**UNIVERSITY OF SOUTHAMPTON**
**SCHOOL OF MEDICINE**

# Relationships between Cardio-Metabolic Risk Factors in Central Obesity and the Effects of High Dose Statin Treatment

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*To my parents for believing, my husband for supporting and my son for inspiring*

UNIVERSITY OF SOUTHAMPTON

ABSTRACT
SCHOOL OF MEDICINE
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RELATIONSHIPS BETWEEN CARDIO-METABOLIC RISK FACTORS IN CENTRAL OBESITY AND THE EFFECTS OF HIGH DOSE STATIN TREATMENT
by Magdalena Joanna Turzyniecka

Central obesity is a complex cardiometabolic entity strongly linked to the constellation of risk factors such as insulin resistance, hypertension, dyslipidaemia and physical inactivity, which when combined lead to an increased risk of type 2 diabetes and cardiovascular disease. The available evidence suggests that these conditions are linked to microvascular dysfunction, which may appear much before the onset of overt cardiovascular and metabolic disease. However, in apparently healthy but viscerally obese subjects, little is known about the interactions between cardiometabolic risk factors, including microvasculature, which could be potential targets for early therapeutic intervention. Statins are attributed to have pleiotropic properties, but their effects on insulin resistance and microcirculation are still uncertain.

The hypotheses for this study were that in centrally obese but non-diabetic subjects:

- Skeletal muscle exchange capacity influences levels of $HbA_{1}c$
- Diminished insulin sensitivity in skeletal muscle is associated with reduced microvascular exchange capacity
- Microvascular functional dilator capacity is independently associated with insulin sensitivity and age
- Six months of treatment with high dose statin improves insulin sensitivity and reverses microvascular dysfunction
- Cardiorespiratory fitness is independently associated with cardiac diastolic function and arterial stiffness

A double-blinded, randomised, placebo controlled trial was conducted in white Caucasians aged 29-69 with abdominal obesity and a cardio-metabolic phenotype. Insulin resistance was assessed by stepped hyperinsulinaemic euglycaemic clamp and fasting insulin sensitivity indices. Microvascular function was examined with venous congestion plethysmography and Laser Doppler Fluximetry.

It was demonstrated that in centrally obese, non-diabetic subjects with modest insulin resistance, skeletal muscle exchange capacity was associated negatively and independently with $HbA_{1c}$, positively and independently of visceral fatness with insulin sensitivity, and that functional dilator capacity was strongly and positively associated with insulin sensitivity and age, independently of each other. Six months of intensive treatment with Atorvastatin did not improve insulin sensitivity or microvascular function. A strong association was shown between cardiorespiratory fitness and measures of diastolic function and arterial stiffness.

In conclusion, this thesis presented novel aspects of cardio-metabolic factors and microvascular relationships, which indicate the early onset of microvascular dysfunction in obesity and the importance of fitness in maintaining arterial flexibility and cardiac diastolic function. Atorvastatin has no role in improving insulin sensitivity and reversing microvascular dysfunction.

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ABBREVIATIONS

| | |
|---|---|
| ACR | Albumin to creatinine ratio |
| AGE | Advanced glycation end products |
| AIx@75 | Augmentation index |
| AngII | Angiotensin II |
| ANOVA | Analysis of variance |
| Apo | Apolipoprotein |
| ATP | Adenosine triphosphate |
| ATP III | Adult Treatment Panel III |
| AU | Arbitrary units |
| AUC | Area under the curve |
| BF% | Body fat percentage |
| BG | Blood flow |
| BMI | Body mass index |
| BMR | Basic metabolic rate |
| CAD | Coronary artery disease |
| cAMP | Cyclic adenosine monophosphate |
| CI | Confidence interval |
| $CIR_{30}$ | Corrected insulin response |
| C-peptide | Connecting peptide (C-peptide |
| CT | Computed tomography |
| CV | Coefficient of variation |
| CVC | Cutaneous vascular conductance |
| CVD | Cardiovascular disease |
| $D_2O$ | Deuterium |
| DCCT | Diabetes Control and Complications Trial |
| DEXA | Dual energy X-ray absorptiometry |
| $\Delta I$ | Difference between fasting and steady-state insulin |
| ED | Ejection duration |
| EE | Energy expenditure |
| EGIR | European group for the study of Insulin Resistance |
| eNO | Endothelium nitric oxide |
| eNOS | Endothelial nitric oxide synthase |
| ERK | Extracellular signal-regulated kinase |
| ET-1 | Endothelin-1 |
| FAS | Fatty acid synthetase |
| FFA | Free fatty acids |
| FMD | Flow-mediated dilatation |
| FSIVGTT | Frequently sampled intravenous glucose tolerance test |
| G | Glucose concentration |
| $G_0$ | Baseline glucose |
| GLUT | Glucose transporter |
| GSK | Glycogen synthase kinase |
| $HbA_{1c}$ | Glycated haemoglobin $A_1c$ |

| | |
|---|---|
| HDL | High density lipoprotein |
| HEC | Hyperinsulinaemic euglycaemic clamp |
| HMGA | High mobility group protein A |
| HMG-CoA | 3-hydroxy-3-methylglutaryl coenzyme A |
| HMGR | 3-hydroxy-3-methylglutaryl coenzyme A reductase |
| HOMA | Homeostasis model assessment |
| HPLC | High pressure liquid chromatography |
| hs-CRP | High sensitivity-C-Reactive Protein |
| HSL | Hormone sensitive lipase |
| $I_0$ | Baseline insulin |
| IDF | International Diabetes Federation |
| IGF | Impaired fasting glycaemia |
| IGF-1 | Insulin-like growth factor-1 |
| IGT | Impaired glucose tolerance |
| IL | Interleukin |
| IR | Insulin receptor |
| IR | Insulin resistance |
| IRS | Insulin receptor substrate |
| IS | Insulin sensitivity |
| ISI | Insulin sensitivity index |
| IST | Insulin suppression test |
| $J_v$ | Fluid filtration rate |
| $K_f$ | Filtration capacity |
| LDF | Laser Doppler fluximetry |
| LDL | Low density lipoprotein |
| LPL | Lipoprotein lipase |
| MAPK | Mitogen-activated kinase |
| MBV | Microvascular blood volume |
| MCP | Monocyte chemoattractant protein |
| MET | Metabolic equivalent of task |
| MetSy | Metabolic syndrome |
| MRI | Magnetic resonance imaging |
| M-value | Whole body glucose disposal |
| NADH | Nicotinamide adenine dinucleotide |
| NCEP | National Cholesterol Education Programme |
| NEFA | Non-esterified fatty acids |
| NHANES | National Health and Nutrition Examination Survey |
| NO | Nitric oxide |
| OGGT | Oral glucose tolerance test |
| PAEE | Physical activity energy expenditure |
| $P_{cuff}$ | Cuff pressure |
| PDE | Phosphodiesterase |
| PDK | PI3-dependent serine/threonine kinase |
| PEPCK | Phosphoenolpyruvate carboxykinase |
| PF | Peak Flux |

| | |
|---|---|
| PFK | Phosphofructokinase |
| PI3K | Phosphatidylinositol 3-kinase |
| PKA | Protein kinase A |
| PORH | Post occlusive reactive hyperaemia |
| PPAR | Peroxisome proliferator-activated receptor |
| PTPase | Protein tyrosine phosphatase |
| $P_{vi}$ | Isovolumetric venous pressure |
| PWA | Pulse-wave analysis |
| PWV | Pulse-wave velocity |
| $Q_a$ | Capillary blood flow |
| $Q_a$rest | Resting capillary muscle blood flow |
| QC | Quality control |
| QUICKI | Quantitative insulin sensitivity check index |
| RAAS | Renin-angiotensin-aldosterone system |
| RF | Resting flux |
| ROS | Reactive oxygen species |
| SBF | Skin blood flow |
| SEVR | Subendocardial viability ratio |
| sICAM-1 | Soluble Intercellular Adhesion Molecule-1 |
| SOCS | Suppressors of cytokine signalling |
| SREBP | Sterol regulatory element binding protein |
| TEE | Total energy expenditure |
| TNFα | Tumour Necrosis Factor |
| VLDL | Very low density lipoprotein |
| $VO_2$ max | Maximal oxygen uptake |
| WHO | World Health Organization |
| WHR | Waist to hip ratio |

DECLARATION OF AUTHORSHIP

I, Magdalena Joanna Turzyniecka, declare that the thesis entitled *"Relationships between Cardio-Metabolic Risk Factors in Central Obesity and the Effects of High Dose Statin Treatment"* and the work presented in the thesis are both my own, and have been generated by me as the results of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for the research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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**Signed:**……....

**Date:**…….

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# 1. Background

## 1.1 Obesity

### 1.1.1 Epidemiology

Obesity, in broad terms, describes the accumulation of excessive amounts of adipose tissue in the body, which usually translates into excessive body weight. The word comes from Latin *obesitas,* which means fatness, corpulence, and is composed of *ob* meaning "over "and *edere* meaning "to eat" pointing towards its commonest cause i.e. overeating. The first documentation of obesity in the form of *Venus of Willendorf* figurines dates back to the Stone Age. Hippocrates pointed out the role of excessive food intake in the pathogenesis of obesity and a few hundred years later Galen documented the benefits of physical activity and moderate food intake on weight maintenance [1]. Furthermore, the existence of the different fat distribution and the role of intra-abdominal fat in the pathogenesis and phenotypes of obesity was described for the first time already in1765 by Morgagni. The worldwide prevalence of obesity has been rapidly increasing over the last century and in 1997 obesity was formally recognised by the World Health Organisation (WHO) as a global epidemic with Sub-Saharan Africa being the only region where obesity was uncommon [2]. As of 2005, the WHO estimated that worldwide there were approximately 1.6 billion overweight adults, with at least 400 million of them clinically obese, and this being an increasing trend with a projected 2.3 billion of overweight adults and over 700 million obese adults by 2015 [3]. In the United States of America, the prevalence of obesity amongst adults increased from 13.4 % to 35.1% between 1960–2 and 2005–6 [4]. The increase in obesity has not been restricted to industrialised countries but is present also in developing countries, where the obesity rates have tripled over the last two decades [5] because of the adoption of Westernized lifestyles. The prevalence of excessive body weight increases with age and is higher in women [6]. Its negative association with physical activity is also well documented [7].

### 1.1.2 Obesity: morbidity and mortality

Obesity has a profound and adverse impact on health, and subsequently leads to reduced life expectancy [2]. Obesity (and overweight) poses not only the major risk for development of type 2 diabetes, dyslipidaemia, hypertension, all of which are recognised to facilitate the onset of cardiovascular disease but also has been associated with many other non-fatal but serious chronic conditions that significantly affect the quality of life [2,

8, 9]. The risk of developing diabetes is 40-fold in obese as compared to normal weight individuals [10]. The link between obesity and cardiovascular disease, first identified in the large, epidemiological Framingham study, is well established by now. It is evident that with the obesity pandemic there has been an increase in the incidence of cardiovascular disease as well as diabetes and hypertension [2, 11, 12]. Moreover, recent report from the National Heart Forum predicts further increases in cardiovascular disease, in particular heart disease, due to obesity over the next 40 years in England [13]. Based on the trends in data collected between 1993 and 2007, the authors predict a 44% rise in obesity-related coronary heart disease, a 34% rise in obesity-related hypertension and a 98% rise in obesity-related diabetes by 2050. Various epidemiological studies have confirmed that excess body fat is strongly associated with an increased risk of death [14]. Moreover, obesity is associated with raised long term total mortality as shown in a 29 year follow-up of Finnish women [15]. The major cause of mortality in obesity and overweight is, unsurprisingly, due to cardiovascular disease. The relative risk of death due to cardiovascular disease in those with body mass index (BMI) over 30 $kg/m^2$ in comparison to normal weight varies from 1.2 to 3.0 depending on the age and gender [16-18]. The other causes of death in the overweight and obese include diabetes, respiratory disease and cancer [17, 19]. There is also evidence of an inverse relationship between physical activity and all causes of mortality in the presence of increased body weight [14]. Hu *et al.* [11] showed in a long term follow-up study of women without cardiovascular disease or cancer, that increased fatness and physical inactivity were strong and independent predictors of death. Moreover, the authors estimated that the BMI in excess of 25 $kg/m^2$ and the regular physical activity of less than 3.5 hours of exercise per week, together could account for 31% of all premature deaths, 59% of deaths from cardiovascular disease, and 21% of deaths from cancer.

### 1.1.3 Classification of obesity

The World Health Organisation (WHO) classification based on Body Mass Index (BMI) criteria is the most widely accepted and used classification, and provides reasonable approximation of adiposity in most people although it does not provide an individual's body composition. According to the 1997 WHO definition, published in 2000 and based on large scale morbidity data, in adults overweight is defined as BMI of 25-29.9 $kg/m^2$ and obesity as BMI $\geq$ 30 $kg/m^2$. Obesity is further categorised into three subgroups, each with associated co-morbidities risk as moderate, high and very high, respectively (Table 1.1). The other commonly used classification, which provides the most practical assessment of

abdominal fat, is based on the association between increased cardiovascular risks and waist circumference adjusted for gender. Since the waist circumference thresholds have been based on the Euro-Caucasian populations, the International Federation of Diabetes (IDF) recommended (for defining metabolic syndrome) [20] the application of different definitions of central obesity depending on the ethnic origins (Table 1.2). Waist/hip ratio (WHR) is another classification of adiposity which accounts for gender differences. Men with WHR>0.9 and women with WHR>0.85 are classified as being centrally obese. It is important to point out that while the individuals may be classified as being centrally obese by waist girth or WHR, their total body fatness may still be classified as overweight according to BMI criteria.

**Table 1.1 WHO classification of obesity**

| BMI (kg/m$^2$) | Classification |
|---|---|
| < 18.5 | underweight |
| 18.5–24.9 | normal weight |
| 25.0–29.9 | overweight |
| 30.0–34.9 | class I obesity |
| 35.0–39.9 | class II obesity |
| ≥ 40.0 | class III obesity |

**Table 1.2 IDF definitions for central adiposity**

| Ethnic group | Waist circumference (cm) | |
|---|---|---|
| | Male | Female |
| Europids | ≥ 94 | ≥80 |
| South Asians | ≥ 90 | ≥ 80 |
| Chinese | ≥ 90 | ≥ 80 |
| Japanese | ≥ 90 | ≥ 80 |
| Ethnic South and Central Americans** | ≥ 90** | ≥ 80** |
| Sub-Saharan Africans | ≥ 94* | ≥80* |
| Eastern Mediterranean and Middle East | ≥ 94* | ≥80* |

* based on European criteria until further data available

** due to heterogeneity of the population this is based on criteria for South Asians until further data available

## 1.1.4 Assessment of obesity

There are several methods of assessing excess body adiposity with different methods appropriate for different circumstances. In everyday clinical practice or in the large cohorts of subjects, there are indirect methods used and these are based on the relation between height and weight, which include BMI, waist circumference and waist-hip ratio. They are all relatively simple and easily implemented on a mass scale. The methods that directly measure the amount of body fat provide more precise information related to its distribution, fat weight or percentage. These include bioimpedance, dual energy X-ray absorptiometry (DEXA), computed tomography (CT), magnetic resonance imaging (MRI), air displacement plethysmography amongst many others, all of which are complex and require specialist equipment and expertise.

Those methods can provide accurate estimates of total or regional body adiposity or both.

### 1.1.4.1 Body mass index

Body mass index (BMI), also known as Quetelet index, is a measure of overall body weight adjusted for height and is calculated as the weight (in kilograms) divided by the height (in metres) squared. BMI estimates only a relative fatness and not the percentage of body fat. It correlates highly, although not perfectly with body adiposity assessed by more sophisticated and complex methods. For this reason, it has been often employed as a screening tool and a method for the estimation of relative disease risk in large populations. Multiple studies have concluded that BMI predicts the risk of developing diabetes [21], and morbidity and mortality from cardiovascular disease [22].

BMI may not correspond with the same degree of fatness in different populations due to differences in body proportions for example height. This may be especially applicable to various ethnic groups. Dudeja *et al.*[23] observed that in northern Indians a higher percentage body fat was associated with a relatively lower BMI. In males, BMI of 21.4 $kg/m^2$ corresponded with 21.3 % of body fat (BF) whereas an equivalent of percentage body fat in Euro-Caucasian equated to a BMI of 25.2 $kg/m^2$ [24]. The comparison of their findings with the body fat data collected among Caucasians, Blacks, Polynesians and other Asians ethnic groups showed further differences [23]. The proposed different cut-off points for defining obesity in different ethnic groups are still under debate [23].

The equation for BMI calculation does not take into account age or different genders. Gallagher *et al.* [24] obtained data from black and white Americans of both genders suggesting that BMI is age and sex dependent when used as an indicator of body fatness. BMI has also other limitations. It does not distinguish between fat or lean mass, which leads to

inaccurate estimations of fatness in certain groups. For example, BMI may provide overestimated values of body adiposity in those with high muscle mass, but low fatness, because of increased total body weight. In addition, it may not be an appropriate tool for defining obesity in elderly population. This is because with increasing age and change in body composition, there is increased loss of lean mass which replaced by adiposity results in maintenance of stable weight and in turn underestimation of overall fatness when measured with BMI [25].

#### 1.1.4.2 Body fat percentage

The body fat percentage (BF%) is the total weight of fat divided by the individual's weight (expressed as a percentage). It is a direct measure of body composition often regarded as a measure of an individual's fitness. It includes the essential and storage adipose tissue in the body. The essential body fat is necessary to maintain life and reproductive functions whereas the storage fat protects internal organs and is required to provide energy supply in times of famine, as well as thermogenesis (in the case of brown fat). The body fat percentage is greater in women to support their childbearing period and other hormonal functions. The American Council on Exercise recommended the essential body fat percentage for women of 10-13% and for men of 2-5%, whereas obesity was classified as body fat percentage of over 32% in women and over 25% in men.

The percentage of body fat can be predicted from BMI with the Deurenberg equations [26], which are based on the estimation of overall body fatness using BMI and adjusted for age and gender. In the formula the age is provided in years, 1 is substituted for male and 0 for female gender: body fat percentage = 1.2 (BMI) + 0.23 (age) - 10.8 (sex) - 5.4. The equation has a standard error of 4% of measured percentage body fatness which is comparable to the prediction error obtained with other methods of estimating BF%, such as skinfold thickness measurements or bioelectrical impedance. The prediction formulae were cross-validated using densitometrically-determined percentage body fat and gave valid estimates of body fat in males and females at all ages. The cut-off thresholds for defining obesity using those formulas were 25 % of body fat for men and 33% for women. The formulas provide linear relationship between BMI and percentage body fatness while the measured relationship is curvilinear therefore they are likely to overestimate obesity at the higher values of BMI.

#### 1.1.4.3 Waist circumference

Waist circumference is often used along with BMI as a screening tool to identify obesity.

It is also an important criterion for defining metabolic syndrome [20]. As it is measured at the position of anatomical waist, between lower margin of ribcage and the iliac crest, which is different from the commonly defined waist, its accurate determination requires appropriate, but not complex, prior training. Although waist circumference provides approximate information about total body fat, it is used more as a simple measure of fat distribution and an index of intra-abdominal fat mass. In men, this is an anthropometric measurement that most uniformly predicts the distribution of adipose tissue among several fat compartments in the abdominal region [27]. Waist circumference measurements play an important role in identifying central obesity in those whose BMI lies within the normal range [28]. Additionally, in some populations for example, such as Asian ethnic groups it may be a better risk predictor than BMI [29, 30]. It is also the best available surrogate estimate of visceral adiposity as validated in studies using MRI and CT for measurement of body fat distribution [31, 32]. Waist circumference is an accurate predictor of the risk of developing type 2 diabetes. Along with the increasing waist size, there is an increase in the rate of metabolic complications and cardiovascular risks [28], and the waist circumference is a stronger predictor of those in comparison to BMI [33]. In people with BMI 25¬35 $kg/m^2$, measurements of waist circumference provide additional to BMI information about the health risks.

Waist circumference to hip circumference ratio (WHR) can also be used for defining c*entral*, known as *visceral adiposity* and describe the overall body shape e.g. apple versus pear-like shape associated with peripheral obesity. Worldwide WHR shows stronger association with the risk of acute myocardial ischaemia than BMI as demonstrated in the INTERHEART study [34]. This was, however, a case-control study of subjects with acute myocardial infarction that assessed relationships between BMI, WHR and waist and hip circumferences to myocardial infarction. WHR is a slightly worse predictor than waist circumference alone in predicting the development of diabetes [10].

#### 1.1.4.4. Skinfold thickness

The Durnin-Womersley method is one of the many methods applied for the measurement of skin fold thickness. Those methods provide not only information about subcutaneous adiposity but also (less accurately) about the body fat percentage. The skinfolds are measured by calipers at the standardized points on the body. Depending on the method applied, three to seven measurements may be required. Although a larger number of measurements is associated with a higher degree of estimation error, it is the individual's unique body fat distribution that influences the results the most. The Durnin-Womersley

method requires 4 measurements at biceps, triceps, sub-scapular and supra-iliac areas. Their logarithmic sum is used for the estimation of the total body fat. Consequently, the calculated fat percentage result carries the cumulative error from the application of two different statistical methods. Moreover, this is a technically difficult method and is sensitive to the type of calipers used leading to considerable variation between observers. The measurements cause particular difficulties in the severely obese with large skinfold thickness and in the elderly because of the wide intra-individual variation found in this group. In addition, there are several different methods applying various equations for the estimation of body density which lead to the differences in the estimation of the subcutaneous fat between 20 to 70%, depending on the equation used. Although skinfold methods are inexpensive, they are not considered to convey any real advantages over the other anthropometric methods such as BMI [35]. There is evidence that they do not correlate well with total body fatness [36]. The other disadvantage of these methods is that they do not estimate visceral adiposity. However, provided the skinfold measurements are carried out by the same person and using the same technique, the method can give a reliable measure of the change in body composition over a period of time.

#### 1.1.4.5 Dual Energy X-ray Absorptiometry

Total and regional body fat can be estimated with total body dual energy X-ray absorptiometry (DEXA). This technique enables rapid and non-invasive estimation of the body fat percentage obtained with exposure to the radiation of much lower magnitude than that of a Chest X-ray (0.37μSv vs 50 μSv) [37]. DEXA implements a three-compartment model that quantifies fat tissue, lean (fat-free, soft) mass and total body mineral (BM).This technique assumes that bone mineral content is directly proportional to the amount of photon energy absorbed by the bone being studied. It uses a whole body scanner with two different types of X-ray, one detecting bone mineral and another detecting soft tissue with the exception of fat. A computer program is used to estimate fat from the recorded readings and the grade of the exposure determines the fat mass. The comparison with a four compartment model for measuring total body fat showed that DEXA is an accurate method although may underestimate percentage body fat in leaner individuals [38]. DEXA is a very reliable technique, provides extremely repeatable results and presents little burden to the subject. Its disadvantage is the high cost of the equipment and problems with providing accurate measurements in the morbidly obese.

#### 1.1.4.6 Total body water measurements

Body composition can be estimated by total body water measurement. This method involves quantification of body water volumes by isotope dilution based on the assumption that the isotope has the same distribution volume and is exchanged in the same manner as water. Deuterium (in the form of $D_2O$) has been the most commonly used non-radioactive isotope and established as a gold standard method. $D_2O$ dilution provides one of the best estimates of overall relative leanness, with fractional body water inversely correlated with the percentage of body fat. This method is very precise but expensive, laborious and restricted to specialized laboratories with access to $D_2O$ produced in nuclear power stations.

#### 1.1.4.7 Bioelectrical impedance

Determination of the body impedance is another method that allows characterisation of the body composition and provides information about body fat percentage. It relies on the fact that various tissues have varying degrees of conductivity (the reciprocal of impedance), which is far greater in the fat-free mass than in the fat mass. Bioimpedance is based on a two-compartment model. It involves reading of the lean mass and application of scientific formulas to determine accurate body composition. Information obtained in this way includes the percent and amount of body fat as well as muscle and water mass. Bioimpedance is a precise technique that has been validated using various standard methods including densitometry [39]. However, the readings may be affected by the hydration status, fasting state, skin temperature, and other factors. Therefore, to obtain accurate results for repeatable measurements, it is crucial to maintain the same reading conditions for each assessment.

#### 1.1.4.8 Total body density measurements

Another method for assessing body fat involves determination of total body density. Hydrodensitometry is one such method and measures body density by determining total body volume based on the Archimedes' Principle of displacement. Hydrodensitometry, also known as underwater weighing, requires weighing the subject prior to and after their complete immersion in water. The calculated body volume is used to determine whole body density using standard formulas. The values of whole body density are used to estimate the percentage of body fat using either Siri or Brozek equations [40]. Although this method is recognised as the gold standard method, it probably underestimates body fat in athletes and overestimates body fat in the elderly with osteoporosis. Hydrodensitometry

is a cumbersome and expensive technique. Nowadays it is of limited availability and used only in small studies for the validation of other techniques.

Air displacement plethysmography is another method that determines body density and estimates body fat using similar standardised calculations in a similar manner to hydrodensitometry. Instead of water, it measures the amount of displaced air while a subject sits in air-tight capsule for 20 seconds. This technique is applied in commercially available BOD POD which is precise and well tolerated by subjects. Its availability in clinical practise is limited because of its high cost and strict requirements regarding temperature stability of the testing environment and subject's body temperature, skin moisture and avoidance of exercise prior the testing.

#### 1.1.4.9 Computed Tomography and Magnetic Resonance Imaging

Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are modern and reliable techniques in measuring not only total but also regional body fat. They provide a ratio of intra-abdominal fat to extra-abdominal fat.

Computed Tomography uses x-ray radiation to produce cross-sectional images that determine the body composition using complex algorithms. CT has demonstrated good spatial resolution in measuring regional adipose tissue distributions. Although non-invasive it is an expensive method, which together with the radiation exposure limits its widespread use [41].

MRI employs a magnetic field and uses its interactions with protons - hydrogen ions abundant in all tissues - to produce cross sectional images of the body [42]. The high-quality images show the amount and distribution of fat in the body. MRI has been extensively validated and it is an accurate method to quantify total body adipose tissue, when compared with isotope ($D_2O$) dilution and bioimpedance [41]. As a single slice technique it can determine the amount of abdominal fat in terms of both visceral and subcutaneous fat [43]. The disadvantage of this technique is the high cost of equipment and analysis, which limits its use to research in small studies.

### 1.1.5 Distribution of Adipose Tissue

There are two distinct patterns of body fat deposition depending on the anatomical location:

- the upper /central body segment also known as apple-shape distribution
- the lower body/ gluteo-femoral segment, often termed pear-shape distribution.

The apple-shape or central distribution is more prevalent, and increases in frequency with age, in men while the peripheral, gluteo-femoral adiposity is more common amongst women. The body fat phenotype, however, may change with age and the tendency towards central rather than peripheral fat distribution has been observed in post menopausal women.

The peripheral fat distribution involves predominantly subcutaneous fat deposition in the buttocks, thighs and hips, although intramuscular adipocyte infiltration may also be present. In the case of central obesity patterns, adipose tissue can be deposited predominantly within the abdominal cavity and referred to as visceral adiposity, or it can be deposited subcutaneously. The visceral abdominal phenotype is characterized by excess fat around viscera such as gut and within internal organs such as the liver. The subcutaneous upper body adiposity is characterized by fat deposits in the subcutaneous tissue of the abdominal and chest wall.

#### 1.1.5.1 Factors affecting body fat distribution

Development of obesity and the distribution of body fat are multifactorial and governed predominantly by genetic [44], environmental [45] and physiological factors.

Fat distribution phenotypes vary between different populations and ethnic groups. Asians have higher body fat and different fat distribution compared to Caucasians [46]. These ethnic differences suggest that body fat distribution may be modulated by various environmental factors that affect the proportion of bone, muscle and fat and result in different behavioural patterns that contribute to weight gain and it may also be influenced by some genetic components shared amongst individuals of the same race. Loos *et al.* suggested the influence of major genes on regional fat distribution phenotypes [47]. To date (in the association studies) there have been 135 different candidate genes identified that are associated or linked with the obesity-related phenotypes [44]. While the majority of these are probably false positive associations, it is possible that at least 20 of the obesity candidate genes will contribute in varying degrees to the risk and type of obesity in human populations.

Some studies in humans demonstrated a strong genetic influence on the development of obesity in the individuals exposed to unhealthy diet or physical inactivity [48] which may suggest the interaction between genetic predisposition and an abnormal environmental factor required in some individuals to gain excess weight. Twin, familial and adoption studies confirmed consistently not only a genetic basis for obesity but also an environmental contribution (physical inactivity, excessive energy diets and alcohol, and

smoking) to its heritability [45, 47]. However, the mechanism behind their involvement in the formation of intra-abdominal fat is unclear.

Several physiological factors have been identified contributing to the pathogenesis of obesity. The storage of fat can be stimulated by the changes in the adipose tissue metabolism. Altered brain signalling for the satiety and hunger control centres is another mechanism contributing to obesity [49]

Sex-steroid hormones play a significant role in the defining of body shape, with their effects beginning at puberty and varying throughout life. Testosterone levels are responsible for the development of the android shape seen predominantly in men but on occasions also presenting itself in some females. In contrast, oestrogens promote fat storage in the buttocks, hips and thighs. Hence, fat levels are higher in women then in men. This is mainly for reproductive and childbearing reasons. Hormonal changes throughout life result in changes in body shape. A significant surge in oestrogens during pregnancy stimulates subcutaneous fat deposition whereas a decline in levels during menopause promotes abdominal fat deposition. Excessive amounts of cortisol, which can be stress-induced or present in Cushing's syndrome, lead to central adiposity [50, 51]. Some studies have confirmed a strong relationship between the disturbances in the hypothalamic-pituitary axis and visceral fat accumulation [51]. There are also many other hormones important for the development of obesity although their influence on the body fat distribution may not be as clearly defined. For example, hypothyroidism, growth hormone insufficiency and the age-related decline of dehydroepiandrosterone (DHEA) levels are all recognised to increase body adiposity. Deficiency in hormones that suppress appetite such as cholecystokinine, ghrelin, PYY3-36 and adrenaline also results in obesity.

#### 1.1.5.2 Cardio-metabolic effects of different body fat phenotypes

The recognition of different body fat deposition patterns such as visceral abdominal, subcutaneous abdominal and peripheral phenotypes is important because of their different metabolic effects and associated health risks. This is because adipose tissue is not only an energy storage for the times of starvation or reproduction but it is also a metabolically and hormonally active tissue. It has been established that central obesity is more strongly associated with metabolic and cardiovascular complications than total body fat [10, 52, 53]. In many epidemiological studies, the increased risk of diabetes, hypertension and cardiovascular disease has been linked to visceral and subcutaneous upper body adiposity [54, 55] and not the subcutaneous fat of the lower body. Moreover, even in lean subjects, visceral fat accumulation remains an independent cardiovascular risk factor [56]. Over the

years many studies have indicated the strong association between visceral fat excess (estimated by a variety of different techniques) and cardiovascular disease such as carotid atherosclerosis or increased carotid intima-media thickness [57-59]. Furthermore, visceral adiposity has been associated with the acceleration of atherosclerosis in previously disease-free men [60].

Several studies have reported a strong relationship between insulin resistance and intra-abdominal fat deposits. A prospective study by Pouliot *et al.* showed that the degree of visceral adiposity in men with stable total body fat or weight-predicted changes in insulin resistance indices over time [61]. Also in women, isolated central obesity has been associated with diminished insulin sensitivity and fasting hyperglycaemia [62]. However, the mechanisms linking visceral fat and insulin resistance are still not completely understood. The association of $\beta_3$-adrenergic receptor gene polymorphism with the presence of increased visceral fat and insulin resistance supported a lipotoxic theory which suggested the increased activity of $\beta_3$-adrenergic receptor was responsible for visceral lipolysis and insulin resistance. Another theory proposed that cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), released from intra-abdominal fat, diminished adiponectin levels resulting in reduced insulin sensitivity [63]. The existing evidence suggests that growing fat mass becomes infiltrated by macrophages and T-lymphocytes, which lead to the release of high levels of inflammatory mediators that alter lipolysis, increase levels of free fatty acids and impair insulin signalling. The research studies showed that the visceral fat does not exclusively produce most of cytokines and adipokines involved in the cardio-metabolic complication of central obesity. Currently, IL-6 is the only documented cytokine to be produced in visceral fat in excess of 50% of its total concentration [64]. Most studies showed that adiponectin concentrations are low in central adiposity. In contrast, a study of obese women showed a positive correlation between adiponectin and peripheral fat [65]. In central obesity and type 2 diabetes there is reduced production of protective factors such as adiponectin and increased production of leptin, resistin, and cytokines such as IL-6, TNF-α, and monocyte chemoattractant protein-1 (MCP-1) [66]. Visfatin, another adipocytokine whose role in obesity and insulin resistance is under scrutiny is predominantly produced in visceral fat [67].

There have been several studies pointing out the association between elevated CRP and obesity as measured by waist circumference [68]. A cross-sectional study of men by Despres *et al.* revealed an association between visceral fat and high levels of CRP, which was independent of total body fat [63]. Furthermore, Forouhi *et al*. demonstrated a strong

correlation between visceral adiposity as measured by CT scanning and waist circumference and CRP across two different ethnic groups [69].

A limited number of studies have addressed the role of peripheral adiposity and showed its negative correlation with glucose and atherogenic lipid profile, and that it is an independent predictor of lower cardiovascular and diabetes-related mortality [62, 70]. In addition, some studies demonstrated that subcutaneous adipocytes were smaller in size and less sensitive to lipolytic stimulation by catecholamines due to the increased number of β-adrenergic receptors and decreased affinity for $\alpha_2$ -adrenergic receptors in the lower body [71, 72] which made them more insulin sensitive. These findings may only in part explain the regional differences in fat metabolic activity. Another possible explanation for this phenomenon may lie in the fact that visceral fat has much greater expression of the genes involved in glucose homeostasis, insulin action and lipid metabolism than the subcutaneous fat.

### 1.1.6 Dyslipidaemia

Dyslipidaemia is a disorder of lipoprotein metabolism including their overproduction or deficiency. The typical pattern of dyslipidaemia in central obesity is that of increased small and dense Low Density Lipoprotein (LDL), triglycerides and apo-B, but decreased levels of High Density Lipoprotein (HDL) [73]. Dyslipidaemia of central obesity undoubtedly plays an important role in development of cardiovascular morbidity and mortality. Each individual component of this dyslipidaemia (except for apoB whose role is not clear although it is a primary apolipoprotein of LDL-cholesterol) has marked atherogenic properties and the pattern of dyslipidaemia is not affected by the gender or ethnicity. The National Health and Nutrition Examination Survey (NHANES) showed that the greater degree of fatness, the more likely is the presence of dyslipidaemia and the greater its severity. The greater the increase in the visceral fat the higher the concentration of triglycerides and the lower the concentration of HDL [62]. A study by Kissebach *et al.* showed that upper body obesity (apple shape) in women was associated with larger fat cells and higher triglyceride concentrations in comparison with lower body obesity (pear shape) and non-obese individuals [71]. The NHANES demonstrated that the level of dyslipidaemia may be modified by age and gender. The central role in the pathogenesis of dyslipidaemia in obesity appears to be played by insulin resistance, strongly associated with visceral adiposity. This has been supported by many population-based studies, which showed association between central obesity, insulin resistance and dyslipidaemia. Studies

such as Framingham, CARDIA and many others confirmed that insulin resistance in the centrally obese is positively associated with triglycerides and negatively with HDL cholesterol concentrations [74, 75].

The triglycerides levels in the obese are elevated in both fasting and the postprandial state. It appears that fasting hypertriglyceridaemia is due predominantly to a primary defect in the hepatic very low density lipoprotein VLDL production. The mechanisms behind postprandial lipaemia are not yet fully understood, but may be related to diminished levels of (insulin-sensitive) lipoprotein lipase and impaired clearance of VLDL produced postprandially in the liver [76, 77]. The modifications in VLDL metabolism lead to the change in the composition of LDL particles, which become enriched in triglycerides and when hydrolysed by lipoprotein lipase, they form small, dense, very atherogenic LDL particles. Similarly, low concentrations of HDL are the result of the abnormal triglyceride metabolism. It is likely that decreased HDL levels are due not only to decreased transfer of apolipoproteins and phospholipids from triglyceride rich lipoproteins to HDL, but also increased exchange of cholesterol esters between those particles, increased HDL clearance and decreased Apo A-I production.

### 1.1.7 Insulin resistance

The association between obesity and the risk of developing insulin resistance resulting in glucose intolerance and diabetes has been well documented. Several studies have shown that with increasing BMI there is also a steep linear increase in the incidence of type 2 diabetes, and that the prevalence of diabetes is much higher in the obese then in normal weight population [18, 78]. Moreover, the risk of insulin resistance progresses as the BMI absolute values increase even within the range defined as normal [79]. Certain ethnic groups such as South Asian Indians or Pima Native Americans are particularly prone to obesity and insulin resistance which may be present even from birth [80].

From various experimental and clinical studies it has become evident that a causal link exists between the insulin resistance and excessive body weight: obesity can contribute to the development of insulin resistance that leads to hyperinsulinaemia, which in turn may contribute to further weight gain and greater escalation of insulin resistance. It is, however, unclear whether insulin hypersecretion or resistance is the primary impulse for the development of obesity. It is thought that the adipose tissue contribution to the pathogenesis of insulin resistance is through the release of high amounts of non-esterified fatty acids, glycerol, cytokines, hormones and many other factors which induce and promote insulin resistance in the liver and muscle.

### 1.1.8 Hypertension

Obesity, in particular abdominal obesity, has been consistently linked to hypertension and increased cardiovascular risk in many epidemiological studies. Its prevalence increases with the increasing degree of fatness regardless of gender [78], being lower in the overweight compared to the obese. Analysis of the cohort from the Framingham study showed that being overweight or gaining weight increased future risk of developing hypertension [81]. Also, a positive correlation between weight and systolic blood pressure has been reported in the normotensive individuals [82]. The mechanisms behind obesity-related hypertension are very complex and are still being investigated. It has been suggested that obesity may lead to hypertension by

(a) activating the sympathetic nervous system through hyperleptinaemia-mediated stimulation of hypothalamic pathways

(b) activation of renin-angiotensin-aldosterone system (RAAS) resulting in volume expansion [83]

(c) other mechanisms, associated with longstanding obesity which include worsening renal function, increased procoagulatory activity and microvascular dysfunction as indicated in the recent studies [84, 85].

## 1.2 Insulin sensitivity and resistance

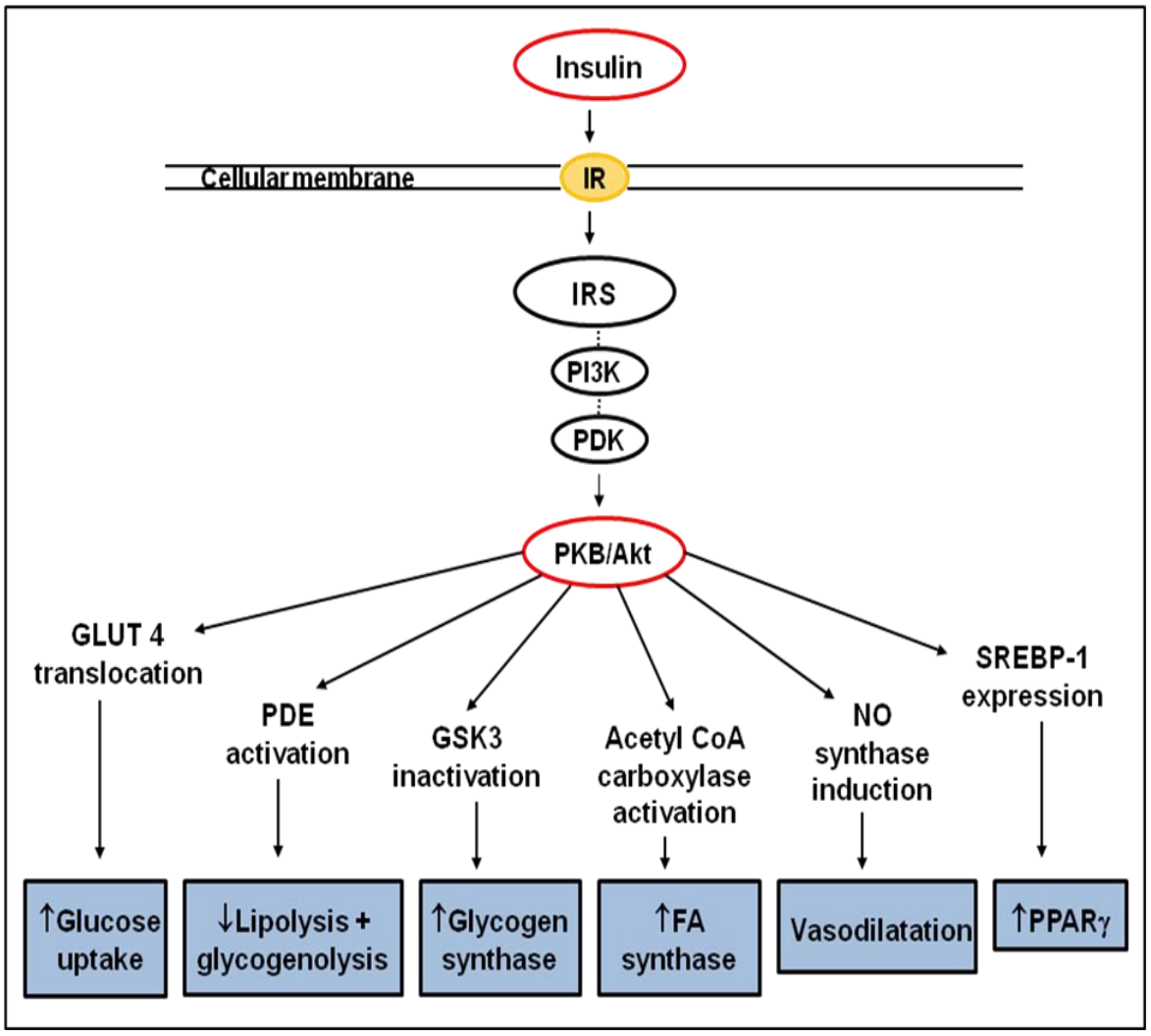
### 1.2.1 The mechanism of action of insulin: signalling pathways for insulin

Insulin is a polypeptide hormone, which consists of an alpha and beta chain of amino acids joined together by two disulphide bonds. It is a product of the proteolytic cleavage of proinsulin to biologically inactive connecting peptide (C-peptide) and insulin. Insulin is released from pancreatic β cells, in response to glucose levels, by exocytosis into the portal venous system and nearly 50% of insulin is taken up directly by liver, with the remainder being distributed throughout the body. Insulin is an anabolic hormone essential for glucose, lipid and protein metabolism. Its main sites of actions include skeletal muscle – where about 80% of glucose is metabolised, liver and adipose tissue. Insulin exerts its actions through widely distributed insulin-specific insulin receptors and a system of intracellular messengers and signalling pathways (Figure 1.1).

The insulin receptor (IR) is a member of the tyrosine kinase superfamily of transmembrane signalling proteins. It is composed of two alpha subunits, which are entirely extracellular and have ligand binding domains, and two beta subunits that penetrate the plasma membrane and with their intracellular portion containing the tyrosine kinase activity [86]. The binding of insulin to the alpha subunits results in receptor activation characterized by autophosphorylation of the intracellular β–subunit, which in turn phosphorylates a number of intracellular proteins altering their activity and thereby triggering signalling cascades. The intracellular proteins belong to the insulin receptor substrate (IRS) family and include IRS1- 4, Gab-1, Shc, $p62^{dok}$, Cbl and APS. A single activated IR can activate multiple IRS proteins. These proteins can also be activated by a variety of the other proteins such as insulin-like growth factor 1 (IGF-1), interleukin-4 (IL-4) and vascular endothelial growth factor (VEGF) [87]. Once IRS proteins are tyrosine phosphorylated they become docking centres for the recruitment and activation of other signalling proteins. The activation of IRS proteins stimulates activity of the downstream signaling molecules, of which one of the most important is phosphatidylinositol 3-kinase (PI3K). PI3K mediates the recruitment of PI3-dependent serine/threonine kinase (PDK1) and protein kinase B (PKB, also known as Akt) from the cytoplasm to the plasma membrane, where PDK1 activates PKB/Akt, which regulates several downstream proteins including components of the glucose transporter 4 (GLUT4) complex, protein kinase C (PKC) isoforms, and glycogen synthase kinase-3 (GSK3), all of which are critical for the insulin-mediated metabolic effects [87, 88].

**Figure 1.1 Schematic insulin signalling pathways**

The binding of insulin to its receptor initiates phosphorylation of insulin receptor substrate which stimulates downstream enzymes crucial for the activation of several proteins indispensible for insulin-mediated metabolic effects.
IR - Insulin Receptor, IRS - Insulin Receptor Substrate, PI3K - Phosphatidylinositol 3-kinase, PDK - PI3-dependent serine/threonine kinase, PKB/Akt - Protein kinase B GLUT4 - Glucose Transporter 4, PDE – Phosphodiesterase, GSK – Glycogen Synthase Kinase 3, NO - Nitric Oxide, FA – Fatty Acids, SREBP-1 - Sterol regulatory element binding protein, PPAR-γ - Peroxisome proliferator-activated receptor



## 1.2.2 Physiological role of insulin

Insulin has several important functions at the whole body and cellular levels. The principle role of insulin is the stimulation of glucose and fat metabolism and storage through the promotion of cellular glucose and fatty acid uptake, and the suppression of the hepatic glucose output. Thus insulin participates in stimulation of glycogenesis, lipogenesis and glycolysis, and inhibition of hepatic gluconeogenesis and glycogenolysis. It also diminishes lipolysis and mediates vasodilatation through the stimulation of nitric oxide synthase. Additionally, insulin promotes intracellular transport of amino acids and protein synthesis. It modulates mRNA transcription and stimulates growth, DNA synthesis and cell replication.

### 1.2.2.1 Regulation of glucose metabolism

The insulin-stimulated increase in the cellular glucose uptake is mediated by plasma membrane glucose transporter type 4 (GLUT4). GLUT4 is regulated by insulin and is found in the insulin-responsive tissues such as skeletal muscle, heart and adipose tissue. Released from the pancreatic cells insulin stimulates, through the activation of IR, IRS, PI3K and PKB/Akt signaling cascade, GLUT4 translocation to the plasma membrane to facilitate glucose uptake.

The activation of insulin-dependant phosphodiesterase (PDE) that induces several key glycolytic enzymes such as glucokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase (PK) results in increased liver glucose uptake. Inhibition of protein kinase A (PKA) by insulin leads to a greater activity of glycogen synthase. Thus the net effect is the increased glycolysis and glycogenesis, which in turn increases glucose content in the hepatocytes and lowers blood glucose.

In addition, it has also been documented that the activation of PKB/Akt in liver and muscle leads to the phosphorylation and inhibition of GSK3. This process promotes glycogen synthase activity, which together with the increased cellular glucose uptake augments glycogen synthesis [88].

### 1.2.2.2 Regulation of lipid metabolism

Insulin, through the activation of PI3K, stimulates activity of insulin-dependent Phosphodiesterase (PDE) which inhibits hormone sensitive lipase (HSL) crucial for lipolysis and release of fatty acids and glycerol into blood. Insulin induces the activity of lipoprotein lipase (LPL) and fatty acid synthetase (FAS) in adipocytes and myocytes, and these are important for lipogenesis.

Insulin enhances the expression and maturation of the transcription factor sterol regulatory element-binding protein-1 (SREBP-1), an important player in FFA metabolism, present in all metabolically active tissues [89], and which regulates adipogenesis and is a mediator of insulin action through the induction of peroxisome proliferator-activated receptor gamma (PPARγ) activity.

### 1.2.3 Insulin resistance definition, aetiology and molecular mechanisms

#### 1.2.3.1. Definition

Insulin resistance is a state in which tissues exhibit inadequate or reduced response to normal amounts of insulin. This may be due to inability of insulin to bind to its receptors or due to abnormalities in the insulin receptors which result in inadequate insulin signaling down the signaling pathway and the imbalance in metabolic homoeostasis. This initiates a compensatory augmentation in insulin synthesis, which subsequently results in hyperinsulinaemia disproportionate to the level of glycaemia [90]. Commonly the end result of this metabolic disharmony is development of variable degrees of dysglycaemia or type 2 diabetes. Since 1988, when Gerald Reaven proposed the hypothesis of insulin resistance syndrome as a link between type 2 diabetes, hypertension and cardiovascular disease, insulin resistance has also been associated with obesity, microvascular dysfunction and inflammation. There have been several various hypotheses regarding the etiology and pathogenesis of insulin resistance and its interactions with other metabolic disease states but the precise mechanisms underlying the development of insulin resistance are still being investigated.

#### 1.2.3.2. Etiology

In 1962, James Neel proposed the "thrifty genotype" hypothesis as an explanation of the rapidly expanding epidemic of insulin resistance and obesity. This hypothesis suggested existence of genes predisposing to thrifty metabolism, which conferred survival advantage to hunter-gatherer societies by allowing increased fat storage in times of plenty for the subsequent utilization in times of famine. However, in the modern times of excess of food and physical inactivity, the thrifty genes became detrimental to the development of obesity, overproduction of insulin and glucose intolerance [91]. The "thrifty genotype" hypothesis was decades later challenged by based on the evidence from epidemiological studies "thrifty phenotype" hypothesis. This hypothesis proposed that the environmental factors during the pre-natal development produced permanent changes in glucose-insulin metabolism, which resulted in the future development of insulin resistance [92]. The "thrifty epigenome" hypothesis combined elements of both thrifty genotype and thrifty

phenotype hypotheses. It suggested that a default "thrifty genome", a primarily result of the complex gene–environment interactions lead to the development of insulin resistance, diabetes and obesity [93]. The most recently proposed "drifty gene" hypothesis, by John Speakman, referred more to obesity rather than insulin resistance *per se*, and suggested that a drift in the genes encoding the regulation system responsible for the control the upper limit of body fatness was the underlying explanation for the modern epidemic of obesity phenotype and its related metabolic complications [94]. The above hypotheses are not mutually exclusive but represent the complexity of the interactions between multiple environmental and genetic factors that have been influencing development of insulin resistance and obesity.

#### 1.2.3.3 Pathogenesis and molecular mechanisms

The pathogenesis, molecular and cellular mechanisms behind insulin resistance are multifactorial and have been under constant debate over the several decades. It has been well established that free fatty acids (FFA) are important players in the pathogenesis of insulin resistance. Randle *et al*. were first to suggested that FFA competed with glucose for mitochondrial substrate oxidation, thus a surplus of FFA present in obesity due to increased lipolysis in adipose tissue resulted in the increased FFA oxidation and decreased glucose utilisation and was responsible for insulin resistance in obesity [95]. The essential component of this hypothesis was that Fatty Acid – Coenzyme A oxidation would increase the ratios of acetyl CoA to CoA and NADH to $NAD^+$ which inhibit the pyruvate dehydrogenase (PDH) complex, and increase citrate which inhibit phosphofruktokinase (PFK) . In turn these changes would diminish oxidation of glucose and pyruvate and increase glucose-6-phosphate (G6P) which would stimulate glycogen storage, inhibit hexokinase (HK) and decrease glucose transport. Findings from a recent study suggested that increased FFA concentrations first inhibit glucose transport and than reduce glycogen synthesis and glucose oxidation in muscle [96]. The intramuscular accumulation of FFA and their metabolites may affect directly GLUT4 activity or indirectly, through alterations in the upstream insulin signalling pathways, it may reduce GLUT4 translocation, thus resulting in diminished intracellular glucose transport [97].

It has been documented that increased intracellular fatty acid metabolites stimulate a serine/threonine kinase cascade in the muscle and subsequent phosphorylation of sites on IRS-1 [98], thus impairing activity of PI3K, and subsequently adversely affecting insulin signal transduction pathways. Similarly, excess of FFA metabolites in the liver activates PKC, leading to diminished phosphorylation of IRS-2 [97, 99].

The evidence from recent studies suggests that the excess of visceral adipocytes, which are less effective in lipid storage then peripheral fat may promote redistribution of fat to intracellular sites in liver and muscle, resulting in the insulin resistance in these tissues. Moreover, studies in transgenic mouse models and humans with severe lipodystrophy suggest that defects in adipocyte fatty acid metabolism may contribute to increased fat delivery to liver and muscle [100]. In addition, reports from studies in the elderly and subjects with diabetes support the notion that the acquired or inherited defects in mitochondrial fatty acid oxidation may also contribute to the pathogenesis of insulin insensitivity [101-103]. Therefore an excess of energy intake over energy expenditure may lead to accumulation of intracellular fatty acids metabolites and induce insulin resistance in muscle and liver through the alterations of adipocyte fatty acid metabolism, or decreased mitochondrial fatty acid oxidation [98]. In addition, as described in the recent human studies, defects at various levels in the insulin signalling pathway may also contribute to the insulin resistance.

### 1.2.4 Assessment of insulin sensitivity/resistance

There is a wide choice of different methods available for the assessment of insulin secretion and sensitivity or resistance. This includes simple baseline tests, specialist techniques performed in the clinical or research setting as well as computed indices based on mathematical equations and modeling. Their application depends on the nature of the studies and often the size of studied population and local expertise and availability. All these methods rely on glucose and insulin measurements and therefore may be influenced by factors affecting those measurements such as oscillatory insulin secretion in fasting and postprandial states, and insulin liver clearance, which can change in both physiological and pathological conditions. Most of the techniques have been validated against the best available standard such as euglycaemic clamp and have been able to reproduce clamp-derived information on insulin resistance in disease states. Methods for the assessment of insulin sensitivity or resistance may be broadly grouped into simple and complex techniques, the latter consisting of simple surrogate insulin sensitivity (IS) markers, surrogate models of IS derived from dynamic tests and indirect and direct measures of tissues sensitivity to insulin.

#### 1.2.4.1 Simple fasting surrogate measures of insulin sensitivity/resistance

##### 1.2.4.1.1 Fasting glucose and insulin

Glucose homeostasis is tightly regulated by feedback mechanisms predominantly linked to insulin secretion and its action. It is well recognized that the higher the fasting hyperglycemia and compensatory hyperinsulinaemia the worse the degree of insulin resistance. Both fasting glucose and insulin concentrations as well as their ratios have been widely used in large population studies as surrogate markers of insulin resistance because of the simplicity in their measurements and availability. Although they all correlate with the clamp-derived insulin sensitivity measurements ($ISI_{clamp}$), the correlation is not a strong and is particularly weak in relation to impaired glucose tolerance [104, 105]. Moreover, because of the lack of a standardized insulin assay, it is difficult to define universal cut-off points for insulin resistance with these simple surrogate indices.

##### 1.2.4.1.2 HOMA-IR and QUICKI

It is well recognized that fasting insulin concentration provides more accurate information about insulin sensitivity if it is interpreted in the context of the concurrent glucose concentration. This knowledge led to the development of mathematical models of glucose-insulin interactions derived from the responses to intravenous glucose tolerance test, hyperglycaemic and euglycaemic clamps. These models were then employed to formulate the equations for the insulin resistance and sensitivity estimation such as Homeostasis Model Assessment (HOMA) and Quantitative Insulin sensitivity Check Index (QUICKI), respectively. Both methods are simple surrogate indices of insulin sensitivity and secretion based on paired fasting glucose ($G_0$, mmol/L) and insulin ($I_0$, mIU/L) concentrations determined in steady-state, basal conditions.

HOMA is a computer-generated model that consists of several non-linear empirical equations and accounts for glucose distribution, production and utilization. It is used to estimate pancreatic β-cell function (HOMA-B) and insulin sensitivity expressed as a relative insulin resistance (HOMA-IR) [106]. To account for pulsatile insulin secretion some researchers recommend using a mean of three measurements spaced five minutes apart in the calculation, but many epidemiological studies, for simplicity, implement only a single measurement. The mathematical approximation of both models is represented in the following equations:

$$\text{HOMA-B} = (I_0 \times 20) / (G_0 - 3.5)$$

$$\text{HOMA-IR} = (I_0 \times G_0) / 22.5$$

where 3.5 and 22.5 are the normalising factors.

HOMA-IR of 1 indicates typically normal insulin sensitivity in an individual. It has been established that a HOMA-IR greater than 2.5 indicates insulin resistance [107, 108]. The CV for HOMA-IR varies depending on the number of fasting samples and type of insulin assay employed [106, 109, 110]. Some studies reported that the index is less accurate in the presence of marked hyperglycemia or poor β-cell function [105]. Both HOMA models have been shown to be strong predictors of the development of diabetes [111, 112]. Log transformed HOMA-IR appears to be especially useful in people with variable degrees of glucose intolerance, possibly because it transforms skewed fasting insulin distribution.
QUICKI is another empirically derived mathematical transformation of fasting insulin and glucose. It is believed to be a better approximation of insulin sensitivity than HOMA because of the log transformation employed in the equation that accounts for the skewed distribution of insulin values [113] and improves the linear correlation with clamp-based insulin sensitivity index ($ISI_{clamp}$). QUICKI is derived by calculating the inverse of logarithmically expressed values of fasting glucose ($G_0$, mmol/L) and insulin ($I_0$, mIU/L):

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

QUICKI provides a reproducible and accurate index of insulin sensitivity with good positive predictive power [114, 115] and it performs best in insulin resistant subjects. With regard to the coefficient of variant (CV) it exceeds that of other simple fasting surrogate markers including HOMA-IR [116] because the employed mathematical transformation of reverse of log transformation accounts for the variability in single fasting insulin and glucose measurements.
Both models have been widely employed in clinical research, since they are easy to use in the epidemiological and population-based studies in place of sophisticated but complex alternative methods. QUICKI appears to have better reproducibility in comparison to HOMA [117]. Although both methods are reported to correlate relatively well with $ISI_{clamp}$, QUICKI has better linear correlation over a wide range of insulin sensitivity/resistance [113]. This is not only because HOMA is a measure of insulin resistance rather than sensitivity [107, 108, 113] but also because of the log transformation of both fasting markers in the calculations.

#### 1.2.4.2 Surrogate models derived from dynamic tests

Oral Glucose Tolerance Test (OGTT) is the most commonly used test for the evaluation of whole-body glucose tolerance *in vivo*. OGTT is the mainstay test for the clinical diagnosis of abnormal glucose metabolism such as impaired fasting glycaemia (IFG), glucose intolerance (IGT) and diabetes mellitus (DM). The WHO diagnostic criteria for glucose

intolerance published in 1998, and reviewed in 2006, (Table 1.3) distinguish the diabetic group as being at significantly increased risk of premature mortality, micro- and macrovascular complications [118]. OGTT is performed after an overnight fast and involves blood sampling at 0 and 120 minutes for clinical purposes or at 0, 30, 60 and 120 minutes for research studies following the standard 75g glucose loads. It reflects the body efficiency at disposing of glucose after a glucose dose or a less commonly used standard meal. Glucose metabolism is influenced not only by glucose absorption but also by complex interactions between insulin secretion and its metabolic actions, and neurohormonal and incretin actions, all of which are primary determinants of glucose homeostasis. Thus OGTT or a test meal provides information about glucose tolerance but not about insulin sensitivity/resistance *per se*. Moreover, the complexity of glucose homeostasis regulatory mechanisms affects its reproducibility. However, the OGTT mimics the physiological conditions of glucose and insulin dynamics more closely than the experimental test for insulin sensitivity estimation such as the glucose clamp, insulin suppression test (IST) or minimal model. The information about insulin secretion and sensitivity can be derived from OGTT / meal test if appropriate mathematical models are applied.

**Table 1.3 WHO criteria for diagnosis of glucose intolerance**

| | **Fasting** | | **120 minutes** |
|---|---|---|---|
| Normal | <6.1 mmol/L | and | < 7.8 mmol/L |
| Impaired fasting glycaemia | ≥ 6.1 mmol/L and <7.0 mmol/L | and | < 7.8 mmol/L |
| Impaired glucose tolerance | <7.0 mmol/L | and | ≥ 7.8 mmol/L and < 11.1 mmol/L |
| Diabetes Mellitus | ≥ 7.0 mmol/L | and / or | ≥ 11.01mmol/L |

These surrogate insulin sensitivity indices (ISI) take into an account fasting steady-state and dynamic post-glucose load plasma glucose and insulin levels. Insulin secretion may be estimated from the area under the curve (AUC) of insulin or C-peptide concentration but this does not show a completely linear correlation with total insulin secretion over a period of time [119]. The other, generally better methods include the ratio of the AUC of insulin and glucose (good for assessing β function in diabetics), insulinogenic index $(I_{30} - I_0)/(G_{30}-G_0)$ calculated using fasting and 30 minutes insulin and glucose results or corrected insulin response index, $CIR_{30} = I_{30}/( G_{30} \times (G_{30} -70))$. All of these simple indices show significant but modest correlation with the clamp-derived insulin sensitivity and acute response to intravenous insulin administration [120].

Several other surrogate ISI derived from OGTT ($ISI_{OGGT}$) employ far more complex mathematical equations. All these methods employ a particular sampling protocol during the glucose or meal tolerance test. In addition, the minimal model approaches have also been applied to assess plasma glucose and insulin dynamics during the glucose load [121]. The surrogate $ISI_{OGGT}$ has been shown to correlate with $ISI_{clamp}$, but only moderately. This may be because the clamp technique is designed to measure peripheral glucose utilization whereas responses to OGTT represent peripheral utilization and hepatic glucose production. In contrast to several ISI models, Soonthornpun's index has much stronger correlation with $ISI_{clamp}$ ($r = 0.869$, $p<0.0001$) as assessed in non-diabetic subjects [105]. This was possibly due to the fact that Soonthornpun and colleagues utilized the area above the glucose curve which represents peripheral glucose disposal instead of the area under the curve which represents hepatic glucogenesis and unused glucose. In addition, the adipose tissue insulin sensitivity can be estimated by incorporating plasma non-esterified fatty acids (NEFA) suppression measured during OGTT [122].

Some surrogate ISI derived from dynamic tests use statistical models employing stepwise linear regression analysis, which allow estimation of both insulin secretion and sensitivity at the same time. These equations may be used in normal and diabetic individuals, and they correlate reasonably well with clamp-measured insulin sensitivity indices [123, 124]. Several epidemiological studies have reported that the surrogate estimates of insulin sensitivity derived from dynamic tests can predict the development of type 2 diabetes [120, 125].

#### 1.2.4.3 Indirect measures of insulin sensitivity/resistance

The minimal model analysis provides an indirect measure of insulin sensitivity/resistance based on glucose and insulin results obtained during a frequently sampled intravenous

glucose tolerance test (FSIVGTT) and incorporated in a simplified mathematical representation of their relationships. This model was developed by Bergman *et al.* in 1979 [126] and involves an intravenous infusion of a bolus of a glucose after an overnight fast. Blood is sampled for glucose and insulin measurements every 1-2 minutes for the first 30 minutes, then every 10 minutes for 90 minutes and then at 160 and 180 minutes. In insulin deficient subjects a modified FSIVGTT is currently applied with an additional intravenous insulin or Tolbutamide bolus given 20 minutes after the glucose bolus to stimulate endogenous insulin secretion [105]. The results are then subjected to minimal model analysis using the computer program MINMOD which generates an index of insulin sensitivity ($S_I$). The described minimal model consists of two differential equations, one that represents glucose kinetics with a single-compartment model for glucose distribution, and the other which represents the insulin effect within the compartment, which is different from plasma. Thus $S_I$ expresses the relationship between insulin levels in the effect compartment and glucose disappearance from the glucose compartment [104]. Additionally, a "glucose effectiveness" index ($S_G$) can be estimated from the minimal model which describes the ability of glucose *per se* to promote its own disposal and inhibit hepatic production at baseline conditions. The minimal model correlates reasonably well with clamp studies in healthy subjects, but this correlation weakens in severe resistance states due to the negative results that the minimal model may yield in those states [105]. Although it is a laborious method it is technically easier to perform than glucose clamp and provides information about insulin sensitivity derived from a single dynamic test.

#### 1.2.4.4 Direct measures of insulin sensitivity/resistance

##### 1.2.4.4.1 Hyperinsulinaemic euglycaemic clamp

The hyperinsulinaemic euglycaemic clamp originally devised by Andres *et al*. was further developed by DeFronzo *et al.* and is recognised worldwide as a gold standard technique for the direct estimation of insulin sensitivity [127]. This technique involves simultaneous intravenous infusion of insulin, first as a priming bolus followed by a constant rate infusion to achieve a pre-set hyperinsulinaemic plateau, and a 20% dextrose set at a variable rate to maintain normal fasting glucose levels (euglycaemia, 5.0mmol/L). Blood for is sampled every 5-10 minutes for glucose measurements to aid glucose rate adjustments and maintenance of euglycaemia. Blood samples for insulin measurements are usually obtained every 30 minutes until the steady-state is reached and then every 10 minutes for the last 30 minutes of the steady state. The steady state is usually achieved after about 2 hours of continuous glucose and insulin infusions.

It is assumed that during the clamp the exogenous hyperinsulinaemia completely inhibits hepatic glucose production, but stimulates skeletal muscle and adipose tissue glucose disposal. Thus, the glucose infusion rate in the steady-state reflects whole body glucose disposal (M-value) and overall insulin sensitivity.

The application of an uncorrected M-value may lead to the overestimation of insulin sensitivity in some groups i.e. men when compared with women [128]. Typically, to estimate insulin sensitivity index, the M-value is normalized to body weight or fat-free mass, although the former may result in exaggerated estimates of insulin resistance in obese individuals [128]. Given that glucose uptake can occur only in lean tissues, the M-value may be best optimised for the fat free mass. Also steady-state whole body glucose disposal rate may be corrected for the rate of the resting energy expenditure (REE), because it is a quantitative measure of all metabolically active tissues, providing REE measurement preceded the clamp procedure within the same session[129]. Regardless of the employed M-value expression, it may further be corrected for hyperinsulinaemia under steady-state conditions (M/I) to account for the small differences in the clamp insulin concentrations.

Alternatively, the clamp data may be used to derive an insulin sensitivity index such as $SI_{clamp} = M/(G \times \Delta I)$, where M-value is normalised for the steady-state glucose concentration (G) and the difference in insulin concentration between fasting and steady-state ($\Delta I$)[113].

The hyperinsulinaemic glucose clamp is a very demanding and laborious technique, and not without risk. It is expensive and requires a high level expertise to perform. The clamp procedure requires maintaining the patency of two intravenous catheters, one of which usually provides an arterial or arterialised blood. The latter is accomplished by retrograde cannulation at the wrist or hand while heating the hand at about 50 $^{o}$C. Arterial or arterialised blood is preferred for glucose measurement because of the arterio-venous differences in blood glucose concentrations, which increase with insulin sensitivity and insulin dose, and which may lead to possible overestimation of insulin sensitivity if the venous blood is sampled. The insulin dosage requires standardisation (either per body surface area or body mass) to allow for results comparison. It needs to be established at an appropriate level to account for the differences in insulin sensitivity between various examined populations. The fine infusion rate adjustments rely on the well calibrated infusion pumps and bed-side glucose analysers. Also, defining the glucose level for clamping at either the fasting or pre-determined levels requires consideration depending on the type of studied population.

However, the clamp can be combined with a number of other procedures to enhance the information obtained on whole body glucose disposal at a given level of insulinaemia under steady-state conditions. Thus, the application of radiolabelled glucose tracers under the clamp conditions allows simultaneous quantification of hepatic glucose production and whole body glucose disposal [104]. Similarly, use of radiolabelled glycerol or amino acids tracers during the glucose clamp facilitates assessment of insulin sensitivity with respect to lipolysis or protein metabolism [104]. The adipose tissue insulin sensitivity may be also estimated with the application of a stepped clamp that consists of low dose insulin infusion, which is exclusively directed at adipose tissue, followed by the high dose insulin infusion.

It has been reported that the glucose clamp has an excellent CV of 0.1% which may be an under-estimate considering the complexity of this procedure and great variability in measured insulin and glucose depending on variety of factors (i.e. subjects preparation), and good reproducibility

[130]. Its use is, however, limited to smaller studies with insulin sensitivity as a primary outcome.

**1.2.4.4.2 Insulin suppression test**

Insulin suppression test (IST) is another technique that directly measures insulin sensitivity and was developed in 1970 by Shen *et al.* and later modified by Harano *et al*. [104]. During this test endogenous glucose release is inhibited by the combined action of hyperinsulinaemia, hyperglycaemia and hypoglucagonaemia, and the steady-state plasma glucose represents peripheral insulin sensitivity. IST involves, after an overnight fast, intravenous infusion of somatostatin or its analogue to suppress endogenous insulin and glucagon secretion, and simultaneous constant infusion of insulin and glucose. Blood for glucose and insulin measurements is sampled every 30 minutes for 2.5 hours and every 10 minutes during a steady-state reached at 150-180 minutes after the initiation of IST.

In the steady state, plasma insulin concentrations are generally similar among subjects but plasma glucose concentrations vary depending on insulin sensitivity (higher in insulin resistant). Thus, the test provides a direct measure of the ability of exogenous insulin to mediate the disposal of an exogenous glucose load under steady-state conditions where endogenous insulin secretion is suppressed. The estimates of insulin sensitivity derived from IST express good and strong correlation with clamp indices in both normal subjects ($r=0.93$) and people with type 2 diabetes ($r=0.91$) [120, 131].

## 1.3 Microvascular circulation

### 1.3.1 Definition and functions

Microvascular circulation is a term applied to describe small vessels of a diameter less then 150 μm such as arterioles, venules, capillaries, terminal lymphatic capillaries and collecting ducts as well as all arterial vessels that are able to contract their lumens in response to increasing internal pressure [132]. Microvasculature vessels are responsible for the distribution of micronutrients, hormones, inflammatory mediators and other stimuli as well as collecting products of tissues metabolism. Arterioles are a major site of systemic vascular resistance due to their high level of responsiveness to sympathetic vasoconstriction. They regulate organ blood flow and in part capillary hydrostatic pressure. Capillaries are characterised by a variable degrees of permeability depending on the site of location and organ. They are the primary site of fluid, electrolyte, gas, and macromolecule exchange. Venules play an important role in fluid and electrolyte exchange as well as regulating capillary hydrostatic pressure through their sympathetic innervation. The primary function of the microcirculation is to ensure optimal delivery of nutrients and oxygen to the tissues in response to variable demand, and removal of $CO_2$ and other metabolic waste products. Microcirculation is responsible for the maintenance of adequate hydrostatic pressure that is invaluable for appropriate capillary exchange and determination of the overall peripheral resistance [133]. It is also involved in inflammatory responses. The number of flowing capillaries per unit volume of tissue (the functional capillary density), perfused capillaries (capillary recruitment), microvascular filtration capacity (exchange capacity) and increased tissue perfusion in response to demand (functional hyperaemia) ensure efficiency of microcirculatory functions [134, 135].

### 1.3.2 Assessment of microcirculation

The available techniques for studying microcirculation differ widely in their application, cost and availability. Many of them, such as micromyographic analysis of isolated arteries or intravital microscopy are invasive and predominantly used in animal studies to examine the structure and properties of microvessels. In humans, skin microvasculature is the most commonly examined part of the microcirculation. This is usually done with the employment of direct visualization techniques such as non-dye microscopy or capillaroscopy with orthagonal polarization spectral imaging, which allow measurement of capillary permeability, density and flow velocity [136-138].

Microvascular studies in human muscle are much less popular due to restricted availability of non-invasive methods. Techniques used to measure tissue perfusion and capillary blood flow such as contrast enhanced ultrasound with the application of microbubbles (microspheres), positron emission tomography with radiolabelled tracers or needle-inserted laser Doppler are very invasive in nature, expensive and often limited by the requirement for high expertise, technical skills and local availability of an appropriate tracer [136, 139, 140]. The alternative methods for assessing muscle microvascular function include non-invasive techniques of plethysmography and laser Doppler flowmetry.

#### 1.3.2.3 Venous Congestion Plethysmography

Venous congestion plethysmography (VCP) is a well established and widely used *in vivo* technique in human studies to investigate the mechanics of vasodilatation and vasoconstriction, and the role of the vascular endothelium [141]. It can be coupled to intra-arterial drug administration for the assessment of vascular pharmacology. It is a non-invasive method for measuring regional blood flow in human limbs. VCP measures the increase in limb volume after cuff inflation to a pressure occluding only a venous return but not arterial inflow. Displacement of fluid or strain gauge around the limb is used for the estimation of volume change which is a result of "venous filling as the pressure rises and fluid filtration in the microvasculature" [142]. VCP enables estimation of fluid filtration capacity ($K_f$), venous pressure ($P_v$), isovolumetric venous pressure ($P_{vi}$)
VCP has been well validated, and provides accurate and reproducible results [141]. It is limited by the need to avoid high inflation pressures to minimize the risk of the arterial flow occlusion.

#### 1.3.2.4 Laser Doppler flux measurement

Laser Doppler fluximetry is another non-invasive method, which involves the measurements of red cell flux in small-volume samples (0.5 $mm^3$) of body and organ surfaces such as the skin as well as muscle [143]. The measured concentration of blood cells and their local speed are referred to as the perfusion and the laser Doppler perfusion monitor records the integrated perfusion within the sampling volume. The shift in the frequency from a defined, stable and single reference point provides the required measurement. The penetration depth of the laser probe is commonly about 0.6 mm but its sensitivity decreases with the increasing depth of dermis, thus in practice the best penetration is achieved at about 0.3mm [136]. However, the generation of two-dimensional perfusion maps and use of higher power and wider separation probes with the laser

Doppler imaging overcomes the heterogeneity in the measured variables and allows for implementing of this technique in the assessment of muscle microvasculature [143]. Laser Doppler fluximetry allows assessment of local responses to a variety of stimuli including hyperaemia, change in body temperature or pharmacological stimuli. The measurements are provided in perfusion units and results are usually expressed as the percentage of

### 1.3.3 Role of Insulin in microvascular regulation

The vasodilatory effects of insulin administration have been known for a long time, but it was only in 2000 that Baron *et al*. reported that insulin dilates, in a dose-dependent fashion, skeletal muscle vasculature [144]. Insulin preferentially regulates skeletal muscle vascular resistance over the microvasculature of other tissues. It has been established that physiological insulin concentrations increase cardiac output, but this mechanism is impaired in central obesity. There is heterogeneity of vascular responses to insulin, with lesser vasodilation in more insulin-resistant subjects. More recent data have shown that insulin also stimulates blood flow and perfusion in both skin [133, 140] and skeletal muscle [145, 146]. It has been suggested that impairment of insulin-mediated microvascular dilator responses in skeletal muscles decreases glucose uptake and thus contributes to insulin resistance [147, 148]. Many studies documented impaired insulin-mediated skeletal muscle vasodilatation in individuals with obesity, diabetes and in hypertension confirming the crucial vasoactive role of insulin in microvascular homeostasis [132].

The proposed mechanism of insulin action by Baron *et al.* [144] suggests that the postprandially raised insulin concentration stimulates pre-capillary arteriolar vasodilatation and reduces skeletal muscle vascular tone, which in turn allows greater cardiac output to be directed towards the skeletal muscles. This in turn improves arteriolar blood flow and facilitates capillary recruitment and perfusion, resulting in greater glucose and insulin delivery to the tissues. The raised capillary recruitment leads to increased functional capillary density and subsequently greater participation of skeletal muscle in insulin-mediated metabolism, thus augmenting insulin action [144, 149]. The molecular mechanism of insulin-mediated vasodilatation has been under constant investigation and it is likely to be multifactorial and synergistic. There is strong evidence that in insulin-stimulated vasodilatation, endothelium-derived nitric oxide plays a crucial role [132, 135]. This vasodilatory mechanism has been well-explored recently and it has become evident that insulin exerts its vasodilatory action through PI3-kinase dependent signalling

pathways. Insulin stimulates PKB/Akt which then directly increases activity of endothelial nitric oxide synthase (eNOS) and nitric oxide production. Insulin also has vasoconstrictor properties that are mediated via endothelin-1 (ET-1), derived from the endothelium in a process involving an intracellular mitogen-activated kinase (MAPK) / the extracellular signal-regulated kinase-1/2 (ERK1/2) mediated pathway [132, 135].

### 1.3.4 Microvascular function in obesity

Over the recent years, there has been a growing interest in the role of microvascular function in obesity and its cardio-metabolic complications. It has been reported that central obesity as measured by WHR is strongly associated with microvascular function, insulin sensitivity and blood pressure [150]. Serne and colleagues showed that microvascular function plays a central role, even within physiological range, in linking insulin sensitivity, blood pressure and abdominal fat in non-obese subjects [151]. In addition, de Jongh *et al*. showed that in obese women, visceral adiposity estimated by MRI is inversely associated with insulin-mediated capillary recruitment [152]. It has been reported that measures of obesity in healthy individuals are strongly related to skin microvascular function. A recent study of young, non-smoking and normoglycaemic women confirmed the presence of microvascular dysfunction in overweight and obese individuals [153]. Also, it has been postulated that obesity-related microvascular impairment may contribute to the development of nephropathy [154] and heart failure [155], and that it plays a role in the development of insulin resistance [156]. There is a body of evidence that obesity alters microvascular function through the changes in microvascular structure and endothelial functions [152]. Animal studies in genetically obese Zucker rats revealed structural re-modelling [148] and capillary rarefaction [139], the latter also found in skeletal muscle of obese humans [157]. With regards to endothelial dysfunction in obese individuals, there have been reports of blunted vasodilatation to several endothelium specific stimuli in skin and resistance vessels [152, 158, 159], diminished capillary recruitment in response to reactive hyperaemia, stress shear and hyperinsulinaemia [152, 160, 161]. The findings of improved endothelial function in obese women after weight loss further support the links between obesity and microvascular function [162]. There have also been reports, in both animal and human studies, of impaired insulin-mediated muscle microvascular function in obesity [161, 163].

The mechanisms underlying obesity-related microvascular dysfunction are complex (Figure 1.2). They revolve predominantly around interconnected intracellular, endocrine and vasocrine signalling. The studies, which looked into the endocrine and paracrine

aspects of adipose tissue, point towards the interactions between adipocytes, macrophages, lymphocyte, endothelial cells and vascular smooth muscle cells which take place within the adipose tissue, and their end-products (inflammatory mediators) such as cytokines, chemokines and hormone-like factors play an important role in the microvascular dysfunction of obesity [66].

In obesity, the imbalance between insulin vasodilatory and vasoconstrictor effects is due to defects in signalling pathways at the cellular level. The excessive amounts of reactive oxygen species (ROS) diminish the concentration of available nitric oxide (NO) and impair endothelium-related vasodilatation through the direct inhibition of NO synthase and the reduced expression and activity of endothelial NO synthase in muscle and kidney. Excessive amounts of free fatty acids (FFA) released from the visceral fat impair phosphorylation of IRS-1 and activation of PI3-kinase resulting in dysfunction in the intracellular insulin signalling transduction pathway and subsequent diminished basal and insulin-induced capillary recruitment and endothelium-dependent vasodilatation [132, 164]. At the same time, insulin-mediated vasoconstriction through MAPK / ERK1/2 activation and production of endothelin-1 remains intact as demonstrated in a study of obese and hypertensive individuals [132].

Cytokines and adipokines produced in fat tissue constitute an important element of the dysfunctional endocrine-intercellular signalling. TNF-α stimulates lipolysis which leads to release of FFA. Thus the excess of TNF-α present in central adiposity, indirectly impairs the balance between endothelium-derived vasodilators and vasoconstrictors by down regulating the expression of NO synthase, inhibiting IRS-1 phosphorylation and up regulating ET-1 expression [132]. Obesity-related hyperleptinaemia augments production of ROS in the endothelium and coexistent leptin resistance leads to decreased leptin-dependent signalling, causing further impairment in insulin-mediated microvascular function and glucose uptake. In contrast, there is diminished release of adiponectin, which leads to reduced IRS-1 phosphorylation in the insulin-signalling pathway thus adversely affecting glucose uptake and vascular endothelium.

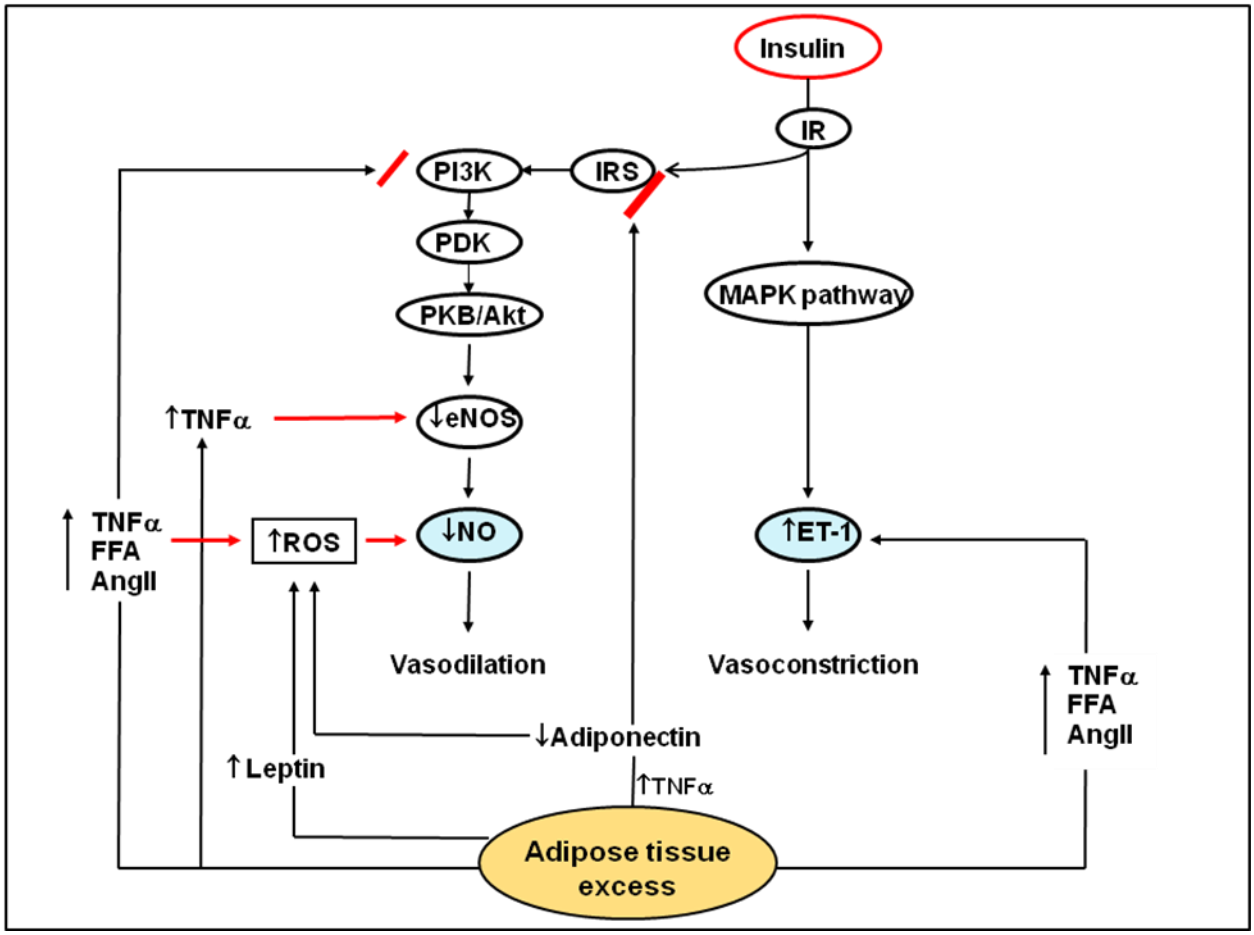
Adipose tissue is also a source of all components of the renin-angiotensin system (RAS), which are necessary for the generation of angiotensin II (AngII). AngII is not only a potent vasoconstrictor itself but it also induces expression of another vasoconstrictor, ET-1. There are reports that ET-1 and AngII mutually but indirectly stimulate their own production thus mediating the enhanced predisposition to vasoconstriction [132, 156]. There is evidence that AngII derived from adipose tissue binds to receptors on adipocytes and pre-synaptic nerve endings and blood vessels [84]. In obesity states, the activity of

RAS is increased both systematically and locally within adipose tissue [84, 165], leading to augmentation of vasoconstriction, decreased total muscle blood flow and capillary recruitment that contribute to lowered insulin-mediated glucose uptake and development of hypertension [156, 166].

The latest evidence suggests that perivascular fat deposition, such as in tunica adiposae, and local fat deposits around the arterioles in skeletal muscle actively secrete adipocytokines, which influence the regulation of microcirculation in obesity. According to the "vasocrine" signalling mechanism proposed by Yudkin *et al*. distal periarteriolar fat has similar vasoactive properties to visceral fat and releases cytokines such as TNF-α. These inhibit insulin-mediated capillary recruitment and thus reduce blood flow and nutrient supply in the local arteriolar-capillary network [167].

**Figure 1.2 Schematic overview of the molecular mechanisms underlying microvascular dysfunction in obesity**

The diagram depicts the effects of excess of the cytokines and adipokines produced in the adipose tissue on Insulin-mediated vasodilatation and vasoconstriction signalling cascade. IR – Insulin Receptor, IRS – Insulin Receptor Substrate, PI3K - Phosphatidylinositol 3-kinase, ET-1 – Endothelin-1,PDK - PI3-dependent serine/threonine kinase, PKB/Akt – Protein kinase B, MAPK - Mitogen-activated kinase, eNOS – endothelial Nitric Oxide Synthase, NO – Nitric Oxide, ROS – Reactive Oxygen Species, TNFα - Tumour Necrosis Factor, FFA – Free Fatty Acids, AngII - Angiotensin II.



## 1.4 Metabolic syndrome

### 1.4.1 Definition

The term "metabolic syndrome" (MetSy) refers to a combination of cardio-metabolic risk determinants, with the main components being central obesity, hyperglycaemia, dyslipidaemia and hypertension. It was first described as a *Syndrome X* by Reaven in 1988, who introduced a concept of insulin resistance as an unifying element for the set of metabolic abnormalities [168]. Since then the syndrome has been the subjects of scientific scrutiny and was only formally recognised by the Centre for the Disease Control- in 2001, and registered as "Dysmetabolic syndrome X".

To date, several definitions of the metabolic syndrome have been postulated. The most commonly used definitions are from the International Diabetes Federation (IDF, 2005) , the National Cholesterol Education Programme Adult Treatment Panel III (NCEP ATP III, 2001), the World Health Organization (WHO, 1999) and the European Group for the study of Insulin Resistance (EGIR, 1999) [169]. Although these definitions use slightly different diagnostic criteria (Table 1.4), they all consist of common, essential features such as central obesity, glucose intolerance (or diabetes), hypertension, elevated triglycerides and reduced HDL-cholesterol, and require the presence of at least three abnormal parameters for the diagnosis. The NCEP ATP III definition requires any three out of the five abnormal parameters for the diagnosis, whereas in the IDF criteria central obesity is the essential feature besides two other parameters. The EGIR criteria require insulin resistance defined as the top quartile of the fasting insulin values among non-diabetic individuals, whereas the WHO classification requires measurement of insulin resistance by oral glucose tolerance test or hyperinsulinaemic–euglycaemic clamp. Microalbuminuria, hyperuricaemia and hypercoagulability are also included in the definitions by the WHO and American Association of Clinical Endocrinologists, with the latter organisation also incorporating into the definition a polycystic ovarian syndrome, vascular endothelial dysfunction and coronary heart disease [170]. The multitude of the definitions for this syndrome leads to the ambiguity and incompleteness of its criteria. Since its aetiology and pathogenesis (which are discussed in further paragraphs) are uncertain some researchers put into doubt its existence. Moreover, the name "syndrome" is inappropriately used for this cluster of metabolic factors since a syndrome means a group of symptoms indicating or characterising a disease and this is not the case here. The medical value of diagnosing patients with this syndrome may also be questionable although it can be used as a screening tool for obesity and other metabolic problems within general practice.

**Table 1.4 Various criteria for the metabolic syndrome definition**

| | IDF 2005 | NCEP ATPIII 2001 | WHO 1999 | EGIR 1999 |
|---|---|---|---|---|
| Required | Obesity and 2 other features | 3 or more features | IGT or DM and 2 other features | Fasting ↑ insulin and 2 other features |
| Central obesity | Waist circumference ≥ 94 cm male ≥ 80cm female (Europids) | Waist circumference ≥ 102 cm male ≥ 88cm female | Waist to Hip Ratio ≥0.9 male ≥0.85 female or BMI >30kg/m$^2$ | Waist circumference ≥ 94 cm male ≥ 80cm female |
| Blood Pressure (mmHg) | SBP ≥ 130 DBP ≥ 85 or treated hypertension | SBP > 130 DBP > 85 or treated hypertension | SBP ≥ 140 DBP ≥ 90 | SBP ≥ 140 DBP ≥ 90 or treated hypertension |
| Dyslipidaemia (mmol/L) | TG ≥ 1.7 HDL < 1.04 male <1.29 female | TG ≥ 1.7 HDL < 1.0 male <1.3 female | TG ≥ 1.7 HDL < 0.9 male <1.0 female | TG ≥ 2.0 HDL < 1.0 or treated dyslipidaemia |
| Dysglycaemia (mmol/L) | Fasting glucose ≥ 5.6 or diagnosed IGT/DM | Fasting glucose ≥ 6.1 | Fasting glucose ≥ 6.1 or/and IGT/DM | Fasting glucose ≥ 6.1 but not diabetic |
| Insulin resistance | Not applicable | Not applicable | Glucose uptake during HEC in lowest quartile | Fasting ↑ insulin (highest 25% of non-diabetics) |
| Other factors | None | None | Microalbuminuria UAER > 20µg/min or ACR>30mg/mmol | None |

SBP – systolic blood pressure, DBP – diastolic blood pressure,
TG –triglycerides, HDL – high density lipoprotein, IGT – impaired glucose tolerance,
DM – diabetes mellitus, HEC – hyperinsulinaemic euglycaemic clamp,
UEAR – urinary albumin excretion rate, ACR – albumin-creatinine ratio

## 1.4.2 Epidemiology

The prevalence of metabolic syndrome has been increasing worldwide over the years and the syndrome is increasingly seen in younger people. This has been driven by the explosion of the obesity epidemic [171, 172]. The prevalence of metabolic syndrome in the USA between 1994-2000 has increased from 23% to 27%, along with an increase in obesity and physical inactivity [173]. The prevalence of metabolic syndrome differs depending on the definition employed as well as the type of population studied including age, ethnicity, region and rural or urban environment. The WHO and EGIR definitions are more restrictive than the NCEP ATP III definition because of the prerequisite requirements regarding defining insulin resistance state. The IDF definition with obesity as a prerequisite feature appears to be least constraining. Indeed, there are several studies which show the prevalence of metabolic syndrome to be the highest with the use of IDF diagnostic criteria [174-176], reaching up to 39%. Many reports from studies conducted in other countries were in keeping with the USA findings, but studies from China and Iran reported lower rates of metabolic syndrome using the IDF criteria [177, 178]. This may be explained by the race-specific waist circumference guideline within the IDF definition. The increasing prevalence of raised BMI positively correlates with the prevalence of the individual components of metabolic syndrome even in environmentally different populations [78, 179, 180]. Metabolic syndrome is more prevalent amongst women than men [173]. Based on NHANES studies it appears that gender-related differences in the prevalence of metabolic syndrome depend largely on the ethnic and racial background of the study's cohorts. The age-adjusted prevalence was lower in white non-Hispanic women than men, but higher in African-American women than men. Rates of metabolic syndrome increase consistently with rising age between 12 and 60 years [181-183] and drop down in the $6^{th}$ or $7^{th}$ decade, which also varies depending on the used definition [182-184].

## 1.4.3. Cardio-metabolic implications

There is a body of evidence suggesting that the presence of metabolic syndrome doubles the cardiovascular risk [185], triples the risk of coronary heart disease and raises the risk of developing type 2 diabetes five-fold [186]. Its presence correlates strongly with the incidence and progression of carotid atherosclerosis [187]. It has been established that the metabolic syndrome can predict total, cardiovascular and coronary heart disease mortality. The presence of even one component of the syndrome increases overall mortality when compared with healthy individuals [175, 188, 189].

However, some researchers reported that the CVD risk based on the full definition did not differ significantly from the risk based on its individual elements [190]. It remains uncertain which of the syndrome's components may be detrimental to this risk.

### 1.4.4. Pathogenesis

The pathogenesis of metabolic syndrome (MetSy) has been under a constant debate for several years. A single unifying mechanism or a single gene responsible for this constellation of cardio-metabolic determinants has not as yet been established. This is also reflected in the large variety of definitions for this syndrome, as discussed in earlier paragraphs. The current concepts of MetSy are based on the understanding that it is a highly complex entity with an aetiology probably linked to genetic defects, mitochondrial mutations, polygenic variability in individuals, pre-natal environment influences, fat distribution phenotypes, physical inactivity, ethnicity and advancing age [185, 191]. The evidence from studies investigating less common disorders associated with obesity supports the genetic bases of the syndrome [192, 193] as an independent factor facilitating metabolic dysfunction. The growing clinical evidence suggests a strong correlation between visceral adiposity, insulin resistance and CVD risk [194], which has been supported by experimental studies and the portal theory of insulin resistance. In this hypothesis, free fatty acids derived from visceral adipose tissue enter the liver directly via the portal vein and negatively affect insulin action [195]. The available evidence from observational studies of the causal links and the presence of hyperinsulinaemia and insulin resistance (IR) in obesity, diabetes dyslipidaemia and hypertension [196-198] strengthens proposed role of insulin resistance as a key player in the pathogenesis of the metabolic syndrome. The proposed mechanisms at a cellular level revolve around a variety of defects in the insulin receptor as well as pre- and post-receptor signalling pathways, and the interactions with microvasculature, skeletal muscles and adipocytes [199-201]. Several studies have confirmed that visceral adiposity promotes insulin resistance through increased lipolysis in response to hormonal stimuli such as glucocorticoids and catecholamines [202-204]. However, adipose tissue also secretes a variety of cytokines and adipokines, which exert endocrine, paracrine and autocrine effects leading to energy imbalance, microvascular dysfunction and impaired glucose uptake and metabolism [205].

# 1.5 Cardiovascular fitness and physical activity

## 1.5.1 Definition

In broad terms, physical activity refers to a variety of behaviours that result in body movement requiring variable levels of energy expenditure. Physical activity, therefore, encompasses a wide range of activities from the undertaking of daily living, to occupational and recreational activities, and the various degrees of exercise. Exercise is comprised of repetitive movements and structured physical activities, often performed at a vigorous intensity with the purpose of improving cardio-respiratory fitness [7]. The increase in physical activity level leads not only to an improvement in cardio-respiratory fitness, but also to an increase in muscle strength and mass, and changes in the body's composition.

## 1.5.2 Methods of assessment

Direct and objective ways of measuring physical activity and / or fitness include motion sensors and measures of fitness and energy expenditure. All these methods differ in the levels of precision and sophistication of measurement, and it is often difficult to employ them separately from each other.

### 1.5.2.1 Motion and physiological response sensors

Motion sensors allow assessment of free-living activity and an indirect estimation of energy expenditure. These methods include accelerometers, heart rate monitors and devices that combine the features of various sensors. Accelerometers measure direct body movement. They provide data on the patterns of physical activity and with the employment of special equations, give estimated energy expenditure. Accelerometers have been validated for assessing the total amount of physical activity using doubly-labelled water, [206], but the laboratory-based energy expenditure prediction equations are not valid for free-living activity. Accelerometers are fairly simple and easy to use but the information they provide needs careful interpretation. This is because during energy expenditure from some movements such as cycling, the upper bodywork is not reflected in the acceleration of the body.

Heart-rate monitors such as Flex HR provide information about heart rate changes in response to body movements. They can provide data on energy expenditure and a pattern

of physical activity. The results from heart-rate monitors can be affected by factors other than physical activity, such as stress or emotions, and consequently their use is limited. The next generation of techniques for the measurement of free-living energy expenditure are methods that combine accelerometers with heart rate monitors, such as Actiheart, or incorporate motion sensors with other types of sensors, such as skin temperature and heat flux sensor (e.g. Armband Pro). As these devices combine different techniques they may be able to provide more accurate information about energy expenditure than separate individual components.

#### 1.5.2.2 Measures of fitness

Measures of fitness can be broadly divided into metabolic and fitness tests. The latter includes a variety of tests measuring muscle endurance, strength, body flexibility, agility, balance and coordination. In contrast with fitness tests, metabolic tests, such as cardiorespiratory fitness, provide an assessment of overall body fitness and cardiovascular health risk [207, 208]. Moreover, physical activity status is the main determinant of cardiovascular fitness and can therefore be used in studies investigating the correlation between physical activity and health [209].

Cardiorespiratory fitness, which is recognised as the gold standard, measures the maximal oxygen uptake ($VO_2$ max) during dynamic exercise such as running or cycling. Maximal oxygen uptake is the largest amount of oxygen a healthy individual can take in during physical activity. With increasing exercise intensity, oxygen uptake increases to a point, until it attains a constant level despite additional increases in the workload, whereas carbon dioxide production increases leading to a rise in the respiratory quotient. This point defines the maximal oxygen uptake. Thus, $VO_2$ max indicates the maximal level of aerobic power output, with the limiting factors being cardiovascular capacity and the rate of oxygen diffusion from haemoglobin to muscle mitochondria [210]. Oxygen uptake ($VO_2$) is measured by indirect calorimetry and is estimated as the difference between inspired and expired oxygen during pulmonary ventilation over a set period. $VO_2$ max is most accurately estimated by measurements of expired air composition and respiratory volume during maximal exertion. These measurements need to be performed at frequent intervals (at least every 15 seconds) to allow accurate assessment of the $VO_2$ response to incremental exercise in order to determine the $VO_2$ max.

The cardiorespiratory fitness assessments are performed with the use of standard exercise protocols usually implementing treadmill or ergonometric bicycle. To minimise the risk to individuals, various sub-maximal exercise protocols have been used in observational and

intervention studies. Although the amount of physical activity undertaken makes a significant contribution, genetic factors can play a major role in an individual's $VO_2$ max, with the heredity accounting for up to 25-50% of the interindividual variance [211]. $VO_2$ max decreases with age at the approximate rate of 10% per decade after the age of 25 [212], which may also be a reflection of increased body weight, without a change in absolute values of oxygen uptake.

#### 1.5.2.3 Measures of energy expenditure

Energy expenditure (EE) is one the three components of total energy expenditure (TEE) besides the basic metabolic rate (BMR) and the thermic effect of food. Energy expenditure can be measured with direct and indirect calorimetry methods, or estimated by non-calorimetric techniques. The latter group of methods applies predictive equations to extrapolate data from physiological measurements and observations obtained using, for example, heart monitors or accelerometers, as discussed above. In contrast to the aforementioned techniques, isotope dilution, also known as the doubly-labelled water method, is an invasive non-calorimetric method. It is recognised as a gold standard method and as it has no applications for free-living energy assessment, it is used for the validation of other, less invasive methods [206].

Direct calorimetry measures the heat lost from the body using isothermal, adiabatic or conductive systems. These are very expensive techniques requiring a high level of expertise, which limits their application to highly specialist laboratories only [213].

In indirect calorimetry, oxygen consumption and/or carbon dioxide production is measured and converted into energy expenditure using specially-designed formulae. There are several different approaches used with this method such as room-open circuit, hood/canopy open circuit, open circuit-expiratory collection, doubly-labelled water and total collection systems. These are all laborious and relatively expensive which limits their use even though they provide accurate and reliable measures of energy expenditure.

### 1.5.3 Cardio-metabolic benefits

It is well known that physical activity has beneficial effects on many systems and disorders of the body, in particular, obesity, diabetes and other components of the metabolic syndrome [214-216]. There is evidence that suggests the more vigorous the activity, the greater the health benefits [207].

Regular physical activity is associated with longevity, in contrast to sedentary lifestyles [217] and good levels of cardiorespiratory fitness are associated with lower risk of all-

cause and cardiovascular mortality [207]. Several studies reported the association of physical inactivity with obesity and development of insulin resistance and type 2 diabetes [218]. It has been well documented that exercise training improves glucose tolerance and insulin sensitivity, even in the absence of weight loss [219]. Exercise exerts its beneficial effects on insulin resistance by increasing glucose uptake in the muscles thorough the augmented recruitment of GLUT4 transporters [219] and activation of insulin signaling pathways [220]. Moreover, exercising boosts muscle capillary density and consequently increases glucose uptake and insulin sensitivity [221]. In addition, a good level of physical activity also contributes to an improved lipid profile. Exercise training leads to an increase in HDL-cholesterol concentrations by 4-18 % and a decrease in triglyceride concentrations by 4-37%, with the greater reductions observed in previously inactive individuals. Benefits of exercise on total cholesterol and LDL-cholesterol are usually associated with a change in body weight and composition, and additional dietary fat reductions [218].

## 1.6 Statins

### 1.6.1 Biochemistry and modes of action

Statins are inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), the first important and rate-limiting enzyme in the biosynthesis of cholesterol via the mevalonate pathway [222] (Figure 1.3). Statins are structurally similar to its endogenous substrate 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), enabling them to compete directly for the active sites on hydroxymethylglutaryl Co-A reductase (HMGR) and block its action (Figure 1.4). This results in the inhibition of *de novo* cholesterol synthesis in the liver and activation of sterol regulatory element binding protein (SREBP), which upregulates expression of the gene encoding the LDL receptor, leading to increased LDL-cholesterol uptake and consequently decreased blood LDL-cholesterol concentration. In addition, this increased expression of LDL receptors stimulates marked reduction in the numbers of ApoB100-containing lipoproteins and consequently improves HDL levels in the blood. At the same time, statins induce ApoA-I production in the liver and the formation of nascent pre-HDL particles. Thus, the end result of the inhibitory actions of statins is to not only lower total and LDL-cholesterol but also to increase the plasma concentration of HDL [223]. The lipid lowering properties of statins extend beyond those described above. Statins also improve hypertriglyceridaemia possibly by stimulating hepatic expression of the peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) gene. The maximum time required for the hypolipidaemic effect of statins to become evident is four to six weeks [223]. The lipid lowering properties of statins extend beyond those described above. Statins also improve hypertriglyceridaemia possibly by stimulating hepatic expression of the peroxisome proliferator-activated receptor $\alpha$ (PPAR$\alpha$) gene. The maximum time required for statins' hypolipidaemic effect to become evident is four to six weeks [223].

All statins are metabolized in the liver, hence their low systemic bioavailability and relatively low potential for serious side effects. They all have the same essential structural components of a ring system and a dihydroxyheptanoic acid unit (Figure 1.4); the latter is responsible for the group's lipid lowering effect. The structural differences in the ring system and substituents define the activity of statins and their pharmacological properties, such as efficacy and lipophilicity. Statins are reported to reduce the concentration of total cholesterol by 20-40%, LDL-cholesterol by 20-60%, triglycerides by 10-30%, and increase HDL-cholesterol by 5-10% depending on the type and dose of statin [224].

**Figure 1.3 Biosynthetic pathway of cholesterol**

Cholesterol is synthesised from Acetyl-CoA in a multistep pathway involving mevalonate. Statins inhibit HMG-CoA reductase which is a rate-limiting enzyme in the cholesterol biosynthesis.

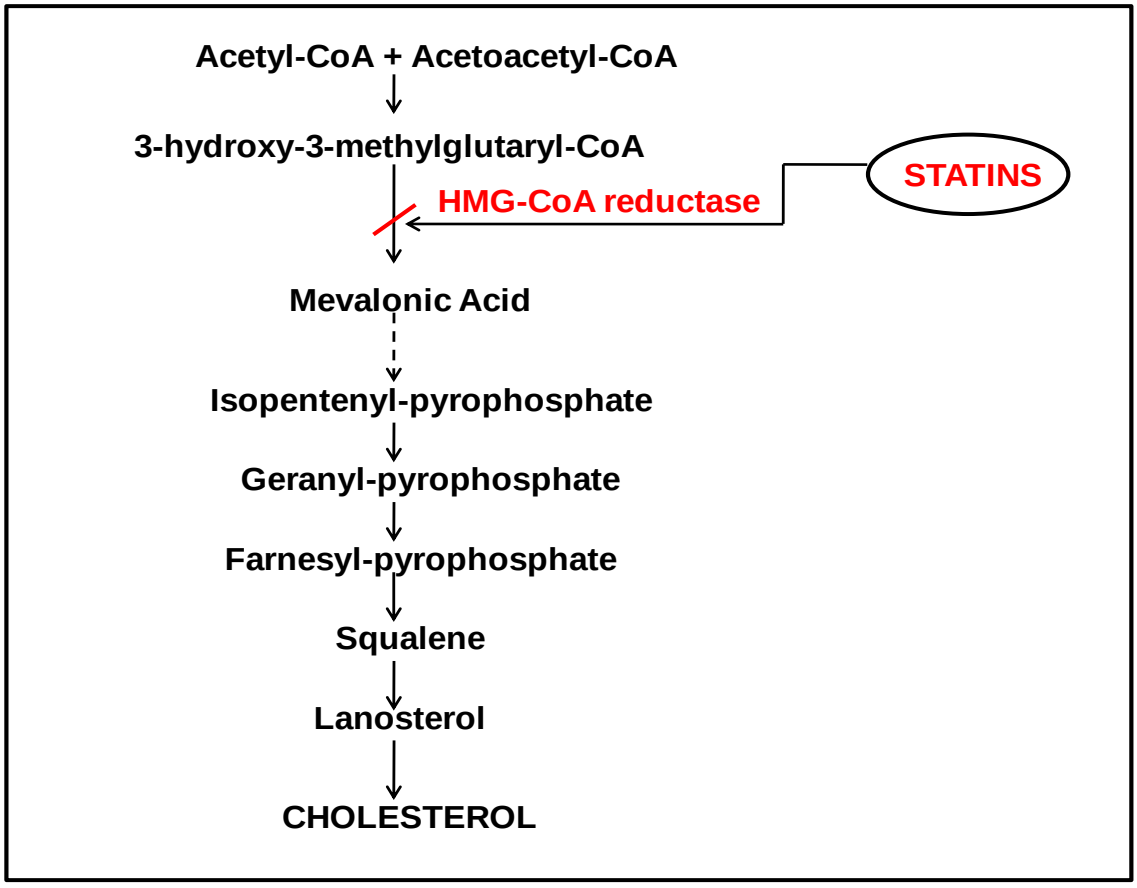
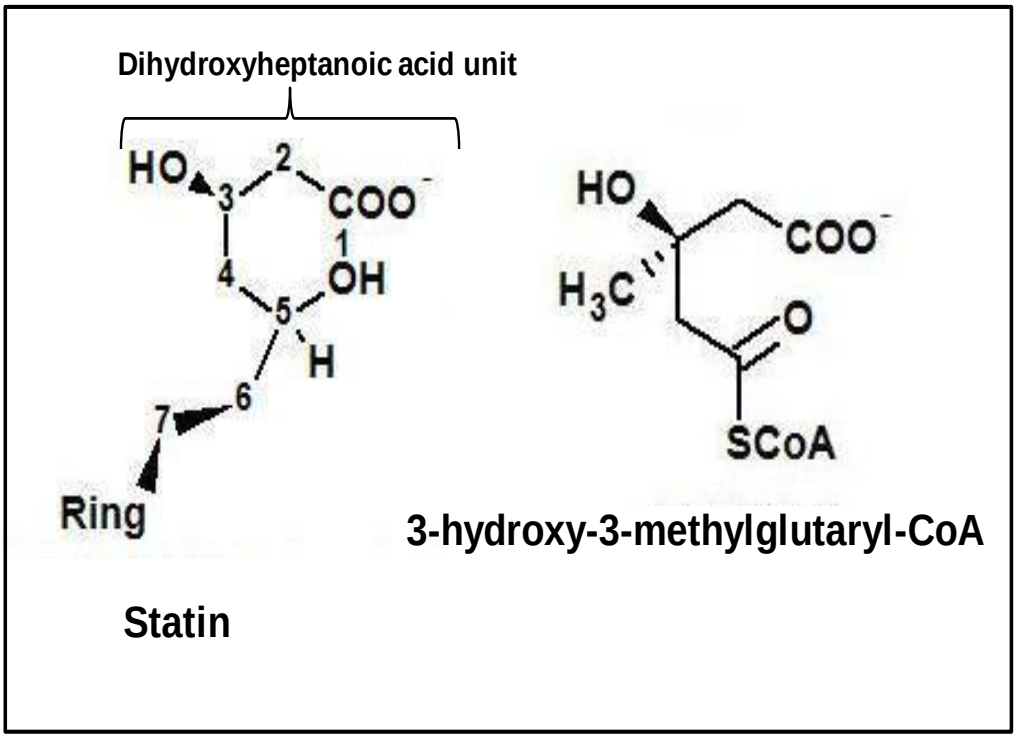


**Figure 1.4 Schematic structure of a statin and HMG-CoA**

Statins exhibit structural similarities to HMG-CoA which allow them to compete for the active sites on HMG reductase.



## 1.6.2 Statins and Macrovascular disease

The profound LDL-cholesterol lowering abilities of statins have been well documented over the last three decades. There is evidence from several large clinical trials that statins reduce cardiovascular morbidity and mortality [223-225] . Five major trials of statins in secondary prevention: 4S, CARE, LIPID, GREACE, and HPS have confirmed the benefits of treatment in terms of reduced vascular events regardless of the type of statin and baseline lipid levels. Four other placebo-controlled trials: WOSCOPS, PROSPER, AFCAPS/TexCAPS and ASCOT proved that statins are beneficial for primary prevention in those with high cardiovascular risk. Trials of statins in diabetes, such as CARDS, confirmed the highly significant favourable cardiovascular risk reduction in parallel with reductions in LDL-cholesterol [223]. The findings from ASTEROID trial additionally showed that statins reduce the formation of atherosclerotic plaques [226] within coronary arteries. Furthermore, the data from the recent METEOR trial showed reduction in progression of carotid intima-media thickness with Rosuvastatin treatment [227].

## 1.6.3 Pleiotropic effects of statin

As shown in many clinical trials, the overall benefits observed with statin therapy occur earlier and are of greater magnitude than would be expected from lipid changes alone. This suggests that statins may have effects beyond cholesterol lowering (Figure 1.5). These extra, non-lipid effects on vascular biology are often referred to as the pleiotropic effects of statins. In support of this notion would be the results from the REVERSAL study, where intravascular ultrasound measured coronary atherosclerosis before and after the intervention with statins showed that despite similar small changes in plaque size there was a marked (over 20%) reduction in the rates of cardiovascular events in these patients [223]. More recently, the JUPITER study showed that in the absence of hyperlipidaemia and atherosclerosis, but in the presence of low grade inflammation, high dose statin therapy reduced the incidence of all major cardiovascular events [228] .

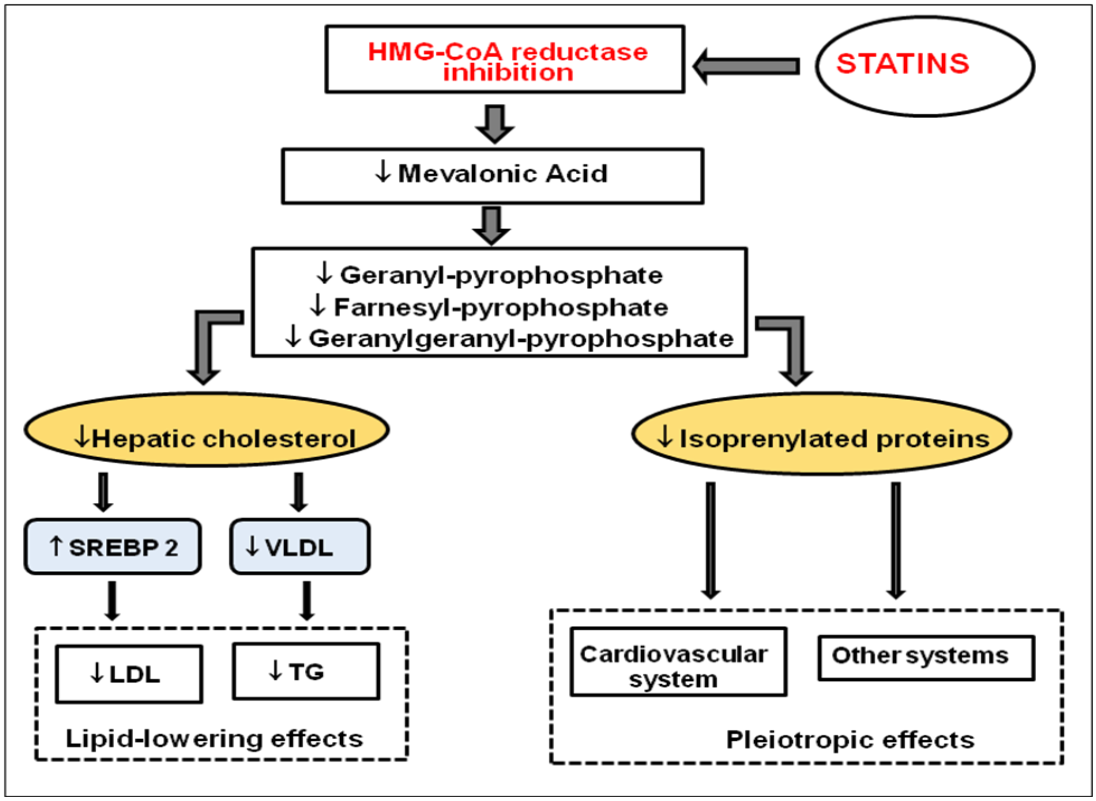
### 1.6.3.1 Endothelial function and inflammatory markers

Animal and cell culture studies have elucidated important cellular mechanisms modulated by statins independently of cholesterol concentrations that affect atherosclerosis and vascular function. These mechanisms include increased bioavailability of nitric oxide (NO) in endothelial cells, isoprenoid-mediated and other specific anti-inflammatory effects on vascular function. Experimental studies have documented increased endothelial availability

of NO due to the statin-mediated increase in protein kinase Akt activity and in turn NO synthase activity, or the decrease in an endothelial cell protein (caveolin), which inactivates NO synthase [229, 230]. Statins also inhibit isoprenoids, which are intermediates in cholesterol synthesis via the mevalonate pathway (Figure 1.3 and 1.5) and serve as attachments for pro-atherogenic intracellular signalling molecules such as small GTP-binding proteins: Rho, Ras and Rac, and cdc42 proteins [229, 231]. It is important to note that those significant lipid-independent statin effects demonstrated in various experiments required statin concentrations several orders of magnitude higher than those used therapeutically in clinical practice. An assessment of the LDL- independent effects of statin therapy in clinical trials is difficult because statins, by default, reduce LDL levels. However, studies comparing statin versus non-statin treatment groups point to smaller improvements in endothelial function in the non-statin intervention group despite lowering LDL to a similar degree as statins [230]. Other clinical trials showed that equipotent doses of various statins reduced hs-CRP in atherosclerosis [232, 233]. Moreover, it appears that in those with low grade inflammation, as evidenced by higher hs-CRP but without atherosclerosis and hyperlipidaemia, statins significantly reduce the risk of cardiovascular events [228]. There have also been reports of reductions in other inflammatory markers such as IL-1, IL-6, TNF$\alpha$, sICAM-1 with statin therapy [234], and that statins exhibit potent antioxidant effects [231]. There is, however, a paucity of data regarding the pleiotropic effects of statins in those with cardio-metabolic risks but without cardiovascular disease.

**Figure 1.5 Diagram of lipid-lowering and pleiotropic effects of statin**

By inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), it is postulated that statins not only lower hepatic cholesterol synthesis but also reduce synthesis of isoprenylated proteins thus exhibiting their pleiotropic effects.



#### 1.6.3.2 Microvascular function

The macrovascular benefits of statin therapy have been well documented over at the last two decades. There is also a significant body of evidence from experimental studies that statins exert marked positive effects on endothelial function [235-237], and some evidence from clinical trials confirming similar findings in the presence of atherosclerosis or diabetes, as described earlier. However, few studies examined the effects of statins on endothelial function in people with obesity or insulin resistance without apparent atherosclerosis and with relatively low cardiovascular risks.

The influence of statins on endothelial function might be translated into indirect effects on microvasculature, but there is a need for studies investigating its effectiveness directly on microvascular function. Parson *et al.* conducted a study investigating subjects with type 2 DM on a daily dose of 10mg Rosuvastatin or diet, and found significant improvement in skin blood flow (SBF) in the statin group as measured by Laser Doppler flowmetry [238]. It was not apparent though whether the increase in SBF was independent of the lipid-lowering effect. Trials with Simvastatin and Ezetimibe in subjects with coronary artery disease (CAD), which measured flow-mediated dilatation (FMD) with either high-resolution ultrasound or venous plethysmography, demonstrated that statin therapy improved endothelial function independently of lowering LDL-cholesterol [230]. Additionally, McGown *et al.* reported enhanced nitric oxide-mediated vessel relaxation thorough increased NO bioavailability with statin treatment [239]. Others also reported reduced oxidative stress and inflammation, inhibition of thrombogenic response, and decreased extracellular matrix remodeling [240, 241].

Overall, there is evidence to suggest that statin use may have direct microvascular benefits, but further evidence is still needed to elicit whether this is a lipid-independent effect.

#### 1.6.3.3 Statins and Insulin resistance

There is far less consensus regarding the influence of statins on insulin resistance than there is on their anti-inflammatory properties. There have been reports of unfavourable [242-244], as well as favourable [245-247] effects of statin treatment on insulin resistance, and additional reports suggesting a lack of benefit [248]. Those conflicting observations might be explained by multiple factors contributing to the outcomes, such as employed investigative methods, type, dose and duration of treatment, in addition to subject selection and changes in body weight and/or body composition during trials which are known to affect insulin action. It may also be possible that there is no drug-class effect with this respect and hence the discrepant results [249]. A study by Koh *et al.* in non-diabetic,

hypercholesterolemic subjects treated with 20 mg Pravastatin daily for 12 weeks resulted in a significant improvement in QUICKI [247]. The same researchers compared in a randomized, single-blinded, placebo controlled trial the effects of 2 months treatment with Pravastatin (40mg daily) and Rosuvastatin (10mg daily), and demonstrated that Pravastatin improved fasting surrogate insulin sensitivity markers and $HbA_1c$, whereas the opposite results were obtained with Rosuvastatin [250]. Sonmez *et al.* investigated a similar cohort of subjects on a daily dose of 40mg Fluvastatin and observed HOMA improvement unrelated to triglyceride decrease [251]. Paolisso *et al.*, in a crossover study of subjects with type 2 diabetes treated with 30mg Simvastatin daily, found significant improvement in insulin sensitivity determined by clamp and reduction in plasma NEFA concentrations by statin therapy [245]. Subsequently the same investigators, in a placebo-controlled study of diabetics, compared 10 mg Simvastatin with 5 mg Atorvastatin daily. Both statins produced significant changes in triglyceride concentration and insulin sensitivity, but the improvements achieved by Atorvastatin were greater, with 26% reduction in triglycerides accompanied by a 13% improvement in insulin action, whereas Simvastatin resulted in a 20% and 9% improvement respectively [246]. However, in a similar cohort of subjects with a similar study duration but using a higher dose of Simvastatin (20mg), Hwu *et al.* did not find any difference in either triglycerides or insulin-mediated glucose disposal as assessed by clamp methodology [248]. In contrast to the aforementioned studies, Ohrvall *et al.* [242] found that in diabetics with hyperlipidaemia, 10 mg of Simvastatin daily increased insulin concentrations by 21% and insulin resistance by 28% as assessed by intravenous glucose tolerance test (IVGTT) and glucose clamp, despite a reduction in VLDL triglycerides [252]. Jula *et al.* also reported a deterioration in fasting insulin and insulin resistance with 20 mg Simvastatin therapy in a randomized, placebo-controlled, crossover study of hypercholesterolemic subjects [252].

A large, primary prevention WOSCOP study provided data indicating that treatment with Pravastatin resulted in a significant, 30% reduction in the onset of new diabetes, suggesting that this statin may have a protective role against development of diabetes. More recently, during an intensive treatment with Rosuvastatin in the JUPITER trial there was observed a small but significant increase in HbA1c and physician reported diabetes rates [228]. Two independent meta-analyses of 6 and 13 clinical trials showed that with the exception of Pravastatin in WOSCOP trial, statins in general tend to increase risk of developing diabetes [253, 254]. A meta-analysis of statin impact on insulin sensitivity in 16 studies also pointed out that only treatment with Pravastatin improved insulin sensitivity, whereas with the other statins there was a trend in increased risk of developing diabetes [249]. It is plausible

that lipophilic statins increase insulin resistance while hydrophilic statins have an opposite effect [247]. Interestingly enough, despite opposite metabolic effects, both Pravastatin and Simvastatin have been reported to improve flow mediated dilatation [247], which could indicated that statins may reduce microvascular complications in diabetes.

### 1.6.5 Atorvastatin

Atorvastatin is one of the most efficacious statins and has been available on the market for clinical use for several years. The proposed mechanism of Atorvastatin metabolism is through cytochrome P450 3A4 hydroxylation and the formation of active metabolites. Although Atorvastatin is lipophilic by nature it has low systemic bioavailability [255] which is the main reason for its good safety record and low incidence of side effects. Atorvastatin, similarly to other statins, reduces LDL-cholesterol and triglycerides, and increases HDL-cholesterol concentration, although at the maximum dose of 80mg daily it has less effect of raising HDL-cholesterol than other statins [255]. It has also been credited with beneficial effects on other aspects of the cardio-metabolic phenotype beyond the typical modification of lipid profile. It reduces LDL particle concentration and Apo B levels [256]. Atorvastatin has been reported to lower hs-CRP concentrations in states of chronic metabolic inflammation, such as atherosclerosis or metabolic syndrome [233, 257]. Orr *et al.* and Karter *et al.* independently demonstrated improvements in arterial stiffness following treatment with Atorvastatin [258, 259] in obesity and prediabetic states. There have been several large clinical trials (ASCOT –ALL, IDEAL, TNT, GREACE, etc) outlining the benefits of high dose Atorvastatin treatment in terms of its efficacy, cardiovascular outcomes and safety record, which may confer advantages over some other statins [255, 260]. Atorvastatin comes in four different doses of 10, 20, 40 and 80mg, and its efficacy increases with increasing dose. Although 80mg of Atorvastatin is the most potent daily dose, there is only a small difference between this and a 40 mg dose with regard to lipid-lowering or cardiovascular effects. However, the maximum dose is associated with higher rates of drug discontinuation. This is because the rate of side effects and potential drug interactions increases in dose dependent manner.

## 1.7 Study hypothesis

The worldwide overwhelming epidemic of obesity is strongly associated with cardiovascular morbidity and mortality, and type 2 diabetes mellitus. The body of evidence collected over the last decades suggests that the visceral obesity is a cardio-metabolic entity interconnected with insulin resistance, microvascular dysfunction and cardiorespiratory fitness but their interactions and relationships require still further detailed investigation. Likewise, there have been several reports on pleiotropic effects of statins used routinely to treat dyslipidaemia, often in the obese subjects, but the effects of statins on microvascular function and insulin sensitivity warrant further research and clarification. The aims of this research study were to investigate in a cohort of viscerally obese but otherwise healthy subjects skeletal muscle microvascular function, in particular exchange/filtration capacity, microvascular blood flow and functional dilator capacity, and insulin sensitivity and to explore their relationships and the relationship with other cardio-metabolic risk factors; to evaluate degree of insulin resistance in non-diabetic but centrally obese subjects and the effects of six months treatment with high dose statin on both insulin sensitivity and microvascular function; to conduct cross sectional assessment of the cardiorespiratory fitness, cardiac diastolic function and arterial stiffness and their relationships in the centrally obese with cardio-metabolic risk factors.

The postulated hypotheses of this study were:

- skeletal muscle exchange/filtration capacity affects levels of $HbA_{1c}$
- decreased microvascular exchange/filtration capacity is associated with reduced skeletal muscle insulin sensitivity
- insulin sensitivity and age are independently associated with microvascular functional dilator capacity
- insulin sensitivity and microvascular dysfunction are improved with six months of treatment with 40 mg of Atorvastatin
- there is independent association between cardiorespiratory fitness and measures of cardiac diastolic function and arterial stiffness

# 2. Study Design

In accordance with research governance the scientific rationale for this study was accepted by the peer review committee. The research project was funded by an educational grant from Pfizer which provided both Atorvastatin and placebo for the study

## 2.1 Ethics approval

This study had the approval of the Southampton General Hospital Research Ethics Committee (LREC05/Q1704/38). It was conducted in accordance with the Declaration of Helsinki [261]. All participants were unpaid volunteers and gave written informed consent.

## 2.2 Research Facility

All metabolic and vascular studies took place on the premises of the Wellcome Trust Clinical Research Facility (WTCRF), based within Southampton General Hospital. This is a modern, state of art facility dedicated for researchers and their volunteers. It is staffed with a team of trained and experienced research nurses and support staff. The unit is equipped with, amongst other things, a ward for conducting metabolic studies, clinical rooms, and environmental, physiology and preparation laboratories. The preparation laboratory allows immediate on site centrifugation, separation, freezing and storage of collected samples. The Department of Densitometry and the Radiology Department, both based at Southampton General Hospital, conducted whole body DEXA scans and abdominal MRI scans.
All data collected during the study have been kept strictly confidential. All written data have been stored, for the period of 10 years from the end of the study, in the clinical trial secure area on Level D and the collected samples have been stored in the designated freezers at -80$^{o}$C in the secure area on Level A at the Institute of Developmental Sciences at Southampton General Hospital. The encoded data was analysed on the code protected computers.

## 2.3 Recruitment

The subjects were recruited through advertisement within Southampton General Hospital, which appealed for volunteers amongst both visitors and hospital and university staff. The enrolment of suitable participants was based on a screening visit. This consisted of a brief interview eliciting the relevant medical and drug history, resting blood pressure assessment, measurements of weight, height and waist circumference, and fasting blood sampling, which included lipid profile, glucose, thyroid, liver and renal function tests and full blood count. Diagrams in Figure 2.1 and 2.2 explain the overview of the study design.

**Figure 2.1 Diagram of study design**

Out of the 136 people who responded to the advertisement recruiting subjects to the study 46 did not wish to proceed to the screening phase and only 90 subjects attended a screening visit. Further 43 subjects dropped out either because they did not fulfill the study criteria or did not wish to proceed further. 47 subjects underwent baseline microvascular and metabolic investigation. 7 subjects declined further participation resulting in 40 subjects being randomised to treatment. 39 subjects completed the study.

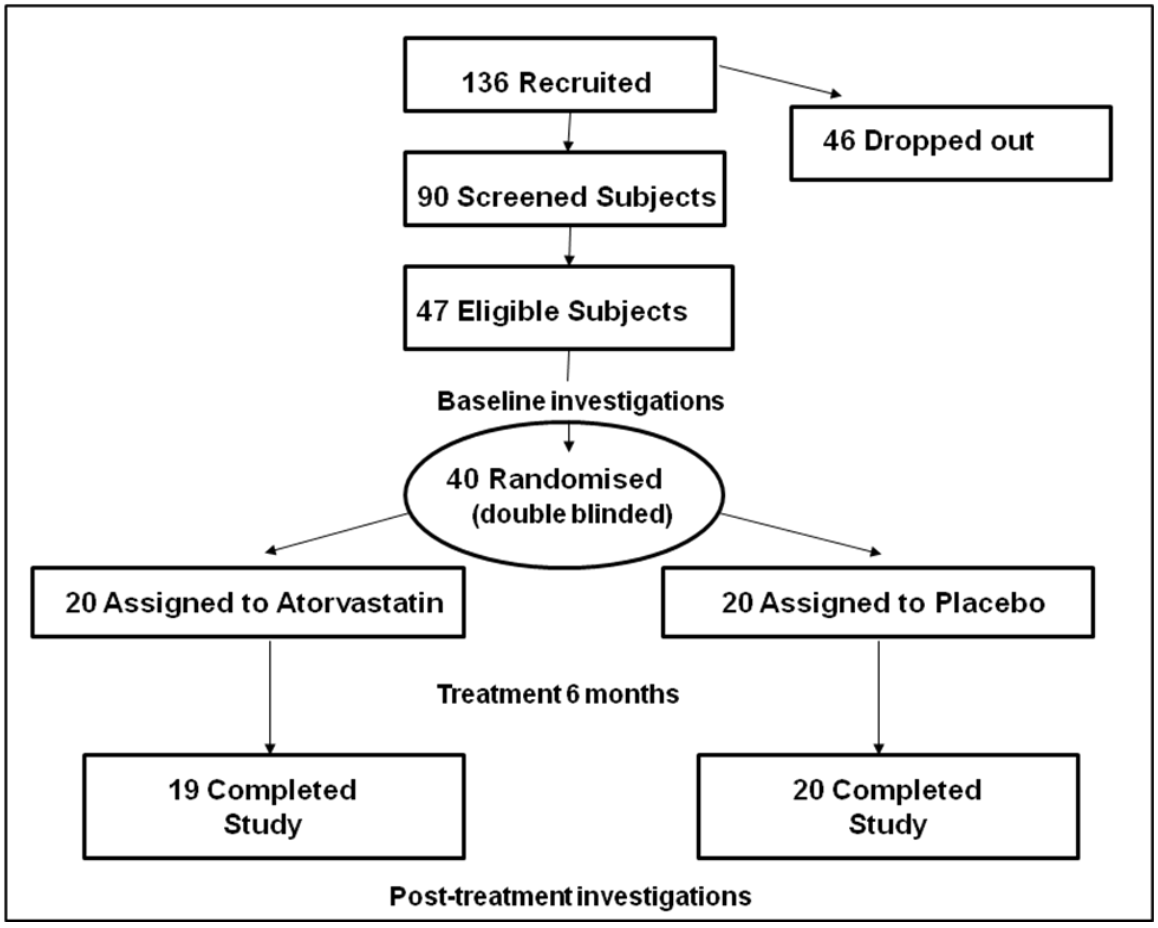
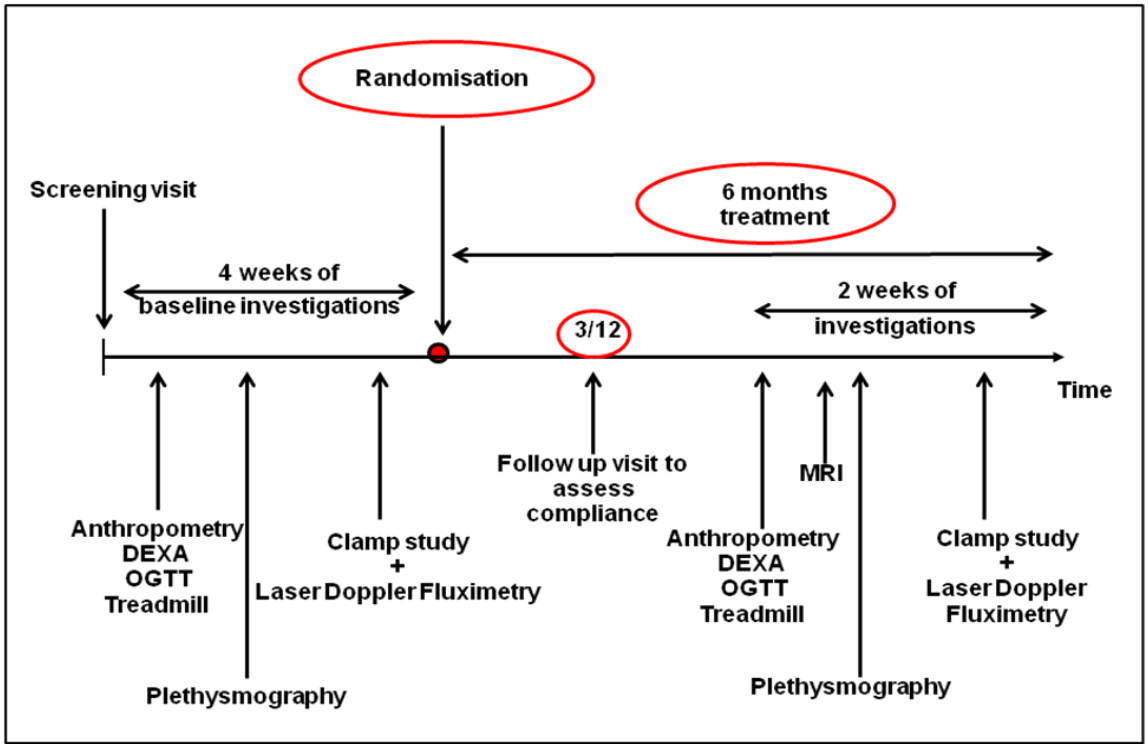


Figure 2.2 Diagram of investigations performed over the course of the study

After a screening visit, all study participants underwent anthropometric, microvascular and metabolic investigations, following which they were randomised (double blind fashion) to placebo or Atorvastatin. All subjects had to attend a follow up visit 3 months after starting treatment to assess their compliance. During the last 2 weeks of the trial, while subjects were still taking their treatment, they all underwent the same investigations as at the beginning of the study.



## 2.4 Subjects

These subjects were white Caucasian men and women aged 18-75 years within the catchment area of Southampton General Hospital and nearby GP surgeries to ensure a minimal drop out rate from the study. Owing to the small number of volunteers, and in order to keep the studied group relatively homogenous, we aimed for a similar number of participants from both genders. The population of Southampton consists mainly of Caucasians and therefore we did not investigate other ethnic groups.

### 2.4.1 Inclusion Criteria

White, Caucasian subjects aged 18-75 years at the time of recruitment were eligible for participation if they had central obesity defined as waist circumference ≥ 94cm for Europid men and ≥ 80 cm for Europid women and any of the other metabolic syndrome features according to International Diabetes Federation criteria [20]. For ethical reasons, subjects were only included into the study, if their estimated cardiovascular risk was less than 20% over 10 years based on the equation derived from the Framingham Heart Study [262]. This was because national guidelines recommend that people at high cardiovascular risk should be treated with statins for primary prevention [263, 264].

### 2.4.2 Exclusion Criteria

The subjects with known diabetes, renal, liver or uncontrolled thyroid disease, uncontrolled hypertension (blood pressure >160/100 mmHg) were excluded from the study. Treatment with antihypertensive medication (such as beta-adrenergic blockers, thiazides), corticosteroids, oral contraceptives or hormone replacement therapy (HRT) and treatment with lipid-modifying drugs within the previous three months served also as exclusion criteria. In order to ensure a low rate of possible treatment complications, subjects with previous history of muscle symptoms, elevated creatinine kinase (CK) or liver function tests while previously on statin therapy were not permitted to participate in the study. Only non-pregnant female volunteers of reproductive age were included in the study.

## 2.5 Medical Assessment

The anthropometric, metabolic and vascular studies were carried out over a course of several days before and after the treatment (Figure 2.2). This was because specific conditions were required to perform each metabolic study. For the screening visit and

thereafter for each metabolic study subjects were required to refrain for 48 hours from alcohol and strenuous exercise, and to fast for 12 hours beforehand. Midway through the study, (during a short visit to the research facility) all participants were assessed by a research nurse through direct questioning with regards to their well-being and compliance. The anthropometric measurements were performed before treatment as a screening tool for the study inclusion and after treatment to document them as possible confounders of results. One volunteer, who was found to be anaemic during the screening process, and another diagnosed with diabetes based on the results of oral glucose tolerance test, were excluded from the study. One participant who was stable on the replacement dose of thyroxine was allowed into the study.

## 2.6 Treatment

The selection of the statin and its strength was based on the considerations of efficacy and tolerability reflecting current clinical practice [255]. Several previous studies documented that the use of a more efficacious statin at a lower dose, rather than a higher dose of a weaker statin, leads to achieving the maximum efficacy with the least side effects.
After completing all baseline measurements and investigations the participants were randomised to either daily Atorvastatin at a dose of 40 mg or placebo, which they took for 26 weeks (Figure 2.2). This was a double-blind, parallel group study design to eliminate possible selection bias and to assess possible pleiotropic effects of Atorvastatin in the most objective way. The randomisation was performed by an independent pharmacist based at the Southampton General Hospital.
There is evidence that weight reduction of 5-10% body weight benefits metabolic traits and health [265, 266]. Intentional or unintentional weight loss could potentially affect the end results of the study. We therefore encouraged all participants to maintain the same diet and lifestyle throughout the whole duration of the study to avoid changes in total body weight greater than 5 % from the baseline to minimise the effect of those confounders on the end results.

## 2.7 Statistical analysis

All statistical analyses were performed using SPSS for Windows version 16.0 (SPSS, Chicago, IL, USA). Means and SD were presented for normally distributed data and medians and ranges were presented for non-normally distributed data. Student's *t* test comparisons were undertaken to compare mean values of normally distributed data. Repeated measurements ANOVA was applied to test for the group effects. Pearson correlation coefficients were presented for univariate regression analyses of normally distributed data and Spearman correlation coefficients were presented for non-normally distributed data. Multivariate linear regression models were developed to describe factors that were independently associated. A $p$ value of $<0.05$ was considered statistically significant for all analyses. The analysis of statistical power of the part of the study that was looking into the statin treatment effects on insulin sensitivity was based on the study of Paolisso *et al.* [245] who demonstrated significant improvement in glucose disposal using the hyperinsulinaemic clamp technique ($p<0.05$) in a small sample size of 12 subjects. It was calculated that to provide 80% power with an alpha of 0.05 between Atorvastatin and placebo groups for M-value (insulin-mediated glucose disposal) the estimated sample size of two ( n=2) for each group was needed (alpha 0.05, power 80%, M1=26.3, M2=19.5, SD1=2.08, SD2=1.73). The fact that data of Paolisso *et al.* [245] related to the elderly subjects with type 2 diabetes and that it was a cross-over and not a parallel study required caution in the extrapolation of their results in to the subjects with obesity and metabolic syndrome phenotype. Therefore it was estimated that a sample size of thirty (n=30) for each treatment group was possibly required. However, this was not possible to achieve because of study time scale design and problems with subjects recruitment and drop outs. Additionally, a retrospective sample size calculation was undertaken for the detection of changes in reactive hyperaemia to hyperinsulinaemia during a glucose clamp. It was calculated that to detect a 2.3 fold improvement in the reactive hyperaemia in response to hyperinsulinaemia with a power of 90% and alpha of 0.05, 12 subjects were required; this was the same number of subjects as in the study reported by de Jongh *et al.* [140] that showed 2.3 fold increase in reactive hyperaemia to hyperinsulinaemia in the tibialis anterior muscle.

# 3. Methods

## 3.1 Anthropometric assessment

All volunteers had their anthropometric assessment conducted using standard procedures.

### 3.1.1 Weight

Weight was determined using Seca electronic scales (Germany) to the nearest 0.1 kilogram (kg). The participants were weighed while wearing lightweight clothing (shirt and trousers) and no shoes. They were asked to stand in the middle of the scale and remain motionless until the measurement was completed.

### 3.1.2 Height

The height was determined using a Seca 220 stadiometer. The measurements were made in meters to the nearest millimetre. All individuals were asked to remove their shoes and stand as straight as possible with both feet flat on the floor, heels together and toes pointing out, and arms held loosely at the sides. The buttocks, shoulder blades and the back of the head were in contact with the vertical board. The head was placed in the Frankfurt plane, which is a horizontal line, parallel to the floor, joining the ear canal with the lower border of the eye's orbit. Once they were correctly positioned, the head board was positioned firmly on top of the head and the reading of the height was taken [267, 268].

### 3.1.3 Body Mass Index

Body Mass Index (BMI) was used as in indicator of subjects' degree of obesity. This index is frequently implemented to estimate prevalence and risks associated with obesity according the World Health Organisation categories [269]. Body mass index was calculated with the weight (kg) divided by the height squared ($m^2$): weight /height$^2$.

### 3.1.4 Waist circumference

Anatomical waist circumference, which is an approximate index of intra-abdominal fat mass and total body fat, was measured against bare skin. Each subject was asked to stand straight, with arms at the sides and relaxed abdomen. The tape was applied in a horizontal plane around the abdomen, halfway between the upper iliac crest and lower rib margin. One measurement was recorded to the nearest 0.1 centimetre (cm) at the end of normal expiration with the tape taut but not pressing the skin.

### 3.1.5 Dual Energy R- ray Absorptiometry

Dual X-ray Absorptiometry (DEXA) method was implemented to assess body composition i.e. fat and lean body mass and distribution. This accurate and precise technique is based on a three-compartment model that divides the body into total body mineral, fat-free soft (lean) and fat tissue mass, and uses two low dose x-rays placed at different sources to simultaneously read bone and soft tissue mass. The standard visual method is applied to divide the images into trunk, limbs and head. Dexa has been widely used to assess the whole body and truncal adiposity [270]. We used DEXA HOLOGIC Delfia W 4500 (Hologic Inc, USA) which has CV of 0.68%. Two experienced technicians from the bone densitometry department performed the measurements. Participants were asked to lie down still on a scanning table, while the scanner passed across their body collecting data at 0.5cm intervals.

### 3.1.6 Magnetic Resonance Imaging

Magnetic Resonance Imaging (MRI) was used to measure abdominal visceral adiposity. This is a validated and very safe technique because it does not use ionising radiation and at the same time it provides high-quality images of fat amount and its distribution [271, 272]. We used Siemens 1.5 T Symphony MR Scanner (software release 4VA15A, Siemens AG, Erlangen, Germany) to take axial images of subjects placed in the supine position. Since we wanted to obtain more detailed information about visceral fat than a conventional, single slice would demonstrate, we acquired 5 non-contiguous slices extending from 5 cm below to 15 cm above the level of L4-L5 [41]. A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111ms, TE = 4.18ms, flip angle = $70^{o}$, slice width = 10 mm, slice spacing = 50 mm) was used to obtain T1 weighted images. Regions of subcutaneous and visceral fat within the cross-sectional abdominal images were identified using a proprietary analytical software package (Mimics, Materialise NV, Belgium). A threshold level for fat pixels was set after examining the histogram of pixel values in each image. Fat tissue was isolated from other tissues in the image with the help of a seed-growing technique, which allows selection of neighbouring pixels of similar values (i.e. within the identified threshold. The areas of subcutaneous fat and visceral fat were calculated, and compared with total cross sectional area [273]. Adipose tissue volume was converted to mass in kilograms using a density of 0.92 for adipose tissue [274].

## 3.2 Blood pressure assessment

Blood pressure was determined using the Omron 705CP blood pressure monitor. The same measurement protocol was followed for the screening visit and each formal vascular assessment performed before and after treatment.

Blood pressure was measured after the participants had acclimatised with the Clinical Research Facility and rested quietly for at least 5 minutes before the actual measurement. The blood pressure was recorded with subjects in a sitting position, on a non-dominant arm. Three measurements separated by 2 minutes intervals were made. They were used for calculation of mean systolic and diastolic blood pressure.

## 3.3 Laboratory techniques

### 3.3.1 Lipid profile

Fasting lipids estimations were used to assess subjects' cardiovascular risk, define metabolic traits and assess response to treatment. Venous blood samples were collected into Lithium Heparin tubes using the Vacutainer system. They were sent immediately to the routine biochemistry laboratory at Southampton General Hospital. There, samples were centrifuged, separated and plasma was analysed on a Beckman Coulter DxC automated analyser using enzymatic colorimetric methods. Owing to practical reasons, samples were not batched for analysis.

#### 3.3.1.1 Total cholesterol

A timed-endpoint method was used to determine concentration of total cholesterol. In the reaction, cholesterol esters are hydrolyzed in the presence of cholesterol esterase to free cholesterol and fatty acids. Free cholesterol is then oxidized to cholestene-3-one and hydrogen peroxide. The latter reacts in the presence of peroxidase with 4-aminoantipyrine and phenol to produce a coloured complex. The change in absorbance measured at 520 nm is proportional to the cholesterol concentration. The "day to day" CV was 1.7% at 5.7mmol/L and 1.7% at 10mmol/ L cholesterol control.

#### 3.3.1.2 HDL-cholesterol

HDL-cholesterol assay utilized a detergent that inhibits LDL, VLDL and chylomicrons reaction with the cholesterol enzymes while allowing release of HDL cholesterol by cholesterol esterase. HDL is then oxidised to cholestenone and hydrogen peroxide, which reacts with 4-Aminophenazone and N, N-bis (4-sulphobutyl)-m-toluidine-disodium (DSBmT chromogen) to produce a quioneimine pigment, similar to that described above for total cholesterol. The change in the absorbance measured at 560 nm is directly proportional to the concentration of HDL-cholesterol in the sample. The "day to day" CV was 2.1% at 1mmol/L and 2.9% at 3mmol/L HDL-cholesterol controls.

#### 3.3.1.3 Triglycerides

Triglycerides assay was based on the generation of hydrogen peroxide and quinoneimine in a timed endpoint method. Lipoprotein lipase hydrolyzes triglycerides to free fatty and glycerol. The latter is converted by glycerol kinase to glycerol-3-phosphate, which is then oxidised to dihydroacetone and hydrogen peroxide. A red quinoneimine dye is produced in

the final reaction between hydrogen peroxide 3,5-dichloro-2-hydroxybenzenesulfonic acid (DHBS) and 4-aminoantipyrine in the presence of horseradish peroxidase. The change in absorbance measured at 520nm is proportional to triglyceride concentration. The "day to day" CV was 6.3% at 1mmol/L and 5.9% at 5mmol/L triglyceride control.

#### 3.3.1.4 LDL-cholesterol

The Friedewald equation [275] was used to calculate LDL-cholesterol concentration providing the triglycerides concentration was ≤ 4.0mmol/l as per hospital's current laboratory practice:

LDL-cholesterol = Total cholesterol – HDL-cholesterol – (Triglycerides÷2.2)

### 3.3.2 Glucose

Glucose results were used for screening, and to define metabolic traits and insulin sensitivity status. Venous blood samples at screening and during the OGTT were collected into Fluoride Oxalate tubes whereas arterialised venous blood samples during hyperinsulinaemic clamp were collected into both Fluoride Oxalate and Lithium Heparin tubes. Screening samples were sent immediately to the routine laboratory where they were processed and analysed on the Beckman DxC. The Fluoride Oxalate samples from OGTT and clamps were placed on ice and centrifuged for 10min at 1500 x g within 1 hour from collection. The plasma was separated and frozen at -80$^{o}$C. They were analysed after thawing on the Beckman Coulter DxC automated analyser in a total of 4 and 5 batches respectively. Beckman DxC analyser utilises the hexokinase method for glucose estimation. In this method, glucose is phosphorylated to glucose-6-phosphate in the presence of ATP, hexokinase and $Mg^{2+}$. Glucose-6-phosphate dehydrogenase reacts then with $NAD^{+}$ to form NADH and 6-phosphogluconate. NADH-induced increase in absorbance at 340nm is directly proportional to glucose concentration in the sample. Baseline and post treatment samples from the same participant were analysed in singleton and in the same batch. The "day to day" CV was 2.8% at 2.5mmol/L and 1.3% at 20mmol/L glucose control.

The Lithium Heparin samples from the clamp were immediately centrifuged for 10 seconds at 19500 x g and the plasma glucose was immediately analysed by the bedside on the YSI 2300 STAT analyser (Yellow Springs Bioanalytical Products). The YSI 2300 STAT uses membrane-bound enzyme electrode methodology [276]. Glucose is oxidised to gluconic acid and hydrogen peroxidase in the presence of glucose oxidase, which is immobilised in a thin membrane between polycarbonate and cellulose acetate layers.

Hydrogen peroxidase then diffuses through a cellulose acetate membrane and is oxidised at a platinum electrode. The generated current is directly proportional to the glucose concentration. The intra-assay CV was 0.2% at 5mmol/L glucose control and 0.3% at 10mmol/L glucose control. The inter-assay CV was 2.5% at the 5mmol/L glucose control and 2.1% at 10mmol/L glucose control.

### 3.3.3 Insulin

Insulin results were used to calculate insulin sensitivity indices. The blood samples were collected into Lithium Heparin tubes during hyperinsulinaemic clamp. They were immediately placed on ice and within 1 hour from collection, centrifuged for 10 min at 1500 x g, separated and plasma frozen at -80$^{o}$C. The sets of pre- and post- treatment samples from each volunteer were analysed in the same batch to minimise the bias from inter-assay variability. The analysis took place in the endocrine section of the biochemistry laboratory in Southampton General Hospital.

The intact Insulin was measured using the dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) which is a solid phase sandwich immunoassay that uses time-resolved fluorometry. A microplate is coated with HUI-018 monoclonal antibodies directed against a specific site on Insulin, which immobilises on the plate after a sample addition. The biotinylated OXI-005 anti-insulin antibody is then added. It is directed to a different Insulin epitope and attaches itself to the solid phase antibody-antigen complex. It then binds to the added Europium-labelled streptavidin. After the addition of each reagent, there is a wash cycle to remove any unbound substances and potential interferents. The next step involves adding the enhancement solution to dissociate Europium ($Eu^{3+}$) from the solid phase bound Europium-labelled antibodies to form a homogenous and long-lived, highly fluorescent Eu-(2-NTA)$_3$(TOPO)$_{2-3}$ chelate solution. Multiple readings are then taken by a time-resolved fluorometer using Wallac 1420 multilabel counter. Europium fluorescence is proportional to the insulin concentration.

All standards, Quality Controls (QC) and samples were analyzed in duplicate. The intra-assay CV was 3.3% at 2mIU/L, 1.4% at 16mIU/L, 1.5% at 55mIU/L and 1.1% at 149mIU/L insulin control. The inter-assay CV was 36.6% at 2mIU/L, 12.3% at 16mIU/L, 8.8% at 55mIU/L and 7.4% at 149mIU/L insulin control.

### 3.3.4 Glycated haemoglobin

Glycated haemoglobin ($HbA_{1c}$) represents glucose concentration to which erythrocytes are exposed during their lifespan. Clinically it is a useful index of mean blood glucose concentration over a period of 16 weeks. $HbA_{1c}$ results were used to investigate its association with microvascular dysfunction. $HbA_{1c}$ was determined by a high pressure liquid chromatography assay using a cation exchange cartridge on Bio-Rad Variant II Turbo (Bio-Rad Laboratories, Irvine, CA, USA). The method was aligned with the Diabetes Control and Complications Trial (DCCT). The intra-assay coefficient of variation (CV) was 2.5% and the inter-assay CV was 3.4%

### 3.3.5 Biochemical markers of endothelial function

Biochemical markers of endothelial function were used to assess a proinflammatory state in relation to microvascular function. Fasting blood samples were collected into SST tubes for hs-CRP, ICAM-1, IL-6 and Adiponectin. The samples were placed on ice and centrifuged for 10min at 1500 x g within 1 hour from collection. The serum was separated and frozen at -80$^{o}$C. Early morning urine samples were collected into plain containers for Albumin to Creatinine Ratio (ACR). They were frozen at -80$^{o}$C. All samples were analysed after thawing at the end of the study. Blood samples for hs-CRP and urine samples for A/C Ratio were analysed in the Southampton General Hospital routine laboratory whereas the samples for the other inflammatory markers were analysed by Debbie Smith in the laboratory of Endocrinology and Metabolism Department at the University of Southampton.

#### 3.3.5.1 High sensitivity - C reactive protein

High sensitivity C reactive protein (hs-CRP) concentration was determined on the Beckman Coulter DxC automated analyser by the highly sensitive near infrared particle immunoassay rate methodology. This assay can measure CRP concentration at extremely low levels thus allowing detection of very early inflammatory states and responses. An anti-CRP antibody-coated particle binds to CRP resulting in the formation of insoluble aggregates that cause increased turbidity. The change in absorbance measured at 940nm is proportional to CRP concentration in the sample. The assay has within run CV at the level of 0.07mg/dL of 3.9% and total CV of 5.1%.

### 3.3.5.2 Albumin to Creatinine Ratio

Microalbumin concentration in urine was measured on the Beckman Coulter DxC automated analyser by a turbidimetric method [277]. In the reaction, a specific anti-albumin antibody binds to albumin present in the urine and forms insoluble albumin-antibody complexes. The change in absorbance measured at 380nm is proportional to albumin concentration in the sample. The estimated within-run CV is 8.7% at 1mg/dL and 3.7% at 3mg/dL.

Creatinine concentration was determined on the Beckman Coulter DxC automated analyser using the modified rate Jaffé method [278, 279]. In an alkaline solution, creatinine directly reacts with the picric acid and forms an orange-red creatinine-picrate complex. The change in absorbance measured at 520nm is directly proportional to creatinine concentration in the sample. The estimated within and total CV for the method is 1.4% at 91 mmol/dL and 1.5% at 244 mmol/dL.

The albumin/creatinine ratio (ACR) is calculated by dividing microalbumin concentration expressed in mg/dL by concentration of creatinine expressed in mmol/dL, which provides the ratio result expressed in mg/mmol creatinine.

### 3.3.5.3 Soluble Intercellular Adhesion Molecule-1

Soluble Intercellular Adhesion Molecule-1(sICAM-1) was determined using ELISA kit from R&D Systems Europe, Ltd. (Abingdon, UK). The assay employs quantitative sandwich enzyme immunoassay technique. A microplate is pre-coated with a monoclonal antibody specific for sICAM-1. 100 µl of a conjugate and 100 µl of standards, samples or controls (two latter pre-diluted) are pipetted into the wells and incubated at room temperature for 1.5 hours. Any sICAM-1 present is sandwiched by the immobilized antibody and an enzyme-linked polyclonal antibody specific for sICAM-1. Any unbound substances and/or antibodies are washed out and a substrate solution is added to the wells, and incubated for 30minutes. When colour develops a stop solution is added and the colour is read using a microplate reader set at 450 nm. The colour is proportional to the concentration of sICAM-1 which is read of the standard curve based on the optical density plotted against concentration of each standard. The intra-and inter assay CVs were <10%.

### 3.3.5.4 Interleukin-6

Interleukin-6 (IL-6) concentration was measured using high sensitivity Quantikine ELISA kit from R&D Systems Europe, Ltd. (Abingdon, UK). It employs quantitative sandwich enzyme immunoassay technique. A microplate is pre-coated with a monoclonal antibody specific for IL-6. 100 µl of samples, standards or controls pipetted into the wells are

incubated at room temperature for 2 hours to allow binding of any IL-6 present to the immobilized antibody. Any unbound substances are then washed away and an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells and incubated for further 2 hours. After another cycle of washes a substrate solution is added to the wells, incubated for an hour following which an amplifier solution is added to initiate colour development. 30 minutes later a stop solution is added and the colour is read using a microplate reader set at 490 nm. The colour is proportional to the concentration of IL-6 which is read of the standard curve based on the optical density plotted against concentration of each standard. The intra-and inter assay CVs were <10%.

### 3.3.6 Non-Esterified Fatty Acids

Non-esterified fatty acids (NEFA) concentration was measured to assess adipose tissue insulin sensitivity. The arterialised venous blood samples were collected into Lithium Heparin tubes during a euglycaemic clamp. The samples were placed on ice and within 1 hour from collection centrifuged for 10 min at 1500 x g, separated and the plasma frozen at -80$^{o}$C. All samples were analysed after thawing. Samples were analysed (by a technician in the Nutrition laboratory at Southampton General Hospital) on Konelab20xTi automated analyzer with Wako NEFA C test kit. NEFA were determined using an enzymatic colorimetric method based on the acylation of Coenzyme A by fatty acids in the presence of added acyl-CoA synthetase. Formed in this way acyl-CoA is oxidised to 2,3-trans-Enoyl-CoA and hydrogen peroxide, which participates in the oxidative condensation of 3-methy-N-ethyl-N-aniline with 4-aminophenazone. The intensity of this reaction product colour is measured colorimetrically at 550nm and is proportional to the concentration of free fatty acids. Grossly haemolysed samples were excluded from analysis. The intra-assay CV was 2.6% and inter-assay CV was 4.3%.

### 3.3.7 Lactate

Throughout the clamp the arterialised venous blood samples were collected at specific time points for the lactate measurements. This was performed to document whether the thigh cuff inflations resulted in any significant level of muscle ischaemia that would be easily identifiable by increased lactate levels, which could affect muscle perfusion and glucose uptake. The blood samples collected into the Lithium Heparin tubes were placed on ice and within 1 hour from collection centrifuged for 10 min at 1500 x g, separated and plasma frozen at -80$^{o}$C. The plasma was analysed (by researcher and a technician) on the

Radiometer ABL 725 blood gases analyser, which employs special biosensors and the lactate oxidase method. This method is based on the lactate conversion to pyruvate and hydrogen peroxide in the presence of lactate oxidase. Electrons released during oxidation of hydrogen peroxide are proportional to the concentration of the lactate in the sample. “Day to day” CV was 2.6% at 1.7mmol/L lactate control.

## 3.4 Indices of insulin sensitivity / resistance

All tests employed for the estimation of the peripheral and the whole body insulin sensitivity and resistance were carried out at 8:00am in most instances, and in the few exceptional cases at 9:00am. All subjects were asked to abstain from alcohol and strenuous exercise for 48 hours and to fast for 12 hours before each assessment took place at WTCRF.

### 3.4.1 Oral Glucose Tolerance Test

On arrival at the WTCRF each volunteer was seated comfortable in a chair and the intravenous cannula was inserted into the vein of one arm to obtain baseline venous blood samples for the measurement of fasting glucose and insulin. After the cannula insertion and baseline blood sampling, participants were asked to drink a loading dose of 75 glucose solution over one minute. This solution was made of 121ml of Polycal (Nutricia, Netherlands) diluted in 69ml of cold water to give a drink of the total 200ml volume. The next blood sample was taken at 30, 60 and 120 minutes later. Both samples were processed and analyzed as described above in chapter 3.3.2. Glucose results from both samples were used to define glucose intolerance according to WHO criteria [118].

### 3.4.2 HOMA-IR and QUICKI

The results of glucose and insulin concentrations, which were obtained from the fasting blood samples taken at the beginning of OGTT, were implemented in the mathematical models of insulin sensitivity. HOMA-IR and QUICKI were calculated using the following formulae:

$$\text{HOMA-IR} = (I_0 \times G_0)/22.5$$

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

where $I_0$ represents fasting insulin concentration in mIU/l and $G_0$ represents fasting glucose concentration in mmol/L.

### 3.4.3 Hyperinsulinaemic euglycaemic clamp

A stepped-hyperinsulinaemic glucose clamp was used to assess whole body and adipose tissue insulin sensitivity [127, 280] at baseline and after intervention while the subjects continued with their study medication. This technique has been widely employed in human

studies investigating the pathophysiology of insulin resistance in obesity, metabolic syndrome or diabetes and it has had an excellent safety record [281].
On arrival at the WTCRF each volunteer was weighed as previously described and asked to take a comfortable, sitting position on a bed. In the same arm there were two intravenous catheters inserted. The first cannulation was performed on hand or wrist using a retrograde technique and the 20-gauge catheter. The hand was warmed up to 50◦C in a hotbox. This improved the oxygenation of the venous blood thus providing so-called "arterialised" venous blood samples [282]. The level of oxygenation was checked at 2-3 time points throughout the clamp by measuring the blood gases. The arterialised venous blood samples were used for the measurements of glucose, insulin, NEFA and lactate concentration. The blood samples for glucose measurements were taken at 5 minute intervals throughout the clamp, centrifuged and plasma was analysed at the subject's bedside using the YSI 2300 STAT as described in chapter 3.3.2. The blood samples for insulin, NEFA and lactate measurements were taken at baseline, 90, 120 minutes and at 10 minutes intervals during the last 30 minutes of each (low and high dose) insulin infusion (Figure 3.1), separated and frozen at -80◦C for analysis as described in previous chapters. The patency of the retrograde intravenous catheter was maintained with 0.9% Normal Saline infusion at a rate of 1 drop per minute.

The 18-gauge catheter was placed in the antecubital fossa, which was used for the exogenous insulin infusion. Human insulin (Actrapid, Novo Nordisk) was given at a rate 0.2mIU/kg/min for one hour followed by 7-minutes of stepped priming infusion and then the continuous insulin infusion at a rate of 1.5mIU/kg/min for two hours. The insulin infusion was prepared in 0.9% NaCl to which 1 ml of subjects' blood was added to prevent adsorption of insulin to the plastic surface of the syringe walls and administered with the syringe driver Syramed usp 6000. Plasma glucose measurements obtained at 5-minute intervals by YSI 2300 STAT, formed the basis for the adjusting the rate of 20% Dextrose infusion (using the variable-speed infusion Baxter pump) to maintain the blood glucose concentrations at the level of about 5mmol/l.

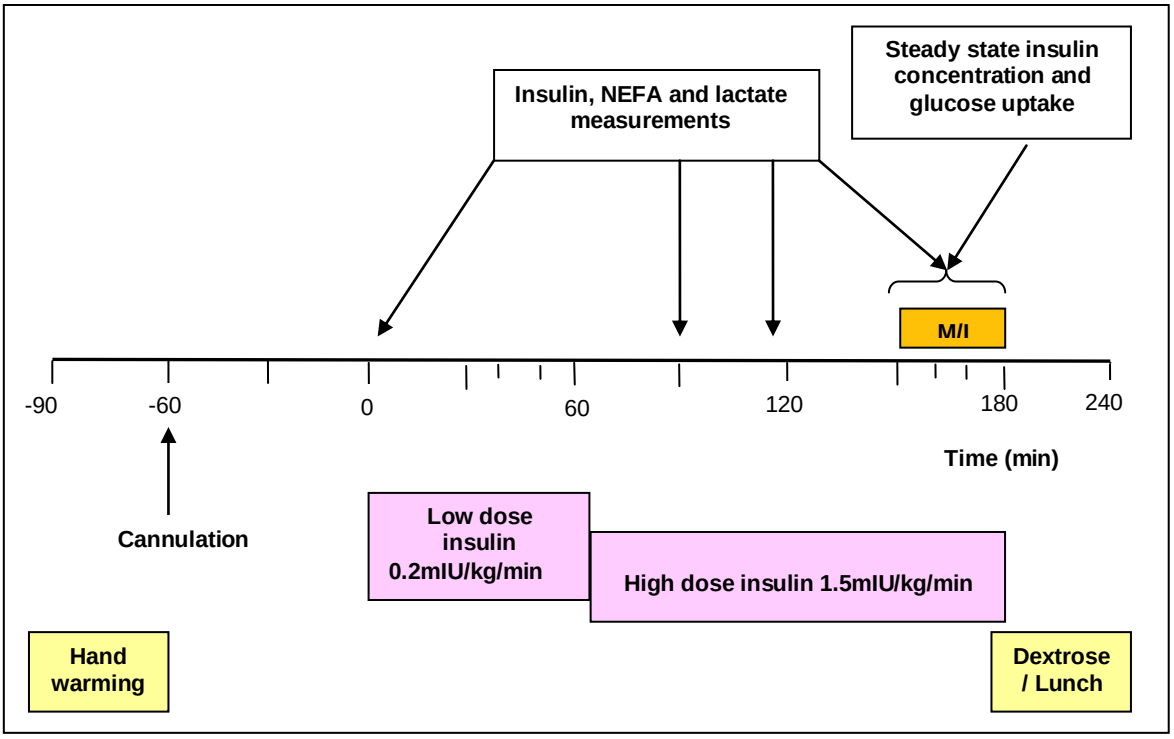
Following the venous cannulation subjects were allowed one hour to adjust to the new environment before the insulin infusion was commenced. At the end of the clamp procedure, the 20% Dextrose infusion was slowly tailed off over about 30 minutes while the subject had lunch to avoid hypoglycaemia. The whole-body insulin-mediated glucose disposal rate (M-value) was estimated from the total amount of glucose infused during the last 30 min of the clamp when steady state insulin concentration had been achieved. M value was expressed in milligrams per body kilogram per minute. The mean of the four

insulin concentration results from the blood samples taken at 150, 160, 170 and 180 minutes determined the steady-state insulin concentration (I value, expressed in milliunits / litre). M value divided by I value defined the insulin sensitivity index (M/I) expressed as m·L/kg of body weight/minute/mIU.

The adipose tissue sensitivity to low dose (0.2mIU/L) insulin infusion was calculated as the percentage of change between the mean NEFA concentration at baseline and after 60 minutes of low dose infusion.

**Figure 3.1 Diagram of stepped hyperinsulinaemic euglycaemic clamp**

Hand warming allows retrograde cannulation which is followed by one hour period of adjustment to a new environment followed by one hour of low dose insulin infusion and two hours of high dose insulin infusion. During the last 30 minutes of high insulin dose infusion, the steady state is reach which allows estimation of M-value and mean insulin concentration (I) which are used to calculate insulin sensitivity index M/I.



## 3.5 Fitness and physical activity

### 3.5.1 Assessment of fitness

Cardiorespiratory fitness of each volunteer was measured in terms of maximum volume of oxygen ($VO_2$ max). It was determined by using a maximal-grading treadmill test and the Cortex Metalyser II (Cortex Biophysics GmbH, Germany). Subjects were asked to avoid strenuous exercise and alcohol for 48 hours beforehand. They were explained the procedure but no practice treadmill was undertaken beforehand. Participants were fitted with the air-tight facemask for gas analysis, blood pressure monitor on a non-dominant arm and a Polar® Hear Rate monitor. After a short period of acclimatisation to the facemask and baseline measurements subjects were asked to perform an incremental treadmill test. The test commenced at 1.3m/s (3mph) at a 0% gradient. It was increased every 2 minutes by either 2% gradient or speed of 0.25m/s (0.5mph), for example: stage 1= 1.3m/s and 0% gradient, stage 2= 1.3m/s and 2% gradient, stage 3= 1.55m/s and 2% gradient, stage 4= 1.55m/s and 4% gradient, etc. Heart rate was monitored continuously and blood pressure was measured at the time of each treadmill adjustment. Unless subjects felt unwell or experienced chest pain or had a drop or excessive rise in blood pressure over 250mmHg, they were asked to continue until exhaustion and until they reached a respiratory quotient over 1.1 and 90% of their target hear rate as determined by formula: 220-age. All subjects completed the treadmill, which was terminated only for the reasons of volitional fatigue. Oxygen uptake and carbon dioxide production was analysed breath-by-breath using metabolic gas analyser system Metalyser II.

### 3.5.2 Assessment of physical activity

Physical activity of each participant was measured in terms of Physical Activity Energy Expenditure (PAEE), Metabolic Equivalent of Task (MET) which is a metabolic equivalent of Kcal/hr/kg. It was assessed using a validated activity monitor SenseWear Armband Pro2 with a software version 6.1 (Bodymedia Interantional, Milan, Italy) [283-285]. This multisensor device contains two accelerometers detecting movement in two planes, a galvanic skin, temperature sensor and a near patient temperature sensors. The physiological body signals recorded by the sensors are used to calculate energy consumption based on predetermined algorithms incorporating free-living activity recognition patterns. The Armband can collect data for a period up to 2 weeks and store it, without the need of recharging the unit, until the transmission of data to the computer using USB connection and manufacturer's software. Before use, the Armbands Pro 2 were

individually programmed with details of each subject's weight, height, hand dominance and smoking status, and identification code. All subjects were asked to wear the armband continuously on the right arm, according to the manufacturers instructions, for a period of 7-10 days. This was to gain the most reliable estimate of mean PAEE for each individual during a typical week. Mean METs were estimated for a 24-hour period of the whole period the device was worn.

## 3.6 Assessment of cardiac diastolic function and arterial stiffness

Aortic Augmentation Index at 75 beats/min heart rate (Aix@75HR), ventricular Ejection Duration (ED) and Subendocardial Viability Ratio (SEVR) were estimated as measures of arterial stiffness and cardiac diastolic function using Pulse Wave Analysis (PWA). This is a non- invasive, based on applanation tonometry technique, which gained popularity in research studies on healthy volunteers because of restriction in the use of cardiac catheterization [286]. PWA has been validated for measurement of diastolic function in terms of myocardial perfusion relative to left ventricular workload which is expressed as SEVR [287, 288].

Pulse wave analysis was performed by a single observer, using SphygmoCor SCOR-PVx version 7.1 (AtCor Medical, Sydney, Australia). The blood pressure result checked immediately before the beginning of measurements and using the standard protocol (as described in the chapter 3.2) was input into the SphygmoCor system. This was to allow accurate computing of data collected during applanation tonometry. Each subject sat comfortably in a chair, with a non-dominant forearm on the table over a pillow for support and their palm facing upwards with wrist in the dorsiflex position. The operator's forearm was also rested on a firm surface to minimise any possible tremor interference. High-fidelity micromanometer-tipped probe with a frequency response of > 2 kHz (Millar Instruments, Houston, Texas) was placed on the wrist. The radial artery was lightly pressed until a consistent, good quality (large, moving across the screen in a steady vertical position) waveform was produced for at least 10 seconds before it was captured for analysis. Waveforms were processed using specialised software to calculate an averaged radial artery waveform and to derive a corresponding central aortic pressure waveform using a previously validated generalized transfer function [289, 290]. The quality index, which represents reproducibility of the waveform, was for all waveform recordings $\geq 95$.

## 3.7 Microvascular function studies

A novel and non-invasive approach was adopted to perform microvascular function studies. This was technically easier and allowed investigation of a larger cohort of subjects then the other currently available but invasive methods. The investigations were performed before and after 6 months of treatment with Atorvastatin.

### 3.7.1 Venous congestion plethysmography

Microvascular filtration capacity ($K_f$) and muscle blood flow were assessed using a venous congestion plethysmography system (Filtrass Angio, DOMED, Munich, Germany) with a passive inductive transducer of $\pm 5$ μm accuracy (Compumedics DWL, Singen, Germany). The investigator followed a small pressure step venous congestion protocol. The studies were conducted in a quiet room, at an ambient temperature of 20 $^{o}$C. Subjects were asked to remove the clothing from the right leg and lay still in a supine position throughout the study. Results of blood pressure checked in this position using standard protocol (as described in the chapter 3.2) were input to Filtrass Angio system. This was necessary for accurate data collection. The subject's right thigh was supported with one pillow and foot with two pillows so that the investigated calf was at the heart level and not touching the bed or covers (Figure 3.2). This was to minimize any possible interference during the recording. Each subject had their calf circumference measured at widest point to select appropriate strain gauge size. The Filtrass strain gauge sensor unit, with its inelastic nylon measuring line in a flexible guide holder, was placed around the calf at the point of maximum circumference and attached to the Filtrass sensor. A congestion cuff was wrapped around the ipsilateral thigh and coupled into the Filtrass unit for automatic inflation during the protocol. At the beginning of the protocol, the strain gauge automatically measured the calf circumference, adjusted its tension and then calibrated itself. Small, 10 mmHg cumulative increases in congestion pressure were applied, starting from zero to 60 mmHg. The maximum cumulative congestion pressure step did not exceed the subject's diastolic blood pressure. Each pressure step was sustained for just over 4 min (Figure 3.3). In addition, to measure resting muscle blood flow ($Q_a$ rest) before the first pressure step, the congestion cuff was rapidly inflated three times up to 50 mmHg. The congestion pressure was maintained for 10 seconds on each occasion and cuff was then deflated (Figure 3.3, 3.4 and 3.6). This procedure was also performed at the end of each subsequent 4-minute pressure step (6 x ~ 10 mmHg) to measure capillary blood flow ($Q_a$) in order to assess integrity of the signalling pathway [141, 291].

At the end of each step, to minimize the pitting, the strain gauge tension was automatically rebalanced to the starting tension. The whole protocol lasted between 30 and 40 minutes. The collected data was analyzed using the manufacturer's software after completion of research project by an independent blinded investigator.

**Figure 3.2 Photograph of Venous Congestion Plethysmography**

The subject lies in supine position in a quiet room and at an ambient temperature with both legs supported on pillows so that the investigated calf is at the level of heart. The electromechanical sensors attached to the limb measure changes in the limb circumference in response to venous congestion using pressure cuff applied on the ipsilateral limb.

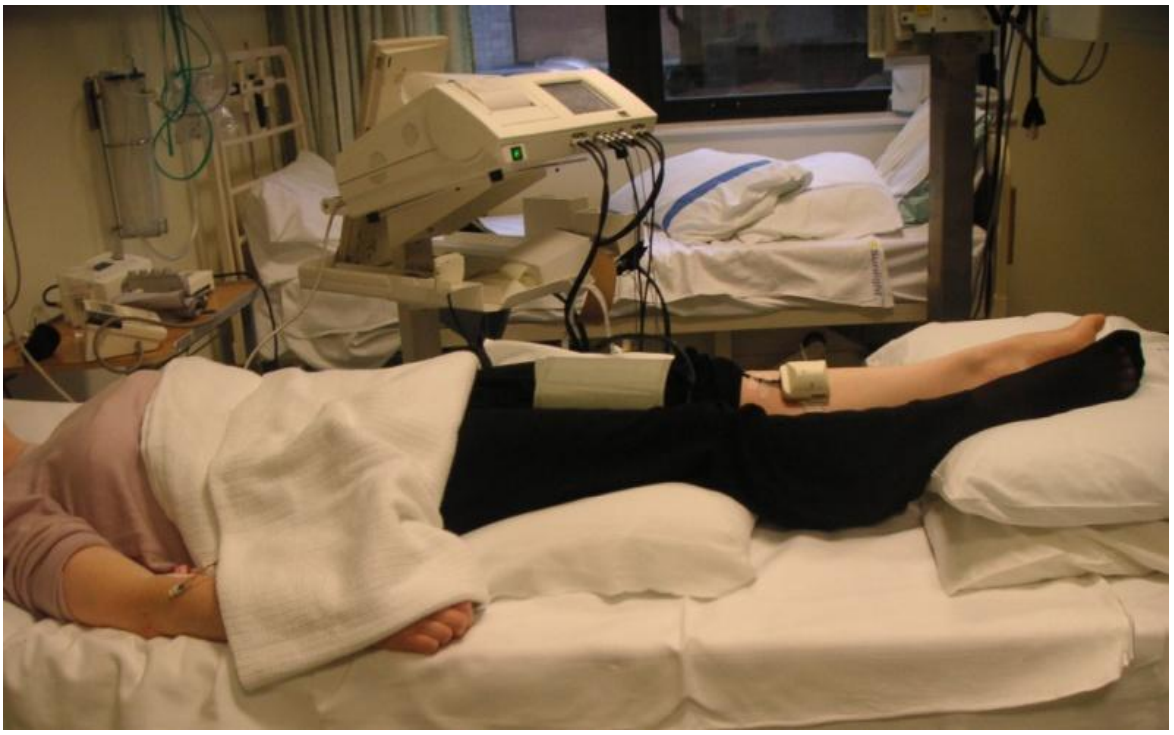


**Figure 3.3 Raw trace of small trace plethysmography to measure $K_f$ with added $Q_a$ measurement at each step**

The trace shows a complete plethysmographic recording from the study. The first three rectilinear peaks represent the 3 blood flow measurements ($Q_a$). They are followed by the progressive rectilinear increases in pressure, representing the cumulative cuff pressure ($P_{cuff}$) increases and the brief increases (single peaks) in pressure to 80 mmHg at the end of each cumulative pressure step, used for the assessment of $Q_a$ at each step. The curvilinear trace reflects the calf volume response to each of these cumulative pressure steps.

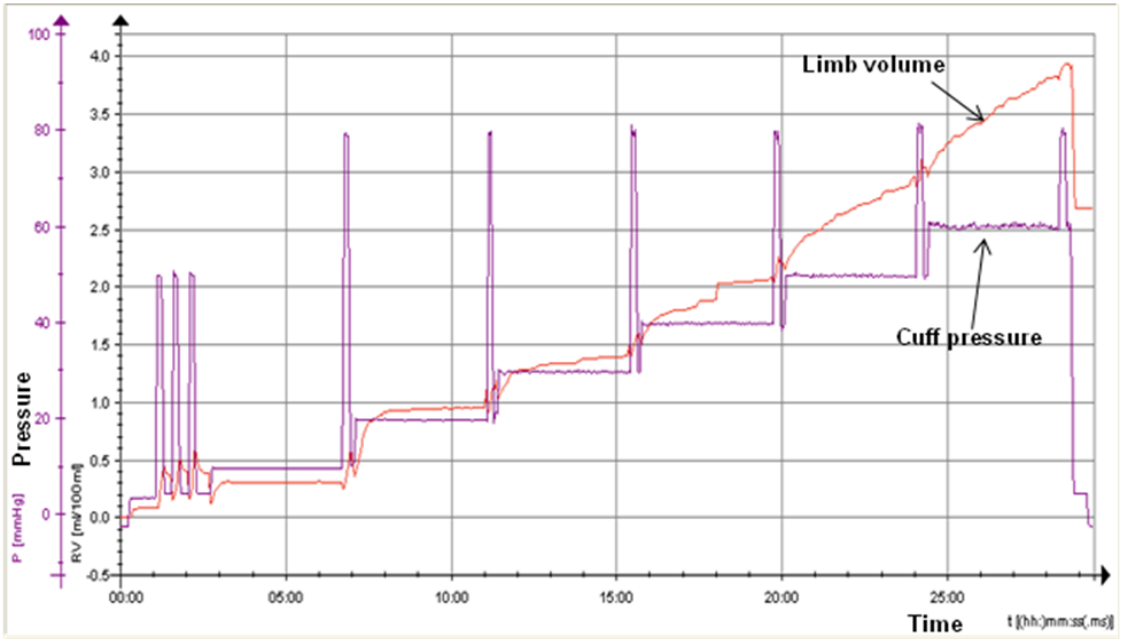



**Figure 3.4 Raw trace of capillary blood flow ($Q_a$) measurement**

The trace depicts the responses to the three initial 50 mmHg pressure steps (each lasting 10 seconds) used for the assessment of blood flow (Qa). The rectilinear traces reflect the pressure steps and curvilinear traces reflect the calf volume responses to the increase in pressure. BF= beginning of pressure cuff inflation, pause = release of pressure cuff

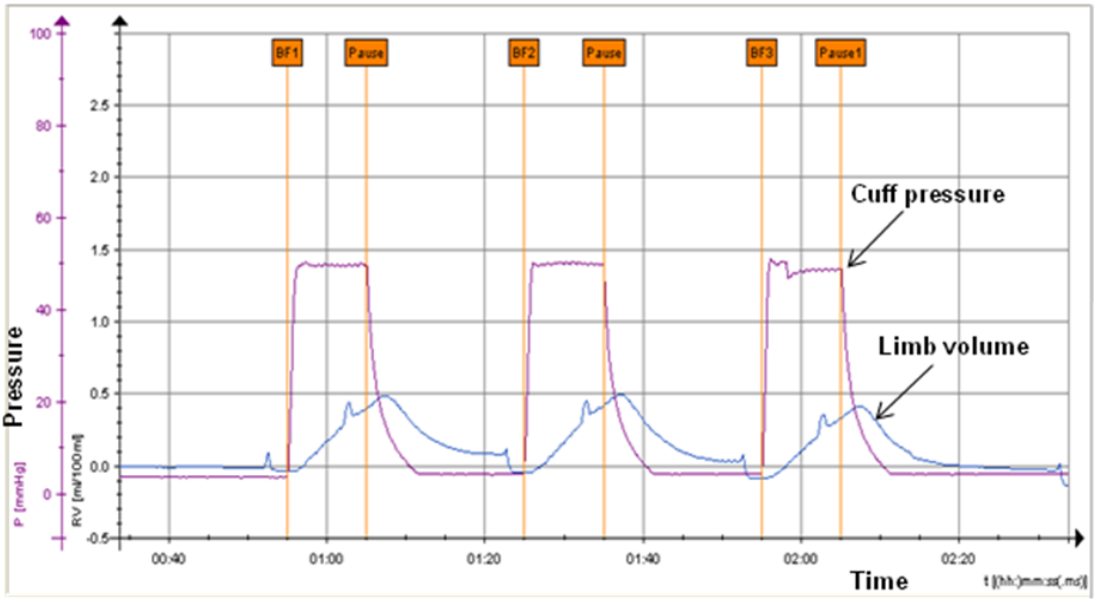


**Figure 3.5 Assessment of muscle filtration capacity ($K_f$) with venous congestion plethysmography**

The cumulative 10mmHg congestion pressure steps result in the increase in limb volume and fluid filtration. Fluid filtration ($J_v$) is measured from the slope of the last 2 minutes of the volume response. $K_f$ is derived from the relationship between $J_v$ and $P_{cuff}$.

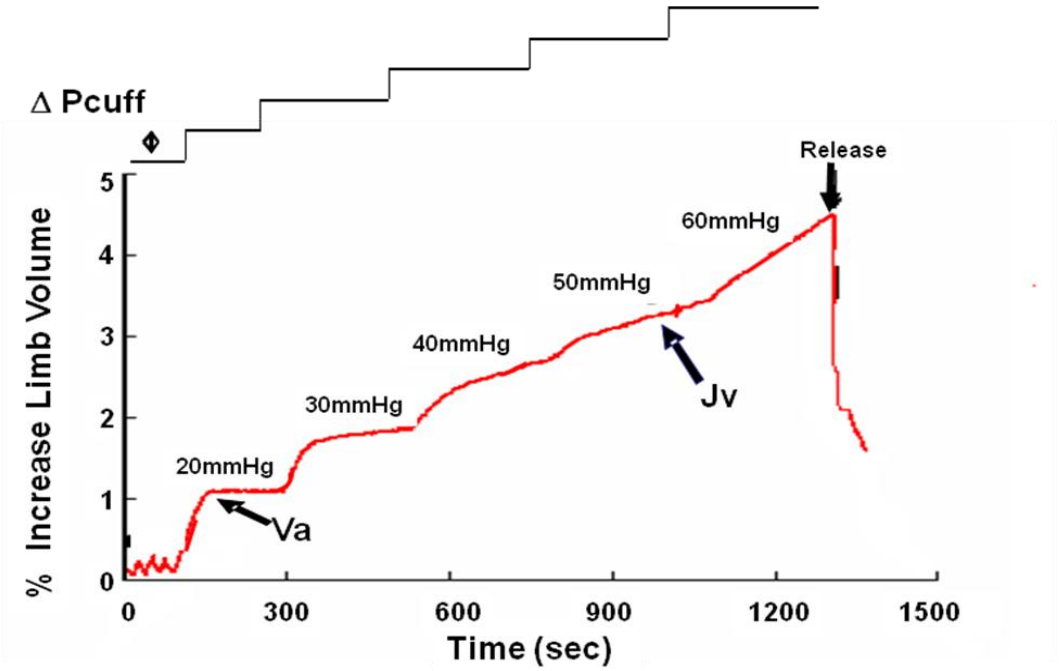
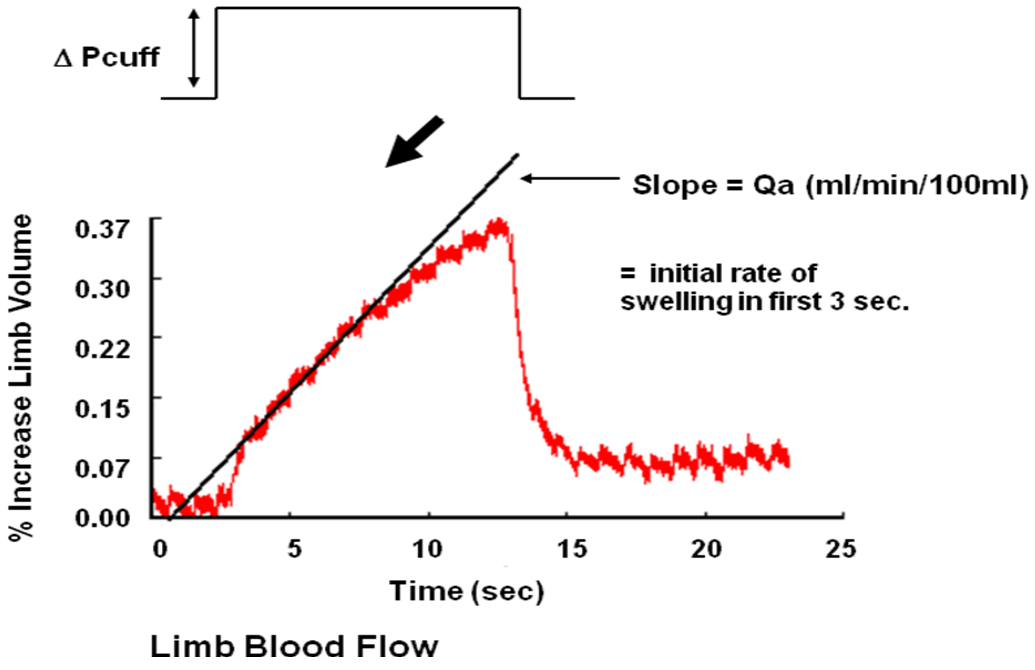



**Figure 3.6 Assessment of muscle capillary blood flow (Qa) with venous congestion plethysmography**

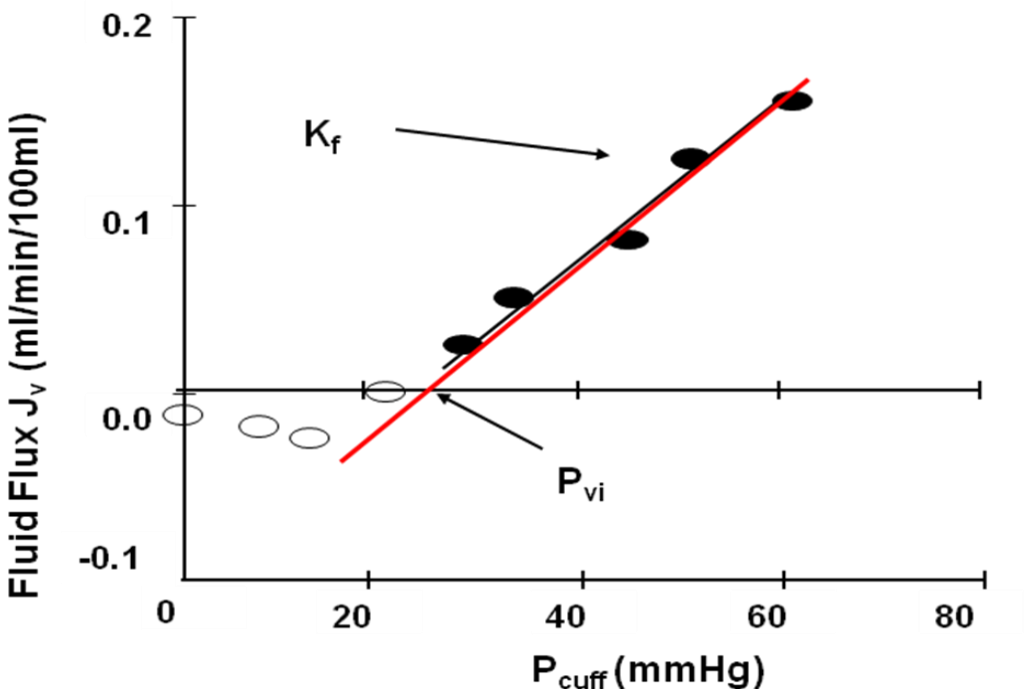
The graph depicts the increase in the limb volume in response to applied single congestion pressure step. The slope of the first 3 seconds of the swelling is used to calculate muscle blood flow. $Q_a$ = muscle blood flow, $P_{cuff}$ = congestion pressure.



The resting capillary muscle blood flow ($Q_a$rest) was calculated from the slope of the first 3 seconds of the volume response to the transient pressure elevations (as described above) before the beginning of the whole protocol [239, 292]. $Q_a$rest was expressed in ml/min/100 ml tissue. Fluid filtration rate ($J_v$) was estimated from the slope of the last 2 minutes of the volume change in response to six, 10 mmHg pressure steps (0 to 60 mmHg, as described above), to allow for the completion of vascular filling. This was plotted against cuff pressure ($P_{cuff}$). $J_v$ was expressed in ml/min/100 ml tissue. Filtration capacity ($K_f$) - a function of exchange surface area and permeability [293, 294] - was calculated from $J_v$ versus $P_{cuff}$ plot as the slope of that relationship and was expressed in ml/min/mmHg/100ml tissue (Figure 3.5 and 3.7). The CV for $K_f$ measurement was 14.5%. Isovolumetric venous pressure ($P_{vi}$) which represents local plasma oncotic pressure/inflammation was estimated as an intercept between the slope representing $K_f$ and congestion pressure ($P_{cuff}$) (Figure 3.7).

**Figure 3.7 Diagram representing calculation of microvascular filtration capacity and isovolumetric pressure based on plethysmographic measurements.**

Net transcapillary fluid movement ($J_v$) is plotted against congestion pressure ($P_{cuff}$) to give a linear relationship. $J_v = K_f$ [(Hydrostatic pressure) – δ(Oncotic pressure)], where δ is the osmotic reflection coefficient and $K_f$ represents microvascular filtration capacity ($K_f$ = permeability x surface area). Intercept $P_{vi}$ represents isovolumetric venous pressure.



### 3.7.2. Laser Doppler fluximetry

Functional microvascular dilatory capacity was assessed with cutaneous Laser Doppler Fluximetry (LDF) using a 785 nm, 20 mW laser and 4 mm separation (DP1-V2-HP) probe (Moor Instruments Ltd, Axminster, UK) with a detection depth of between 3.5 and 4.5 mm [143]. Vasomotor control was estimated using decomposition of low frequency periodic oscillations within the LDF signal [295].

The studies were conducted in a designated room at an ambient temperature of 20 $^{o}$C. All subjects were asked to expose their non-dominant leg and stay still, in a reclining position on a bed. Occlusion thigh cuff with the attached sphygmometer was tightly wrapped round the non-dominant thigh and secured with Velcro. The leg, slightly flexed at the knee, was placed on a mouldable cushion to ensure individual's comfort. The high power probe, which was directed at the muscle, was secured with sticky O ring over the lateral aspect of anterior tibialis muscle. Fibre optic cables sat perpendicular to limb and were directed laterally away from leg. The measurements were made three times within duration of the euglycaemic hyperinsulinaemic clamp: in pre-insulin stage - during the 30 minutes just before the beginning of insulin infusion- and during the last 30 minutes of low and high insulin dose (Figure 3.7).

Within each stage of the clamp, the baseline blood flux was recorded for 10 minutes at rest (Resting Flux = RF), three minutes during arterial occlusion (dynamic test) and 10 minutes during a reactive hyperaemia to arterial occlusion (PORH). PORH was created by inflating, over less then 40 seconds, thigh cuff to 200 mmHg, maintaining the pressure for 3 minutes and then releasing it (Figure 3.8). The recordings with any significant movement artefacts during any stage laser Doppler fluximetry were excluded from analysis. During each stage of clamp, just before the blood flux measurements, participant's blood pressure was measured using standard protocol as described in chapter 3.2.

Mean resting LDF was determined in arbitrary units (AU) at baseline over the final 5 min before the cuff inflation (RF), during the 3 min of arterial occlusion to assess effectiveness of the occlusion, and at peak flux value above baseline after release of the cuff (Peak Flux = PF). The cutaneous vascular conductance (CVC) was calculated as flux in AU divided by the mean arterial pressure in mmHg. This calculation was done to account for any changes in blood pressure that may have occurred during the clamp.

The area under the flux response from baseline curve (AUC) was estimated as previous studies showed that the PORH response to ischaemia presented distinct patterns associated with cardiovascular risk [236, 296] .

Since there is no consensus about expression of PORH flux data [297], the pre-insulin values of RF, the percentage increase in PF relative to RF (PF%RF) and AUC were compared to the values measured during insulin infusion. This comparison was used to assess the insulin-induced change in dilator capacity.

**Figure 3.8 Diagram of LDF measurements during stepped hyperinsulinaemic euglycaemic clamp**

Laser Doppler Fluximetry (LDF) to assess functional microvascular dilatory capacity was performed for half an hour just before insulin infusion and during the last 30 minutes of both low and high dose insulin infusions. M/I = Insulin Sensitivity Index

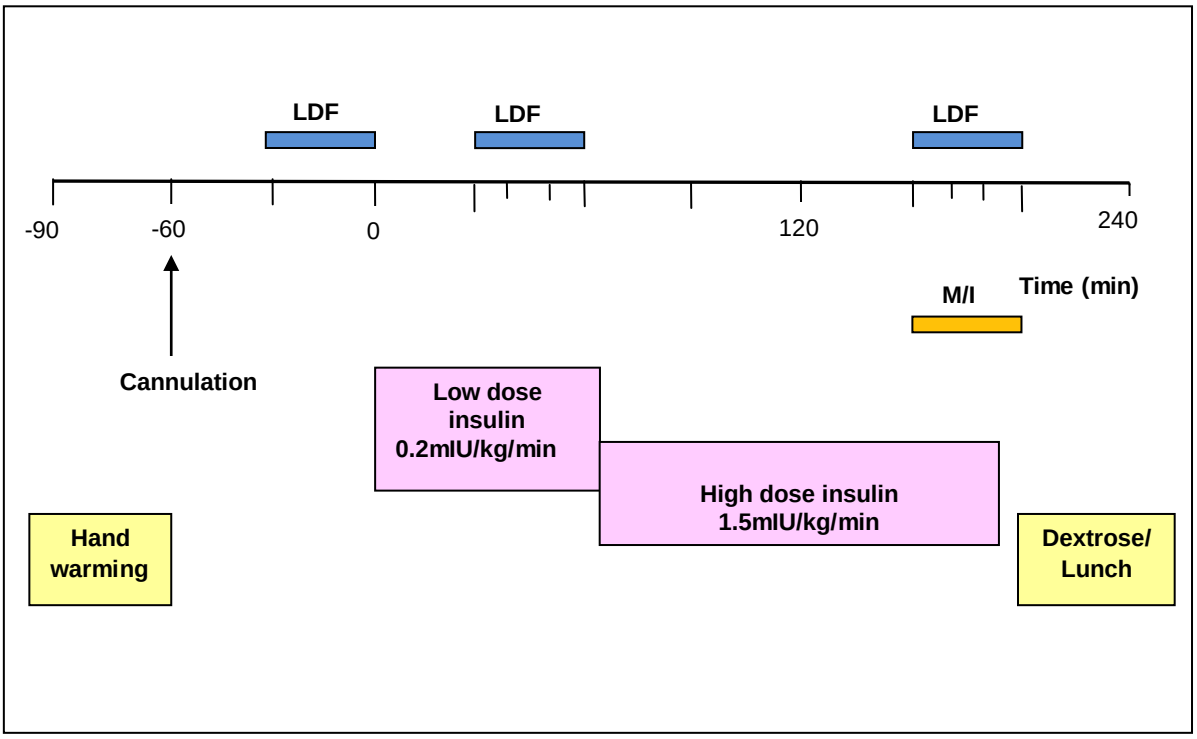
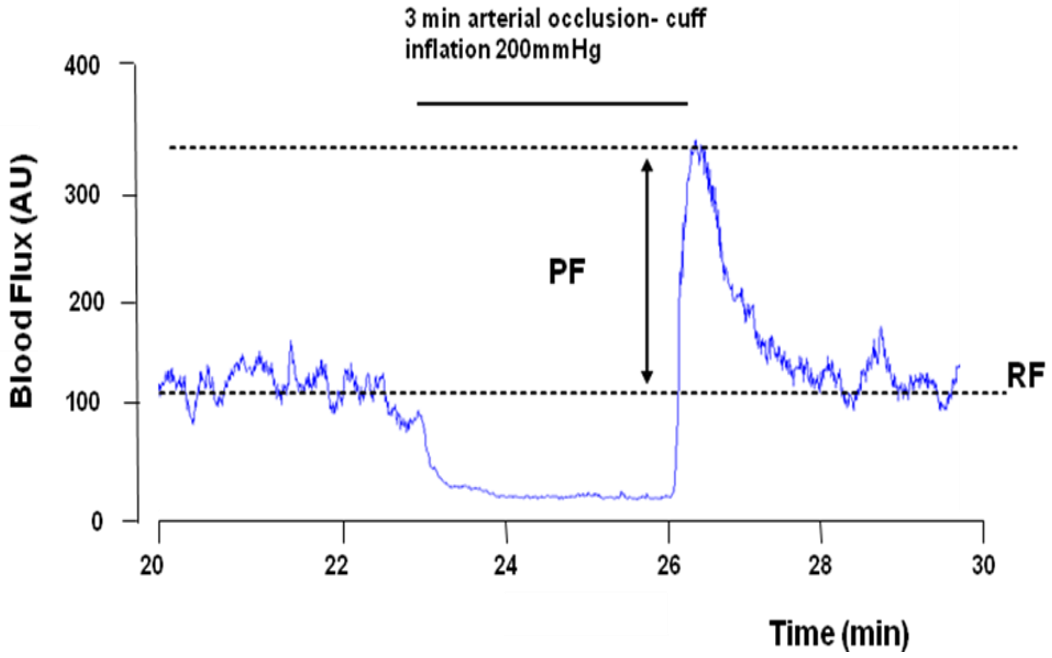


**Figure 3.9 Raw trace of blood flux measured by Laser Doppler Fluximetry before, during and after 3 minutes of arterial occlusion**

The trace shows part of the Laser Doppler Fluximetry recording from the study. The blood flux was measured for 10 minutes during the rest (on the left hand side), for 3 minutes during the arterial occlusion achieved by inflation of the pressure cuff up to 200mmHg and for 10 minutes during reactive hyperaemia to arterial occlusion (PORH); PF = peak flux, RF = resting flux



# 4. Central adiposity and the relationships between measures of glycaemia and microvascular function

## 4.1 Introduction

The links between obesity, diabetes and cardiovascular disease are well established by now [2, 11, 12]. There is also a body of evidence linking visceral adiposity with an increased risk of developing insulin resistance, type 2 diabetes and cardiovascular complications [52, 54-56, 61]. Central obesity and hyperglycaemia are recognised features of metabolic syndrome and pre-diabetic states [298, 299]. Hyperglycaemia is a widely accepted biochemical marker for the diagnostic criteria of type 2 diabetes, which are based on a threshold in the risk of microvascular complications [118]. In clinical practice, the assessment of glycaemia in diabetics is commonly based on the measurement of glycated haemoglobin ($HbA_{1c}$) rather than glucose concentrations. Moreover, new clinical recommendations from the American Diabetes Association advocate the use of $HbA_{1c} \geq$ 6.5% (47.5mmol/mol Hb) for the diagnosis of diabetes and the levels of 5.7-6.4% (38.8-46 mmol/mol Hb) to define pre-diabetes [300]. Glycated haemoglobin - a surrogate marker for the chronic hyperglycaemia - circulates for the lifespan of a red blood cell and reflects the prevailing blood glucose during the preceding twelve weeks. For this reason and because it does not require fasting and has low intra-individual variability [301], $HbA_{1c}$ may also be useful for the assessment of exposure to glucose among those without diabetes. Furthermore, the use of $HbA_{1c}$ is being recommended for the estimation of mean blood glucose using derived equations [302].

Several epidemiological studies showed an association between $HbA_{1c}$ and increased cardiovascular disease and mortality among type 2 diabetics [303-306]. Moreover, a positive linear association between CVD risk and glucose concentrations extends well below the glucose threshold for the diagnosis of type 2 diabetes, i.e. to the levels for glucose intolerance and impaired fasting glycaemia [306-310]. There is growing evidence that blood glucose concentrations in the high-normal range are also associated with an increased risk of cardiovascular disease [304, 311] and that there is an independent association between glycated haemoglobin levels and cardiovascular disease in non-diabetic populations [227, 301]. Most recently the ARIC study provided additional data on the predictive value of $HbA_{1c}$ in non-diabetic but overweight/centrally obese population. The results from this large, prospective (median 14-years follow up) study demonstrated that $HbA_{1c}$ was not only a strong predictor of developing diabetes but also an important marker of cardiovascular risk in non-diabetics after adjusting for age, sex and race even

within the normal (5.0 - 5.5%) range of glycated haemoglobin and also at its lower concentrations [301]. This association did not change after adjusting for fasting glucose. The explanation of those associations is unclear and requires further research.
Since the diagnostic criteria for diabetes are largely based on the evidential links between $HbA_{1c}$ and microvascular disease such as retinopathy [300], it is possible that in the non-diabetic population glycaemia levels relate in some way to microvascular dysfunction. Glycated haemoglobin is strongly influenced by levels of insulin secretion and insulin sensitivity among people with type 2 diabetes [312]. It is well recognised that insulin has a vasodilatory effect on the resistance vessels. It has been shown that insulin increases blood flow and microvascular perfusion in the skin [133, 140] and more importantly in skeletal muscle [145, 146] thus improving glucose delivery to the myocytes [149]. It was suggested by Baron *et al.* that the defective action of insulin to increase blood flow to the insulin-sensitive tissues is a main contributor to insulin resistance [313]. More recent studies have proposed that a diminution in glucose uptake by skeletal muscle in obesity is due to impaired insulin-dependent microvascular dilatory responses [147, 148] and capillary recruitment [152, 164]. There is also evidence for non-insulin mediated mechanisms regulating microvascular function such as exercise-induced functional capillary recruitment [156]. However, the effects of microvascular exchange capacity on the efficacy of glucose uptake in tissues have not been explored in any depth.
It is plausible that in centrally obese people at risk of developing type 2 diabetes, factors affecting peripheral tissue glucose delivery such as microvascular dysfunction could decrease glucose uptake in skeletal muscle through impaired nutrient exchange and thereby have an impact on $HbA_{1c}$. Thus an association between the levels of glycated haemoglobin and cardiovascular disease could be mediated by microvascular dysfunction in skeletal muscles - this association that has not been described to date. Additionally, factors such as physical inactivity, reduced fitness, visceral adiposity or decreased skeletal muscle insulin sensitivity could influence both skeletal muscle microvascular function, in particular microvascular exchange capacity, and $HbA_{1c}$. A better understanding of the factors contributing to the variance in $HbA_{1c}$ may provide explanations for the mechanism underlying the association between glycaemia and risk of CVD in non-diabetic but obese people.

The aim of this study was to investigate the relationship between skeletal muscle microvascular function and $HbA_{1c}$ in people with central obesity. In particular, we aimed to assess the effects of skeletal muscle microvascular exchange capacity, based on the

notion that filtration capacity in skeletal muscle would regulate tissue exchange of glucose and thereby influence $HbA_{1c}$. We tested this hypothesis taking care to estimate the impact of potential mediators such as muscle insulin sensitivity, physical inactivity and cardiorespiratory fitness, which are known to affect plasma glucose concentrations as well as microvascular exchange capacity [221]. In addition, we intended to assess the proportion of the variance in $HbA_{1c}$ that could be explained by physiological and biochemical measurements of factors known to influence glucose tolerance.

## 4.2 Methods

White, Northern European subjects with central obesity defined as waist circumference ≥ 94 cm for men and ≥ 80 cm for women, according to IDF metabolic syndrome diagnostic criteria, participated in this study. All subjects underwent oral glucose tolerance testing with a 75-g glucose load and samples were collected in the fasting state, at 30, 60 minutes and 2 hours. Only non-diabetic subjects were included into the study. In order to examine individuals at modest risk of future diabetes and cardiovascular disease, only volunteers with an estimated CVD risk of less than 20% over 10 years, based on the equation derived from the Framingham Heart Study, were eligible to participate in this study.

$HbA_{1c}$ was measured by a DCCT-aligned high pressure liquid chromatography (HPLC) method. Fasting lipid profiles were measured in relation to cardiovascular risk assessment and glycaemia, and plasma IL-6, sICAM-1 and urinary ACR to assess proinflammatory state in relation to microvascular function. Detailed information about the laboratory analysis was provided in the chapter 3.3.

Skeletal muscle microvascular function was assessed with regards to filtration and functional dilator capacity. Venous congestion plethysmography employing a Filtrass system was performed to measure baseline blood flow $Q_a$, isovolumetric venous pressure ($P_{vi}$) and filtration rate ($J_v$), and to establish skeletal muscle exchange capacity ($K_f$). Laser Doppler fluximetry was used for recording functional dilatory capacity, which was assessed by measuring post occlusive reactive hyperaemia (PORH) during a hyperinsulinaemic clamp. PORH was expressed as a percentage increase in pressure flow relative to resting flow (PF%RF). The detailed description of both measurements and calculations was provided in the methods chapter.

Anthropometry, body composition and visceral fat estimation were performed using methods previously described in detail in chapter 3.1.

In order to assess subjects' insulin sensitivity and microvascular function response to insulin, a hyperinsulinaemic euglycaemic clamp was conducted. We calculated a ratio of glucose disposal rate (M-value) and mean insulin concentration during the last 30 minutes of the clamp as an index of insulin sensitivity (M/I).

Fitness was estimated with the standard maximal oxygen uptake technique during treadmill testing and physical activity was assessed in terms of mean MET during a period of seven to ten days (representative of a typical week) of wearing an activity monitor (SenseWear Armband Pro2).

All statistical analyses were performed using SPSS for Windows version 16.0. The normally distributed data were presented as means and SD, and Pearson correlation

coefficients were used for univariate regression analyses. For the skewed data presentation we used medians, ranges and Spearman correlation coefficients. Student's *t* test was undertaken to compare mean values of normally distributed data.

The $HbA_{1c}$ data were categorised into tertiles to facilitate its presentation and interpretation. In order to examine differences between $HbA_{1c}$ tertiles and other variables as well as the linear trends across tertiles we applied one way ANOVA. We also used multivariate linear regression models with $HbA_{1c}$ as the outcome variable to describe factors that were independently associated with $HbA_{1c}$. Factors used in this model as explanatory (independent) variables were chosen from the results of univariate analysis providing they had statistically significant associations with $HbA_{1c}$. Additionally, age and sex were included into multivariate analysis. A $p$ value of $<0.05$ was considered to be statistically significant for all analyses.

## 4.3 Results

The baseline characteristics of 47 subjects studied in this research project are presented in Table 4.1 and the characteristics stratified by gender are shown in Table 4.2. The majority of participants were middle aged, normotensive and with a normal fasting lipid profile, in particular with normal or mildly increased triglycerides. All participants were normoglycaemic with mean ± SD glycated haemoglobin of 5.3 ± 0.5%. Fifteen subjects had $HbA_{1c}$ in the pre-diabetic range of 5.7-6.3%, according to ADA new diagnostic criteria and of those, 2 subjects had impaired fasting glycaemia and 5 had glucose intolerance. Six more subjects with abnormal glucose tolerance test had $HbA_{1c}$ less than 5.7%. The levels of glycated haemoglobin were similar between men and women, but the latter group had significantly lower mean fasting glucose concentration. Although all subjects had central obesity as defined by IDF criteria, women had statistically significantly lower waist circumferences and amount of visceral fat, but greater total body adiposity. CVD risk in women was half of the risk in men. Women were significantly less fit and had higher HDL-cholesterol levels than men. The microvascular function as well as IL-6 and sICAM1 levels were similar amongst both groups.

**Table 4.1 Baseline characteristics of study population, n=47**

| Variable | Mean ± SD | Range |
|---|---|---|
| Age (years) | 51.5 ± 9.3 | 29.0 - 69.6 |
| Waist circumference (cm) | 104.5 ± 12.3 | 86.5 - 151.0 |
| BMI ($kg/m^2$) | 31.8 ± 4.6 | 25.3 - 47.9 |
| DEXA total body fat (kg) | 32.1 ± 9.2 | 18.7 - 58.5 |
| DEXA total body fat (%) | 36 ± 7.2 | 21.2 - 47.5 |
| DEXA trunk fat (kg) | 16.5 ± 4.7 | 8.9 - 31.9 |
| DEXA trunk fat (%) | 18.5 ± 3.2 | 10.2 -24.8 |
| MRI visceral fat (kg) | 3.6 ± 1.6 | 1.1 - 7.1 |
| Blood pressure systolic (mmHg) | 133 ± 130 | 93 - 155 |
| Blood pressure diastolic (mmHg) | 82 ± 9 | 64 - 104 |
| CVD risk (%) | 7.2 ± 5.1 | 0 - 17.3 |
| Total cholesterol (mmol/L) | 5.8 ± 1.0 | 3.2 - 9.3 |
| LDL-cholesterol (mmol/L) | 3.7 ± 0.9 | 1.7 - 7.0 |
| HDL-cholesterol (mmol/L) | 1.4 ± 0.3 | 0.9 - 2.5 |
| Triglycerides (mmol/L) | 1.4 ± 0.7† | 0.4 - 2.9 |
| $HbA_{1c}$ (%) | 5.5 ± 0.3 | 4.9 - 6.3 |
| Fasting Glucose (mmol/L) | 5.3 ± 0.5 | 4.2 - 6.6 |
| $VO_2$ max (ml/min/kg) | 22.4 ± 7.65 | 8.35 - 52.45 |
| PAEE (MET) | 1.27 ± 0.17 | 0.83 - 1.55 |
| M/I ((mg·L)/kg/min/mIU) | 3.21 ± 1.26 | 0.97 - 6.26 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml tissue) | 3.86 ± 1.13 | 3.86 ± 1.13 |
| $Q_a$ resting (ml/min/100ml tissue) | -1.70 ± 2.23 | -5.0 - 4.0 |
| $P_{vi}$ (mmHg) | 20.31 ± 7.0 | 9.24 - 38.3 |
| Albumin:Creatinine ratio (mg/mmol) | 0.43 ± 0.51† | 0 - 2.3 |
| sICAM-1 (ng/L) | 0.23 ± 0.04 | 0.16 - 0.32 |
| IL-6 (pg/ml) | 2.1 ± 1.0 | 0.8 – 5.1 |

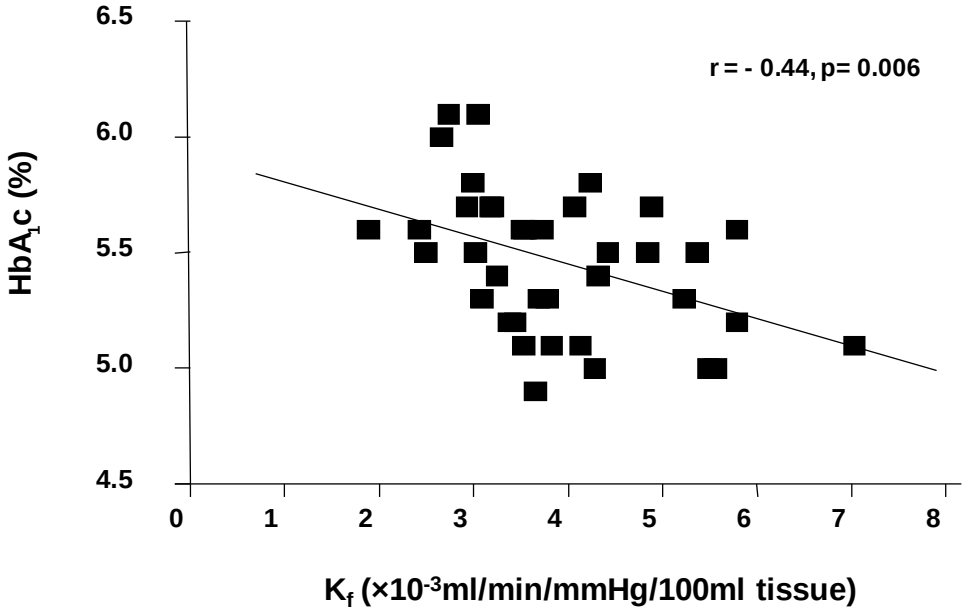
† median range

**Table 4.2 Baseline characteristics of study population by gender, n=47**

| Variable | Mean ± SD | | p- value |
|---|---|---|---|
| | Men (n=19) | Women (n=28) | |
| Age (years) | 54.8 ± 8.9 | 49.1 ± 8.7 | 0.03* |
| Waist circumference (cm) | 111.0 ± 12.7 | 101.4 ±10.6 | 0.006* |
| BMI ($kg/m^2$) | 32.1 ± 5.0 | 31.9 ± 4.4 | 0.86 |
| DEXA total body fat (%) | 29.5 ± 6.0 | 40.6 ± 3.8 | 0.0001* |
| DEXA trunk fat (kg) | 15.8 ± 5.4 | 17.2 ± 4.2 | 0.34 |
| MRI visceral fat (kg) | 4.6 ± 1.5 | 2.9 ± 1.3 | 0.001* |
| Blood pressure systolic (mmHg) | 136 ± 10 | 132 ± 3 | 0.38 |
| Blood pressure diastolic (mmHg) | 83 ± 9 | 81 ± 8 | 0.47 |
| CVD risk (% per 10 years) | 10.6 ± 4.4 | 5.0 ± 4.0 | 0.0001* |
| Total cholesterol (mmol/L) | 5.5 ± 0.7 | 5.9 ± 1.2 | 0.22 |
| LDL-cholesterol (mmol/L) | 3.6 ± 0.7 | 3.8 ± 1.1 | 0.63 |
| HDL-cholesterol (mmol/L) | 1.3 ± 0.2 | 1.5 ± 0.4 | 0.01* |
| Triglycerides (mmol/L) | 2.1 (0.5-2.6)† | 1.3 (0.4-2.9)† | 0.57 |
| $HbA_{1c}$ (%) | 5.5 ± 0.3 | 5.5 ± 0.3 | 0.77 |
| Fasting Glucose (mmol/L) | 5.4 ± 0.7 | 5.0 ± 0.6 | 0.03* |
| $VO_2$ max (ml/min/kg) | 25.8 ± 8.8 | 19.1 ± 6.6 | 0.006* |
| PAEE (MET) | 1.31 ± 0.15 | 1.25 ± 0.19 | 0.22 |
| M/I ((mg·L)/kg/min/mIU) | 3.09 ± 1.32 | 3.31 ± 1.24 | 0.60 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | 3.8 ± 1.2 | 4.0 ± 1.1 | 0.54 |
| $Q_a$ resting (ml/min/100ml tissue) | -1.64 ± 2.2 | -2.29 ± 2.4 | 0.39 |
| $P_{vi}$ (mmHg) | 17.4 ± 5.7 | 23.3 ± 7.05 | 0.009* |
| Albumin:Creatinine ratio (mg/mmol) | 0.20 (0.0-1.5) † | 0.40 (0.0-2.3) † | 0.39 |
| sICAM 1 (ng/L) | 0.24 ± 0.04 | 0.23 ± 0.05 | 0.23 |
| IL-6 (pg/ml) | 2.3 ± 1.4 | 1.9 ± 0.7 | 0.19 |

* $p < 0.05$, statistically significant, † median range

**Figure 4.1 Relationship between glycated haemoglobin and microvascular exchange capacity**



The univariate analysis indicated that age was not significantly associated with $HbA_{1c}$ but the degree of visceral adiposity strongly correlated with glycated haemoglobin. As expected, there was a positive and statistically significant association between $HbA_{1c}$ and all glucose measurements during an oral glucose tolerance test (Table 4.3). Strong negative correlations were present between $HbA_{1c}$ and measures of fitness, physical activity, insulin sensitivity and microvascular exchange/filtration capacity. The scatter plot of the relationship between $HbA_{1c}$ and $K_f$ (Figure 4.1) confirmed a good and strong linear correlation (r=-0.44, p=0.006). Filtration capacity did not correlate with $VO_2$ max (r=0.21, p=0.20), whereas functional dilatory capacity showed positive association (r=0.43, p=0.03). There was a lack of significant association with $HbA_{1c}$ and the other estimates of microvascular function, i.e. resting blood flow (r=0.21, p=0.21) and dilatory capacity (r=-0.26, p=0.22). The statistically significant associations were noted between glycated haemoglobin and sICAM-1and IL-6 but not with ACR. The data were further examined for the differences and linear relationships after it was categorised into 3 groups stratified by tertiles of $HbA_{1c}$ following ranges: <4.9%, 5.0-5.69%, 5.7-6.29% (Table 4.4). Statistically significant linear trends were observed across $HbA_{1c}$ tertiles and filtration capacity, insulin sensitivity, central adiposity, physical activity, fitness, sICAM-1 and IL-6.

**Table 4.3 Univariate associations with $HbA_{1c}$**

| Variable | r value | p-value |
|---|---|---|
| Age (years) | 0.20 | 0.17 |
| Waist circumference (cm) | 0.46 | 0.001* |
| BMI ($kg/m^2$) | 0.58 | 0.000* |
| DEXA total body fat (kg) | 0.37 | 0.009* |
| DEXA trunk fat (kg) | 0.47 | 0.001* |
| MRI visceral fat (kg) | 0.43 | 0.004* |
| Blood pressure systolic (mmHg) | 0.01 | 0.50 |
| Blood pressure diastolic (mmHg) | -0.09 | 0.52 |
| CVD risk (%) | 0.28 | 0.06 |
| Triglycerides (mmol/L) | 0.28 | 0.048 |
| HDL-cholesterol (mmol/L) | -0.25 | 0.08 |
| Glucose, 0 min (mmol/L) | 0.51 | 0.0001* |
| Glucose, 30 min (mmol/L) | 0.50 | 0.0001* |
| Glucose, 60 min (mmol/L) | 0.54 | 0.0001* |
| Glucose, 120 min (mmol/L) | 0.50 | 0.0001* |
| $VO_2$ max (ml/min/kg) | -0.41 | 0.008* |
| PAEE (MET) | -0.34 | 0.02* |
| M/I ((mg·L)/kg/min/mIU) | -0.43 | 0.007* |
| $K_f$ ($\times 10^{-3}$ ml/min/mmHg/100ml tissue) | -.44 | 0.006* |
| $Q_a$ resting (ml/min/100ml tissue) | 0.21 | 0.21 |
| $P_{vi}$ (mmHg) | 0.08 | 0.63 |
| PF%RF | -0.26 | 0.22 |
| Albumin:Creatinine ratio (mg/mmol) | 0.18 | 0.23 |
| sICAM-1 (ng/L) | 0.48 | 0.001* |
| IL-6 (pg/ml) | 0.35 | 0.03* |

† Spearman correlation coefficient, * $p < 0.05$, statistically significant

**Table 4.4 Body fatness, physical activity, insulin sensitivity, microvascular function and adipokines stratified by tertiles of HbA1c**

| $HbA_{1c}$ (%) tertiles | 4.90 - 5.29 | 5.30 - 5.69 | 5.70 – 6.3 | ANOVA p-value | Linear trend p-value |
|---|---|---|---|---|---|
| n | 13 | 19 | 15 | | |
| Age (years) | 46.3 ± 9.6 | 54.2 ± 8.2 | 51.8 ± 8.9 | 0.04 | 0.13 |
| Waist circumference (cm) | 99.1± 10 | 104.8 ± 8.7 | 107.2 ± 12.2 | 0.12 | 0.045* |
| BMI ($kg/m^2$) | 28.7 ± 2.4 | 32.3 ± 3.9 | 33.2 ±4.5 | 0.011 | 0.005* |
| DEXA total body fat (kg) | 28.0 ± 6.9 | 33.1 ± 9.2 | 33.0 ± 8.0 | 0.15 | 0.06 |
| DEXA trunk fat (kg) | 13.5 ± 2.8 | 17.3 ± 4.7 | 17.3 ± 3.4 | 0.03 | 0.02* |
| MRI visceral fat (kg) | 2.70 ± 1.17 | 4.06 ± 1.52 | 4.11 ± 1.72 | 0.04 | 0.03* |
| PAEE (MET) | 1.39 ± 0.13 | 1.22 ± 0.18 | 1.24 ± 0.17 | 0.019 | 0.029* |
| $VO_2$ max (ml/min/kg) | 27.3 ± 9.9 | 20.7 ± 5.9 | 18.6 ± 7.9 | 0.018 | 0.007* |
| M/I ((mg·L)/kg/min/mIU) | 3.88 ± 1.10[a] | 3.27 ± 1.30[b] | 2.42 ± 0.96[c] | 0.02 | 0.006* |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | 4.55 ± 1.21 | 3.79 ± 1.09 | 3.39 ± 0.74 | 0.045 | 0.016* |
| sICAM-1 (ng/L) | 1.98 ± 0.27 | 2.41 ± 0.37 | 2.55 ± 30.51 | 0.004 | 0.002* |
| IL-6 (pg/ml) | 1.51 ± 0.41 | 2.14 ± 0.91 | 2.44 ± 1.40 | 0.07 | 0.03* |

* $p < 0.05$, statistically significant, [a] n=11, [b] n=17, [c] n=13

In order to describe the relationships between the variables and glycated haemoglobin observed in the univariate analysis we conducted multiple linear regression analyses. The factors that were significantly associated with $HbA_{1c}$ but not co-linear with each other, together with age and sex, were selected as explanatory variables and $HbA_{1c}$ was selected as the outcome variable. The initial model containing only age and glucose as dependent variables explained 29% of the variance in $HbA_{1c}$ ($r^2$=0.29, p<0.0001) with fasting glucose independently associated with $HbA_{1c}$ (β coefficient=0.26, 95% CI: (0.13, 0.39), p<0.0001; age: p=0.65). Similarly, the model with age, sex and glucose explained 31% of the variance ($r^2$=0.31, p=0.0001), again with glucose independently associated with $HbA_{1c}$ (β coefficient=0.56, 95% CI: (0.14, 0.40), p=0.0001). An additional 26% of the variance in $HbA_{1c}$ was explained by incorporating into the same model $K_f$ ($r^2$=0.57, p= 0.0001), which was independently associated with $HbA_{1c}$ (β coefficient=0.45, 95% CI: (0.19, 0.06), p= 0.001) as presented in Table 4.5. After replacing glucose with M/I as an insulin sensitivity measure (Table 4.6), the new model explained 43% of the $HbA_{1c}$ variance ($r^2$= 0.43, p= 0.002) with only $K_f$ (β coefficient=-0.37, 95% CI: (-0.18, -0.02), p=0.02) and age (β coefficient=0.14, 95% CI: (-0.101, 0.273), p=0.008) being independently associated with glycated haemoglobin. There was no significant association between M/I and $HbA_{1c}$ (p=0.19) in this model. In order to explore further the effect of fasting glucose and visceral fat mass, those variables were added as explanatory variables to the model generated in Table 4.6. In this model, again there was no association between $HbA_{1c}$ and M/I (p=0.90) or visceral adiposity (p=0.31). $K_f$, as the only variable, remained independently associated with $HbA_{1c}$ (Table 4.7). In yet another model examining effects of visceral fat mass, physical activity (MET), fitness ($VO_2$ max) and sICAM-1 in addition to age and fasting glucose, 55.6% of the variance in $HbA_{1c}$ (p<0.0001) was explained by those variables. When $K_f$ was added to this model, it explained 65.6% of the variance in $HbA_{1c}$ (p<0.0001). Thus $K_f$ explained an important additional 10% of the variance in $HbA_{1c}$ that had previously not been explained by the other factors in the model. In this final regression model the only factors that were independently associated with $HbA_{1c}$ were again $K_f$ (β coefficient=-0.34, 95% CI: (-0.181, -0.006), p=0.038) and fasting glucose (β coefficient= 0.262, 95% CI: (0.104, 0.421), p=0.002).

**Table 4.5 Multiple linear regression model with $HbA_{1c}$ (%) as an outcome variable and age, sex, $K_f$ and fasting glucose as independent factors**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | 0.009 | 0.004 | 0.287 | 0.001 - 0.018 | 0.036 |
| Sex | 0.089 | 0.075 | 0.147 | -0.063 - 0.24 | 0.24 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | -0.123 | 0.032 | -0.451 | -0.187 - -0.058 | 0.001 |
| Glucose (mmol/L) | 0.243 | 0.070 | 0.447 | 0.101 - 0.385 | 0.001 |

$R^2 = 0.57$, $p < 0.0001$

**Table 4.6 Multiple linear regression model with HbA1c (%) as an outcome variable and age, sex, Kf and M/I as independent factors**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | 0.014 | 0.005 | 0.43 | -0.004 - 0.024 | 0.008 |
| Sex | 0.086 | 0.091 | 0.14 | -0.101 - 0.273 | 0.35 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | -0.099 | 0.041 | -0.37 | -0.183- -0.015 | 0.02 |
| M/I ((mg·L)/kg/min/mIU) | -0.356 | 0.265 | -0.21 | -0.898 - 0.186 | 0.19 |

$R^2 = 0.43$, $p = 0.002$

**Table 4.7 Multiple linear regression model with $HbA_{1c}$ (%) as an outcome variable and age, sex, $K_f$, M/I, fasting glucose and fatness as independent factors**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | 0.008 | 0.005 | 0.238 | -0.004 - 0.024 | 0.13 |
| Sex | 0.141 | 0.106 | 0.218 | -0.101 - 0.273 | 0.20 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | -0.100 | 0.044 | -0.354 | -0.183 – -0.015 | 0.03 |
| M/I ((mg·L)/kg/min/mIU) | 0.049 | 0.378 | 0.025 | -0.898 - 0.186 | 0.90 |
| Glucose (mmol/L) | 0.239 | 0.081 | 0.422 | -0.88 – 0.016 | 0.007 |
| MRI visceral fat (kg) | 0.048 | 0.046 | 0.234 | -0.016 – 0.004 | 0.31 |

$R^2 = 0.62$, $p = 0.001$

Summary points:

- A strong, negative and independent linear relationship between skeletal muscle microvascular exchange capacity and $HbA_{1c}$ was present as demonstrated by several different regression models.

- Microvascular functional dilatory capacity was not significantly associated with $HbA_{1c}$ levels.

- As expected, an independent association between fasting glucose and glycated haemoglobin was present.

- An association between $HbA_{1c}$ and insulin sensitivity measure M/I was not independent of visceral fat, fitness and physical activity.

- A strong and positive association between $HbA_{1c}$ and sICAM-1 existed but it was not independent of $K_f$.

## 4.4 Discussion

We have demonstrated for the first time a negative association between a measure of skeletal muscle microvascular exchange/filtration capacity - $K_f$ and glycated haemoglobin in centrally obese and insulin resistant but not diabetic subjects. We used a simple regression analysis containing age, sex and fasting glucose, which described 31% of variance in $HbA_{1c}$. A further 26% in $HbA_{1c}$ variance was explained by adding $K_f$ into this model. Importantly, the association was independent of potential mediators such as muscle insulin sensitivity, sex, age, visceral fat mass and measures of physical activity and cardiorespiratory fitness. M/I, visceral fat, $VO_2$ max and MET were not associated with $HbA_{1c}$ when $K_f$ was included in the regression models, suggesting that any effect of these factors to influence $HbA_{1c}$ may be mediated through microvascular filtration capacity. These findings potentially suggest that altered skeletal muscle microvascular function could be an early and important factor mediating the cardiovascular risk attached to $HbA_{1c}$ levels in people with central obesity.

It is well accepted that microvascular dysfunction can occur in type 2 diabetes, but much less is known about microvascular function in people at risk of diabetes such as those with central obesity. Microvascular disease underpins long-term complications of type 2 diabetes. It is thought that one of the main mechanisms behind microvascular dysfunction in diabetes involves excessive hyperglycaemia and formation of advanced glycation end-products (AGEs), which cause cross-linking of collagen molecules in arterial walls that leads to loss of collagen elasticity and a subsequent increase in arterial stiffness [314]. Also, chronic hyperglycaemia and resultant hyperinsulinaemia may increase the local activity of the renin–angiotensin–aldosterone system and expression of angiotensin type I receptor in vascular tissue, promoting development of wall hypertrophy and fibrosis [315], which contribute to vascular dysfunction. Impaired endothelium-dependent vasodilatation, reduced substrate delivery and lower capillary density in insulin-sensitive tissues have also been reported as important contributors to the microvascular dysfunction in type 2 diabetes [316]. However, it is not clear whether similar changes occur in non-diabetic but insulin - resistant obese people.

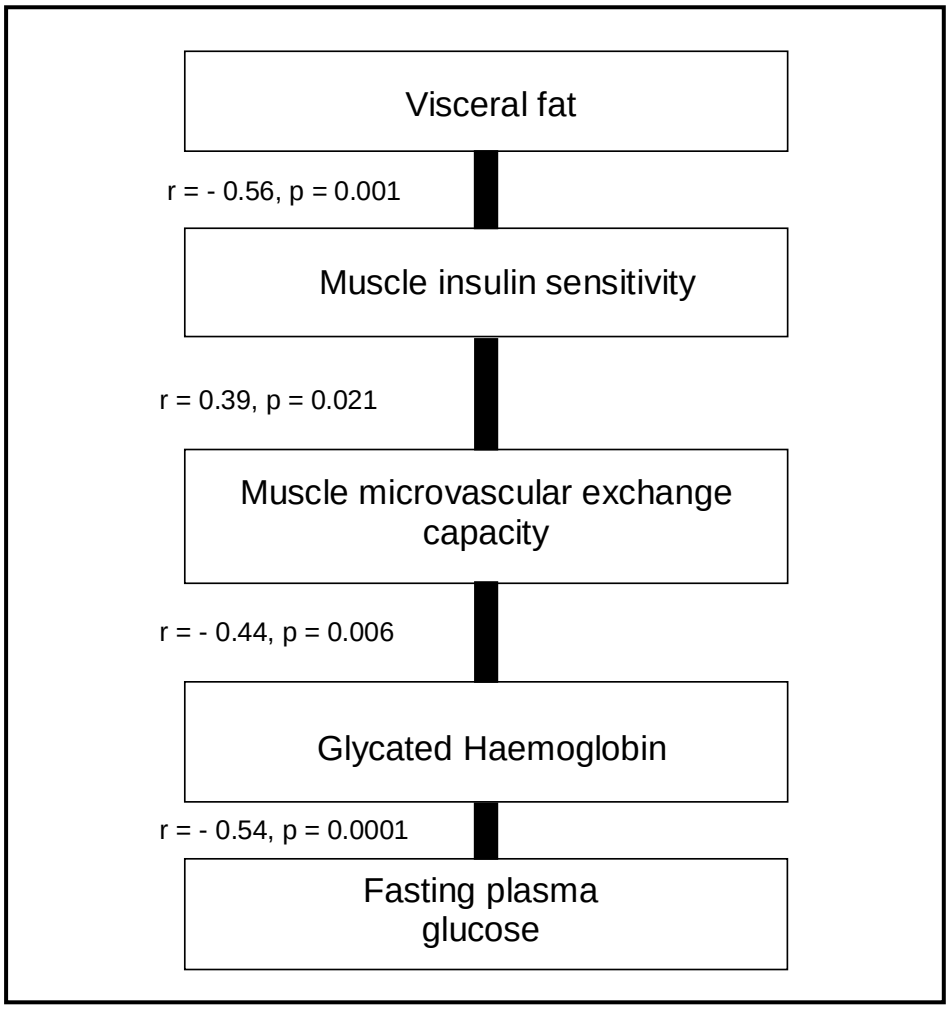
It has been demonstrated that in health, insulin increases blood flow and microvascular perfusion in skin [133, 140] and skeletal muscle [145, 146]. In animal studies, Clark and colleagues [156] extensively investigated insulin-mediated functional capillary recruitment and demonstrated its independence from total blood flow effects on the skeletal muscle glucose uptake and the impairment of these mechanisms in the insulin-resistant subjects

[147, 148]. Studies in obese humans with insulin resistance showed reductions in both insulin-mediated muscle microvascular perfusion and glucose uptake [163, 164, 317]. A recent study employing nailfold videocapillaroscopy demonstrated that in subjects with metabolic syndrome there were structural defects in capillaries besides the changes in capillary functional density and red blood cell velocity (RBCV) [138], thus providing evidence that nutritive skin microvascular dysfunction occurs in pre-diabetic stage. It is plausible that in insulin-resistant individuals both functional and structural changes, through capillary rarefaction or remodelling, could contribute to a reduced microvascular exchange capacity. In our study we measured filtration capacity under the resting conditions using congestion plethysmography, which produced an estimate of microvascular function independent of blood flow and therefore suggestive of capillary remodelling or rarefaction. Moreover, our data extend the results of studies described above because, unlike those human and animal studies, we accounted for additional factors that regulate skeletal muscle glucose uptake and blood glucose concentrations such as fitness, physical activity and visceral adiposity.

The mechanisms behind the relationship of microvascular dysfunction and obesity remain uncertain [139, 148, 316, 317]. It may be that the altered vasodilatory responses, oxidative stress, platelet adhesion and abnormalities of vasoconstriction, which are all linked to the increased body adiposity could potentially influence solute delivery via the microvasculature [317]. However, in our subjects as shown by the regression model (Table 4.7) we failed to demonstrate an association of visceral fat and $HbA_{1c}$. This could suggest that the influence of visceral adiposity on $HbA_{1c}$ is mediated via an effect on microvascular filtration capacity or insulin sensitivity (Figure 4.2).
Whether microvascular function regulates the degree of skeletal muscle insulin sensitivity or vice versa, or whether microvascular dysfunction is independent of the insulin sensitivity properties to regulate glucose concentrations is unclear. Baron *et al.* [144] showed that infusion of an endothelium-dependent vasodilator (methacholine chloride) increased insulin-mediated skeletal muscle glucose uptake, indicating that microvascular function can directly affect insulin sensitivity. Their data demonstrated that muscle perfusion is a potent determinant of glucose uptake in insulin sensitive subjects and insulin stimulation is associated with a change in the pattern of microcirculatory perfusion allowing for the modulation of glucose uptake. Our data are largely in agreement with Baron and colleagues [144] although in contrast to these authors we have recruited centrally obese, more insulin resistant and older subjects. In our obese subjects, muscle

microvascular exchange capacity was negatively associated with glycated haemoglobin independently of low levels of insulin sensitivity. This may suggest that in insulin-resistant subjects the major influence on $HbA_{1c}$ comes from the baseline microvascular filtration capacity, rather than muscle insulin sensitivity *per se*. In these subjects insulin-stimulated/determined microvascular mechanisms such as functional capillary recruitment are potentially defective or ineffective thus allowing for the cellular permeability of glucose to regulate overall glucose uptake. Supporting this notion is our observation of the lack of significant association between functional dilation capacity and glycated haemoglobin in our obese subjects.

**Figure 4.2 Schematic illustration of relationships between factors associated with glycated haemoglobin**



$HbA_{1c}$ is used worldwide as a measure of glucose control in people with diabetes but the factors influencing $HbA_{1c}$ levels in people at risk of type 2 diabetes have not been investigated in depth. The results of our multivariate regression analyses (Table 4.6 and 4.7) illustrate the relationships between each of the major factors that are associated with $HbA_{1c}$ in people with central obesity and insulin resistance. These relationships are presented in Figure 4.2 without indicating the direction of causality. Our data suggest that glucose and $K_f$ are strongly associated with $HbA_{1c}$ as a regression model containing age, sex and fasting glucose explained 31% of the variance in $HbA_{1c}$, ($r^2$=0.31, p<0.0001) and adding $K_f$ to this model explained 57% of the variance in $HbA_{1c}$ ($r^2$=0.57, p<0.0001) (Table 4.5). The model did not change after the addition of insulin sensitivity measure (M/I). More importantly, neither M/I nor visceral fat were associated with $HbA_{1c}$ independently of age, sex, glucose and $K_f$. In another regression model comprising age, fasting glucose, sICAM-1, visceral fat mass, and measures of physical activity and fitness, incorporation of $K_f$ explained additional 10% of the variance in $HbA_{1c}$, with the whole model explaining two thirds of the variance in $HbA_{1c}$ in non-diabetic, centrally obese participants.

The association between glucose and $HbA_{1c}$ in these regression analyses is not unexpected, because glycated haemoglobin represents average long term blood glucose concentrations including fasting levels. The reasons for an independent association between $K_f$ and $HbA_{1c}$ are less certain though. It is plausible that a decrease in microvascular nutrient exchange capacity reduces glucose exchange, resulting in increased plasma glucose concentrations and an increase in $HbA_{1c}$.

This cross-sectional study does not provide an answer as to the direction and causal nature of the association between $HbA_{1c}$ and $K_f$ or other factors. Although further research is merited, the evidence from previous studies allows some speculation about the causal links between glycated haemoglobin, microvascular filtration capacity and other factors playing part in those interactions. There is considerable evidence that in people with diabetes, the effect of the disease *per se* causes deterioration in microvascular structure and function because of hyperglycaemia and consequent formation of AGEs [314]. In our study, we excluded diabetes by oral glucose load testing; the mean plasma fasting glucose concentration was 5.2 mmol/L and mean $HbA_{1c}$ was 5.5%, which would support our earlier speculation that impaired filtration capacity results in increased $HbA_{1c}$. However, it is still possible that relative hyperglycaemia i.e. postprandial hyperglycaemia could directly cause both microvascular dysfunction and elevated $HbA_{1c}$, rather than impaired microvascular exchange capacity leading to increased glucose and $HbA_{1c}$ as illustrated in Figure 4.2. A further

possibility is that a bi-directional relationship exists contributing to a vicious circle of hyperglycaemia and microvascular dysfunction.

If future research confirms the casual link between $HbA_{1c}$ and $K_f$, then the remaining question is whether our finding of an independent association between $K_f$ and $HbA_{1c}$ is clinically relevant. The existing data suggest that it is possible to produce modest changes in $K_f$, which are likely to be physiologically relevant. Charles *et al.* [318] studied 12 individuals in a 14 week training programme during which lower-limbs were trained for endurance exercise and showed a 79% improvement in $K_f$ (from 2.4 ± 0.8 to 4.3 ± 0.9 ml/min/100 ml/mmHg; $p<0.05$). Brown and colleagues [221] showed about 100% increase in $K_f$ from 3.38 ± 0.38 to 6.68 ± 0.62 ml/min/100 ml/mmHg ($p<0.05$) using for 4 weeks electrical stimulation in 5 sedentary individuals (8 Hz, 3 x 20 min/day, 5 days/week). The results of the regression models (Tables 4.5, 4.6 and 4.7), in which we examined the relationship between $HbA_{1c}$ and potential explanatory factors showed that the adjusted β coefficient for the regression line in the relationship between $HbA_{1c}$ and $K_f$ was -0.45, -0.37 and -0.35 respectively. Therefore, if the existence of a causal relationship is confirmed by larger studies including interventional studies, an achievable change in microvascular exchange capacity as described above could be expected to be associated with a clinically relevant change in $HbA_{1c}$ of about 0.6-0.7%, even after adjusting for factors such as M/I, visceral fat, age, sex and fasting glucose.

In addition, our study interestingly showed a strong association between the percentage of $HbA_{1c}$ and sICAM-1 levels (Table 4.3), the latter being a marker of endothelial cell activation. Sattar *et al.* [319] recently demonstrated an association between increased sICAM-1 concentrations and incident diabetes. These authors showed that sICAM-1 levels were higher in those who developed diabetes, and remained independently associated with new-onset diabetes (HR: 1.84, 95% CI: (1.26, 2.69), $p=0.0015$) per unit increase in log(sICAM-1) after adjusting for classical risk factors and C-reactive protein. To date it is uncertain why sICAM-1 should predict incident diabetes but Sattar and colleagues speculated that "there is much more microvascular than large artery endothelium and one could assume that concentrations of sICAM-1 are determined more by microcirculatory function". Our data substantiate this speculation since regression analysis showed that the relationship between $HbA_{1c}$ and sICAM-1 was not independent of $K_f$, and $K_f$ potentially confounded this relationship. Thus these data lead us to suggest that increased sICAM-1 levels may reflect skeletal muscle microvascular endothelial dysfunction.

There are several limitations of this study, and a relatively small sample size is the one of them. Power calculations show that a study of 46 subjects has a 95% power to detect a correlation of r=0.5 and a study of 47 subjects has a power of 80% to detect a correlation of r=0.4. In our study, $HbA_{1c}$ and $K_f$ correlation was of r=-0.44, p=0.006. While undertaking the linear regression analyses we assumed a uniform linear relationship between the variables. However, it is biologically feasible that between individuals there may be differential impact of individual variables but our sample size is too small to stratify our analyses. For this reason, we were also unable to determine in our analyses any possible "stepped" or "hierarchical" effects of variables that may occur in nature otherwise.

Although we have examined in regression analyses the independent nature of the relationship between $K_f$ and $HbA_{1c}$, we were unable to depict in this cross sectional study the direction of the described associations. These results cannot be extrapolated to other population groups such as the non- centrally obese or those of different ethnicity. Also, this study did not investigate the effects of any intervention known to change $K_f$. The factors such as anaemia, high turnover or premature haemolysis of red blood cells can all result in a misleadingly low $HbA_{1c}$. Although we have no measurements of RBC turnover or reticulocyte count in our volunteers, all subjects were well, without biochemical, haematological or radiological evidence of haemolysis or anaemia. All had normal haemoglobin, red cell count, mean corpuscular volume and bilirubin levels. There was no evidence of splenomegaly on abdominal ultrasound.

In conclusion, we demonstrated that in people without diabetes but with central obesity, a simple measure of skeletal muscle microvascular exchange/filtration capacity ($K_f$) is associated with $HbA_{1c}$ independently of age, fasting glucose, visceral fat mass, levels of physical activity and fitness. A model containing all of these factors explains two thirds of the variance in $HbA_{1c}$. We showed that $K_f$ explains 26.5% of the variance in $HbA_{1c}$, beyond that explained by age, sex and glucose. These findings, in conjunction with other data demonstrating achievable improvements in $K_f$ [221, 318] may suggest that enhancing $K_f$ could produce clinically relevant decreases in plasma glucose concentrations in people at risk of type 2 diabetes, and that the relationship between $HbA_{1c}$ and incident cardiovascular disease could be related to skeletal muscle microvascular dysfunction. The increased and positively correlated with $HbA_{1c}$, levels of sICAM-1 may reflect skeletal muscle microvascular endothelial dysfunction.

The finding of strong independent association between $K_f$ and $HbA_{1c}$ warrants further investigations exploring the causal associations and interactions.

Summary points:

- In centrally obese and insulin resistant subjects, there is present a strong negative association between skeletal muscle microvascular exchange/filtration capacity ($K_f$) and $HbA_{1c}$ independent of age, gender, fasting glucose, visceral fat mass, peripheral insulin sensitivity, level of physical activity and fitness and sICAM-1.

- $K_f$ explains almost one third of the variance in $HbA_{1c}$ beyond age, sex and fasting glucose.

- Improvements in $K_f$ may produce clinically relevant decreases in plasma glucose concentrations in centrally obese Caucasians at risk of type 2 diabetes.

- Increased plasma sICAM-1 concentrations may reflect skeletal muscle microvascular endothelial dysfunction.

- Skeletal muscle microvascular dysfunction may be a link between $HbA_{1c}$ and incidence of cardiovascular disease.

# 5. The relationship between microvascular function and insulin sensitivity in obese subjects

## 5.1 Introduction

The body of evidence confirms that microvascular dysfunction is associated with central obesity and insulin insensitivity [316, 320], the main players in the development of metabolic syndrome and its cardiovascular complications. Studies in type 2 diabetics, who invariably have obesity, linked microvascular dysfunction with factors such as impaired endothelium–dependent vasodilatation, reduced substrate delivery, and capillary density in insulin-sensitive tissues. The excessive glycation of erythrocyte membrane proteins present in diabetes results in significant changes in erythrocyte deformability and aggregation, which in turn may affect flow of blood through the microcirculation [321]. Concomitant changes in endothelial cell surfaces due to the activation of receptors of advanced glycation end products (AGE) may influence not only vascular permeability but also exchange surface area [322]. Microvascular dysfunction was also reported in obese subjects without type 2 diabetes [317]. Increased body fatness may lead to molecular changes that can modulate oxidative stress, vasodilatory and vasoconstrictive responses as well as affect platelet adhesion. Some studies in obese, non-diabetic subjects showed reductions in the dilator response to endothelial-dependent and independent agonists [152, 323] while others reported attenuation of insulin-dependent microvascular perfusion and glucose uptake [163, 324]. All of those factors could adversely influence solute delivery via microcirculation, but it is still uncertain which components of the obesity-based pathophysiology lead exactly to microvascular dysfunction [139, 148, 316, 317].

It has been documented that insulin increases blood flow and microvascular perfusion in skin [133, 140] as well as in skeletal muscle [145, 146]. Studies in obese humans with impaired microvascular function showed presence of reduced insulin-mediated muscle microvascular perfusion and glucose uptake [163, 164, 317]. Similarly, studies of insulin resistance in animal models suggested that altered insulin-mediated microvascular dilator responses in skeletal muscles are key factors in reducing glucose uptake, even at basal insulin concentrations [147, 148]. Reports from studies in obese Zucker rats indicated diminished insulin-stimulated muscle microvascular perfusion recruitment [139], which could be due to increased production of reactive oxygen species (ROS) and reduced nitric oxide availability.

It has been advocated, based on the animal models, that by dilating arterioles in the capillary beds [325] insulin increases substrate delivery [191], which precedes and is independent of

the increase in total blood flow and glucose disposal [325] resulting from dilatation of upstream arteriolar vessels [326]. In support of this hypothesis and based on wide literature review, Clark *et al.* proposed insulin-mediated redistribution of blood flow. He suggested that insulin through its vasodilatory action, stimulates redirection of blood flow from non-nutritive routes to so-called nutritive vessels [156] and thus increases microvascular perfusion. Many experimental studies in healthy humans showed that effects of insulin on capillary blood flow are dose- and time-dependent, and that the increase in blood flow parallels glucose disposal [327]. However, it remains uncertain whether the capacity of insulin to boost the capillary recruitment is impaired in insulin resistant states, such as obesity [328].

In obese subjects changes in the microvascular function were reported, such as impaired dilator responses to endothelial dependent and independent stimuli, and reduced insulin-mediated microvascular perfusion may occur early during development of insulin resistance and before the onset of macrovascular disease [138, 320, 323, 329]. Changes in microvascular responsiveness have been linked to increasing age [330, 331] which is also associated with insulin resistance. However, further clarification is required as to whether the impact of age and insulin resistance on microcirculation occurs via a common endothelial signalling pathway [332] or whether those factors independently influence microvascular function.
Although it is known that microvascular dysfunction occurs in obese, non-diabetic individuals, the nature of the relationship between glucose disposal and microvascular function in insulin-sensitive tissues such as skeletal muscle, and the role of potential confounders like physical activity and fitness have not been fully clarified.

The purpose of this study was to investigate in centrally obese subjects the relationship between insulin-mediated glucose disposal and measures of microvascular function such as microvascular exchange capacity ($K_f$), microvascular integrity represented by isovolumetric pressure ($P_{vi}$), resting blood flow ($Q_a$) and functional microvascular dilator capacity. We tested the hypothesis that diminished insulin-mediated glucose disposal in skeletal muscle is associated with reduced filtration capacity ($K_f$) and that the functional dilator capacity is independently associated with insulin sensitivity and age. We also assessed for potential confounders of $K_f$ such as physical activity and cardiorespiratory fitness [221].

## 5.2 Methods

Non-diabetic and non-hypertensive subjects with central adiposity as defined by IDF criteria were recruited into this study as previously described. All had estimated cardiovascular risk less than 20% over 10 years. All volunteers had measured fasting lipid profile, glucose, hs-CRP, sICAM-1 and $HbA_{1c}$ as described in the laboratory analysis section. Detailed information about anthropometric assessment, body composition using DEXA and visceral fat estimation using MRI was provided in chapter 3.1
Measures of microvascular function such as exchange capacity ($K_f$), resting blood flow ($Q_a$) and isovolumetric venous pressure ($P_{vi}$) were assessed using venous congestion plethysmography employing Filtrass system with subjects at rest and lying down. Functional dilator microvasculature capacity in skeletal muscles and its response to insulin was assessed with Laser Doppler Fluximetry (LDF) during a hyperinsulinaemic euglycaemic clamp by measuring post occlusive reactive hyperaemia (PORH) before and during the final 30 minutes of insulin infusion. PORH was expressed as a percentage of increase in pressure flow (PF) relative to resting flow (PF%RF). The mean RF was established 5 minutes before and during the three minutes of dynamic test to assess the effectiveness of occlusion of the pressure cuff and as PF rose above baseline RF after the release of cuff. We also accounted for any possible change in blood pressure by calculating cutaneous vascular conductance (CVC). The description of all measurements and calculations was detailed in the chapter 3.7.
Insulin sensitivity (M/I) was assessed during hyperinsulinaemic euglycaemic clamp which was described together with all relevant calculations of M/I in the chapter 3.4.
We estimated cardiorespiratory fitness with the standard maximal oxygen uptake technique during treadmill and physical activity with an activity monitor (SenseWear Armband Pro2) as possible confounders of skeletal muscle exchange capacity. The detailed description of those techniques was presented in chapter 3.5.

All statistical analyses were performed using SPSS for Windows version 16.0.
Normally distributed data were expressed as mean ± SD, with Student's *t* tests employed to compare mean values and Pearson correlation coefficients for univariate regression analyses. Non-normally distributed variables were log transformed to normalise the distribution and expressed as medians and ranges. Multivariate linear regression models were used to describe factors that were independently associated with $K_f$ or PORH as the

dependent variable. A $p$ value of $<0.05$ was considered to be statistically significant for all analyses.

## 5.3 Results

Forty centrally obese subjects were studied. One individual was unable to complete the study and therefore the baseline characteristics of thirty nine subjects are shown in Table 5.1. The subjects were insulin resistant but did not have diabetes. Amongst the recruited subjects there were 17 men and 22 females who had a similar number of the features of metabolic syndrome. There were no significant differences between men and women with regards to BMI, blood pressure, insulin sensitivity index or lipids, except for HDL cholesterol. Men were significantly older and had greater cardiorespiratory fitness than females as presented in Table 5.2.

The baseline measurements of filtration capacity, blood flow and endothelial integrity, obtained during venous congestion plethysmography, are presented in Figure 5.1. The mean value of $K_f$ was $3.91 \pm 0.18 \times 10^{-3}$ml/min/mmHg/100ml, the mean value of $Q_a$ was 4.01 ± 0.48 ml/min/100ml and the mean value of $P_{vi}$ was 20.5 ± 1.1 mmHg. There was an approximately 3-fold difference in $K_f$ levels between subjects and the other measures also showed considerable variability within the study cohort.
During the stepped increases of venous congestion pressure there was evidence of reduced functional vasodilator capacity. Data free from movement artefacts were available on thirty five subjects. As the venous congestion cuff pressure increased (Figure 5.2) the calf blood flow relative to baseline blood flow ($Q_a$ rest) was not sustained, whereas a sustained blood flow has been previously reported in healthy subjects even as the congestion pressure approached mean arterial pressure [291].

Free from movement artefacts, satisfactory recordings of functional dilator capacity measured by LDF were obtained on thirty six subjects. The results showed the resting flow of 71 (35) AU and pressure flow of 203 (67) AU (median (IQR)). The relative change in PF during post occlusive reactive hyperaemia response was consistent with the response reported in healthy individuals by Clough *et al*. [143].

During the insulin infusion there was a significant increase in resting flow to 87 (69) AU, $p=0.0012$ but pressure flow did not significantly change and was 185 (85) AU, $p>0.05$. There was no change in the mean blood pressure before insulin infusion (95 (13) mmHg) or during insulin infusion (90 (13) mmHg). The peak CVC did not change during the clamp (pre-insulin 2.2 (0.9) AU/mmHg and during insulin 2.1 (0.85) AU/mmHg). The area

under the curve of the PORH response was also unaffected by insulin; pre-insulin 8.6 (4.1) ($\times 10^3$ AU×sec; insulin 8.9 (4.7) $\times 10^3$ AU×sec).

**Table 5.1 Baseline characteristics of study population, n=39**

| Variable | Mean ± SD | Range |
|---|---|---|
| Age (years) | 51.4 ± 9.0 | 29.0 - 69.6 |
| Waist circumference (cm) | 105.3 ± 12.9 | 86.5 - 151.0 |
| BMI ($kg/m^2$) | 32.1 ± 4.6 | 25.3 - 47.9 |
| DEXA total body fat (%) | 35.6 ± 7.4 | 21.2 - 47.5 |
| DEXA trunk fat (%) | 16.8 ± 4.6 | 9.0 -31.9 |
| Blood pressure systolic (mmHg) | 133 ± 14 | 93 - 155 |
| Blood pressure diastolic (mmHg) | 85 ± 9 | 64 - 104 |
| CVD risk (%) | 7.3 ± 5.1 | 0 - 17.3 |
| Total cholesterol (mmol/L) | 5.7 ± 1.1 | 3.2 - 9.3 |
| LDL-cholesterol (mmol/L) | 3.7 ± 0.9 | 1.7 - 7.0 |
| HDL-cholesterol (mmol/L) | 1.6 ± 0.4 | 0.9 - 2.5 |
| Triglycerides (mmol/L) | 1.4 ± 0.6 | 0.4 - 2.7 |
| $HbA_{1c}$ (%) | 5.5 ± 0.3 | 4.9 - 6.3 |
| Fasting Glucose (mmol/L) | 5.2 ± 0.7 | 4.0 – 6.6 |
| M/I ((mg·L)/kg/min/mIU) | 3.36 ± 1.25† | 0.97 - 6.26† |
| $VO_2$ max (ml/min/kg) | 23.2 ± 7.8 | 8.35 - 52.5 |
| PAEE (MET) | 1.3 ± 0.2 | 0.8 - 1.6 |
| hs-CRP (mg/dL) | 2 (1.09 -10.47) †† | 0.0 - 39.8 |
| sICAM-1 (ng/L) | 2.2 ± 0.4 | 1.6 – 3.1 |

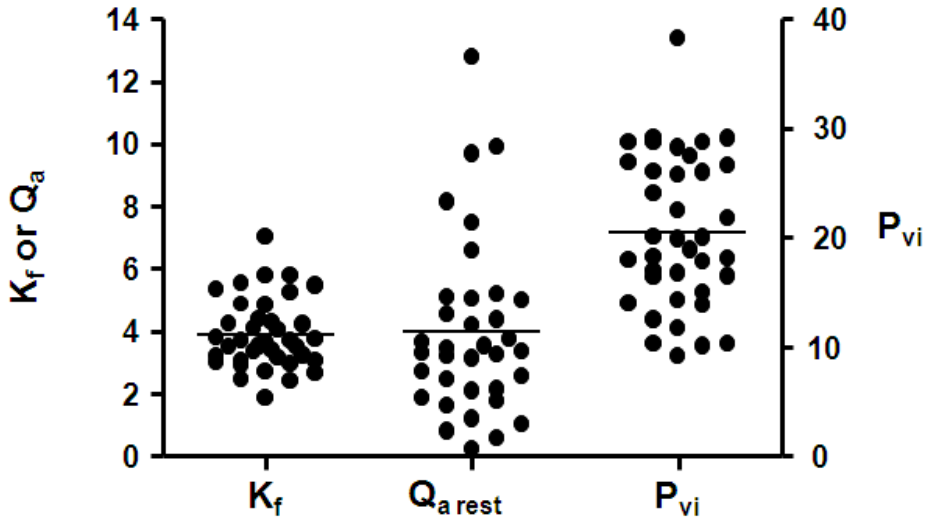
† n = 37, †† median (95%CI)

**Table 5.2 Baseline characteristics of study population by gender, n=39**

| Variable | Mean ± SD | | p- value |
|---|---|---|---|
| | Men (n=17) | Women (n=22) | |
| Age (years) | 55.3 ± 9.1 | 48.4 ± 7.9 | 0.016* |
| Waist circumference (cm) | 110.5 ± 13 | 101.3 ±11.6 | 0.025* |
| BMI ($kg/m^2$) | 31.7 ± 5.2 | 31.6 ± 4.6 | 0.93 |
| Blood pressure systolic (mmHg) | 135 ± 10 | 131 ± 17 | 0.49 |
| Blood pressure diastolic (mmHg) | 84 ± 9 | 81 ± 8 | 0.36 |
| CVD risk (%) | 11 ± 5 | 5 ± 4 | 0.0001* |
| Total cholesterol (mmol/L) | 5.5 ± 0.8 | 5.9 ± 1.3 | 0.24 |
| LDL-cholesterol (mmol/L) | 3.5 ± 0.6 | 3.7 ± 1.1 | 0.53 |
| HDL-cholesterol (mmol/L) | 1.3 ± 0.2 | 1.6 ± 0.4 | 0.007* |
| Triglycerides (mmol/L) | 1.5 ± 0.7 | 1.3 ± 0.6 | 0.23 |
| $HbA_{1c}$ (%) | 5.5 ± 0.4 | 5.4 ± 0.3 | 0.52 |
| Fasting Glucose (mmol/L) | 5.4 ± 0.8 | 5.0 ± 0.6 | 0.08 |
| M/I ((mg·L)/kg/min/mIU) | 3.26 ± 1.28 | 3.44 ± 1.22 | 0.48 |
| $VO_2$ max (ml/min/kg) | 25.8 ± 9.1 | 20.4 ± 7.3 | 0.048* |
| PAEE (MET) | 1.31 ± 0.15 | 1.26 ± 0.18 | 0.04* |

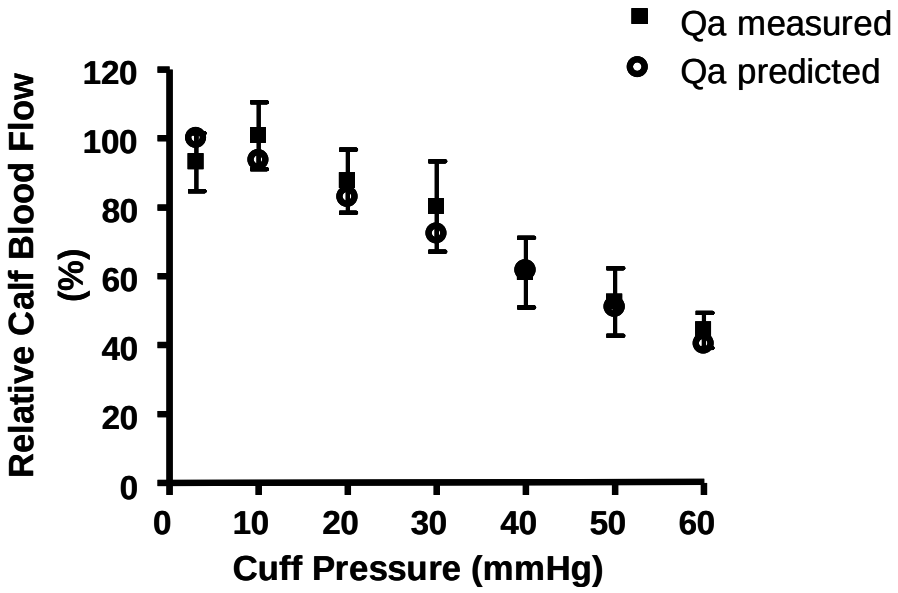
* $p < 0.05$, statistically significant

**Figure 5.1 Baseline measurements of filtration capacity, resting limb blood flow and endothelial integrity**




$K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml), $Q_a$ resting (ml/min/100ml), $P_{vi}$ (mmHg)

**Figure 5.2 Calf blood flow expressed as a % of baseline values measured during increasing venous congestion**




n = 35, data presented as mean ± SEM

The univariate analysis of the relationships between microvascular exchange capacity and metabolic parameters, measures of obesity, physical activity and fitness (Table 5.3) showed that $K_f$ was significantly associated with waist circumference (r=-0.36, p=0.025), but not the other easily measurable features of metabolic syndrome (blood pressure, glucose, HDL-cholesterol and triglyceride concentrations). As represented also in Figures 5.3 and 5.4, $K_f$ was also significantly and positively associated with M/I (r=0.39, p=0.02) and negatively associated with visceral fat (r=-0.43, p=0.015). $K_f$ showed negative associations with plasma hs-CRP (r=-0.32, p=0.04) and soluble ICAM-1 (r=-0.31, p=0.05). There were no significant associations between $P_{vi}$ or $Q_a$ and measures of obesity, insulin sensitivity, physical activity, fitness, inflammatory markers or any of the metabolic syndrome features.

We further explored the factors associated with the microvascular exchange capacity using multiple regression modelling. The model containing $K_f$ as the outcome variable and $HbA_{1c}$, M/I and visceral fatness as the explanatory variables explained 38% of variance in $K_f$. In order to determine whether the association between $K_f$ and insulin sensitivity observed in univariate analysis (Table 5.3) was independent of visceral fat we used $K_f$ as the outcome and M/I and visceral fat as explanatory variables. The index of insulin sensitivity and visceral fat explained 30% of the variance in $K_f$ ($r^2$=0.30, p=0.008). Interestingly, M/I was associated with $K_f$ independently of visceral fat (β coefficient 3.13, (95%CI: 0.22-6.02), p=0.036), whereas visceral fat was not associated with $K_f$ independently of M/I (β coefficient=-0.09 (95% CI: -0.40-0.22, p=0.55). Gender did not have any effect in these models.

**Table 5.3 Univariate associations with $K_f$**

| Variable | r value | p- value |
|---|---|---|
| Age (years) | 0.02 | 0.89 |
| Waist (cm) | -0.36 | 0.025* |
| MRI Visceral fat (kg) | -0.43 | 0.015* |
| MRI Subcutaneous fat (kg) | -0.28 | 0.12 |
| Blood pressure systolic (mmHg) | -0.15 | 0.36 |
| Blood pressure diastolic (mmHg) | 0.08 | 0.63 |
| CVD risk (%) | -0.15 | 0.38 |
| HDL-cholesterol (mmol/L) | 0.13 | 0.43 |
| Triglyceride (mmol/L) | -0.30 | 0.07 |
| Glucose (mmol/L) | -0.05 | 0.77 |
| PAEE (METS) | 0.20 | 0.24 |
| $VO_2$ max (ml/min/kg) | 0.21 | 0.20 |
| M/I ((mg·L)/kg/min/mIU) | 0.39 | 0.021* |
| hs-CRP(mg/dL) | -0.32 | 0.04* |
| sICAM-1(ng/L) | -0.31 | 0.05* |

* $p < 0.05$, statistically significant

**Figure 5.3 Relationship between insulin sensitivity and microvascular exchange capacity**

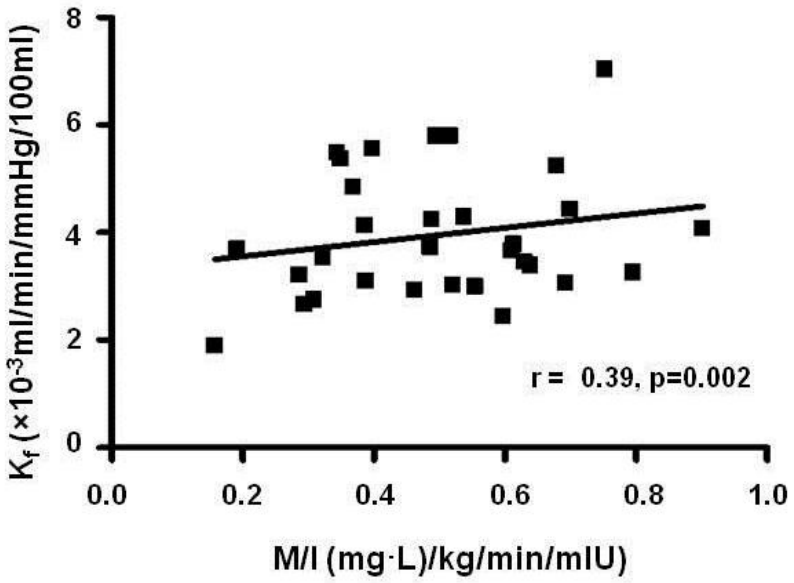
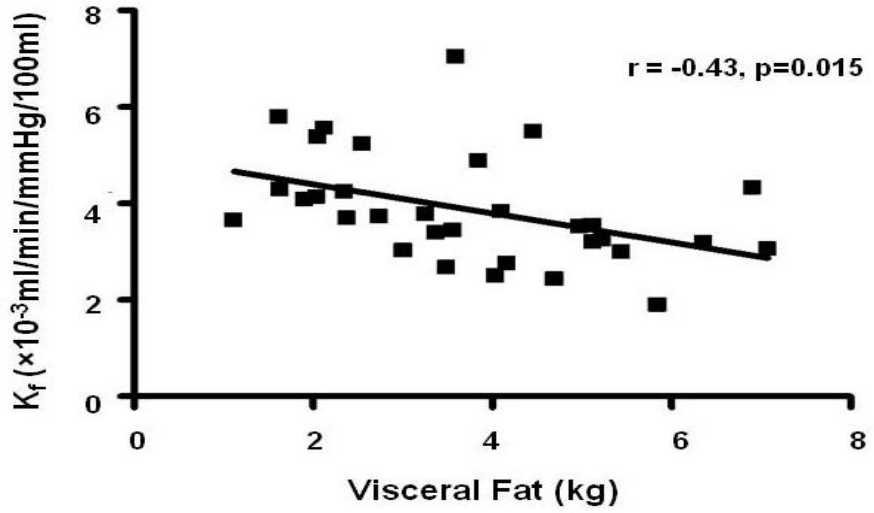



**Figure 5.4 Relationship between visceral fat and microvascular exchange capacity**



We also assessed the relationships between functional dilator microvasculature capacity and metabolic and inflammatory parameters, insulin sensitivity, age and fitness as presented in Table 5.4. There was a statistically significant positive association between insulin-induced change in functional hyperaemia (PF%RF) and insulin sensitivity (r=0.46, p=0.024). PORH was strongly and negatively correlated with the age (r=-0.46, p=0.02), sICAM-1 (r=-0.43, p=0.033) and LDL-cholesterol (r=-0.437, p=0.03). There was also a positive association between cardiorespiratory fitness and PF%RF (r=0.43, p=0.03). However no significant associations were found between PORH and measures of body fatness.

The variables which significantly correlated with PORH in univariate analysis (Table 5.4) were selected for inclusion into multiple regressions modelling to investigate further the association between insulin-induced change in PF%RF and insulin sensitivity, and to explore the potential effect of any confounders in this relationship. Over a half (53%) of the variance in PF%RF was explained by age, M/I, cardiorespiratory fitness and LDL cholesterol used as independent explanatory variables ($r^2$=0.53, p=0.005). A regression model containing age, M/I and sex as explanatory variables identified 44% of variance in the insulin-induced change in dilator capacity (Table 5.7). Stepwise linear regression modelling was undertaken to investigate the proportion of the variance in PF%RF that was identified by the explanatory factors. Age was the most important explanatory factor identifying 27% of the variance (Table 5.5). Inclusion of M/I into the model (Table 5.6) identified an additional 15% of the variance, and this final model explained 42% of the variance in the outcome variable. Only age and M/I were independently associated with an insulin-induced change in PORH (Table 5.7).

**Table 5.4 Univariate associations with PORH**

| Variable | r value | p- value |
|---|---|---|
| Age (years) | -0.460 | 0.02* |
| BMI ($kg/m^2$) | -0.26 | 0.21 |
| MRI Visceral fat (kg) | -0.376 | 0.11 |
| CVD risk (%) | -0.05 | 0.75 |
| LDL-cholesterol | -0.437 | 0.03* |
| Glucose (mmol/L) | -0.414 | 0.04* |
| $HbA_{1c}$ (%) | -0.26 | 0.22 |
| $VO_2$ max (ml/min/kg) | 0.431 | 0.03* |
| M/I ((mg·L)/kg/min/mIU) | 0.46 | 0.024* |
| sICAM-1 | -0.43 | 0.033* |

* $p < 0.05$, statistically significant

**Table 5.5 Multiple linear regression model with PORH (PF%RF) as an outcome variable and age as independent factor**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | -12.6 | 4.4 | -0.52 | -21.8 - -3.4 | 0.009 |

$R^2 = 0.27$, $p < 0.009$

**Table 5.6 Multiple linear regression model with PORH (PF%RF) as an outcome variable and age and M/I as independent factors**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | -11.15 | 4.1 | -0.46 | -19.6 - - 2.7 | 0.013 |
| M/I ((mg·L)/kg/min/mIU) | 422.5 | 180.8 | 0.39 | 46.5 - 798.5 | 0.029 |

$R^2 = 0.42$, $p = 0.003$

**Table 5.7 Multiple linear regression model with PORH (PF%RF) as an outcome variable and age, sex and M/I as independent factors**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | -12.1 | 4.3 | -0.50 | -21.0 - - 3.2 | 0.01 |
| Sex | -66.3 | 78.2 | -0.15 | -229.5 - 96.9 | 0.20 |
| M/I ((mg·L)/kg/min/mIU) | 406.8 | 182.9 | 0.38 | 25.1 - 788.5 | 0.04 |

$R^2 = 0.44$, $p = 0.008$

Summary points:

- A strong and independent linear association between skeletal muscle microvascular exchange capacity and insulin sensitivity index was present as demonstrated by multiple regression modelling.

- The negative and linear relationship between skeletal muscle microvascular exchange capacity and visceral fat was not independent of insulin sensitivity.

- A significant negative, but not independent association between $K_{f,}$ hs-CRP and sICAM-1 was present.

- A lack of sustained blood flow in the face of increasing venous congestion pressure was observed during plethysmography.

- A strong and independent relationship was present between insulin-induced change in microvascular functional dilator capacity, age and insulin sensitivity.

## 5.4 Discussion

In our study we have demonstrated that centrally obese, insulin resistant but not diabetic adults had impaired skeletal muscle microvascular function. Our data demonstrated that impaired muscle microvascular exchange capacity ($K_f$) was associated with attenuated insulin mediated glucose uptake in skeletal muscle, independently of visceral fatness. It also showed that the muscle filtration capacity was adversely associated with body fatness but this was not independent of the level of insulin sensitivity. These relationships were not confounded by a low level of physical activity or cardiorespiratory fitness.

The fact that the insulin sensitivity (M/I) was positively associated with the muscle microvascular filtration capacity ($K_f$) may suggest that greater muscle exchange capacity and in turn nutrient delivery is present in the more insulin sensitive subjects. The values of $K_f$ in our centrally obese cohort showed considerable variability but they were similar to the values previously reported in individuals of similar age [318, 333] and prediabetic or diabetic individuals without microvascular complications [334-337].

Additionally, $K_f$ was also inversely associated with inflammatory markers such as hs-CRP and sICAM-1, the latter a marker of endothelial cell activation. This is in keeping with the reports that microvascular dysfunction is associated with low grade systemic inflammation in obese [338] and is related to changes in endothelial cell activation [339]

The assessment of blood flow in the skeletal muscle microvascular bed by plethysmography in our study showed the mean resting limb blood flow ($Q_a$rest) to be higher than that reported previously in young healthy [141] or in older overweight and obese individuals [340]. Moreover, our subjects with central obesity were unable to maintain calf blood flow in the face of increasing venous congestion pressure unlike the young, healthy individuals in whom $Q_a$ remained constant possibly because of a progressive reduction in pre-capillary resistance due to retrograde transmission of vasodilatory signals through the endothelium [156]. Although the slope of the relationship between blood flow and venous congestion pressure followed, it was considerably lower in our study of obese subjects than the reported in other patient groups [291]. The muscle microvascular perfusion at rest is modulated by local capacity for vasodilatation. It is plausible that in people with central adiposity and insulin resistance, metabolites released from excess body fat down-regulate the vasodilator and up-regulate the vasoconstrictor pathway. These data suggest that both muscle microvascular perfusion and exchange surface area are reduced in the centrally obese and insulin resistant individuals with consequent effects on important aspects of muscle function relating to nutrient handling.

With the employment of Laser Doppler Fluximetry we showed an independent association between age, insulin sensitivity and the functional hyperaemic response to physiological stimuli such as venous occlusion and hyperinsulinaemia. Interestingly, the functional hyperaemic response was not associated with the measures of body fatness or other cardiovascular risk factors. Functional hyperaemia represents an increase in tissue perfusion in response to an increased metabolic demand. It can be modified in response to local or circulating metabolic factors, including insulin, through both endothelium-dependent and -independent mechanisms [341] all of which may contribute to an altered vascular response [342]. We showed in our study that under basal conditions which constitute low metabolic demand, the capacity to increase functional tissue perfusion was lower in individuals at increased cardio-metabolic risk associated with central obesity. Moreover, we also showed that the capacity of functional hyperaemia was not ameliorated or overcome by insulin. De Jongh *et al*. reported that in the obese subjects PORH capillary recruitment in the skin of the nailfold at baseline and during an insulin clamp was impaired in comparison to lean controls [152]. The findings from our study are consistent and extend the findings of de Jong's and colleagues. Furthermore, our data also extends the findings reported by Clerk *et al*. from a study investigating muscle microvascular blood volume (MBV) with contrast ultrasound in obese subjects [163]. In this study, the authors demonstrated a relationship between the insulin-induced change in muscle MBV and BMI only, whereas we showed that both, age and insulin sensitivity index explained independently nearly half of the variance in functional hyperaemia ($r^2$=0.42, p=0.003) (Table 5.6).

Additionally, by utilizing laser Doppler fluximetry to assess blood flux at rest, during post occlusive reactive hyperaemia and acute hyperinsulinaemia we were also able to show that dysregulation of the microvascular function in central obesity manifests early prior to the onset of overt macrovascular disease. The age-related alterations in microvasculature and the mechanisms behind the ameliorated with age dilator responses were extensively studied in the healthy subjects [330]. However, the mechanisms underlying the attenuated microvascular dilator responses in centrally obese individuals at increased cardio-metabolic risk were less explored and understood. Moreover, up to date it has been uncertain whether the adverse effect of age and insulin resistance on microvascular function was independent, considering that insulin resistance may increase with age. Data from our study, however, indicates that age and insulin resistance independently of each other adversely affect muscle microvascular function as demonstrated by altered PORH responses.

Within a range of techniques available for the studying microcirculation, the number of methods for direct investigation of muscle microvasculature are not only limited, but they are also invasive, time consuming and expansive such as radiolabelled imaging techniques [343], contrast enhanced ultrasound using albumin microbubbles [146] or needle-inserted laser Doppler probes [140]. The alternative way to assess muscle microvascular function is to non-invasively quantify the capacity of the microvascular bed to filter fluid using plethysmography. This is a well validated technique which uses measurements of the change in limb volume resulting from small step increases in venous occlusion pressure to provide a measure of muscle microvascular filtration capacity ($K_f$) [141]. $K_f$ has been shown to be differentially sensitive to increases in capillary perfusion [221] as well as to increases in capillary surface area [318]. Since $K_f$ is an important and sensitive measure of microvascular function we elected to use this non-invasive technique in our study, as we felt it would provide better compliance and be acceptable to non-paid volunteers returning for re-testing at the end of extensive study. It is important to stress that plethysmography allows for the measurement of the rate of fluid exchange across the whole muscle microvascular bed thus addressing the issue that many of the invasive and direct methods rely upon visualization of erythrocyte movement or their particulate surrogates which is often difficult to visualise.

For similar reasons as the above, we also employed Laser Doppler Fluximetry with a high power probe to estimate changes in microvascular function under basal conditions and during functional demand. This is another non-invasive and novel technique, and the deeply penetrating laser probe samples a large volume of nutritive vasculature [143]. Glough *et al.* demonstrated previously that in lean individuals over a 65% of the probe signal is derived from sub-dermal tissue [143]. Therefore the use of this LDF probe in our obese subjects may have lead to a greater variability in the signal due to a different sub-dermal thickness between individuals. Nevertheless, the variance in signal reported in our study did not differ significantly from those reported in other studies where a lower laser power and a standard probe were used [297].

A criticism of this study may be that we have not recruited a control group. However, the study was designed to investigate microvascular function in a cohort of individuals with a spectrum of cardiovascular risk determinants but without overt vascular disease. Therefore we did not aim to compare microvascular function in normal weight versus obese individuals. A further criticism is that our study may have lacked power to detect small changes in measures of reactive hyperaemia during hyperinsulinaemia. However, using the study results we conducted a retrospective sample size calculation. We calculated that

measurements in 12 subjects would detect a 2.3 fold improvement in reactive hyperaemia during the insulin infusion providing our study with 90% power at a 5% significance level. The same, 2.3 fold increase in reactive hyperaemia during hyperinsulinaemia in the tibialis anterior muscle was reported previously by de Jongh *et al.* [140]. Unlike de Jongh who used a placebo clamp in the healthy individuals as a comparison, we were unable to include a placebo clamp in this study. The fact, however, that we did not observe an increase in blood pressure would suggest that the attenuated vasoresponse to insulin reported in our cohort was unlikely to be due to differences in baseline sympathetic tone during the experimental protocol.

In conclusion, we have shown that in centrally obese, non diabetic men and women, a strong, independent of visceral fatness, association between skeletal muscle microvascular exchange capacity ($K_f$) and decreased insulin sensitivity in skeletal muscle. Also, a strong but not independent of insulin sensitivity, adverse relationship between $K_f$ and visceral fatness was documented. Both associations were not confounded by factors such as physical inactivity or low fitness level. We have also demonstrated that skeletal muscle microvascular dilator capacity is independently associated with both age and insulin sensitivity, which together explain almost half of the variance in this measure of microvascular function. The assertion that central obesity blunts microvascular dilator response and constrains functional hyperaemia which can not be overcome by acute hyperinsulinaemia is supported by our results. The presented data emphasises that two key elements of microvascular function, the filtration/exchange capacity and functional dilator response, are negatively associated with insulin insensitivity in individuals with central adiposity and in the absence of type 2 diabetes. All these factors may give rise to impaired delivery and handling of nutritive solutes to insulin sensitive tissues such as skeletal muscle during an increased metabolic demand. Moreover, the results of this study support the notion that the impaired microvascular function is an early indicator of cardio-metabolic risk in subjects with central obesity and features of metabolic syndrome.

Summary points:

- In centrally obese, insulin resistant but not diabetic individuals, there is present strong positive association between skeletal muscle microvascular exchange capacity/filtration ($K_f$) and level of insulin sensitivity, independent of visceral fatness and not confounded by physical inactivity and poor cardiorespiratory fitness.

- $K_f$ is also strongly and adversely, but not independently of insulin sensitivity associated with visceral fatness.

- In centrally obese, insulin resistant but not diabetic individuals, there is present a strong positive association between skeletal muscle microvascular dilator capacity (PORH) and age and insulin sensitivity, independent of each other; both together explain almost half of the variance in PORH.

- Microvascular function is negatively associated with insulin insensitivity in individuals with central adiposity and in the absence of type 2 diabetes.

- Central obesity blunts microvascular dilator response and constrains functional hyperaemia in response to metabolic demand.

- The impairment of microvascular function in centrally obese, insulin resistant but not diabetic individuals precedes the onset of overt macrovascular disease.

# 6. The effects of six months treatment with Atorvastatin on the insulin sensitivity and microvascular function in healthy but viscerally obese subjects

## 6.1 Introduction

It is well documented that obesity is associated with the risk of developing insulin resistance [18, 78] and that central obesity is more strongly associated with metabolic and cardiovascular complications than total body fat [52, 344]. Many studies reported a strong relationship between attenuated insulin sensitivity and intra-abdominal adiposity [61]. Also, research studies showed that obesity is associated with microvascular function, and that the latter may be a potential link between visceral adiposity and insulin sensitivity [151, 152]. Both central obesity and insulin resistance play a central role in the development of the metabolic syndrome [168, 345, 346], for which early identification and effective treatment is a major therapeutic goal. However, a clear drug of choice for individuals at high cardio-metabolic risk has not been identified as yet.

Statins, the 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitors, lower blood cholesterol levels by increasing expression of low density lipoprotein (LDL) receptors and improving LDL-cholesterol uptake by hepatocytes. About four to six weeks are required for their hypolipidaemic effect to become evident. The profound effects of statins on total and LDL cholesterol are well documented and proven to reduce cardiovascular risk in primary [223, 225] and secondary prevention [223, 224]. They were shown to be effective in reducing vascular risk in people with metabolic syndrome but no diabetes [347].

It is also well accepted that statins have pleiotropic actions. However, there is conflicting evidence regarding the effects of statin therapy on insulin resistance. Ohrvall *et al.* [242] reported that Simvastatin 10 mg daily for 4 months increased plasma insulin concentrations by 21% and decreased insulin sensitivity by 28% as assessed by glucose clamp in patients with type 2 diabetes mellitus. Similarly, Jula *et al.* demonstrated deterioration in surrogate insulin sensitivity markers in a double-blinded, placebo controlled trial with 20mg daily Simvastatin for 3 months in hypercholesterolaemic but not obese or diabetic subjects [252]. No change in insulin sensitivity was found in a placebo-controlled study with 20 mg of Simvastatin for 3 months in type 2 diabetes mellitus [248]. In contrast to those studies, Paolisso *et al.* compared Simvastatin 10 mg daily with Atorvastatin 5 mg and placebo in subjects with type 2 diabetes and documented a 13% improvement in insulin action with Atorvastatin and 9% with Simvastatin [246]. Interestingly, Koh *et al*. demonstrated

improved insulin sensitivity, as measured by fasting insulin and QUICKI, with 2 months of treatment with 40 mg daily of Pravastatin [247] and deterioration in the same markers with 20mg daily of Simvastatin.

These discrepant findings may be due to different patient selection, baseline lipid profiles, investigative methodology, statin type, dosage and duration of treatment implemented in various research studies. Additionally changes in body weight and/or body composition during the investigations could have potentially played a role as confounding factors on insulin action. Moreover, to date there have been no studies investigating the effects of statins on insulin sensitivity in subjects without type 2 diabetes but with central adiposity and insulin resistance. Although the recent meta-analysis of clinical trials with statins provided information on the increased risk of incident diabetes with all statins [253], analysed studies consisted of various patient cohorts, who were likely to be on multiple medications. The mechanisms behind this increased diabetes risk have been not elucidated as yet and remain uncertain.

The reported pleiotropic effects of statin within macrovasculature include improvement of endothelial function and attenuation of endothelial dysfunction in the presence of atherosclerotic risk factors. Those effects were shown to be achieved through increased bioavailability of nitric oxide (NO) [231, 239], apparent after 6 months of therapy [241], and also through reduced oxidative stress and inflammation, and increased recruitment of endothelial progenitor cells [229, 230, 240]. However, the potential for statins to modulate endothelial function in human microvasculature via the postulated pleiotropic effects and independently of lipid-lowering benefits remains uncertain [138, 332, 348, 349].

The purpose of this study was to assess the level of insulin sensitivity in people with central adiposity and other metabolic traits and to evaluate the effects of statins on insulin resistance and measures of skeletal muscle microvascular function including functional microvascular dilator capacity (PORH), microvascular filtration capacity ($K_f$), a measure of microvascular integrity ($P_{vi}$) and resting limb blood flow ($Q_a$). We tested the hypothesis that in centrally obese subjects without diabetes, six months intensive statin treatment with Atorvastatin 40mg daily would improve insulin sensitivity and reverse microvascular dysfunction via the potential pleiotropic properties of statins to modulate nitric oxide production.

In order to test the effects of high dose statin treatment we estimated insulin sensitivity and microvascular function at baseline and again after randomisation to six months of either Atorvastatin 40 mg daily or placebo. We have chosen Atorvastatin because it was credited

with beneficial effects on central obesity and several aspects of the metabolic syndrome, and its potency, efficacy and safety record may confer advantages over other statins [255, 350]. Since the level of physical activity may potentially influence insulin sensitivity and skeletal muscle microvascular function [221], we investigated the influence of this potential confounder on those variables.

## 6.2 Methods

White Caucasian, non-diabetic and non-hypertensive unpaid volunteers aged 29-69 years were recruited into the study. All subjects had central adiposity defined according to IDF criteria as a waist circumference ≥ 94cm in men and ≥ 80 cm in women. Only individuals who had low or moderate cardiovascular risk of less than 20% over 10 years, as calculated using equation derived from the Framingham Heart Study, were included into the study. All participants were statin naïve. All investigations were performed at baseline and after interventions while the subjects continued with their study medication. They all underwent an oral glucose tolerance test with a 75g glucose load and had measured fasting lipid profile, insulin, NEFA, hs-CRP and $HbA_{1c}$. Lactate was measured throughout the clamp studies to assess for any adverse effects of thigh cuff inflations. Description of the analytical methods for the above parameters was provided in the laboratory methods chapter.

All individuals were subjected to anthropometric assessment and their body composition was measured with DEXA before and after intervention, of which a detailed description was provided in chapter 3.1.

The stepped hyperinsulinaemic euglycaemic clamp was employed to assess insulin sensitivity. The whole-body glucose disposal rate (M-value) was determined during the last 30 minutes of clamp as well as mean insulin level. Both parameters were used to calculate insulin sensitivity index (M/I). Adipose tissue response to low dose insulin infusion was estimated by calculating the percentage of change between the mean NEFA concentration at baseline and after 60 minutes of low dose infusion. The details of clamp and calculations were presented in chapter 3.4.

Skeletal muscle microvascular function was assessed in terms of functional dilator capacity during a hyperinsulinaemic euglycaemic clamp by measuring post-occlusive reactive hyperaemia (PORH) before and during the final 30 minutes of insulin infusion using Laser Doppler fluximetry. PORH was calculated as a percentage increase in pressure flow relative to resting flow (PF%RF). Exchange/filtration capacity ($K_f$), resting blood flow ($Q_a$) and isovolumetric venous pressure ($P_{vi}$) as a measure of endothelial integrity were assessed using venous congestion plethysmography employing Filtrass system. All measurements and calculations were detailed in chapter 3.7.

Physical activity was measured with an activity monitor (SenseWear Armband Pro2) and cardiorespiratory fitness with the standard maximal oxygen uptake technique during treadmill exercise as they were considered as possible confounders of skeletal muscle exchange capacity. Those techniques were described in detail in chapter 3.5.

The participants were randomised to either Atorvastatin (40 mg daily) daily or placebo for 6 months in a double-blind, parallel group study design. The selection of a daily dose of 40 mg Atorvastatin was based on considerations of efficacy and tolerability [255, 351].
The subjects were encouraged to maintain the same diet and lifestyle throughout the duration of the study to avoid changes in total body weight greater than 5 % from the baseline and thus to minimise the effect of those confounders on the end results.
The statistical analysis was performed using SPSS for Windows version 16.0. No statistical correction was undertaken for the performed multiple measurements..Paired and unpaired student *t*-test was used for within- and between-group analysis, and repeated measurements ANOVA to test for the group effect. Normally distributed data were expressed as mean ± SD. To test the effect of statin on measures of microvascular function we analysed microvascular function at the end of the trial, adjusting for randomization and baseline microvascular measures and in case of functional dilator capacity additionally for age, by factorial ANOVA. A $p$ value of $\leq 0.05$ was considered to indicate statistical significance.

## 6.3 Results

Out of the forty-nine participants recruited to this study, eight declined further participation and one was excluded due to severe anaemia before commencing the treatment phase. Another subject withdrew after randomisation. The baseline characteristics of thirty nine subjects who completed the treatment and all microvascular studies are presented in Table 6.1. They included 17 men and 22 women and their mean age was 51.4 ± 9 years. All participants had central adiposity with a mean waist circumference of 105.3 ± 12.9cm. Of those, twenty fulfilled IDF criteria for the metabolic syndrome: 13 had 3 features, 6 had 4 features and 1 individual had all 5 features. Nine subjects fulfilled both waist and blood pressure criteria, while the remaining ten only fulfilled the waist criterion. The summary of the metabolic traits in our cohort is presented in Table 6.2. All participants managed to maintain their lifestyle throughout the study as indicated by their body weight, which remained within 5% from the baseline (mean weight difference between baseline and end of the study was 3% ± 2%). Their level of physical activity, measured by activity monitor over an average period of seven days before randomisation and at the end of treatment, had not significantly changed during the course of study with the mean daily METs before treatment of 1.28 ± 0.17 and after treatment of 1.26 ± 0.23, p=0.58.
Twenty volunteers were randomised to the placebo arm, which included an equal number of men and women. Within the active treatment group there were seven men and twelve women. The characteristics of the study cohort based on the randomisation at the baseline and at the end of intervention are shown in Table 6.3. We collected completed sets of results from clamp studies (before and after randomisation) on thirty two participants.

Subjects randomised to placebo had waist circumference of 106.5 ± 2.5 cm and those within the Atorvastatin group of 103.0 ± 2.7 cm (p=0.355). There was no significant difference between both groups with regards to baseline weight and BMI, neither was there a significant difference between anthropometric parameters after 6 months of treatment within and between groups (Table 6.3 and 6.4). Similarly, the body fat composition remained similar between and within both groups at the baseline and after intervention (Table 6.3 and 6.4).

Although the systolic blood pressure was significantly lower in the treatment group (134 ± 8.7 mmHg vs 124 ± 17.2 mmHg, p=0.026), which may be related to slightly lower (but statistically insignificant) body weight (Table 6.3), this did not changed after intervention

and there was no significant change in systolic blood pressure within each group (placebo: 134 ± 8.7 vs 131 ± 13.1mmHg, p=0.314; statin: 124 ± 17.2 vs 120 ± 12.5, p=0.130).

As expected there was a dramatic change in the fasting lipids in the treatment arm of the study as opposed to the placebo arm (n=20) in which the mean baseline LDL-cholesterol was 3.76 ± 1.02 mmol/L and at follow up was 3.69 ± 0.87 mmol/L (p=0.566), and baseline triglycerides were 1.36 ± 0.69 mmol/L and after 6 months: 1.25 ± .65 mmol/L (p=0.361). In contrast, in the treatment group (n=19), the baseline LDL-cholesterol decreased from 3.53 ± 0.82 mmol/L to 1.63 ± 0.58 (p<0.0001) at follow up and triglycerides from 1.36 ± 0.60 mmol/L at baseline to 0.92 ± 0.49 mmol/L (p<0.0001) after treatment (Table 6.4 and 6.5). The alteration in lipids concentration within the treatment group resulted in a diminished by 50% CVD risk, from 6.1% to 2.88% (p<0.0001), while in the placebo group the CVD risk remained similar over 6 months period (8.4% vs 9.1%, p=0.092).

**Table 6.1 Baseline characteristics of study population, n=39**

| Variable | Mean ± SD | Range |
|---|---|---|
| Age (years) | 51.4 ± 9.0 | 29.0 - 69.6 |
| Waist circumference (cm) | 105.3 ± 12.9 | 86.5 - 151.0 |
| BMI ($kg/m^2$) | 32.1 ± 4.6 | 25.3 - 47.9 |
| DEXA total body fat (%) | 35.6 ± 7.4 | 21.2 - 47.5 |
| DEXA trunk fat (%) | 16.8 ± 4.6 | 9.0 -31.9 |
| Blood pressure systolic (mmHg) | 133 ± 14 | 93 - 155 |
| Blood pressure diastolic (mmHg) | 85 ± 9 | 64 - 104 |
| CVD risk (%) | 7.3 ± 5.1 | 0 - 17.3 |
| Total cholesterol (mmol/L) | 5.7 ± 1.1 | 3.2 - 9.3 |
| LDL-cholesterol (mmol/L) | 3.7 ± 0.9 | 1.7 - 7.0 |
| HDL-cholesterol (mmol/L) | 1.6 ± 0.4 | 0.9 - 2.5 |
| Triglycerides (mmol/L) | 1.4 ± 0.6 | 0.4 - 2.7 |
| $HbA_{1c}$ (%) | 5.5 ± 0.3 | 4.9 - 6.2 |
| Fasting Glucose (mmol/L) | 5.2 ± 0.7 | 4.0 – 6.6 |
| M/I ((mg·L)/kg/min/mIU) | 3.36 ± 1.25† | 0.97 - 6.26† |
| PAEE (MET) | 1.3 ± 0.2 | 0.8 - 1.6 |
| hs-CRP (mg/dL) | 2 (1.09 -10.47) †† | 0.0 - 39.8 |

† n=32, †† median (95%CI)

**Table 6.2 Summary of the features of the metabolic syndrome in the cohort**

| Features of metabolic syndrome | Total number of subjects |
|---|---|
| Waist circumference: ≥ 94cm in men<br>≥ 80cm in women | 39 |
| Blood pressure: systolic ≥ 130mmHg or<br>diastolic ≥ 85mmHg | 22 |
| Fasting triglycerides ≥ 1.7 mmol/L | 12 |
| Fasting glucose ≥ 5.6 mmol/L | 10 |
| HDL-cholesterol: ≤ 1.03mmol/L in men<br>≤1.29 mmol/L in women | 6 |

**Table 6.3 Characteristics of study population based on randomisation, at baseline and after intervention**

| | 0 months | | p-value | 6 months | | p-value |
|---|---|---|---|---|---|---|
| | Placebo | Atorvastatin | | Placebo | Atorvastatin | |
| Age (years) | 53.3 ± 9.3 | 52.9 ± 8.7 | 0.927 | | | |
| Waist circumference (cm) | 108.4 ± 14.3 | 102.0 ± 10.7 | 0.126 | | | |
| Body weight (kg) | 94.4 ± 20.` | 89.3 ± 14.9 | 0.376 | 95.5 ± 21.84 | 89.7 ± 16.43 | 0.356 |
| BMI ($kg/m^2$) | 32.30 ± 5.5 | 30.9 ± 3.9 | 0.357 | 32.6 ± 6.12 | 31.1 ± 4.79 | 0.383 |
| DEXA total body fat (kg) | 33.3 ± 10.95 | 30.39 ± 7.95 | 0.351 | 34.65 ± 12.79 | 30.97 ± 8.52 | 0.298 |
| DEXA trunk fat (kg) | 17.36 ± 5.78 | 15.32 ± 3.68 | 0.200 | 18.54 ± 7.65 | 15.72 ± 4.11 | 0.164 |
| Blood pressure systolic (mmHg) | 134.2 ± 8.7 | 124.2 ± 17.2 | 0.026* | 131.9 ± 13.1 | 120.8 ± 12.5 | 0.011* |
| Blood pressure diastolic (mmHg) | 81.7 ± 8.5 | 79.7 ± 10.2 | 0.526 | 82.5 ± 7.5 | 76.1 ± 8.8 | 0.020* |
| Total cholesterol (mmol/L) | 5.8 ± 1.2 | 5.7 ± 1.0 | 0.887 | 5.49 ± 1.03 | 3.49 ± 0.66 | 0.000* |
| LDL-cholesterol (mmol/lL) | 3.7 ± 1.02 | 3.53 ± 0.8 | 0.446 | 3.69 ± 0.87 | 1.63 ± 0.58 | 0.000* |
| HDL-cholesterol (mmol/L) | 1.4 ± 0.3 | 1.5 ± 0.4 | 0.397 | 1.25 ± 0.30 | 1.44 ± 0.30 | 0.048 |
| Triglycerides (mmol/L) | 1.4 ± 0.7 | 1.4 ± 0.6 | 0.992 | 1.25 ± 0.65 | 0.92 ± 0.49 | 0.086 |
| $HbA_{1c}$ (%) | 5.5 ± 0.36 | 5.4 ± 0.32 | 0.302 | 5.7 ± 0.58 | 5.6 ± 0.24 | 0.256 |
| Fasting Glucose (mmol/L) | 5.3 ± 0.89 | 5.1 ± 0.47 | 0.478 | 5.6 ± 1.03 | 5.40 ± 0.46 | 0.493 |
| Fasting Insulin (mIU/L) | 10.88 ± 7.76 | 8.83 ± 3.17 | 0.293 | 11.65 ± 8.57 | 11.52 ± 3.99 | 0.951 |
| HOMA-IR | 2.73 ± 2.56 | 2.12 ± 0.74 | 0.331 | 3.10 ± 3.44 | 2.73 ± 1.05 | 0.662 |
| QUICKI | 0.60 ± 0.09 | 0.61 ± 0.07 | 0.686 | 0.58 ± 0.07 | 0.57 ± 0.05 | 0.438 |
| M value † (mg/kg/min) | 4.30 ± 1.16 | 5.48 ± 1.25 | 0.005* | 4.57 ± 1.09 | 5.04 ± 1.13 | 0.231 |
| M/I † ((mg·L)/kg/min/mIU) | 2.90 ± 1.27 | 3.85 ± 0.99 | 0.014* | 2.97 ±1 .04 | 3.83 ± 1.41 | 0.054 |
| PAEE (MET) | 1.29 ± 0.19 | 1.28 ± 0.13 | 0.895 | 1.22 ±0.19 | 1.31 ± 0.25 | 0.198 |

* $p < 0.05$, statistically significant, † n=32

**Table 6.4 Anthropometric measurements, within-group comparison**

| | Placebo | | p-value | Atorvastatin | | p-value |
|---|---|---|---|---|---|---|
| | 0 months | 6 months | | 0 months | 6 months | |
| Body weight (kg) | 94.4 ± 20.1 | 95.5± 21.8 | 0.205 | 89.3 ± 14.9 | 89.6 ± 16.4 | 0.607 |
| BMI ($kg/m^2$) | 32.3 ± 5.5 | 32.6 ± 6.1 | 0.375 | 30.9 ± 3.9 | 31.0 ± 4.8 | 0.573 |
| DEXA total body fat (kg) | 33.3 ± 10.9 | 34.6 ± 12.7 | 0.47 | 30.3 ± 7.9 | 30.9 ± 8.5 | 0.273 |
| DEXA trunk fat (kg) | 17.4 ± 5.8 | 18.5 ± 7.6 | 0.065 | 15.3 ± 3.7 | 15.7 ± 4.11 | 0.199 |

* $p < 0.05$, statistically significant

**Table 6.5 Fasting lipid profile before and after treatment with Atorvastatin**

| | Baseline | After treatment | p- value |
|---|---|---|---|
| Total cholesterol (mmol/L) | 5.71 ± 0.98 | 3.49 ± 0.66 | 0.000* |
| LDL-cholesterol (mmol/L) | 3.53 ± 0.82 | 1.63 ± 0.58 | 0.000* |
| HDL-cholesterol (mmol/L) | 1.50 ± 0.37 | 1.44 ± 0.30 | 0.209 |
| Triglycerides (mmol/L) | 1.36 ± 0.60 | 0.92 ± 0.49 | 0.002* |

* $p < 0.05$, statistically significant

Atorvastatin also reduced hsCRP significantly from a median baseline concentration of 2.0mg/dL (95% CI: (1.31, 5.59)) to 0.5mg/dL (95% CI: (0.35, 4.65)) at the follow up (p=0.02), whereas the median level of hsCRP in the placebo group was 2.0 mg/dL (95% CI: (1.09, 10.47)) compared with 3.0 mg/dL (95% CI: (1.62, 6.35)) after 6 months (p=0.73). There was a positive correlation between the change in LDL-cholesterol and hsCRP (r=0.27, p<0.002).

Oral Glucose Tolerance Testing identified 5 subjects with impaired glucose tolerance (IGT) both before and after intervention, 3 of whom were in the placebo group. An additional subject in the placebo group, who at 0 months had impaired fasting glycaemia (IFG), was found to have IGT (borderline) at 6 months and one subject from intervention group who previously had a normal test was found to have IFG at the end of study. Three subjects in the placebo group who had initially normal glucose tolerance tests were found to have IGT after 6 months while the opposite was seen in the case of one subject from the placebo and 1 subject from the statin group.

The baseline levels of fasting insulin sensitivity indices such as glucose, insulin, HOMA-IR and QUICKI were similar between both groups. All those parameters were significantly altered with Atorvastatin treatment – concentrations of glucose, insulin and HOMA increased whereas the QUICKI level proportionally decreased (Table 6.3 and 6.6). There was also a small, significant increase in fasting glucose in the placebo group. However repeated measures ANOVA did not show a group effect with *p*-value of 0.467.

The concentrations of glucose and insulin measured throughout the clamp studies did not differ significantly between the placebo and statin group at any time point, both at baseline and 6 months later, except for the 170 minute time point at baseline (t (30)=2.216, p=0.034) and 150 minute time point at 6 months (t (30)=2.173, p=0.038) as presented in Figure 6.1 and 6.2. This suggests that the tests were comparable and reproducible between and within the groups.

Subjects randomised to active treatment were at baseline more insulin-sensitive than those in the placebo group as defined by the mean insulin-mediated glucose disposal rate (M-value) and insulin sensitivity index (ISI) expressed as M/I value. Although the insulin sensitivity markers deteriorated over the course of study in the statin group, the difference was not statistically significant when using Student *t*-test. The repeated measures ANOVA, however, indicated borderline significant change for M-value (p=0.054) and statistically significant group effect for M/I with p=0.032. $HbA_1c$ concentrations showed small but

significant increase in both groups over the time with pre-treatment level of 5.5% and post-treatment level of 5.7% ($p<0.009$) in placebo arm and 5.4% versus 5.6% ($p<0.0001$) respectively in the intervention arm, but repeated measures ANOVA did not show a group effect for $HbA_1c$ ($p=0.246$).

NEFA concentrations, measured to assess lipolysis in the adipose tissue during the clamps, were similar amongst both groups before randomisation, and they were higher than those in non-obese populations (Table 6.8). There was 46% and 42% NEFA suppression in the statin and placebo groups respectively in response to low dose (0.2mIU/kg/min) insulin infusion and this remained unchanged by treatment in either group. The degree of NEFA suppression to high dose (1.5mIU/kg/min) insulin infusion reached 90% in both groups of subjects and was not altered by intervention (Figure 6.3).

Thigh cuff inflation at 44 and 164 minutes during the clamp did not affect lactate concentrations (Table 6.8). There was no significant difference in lactate levels at any time point during the low dose and during the last 30 minutes of high dose insulin infusion. Lactate concentration increased by 35% during high dose in comparison to the low dose insulin infusion (Figure 6.4).

**Table 6.6 Fasting insulin sensitivity indices – comparison within the groups**

| | Placebo | | p-value | Atorvastatin | | p-value |
|---|---|---|---|---|---|---|
| | 0 months | 6 months | | 0 months | 6 months | |
| Glucose (mmol/L) | 5.26 ± 0.89 | 5.58 ± 0.03 | 0.007* | 5.09 ± 0.47 | 5.40 ± 0.46 | 0.006* |
| Fasting Insulin (mIU/L) | 10.9 ± 7.77 | 11.6 ± 8.57 | 0.152 | 8.84 ± 3.17 | 11.52 ± 3.99 | 0.003* |
| HOMA-IR | 2.72 ± 2.56 | 3.10 ± 3.44 | 0.145 | 2.12 ± 0.74 | 2.74 ± 1.04 | 0.008* |
| QUICKI | 0.60 ± 0.09 | 0.58 ± 0.07 | 0.107 | 0.61 ± 0.06 | 0.56 ± 0.04 | 0.003* |

* $p < 0.05$, statistically significant

**Figure 6.1 Glucose concentrations during stepped hyperinsulinaemic euglycaemic clamp; pre tx = pre treatment, post tx = post treatment**

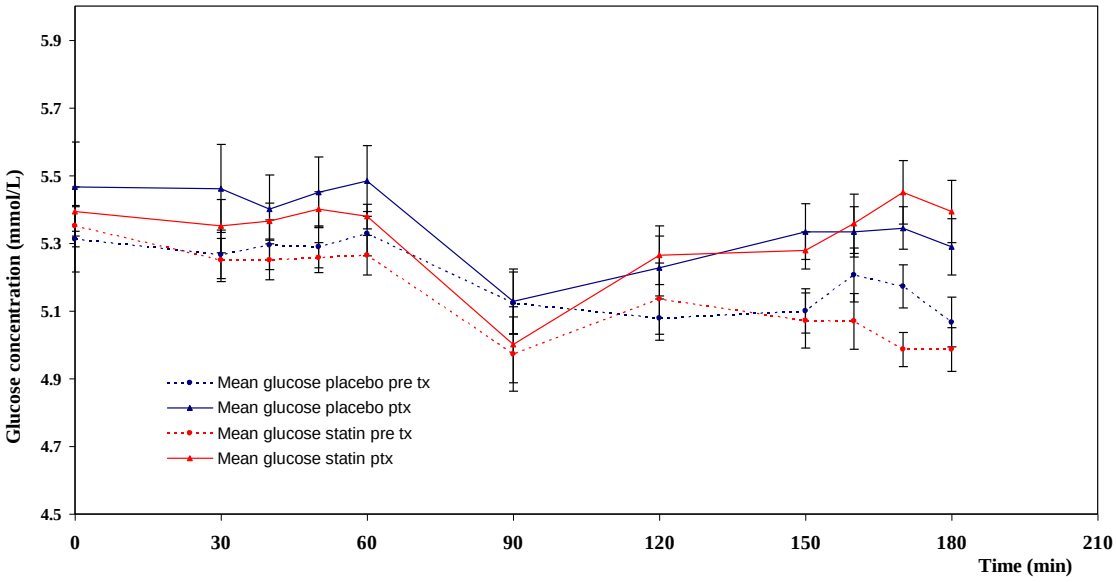


**Figure 6.2 Insulin concentrations during stepped hyperinsulinaemic euglycaemic clamp; pre tx = pre treatment, post tx = post treatment**

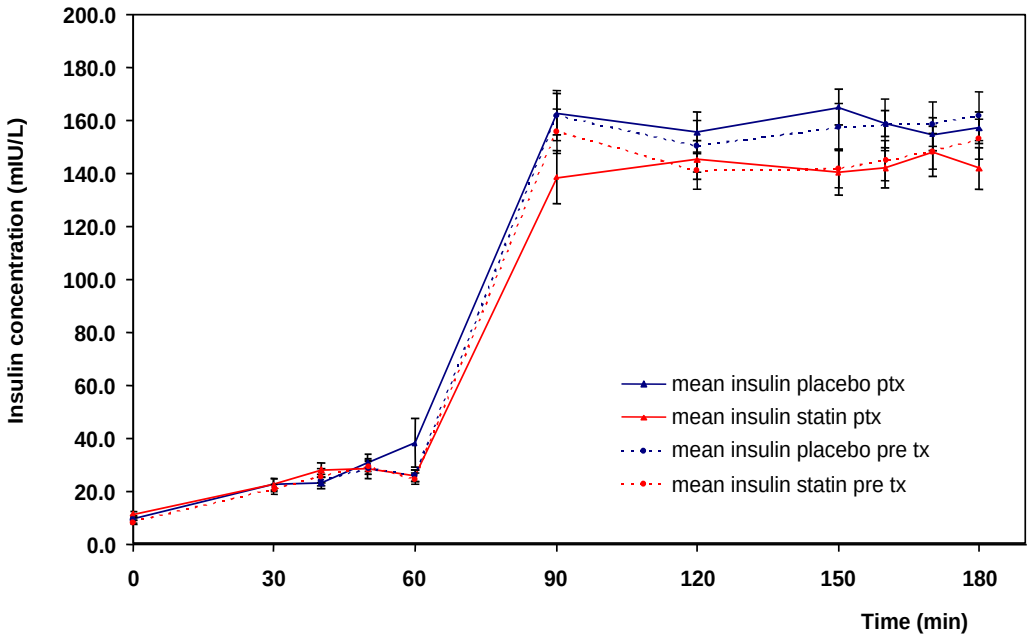



**Table 6.7 Clamp-derived insulin sensitivity indices – within-group comparison**

| | Placebo | | p-value | Atorvastatin | | p-value |
|---|---|---|---|---|---|---|
| | 0 months | 6 months | | 0 months | 6 months | |
| M value (mg/kg/min) | 4.42 ± 1.16 | 4.56 ± 1.09 | 0.490 | 5.41 ± 1.23 | 5.09 ± 1.15 | 0.247 |
| M/I (mg/kg/min/mIU/L) | 3.00 ± 1.26 | 2.97 ± 1.04 | 0.849 | 3.90 ± 1.07 | 3.83 ± 1.41 | 0.756 |

* $p < 0.05$, statistically significantly

**Table 6.8 NEFA concentrations during the stepped hyperinsulinaemic euglycaemic clamp: at baseline, after 60 minutes of low dose insulin infusion and during the steady state of high-dose insulin infusion**

| | Placebo | | p-value | Atorvastatin | | p-value |
|---|---|---|---|---|---|---|
| | 0 months | 6 months | | 0 months | 6 months | |
| Basal NEFA (μmol/L) | 640 ± 40 | 571 ± 46 | 0.008* | 679 ± 63 | 595 ± 46 | 0.120 |
| Low-dose insulin infusion (μmol/L) | 372 ± 46 | 375 ± 38 | 0.925 | 368 ± 34 | 328 ± 38 | 0.458 |
| High-dose insulin infusion (μmol/L) | 39 ± 4 | 40 ± 3.5 | 0.780 | 38 ± 5 | 31 ± 4 | 0.143 |

* $p < 0.05$, statistically significant

**Figure 6.3 NEFA concentrations during stepped hyperinsulinaemic euglycaemic clamp; pre tx = pre treatment, post tx = post treatment**

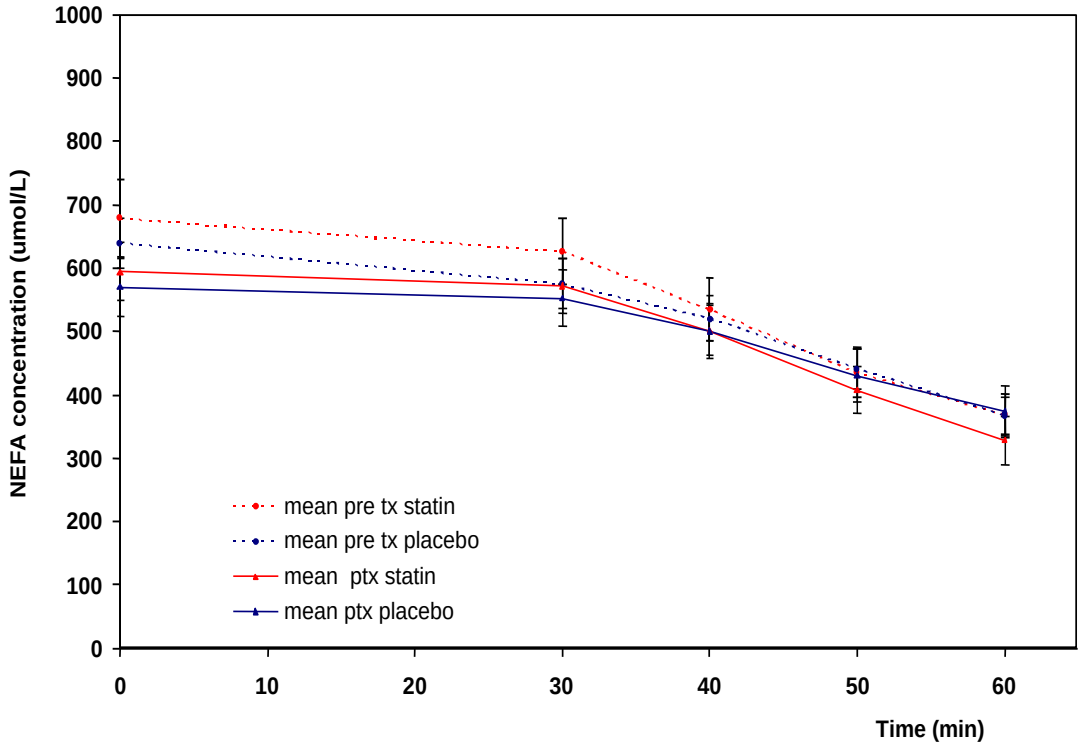
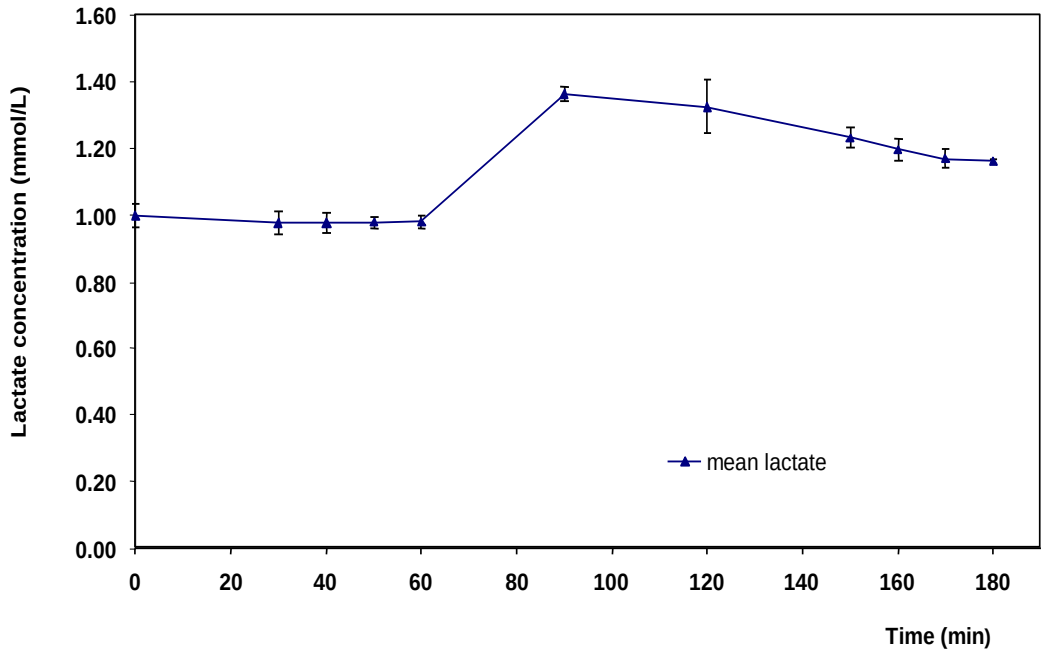


**Table 6.9 Changes in lactate concentrations at the time points of thigh cuff inflation during the hyperinsulinaemic euglycaemic clamp**

| Insulin Infusion | Time point (minutes) | Δ Lactate (mmol/L) | p- value |
|---|---|---|---|
| Low dose | 40 -50 | - 0.155 | 0.293 |
| Low dose | 40 -60 | - 0.103 | 0.545 |
| Priming for high dose /high dose | 60-90 | - 0.378 | 0.000* |
| High dose | 160-170 | 0.013 | 0.234 |
| High dose | 160-180 | 0.028 | 0.030 |

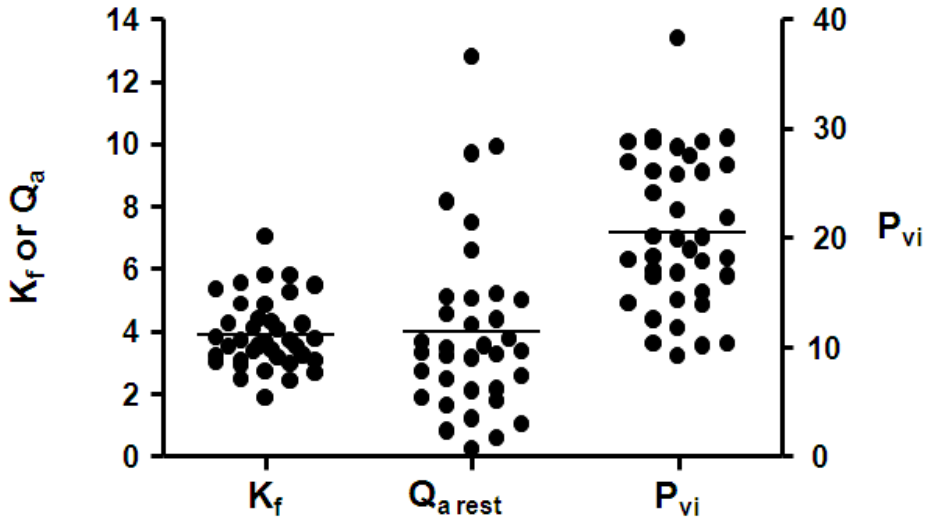
**Figure 6.4 Lactate concentrations during the stepped hyperinsulinaemic euglycaemic clamp; pre tx = pre treatment, post tx = post treatment**



The baseline microvascular function measurements are presented in Figures 6.5 (this figure was also shown in chapter 5) and Figure 6.6. There was considerable variability in all measures of microvasculature within both groups which remained unchanged throughout the study as presented with the example of functional dilatory capacity in Figure 6.6. The characteristics and relationships of microvascular function in our obese subjects were described in this dissertation in chapters 4 and 5.

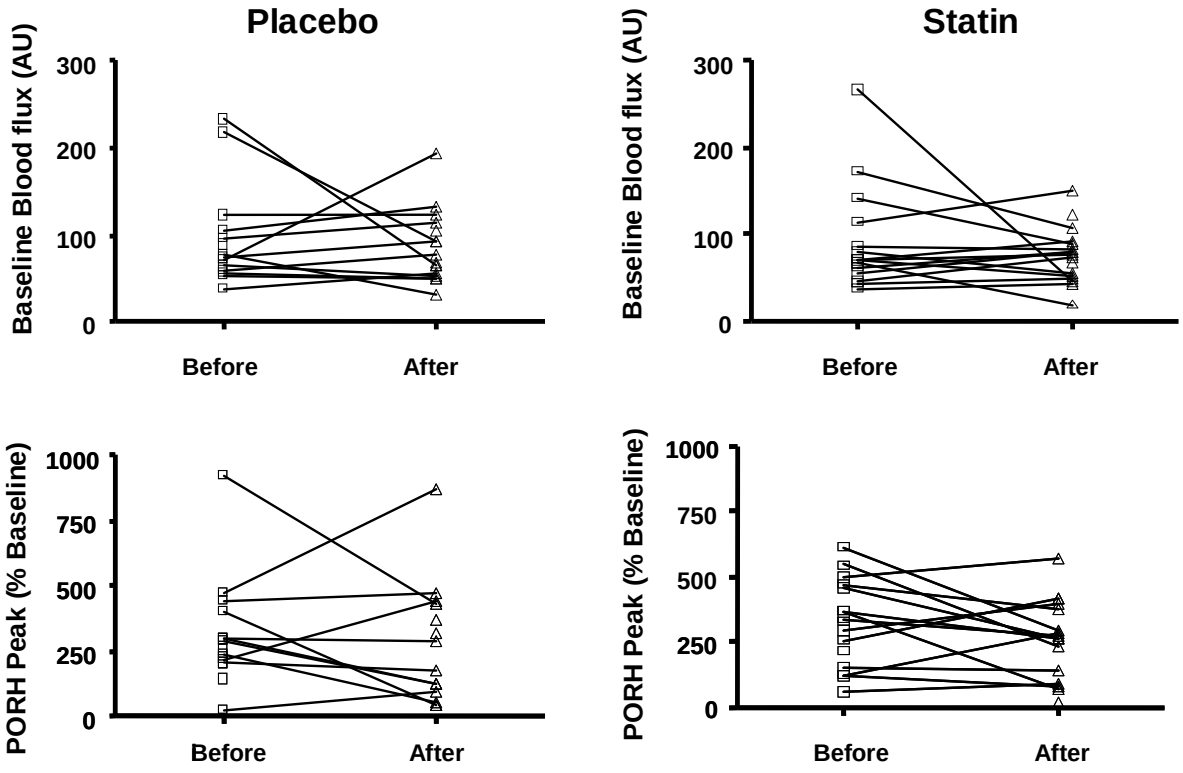
Six months of treatment with Atorvastatin did not significantly alter any of the microvascular function parameters measured with the venous congestion plethysmography or LDF during the clamp studies (Figures 6.6, 6.7 and 6.8). There were no significant changes in $K_f$ ($p=0.99$), $P_{vi}$ ($p=0.28$) and $Q_a$ ($p=0.29$) in the intervention group after adjusting for baseline measurements, age and gender. Similarly, therapy with Atorvastatin did not influence the resting blood flux (RF) when compared with the baseline ($p=0.15$). The microvascular dilator capacity to arterial occlusion (PF%RF) (Figure 6.8) and insulin-induced change in PF%RF were not significantly altered by statin treatment compared with placebo group.

**Figure 6.5 Baseline measurements of filtration capacity, resting limb blood flow and endothelial integrity**



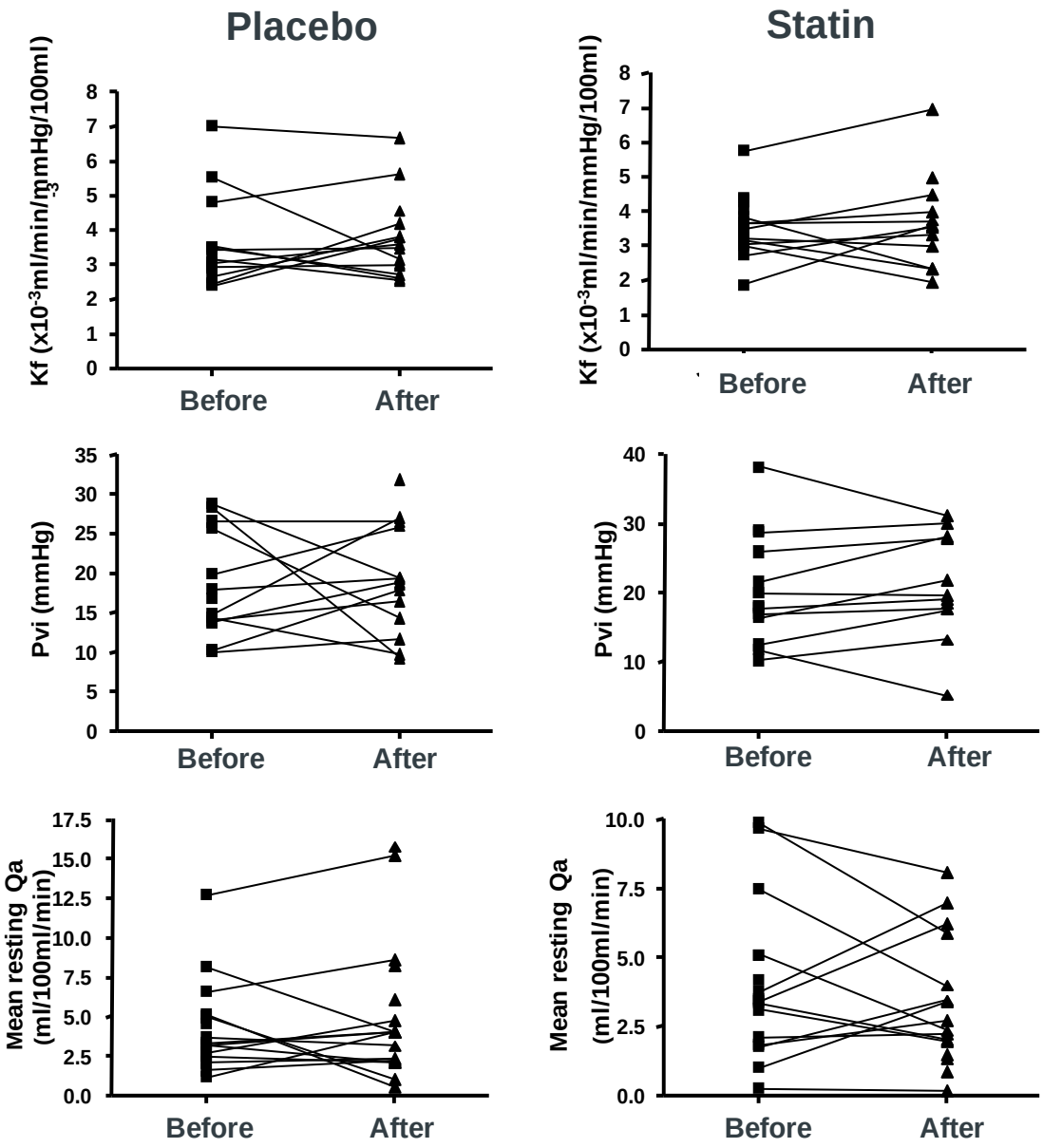

$K_f$ - exchange capacity ($\times 10^{-3}$ml/min/mmHg/100ml tissue), $Q_a$ rest - resting blood flow (ml/min/100ml tissue), $P_{vi}$ - isovolumetric venous pressure (mmHg)

**Figure 6.6 Baseline and post treatment results of blood flux and PORH peak measured by Laser Doppler Fluximetry during hyperinsulinaemic clamp**



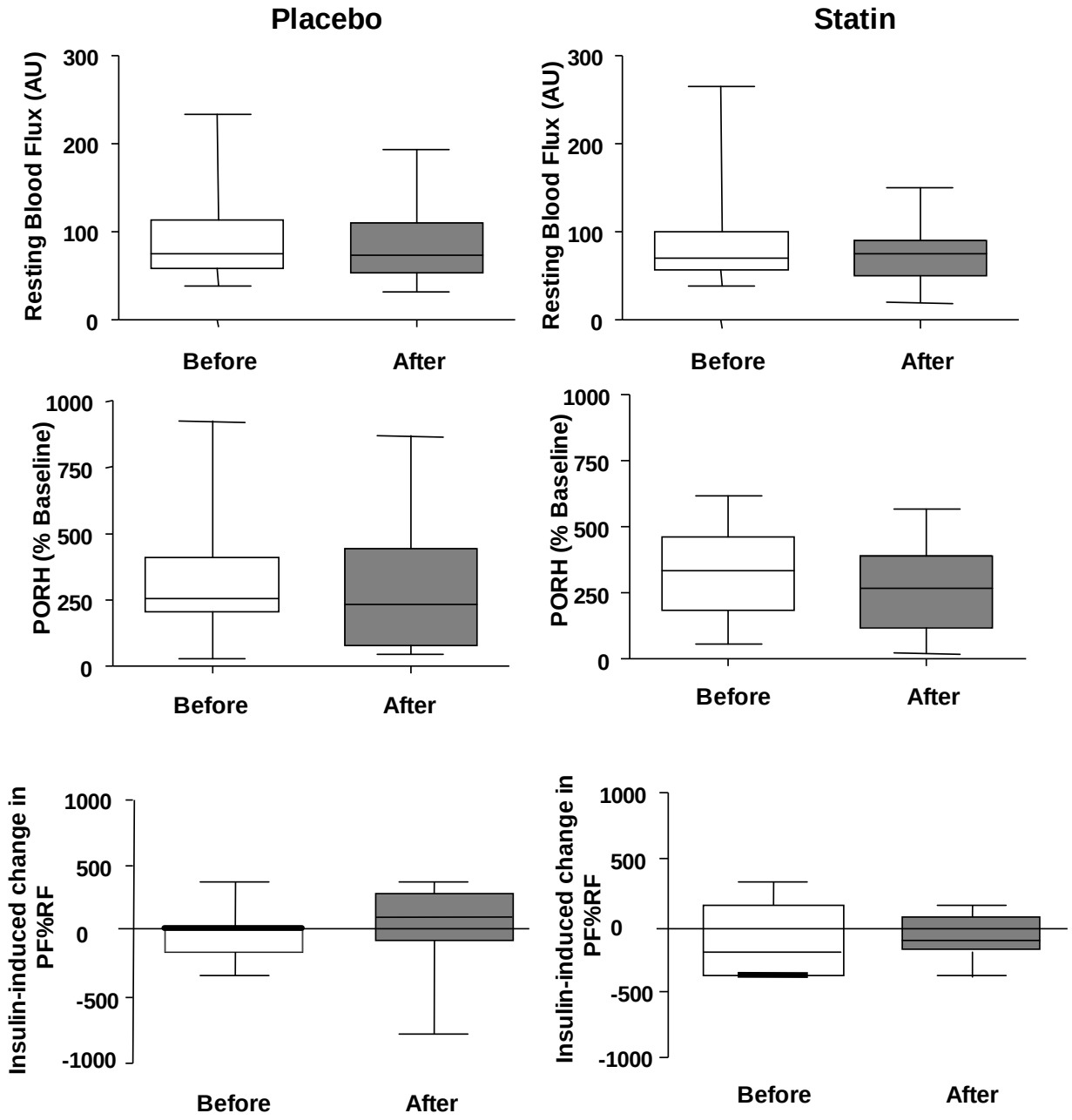

Before = pre-treatment, After = post-treatment

**Figure 6.7 Effects of six months treatment with statin and placebo on exchange capacity (Kf), isovolumetric venous pressure (Pvi) and baseline blood flow**




Before = pre-treatment, After = post-treatment

**Figure 6.8 Effects of six months treatment on resting blood flux (RF), functional dilator capacity (PF%RF) and insulin-induced change in PF%RF**




Before = pre-treatment, After = post-treatment

Summary points:

- All subjects had central obesity defined by waist circumference according to IDF criteria and a modest degree of insulin resistance as assessed by stepped hyperinsulinaemic clamp.

- There were no significant differences in the anthropometric measurements and body fat estimation between the placebo and active treatment group at baseline and after intervention.

- Subjects' level of physical activity remained stable throughout the study.

- There was a significant reduction in total and LDL-cholesterol, triglycerides and hs-CRP with Atorvastatin.

- Fasting glucose, insulin and HOMA-IR significantly increased and QUICKI decreased within the intervention group as compared to placebo group where only fasting glucose increased while other parameters remained unaffected.

- There was a small decrease in the insulin sensitivity markers (M-value and M/I) but this was not statistically significant.

- Atorvastatin treatment did not alter any of the microvascular function parameters.

## 6.4 Discussion

In this study we showed that our cohort of centrally obese, non-diabetic participants but with other metabolic traits and at cardiovascular risk, had a modest degree of insulin resistance as characterized by fasting surrogate and direct markers of insulin sensitivity. It has previously been shown that hyperinsulinaemia in the presence of normoglycaemia provides evidence of insulin resistance and in general, fasting insulin concentrations greater than the upper limit for an assay might indicate insulin resistance [352]. Based on this criterion, a fasting insulin level above 10 mIU/L suggested that 44 % of the study population had insulin resistance of variable degrees. However, fasting insulin concentrations may overestimate insulin resistance due to the considerable overlap in insulin concentration between normal and insulin resistant states [353]. Only half of individuals who are hyperinsulinaemic are actually insulin resistant on a clamp [354]. When we defined insulin resistance as HOMA-IR > 2.5 again over 40% of the study met the criteria. The similarity in those results may be due to the use of a single fasting insulin for HOMA-IR calculations, which has previously been done by researchers for the sake of simplicity [355]. However, this does not take into account the pulsatility of insulin secretion and blood glucose levels and while it may not influence the results in larger, diabetic populations, in a smaller and non-diabetic population such as in our study, HOMA-IR based on a single measurement may misrepresent the actual insulin resistance status. The variable degrees of obesity and fat distribution (visceral versus subcutaneous) could potentially also influence the characteristics of the subjects' insulin sensitivity.

It has previously been reported that the rate of glucose disposal in the steady state expressed as M-value > 7.5mg/kg/min indicates insulin sensitivity, while the M-value < 4mg/kg/min indicates relative body resistance to insulin action. Levels between 4.0 and 7.5 are regarded as a grey area, possibly a pre-diabetic state [104, 356]. Using these criteria, we found that at the baseline about 23% of the study population had insulin resistance, whereas the rest of the subjects fell into the "pre-diabetic" category. There is evidence from other studies that glucose and insulin concentrations during the steady phase of a euglycaemic clamp differ between normal, obese as well as diabetic subjects [113, 356]. Within the obese population there may be additional variability in the level of insulin resistance depending on the fat distribution resulting in various grades of metabolic disturbances and therefore it is likely that application of the general criteria defined above may have underestimated the subjects' insulin sensitivity. Throughout the clamps we performed, as part of microvascular studies, periodic thigh cuff inflations, which were designed to cause complete arterial occlusion and would be expected to result in a degree

of leg ischaemia with consequent increase in plasma lactate. Since it has previously been reported that elevated lactate levels induce insulin resistance in skeletal muscle [357] this could have potentially affected the results of M-value and M/I. However, we showed that thigh cuff inflations did not alter plasma lactate concentrations in our subjects throughout the clamps and since the timing of blood sampling followed a similar pattern to the one during a standard ischaemic forearm exercise test, it is unlikely that any significant increase in lactate was not detected. The observed 36% increase in lactate concentration following a high dose primer infusion and maintained throughout the clamp is likely to reflect body compensatory mechanisms involving increased output through the Cori cycle in response to the infusion of large amounts of dextrose required for maintaining euglycaemia in the presence of exogenous hyperinsulinaemia. It is not clear whether the acute increase in lactate could affect insulin signalling at peripheral tissues and this may require further investigation.

Furthermore, there are no standard protocols for performing hyperinsulinaemic clamps and many modifications of the original clamp exist, but there have been no studies investigating the effects of variable methodology on the interpretation of the results. All of these factors require consideration when interpreting and comparing results from different studies and of various insulin sensitivity markers. Additionally, it is important to note that HOMA-IR and other fasting indices provide an estimation of hepatic, not peripheral/whole body insulin sensitivity, whereas the standard clamp technique assesses sensitivity to insulin at stimulated (non-physiological) extremes and estimates peripheral sensitivity, mainly derived from the muscle such as in the present study. This may explain the differences in the estimation of insulin resistance in our subjects using the various methods.

The baseline NEFA results found in our obese cohort were in keeping with previous reports that post-absorptive plasma NEFA concentrations are higher in obese than in normal subjects, in whom circulating NEFA concentrations are around 500 μmol/L [358]. This may be due to enhanced lipolysis from increased fat mass [359] present in obese subjects. It is also thought that raised plasma NEFAs may cause a reduction in peripheral insulin sensitivity via the Randle cycle, which may in part contribute to the explanation of our findings [95, 360, 361]. The approximate 40% suppression of NEFA after 60 minutes of low dose insulin infusion suggests a relatively good, but perhaps less than expected response when compared with the results of Brackenridge *et al*. [280] who showed 70% suppression of NEFA by the end of a 2 hour low dose (0.3mIU/kg/min) infusion in

overweight subjects. The maximal suppression of NEFA to high dose insulin infusion is consistent with previous findings [280].

Our data demonstrated that in centrally obese and non-diabetic subjects, six months of treatment with high dose statin did not improve insulin sensitivity, however, there was a trend towards an increase in insulin resistance. While we showed that the statin therapy significantly increased fasting surrogate measures of insulin resistance when adjusting for baseline, we did not confirm a group effect with repeated measures ANOVA. We observed the opposite with direct measures of insulin sensitivity, that is no significant alteration in the results with active treatment when adjusting for baseline but when adjusted for placebo there was present a significant effect of statin therapy. These, at first glance, paradoxically discrepant results may be explained by a relatively small number of subjects recruited into the study and the fact that we were unable to obtain repeated measurements on M-value and M/I index for all participants both, before and after completed therapy. Previous unfavorable reports for Simvastatin and Atorvastatin by Orhvall *et al.* [242] and Koh *et al.* [243] respectively were based on studies with a little higher number of subjects (n=29 and n=43 for statin, respectively) but who had already type 2 diabetes. Therefore it is plausible that in those populations it would be far easier to elicit any changes in insulin sensitivity than in a population with much higher insulin sensitivity reserve. This speculation concurs with the findings by Sukhija *et al*. [362] who reported a significant increase in fasting glucose with use of any statin after adjustment for age and concurrent medication in a study with over three hundred thousand subjects, which included both diabetic and non-diabetic.

The small and significant deterioration in $HbA_1c$ levels in a placebo group may be explained by the fact that subjects in this group had 16% higher insulin resistance, which over a period of time was far more likely to deteriorate than otherwise. Since both groups showed deterioration in $HbA_1c$ when adjusted for baseline, it is not unexpected that we did not demonstrate statistically significant changes after adjustment for placebo. However, the increase in glycated haemoglobin with active treatment led to reclassification of those subjects from normal to a prediabetic category. At the same time our data showed that Atorvastatin did not significantly affect peripheral insulin resistance in the middle aged, centrally obese and modestly insulin resistant subjects when adjusted for baseline results. Although the peripheral glucose disposal deteriorated in the intervention group, while it remained unchanged in the placebo group, which was at baseline 16% more insulin resistant than the active treatment group, the change in M-value and M/I index was not

statistically significant. However, there was a borderline change for M-value and significant change for M/I index after adjusting for randomization. Again, it could be speculated that our subgroups consisted of relatively small number of participants which were insufficient to detect significant effects of statin on peripheral insulin sensitivity. Although other studies with statins that implemented clamps for assessment of insulin resistance and showed significant changes in the outcome results used even lower number of participants [245, 248], their populations were far more insulin resistant and older than our cohort, which could in part explain our findings: our subjects have a large enough reserve of peripheral (skeletal muscle) insulin sensitivity to protect them from marked deterioration in peripheral insulin resistance with statin treatment. Since we had a relatively smaller number of subjects in active treatment group, which was also much younger than in the other study, we could not easily demonstrate statistically significant change. In support of this speculation is a recently reported meta-analysis by Sattar *et al.* [253] which indicated that the risk of incident diabetes with statin is the highest in older populations, over 65 years, which by default will have a much lower reserve of insulin sensitivity.

The mechanism by which statins may increase the fasting markers of insulin sensitivity is unclear. Sukhija *et al*. [363] proposed that statins may decrease various metabolites including ubiquinone, which enhance glucose uptake via glucose transporter type 4 (GLUT4) in adipocytes thus impairing insulin release and altering glycaemic control. However some researchers suggested that while lipophilic statins such as Atorvastatin worsen insulin sensitivity, hydrophilic statins on the other hand seem to improve it. We have not been able to investigate the mechanisms by which treatment with Atorvastatin increases fasting surrogate markers of insulin sensitivity and this certainly requires further exploration.

The maintenance of subjects' body weight and fat composition, and level of physical activity throughout the study makes it unlikely that any major changes in the participants' lifestyle confounded the results.

While statins have been shown to have beneficial pleiotropic effects within the macrovasculature through regulation of endothelial function and blood flow, our data from a randomised double blind placebo controlled trial with 6 months of treatment with high dose of Atorvastatin has unequivocally shown no effect of statin on any of the measured aspects of skeletal muscle microvascular function. This finding is consistent with that of Fegan *et al.* [348] who reported a lack of improvement in cutaneous vascular response

after 3 months of single or combined lipid-lowering therapy in type 2 diabetics. Similarly, no effect of 4 week therapy with a small dose of Atorvastatin was seen on vasomotor function investigated with high-resolution ultrasound over the brachial artery [231]. However, Haak *et al.* [349] demonstrated that 12 weeks of 80mg daily Fluvastatin in patients with hyperlipidaemia improved time to peak reactive hyperaemia in the capillaries of the nailfold and that this was positively correlated with post treatment LDL-cholesterol levels. Statins have also been shown to exert a lipid-independent amelioration of endothelial dysfunction in several animal models via increases in NO bioavailability, attenuation of NADPH oxidase-mediated superoxide production and down regulation of COX-2 dependent 8-isoprostane generation, or possibly by lessening the severity or rarefaction through a proangiogenic action [235-237]. It is therefore plausible that the noted beneficial effects of statins on endothelial function may be statin-specific or that their pleiotropic effects do not play a major role in improving microvascular function [140]. Furthermore, it is possible that a 6 month treatment with statins may be not sufficient for the necessary neovascularization or endothelial cell turnover to occur and improve the aspects of microvascular function measured in our study.
It is plausible that our study lacked power to detect small changes with high dose statin treatment. There have been no similar studies examining effects of statins on microvascular function, to our best knowledge. However, a retrospective sample size calculation using our data-set estimated that our study would have had 90% power, at a 5% significance level, to detect a 68% improvement in PORH with 14 subjects in our statin trial. A 68% improvement in PORH with exercise was noted by Pasqualini *et al.* [364] who recently undertook a study in overweight and hypertensive patients, testing the effects of exercise on reactive hyperaemia. Also, our study was powered to detect relatively modest changes in $K_f$ such as an increase in mean $K_f$ from 3.9 to 5.0 $x10^{-3}$ml/min/mmHg/100ml (97% power at 5% significance level). More marked changes in $K_f$ have been reported with electrical stimulation for 4 weeks, which increased $K_f$ from, $3.38 \pm 0.38$ to $6.68 \pm 0.62$ ($p<0.05$) [221].

In conclusion, our findings showed that in the centrally obese subjects without type 2 diabetes but with other moderate metabolic traits, the level of insulin resistance was modest, predominantly within the pre-diabetic range as assessed by hyperinsulinaemic euglycaemic clamp and M-value. They also had, as expected, enhanced lipolysis but reduced adipose tissue response to low dose of insulin infusion. We also demonstrated that 6 months of treatment with high dose of Atorvastatin resulted in reduced hepatic insulin

sensitivity as expressed by increased fasting surrogate markers of insulin resistance. However we failed to show a significant effect of statin on peripheral insulin sensitivity. Furthermore we demonstrated that despite marked decreases in LDL-cholesterol and hs-CRP concentrations, 6 months of therapy with Atorvastatin did not improve any of the measures of microvascular function investigated in our study.

Summary points:

- Our cohort of middle-aged, centrally obese but non diabetic subjects had a modest degree of insulin sensitivity and impaired peripheral lipolysis as assessed by hyperinsulinaemic euglycaemic clamp.

- Six months of daily treatment with 40mg of Atorvastatin did not improve insulin sensitivity but we observed a trend towards deterioration in insulin sensitivity markers.

- As expected Atorvastatin significantly reduced total and LDL-cholesterol as well as triglycerides concentrations leading to significantly lowered cardiovascular risk in subjects with features of metabolic syndrome.

- Although intensive treatment with statin reduced hs-CRP, it did not alter any of the measures of microvascular function.

Part of this chapter / data were submitted in 2008 for FRCPath in a dissertation titled: “The Effect of Atorvastatin on Insulin Resistance of the Metabolic Syndrome”.

# 7. The relationship between cardiorespiratory fitness, cardio-metabolic risk factors and cardiac diastolic function in central obesity

## 7.1 Introduction

Cardiorespiratory fitness is a well-recognised predictor of cardiovascular outcome in normal individuals as well as in those with underlying cardiovascular disease. Fitness is related to several factors such as age, weight, nutrition [7, 365], gender [7], obesity, quantity and quality of physical activity [7] and genetics [211]. Bouchard *et al*. reported that heredity accounts for 25-50 % of variance in aerobic power [211]. Studies have shown that a low level of cardiorespiratory fitness in the overweight results in an increased risk of type 2 diabetes [214, 215, 217], cardiovascular morbidity and all cause mortality [208]. A large cohort prospective study demonstrated that the association between obesity, metabolic syndrome and all cause and cardiovascular mortality is largely explained by cardiorespiratory fitness [216]. The modern literature suggests that high aerobic fitness protects against cardiovascular disease in non-obese and obese individuals. Recently, Fogelholm conducted a systematic review of 36 publications examining the health risks of poor aerobic fitness in normal weight and good aerobic fitness in obese individuals with BMI less than 35 [207]. The data indicated that the risk of all-cause and cardiovascular mortality was lower in individuals with good cardiorespiratory fitness and high body mass index, compared to those with normal BMI but low levels of fitness. Additionally, the data showed that obese subjects, despite high levels of physical activity, were still at a higher risk of developing type 2 diabetes than those with normal BMI and low physical activity level. These findings indicate that relationships between fitness, fatness and cardio-metabolic factors are far more complicated than they may appear at first glance. This also illustrates the importance of understanding which determinants contribute to fitness-induced cardiovascular protection in obese people at risk of cardiovascular disease and type 2 diabetes.

There are still many uncertainties about the exact determinants of fitness in middle-aged individuals with central adiposity and other metabolic traits. The maximal oxygen consumption ($VO_2$ max) is the single best measure of cardiorespiratory capacity to deliver oxygen to the skeletal muscles during physical activity. $VO_2$ max is determined by the oxygen supply and its utilisation. Convective oxygen delivery is largely dependent on cardiac function and peripheral vasodilatory response. Crucial for oxygen utilisation is the

skeletal muscle diffusing capacity, which is regulated by the muscle functional capillary surface area [212]. Regular exercise appears to be the most important physiological stimulus for myocardial oxygen demand and ensuring system efficiency. It modulates cardiac diastolic function which predetermines cardiac output, promotes muscle capillary development that is essential for oxygen diffusion and utilisation, and improves insulin-mediated whole body glucose uptake [212].

Studies have shown that cardiac diastolic function correlates with exercise capacity in normal subjects and that left diastolic dysfunction plays a key role in the development and progression of cardiovascular disease [366]. Physiological insulin levels have been shown to increase cardiac output and stimulate peripheral vasodilatation preferentially into skeletal muscle microvasculature [151, 313], whereas those mechanisms seem to be impaired in central obesity [156, 313].

Thus, it becomes apparent that cardiorespiratory fitness may be influenced by several factors such as the energy expenditure derived from physical activity, insulin sensitivity, skeletal muscle microvasculature and cardiac function. Their relative impact on the $VO_2$ max however, is unclear and requires further research that would provide a better understanding of the individual contribution of the key regulators of $VO_2$ max variance and an insight into the new ways of ameliorating the cardiorespiratory fitness.

The use of cardiac catheterizations, an invasive technique, is restricted in research on healthy volunteers and echocardiography provides measures of cardiac function but not other vascular parameters. With advances in the modern technology, cardiac function together with arterial stiffness can be reliably assessed in healthy individuals using the well-validated, non-invasive method of pulse wave analysis [289, 290]. This technique allows estimation of the subendocardial viability ratio (SEVR) which represents myocardial perfusion relative to the left ventricular workload and is a proxy measure for diastolic function. We therefore used pulse wave analysis as an indirect measure of diastolic function and arterial stiffness.

In this study we aimed to elucidate the factors independently associated with $VO_2$ max and determine the proportion of the variance in $VO_2$ max that they explained. We therefore examined the relationships between cardiorespiratory fitness and insulin sensitivity, fatness, physical activity energy expenditure, skeletal muscle microvasculature and cardiac diastolic function. Since the myocardial perfusion occurs during diastole, we tested the hypothesis that $VO_2$ max is independently associated with diastolic function when

controlled for potential confounders such as physical activity levels, insulin sensitivity, skeletal muscle microvascular function and visceral and total adiposity.

## 7.2 Methods

Forty-seven white Caucasian, non-diabetic men and women aged 29 – 69 were recruited. All subjects had central obesity defined as waist circumference ≥ 94 cm for men and ≥ 80 cm for women and were assessed with the regards to cardiorespiratory fitness and physical activity.

Fasting lipid profiles, glucose and $HbA_1c$ were measured to establish the presence of any other metabolic traits, and plasma IL-6 and urinary ACR to assess proinflammatory state in relation to microvascular function. Detailed information about laboratory analysis and subject recruitment was provided in the methods chapter.

Fitness was estimated as maximal oxygen uptake during a maximal-grading treadmill test as described in the methods chapter. Physical activity was assessed in terms of mean energy expenditure expressed as MET during a period of seven to ten days (representative of a typical week) of wearing an activity monitor as detailed in the methods section.

Body composition in terms of fat and lean body mass and visceral fat mass was measured using DEXA and abdominal MRI respectively as described previously in methods chapter.

Data from forty two subjects was collected during pulse wave analysis studies. PWA was performed using radial artery applanation tonometry to obtain measures of arterial wave reflection (Alx@HR75) as a surrogate measure of arterial stiffness and peripheral arteriolar resistance, diastolic function/myocardial perfusion (SEVR) and percentage of ejection duration (ED%), which indirectly influences SEVR. Details of method principles and measurements were previously provided in chapter 3.6.

Skeletal muscle exchange capacity ($K_f$) was measured during venous congestion plethysmography to assess skeletal muscle microvascular function. Insulin sensitivity was estimated using a hyperinsulinaemic euglycaemic clamp and a ratio of M value and mean insulin concentration during the last 30 minutes of the clamp.

All statistical analyses were performed using SPSS for Windows version 16.0. Student's *t* test was undertaken to compare mean values. Pearson correlation coefficients were used for univariate regression analyses of normally distributed data. The $VO_2$ max data was categorised into tertiles to facilitate its presentation and interpretation. Differences and linear trends across $VO_2$ max tertiles were examined with the application of one way ANOVA. In order to describe factors independently associated with $VO_2$ max or SEVR% as outcome variables, multivariate linear regression models were developed, for which explanatory variables were chosen from the results of univariate analyses.

## 7.3 Results

The mean ± SD of age of the forty-seven volunteers, which included 19 men and 28 women, was 51.5 ± 9.3 years. The mean ±SD of $VO_2$ max for all subjects was 22.6 ± 8.7 $ml \cdot min^{-1} \cdot kg^{-1}$ with significantly higher mean $VO_2$ max values for men (26.1 ± 9.0 $ml \cdot min^{-1} \cdot kg^{-1}$) than for women (19.1 ± 6.6 $ml \cdot min^{-1} \cdot kg^{-1}$), p=0.005. The baseline characteristics of subjects are presented in the Table 7.1.

The results of univariate associations analysis between $VO_2$ max and estimates of cardiac diastolic function, arterial stiffness, microvascular function, insulin sensitivity, body composition and physical activity are shown in Table 7.2. $VO_2$ max was significantly and inversely correlated with $HbA_1c$, total and truncal fat. However, there was no significant association with whole body insulin sensitivity (p=0.11) or mean physical activity energy expenditure (p=0.26). The measures of diastolic function and arterial wave reflection showed a statistically significant association with $VO_2$ max (SEVR: r=0.50, p=0.001; Alx@ HR 75: r=-0.46, p=0.002). The scatter plots of the relationships between $VO_2$ max and SEVR (Figure 7.1) and $VO_2$ max and Alx@HR75 (Figure 7.2) confirm a good linear correlation. In order to examine the relationship between each measure and $VO_2$ max in more detail, the $VO_2$ max data was categorised into tertiles (Table 7.3). The strong and linear relationship between maximal oxygen uptake and measures of diastolic function and arterial stiffness has been maintained across all $VO_2$ max tertiles. Similarly, total, truncal and visceral fat showed significant linear trends across all $VO_2$ max tertiles.

**Table 7.1 Baseline characteristics of study population, n=47**

| Variable | Mean ± SD | Range |
|---|---|---|
| Age (years) | 51.5 ± 9.3 | 29.0 – 69.6 |
| Waist circumference (cm) | 104.5 ± 12.3 | 86.5 – 151.0 |
| BMI ($kg/m^2$) | 31.8 ± 4.6 | 25.3 – 47.9 |
| DEXA total body fat (kg) | 32.1 ± 9.2 | 18.7 – 58.5 |
| DEXA total body fat (%) | 36 ± 7.2 | 21.2 – 47.5 |
| DEXA trunk fat (kg) | 16.5 ± 4.7 | 8.9 – 31.9 |
| DEXA trunk fat (%) | 18.5 ± 3.2 | 10.2 –24.8 |
| MRI visceral fat (kg) | 3.6 ± 1.6 | 1.1 – 7.1 |
| Blood pressure systolic (mmHg) | 133 ± 13 | 93 – 155 |
| Blood pressure diastolic (mmHg) | 82 ± 9 | 64 – 104 |
| CVD risk (%) | 7.2 ± 5.1 | 0 – 17.3 |
| Total cholesterol (mmol/L) | 5.8 ± 1.0 | 3.2 – 9.3 |
| LDL-cholesterol (mmol/lL) | 3.7 ± 0.9 | 1.7 – 7.0 |
| HDL-cholesterol (mmol/L) | 1.4 ± 0.3 | 0.9 – 2.5 |
| Triglycerides (mmol/L) | 1.4 ± 0.7 | 0.4 – 2.9 |
| HbA1c (%) | 5.5 ± 0.3 | 4.9 – 6.3 |
| $VO_2$ max (ml/min/kg) | 22.4 ± 7.65 | 8.35-52.45 |
| PAEE (MET) | 1.27 ± 0.17 | 0.83-1.55 |
| M/I ((mg·L)/kg/min/mIU) | 3.21 ± 1.26 | 0.97-6.26 |
| Kf ($\times 10^{-3}$ ml/min/mmHg/100ml tissue) | 3.86 ± 1.13 | 3.86 ± 1.13 |
| ED (%) | 34.3 ± 4.0 | 26.0 - 48.0 |
| AIx@ HR 75 | 18.4 ± 10.6 | -12.0 - 41.0 |
| SEVR (%) | 169.6 ± 32.1 | 102.0 - 247.0 |

**Table 7.2 Univariate associations with maximal oxygen consumption**

| Variable | r value | p- value |
|---|---|---|
| Age (years) | -0.18 | 0.24 |
| Waist circumference (cm) | -0.12 | 0.43 |
| BMI ($kg/m^2$) | -0.31 | 0.04* |
| DEXA total body fat (kg) | -0.39 | 0.008* |
| DEXA trunk fat (kg) | -0.37 | 0.013* |
| MRI visceral fat (kg) | -0.07 | 0.70 |
| Blood pressure systolic (mmHg) | -0.10 | 0.54 |
| Blood pressure diastolic (mmHg) | 0.11 | 0.45 |
| CVD risk (%) | -0.05 | 0.75 |
| Triglycerides (mmol/L) | -0.17 | 0.28 |
| HbA1c (%) | -0.35 | 0.018* |
| PAEE (MET) | 0.18 | 0.26 |
| M/I ((mg·L)/kg/min/mIU) | 0.26 | 0.11 |
| Kf ($\times 10^{-3}$ ml/min/mmHg/100ml tissue) | 0.21 | 0.20 |
| ED (%) | -0.45 | 0.002* |
| AIx@ HR 75 | -0.46 | 0.002* |
| SEVR (%) | 0.50 | 0.001* |

* $p < 0.05$, statistically significant

**Figure 7.1 Relationship between maximal oxygen uptake and subendocardial viability ratio**

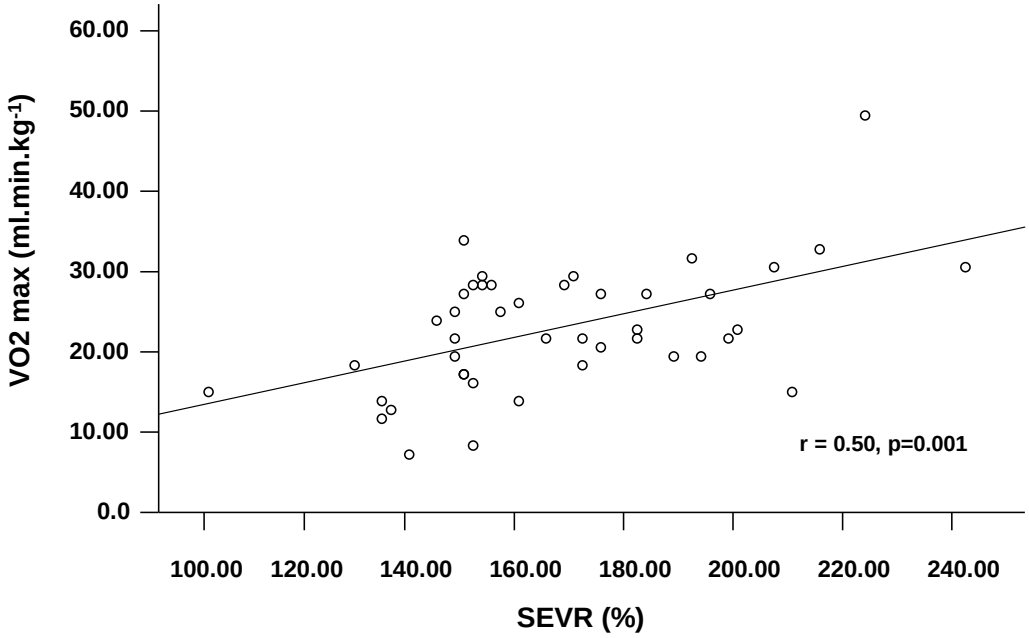



**Figure 7.2 Relationship between maximal oxygen uptake and augmentation index at heart rate of 75 beats/min**

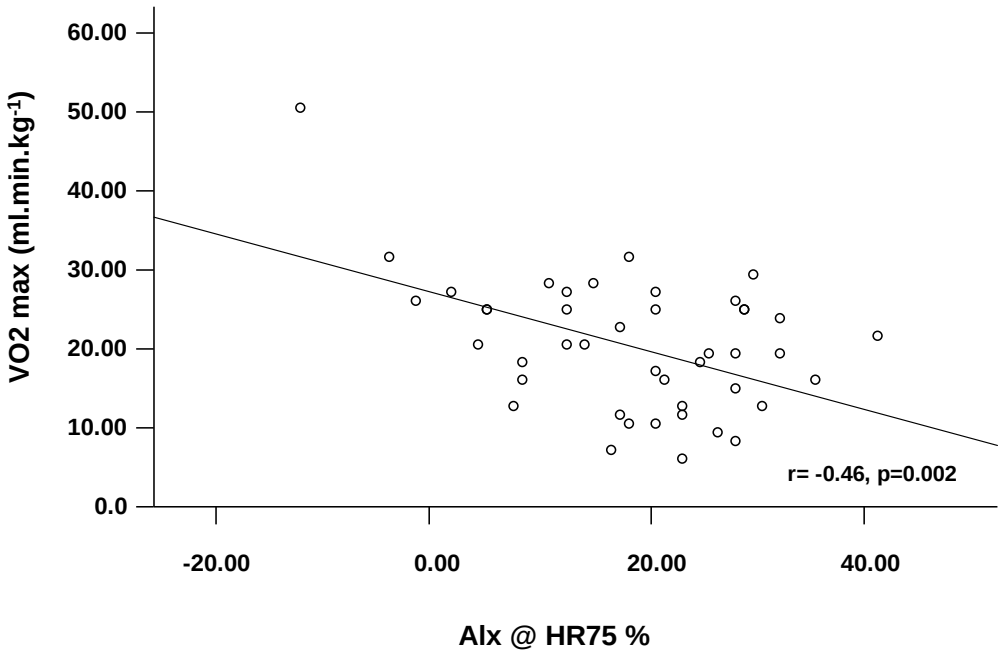


**Table 7.3 Body fatness, physical activity, insulin sensitivity, arterial stiffness and diastolic function stratified by tertiles of $VO_2$**

| $VO_2$max (ml/kg/min) tertiles | 8.3-19.6 | 20-27 | 28.3-52.5 | ANOVA p-value | Linear trend p-value |
|---|---|---|---|---|---|
| n | 16 | 16 | 15 | | |
| Age (years) | 51.7 ± 8.3 | 53.8 ± 8.6 | 48.3 ± 10.1 | 0.24 | 0.30 |
| Waist circumference (cm) | 105 ± 12 | 107 ± 15 | 103 ± 11 | 0.74 | 0.71 |
| BMI ($kg/m^2$) | 32.7 ± 4.7 | 32.0 ± 4.7 | 31.0 ± 3.8 | 0.57 | 0.30 |
| DEXA total body fat (kg) | 35.2 ± 10.0 | 32.5 ± 9.1 | 28.5 ± 7.1 | 0.12 | 0.04 |
| DEXA trunk fat (kg) | 17.9 ± 5.0 | 17.2 ± 4.8 | 14.4 ± 3.5 | 0.09 | 0.04 |
| MRI visceral fat (kg) | 3.75 ± 1.49 | 3.58 ± 1.80 | 3.51 ± 1.56 | 0.04 | 0.03 |
| PAEE (MET) | 1.23 ± 0.22 | 1.31 ± 0.15 | 1.24 ± 0.18 | 0.69 | 0.68 |
| M/I ((mg·L)/kg/min/mIU) | 0.43 ± 0.22 | 0.47 ± 0.15 | 0.52 ± 0.16 | 0.44 | 0.21 |
| $K_f$ ($\times 10^{-3}$ml/min/mmHg/100ml) | 3.62 ± 1.27 | 4.05 ± 0.86 | 4.05 ± 1.22 | 0.54 | 0.34 |
| ED (%) | 36.2 ± 4.6 | 33.1 ± 2.7* | 33.4 ± 3.9† | 0.049 | 0.45 |
| AIx@ HR 75 | 21.7 ± 7.0 | 20.3 ± 10.6* | 12.2 ± 11.7† | 0.02 | 0.01 |
| SEVR (%) | 153.3 ± 27.6 | 172.6 ± 19.6* | 177.4 ± 32.2† | 0.037 | 0.016 |

* n=14, † n=12

Several multiple linear regression analyses were performed to identify the factors that were independently associated with $VO_2$ max and to assess how much they contributed to the $VO_2$ max variance. We used maximal oxygen uptake as an outcome variable, whereas the factors that were significantly associated with $VO_2$ max in the univariate analysis were selected as the explanatory factors. 29% of the variance in $VO_2$ max ($r^2$=0.29, p=0.001) was explained in the regression models when age, sex and truncal or visceral fat were used as explanatory variables. Incorporating SEVR as an additional explanatory variable to the regression model with age, sex and truncal fat (Table 7.4), explained 46% of the variance in $VO_2$ max ($r^2$=0.46 p=0.0001). This regression model showed that SEVR was independently associated with $VO_2$ max with a standardised coefficient β of 0.37 (95%CI: (0.003, 0.18)), p=0.007). The association between SEVR and $VO_2$ max remained independent and statistically significant despite replacing truncal fat with other measures of the body fatness such as visceral and total body fat or lean body mass in the further analyses.

$HbA_{1c}$ was another variable that showed significant association with $VO_2$ max (Table 7.2) and therefore was built into the regression model with age, sex, truncal fat and SEVR as independent variables. Although this regression model explained 49% of the variance in $VO_2$ max ($r^2$=0.49, p=0.0001), it influenced the nature of some associations between variables. Incorporating $HbA_{1c}$ into the model resulted in the loss of the association between age and $VO_2$ max. However, it did not alter significantly the correlation between SEVR and $VO_2$ max (standardised coefficient β=0.35, p=0.009). Also, the association between sex and $VO_2$ max (standardised coefficient β=-0.37, p=0.009) was still present as shown previously.

We also explored the relationship between $VO_2$ max and augmentation index, a proxy measure for arterial stiffness, which in the univariate analyses was associated with $VO_2$ max (r= -0.46, p=0.002) and age (r=0.35, p=0.017). In the linear regression model with age, sex, truncal fat and SEVR, we replaced SEVR with Alx@HR75. This model explained 40.5% of variance in $VO_2$ max ($R^2$=0.41, p=0.001). This analysis showed a borderline significantly independent association between Alx@HR75 and $VO_2$ max (standardised coefficient β=-0.32, p=0.054). As expected from the previous analyses, there was a negative association between $VO_2$ max and truncal fatness (standardised coefficient β=-0.28, p=0.04).

Finally, we investigated which factors may be associated with SEVR, since we showed earlier that this proxy measure of cardiac diastolic function was strongly and independently associated with $VO_2$ max in subjects with central obesity. In particular we wanted to determine whether diastolic function was independently associated with fatness. Diastolic function results (mean ± SD) were not statistically different between the genders, with SEVR (%) of 175±34 in men and 162±23 in women (p=0.18). The univariate analyses showed lack of association between SEVR and physical activity energy expenditure expressed in mean MET (r=0.18, p=0.26) or age (r=0.05, p=0.73). However, there was a statistically significant inverse correlation between SEVR and fatness (r=-0.33, p=0.026) and IL-6 (r=-0.36, p=0.014). To identify which of those factors were independently associated with SEVR we built linear regression models with SEVR as the outcome variable. Age, sex, IL-6 and truncal or total or visceral fat were used in subsequent regressions as explanatory variables. In none of those analyses were measures of fatness, age or gender independently associated with SEVR as a proxy measure of diastolic function. However, as presented in Table 7.5, IL-6 was inversely and independently associated with SEVR.

**Table 7.4 Multiple linear regression model of factors independently associated with $VO_2$ max as an outcome variable**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | 33.6 | 10.99 | -0.31 | -0.50 - -0.04 | 0.02 |
| Sex | -0.27 | 2.15 | 0.40 | -11.01 - -2.30 | 0.004 |
| DEXA trunk fat (kg) | -0.30 | 0.22 | -0.17 | -0.74 - 0.15 | 0.18 |
| SEVR (%) | -6.65 | 0.04 | 0.37 | 0.03 - 0.18 | 0.007 |

CI: Confidence Intervals, $R^2 = 0.46$, $p = 0.0001$

**Table 7.5 Multiple linear regression model of factors independently associated with SEVR as an outcome variable**

| Independent Variables | Unstandardised coefficient | | Standardised coefficient β | 95% CI | p-value |
|---|---|---|---|---|---|
| | B | SE | | | |
| Age (years) | -0.10 | 0.46 | -0.03 | -1.02 - 0.83 | 0.83 |
| Sex | -13.8 | 9.28 | -0.24 | -32.5 - 4.98 | 0.15 |
| DEXA trunk fat (kg) | -1.03 | 1.07 | -0.15 | -3.19 - 1.13 | 0.34 |
| IL-6 | -10.4 | 4.46 | -0.36 | -19.4 - -1.40 | 0.025 |

$R^2 = 0.23$, $p = 0.03$

Summary points:

- A significant and inverse correlation between cardiorespiratory fitness and $HbA_{1c}$, total and truncal fatness was present.

- A linear association between measures of diastolic function (SEVR) and cardiorespiratory fitness ($VO_2$max) was demonstrated.

- There was lack of significant relationship between cardiorespiratory fitness and measure of insulin sensitivity (M/I) or physical activity energy expenditure (PAEE).

- $VO_2$ max and SEVR showed a strong, positive and independent association in several regression models.

- $VO_2$ max and Alx@HR75 inverse correlation was borderline independent of other factors.

- There was a significant inverse correlation between SEVR, fatness and IL-6, but only the latter was independently associated with measurement of diastolic function.

## 7.4 Discussion

The novel aspect of our study is that in centrally obese subjects we have demonstrated a strong and independent association between cardiorespiratory fitness and a measure of diastolic function closely related to left ventricular load. Regression modelling showed that a simple model containing age, sex and the proxy measure of diastolic function identified almost half of the variance in $VO_2$ max. Moreover, the relationship between fitness and diastolic function was independent of confounding factors that are recognised to influence level of fitness such as age, gender, visceral, truncal or total body adiposity and physical activity. Additionally, other potential confounders of fitness such as whole body insulin sensitivity and skeletal muscle microvascular exchange capacity did not have any significant influence on this association.

We did not use the conventional methods of cardiac function assessment such as cardiac catheterisation because its invasive nature restricts its use in research on healthy volunteers. Instead we have opted for the non-invasive, arterial applanation tonometry, which over recent years has been commonly used in vascular research studies in healthy individuals [286]. This technique can readily be applied to study individuals with central obesity, because the arterial waveform is reproducibly measured over the radial artery at the wrist, which is easily accessible in obese individuals. Pulse wave analysis allows a validated estimation of subendocardial viability ratio, which would usually be derived from cardiac catheterisation, and pulse wave augmentation index, both of which are the proxy measures for cardiac diastolic function and arterial stiffness [367, 368]. Although SEVR measured with applanation tonometry does not take account of the left ventricular end diastolic pressure, it is a good estimate of the subendocardial viability index in people without evidence of ischaemic heart disease and normal left ventricular end diastolic pressure [367, 368]. The reason for using SEVR as a measure of cardiac diastolic function is that it is a non-invasive estimate of myocardial blood supply, i.e. myocardial perfusion relative to left ventricular workload and thus myocardial demand [287, 288, 369]. Myocardial oxygen demand depends mainly on the heart rate, myocardial contractility and ejection pressure, which all contribute to cardiac output and oxygen demand during physical activity [370-372]. The increase in the left ventricular oxygen demand is predominantly met by increasing coronary blood flow [373]. However, coronary blood flow is hindered during systole. Therefore the duration of cardiac diastole is a crucial determinant of myocardial perfusion at a given diastolic perfusion pressure [374, 375].

Diastolic function is also an important determinant of aerobic exercise capacity [366], because the enhanced systolic function which is fundamental for high levels of cardio-respiratory fitness has to be matched by changes in left ventricular filling. Although diastolic function has been reported to be related to high levels of cardio-respiratory fitness [376, 377], it has been uncertain whether diastolic function in sedentary obese individuals correlated with levels of fitness, especially when adjusted for the potential confounding factors such as fatness, insulin sensitivity and levels of energy expenditure during activity. In our study we assessed levels of physical activity energy expenditure in free living individuals, using a validated multi-sensor Sensewear Pro device. To obtain as precise an estimate of PAEE as possible, subjects were asked to maintain their usual activity behaviour pattern over the period of 7-10 days during which period they wore the device continuously. This was because we expected that a relationship between $VO_2$ max and diastolic function may be influenced by the level of physical activity. The results obtained from the activity monitor showed that our subjects were generally physically inactive (mean PAEE estimated from 7-10 days record = 0.83 – 1.55 MET). Interestingly, there was a lack of strong relationship between both PAEE and the measure of diastolic function, SEVR ($r=0.18$, $p=0.26$), and PAEE and $VO_2$ max ($r=0.18$, $p=0.26$). Given that neither of these relationships came close to reaching statistical significance, it is clear that the association between cardiorespiratory fitness and diastolic function is not confounded by levels of physical activity in this centrally obese and sedentary cohort.

Therefore, the question what mechanisms may explain the association between $VO_2$ max and SEVR remains to be answered. Our data supported the previous reports that the level of fitness was related to the degree of fatness [7]. We showed in the univariate analyses that measures of fatness were inversely associated with diastolic function, and there was even a stronger inverse association between diastolic function and IL-6. The study of Fontana *et al*. reported that IL-6 is mainly produced in the visceral fat and that its concentration correlated directly with CRP levels, suggesting a potential link between visceral adiposity and systemic inflammation [64]. There is also some evidence that perivascular adipocytes influence vasocrine signalling mechanisms [167, 378]. Moreover, it is now apparent that the epicardial fat which lies directly over the myocardium and shares its microcirculation is also metabolically active and may interact with the myocardium through epicardial fat paracrine and vasocrine signalling mechanisms [379]. It is therefore possible that cardiac diastolic function may be directly affected in central adiposity by the physical contact between an increased layer of epicardial fat and

myocardium- or adipocyte-derived cytokines such as IL-6, or both. A study of epicardial fat function in subjects with cardiovascular disease demonstrated higher levels of inflammatory cytokine expression, including IL-6, in epicardial fat than in matched subcutaneous fat samples independent of other metabolic factors such as obesity or diabetes [380].

Interestingly in our study, adding cytokine IL-6 into the linear regression model adversely affected the association between fatness and diastolic function identified previously in univariate analyses; this model showed that only IL-6 and not any measure of fat quantity, was independently and inversely associated with diastolic function. A recent study showed that increased plasma levels of IL-6 and TNF-$\alpha$ had cardio-depressive effects and were negatively associated with left ventricular diastolic function [381] suggesting the importance of proinflammatory processes in the pathogenesis of diastolic dysfunction. In another study IL-6 was reported to accentuate already present systemic low grade inflammation.

It is plausible that a subclinical grade of inflammation in centrally obese individuals adversely affects diastolic function, which in turn influences $VO_2$ max. Studies looking into the effects of weight reduction and left ventricular structure and/or function in obese individuals with and without ischaemic heart disease demonstrated that weight loss improves cardiac structure together with systolic and diastolic function [382, 383]. Recently, a large study of middle aged obese subjects showed that significant weight reduction and a decrease in $HbA_{1c}$ were independent predictors of left ventricular systolic and diastolic function improvement [382]. Whether the benefit of decreasing body fat on cardiac function in these studies was mediated by altered visceral adipocyte function and specifically modified adipocyte-derived paracrine signalling mechanisms induced by weight loss remains uncertain. Therefore further research is required to investigate the mechanisms behind the proinflammatory state and diastolic function interaction and its effects on maximal oxygen uptake in obese individuals at low cardiovascular risk.

We found that in people with central obesity and without clinical evidence of cardiovascular disease, the level of cardiorespiratory fitness was strongly correlated with the augmentation index, corrected for heart rate to minimise possible confounding. This index estimates arterial wave reflection, which depends amongst other parameters on pulse-wave velocity. Therefore the augmentation index is classified as an indirect, surrogate measure of arterial stiffness [384]. Several studies reported that the augmentation index increases with age because of the faster wave reflection due to loss of arterial

elasticity. Increased arterial stiffness was reported to relate with visceral adiposity in a large study of elderly adults [385]. However to date, there have been no reported studies investigating wave reflection in relation to obesity and fitness [384]. We demonstrated that the augmentation index significantly decreased across tertiles of increasing $VO_2$ max, that is, with increasing aerobic exercise capacity. We used adjusted augmentation index for heart rate (AIx@750, which allowed the elimination of potential confounding associated with the variability in heart rate between and within subjects. The slower the wave reflection, the lower the augmentation index as the more elastic the arteries are [384]. This would mean that in our obese individuals, higher levels of fitness were potentially associated with significantly less stiff arteries. Increased arterial stiffness causes a premature return of reflected waves in late systole, increasing the workload of left ventricle and myocardial oxygen demand, thus influencing diastolic function and aerobic fitness. Recently, the relationship between visceral adiposity and carotid arterial stiffness has been examined in a study of 459 patients in whom epicardial fat was assessed by echocardiography as a measure of visceral fat that may impair diastolic function. The authors found a positive correlation between epicardial fat and arterial stiffness parameters and a negative correlation was found with diastolic parameters [386]. This may suggest that the visceral fat is adversely influencing cardiovascular function. Over the last decade studies have confirmed not only the deleterious effects of subclinical inflammation resulting in atherosclerosis and arterial stiffness, but also its strong links with metabolically active visceral fat [64, 66, 384]. It is therefore possible that the strong association between level of cardiorespiratory fitness, diastolic function and arterial stiffness, and the negative correlation between diastolic function, body fatness and IL-6 found in our viscerally obese subjects is more likely due to the paracrine function of fat than its mechanistic effects.

The limitation of this study is that the estimations of central aortic systolic and pulse pressures depend on the validity and applicability of the generalized transfer function used to generate the central aortic waveforms from peripheral readings. However, the correspondence between calculated central aortic and directly recorded systolic and pulse pressures has been found to be within 1 mmHg [290, 387, 388]. The transfer function used to derive the central aortic pressures is based on observation that pressure wave transmission in the upper limb is remarkably consistent despite effects of aging, disease, drug therapy, and variation in heart rate. This allows a generalized transfer function to be used to convert the radial to an aortic pressure wave [289], which was implemented in the SphygmoCor device used in the CAFE study [389] and gained US Food and Drug

Administration approval in 2001. However, a potential weakness of this technology is that the calibration of central aortic pressures depends on the accuracy of the brachial pressure measurements [390]. Additionally, we extrapolated PWA data on augmentation index for assessment of arterial stiffness, whereas pulse-wave velocity (PWV) is the preferred non-invasive method for the estimation of arterial stiffness. PWV provides direct information about the intrinsic wall stiffness unlike augmentation index which only indirectly provides the information about arterial stiffness and may be affected by pathophysiological conditions and drugs while aortic PWV remains unchanged. However, precise measurements over the carotid and femoral arteries required in PWV analysis are technically difficult and time consuming in obese individuals thus potentially leading to inaccurate results [384]. This is not the issue with PWA, which requires only detection of easily accessible, even in the obese, radial pulses and therefore has been used as a proxy measure for arterial stiffness in large studies such as Hoorn study [391]. Also, our subjects were healthy and on no medication therefore eliminating the effects of those factors on the results and their interpretation.

In conclusion, we demonstrated in people with central obesity and low cardiovascular risk a strong association between cardiorespiratory fitness ($VO_2$ max) and a measure of diastolic function (SEVR ) which is independent of age, gender, measures of body adiposity, level of physical activity, skeletal muscle microvascular exchange capacity and whole body insulin sensitivity. We showed that cardiac diastolic function in those obese subjects is strongly and independently correlated with IL-6, which may suggest that the low-grade proinflammatory state present in obesity may be an important link between cardiac diastolic function and aerobic exercise capacity, and may contribute to the pathogenesis of diastolic dysfunction. Additionally, for the first time, we documented strong correlation between arterial wave reflection and cardiorespiratory fitness, indirectly showing the important relationship of arterial stiffness and fitness in centrally obese middle-aged people.

Summary points:

- In centrally obese, sedentary and at low cardiovascular risk subjects, there is present a strong positive association between proxy measure of diastolic function (SEVR) and cardiorespiratory fitness independent of age, gender, fatness, physical

activity energy expenditure, insulin sensitivity and skeletal muscle microvascular function.

- Subclinical inflammation present in subjects with visceral adiposity may contribute to the pathogenesis of cardiac diastolic dysfunction.

- Low - grade proinflammatory state may be a link between diastolic function and cardiorespiratory fitness capacity in sedentary people with visceral adiposity.

- Higher level cardiorespiratory fitness is strongly associated with reduced arterial stiffness in obese.

# 8. Conclusions

In this study of individuals with central obesity we have explored several aspects of the relationships between cardio-metabolic risk factors and the effects of treatment with 40mg daily of Atorvastatin on insulin sensitivity and microvascular function.

In the cohort of non-diabetic subjects with central obesity defined by waist circumference according to IDF criteria, with visceral adiposity confirmed on MRI and in the presence of a mild metabolic syndrome phenotype we found modest levels of peripheral insulin resistance. Both fasting surrogate markers and direct measures confirmed variable levels of insulin resistance. The degree of insulin sensitivity assessed by the gold standard stepped hyperinsulinaemic euglycaemic clamp was predominantly within a pre-diabetic range, as were the concentrations of glycated haemoglobin. We also demonstrated that the subjects had low levels of daily physical activity METs and cardiorespiratory fitness, which would suggest that in the centrally obese individual with low cardiovascular risk and with minimal metabolic disturbances with a sedentary lifestyle, a degree of insulin resistance is already present.

We showed that in central obesity there is a greater variability in the measures of microvasculature such as filtration capacity, blood flow, endothelial integrity and functional vasodilator capacity. With the use of venous congestion plethysmography and Laser Doppler fluximetry we demonstrated in subjects with a low to moderate cardiovascular risk the presence of microvascular dysfunction. With plethysmography, we provided evidence that in the face of even a modest degree of peripheral insulin resistance, skeletal muscle exchange capacity is altered, resting limb blood flow is increased and the functional vasodilator capacity is reduced. We produced an estimate of filtration capacity independent of blood flow suggesting that capillary rarefaction or remodelling may contribute to skeletal muscle microvascular dysfunction in obesity. We also showed that in the centrally obese the capacity to increase functional tissue perfusion during low metabolic demand was attenuated, and the capacity for functional hyperaemia was not ameliorated by insulin. All these findings would provide an argument that the functional and structural changes contribute to microvascular dysfunction, which may be present without clinically overt insulin resistance and which precedes, well in advance, the onset of macrovascular disease in those with central obesity and features of the metabolic syndrome.

We have gone on to explore the relationship between the skeletal muscle microvascular function, namely skeletal muscle microvascular exchange capacity, and the measure of long term glycaemia - $HbA_1c$. The evidential links between $HbA_1c$ and microvascular disease have for decades provided a basis for the diagnostic criteria for diabetes mellitus but recently published data have suggested that $HbA_1c$, even within the high-normal range, is not only a strong predictor of diabetes but also a marker for cardiovascular disease risk in non-diabetic but centrally obese individuals. We have demonstrated for the first time a strong negative association between skeletal muscle microvascular exchange/filtration capacity ($K_f$) and $HbA_1c$, which was independent of potential confounders such as age, gender, fasting glucose, peripheral insulin sensitivity, visceral fat mass, level of physical activity and fitness, and sICAM-1. Moreover, $K_f$ explained almost a third of the variance in $HbA_1c$ thus emphasising its important role in peripheral glucose uptake. The fact that microvascular exchange capacity, independently of modest levels of insulin sensitivity, was associated negatively with glycated haemoglobin would point towards skeletal muscle microvascular filtration capacity rather than muscle insulin sensitivity as being an important influence of $HbA_1c$ concentrations. Those findings also indicate a potential for skeletal muscle microvascular dysfunction to be an early, important mediator of cardiovascular risk associated with $HbA_1c$. At the same time we did not demonstrate an independent association between visceral adiposity and $HbA_1c$, which means that the effects of visceral fat on long term glycaemic control may be modulated via skeletal muscle microvascular exchange capacity or insulin sensitivity. Interestingly, we found a strong positive association between sICAM-1 and $HbA_1c$ concentrations which were potentially confounded by $K_f$, and this could indicate that increased levels of sICAM-1 may be a reflection of skeletal muscle endothelial dysfunction.

Although in our cross-sectional study we were unable to provide an explanation as to the causative nature of the relationships between $K_f$, $HbA_1c$ and other interconnected factors, it is plausible that either the impaired exchange capacity modulates levels of $HbA_1c$, or relative glycaemia directly alters microvascular exchange capacity and raises levels of $HbA_1c$. Alternatively, it may be that bi-directional interactions exist between microvascular dysfunction and relative hyperglycaemia resulting in increased long term glycaemia.

We have further examined in centrally obese but non-diabetic individuals the associations between insulin sensitivity and several measures of skeletal muscle microvascular function such as exchange/filtration capacity ($K_f$), resting blood flow ($Q_a$), isovolumetric venous

pressure ($P_{vi}$) and functional dilator capacity (PORH). We found that out of all the investigated measures of microvasculature only filtration capacity and functional dilator capacity were associated with insulin resistance status. We showed that diminished microvascular filtration capacity was strongly and independently (of visceral adiposity) associated with insulin resistance and that the negative association of $K_f$ with visceral fat was not independent of insulin sensitivity; and these associations were not confounded by levels of physical activity and fitness. These findings together with the body of evidence from research in animal models and obese humans without diabetes may suggest mutual interaction between insulin sensitivity and microvascular filtration capacity in obesity; that is, the greater the insulin sensitivity the better the microvascular exchange capacity, and the greater microvasculature exchange capacity the lesser the insulin resistance, which in turn allows greater nutrient delivery to skeletal muscle and greater glucose uptake.

We also demonstrated a strong and independent relationship between insulin-induced changes in microvascular functional dilator capacity, age and insulin sensitivity, which indicates that age and insulin resistance independently of each other can adversely affect microvascular function. This is an important finding because for the first time the independent influence of age and insulin sensitivity on microvasculature became apparent. Additionally our findings support the notion that central obesity blunts the microvascular dilatory responses at a given metabolic demand.

Furthermore, we have examined the effects of six months of treatment with high dose Atorvastatin on insulin sensitivity and measures of microvascular function in a double-blinded randomised trial with placebo. Importantly our results were not confounded by change in body composition, weight or level of physical activity.
We demonstrated that 40mg daily of Atorvastatin did not improve insulin sensitivity in centrally obese non-diabetic individuals with features of metabolic syndrome despite significantly lowering cholesterol (total and LDL fraction), triglycerides and hs-CRP Nevertheless, we showed a trend towards deterioration in insulin sensitivity markers, especially fasting surrogate measures. To date, there has been conflicting evidence regarding the effects of statins on insulin resistance [242, 245, 246, 248]. However, a recent large trial reported by Sukhija *et al*. [363] demonstrated a significant increase in fasting blood glucose in both diabetic and non-diabetic populations with any statin, and a smaller study of hypercholesterolaemic patients on Atorvastatin by Koh *et al*. [243] showed a significant increase in fasting insulin and $HbA_1c$ concentrations. This has been further supported by the recently published meta-analysis by Sattar *et al*. [253]

documenting the detrimental effects of statins on incident diabetes. Our study is in part in keeping with those reports and also allows for additional speculation. It is plausible that in individuals with modest insulin resistance, like those in our study, who have large insulin sensitivity reserves in the peripheral tissues such as skeletal muscle, statins may exert adverse effects on hepatic insulin sensitivity before the peripheral insulin insensitivity becomes identifiable with direct measures of insulin sensitivity. However, a much larger study, with perhaps a more homogenous population of centrally adipose non diabetics is required to confirm this.

We have also presented evidence that six months of treatment with high dose statin did not alter any of the investigated measures of skeletal muscle microvascular function despite lowering lipids and hs-CRP concentrations. Likewise, with the effects of statins on insulin sensitivity, there have been conflicting reports regarding their influence on microvascular function, with more recent animal studies demonstrating that statins, independently of their lipid-lowering effect, ameliorate endothelial dysfunction [235-237, 348]. It is possible that either the beneficial effects of statins on microvasculature are statin-specific or that they require a much longer period than six months, as in our study, to exert their effects through revascularisation of endothelial cells.

Finally, we elucidated the relationship between cardiorespiratory fitness, cardiac diastolic function, and arterial stiffness using a novel Pulse Wave Analysis (PWA) technique. For the first time we demonstrated that cardiorespiratory fitness in centrally obese individuals was strongly and independently associated with SEVR - a measure of diastolic function closely related to left ventricular load - this relationship was not confounded by age, gender, level of physical activity, body adiposity, microvascular function or insulin sensitivity. Based on recent reports [64, 167, 379, 380] and our data showing inverse associations between diastolic function, fatness, and concentration of IL-6, which is mainly produced in visceral fat, it is plausible that subclinical inflammation alone or together with mechanistic effects from excessive accumulation of epicardial fat [378] may be one of the key players in the pathogenesis of cardiac diastolic dysfunction that in turn adversely affects cardiorespiratory fitness. We also showed significant improvement in a surrogate measure of arterial stiffness - Augmentation Index (AIx@75) - with increased cardiorespiratory fitness. This additional finding could suggest that in centrally obese **subjects**, greater fitness translates into lesser arterial stiffness and thus may also positively influence diastolic function. However, further research is required into the mechanisms behind the interactions between adiposity, pro-inflammatory state, arterial stiffness and

$VO_2$max in centrally obese, non-diabetic but insulin-resistant subjects. A potential criticism of this study may be that we did not use the conventional techniques for measurement of cardiac diastolic function and arterial stiffness but used surrogate measures with the application of PWA. However, this technique, in contrast to cardiac catheterisation and pulse wave velocity, is easy to perform in obese subjects and is without health risk unlike cardiac catheterisation, whose use is limited to research on healthy volunteers; it is also a well-validated technique and has been used for similar assessments in much larger studies [391].

In conclusion, our study uncovered several novel aspects of the relationships between cardio-metabolic risk factors in subjects with visceral adiposity and modest levels of insulin sensitivity which strongly suggest that obesity blunts microvascular responses even in apparently otherwise healthy individuals; that a good level of cardiovascular fitness benefits obese people by ensuring lesser arterial stiffness and better cardiac diastolic function. Our findings adds credence to the argument that microvascular dysfunction is an early marker of cardio-metabolic risk in people with obesity at risk of CVD and diabetes. Our data provided new insights into the relationships between skeletal muscle microvascular function, ambient glycaemia, pro-inflammatory state, insulin sensitivity and visceral fatness suggesting mutual interactions between those factors, but their directional/causality links require further research. Furthermore, considering all the relationships discussed above between cardiometabolic factors and measures of microvascular status, it appears that microvascular dysfunction should be included within vascular implications of the cardio-metabolic phenotype associated with central obesity. Our study documented a lack of beneficial pleiotropic effects of statin on insulin sensitivity or microvascular function, emphasising the need for investigating other agents and measures to improve the factors that are crucial in the development of metabolic syndrome. Further research in a larger population, employing both surrogate and direct measures of insulin sensitivity is required to confirm the observed trend towards deterioration in insulin sensitivity with high dose statin therapy.

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