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A study of insulin sensitivity in men: the association with fatty liver, cortisol metabolism and fitness

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ABSTRACT

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A STUDY OF INSULIN RESISTANCE IN MEN: THE ASSOCIATION WITH FATTY LIVER, CORTISOL METABOLISM AND FITNESS

by Helen Bridget Holt

Obesity prevalence is increasing and with it the prevalence of insulin resistance, diabetes and metabolic syndrome. The link between obesity and insulin resistance is not fully understood.

In a group of middle-aged men we measured blood pressure, fasting lipids and glucose, BMI, waist circumference, skin fold thickness, total fat on DEXA, bioimpedance, and plethysmography and visceral fat on MRI. We measured insulin sensitivity in muscle and liver using a euglycaemic, hyperinsulinaemic clamp technique with deuterated glucose, and suppression of non-esterified fatty acids during an oral glucose tolerance test. We demonstrated that BMI and waist correlate strongly with measures of visceral and truncal fat and also to insulin sensitivity and other metabolic syndrome features.

We measured hepatic steatosis using ultrasonography and demonstrated that fatty liver is associated with impaired suppression of non-esterified fatty acids, independently of body fat, indicating that men with fatty liver have abnormal adipocyte function.

We found a strong correlation between metabolism of cortisol measured by clearance of a tracer bolus of deuterated cortisol and insulin sensitivity in muscle and adipose tissue which was independent of body fat, suggesting that exposure to cortisol is higher in insulin-resistant individuals.

We measured fitness by oxygen uptake during maximal exercise, and physical activity energy expenditure using heart-rate monitoring. We did not find that either fitness or energy expenditure were related to insulin sensitivity independently of body fat suggesting that the beneficial effects of exercise are mediated through changes in body composition.

In conclusion we found that insulin resistance in fat is associated with the development of fatty liver and clearance of cortisol independently of fat mass but that total fat is associated with whole body insulin resistance and is more important than exercise. Abnormal adipocyte function and exposure to cortisol may underlie development of whole body insulin resistance in obese subjects.

TABLE OF CONTENTS

1 INTRODUCTION 11

2 BACKGROUND 14

2.1 Obesity 14
2.1.1 Epidemiology and metabolic consequences 14
2.1.2 Assessment of obesity 15
2.1.2.1 Measures of overall fatness 15
2.1.2.1.1 Body mass index (BMI) 16
2.1.2.1.2 Waist circumference 17
2.1.2.1.3 Skin fold thickness measurement 17
2.1.2.1.4 Dual energy x-ray absorptiometry (DEXA) 18
2.1.2.1.5 Bioimpedance and other measurements of total body water 18
2.1.2.1.6 Hydrostatic weighing and air displacement plethysmography 19
2.1.2.2 Measures of regional fatness 19
2.1.2.2.1 Waist circumference and waist hip ratio (WHR) 20
2.1.2.2.2 Magnetic resonance imaging (MRI) and computerised tomography (CT) 20

2.2 Insulin resistance 20
2.2.1 Definition, pathogenesis and molecular mechanisms 20
2.2.2 Assessment of insulin sensitivity 24
2.2.2.1 Whole body insulin sensitivity 24
2.2.2.1.1 Model assessments of insulin resistance 24
2.2.2.1.2 Clamps 24
2.2.2.1.3 Oral glucose tolerance test 26
2.2.2.2 Assessment of hepatic insulin sensitivity 26
2.2.2.3 Suppression of non-esterified fatty acids 27

2.3 Metabolic syndrome 27
2.3.1 Definition, epidemiology and metabolic consequences 27
2.3.2 Features of the metabolic syndrome 28
2.3.2.1 Insulin resistance 28
2.3.2.2 Central obesity 29
2.3.2.3 Dyslipidaemia 29
2.3.2.4 Hypertension 30

2.4 Non-alcoholic fatty liver disease (NAFLD) 30
2.4.1 Definition, pathogenesis and epidemiology 30
2.4.2 Assessment of non-alcoholic fatty liver disease 31
2.4.2.1 Ultrasonography 31
2.4.2.2 MRI spectroscopy 31
2.4.2.3 Liver enzymes 32
2.4.2.4 Liver biopsy 32
2.4.3 Interplay between the features of the metabolic syndrome 32

2.5 Hypothalamic-pituitary-adrenal axis (HPAA) 33

2.6 Physical activity and fitness 35
2.6.1 Assessment of cardiorespiratory fitness and energy expenditure..... 36
2.6.1.1 Maximal oxygen uptake 37
2.6.1.2 Energy expenditure 38

3 METHODS 39

3.1 Ethical approval 39

3.2 Research Facility 39

3.3 Recruitment 39

3.4 Subjects 40
3.4.1 Inclusion criteria 40
3.4.2 Exclusion criteria 40
3.4.2.1 Medical history and examination 41

3.5 Anthropometry and blood pressure 41
3.5.1 Weight 41
3.5.2 Height 41
3.5.3 Body Mass Index (BMI) 41
3.5.4 Waist circumference 41
3.5.5 Hip circumference 42
3.5.6 Skin fold thickness 42
3.5.6.1 Biceps and Triceps 43
3.5.6.2 Subscapular 43
3.5.6.3 Suprailiac 43
3.5.6.4 Calculating body fat percentage from skin fold thickness 43
3.5.7 Bioelectrical impedance 44
3.5.8 Air displacement plethysmography 44
3.5.9 Dual energy x-ray absorptiometry (DEXA)) 44
3.5.10 Magnetic Resonance Imaging (MRI) 44
3.5.11 Blood pressure 46

3.6 Lipid profile 46

3.7 Non-alcoholic fatty liver disease (NAFLD) 46
3.7.1 Ultrasonography 46
3.7.2 Liver enzymes 47

3.8 Whole body insulin sensitivity 47
3.8.1 Oral glucose tolerance test (OGTT) 47
3.8.2 Euglycaemic hyperinsulinaemic clamp with deuterated glucose 47

3.9 Insulin sensitivity in different tissues 49
3.9.1 Insulin sensitivity in muscle and liver 49
3.9.2 Insulin sensitivity in adipose tissue 49

3.10 Hypothalamic pituitary adrenal axis (HPAA) studies 50
3.10.1 Morning cortisol 50
3.10.2 Low dose ACTH test 50
3.10.3 Corticosteroid binding globulin (CBG) 50
3.10.4 Free cortisol 50
3.10.5 Cortisol clearance 51
3.10.5.1 Preparation of deuterated cortisol 51
3.10.5.2 Clinical study 51
3.10.5.3 Lipid extraction 51
3.10.5.4 Internal standards 52
3.10.5.5 Mass spectrometry 52

3.11 Physical activity and fitness 53
3.11.1 Physical activity 53
3.11.1.1 Physical activity energy expenditure (PAEE) 53
3.11.1.2 Physical activity questionnaires 54
3.11.2 Cardiorespiratory fitness 54
3.11.2.1 Treadmill assessment of VO_2 max 54

4 BODY COMPOSITION – COMPARISON OF METHODS AND ASSOCIATION WITH METABOLIC SYNDROME FEATURES 56

4.1 Introduction 56

4.2 Methods 57

4.3 Results 58

4.4 Discussion 74

5 THE ASSOCIATION BETWEEN FATTY LIVER AND INSULIN RESISTANCE IN OBESE SUBJECTS 78

5.1 Introduction 78

5.2 Methods 80

5.3 Results 81

5.4 Discussion 91

6 THE ROLE OF CORTISOL IN THE RELATIONSHIP BETWEEN INSULIN SENSITIVITY AND OBESITY 96

6.1 Introduction 96

6.2 Methods 98

6.3 Results 99

Discussion 107

7 THE ROLE OF BODY FAT, FITNESS AND PHYSICAL ACTIVITY IN INSULIN RESISTANCE AND THE METABOLIC SYNDROME 114

7.1 Introduction 114

7.2 Methods 115

7.3 Results 116

Discussion 126

8 CONCLUSIONS 132

TABLE OF FIGURES

Figure 4-1: Distribution of BMI amongst the subjects 63
Figure 4-2 Bland-Altman plots comparing percentage fat measured by DEXA, Bod Pod, bioimpedance and skin fold measurement..... 64
Figure 4-3 Scatter plots of percentage fat measured by DEXA, Bod Pod, bioimpedance and skin fold measurement against BMI 66
Figure 4-4 Relationships between DEXA, Bod Pod, bioimpedance and skin fold measured percentage total body fat and BMI..... 67
Figure 4-5 Scatter plot of waist circumference against BMI..... 68
Figure 4-6 Bland Altman plot standardised BMI and waist 69
Figure 4-7 Bland Altman plot standardised BMI against total fat on DEXA 70
Figure 4-8 Scatter plot of standardised trunk fat on DEXA against standardised total fat on DEXA, BMI and waist circumference 71
Figure 5-1 Plasma glucose during the clamp 88
Figure 5-2 Plasma insulin during the clamp..... 88
Figure 5-3 NEFA suppression during the OGTT in lean men and obese men with and without fatty liver..... 89
Figure 5-4 Suppression of hepatic glucose output on commencing insulin infusion 90
Figure 6-1 The relationship between cortisol clearance and glucose disposal. 105
Figure 7-1 Distribution of fitness in 25 subjects 122
Figure 7-2 Distribution of physical activity energy expenditure in 19 subjects.. 123
Figure 7-3: Scatter plot of PAEE against fitness..... 124

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
11βHSD	11 beta hydroxysteroid dehydrogenase
BMI	body mass index
DEXA	dual energy x-ray absorptiometry
HDL	high density lipoprotein
HPAA	hypothalamic pituitary adrenal axis
IL-6	Interleukin-6
LDL	low density lipoprotein
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NEFA	non-esterified fatty acid
PAEE	physical activity energy expenditure
PEPCK	phosphoenolpyruvate carboxykinase
PAI	plasminogen activator inhibitor
PI3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
PPAR	peroxisome proliferator-activated receptor
TNFα	tumour necrosis factor alpha

1 INTRODUCTION

Obesity has been increasing in prevalence over recent decades, and although it is now well established that obesity is associated with increased risk of cardiovascular disease, many aspects of this association are not well understood. Obese subjects are at increased risk of developing insulin resistance and diabetes, hypertension and having high plasma levels of triglycerides with low levels of protective high-density lipoprotein, all of which lead to atherosclerosis. It was originally Reaven's observation that these cardiovascular risk factors tend to cluster together, in what is now called the metabolic syndrome [1-4], a condition which has subsequently undergone many definitions by different health organisations. It remains poorly understood why some subjects develop obesity and associated health problems, whilst others seem to remain metabolically normal by comparison. Furthermore the metabolic syndrome now encompasses the development of non-alcoholic fatty liver disease and it is not yet clear how this condition fits into the picture of obesity and insulin-resistance.

The aim of these studies was to carefully define a small group of men with regard to body composition and insulin resistance, to determine:

- the best method to assess body composition to identify subjects at increased cardiovascular risk and whether BMI remains useful;
- the relationship between fatty liver disease, obesity and insulin resistance;
- the role of the hypothalamic pituitary adrenal axis, with particular reference to the clearance of cortisol, to determine whether excess exposure to cortisol plays a role in the development of the metabolic syndrome;
- whether increased exercise and fitness are protective against cardiovascular risk factors independently of their effect on body composition..

There has been very much controversy recently about the best method to use to identify obese subjects at risk of developing cardiovascular disease. In particular it has been suggested that waist measurement, as a proxy for central obesity, is

used in preference to body mass index to identify individuals at risk [5 6]. Body composition was measured in detail, and because many methods are used for the evaluation of fatness and lean mass, the first aim of this study was a comparison of the different methods available, particularly because there has been much controversy regarding the best method to identify individuals with increased metabolic risk [6-9].

Body composition and insulin resistance were measured in as precise detail as possible, to determine as far as possible the relationships between aspects of these measurements and the other modalities of interest. A literature search was undertaken to determine the best methods of measuring insulin resistance in this group of subjects. A euglycaemic, hyperinsulinaemic clamp was used with labelled glucose to allow measurement of suppression of endogenous glucose production [10,11] and suppression of non-esterified fatty acids during an oral glucose tolerance test was used to measure insulin sensitivity in adipose tissue [12].

Non-alcoholic fatty liver disease (NAFLD) is now recognised as part of the metabolic syndrome [13-15], and describes a range of disease through simple steatosis, or fatty infiltration, through non-alcoholic steatohepatitis (NASH) to cirrhosis. The underlying cause of NAFLD is uncertain but it is strongly associated with obesity and type 2 diabetes [14,16,17]. Establishing whether insulin resistance or obesity is the more important factor for the development of fatty liver is difficult because of the strong co-linear association between obesity and insulin resistance [14,18-20]. Recent literature has suggested that insulin resistance may be a more important factor than obesity for the development of NAFLD [13,19,21] and so the second aim of this study was to determine the relationship between fatty liver, fatness and insulin resistance.

It has been suggested that abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis underlie the link between central obesity and insulin resistance because these features also occur in Cushing's syndrome, when a pathological

excess of cortisol results in a severe metabolic syndrome phenotype [22] and because cortisol increases gluconeogenesis [23]. In fact circulating cortisol levels are low or normal in obesity [24-27], however ACTH and cortisol responses to stress increase with both obesity and impaired glucose disposal [28]. Urinary cortisol is increased in obesity suggesting that clearance is increased [29,30] and increased urinary cortisol has also been demonstrated to correlate with fasting glucose and insulin [31]. Cortisol clearance has not previously been studied directly in relation to detailed measurements of insulin sensitivity. A paradox arises because although morning cortisol is low in obesity, increased morning cortisol is associated with reduced insulin sensitivity [27,32] but there is a strong relationship between obesity and reduced insulin sensitivity. The third aim of this study was to determine the relationship between fatness, insulin sensitivity and cortisol clearance, and to determine its relationship with fatty liver, because it has been suggested that there may also be a subtle, chronic activation of the HPA axis in NAFLD [33].

High levels of cardiorespiratory fitness and physical activity offer some protection against the excess cardiovascular risk associated with insulin resistance and the metabolic syndrome [34], however there is still not consensus regarding the relative importance of fitness and physical activity to influence these factors. Furthermore it is unclear whether the relationship between insulin sensitivity, fitness and physical activity is conferred through alterations of body composition. The fourth aim of this study was to determine the relationship between fitness, physical activity, body composition and insulin sensitivity.

2 BACKGROUND

2.1 Obesity

2.1.1 Epidemiology and metabolic consequences

Obesity is defined as a BMI greater than 30kg/m^2 and its prevalence is increasing, standing at 15-20% across most established market economies [35]. In America the prevalence of obesity increased from 22.9% to 30.5% between 1994 and 2000 according to the National Health and Nutrition Examination Survey (NHANES III), with well over half of adults in the overweight or obese categories [36].

The original Framingham epidemiological studies identified a link between obesity and the development of cardiovascular disease [37], and this link is now well established, with increasing obesity thought to be responsible for slowing the decline in cardiovascular disease incidence seen in the Nurses' Health Study [38]. Obese individuals have a 40-fold increased risk of diabetes compared with lean individuals [4]. Obesity was not included in Reaven's original description of the metabolic syndrome [1] but it is the best predictor of subsequent development of metabolic syndrome [39], and is often now considered to be the common factor in the development of the metabolic syndrome [40,41].

It has been well described that obese women with the typical female pattern of peripheral fat accumulation are relatively insulin sensitive compared to those with central fat accumulation [42]. There is some evidence that the same association may be seen in men although the degree of variation in fat distribution is smaller [4]. One study showed that central fat accumulation is a risk factor beyond increased BMI for the development of diabetes in men [43].

Abdominal fat is divided between the subcutaneous and visceral compartments, and there has been evidence presented suggesting a causal link between visceral fat and development of the dyslipidaemia and insulin resistance seen in the metabolic syndrome [44-46], although this is not yet a universally accepted

mechanism [47]. Amount of visceral fat is strongly associated with adverse lipid profile, namely increased plasma triglycerides and Apo B cholesterol and reduced HDL cholesterol [48,49]. Visceral fat releases fatty acids directly to the portal circulation and Randle first introduced the concept that non-esterified fatty acids (NEFAs) may be causal in type 2 diabetes by resulting in impaired glucose oxidation and utilisation [50] This effect of visceral fat on dyslipidaemia seems to be independent of the effect of body mass index. Fat mass is increased by a combination of increased adipocyte size and number [51] and large abdominal adipocyte size also predicts the development of diabetes [52].

2.1.2 Assessment of obesity

Different methods are appropriate for defining fatness depending on the aims of measurement.

2.1.2.1 Measures of overall fatness

There is clear evidence that overall obesity puts individuals at health risk. Obesity is defined by BMI, which does not define an individual's body composition, and it has been suggested that waist circumference rather than BMI is used for population screening in identifying individuals at cardiovascular risk [5 53], although evidence that it is a more useful measure than BMI is lacking. As neither measure is used alone as a basis for instituting treatment, it may not matter which method is used to determine who should have plasma lipid and glucose and blood pressure measured. For small studies aimed at understanding more about how obesity relates to the risk factors for atherosclerosis however, much more detailed measurements of body composition are required. The methods which are used for assessing overall obesity are BMI, waist circumference, skin-fold thickness, DEXA scanning, bioimpedance and other measurements of total body water, hydrostatic weighing and air displacement plethysmography. Each of these is considered below.

2.1.2.1.1 Body mass index (BMI)

The simplest and most widely-accepted measure of overall obesity is a measure of weight adjusted for height, the body mass index (Quetelet index), defined as weight divided by height squared (kg/m^2). The BMI does not give any indication of body composition but is closely correlated with more sophisticated measures of obesity and as such is a useful screening tool [54]. It has been widely used in population studies and predicts the development of diabetes [4,55,56], ischaemic heart disease [57,58] and premature death [59].

The cut-offs for overweight and obesity have varied in different studies. The WHO definition of overweight is BMI>$25kg/m^2$ and obesity BMI>$30kg/m^2$ [60] although it is recognised that even within the normal weight category there is progressive risk of disease from BMI 20-$22kg/m^2$ [61].

The normal range of BMI should be modified for different populations, according to body composition and degree of risk associated with a high BMI in that population. In a North Indian population it was shown that in men a BMI of 21.4 kg/m^2 was associated with percentage body fat 21.3 %, whereas in a European population an equivalent percentage body fat equated with a BMI of 25.2 kg/m^2. In women a BMI of 23.3 kg/m^2 was associated with percentage body fat 35.4 %. It has been suggested therefore that BMI over 22.5 kg/m^2 is used to define overweight in South Asian males and BMI over 19 kg/m^2 to define overweight in South Asian females [62]. Across other Asian populations, namely Indonesians, Singapore Chinese, Malays, and Indian and Hong Kong Chinese BMI was 3-4 units lower for the same percentage body fat compared to individuals of European origin, with further slight variation between these populations and suggested definitions for obesity have ranged from BMI over $25kg/m^2$ to BMI over $27.5kg/m^2$ [63].

In contrast, although there are slight differences in body composition between individuals of Afro-Caribbean and European origin there is close correlation

between BMI and total fat percentage so it is reasonable to use the same BMI indices of overweight and obesity in these populations [64,65].

BMI becomes a less accurate measure of fatness in the elderly due to the changes in body composition which occur with age, namely loss of muscle mass and increased adiposity. Weight may remain stable or even decrease despite increasing obesity [66-68] and BMI is probably not an ideal tool to use in this group. There is also a minority of very muscular individuals in whom BMI is not a good measure of fatness.

In summary BMI is a simple and useful measure of fatness for population studies and to identify individuals who may be at risk of cardiovascular disease but appropriate normal values must be used for the ethnicity of the population or individual being studied and it is less useful in elderly or extremely muscular individuals.

2.1.2.1.2 Waist circumference

Waist circumference measurement has been suggested as an alternative screening method to BMI to identify obese individuals [5,8]. It does have the potential advantage of combining a measure of overall fatness with an assessment of regional adiposity. Waist circumference is arguably more difficult to reproduce, requiring training in a standardised technique, usually measured at the midpoint between the lowest rib and iliac crest [5]. The levels which predict increased cardiovascular risk are greater than 94cm in men and greater than 80cm in women [53]. Its potential use in predicting visceral obesity will be discussed below but at present waist circumference is usually used in addition to rather than in place of BMI as a measure of overall fatness.

2.1.2.1.3 Skin fold thickness measurement

Subcutaneous fat can be measured using calipers to determine skin fold thickness and a logarithm sum of four measurements located at the sub-scapular, supra-iliac, biceps and triceps areas used to estimate total body fat by

the method of Durnin and Wormersley [69]. This method has the advantage of being relatively inexpensive but the disadvantages of this method are that it is technically difficult and shows considerable variation between observers. It has been suggested that because of these disadvantages, and as it does not provide a measure of intra-abdominal fatness, skin-fold thickness measurement offers no advantages over BMI [70]. Individual skin fold measurements have been used to estimate fat distribution, for example using the subscapular: triceps ratio as a marker for central obesity, and this measure has been shown to be weakly correlated to cardiovascular risk [71]. The method also causes particular difficulties in obese individuals in whom skin fold thicknesses are large and in elderly individuals in whom there is wide intra-individual variation in subcutaneous fat deposition [72].

2.1.2.1.4 Dual energy x-ray absorptiometry (DEXA)

The unique x-ray absorption properties of fat allow DEXA scanning to be used to determine total body fat. It can also be used to give some information on regional distribution, which correlates with glucose intolerance [73] although it cannot be used to distinguish between visceral and subcutaneous fat. This method was shown to be very accurate measuring total fat using a four component model using body density, total body water and total body mineral in young individuals [74].

2.1.2.1.5 Bioimpedance and other measurements of total body water

Total fat may be calculated from lean mass using methods which measure total body water. The gold-standard method uses isotope dilution but is now predominantly used only for validating other techniques [74]. The measurement of bioimpedance depends on the properties of body water to conduct an electrical current, and provides an indirect measurement from which total body fat can be estimated. It is readily measured and widely available but has a high degree of variance [75]. It does not measure fat or predict metabolic abnormalities any better than BMI and waist circumference [69].

2.1.2.1.6 Hydrostatic weighing and air displacement plethysmography

These methods allow calculation of total fatness from measurements of density, using equations derived from cadaver-dissection methods based on a small number of Caucasian males [76]. Density can be determined from hydrostatic weighing under water but this method is generally only used in small studies for validation of other techniques. A new method is air displacement plethysmography using the commercially available 'Bod Pod'. This is more acceptable to the subject but may underestimate density resulting in overestimation of fat percentage [77]. The values obtained correlate closely with those from hydrostatic weighing in obese but not lean individuals so it is probably a more useful tool for obese subjects [78]. Air displacement plethysmography ideally requires residual lung volume to be determined using inert gas dilution [78], although an estimate may be used for individuals with normal lung function.

2.1.2.2 Measures of regional fatness

Central fat accumulation is believed to confer additional metabolic risk over total fatness in determining the metabolic consequences of obesity [79]. Women with lower body obesity show increased suppression of lipolysis, compared with those with upper body fat [80]. Furthermore central fat may be divided into visceral and subcutaneous fat. In one study obese but metabolically normal post-menopausal women had 49% less visceral fat than metabolically abnormal women despite comparable total fat mass [81] and in men visceral fat correlates with insulin resistance and dyslipidaemia independently of total fat, subcutaneous abdominal fat and fitness [82-84]. The methods used to assess regional obesity are waist circumference, waist hip ratio and magnetic resonance imaging (MRI) and computerised tomography (CT), and these are considered below, with MRI and CT additionally able to provide information on the ratio of visceral to subcutaneous fat deposition.

2.1.2.2.1 Waist circumference and waist hip ratio (WHR)

The simplest measures of central obesity are waist circumference and waist/hip ratio. Waist circumference is particularly important in identifying a small sub-group of the population with central obesity despite a BMI in the normal range [53]. Waist circumference is the best surrogate for visceral fat as measured by MRI or CT scanning [7,54] and identifies individuals with other features of the metabolic syndrome [53].

WHR in men largely reflects increasing waist circumference [7], and is less useful than waist circumference alone in predicting the subsequent development of diabetes [4].

2.1.2.2.2 Magnetic resonance imaging (MRI) and computerised tomography (CT)

These cross-sectional imaging techniques can be used to measure total body fat, or can be used as single-slice techniques to determine how much abdominal fat is stored viscerally and how much is subcutaneous [85-89]. MRI is the preferred method as subjects are not exposed to ionising radiation. Visceral fat as determined by these methods correlates with features of the metabolic syndrome [90].

2.2 Insulin resistance

2.2.1 Definition, pathogenesis and molecular mechanisms

Insulin resistance is a state where there is reduced biological effect of insulin, and is the reciprocal of insulin sensitivity. Type 2 diabetes results from insulin resistance in peripheral tissues, compounded by inadequate insulin secretion by pancreatic β-cells. When a normal concentration of insulin produces a less than normal biological response the dose-response curve of effect against concentration is described as being shifted to the right, but a reduction in maximal effect is usually also seen. Insulin lowers blood glucose by facilitating glucose uptake, mainly by muscle and fat tissue and by inhibiting endogenous

glucose production by the liver, and defects in all three tissues may co-exist in insulin resistant individuals [91].

In muscle, abnormalities of glucose uptake may precede the development of diabetes by 10-20 years and muscle insulin resistance has been demonstrated in non-diabetic relatives of patients with diabetes [92,93]. About 75% of insulin-dependent glucose disposal occurs into skeletal muscle [94]. The cellular and molecular basis for insulin resistance is multifactorial. Abnormalities have been described in insulin receptor number and function. The insulin receptor consists of an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. Binding of insulin to the extra-cellular portion activates its kinase activity resulting in phosphorylation of scaffolding proteins such as insulin receptor substrate (IRS) proteins [91]. Muscle IRS dysfunction may be caused by factors associated with adipocyte dysfunction such as increased circulating non-esterified fatty acids, and TNFα [91,93,95]. Recent studies using nuclear magnetic resonance spectroscopy have demonstrated that defects in intracellular glucose transport are the rate-limiting step in insulin resistant states. Downstream of IRS proteins activation of phosphoinositide-3-kinase (PI-3-kinase) leads to activation of protein kinase B (PKB). In muscle PKB mediates the effects of insulin on glucose uptake via facilitated glucose transporters (GLUTs) and intracellular glucose metabolism. Mutations in PKB have been detected in subjects with insulin resistance [91].

Functional abnormalities have been identified in muscle cell GLUT 4, the major glucose transporter which moves to the cell membrane in response to insulin [96], and in glycogen synthase and hexokinase [97]. Recently the PPARγ co-activator-1 (PGC-1) has been recognised as playing a major role in glucose homeostasis in regulating GLUT4 expression in muscle cells and PGC-1α may also play a role in the genes involved in the process of oxidative phosphorylation which commonly show reduced expression in the muscle of diabetic individuals [91].

Non-esterified fatty acids have been implicated in the pathogenesis of muscle insulin resistance [98]. Randle described how glucose conservation is regulated by phosphorylation of pyruvate dehydrogenase in the mitochondria and that when glucose oxidation is turned off during starvation, non-esterified fatty acids are used instead as fuel [99]. In obesity circulating non-esterified fatty acid levels are high as a result of increased release from adipocytes, and acetyl CoA and NADH formed by fatty acid oxidation cause phosphorylation of the pyruvate dehydrogenase complex and reduced glucose utilisation. It has more recently been found that glucose uptake is the rate limiting step rather than intracellular glucose metabolism in fatty acid-induced insulin resistance [91], indicating a mechanism whereby accumulation of intracellular fatty acids or their metabolites results in impairment of signalling pathways resulting in reduced translocation of GLUT4 into the plasma membrane. It has also been shown that NEFAs increase serine phosphorylation of IRS proteins, impairing insulin signal transduction [91].

In the liver, insulin suppresses hepatic glucose output partly through direct effects on gluconeogenesis and glycogenolysis [45,100,101]. Fasting blood glucose levels are predominantly determined by the rate of endogenous glucose production [102]. One study has shown that 56% of the variation in area under the glucose tolerance curve could be explained by impaired suppression of hepatic glucose output amongst a group of men with normal glucose tolerance, impaired glucose tolerance or 'mild' diabetes [103]. In subjects with type 2 diabetes there is impaired suppression of hepatic glucose output [11]. This is a result of the lack of inhibition of two key gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and the glucose-6-phosphatase catalytic subunit (G6Pase). Insulin inhibits the expression of both of these enzymes and it is widely accepted that this process is mediated via activation of PKB [91]. Impaired hepatic glycogen storage and glycogen synthase activity is also seen in insulin resistance as a result of impaired phosphorylation of glycogen synthase kinase 3 (GSK3) by PKB. There is also now emerging evidence for the role of cytokines secreted by adipocytes in regulating hepatic insulin sensitivity: Adiponectin, an adipocytokine which regulates energy homeostasis and glucose and lipid

metabolism, causes a reduction in molecules involved in gluconeogenesis in the liver [104]. In-vitro experiments with human hepatocytes have shown that exposure to leptin, another adipocytokine, caused attenuation of several insulin-induced activities, including tyrosine phosphorylation of the insulin receptor substrate-1 (IRS-1), association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, and down-regulation of gluconeogenesis [105]. The adipocytokine resistin decreases hepatic glucose production as demonstrated by reduced mRNA of two key gluconeogenic enzymes glucose-6-phosphatase (G6Pase) and AMP-activated protein kinase (AMPK) in resistin knockout mice [91].

There is increasing evidence for the role of adipose tissue in the development of insulin resistance and type 2 diabetes. Although insulin does stimulate glucose uptake in adipose tissue via increased translocation of GLUT4 to the plasma membrane [100], the predominant effect of insulin in adipose tissue is on suppression of lipolysis [106,107]. Because both obesity and lipodystrophy lead to insulin resistance in muscle, and the latter can be reversed through transplanting adipose tissue, fat seems to influence insulin resistance through different mechanisms than just taking up glucose itself. Obesity is associated with increased NEFA release [45] and there is increasing evidence for the important role of non-esterified fatty acids in mediating insulin resistance. In keeping with this argument, organ-specific interruption of adipose GLUT 4 function in mice leads to insulin resistance in muscle and liver [91]. Whole body glucose metabolism is altered by levels of non-esterified fatty acid production by adipose tissue through the substrate competitive mechanism described by Randle [50]. In addition the cytokines TNFα, adiponectin, resistin and leptin described above are secreted by adipocytes and have various effects on insulin sensitivity in muscle and liver as well as adipocytes. TNFα expression is increased in adipocytes of obese individuals and correlates with insulin resistance [108]. TNFα inhibits insulin signal transduction at the level of the insulin receptor tyrosine kinase and the subsequent phosphorylation of insulin receptor substrate 1 (IRS-1) and impairs insulin action resulting in increased lipolysis.

2.2.2 Assessment of insulin sensitivity

Many different methods have been used, mostly which measure whole body insulin sensitivity but there are also techniques available which assess, as far as possible, insulin sensitivity in muscle, liver and fat.

2.2.2.1 Whole body insulin sensitivity

2.2.2.1.1 Model assessments of insulin resistance

The homeostasis model assessment (HOMA) is a mathematical model which allows values for insulin sensitivity and β-cell function (expressed as a percentage of normal) to be obtained from simultaneously measured fasting glucose, insulin and C-peptide (as a measure of insulin secretion) [109]. Since insulin secretion is pulsatile, the optimal sample should be the mean of three results at 5-minute intervals, however a single measurement is often used in epidemiological studies [109]. Estimates of insulin resistance using HOMA correlate well with results from the euglycaemic clamp [110,111].

Minimal models based on results from frequently sampled intravenous glucose tolerance tests (IVGTT) have also been used. Since insulin causes glucose to fall and glucose causes insulin to rise, the complex feedback loop can be mathematically analysed to partition β-cell function and insulin resistance [112].

2.2.2.1.2 Clamps

The euglycaemic hyperinsulinaemic clamp was first used by De Fronzo in 1979 and has been referred to as the 'gold standard' method for measuring insulin sensitivity [10]. Glucose is 'clamped' at a predetermined level (e.g. 5 mmol/l) by titrating a variable-rate infusion of glucose against a fixed-rate infusion of soluble insulin (priming dose followed by a maintenance dose). Insulin infusion rates can be given as dose per surface area or body weight [113-115]. The glucose infusion rate is computed on the basis of blood glucose concentrations measured at 3 to 5 minute intervals throughout the clamp. Once a steady state has been reached the degree of insulin resistance is inversely related to the amount of glucose

required to maintain the stated blood glucose concentration. For diabetic subjects, normoglycaemia is not representative of the usual physiological state so isoglycaemic clamps may be used instead. Hepatic glucose output may be largely suppressed by the doses of insulin used and can be traced using radio-isotope-labelled glucose. Criticisms of the clamp technique are that they are labour-intensive and expensive [112], that they are non-physiological, particularly as high insulin doses are required, and they have a relatively high co-efficient of variability [116,117].

A variety of different clamp techniques have been used under different circumstances. The areas of contention relate to insulin dosing and hand-warming to produce arterialised samples. The clamp technique, although considered the 'gold standard' for measuring insulin sensitivity, is a highly artificial technique. One of the main criticisms of the technique is the non-physiological insulin doses required, and for this reason a range of different insulin doses have been used, the aim being to use as low a dose as possible to achieve the required glucose level, and generally this will be larger the more insulin-resistant the study population under investigation. Some investigators have used two-stage clamps with a low dose insulin dose stage, arguing that hepatic insulin sensitivity can be measured more physiologically using this technique [103,118]. Hand-warming using a heating box or pad is used to cause local vasodilation, which results in arterialisation of the venous blood, thereby reducing peripheral glucose uptake. Without arterialisation, peripheral glucose readings are 0.2-0.5mmol/l lower because of increased tissue uptake of glucose [119] and glucose uptake (M value) is higher [116]. However hand-warming also causes measurable alterations in blood pressure and heart rate resulting from systemic vasodilation [116] which may also affect measured insulin sensitivity. For this reason hand warming was not used in this study.

2.2.2.1.3 Oral glucose tolerance test

The oral glucose tolerance test (OGTT) has traditionally been used to diagnose diabetes but its possible role in the diagnosis of insulin resistance has been examined. An estimate of insulin resistance has been calculated from the logarithm of the plasma insulin concentration two hours after a 75g oral glucose load [120]. More recently an insulin sensitivity index for glycaemia (ISI(gly)) has been calculated using the formula ISI(gly) = 2/[(INSp x GLYp) +1], in which INSp and GLYp, = insulinaemic and glycaemic areas during a 75g OGTT (sampling time 0, 1, and 2 hours). A slight modification of this formula allows the calculation of insulin resistance indices (IRI). ISI and IRI are complementary, as their sum is always equal to two, so that IRI can be deduced from ISI and vice versa [121].

2.2.2.2 Assessment of hepatic insulin sensitivity

Hepatic glucose output can be quantified during the clamp using the isotope dilution technique proposed by Steele in 1959 [11,122]. An isotope dilution technique is used to determine endogenous glucose production using a labelled infusion, usually of deuterated glucose, a stable isotope. Criticisms of the technique are that calculated rates of total glucose appearance (Ra) as estimated by the dilution of tracer in the plasma, may be less than the measured endogenous glucose infusion rates resulting in negative values for hepatic glucose production [11]. Tracer studies require that there is no discrimination between native glucose and the labelled isotope species and if this does not occur then this is described as an 'isotope effect'. Other problems which may occur with tracers are tracer-recycling, inability of the simple mathematical model to cope with complex glucose physiology and loss of label. Maintaining the plasma enrichment with labelled glucose by spiking the variable rate glucose infusion with isotope label in addition to the constant infusion, and improved purity of labelled product have minimised these errors [11]. It has also been found that hepatic glucose output has been underestimated and negative values have been reported during studies of suppression of hepatic glucose output, although this has also been minimised by

spiking the variable rate infusion with deuterated glucose [123]. Because lower doses of insulin are required for suppression of gluconeogenesis and glycogenolysis than are required for glucose uptake in muscle, hepatic glucose output may be completely suppressed during the euglycaemic clamp and so some investigators have used a two-stage clamp with a first stage of low dose insulin infusion (e.g. 0.25mu/kg/min) in an attempt to make the measurement more physiological [103,118].

2.2.2.3 Suppression of non-esterified fatty acids

Suppression of lipolysis by insulin in adipose tissue can be measured by response to an oral glucose load or to a meal, or can be measured during a low-dose insulin infusion or during a euglycaemic hyperinsulinaemic clamp. Like hepatic glucose output, lipolysis is suppressed by relatively low doses of insulin even in insulin-resistant individuals, compared with the doses for peripheral glucose disposal [106,124], so if a traditional clamp method is used, a low-dose insulin stage is usually required [45]. The oral glucose tolerance test has been used to measure suppression of NEFA production [125,126], and Belfiore described a method to calculate the insulin sensitivity index for non-esterified fatty acid (ISI(NEFA)) using the area under the curve method in the same way as described for ISI(GLY) [121]. To measure suppression of non-esterified fatty acids after a meal, a standard meal can be used [127]. Labelled fatty acids such as palmitate can be used in combination with these techniques to examine oxidative and non-oxidative fatty acid metabolism [45].

2.3 Metabolic syndrome

2.3.1 Definition, epidemiology and metabolic consequences

Reaven first described the co-existence of insulin resistance, hypertension and dyslipidaemia in 1988 and postulated that increased non-esterified fatty acids (NEFAs) resulting from resistance to suppression of NEFAs by insulin, in the presence of failure to maintain hyperinsulinaemia, might mediate the development of diabetes, by increasing endogenous glucose production [1]. There

are now various different definitions of the metabolic syndrome, each with slightly different diagnostic criteria. The essential features are obesity, impaired glucose tolerance (or diabetes), hypertension, raised triglycerides and reduced high density lipoprotein (HDL) cholesterol (with a predominance of atherogenic small dense LDL and apolipoprotein B). Additional features are microalbuminuria, fatty liver, and clotting factor activation. Definitions of the metabolic syndrome produced by the World Health Organisation (WHO), National Cholesterol Education Programme Adult Treatment Programme III (NCEP ATP III) [128] and European Group for the Study of Insulin Resistance (EGIR) [129] are the most commonly used.

The prevalence of the metabolic syndrome varies slightly according to the definition chosen, and different definitions may be more appropriate for different populations. In the United States the NHANES III 1988-1994 study, the prevalence of the metabolic syndrome in the representative sample of 8814 was found to be 43.5% amongst individuals aged 60-69 years and 6.7% amongst individuals aged 20-29 years, with age-adjusted prevalence of 24% suggesting around 47 million US residents have the metabolic syndrome [130]. The WHO and ATP III criteria show close agreement in Caucasian populations but poor agreement in Asian and Chinese populations [131], possibly because ATP III defines glucose intolerance by fasting glucose alone which may underestimate the prevalence of impaired glucose tolerance in these populations.

There is clear evidence that this cluster of metabolic abnormalities results in increased cardiovascular risk [132,133], with around a three-fold risk for the development of coronary heart disease [134].

2.3.2 Features of the metabolic syndrome

2.3.2.1 Insulin resistance

Insulin resistance is the most commonly occurring feature of the metabolic syndrome, and together with central obesity has been shown in mathematical modelling to be the most important feature of the metabolic syndrome. [41]. Plasma

pro-insulin level (as well as waist circumference and plasma triglycerides) is a predictor if incident metabolic syndrome, indicating that insulin resistance predicts the development of metabolic syndrome [39].

2.3.2.2 Central obesity

Although Reaven's original description did not describe central obesity, Vague described a relationship between body fat distribution and cardiovascular risk factors in 1947 [135]. It is now recognised that obesity correlates strongly with metabolic syndrome [40,136] and that waist circumference is a good predictor of subsequent development of metabolic syndrome [39]. Mathematical modelling has shown that of the metabolic syndrome features, the insulin resistance and central obesity are the primary features [41], and another study has shown that intra-abdominal fat is independently associated with all the other features of the metabolic syndrome, suggesting there may be a pathophysiological role for central fat [40]. There is a body of opinion that central obesity is pathogenic in the metabolic syndrome [47,137]. The theory proposed is that abdominal adipocytes are loaded with triglyceride, reducing fat depot capacity to protect other tissues from utilising a large proportion of dietary fat. In contrast to subcutaneous adipocytes, the central adipocytes exhibit a high rate of basal lipolysis and are highly sensitive to fat mobilising hormones, but show poor suppression of lipolysis by insulin. The enlarged visceral adipocytes flood the portal circulation with non-esterified fatty acids (NEFAs) at metabolically inappropriate times, when NEFA should be oxidised, thereby exposing non-adipose tissues to fat excess. This leads to triglyceride accumulation in muscles, liver and pancreatic β-cells, resulting in insulin resistance and β -cell dysfunction. [44]

2.3.2.3 Dyslipidaemia

The dyslipidaemia seen in type 2 diabetes and metabolic syndrome is of raised plasma triglycerides and low levels of circulating high-density lipoprotein (HDL) cholesterol. Abnormalities of the lipoprotein subfraction are also seen with increased concentration of atherogenic small dense low-density lipoprotein (LDL)

cholesterol and large very-low-density lipoprotein (VLDL) size [138,139]. Small HDL particles are also found [140].

2.3.2.4 Hypertension

Whilst hypertension is very frequently present in subjects with type 2 diabetes, it is the least commonly occurring feature of the metabolic syndrome [41]. There has been much debate about its role in the metabolic syndrome, and speculation about mechanisms [133 141]. In the presence of insulin resistance, insulin has a reduced effect on the phosphatidylinositol 3 kinase (PI3K) pathway, whereas mitogen-activated protein kinase activity is increased. This results in an exaggeration of the mitogenic actions of insulin leading to vascular smooth muscle proliferation and elevated plasminogen activator inhibitor (PAI)-1. Nitric oxide-mediated vasodilation is also impaired, further contributing to atherogenesis [142].

2.4 Non-alcoholic fatty liver disease (NAFLD)

2.4.1 Definition, pathogenesis and epidemiology

There is a very high prevalence of fat accumulation within the liver associated with obesity, and a proportion of affected patients develop inflammation and fibrosis within the liver. Described as non-alcoholic fatty liver disease (NAFLD) because of its histological similarity to alcoholic liver disease, it is now recognised as part of the metabolic syndrome. [13,14,18]. NAFLD represents a spectrum from simple steatosis, which is present in up to 80% of obese individuals [18,143], through non-alcoholic steatohepatitis (NASH) to fibrosis and cirrhosis. Although only a small proportion of affected patients go on to develop cirrhosis, it is estimated that NASH will become the leading cause of cirrhosis because of increasing obesity prevalence.

The pathogenesis of NAFLD is not fully understood but probably represents a two-stage process where fat accumulation is the first step and the development of inflammation the second. Increased non-esterified fatty acid influx to the liver resulting from insulin resistance to suppression of lipolysis in visceral adipocytes

and the absence of peripheral utilisation results in excess hepatic fatty acids. These fatty acids are not oxidised but converted to diacyl- and triacyl-glycerols and stored in the hepatocyte cytoplasm, leading to steatosis. This effect is further aggravated by insulin resistance affecting activity of the nuclear peroxisome–proliferator-activated receptors PPAR-α, PPAR-γ and SREBPs.

The mechanisms underlying development of NASH are complex but increasing data suggests that defects of hepatic fat oxidation are pivotal in the pathogenesis. Oxidative stress involves pro-inflammatory cytokines such as TNF α and endotoxin.

2.4.2 Assessment of non-alcoholic fatty liver disease

Imaging techniques such as ultrasonography and MRI spectroscopy can be used to assess degree of fat accumulation within liver. Diagnosis of NASH requires liver biopsy so that fibrosis can be demonstrated histologically, but circulating plasma liver enzymes are also used as a marker of degree of inflammation.

2.4.2.1 Ultrasonography

Ultrasonography has been shown to identify steatosis with a sensitivity of 94% and specificity of 88% when compared to histological findings [144], although this technique is operator-dependent. Other imaging methods such as computer tomography (CT) and magnetic resonance imaging (MRI) have also been used [145-147] but have not been shown to be superior to ultrasonography [148]. Ultrasonography is widely accepted for identification of subjects with fatty liver [148,149].

2.4.2.2 MRI spectroscopy

MRI spectroscopy is a new method which allows quantification of hepatic fat content [150-152]. However this technique is expensive and invasive and this method was not available for this study.

2.4.2.3 Liver enzymes

Abnormal liver enzymes are found in association with NAFLD although levels of liver transaminases are not closely correlated with staging or grading of NAFLD and patients with normal alaninine transaminase (ALT) levels have been demonstrated to have advanced fibrosis [153,154].

2.4.2.4 Liver biopsy

Liver biopsy allows quantification of hepatocyte fat accumulation and degree of inflammation or fibrosis. Biopsy is necessary to diagnose non-alcoholic steatohepatitis (NASH) as this is a histological diagnosis [155] but liver biopsy is not without risk and undertaking this procedure in healthy volunteers is therefore often considered unacceptable. There is close correlation between degree of hepatic steatosis detected radiologically and amount of fat found on histological examination [144] and therefore liver ultrasound is often used in the diagnosis of NAFLD [148,149]. Liver biopsy was not undertaken in this study.

2.4.3 Interplay between the features of the metabolic syndrome

Excess accumulation of intracellular lipid in muscle and liver has been demonstrated in type 2 diabetes, in subjects who go on to develop the condition later, and in relatives of subjects with type 2 diabetes [92,156]. Substrate competition between lipid and glucose suggests that this lipid accumulation contributes to the insulin resistance [50,157]. Abnormal fatty acid metabolism has been demonstrated in type 2 diabetes, and increased NEFAs and intramyocellular lipid has been identified in offspring of subjects with type 2 diabetes, indicating that abnormalities of fatty acid metabolism may underlie the development of insulin resistance [92,158,159].

A relationship has been demonstrated between plasma non-esterified fatty acids and blood pressure, which is not explained by obesity, in a longitudinal study in which baseline blood pressure predicted plasma non-esterified fatty acids, suggesting the possibility of a causative role for blood pressure. Furthermore the effect was most marked for subjects taking diuretics rather than beta blockers

raising the possibility of a role for the sympathetic nervous system in explaining the relationship [126].

2.5 Hypothalamic-pituitary-adrenal axis (HPAA)

Patients with Cushing's syndrome develop central obesity and a severe form of the metabolic syndrome phenotype with insulin resistance or diabetes, hypertension and dyslipidaemia as a result of cortisol excess. This observation raises the question of whether a more subtle abnormality of the hypothalamic-pituitary-adrenal (HPA) axis also causes the metabolic syndrome to develop in some obese individuals. It has long been suggested that cortisol may play a role in the development of the metabolic syndrome [160-162] but the relationship between the HPA axis, obesity and the metabolic syndrome as described in the literature is not clear.

Paradoxically, circulating cortisol levels have usually been found to be low or normal in obesity [24-26]. Morning cortisol levels increase with fasting glucose and insulin, however [32,162], and ACTH and cortisol responses to stress increase with both obesity and impaired glucose disposal [28]. Cortisol clearance is increased in obesity [24,30,31] indicating that overall exposure to corticosteroids is increased, and increased cortisol clearance has also been demonstrated to correlate with fasting glucose and insulin [29].

In obese men with the metabolic syndrome an epidemiological study has shown that cortisol response to a stressful event (venepuncture) is high, and furthermore that repeated stresses do not cause the blunting of stress response seen in normal individuals[163]. Another epidemiological study showed that cortisol level after stimulation with ACTH was higher in obese individuals with features of the metabolic syndrome [164]. Another study showed that in middle-aged men a flattened diurnal curve and less suppression after dexamethasone was seen in individuals with high waist hip ratio, associated with other cardiovascular risk factors, and furthermore that these individuals showed increased cortisol secretion in response to perceived stress and also to the physiological stress of

eating [165]. Using salivary cortisol measurement, Bjorntorp identified a group of individuals with obesity and insulin-resistance who demonstrated a flat, rigid day curve and poor feedback control. A pattern is therefore suggested in individuals with central obesity and other features of the metabolic syndrome of blunted HPA axis activity but hypersensitivity of the axis such that in obese individuals with the metabolic syndrome cortisol exposure may in fact be increased.

The whole pattern of HPA axis activity is therefore apparently altered in obesity and insulin resistance. However because of the significant co-variation of obesity and insulin resistance it is not known whether the abnormalities of the HPA axis found in association with insulin resistance are simply a reflection of the abnormalities found in obesity or whether a separate relationship exists between insulin resistance and abnormalities of the HPA axis.

Cortisol is metabolised irreversibly by A-ring reduction and reversibly to inactive cortisone by 11 β hydroxysteroid dehydrogenase (HSD) enzymes. The irreversible A-ring metabolism of cortisol to inactive metabolites by 5α and 5βreductase enzymes occurs within fat and liver. Heterogeneity in expression of these enzymes may explain individual variation in glucocorticoid exposure and metabolism. In obesity there is an increased ratio of 5α to 5β reductase activity which probably reflects the 5α reductase activity of adipose tissue [29] although it is not known whether this is of biological importance. There has been considerably more interest in the expression of expression of 11 βHSD1 which regenerates cortisol from cortisone, predominantly in adipose tissue and liver, and altered expression of these hormones has been found in obese and insulin resistant states [166] [167], which may change cortisol exposure at tissue level. One suggested explanation for increased urinary cortisol found in obesity is that expression of this enzyme is decreased in liver [167], which results in a compensatory increase in production by stimulating the HPA axis, but increased in adipose tissue [167]. This may then result in insulin resistance at the level of the adipocyte which leads in turn to central adiposity as well as insulin resistance in other tissues, in a condition described by Bujalska as 'Cushing's disease of the omentum' because

activity of 11 βHSD1 is higher in visceral than subcutaneous adipocytes in vivo [166].

Cortisol is reversibly metabolised by dehydrogenation to inactive cortisone by the other isoform of this enzyme, 11 βHSD 2, in the kidney. This has been demonstrated to be abnormal in some individuals with hypertension through the mineralocorticoid effect of excess cortisol in the kidneys but is not thought to be pathogenic in the metabolic syndrome [168].

2.6 Physical activity and fitness

Early evidence that physical activity might confer health benefits was presented by Morris in a study of bus drivers and conductors after World War II, which showed that drivers had an annual incidence of coronary heart disease of 0.27% per year compared with 0.19% per year in the more physically active conductors. Similar results were found in a comparison of postal clerks and postmen. Morris later showed in the Whitehall Study that in men who undertook vigorous exercise such as swimming, hill-climbing, gardening, building work, brisk walking or cycling for more than 30 minutes a day had a risk of developing coronary heart disease around a third of those who did not [169].

Physical activity has been shown in epidemiological studies to be protective against the development of the metabolic syndrome [170-172]. Moderate regular exercise is associated with lower systolic blood pressure, raised HDL cholesterol and lower fasting glucose and insulin levels compared with low levels of exercise [173]. Both vigorous physical activity and regular walking were found to significantly reduce the risk of future onset of diabetes in the Nurses' Health Study [174]. Both fitness and physical activity have been shown in one study to be more important in predicting mortality than smoking, hypertension, obesity and diabetes [175].

The benefits of physical activity are mediated partly through improved body composition but may also have independent effects on cardiovascular risk factors. Physical activity reduces visceral, and to a lesser extent, subcutaneous

fat mass [176]. Samaras showed that increased physical activity is associated with a reduction in total and abdominal fat accumulation and that this is more important than dietary intake, smoking or socio-economic status in determining obesity [177]. Regular walking causes weight loss and specifically loss of intra-abdominal body fat [178]. It has been suggested that loss of fat mass may be responsible for most of the benefits associated with increased exercise [179]. Resting metabolic rate is higher in trained individuals and therefore resting calorie expenditure is higher than in untrained individuals, augmenting the loss of fat mass in these individuals [180].

Exercise does appear to confer apparent cardiovascular benefits beyond those mediated by reduction in obesity. High intensity exercise in particular has a beneficial effect on LDL concentration and particle size resulting in increased average LDL particle size and HDL concentration is also increased by high intensity exercise [181]. Even a modest amount of low intensity exercise has a benefit in reducing circulating triglycerides [182]. The mechanism of action by which exercise improves lipid profile is by activation of muscle AMP-activated protein kinase (AMP kinase), which upregulates lipid metabolism and increases fatty acid oxidation and decreases re-esterification through direct effects on enzymes such as Malonyl-CoA Decarboxylase [183].

2.6.1 Assessment of cardiorespiratory fitness and energy expenditure

In examining the effects of physical activity on metabolic profile, there are two aspects to consider, namely cardiorespiratory fitness and energy expenditure. These elements of physical activity are not entirely independent of one another but may contribute differently to the metabolic profile and so it is helpful to consider them independently [184]. For example it has been shown in children that fitness is more important than energy expenditure in determining development of obesity [185], whereas there is evidence to suggest that physical activity is more important in determining metabolic syndrome and insulin sensitivity [184,186,187].

Cardiorespiratory fitness is measured by maximal oxygen uptake during maximal exercise testing or calculated from sub-maximal exercise testing. Energy expenditure is more difficult to measure but may be determined through the use of questionnaires or through heart-rate monitoring.

2.6.1.1 Maximal oxygen uptake

Maximal oxygen uptake (VO_2max) is the greatest amount of oxygen a person can take in during physical work and is a measure of his maximal capacity to transport oxygen to the tissues of the body [188]. Oxygen uptake (VO_2) is determined by indirect calorimetry, where VO_2 is equal to pulmonary minute ventilation (V_E) multiplied by the difference in the inspired and expired oxygen percentages:

VO_2 (l/min) = V_E (l/min) x (inspired fraction of O_2 - expired fraction of O_2)

Oxygen uptake at rest is approximately 0.25 l/min and maximum average oxygen uptake of a sedentary 70kg man is 3.0 l/min (42.8ml/kg/min). At VO_2max additional increases in workload produce no further increase in oxygen uptake and the respiratory quotient CO_2 produced/ O_2 consumed starts to rise. It is determined during graded exercise on a cycle ergometer or treadmill and indicates the maximal level of aerobic power output. The limiting factors to VO_2max are central circulatory factors and the rate at which oxygen is moved by diffusion from haemoglobin to muscle mitochondria [189].

Cardiorespiratory functional capacity is most strongly influenced by the amount of physical activity undertaken, although there is also a strong inherited component [190]. Endurance training produces an increase in VO_2max and the rate and magnitude of these increases are related to the age of the individual, previous fitness, and rate, intensity and duration of the exercise training programme. The VO_2max of sedentary individuals is usually less than 40ml/kg/min whereas that of world-class male endurance athletes can exceed 80ml/kg/min. Increase in VO_2max occurs as a result of increased mitochondrial mass, haemodynamic adjustments such as increased maximal cardiac output (heart rate for a given

power output decreases but stroke volume increases) and increased arteriovenous oxygen differential. Intracellular metabolic adaptations occur to maximise oxygen utilisation within mitochondria [189].

2.6.1.2 Energy expenditure

Energy expenditure can be measured using invasive methods such as indirect calorimetry using doubly labelled water but these do not allow for habitual expenditure in free-living individuals so are usually only used for validating less invasive methods [191,192]. Energy expenditure has traditionally been assessed through the use of questionnaires [169,193] but more recently heart-rate monitoring has been used, usually over a period of several days, and used together with individual calibration from exercise testing to determine energy expenditure [184,186,187]. Motion sensing using accelerometers placed on the wrist, hip or lower back provides an assessment of energy expenditure which correlates well with indirect calorimetry using doubly labelled water [191] but these have not yet been widely used.

3 METHODS

3.1 Ethical approval

The study had the approval of the local research ethics committee

3.2 Research Facility

All studies took place in the Clinical Research Facility located within Southampton General Hospital. This unit is staffed with trained research nurses and is dedicated to full time research. It was set up with funds from the Wellcome Trust but staffed by NHS research and development funding. It is equipped with a ward for metabolic studies, clinical rooms and a physiological laboratory. There is a preparation laboratory on site where blood samples can be centrifuged, separated and frozen.

3.3 Recruitment

Subjects were recruited through advertisement in the hospital and local press, and using a direct approach by three local GP surgeries who were able to identify individuals by body mass index and medical history, and who agreed to send out letters inviting volunteers to participate. Suitable individuals were identified by individual GP practices after agreement of all partners in the practice. Letters which had been pre-signed were then sent out by the practice clerical staff, with self-addressed envelopes. Local large companies such as Ford Motors and Ordnance Survey were also approached and a recruitment day was held in a local shopping centre. Volunteers then had a brief preliminary interview over the telephone prior to enrolment. Eighty-two subjects expressed an interest in participating and the proportion of subjects who agreed to participate or who were suitable for the study was as follows:

Table 3-1: Recruitment to the study

82 subjects expressed an interest in taking part in the study				
Number of subjects included in study	Number of subjects who declined inclusion after further information supplied or did not attend appointment	Number of subjects who were excluded from the study on the basis of known pathology or medication	Number of subjects who were excluded from the study on the basis of race or gender	Number of subjects who were excluded because of BMI in range 25-30kg/m^2 to reduce numbers in this group later during recruitment
34	17	21	4	6

3.4 Subjects

This study was of middle-aged white Western European men. There is evidence that there is both gender and racial variation in many of the aspects of our study, and in women changes related to the menopause. Ideally this study would have encompassed all groups, but because our numbers were small, the priority was to keep the group of subjects studied as homogenous as possible. For this reason men were chosen, with the acceptance that the results might not apply to women or other ethnic groups.

3.4.1 Inclusion criteria

All subjects were white males who were aged 40-65 years at the time of recruitment. Subjects across the range of BMI from 19kg/m^2 to 46kg/m^2 were studied. Subjects were specifically recruited who had BMI greater than 30kg/m^2 to ensure this group who were of particular interest for this study were well-represented.

3.4.2 Exclusion criteria

Subjects had no significant past medical history and were on no regular medication apart from simple analgesics. Subjects with a history of clinical

depression or alcohol abuse were excluded. No subject had a history of treatment with corticosteroids.

3.4.2.1 Medical history and examination

A medical history and examination were performed. Subjects were excluded if they had taken corticosteroids within the last year or if they had suffered with major depression or drank more than 25 units of alcohol per week. One subject who was stable on replacement dose thyroxine was included in the study. One subject was excluded after the first day on the basis of severe hypertension as it was not felt ethical to withhold treatment for the duration of the study, and therefore 33 subjects were included after the first day.

3.5 Anthropometry and blood pressure

3.5.1 Weight

Individuals were weighed wearing light indoor clothing (shirt and trousers) without shoes. Measurements were made using digital scales (Taneta, Japan) and recorded in kilograms to the nearest 0.1 kg.

3.5.2 Height

Height was measured using a stadiometer. The subject was asked to stand as tall and straight as possible with feet together and arms held loosely at the side and shoulders relaxed. The head was placed in the Frankfurt Plane in which an imaginary line joining the upper border of the external auditory meatus and the lower border of the orbit of the eye is horizontal. Height was measured in meters to the closest 0.1cm [194].

3.5.3 Body Mass Index (BMI)

Body mass index was calculated using the equation weight / (height ^{2})

3.5.4 Waist circumference

Waist measurement was measured over bare skin. The tape was applied midway between the costal margin and the iliac crest, taking care to ensure the tape was

horizontal. The subject was asked to relax and look straight ahead with his arms at his sides. Measurement was made at the end of expiration, with the tape resting on the skin but not indenting it. One measurement was recorded.

3.5.5 Hip circumference

The measuring tape was applied at the widest part between the greater trochanter and lower buttock level, with the legs together, ensuring the tape was horizontal. Several measurements were made and the maximum single reading was recorded.

3.5.6 Skin fold thickness

These measurements were made by one of two research nurses who were trained in the technique, and who regularly attended for re-evaluation to ensure reliability and repeatability. I attended training in this technique by Medical Research Council nurses who have considerable experience in these techniques from epidemiological research but the subject measurements for this study were undertaken by the two CRF nurses. Skin fold measurements were made using skin fold calipers (Harpenden, UK) at four standard sites: triceps, biceps, subscapular and upper suprailiac [195]. Measurements were made on the non-dominant side using the thumb and index finger in a sweeping movement to collect the subcutaneous tissue away from the underlying muscle fascia. Two hands were used initially and then one hand held the skin fold whilst the calipers were used with the other hand. The positioning of the blades of the calipers was generally at least one blade-breadth from the apex of the skin fold. The calipers were fully released and then the dial read at five seconds (even if the dial was still moving). Three measurements were taken at each site and the average value used. If very different readings were obtained further readings were taken until three closely matching readings were obtained. All measurements were performed by one of two trained nurses as previously.

3.5.6.1 Biceps and Triceps

The subject was asked to stand with his back to the person measuring, arms hanging by his sides. With the subject's arm flexed at 90^{o} a mark was made at the midpoint between the acromion and olecranon. The subject was then asked to relax with his arm hanging by his side and a tape measure was placed with the upper border of the tape on the mark. A horizontal line was made at the anterior and posterior surface of the arm, and then vertical lines were made to form a cross at the anterior midpoint over the belly of the biceps and at the most dorsal point. These points were used for the measurement of biceps and triceps skin fold thicknesses respectively using the methods described above.

3.5.6.2 Subscapular

The subject stood with shoulders and arms relaxed. The lowest tip of the scapula was identified and identified by marking with a cross. The skin fold was picked up obliquely, in the natural cleavage of the skin and the calipers applied with the cross at the apex of the fold.

3.5.6.3 Suprailiac

The subject was asked to stand straight and relaxed with his arms folded in front of him. From behind the subject the iliac crest was identified and a horizontal line marked just above the iliac crest at the side. A vertical line was then made at the mid axilliary line to form a cross. The skin fold was then picked up in the natural crease of the skin and the callipers applied at the level of the cross with the cross on the apex of the fold to measure the skin fold thickness.

3.5.6.4 Calculating body fat percentage from skin fold thickness

The equations of Durnin and Womersley [72] were used to calculate percentage body fat according to the equation:

body fat % = (4.95/ density - 4.5) x 100

where density = c-[m-log total skin folds], where c and m are constants according to age and total skin folds = average triceps + average biceps skin fold + average subscapular skin fold + average upper suprailiac skin folds)

3.5.7 Bioelectrical impedance

Bioelectrical impedance was measured between the subject's left wrist and ankle according to the manufacturer's instructions using a Bodystat 1500 instrument (Bodystat Ltd, Isle of Man, UK). The subject's weight, height and age were entered into the instrument and the calculated percentage body fat was recorded.

3.5.8 Air displacement plethysmography

Air displacement plethysmography was performed according to the manufacturer's instruction using the Bod Pod (Life Measurements Incorporated, USA). Subjects were asked to wear tight-fitting underpants and a swimming cap but body hair was not shaved. Each subject's age and height were entered and the machine incorporates its own weighing device, which was calibrated on each day of use and subjects weighed according to the instructions. The machine makes a correction for lung volume and all subjects were free of respiratory disease.

3.5.9 Dual energy x-ray absorptiometry (DEXA))

Dual energy x-ray absorptiometry was undertaken on a Delphi W instrument (Hologic Inc, USA) using a standard visual method to divide images into trunk, limb and head. Subjects were weighed and measured on the day of the assessment and total fat, regional fat and lean mass were calculated. This measurement was performed by a technician from the bone densitometry department where the machine is located.

3.5.10 Magnetic Resonance Imaging (MRI)

Subjects with BMI greater than 30kg/m^2 had magnetic resonance imaging of the abdominal region. Lean and overweight individuals were not subjected to MRI because of resource implications and because it was considered that there would

be much less range of visceral fat amongst non-obese individuals. The scans were performed by one of two radiographers within the radiology department of the hospital and the method for calculating fat volume from the images was developed and undertaken by a scientist from the medical physics department after we had performed a full literature review [85,86,196,197].

We acquired five non-contiguous slices extending from 5cm below to 15cm above L4-L5 to obtain more detailed information about visceral fat than a single slice would demonstrate [197]. Axial scans were taken with subjects in the supine position (with the exception of one very obese subject in whom better images were obtained through scanning prone). Two subjects were unable to be scanned because of claustrophobia. Subjects were scanned on a Siemens 1.5 T Symphony MR scanner (software release 4VA15A, Siemens AG, Erlangen, Germany). A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111ms, TE = 4.18ms, flip angle = 70°, slice width = 10 mm, slice spacing = 50 mm) was used to obtain T1 weighted images. In order to accommodate the circumference of the individual being scanned within the image, the field of view was varied.

MR images were analysed using a proprietary software package (Mimics, Materialise NV, Belgium) to identify regions of subcutaneous and visceral fat within the cross-sectional abdominal MR images. By examining the histogram of pixel values in each image a threshold level was set for fat pixels. By using a seed-growing technique, where neighbouring pixels of similar values (i.e. within the identified threshold) are selected, fat tissue could be segmented out from other tissue in the image. Some manual intervention was required because of variation in signal intensity across the image, which is the case in many MR images. Three different 'masks' were created, one comprising the whole cross section of the body, one containing the visceral fat region and one containing subcutaneous fat region. It was possible to extract the number of pixels contained within each of these masks, and hence calculate the areas of subcutaneous fat and visceral fat, and compare them with total cross sectional

area. Adipose tissue volume was converted to mass in kilograms using a density of 0.92 for adipose tissue [82].

3.5.11 Blood pressure

Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, UK) on the non-dominant arm after subjects had become acclimatised to the Clinical Research Facility and had rested for at least 60 minutes. Measurements were made with the subject seated quietly after the glucose tolerance test. Three measurements were made 5 minutes apart and mean values of systolic and diastolic blood pressure calculated. If there was significant variation between the readings (>5mmHg) repeat measurements were taken until three similar readings were obtained.

3.6 Lipid profile

Total cholesterol, HDL cholesterol and triglycerides were measured on the day of study within the biochemistry laboratory on fasting plasma samples using *in vitro* enzymatic colorimetric kit methods according to the manufacturers' instructions (Advia 1650 Chemistry System, Bayer Diagnostics, Germany).

3.7 Non-alcoholic fatty liver disease (NAFLD)

3.7.1 Ultrasonography

Abdominal ultrasonography was performed by one of two experienced radiologists and an assessment of hepatic steatosis was made by visual assessment of degree of echogeneity. The criteria used for assessment of the liver scans were: a) comparison of reflectivity with the right kidney, b) visualisation of the portal tracts and c) attenuation of beam. The measure of agreement of scoring between both radiologists was analysed and a kappa statistic is presented. A grade of mild, moderate or severe fatty liver was assigned by the radiologists, but for some analyses subjects were divided into those with or without ultrasonographic evidence of fatty liver alone.

3.7.2 Liver enzymes

ALT and GGT were measured in the biochemistry laboratory on fasting plasma using *in vitro* enzymatic colorimetric kit methods according to the manufacturers' instructions (Advia 1650 Chemistry System, Bayer Diagnostics, Germany).

3.8 Whole body insulin sensitivity

3.8.1 Oral glucose tolerance test (OGTT)

Subjects were asked to attend the CRF at 9am having fasted from 10pm the previous night. They were asked to avoid strenuous exercise or alcohol on the day prior to attending. On the morning of the test an intravenous cannula was inserted. Blood was taken for measurement of glucose and insulin, and the subject was given a 75g oral glucose load (121ml Polycal [Nutricia Clinical, UK] made up to 200ml with water), which they were asked to drink over one minute. Blood was then taken at 10, 20, 30, 40, 60, 90 and 120 minutes. Samples were stored on ice and separated within one hour for measurement of glucose, insulin and non-esterified fatty acids. Plasma glucose was measured on fluoride samples by the glucose oxidase method using a Yellow Springs Glucose Analyser (YSI, USA).

3.8.2 Euglycaemic hyperinsulinaemic clamp with deuterated glucose

Insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique with deuterated glucose infusion to determine hepatic insulin sensitivity determined by insulin-mediated suppression of endogenous glucose production [10,11]. Deuterated (6,6) glucose was used as a steady state infusion prior to the clamp, and during the clamp to determine hepatic glucose output and hepatic insulin sensitivity respectively. The deuterated glucose was supplied by St Thomas' Hospital pharmacy department.

Subjects attended having abstained from vigorous exercise for 48 hours, and having fasted from 10pm the night before. They were weighed as previously described on arrival. A cannula was placed into a large vein in the anterior cubital

fossa of each arm or forearm. Venous blood samples were not arterialised as it was felt that the advantages of hand-warming would be off-set by the effects of warming on blood pressure and heart rate [116]. Baseline blood samples were taken and then a 170mg bolus of deuterated glucose was administered prior to commencing an infusion of deuterated glucose. The infusion was 7ml deuterated glucose 100mg/ml in 43ml 0.9% saline and was infused at 7.3ml/min (1.7mg deuterated glucose per minute). The infusion was continued for 150 minutes to reach a steady state and blood samples were taken from the contralateral arm at 30, 60, 90, 120, 125, 130, 140 and 150 minutes. Blood samples were stored in ice and centrifuged at 2000G at 4° C. Fluoride samples were used to determine plasma glucose by the glucose oxidase method using a Yellow Springs Glucose Analyser (YSI, USA). Fluoride and heparinised samples were subsequently stored at –70 °C for later analysis of deuterated-glucose enrichment, and insulin respectively.

At 150 minutes the euglycaemic hyperinsulinaemic clamp was commenced. An infusion of insulin was prepared by adding 50 units of soluble insulin (Actrapid, Novo Nordisk, Denmark) to 48 ml 0.9% saline mixed with 2 ml of the subject's blood (to prevented insulin adhering to the syringe). The infusion was commenced at 1.5mU/kg/min and blood was taken at 5-minute intervals. The deuterated glucose infusion continued throughout the test. A 20% glucose infusion which had been enriched with 800mg deuterated glucose was commenced at a variable rate once the whole blood glucose began to drop, and the whole blood glucose was clamped at 4.5-5mmol/l. The clamp continued for 3 hours with blood sample taken as previously for plasma glucose, isotope enrichment and insulin at 180, 210, 240, 270, 300, 305, 310, 320 and 330 minutes from the beginning of the test. The M value of insulin sensitivity was defined as the glucose infusion rate during the final 30 minutes of the test in mg/kg/minute.

3.9 Insulin sensitivity in different tissues

3.9.1 Insulin sensitivity in muscle and liver

The isotopic enrichment of glucose was measured by gas chromatography-mass spectrometry on a HP 5971A MSD (Agilent Technologies, Berkshire, UK) using the penta-O-trimethylsilyl-D-glucose-o-methoxime derivative analysed by selected ion monitoring of the ions at m/z 319 and 321. The total appearance of glucose (Ra), endogenous glucose production and disappearance of glucose (Rd) were calculated using a modified version of the equations formulated by Steele [122]. Tracer to tracee ratios (TTR) were calculated as the ratio of the two areas from the mass spectrometry data. TTR and plasma glucose values were smoothed using the method of optical segments [198]. For the Steele equations, 65% was used as the effective fraction and 0.22 l/kg as the distribution volume of glucose to calculate Ra and Rd [11,122,199]. Glucose disappearance (Rd) during the steady state was used as the measure of insulin sensitivity in muscle. These measures were corrected for total body weight. Hepatic insulin sensitivity was measured as insulin mediated suppression of glucose output in the early part of the clamp and was expressed as percentage suppression 60 min after commencing the insulin infusion, when suppression was linear to this time point. This measurement and calculation were undertaken by collaborators in St Thomas' Hospital.

3.9.2 Insulin sensitivity in adipose tissue

NEFAs were measured fasting at 0, 10, 20, 30, 40, 0, 90 and 120 min during the OGTT to allow measurement of insulin-mediated suppression of NEFA concentration [200,201]. NEFA concentrations were measured in heparinised plasma samples with an autoanalyser by a post-graduate scientist in our laboratory (Konelab 20; Thermo Electon, Waltham, MA, USA) using reagents from Wako Chemicals (Richmond, VA, USA). Insulin sensitivity indices for glycaemia [ISI (gly)] and NEFAs [ISI (NEFA)] were estimated using data from fasting, one hour and two hour samples for insulin, glucose and NEFA concentrations, according to the method proposed for calculating insulin sensitivity in fat from data obtained

during an OGTT [12]. Percentage suppression from baseline and area under the NEFA curve was also calculated using a trapezoid method [187].

3.10 Hypothalamic pituitary adrenal axis (HPAA) studies

3.10.1 Morning cortisol

Fasting blood was taken immediately on cannulation at 9am in a lithium heparin tube and cortisol levels were measured on the day of testing in the endocrine laboratory by an in-house direct assay using an Iodine-125 tracer [202].

3.10.2 Low dose ACTH test

A low dose ACTH test was performed using an ACTH analogue tetracosactrin (Synacthen, Alliance, UK) to determine the adrenal sensitivity. A physiological dose of one microgram of freshly prepared ACTH was used [164]. Tests were performed in the afternoon, one hour after eating, via an indwelling cannula which had been inserted in the morning [164] using 1mcg Synacthen, which was freshly prepared by adding 250mcg Synacthen to 500ml normal saline, shaking vigorously for 2 minutes and then drawing up 2ml of this solution. Subjects had been cannulated on the morning of the test and Synacthen testing was undertaken at 14.00. Synacthen solution was administered as a bolus and 10ml venous blood taken from the in-dwelling cannula at 0, 30 and 60 minutes. Blood was transferred to lithium heparin plasma tubes and cortisol levels were measured in the endocrine laboratory as above.

3.10.3 Corticosteroid binding globulin (CBG)

CBG was measured in the endocrine laboratory using a commercial RIA assay kit (code CBG-RIA-100) (Biosource, Nivelles, Belgium).

3.10.4 Free cortisol

Free cortisol was calculated from measured cortisol and CBG using Coolens' method [203].

3.10.5 Cortisol clearance

3.10.5.1 Preparation of deuterated cortisol

Deuterated Cortisol (9,12,12-D3) was obtained from Cambridge Isotope Laboratories, Massachusetts, USA. Purity was 98%. Stock solution was prepared in pharmacy by dissolving 5 mg deuterated cortisol in 2.5mg pharmaceutical grade absolute ethanol to a concentration of 2mg/ml. The bolus dose of 200mcg/m^2 was prepared freshly in pharmacy on the day of investigation according to subject's surface area by diluting stock solution to 1% alcohol with normal saline. The bolus dose was then filtered using a Millipore Millex GV PDVF filter. The methods were validated by analysing a test dose in biochemistry and using tandem mass-spectrometry.

3.10.5.2 Clinical study

Subjects were asked to eat a light breakfast of toast or cereal and without caffeine prior to 8am and to attend the clinical research facility at 9am where they were cannulated in each arm and weighed as previously. Patients were cannulated at least 30 minutes prior to receiving the bolus dose. The 200mcg/m^2 dose of deuterium-labelled cortisol was given at 10am over 30 seconds. Blood was taken for measurement of deuterated and native cortisol from the contralateral arm at times 0, 5, 10, 15, 20, 30, 60, 90, 120, 150, 180, 210 and 240 minutes in EDTA. Subjects were asked to sit quietly during this time and were not given anything else to eat until after 240 minutes. Blood was centrifuged as previously and stored at –70°C until required for analysis. Urine was collected at time 0, 2, 4, 6 and 8 hours and the volume was measured and a sample stored at -20°C for analysis of cortisol metabolites [204].

3.10.5.3 Lipid extraction

Serum samples were thawed in a water bath at 20°C, vortexed and 0.8ml was added to 4 ml dichloromethane in a glass tube under an extraction hood. The solution was shaken vigorously and vortexed, then centrifuged at 3,500 rpm at 20°C for 10 minutes. The top layer was removed using a glass pipette. A 3ml

aliquot was dried under nitrogen on a heating block at 20°C. This was then reconstituted in approximately 50mcg dichloromethane with two further washes to ensure no sample remained, and the solution was dried down as previously in glass microvials for mass spectrometry analysis. This method was validated prior to testing using samples of known concentration, and repeated sampling on two subjects. During the validation of this method a fourth wash was subjected to analysis using mass spectrometry confirming that no cortisol remained. Once the methods had been validated the lipid extractions were performed by a laboratory technician.

3.10.5.4 Internal standards

For each time point the same method was used for samples which had been spiked with native cortisol of a known concentration of 300nmol/l to act as an internal standard. This internal standard was made by adding 8.5mg cortisol to 78.182ml ethanol (molar weight of cortisol 362.47g). One millilitre of this solution was added to each glass vial and dried down prior to addition of the plasma samples. This method was also validated as above and then performed by the same laboratory technician as above.

3.10.5.5 Mass spectrometry

The lipid-extracted samples were measured by LC/MS/MS using a symmetry C18 3.5ìm 2.1x50mm column (Waters) and an isocratic solvent consisting of 530:470:002 (v:v) methanol: water: glacial acetic acid. Each measurement was repeated about 10 times to decrease errors. Eluted sterols were quantified on a Micromass Quatro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshaw, UK) by multiple reaction monitoring (MRM) scans using the following transitions: 361.20 to 163.00 for cortisone; 363.20 to 121.00 for cortisol; 364.20 to 163.00 for d3cortisone; 366.20 to 121.00 for d3 cortisol. The mass spectrometry measurements were performed by a post-graduate scientist. A software programme (Mass Lynx) was used to integrate areas for each eluted sterol, results were manually checked and the mean of 6 replicate measurements

was used for each data point. Where one or more sample showed a significant deviation (>20%) from the other readings this sample was omitted from the calculation of the mean and a different reading was used. Clearance of cortisol was calculated from the cortisol measurements and spiked sample measurements using a two pool method as described by Tait [205].

3.11 Physical activity and fitness

3.11.1 Physical activity

3.11.1.1 Physical activity energy expenditure (PAEE)

PAEE was assessed with heart rate monitoring using the HRFlex method. The HRFlex method uses an individual calibration of the relationship between energy expenditure and heart rate on exertion together with measurement of resting energy expenditure and estimation of heart rate at which the linear assumption does not hold (at low energy expenditure) the 'FLEX' point. This FLEX heart rate is taken empirically as the mean of the highest pulse rate at rest and lowest during exercise. The HRFlex method has been compared in validation studies with the 'gold standard' techniques of doubly labelled water (for references see Wareham et al 1997 [206]) which suggest that the technique provides an accurate estimate of energy expenditure with a mean error for estimating total energy expenditure of as little as 0.6%).

Volunteers were asked to wear a heart rate (HR) monitor (Polar instruments) for all waking hours during three typical days, usually 2 weekdays and a weekend day. Volunteers were shown how to fit the chest electrodes and transmitter with a recording device worn on the wrist. They were asked to wear the monitor from after washing in the morning until they went to bed at night. The monitor recorded heart rate every 15 seconds and the data were downloaded via infrared signal to a computer with Polar software installed. The readings were analysed after a manual check on each file, and excluding all HR readings of zero beats per minute and greater than 200 beats per minute. Physical activity energy expenditure (PAEE) over three days was calculated using the individual

relationship between HR and average VO_2 for each workload, then using this relationship to calculate average daily PAEE for all free-living HR readings greater than the lowest HR during light exercise and averaged over the three day period. Data on free-living PAEE are expressed in relation to body weight (i.e. kcal/kg/day). PAEE was calculated using locally developed software and methods by collaborators in Cambridge.

3.11.1.2 Physical activity questionnaires

Subjects completed a well-validated questionnaire to assess physical activity, the Baecke questionnaire of habitual activity [193,207] which has a total of 16 questions in three categories (work, sport and leisure), and responses are graded 1-5. An average score is then calculated for each of three categories, to give a work index, sports index, and non-sports leisure index so that a score close to zero indicates a low level of activity and a score close to 5 indicates a high level of activity. A total score out of 15 is determined by adding together the three scores.

3.11.2 Cardiorespiratory fitness

3.11.2.1 Treadmill assessment of VO_2 max

All tests were performed fasting after subjects had avoided strenuous exercise for 24 hours. Subjects were asked to avoid alcohol on the day prior to testing. On arrival in the CRF volunteers were weighed and then asked to rest quietly for 30 minutes prior to testing. Subjects were then fitted with the air-tight facemask for gas analysis, Polar heart rate monitor and ECG leads. Resting measurements were taken for 20 minutes prior to commencing measurement to allow subjects to become acclimatised to the facemask and so that resting metabolic rate could be determined. Breath-by-breath analysis of oxygen consumption and CO_2 production was made using a Metalyser instrument which was calibrated daily using gases of known composition prior to testing.

Subjects were asked to perform an incremental treadmill test commencing at 1.3 m/s (3 miles/hour) at a gradient of 0% and increased by either 2% gradient or 0.25m/s (0.5miles/hour) every 2 minutes (i.e. stage 1: 1.3m/s, 0% gradient, stage

2: 1.3m/s, 2% gradient, stage 3:1.55 m/s, 2% gradient) (Woodmill treadmill)[178]. ECG was recorded throughout and blood pressure was measured intermittently. Subjects were asked to continue to exhaustion unless they experienced chest pain or felt unwell, and were encouraged to continue until the respiratory quotient was >1.1 and they had reached 90% of their target heart rate (as determined by 220-age).

Three subjects who were unable to run on the treadmill performed a cycle ergometer test. VO_2 max was determined using an incremental workload test using an electromagnetic brake ergometer (SECA 545, Volga & Holke, Hamburg, Germany). Subjects began pedalling at a comfortable rate at a workload of 20 watts. They were asked to continue cycling at the same rate and workload was increased by 20 watts every two minutes until they had exercised for ten minutes. The workload was then increased by 20 watts every one minute to ensure cardiovascular exhaustion was achieved before muscle fatigue.

4 BODY COMPOSITION – COMPARISON OF METHODS AND ASSOCIATION WITH METABOLIC SYNDROME FEATURES

4.1 Introduction

Obesity prevalence is increasing and with it the number of people affected by type 2 diabetes and the other metabolic consequences of obesity, namely hypertension and dyslipidaemia, which together make up the metabolic syndrome [3,39,113,136]. There has been much debate over the importance of total fat, truncal fat and visceral fat in the pathogenesis of the metabolic syndrome and consequently extensive discussion of the best methods to use in assessing obesity for screening purposes, for population studies and for clinical studies [5,53]. The aim of this study was to determine how BMI and waist circumference compare with more technical measures of body composition for association with insulin resistance and other cardiovascular risk factors.

Because of evidence that abdominal visceral fat deposition is associated with increased risk of the metabolic consequences of obesity [208] it has been suggested that BMI, which does not identify abdominal fat, is left aside for identifying these individuals for lipid-lowering and blood pressure intervention [6] and that more technical methods which do provide an estimate of visceral fat such as DEXA may be better [73], or at least that waist circumference is used instead [5] However there are two arguments against this approach. Firstly it is not universally accepted that visceral fat is causative in the metabolic syndrome, or that subcutaneous fat may not also be pathological [47 748,209]. Secondly, as identifying these individuals is a two-step process, where the second step is simple blood pressure and blood lipid and glucose measurement, there is an argument for keeping the first step as simple as possible.

BMI measurement does not provide information about body composition but is very easily measured in a clinical setting and requires a minimum of equipment

and training. It is closely correlated with other measures of body composition and as such is a useful screening tool [54]. It has been widely used in population studies and predicts the development of diabetes [4,55,56], ischaemic heart disease [57,58] and premature death [59]. Waist circumference measurement can be used instead of BMI to identify obese individuals [5,8] and has the advantage of combining a measure of overall fatness with an assessment of regional adiposity but is more difficult to reproduce and requires training in a standardised technique [5]. Waist circumference is particularly important in identifying a small sub-group of people who have central obesity despite a BMI in the normal range [53] and also to exclude the even smaller subgroup of individuals who have a high BMI because of increased muscle mass through intensive physical training.

In a group of healthy middle-aged men with a wide range of body fat quantity we have assessed body composition using BMI, waist and hip circumference, DEXA, bioimpedance, Bod Pod plethysmography and skin fold measurement and in obese subjects we have also measured visceral fat using 5-slice MRI to evaluate how BMI compares with these more technical measurements. We have measured metabolic syndrome features and tissue insulin sensitivity using a euglycaemic hyperinsulinaemic clamp technique with deuterated glucose and compared the methods to determine which measure of fat correlates best with metabolic syndrome features and insulin sensitivity.

4.2 Methods

Thirty-three male subjects aged 42-64 were recruited from general practice and advertisement as previously described. They were recruited to have a wide range of body mass index and subjects on treatment for known cardiovascular risk factors were specifically excluded from this study to avoid potential confounding by lipid, glucose or blood-pressure lowering agents. For this reason our obese subjects might have been uncharacteristically healthy compared with other obese subjects of similar age. During the recruitment process many volunteers were rejected from the study on the grounds of known medical complications of obesity (see Methods chapter).

Subjects were asked to attend the CRF at 9am having fasted from 10pm the previous night. They were asked to avoid strenuous exercise or alcohol on the days prior to attending. Subjects had anthropometry measurements as described in the methods section and 29 subjects attended on a second occasion for the euglycaemic hyperinsulinaemic clamp as previously described. Subjects with BMI greater than 30kg/m^2 had visceral and subcutaneous fat measured by 5-slice abdominal MRI.

All statistical analyses were undertaken using SPSS for Windows version 14.0. For each method the mean, range and standard deviation were calculated. The methods were compared by plotting the mean of each pair of values on the x-axis against the difference between each pair on the y-axis, to provide a Bland-Altman plot of the agreement between the measurements. Perfect agreement would be represented by a scatter plot on a line parallel to the x-axis.

The different measurements were then plotted against BMI, as BMI is usually used to define obesity, and the percentage fat for each measurement method was compared to a BMI of 30kg/m^2.

Pearson Correlation was used to compare each method with fasting plasma glucose, 2-hour plasma glucose after 75g OGTT, systolic and diastolic blood pressure, fasting plasma triglycerides and HDL cholesterol. If distribution of a variable was not normal the variable was normalised using logarithmic transformation prior to comparison. Where measurements were made in different units standardised z-scores were used for comparison.

4.3 Results

Thirty-three men were studied: their characteristics are shown in table 4.1. None were known to have diabetes or to be on treatment for blood pressure or dyslipidaemia. Two men had 2-hour glucose levels just above the cut-off for diabetes but were asymptomatic; four further individuals had impaired glucose tolerance and one subject had impaired fasting glucose.

The six methods used to determine total body fat were BMI, waist circumference, DEXA, bioimpedance, Bod Pod plethysmography and skin fold measurement. The mean, range and standard deviation for DEXA, bioimpedance, Bod Pod and skin fold measurement can be expressed as fat percentage and are compared in table 4.2. It can be seen that there is close agreement between the results obtained from DEXA and bioimpedance (mean 28.1, range 12.0-39.8 and mean 28.1, range 13.6-45.7 respectively), and from the Bland-Altman plots seen in figure 4.2 it can be seen that there is reasonable agreement between the individual measurements represented by relatively little scatter about the line and narrow 95% confidence intervals. For the calculated total fat using the Bod Pod and skin fold measurement the mean values are higher, (mean 32.0 range 4.5-45.3 and mean 32.2, range 17.1-41.4% respectively) and in the case of the Bod Pod the range of measurements is large. In particular there are some individuals with very low measurements for body fat as seen in the plot of BMI against Bod Pod measurement (figure 4.3). It can be seen from the plots of Bod Pod against DEXA and bioimpedance (figure 4.2) that there is a strong negative relationship between these values indicating that as the values get larger the differences between them get smaller suggesting that the Bod Pod gives lower readings than other measurements at low values.

The relationships between BMI and the other measures of fatness are presented in figure 4.3 and table 4.3. It can be seen that the strongest relationship is between BMI and waist circumference, although when total fat on DEXA is used rather than percentage this relationship is slightly stronger than that with waist circumference. The relationships between BMI and Bod Pod and skin fold measured fat are not linear as seen in figure 4.4. The close relationship between BMI and waist is shown in the scatter plot (figure 4.5) and in the Bland-Altman plot of standardised (z score) BMI and waist mean and difference (figure 4.6). A Bland-Altman plot is also shown for standardised BMI against total fat on DEXA (figure 4.7).

Pearson correlation co-efficients were calculated for features of the metabolic syndrome with the measurements of total fatness (table 4.4). No one measurement correlated better than the others with metabolic syndrome features. The strongest correlation with fasting glucose was with BMI ($r=0.588$, $p<0.001$), the strongest relationship with HDL cholesterol was with waist circumference ($r=-0.541$, $p=0.001$) and the strongest relationship with plasma triglycerides was with DEXA fat percentage ($r=0.531$, $p=0.002$), the strongest relationship with systolic blood pressure was with the bioimpedance measurement ($r=0.449$, $p=0.009$) and with diastolic blood pressure was with Bod Pod calculated fat ($r=0.508$, $p=0.005$). BMI correlated well with metabolic syndrome features although the relationship with BP did not reach statistical significance.

The methods used to determine regional fat were waist and hip circumference, truncal fat measured on DEXA and visceral and subcutaneous trunk fat measured by MRI. Cross-sectional MRI imaging was performed only on the individuals with BMI greater than 30kg/m^2. Truncal fat on DEXA correlates strongly with the measurements of total fat on DEXA, BMI and waist circumference as seen in figure 4.5. Both visceral and subcutaneous fat correlated strongly with truncal fat on DEXA and the measures of total fat including BMI and waist (Table 4.6).

Truncal fat and visceral fat correlated strongly with features of the metabolic syndrome (Table 4.7), but interestingly subcutaneous fat correlated equally strongly, and none of these measures showed a better overall correlation co-efficient than the measures of total fatness. The only exception being the negative relationship between HDL cholesterol and visceral fat, which was slightly stronger than the relationship with total fatness ($r=-0.629$, $p=0.003$ compared with $r=-0.550$, $p=0.001$ for waist circumference). Waist-hip ratio showed relatively poor correlation with the metabolic syndrome features.

We compared the relationships between the different measurements of fatness and tissue insulin sensitivity in muscle liver and fat (table 4.8) and there was minimal difference between the correlation between insulin sensitivity and BMI and the correlation with trunk or total fat on DEXA.

Table 4-1 Subject characteristics

	Mean	SD	Range
Age (years)	53	5.8	42-64
BMI (kg/m^2)	31.5	6.5	21-49
Waist circumference (cm)	108.6	17.1	80-150
DEXA fat (%)	28.1	6.9	12-40
Visceral fat MRI (kg)	6.1	1.4	3.9-9.7
Systolic BP (mmHg)	132	15	102-188
Diastolic BP (mmHg)	77	9	58-103
Fasting glucose (mmol/l)	5.4	0.6	4.5-7.0
HDL cholesterol (mmol/l)	1.4	0.4	0.8-2.6
Triglycerides (mmol/l)	1.7	1	0.7-4.9
Glucose disposal (mg/kg/min)	6.7	3.3	2.2-13.9
RD mean steady state (umol/kg/min)	37.6	16.3	12.4-73.3
Suppression hepatic glucose output by 60 minutes insulin (%)	67.9	24.1	21-117
NEFA percentage suppression by 60 minutes (%)	58.3	20.2	14.7-88.3

Figure 4-1: Distribution of BMI amongst the subjects

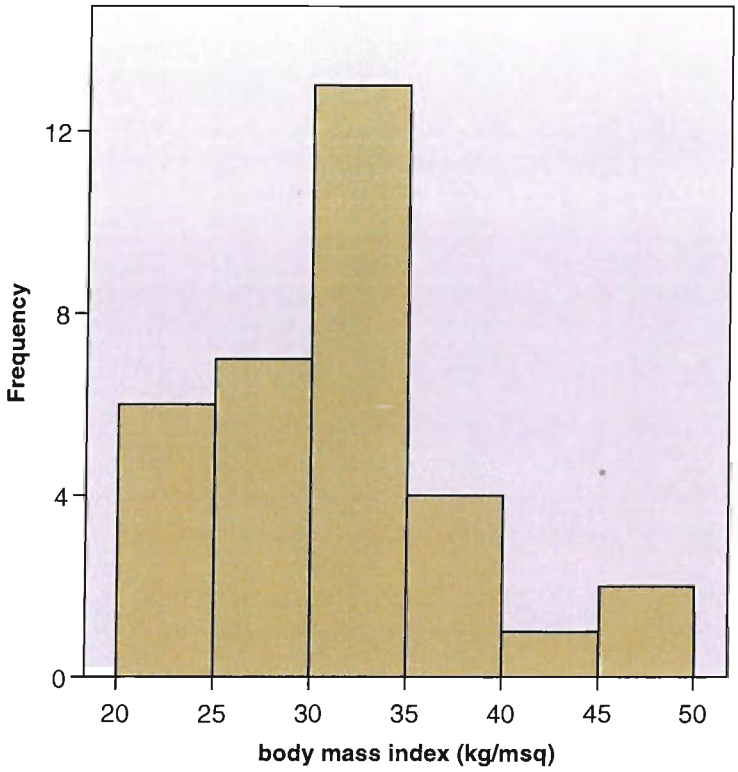


Table 4-2 Comparison of fatness measured by DEXA, bioimpedance, Bod Pod and skin fold thickness

	DEXA (%) (n=32)	Bioimpedance (%)	Bod Pod (%) (n=29)	Skin fold calculated fat (%)
Mean	28.1	28.1	32.0	32.2
Median	29.0	27.8	35.0	33.5
Std. Deviation	6.9	7.6	10.8	5.6
Range	12.0-39.8	13.6-45.7	4.5-45.3	17.8-41.4

Figure 4-2 Bland-Altman plots comparing percentage fat measured by DEXA, Bod Pod, bioimpedance and skin fold measurement

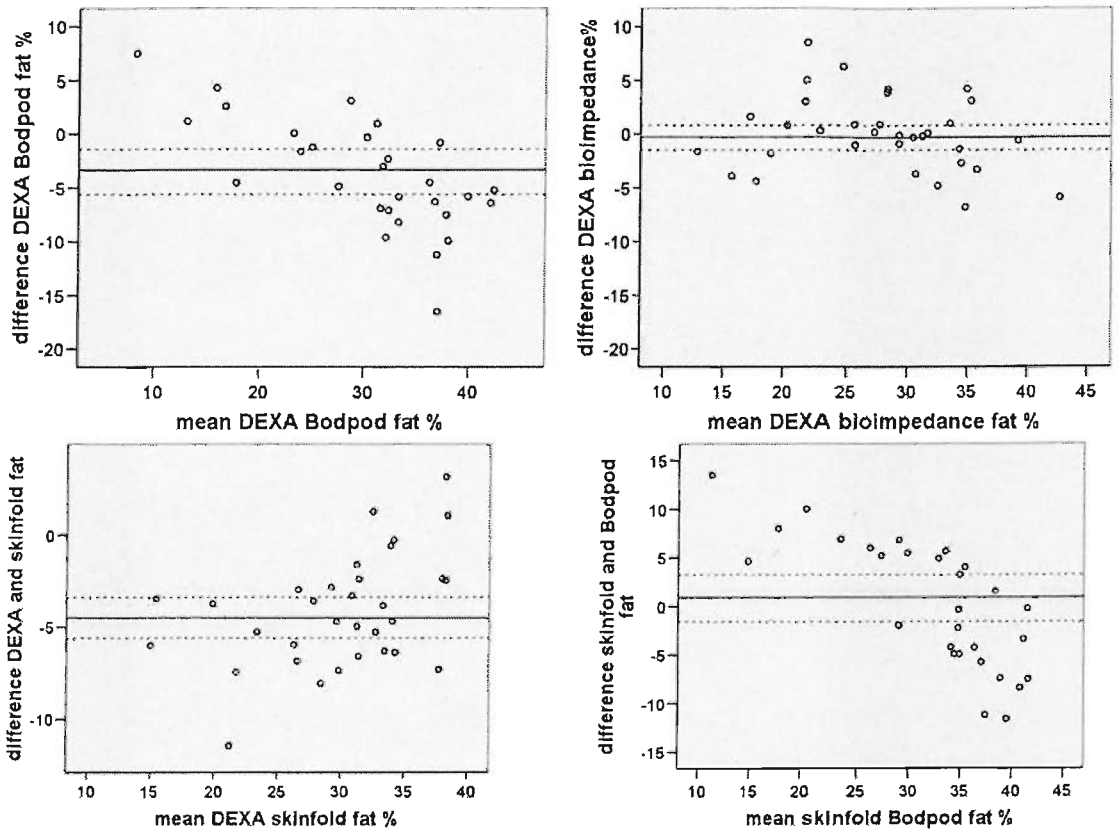


Table 4-3 Correlation between BMI and fat measured by bioimpedance, Bod Pod, DEXA, skin fold thickness and waist circumference

	Bio-impedance	Bod Pod	DEXA % fat	Total fat on DEXA	Skin fold calculated fat	Waist circum-ference
Correlation	0.929	0.865	0.881	0.973	0.760	0.969
Significance	0.000	0.000	0.000	0.000	0.000	0.000

Table 4-4 Correlation between measures of body fat and features of the metabolic syndrome

		Systolic BP	Diastolic BP	Fasting plasma glucose	HDL cholesterol	Triglycerides
Body mass index (n=33)	Correlation	0.327	0.316	0.588	-0.481	0.464
	Sig	0.063	0.074	0.000	0.005	0.007
Bioimpedance fat (=33)	Correlation	0.449	0.374	0.509	-0.449	0.455
	Sig	0.009	0.032	0.002	0.009	0.008
Bod Pod fat (n=29)	Correlation	0.355	0.508	0.524	-0.467	0.391
	Sig	0.059	0.005	0.004	0.011	0.036
DEXA fat (%) (n=32)	Correlation	0.368	0.438	0.578	-0.487	0.531
	Sig	0.038	0.012	0.001	0.005	0.002
Skin fold calculated fat (n=33)	Correlation	0.315	0.461	0.420	-0.378	0.406
	Sig	0.075	0.007	0.015	0.030	0.019
Waist circumference (n=33)	Correlation	0.433	0.371	0.572	-0.541	0.517
	Sig	0.012	0.034	0.001	0.001	0.002
Waist hip ratio (n=33)	Correlation	0.330	0.217	0.298	-0.451	0.355
	Sig.	0.060	0.225	0.093	0.008	0.042

Table 4-5 Correlation between visceral and subcutaneous fat on MRI and trunk and total fat on DEXA, BMI and waist circumference

		Trunk fat on DEXA	Waist circumference	Total fat measured on DEXA	Body mass index
Visceral fat MRI (n=19)	Pearson Correlation	0.718	0.757	0.657	0.670
	Sig	0.001	0.000	0.002	0.001
Subcutaneous fat MRI (n=19)	Pearson Correlation	0.656	0.810	0.743	0.799
	Sig	0.002	0.000	0.000	0.000

Figure 4-3 Scatter plots of percentage fat measured by DEXA, Bod Pod, bioimpedance and skin fold measurement against BMI

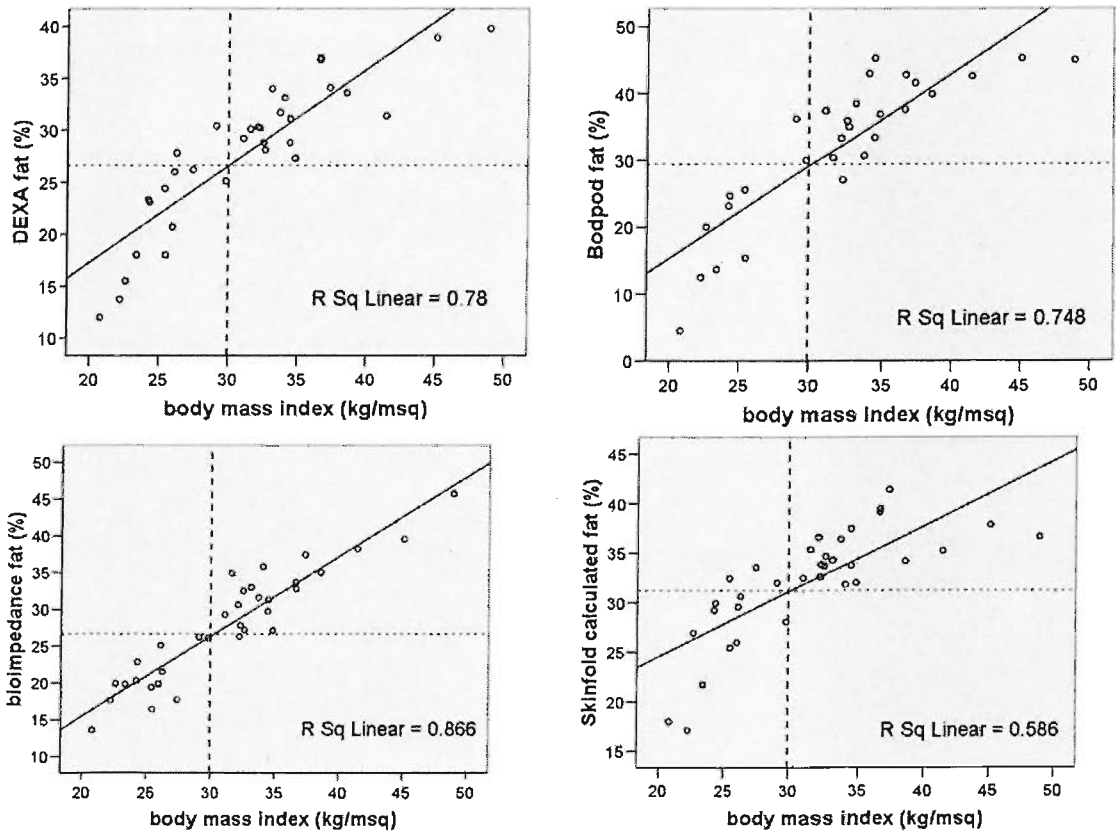


Figure 4-4 Relationships between DEXA, Bod Pod, bioimpedance and skin fold measured percentage total body fat and BMI

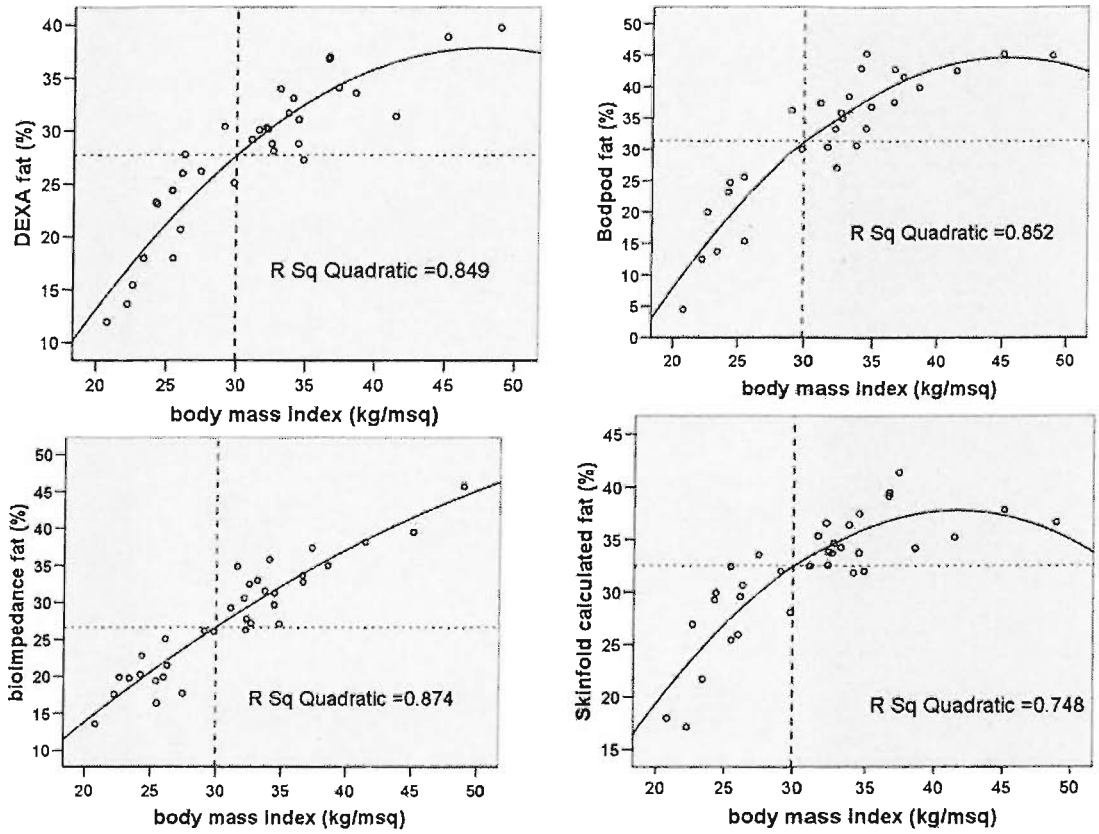


Figure 4-5 Scatter plot of waist circumference against BMI

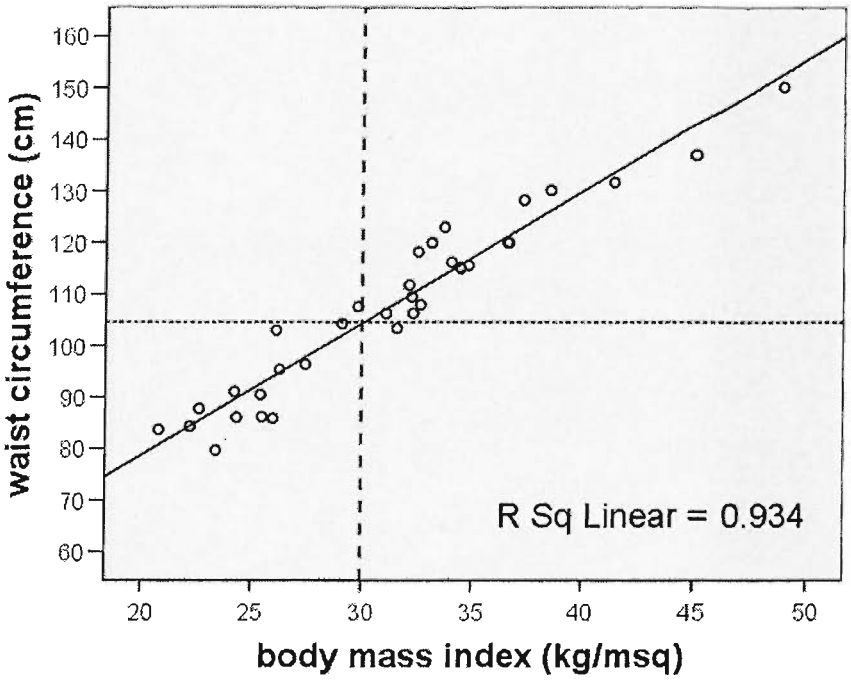


Figure 4-6 Bland Altman plot standardised BMI and waist

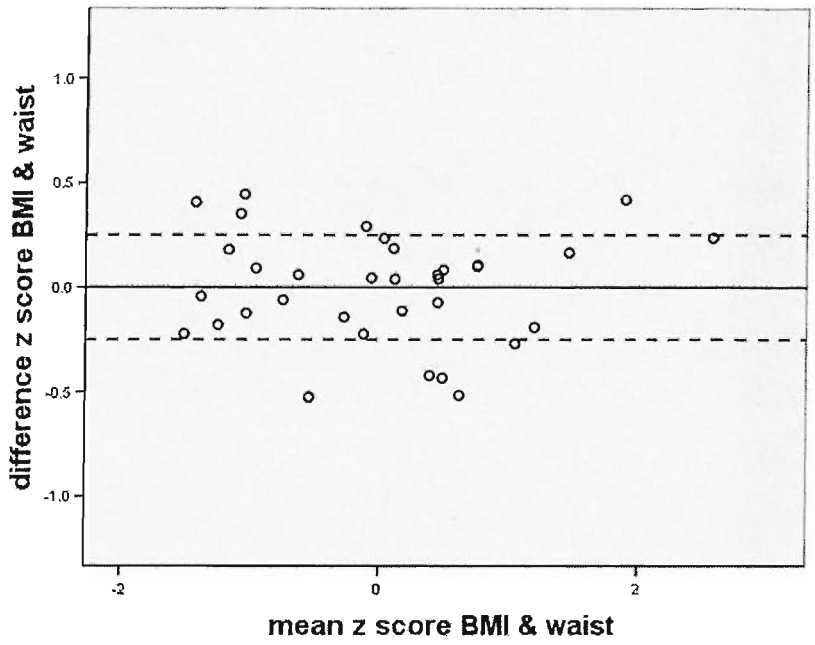


Figure 4-7 Bland Altman plot standardised BMI against total fat on DEXA

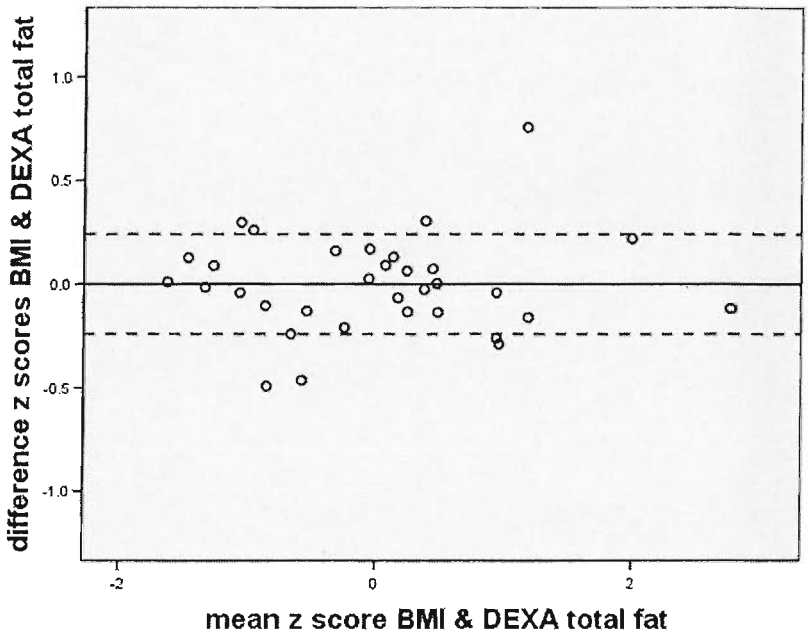
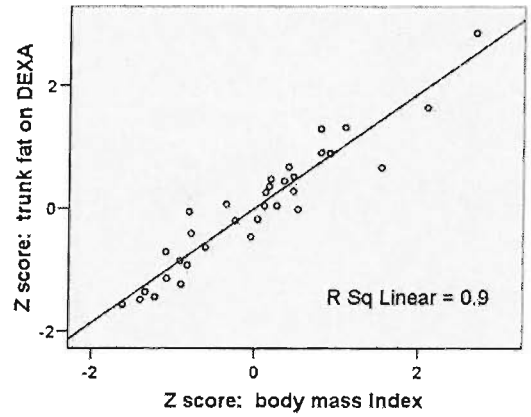
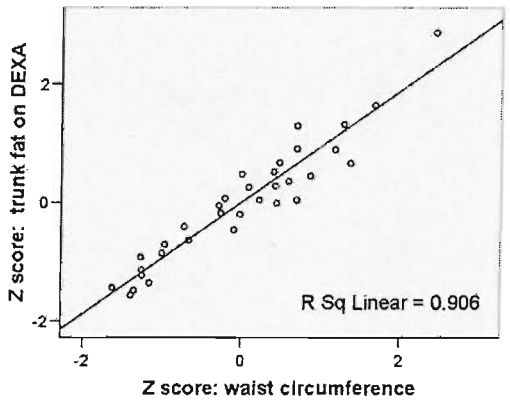


Figure 4-8 Scatter plot of standardised trunk fat on DEXA against standardised total fat on DEXA, BMI and waist circumference





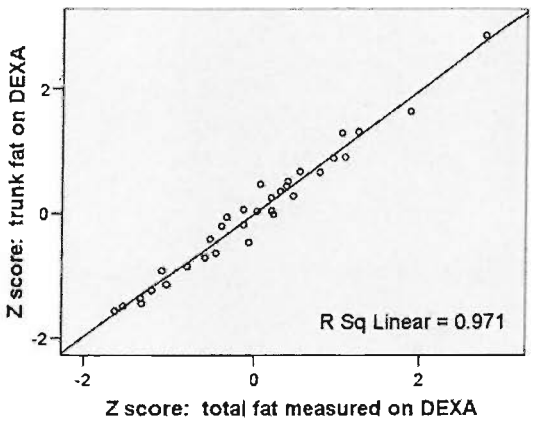


Table 4-6 Pearson Correlation coefficients between visceral and subcutaneous fat and trunk fat on DEXA, waist circumference, total fat on DEXA and BMI

		Trunk fat on DEXA	Waist circumference	Total fat measured on DEXA	Body mass index
Visceral fat MRI (n=19)	Pearson Correlation	0.718	0.757	0.657	0.670
	Sig	0.001	0.000	0.002	0.001
Subcutaneous fat MRI (n=19)	Pearson Correlation	0.656	0.810	0.743	0.799
	Sig	0.002	0.000	0.000	0.000

Table 4-7 Correlation between measurements of trunk fat and metabolic syndrome features

		Systolic BP	Diastolic BP	Fasting plasma glucose	HDL cholesterol	Triglycerides
Trunk fat on DEXA n=33	Pearson Correlation	0.400	0.347	0.629	-0.525	0.577
	Sig	0.023	0.051	0.000	0.002	0.001
Visceral fat MRI n=20	Pearson Correlation	0.405	-0.042	0.343	-0.629	0.521
	Sig	0.077	0.860	0.139	0.003	0.018
Subcutaneous fat MRI n=20	Pearson Correlation	0.213	-0.072	0.133	-0.521	0.211
	Sig	0.366	0.763	0.576	0.018	0.371
Waist circumference n=33	Pearson Correlation	0.433	0.371	0.572	-0.541	0.517
	Sig	0.012	0.034	0.001	0.001	0.002
Waist/hip ratio n=33	Pearson Correlation	0.330	0.217	0.298	-0.451	0.355
	Sig	0.060	0.225	0.093	0.008	0.042

Table 4-8 Correlation between BMI, waist, total and trunk fat on DEXA and whole body insulin sensitivity, and insulin sensitivity in muscle, adipose tissue and liver

		M value (n=29)	RD steady state (n=29)	NEFA percentage suppression by 60 minutes (n=33)	Suppression hepatic glucose output by 60 minutes insulin (n=29)
Body mass index	Pearson Correlation	-0.747	-0.765	-0.621	-0.473
	Sig	0.000	0.000	0.000	0.010
Waist circumference	Pearson Correlation	-0.776	-0.795	-0.623	-0.564
	Sig.	0.000	0.000	0.000	0.001
Total fat measured on DEXA	Pearson Correlation	-0.797	-0.813	-0.671	-0.525
	Sig.	0.000	0.000	0.000	0.003
Trunk fat on DEXA	Pearson Correlation	-0.811	-0.817	-0.656	-0.544
	Sig.	0.000	0.000	0.000	0.002
Visceral fat on MRI (n=19)	Pearson Correlation	-0.411	-0.352	-0.316	-0.349
	Sig.	0.080	0.140	0.175	0.142

4.4 Discussion

We found that in men the simple methods of measuring fatness, namely BMI and waist, correlate equally strongly with plasma glucose, triglycerides and HDL cholesterol, and in the case of waist circumference, blood pressure; compared with more technical methods, namely DEXA, skin fold measurement, Bod Pod and bioimpedance. We also found that there was considerable variation in body fat measured by these different methods.

We found a close inverse correlation between BMI and waist and insulin sensitivity in the individual tissues. We found that truncal and visceral fat measured by DEXA and MRI were slightly more strongly associated with metabolic syndrome features than BMI but waist circumference correlated equally strongly as DEXA and MRI. We found a very strong correlation between BMI and waist circumference. Both BMI and waist were more strongly associated than visceral fat with insulin sensitivity in the individual tissues, although this finding is treated with caution because only 20 obese subjects had MRI scans.

Since the recognition of obesity as an increasing health issue there has been much emphasis in the literature on how to identify individuals as risk, and the importance of visceral fat [6 5]. Although it has been well described that obese women with the typical female pattern of peripheral fat accumulation are relatively insulin sensitive compared to those with central fat accumulation [42], there is little evidence that the same association occurs in men [51]. There has long been discussion in the literature about the importance of identifying 'metabolically normal' obese individuals, but these individuals are in fact predominantly female [81,135].

There is some evidence that central fat accumulation is a risk factor beyond increased BMI for the development of diabetes in men [43] and there is also evidence that visceral fat is metabolically different to subcutaneous fat and may be pathogenic in obese individuals by releasing non-esterified fatty acids directly

into the portal circulation [44-46,50]. However Frayn has argued that the evidence for a causal link between visceral fat and the features of the metabolic syndrome is weak, pointing out that if excess fatty acid release from visceral fat is responsible for the other features this should result in disappearance of the visceral fat mass unless there is also increased fat deposition which has not been demonstrated [47]. He suggests that other mechanisms should be considered such as the importance of the subcutaneous fat mass which outweighs the visceral fat mass several-fold. This would be consistent with our finding of the importance of total fat mass in men. Although waist circumference is better than BMI in predicting visceral fat [7,210], BMI has been demonstrated by others to be better than waist circumference at predicting non-abdominal fat [9].

Even accepting that visceral fat is more metabolically active than subcutaneous fat [208],and in keeping with our findings, the literature lacks convincing evidence that in Caucasian men visceral adiposity exists in the absence of a raised BMI or similarly evidence for the existence of obese individuals without significant quantities of visceral fat. It has been argued that waist measurement provides further information within BMI categories for determining cardiovascular risk factors [8], but the numbers of subjects with raised BMI but low waist circumference, and conversely low BMI but high waist circumference are very small [8]. Others have also found that in men there is a strong correlation between BMI and other measures of fatness. [7,210].

In a recent meta-analysis of 40 studies, Romero-Corral and colleagues found an increased mortality risk for subjects with normal BMI compared with overweight individuals [211]. However, in addition to the problems inherent to meta-analysis, such as lack of consistency between studies, this was a study of subjects with known cardiovascular disease and the subjects with low and normal BMI were more likely to be smokers and were significantly older than those with higher body mass. Recent findings from the Interheart Study have re-emphasised the importance of tobacco use as risk of myocardial infarction [212], suggesting that the link between tobacco use and heart disease is even stronger than previously

thought. However perhaps more importantly Romero-Corral's study is an analysis of secondary prevention, and patients with existing cardiovascular disease were recruited after myocardial infarction, coronary artery bypass grafting or percutaneous coronary intervention, and having a lower BMI could indicate more severe cardiovascular disease or other pathology. In an accompanying editorial Franzoni suggests that 'BMI is laid aside as a clinical and epidemiological measure of cardiovascular risk for both primary and secondary prevention' [6], however this conclusion should not be based on a study of secondary prevention and our findings indicate that valuable information can be obtained from BMI in men.

Laying aside BMI is not without resource implications. Patients can measure and calculate their own BMI fairly accurately without need for training or specialised equipment, whereas even waist measurement requires training, and there exists widespread familiarity with both measurement and interpretation in primary care. Men with a high BMI but low waist circumference are rare compared with women in whom there is much more variation in body composition.

The relationship between BMI and blood pressure did not reach statistical significance although this could be a type 2 statistical error because of the small numbers in our study. This was a small study and as such there is a risk that weaker correlations will be missed. For these associations larger epidemiological studies are better and using mathematical modelling it has been shown that the associations with blood pressure show the weakest association with the other features of the metabolic syndrome [41]. Because blood pressure would usually be measured at the time BMI is measured in a clinical setting it is not so important for clinical practice that the measure of fatness correlates well with blood pressure.

A criticism of this study is that only individuals without known cardiovascular risk factors were recruited. Individuals with a wide range of BMI were sought and during recruitment a number of obese subjects with diabetes and on treatment

for hypertension and dyslipidaemia were excluded. Although this meant that the obese subjects might have been exceptionally healthy, the fact that there were still strong correlations between BMI and cardiovascular risk factors suggests that in the general population this finding would be stronger. A further criticism is that this study did not include women or non-Caucasians and further research is needed to determine how useful BMI is in women and other ethnic groups as our findings cannot be extrapolated to other populations.

In summary we have found very close correlation between BMI and other measures of fatness, but more importantly shown that the correlation between BMI and metabolic syndrome features is as strong as fat measurement by DEXA, Bod Pod, bioimpedance, skin-fold measurement in men. We found minimal evidence that more technical methods of measuring fatness would provide a better measure of cardiovascular risk factors. Waist circumference correlates slightly more strongly with metabolic syndrome features than BMI but because blood pressure measurement and plasma glucose and lipid profiles are easily available, BMI remains useful for screening for cardiovascular risk in men.

5 THE ASSOCIATION BETWEEN FATTY LIVER AND INSULIN RESISTANCE IN OBESE SUBJECTS

5.1 Introduction

Non-alcoholic fatty liver disease (NAFLD) is a newly described feature of the metabolic syndrome [213] [13], but it is not yet clear whether it relates predominantly to obesity, or is a part of the picture of insulin resistance. NAFLD forms a spectrum of disease from simple steatosis, or fatty infiltration through non-alcoholic steatohepatitis (NASH) to cirrhosis and is now recognised as the cause of many previous cases of 'idiopathic' cirrhosis [155]. The underlying cause of NASH is uncertain but it is strongly associated with obesity and type 2 diabetes [14,16,17]. The aim of this study was to determine the relationship between fatty liver, tissue insulin resistance and quantity and location of adipose tissue with a view to elucidating mechanisms of pathogenesis.

The worldwide prevalence of NAFLD is likely to increase because the global prediction for 2030 suggests a profound rise in numbers of individuals with diabetes [3] and NAFLD is already thought to be the most common of all liver disorders in developed nations. This has important health and resource implications for these countries [214] because up to 50% of subjects develop liver fibrosis, 15-17% develop cirrhosis and 3% develop end-stage liver failure requiring transplantation [17,18,215]. Establishing whether insulin resistance or obesity is the more important factor for the development of fatty liver is difficult because of the strong co-linear association between obesity and insulin resistance [14,18-20], however this is an important question to answer because of identifying individuals at risk of developing liver disease and to determine which therapeutic interventions may prevent progression.

Recent literature has suggested that insulin resistance may be a more important factor than obesity for the development of NAFLD [13,19,21]. In healthy subjects

Johanson et al [21] demonstrated that fatty liver was associated with insulin sensitivity (measured during a euglycaemic hyperinsulinaemic clamp) independently of total or visceral fat on CT. A further question is whether insulin resistance in a specific tissue is specifically associated with the development of fatty liver. Kelley et al [19] compared insulin sensitivity in different tissues in diabetic subjects with and without fatty liver on CT with lean and obese subjects and in this study subjects with fatty liver were considerably more insulin resistant in muscle and fat but not liver.

To our knowledge there has been no single study comparing insulin sensitivity in skeletal muscle, liver and adipose tissue in healthy obese subjects with and without fatty liver and there is no study that has in parallel undertaken precise measurements of quantity and location of body fat. Again this question is important to answer because different pharmaceutical agents affect insulin sensitivity in different tissues, and so if insulin resistance in a specific location may contribute to the development of fatty liver disease then improving insulin sensitivity in that tissue may also improve fatty liver disease.

Insulin sensitivity in skeletal muscle is assessed during the euglycaemic hyperinsulinaemic clamp [10,116], and hepatic insulin action can be measured by insulin-induced suppression of hepatic glucose output [13,19,122,216]. In adipose tissue, insulin-mediated suppression of lipolysis is often used as a measure of insulin action [13,19,106,217]. Impaired suppression of non-esterified fatty acids (NEFAs) to an oral glucose load is also a marker of insulin resistance in adipocytes [107,124,141,218-221]. Evaluation of insulin sensitivity in adipose tissue using suppression of NEFAs in subjects with fatty liver [13,152,222] has been undertaken but no consensus has been reached regarding insulin sensitivity in fat . Although there are correlations between measures of insulin sensitivity in skeletal muscle, liver and adipose tissue [223] correlations between these measures are relatively weak. Conclusions about insulin action in one tissue should not be extrapolated from data obtained for another organ and direct measurement of insulin action in each tissue is the ideal.

Ultrasonography has been shown to identify steatosis with a sensitivity of 94% and specificity of 88% when compared to histological findings [144]. Other imaging methods such as computer tomography (CT) and magnetic resonance imaging (MRI) have also been used [145-147] but have not been shown to be superior to ultrasonography [148]. MRI spectroscopy is a new method which allows quantification of hepatic fat content and therefore enables correlations to be made with associated features [150-152], however this technique is expensive and invasive and was not available for this study. We therefore used ultrasonography rather than MRI spectroscopy to identify subjects with and without fatty liver as this method is widely accepted for this purpose [148,149].

The aim of our study was to measure insulin sensitivity in muscle, liver and fat in obese men with and without fatty liver, in whom body composition has been precisely determined. We have used a euglycaemic clamp technique with deuterated glucose tracer, and suppression of NEFAs during an oral glucose tolerance test to determine insulin sensitivity in muscle, liver and adipose tissue and we have used anthropometry, DEXA, bioelectrical impedance, air displacement plethysmography and horizontal 5-slice cross-sectional MRI to evaluate in detail total body fat, truncal fat and visceral fat. We have also studied a group of lean subjects without fatty liver for further comparison as additional controls.

5.2 Methods

Subjects with BMI greater that 30kg/m^2 were categorised as those with and without fatty liver on ultrasound, and were compared with lean men (BMI ≤ 25kg/m^2), none of whom had fatty liver.

Anthropometry was performed and MRI scanning of obese subjects as described in the methods chapter. Liver ultrasonography was performed as described previously: although we had a radiologist-assigned score for steatosis from mild through moderate to severe, for this analysis subjects were divided into those

with or without any evidence of steatosis, particularly as only one subject had been assigned a score of 'mild'.

Insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp technique with deuterated glucose infusion to determine hepatic insulin sensitivity determined by insulin-mediated suppression of endogenous glucose production [10,11]. One obese subject was unable to attend for this study.

For measurement of insulin sensitivity in adipose tissue, NEFAs were measured for each time point during the OGTT. After reviewing the plots of NEFA levels against time, NEFA suppression was measured as determined by the area under the NEFA curve from 30 minutes to 120 minutes and by percentage NEFA suppression from baseline during the OGTT.

All statistical analyses were performed using SPSS for Windows version 14.0. Comparison of data from the two obese groups was undertaken using Student's t-test. Where variables were not normally distributed log transformation was undertaken to normalise the distribution. Analysis of variance was used for comparison of normally distributed data from the three groups where lean controls were included in the analysis. Pearson correlation coefficients are presented for univariate regression analyses of normally distributed data. A p-value of <0.05 was considered to be statistically significant.

5.3 Results

22 obese men (mean BMI 35.1 ± 4.7 kg/m^2) and 8 lean men (mean BMI 23.6 ± 1.6 kg/m^2) were studied (table 5.1). Of the obese subjects, 15 (68%) had fatty liver on ultrasonography. Although steatosis was graded as mild, moderate or severe subjects were categorised as either having fatty liver (defined as any level of hepatic steatosis) or not having fatty liver. None of the lean controls had fatty liver. There was no difference in reported alcohol consumption between obese with fatty liver and obese subjects without fatty liver (11.8 v 14.1 units per week p=0.76, Table 5.1).

GGT was increased in the obese subjects with fatty liver compared to obese subjects without fatty liver (57.5 v 30.4 mmol/l p=0.02, Table 5.1). ALT was also higher in the subjects with fatty liver but the difference did not reach statistical significance (37.1 v 28.3 mmol/l p=0.13, Table 5.1). There was no significant difference between obese subjects with and without fatty liver in any of the other features of the metabolic syndrome (table 5.1). Lean controls had significantly lower diastolic blood pressure (p=0.006) and fasting plasma triglyceride (p=0.024), and higher fasting HDL cholesterol concentrations (p=0.026) than obese subjects (table 5.1).

Obese subjects with fatty liver had slightly more total body fat than obese subjects without fatty liver (33.2kg v 29.3kg, p=0.027, table 5.2). There was close correlation between all four measures used to determine percentage body fat (Pearson correlation coefficients from 0.77 to 0.93 $p<0.001$). Importantly, the obese fatty liver group had slightly more truncal fat on DEXA scanning (20.9kg v 15.6kg, p=0.038). Interestingly there was no difference in the amount of either abdominal visceral fat (p=0.42) or subcutaneous fat (p=0.62) as measured by 5-slice MRI (table 5.3). Because this finding was somewhat unexpected and since most other investigators have assessed visceral fat by single horizontal MRI scanning, we examined correlations between each of the single slices and the composite measure obtained from all 5 horizontal slices. R2 values varied considerably from 0.47 to 0.85 depending on which single slice was chosen. Given that the calculated value from the composite of 5 slices is most likely to reflect quantity of total visceral fat, the relatively poor correlation coefficients obtained with a single slice may be interpreted to mean that use of a single slice only, may result in an imprecise value for total visceral fat. As expected obese subjects with fatty liver also had a trend toward slightly higher lean mass than obese subjects without fatty liver (67.4 v 56.8kg, p=0.15).

During the clamp steady state was obtained for glucose and insulin levels (see figures 5.1 and 5.2), with co-efficients of variation for steady state glucose in lean subjects (CV) 0.070 ± (SD) 0.018 mmol/l, obese subjects without fatty liver 0.068 ± 0.025 mmol/l, and obese subjects without fatty liver CV 0.053 ± 0.028 mmol/l.

Obese subjects with fatty liver were more insulin resistant in muscle than obese subjects without fatty liver (see table 5.4), as demonstrated by reduced whole body glucose disposal in the euglycaemic hyperinsulinaemic clamp (4.4 ± 1.8 mg/kg/min v 6.7 ± 0.43 mg/kg/min p=0.008). Both obese groups were markedly more insulin resistant than the lean controls (glucose disposal 10.8mg/kg/min p<0.001). There was no difference in hepatic insulin sensitivity in obese subjects with, and obese subjects without fatty liver (percentage suppression at 60 minutes 58.7 ± 20.4% v 64.8 ± 20.5% respectively, table 5.4, figure 5.4). Lean subjects appeared to have higher hepatic glucose output prior to insulin infusion compared with obese subjects although when hepatic glucose output was corrected for lean body mass instead of weight there was no significant difference between lean and obese subjects (14.3 ± 2.7 umol/kg/min v 12.7 ± 1.8umol/kg/min, p=0.09). Both obese groups showed markedly impaired suppression of hepatic glucose output compared with the lean controls (p=0.002, table 5.4). There was impaired NEFA suppression in obese subjects with fatty liver compared to obese subjects without fatty liver (figure 5.3, table 5.4) as measured by a marked difference in both area under the NEFA suppression curve from 30 minutes (419 ± 169.5 v 271 ± 94.4 hr.nmol/l, p=0.048) and percentage suppression from baseline (percentage suppression at 60 minutes 46.9 ± 17.3% v 62.5 ± 11.5%, p=0.044), indicating insulin resistance in adipose tissue in the obese group with fatty liver compared with the obese group without fatty liver. Both groups were significantly more insulin resistant in adipose tissue than the lean controls (p<0.001, table 5.4).

Because the obese group with fatty liver had more total and truncal fat than the obese group without fatty liver, a sub-analysis of subjects matched for total fat on

DEXA was undertaken to determine whether the relationship with insulin sensitivity was independent of fatness. Six subjects without fatty liver were matched with six patients with fatty liver for total fat (30.6 ± 4.4kg v 31.7 ± 4.6 kg p=0.68) and trunk fat (16.0 ± 2.7 kg v 17.5 ± 2.8 kg p=0.35) and there was still a marked and significant difference in whole body insulin sensitivity (M value 6.4 ± 0.8 mmol/min v 4.4 ± 1.7 mmol/min, p= 0.032; fasting insulin 9.8 ± 1.8 mu/l v 20.4 ± 8.7 mu/l p=0.014) and insulin sensitivity in muscle (RD = 37.0 ± 4.1 mg/kg/hr v 26.5 ± 6.3 mg/kg/hr, p=0.007). However in this small number of subjects no significant difference was seen in insulin sensitivity in liver (percentage suppression HGO 64.1 ± 22.3% v 60.5 ± 14.6 % p=0.75) or adipose tissue (area under NEFA curve 486 ± 155 hr.ng/l v 513 ± 66.9 hr.ng/l, p=0.39).

Table 5-1 Characteristics of the lean men, obese men without fatty liver and obese men with fatty liver.

	Lean men (n=8)	Obese men without fatty liver (n=7)	Obese men with fatty liver (n=15)	p value
Body mass index (kg/m^2)	23.6 ± 1.6	32.9 ± 4.2	36.1 ± 4.9	0.14
Age (years)	51.8 ± 5.4	54.0 ± 7.1	53.1 ± 5.7	0.75
Weight (kg)	72.5 ± 8.7	102.3 ± 13.7	114.3± 18.3	0.14
Alcohol consumption (units/week)	15.9 ± 11.8	11.9 ± 12.7	14.1 ± 17.2	0.76
Systolic Blood Pressure (mmHg)	121 ± 13	136 ± 10	136 ± 18	0.99
Diastolic Blood Pressure (mmHg)	68 ± 9	78 ± 6	80 ± 8	0.56
Fasting plasma glucose (mmol/l)	5.1 ± 0.4	5.5 ± 0.26	5.6 ± 0.66	0.66
HDL cholesterol (mmol/l)	1.71 ± 0.59	1.25 ± 0.28	1.25 ± 0.0.26	0.99
Triglycerides (mmol/l)	0.95 (0.7-1.1)	1.62 (1.07-2.47)	1.87 (1.38-2.55)	0.60
Fasting plasma insulin (mu/l)	5.1 (3.5-6.6)	9.4 (8.0-11.1)	18.4 (13.6-24.8)	0.005
Gamma GT (mmol/l)	23.5 (12-41)	30.4 (18.4-50.3)	57.5 (41.4-79.9)	0.02
Alanine Aminotransferase (mmol/l)	22.3 (16-25)	28.3 (20.7-38.9)	37.1 (29.9-46.1)	0.131
Fasting NEFA (nmol/l)	535 ± 241	452 ± 127	574 ± 127	0.049

Values are mean ± SD, where data are not normally distributed geometrical mean and 95% CI are shown. P-value comparisons are between obese subjects with and without fatty liver.

Table 5-2 Comparison of total adiposity between lean men, obese men without fatty liver and obese men with fatty liver

	Lean men (n=8)	Obese men without fatty liver (n=7)	Obese men with fatty liver (n=15)	p value
Body mass index (kg/m^2)	23.6 ± 1.6	32.9 ± 4.2	36.1 ± 1.3	0.14
Bioimpedance total fat (%)	18.7 ± 2.8	29.5 ± 4.2	33.7 ± 4.8	0.065
DEXA total fat (%)	18.5 ± 4.7	29.3 ± 2.1	33.2 ± 3.9 (n=14)	0.027
Bod Pod total fat (%)	17.5 ± 7.3	36.0 ± 6.5	38.3 ± 4.9 (n=14)	0.38
Skin fold calculated total fat (%)	25.1 ± 5.6	33.0 ± 2.7	35.9 ± 2.8	0.032

Values are mean ± SD. P-value comparisons are between obese subjects with and without fatty liver.

Table 5-3 Comparison of regional adiposity between lean men, obese men without fatty liver and obese men with fatty liver.

	Lean men (n=8)	Obese men without fatty liver (n=7)	Obese men with fatty liver (n=15)	p value
Waist circumference (cm)	86.2 ± 3.7	112.4 ± 9.2	120.8 ± 12.1	0.12
MRI visceral fat (kg)		4.62 ± 1.1	5.04 ± 1.5 (n=13)	0.42
MRI subcutaneous fat (kg)		4.82 ± 2.5	5.24 ± 2.08 (n=13)	0.62
Trunk fat on DEXA (kg)	5.83 ± 2.3	15.60 ± 2.7	20.86 ± 5.9 (n=14)	0.038
Lean Body Mass (kg)	55.01 ± 5.2	56.75 ± 11.6	67.44 ± 16.6 (n=14)	0.15

Values are mean ± SD. P-value comparisons are between obese subjects with and without fatty liver.

Table 5-4 Comparison of insulin sensitivity in lean men, obese men without fatty liver and obese men with fatty liver

	Lean Controls (n=8)	Obese subjects without fatty liver (n=7)	Obese subjects with fatty liver (n=14)	p value
Insulin sensitivity in fat (area under NEFA suppression curve 30-120 minutes OGTT) (hr.nmol/l)	147.0 ± 77.4	271.6 ± 94.4	418.8 ± 169.5	0.046
Insulin sensitivity in muscle (gluc disposal during steady state insulin infusion) (mg/kg/hr)	10.80 ± 2.8	6.69 ± 0.43	4.44 ± 1.8	0.008
Insulin sensitivity in liver (% suppression of endog glucose production by insulin at 60 minutes)	86.6 ± 24.5	64.8 ± 20.5	58.7 ± 20.4	0.675

Figure 5-1 Plasma glucose during the clamp

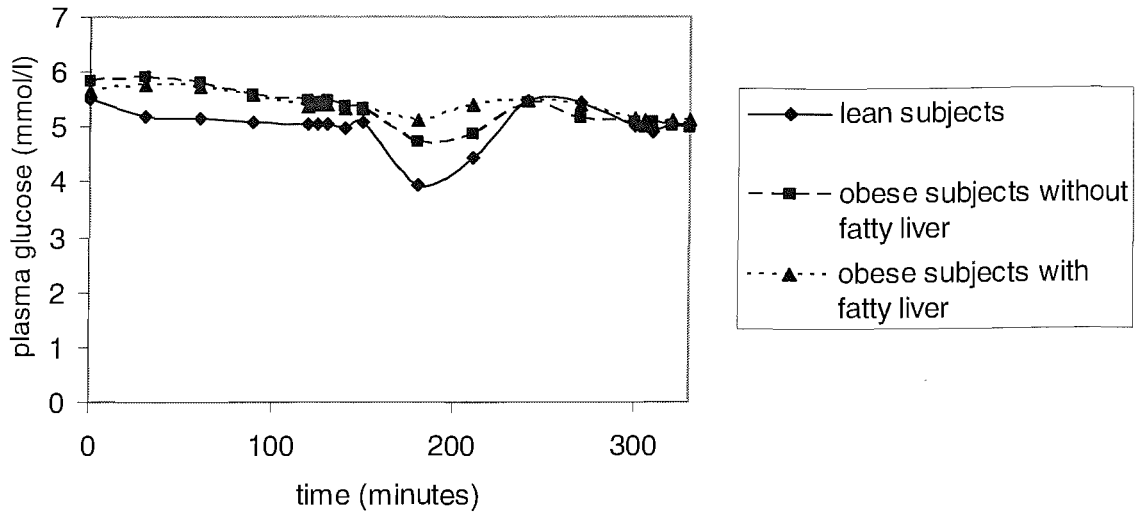


Figure 5-2 Plasma insulin during the clamp

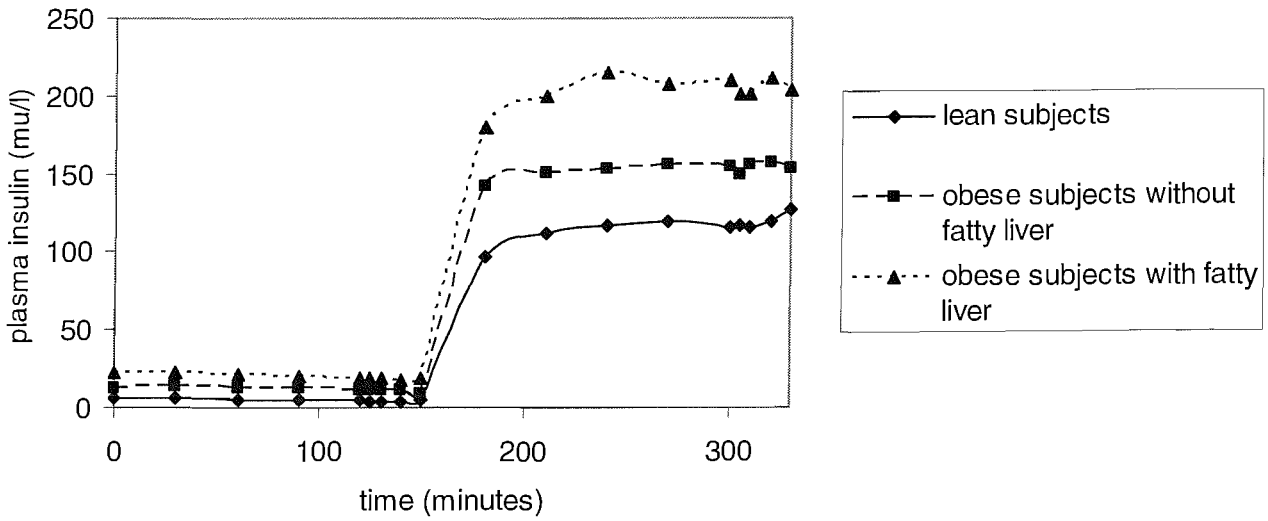
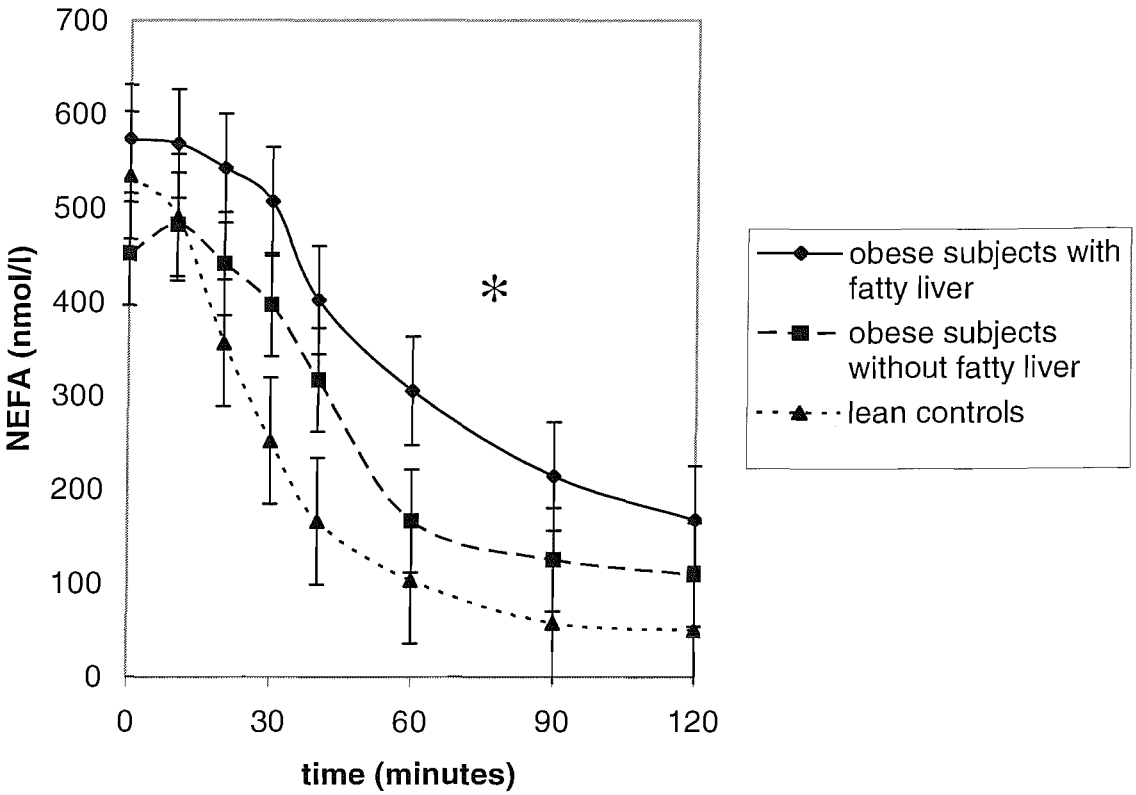
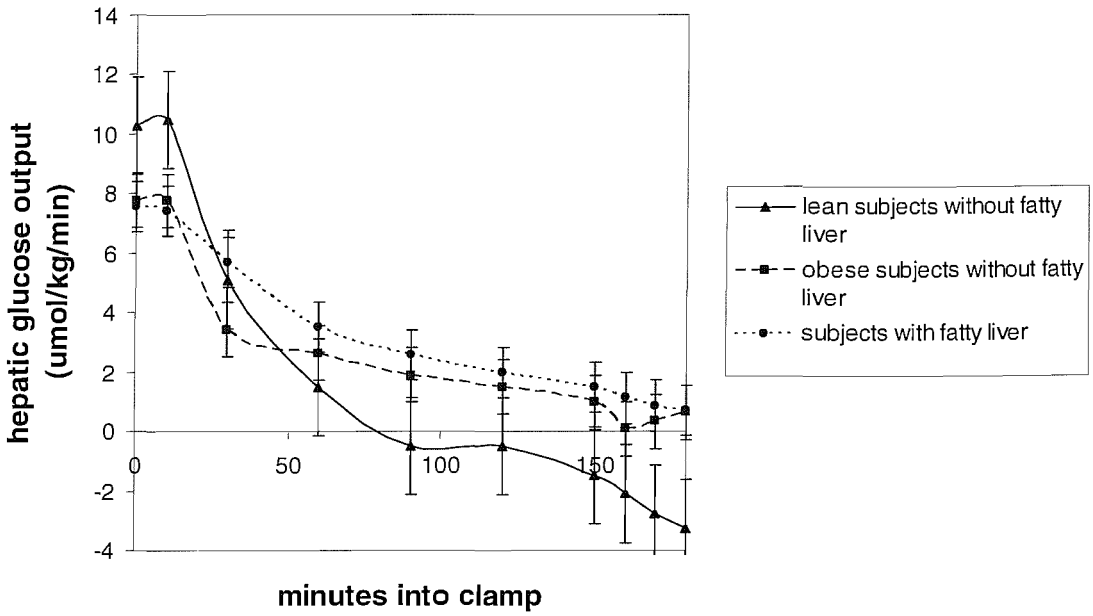


Figure 5-3 NEFA suppression during the OGTT in lean men and obese men with and without fatty liver



Student's t-test is used to compare obese subjects with fatty liver and obese subjects without fatty liver for area under the curve. P-value comparisons are between obese subjects with and without fatty liver * $p<0.05$. Error bars +/- 1 SE

Figure 5-4 Suppression of hepatic glucose output on commencing insulin infusion



5.4 Discussion

The novel results of our study are that in obese subjects with fatty liver we have demonstrated a marked increase in insulin resistance in both muscle and adipose tissue. Our subjects with fatty liver had slightly more overall fat and markedly more trunk fat although no difference in visceral fat was demonstrated by detailed abdominal MRI scanning. When subjects with and without fatty liver were matched for total and trunk fat, there was still a difference in muscle insulin sensitivity.

We have demonstrated marked differences in insulin sensitivity in obese individuals with fatty liver compared to those without, with fasting insulin levels two-fold higher in the fatty liver group (table 5.1). We have shown clear differences in tissue-specific insulin resistance in muscle measured by glucose disposal in a euglycaemic hyperinsulinaemic clamp and adipose tissue measured by NEFA suppression during an OGTT.

Whilst other researchers have demonstrated whole body insulin resistance in subjects with fatty liver [13,15,19,21,224] our study is the first to show tissue-specific differences in insulin sensitivity in non-diabetic obese subjects with fatty liver compared to obese non-diabetic subjects without fatty liver. Interestingly, we did not demonstrate a difference in hepatic insulin sensitivity with the presence of fatty liver. However, a criticism of our study is that we did not use a two-stage clamp, and furthermore that insulin levels were higher in the fatty liver group than in the non-fatty liver group throughout the clamp (see figure 5.2). Because the same amount of suppression of hepatic glucose output was seen but at higher insulin levels our findings may represent a shift in the dose-response curve indicative of impaired suppression of hepatic glucose output in the fatty liver group [225].

We also found, contrary to expectation, that fasting hepatic glucose output was higher in the lean individuals. During the clamp negative values were seen in hepatic glucose output for the lean individuals and whilst this does not make

physiological sense it is an acknowledged finding in this method of measuring hepatic glucose output [123]. A potential criticism of our clamp method was that we did not use arterialised venous blood. This method is now widely accepted in the literature for the same reason as ours, namely that there are physiological effects of hand-warming which alter the measured insulin-sensitivity [226, 227, 116]; however we acknowledge that glucose utilisation in the peripheries is increased without hand-warming, and that there may be a differential effect between insulin-sensitive and insulin-resistant individuals.

Marchesini et al did demonstrate impaired insulin sensitivity in liver in overweight subjects with NAFLD to a degree comparable with diabetic subjects without fatty liver although the controls in that study were not overweight let alone obese [13]. Kelley et al demonstrated increased insulin resistance in muscle and fat in obese diabetic individuals with fatty liver compared to those without, but in that study also, those subjects with fatty liver were significantly more obese than those without fatty liver [19].

The effect of increased NEFA concentrations on hepatic insulin sensitivity is uncertain. It has been suggested that increased non-esterified fatty acids supplied directly to the liver impair hepatic insulin sensitivity by upregulating glucose-6-phophate and stimulating gluconeogenesis [98]. In a study of healthy non-obese men, there was impaired hepatic insulin sensitivity and suppression of non-esterified fatty acids in those with high liver fat compared with those with low liver fat using very low levels of insulin infusion (0.3mU/kg/min) [152]. Thus it is possible that the higher amounts of insulin which are often used in studies of muscle insulin sensitivity cannot detect subtle difference in hepatic insulin sensitivity.

It is an attractive argument that hepatic insulin resistance might be expected to result from fat accumulation within hepatocytes, although the evidence to support this assertion is not strong. In mice, hepatic over-expression of suppressors of cytokine signaling (SOCS)-1 and SOCS-3 causes insulin resistance and an

increase in fatty acid synthesis in liver. Inhibition of these signaling proteins improves insulin sensitivity, normalises fatty acid synthesis and dramatically ameliorates hepatic steatosis [228]. Another recent study suggests that hepatic fat accumulation is not sufficient to impair hepatic insulin action [229]. The authors showed that in rats, high-fat feeding resulted in liver fat accumulation but did not cause hepatic insulin resistance. In human subjects Carey demonstrated reduced intrahepatic triglyceride using magnetic resonance spectroscopy by the administration of acipimox which resulted in lower post-prandial glucose but not via an effect on endogenous glucose production, which was unaffected [230]. There is some evidence therefore that regulation of hepatic insulin sensitivity is not directly affected by accumulation of liver fat, but this is an area which needs further study.

We have studied obese men with and without fatty liver and found minimal difference in total fat and demonstrated no difference in amount of visceral fat between the obese groups. Because fatness is often imprecisely measured our study was designed to characterise body composition in our subjects in as much detail as possible. We have used four methods in addition to BMI to measure body fat quantity (skin-fold measurements, DEXA, bioimpedance, air-displacement plethysmography) and three methods to determine body fat distribution (waist circumference, percentage of total fat which is truncal on DEXA, and MRI for visceral and subcutaneous fat). We have shown a small increase in total fat in the obese group with fatty liver compared to the obese group without fatty liver but importantly there was no difference in amount of visceral fat despite a marked difference in truncal fat between these two groups. We deliberately chose to use a five slice MRI method, as described by Ross [82] to allow detailed evaluation of visceral fat. We were concerned that an image taken using a single slice methodology, as used in many studies [84,231-233] was inadequate to evaluate precisely amount of visceral fat. In support of our approach Thomas et al found that individual visceral fat content could not be predicted from a single-slice MRI sampling strategy, in a study using contiguous, whole body multi-slice magnetic resonance imaging, [197].

Our finding that obese subjects with and without fatty liver have similar amounts of visceral fat has been confirmed in other studies [222,234] and suggests that quantity of visceral fat is not specifically important to fatty liver. Although some studies have reported increased quantity of visceral fat in association with fatty liver [19,127,235], Frayn has argued convincingly that a quantitive increase in visceral fat is unproven, even in association with the metabolic syndrome [47]. The fact that when we matched subjects for amount of total and trunk fat measured on DEXA there was still a marked and significant difference in insulin sensitivity in muscle suggests that insulin sensitivity is related to fatty liver independently of fatness.

Our finding of impaired NEFA suppression in subjects with fatty liver suggests there may be an abnormality in adipocyte metabolism, although this finding was not independent of amount of body fat. Post-prandial plasma NEFAs are largely determined by the action of insulin on adipocytes to suppress lipolysis of triglycerides by hormone-sensitive lipase and promotion of re-esterification of fatty acids to triglyceride [107,124,141,218-221]. In the presence of insulin resistance there is increased NEFA release from adipocytes as a result of decreased inhibition of hormone-sensitive lipase [107]. In support of the importance of hormone sensitive lipase and the role of fatty acids in the pathogenesis of fatty liver, the hormone-sensitive lipase knock out mouse has a reduction in liver fat [101]. Our results also support the notion that there is a qualitative defect in adipose tissue function that is important in the pathogenesis of fatty liver. We did not measure NEFA levels during the clamp but this might have provided further supporting evidence for our findings.

Disturbed adipocyte function might result in NASH through a contribution to the development of fatty liver. Hepatic steatosis has been described as the 'first hit' in a two-hit model for the progression of NAFLD, where the second hit is the resultant oxidative stress from pro-inflammatory cytokines [236,237]. Fatty liver may develop as a result of either increased delivery of NEFA from insulin-resistant adipocytes or from increased hepatic fatty acid synthesis [236]. Increased fatty acid synthesis may occur in insulin resistant states where plasma insulin

concentrations are high and dietary substrate is provided in excess. If dietary carbohydrate intake is high and physical activity levels are low, excess carbohydrate is converted into fatty acid via acetyl CoA, with insulin facilitating the rate limiting conversion in fatty acid synthesis, namely conversion of acetyl CoA to malonyl CoA. Thus in post-absorptive states when excess dietary glucose cannot be converted to glycogen, nor completely oxidised, glucose provides a substrate for fatty acid synthesis and hepatic fat accumulation. Fatty acids have recently been proposed to have a pathogenic role in the development of NASH [236,238]. Non-esterified fatty acids may indirectly promote hepatic lipotoxicity by stimulating TNFα [238] and in vitro some NEFAs have been shown to cause direct hepatotoxicity [239]. By stimulating cytokine P450 Cyp2E1/Cyp4A enzyme activity and increasing oxidative stress and lipid peroxidation [240,241], NEFAs may cause direct cell necrosis or apoptosis [236].

In summary, our study has demonstrated for the first time that obese individuals with fatty liver have marked insulin resistance in muscle and fat compared with obese individuals without fatty liver. We propose that in obese subjects an abnormality of adipose tissue function occurs that is linked to insulin resistance. This functional abnormality results in increased NEFA supply to liver and muscle, facilitating hepatic fat accumulation and skeletal muscle insulin resistance. Subjects with fatty liver were more insulin resistant in muscle than subjects without fatty liver even when subjects were matched for body fat, indicating that there is an independent relationship between insulin sensitivity and fatty liver. These findings are consistent with the finding that thiazolidinediones, which amongst other actions, improve insulin sensitivity in muscle [118], also appear to reduce fatty infiltration of the liver [242].

6 THE ROLE OF CORTISOL IN THE RELATIONSHIP BETWEEN INSULIN SENSITIVITY AND OBESITY

6.1 Introduction

Cushing's syndrome results from excess glucocorticoid exposure and is characterised by central obesity and diabetes. Cortisol activates glycogen synthase, inactivates glycogen phophorylase and increases gluconeogenic enzymes such as PEPCK as well as inhibiting peripheral glucose uptake and utilisation, and activating lipolysis in adipose tissue [243]. It has been suggested that more subtle abnormalities of the hypothalamic-pituitary-adrenal (HPA) axis, which regulates cortisol exposure, may therefore also underlie the metabolic syndrome [22].

Various abnormalities of the HPA axis have been reported in both obesity and metabolic syndrome [29,244,245], but the abnormalities described do not point clearly to increased exposure. Although increased morning cortisol levels are associated with metabolic syndrome features [27,32], in obesity morning and average circulating cortisol levels are low or normal [24-27] which presents a paradox given the strong association between obesity and metabolic syndrome. Furthermore cortisol production and clearance are apparently higher in obesity [30] and stress response is also increased [29]. It has been shown that some indices of HPA axis activity, including raised fasting cortisol concentrations and an increased adrenal response to adrenocorticotropic hormone (ACTH), are associated with increased insulin resistance, independently of the effects of obesity [27,164], and it may be that abnormalities of the HPA axis contribute independently to the development of insulin resistance. By measurement of salivary cortisol, Bjorntorp described how individuals with metabolic syndrome features had a flat, rigid day curve of cortisol compared to the high plasticity and sensitive feedback control seen in healthy individuals [244].

Non-alcoholic fatty liver disease is a newly recognised feature of the metabolic syndrome which is strongly related to insulin resistance [19,240]. It is plausible that abnormalities of cortisol metabolism may contribute to the development of fatty liver as well as insulin resistance. It has been shown that NAFLD is associated with the presence of subclinical, chronic activation of the HPA axis with increased urinary free cortisol [33], increased 5β reductase activity [20] but it is not known whether these disturbances contribute to insulin resistance independently of body fat. Furthermore the mechanism for the marked insulin resistance in NAFLD remains uncertain [246], as does the relationship between cortisol metabolism and insulin resistance.

Much of the previous work on the association between NAFLD, insulin resistance and cortisol metabolism has used the dexamethasone suppression test to assess cortisol metabolism. However the findings of these studies may have been confounded by obesity. Some obese subjects have increased suppression of cortisol during a dexamethasone suppression test [247] but it has been demonstrated that the rate of dexamethasone metabolism is reduced in people who have increased suppression of cortisol during the test [247,248] and also that dexamethasone metabolism is associated with parameters of liver function (ALT, AST and GGT) [249]. The results of this test need to be treated with caution in this group of subjects and studies of cortisol metabolism in obese individuals or those with fatty liver need to include additional tests of cortisol metabolism beyond dexamethasone suppression studies. Furthermore, only by controlling for body fat, or by undertaking studies in which subjects are matched for body fat, is it possible to identify independent effects of cortisol metabolism in NAFLD.

The purpose of our study was to define the relationship between insulin sensitivity, cortisol clearance and obesity and to determine whether the relationships found were influenced by the presence of fatty liver. in a group of middle-aged men with a wide range of body mass indices, we have used a tracer dose of deuterium-labelled cortisol measured by tandem mass spectrometry, to determine cortisol clearance without perturbing the HPA axis. We have also

measured 9am cortisol and cortisol response to low-dose ACTH to determine adrenal sensitivity. We have also measured corticosteroid binding globulin (CBG) as there is evidence that this is a negative acute phase protein, which we postulate, may be protective against diabetes. We have used ultrasonography to determine hepatic steatosis and we have measured insulin sensitivity in muscle, fat and liver using a euglycaemic hyperinsulinaemic clamp and suppression of non-esterified fatty acids during an oral glucose tolerance test (OGTT).

6.2 Methods

Data from 23 subjects were available for this study. Cortisol clearance was measured in 29 subjects but data were not available on six subjects because of technical difficulties with the preparation of the samples for tandem mass spectroscopy. Statistical comparisons showed that there were no differences in body fat, CBG, or insulin sensitivity between these 6 subjects and the 23 subjects in whom cortisol clearance measurements were undertaken. Body composition, metabolic syndrome features and insulin sensitivity using a euglycaemic hyperinsulinaemic clamp technique were measured as previously described. The HPA axis was studied using fasting 9am cortisol, response to 1mcg $ACTH_{1-24}$, cortisol clearance using a tracer dose deuterated cortisol and measurement of CBG. Fatty liver was assessed using ultrasonography.

All statistical analyses were performed using SPSS for Windows version 14.0. Student's t tests were undertaken to compare mean values of normally distributed data. Where variables were not normally distributed, log transformation was undertaken to normalise the distribution. Multivariate linear regression modelling was undertaken to describe the measures that were independently associated with M value as the dependent (outcome) variable. Independent (exposure) variables selected from the univariate analyses were entered into the regression models to examine the factors that were independently associated with M value. The scatter plot between cortisol clearance and insulin sensitivity suggested that the relationship was not linear and therefore other non-linear models were examined. A quadratic model gave a

better fit than a linear model. To avoid collinearity the cortisol clearance data were transformed by the simple mathematical manipulation of centering i.e. subtracting a constant from the data values, and tests to check for collinearity were undertaken [250]. Further comparisons were made using t-tests between mean values of a variety of factors between top and bottom groups as defined from tertiles of insulin sensitivity.

6.3 Results

Characteristics of the 23 subjects included in this study are shown in table 6.1. Mean 9am fasting plasma cortisol concentration in the 23 men was 336.5 nmol/l (range 189-577nmol/l) and mean plasma cortisol after 1mcg $ACTH_{1-24}$ was 596.0 nmol/l (382-839 nmol/l). The mean cortisol clearance was 202 ml/min (99 – 470 ml/min) and mean CBG concentration was 45.3 mg/l (23-60mg/l).

There was a strong inverse correlation between cortisol clearance and whole body insulin sensitivity (M value) as shown in figure 6.1 ($r = -0.61$, $p=0.002$). There were also strong inverse relationships between cortisol clearance and insulin sensitivity in muscle ($r=-0.53$, $p=0.009$) and fat ($r=-0.42$, $p=0.045$). Cortisol clearance was 270 +/- 100 mls/minute in the most insulin resistant tertile and 150+/-30 mls/minute in the most insulin sensitive tertile of whole body glucose disposal (M value) (mean +/-SD), $p=0.004$. Cortisol clearance did not show a significant relationship with total body fat or truncal fat ($r=0.27$, $p=0.207$, $r=0.31$, $p=0.155$ respectively) but when regression analysis was undertaken with cortisol clearance as the outcome variable, with insulin sensitivity and body fat (either total or truncal) as independent variables, both insulin sensitivity and body fat were independently associated with cortisol clearance ($B=-1.16$, $p<0.001$ for cortisol clearance, $B= -0.67$, $p=0.021$ for total fat) . There were no significant associations between cortisol clearance, 9am total or free cortisol concentrations, or the cortisol response to $ACTH_{1-24,}$ and individual components of the metabolic syndrome (blood pressure, fasting glucose, plasma triglycerides or HDL cholesterol concentrations and waist circumference).

Since there was a strong inverse univariate association between cortisol clearance and whole body insulin sensitivity (figure 6.1), we studied the relationships between cortisol clearance and insulin sensitivity in multivariate models. Multiple regression analyses showed that the relationship between cortisol clearance and insulin sensitivity was strong and independent of CBG concentrations. In a model with M value as the dependent variable (as a measure of whole body glucose disposal), and both cortisol clearance and CBG as independent variables, only cortisol clearance remained statistically significantly independently associated with M value (B -1.19 S.E. 0.50, p=0.026).

We studied the relationship between cortisol clearance and CBG and insulin sensitivity in muscle, liver and fat (see table 6.3). There was a significant negative relationship between cortisol clearance and insulin sensitivity in muscle and adipose tissue but not liver. CBG was associated with increased insulin sensitivity in all three tissues. The relationship between cortisol clearance and insulin sensitivity in muscle was independent of body fat and CBG on regression analysis with RD as the outcome variables and body fat, cortisol clearance and CBG as independent variables (B=-0.263, p=0.025). The relationship between cortisol clearance and insulin sensitivity in adipose tissue was not independent of total fat and CBG when both of these were included in a model with NEFA suppression as the outcome, but when CBG was excluded from the model cortisol clearance was independently associated with NEFA suppression (B=-0.321, p=0.039). In both of these relationships body fat remained independently associated with insulin sensitivity with cortisol clearance and CBG in the model. CBG was not independently associated with any of the measures of tissue insulin sensitivity when cortisol clearance was included in the regression analyses.

Twelve of the 23 men had fatty liver. Liver ultrasound scoring between the two radiologists, independently scoring the scans, showed that agreement on scoring was 96%, kappa statistic = 0.93, (S.E 0.14), p<0.001. Cortisol clearance was increased in men with fatty liver, who were of a similar age but were more obese, had higher blood pressure and poorer glucose tolerance compared with those

without fatty liver (see table 6.2). The men with fatty liver were markedly more insulin resistant in muscle, liver and fat. There was a trend towards higher triglyceride levels and lower HDL cholesterol concentrations among men with fatty liver compared to those without fatty liver, but these differences were not statistically significant. There was no difference in alcohol intake between the two groups.

To determine the impact of total and regional fat and fatty liver on the relationship between cortisol clearance and insulin sensitivity (M value), multiple regression modelling was undertaken with M value as the dependent variable with inclusion of CBG, body fat, fatty liver and cortisol clearance as explanatory variables. Variation in total fat and cortisol clearance explained 82% (R^2=0.82) of the variance in M value, whereas variation in truncal fat and cortisol clearance explained 85% (R^2=0.85) of the variance in M value. Because the scatter plot between cortisol clearance and insulin sensitivity suggested that the relationship was not linear (figure 6.1) and a quadratic model gave a better fit than a linear model, tests for collinearity between explanatory variables were undertaken. These tests showed that fatty liver and insulin sensitivity were not collinear variables in the regression model and the use of a quadratic model was unlikely to decrease the precision of the model. Thus the regression modelling analyses showed that body fat and cortisol clearance were both independently associated with insulin sensitivity and, with inclusion of body fat and cortisol clearance as explanatory variables, fatty liver was not independently associated with insulin sensitivity.

Although the subjects with fatty liver had markedly increased cortisol clearance compared to those without, fatty liver was not an independent predictor of cortisol clearance. To explore further the relationship between fatty liver and insulin sensitivity the regression analyses were repeated. We omitted in turn and singly, first cortisol clearance and then the measure of body fat. These results showed that: 1) with cortisol clearance omitted, fatty liver was independently associated with M value (B=-0.13, 95%CI -0.25, -0.01, p=0.035) and 2) when cortisol

clearance was re-inserted and a measure of body fat removed (truncal or total), fatty liver was independently associated with M value [(B=-0.30, 95%CI -0.43, -0.17, $p<0.001$ (for total fat)). Thus these two regression models suggested that the relationship between fatty liver and insulin sensitivity was influenced via a contribution from both fatness and cortisol clearance.

Thus the regression modelling analyses showed that body fat and cortisol clearance were both independently associated with insulin sensitivity and the relationship between insulin sensitivity and fatty liver was influenced by cortisol clearance.

Table 6-1 Data for all 23 subjects

	Means (SD)	Range
Age (years)	52.8 (5.1)	42-61
BMI (kg/m^2)	31.3 (7.2)	20.8-48.9
Waist circumference (cm)	107.9 (19.3)	79.7-150.3
DXA fat (kg)	28.0 (14.0)	7.7-62.9
DXA truncal fat (kg)	15.0 (8.6)	3.3-36.1
Systolic blood pressure (mmHg)	132.2 (17.0)	102-188
Diastolic Blood pressure (mmHg)	75.3 (10.3)	58-103
Fasting plasma glucose (mmol/l)	5.4 (0.5)	4.5-7.0
Fasting triglyceride (mmol/l)	1.8 (1.2)	0.7-4.9
Fasting HDL (mmol/l)	1.4 (0.47)	0.8-2.6
Glucose disposal (mg/kg/min) (M value)	6.9 (3.6)	2.2-13.9
Rd steady state (µmol/kg/min)	38.7 (17.5)	17.1-73.3
Insulin sensitivity index NEFA (ratio)	0.68 (0.41)	0.2-1.6
Suppression endogenous glucose output (%)	69.5 (25.1)	21-100
ALT (IU/L)	31.6 (11.8)	16-58
Cortisol 9a.m.(nmol/l)	336 (107)	189-577
Cortisol 0 (nmol/l)	281 (136)	138-752
Cortisol 30 (nmol/l)	595 (110)	382-839
Cortisol 60 (nmol/l)	492 (174)	245-876
Cortisol binding globulin (mg/l)	45.3 (8.8)	23.3-59.8
Cortisol MCR (ml/min) *	202 (89)	99-467

Table 6-2 Data comparing the men with fatty liver with those without fatty liver (means and S.D. or geometric means and 95%CIs)

	Fatty liver (n=12)	No fatty liver (n=11)	P-value
Age(years)	53.6 (4.5)	52.0 (5.8)	0.47
BMI (kg/m^2)	36.5 (5.4)	25.6 (3.8)	<0.001
Waist to hip ratio	1.01 (0.06)	0.90 (0.06)	<0.001
Waist circumference (cm)	122.5 (12.7)	92.1(10.6)	<0.001
DEXA fat (kg)	38.1 (10.5)	17.0 (7.5)	<0.001
DEXA truncal fat (kg)	21.3 (6.1)	8.0 (4.4)	<0.001
Systolic blood pressure (mm)	139.1 (17.7)	124.7 (13.3)	0.04
Diastolic Blood pressure (mm)	80 (9.0)	70.0 (9.2)	0.02
Fasting plasma glucose (mmol/l)	5.5 (0.67)	5.2 (0.36)	0.14
2 hr glucose (mmol/l)	7.3 (2.4)	5.4 (1.1)	0.03
Fasting triglyceride (mmol/l)	2.2 (1.3)	1.3 (0.77)	0.06
Fasting HDL (mmol/l)	1.2 (0.28)	1.5 (0.59)	0.09
Glucose disposal (mg/kg/min)	4.3 (1.4)	9.8 (3.0)	<0.001
Rd steady state (μmol/kg/min)	25.2 (6.1)	53.4 (13.2)	<0.001
Insulin sensitivity index NEFA	0.36 (0.11)	1.02 (0.33)	<0.001
Suppn endogenous glucose output (%)	57.8 (22.0)	82.2 (22.7)	0.016
Alcohol consumption (units/week)	13.3 (15.2)	14.5 (12.6)	0.84
ALT (IU/L)	36.3 (10.9)	26.4 (11.0)	0.04
TNFα (pg/ml)	0.91 (0.46,1.37)	0.58 (0.22,0.94)	0.08
IL-6 (pg/ml)	2.51 (1.45,3.57)	1.22 (0.27,2.17)	0.004
IL-1β (pg/ml)	0.05 (0.03,0.07)	0.05 (-0.02,0.12)	0.56
Adiponectin (g/l)	3.85 (2.80,4.90)	7.43 (4.44,10.42)	0.04
Cortisol MCR (ml/min)	242.6 (105.0)	157.6 (36.2)	0.02
CBG concentration (mg/l)	41.7 (9.5)	49.3 (5.8)	0.03

Figure 6-1 The relationship between cortisol clearance and glucose disposal

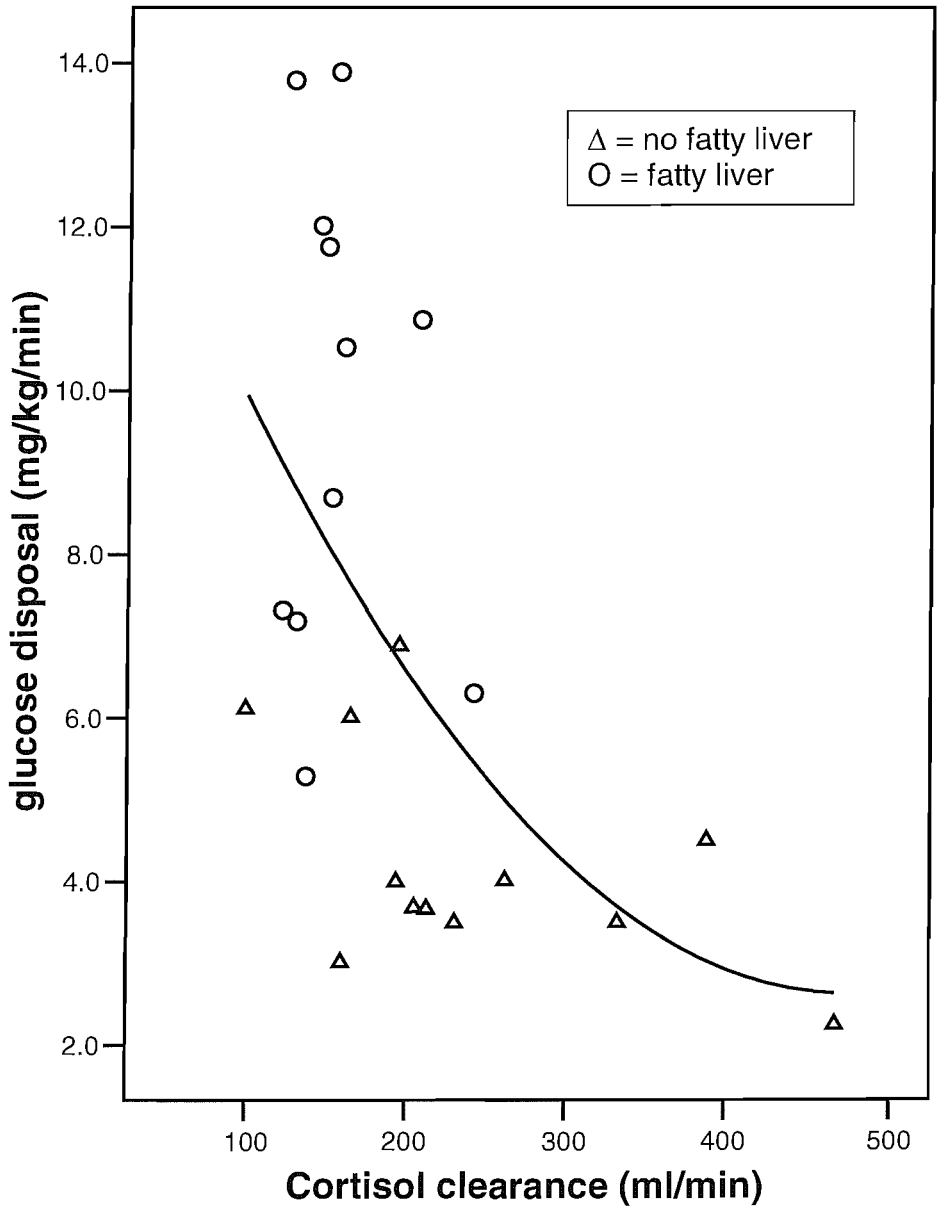


Table 6-3 Correlation between cortisol clearance and CBG with total glucose disposal (m value), insulin sensitivity in muscle (RD), adipose tissue (insulin sensitivity index for NEFAs) and liver (suppression of hepatic glucose output)

		M value	RD steady state	ISI NEFA	Suppression hepatic glucose output by 60 minutes insulin
Cortisol MCR	Pearson Correlation	-0.609	-0.531	-0.493	-0.210
	Sig	0.002	0.009	0.017	0.337
CBG	Pearson Correlation	0.520	0.458	0.396	0.433
	Sig.	0.004	0.012	0.019	0.030

Discussion

We have shown that increased cortisol clearance is strongly associated with decreased insulin sensitivity independently of amounts of total or truncal body fat. To our knowledge our study is the first to show this finding and to assess whole body insulin sensitivity together with insulin sensitivity in each of the key tissues affecting whole body insulin sensitivity namely muscle, liver and fat. We have shown a relationship between cortisol clearance and insulin sensitivity in muscle which is independent of amount of body fat and CBG, and a relationship between cortisol clearance and insulin sensitivity in adipose tissue which is independent of body fatness. We did not demonstrate an independent relationship between cortisol clearance and hepatic insulin sensitivity. We have also assessed total and truncal fat and the presence of fatty liver and shown that the relationship between fatty liver and insulin sensitivity is influenced by cortisol clearance. Previous studies have not clarified whether increased cortisol clearance is predominantly associated with insulin resistance or with body fat, and our study clearly demonstrates a strong and independent relationship between cortisol clearance and insulin resistance.

We had insufficient patient numbers with MRI studies to evaluate the relationship between visceral fat and cortisol clearance but DEXA measurement of truncal fat is an excellent proxy measure for visceral fat quantity as estimated by CT scanning [317]. In this study we have controlled for truncal fat rather than visceral fat. However, controlling for truncal fat did not alter the relationship between cortisol clearance and insulin resistance. These data support the findings of a previous study showing visceral adipose fat mass was not associated with indices of cortisol metabolism, after adjusting for the effects of whole-body and liver fat [20]. Moreover, it has recently been shown in a study of portal vein and peripheral vein cortisol concentrations obtained in obese subjects undergoing bariatric surgery, that cortisol concentration was not increased in the portal circulation [251], suggesting that visceral fat does not generate markedly greater amounts of cortisol than subcutaneous adipocytes in obesity. Although in

contrast to these findings, Lottenberg et al demonstrated that increased cortisol clearance after an intravenous infusion of cortisol was associated with central fat accumulation amongst obese subjects [252].

Previous research provides supporting evidence for our finding that cortisol clearance is increased with insulin resistance. Twenty-four hour urinary free cortisol (24-h UFC) is associated with fasting glucose and insulin [31] and is increased in obese subjects with type 2 diabetes [253] in comparison with control subjects. We also found that fat mass was independently associated with cortisol clearance in keeping with previous studies [29]. Vicennati found an increased ACTH response to CRH/AVP associated with HOMA in obese women, although in contrast with other studies in this study no relationship was found between UFC and HOMA [254]. These contrasting findings indicate the importance of measuring cortisol clearance directly as we have done, as well as the importance of measuring tissue insulin sensitivity.

Our study adds to these previous findings because cortisol clearance is measured directly and the HPA axis is not perturbed by non-physiological doses of dexamethasone in a suppression test which is subject to confounding error in obese subjects because of altered metabolism as discussed previously [247,248]. It has been suggested that the increased UFC in obesity is appropriate for mass and does not represent increased tissue exposure [30] but our finding of an independent relationship with insulin resistance suggests that the increased cortisol clearance represents more exposure to cortisol than is appropriate for fat mass.

We did not demonstrate significant variation of 9am cortisol or response to $ACTH_{1-24}$ with fatness, insulin resistance or other features of the metabolic syndrome, suggesting that abnormalities of these aspects of HPA activity are subtle. This negative finding was not surprising given the small number of subjects studied and the relatively low correlation co-efficients ($r=0.20-0.26$, $p<0.001$) demonstrated in previous epidemiological studies [32,162]. When data was

examined on all 31 subjects for 9am cortisol and response to $ACTH_{1-24}$ there were still no significant relationships identified. Because we demonstrated such strong independent relationships between cortisol clearance and insulin sensitivity in muscle and fat, but did not demonstrate other abnormalities of the axis, this suggests that increased cortisol clearance is an important abnormality in these subjects.

It is not possible from our study to determine the direction of the relationship between insulin resistance and cortisol clearance, nor is it possible to know whether increased cortisol clearance is the primary abnormality of the HPA axis, or whether it is a response to other abnormalities of the axis. It is hard to understand how a primary increase in cortisol clearance rate would result in increased tissue exposure to cortisol. Increased cortisol clearance must be matched by increased production and a rapid turnover might be expected to lead to a very responsive HPA axis rather than the blunted profile described in obese subjects with insulin resistance [244]. It is possible that increased production and clearance represent loss of diurnal rhythm as seen in early Cushing's syndrome and the result is increased overall exposure. It is known that the nocturnal nadir of cortisol secretion is not as low during aging, which fact has been attributed to the 'wear-and-tear' effect of long tem exposure to stress [255] and it is plausible that a similar effect is seen in insulin resistant states.

Alternatively increased cortisol clearance may represent a compensatory mechanism whereby increased tissue exposure to cortisol results in both insulin resistance and increased cortisol clearance. Stewart has argued that tissue exposure to hormone levels is of more biological importance than circulating levels [245]. Rask showed that in obese men there was reduced 11 β HSD activity in liver but increased activity in subcutaneous adipose tissue and proposed that increased regeneration of cortisol from cortisone occurs as a response to increased deactivation in liver but that exposure of adipose tissue to cortisol was then high, exacerbating the effects of obesity [256]. Andrews also showed reduced 11β HSD activity in liver but showed normal activity in gluteal fat [167], and

suggested that this would represent increased exposure of adipose tissue to cortisol, especially if there was compensatory upregulation of the HPA axis. In 11 β HSD-1 deficient mice adipose tissue levels of messenger RNA for resistin and TNFα are lower and PPARγ, adiponectin, and uncoupling protein-2 are higher, indicating insulin sensitisation; glucose uptake is higher and there is reduced visceral fat accumulation upon high-fat feeding. High-fat-fed, β HSD-1 deficient mice also resisted diabetes and weight gain despite consuming more calories [257], indicating the important role of 11β HSD in insulin resistance, and possibly also in regulating weight gain. Inhibition of hepatic 11β HSD results in reduced expression of hepatic glucocorticoid-inducible genes such as PEPCK, the rate-limiting step in gluconeogenesis indicating that this might be a useful therapeutic target for the treatment of insulin resistance and obesity [258].

It is possible that a primary abnormality of cortisol clearance underlies the development of insulin resistance. As well as the reversible metabolism of cortisol to cortisone, irreversible A-ring metabolism of cortisol to inactive metabolites by 5α and 5β reductase enzymes occurs, predominantly within fat and liver. Enhanced metabolism by these enzymes is another potential mechanism for increased clearance, possible representing the primary abnormality of the HPA axis. In obesity increased 5α reductase activity has been found [20,29] but is offset by impaired 5β reductase activity in women and enhanced11β HSD activity in men, which Andrew suggests results in activation of the HPA axis, increased exposure of key tissues to cortisol, and contributing to the adverse effects of obesity [29].

It is plausible that abnormalities of the HPA axis underlie the development of insulin resistance and obesity. It is known that there is a strong genetic influence in the development of type 2 diabetes, and also to a lesser extent in the development of obesity [259-262], and so it is possible that abnormalities of the HPA axis underlie the development of obesity and insulin resistance. In a twin study Inglis has shown family patterns in the activity of 11βHSD, and a closer correlation in monozygotic than dizygotic twins in urinary cortisol metabolites

indicating a genetic as well as familial influence in cortisol clearance [263]. The 'thrifty phenotype' is a term which has been used to describe how some individuals develop obesity and insulin resistance in a plentiful environment when they have been exposed to reduced nutrient supply in early life [264,265]. Programming of the HPA axis as a result of a deprived intrauterine environment may result in abnormal functioning of the HPA axis [162] and subsequent development of obesity and insulin resistance.

We found that cortisol clearance contributed to the relationship between fatty liver and insulin resistance. It is possible that fatty liver might cause increased cortisol metabolism. In alcoholic liver disease circulating cortisol levels are higher and correlate with hepatic function indicating that impaired cortisol clearance occurs in these patients [266]. However our subjects had normal hepatic function and it is more likely that fatty liver develops either as a consequence of insulin resistance (because we have shown marked insulin resistance in subjects with fatty liver [201]) or as a direct result of the abnormalities of cortisol clearance. It is known that in Cushing's syndrome around 20% of patients develop fatty liver [266]. Westerbacka et al found an increase in cortisol metabolites in fatty liver, which was independent of visceral fat [20]. Together with our present results, these data suggest that alterations in hepatic 11 β HSD1 and hepatic 5α -reductase activity in NAFLD may contribute to insulin resistance, rather than any increase in the amount of visceral fat.

We found CBG concentration was inversely related to cortisol clearance and positively associated with insulin sensitivity. Others have also found CBG is decreased in insulin resistance [267,268]. CBG is important in determining tissue exposure to cortisol [203,269] and so it is possible that CBG may have a protective role against insulin resistance. Other researchers have found a positive relationship between insulin sensitivity and CBG concentration using HOMA [267,268]. Low levels of CBG are associated with an increase in free cortisol concentration [203,269] and CBG shows a diurnal rhythm with increased afternoon CBG production partly responsible for the afternoon dip in cortisol [268]. It is

plausible therefore that low CBG in insulin resistant individuals is a primary defect resulting in increased tissue cortisol exposure and further studies are needed to determine whether CBG is protective against the development of metabolic syndrome.

CBG is a negative acute phase protein which is reduced in inflammatory conditions such as sepsis and pancreatitis [269,270] and which is associated with low levels of inflammatory cytokine interleukin 6 [267]. It has been suggested that obesity is a pro-inflammatory state, and because CBG is reduced in subjects with insulin resistance, a protective role has been postulated for CBG [271]. Adipose tissue secretes cytokines, including adiponectin which is associated with serum CBG in men ($r=0.26$, $P=0.03$, $n=79$) [271] and CBG concentration has now been shown to be inversely related to measures of inflammation [267]. In our study there were similar correlation coefficients to the above study between CBG and a) adiponectin, b) TNFα, c) IL-6 and d) IL-1β but with much the smaller numbers in our study these associations were not significant ($r=0.32$, $p=0.14$, $r=-0.30$, $p=0.17$, $r=-0.31$, $p=0.15$, and $r=-0.23$, $p=0.29$ respectively for adiponectin, TNFα, IL-6 and IL-1β). As our study was cross sectional it is not possible to comment on the direction of these relationships, and further studies are needed to explain whether inflammation plays a role in the association between CBG and insulin resistance.

Our study was of middle-aged men and caution should be exercised in extrapolating our findings to women. There are many differences between the HPA axis in men and women [29,247,255,272], and indeed one of the reasons for studying men rather than women is that there is also subtle variation in the HPA axis during the menstrual cycle, especially in women with PCOS [273]. An inclusive study of the HPA axis should include three groups – men, post-menopausal women and pre-menopausal women ideally studied at the same phase of the menstrual cycle. Because this was a small study the former group was chosen, but further studies are needed to determine whether the same abnormalities of cortisol metabolism are present in women.

In summary we have found increased cortisol clearance and reduced CBG concentration with increasing insulin resistance amongst middle-aged men. It is possible that abnormalities of the HPA axis may underlie the development of insulin resistance and fatty liver disease, either directly through a primary increase in cortisol clearance or more probably through increased activity of hepatic 11β HSD leading to upregulation of the HPA axis and thereby increasing tissue exposure in adipose tissue with compensatory increase in cortisol clearance. Low levels of CBG may then occur as a result of a pro-inflammatory obese state, which further increase corticosteroid exposure.

7 THE ROLE OF BODY FAT, FITNESS AND PHYSICAL ACTIVITY IN INSULIN RESISTANCE AND THE METABOLIC SYNDROME

7.1 Introduction

It has been shown that high levels of fitness and increased physical activity are associated with lower prevalence of insulin resistance [274] and metabolic syndrome [275,276] and lower mortality [277]. It has been suggested that these factors may be more important than body fatness in determining insulin resistance and metabolic syndrome [278-280]. Both fitness and physical activity are often negatively related to body fatness [277] and many studies have not investigated these factors separately, although it has also been suggested that fitness attenuates metabolic risk independently of its effect on body fatness [281].

Exercise improves glucose uptake acutely by insulin-independent mechanisms as well as through the increased expression of GLUT 4 on muscle cell membranes [282] and an increase in whole body insulin sensitivity is seen relative to the intensity and duration of the exercise [283]. However weight loss through dietary restrictions results in similar improvement in insulin sensitivity to weight loss through exercise [284]. Furthermore although exercise intervention without weight loss does result in an improvement in insulin sensitivity, changes in body composition are seen in response to exercise which may account for the improvement in insulin sensitivity, namely reduced abdominal fat [284] or increased musculature [278]. Although in young women insulin sensitivity (measured by homeostasis modelling) has been demonstrated to be more closely related to VO_2 max than to fatness [278,279] the same effect has not been found in men.

Physical activity and fitness are terms which are sometimes used interchangeably in considering the impact of training and habitual exercise on

body composition, cardiovascular risk factors and cardiovascular disease. However it is important to attempt to differentiate between the effects of the different components of physical activity, as well as from the effects mediated by changes in body composition. It is helpful to consider the components of physical activity separately as cardiorespiratory fitness and habitual energy expenditure, although these components are clearly not entirely independent of one another [184].

The aim of this study was to determine whether fatness, fitness or physical activity is more closely related to insulin resistance and metabolic syndrome in men.

7.2 Methods

Twenty-five men aged 42-64 years participated in the study of physical activity and fitness. Cardiorespiratory fitness was determined after volunteers had avoided strenuous exercise for 24 hours and fasted for 8 hours. Physical activity was assessed by three days wearing a heart rate monitor and individual calibration using the treadmill or ergometer data, and using a questionnaire as described in the methods section. Tissue insulin sensitivity was measured using the euglycaemic hyperinsulinaemic clamp with di-deuterated glucose and NEFA suppression during the oral glucose tolerance test as previously described.

All statistical analyses were performed using SPSS for Windows version 14.0. Student's t test comparisons were undertaken to compare mean values of normally distributed data. Pearson correlation coefficients are presented for univariate regression analyses of normally distributed data. Where variables were not normally distributed log transformation was undertaken to normalise the distribution. Multivariate linear regression modelling was undertaken to describe the measures that were independently associated with insulin sensitivity and metabolic syndrome features as the dependent (outcome) variables. Independent (exposure) variables that were selected from the univariate analyses were entered into the models to examine the relationships with fatness

and to determine whether measures of physical activity and fitness were associated with insulin sensitivity independently of fatness. A p-value of <0.05 was considered to be statistically significant for all analyses.

7.3 Results

Twenty-five white, middle-aged men were studied and relevant characteristics are given in table 7.1. Distribution of fitness and physical activity energy expenditure amongst the subjects is shown in figures 7.1 and 7.2, and a scatter plot of fitness and PAEE is shown in figure 7.3. Fitness, as measured by VO_2 max measured on the treadmill or on a cycle ergometer (in three subjects), correlated with insulin sensitivity measured by glucose disposal during the clamp (M value) ($r=0.576$, $p=0.003$), and also with the measures of tissue insulin sensitivity: RD, suppression of endogenous glucose output and NEFA suppression (see table 7.2). However, the relationships between fatness and insulin sensitivity were much stronger ($r=-0.812$, $p<0.001$ for M value). Linear regression analysis showed that fitness was not independently related to any of the measures of insulin sensitivity, whilst fatness was a strong independent predictor of reduced insulin sensitivity. These analyses were repeated excluding the subjects who had exercised on the ergometer with very similar results.

To further explore the contribution of fitness to insulin sensitivity, fitness was added in to a regression model with fatness as the independent variable and M value as the outcome variable. The adjusted R square for the model without fitness was 0.646 indicating that fatness explains 65% of the variation in insulin sensitivity. Adding fitness into the model only increased the adjusted R square to 0.666 suggesting that fitness only contributes a very small amount to insulin sensitivity. This analysis was repeated for insulin sensitivity in individual tissues; and for RD, the measure of glucose disposal in muscle, the R square value was 0.665 increasing to 0.696 with fitness in the model. For suppression of endogenous glucose output R square was 0.249 only increasing to 0.268 with fitness in the model. However for NEFA suppression R square was 0.432 increasing to 0.682 indicating that fitness explains 25% of the variability in insulin

sensitivity in fat and fatness contributes 43%. These analyses were repeated using trunk fat in place of total fat with very similar results.

The relationship between VO_2 max and metabolic syndrome features was explored and VO_2 max was negatively associated with systolic and diastolic blood pressure, and positively associated with HDL cholesterol (see table 7.3). When fatness and VO_2 max were entered into a linear regression model neither fatness nor fitness was independently associated with blood pressure and only fatness was independently reciprocally associated with HDL cholesterol.

Physical activity energy expenditure (PAEE) data were available on 21 subjects. PAEE was not significantly associated with M value or RD, the measure of insulin sensitivity in muscle (see table 7.2) but was positively associated with suppression of endogenous glucose production ($r=0.551$, $p=0.01$) and suppression of NEFAs ($r=0.459$, $p=0.036$). Linear regression analysis was performed as previously and the relationships between PAEE and insulin sensitivity were not independent of body fat, with only fatness independently associated with the measures of insulin sensitivity.

To further explore the contribution of PAEE to suppression of endogenous glucose output and NEFA suppression PAEE was added in to a regression model with fatness as the independent variable and the measures of insulin sensitivity as previously. With suppression of endogenous glucose production as the outcome variable the adjusted R square for the model without PAEE was 0.269 and adding in PAEE increased the adjusted R square to 0.380 indicating that physical activity contributes 11% to hepatic insulin sensitivity. Repeating the analysis for NEFA suppression, the adjusted R square for fatness alone was 0.696 increasing to 0.729 when PAEE was added in to the model indicating that physical activity contributes only 3% to NEFA suppression. PAEE was not significantly associated with features of the metabolic syndrome (table 7.3).

Two individuals with low fitness had surprisingly high PAEE (figure 7.3). Although these results were thought to be valid, all analyses were repeated excluding

these individuals, in case of the possibility of confounding in the data, especially as one of the individuals had had oxygen uptake measured on the ergometer. Comparison with the Baecke questionnaire data did indicate that these PAEE results were surprisingly high in these subjects. When these two individuals were excluded from the analysis results were similar except that in the regression analysis with PAEE and total body fat, and suppression of hepatic glucose output as the outcome variable, PAEE was then independently associated with suppression of hepatic glucose output whereas fatness was not (standardised beta coefficient 0.576 p=0.022).

Physical activity was also assessed using the Baecke questionnaire. Suppression of hepatic glucose output was positively associated with both the total score (r=0.418, p= 0.042) and the score for sport (r=0.580, p=0.002) using this questionnaire. The relationship between the score for sport on the Baecke questionnaire and suppression of hepatic glucose output was independent of body fatness (standardised Beta coefficient 0.461, p=0.08). None of the other measures of insulin sensitivity or metabolic syndrome features were significantly associated with any of the scores from the Baecke questionnaire.

There was no statistically significant relationship between VO_2 max and PAEE, although there was a significant relationship between these variables when the two subjects with outlying results for PAEE were excluded from the analysis (r=0.56, p=0.013 see figure 7.3). There was no relationship between either VO_2 max or PAEE and the score on the Baecke questionnaire. Fitness was related to both total fatness and truncal fatness on DEXA and interestingly the relationship with total fatness was stronger than the relationship between truncal fatness (r= -0.574, p=0.003 and r= -0.544, p= 0.005 respectively). PAEE was also related to both total and truncal fatness (r= -0.448, p=0.042 and r= -0.469, p= 0.032 respectively). No significant relationship was demonstrated between score on the Baecke questionnaire and fatness. There was no significant relationship demonstrated between visceral or subcutaneous fat on MRI and VO_2max or PAEE.

Subjects with fatty liver were significantly more fit than subjects without fatty liver (VO_2max 32.6 v 24.4 ml/kg/min, p= 0.018), however subjects with fatty liver were also considerably fatter (total fat 20.9 v 36.9 kg, $p<0.001$) and more insulin resistant (M value 8.88 v 4.44 mg/kg/min, $p<0.001$) that those without. There was no significant difference in PAEE or score on the Baecke questionnaire for subjects with and without fatty liver.

There was no statistically significant relationship between cortisol clearance and either VO_2 max or PAEE.

Table 7-1 Characteristics of the 25 subjects in whom fitness was measured

	Mean ± SD	Range
Age (years)	52.6 ± 5.7	42.0-64.0
Body mass index (kg/m^2)	31.9 ± 7.3	20.8-48.9
Total fat measured on DEXA (kg)	28.8 ± 13.9	7.7-62.9
Trunk fat on DEXA (kg)	15.4 ± 8.3	3.3-36.1
Waist circumference (cm)	109 ± 19	80-150
Systolic BP (mmHg)	129 ± 12	102-147
Diastolic BP (mmHg)	74 ± 7	58-83
Fasting plasma glucose (mmol/l)	5.4 ± 0.56	4.5-7.0
HDL cholesterol (mmol/l)	1.38 ± 0.46	0.80-2.55
Triglycerides (mmol/l)	1.66 ± 1.0	0.70-4.90
Glucose disposal (mg/kg/min)	7.0 ± 3.5	2.2-13.9
RD mean steady state (micromol/kg/min)	38.9 ± 16.8	12.4-73.3
Suppression hepatic glucose output by 60 minutes insulin (%)	69.0 ± 23.9	24.4-100.0
NEFA percentage suppression 60 minutes after 75g OGTT (%)	59.0 ± 19.9	14.7-88.3
VO2 max (ml/min/kg)	29.0 ± 8.9	13.8-43.5
Mean physical energy expenditure (kcal/kg/day) n=21	9.04 ± 8.9	0.45-40.0
Baecke work score	2.5 ± 0.7	1.3-4.1
Baecke sport score	2.8 ± 0.9	1.0-4.5
Baecke leisure score	3.1 ± 0.7	2.0-4.5
Baecke total score	8.4 ± 1.7	4.3-10.3

Table 7-2 Pearson correlation coefficients between fatness, fitness and PAEE and insulin sensitivity

		M value	RD steady state	NEFA suppression by 60 minutes	Suppression hepatic glucose output by 60 minutes insulin
Total fat measured on DEXA	Pearson Correlation	-0.812	-0.831	-0.842	-0.547
(n=25)	Sig.	0.000	0.000	0.000	0.005
VO2 max	Pearson Correlation	0.576	0.602	0.524	0.475
(n=25)	Sig.	0.003	0.001	0.007	0.016
Physical activity energy expenditure	Pearson Correlation	0.172	0.076	0.459	0.551
(n=21)	Sig.	0.455	0.743	0.036	0.010

Figure 7-1 Distribution of fitness in 25 subjects

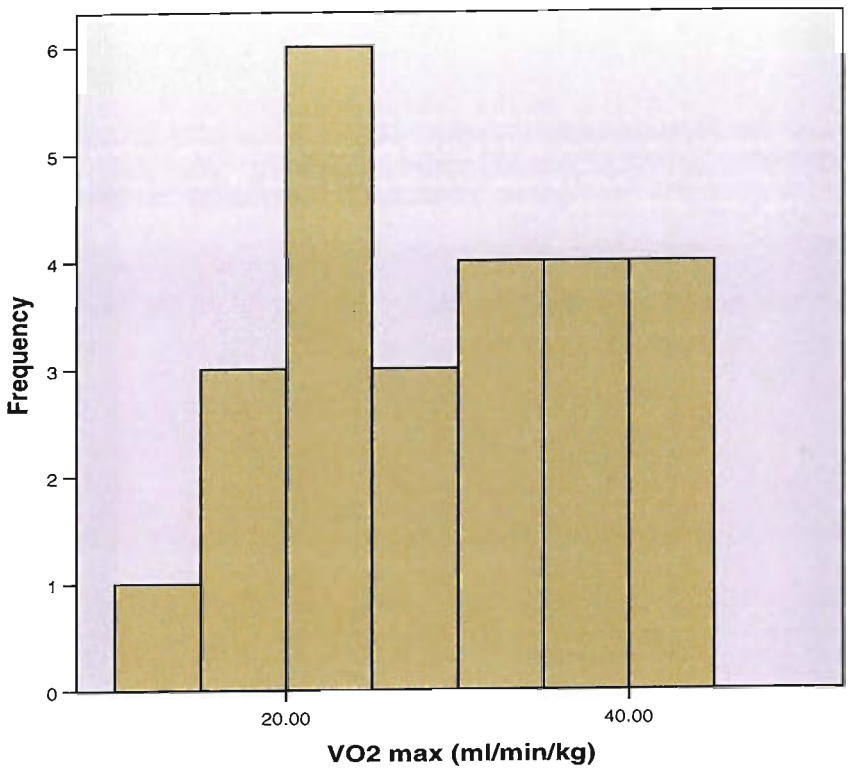


Figure 7-2 Distribution of physical activity energy expenditure in 19 subjects

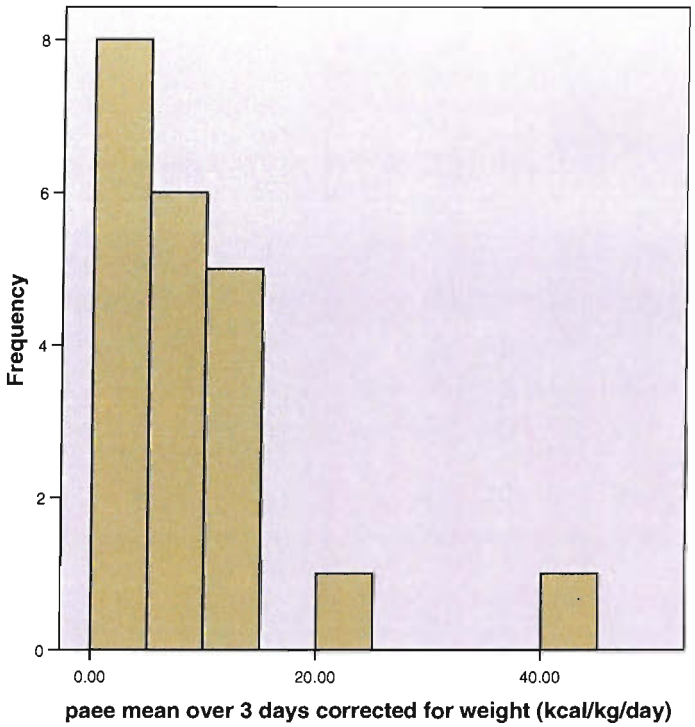


Figure 7-3: Scatter plot of PAEE against fitness

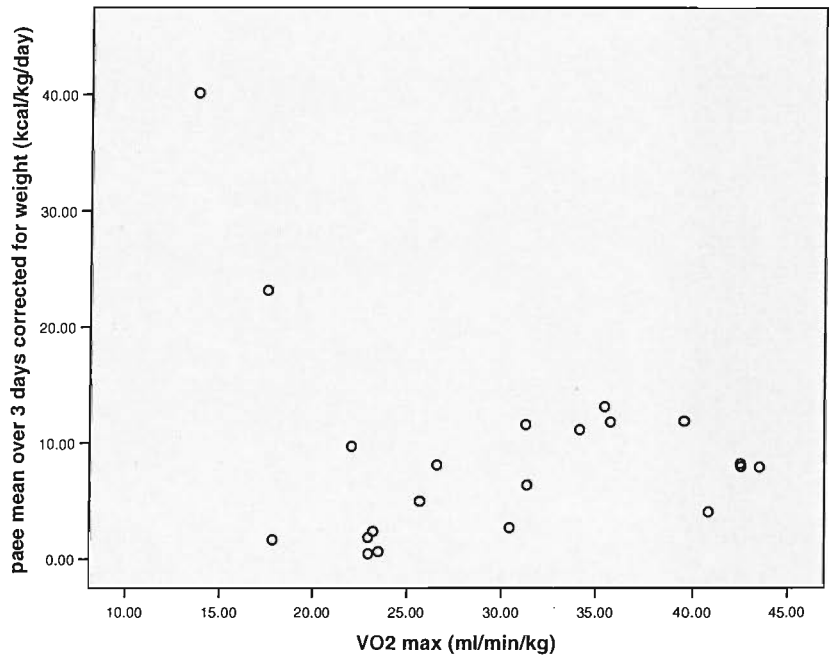


Table 7-3 Correlation between total fat, fitness and PAEE, and metabolic syndrome features

		Systolic BP	Diastolic BP	Fasting plasma glucose	Triglycerides	HDL chol
Total fat measured on DEXA n=25	Pearson Correlation	0.488	0.507	0.690	0.679	-0.557
	Sig. (2-tailed)	0.013	0.010	0.000	0.000	0.004
VO2 max n=25	Pearson Correlation	-0.474	-0.383	-0.286	-0.331	0.408
	Sig. (2-tailed)	0.017	0.059	0.166	0.106	0.043
Physical activity energy expenditure n=21	Pearson Correlation	-0.354	-0.220	-0.237	-0.409	0.351
	Sig. (2-tailed)	0.115	0.338	0.301	0.066	0.119

Discussion

We have demonstrated a relationship between fitness and insulin sensitivity but the correlation between fitness and insulin sensitivity is much less strong than the relationship between fatness and insulin sensitivity, and is not independent of fatness. The strength of our study is that we have measured body fatness in detail in addition to measuring fitness. We have demonstrated a strong and consistent negative correlation between fatness and insulin sensitivity, and so our results suggest that the relationship between fitness and insulin sensitivity is far less important than that between fatness and insulin sensitivity. Using multiple regression analysis we have shown that fitness contributes only 2-3% to insulin sensitivity in muscle and liver, when fatness contributes 65% and 23% respectively. We found that fitness contributed 25% to NEFA suppression with fatness contributing 43%, but fitness did not independently predict NEFA suppression either. Because our study is small, it is possible that we failed to demonstrate an independent relationship between fitness and insulin sensitivity which does exist, although the strength of our study is that we measured both fatness and insulin sensitivity in very careful detail, as well as measuring VO_2 max rather than using a sub-maximal test. We found an inverse relationship between fatness and fitness, which has also been demonstrated in larger studies [285].

Although it has been demonstrated that for a given level of abdominal fat, reduced fitness has a relative risk of being associated with the metabolic syndrome of around 1.8 [281], this risk is small compared with the relative risk of developing diabetes of 42 for men with BMI greater than 35kg/m^2 compared with those with BMI less that 23kg/m^2 [4], and when it is considered that up to 70% of individuals with type 2 diabetes have the metabolic syndrome [286]. Both low fitness and high fatness have been demonstrated to increase overall and cardiovascular mortality to a similar degree but it remains unclear to what extent these risks are independent of one another [277,287-289]. It has been suggested that low fitness is largely responsible for the increased mortality risk associated with

obesity [288] but a recent review of the literature emphasises the lack of evidence for improvements in metabolic profile in the absence of weight loss [181].

Interventional studies to improve fitness have shown benefit to cardiovascular risk factors but have also resulted in improvements in body composition [290,291] and regression analysis showed that improvement in body composition was responsible for improved insulin sensitivity, rather than improved fitness [291]. Only in very young females has exercise intervention been shown to improve insulin resistance without changes in body composition [278].

Physical training reduces visceral and subcutaneous fat mass when combined with dietary restriction, with no difference between aerobic exercise and resistance exercise [176]. We have demonstrated an inverse relationship between fitness and fatness, and energy expenditure and fatness, though interestingly we have not demonstrated a relationship between either component of physical activity and visceral fat on MRI. A study using questionnaires has shown that increased moderate intensity, weight-bearing exercise is associated with a reduction in total and abdominal fat accumulation and that this is more important than dietary intake, smoking or socio-economic status in determining obesity [177]. Resting metabolic rate is higher in trained individuals and therefore resting calorie expenditure is higher than in untrained individuals, augmenting the loss of fat mass for a given calorie intake in these individuals [180].

We found that fitness contributed 25% to the relationship between fatness and insulin sensitivity in adipose tissue compared with a minimal contribution to insulin sensitivity in muscle and liver. It has been suggested that exercise leads to improved insulin sensitivity in fat by increasing interleukin-6 (IL-6) release from muscle cells which increases lipolysis and induces gene transcription for lipid oxidation in adipocytes, and reduces TNFα [292], and it has been shown that inflammatory cytokines exert an effect on insulin sensitivity in vivo and in animal models [293,294]. However other studies have shown minimal effects of exercise on adipocytokines [295-297].

We demonstrated a negative correlation between VO_2 max and blood pressure, and neither fatness nor fitness were independently associated with blood pressure on regression analysis. The correlation co-efficients with fatness were much lower than those between fatness and insulin sensitivity and it is likely therefore that both fatness and fitness contribute to blood pressure independently, and that we were unable to demonstrate this because of our small sample size. Blood pressure is strongly associated with BMI [298] but a study of sympathetic nervous system activation, measured as extravascular norepinephrine release rate in response to aerobic physical training in hypertensive individuals showed a correlation between reduction in sympathetic nervous system activity which correlated to reduction in mean arterial blood pressure in response to training without an increase in insulin sensitivity, suggesting that there is an independent link between blood pressure and fitness [299] which we have been unable to demonstrate because of our small numbers.

We did not show an association between PAEE and whole body insulin sensitivity, tissue insulin sensitivity or metabolic syndrome features. There are much fewer data available on whether habitual physical activity is beneficial than there are for fitness, and this is presumably because of the difficulty in measuring physical activity. Many studies are questionnaire-based, relying on self-reported physical activity levels, but there is evidence that physical activity is beneficial, although again, there is little evidence that the benefits are independent of the improvement in body composition [170-173,178]. We used an accepted and well-validated method using energy expenditure data from the treadmill or ergometer combined with three days wearing a heart-rate monitoring device [300,301], but it is possible that the days during which the subjects wore the monitoring device were not typical of their normal habits, or that wearing the device altered behaviour. Additionally we did not use a device combined with a movement sensor to ensure that increased heart rate was associated with physical activity, which might have improved accuracy [302]. In addition there was surprisingly little spread in the PAEE data (figure 7.2), which may have reduced the likelihood of finding a relationship with insulin sensitivity.

It was of concern that there were two significantly outlying subjects with high PAEE results and low fitness (figure 7.3). The subject with the highest PAEE was one of the subjects who had his VO_2 measured on the ergometer, rather that the treadmill, and so it is possible that overestimation of workload at specific heart rates occurred compared to subjects who had exercised on the treadmill. Comparison with data from the Baecke questionnaire suggested that these PAEE readings were surprisingly high for these individuals, again raising the possibility of confounding in the data for these subjects. The only result which was significantly different on excluding these individuals was the relationship with fitness, which showed a significant positive correlation.

These factors combined with a relatively small sample size mean it is possible that we have not detected a difference which does exist. The relationships described between PAEE and metabolic syndrome features have been on larger population-based studies rather than small, very detailed studies such as this [184,301]. Our most notable finding, as with fitness, is that the relationship with these features is much stronger with fatness than with physical activity.

We did find a significant relationship between PAEE and NEFA suppression and suppression of hepatic glucose output. These relationships were not independent of fatness and regression analysis indicated that PAEE contributed a very small amount to suppression of NEFAs (3%) but 11% to suppression of endogenous glucose production by the liver. This was surprising given our failure to demonstrate a relationship with whole body or muscle insulin sensitivity, however our findings were supported by the finding that the Baecke questionnaire score for sport, which comprises information on number of sports and amount of time spent in sporting activity as a measure of physical activity, was positively and independently associated with suppression of endogenous glucose production by the liver. When the two outlying subjects were excluded from the analysis there was also an independent relationship between PAEE and suppression of hepatic glucose output, although these results are treated with caution.

There is little information in the literature on the direct effect of physical activity levels on hepatic insulin sensitivity [303], although hepatic insulin sensitivity correlates closely with abdominal fat stores [114]. Hormone sensitive lipase (HSL) catalyses lipolysis of triglycerides in adipocytes [304] and this enzyme is up-regulated by exercise [305]. It is possible therefore that reduced HSL associated with low levels of habitual exercise increases intra-abdominal fat stores and thereby increases overall fatty acid supply to the liver resulting in reduced hepatic insulin sensitivity [152]. It is surprising, if this is the case, that there was not a stronger association between NEFA suppression and energy expenditure. Interestingly VO_2 max did contribute more significantly to NEFA suppression than to the other measures of insulin sensitivity (25%).

We demonstrated an association between VO_2 max and HDL cholesterol which was not independent of fatness, and a relationship between PAEE and triglycerides which did not quite reach statistical significance ($p=0.066$). Improvement in lipid profile has been demonstrated with both high fitness and increased physical activity [182,306]. High intensity exercise in particular has a beneficial effect on LDL concentration and particle size resulting in increased average LDL particle size and HDL concentration is also increased by high intensity exercise [181]. Even a modest amount of low intensity exercise has a benefit in reducing circulating triglycerides [182]. The putative mechanism of action by which exercise improves lipid profile is by activation of muscle AMP-activated protein kinase (AMP kinase), which upregulates lipid metabolism and increases fatty acid oxidation and decreases re-esterification through direct effects on enzymes such as Malonyl-CoA Decarboxylase [183], although changes in body composition are also contributory [181,307].

In summary we have found that although greater levels of fitness are associated with higher insulin sensitivity in all tissues, and greater physical activity energy expenditure is associated with higher insulin sensitivity in liver and adipose tissue, these finding are not independent of body fat. Because the associations between insulin sensitivity and fatness are so strong and independent of VO_2

max and PAEE we believe that fatness is much more important than fitness or habitual physical activity in determining insulin resistance, and that some of the benefits of exercise may be mediated through improvements in body composition. Further interventional exercise studies are needed to determine whether improved insulin sensitivity is mediated by a reduction in body fat.

8 CONCLUSIONS

We have explored four aspects of the association between obesity and insulin sensitivity. We have shown firstly, that in men there is very close correlation between the simplest measurements of body composition, namely BMI and waist, and the more detailed measurements of body composition, as well as with insulin resistance and the other features of the metabolic syndrome. This is important because as obesity prevalence increases the very simplest tools can be used to identify which individuals need the secondary screening measurements of blood pressure, lipid and glucose measurement.

In our study we have found that there is no biologically significant difference between visceral fat measured on MRI, or trunk fat on DEXA, compared with waist circumference or BMI in the associations with insulin resistance and metabolic syndrome features. Although the numbers are small for the visceral fat data, we have used a five-slice abdominal MRI technique which is more detailed than that used in many studies. We have concluded that the important associations with insulin sensitivity and metabolic syndrome features demonstrated in our subjects are with total fat mass, and that even if visceral fat is more biologically active than subcutaneous fat as has been suggested [96,161], visceral and abdominal fat are markers of total fat mass in men.

We went on to explore the relationship between fatty liver disease and fatness and insulin sensitivity. Non-alcoholic fatty liver disease is the most recently described of the metabolic syndrome features, with huge implications for patient morbidity and mortality, in addition to resource implications, and we showed that this is closely linked to insulin sensitivity in muscle and fat, independently of the association with body fat. We did not demonstrate a difference in hepatic insulin sensitivity in the subjects with fatty liver but clamp insulin levels were higher in the subjects with fatty liver for the same degree of suppression of hepatic glucose output so it is possible that our findings also represent impaired hepatic insulin sensitivity in these subjects.

We showed no significant difference in visceral fat between the subjects with and without fatty liver, despite the subjects with NAFLD having more total and trunk fat. We have concluded that in insulin resistant subjects there is an abnormality of adipocyte function, not necessarily specific to visceral fat, which results in increased non-esterified fatty acid supply to the liver and the release of inflammatory cytokines, and together these factors lead to the development of fatty liver.

We have examined the role of the HPA axis in insulin resistance. We measured cortisol clearance using a tandem mass spectrometry technique to detect clearance of a tracer dose of deuterated cortisol. We found a strong inverse correlation between the clearance of cortisol and whole body insulin sensitivity and insulin sensitivity in muscle and adipose tissue, which was independent of body fat. We did not find a relationship between other aspects of the HPA axis, namely morning cortisol or response to $ACTH_{1-24}$ and insulin sensitivity, although we did find a relationship between CBG and insulin sensitivity which was not independent of cortisol clearance or fatness. We have concluded that it is possible that abnormalities of the HPA axis may underlie the development of insulin resistance and fatty liver. There is evidence of increased 11βHSD activity in adipose tissue of insulin resistant individuals [308] but downregulation in liver [256,309] which may lead to adipose tissue-specific increased glucocorticoid exposure with simultaneous upregulation of the axis because of decreased levels in liver. These abnormalities may be familial or contributed to by the intrauterine environment, both of which have an impact on the HPA axis [162,263]. Down-regulation of 11βHSD in liver may also explain why we found that insulin sensitivity in liver was not related to cortisol clearance but tissue insulin sensitivity in muscle and adipose tissue was.

We explored the role of fitness and physical activity in insulin resistance and metabolic syndrome. We found that both fitness and physical activity level were inversely related to fatness, with no closer relationship between trunk fatness than total fatness and no relationship between visceral fat and either fitness or

physical activity level. Although we did demonstrate a relationship between fitness and insulin sensitivity this was much less strong than the relationship between total or trunk fatness and insulin sensitivity and was not independent of fatness. Similarly physical activity level was not related to insulin sensitivity independently of fatness. Neither fitness nor fatness was independently related to other metabolic syndrome features. Multiple regression analyses indicated that fitness did contribute 25% to insulin sensitivity in adipose tissue and that physical activity levels contributed 11% to insulin sensitivity in liver, but that the contributions to insulin sensitivity in other tissues was minimal.

We have concluded that fatness is much more important than either fitness or physical activity in determining insulin sensitivity and that it is likely that the predominant effect of components of physical activity on insulin resistance is mediated through its effect on body composition. It is interesting that we did find that fitness contributes to insulin resistance in fat, because it has been suggested that IL-6 released from exercising muscle may exert a beneficial effect by inducing lipolysis and gene transcription in adipose tissue and inhibiting TNFα [292], and inflammatory cytokines also contribute to the development of insulin resistance in adipocytes [293,294]. We found that physical activity levels contributed to insulin sensitivity in liver. One explanation for this would be that exercise increases levels of hormone sensitive lipase [305] which catalyses lipolysis of triglycerides in adipocytes [304]. A smaller adipose tissue mass may therefore result in increased hepatic insulin sensitivity through reduced fatty acid supply to the liver [152].

We undertook 5-slice MRI studies of the obese subjects to evaluate visceral fat and also had DEXA data on trunk fat for all subjects. Where information was available visceral fat did not show a closer relationship with any of the factors we studied than trunk fat, namely insulin sensitivity in muscle, fat and liver; fatty liver, fitness or energy expenditure. Trunk fat did show a very slightly stronger relationship than total fat with blood lipid and glucose levels and some aspects of insulin sensitivity but the differences were very small, even when compared with

BMI and waist circumference. We did not demonstrate a stronger relationship between trunk fat and fatty liver, cortisol clearance, fitness or physical activity. We found that trunk fat and visceral fat correlated very strongly with total fat and our findings suggest that it is total fat rather than visceral fat which is associated with metabolic abnormalities in men.

There is a considerable literature linking insulin resistance to visceral fat [48,82,84] although Frayn has pointed out that the subcutaneous abdominal fat mass, which is very much larger than the visceral fat mass, may also be a source of high levels of non-esterified fatty acids [47]. Smith found that total fat mass and deep subcutaneous fat were more important than visceral fat in predicting insulin resistance [209]. Interestingly the studies of 11βHSD in which increased regeneration of cortisone to cortisol was inferred from 11βHSD expression in adipose tissue biopsies were of subcutaneous fat [167,310], and in the case of one study from the gluteal region [167], indicating that subcutaneous fat may well also be important in the development of insulin resistance.

It is also true that visceral fat to subcutaneous fat ratio is increased in subjects with Cushing's syndrome [311], and Bjorntorp has argued that visceral fat accumulation related to abnormalities of the HPA axis underlies the metabolic syndrome [312], although there have been contrasting findings that cortisol metabolism is lower in association with visceral fat accumulation after adjustment for total fat mass [20]. Discussing an alternative view, Drapeau has pointed out that visceral fat has more cells per mass unit, higher blood flow and more glucocorticoid receptors than other adipose tissue and abdominal obesity may therefore develop in response to increased circulating cortisol [313,314]. Our findings are that cortisol clearance is related to insulin sensitivity independently of fat mass, and that fat mass is also independently associated with cortisol clearance and we believe that abnormalities of the HPA axis may underlie the development of insulin resistance and obesity. Because we found that total fat was strongly linked to whole body insulin sensitivity and to insulin resistance in muscle, liver and fat it is possible that abnormal adipocyte function leading to insulin

resistance in adipocytes leads in turn to whole body insulin resistance and the concurrent development of obesity.

Our finding that fatty liver is independently associated with insulin resistance in adipose tissue independently of total, truncal or visceral fat mass suggests that an abnormality of adipose tissue function rather than quantity underlies the development of fatty liver in insulin resistant states. Our findings suggest that increased lipolysis and secretion of pro-inflammatory cytokines from adipocytes leads to the development of fatty liver disease.

It is tempting at this point to bring the main findings together and link the abnormal adipocyte function suggested by the fatty liver and cortisol clearance results to the close association between insulin resistance and whole body obesity by suggesting that abnormalities of adipocyte function lead to obesity. However is recognised that obesity is associated with excess calorie intake [315,316] and less physical activity [185] [56], and our own findings also indicate that fitness and energy expenditure are linked to obesity. It is probable therefore that insulin resistance develops through a combination of abnormal adipocyte function and increased fat mass related to excess calorie intake and reduced energy expenditure and fitness. It is yet to be determined what the relative contributions of these factors are, and as fascinating animal data suggests that rats deficient in adipose tissue 11βHSD1 fail to develop obesity in the face of increased calorie intake [257] it remains possible that the two abnormalities may be linked.

In conclusion our study suggests that abnormal function of adipocytes, as well as increased total fat underlie insulin resistance and the metabolic syndrome, with increased exposure to cortisol, increased fatty acid release and release of pro-inflammatory cytokines leading to the development of insulin resistance in other tissues, as well as the development of fatty liver disease.

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INDEX

Air displacement plethysmography 44
Anthropometry and blood pressure 41
Assessment of cardiorespiratory fitness and energy expenditure 36
Assessment of hepatic insulin sensitivity 26
Assessment of insulin sensitivity 24
Assessment of non-alcoholic fatty liver disease 31
Assessment of whole body insulin sensitivity 47
Baecke Questionnaire 54
Bioelectrical impedance 44
Bioimpedance and other measurements of total body water 18
Blood pressure 46
Body composition 56
Body Mass Index (BMI) 16, 41
Calculating body fat percentage from skin fold thickness 43
Cardiorespiratory fitness 54
Central obesity 29
Corticosteroid binding globulin (CBG) 50
Cortisol clearance 51
Dual energy x-ray absorptiometry (DEXA) 18, 44
Dyslipidaemia 29
Energy expenditure 38
Ethical approval 39
Euglycaemic hyperinsulinaemic clamp 24
Features of the metabolic syndrome 28
Fitness 114
Free cortisol 50
Glucocorticoid 96
HDL cholesterol 46
Height 41
Hip circumference 42
Homeostasis model assessment (HOMA) 24
HRFlex method 53
Hydrostatic weighing and air displacement plethysmography 19
Hypertension 30
Hypothalamic pituitary adrenocortical axis (HPAA) studies 50
Hypothalamic-pituitary-adrenocortical axis (HPAA) 33, 96
Inclusion criteria 40
Insulin resistance 20, 28
  Definition, pathogenesis and molecular mechanisms 20
Insulin sensitivity in adipose tissue 49
Insulin sensitivity in muscle and liver 49
Interplay between the features of the metabolic syndrome 32
Lipid profile 46

Liver biopsy 32
Liver enzymes 32, 47
Low dose ACTH test 50
Magnetic Resonance Imaging (MRI) 20, 44
Mass spectrometry for deuterated cortisol 52
Maximal oxygen uptake 37
Measures of regional fatness 19
Medical history and examination 41
Metabolic syndrome 27
  Definition, epidemiology and metabolic consequences 27
Morning cortisol 50
MRI spectroscopy 31
NAFLD
  Definition, pathogenesis and epidemiology 30
  Ultrasonography 31, 46
Non-alcoholic fatty liver disease (NAFLD) 30, 46, 78
Obesity 14
  Assessment of Obesity 15
  Definition, epidemiology and metabolic consequences 14
Oral glucose tolerance test 26
Oral glucose tolerance test (OGTT) – 75g 47
Physical activity 114
Physical activity and fitness 35
Physical activity energy expenditure (PAEE) 53
Physical activity questionnaires 54
Preparation of deuterated cortisol 51
Recruitment of subjects 39
Research facility 39
Skin fold thickness 42
Skin fold thickness measurement 17
Subject characteristics 40
Suppression of free fatty acids 27
Tissue insulin sensitivity 49
Total cholesterol 46
Treadmill assessment of VO_2 max 54
Triglycerides 46
VO_2max 37
Waist circumference 17, 20, 41
Waist hip ratio 20
Weight 41