who were diagnosed diabetes at the time of first CVD event (n=12) were excluded from the analysis.

2.6.2.12 Selection of cohort

Inclusion: Only people with Type 2 diabetes having follow-up data (either hospital admission or any data for outpatient clinical visits) between Jan 2005 – December 2011 and available ethnicity data were included in the study. Patients having no follow-up data for clinical variables after 2005 (n=4557, 1.6%) were excluded with the assumption that they may have left the area (Figure 7.2)

Exclusion: People with other types of diabetes, missing type of diabetes or who developed type 2 diabetes before age of 17 (n = 109) were excluded from analysis. Children aged <17 were excluded as the SCI- Diabetes system is used mainly for adults and has less complete coverage in the paediatric population Patients with missing ethnicity data (n=67,994, 24%) were also excluded from the main analysis. Patients with inconsistent data (n=12) were also excluded- i.e. dates of examination after date of death.

Entry Date: For the analysis, entry date was taken as 1st Jan 2005 or the date of diabetes diagnosis if later

Exit date: was recorded as 31st Dec 2011 or date of 1st CVD event or death if earlier

Follow-up time: exit date – entry date.

2.6.2.13 Prevalent CVD at baseline

Prevalent CVD was defined as hospital admission for CVD (using codes above and equivalent ICD9 codes for earlier data) in retrospective data to 1992 with similar “lookback” time for the different ethnic groups. Lookback time is the time in years (retrospective) between entry date and date of CVD incidence. The lookback time for different ethnic groups was e.g. White: mean±SD 4.7±2.8 years, Multiple Ethnic: 4.9±3.0, Indian: 4.6±2.5, Other Asian: 4.7±2.3, Pakistani: 4.2±2.6 years, African-Caribbean: 4.2±2.8 years and Other Ethnic: 4.7±2.7.

2.6.2.14 Population used in analysis

After following the exclusion criteria above and also excluding the people with prevalent CVD, a total of 121,523 people who did not have any history of CVD at baseline were followed for development of CVD.

2.6.2.15 Incident CVD events

Incident CVD events (cardiovascular and cerebrovascular) were obtained from SMR-01 records using the ICD 10 codes: I20–I25, I60–69 (excluding I62 and I68). A person was labelled as having an event if he or she experienced the event between 1 Jan 2005 and 31 Dec 2011. Details of ICD 10 codes used in analysis are attached as Appendix C. Individuals with stable angina are not included in this category. Other people who may have been missed would be of old age.

2.6.2.16 Confounding Variables

Age of diabetes diagnosis: Calculated as date of diabetes diagnosis – Date of birth

Age at baseline: Calculated as entry date – Date of birth

Anthropometric and other variables: For baseline anthropometric and biochemical measures (BMI, BP, total cholesterol, HDL-c, LDL-c, triglycerides, HbA1c, creatinine), the values nearest to the entry date (and within past 9 months) were obtained. Following preference was used to obtain the value.

1 = same day as opening date.

2 = up to 90 days (3 months)

3 = 91 – 180 days (4-6 months)

4 = 181 – 270 days (7-9 months)

3 Euglycaemic clamp insulin sensitivity and longitudinal systolic blood pressure: role of gender

3.1 Introduction

A relationship between insulin resistance, hyperinsulinaemia and blood pressure/hypertension was suggested nearly three decades ago (196) but the nature of this relationship and its pathophysiological basis remains unclear. Most (871-884) but not all studies (885-889) have reported a relationship between insulin resistance and future BP rise and/or the development of hypertension. The majority of studies demonstrating this relationship have used surrogate markers for measuring insulin sensitivity. The few which used direct measures of insulin sensitivity have been either small (890), or cross-sectional(202;891). As explained in the Introduction (Section 2.2.6 and 2.2.7), these surrogate measures are critically dependent on insulin immunoassays. They correlate with clamp-measured insulin sensitivity (892) but do not take account of body mass or body composition.

The hyperinsulinaemic euglycaemic clamp (HEC) technique, is the gold standard for the assessment of insulin sensitivity(776) but as it is an invasive and labour-intensive procedure, it has not been applied in adequately-sized cohorts for evaluating the risk of developing hypertension. In this chapter I analysed HEC data from the large healthy RISC cohort to evaluate the hypothesis that insulin sensitivity is an independent predictor of BP rise or development of hypertension. In addition, I examined whether sex influenced this relationship.

3.2 Statistical analysis

Distribution of all continuous variables was checked at baseline and year 3 follow-up: age, height, waist, hip, waist, BMI, fat free mass, fat mass, SBP, DBP, Percent change in BMI, Glucose, LDL, total cholesterol, HDL, triglycerides (TG), insulin sensitivity (M/I), alcohol intake and physical activity (Appendix A: Figure 1.1-1.6). All variables exhibited a normal distribution except for TG, alcohol intake, physical activity, hsCRP and IL-6 which were log transformed for analysis (Appendix A: Figure 1.6 and 1.7). The histogram for fat free mass at baseline and year 3 showed two peaks (Appendix A: Figure 1.3): on further investigation, it was found that this reflected differences in fat free mass between males and females (Appendix A: Figure 1.3). Log M/I showed a significant negative linear correlation with systolic BP at year 3 (r= -0.207, p

=<0.001) (Appendix A: Table 1.1). Further exploration revealed a significant gender*log M/I interaction term for SBP at Year 3 in pooled regression with all of the data (p=0.003). This effect of gender on the relationship of insulin sensitivity and SBP was also apparent in scatterplot showing a negative linear relationship in females only (Appendix A: Figure 1.8). The analysis in this chapter was therefore conducted separately by gender owing to this finding of a significant interaction term in the early analyses. The correlation matrix for the different measures in participants of the RISC cohort is shown in Appendix A- Table 9.1.

Baseline measurements are shown in Table 3.1 and 3.2, with univariate Pearson correlation coefficients between change in systolic and diastolic BP and other covariates in Table 3.3. Multiple linear regression analysis was used to determine whether insulin sensitivity (M/I) predicted systolic and/ or diastolic BP at three years with covariates including age, recruitment centre (using indicator variables), baseline BP, BMI, change in BMI, blood glucose, lipid profile and lifestyle factors. Given that baseline BP and change in BP are usually highly correlated, the relationship between log M/I and systolic BP at Year 3 adjusted for baseline systolic BP was used, in preference to the unadjusted relationship with change in systolic BP.

The quadratic term (LogM/I* LogM/I) was used to check the linearity of the association between BP and M/I in both genders. The relationship was linear for both SBP and DBP after adjustment for centre only or centre and age. β-coefficients for insulin sensitivity are shown along with R^2 for the coefficient of determination for the model. Due to multicollinearity of waist, fat mass and weight with BMI ($r > 0.7$ for all correlations), only BMI was used in regression models. However multiple regression analysis was repeated by substituting waist for BMI. Multiple regression was also used to check the contribution of M/I in the model in predicting risk. Binary logistic regression was used to assess prediction of hypertension defined according to ESH/ JNCVII as ≥140/≥90 mmHg (104;860) (or by its treatment). Odds ratios (OR) with 95% confidence intervals (CI) are shown as the odds of developing hypertension in relation to M/I.

Change in BP between baseline and the follow-up examination was then evaluated in relation to baseline insulin sensitivity. The M/I was used as a continuous variable in all the correlation and regression analyses. Insulin

sensitivity was then split into tertiles in order to visually inspect the relationship with change in BP.

ANOVA was used to examine change in BP across sex-specific tertiles of baseline insulin sensitivity with Tukey *post hoc* testing (corrected for multiple comparisons) to examine changes between tertiles. Change in BP between baseline and follow-up was compared within each tertile of insulin sensitivity using paired t-tests. SPSS version 18 was used in all analyses.

3.3 Results

At three years, BP had reached a diagnostic threshold for hypertension in 11.6% of all participants (n=125; 75 men, 50 women). A further 4.3% (n=46; 23 men, 23 women) had been commenced on antihypertensive treatment in routine care i.e. 16.0% (n= 171; 98 men, 73 women) had developed incident hypertension.

Mean age in both males and females was approximately 44 years. SBP, DBP, waist, weight, BMI, fat free mass, glucose, LDL, TG and alcohol intake were significantly higher in men compared with women (Table 3.1). However, men had lower insulin sensitivity (M/I) (112 vs 141) and less fat mass (18.6 vs 22.7) than women. Smoking rates and physical activity levels were similar in men and women (Table 3.1). Insulin concentrations achieved at steady state in the clamp procedures (mean±SD) were 416±111 pmol/L in men and 406±112 pmol/L in women.Except for lean body (fat free) mass, anthropometric measures increased over three years of follow-up for the whole cohort and also in males and females when considered separately (Table 3.2). Although SBP, DBP, weight and BMI decreased in a few individuals; mean BP and BMI increased over three years.

In univariate analyses (Table 3.3), change in both systolic and diastolic BP correlated in the expected manner with baseline values in both genders. Change in both systolic and diastolic BP from baseline was correlated with insulin sensitivity (log M/I) in women (r= -0.132 for SBP) but not in men (r=-0.054 for SBP). Weight correlated with change in diastolic but not systolic BP in both genders. Other correlations were of borderline statistical significance.

When key covariates (including recruitment centre, age, baseline systolic BP, BMI, change in BMI, lipid profile, smoking status, and fasting glucose) were included in multiple regression analyses (Table 3.4), low insulin sensitivity (log M/I) significantly and independently predicted systolic BP at three years in women (β = -0.214, P<0.001) but not in men. However, no relationship between M/I and SBP was detected in males, even in univariate analyses. Following adjustment for baseline DBP, Insulin sensitivity did not predict longitudinal DBP rise after adjustment for BMI in either males or females (Table 3.5). When regression analyses were repeated substituting waist for BMI, results were very similar. There was no relationship between insulin sensitivity and systolic BP in either gender when HOMA was substituted for LogM/I as an independent variable.

Pearson correlation and multiple regression analyses showed significant relationships between insulin resistance and BP to be present only in females. For the purposes of visual inspection and presentation, baseline and follow-up SBP were expressed according to sex-specific tertiles of insulin sensitivity (Figure 3.1). M/I tertiles were as follows, for men: *low* 16.2-90.8; *intermediate* 90.8-137; *high* 137-454 $\mu mol \cdot min^{-1} \cdot kgffm^{-1} \cdot nM^{-1}$, and for women: *low* 21.4-120.2; *intermediate* 120.2-173.8; *high* 173.8-977.2 $\mu mol \cdot min^{-1} \cdot kgffm^{-1} \cdot nM^{-1}$. There were no differences between tertiles amongst men; mean (SD) [123±11 (low); 121±11 (intermediate); 123±10 mmHg (high)]. However, women with low baseline M/I had higher baseline systolic BP: 117±13 mmHg (low), 111±12 mmHg (intermediate), 114±12 mmHg (high) [low *vs* intermediate, 6.0 (95% CI 3.0, 9.0) mmHg, *P*<0.001; low *vs* high 3.0 (95% CI -0.1, 5.9) mmHg, *P*=0.06].

Over three years of follow-up, SBP increased from baseline in all tertiles for men (*P*<0.05) (Figure 3.1). In women, SBP rose in those with low and intermediate M/I (*P*<0.05), but no change was observed in the high insulin sensitivity tertile (114±12 *vs* 114±14 mmHg; *P*=0.791, 0.2 (95% CI -1.8, 1.4)). Comparing unadjusted 3 year SBP between insulin sensitivity tertiles within each gender (Tukey *post hoc* testing), no statistically significant differences were observed in men: [127±13 (low); 126±13 (intermediate), 126±14 mmHg (high)]. However, in women 3 year SBP in the low M/I tertile (121±16 mmHg) was significantly higher than in the intermediate (116±16 mHg) (*P*=0.001, 5.6 (95% CI 1.93, 9.26)) and

high (114±14 mmHg) M/I tertiles [*P*=<0.001, 7.1 (95% CI 3.47, 10.79)]. Test for trend for three year SBP across the M/I tertiles was significant in women ($p<0.001$) but not in men ($p=0.307$). When these analyses were repeated excluding individuals on antihypertensive treatment (as a sensitivity analysis), similar results were obtained.

As log M/I was a significant predictor for SBP rise only in females, its specific contribution was further explored: this showed that it explained only 0.4 to 0.5 % of the variance of the combined model; shown in model 5 and 6 (Table 3.6).

The test for trend across the M/I tertiles was significant for the development of hypertension in women ($p<0.01$) but not in men ($p=0.260$). In terms of odds ratios, unadjusted M/I was associated with a lower risk of developing hypertension in total [$p<0.001$, OR= 0.201 (95% CI 0.09, 0.43)] and in females [$p<0.001$, OR= 0.106 (95% CI 0.03, 0.36)]. However, following adjustment for baseline SBP, age and BMI, there was no longer a significant association in either gender or in the total population (Table 3.7). This was the case whether M/I was used as either a continuous or categorical variable.

Table 3.1 Baseline characteristics of men and women in the RISC Study

	Men (n=486)	Women (n=587)	P
Age (years)	43.8 ±8.5	44.9 ±8.0	0.04
Systolic BP (mmHg)	126 ±14	117 ±16	<0.001
Diastolic BP (mmHg)	77 ±7	73 ±8	<0.001
Waist (cm)	93 ±10	81 ±11	<0.001
Weight (Kg)	83.4 ±12.3	67.5 ±12.0	<0.001
BMI (Kg/m²)	26.2 ±3.4	24.8 ±4.2	<0.001
Fat Free Mass (Kg)	64.8 ±7.0	44.8 ±4.3	<0.001
Fat Mass (Kg)	18.6 ±7.6	22.7 ±9.0	<0.001
Glucose (mmol/L)	5.2 ±0.5	5.0 ±0.5	<0.001
Total Cholesterol (mmol/L)	4.9 ±0.9	4.8 ±0.9	0.03
LDL Cholesterol (mmol/L)	3.1 ±0.8	2.8 ±0.8	<0.001
HDL Cholesterol (mmol/L)	1.3 ±0.3	1.6 ±0.4	<0.001
Triglycerides (mmol/L)*	1.12 [1.07-1.17]	0.86 [0.83-0.89]	<0.001
Clamp Insulin Sensitivity (M/I) *	112 [107-117]	141 [135-148]	<0.001
Smoker (%)	26	26	0.91
Alcohol grams/week*	81 [76-89]	47 [43-50]	<0.001
Phys. Activity (Counts per min)*	339 [316-355]	324 [316-339]	0.43
Menopause (Y/N)	-	153/ 434	-
Creatinine µmol/L	75 ±12	59 ±12	<0.001
eGFR (ml/min/ 1.73m^2)	110 ±29.5	107 ±36.5	0.17

Data shown are as mean ± standard deviation (SD), or geometric means and confidence intervals [CI]. BP= blood pressure, BMI= body mass index, HDL= high density lipoprotein, LDL= low density lipoprotein, eGFR= estimated glomerular filtration rate
***log-transformed for analysis**

Table 3.2 Baseline and three year follow-up measures in the RISC cohort

	Total (n=1073)		**Men (n=486)**		**Women (n=587)**	
	Y0	**Y3**	**Y0**	**Y3**	**Y0**	**Y3**
Systolic BP (mmHg)	118± 12***	121±15***	122± 10***	126± 13***	114± 13***	117± 16***
Diastolic BP (mmHg)	75±8***	76 ±9***	76±7***	79±9***	73±8***	74±9***
Waist (cm)	87 ±12***	88±13***	93±10***	95±11***	81±11***	82±12***
Weight (Kg)	74.7±14.5***	75.7±15.3***	83.4±12.3***	84.5±13.2***	67.5± 12***	68.4±12.9***
BMI (Kg/m²)	25.4±3.9***	25.8±4.2***	26.2± 3.4***	26.5± 3.7***	24.8± 4.2***	25.2± 4.5***
Fat free Mass(Kg)	53.9±11.5	53.8±11.7	64.8±7	64.7±7.4	44.8±4.3	44.7±4.9
Fat Mass (Kg)	20.8±8.6***	21.9±9.5***	18.6±7.6***	19.8±8.9***	22.7±9***	23.6±9.7***

Values are mean ± SD. Y0= Baseline (year 0), Y3= 3 year follow-up. T test for comparison between baseline and three year follow-up. ***p<0.001

Table 3.3 Pearson correlation (r) between baseline characteristics and change (Δ) in systolic and diastolic BP over three years

Characteristic	ΔSBP		ΔDBP	
	Male	Female	Male	Female
	r	r	r	r
Insulin Sensitivity (log M/I)	-.054	**-.132****	-.045	**-.110****
Age (years)	.162***	.027	.074	-.068
Baseline Systolic BP (mm Hg)	-.293***	-.273***	-.162***	-.163***
Baseline Diastolic BP (mmHg)	-.293***	-.196***	-.340***	-.339***
Waist (cm)	.056	.055	.067	.008
Weight (Kg)	.077	.073	.113*	.134**
BMI (Kg/m²)	.034	.081*	.086	.100*
Fat Free Mass (Kg)	.092	.050	.079	.162***
Fat Mass (Kg)	.040*	.074	.110*	.103*
Glucose (mmol/L)	-.008	.016	-.005	-.037
Total Cholesterol (mmol/L)	.029	.076	-.041	-.012
LDL Cholesterol (mmol/L)	.061	.080	-.044	-.017
HDL Cholesterol (mmol/L)	-.153**	-.009	-.100*	-.007
TG (mmol/L)	.075	.016	.058	.006
Smoker (%)	.019	-.004	-.010	-.022
Alcohol (g/week)	.021	.044	.012	.057
Physical Activity (Counts per minute worn)	-.014	.000	-.024	-.103*
Creatinine μmol/L	-.041	-.031	.052	.080
eGFR (ml/min/ 1.73m^2)	.055	.030	-.035	-.067

***p<0.05, **p<0.01, ***p<0.001. M/I used as a continuous variable**

Table 3.4 Standardised beta coefficients for predicting systolic BP at three year follow-up from log M/I as independent variable (with various adjustment factors)

Model	Adjustment factors	Men (n=486)			Women (n=587)		
		R^2	Beta	*P*	R^2	Beta	*P*
1	Unadjusted	0.005	-0.069	0.13	0.046	-0.214	<0.001
2	Centre & Age	0.147	-0.049	0.30	0.250	-0.194	<0.001
3	Model 2 + baseline systolic BP	0.365	-0.034	0.40	0.466	-0.121	<0.001
4	Model 3 + BMI	0.368	-0.007	0.87	0.474	-0.081	0.03
5	Model 4 + %change BMI	0.385	-0.003	0.95	0.481	-0.078	0.03
6	Model 5 + baseline eGFR	0.386	-0.004	0.94	0.481	-0.077	0.04
7	Model 6 + glucose, Chol, LDL, HDL, log TG	0.397	0.030	0.54	0.486	0.073	0.05
8	Model 7 + baseline HRT & OCP use	0.397	0.030	0.54	0.488	-0.079	0.04
9	Model 8 + smoking	0.398	0.031	0.52	0.488	0.079	0.04
10	Model 9 + phys. Activity	0.396	0.026	0.68	0.482	-0.086	0.09
11	Model 10 + log Alcohol	0.396	0.026	0.69	0.483	-0.088	0.13

M/I used as a continuous variable

Table 3.5 Standardised beta coefficients for predicting diastolic BP at three year follow-up from log M/I as an independent variable (with various adjustment factors)

Model	Adjustment factors	Men (n=486)			Women (n=587)		
		R^2	Beta	*P*	R^2	Beta	*P*
1	Unadjusted	0.029	-.169	<0.001	0.045	-.213	<0.001
2	Centre & Age	.202	-.126	.01	.214	-.185	<0.001
3	Model 2 + baseline diastolic BP	.368	-.043	.30	.446	-.100	<0.01
4	Model 3 + BMI	.380	.005	.90	.463	-.042	.26
5	Model 4 + %change BMI	.399	.010	.82	.477	-.038	.30
6	Model 5 + baseline eGFR	.399	.011	.81	.477	-.041	.26
7	Model 6 + glucose, Chol, LDL, HDL, log TG	.408	.044	.37	.482	-.038	.32
8	Model 7 + baseline HRT & OCP use	.408	.044	.37	.483	-.042	.27
9	Model 8 + smoking	.409	.047	.33	.483	-.043	.27
10	Model 9 + phys. activity	.409	.047	.45	.483	-.025	.61
11	Model 10 + log Alcohol	.410	.048	.46	.483	-.028	.63

M/I used as a continuous variable

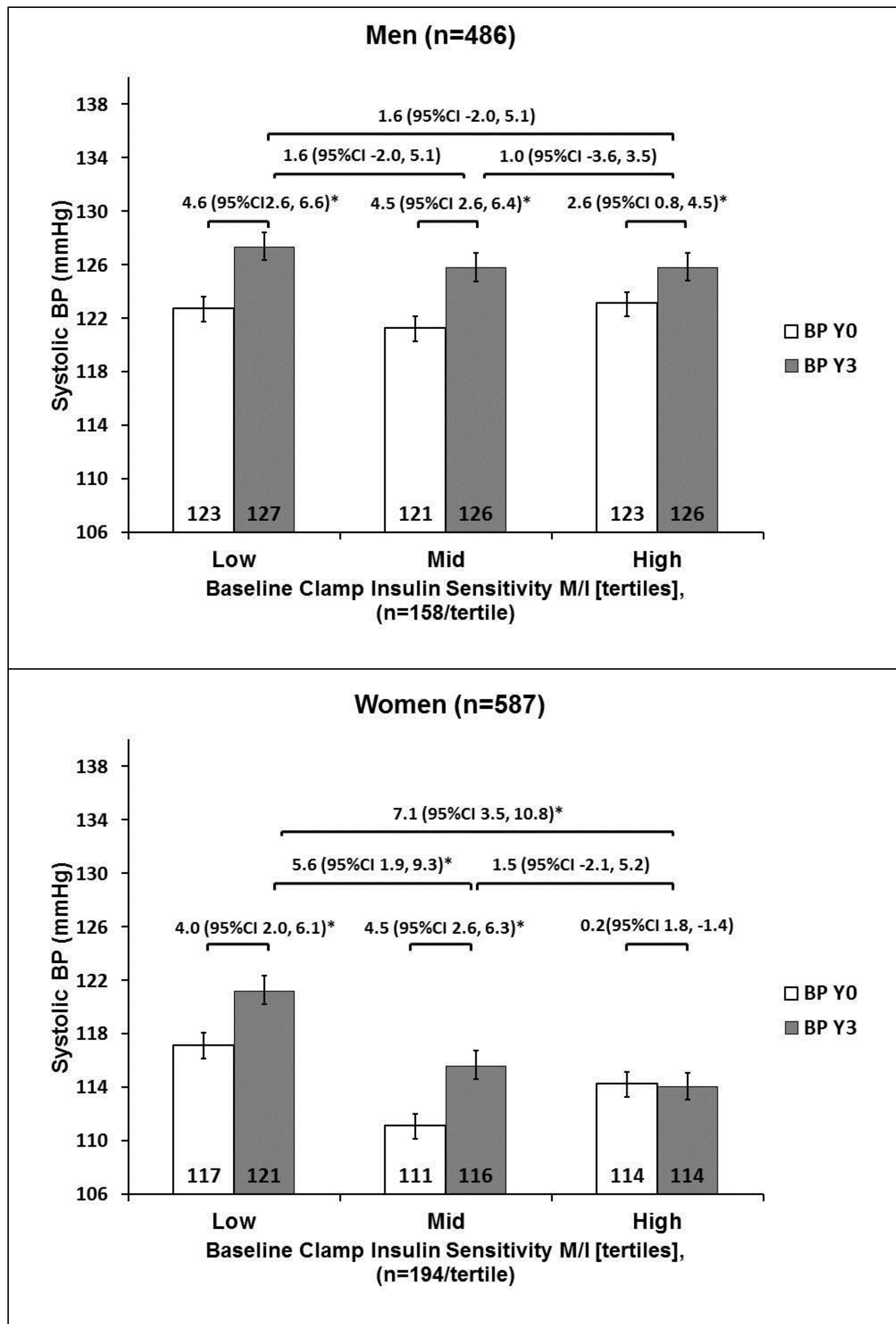


Figure: 3.1 Unadjusted systolic BP (mean±SEM) at baseline and three year follow up in men (upper panel) and women (lower panel) by tertiles of baseline insulin sensitivity.
Values inside the base are of BP in mmHg. Comparison between tertiles by ANOVA with Tukey post hoc testing with 95% confidence intervals for the difference in means between tertiles. Differences in means between baseline and 3 year follow-up SBP by paired sample t tests.
BPY0 = baseline systolic BP, BPY3 = systolic BP at three years (mean±SEM),
†= P<0.05, ** = P<0.01, ***= P<0.001, NS= not significant.
M/I tertile range; Men: low 16.2-90.8; intermediate 90.8-137; high 137-454 μmol·min-1·kgffm-1·nM-1, and Women: low 21.4-120.2; intermediate 120.2-173.8; high 173.8-977.2 μmol·min-1·kgffm-1·nM-1

Table 3.6 Estimation of contribution of insulin sensitivity in prediction of SBP in females

	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
	Baseline SBP	Model 1 + Age	Model 2 + BMI	Model 3 + % change BMI	Model 3 + Log M/I	Model 4 + Log M/I
Model R^2	.435	.454	.469	.477	.474	.481

Model R^2 calculated by multiple regression. Each subsequent model shows increase in R^2 with addition of another variable. Log M/I contributed only 0.5% in model 5 (ΔR^2= 0.474-0.469) and 0.4% in model 6 (ΔR^2= 0.481- 0.477). M/I used as a continuous variable

Table 3.7 Odds Ratio of Log M/I for the development of hypertension in total, male and female population

		Total		Males		Females	
Model	Adjustment factor	OR	P	OR	P	OR	P
1	Unadjusted	.201	<.001	.474	.156	.106	<.001
2	Centre & Age	.229	<.001	.509	.243	.122	.002
3	Model 2 + baseline SBP	.396	.038	.509	.253	.236	.050
4	Model 3 + BMI	.594	.284	.588	.416	.535	.434
5	Model 4 + %change BMI	.590	.283	.577	.405	.516	.413
6	Model 5 + glucose, Chol, LDL, HDL, log TG	.545	.266	.629	.545	.427	.329
7	Model 6 + baseline HRT & OCP use + smoking + phys. Activity + log Alcohol	1.45	.690	2.08	.569	.239	.445

OR = odds ratio, M/I used as a continuous variable. BP= blood pressure, BMI= body mass index, HDL= high density lipoprotein, LDL= low density lipoprotein, Chol= cholesterol, TG= triglycerides, HRT= hormone replacement therapy, OCP= oral contraceptive pills and Phys= physical.

3.4 Discussion

In this analysis of 1,073 healthy European adults, low insulin sensitivity measured using a robust and standardised euglycaemic clamp technique predicted rise in systolic BP at three years in women but not in men. Systolic BP was higher at baseline in women with low insulin sensitivity than it was in those with intermediate or high insulin sensitivity. It increased over three years in all groups studied except women with high baseline insulin sensitivity. Insulin sensitivity predicted change in systolic BP independently of key covariates (including age, baseline BP, BMI and change in BMI) in women only. As the

overall contribution in prediction was minimal, this observation is of mechanistic rather than clinical relevance. The findings of the study therefore offer new insights into the relationships between metabolic factors and BP by gender, clarifying and extending cross-sectional data previously reported from the RISC cohort (893). In general they imply a less important role for insulin resistance in the pathogenesis of hypertension than has been suggested by previous investigators. However, the duration of follow-up was short and only 16% of individuals in the cohort developed incident hypertension: ideally longer follow-up is required.

The most comprehensive previous study on this topic was the Framingham Offspring study in which insulin sensitivity was assessed in 1,933 healthy adults using an insulin sensitivity index based on fasting and post-load insulin and glucose levels.(811). In this report, insulin sensitivity (expressed categorically in sex-specific quartiles and stratified by age) was independently associated with BP over time in younger, leaner individuals of both genders but not in those who were older, overweight or obese.

A unique feature of the present analysis is that it is based on data from a large number of individuals undergoing a standard euglycaemic clamp. Insulin sensitivity was directly derived from the glucose infusion rate during steady state euglycaemia adjusted only for centrally- measured insulin concentrations and lean body mass measured using a standard device.

Only two previous investigations into the longitudinal relationship between insulin sensitivity and BP in adults have incorporated direct measures of insulin sensitivity (882;890), and only one used the euglycaemic clamp. One of these studies, was relatively small (n=54) and in men only: no effect was demonstrated.(890) The other used a modified frequently-sampled intravenous glucose tolerance test in a tri-ethnic population (n=840) and reported a modest protective association of insulin sensitivity on the risk of hypertension (882).

Other longitudinal studies reporting a relationship between insulin sensitivity and BP (872-884) have been based on surrogate measures of insulin resistance including either fasting insulin (872-877;879;883;884) or fasting insulin and glucose concentrations (HOMA) (880;881). They have been conducted in a variety

of populations [Scandinavian(873;877;884); Mexican-American(878;879); Japanese individuals(875;876)] with some including only men (872;873;881;887;890) but none only women. Some studies of reasonable size and duration have reported no relationship between insulin sensitivity and BP (871;885;886;888;889) after adjustment for baseline BP and weight/ adiposity, although it is difficult to interpret from some studies whether data were examined separately by gender.

Validation studies of HOMA against clamp insulin sensitivity report Pearson *r* values of between 0.5 and 0.6 but are based on small numbers of participants pooled for gender (782;892). On average, women have a lower percentage of lean body mass compared with men. Therefore, in the presence of intact ß-cell function a given absolute value of fasting insulin (or HOMA) in women reflects a greater level of *tissue* insulin resistance than in men. Women in the Framingham Offspring cohort had (on average) lower BMI and lower fasting insulin than men, but a similar insulin sensitivity index. If BP tracking over time is related to tissue rather than whole body insulin sensitivity, euglycaemic clamp data are likely to be more precise by gender than indices based on fasting insulin. Differences in the relationships among insulin sensitivity and BP according to gender may reflect higher fat mass as a percentage of body weight in women, particularly with ageing (648).

In summary, these prospective data from the Europe-wide RISC cohort of healthy adults indicate that low insulin sensitivity measured using the euglycaemic clamp technique is an independent predictor of longitudinal change in BP over time in women but not in men. Women with high insulin sensitivity may be protected against rise in systolic BP over time. The physiological basis for the gender difference I report in the RISC cohort and its implications for the role of insulin resistance in the pathophysiology of hypertension remain uncertain. Further insights may be gained by further follow up of the cohort.

The strengths, limitations and final conclusion are discussed in the final discussion (chapter 8)

4 Systematic review: relationship between CRP/IL-6 and blood pressure

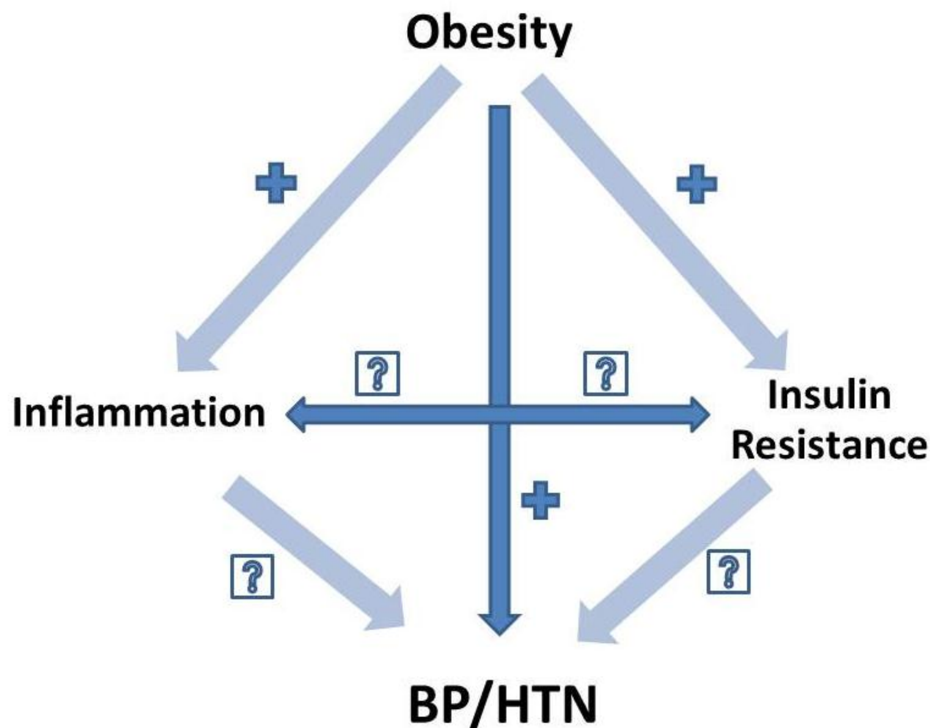
4.1 Introduction

Obesity (894), inflammatory markers (895;896), insulin resistance (196), family history and race (2;897) have been associated with blood pressure and the development of hypertension. Some studies show independent relationships between CRP, IL-6 and BP, while others show more complex relationships amongst the risk factors. The relationship between BMI or obesity with BP is the most studied pathway and has been demonstrated in many large studies (894;898).Holland et al showed over a long follow-up of 36 years (n= 3332) that BMI was directly related to BP from childhood to adult age (899) and the associations of weight/BMI and weight change with BP/hypertension have also been shown in many studies (900-903). Similarly weight loss achieved through bariatric surgery in morbidly obese patients results in a decrease in BP along with resolution or improvement of hypertension in 60-80% of cases (904-906)

The relationship between CRP and CVD (MI and stroke) has been reviewed above (see Section 1.6.3 and 1.13.1), however, the relationship between inflammatory markers and the development of hypertension is more controversial. Inflammatory markers have been associated with the development of hypertension in studies published over the last two decades (895;896). Some epidemiological studies support a relationship between high levels of CRP and hypertension (827). However, the evidence of a causal association in humans is not strong (818-820;828). It remains uncertain whether CRP or another related inflammatory mediator e.g. IL-6 could increase BP directly, whether the relationship is mediated by some other mechanism (e.g. obesity, insulin resistance) or whether both are affected by some other feature of the metabolic syndrome; e.g. insulin resistance (829). The association of chronic low grade inflammation with HTN is widely documented in experimental and clinical results and inflammatory activation is implicated in the development of the cardiovascular consequences of HTN. However, it still remains unclear whether inflammation is a pathogenic inducer of HTN or whether HTN precedes the inflammatory events of atherosclerosis (282) (reverse causality).

As discussed in Chapter 3, insulin resistance (IR) has an association with rise of BP over time which may have some pathophysiological relevance to the development of hypertension (196;811;871;882;883). This association is weaker

than that between IR and dyslipidaemia: for example, only about 50% of hypertensive subjects are insulin-resistant (198). Insulin resistance is also related to obesity (907) and inflammation (908). My aim in this Chapter was therefore to conduct a systematic review of the evidence linking inflammatory markers (CRP and IL-6) with blood pressure and the development of hypertension independent of insulin resistance. Both CRP and IL-6 were evaluated instead of only one marker as a check of internal validity i.e. if a relationship was shown with both markers it would provide reassurance of a robust relationship.



Hypothesis: low grade inflammation is associated with BP and incident hypertension independently of adiposity and insulin resistance

4.2 Objectives/Outcome:

To evaluate the relationships between IL-6 and CRP with BP (SBP and DBP) and hypertension:

Primary: are these relationships independent of insulin resistance?

Secondary: are these relationships independent of BMI and adiposity?

Tertiary: Do age and sex play a role in these relationships?

Search Strategy and Selection Criteria

A systematic review of published studies was conducted in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (http://www.prisma-statement.org/). Searches were conducted of PubMed (Medline), Embase, Web of Science using MeSH terms and text words as follows: "hypertension OR high blood pressure OR blood pressure"; and "C-Reactive protein OR C Reactive protein OR high sensitivity C-reactive protein OR Interleukin 6 OR IL-6." Each term or text word was mapped to a subject heading. The last search was under taken on 20 December 2011. Searches were done separately in all databases: Medline and Embase were not combined in OVID in order to maximise sensitivity. Searches were conducted separately for CRP and IL-6. The reference lists of retrieved articles were manually searched through Web of Science.

Limitations of Search: Search was limited to articles in English and Humans only.

4.3 Eligibility Criteria

The studies included in the review were those:

1. conducted in humans,

2. written in the English language (as the scientific and technical community predominantly uses English as its common language and limited information can be extracted from papers in other languages),

3. containing original research data, i.e., not a review, abstract, editorial, letter, commentary or duplicate publication.

4. with study designs including observational (both cross sectional (CS) and longitudinal), case control studies and randomised control trials (RCT). Longitudinal studies included both prospective and retrospective studies.

5. evaluating only healthy people (including hypertensives) and also if the population had less than 20% diabetic people and the analysis were adjusted for blood glucose or diabetes.

6. including only adult populations.

7. analysing blood or serum levels of CRP and IL-6 (not tissue levels or genetic polymorphisms).

8. stating clear definition/values of SBP, DBP, Change in SBP, change in DBP and hypertension. Hypertension was defined by SBP, DBP or use of anti-hypertensive medication.

9. with results expressed as correlations, beta coefficient of regression, odds ratio (OR), risk ratio (RR), Prevalence ratio (PR), hazards ratio (HR) or relative risk (RR).

Exclusion Criteria

Studies were excluded if cohorts only included children or young adults (age less than 20 years), included pregnant women only, included those on hormone replacement therapy (HRT) (>50%), had a high prevalence of type 2 diabetes people (more than 20%) or used CRP assays which were not characterised by high sensitivity. Studies were also excluded if only univariable association was reported or in multivariate if the relation was not adjusted for BMI or any other body adiposity measures like weight, waist circumference, waist hip ratio, fat mass, fat free mass etc. The following acute or chronic conditions which may interfere with inflammatory markers were excluded:

Acute Conditions:
Acute aortic dissection
Acute bacterial infection, pneumonia

Chronic disease Conditions:
Myocarditis, cardiomyopathy
Hypertension with diastolic dysfunction
Atrial fibrillation, arrhythmia

Chronic obstructive pulmonary disease (COPD)
Diabetes mellitus with complications like nephropathy, retinopathy, neuropathy, ketoacidosis, diabetic foot,
People with type 1 diabetes
All people with type 2 diabetes
Women having gestational diabetes
Chronic kidney disease
Kidney dysfunction
Sepsis with or without septic shock
Any type of transplant; renal, liver
People undergone any type of cardiac procedure like coronary angioplasty, coronary artery bypass graft (CABG)
Organ failure for example heart, kidney and liver
Sleep disorders, also including sleep deprivation
Sleep apnoea or obstructive sleep apnoea
Angina, Myocardial infarction or any other ischemic heart disease
Pulmonary hypertension
Stroke; both ischemic and haemorrhagic
Hormonal problems like hyperaldosteronism, hypothyroidism, hyperthyroidism (Graves' disease), pheochromocytoma, Cushing's disease
Chronic inflammatory conditions like Rheumatoid Arthritis (RA), Systemic lupus erythematosus (SLE)
Mental conditions like bipolar disorders, depression and any other stress related disorder, Alzheimer disease, fibromyalgia
Brain White or Grey matter lesion
Kawasaki disease
Dyslipidaemia like hypertriglyceridemia, Apo lipoprotein abnormalities
Gestational hypertension, pre-eclampsia or eclampsia
Any type of surgery e.g. abdominal, periodontal, cardiac,
Morbid obesity i.e. BMI >= 35
Patients undergoing dialysis like blood, peritoneum
Cirrhosis of liver
Structural abnormalities of heart; atrial septal defect, valvular abnormalities and aortic aneurysm

4.4 Data Collection and Critical Appraisal

Two individuals (Muhammad Omar Malik (MOM) and Zia-ul-Haq (ZH) independently marked the title of each article for eligibility criteria and to exclude those that were clearly not relevant. All disagreements were resolved by discussion. Abstracts of the remaining articles were then reviewed to eliminate those that did not meet the inclusion criteria. The full text of the remaining articles was appraised independently and compared afterwards. Disagreements were found for three articles and resolved by consensus.

The following information was extracted for each relevant study: (1) study type (CS, longitudinal, case control); (2) region and country; (3) year published; (4) ethnic group; (5) number of individuals in the population; (6) age range and mean/median age of participants; (7) sex distribution; (8) outcome variable [SBP, DBP, change in SBP (ΔSBP), change in DBP (ΔDBP), hypertension (HTN)]; (9) inflammatory marker (CRP or IL-6); (10), inflammatory marker used as a continuous or categorical variable; (11) assay used for measurement of inflammatory marker; (12) adjusted for measure of insulin sensitivity (HOMA-IR, insulin, fasting insulin); (13) measure of adiposity (BMI, weight, fat mass etc.); (14) effect sizes (β, OR, RR, PR, or HR); and (15) lists of variables for which statistical adjustment was performed.

The data collection form is shown in Appendix B

4.5 Results

Searches for CRP and IL-6 were conducted separately.

For CRP, 7072 potentially relevant studies were initially retrieved (See Figure 4.1). Duplicate studies (n=1392) were removed with the help of Reference Manager software with the following preference: Medline, Embase and Web of Knowledge. After removal of duplicates 5748 studies were screened by scanning the titles; from which 4709 were excluded. The abstracts of the remaining 1039 were reviewed to eliminate any that did not meet the inclusion criteria (876 removed). The remaining 163 articles were appraised. This led to a further exclusion of 121 articles that did not meet our eligibility criteria (chronic

disease, diabetes, crude assay, unclear or no direct relation, not adjusted for adiposity, children, review and letter to editor, commentary).

For IL-6, 1902 potentially relevant studies were retrieved (see Figure 4.2). Duplicate studies were removed with the help of Reference Manger software with the following preference hierarchy: Medline, Embase, then Web of Knowledge. After removal of duplicates 1529 studies were screened by scanning the titles; from which 927 were excluded. The abstracts of the remaining 602 were reviewed to eliminate any that did not meet the inclusion criteria (544 removed). The remaining 58 articles were appraised. This led to a further exclusion of 45 articles that did not meet our eligibility criteria (diabetes, unclear or no direct relation, not adjusted for adiposity, children, review and letter to editor, commentary or editorial). The search details are also attached as Appendix B.

42 studies were identified in relation to CRP and 13 were identified in relation to IL-6. For the purposes of presentation, the relationship between inflammatory markers and blood pressure (both SBP and DBP) and the relationship with hypertension *per se* are shown in separate tables. In addition, results are presented in separate tables for cross sectional, longitudinal and case control studies and also whether adjusted for insulin sensitivity or not.

In total, eight studies were identified which included a measurement of insulin sensitivity and also evaluated the relationship between CRP with BP as an outcome variable; for hypertension as an outcome there were only three such studies. In the case of IL-6 only two studies were identified in which data were adjusted for IR (one for BP and one for hypertension). The sample size of studies for CRP ranged from n= 95 to n=16966, and for IL-6 sample size ranged from n=196 to n=3543. The mean age range for studies evaluating CRP was 31-76 years and the mean age range in IL-6 studies was from 40-60 years. Regarding sex distribution, five studies were of female-only populations while three were of male-only populations. The case control studies for both CRP and IL-6 were all conducted in females.

CRP-Cross sectional studies

Seven cross sectional studies were identified showing relationships between CRP and BP, adjusting for a measure of insulin sensitivity (Table 4.1): five reported significant association with SBP and two detected no relationship. The negative (showing no association of SBP) studies (909;910) were conducted in a predominantly male population (>50% population). In addition both of these showed significant relation of DBP with CRP. For DBP, of 6 studies, 4 showed significant associations and two did not.

In cross sectional analysis, CRP was positively associated with development of hypertension after adjusting for a measure of insulin sensitivity (Table 4.2).

In cross sectional studies not considering insulin sensitivity, SBP was associated with CRP in half of the studies (7/14), while DBP was not related to CRP in any of the studies (0/10) (Table 4.3). Within the same group of studies; larger cohorts (>1000 people) also showed that SBP was related to CRP in 2 out of 5 studies (Table 4.3).

Table 4.4 shows the relation of CRP with the development of hypertension, not accounting for insulin sensitivity. Out of the 11 studies identified only 5 showed a relationship with CRP. There was no ethnic, age, sex and size of cohort pattern in the studies showing relation with CRP and hypertension.

CRP-Longitudinal Studies

Only one longitudinal study (911) evaluated the relation of CRP (categorical) with BP and hypertension; also adjusting for insulin sensitivity (Table 4.5 and 4.6). It showed no relation of CRP with BP but there was a significant relation with hypertension over a 10 year of follow-up. However, the same study showed the relation to be significant for both SBP and DBP in cross sectional analysis; for both males and females.

Two studies evaluated the effect of CRP on longitudinal prediction of BP (not adjusting for insulin sensitivity) and only one showed significant relation with SBP but none with DBP (Table 4.7). Out of the eight longitudinal studies evaluating the association of CRP with the development of hypertension (not adjusting for insulin sensitivity), six presented significant association while two

had negative results (Table 4.8). No influence of age, sex, ethnicity and size was seen on this relation with hypertension.

CRP-Case control studies

Two case control studies were identified and both of them did not show any relation of CRP with longitudinal development of hypertension in females (Table 4.9).

IL-6- Cross sectional studies

The relation of IL-6 with BP and hypertension is shown in Tables 4.10- 4.15. Only one study was identified: with no relation of IL-6 with BP when adjusting for HOMA-IR (Table 4.10). However, the development of hypertension was related to IL-6 in females only independently of insulin resistance (Table 4.11). All (3/3) cross sectional studies evaluating association of IL-6 with SBP and DBP showed significant association (Table 4.12). Similarly IL-6 was related to hypertension development in cross sectional analysis (Table 4.13).

IL-6-longitudinal studies

In longitudinal analysis IL-6 was related to development of hypertension over a 5 year of follow-up (Table 4.14).

IL-6- Case Control studies

Both the case control studies did not show any relation with HTN in females, over a long follow-up (Table 4.15).

4.5.1 Influence of age and sex in relation of CRP with BP

Another angle of viewing these studies was to check if age and sex influenced the relationship of inflammatory markers with BP and hypertension. We only reviewed CRP relations as studies with IL-6 measurement were few. For age, we separated the studies with mean age less than 50 (younger group), and more than equal to 50 (older group).

4.5.1.1 For DBP

Younger group: All studies which adjusted for insulin sensitivity (n=3) showed a significant relationship between CRP and DBP ((909;910;912)). These studies included both sexes but with a higher proportion of males. The studies in this group which did not adjust for insulin sensitivity showed no relation between CRP and DBP (913-917).

Older group: No studies in this group reported a relationship between CRP and DBP except for Cheung et al. (911). However, Cheung et al. showed a relationship only in cross sectional analysis (and not in an accompanying 10 year longitudinal analysis) (911). In summary DBP, was associated with CRP in a younger population with male predominance when analysis was adjusted for insulin sensitivity.

4.5.1.2 For SBP

Younger group: Considering the three studies which also adjusted for insulin sensitivity (909;910;912), only one reported a significant relationship between SBP and CRP (912). These studies (showing no relationship) were of predominantly male populations. Among the studies in the younger group that were not adjusted for insulin sensitivity, four showed a significant relationship with CRP (913;916-918), while two showed none (914;915). No sex pattern was obvious in these relationships.

Older group: Considering the studies which adjusted for insulin sensitivity, all four cross sectional studies showed a significant relationship with CRP (911;919-921); only the longitudinal study did not show an association (911). Again, there was no obvious difference according to sex. Three cross sectional studies which did not adjust for insulin sensitivity showed a relationship with CRP (922-924). However, five cross sectional (925-929) and one longitudinal (930) studies (not adjusting for insulin sensitivity) did not show any relation with CRP. No sex association was seen in these relations.

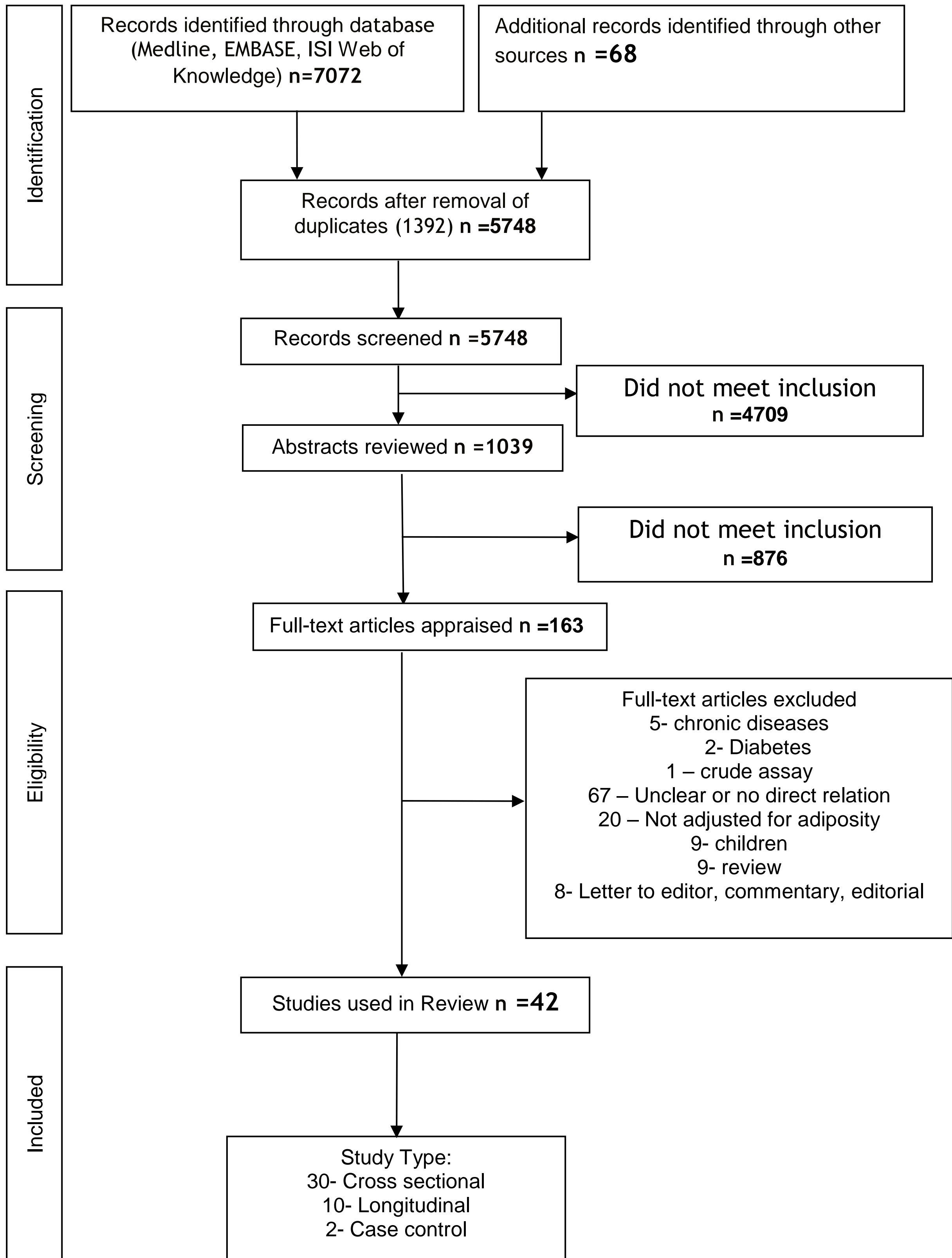


Figure 4.1 PRISMA flowchart of CRP search

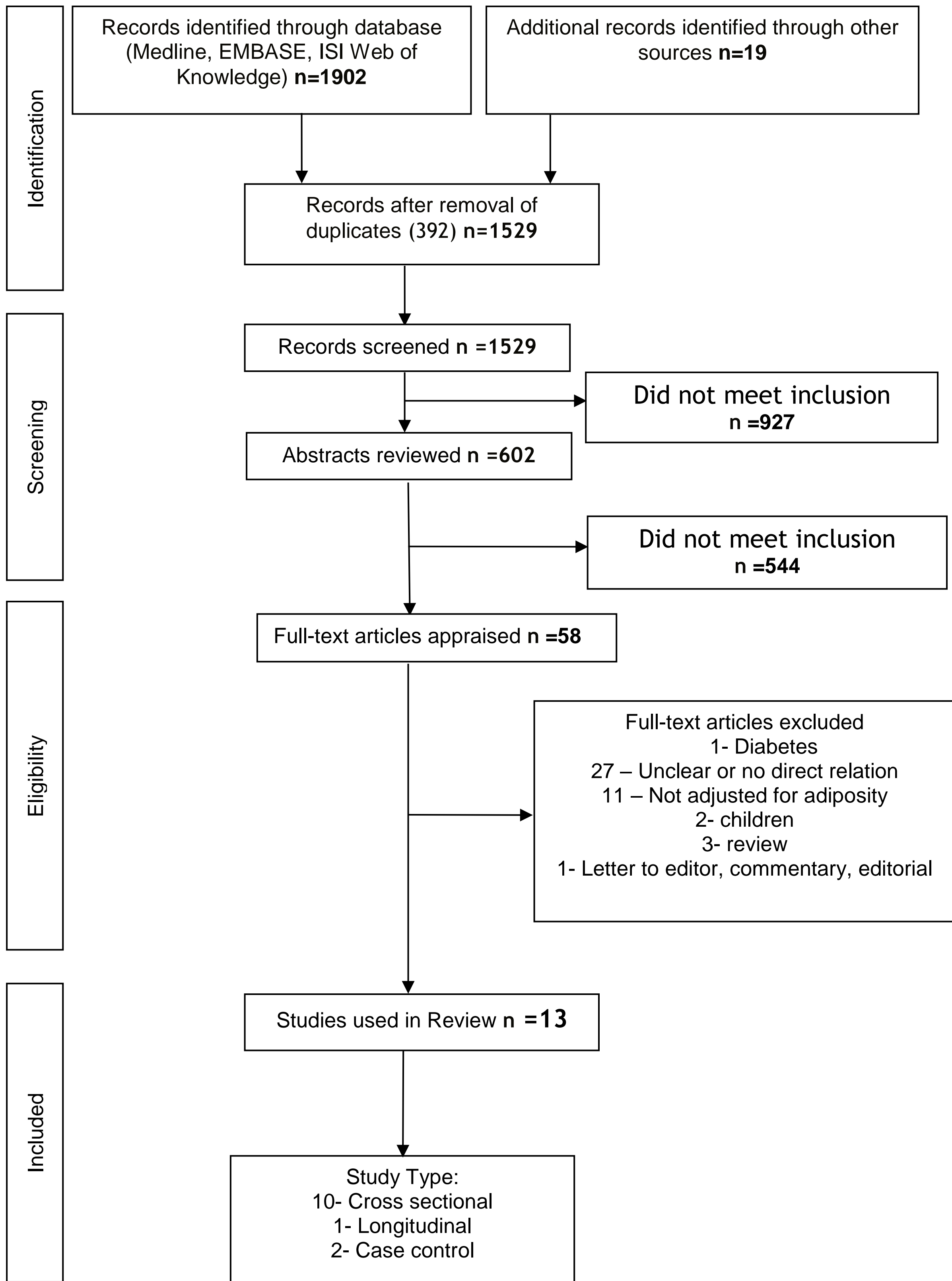


Figure 4.2 PRISMA flowchart of IL-6 search

4.5.2 C reactive protein and BP/Hypertension

Table 4.1 Relationship of CRP with Blood pressure, adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay	Adj IS
CRP as Continuous											
Festa A (919)	2000	Multiethnic/ USA	1008	43% male	40-69/ 55	SBP/DBP	Yes/No	age, sex, clinic, Eth, Smok, BMI, F glucose, F insulin, ISI, proinsulin	Beta	ultrasensitive competitive immunoassay	ISI (MINMOD)
Yamada S (920)	2001	Japanes/ Japan	6107	37% males	≥30/ 55.5	SBP	Yes	age, SBP, T Chol, HDL, TG, Glucose, BMI, fibrinogen, Smok, insulin, WHR	OR	particle enhanced nephelometry	insulin
Aldaghri NM (910)	2010	Arabs/ Saudi Arabia	330	56% Males	48	SBP, DBP	NO (in all F & M gps), Yes (in IR males only)	age, BMI, WC, glucose, insulin, HOMA-IR, HDL, , LDL, TG, T Chol, CRP, TNF-α	Beta	Elisa	HOMA IR
Kawamoto R (921)	2011	Japanese/ Japan	1919	43% Males	20-89/ 62	SBP/DBP	Yes women/No	Age, BMI, Smok, alcohol, Hx of CVD, anti HTN Rx, TG, HDL, LDL, statins, uric acid, F glucose, F insulin, adiponectin, antiDM Rx	Beta	nephelometer	Fasting insulin
Labonte ME (912)	2012	Caucasians/ Canada	801	46% males	36	SBP, DBP	Yes, Yes	Age, sex, WC, smoking	OR	hsCRP-nephelometer	Insulin
CRP as Categorical											
Cheung BMY (911)	2012	Chinese/ Hong Kong	1925	46% males	25-74/ 54	ΔSBP/ ΔDBP	Yes/Yes	Age, sex, BMI, TG, HDL, glucose, HOMA-IR, Smok, Hx of CV disease, BP, Rx of hypercholesterolemia	OR	sandwich ELISA	HOMA-IR
Bautista LE (909)	2004	Caucasians/ USA	904	86% males	39-50/ 43.1	SBP/DBP	No/Yes	sex, BMI, insulin, Eth, family Hx	Beta	immunoturbidimetric latex agglutination method	serum insulin levels

Table 4.2 Relationship of CRP with hypertension, adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay	Adj IS
CRP as Categorical											
LI Hongmei (931)	2012	Mangolian/ Japanese	2553	41% males	45	HTN	Yes	age, sex, familoy history, smoking, alcohol, overweight, TG	OR	immunoturbidimetry	HOMA IR
Bautista LE (909)	2004	Caucasians/ USA	904	86% males	39-50/ 43.1	HTN	Yes	sex, BMI, insulin, Eth, family Hx	OR	immunoturbidimetric latex agglutination method	serum insulin levels

Following abbreviations will be used in the following tables also.
F= females, M= Males, Eth= ethnicity, BMI= body mass index, T Chol= total cholesterol, SBP= systolic blood pressure, DBP= diastolic blood pressure, F= fasting, HDL= high density lipoprotein, LDL= low density lipoprotein, TG= triglyceride, Smok= smoking, WC= waist circumference, WHR= waist hip ratio, Hx= history, Rx= treatment, DM= diabetes, HRT= hormone replacement therapy, SES= socio economic status, Phy Act= physical activity, alcohol= consumption of alcohol, CRP= C reactive protein, IL-6= interleukin 6, TNFα= Tumour necrosis factor-alpha, HTN= hypertension, CAF= central abdominal fat, apo= apolipoprotein, Edu= education, HOMA-IR= homeostasis model for assessment of insulin resistance, ISI= insulin sensitivity index,
RR= Relative risk, OR= Logistic regression (odds ratio), HR= Hazard ratio, Beta= Multiple regression (Beta), c=categorical (both CRP and BP), Corr= Correlation, PR= Prevalence ratio, g= genes related to hypertension

Table 4.3 Relationship of CRP with Blood pressure, not adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay
CRP as Continuous										
Onat A (925)	2001	Turkish/ Turkey	1046	50% males	≥30/51	SBP/DBP	No/No	age, sex, clinic, Eth, Smok, BMI, DBP, SBP, F glucose, income	Beta	particle enhanced immunonephelometry
Bermedez Edmund A (922)	2002	Caucasians/ USA	340	100% females	60	SBP	Yes	age, BMI, smoking, Alcohol, DM, Exercise, HRT, HDL, T chol	Beta	hsCRP-BN II analyzer
Schillaci G (913)	2003	Caucasians/ USA	135	44% males	47	SBP, DBP	Yes, NO	age, sex, Smok, BMI, T Chol, LDL, HDL, TG, heart rate	Beta	hs nephelometer
Greenfield JR (926)	2004	Caucasians/ UK	194	100% females	57	SBP, DBP	NO, NO	CAF, TG, apo B, HRT, LDL, HDL, apo A1, Phy Act, alcohol	Corr for SBP, Beta for DBP	hs automated microplate capture enzyme immunoassay
Schutte AE (914)	2006	African and Caucasian/ South Africa	217	100% females	31	SBP, DBP	NO, NO	age, BMI, WC	Corr	hs immunochemistry
Wong LYF (923)	2007	Hong Kong Chinese/ China	502	53% mlaes	55	SBP	Yes	age, WC, LDL	Beta	hs ELISA
Sung SH (927)	2008	Chinese/ Taiwan	2045	34% males	56	SBP, DBP	NO, NO	Age, sex, BMI, WC	Beta, OR	Particle enhanced immunoturbidimetry

Asferg Camilla (928)	2009	Danish/ Denmark	487	48% males	36-80/ 62	SBP/DBP	No/No	age, sex, DM, T Chol, HDL, TG, glucose, HbA1c, WC, BMI, WHR, creatinine, fibrinogen, alcohol, Smok, Phy Act	Beta	nephelometric assay
Torun D (915)	2012	Turkish/ Turkey	95	43% males	37-69/ 48	SBP/DBP	No/No	BMI, fibrinogen, urinary albumin, Lt ventricular mass index	Beta	Nephelometric method
CRP as Categorical										
Abramson JL (916)	2002	Caucasians/ USA	9867	51% males	38	SBP, DBP	Yes, NO	age, sex, race, Edu, T Chol, BMI, WHR, Smok, alcohol, Phy Act, anti HTN Rx	OR	latex enhanced nephelometry
Niu Kaijun (924)	2005	Japanese/ Japan	643	49% males	76	SBP	YEs	age, sex, BMI, Smok, DM, HDL, hypercholesterolemia, gout, Hx of CVD, alcohol	Beta	hs immunotechnique
Davey Smith G (929)	2005	British/ UK	3529	100% Females	60-79/ 69	ΔSBP/ΔDBP	No/No	Age, BMI, Phy Act, Smok, DM, alcohol, HRT, Family Hx of CVD, TG, HDL, Height, WHR, FEV1, SES.	OR	hs immunonephelometric assay
Sorensen MV (918)	2006	Siberian/ Siberia	265	33% males	45	SBP	Yes males only	age, WC, smoking	ANCOVA	hs immunoturbidimetric
King Dana E (917)	2004	Caucasians/ USA	16966	43% males	40	SBP, DBP	Yes, NO	age, sex, Eth, Smok, BMI, Phy Act, Rx, DM	OR-c	hs nephelometer

Table 4.4 Relationship of CRP with hypertension, not adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay
CRP as Continuous										
Sung SH (927)	2008	Chinese/ Taiwan	2045	34% males	56	HTN	No	Age, sex, BMI, WC	OR	Particle enhanced immunoturbidimetry
Chamarthi B (932)	2011	Multiethnic/ USA and France	581	50% males	44	HTN	No	age, BMI, sex, Eth	OR	hsCRP enzyme linked assay
Wang Guiyan (933)	2011	Mangolians/ China	2589	41% males	47	HTN	Yes	Age, sex, BMI, WHR, F Glucose, Smok, alcohol, LDL, T chol, TG and family Hx	OR	immunoturbidimetry
CRP as Categorical										
Niu Kaijun (924)	2005	Japanese/ Japan	643	49% males	76	HTN	Yes	age, sex, BMI, Smok, DM, HDL, hypercholesterolemia, gout, Hx of CVD, alcohol	OR	hs immunotechnique
Davey Smith G (929)	2005	British/ UK	3529	100% Females	60-79/ 69	HTN	no	Age, BMI, Phy Act, Smok, DM, alcohol, HRT, Family Hx of CVD, TG, HDL, Height, WHR, FEV1, SES.	OR	hs immunonephelometric assay

Lakoski SG (934)	2005	Caucasians/ USA	6814	50% males	45-84/ 63	HTN	yes	age, sex, BMI, LDL, HDL, DM, Phy Act, Smok, alcohol, use of HMG-CoA reductase inhibitors, oestrogen Rx and aspirin	OR	hs nephelometer
Bautista LE (935)	2005	Caucasians/ Colombia	196	37% males	30-64/ 44	HTN	No	Age, sex, BMI, family Hx, other inf marker (CRP, IL6 or TNFα)	PR	High Sen ELISA
Imatoh Takuya (936)	2007	Japanese/ Japan	249	100% males	23-70/ 58	HTN	No	age, BMI, Smok, alcohol,	OR	hs immunonephelometric assay
Xu Tan (937)	2008	Chinese/ China	1529	47% males	30-84/ 50	HTN	Yes	Overweight, alcohol, Family Hx	OR	immunoturbidimetry
Huffman FG (938)	2009	Cubans/ USA	161	34% males	62	HTN	No	age, sex, BMI, Smok, cholesterol Rx, antiinflammatory Rx, family Hx of DM and CVD	OR	immulite method
Komurcu BE (939)	2009	Turkish/ Turkey	1987	49% males	54.3	HTN	yes	Age, BMI, Smok, glucose, menopausal status	OR. CRP-g	particle enhanced immuno nephelometry

Table 4.5 Relationship of CRP with Blood pressure, adjusted for insulin sensitivity in longitudinal studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay	Adj IS
Cheung BMY (911)	2012	Chinese/ Hong Kong	1115	44% males	25-74/ 50	ΔSBP/ΔDBP	No/No	Age, sex, BMI, TG, HDL, glucose, HOMA-IR, Smok, Hx of CV disease, BP, Rx of hypercholesterolemia	10	OR	sandwich ELISA	HOMA-IR

CRP was used as categorical variable

Table 4.6 Relationship of CRP with hypertension, adjusted for insulin sensitivity in longitudinal studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay	Adj IS
Cheung BMY (911)	2012	Chinese/ Hong Kong	1115	44% males	25-74/ 50	**HTN**	Yes	Age, sex, BMI, TG, HDL, glucose, HOMA-IR, Smok, Hx of CV disease, BP, Rx of hypercholesterolemia	10	OR	sandwich ELISA	HOMA-IR

CRP was used as categorical varibale

Table 4.7 Relationship of CRP with Blood pressure, not adjusted for insulin sensitivity in longitudinal studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay
CRP as continuous											
Lieb Wolfgang (930)	2008	Caucasians/ USA	1029	46% males	54	ΔSBP	Yes	Age, sex, BMI, SBP, DBP, total:HDL cholesterol ratio, TG, lipid lowering Rx, HRT, Smok	4	Beta	nephelometer
Lakoski SG (940)	2006	White, African American/ USA	5115	50% males	32	ΔSBP/ ΔDBP	No/No	age, Eth, BMI, Smok, alcohol , LDL, HDL, Hx of DM, cholesterol-lowering medication, Phy Act, clinical site	7	Beta	hs CRP

Table 4.8 Relationship of CRP with hypertension, not adjusted for insulin sensitivity in longitudinal studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay
CRP as continuous											
Wang TJ (941)	2007	Caucasians/ USA	1456	42% males	56	**HTN**	Yes	age, sex, BMI, % weight change, DM, Smok, SBP, DBP, serum creatinine, PAI-1 and UACR	3	OR	hs nephelometer
Dauphinot V (942)	2009	French/ France	160	35% males	≥65/ 65.6	**HTN**	yes	CRP, change in CRP, BMI, 24 hr Sys ABPM(ambulatory BP measurement), change in 24 hr sys ABPM	2	OR	turbidimetric immunoassay
Mattace Raso (943)	2010	Caucasians/ Netherland	1637	41% males	64	**HTN**	Yes	age, Sex, SBP, BMI, T chol, HDL, DM, Smok, Leucocyte count	12	OR	infrred particle immunoassay
Pitsavos C (944)	2008	Caucasians/ Attica-Greece	782	54% males	45	**HTN**	Yes	Age, sex, Edu, WC, SBP, T chol, Phy Act	5	OR	particle enhanced immunonephelo metry
CRP as categorical											
Sesso HD (895)	2003	Caucasians/ USA	11605	100% females	≥45/ 53.7	**HTN**	Yes	Age, Rx assigned (aspirin), BMI, Smok, Phy Act, alcohol, Family Hx, DM, Cholesterolemia	7.8	RR	hs assay

Niskanen L (896)	2004	Caucasians/ Finland	379	100% males	50	**HTN**	Yes	Age, BMI, WC, Lipid profile, Glucose, Smok, Phy Act	11	OR	immunometric assay
Lakoski SG (940)	2006	White, African American/ USA	5115	50% males	32	**HTN**	No	age, Eth, BMI, Smok, alcohol , LDL, HDL, Hx of DM, cholesterol-lowering medication, Phy Act, clinical site	7	OR	hs CRP
Lakoski SG (945)	2011	Multiethnic/ USA	3543	49% males	45-84/ 60	**HTN**	No	age, BMI, Eth, Smok, DM, statin use, aspirin use, alcohol, study site	5	HR	BNII Nephelometer

Table 4.9 Relationship of CRP with hypertension, not adjusted for insulin sensitivity in Case Control Studies

Author	**year**	**Race/ country**	**Sample size**	**sex**	**Age range/ mean**	**Outcome variable**	**Sig Ass**	**Adjusted for**	**FU Yr**	**Stats used**	**Assay**
CRP as continuous											
Wang Lu (946)	2011	White and Black/ USA	800 HTN, 800 control	100% females	60	**HTN**	No	age, BMI, clinical centre, and time of enrollment, Smok, alcohol, Phy Act, HRT use	5.9	RR	ultrasensitive immunotech
CRP as categorical											
Sesso HD (947)	2007	Caucasian s/ USA	400 cases, 400 control	100% females	45+/ 54.5	**HTN**	No	BMI, Smok, alcohol, Phy Act, menopausal status, family Hx	10	RR	High sensitive assay

Both studies were prospective

4.5.3 Interleukin-6 and BP/Hypertension

Table 4.10 Relationship of IL-6 with blood pressure, adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay	Adj IS
Aldadhri NM(910)	2010	Arabs/ Saudi Arabia	330	56% Males	48	SBP, DBP	NO, NO (in all M & F gps)	age, BMI, WC, glucose, insulin, HOMA-IR, HDL, LDL, TG, T Chol, CRP, TNF-α	Beta	Elisa	HOMA IR

Table 4.11 Relationship of IL-6 with hypertension, adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay	Adj IS
Cheung BMY (948)	2011	Chinese/ Hong Kong	831	50% males	55	**HTN**	yes (in F)	Age, BMI, glucose, HOMA IR, sex, Smok	OR	ELISA	HOMA-IR

Table 4.12 Relationship of IL-6 with blood pressure, not adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay
IL-6 as continuous										
Fernandez-Real JM (949)	2001	Caucasians/ Spain	228	58% males	40	SBP, DBP	Yes, Yes (in total, females and non-smokers only)	BMI or fat mass	Beta	Immunoassay Elisa
Bermedez Edmund A (922)	2002	Caucasians/ Usa	340	100% females	60	SBP	Yes	age, BMI, Smok, alcohol, DM, Phy Act, HRT, HDL, T chol	Beta	ELISA

Chae CU (950)	2001	Caucasians/ USA	587	100% males	59.2	SBP[c]/ DBP[c]	Yes/Yes	age, Hx of DM, high cholesterol, alcohol, family Hx of MI, aspirin use, Smok, Phy Act, BMI	Beta	ELISA

[c] =BP used as a categorical variable

Table 4.13 Relationship of IL-6 with hypertension, not adjusted for insulin sensitivity in Cross sectional studies

Author	year	Race/ Country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	Stats used	Assay
IL-6 as continuous										
Chamarthi B (932)	2011	Caucasians/ USA and France	581	50 % males	45	**HTN**	Yes	age, BMI, sex, Eth	OR	ELISA
IL-6 as categorical										
Bautista LE (935)	2005	Caucasians/ Colombia	196	37 % male	30-64/44	**HTN**	Yes	Age, sex, BMI, family Hx, other inf marker (CRP, IL6 or TNFα)	PR	High Sen ELISA

Table 4.14 Relationship of IL-6 with hypertension, not adjusted for insulin sensitivity in longitudinal studies

Author	year	Race country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay
Lakoski SG (945)	2011	Multi-ethnic/ USA	3543	49% males	45-84/ 60	**HTN**	Yes	age, BMI, sex, Eth, Smok, DM, statin use, aspirin use, alcohol use, MESA study site	5	HR	ultrasen sitive ELISA

Study was prospective, CRP was used as a categorical variable

Table 4.15 Relationship of IL-6 with hypertension, not adjusted for insulin sensitivity in case control studies

Author	year	Race/ country	Sample size	sex	Age range/ mean	Outcome variable	Sig Ass	Adjusted for	FU Yr	Stats used	Assay
CRP as continuous											
Wang Lu (946)	2011	White and Black/ USA	800 HTN, 800 control	100% females	60	**HTN**	No	age, BMI, clinical centre, time of enrollment, Smok, alcohol, Phy Act, HRT	5.9	RR	ELISA
CRP as Categorical											
Sesso HD (947)	2007	Caucasians/ USA	400 cases, 400 control	100% females	45+/ 54.5	**HTN**	No	BMI, Smok, alcohol, Phy Act, menopausal status, family Hx	10	RR	ELISA

Both studies were prospective

Abreviations:
F= females, M= Males, Eth= ethnicity, BMI= body mass index, T Chol= total cholesterol, SBP= systolic blood pressure, DBP= diastolic blood pressure, F= fasting, HDL= high density lipoprotein, LDL= low density lipoprotein, TG= triglyceride, Smok= smoking, WC= waist circumference, WHR= waist hip ratio, Hx= history, Rx= treatment, DM= diabetes, HRT= hormone replacement therapy, SES= socio economic status, Phy Act= physical activity, alcohol= consumption of alcohol, CRP= C reactive protein, IL-6= interleukin 6, TNFα= Tumour necrosis factor-alpha, HTN= hypertension, CAF= central abdominal fat, apo= apolipoprotein, Edu= education, HOMA-IR= homeostasis model for assessment of insulin resistance, ISI= insulin sensitivity index,
RR= Relative risk, OR= Logistic regression (odds ratio), HR= Hazard ratio, Beta= Multiple regression (Beta), c=categorical (both CRP and BP), Corr= Correlation, PR= Prevalence ratio, g= genes related to hypertension

4.6 Discussion

In this systematic review, I examined relationships between inflammatory markers (CRP and IL-6) and blood pressure (systolic BP, diastolic BP and the development of hypertension) focusing on studies which also measured insulin sensitivity and adiposity.

Adipose tissue dysfunction is associated with overweight and obesity and also alters adipokine production resulting in increased production of leptin, TNF-α and IL-6 but decreased adiponectin. It is characterized by hypertrophied adipocytes and infiltration by macrophages (325). Healthy adipose tissue is populated with 5–10% macrophages but this macrophage infiltration increases up to 60% in obesity (507). Inflamed macrophages release TNF-α and IL-6 which have been shown to impair insulin sensitivity and increase blood pressure (360;835). Obesity leads to activation of inflammatory pathways in all insulin target tissues, including fat, liver and muscle, signifying a role for inflammation in driving the pathogenesis of systemic insulin resistance (831). Proposed mechanisms leading to inflammation in obesity include oxidative stress, lipotoxicity (increased free fatty acids), glucotoxicity, endoplasmic reticulum stress, hypoxia, amyloid and lipid deposition (831). Decrease in adiponectin (an anti-inflammatory adipokine) also adds to insulin resistance as adiponectin receptors on liver and muscle cells mediate ß-oxidation of fatty acids, glucose uptake, gluconeogenesis and peroxisome proliferator activated receptor-γ activation (325;330).

Insulin resistance may have a biological relationship with inflammation: for example, insulin sensitising agents have been shown to reduce serum levels of inflammatory markers, and a decrease in IR (as measured by HOMA) causes a decrease in inflammation (908;951). Conversely, some anti-inflammatory agents (salsalates) have been shown to improve glucose utilization and insulin sensitivity in euglycaemic hyperinsulinaemic clamp studies (612). Salsalates also improve glycaemia in patients with type 2 diabetes (952).

From the analysis in the present Chapter, it is clear that these relationships have been evaluated in many studies, including large cohorts, but the cumulative evidence is not supportive of a robust and consistent relationship. There is a

weak evidence of a relationship between CRP and BP (as a continuous variable) in cross sectional studies but few longitudinal studies are available for evaluation. However, there is evidence for an association between CRP and hypertension *per se* (as a categorical variable) not only in some cross sectional reports but also in some longitudinal cohort studies.

In the present review, more studies reported a significant relationship between CRP and BP/hypertension when analyses were adjusted for insulin sensitivity (and not just adiposity). However, none of the studies measured insulin sensitivity by the gold standard euglycaemic clamp technique.

Although fewer studies are available considering IL-6 as an inflammatory marker, all cross sectional and longitudinal studies except one (910) showed that IL-6 was associated with SBP, DBP and HTN. This relationship was not affected by age, sex, ethnicity or the size of the cohort. However, both case control studies did not show any relationship between IL-6 and hypertension.

The studies reviewed were clearly heterogeneous in terms of ethnicity, age, sex distribution and sample size; however, none of these factors had a profound impact on the relationship between inflammatory markers and BP/ hypertension. There was less heterogeneity in relation to the outcome variable: BP or ΔBP as continuous variables or presence or absence of hypertension as a categorical variable. Only one study used categorical BP as an outcome variable (950). The inflammatory markers (both CRP and IL-6) were used both as a continuous or categorical variable but this did not have a substantial effect and both had similar results.

Considering sample size and the relationship between CRP and SBP, large studies by Yamada et al (n=6107) (920) and King et al (n=16966) (917) showed a relationship; however, this was not the case in other large studies by Sung (n=2045) (927) and Davey et al (n=3529) (929). Similarly for HTN, Wang et al (n=2589 (933) and Lakoski et al (n= 6814) (934) showed a relationship between CRP and HTN but this was not demonstrated by Davey et al (n=3529) (929) and Sung et al (n=2045) (927). In relation to sex, only three studies specifically showed a significant positive relationship between CRP and BP/HTN in females (895;921;922) and only two studies showed a relationship between CRP and

BP/HTN in males (896;910); all these studies had a small sample size (< n= 400) except for two; Sesso et al. (n=11605) (895) and Kawamoto et al. (n= 1097) (921). Most of the studies did not show results stratified by males and females and so the type of relationship in each sex cannot be determined from pooled population results. For those studies that were positive, it is not clear whether the results were significant in either sex when considered separately.

Regarding age, the relationship varied in younger and older individuals. For DBP, there was a relationship with CRP in younger and predominantly male populations following adjustment for insulin sensitivity. For SBP, some studies showed a relationship between CRP and SBP in older populations but this was not robust. Taking age and sex together, the studies I reviewed were not conclusive overall but it would be valuable if future studies consistently reported results stratified by sex and age.

Considering heterogeneity in the results, one possible approach was to meta-analyse by age categories. However, this was not possible as the age distribution was wide in most of the studies (from around 20-89 years) with only one study (909) clearly showing a younger age distribution (age range 39-50); other studies documenting younger mean age did not clearly state age distribution. Similarly, meta-analysis by sex was also not possible as only a few studies reported sex stratified results, which were also heterogeneous in outcome (SBP, DBP and hypertension) and study type (cross sectional, longitudinal or case control). This analysis did not provide any robust findings: individual participant data meta-analysis might provide some evidence but would depend upon obtaining the original datasets from the authors of all the published papers.

The relationship between BMI and BP or HTN has been demonstrated in many studies and is widely accepted (899-906). One explanation for the findings may be that the chain of causation between obesity and high BP involves both inflammation and insulin resistance, and not inflammation alone. This argument is supported by the trials (953-955) in which insulin sensitizers lowered BP and also affected CRP: Sanchez et al. showed that telmisartan (an angiotensin receptor blocker which also decreases insulin resistance through partial agonist effect on peroxisome proliferators-activated receptor-γ) reduced BP as well as CRP and HOMA-IR (954). In addition Chujo et al. showed that telmisartan

reduced BP, HOMA-IR, IL-6 and CRP despite no change in BMI (953). Similarly Yano et al compared Valsartan (an angiotensin receptor blocker without insulin sensitising action) with telmisartan and showed that both drugs lowered BP, but only telmisartan additionally lowered CRP and microalbuminuria in and increased adiponectin (955).

In summary, this systematic review showed evidence of considerable variation in the relationships amongst low grade inflammation, adiposity, insulin resistance and the development of hypertension. However, CRP and DBP appear to be associated in younger age. It appears from the literature that inflammation and insulin resistance are closely related, with evidence that reducing inflammation lowers insulin resistance and that insulin sensitisation can decrease low grade inflammation. From a clinical perspective, given the knowledge that obesity increases insulin resistance, low grade chronic inflammation and BP, it makes sense to focus on weight reduction when designing interventions to improve cardio metabolic health (rather than specifically targeting inflammation). Decreasing BMI by bariatric surgery (904-906) or very low caloric diet (VLCD) (956) decreases both inflammatory markers and insulin resistance: these effects are sustained provided weight loss is maintained. If inflammation or insulin resistance is decreased without decreasing BMI, the effect on BP may be only short-term.

5 Inflammatory markers, blood pressure, incident hypertension and insulin resistance in a healthy European population – RISC study.

5.1 Introduction

As seen in chapter 3, and as has reported in the literature, insulin resistance (IR) has been associated with a rise in BP over time which may be relevant to the development of hypertension, particularly in women (196;811;871;882;883). However, insulin resistance is also related to obesity (907) and inflammation (908). In chapter 3 it is also the case that insulin sensitivity was related to BP when estimated by HEC, but not when estimated by HOMA. This gives an indication that surrogate markers may lack sensitivity for assessing relationships between IR and different biomarkers or pathways. Mendelian randomisation can be used to determine the causality or otherwise of specific hormones and mediators in the pathogenesis of disease. This approach has been used for CRP as gene variants have been identified which link with blood CRP concentration. A Mendelian randomisation meta-analysis of four CRP single nucleotide polymorphisms (SNP) showed them to be related to concentration of CRP but not to BP or cardiovascular disease (723), suggesting that any relationship between inflammatory markers and BP/hypertension is unlikely to be causal. However mechanistic links between insulin resistance, inflammation, obesity, and blood pressure remain poorly understood and requires further exploration.

The systematic review in chapter 4 showed no clear evidence of a significant independent relationship between inflammatory markers and BP/hypertension in studies which adjusted for insulin sensitivity and markers of adiposity. However, these studies were either small or used surrogate markers of insulin sensitivity. These findings identified the need for a large study in this area using the hyperinsulinaemic euglycaemic clamp technique, the acknowledged gold standard for the assessment of insulin sensitivity (IS) (776). Chapter 4 also identified the importance of age and sex stratification. Therefore, in this chapter I have evaluated the relation between inflammatory markers (hsCRP and IL-6) and hypertension/BP in the RISC cohort, which is well-characterised for insulin sensitivity and obesity. The objective was to examine the effect of age and sex on any relationship, given their effects on body composition and especially fat mass (which in turn is related to vascular pathophysiology).

5.2 Methods

The methods for the RISC study were as in Chapter 2 above.

Of the total 1,563 participants, 946 (502 women and 444 men) had complete data at the three year follow up for inflammatory markers and so were included in the study. Individuals with hsCRP more than 10 mg/L at baseline (n=19) were excluded in order to avoid overestimating long term exposure to low grade inflammation. HsCRP levels in healthy people are less than 10mg/L and levels more than 10mg/L are likely to suggest a possible acute phase response (957;958): CRP returns to normal quickly after the acute phase reaction subsides (957). Women taking oral contraceptive pills (OCPs) or on hormone replacement therapy (HRT) were also excluded from the analysis (n= 45). It has been shown that young, healthy, fertile, non-obese women taking OCPs have 4 times higher odds of having a high CRP (> 3mg/L) as compared to non-OCP users (959).Moreover HRT are also shown to increase CRP (960).

5.2.1 Statistical Analysis

In the Introduction (Section 1.11), I have reviewed the evidence that changes in body composition take place around 40-45 years of age across different ethnic populations and that sex (Section 1.12) has a strong impact on this change. Gender and age interactions with change in BP over 3 years as the dependent variable were checked for hs-CRP and IL-6 as follows: gender*log(hs-CRP), age*log(hs-CRP), gender*log(IL-6) and age*log(IL-6). Taking $p<0.10$ for significance, given the limited statistical power of interaction terms (961), the age interaction term (*CRP) for ΔDBP ($p= 0.069$) was significant in pooled analysis. The data were therefore split by median age of the RISC cohort (<45 years; ≥45 years) for further DBP-CRP analysis. The Framingham Predictors study showed that young age and male sex are predictors of isolated diastolic hypertension, and that older age and female sex are predictors of isolated systolic hypertension (72). In view of this finding from the literature, I conducted exploratory analysis for the interaction term age*log(hs-CRP) in males only and found it to be significant for ΔDBP ($p=0.012$).

The quadratic terms of hs-CRP and IL-6 were used to check the linearity of association with BP, and hs-CRP and IL-6 data met the assumptions for linear analysis (962). Baseline measurements were compared by t-test and chi-square (χ^2) according to hypertensive status at the three year follow-up. Multiple linear regression analysis was used to determine whether baseline hs-CRP and IL-6 predicted change in BP at three years, including covariates at baseline: age, recruitment centre (using indicator variables), systolic/diastolic blood pressure, BMI, change in BMI, as well as log M/I with subsequent adjustment for blood glucose, lipid profile and lifestyle factors. Binary logistic regression was used to assess prediction of hypertension from hs-CRP and IL-6 as continuous variable. A test for trend was used to analyse the development of hypertension across tertiles of CRP and IL-6 at baseline. SPSS version 18 was used for all analyses.

5.3 Results

At three years, median BP had reached a diagnostic threshold for hypertension in 12.1% of all participants (n=114; 70 men, 44 women); a further 4.2% (n=40; 21 men, 19 women) had been commenced on antihypertensive treatment in routine care i.e. a total of 16.3 % (n= 154; 91 men, 63 women) with incident hypertension.

Table 5.1 shows the baseline characteristics according to hypertension status at follow-up. Almost all were different between normotensives and hypertensives except IL-6, alcohol intake and smoking status. Insulin sensitivity was higher in those who remained normotensive, whereas hsCRP and IL-6 were lower. Weight, BMI, fat mass and fat free mass were all significantly lower in the normotensive group.

Table 5.2 shows that baseline CRP predicted unadjusted ΔSBP (ß = 0.071, P = 0.031), but not following adjustment for BMI and other variables. Table 5.3 shows the prediction of ΔDBP (ß = .113, P = 0.001) by CRP: ß-values were significant after adjustment for BMI, insulin sensitivity (IR) and other variables. When the cohort was split by median age (45 years), CRP was a predictor for ΔDBP in younger adults (ß = 0.171 and p = <0.001) but not those who were middle-aged (age≥45, ß= 0.049, p = 0.298). The relation in younger adults remained significant following adjustment for BMI, IR, lipid profile and life style

factors. In younger males CRP predicted ΔDBP (ß = 0.263, p = <0.001) and it remained significant after adjustment for all the confounding variables (Table 5.4). Hs-CRP neither predicted ΔDBP in younger and older females, nor predicted ΔSBP in younger and older females (Framingham predictors) after adjustment for BMI. IL-6 predicted ΔSBP in univariate analysis (ß = 0.077, p = 0.019) but not in multivariate analysis with adjustment. IL-6 did not predict change in DBP (ΔDBP)(Table 5.5).

As hs-CRP was a significant predictor for only DBP and in young age (≤44 years), its specific contribution in explaining the variance was further explored. It showed that hs-CRP explained 1.3-1.8 % of the variance; this is shown in model 3 for the whole population and also for the males only (Table 5.7). The main contributors to prediction of risk were baseline DBP and BMI.

Test for trend for the development of hypertension across tertiles of baseline CRP and IL-6 (used as a categorical variable) was significant (p<0.001, p = 0.009 respectively, unadjusted data) as shown in Figure: 5.1. However, when using logistic regression, hs-CRP and IL-6 (used as continuous variable) were not significant predictors of hypertension following adjustment for baseline BP and/or BMI (Table 5.6)

Table 5.1 Baseline Characteristics according to hypertension status at follow-up

	Total (946)	Normotensives (792)	Hypertensives (154)	P
Age (years)	44.4 ± 8.3	43.9 ± 8.2	47.3 ± 8.2	<0.001
Sex n (% males)	445 (47)	353 (45)	91 (59)	<0.01
Systolic BP (mmHg)	118 ±13	116 ± 12	128 ± 10	<0.001
Diastolic BP (mmHg)	74 ± 8	73 ± 8	80 ± 7	<0.001
BMI (Kg/m²)	25.4 ± 3.9	25.0 ± 3.7	27.2 ± 4.1	<0.001
Weight (Kg)	74.7 ± 14.4	73.5 ± 14	81.2 ± 14.2	<0.001
Fat Free Mass (Kg)	54.2 ± 11.5	53.5 ± 11.4	57.7 ± 11.8	<0.001
Fat Mass (Kg)	20.6 ± 8.5	20.0 ± 8.1	23.5 ± 9.5	<0.001
Clamp Insulin Sensitivity (M/I) *	127.8 ± 1.6	131.2 ± 1.6	111.5 ± 1.7	<0.001
CRP (mg/L)	0.7 ± 3.2	0.6 ± 3.2	0.9 ± 3.2	<0.01
IL6 (pg/ml)	0.8 ± 1.9	0.8 ± 1.9	0.9 ± 1.8	0.05
Glucose (mmol/L)	5.1 ± 0.5	5.0 ± 0.5	5.2 ± 0.5	0.01
Total Cholesterol (mmol/L)	4.9 ± 0.9	4.8 ± 0.9	5.1 ± 0.9	<0.01
LDL Cholesterol (mmol/L)	3.0 ± 8	2.9 ± 0.8	3.2 ± 0.8	<0.01
HDL Cholesterol (mmol/L)	1.4 ± 0.4	1.4 ± 0.4	1.3 ± 0.4	0.01
Triglycerides (mmol/L)*	1.0 ± 1.6	1.1 ± 1.7	1.1 ± 1.9	<0.01
Family Hx of HTN n(%)	409 (44)	329 (42)	80 (53)	0.01
Alcohol grams/week*	62.4 ± 2.5	62.1 ± 2.4	63.9 ± 2.6	0.75
Smoker n(%)	241 (26)	202 (26)	39 (26)	0.35
Phys. Activity (Counts per min)*	330.2 ± 1.6	335.8 ± 1.5	301.7 ± 1.7	0.03

Table 5.2 Beta coefficients for predicting change in SBP (ΔSBP) over 3 years from log hs-CRP as independent variable, with various adjustment factors

Model	Independent: CRP	Dependent: ΔSBP (946)		
		Total population (946)		
	Adjustment factors	R^2	Beta	P
1	-	.005	.071	.03
2	Centre & Age	.111	.047	.15
3	Model 2 + baseline SBP	.191	.088	<0.01
4	Model 3 + BMI	.201	.049	.14
5	Model 4 + % change BMI	.216	.043	.19
6	Model 5+ Log M/I	.219	.038	.26
7	Model 6 + Glucose, LDL, HDL ,Chol, TG	.223	.037	.27
8	Model 7 + Family Hx	.225	.038	.25
9	Model 8 + Log Alcohol	.227	.041	.26
10	Model 9 + smoking	.227	.041	.26
11	Model 10 + physical activity	.224	.041	.37
12	Model 11+ IL6	.224	.045	.35

Table 5.3 Beta coefficients for predicting change in DBP (ΔDBP) over 3 years from log hs-CRP as independent variable, with various adjustment factors

All by gender	Independent: CRP	Dependent: ΔDBP			Dependent: ΔDBP					
		Total population (946)			Age ≤ 44 (482)			Age ≥ 45 (462)		
Model	Adjustment factors	R^2	Beta	P	R^2	Beta	P	R^2	Beta	P
1	-	.013	.113	<0.01	.029	.171	<.001	.002	.049	.30
2	Centre & Age	.148	.094	<0.01	.170	.136	<0.01	.157	.038	.40
3	Model 2 + baseline DBP	.270	.135	<.001	.303	.180	<.001	.268	.080	.06
4	Model 3 + BMI	.286	.086	<0.01	.326	.123	<0.01	.276	.043	.35
5	Model 4 + % change BMI	.302	.079	.01	.329	.118	<0.01	.319	.038	.39
6	Model 5+ Log M/I	.303	.077	.01	.329	.117	<0.01	.319	.038	.40
7	Model 6 + Glucose, LDL, HDL ,Chol, TG	.306	.076	.02	.341	.118	<0.01	.330	.040	.37
8	Model 7 + Family Hx	.308	.077	.02	.342	.119	<0.01	.332	.040	.37
9	Model 8+ Log Alcohol	.309	.079	.02	.347	.129	<0.01	.332	.040	.41
10	Model 9 + smoking	.309	.079	.02	.349	.126	.01	.333	.041	.41
11	Model 10 + Physical Activity	.308	.080	.06	.357	.131	.04	-	-	-
12	Model 11+ IL6	.309	.088	.05	.357	.132	.05	.335	.059	.26

Table 5.4 Beta coefficients for predicting ΔDBP over 3 years from log hs-CRP as independent variable in males only, with various adjustment factors

Males	Independent: CRP	Dependent: ΔDBP					
		Age ≤ 44			Age ≥ 45		
Model	Adjustment factors	R^2	Beta	P	R^2	Beta	P
1	-	.069	.263	<.001	.000	.006	.94
2	Centre & Age	.180	.189	<0.01	.202	.030	.69
3	Model 2 + baseline DBP	.312	.176	<0.01	.306	.043	.53
4	Model 3 + BMI	.327	.137	.03	.307	.026	.72
5	Model 4 + % change BMI	.333	.128	.04	.334	.021	.77
6	Model 5+ Log M/I	.333	.128	.04	.336	.026	.73
7	Model 6 + Glucose, LDL, HDL ,Chol, TG	.355	.140	.03	.363	.036	.64
8	Model 7 + Family Hx	.357	.141	.03	.366	.036	.64
9	Model 8+ Log Alcohol	.370	.154	.03	.382	.033	.68
10	Model 9 + smoking	.370	.155	.02	.382	.033	.68
11	Model 10 + Physical Activity	-	-	-	-	-	-
12	Model 11+ IL6	.371	.170	.02	.397	.089	.29

Table 5.5 Beta coefficients for predicting ΔSBP and ΔDBP over 3 years from log IL-6 as independent variable, with various adjustment factors

All by gender	Independent: IL-6	Dependent: ΔSBP			Dependent: ΔDBP		
Model	Adjustment factors	R^2	Beta	P	R^2	Beta	P
1	-	.006	.077	.02	.001	.029	.37
2	Centre & Age	.110	.027	.40	.140	.018	.58
3	Model 2 + baseline BP	.186	.046	.14	.255	.055	.07
4	Model 3 + BMI	.199	.013	.70	.280	.011	.71
5	Model 4 + % change BMI	.214	.004	.91	.297	.002	.96
6	Model 5+ Log M/I	.218	-.002	.96	.298	.000	1.0
7	Model 6 + Glucose, LDL, HDL ,Chol, TG	.222	-.005	.88	.301	.000	1.0
8	Model 7 + Family Hx	.224	-.006	.85	.303	-.001	.97
9	Model 8+ Log Alcohol	.226	-.006	.86	.304	-.001	.98
10	Model 9+ smoking	.226	-.006	.86	.304	-.001	.97
11	Model 10 + physical activity	.222	.003	.94	.303	.002	.96

Table 5.6 Odds Ratio (CI) of hs-CRP and IL-6 for development of hypertension in total population

	Univariate	Model 1	Model 2	Model 3
hsCRP	1.81(1.27,2.59)**	1.19 (0.77, 1.84)	1.13 (0.72, 1.76)	0.69 (0.31, 1.52)
IL6	1.76 (0.99, 3.15)	0.88 (0.42, 1.85)	0.80 (0.37, 1.72)	0.27 (0.07, 1.05)

****= p <0.01,**
Model 1: Adjusted for age baseline BP, BMI.
Model 2: Adjusted for Model 1 + % change in BMI and insulin sensitivity (M/I).
Model 3: Adjusted for model 2 + glucose, total cholesterol, LDL, HDL, TG, family history, alcohol intake, smoking and physical activity

Table 5.7 Estimation of contribution of hs-CRP in prediction of DBP in < 45 years age: total population and males only

		Model 1	Model 2	Model 3	Model 4	Model 5
		Baseline DBP	Model 1 + BMI	Model 2 + Log hs-CRP	Model 3 + Age	Model 4 + % change BMI
Total	R^2	.264	.308	.321	.326	.329
Males	R^2	.261	.295	.313	.327	.333

Model R^2 calculated by multiple regression. Each subsequent model shows increase in R^2 with addition of another variable. Hs-CRP contributed only 1.3 % in model 3 (ΔR^2= 0.321-0.308) in total population, and 1.8 % in model 3 (ΔR^2= 0.313- 0.295) in males. Addition of age and % change in BMI contributed minimally to the model.

Table 5.8 Differences in BMI, fat free mass and fat mass by age

	Total population			Males			Females		
	Age≤ 44	Age≥ 45	P	Age≤ 44	Age≥ 45	P	Age≤ 44	Age≥ 45	P
BMI(kg/m2)	25.1	25.7	0.02	26.0	26.1	0.70	24.2	25.4	<0.01
Fat free mass(Kg)	55.8	52.4	<.001	66.2	62.9	<.001	45.2	44.4	0.05
Fat mass (Kg)	19.4	21.8	<.001	17.7	19.0	0.05	21	24	<.001

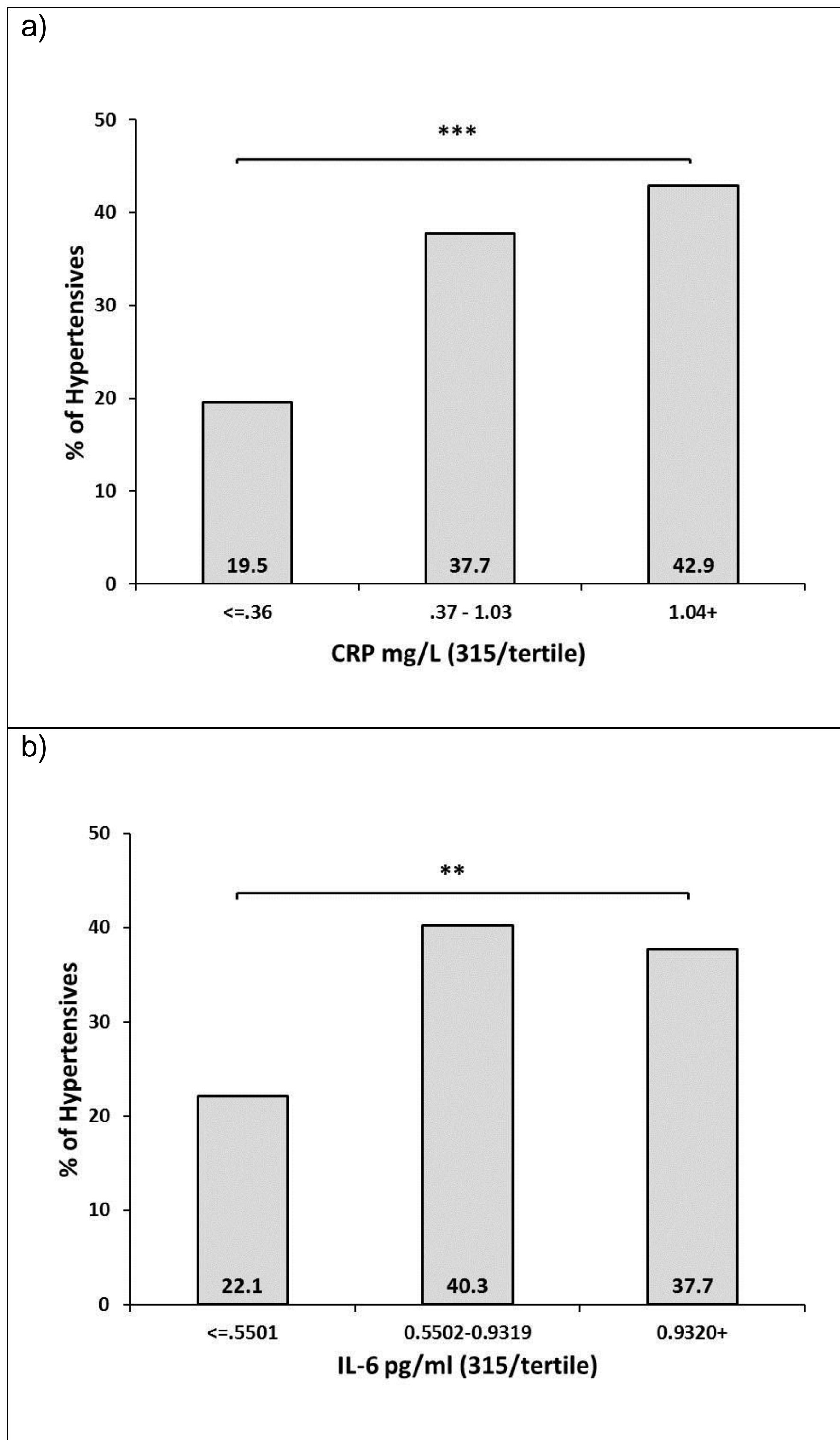


Figure: 5.1 Trend test for development of hypertension over three years in the whole RISC population by a) CRP; and b) IL-6 (unadjusted analyses).
****= P <.01, *** = P <.001**

5.4 Discussion

In this longitudinal study of an initially healthy population, I found that hs-CRP predicted change in DBP (ΔDBP) over three years independently of obesity and clamp-derived insulin sensitivity. The relationship was most evident in younger

adults (age <45) rather than in middle aged participants (age ≥ 45) and in males. The present analysis showed that hs-CRP explained 1.3-1.8 % of the variance in predicting DBP in younger (<44 years) individuals. There was no relationship between CRP or IL-6 and change in SBP (ΔSBP). Neither CRP nor IL-6 predicted the development of hypertension after adjustment for BMI.

Many cross sectional studies have reported the relationship between inflammatory markers and BP (909;915;919;921;963-966) or hypertension (935;937;963;965). Cheung BMY et al reported a relationship between IL-6 and hypertension in a cross sectional analysis but when evaluated longitudinally within the same population over 10 years of follow-up this was not detected (948). Other longitudinal studies assessing the relationship between IL6 and hypertension used trend analysis (945;947) and only one study adjusted for insulin resistance by using HOMA-IR (948). Most of the longitudinal studies reporting the relation of CRP with hypertension used trend test analysis or CRP as a categorical variable (895;896;947;967). Eight longitudinal studies used multivariate logistic regression (Table 5.8) but only one study additionally adjusted for insulin sensitivity by HOMA-IR (911). None of these studies adjusted for IS measured using the clamp method.

In many studies, the relationship between hypertension and inflammatory markers was only significant when examined using test for trend. The same was true in our data in that the test for trend was significant for both CRP and IL-6 using unadjusted data; however, when evaluated using multivariate logistic regression with development of hypertension over three years as the (binary) outcome variable, the relationship was no longer significant. However, one limitation in our cohort is that relatively few participants developed hypertension (n = 154) and follow-up was short (three years). It will be interesting to examine the relationship over 10 years of follow-up (currently in progress).

The relationship between CRP and DBP was significant in the RISC study only in younger individuals, particularly males. As discussed in Chapter 4, CRP was related to DBP in previous studies of younger (<50 years) populations (909;910;912), but these studies did not present results stratified by sex, or use clamp-derived measurements of IS. Here I have shown the results in younger

population and males only. Only one longitudinal study showed contrasting results of CRP and DBP relation; the CARDIA study (940). The CARDIA cohort age range was 18-36 years (mean age 32 years), did not adjust for IR and also did not show sex stratified results (940). I excluded patients with CRP > 10mg/L but some studies reporting a negative relationship used CRP values as high as 50 or 100 mg/L (929;965).

No clear relationship between inflammatory markers and SBP was shown in any group defined by age or sex, consistent with the review of the literature presented in Chapter 4: here I have also shown in the RISC cohort that after adjusting for IS by clamp, there was no significant relation of SBP with CRP or IL-6 in any age or sex group.

Aggregating the findings of Chapters 4 and 5, I have shown evidence from the literature and the RISC cohort that CRP may be statistically related to rise in DBP over time in younger age groups and particularly in males. However, this finding is of limited clinical significance and should be confirmed in other studies. Inflammatory markers are significantly related to obesity, and their levels are dependent on the amount of fat mass in the body. When fat mass is low, as in young males, a small influence of inflammatory markers on BP over time may be easier to detect. In keeping with this notion, in older individuals (and females) with higher fat mass, the influence of fat mass itself may be dominant over any effect of inflammatory markers. Thus, higher fat mass may confound any direct relationship between CRP and BP.

An increase in fat mass and a decrease in lean body mass with age has been reported in many studies; see Section 1.11 (645;648). I also compared fat free mass, fat mass and BMI between younger adults and middle aged groups (in total and sex stratified population) and there was a significant increase in fat mass and BMI and decrease in fat free mass (Table 5.8). Khera et al. evaluated sex differences in the relationship between CRP and body fat in adults to middle aged population (30-65 years) (968). They found that BMI and total fat mass were higher in women and total fat mass had a positive and a steeper slope association with CRP in women. Moreover, CRP increased to a greater degree with increasing truncal, intraperitoneal or subcutaneous fat in women as

compared to men. Fat distribution (truncal/lower body) was also strongly associated with CRP in women (968).

In mechanistic terms, a rise in DBP can be attributed either to cardiac or vascular causes. Cardiac causes manifest as impairment of relaxation of the left ventricle, leading to raised pressure during diastole. Diastolic BP reflects the systemic resistance offered by small arterioles and diastolic hypertension relates to arteriolar vasoconstriction (130). In blood vessels, endothelial dysfunction leads to reduction in nitric oxide (NO) bioavailability which increase peripheral resistance and rise in diastolic BP (969). The latter mechanism seems more likely to be relevant in younger adults (970;971). In the Framingham Heart Study it was reported that predictors of isolated diastolic hypertension (IDH) were young age and male sex (72). It should be noted that 82.5% of persons with IDH developed new onset systolic diastolic hypertension (SDH) during the ensuing 10 years (72). It has been reported that increased arterial stiffness or vascular resistance in people of age <50 is more closely related to DBP than SBP (970;971).

CRP is an inflammatory marker and inflammation has been shown to be associated with endothelial dysfunction, vascular hypertrophy, increase in angiotensin II and vasoconstriction leading to hypertension (282). Previous studies have reported that increased CRP alters endothelial dysfunction leading to hypertension (969;972;973), however this may not be a direct mechanism. Bhagat and Vallance demonstrated that inflammatory cytokines disturb endothelium dependent venous relaxation in humans due to impaired production of nitric oxide and prostacyclins (974). In addition, Guan et al demonstrated that chronic *in vivo* increase in CRP, as mediated by C-reactive protein gene delivery in rats, decreases vessel relaxation and NO production and increases vessel elastance and BP (975). In contrast, recent studies in humans did not show a pro-inflammatory role of CRP (976).

Recently Mark Pepys group showed that infusion of pharmaceutical grade natural human CRP is neither pro-inflammatory nor pro-atherogenic in healthy humans (976). The authors reported no changes in neutrophils, platelet counts heart rate and BP. However the same group has showed pro-inflammatory role of CRP in individuals with pre-existing tissue damage. They showed the effects in rat models of acute myocardial infarction (977) and stroke (978). The proposed

mechanism is that human CRP binds avidly to the dead and damaged cells and activates the classic complement pathway; triggering increased inflammation and further exacerbating tissue damage. As a limitation to this analysis it should be noted that circulating CRP may only be a crude marker of low grade inflammation at tissue level. However, as already noted, CRP was chosen for this analysis as it is a stable protein which is routinely analysed at reasonable cost in stored samples.

In conclusion, genetic epidemiology (mandelian randomisation) and human experimental studies do not support a causal association of CRP with BP or hypertension in healthy animals and humans. However, in the presence of any tissue damage due to obesity, ageing, prehypertension or any other chronic inflammatory condition there are data to suggest that CRP may enhance lesion severity (979). Body adiposity and its change is significantly correlated (r= >0.5) with CRP and change in CRP (980). Higher fat mass as in females and older individuals may confound the relationship between CRP and DBP. However CRP can contribute to prediction of rise in diastolic BP in healthy younger males with lower fat mass.

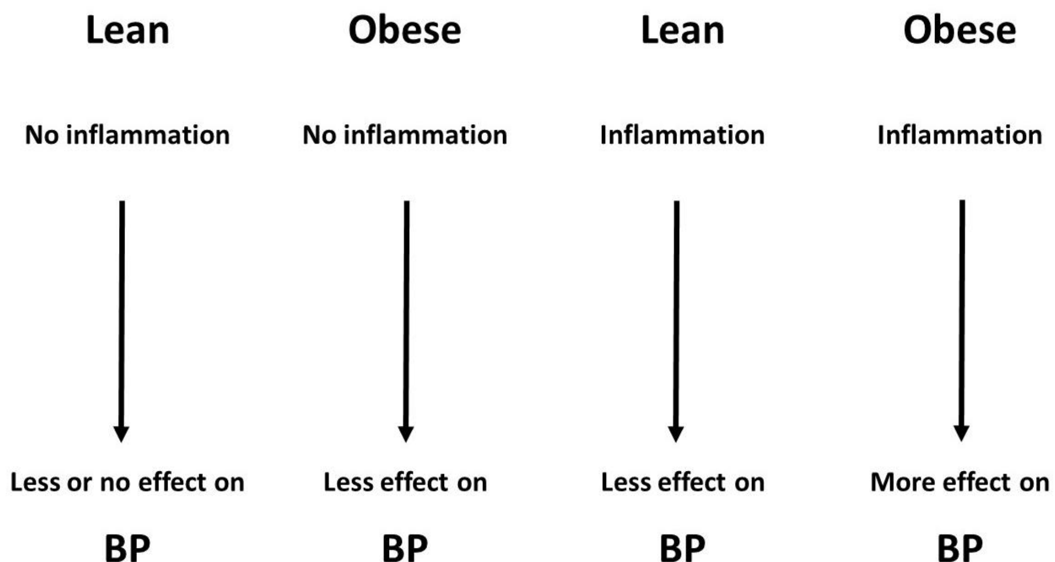


Figure: 5.1 Proposed link between obesity, inflammation and blood pressure.

In summary, this study showed a statistical (if not clinically important) relationship between CRP and DBP in sex and age stratified and clamp adjusted data in a large European cohort. More studies would be required to examine this relationship in other ethnic groups.

6 Predictors for follow-up BP in healthy European population – Role of sex and age

6.1 Introduction

Worldwide, there are 9.4 million deaths per year due to complications of hypertension (981). A number of anthropometric, metabolic, inflammatory, lifestyle and genetic factors have been linked with BP and hypertension in many studies. Some studies show independent relationships of these factors with BP and have proposed different underlying mechanisms. The relationship between weight, BMI or obesity and BP is the most studied and has been demonstrated in many large studies (894;898). Moreover, inflammatory markers (895;896), insulin resistance (196), family history and race (2;897) have been associated with the development of hypertension. Some of these studies have demonstrated independent relationships between lifestyle factors and BP (982;983) while others have shown that these are attenuated following adjustment for weight and/or BMI (984;985). In chapter 3 and 5, we found the association of risk factors with BP/hypertension to be different in different sex and age groups. As explained in Chapter 5, the Framingham study has also reported different gender and age associations with the development of isolated systolic (females, older age and increase in BMI) or diastolic (males, younger age and baseline BMI) hypertension (72). The different association of risk factors with hypertension may be due to sex related differences in body composition and also change in body composition with age (see Introduction Section 1.11, 1.12 and Chapter 5).

Therefore, in the present chapter I have used the RISC cohort to explore which risk factors have an independent relationship with rise in blood pressure over time and to what extent their effect is mediated by associated phenotypes. All phenotypes were measured using standardized techniques with strict quality control (see Chapter 2) and were entered together into the analysis. I investigated differences in predictors of BP rise, according to age, gender and menopausal status with the hypothesis that Predictors for systolic and diastolic BP change with age and sex in relation to ageing and age related changes in body composition.

6.2 Methods

The methods of the RISC study were as described in Chapters 2 and 5.

6.2.1 Statistical Analysis

Altogether, 16 anthropometric and life style factors (baseline BP, age, BMI, percent change in BMI, log M/I, glucose, LDL cholesterol, HDL cholesterol, TG, IL-6, CRP, family history of hypertension, physical activity, smoking and alcohol intake) previously linked with blood pressure and hypertension in the literature were included in this analysis. Only BMI was used as a measure of adiposity in regression models to avoid multicollinearity as correlation (Pearson's r) with waist circumference (r= 0.775), weight (r=0.803), hip (r=0.726) and fat mass(r=0.820) was high. Data were split according to gender, age and menopausal status to find the predictors within each sex and age range as well as according to menopausal status. As explained in Chapter 5 methods, the data were split by median age of our RISC cohort [<45 years (adults); >45 years (middle-aged)] for the analysis. The following age and sex interaction terms were checked in relation to BP: BMI*Age (p = 0.007), percent change in BMI*Age (p = 0.082), log CRP*sex (p = 0.039), log Alcohol*Sex (p = <.001), log M/I*Sex (p = <.001).

Multiple regression and stepwise multivariate regression were performed within all groups to identify significant associations with follow-up BP. The variables segregated through stepwise regression were again used in multivariate linear regression to derive regression equations for each category. Each age and sex group has two models; FM (full model) containing all the variables and SM (selective model) containing only significant variables identified from the full model and stepwise regression. R^2 and adjusted R^2 are compared for both models in order to demonstrate the utility of the selective model.

Significant predictors were further evaluated by t test, chi-square and trend test for their influence on follow-up BP. Differences in baseline measurements between males and females were assessed by t-test (or Chi-square for categorical variables).

6.3 Results

Table 6.1 shows the baseline characteristics of whole population and also males and females separately. SBP and DBP values were in the optimal range in

females and normal in males according to JNC 6 guidelines (986). There were significant differences in all measurements between males and females except for total cholesterol, hs-CRP, IL-6, physical activity count and smoking status. Table 6.2 shows the significant predictors of SBP Y3 and DBP Y3 from stepwise regression. Baseline BP is significant in all the models. Percent change in BMI was a significant predictor of SBP and DBP at the year 3 follow up in the whole population and within all males and all females. In subgroups defined by age and menopausal status, percent change in BMI was a significant predictor in middle aged (both males and females) and postmenopausal groups. However, baseline BMI was a significant predictor in adults (both males and females) and premenopausal females. Alcohol intake and hs-CRP were predictors of BP rise in males only while insulin sensitivity was a predictor of BP rise in females only.

Tables 6.3, 6.4, 6.5, 6.6 and 6.7 show individual predictors of year 3 BP (both SBP and DBP) in different sex and age groups. Each group shows results for two models; FM (full model) and SM (selective model) as explained in the methods.

Table 6.3 shows predictors of year 3 SBP in the whole population and within males and females respectively. Baseline SBP, age and percent change in BMI were significant predictors in all models. However, BMI *per se* was not a significant predictor in males. Insulin sensitivity (M/I) was a significant predictor in females only.

Table 6.4 shows that baseline DBP, BMI and percent change in BMI were significant predictors of year 3 DBP in all models. Age was a significant predictor in FM but had no role in SM. Hs-CRP was a significant predictor in total SM independent of other adjustment variables as seen in the previous chapter.

Table 6.5 and 6.6 show predictors of SBP and DBP in females by age and menopausal status subgroups. In all these groups baseline BP was a significant predictor for follow-up BP. BMI *per se* was a significant predictor in younger and premenopausal females, whereas percent change in BMI was significant in middle aged and post-menopausal females. M/I was a significant predictor for SBP Y3 only in middle aged females. Other significant predictors were cholesterol (SBP Y3) in premenopausal and smoking (DBP Y3) in younger females.

Table 6.7 shows that baseline BP was a significant predictor in all models (SBP and DBP). BMI was significant (for DBP Y3) only in younger males. Percent change in BMI was significant for both SBP and DBP in middle aged males. Alcohol intake was a significant predictor for DBP Y3 but having different associations in the age groups; positive in younger and negative in older. For DBP Y3 hs-CRP was a significant predictor in younger males only. Age was associated with SBP Y3 in middle aged males only.

Figure 6.1 shows the development of hypertension across SBP categories (986) at baseline; Optimal (≤119), Normal (120-129), High Normal (≥130). Development of hypertension was frequent in the high normal and normal groups compared to the optimal group, as baseline BP was most significant predictor in all the models.

I then undertook an exploratory analysis within the optimal BP group [according to JNC 6: (986)] for change in BP/development of hypertension in order to test the predictors derived in the wider population. Within the optimal group, I compared participants according to hypertensive status at follow-up (Table 6.8). Even within this subgroup, almost all anthropometric measurements were significantly different between those who remained normotensive and those who later developed hypertension. This signifies the importance of BMI, waist, weight, fat and fat free mass in addition to baseline BP as predictors of BP rise over time and the development of hypertension. In addition insulin sensitivity was lower, and per cent change in BMI, CRP and IL-6 were higher in those group who developed hypertension

As baseline BMI and percent change in BMI appeared to behave differently as predictors in the above analyses, I plotted the distribution of BMI across tertiles of % change in BMI tertiles (Figure 6.2). This allows easy visualisation of the finding that % change in BMI is not simply a function of baseline BMI. Each category of change in BMI had almost equal percentage of Obese, overweight and normal BMI people.

Percent change in BMI was a significant predictor of both SBP and DBP in all middle aged groups and so its relation with change in BP was further explored. For presentation purpose, change in BMI was stratified into tertiles. Change in

BP (both SBP and DBP) from baseline to year 3 according to tertiles of percent change in BMI (Figure 6.3), and according to decrease or increase in weight (Figure 6.4) was calculated in both sexes. There was no change in either SBP or DBP if BMI remained stable or decreased in either sex. Baseline BP was higher or the same in the lower tertile as compared to mid and high tertiles. However 3 year SBP and DBP were higher in the mid and high tertiles in both sexes, signifying a consistent role for change in BMI in addition to baseline BP.

In Tables 6.5, 6.6 and 6.7, percent change in BMI was a significant predictor of SBP and DBP in middle aged groups, but BMI *per se* did not show any relationship; contrary to the usual finding of a relation of BMI with BP/hypertension. We replaced baseline BMI with BMI at year 3 in all the models to check if percent change in BMI was still a significant predictor. It showed that 3 year SBP and DBP were more strongly related to percent change in BMI in comparison to baseline or 3 year BMI in middle aged population.

Table 6.1 Baseline Characteristics of RISC population and comparison between males and females.

	Total (946)	Male (444)	Female (502)	P
Baseline SBP (mmHg)	118.0 ±12.5	122.4 ±10.5	114.1 ±12.8	<0.001
Baseline DBP (mmHg)	74.4 ± 8.0	76.4 ± 7.4	72.7 ± 8.0	<0.001
Male %	47	-	-	
Age (years)	44.4 ± 8.3	43.8 ± 8.6	45.0 ± 7.9	.03
BMI	25.4 ± 3.9	26.0 ± 3.3	24.8 ± 4.2	<0.001
Waist (cm)	87.0 ± 12.2	93.2 ± 9.6	81.4 ± 11.5	<0.001
Weight (Kg)	74.7 ± 14.4	83.0 ± 12.0	67.4 ± 12.1	<0.001
Glucose (mmol/L)	5.1 ± 0.5	5.2 ± 0.5	5.0 ± 0.5	<0.001
Total Cholesterol (mmol/L)	4.9 ± 0.9	4.9 ± 0.9	4.8 ± 0.9	0.07
HDL Cholesterol (mmol/L)	1.4 ± 0.4	1.3 ± 0.3	1.6 ± 0.4	<.001
LDL Cholesterol (mmol/L)	3.0 ± 0.8	3.1 ± 0.8	2.8 ± 0.8	<.001
Triglycerides (mmol/L)*	1.0 (0.9, 1.0)	1.1 (1.1, 1.2)	0.9 (0.8, 0.9)	<.001
Clamp Insulin Sensitivity (M/I)*	127.8 (123.9, 131.9)	112.6 (107.5, 118)	142.8 (137.2, 148.6)	<.001
CRP (mg/L)*	0.7 (0.6, 0.7)	0.7 (0.6, 0.8)	0.6 (0.5, 0.7)	0.10
IL6 (pg/ml)*	0.8 (0.8, 0.8)	0.8 (0.8, 0.9)	0.8 (0.7, 0.8)	0.16
Alcohol grams/week*	62 (59, 66)	82 (75, 89)	47 (43, 51)	<.001
Family History of Hypertension %	44	39	49	<0.01
Phys. Activity (Counts per min)*	330 (319, 342)	333 (316, 351)	328 (313, 344)	0.68
Smoker %	26	27	25	0.81

***= Log transformed for analysis; values are geometric means (CI)**

Table 6.2 Significant predictors of SBP and DBP according to stepwise regression in the total population and within groups according to , sex, age and menopausal status

SBP at year 3				DBP at year 3			
Total Population Baseline SBP BMI Age % Change in BMI				**Total population** Baseline DBP BMI % Change in BMI Hs-CRP			
Males Baseline SBP Age % Change in BMI		**Females** Baseline SBP Age % Change in BMI BMI LogMI		**Males** Baseline DBP BMI % Change in BMI		**Females** Baseline DBP BMI % Change in BMI	
Adults(Age≤44)	**Middle age(Age≥45)**	**Adults(Age≤44)**	**Middle age(Age≥45)**	**Adults(Age≤44)**	**Middle age(Age≥45)**	**Adults(Age≤44)**	**Middle age(Age≥45)**
Baseline SBP TG	Baseline SBP % Change in BMI Age	Baseline SBP BMI	Baseline SBP LogM/I % Change in BMI	Baseline DBP Hs-CRP Alcohol BMI	Baseline DBP Alcohol % Change in BMI	Baseline DBP BMI Smoker	Baseline DBP BMI % Change in BMI
		Premenopausal	**Postmenopausal**			**Premenopausal**	**Postmenopausal**
		Baseline SBP BMI Cholesterol	Baseline SBP % Change in BMI			Baseline DBP BMI	Baseline DBP % Change in BMI

Table 6.3 Models for predicting follow-up SBP

Dependent: SBP at year 3						
	Total (n=946)		**Males (n=444)**		**Females (n=502)**	
Model	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**
Model R^2	0.465	.458	0.398	.378	0.482	.471
Model Adjusted R^2	0.428	.446	0.310	.350	0.434	.447
	Beta	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**
Age	.119**	.123***	.180**	.152***	.120*	.151***
BMI	.063	.115***	.050		.098	.115**
% Change in BMI	.104**	.108***	.123*	.121**	.088*	.100**
Log M/I	-.050		.037		-.081	-.083*
Glucose	.024		-.055		.019	
Cholesterol	.163		.654		.195	
HDL	-.099		-.302		-.014	
LDL	-.100		-.523		-.132	
Log TG	-.050		-.218		-.028	
Log Alcohol intake	.043		-.013		.043	
Log hs-CRP	.037		.045		.065	
Log IL-6	-.009		-.048		.005	
Family History of Hypertension	.029		.069		.032	
Log Physical activity	.043		.049			
Smoker	.007		.015		-.004	
Baseline SBP	.519***	.537***	.484***	.502***	.471***	.483***

FM= full model, SM= selective model. P = <.05*, p=<0.01, p=<.001*****

Table 6.4 Models for predicting follow-up DBP

Dependent: DBP at year 3						
	Total (n=946)		**Males (n=444)**		**Females (n=502)**	
Model	FM	SM	FM	SM	FM	SM
Model R^2	0.473	.464	0.425	.393	0.491	.478
Model Adjusted R^2	0.437	451	0.341	.365	0.444	.456
	Beta	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**
Age	.082*		.146*		.047	
BMI	.124**	.142***	.113	.144***	.155**	.190***
% Change in BMI	.112**	.114***	.109*	.117**	.115**	.121***
Log M/I	.001		.053		-.037	
Glucose	.000		-.037		-.021	
Cholesterol	.259		.618		-.003	
HDL	-.128		-.263		.061	
LDL	-.248		-.562		-.001	
Log TG	-.050		-.177		.029	
Log Alcohol intake	.040		.010		.029	
Log hs-CRP	.076	.073**	.106		.067	
Log IL-6	-.021		-.071		.015	
Family History of Hypertension	.038		.063		.038	
Log Physical activity	-.024		.014			
Smoker	.005		-.005		.025	
Baseline DBP	.478***	.498***	.442***	.436***	.494***	.519***

FM= full model, SM= selective model. P = <.05*, p=<0.01, p=<.001*****

Table 6.5 Models for predicting follow-up SBP in females by age and menopausal status

Dependent: SBP at year 3 in females								
	Age≤44 (n=239)		Age≥45 (n=263)		Premenopausal (n=378)		Postmenopausal (n=124)	
Model	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**
Model R^2	0.543	.495	0.465	.437	0.503	.477	0.484	.437
Model Adjusted R^2	0.440	.451	0.365	.393	0.439	.447	0.234	.352
	Beta	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**
Age	.064		.074		.092		.185	
BMI	.136	.193**	.049		.104	.171***	.104	
% Change in BMI	.028		.137*	.145**	.004		.237*	.206**
Log M/I	.006		-.139	-.149**	-.073		-.016	
Glucose	-.029		.051		.020		-.010	
Cholesterol	-1.171		.530		.696	.135**	-.305	
HDL	.612		-.187		-.254		.304	
LDL	1.166		-.500		-.548		.221	
Log TG	.235		-.076		-.163		.191	
Log Alcohol intake	.005		.094		.045		.010	
Log hs-CRP	.120		.062		.090		.063	
Log IL-6	.065		-.036		.025		-.044	
Family History of Hypertension	.063		-.015		.067		.034	
Smoker	.100		-.028		-.002		-.072	
Baseline SBP	.504***	.498***	.464***	.508***	.448***	.477***	.494***	.568***

FM= full model, SM= selective model. P = <.05*, p=<0.01, p=<.001*****

Table 6.6 Models for predicting follow-up DBP in females by age and menopausal status

Dependent: DBP at year 3 in females								
	Age≤44 (n=239)		Age≥45 (n=263)		Premenopausal (n=378)		Postmenopausal (n=124)	
Model	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**
Model R^2	0.517	.484	0.539	.518	0.511	.487	0.545	.506
Model Adjusted R^2	0.408	.436	0.453	480	0.448	.459	0.325	.431
	Beta	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**
Age	-.001		.056		.019		.052	
BMI	.188*	.204***	.106	.151**	.154*	.203***	.135	
% Change in BMI	.025		.190**	.204***	.032		.280**	.275***
Log M/I	.015		-.061		-.026		-.012	
Glucose	-.046		.013		-.037		.027	
Cholesterol	-1.050		-.113		.175		-.663	
HDL	.564		.089		-.044		.512	
LDL	1.044		.011		-.131		.542	
Log TG	.226		.096		-.022		.256	
Log Alcohol intake	-.069		.107		.027		.069	
Log hs-CRP	.099		.058		.098		.028	
Log IL-6	.049		-.003		.032		-.050	
Family History of Hypertension	.045		.012		.074		-.022	
Smoker	.141*	.105*	-.007		.042		-.044	
Baseline DBP	.468***	.488***	.499***	.531***	.467***	.493***	.526***	.562***

FM= full model, SM= selective model. P = <.05*, p=<0.01, p=<.001*****

Table 6.7 Models for predicting follow-up SBP and DBP in males by age-

	SBP Y3 in males				DBP Y3 in males			
	Age≤44 (n=244)		Age≥45 (n=200)		Age≤44 (n=244)		Age≥45 (n=200)	
Model	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**	**FM**	**SM**
Model R^2	0.418	.384	0.480	.431	0.442	.406	0.507	.460
Model Adjusted R^2	0.318	.335	0.369	.371	0.346	.344	0.402	.396
	Beta	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**	**Beta**
Age	.086		.130*	.126*	.109		.082	
BMI	.047		.023		.124	.158*	.074	
% Change in BMI	.106		.164*	.156**	.074		.167*	.160**
Log M/I	.014		.056		.062		.030	
Glucose	-.002		-.092		.028		-.047	
Cholesterol	-.311		1.554		.341		1.115	
HDL	.044		-.649		-.098		-.569	
LDL	.313		-1.399		-.298		-1.087	
Log TG	.227	.180**	-.610*		.005		-.467	
Log Alcohol intake	.065		-.149*		.130	.146*	-.141*	-.136*
Log hs-CRP	.073		.050		.156*	.152*	.078	
Log IL-6	-.064		-.081		-.036		-.143	
Family History of Hypertension	.082		.058		.053		.045	
Smoker	.021		.053		-.016		.010	
Baseline BP	.491***	.525***	.502***	.502***	.408***	.410***	.505***	.499***

FM= full model, SM= selective model. P = <.05*, p=<0.01, p=<.001*****

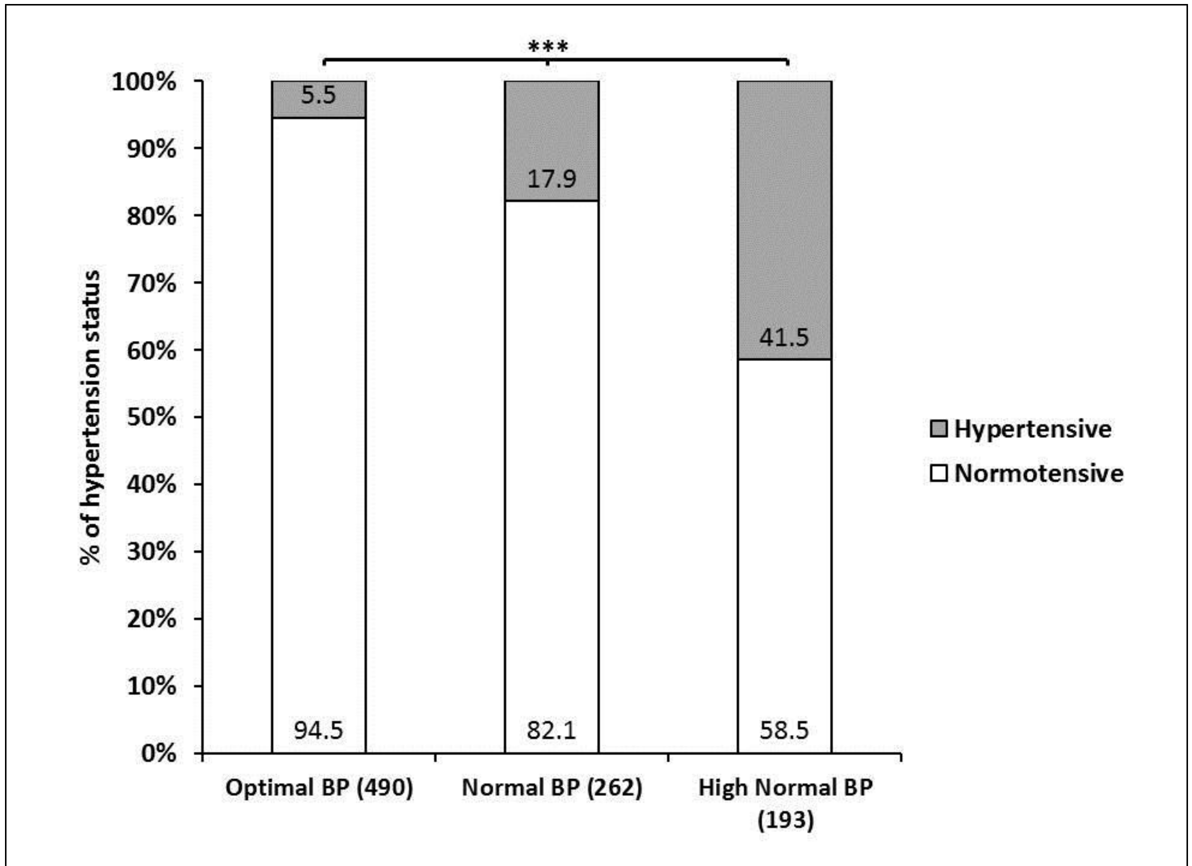


Figure: 6.1 Development of hypertension across BP categories at baseline.
Values according to % of normotensives and hypertensives. Groups compared by Chi-square and trend test across the categories. p=<.001***
Optimal (≤119), Normal (120-129), High Normal (≥130)

Table 6.8 Comparison of baseline measurements according to hypertension status at 3 year follow-up within the optimal BP group (n=490) at baseline

	Normotensive (n=463)	Hypertensive (n=27)	P
	Mean ± SD	Mean ± SD	
Baseline SBP (mmHg)	108.2 ± 8.2	111.4 ± 7.4	0.05
Baseline DBP (mmHg)	69.4 ± 6.4	73.5 ± 6.9	<0.01
Males n (%)	154 (33)	16 (59)	<0.01
Age (Years)	43.3 ± 8.0	48.1 ± 6.5	<0.01
BMI	24.3 ± 3.5	27.0 ± 3.7	<0.01
Waist (cm)	82.8 ± 11.6	94.1 ± 13.4	<0.01
Weight (Kg)	69.9 ± 12.7	79.4 ± 13.9	<0.01
Fat mass (Kg)	19.3 ± 7.7	22.1 ± 8.3	0.06
Fat free mass (Kg)	50.6 ± 10.1	57.3 ± 12.1	<0.01
Clamp Insulin Sensitivity (M/I)*	138.0 (132.5, 143.9)	108.4 (89.5, 131.4)	0.01
% Change in BMI	1.5 ± 6.3	3.5 ± 5.4	0.11
CRP (mg/L)*	0.6 (0.5, 0.6)	0.9 (0.5, 1.5)	0.08
IL6 (pg/ml)*	0.7 (0.7, 0.8)	1.0 (0.8, 1.2)	0.04
Alcohol grams/week*	57 (52, 62)	53 (35, 80)	0.70
Physical activity (counts per minute)*	338 (322, 355)	374 (289, 485)	0.39
Family history of HTN n (%)	184 (41)	14 (54)	0.19
Smoking status n (%)	134 (30)	8 (31)	0.92

***= Log transformed for analysis; values are geometric means (CI)**

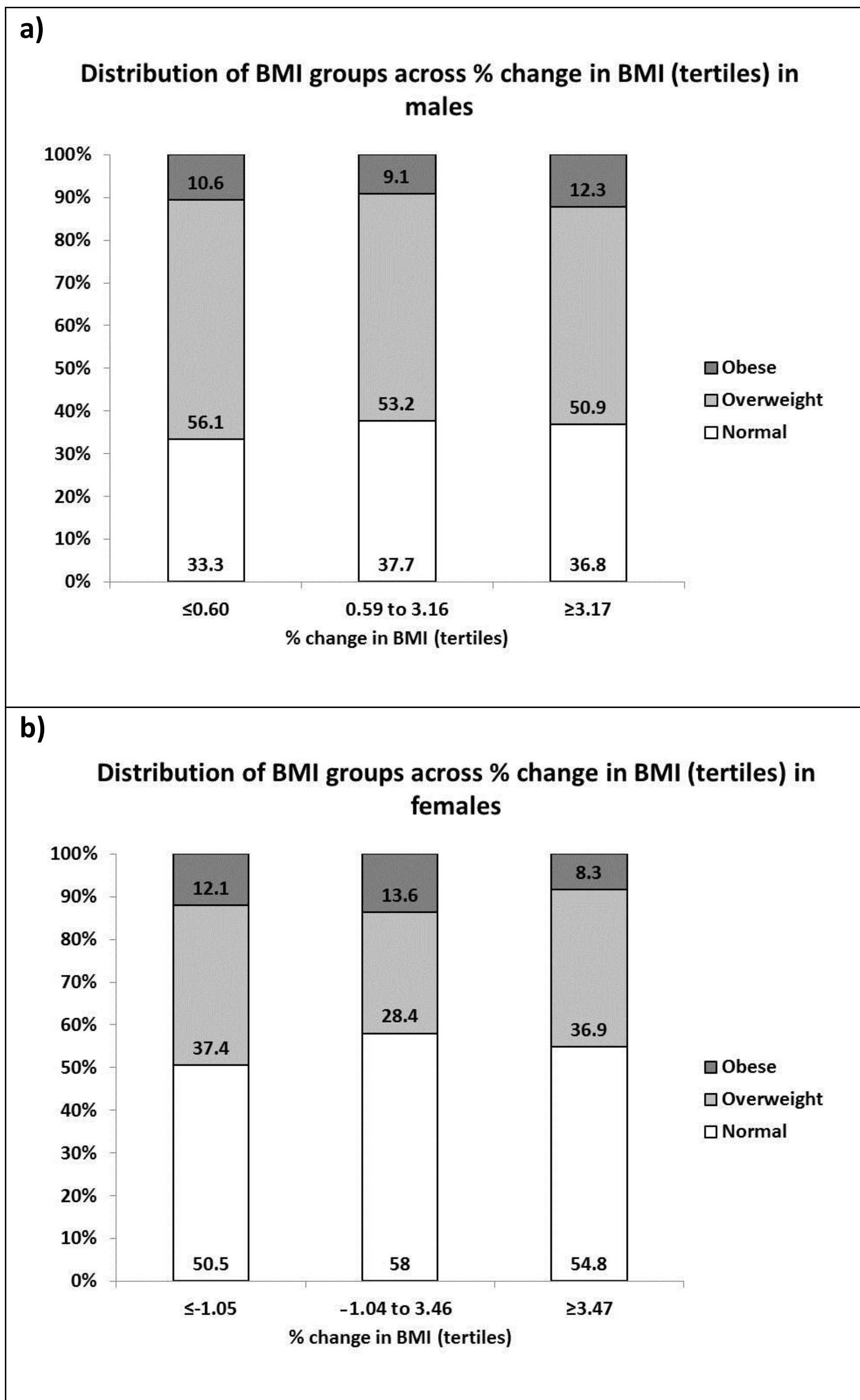


Figure: 6.2 Distribution of BMI groups across % change in BMI tertiles in people having optimal BP at baseline. a) in males, b) in females

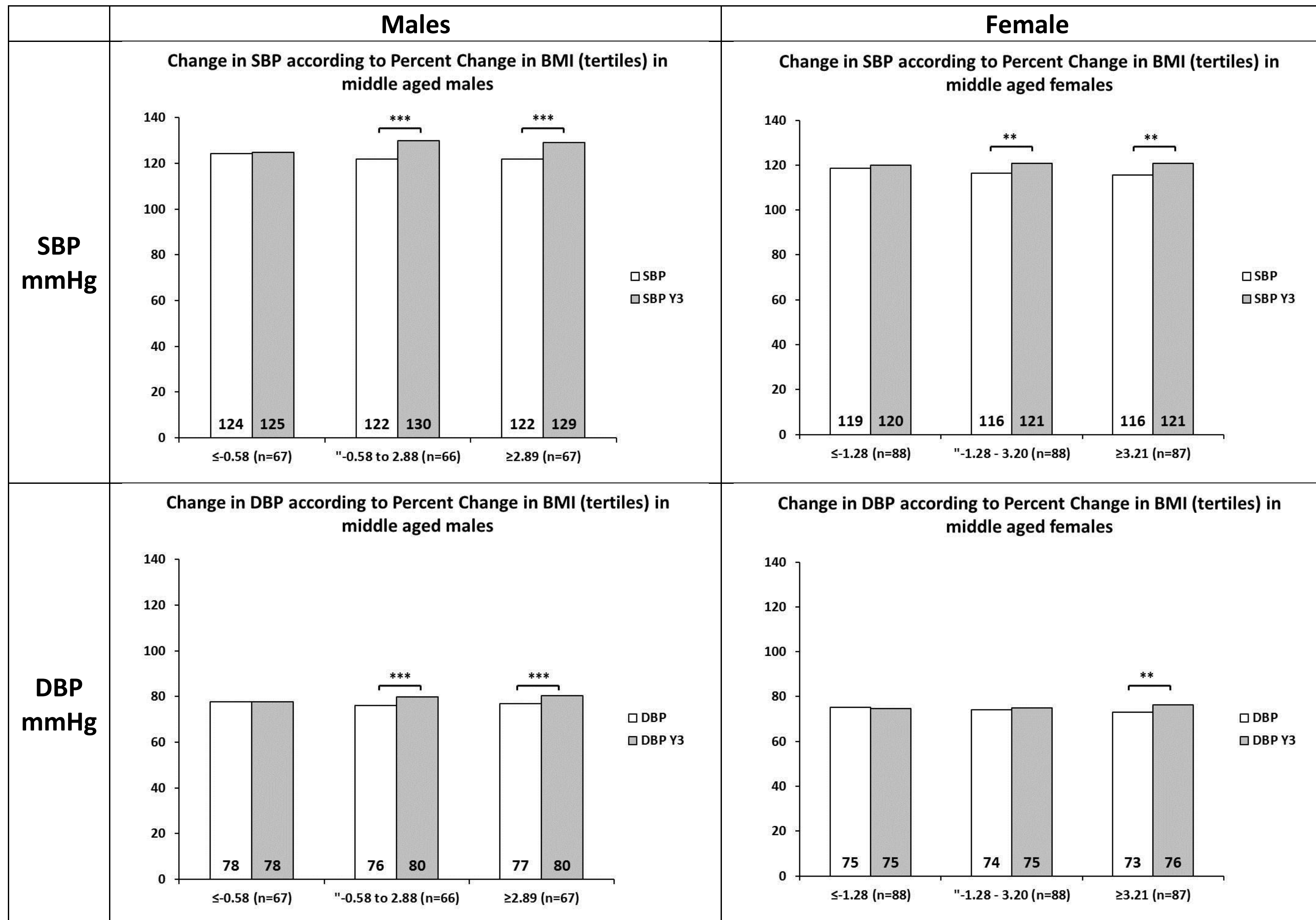


Figure: 6.3 Change in SBP and DBP across % change in BMI (tertiles) in males and females. T test for comparison between baseline and three year follow-up BP. p=<0.01, p=<.001*****

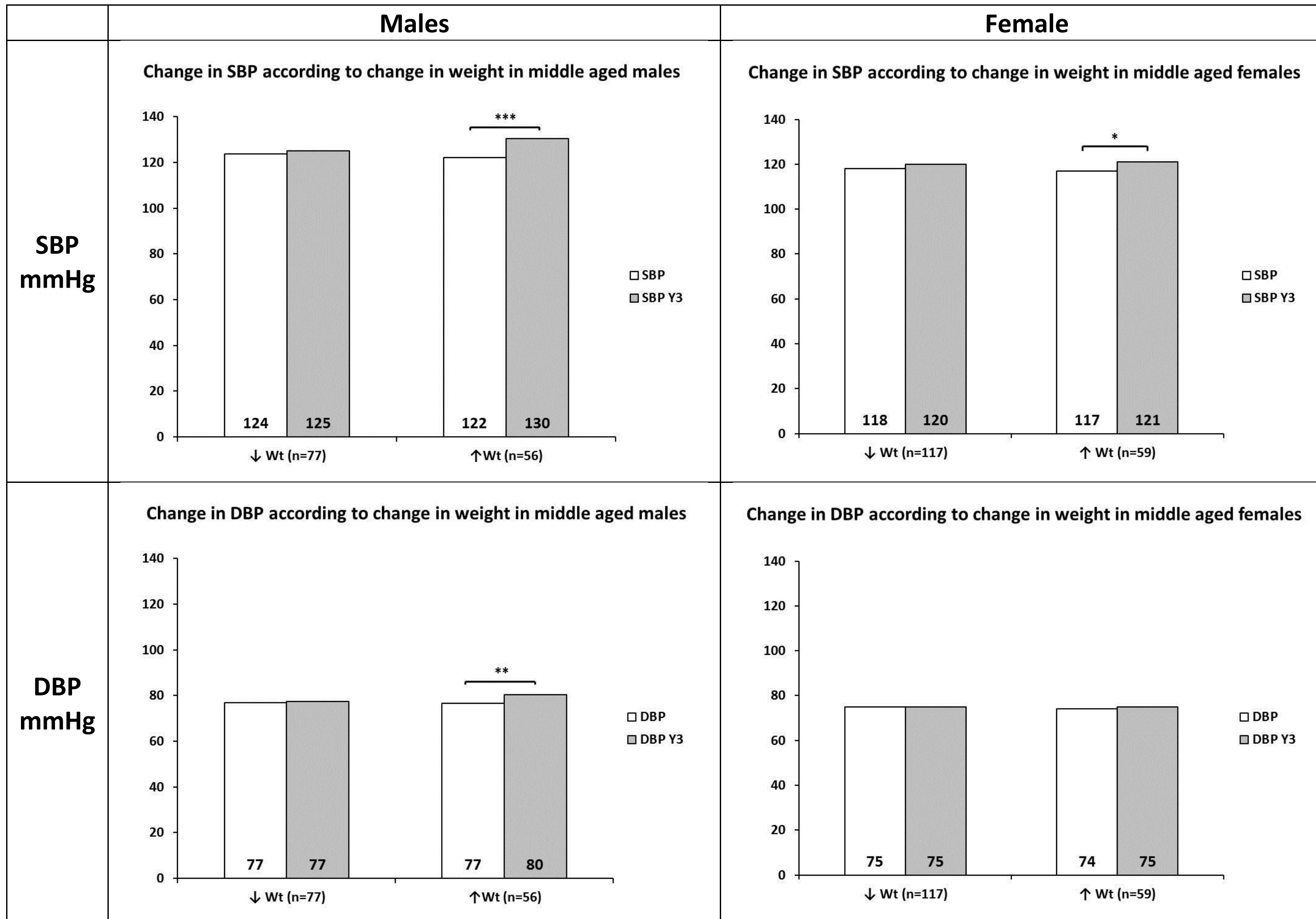


Figure 6.4 Change in SBP and DBP according to decrease or increase in weight over 3 years in males and females. T test for comparison between baseline and three year follow-up BP. p= <0.05, p=<0.01, p=<.001*****
↑= Increase, ↓= decrease, Wt= weight. In males: ↓= range of decrease in weight (kg); -14.40 to -0.10 and ↑= range of increase in weight (kg); 0.10 to 3.30
In females: ↓= range of decrease in weight (kg); -15.5 to -0.10 and ↑= range of increase in weight (kg); 0.10 to 3.50

6.4 Discussion

Baseline BP at all ages and in both sexes is the most important predictor for future or follow-up BP. In the younger age group (30-44 years), baseline BMI *per se* was the second most important predictor. However, in middle age, change in BMI (expressed as a percentage of the baseline value) was found to be more important. Similar findings of the role of weight and weight change in development (894;987;988) or resolution/improvement of hypertension have been demonstrated previously (906). The other important finding is that increasing BMI in middle age is associated with a rise in BP independent of the baseline or present state of BMI i.e. whether one is normal, overweight or obese. Conversely only 2-3% increase in weight was associated with a rise in BP, even in middle aged people with normal BMI. Taken with the findings from the literature, these results permit speculation that in middle age hypertension might best be prevented by avoiding an increase in BMI.

I entered all measured variables in the models to identify these effects and then identified key contributing variables using stepwise regression. This analysis showed that a selective model could be developed which explained a similar proportion of the variance (in terms of adjusted R^2) as a full model. To account for data stratification, age and sex interaction were checked for significant variables: these analyses demonstrated that factors predicting BP were different within the subgroups defined by age and sex.

Baseline SBP and DBP were significant predictors of follow up BP in all age, sex and menopausal status groups: a higher baseline BP gives a higher follow-up BP (i.e. BP "tracking"). Prehypertension has been associated with arterial stiffness, and increased arterial stiffness is associated with hypertension and increased risk of CVD (106). Tomiyama et al. showed in a large longitudinal study that the change of the arterial stiffness (brachial-ankle PWV) during 6 years was higher in the prehypertensive subjects than in those with persistent normal blood pressure (109). It has also been shown that recovery from hypertension after weight loss surgery depends on preoperative SBP (989). In conclusion baseline BP through “tracking phenomenon” and arterial stiffness leads to development of hypertension. The TROPHY study showed that controlling BP in pre-hypertensive stage prevents the development of hypertension (117). More recently Zoungas et

al. also showed that intensive BP control decreases death from cardiovascular disease (990).

The associations of weight/BMI and weight change with BP/hypertension have been shown in many studies (900-903). Mendelian randomization in relation to the *FTO* gene also demonstrates a relationship between BMI and both SBP and DBP (894). Holland et al showed over follow-up of 36 years that BMI was directly related to BP from childhood to adult age. They also showed that adult age BP was more closely related to current BMI than childhood BMI (899). Similar findings were also provided by Petkeviciene et al from a 35 year follow-up study which showed that change in BMI from childhood (age10-12) to middle age (age 48-49) was a significant predictor of the development of hypertension (childhood BMI was not a significant predictor) (991). In this study, people who developed hypertension in middle age experienced a greater increase in BMI over 35 years than people who remained normotensive (991). Similar findings of increase in BMI in middle age predicting hypertension were also reported in Isfahan Cohort Study (992). Wills et al have showed similar findings in a British cohort of 3035 males and females which demonstrated that becoming overweight in early adult life leads to higher SBP and DBP in midlife (988).

Diet and exercise have previously been linked with change in BP (983;993;994). In our study we did not collect dietary/nutritional information. However, there was no significant contribution of the lifestyle factors measured (smoking, alcohol intake and physical activity) following adjustment for BMI and percent change in BMI. This was also the case in the National Runners Health survey, USA (985) which evaluated the role of weight and increase in weight on development of hypertension in lean physically active individuals (n = 34661). It was demonstrated that higher body weight or increase in weight was associated with an increase in the risk of hypertension with no advantage of having previously been lean in either sex. It was also shown that in middle age, the odds of developing hypertension are significantly related to *current* BMI and not to adult age BMI (985). Similar findings were reported in a recent study in which Masuo et al showed that reduction of BMI by diet or exercise (or a combination of diet and exercise) leads to decrease in BP in obese hypertensive men. In this study, reduction of fat mass, insulin resistance (HOMA-IR) or plasma nor-epinephrine (NE) alone did not decrease BP. However when lifestyle modifications were

sufficient to decrease BMI, then BP did decrease (even when there was no change in WHR or HOMA-IR) (995). Similarly Obarnazek E et al showed that the effect of lifestyle factors was attenuated after considering weight change (984). My analysis is in accordance with these studies in predicting that lifestyle factors *per se* do not influence BP directly but rather via altering BMI in young people and percent change in BMI in middle aged people.

The analysis presented in this chapter suggests that change in BMI predicts BP in middle age. There is support from the literature for this proposition: studies performed in middle aged populations (40-60 years of age) also show a relationship between weight gain and increase in BP or development of hypertension (902;903;987;996;997). Conversely, weight loss achieved through bariatric surgery in morbidly obese patients results in a decrease in BP along with 60-80% resolution or improvement of hypertension (904-906). Bariatric surgery- related weight loss increases adiponectin and expression of adiponectin receptors (502;998). The increase in adiponectin is strongly correlated with percentage decrease in BMI (499). Bariatric surgery also improves the inflammatory profile of obese individuals by decreasing expression of IL-6 and TNF-α mRNA (502;998), along with restoring perivascular adipose tissue (PVAT) anti-contractile activity (504). However, it should be noted that in some studies this improvement in hypertension is not sustained at long term post operation follow-up (8-10 years) of these patients (999;1000). Kuller LH et al also reported that weight loss through lifestyle intervention had beneficial effects on BP for the first 18 months but the improvement was not sustained in long term follow-up over 4 years (982). All of the studies which showed long term attenuation of the beneficial effects on BP and hypertension of weight loss (982;999;1000) have one common feature: after initial weight loss and decrease in BP, there was an increase in weight over the follow-up time. On the other hand, studies in which initial weight loss was maintained after lifestyle or surgical intervention showed that the beneficial effects of intervention on BP and resolution/improvement of hypertension were still present even after 10 years of follow-up (902;989;1001-1003).

It has been documented previously that surgical intervention did not correct hypertension in older patients (989): the longer that obesity and hypertension persist the more permanent the accompanying functional and structural changes

(1001;1004). Chronic high blood pressure is associated with structural and functional alteration (rarefactions) of micro vessels. In the beginning micro vascular rarefaction is mostly functional, but progresses to structural rarefaction with persistent high BP (also see Sections 1.3.3.1 and 1.3.7.4). It has been shown in never treated patients of essential hypertension that 62% of rarefaction was explained by structural defects, and 38% was explained by functional defects (84). In addition it has been demonstrated both clinically and experimentally that obesity induces renal haemodynamic changes (glomerular hyperperfusion and hyperfiltration) which lead to proteinuria, glomerulosclerosis and progressive renal failure. The renal modifications due to high BP and chronic kidney disease (CKD) do not reverse completely as some changes lead to apoptosis, nephron loss and fibrosis (185-188).

The other important finding is that avoiding an increase in BMI in middle age prevents rise in BP, independent of current BMI and the baseline state of BMI; whether one is normal, overweight or obese. There are two plausible explanations for these findings; the obesity paradox, and physiological changes in body composition with ageing. The "obesity paradox" is a term used to summarise observation of lower CVD related mortality in people who are overweight or obese in comparison to normal BMI people. Uretsky et al have shown that CVD associated mortality was 30% less in overweight and obese patients (1005). The same finding was also reported in a recent meta-analysis of 26 studies (1006). Niedziela et al. reported that one of the reasons of obesity paradox may be that obese people were young as compared to older normal weight people (1006). In addition Carnethon et al. also showed that onset of diabetes at a young age occurs mostly in obese people, in contrast to the situation in older individuals (1007). The effect of ageing may have a stronger impact than obesity alone. It has been shown that ageing itself is a pro-inflammatory state.

In middle age health is threatened by two important physiological changes: ageing and change in body composition. These changes in body composition occur even without a change in diet and lifestyle (645-648;650;651). The British birth cohort study showed that participants who were in the normal range of BMI until the age of 43 years had a rise in BMI after 43. There was also an increase in BMI after age 43 in people who were already overweight (988). Thus, basal

metabolic rate slows down and the hormonal changes responsible for this; result in weight gain. For those who maintain weight this is a physiological transition of body composition; but for those who gain weight the fat mass can disturb metabolic balance and lead to obesity-related vascular dysfunction and rise in BP. It can be speculated that if metabolism and body composition are in homeostasis with current BMI (whether normal, overweight or obese) and maintains the BMI, the pro-inflammatory component caused by change in BMI will be absent; ageing-related inflammation will still be present. When the pro-inflammatory stimulus of increasing BMI is superimposed on normal ageing, it will accentuate age-related inflammation and lead to increase in BP. Concluding, it could be proposed that obesity *per se* is relatively harmless, at least in cardiovascular terms. It becomes more harmful when associated with chronic low-grade inflammation. Recent results from the 1946 British birth cohort also show that reduction of BMI was associated with decreases in cIMT and improvements in cardiovascular risk profile (1008).

It is proposed on the basis of the present analysis of the RISC cohort that maintaining BMI in middle age (or early intervention if BMI is increased) can prevent rise in BP. Mechanistically, control of weight can prevent the initiation of early structural changes in the kidneys and vascular system. This finding is in keeping with previously published literature. Strength of this study is the availability of data for percent change in BMI which take into accounts the baseline BMI. For example, a 3 kg increase in weight will have different effects in people with different baseline BMI and is equivalent to a 14% increase in a person with a BMI = 21.5 kg/m^2 (3/21.5 * 100) but only a 10.4% increase in a person with a BMI = 29 (3/29 * 100) kg/m^2. Thus even when a person gains weight (e.g. from BMI 21.5-24.5) within the normal range, there will be a higher risk of rise in BP as percent change in BMI is more (14%).

These prospective data from the Europe-wide RISC cohort of healthy adults indicate that BP depends on BMI in adult age and change in BMI in middle age in both men and women. This age associated differences in predictors need to be confirmed in larger studies and have the potential of clinical significance. People who maintain their BMI in middle age are protected from increase in both SBP and DBP over 3 years. Percent change in BMI over time is a useful marker for

monitoring/assessing cardiovascular risk and the effectiveness of lifestyle (diet, exercise) and other (drugs, surgery) interventions.

The strengths, limitations and final conclusion are discussed in the final discussion (chapter 8)

7 Ethnicity and risk of cardiovascular disease (CVD): 4.8 year follow-up of patients with Type 2 diabetes living in Scotland

7.1 Introduction

For detailed methodology see Chapter 2 (Section 2.6)

Type 2 diabetes (T2DM) is associated with increased risk of premature mortality with vascular death, the most common cause for mortality (617). More than 60% of people with type 2 diabetes die of cardiovascular diseases (CVD) such as MI or stroke (1009). In developed countries, the rate of cardiovascular deaths is decreasing, but for people of South Asian origin living in those countries the rate is stable (1010) or even increasing when compared to White populations living in those countries (1011;1012).

South Asians are at an increased risk of CVD (1013). Cardiovascular risk factors are increased with higher waist hip ratio (1014;1015), increased risk of diabetes (10), with earlier onset (711;1016) and greater likelihood of suboptimal glycaemic control (734;1017) than white populations. In the UK the substantially higher rate of CVD in South Asians is in part explained by higher rates of diabetes (709). At the same time within the population with diabetes, the rate of coronary heart disease (CHD) deaths remained double than that in White, an increase which was not explained by conventional risk factors- albeit with relatively small numbers (311 South Asians, 51 deaths) (1014). In addition, South Asians are considered a single group in most of the studies (709;1014), despite the fact that ethnic differences in risk of CVD within South Asians countries have been reported (679;1012;1018-1020).

Here I use a large prospective cohort of people with clinically diagnosed diabetes to examine whether there is an ethnic difference in rates of CVD, and if so whether this is independent or explained by onset, duration and severity of diabetes or conventional CVD risk factors.

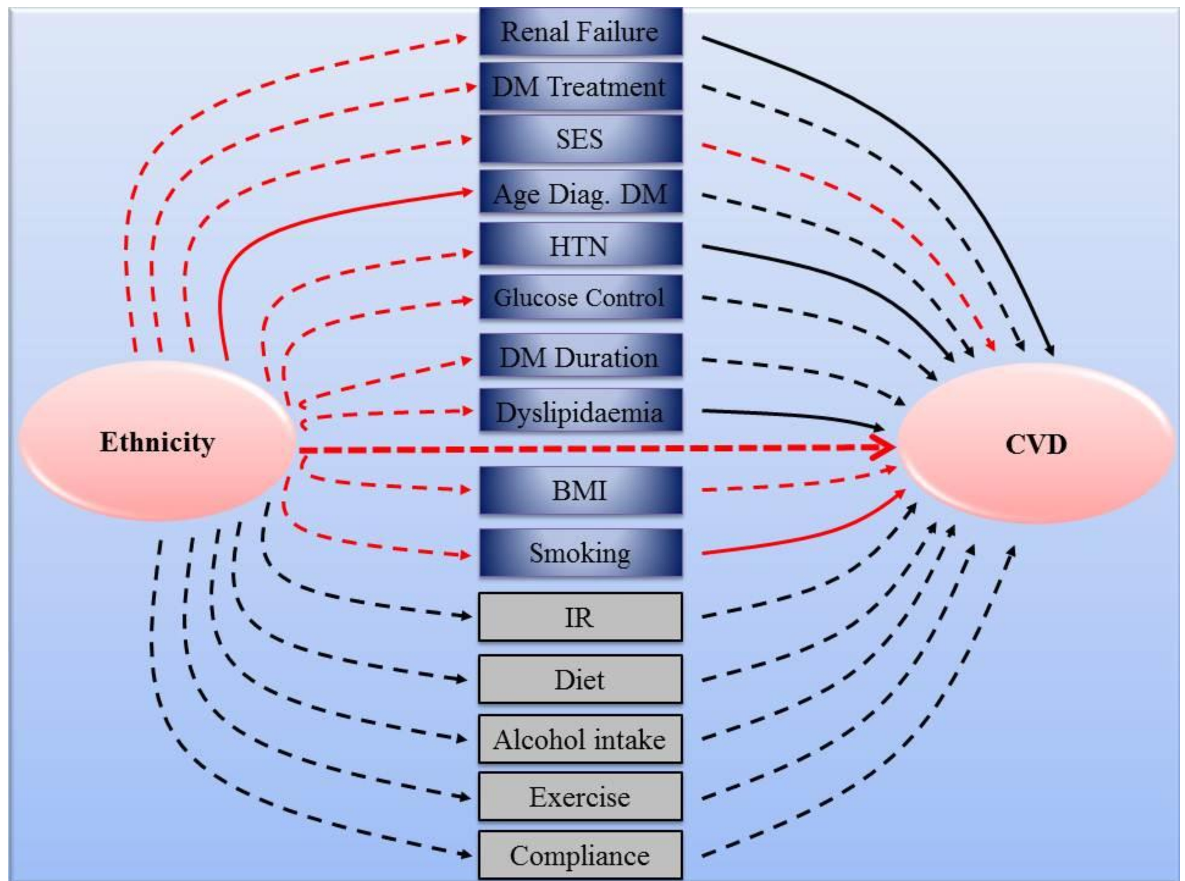


Figure 7.1 Relationship between ethnicity and cardiovascular disease in people with type 2 diabetes
Ethnicity influences many cardiovascular risk factors and also development of CVD. We checked if ethnicity independently (i.e. not confounded by other CV risk factors) influences occurrence of CVD (thick dashed line). Another objective was to check if the effect of other CV risk factors was influenced by ethnicity. Solid lines= established relationship, dashed lines = ambiguous relationship, red lines= relationships checked in this chapter, purple blocks= data available, grey blocks= plausible associations but data not available. Age Diag DM= age of diabetes diagnosis, HTN= hypertension, DM= diabetes, SES= socio economic status

7.1.1 Statistical Analysis

All data analyses were performed using STATA 12.

7.1.1.1 Normality

Normality was checked for all continuous variables: age at baseline, age at diabetes diagnosis, BP, BMI, HBA1c, total cholesterol, HDL-c, LDL-c and creatinine (Appendix C: Figure 2.1 and 2.2). All variables were normally distributed except for triglycerides (TG) and duration of diabetes which were log transformed for analysis (Appendix C: Figure 2.3).

In univariate analysis continuous variables were compared across different ethnic groups by ANOVA with Bonferroni adjustment. Categorical variables

(incident CVD event, gender, baseline renal impairment, smoking, diabetes treatment, hypertension treatment, high cholesterol treatment and socio-economic status (SES)) were compared across different ethnic groups by chi square test. Logistic regression was used for individual group comparison with White as the reference category. Where previously recorded as percentage, HbA1c was expressed as IFCC units.

7.1.1.2 Survival analysis

A Cox proportional-hazards regression model was used to examine the relationship of ethnicity and CVD incidence expressed as Hazard ratios (HR) with corresponding 95% confidence interval (CI) with and without adjustment for other covariates [age, sex, HbA1c, duration of diabetes, socioeconomic status, severe renal impairment (defined as serum creatinine ≥200μmol/L), cholesterol lowering treatment (defined as drug use with British National Formulary-BNF code 02.12), antihypertensive treatment (defined as drug use with BNF code 02.05), smoking status, BMI, BP (both systolic and diastolic), total cholesterol and HDL-c]. To explore the effects of residual BP and cholesterol on CVD risk, both BP and cholesterol levels were added in the same model along with hypertension and cholesterol lowering treatment. Cox regression was performed, both by including and excluding BP and cholesterol in the model. The proportional hazards model carries the assumption that hazards in different groups (ethnic groups in our study) are proportional over time (i.e. constant relative hazards). Survival in the different groups is illustrated by minus log-log plot, Kaplan–Meier and predicted survival plot and also by using Schoenfeld residuals (Appendix C: Figures 2.4 -2.7). For clarity, groups with smaller numbers of participants (Chinese, Other Asian, African- Caribbean and Other Ethnic) were not shown in some log- log and predicted survival plots (Appendix C: 2.5 and 2.7). To check for multicollinearity, we plotted a correlation matrix of all the variables used in the SDRN dataset (Appendix C: Table 2.1). Due to multicollinearity with age (0.90, p= <.001), age of diabetes diagnosis was omitted from Cox-regression (Appendix C: Table 2.1).

7.1.1.3 Ethnicity Interaction with cardiovascular risk factors

To further explore potential effects of ethnicity on the relationship of known cardiovascular risk factors to CVD events, separate models incorporating interaction terms (sex, HbA1c, hypertension treatment, cholesterol treatment, total cholesterol, renal impairment, smoking, age, age at diagnosis and diabetes treatment) were examined.

7.2 Results

The differences in anthropometric and metabolic variables, and risk factors, between different ethnic groups are shown in Table 7.1. White, Multiple Ethnic and Chinese were older at baseline and when their diabetes was diagnosed. By contrast, Pakistanis, African-Caribbean and Other Asian were younger at baseline and their diabetes was diagnosed at a younger age. Pakistanis, Other Asian and Chinese had lower BP, BMI and total cholesterol than White. Pakistanis and African Caribbean had the highest HbA1c in the groups. White and Multiple Ethnic had the highest percentage of ever smokers and of being on anti-hypertensive treatment. Pakistanis, Indian and Chinese had the highest mean duration of diabetes at baseline (Table 7.1). Socioeconomic distribution was also different in different ethnic groups, with people of African-Caribbean origin having the highest percentage (41%) of most deprived people and Indian’s having the highest percentage (31%) of the most affluent group. White individuals formed the highest percentage of the population (94.2%) at baseline (Table 1). For those excluded from the analysis due to existing CVD the ethnicity distribution was similar to those included i.e. White (95.2%), multiple Ethnic (2%), Indian (0.5%), Other Asian (0.14%), Pakistani (1.54%), Chinese (0.17%), African Caribbean (0.10%) and Other Ethnic (0.28%).

In total, 16,265 (13.4%) patients developed CVD in the follow-up period (Table 1.2). Incidence of new CVD events was less in Indians (74 events, 9.3 % of Indians), Other Asians (27, 8.5 %), Chinese (35, 9 %) and Afrcan-Caribbean (24, 8 %) as compared to White (15394, 13.4%), Pakistanis (290, 13%) and Multiple Ethnic (358, 14%). Follow-up duration was slightly different in the ethnic groups with Pakistanis (5.2), Indians (5.2) and Chinese (5.3) the highest. The differences in events per 1000 person years follow-up and duration of diabetes until the end

of follow-up are shown in Table 7.2. Notably, Pakistanis (63 years) and Other Asians (64 years) were almost nine years younger when they had a CVD event as compared to White (72 years) (Table 7.2). In keeping with this, for the same age and age of diagnosis, Pakistanis had a higher incidence of CVD events as compared to White and Chinese (Figure 7.3).

In the univariate (unadjusted) model the risk of having a CVD event was less in all ethnic groups in comparison to White, except Other Ethnic group (HR 1.01, CI 0.79- 1.29). After adjustment for age and sex, risk of CVD was increased in Pakistanis (HR= 1.31, CI 1.17-1.47, p=<0.01) and decreased in Chinese (HR= 0.66, CI 0.47-0.92, p=0.01) as compared to White. Adjustment for other cardiovascular risk factors made little difference to the estimate of hazard ratio for Pakistanis: after inclusion of all relevant risk factors Pakistanis remained at higher CVD risk (HR=1.45, CI 1.14–1.85, p=<0.01)(Table 7.3), but the results for Chinese origin and other ethnic groups were no longer significant (Table 7.3). The Risk of CVD was also significant in Pakistani males (HR= 1.33, CI 1.01-1.75, p=0.04) and females (HR=1.67, CI 1.25-2.24, p=<0.01) (Table 7.4). The main finding (HR significant for Pakistani population only) remained unchanged whether or not BP was included in the model. Findings were similar for total cholesterol and cholesterol lowering treatment. In addition removing both blood pressure and total cholesterol did not change the main finding.

None of the CV risk factors had significant interactions with ethnicity (sex p=0.67, hypertension treatment p=0.23, high cholesterol treatment p=0.47, total cholesterol p=0.90, HbA1c p=0.39, renal impairment p=0.35, smoking p=0.36, age p=0.44, age of diabetes onset p=0.14 and diabetes treatment p=0.79).

Smoking, renal failure, treatment for hypertension and high cholesterol had increased HR for CVD event with renal failure having highest risk [HR (CI) 1.46(1.62, 1.83)] (Table 7.5). More affluent socio economic class was associated with lower risk of CVD event (most affluent 0.81 vs. most deprived 1.0).

For a given quintile of HbA1c, people of Pakistani origin had higher odds for the development of CVD compared to White. Similarly Pakistanis had higher odds for CVD in almost all the socio economic status categories as compared to White (Figure 7.4). Odds ratios for the development CVD in relation to development of

renal failure, hypertension and high cholesterol are shown in Figure 7.5. It also shows higher odds for Pakistanis compared to white.

CVD morbidity in different ethnic groups was also explored and it revealed that Other Asians and Pakistanis had the highest percentage of Ischemic heart disease. Whereas African Caribbean and Chinese had the highest rate for cerebrovascular disease (Table 7.6). In total 17,637 people died during the follow-up and deaths due to CVD were 3,722 (21% of total deaths). The number of deaths due to CVD and other causes according to different ethnic groups is shown in Table 7.6.

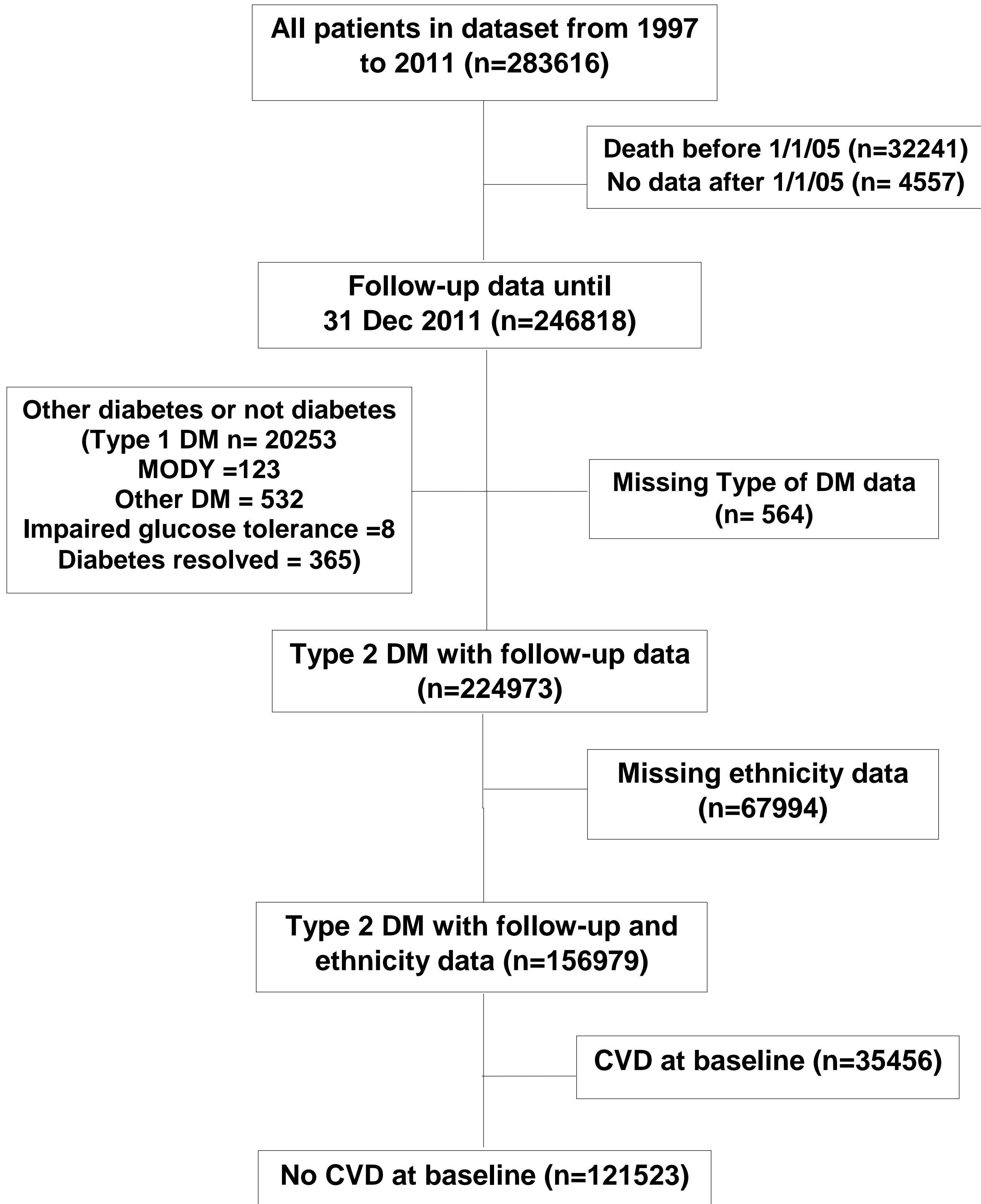


Figure 7.2 CVD in People with type 2 diabetes in Scotland: Numbers of patients excluded CVD= cardiovascular disease, DM= diabetes mellitus, MODY = Maturity onset diabetes of the young.

Table 7.1 Baseline measures and risk factors by ethnic group.

Variable (n=)	White(114450)	Multiple Eth (2 554)	Indian (797)	Other Asian (3 19)	Pakistani (2249)	Chinese (387)	African Carr (3 01)	Other Eth (466)	P
N =121535 (% of total)	114450(94.2)	2554 (2.1)	797 (0.7)	319 (0.3)	2249 (1.9)	387 (0.4)	301 (0.3)	466 (0.4)	
N % males (121523)	59469 (52)	1274(49.9)[b]	464(58.2)[b]	167(52.3)	1157(51.5)	194(50.1)	157(52.2)	251 (53.9)	0.01
Age at baseline (YR) (121476)	63.3± 12.6	64.0± 12.9	56.2± 2.8[a]	52.5±11.8[a]	53.8±12.0[a]	61.7±12.8	51.8±13.0[a]	57.6±14.0[a]	<.001
Age at DM diagnosis (Yr) (121476)	59.3± 12.7	60.2± 13.2[a]	51.2±12.2[a]	48.7±11.2[a]	48.8±11.6[a]	56.7±12.7[a]	48.5±12.2[a]	54.1±13.6[a]	<.001
BP baseline mmHg(64160)	141/79	140/79	136/79[a]	131/78[a]	134/79[a]	135/77[a]	140/82	139/79	<.001
BMI (per kg/m2) (85720)	31.8± 6.6	31.8± 6.7	28.8± 5.2[a]	28.7± 5.2[a]	30.2± 5.8[a]	26.0± 3.9[a]	31.1± 6.8	30.2± 6.2[a]	<.001
Total Cholesterol (mmol/L)(90839)	5.0± 1.4	5.1± 1.4	4.9± 1.2	4.9± 1.1	4.9± 1.6	4.7± 1.0[a]	5.0± 1.3	5.0± 1.2	<.001
HDL-c (mmol/L) (61525)	1.3± 0.4	1.3± 0.4	1.3± 0.4	1.2± 0.3	1.3± 0.4	1.3± 0.4	1.3± 0.4	1.3± 0.5	0.08
LDL-c (mmol/L) (18988)	2.8± 1.0	2.8± 1.0	2.8± 1.0	2.8± 1.1	2.9± 0.9	2.7± 1.0	2.8± 1.1	2.8± 1.0	0.656
TG (mmol/L) (36343)[c]	2.11(2.10-2.12)	2.05(1.99-2.12)	1.93(1.78-2.09)	2.01(1.79-2.27)	2.00(1.91-2.10)	1.74(1.55-1.97)[a]	1.74(1.52-1.98)	2.12(1.93-2.32)	<.001
Creatinine (µmol/l) (91612)	91.0± 32.7	91.2± 30.9	86.9± 24.0	82.0±19.3[a]	83.3±37.0[a]	93.2± 37.0	89.0± 23.8	86.4± 22.8	<.001
Duration of DM at Base (Yr) (121476)[c]	4.14(4.10-4.17)	4.13(3.90-4.38)	4.80(4.32-5.32)	4.20(3.51-5.01)	4.75(4.45-5.07)[a]	4.72(4.07-5.47)	3.93(3.14-4.93)	3.78(3.25-4.41)	<.001
HBA1c % (mmol/mol) (79508)	7.8±1.8(61.75)	7.7±1.8(60.66)	8.0±1.7(63.94)	7.8±1.7(61.75)	8.5±2.0(69.41)[a]	7.8±1.6(61.75)	8.4±2.1(68.31)[a]	8.0±1.8(63.94)	<.001
Renal impairment (91612)	601 (0.7)	9 (0.4)	2 (0.4)	0 (0)	11 (0.7)	8 (3)[b]	1 (0.5)	1 (0.3)	0.001
Ever Smokers N(%) (121355)	83189 (72.8)	1983(77.7)[b]	386(48.4)[b]	164(51.6)[b]	1084 (48.2)[b]	186(48.3)[b]	142(47.2)[b]	287(61.6)[b]	<.001

Diabetes treatment (63704)	Diet	8144 (13.6)	195 (13.4)	40 (9.1)	16 (10.5)	71 (5.5)	25 (12.1)	4 (2.9)	22 (8.9)	<.001
	Biguanide alone	34270 (57.3)	562 (38.5)	291 (66)	89 (58.6)	863 (66.7)	126 (61.2)	89 (64.5)	155 (62.8)	
	Other AD Drugs	6972 (11.7)	103 (7.1)	34 (7.7)	16 (10.5)	110 (8.5)	31 (15.1)	13 (9.4)	29 (11.7)	
	Insulin	10381 (17.4)	600 (41.1)[b]	76 (17.2)	31 (20.4)	249(19.3)[b]	24 (11.6)	32 (23.2)[b]	41 (16.6)	
Hypertension treatment (121523)		55943 (48.9)	1206 (47.2)	361(45.3)[b]	94 (29.5)[b]	986(43.8)[b]	177 (45.7)	100(33.2)[b]	178(38.2)[b]	<.001
High Cholesterol treatment (121523)		66938 (58.5)	1453 (56.9)	477 (59.8)	138(43.3)[b]	1333(59.2)	219 (56.6)	123(40.9)[b]	252 (54.1)	<.001
SIMD quintile (12 1276)	Most deprived (5)	31955 (28)	318 (12.5)	105 (13.2)	70 (22)	484 (21.6)	73 (18.9)	123 (41)	129 (27.8)	<.001
	Deprived	26277 (23)	540 (21.2)	147 (18.5)	72 (22.6)	575 (25.7)	76 (19.6)	53 (17.7)	77 (16.6)	
	Middle	22094 (19.3)	647 (25.4)	125 (15.7)	44 (13.8)	385 (17.2)	72 (18.6)	40 (13.3)	77 (16.6)	
	Affluent	18548 (16.2)	595 (23.3)	170 (21.4)	54 (17)	367 (16.4)	63 (16.3)	37 (12.3)	77 (16.6)	
	Most Affluent (1)	15347 (13.4)	450 (17.6)[b]	248(31.2)[b]	78 (24.5)[b]	430 (19.2)[b]	103(26.6)[b]	47 (15.7)[b]	104(22.4)[b]	

Values are n (%age). For age, BP, BMI, HbA1c, cholesterol, and creatinine; values are mean (SD). For TG and duration of diabetes values are geometric mean (Confidence interval-CI).
CVD = Cardio vascular disease, DM = Diabetes Mellitus, AD = anti-diabetic, SES = Socio-economic status. P = p value for ANOVA (continuous variable) or Chi square test (categorical variable) a = p <.05 for the post hoc (Bonferroni) difference between white and other ethnicities. b = p <.05 for the logistic regression comparison of ethnicities with white as reference category. c= log transformed for analysis.

Table 7.2 Cardiovascular disease events: Percent of events, average follow-up time, duration of diabetes and age at events by Ethnicity.

	White(114450)	Multiple Eth (2554)	Indian (797)	Other Asian (319)	Pakistani (2249)	Chinese (387)	African Carr (301)	Other Eth (466)	P
CVD events (% in group)	15394 (13.4)	358 (14)	74 (9.3) [b]	27 (8.5) [b]	290 (12.9)	35 (9) [b]	24 (8) [b]	63 (13.5)	<.001
Follow-up (Yr)	4.8	4.8	5.2[a]	5.0	5.2[a]	5.3[a]	4.6	4.8	<.001
Events per 1000 person Years follow-up	27.92	29.17	17.88	17.00	24.81	16.98	17.39	28.13	
Duration of DM (Yr)[c]	6.02(5.98-6.05)	5.83(5.61-6.07)	7.17(6.73-7.64)[a]	5.72(5.09-6.43)	6.98(6.69-7.28)[a]	7.34(6.70-8.05)[a]	5.04(4.50-5.65)	5.53(5.03-6.07)	<.001
Age at CVD event (Yr)	71.6±11.2	72.4±11.2	67.9±10.9	63.7±10.8[a]	62.8±11.5[a]	69.6±12.1	69±11.4	68.7±12.3	<.001

Yr = Years. p value shows ANOVA for the difference between White and other ethnicities. a = p <.05 for the post hoc (Bonferroni) difference between white and other ethnicities. b = p <.05 for the logistic regression comparison of ethnicities with white as reference category. c= log transformed for analysis and values are geometric mean (CI).

Table 7.3 Cardiovascular disease events: Hazard ratios (Confidence Intervals) for time to first CVD event (Fatal and non-fatal) by ethnicity with White as reference group, up to 7 years follow-up in people with type 2 diabetes.

	Multiple Eth	Indian	Other Asian	Pakistani	Chinese	African Carr	Other Eth
Unadjusted	1.04(0.93-1.15)	0.63(0.50-0.79)[a]	0.61(0.42-0.89)[a]	0.88(0.79-0.99)[a]	0.61(0.44-0.85)[a]	0.63(0.42-0.94)[a]	1.01(0.79-1.29)
Adjusted for: Age	0.99(0.89-1.10)	0.84(0.67-1.06)	0.93(0.64-1.36)	1.30(1.16-1.46)[a]	0.66(0.47-0.91)[a]	0.98(0.66-1.46)	1.20(0.94-1.54)
Age and sex (model 1)	1.0(0.90-1.11)	0.83(0.66-1.04)	0.93(0.64-1.36)	1.31(1.17-1.47)[a]	0.66(0.47-0.92)[a]	0.97(0.65-1.45)	1.22(0.95-1.56)
Model 1+ duration of DM	0.98(0.88-1.09)	0.95(0.76-1.20)	1.00(0.69-1.47)	1.56(1.39-1.75)[a]	0.74(0.53-1.03)	1.00(0.67-1.49)	1.20(0.94-1.54)
Model 1 + Ever Smoke	0.98(0.88-1.09)	0.88(0.70-1.10)	0.98(0.67-1.42)	1.38(1.23-1.55)[a]	0.68(0.48-0.95)[a]	1.03(0.69-1.53)	1.24(0.97-1.59)
Model 1 + Renal Imp	0.99(0.88-1.12)	0.76(0.57-1.01)	0.98(0.63-1.54)	1.28(1.12-1.47)[a]	0.55(0.36-0.84)[a]	1.08(0.68-1.71)	1.20(0.90-1.62)
Model 1 + SES	1.03(0.93-1.15)	0.89(0.71-1.12)	0.94(0.64-1.38)	1.35(1.20-1.52)[a]	0.68(0.49-0.95)[a]	0.97(0.65-1.45)	1.24(0.96-1.59)
Model 1 + HTN Rx	1.00(0.90-1.12)	0.84(0.67-1.05)	0.99(0.68-1.44)	1.33(1.19-1.50)[a]	0.67(0.48-0.94)[a]	0.99(0.66-1.48)	1.26(0.98-1.62)
Model 1 + T Chol Rx	1.00(0.90-1.11)	0.83(0.66-1.04)	0.97(0.67-1.42)	1.32(1.17-1.48)[a]	0.66(0.47-0.92)[a]	1.01(0.67-1.50)	1.23(0.96-1.58)
Model 1 + HbA1c	1.02(0.90-1.16)	0.72(0.54-0.97)[a]	0.89(0.55-1.44)	1.24(1.08-1.42)[a]	0.57(0.38-0.85)[a]	1.00(0.61-1.64)	1.31(0.98-1.75)
Model 1 + BMI	1.01(0.89-1.13)	0.71(0.53-0.96)[a]	0.82(0.49-1.36)	1.31(1.14-1.50)[a]	0.58(0.38-0.88)[a]	0.90(0.54-1.50)	1.26(0.93-1.69)
Model 1 + T Chol	0.99(0.87-1.12)	0.68(0.51-0.90)[a]	0.97(0.62-1.52)	1.34(1.18-1.53)[a]	0.59(0.39-0.89)[a]	0.94(0.57-1.53)	1.24(0.92-1.67)
Model 1 + HDL	0.92(0.79-1.07)	0.66(0.46-0.95)[a]	0.85(0.48-1.50)	1.33(1.13-1.56)[a]	0.41(0.22-0.77)[a]	1.49(0.91-2.44)	1.11(0.76-1.64)
Model 1 + SBP	1.04(0.88-1.21)	0.73(0.55-0.96)[a]	0.94(0.58-1.51)	1.26(1.10-1.44)[a]	0.63(0.42-0.93)[a]	0.98(0.60-1.61)	1.22(0.91-1.65)
Model 1 + Drugs	0.93(0.82-1.05)	0.80(0.60-1.07)	1.10(0.70-1.73)	1.18(1.02-1.37)[a]	0.60(0.38-0.94)[a]	0.90(0.54-1.50)	1.10(0.80-1.52)
Model 1 + All[b]	0.91(0.67-1.23)	0.91(0.55-1.51)	0.89(0.37-2.15)	1.45(1.14-1.85)[a]	0.58(0.24-1.40)	1.25(0.62-2.51)	1.34(0.76-2.36)

DM= diabetes, HTN= hypertension, Rx=treatment, T Chol= total cholesterol. a = p <.05, b = Model adjusted for age, sex, HbA1c, diabetes treatment/drugs, duration of diabetes, socioeconomic status, renal failure, cholesterol lowering treatment, antihypertensive treatment, smoking, BMI, BP, total cholesterol, HDL.

Table 7.4 Cardiovascular disease events: Hazard ratios (Confidence Intervals) for time to first CVD event (Fatal and non-fatal) by ethnicity and gender with White as reference group, during 7 years follow-up in people with type 2 diabetes.

	Multiple Eth	Indian	Other Asian	Pakistani	Chinese	African Carr	Other Eth
Female							
Unadjusted	0.95(0.81-1.11)	0.71(0.50-1.00)[a]	0.55(0.31-1.00)[a]	0.85(0.71-1.01)	0.57(0.35-0.93)[a]	0.91(0.55-1.51)	0.76(0.51-1.15)
Adjusted for age	0.91(0.78-1.07)	1.01(0.72-1.43)	0.94(0.52-1.70)	1.36(1.14-1.62)[a]	0.62(0.38-1.01)	1.68(1.01-2.79)[a]	0.87(0.58-1.32)
Adjusted for all	0.89(0.60-1.31)	0.83(0.41-1.66)	0.91(0.29-2.84)	**1.67(1.25-2.24)[a]**	0.47(0.15-1.47)	1.81(0.86-3.81)	0.91(0.38-2.19)
Male							
Unadjusted	1.13(0.98-1.30)	0.58(0.42-0.78)[a]	0.66(0.41-1.08)	0.92(0.79-1.08)	0.65(0.42-1.03)	0.41(0.22-0.80)[a]	1.24(0.91-1.69)
Adjusted for age	1.07(0.93-1.23)	0.73(0.53-0.99)[a]	0.93(0.57-1.52)	1.28(1.09-1.49)[a]	0.70(0.44-1.09)	0.57(0.30-1.10)	1.57(1.15-2.15)[a]
Adjusted for all[b]	1.11(0.80-1.54)	0.90(0.52-1.55)	0.84(0.31-2.24)	**1.33(1.01-1.75)[a]**	0.50(0.19-1.32)	0.79(0.25-2.45)	2.07(1.22-3.49)[a]

a = p <.05, b = Model adjusted for age, HbA1c, diabetes treatment/drugs, duration of diabetes, socioeconomic status, renal failure, cholesterol lowering treatment, antihypertensive treatment, smoking, BMI, BP, total cholesterol, HDL

Table 7.5 Hazard Ratios for CVD event in population with no CVD at baseline

	Model A	P value	Model B	P value
	HR (95%CI)		HR (95%CI)	
Age of DM diagnosis (years):	1.03 (1.03- 1.03)	<0.001	0.93 (0.92- 0.93)	<0.001
Age at baseline (years):	1.04 (1.04- 1.05)	<0.001	1.12 (1.12- 1.13)	<0.001
Sex m vs F	1.09 (1.06- 1.13)	<0.001	1.14 (1.08- 1.20)	<0.001
Ever Smoked	1.31 (1.26- 1.36)	<0.001	1.24 (1.17- 1.33)	<0.001
Renal impairement	2.67 (2.30- 3.09)	<0.001	1.46 (1.62- 1.83)	<0.01
SES: Most deprived (Ref)				
Deprived	0.94 (0.90- 0.98)	<0.01	0.91 (0.84- 0.97)	0.01
Middle	0.93 (0.89- 0.98)	<0.01	0.91 (0.84- 0.98)	0.02
Affluent	0.88 (0.84- 0.93)	<0.001	0.91 (0.84- 0.99)	0.02
Most Affluent	0.82 (0.78- 0.86)	<0.001	0.81 (0.74- 0.88)	<0.001
Anti-hypertensive Rx	1.64 (1.58- 1.69)	<0.001	1.44 (1.35- 1.55)	<0.001
Cholesterol lowering Rx	1.34 (1.29- 1.39)	<0.001	1.26 (1.17- 1.36)	<0.001
HBA1c	1.02 (0.97- 1.08)	0.41	1.06 (1.04- 1.08)	<0.001
BMI	0.98 (0.97- 0.98)	<0.001	1.01 (1.00- 1.01)	<0.01
Total Cholesterol	0.91 (0.90- 0.93)	<0.001	0.99 (0.97- 1.01)	0.20
HDL	1.02 (0.97- 1.08)	0.41	0.90 (0.83- 0.96)	<0.01
SBP	1.00 (1.00- 1.01)	<0.001	1.00 (1.00- 1.00)	0.08

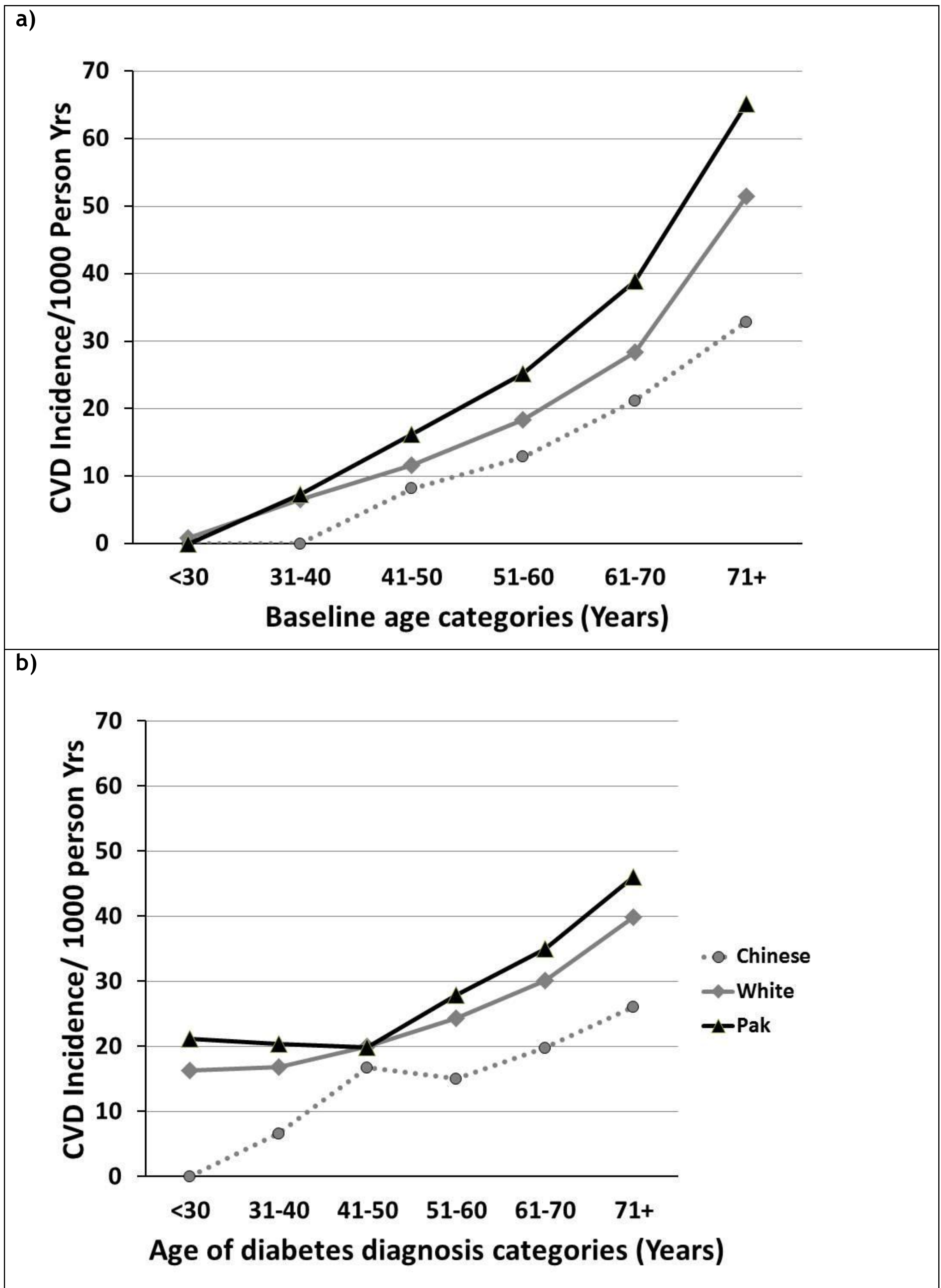
Model A: Univariate (unadjusted),
Model B: adjusted for ethnicity, duration of diabetes and all others in the model.
Reference Categories: Sex: female = 0 and male = 1, ever smoked = 1, SES: 1 = most deprived & 5 = most affluent, antihypertensive treatment = 1, Cholesterol lowering treatment = 1, Rx = treatment

Table 7.6 Types of cardiovascular diseases and mortality in different ethnic groups during the follow-up

	White	Multiple Eth	Indian	Other Asian	Pakistani	Chinese	African Carr	Other Eth	Total
CVD morbidity									
Angina	2,826(18)	75(21)	12(16)	6(22)	61(21)	3(9)	4(17)	4(6)	2,991(18)
MI	1,774(12)	47(13)	5(7)	4(15)	37(13)	2(6)	2(8)	8(13)	1,879(12)
Other IHD	6,206(40)	140(39)	38(52)	14(52)	136(47)	19(54)	5(21)	34(54)	6,592(40)
Peripheral VD	238(2)	3(1)	1(1)	0	4(1)	0	0	0	246(2)
Cerebral Haemorrhage	288(2)	10(3)	0	0	6(2)	3(9)	1(4)	2(3)	310(2)
Cerebral Infarction	1,412(9)	36(10)	8(11)	1(4)	19(7)	4(11)	5(21)	3(5)	1,488(9)
Unspecified Stroke	966(6)	22(6)	4(5)	1(4)	12(4)	2(6)	5(21)	5(8)	1,017(6)
Other Cerebro VD	1,551(10)	22(6)	5(7)	1(4)	15(5)	1(3)	2(8)	6(10)	1,603(10)
Sequelae of Cerebro VD	133(1)	3(1)	1(1)	0	0	1(3)	0	1(2)	139(1)
Total	15,394	358	74	27	290	35	24	63	16,265
Mortality									
CVD	3,582(21)	71(21)	18(31)	4(33)	27(21)	10(25)	5(28)	5(12)	3,722(21)
Other causes	13,418(79)	265(79)	40(69)	8(67)	104(79)	30(75)	13(72)	37(88)	13,915(79)
Total	17,000	336	58	12	131	40	18	42	17,637

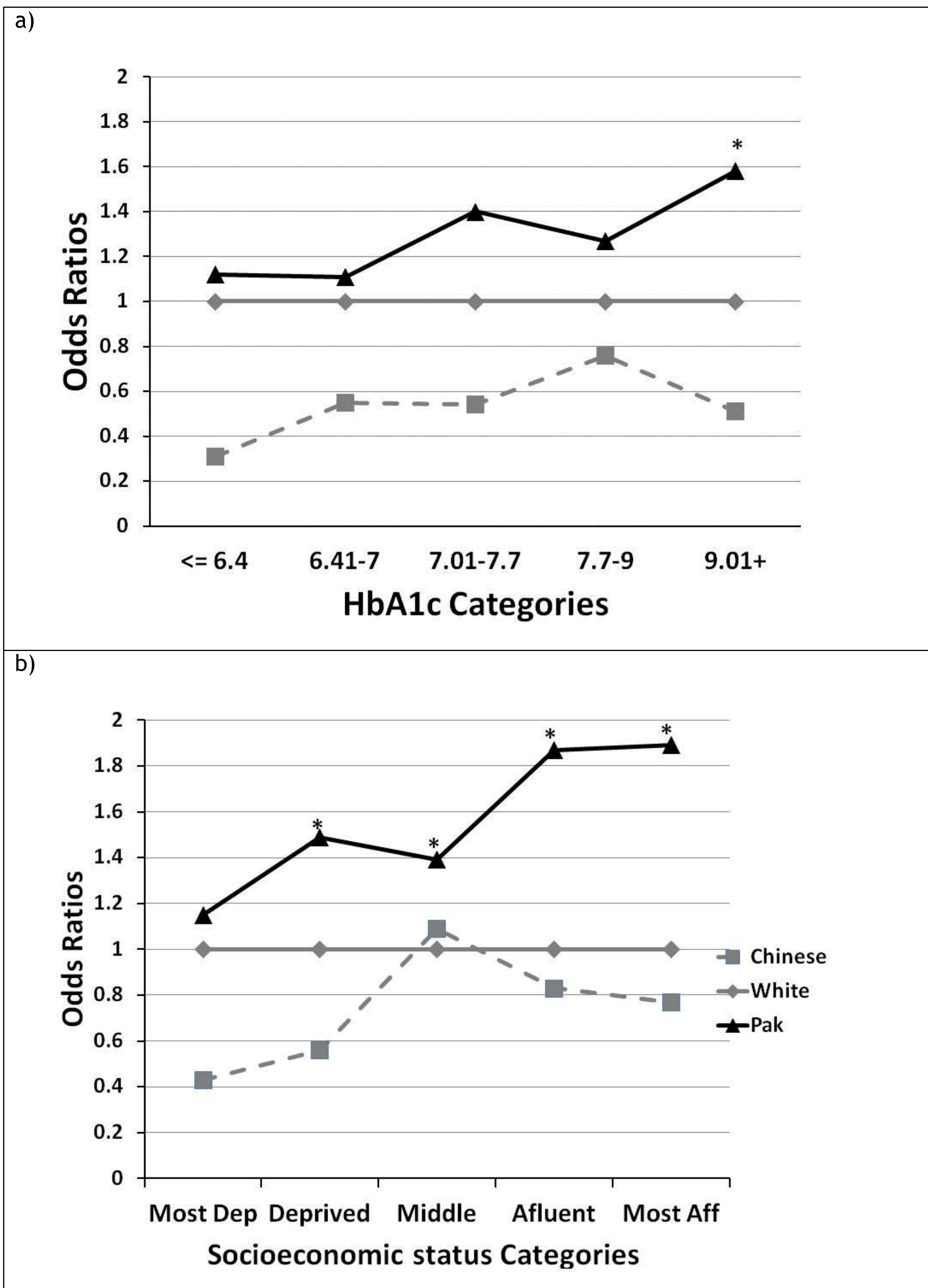
Values are n (%) within each ethnic group. MI= Myocardial infarction, IHD= Ischemic heart disease, VD= vascular disease, CVD= cardiovascular disease.

1

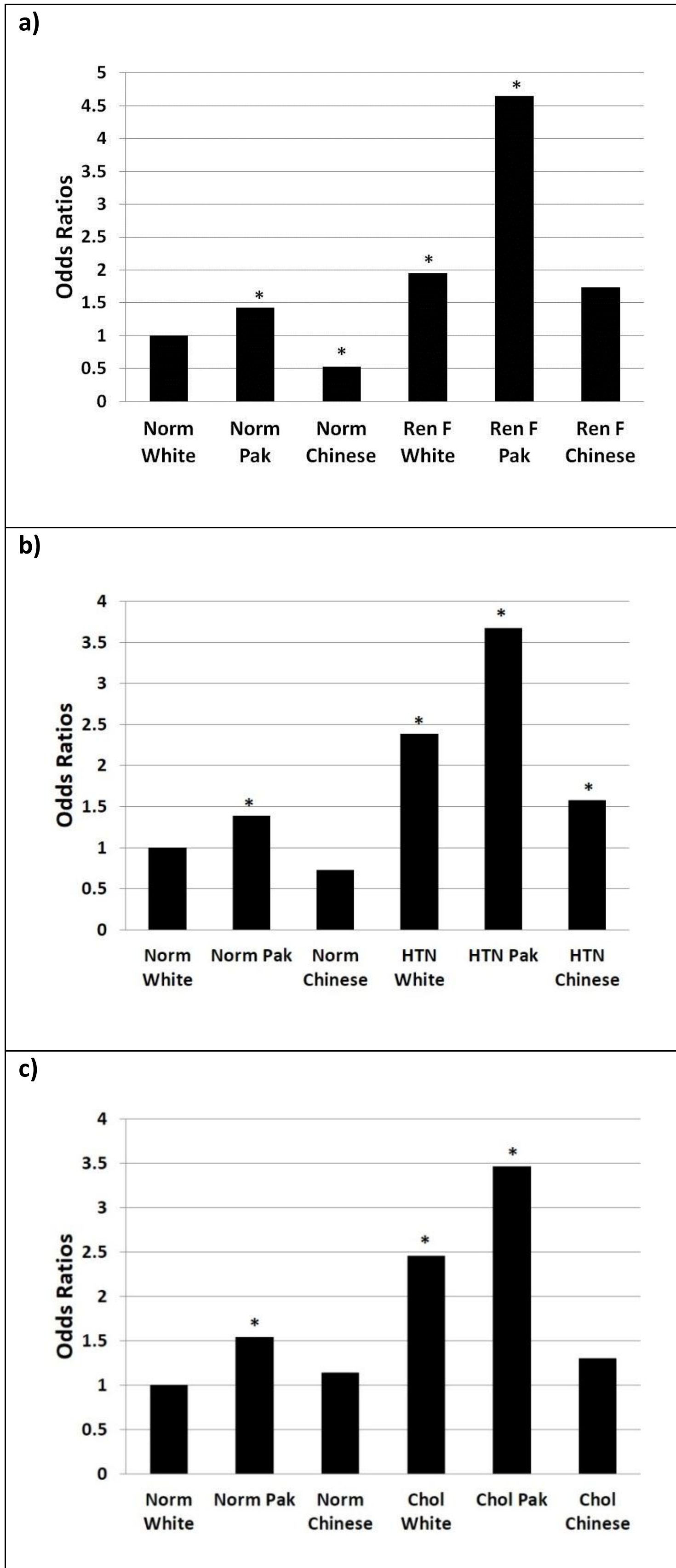


2 **Figure 7.3 Incidence of CVD per 1000 person years in age and age of diabetes diagnosis**
3 **categories**
4 **Incidence of CVD/1000 person years in (a) Baseline age categories, (b) Age of diabetes**
5 **diagnosis categories.**
6

1



2 **Figure 7.4 Odds ratios for development of CVD in HbA1c Quintiles and SES categories**
3 **Odds ratios for development of CVD with White as Reference in HbA1c Quintiles (a), SES**
4 **categories (b). All analysis were adjusted for age at baseline. Pak = Pakistani. * = P <0.05 for**
5 **the difference in odds from White.**



1 **Figure 7.5 Odds ratios for development of CVD with White as Reference in people**
2 **developing renal failure or hypertension or on treatment for high cholesterol**
3 **Odds ratios for development of CVD with Normal White as Reference in renal failure (a),**
4 **hypertension treatment (b), and treatment for high cholesterol (c). All analysis were**
5 **adjusted for age at baseline. Norm = Normal, Pak = Pakistani, Ren F = Renal impairment,**
6 **HTN = Treatment for hypertension, Chol = Cholesterol lowering treatment. * = P <0.05 for the**
7 **difference in odds from Normal White.**

7.3 Discussion

In this analysis, we demonstrate increased CVD risk in Pakistanis compared to White individuals with type 2 diabetes living in Scotland. Similar findings have previously been reported in the general population (727;1021;1022); here we show this for the first time in a population with diabetes and demonstrate that the excess risk persists even after statistical adjustment for BMI, metabolic, lifestyle, socioeconomic and disease factors. Independent of the effect of age and age of diabetes diagnosis, rates of CVD differed in the various ethnic groups. Once they had developed diabetes, those of Pakistani origin had an increased rate of CVD at all ages compared to White and Chinese individuals with diabetes. Within the same age category, Pakistanis had an increased risk of CVD relative to the white population (HR=1.45, CI 1.14–1.85, p=0.002) and this was apparent at all age groups over the age of 41 (Figure 1.3). Also Pakistanis with diabetes appear to have an incidence of CVD comparable to White or Chinese people 10 years older.

Cardiovascular disease is one of the leading causes of death in people with type 2 diabetes and more than 60% of people with type 2 diabetes die of CVD (MI or stroke) (1009). Differences in diabetes incidence rates (1014;1016;1023) and CVD rates (1014;1022;1024) have been evaluated in many studies. Ethnic differences in age of diabetes diagnosis (711;1016;1025), diabetes prevalence (679;1014), HbA1c levels (1026;1027) , body composition (712;1014;1015), smoking (1026;1028), physical activity (713;1016;1029) and diet (1013;1030) have all been reported previously. Many studies have also reported effects of socioeconomic status on DM (1031;1032) and CVD incidence (1033). The present study adds to this literature in showing an increased risk of CVD in Pakistanis after full adjustment for duration of diabetes and socioeconomic status (SES). The increased risk of CVD by ethnicity in Pakistanis was not dependent on known risk factors as most of the cardiovascular risk factors (BP, BMI, total cholesterol, TG, creatinine, smoking status and hypertension) were indicative of lower risk compared to levels found in White people. In addition we also show that increased risk is not accounted for by an increased effect of risk factors in the Pakistani group as none of the CV risk factors had significant interaction with ethnicity.

A number of studies have observed differing rates of T2DM and CVD development in differing environments and ethnicities. There are a number of potential explanations including the thrifty genotype, thrifty phenotype, fetal insulin hypothesis and overflow hypothesis. South Asians are proposed to have “thrifty genotype” which states that their predisposition to diabetes may have evolved as an adaptive trait in certain environmental conditions but later turned disadvantageous due to change in lifestyle (sedentariness and excess of food) (1034). The increased prevalence of IR in SA is also associated with thrifty genotype (691). The fetal insulin hypothesis states that common genetic factors are related to growth of the fetus (birth weight), adult insulin resistance and to the risk of diabetes and other vascular disease in later life (1035). To date no clear genetic differences have been found between SA and Caucasians (683;1034). However, an exceptionally high percentage of positive family history of type 2 diabetes in SA makes it likely that there may be an excess of risk alleles in SA. Another proposed mechanism is the “thrifty phenotype” which states that a disadvantageous intrauterine environment induces thrifty mechanisms that set the body’s metabolism to cope with potential future food shortage (1034). This change is beneficial for early survival but increases the risk of diabetes in nutrient rich environment. This is evidenced by low birth weight in SA and later increased weight gain in childhood (691;1036). The potential importance of low birth weight is further supported by the work of Bergvall et al. who showed in both monozygotic and dizygotic twins that birth weight was independently associated with development of hypertension; independent of genetic factors, environmental factors and adult life risk factors (including BMI) (1037). It further support the concept that the link between low birth weight and later disease development as an environmental rather than genetic factor. Finally with regard to birth weight others have examined whether low birth weight explains ethnic differences. A recent study by Nightingale et al. suggests that birth weight was inversely related to insulin, glycaemia and urate. They further showed that birth weight was lower in SA and African-Caribbean as compared to Europeans, however, birth weight did not explain the ethnic differences in risk markers for diabetes and CVD (1038).

Beyond these hypotheses a number of mechanistic explanations have been made for the increased propensity of SA to metabolic disease. These include the

"overflow hypothesis". This states that when superficial subcutaneous adipose compartment is less well developed, it results in earlier expansion of the secondary compartments (deep subcutaneous and visceral adipose tissues) (1034). It has been shown that SA have higher levels of deep subcutaneous and visceral adipose tissues (686;687). These deep adipose compartments are shown to be metabolically more active and may contribute to diabetes and also increase cardiovascular risk (686;687). SA have also been shown to have more efficient mitochondria; producing more energy and generating less heat (697;698). This adaptation is useful in hot climates and periods of starvation, however, it is disadvantageous in environment of excess food and low physical activity. Another important difference in SA is the presence of less lean or muscle mass as compared to White (682;683;686). This difference is also present in healthy young SA men compared with BMI matched White (686;1034). Moreover, low muscle mass is also associated with reduced insulin sensitivity (694). In spite of IR, the muscle oxidative capacity and expression of oxidative and lipid metabolism genes in SA is not different from Whites (683;1034). Muscles are the main energy organs for glucose disposal and also the main energy using organs. Low muscle mass in SA may have metabolic effects but this hypothesis need further exploration as limited studies are available.

South Asians are considered a single group in most studies, and here we report a significant difference only in Pakistanis. Importantly people of Pakistani origin were different in a number of key characteristics. Previous work from our group has shown that Pakistanis living in Scotland have higher average levels of glycaemia and higher levels of social deprivation than Indians (734). When Pakistanis were compared with Indians in our cohort, they were younger at baseline (53.8±12 vs Indians 56.2±13 years, p= <.001), younger at diabetes diagnosis (48.8±11.6 vs Indians 51.2±12.2 years, p= <.001), had higher BMI (30.2 vs 28.8, p= <.001), lower BP (134/78 vs 137/79, p=0.04), higher HbA1c % (8.5 vs 8.0, p=<.001), lower creatinine (83.3 vs 87 μmol/L, p=0.04) and lower SES (p=<.001). These findings are consistent with a number of previous studies, but none of these specifically examined CVD and its risk factors according to ethnicity in a large population of individuals with type 2 diabetes (1012;1018-1020). It was noteworthy that following adjustment for all of these factors in Cox regression, increased risk was demonstrated only in Pakistanis. However, not

1 all previously-published studies have been consistent with our findings: four
2 reported similar or lower prevalence of CVD in Pakistanis or South Asians with
3 diabetes as compared to White. One of these was longitudinal but had a very
4 small South Asian sample size of 86 patients (1039). The other three were cross
5 sectional (1018;1026;1029) and two of these reported pooled data on South
6 Asians rather than the individual ethnicities group (1026;1029).

7 Pakistanis and Indians are not reported to vary greatly in their genetic makeup
8 and so the differences in cardiovascular risk reported here must be interpreted
9 with caution. It has been reported that South Asians are more likely to have
10 cardiovascular risk factor (BMI, HbA1c, BP, hypertension and retinopathy)
11 evaluation and access to primary care as compared to White people (1017) but
12 to date we are not aware of any such differences having been reported between
13 Pakistanis and Indians in Scotland. Our data show that Indians are less deprived
14 than Pakistanis and it has been reported recently that SES evaluation measures
15 are inconsistent across different ethnic and sex groups (1020) and may be a
16 possible reason for the difference. There may be differences in access to
17 healthcare, compliance with medication, improvement of cardiovascular risk
18 factors over time and other health behaviours. These factors could in theory
19 explain some of the differences we observed in rates of cardiovascular disease
20 between Indians and Pakistanis which generates an important hypothesis for
21 future work.

22 The population we studied included a much smaller group of individuals of
23 Chinese origin. Our finding of a decreased risk of CVD in Chinese individuals with
24 type 2 diabetes is also in keeping with the literature in the general population
25 (730;1040)

26 7.3.1 Strengths and Limitations

27 Our study is one of the largest longitudinal studies of people with type 2
28 diabetes (n=121,523) free of CVD at baseline and followed for an average of 4.8
29 years for the development of CVD. Other studies reporting ethnic differences
30 were conducted in the general population and used diabetes as an adjustment
31 factor (727;1014;1021;1022) rather than investigating ethnic differences among
32 people with diabetes. Studies performed in people with type 2 diabetes, were

1 small to modest cohort studies (n=4974) (711;1016;1026;1039;1041;1042); or
2 combined different ethnic groups (Pakistani, Indian, Bangladeshi and Sri Lankan)
3 as one group of South Asians (1014;1016;1022;1024;1026;1039;1042). In order to
4 avoid missing differential effects within this group, and because of the much
5 larger size of our sample of people with type 2 diabetes we were able to
6 evaluate the ethnic groups Pakistani, Indian, and Other Asians separately, rather
7 than merging them into one group of South Asians. This was justified by the
8 existing literature showing differences between separate ethnic groups in the
9 general population (679;1012;1018;1019;1024). Most of the ethnic groups in our
10 cohort comprised participants from one country of origin, except for Other
11 Asians (which included Bangladeshi), and African and Caribbean blacks which
12 were combined due to the relatively small sample size in these groups.

13 Strengths of this study include the population-based nature of the electronic
14 record of diagnosed diabetes in Scotland. SCI-Diabetes has many distinguishing
15 features such as a single shared electronic record, real time data entry (updated
16 immediately), over 99% completeness along with patient contact and care
17 record. SES was assessed on an area-based measure and considers several
18 different aspects of deprivation such as employment, income, health, education,
19 skills and training, geographic access to services, crime and housing. Individual-
20 based measures for SES were not available but each individual area assessed
21 using SIMD was small (median, 800 people). Patients were asked to identify their
22 ethnic group from a standard list used in the 2001 Census in Scotland (734),
23 which is well tested over the years and has been shown to be acceptable for use
24 in the general population.

25 One of the main limitations of our study is that 67,994 people (24% of total) had
26 missing ethnicity information. There is a potential for bias here: for example if
27 those who were most healthy were less likely to be in contact with medical
28 services and in turn not have ethnicity coding. In addition, if this group remained
29 disease free then the cardiovascular risk in our results may be an overestimate.
30 To examine this we explored this missing ethnicity group and it revealed that
31 50,410 were free of CVD at baseline and 7810 (15.5%) developed CVD in the
32 follow-up period giving a rate similar to that in those with known ethnicity
33 (13.4%). We excluded the people having a prior CVD event. In keeping with our
34 results showing a higher CVD risk in Pakistanis, there was a possibility that we

1 may have excluded more Pakistanis as having prevalent CVD. So our results may
2 be an underestimate of the actual cardiovascular risk. To address this, the
3 percentage of each ethnic group was checked in the prevalent CVD group and
4 was found to be similar to the people included in the analysis.

5 Another limitation was that we were unable to adjust our models for physical
6 activity, diet and family history of DM and CVD (which are known to vary by
7 ethnicity) (728;1013;1029). At the same time the effects of many of these
8 factors may act through BMI, BP, total cholesterol, HDL-c, hypertension, and age
9 of diabetes diagnosis, which we have adjusted for in the analysis. CVD events
10 were coded using ICD-10 codes recorded by, record linkage to Scottish Morbidity
11 Records (SMR01). Smoking was coded as "never smoked" and "ever smoked" and
12 a larger effect of smoking may have been found with a more granular measure.

13 In our study, we have not checked for genetic and birth weight differences
14 between different ethnic groups as these are not available from routine data in
15 SCI-Diabetes. We did not check for the differences in central and peripheral
16 obesity, mitochondrial oxidative capacity, fat and fat free mass in different
17 ethnic groups and so cannot confirm the findings in our data. In addition, we did
18 not check for time-dependent differences in healthcare, compliance with
19 medication, deterioration/improvement of risk factors in different ethnic
20 groups. All of these data could further augment the findings reported here.

21 Another important issue is the extent to which our data are generalizable to
22 other populations. We have stressed that considering people of South Asian
23 origin as a single group may miss important detail. It is not possible to know
24 whether the increased risk we have observed in those of Pakistani origin living in
25 Scotland would be shared by people of Pakistani origin living in other countries,
26 and therefore whether it might be explained by genetic differences, the
27 particular characteristics of people of Pakistani origin migrating to Scotland,
28 environmental factors or a combination of these factors.

7.3.2 Potential limitations of secondary analysis of electronic database

Listed below are the potential limitations of secondary analysis of electronic databases mentioned in the literature (1043;1044) and which were checked in our dataset and analysis.

Non generalizability: Some secondary analyses do not include whole population data and include selective health care facilities or over represent some data (e.g. younger physicians' data). SCI-Diabetes includes all health care facilities, GP practices and is now implemented in all health boards of Scotland. The Scottish population consists of people from different ethnicities, with different immigration status (new immigrant to 2nd and 3rd generation immigrant) and also have different cultural and social habits, and life style. However the climate, lifestyle, working hours, food availability, health care facilities, education, employment and other facilities are same in all Scotland and in different ethnicities. These population characteristics are similar to the population characteristics in other parts of UK, western European countries and some states of America and Canada, and thus can generalized to those parts of the world; or study planned to confirm the findings. These findings cannot be generalized to the people living in countries like Pakistan, India, Bangladesh etc; due to different population and environmental characteristics.

Data Quality: the data quality is affected by incorrect coding, missing data, abnormal or unrealistic values and incomplete data base. In SMR-01 the ICD-10 codes are used for CVD. For the prevalent CVD we checked the hospital admissions for CVD using ICD- 10 codes but as the SMR-01 started using ICD-10 codes from April 1996, and our look back period was up to 1992 so we used equivalent ICD- 9 codes. About 67,994 people (24% of total) had missing ethnicity information and are discussed in detail in the limitations of discussion. The data were complete (almost 100%) for most of the variables like age, sex, age of diabetes diagnosis, duration of diabetes, ever smoking status, treatment for hypertension, cholesterol lowering drugs and SIMD. Data were not complete (50-75 %) at baseline for some of the variables like BMI, BP, total cholesterol, creatinine, HbA1c, treatment for diabetes and HDL cholesterol. Missing data were not imputed from values after the date of diabetes diagnosis.

1 **Diagnosis of diabetes:** Some patients can be misdiagnosed with diabetes in
2 databases from population studies. In SDRN, general practitioners (GP) make the
3 diagnosis (this is at a clinical level and patients are therefore informed).
4 Diagnosis is based on fasting glucose and HbA1c with OGTT at times for
5 confirmation. Patients are added to the database only once their diabetes is
6 confirmed. In our dataset only 365 people (0.1% of total) appeared to have been
7 wrongly diagnosed and the diagnosis was clinically revised. After revision of
8 diagnosis, these individuals were removed from the analysis (Figure 7.1). Data in
9 SCI-diabetes is routinely validated for irregularities. Cross referencing of
10 diabetes diagnosis coding between routine hospital discharge information
11 (SMR01) and SCI-DC in 2008 showed that 0.6% of total diabetes cases were not
12 found in SCI-DC (1045). However, at present SCI-Diabetes includes all health
13 boards of Scotland, including GP practices and also including adjacent isles.

14 **Type of diabetes:** Most diabetes diagnoses in adults are treated as type 2, but in
15 general practice it is not always easy to distinguish type 1 from type 2 diabetes,
16 especially in new onset diabetes. In our analysis type of diabetes was assigned
17 by the clinician and was also confirmed by an algorithm using age at diagnosis
18 and use and timing of treatment with oral hypoglycaemic agents and insulin.
19 Details of the other forms of diabetes are shown in Figure 7.1.

20 **Follow-up:** In longitudinal analysis, some of the people do not have a follow-up
21 data and if this proportion is large, it may affect data quality and influence
22 results. In our analysis n= 4557, 1.6% of total population did not had follow- up
23 data and were excluded from the analysis with the assumption that they may
24 have left the area. This assumption was made as it is unlikely for a person with
25 diagnosed diabetes and registered in SDRN to have no data for any clinical
26 variable in 7 years of follow- up (2005-2012).

27 **Disease documentation:** Coding for some complications of diabetes (e.g.
28 retinopathy) is still variable and depends on free- text comments and different
29 codes. In our analysis we used ICD-10 codes for CVD which have been validated
30 in many studies. See Appendix C for details of ICD codes used in this study.

1 ### 7.3.3 Conclusion

2 Pakistani ethnicity is an independent risk factor for CV disease among people
3 with type 2 diabetes. Our finding confirms and extends existing literature
4 demonstrating that some ethnic groups (especially South Asians) are at higher
5 risk of CVD. This is reflected in some (1046-1048) but not all (1049;1050)
6 guidelines targeting cardiovascular risk; only one currently advises recording of
7 ethnicity but does not recommend different specific treatment or prevention
8 strategies (1048). The QRISK2 risk calculator (http://www.qrisk.org/index.php)
9 already considers Pakistani, Indians and other groups separately. As those of
10 Pakistani origin had an earlier age of onset of diabetes and poorer metabolic
11 control but apparently similar control of other risk factors such as blood
12 pressure, cholesterol and smoking, we suggest that programs designed to
13 prevent or delay onset of diabetes in this group might be of particular
14 importance.

8 Conclusion/future recommendations

My thesis analysed the inter-relationships amongst obesity, insulin resistance, inflammation, and ethnicity in relation to the pathogenesis and progression of cardiovascular disease and diabetes. CV risk factors play common roles in populations across groups defined by age and sex but also have subtly different implications within genders and at different times in the lifespan. I have discussed the conclusions specific to each chapter in the relevant sections; I focus here on the overall conclusions, mechanisms, limitations and recommendations.

In this thesis I have been fortunate to work with two quite different data sets. Both are observational, with no study intervention. There are a variety of differences in that RISC study data result from an independent scientific project whereas SDRN data are routinely generated (anonymised) from care activity within the National Health Service. Taken together there are different strengths and weaknesses. Notably both are longitudinal studies and not randomised controlled trials (RCT). Clearly, RCTs are considered the gold standard for establishing causal linkage and so the relationships found should be considered "hypothesis-generating" rather than definitive experiments. One of the points of strength is that both are human studies.

The RISC cohort constitutes a healthy population (by selection) with no chronic disease (high BP, hyperglycaemia, cardiovascular disease, cancer and any chronic diseases): thus disease-associated pathophysiological changes are absent. RISC is a scientific study; therefore more able to control and standardise study procedures and protocols. However, in this study design there will be fewer major end points (MI or stroke) in follow-up. The RISC cohort age range is between 30-60 years. Most conditions (e.g. diabetes, hypertension and CVD) are acquired in this age, or at least the pathology (disease process) starts in this age. Studying risk factors in a healthy population is interesting and can give insights into how diseases develop and which factors are involved. Thus the RISC study is well-placed to investigate how body anthropometric measures and their changes over time (age, BMI, physical activity, BP etc.) are related to the metabolic parameters in the blood (including M/I, CRP, IL-6, lipids). It is also useful for studying the early development of atherosclerosis (cIMT), diabetes and change in BP (hypertension). However as the individuals recruited were healthy at baseline and there was a maximum age limit at entry of 60 years, only 21

people developed diabetes and/or symptomatic cardiovascular disease in three years. This indicates that a much longer follow-up (10-15 years) will be required to evaluate cardiovascular events and outcomes.

The main shortcoming of the RISC dataset is that it derives from a relatively small and selected cohort of consented individuals (inevitable given the invasive nature of assessments and strict quality control). The level of data collection is appropriate for a cohort study but more intense and invasive than could be applied to a clinical population like that of the SDRN. For example, the use of a gold standard assessment of insulin sensitivity by clamp technique while highly informative in RISC is not practical in a population or large cohort. The RISC population is not truly population-based so the results may not all be generalizable to a wider population. The RISC cohort is a healthy population; this is strength in some respects, particularly for physiological investigation, but it is also a limitation for studies intending to investigate disease processes. Both approaches (selected cohort/ healthy population) have limitations when results are applied to the general population; some members will have the disease processes in question at any one time point.

As only European centres were included with almost all Caucasian population the findings cannot be generalized to other ethnic groups. However, they can be used as a template for designing a study in a different population. Another limitation of RISC is the absence of dietary history and socioeconomic status in the analysis, the latter being an important cardiovascular risk factor.

By contrast, the SDRN cohort is a large population-based cohort with no obvious bias; a major strength of the study. It reflects real clinical practice, i.e. if we detect an increase in risk of MI in people of Pakistani origin; it is a potential clinical issue. However as it is a large cohort, measures like insulin sensitivity, CRP, IL-6, cIMT, physical activity have not been assessed. More notably in comparison to the RISC cohort, the SDRN cohort is a disease cohort with all members having type 2 diabetes and some additionally having hypertension or kidney disease. As there is no maximum age limit restriction it is a good cohort in which to examine incident cardiovascular disease as an outcome. As SDRN is a population cohort with people from other ethnic groups, its results are generalizable to other parts of UK and Europe. However the results of ethnic

groups other than White cannot be applied in the country of origin due to different environmental and other conditions.

Medical knowledge is derived from different types of studies, giving different types of information, complementing each other in drawing a conclusion. Here I was able to utilise two different cohorts with different characteristics resulting in different types of information. The findings do not add enough information to influence clinical management, but do add to available knowledge of cardiovascular risk and identify new areas for future work.

The economic and other (social, psychological) burden of disease is not due to CVD alone but also due to risk factors like obesity, diabetes and hypertension. Over the past century, researchers have worked to find the main pathology linking all of these diseases and risk factors, but to date have reached no conclusive answer. The main management strategies are to control obesity and modify lifestyle to prevent/delay the development of disease and/or to control progression of disease and associated complications. Regarding prevention of disease, the first step is to find the appropriate relationship between predictors and outcome. The next step is to check if the predictor is causally related to the disease and if modifying the predictor alters the disease pathway or progress. For example the Diabetes Prevention Programme (DPP) trial showed that lifestyle changes and treatment with metformin both reduced the development of type 2 diabetes in a high risk group (1051).

Risk scores are algorithms used to estimate the 10-year cardiovascular risk of an individual. Many have been developed to categorise people in low, medium or high risk categories in relation to development of CVD – for example, Framingham and QRISK scores. Currently the Framingham score (http://cvdrisk.nhlbi.nih.gov/) includes age, total cholesterol, smoking status, HDL and SBP. The QRISK score (http://www.qrisk.org/index.php) additionally includes BMI, ethnicity, measures of deprivation, family history, chronic kidney disease, rheumatoid arthritis, atrial fibrillation, diabetes and treatment for hypertension. These risk scores are not definitive as the QRISK2 algorithm explains 43% of risk variation in women, 38% in men; the modified Framingham risk score explains 39% in women and 35% in men (1052). Moreover, there is still a discrepancy between different scores, in terms of whether people are

classified as high or low risk (1052). Many novel CVD risk factors like insulin resistance, CRP, IL-6, fibrinogen, augmentation index, pulse wave velocity, lipoprotein (a), homocysteine, small dense LDL-c, urinary albumin to creatinine ratio, renin, B type natriuretic peptide and testosterone levels have been studied in the last three decades to find out if they improve risk prediction. Moreover, there has been a lot of work to evaluate if blocking or modifying any risk factor may prevent or delay cardiovascular complication. Some drugs have been found to result in significant improvements: ARBs, ACEIs and statins. Some newer work is focusing on nutrition-based interventions: e.g. antioxidants, vitamins and minerals.

As the existing risk factors fall some way short of explaining all of the risk of CVD, I have further examined other novel risk factors. In the RISC chapters I examined whether insulin sensitivity and inflammation make independent statistical contributions to the change of BP or the development of hypertension. The results showed that both add very little to predicting risk in the presence of the important risk factors. This is in keeping with results on several other risk factors: a recently-published study suggested that CRP improves risk classification by only 1.52%; the main contributors are of course conventional risk factors: age, sex, smoking status, BP, history of diabetes, cholesterol and HDL levels (1053). Insulin resistance and inflammation are not generalized predictors in all age and sex groups and are of limited utility in clinical practice. However, we found that the associations of these risk factors does change with age and is also different in different age groups. The data from my thesis suggest that it is not sufficiently important routinely to measure insulin sensitivity particularly given the expertise required, labour and cost for assessment.

I found a relationship between CRP and diastolic BP in an adult population. It should be noted that this is not sufficient evidence to recommend routine measurement of CRP in all young people. Moreover, I identified percentage change in BMI to be strongly linked to both systolic as well as diastolic BP, suggesting that risk of hypertension may increase even if a person gains weight within the normal range. The importance of change in BMI was apparent in all BMI categories, from healthy to obese. Furthermore, percentage change in BMI appeared to be more important than BMI as a predictor of BP rise in middle-aged individuals.

Considering CRP, BMI category and percent change in BMI together; a possible explanation for the influence of modest rises in weight on blood pressure is as follows. I found that there was no change in BP (SBP and DBP) if a participant maintained or decreased his weight, even if they were overweight or obese. In contrast only 2-3% increase in weight was associated with a rise in BP, even in middle aged people with normal BMI. This may be due to the apparent metabolic health of these overweight and obese people: their adipose tissue is not causing chronic inflammation. When there is any increase in adipose tissue (BMI), it disturbs homeostatic balance and leads to chronic low grade inflammation, which then influences BP. CRP is a marker of chronic low-grade inflammation: it may not in itself be pathological (976) but it can at least flag the existence of other ongoing inflammatory conditions. Summing-up, it can be easily evaluated if change in BMI is additionally linked with change/rise in CRP. In clinical settings measurement of CRP is costly and involves sampling, transportation and analysis. In contrast, change in BMI can be easily monitored in GP settings and can be easily done at routine visit. The recent follow-up results of the DPP study show similar findings i.e. that participants who maintain long term weight loss over two years have a reduced risk of developing diabetes as compared to people who regained weight or undergo weight cycling (1054). Similar to the DPP study, change in BMI is an excellent potential risk factor in the development of hypertension and can easily be explored in larger/populations and other cohorts as it does not require any laboratory test or special skills.

In most of the groups I studied, adjustment for BMI weakened the relationship between insulin resistance, inflammation and BP. Awareness of BMI should continue as a main focus of future disease management and planning worldwide. This supports the obvious public health message that all individuals (regardless of age and sex) should aim to control body weight within healthy limits.

I studied the importance of the predictors in relation to hypertension, but not the relationships amongst these factors and atherosclerosis (owing to the short duration of the follow up). Atherosclerosis is a key step in the development of cardiovascular disease and can be assessed *in vivo* by measurement of carotid-intima media thickness (cIMT). All of the risk factors I have studied in relation to age and sex stratified groups could be studied in relation to cIMT/atherosclerosis

once further follow of the RISC cohort is available (as is now ongoing in many centres).

The evaluation of ethnicity I conducted in Chapter 7 shows it to be an important risk factor which can be explored further and with the potential to be included in risk scores. South Asians are more likely to have cardiovascular risk factor (BMI, HbA1c, BP, hypertension and retinopathy) evaluation and access to primary care as compared to White (1017). I found that despite having almost similar blood pressure, cholesterol and smoking, Pakistanis still had an earlier age of onset of diabetes and poorer metabolic control. To date, no current management guidelines consider specific treatment for different ethnic groups; this is an important area of work for the future. I have not checked for differences in access to healthcare, adherence to medication, improvement of cardiovascular risk factors over time and other health behaviours between Pakistanis and White. These factors could in theory explain some of the differences and generates important hypotheses for future work.

In my study, the ethnic groups (other than White) were population-based but few in number; more studies should be planned with larger ethnic minority groups from developed countries. Most developed countries have different climate, culture, food, working hour and leisure activities compared with developing countries. Many ethnic minorities in developed countries are from developing countries: studies should be planned within developing countries to evaluate the association and impact of risk factors in these environmental settings. Moreover, type 2 diabetes occurs more frequently in children and young adults of ethnic minority groups as compared to White. The relation of this early development of diabetes with cardiovascular disease needs evaluation.

Sex differences in relation to socioeconomic status (SES), diet and physical activity have been identified previously. These sex differences are also present in ethnic minority groups. I identified some areas for future work in relation to this finding. The role of socio economic status in cardiovascular outcomes in people with diabetes should be evaluated along with consideration of sex. This relation should also be checked in different ethnic groups.

With the development of diabetes there is persistent vascular damage in spite of intensive glycaemic control. One of the proposed mechanisms is the concept of glycaemic memory (641). SDRN is a population cohort with age of diabetes diagnosis has been documented. A potential area for study is evaluation of age of diabetes diagnosis with cardiovascular disease outcome in people with type 2 diabetes taking account of previous control of the condition.

In summary this thesis showed that the relationships of insulin resistance, inflammation and measures of body composition with CV risk vary in different age and sex groups. Insulin resistance and inflammation add very little to prediction of risk in presence of important risk factors. In contrast, we showed that change in weight (BMI) and ethnicity are important independent (of other major CV risk factors) predictors of CV risk and can be a useful addition to the available risk scores.

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