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UNIVERSITY OF GLASGOW

Metabolic and Vascular Effects of Intentional Weight Loss in Type 2 Diabetes

Submitted for the Degree of Doctorate of
Medicine

Dr Claire McDougall

October 2010

Work undertaken in the Division of Cardiovascular and Medical Sciences, Faculty of
Medicine, University of Glasgow.

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List of Abbreviations

ACH	Acetylcholine
ADP	Air displacement plethysmography
AI	Augmentation index
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adult Treatment Panel
AUC	Area under curve
BMI	Body Mass Index
BP	Blood Pressure
BRFSS	Behavioural Risk Factor Surveillance System
CAD	Coronary artery disease
CRP	C-reactive protein
CT	Computerised tomography
CVD	Cardiovascular disease
DAG	Diacylglycerol
D _b	Body density
DPP	Diabetes Prevention Program
DPS	Diabetes prevention Study
DXA	Dual energy x-ray absorptiometry
EDHF	Endothelium-derived hyperpolarizing factor
EGIR	European Group on Insulin Resistance
EID	Endothelium-independent dilatation
ENOS	Endothelial nitric oxide synthase

ET-1	Endothelin-1
FFAs	Free fatty acids
FFM	Fat-free mass
FIRI	Fasting Insulin Resistance Index
FMD	Flow-mediated dilatation
FPG	Fasting plasma glucose
GGT	Gamma-glutamyl peptidase
GLUT4	Glucose transporter 4
GTN	Glyceryl trinitrate
HbA1c	Glycosylated haemoglobin
HDL-C	High-density lipoprotein cholesterol
HOMA	Homeostasis Model Assessment
hsCRP	High sensitivity c-reactive protein
HTA	Health Technology Assessment
IA	Intraarterial
ICAM	Intercellular adhesion molecule
IGF1	Insulin-like growth factor 1
IL-6	Interleukin-6
IMT	Intima media thickness
IRS	Insulin receptor substrate
LDI	Laser Doppler Iontophoresis
LDL-C	Low density lipoprotein cholesterol
L-NMMA	L-N ^G -monomethyl arginine citrate
MAP	Mitogen-activated protein
MI	Myocardial infarction

MRI	Magnetic resonance imaging
NAFLD	Non-alcoholic fatty liver disease
NF- κ B	Nuclear factor κ B
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
NOS	Nitric oxide synthase
PAI	Plasminogen-activator 1
PI3-K	Phosphatidylinositol 3-kinase
PU	Perfusion units
PWA	Pulse wave analysis
PWV	Pulse wave velocity
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SEM	Standard error of the mean
SNP	Sodium nitroprusside
TBW	Total body water
TC	Total cholesterol
TGs	Triglycerides
TNF α	Tumour necrosis factor alpha
UKPDS	United Kingdom Prospective Diabetes Study
UWW	Underwater weighing
VAT	Visceral adipose tissue
VCAM	Vascular cell adhesion molecule
VLCD	Very low calorie diet
VLDL-c	Very low density lipoprotein cholesterol

vWF	von Willebrand factor
WC	Waist circumference
WHO	World Health Organisation
WHR	Waist-hip ratio

SUMMARY

Obesity is increasing in prevalence, in both the developed and the developing worlds. This is due to an increase in the availability of energy dense food and globally reduced levels of physical activity. Obesity would appear to be the driving force for increased prevalence of type 2 diabetes.

The processes that lead to the development of insulin resistance and ultimately type 2 diabetes may also play a significant role in the development of endothelial function. Biological changes leading to insulin resistance may also cause impairment of endothelium-mediated vasodilatation. This is thought to be one of the first manifestations of atherosclerotic cardiovascular disease (CVD), also a major burden on individuals and health services. Endothelial dysfunction has been shown to be a surrogate marker of underlying risk for atherosclerotic cardiovascular disease.

The effects of intentional weight loss on reducing the burden of cardiovascular disease remain unclear. Although it is recognised that intentional weight reduction can ameliorate insulin resistance, hypertension and abnormalities of lipoprotein metabolism, there is little evidence to suggest that weight loss reduces CVD-related morbidity. A number of studies have attempted to address this issue by assessing the effects of weight loss on endothelial function. However, few of these studies have included subjects with type 2 diabetes, and a number of them have included exercise interventions, which have been shown to have effects on vasculature over and above the effects of weight reduction.

Given the increased risk of cardiovascular morbidity and mortality associated with type 2 diabetes, and the need for further strategies addressing this issue, this study aimed to assess the effects of intentional weight loss using solely dietary manipulation in a cohort of subjects with type 2 diabetes.

Fifteen male and female subjects with type 2 diabetes were recruited from local diabetes clinics and advertisements in local press. A six-week period of dietary intervention in the form of a 1200kal/day liquid diet was prescribed, with subjects being closely monitored by a dietician during this period. Vascular and metabolic assessments were performed at baseline and following a one-week dietary washout period at the end of the intervention.

This dietary intervention led to significant reductions in body weight, waist and hip circumferences and body fat percentage, measured by three distinct methods. Change in body weight correlated with change in waist circumference, but there were no correlations between change in body composition or any of the other anthropometric measurements. The probable reason for this was the small numbers involved.

Weight reduction was associated with improvements in metabolic parameters. Significant reductions in fasting plasma glucose, glycated haemoglobin and whole body insulin sensitivity as measured by the isoglycaemic hyperinsulinaemic clamp technique were noted after the intervention. In addition, there were significant reductions in liver enzymes (surrogate markers of hepatic steatosis), and in serum

total cholesterol concentrations. Changes in glycaemic control were closely correlated with changes in liver enzymes.

With the exception of serum intercellular adhesion molecule-1 (ICAM-1), there were no improvements in any of the measured concentrations of adipocytokines or inflammatory markers following weight reduction. Serum concentrations of ICAM-1 were significantly reduced as a result of the intervention, with no changes in C-reactive protein, interleukin-6 or adiponectin being observed. The possible reasons for these results are multifactorial.

Weight loss was associated with variable effects on vascular function. Three modalities were employed for the assessment of the vasculature; wire myography, laser Doppler inotophoresis and pulse wave analysis / velocity measurements. Significant reductions in endothelium-dependent vasodilatation as measured by both wire myography and laser Doppler iontophoresis were observed after weight reduction. In contrast, however, there was a significant improvement in pulse wave velocity measurements.

In summary, weight reduction in a cohort of subjects with type 2 diabetes led to significant improvements in metabolic parameters, little change in markers of inflammation and a deterioration in endothelium-dependent vasodilatation.

CHAPTER 1

INTRODUCTION AND BACKGROUND

1.0 Introduction

Obesity, defined as a body mass index (BMI) of $> 30\text{kg/m}^2$ is increasing in prevalence, in both the developed and the developing worlds. This is most probably due to an increase in the availability of energy dense, inexpensive food that is of low nutritional quality and high in fat and globally reduced levels of physical activity. Obesity would appear to be the driving force for increased levels of type 2 diabetes (Gregg et al, 2004), the prevalence of which for all age groups worldwide was estimated to be 2.8% in 2000 and is predicted to rise to 4.4% in 2030. The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al, 2004).

Biological changes leading to insulin resistance may also play a role in the development of endothelial dysfunction, which is thought to be one of the first manifestations of atherosclerotic cardiovascular disease (CVD), also a major burden on individuals and health services. The effects of intentional weight loss on reducing this burden remain unclear.

1.1 Epidemiology of obesity

1.1.1 Obesity in Europe and the USA

Data on the prevalence of obesity in the USA comes from two sources; the Behavioural Risk Factor Surveillance System (BRFSS) and the National Health and Nutrition Examination Survey (NHANES). The BRFSS conducts annual telephone surveys of randomly selected individuals who are representative of the general population, and subjects are asked to self-report on height and weight. The NHANES population have height and weight measured by a health professional: the latest survey suggested a prevalence of BMI of $>30\text{kg/m}^2$ of 32.2% in North American adults over the age of 20 years (Ogden et al 2006). Self-reported BRFSS data suggest a figure about 8% lower than the NHANES data.

In Europe, the most reliable estimates of obesity prevalence come from the UK. The Health Surveys for England database examines long-term trends in body weight and other demographic variables. In the most recent survey from 2003, it was estimated that 22% of men and 23% women were overweight. A further report from this organisation has estimated that if current trends continue, a further 3.5 million people in the UK will be obese by 2010 (Health Survey for England / DOH).

1.1.2 Obesity in the Developing World

The prevalence of non-communicable diseases is increasing at an alarming rate in the developing world, with worldwide figures of 18 million deaths per year from CVD in 2004. Projected figures suggest that this will increase to around 24 million by

2030. It has been estimated that cardiovascular mortality rates will increase further in low and medium income countries than in high-income countries (The global burden of disease report update: 2004 update; WHO publications). The main driving force for this significant increase in CVD mortality is the global pandemic of type 2 diabetes, which is associated with increased rates of obesity. The estimate of worldwide obesity currently stands at 312 billion (Hossain et al, 2007).

Rates of obesity in developing countries have tripled over the past 20 years as a result of reduced energy expenditure and the consumption of inexpensive energy-dense food. People from the Middle East, Pacific Islands, Southeast Asia and China seem to be at greatest risk of obesity and its complications (Hossain et al, 2007). These developing countries in particular face the paradox of families in which the children are underweight and the adults are overweight (Hossain et al, 2007). This combination has been attributed to intrauterine growth retardation and resulting low birth weight, which apparently confer a predisposition to obesity later in life through a "thrifty" phenotype that, when accompanied by rapid childhood weight gain, is conducive to the development of insulin resistance and the metabolic syndrome (Hales et al 1992, Diabetologia).

1.1.3 Risk factors for obesity

Several epidemiological studies have shown that certain demographic, sociocultural and behavioural factors are associated with overweight and obesity. For example, the Health Survey for England illustrates associations between increasing age,

female gender and obesity. Obesity and overweight also seem to be associated with lower socio-economic status in industrial societies, although this relationship may not be so striking in the developing world, where middle income individuals seem to be at higher risk of obesity. The long-term effects of lifestyle in adults have been more difficult to assess. However, one retrospective questionnaire-based study has suggested some lifestyle factors that are associated with obesity in older adults. Data was analysed from the National Health Interview Survey, which is distributed to 40,000 households across all states in the US. In males aged older than 50 years, obesity was associated with previous smoking history, eating less than two portions of fruit / vegetables per day, and light to moderate alcohol intake. Obese males were less likely to walk for leisure or transportation purposes than overweight or normal weight males. Risk factors were noted to be similar for older females, with the exception that women who drank no alcohol were more likely to be overweight than those with light or moderate alcohol consumption (Kruger et al, 2009).

1.2 MEASURES OF BODY COMPOSITION

1.2.1 Anthropometric measures of body composition

a. Body mass index

Body mass index (BMI) is the most commonly used parameter of underweight or obesity, and is calculated by dividing body weight (in kilograms) by the square of height (in metres). This measurement is commonly used both in clinical practice and in the clinical trial setting. BMI is a useful tool for observing secular trends and regional variations in countries. In population settings there is good correlation between BMI and percentage body fat. The measurement has formed the basis for worldwide classifications of obesity, with a BMI of 20-24.9 kg/m² being classed as normal, with the “ideal” suggested as 23 kg/m². ‘Overweight’ (grade 1 obesity) is defined as 25-29.9 kg/m², obesity (grade 2) as BMI 30-39.9 kg/m², and morbid obesity (grade 3) as >40 kg/m² (World Health Organization, 1995).

Associations between BMI and mortality have been repeatedly demonstrated in studies (Engeland A et al. 2003; Zhu S et al 2003). These studies have demonstrated that the relationship between BMI and all-cause mortality is U or J shaped – mortality is lowest at approximately 23 kg/m² and increases both with increasing obesity and under-nourished states. BMI is therefore useful in population assessments to document trends in obesity, or to assess health inequalities between populations.

However, BMI is relatively less useful in the practice of obesity management. Its main drawback is that it gives no estimate of body composition or excess fat mass. It may therefore overestimate body fat in certain clinical scenarios such as reduced height due to kyphosis, or increased body weight due to oedema or increased muscularity.

Ageing leads to a progressive increase in the ratio between fat and lean body mass, and this trend has been shown in subjects whose BMI remains constant throughout life, suggesting that BMI cannot be a true indication of body fat mass throughout life. A number of differences in the relationship between BMI and body fat between ethnic groups have also been demonstrated. Asians have been shown to have a higher percentage of body fat for a given BMI (WHO Expert Consultation, 2004), while the reverse is true for African-Americans (Banerji et al, 1999; Deurenberg et al, 1995). In addition, certain groups in society such as athletes and military and civil forces personnel are often considerably leaner than suggested by their BMI.

b. Skinfold thickness

The measurement of skinfold thickness at a number of body sites to estimate body fat stores has been employed for many years. Subcutaneous fat is measured using callipers that exert a standard pressure, and from these measurements body density can be predicted. Percentage body fat can then be estimated using either the Brožek (1963) or Siri (1961) equation. Skinfold thickness is commonly measured at four sites: triceps, biceps, subscapular and suprailiac. After log transformation of the data, there is a linear relationship between the sum of the skinfold thickness at these sites and body density, which is age and gender specific (Durnin et al 1973, 1974).

Fuller et al (1992) have shown that measurements made by a single observer can give good agreement with reference methods. However the technique contains the error associated with the prediction of body density. Although the density of fat mass is reasonably constant, that for the fat-free mass is variable, due to inter-individual differences in bone mineral mass and total body water. In addition the technique has been shown to be poorly reproducible between observers (Fuller et al 1991). There may also be specific problems in the obese including the compressibility of large subcutaneous fat pads, and the presence of oedema.

1.2.2 Other methods of assessing body composition

A number of methods of measuring body composition have been developed over the past few decades. These methods are described as two, three, or four compartment models, depending on the amount of information they provide on the different components of the body's lean or "fat free" mass (FFM).

a. Two compartment models

Development of the classic two compartment (2-C) model of body composition has accelerated in recent years because of the association of excess body fat with increased risk for cardiovascular disease. In the basic 2-C model, the body is divided into two parts: body fat and fat free mass (FFM). There are a number of methods of measuring body composition based on this model.

Underwater weighing

Underwater weighing was the first 2-C model of body composition to be used, and remains the gold standard for measuring body composition. This method involves fully submerging the subject in water; the volume of water being displaced and the subject's body weight are used to calculate the body density (D_b). Studies have shown that the density of fat is relatively constant in humans. However, because of its heterogeneous nature, the density of fat-free mass varies depending on the amount of water (Forslund et al 1996) or bone mineral (Martin and Drinkwater 1991) it contains. Therefore, there is a degree of error associated with this method in estimating body fat percentage. In addition it is an impractical and cumbersome procedure to perform or undergo.

Air displacement plethysmography

Air displacement plethysmography (ADP) has been in use since the early 1900's but has only recently started to replace underwater weighing as a means of measuring body composition. The subject is not immersed in water, but in a closed air-filled chamber. The system consists of two chambers: one for the subject and the other serving as a reference volume. The volume of an object is measured indirectly by measuring the volume of air it displaces within a closed chamber. Body volume is calculated indirectly by subtracting the volume of air remaining inside the chamber when the subject is inside, from the volume of air in the chamber when it is empty. The air inside the chamber is measured by applying relevant physical gas laws. Boyle's Law states that at a constant temperature, volume (V) and pressure (P) are inversely related:

$$P_1 / P_2 = V_1 / V_2$$

However, the requirement for constant temperatures within these systems previously made measurements arduous and impractical. Difficulties in maintaining isothermal conditions arose as a result of rapid fluctuations in temperature, humidity and pressure generated by humans entering the enclosed chamber (Friis Hansen et al, 1963; Gundlach et al, 1986).

In the mid 1990's the BOD POD became the first commercially available air-displacement plethysmograph. The design and operating principles of this system are described in detail elsewhere (Dempster 1995; Life Measurement, Inc. BOD POD body composition system: operator's manual. Concord, CA: Life Measurement, Inc, 1997). With this system, it is unnecessary to conduct measurements under isothermal conditions, the air being allowed to compress and expand adiabatically (i.e. freely gaining and losing heat during compression and expansion). The BOD POD makes use of Poisson's law, which describes the pressure-volume relationship under adiabatic conditions:

$$P_1 / P_2 = (V_1 / V_2)^\gamma$$

where γ is the ratio of the specific heat of the gas at constant pressure to that of constant volume and is equal to 1.4 for air. Body density and body fat can then be estimated by a variety of calculations.

The BOD POD has been shown in a number of studies to be reliable in assessing both body volume and body fat percentage (Sardinha et al, 1998; Biaggi et al, 1999) and shows excellent comparison with UWW, with improved reproducibility and enhanced subject acceptability (Fields et al, 2002). Although this method presents similar problems to UWW with respect to the heterogeneous nature of the fat-free

mass, it is a useful method in assessing changes in body composition after a period of weight loss.

b. Three compartment models

The theory behind three compartment (3-C) models of body composition is that fat-free mass is divided into two components: its water content and the remaining solids (predominantly proteins and minerals). Three compartment models tend to be based on dilution techniques measuring total body water, in combination with a classical 2-C measure of body density. The basic principle behind these methods is that the volume of a compartment can be defined as the ratio of the dose of a tracer to its concentration in that body compartment within a short time after the dose is administered.

The most commonly used method is the estimation of total body water (TBW). This is measured using a tracer dose of labelled water (deuterium, tritium or oxygen-18) and collection of body fluid samples pre and post ingestion. The estimated error for a TBW is typically <1kg, which translates to an error of roughly 10% for the average man for absolute fat mass.

Alternative dilutional methods for estimation of body composition include measurements of extracellular and intracellular water.

c. Four compartment models

The development of dual energy X-ray absorptiometry (DXA) to measure whole body bone mineral content has led to increasing accuracy in the measurement of body composition. Four-compartment models combine measurements of BMD with total body water, and allow calculation of the true density of fat-free mass. This is probably the most accurate way to date of assessing body composition (Fuller et al 1992), but has not yet been widely applied because of certain practical issues, especially in obese individuals. Subjects often exceed the weight capacity of machines, and the scanning area is relatively small and usually inadequate for most obese people.

1.2.3 Measuring body fat distribution

In addition to body weight, body fat distribution is also an important risk factor for obesity related diseases. Excess abdominal or central fat is associated with an increased risk of cardiometabolic disease such as type 2 diabetes (Ohlson et al, 1985).

a. Waist circumference

Waist circumference (WC) is often used as a surrogate marker of abdominal fat mass, as it correlates well with both subcutaneous and intra-abdominal fat as measured by CT scanning and MRI scanning respectively (Pouliot et al, 1994; Chan et al, 2003), and is associated with cardiometabolic risk (Kissebah et al, 1982). Men and women who have waist circumferences greater than 102cm and 94cm

respectively are considered to be at increased risk of cardiometabolic disease (Wang et al, 2005). The World Health Organisation has adopted these cut-off points, although some are of the opinion that these are rather arbitrary (Stevens et al, 2001).

Waist circumference is closely associated with BMI, and both BMI and WC correlate strongly with total body adipose tissue, but there is evidence to suggest that WC is a stronger predictor of intra-abdominal adipose tissue than is BMI (Shen et al, 2004).

There is consistent evidence that WC is strongly correlated with the development of type 2 diabetes (Carey et al, 1997; Chan et al, 1994; Ohlson et al, 1985), and that it is a stronger predictor than BMI (Wei et al; 1997). Some investigators have suggested that increasing waist circumference is an independent risk factor for CHD and mortality, even after correction for other “traditional” risk factors such as hypercholesterolaemia (Dagenais et al, 2005).

b. Waist Hip Ratio

Waist hip ratio has been routinely used as a clinical and research tool in the measurement of abdominal obesity. This measurement correlates well with BMI, but it has been demonstrated that a simple measurement of waist circumference is a better correlate of visceral adipose tissue accumulation (Pouliot et al, 1999; Onat et al, 2004; Kamel et al, 1999). However, because of the negative correlation of hip circumference with traditional risk factors, there is evidence to suggest that WHR is a better predictor of cardiovascular events (de Koning et al 2007).

c. Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI)

Imaging techniques such as MRI and CT scanning have taken an anatomical approach to body composition analysis. It is possible using both of these techniques to measure total body composition by interpolating multiple slices. However, this is impractical because of limited availability of resources and time for reporting, and the amount of exposure to radiation in the case of CT. Both of these techniques, however, can be used to measure body composition at specific sites, usually intra-abdominal fat. The other benefit of this is that subcutaneous fat can be differentiated from visceral fat. There is also evidence of good correlation between fat mass measured in a single CT slice measured at L4 to L5 and total visceral fat volume (Kvist et al 1986), thus reducing time and radiation exposure to the subject.

1.3 Obesity and insulin resistance

1.3.1 Insulin and the insulin receptor

Insulin consists of two linked polypeptide chains and is secreted by the beta cells of the pancreas in response to glucose and amino acids. Insulin is a potent anabolic hormone and promotes the synthesis and storage of carbohydrates, lipids and proteins while inhibiting their degradation and release back into the circulation. The major sites of insulin action are skeletal muscle and adipocytes, although it is now recognised that insulin receptors are present in many other tissues, including beta cells of the pancreas and the vascular endothelium. The cellular actions of insulin are mediated by the membrane-located insulin receptor.

The insulin receptor is a heterotetramer that consists of two alpha-beta dimers made up by 3 disulphide bonds. Tyrosine kinase catalyses the phosphorylation of several intracellular substrates, including the insulin receptor substrate (IRS) proteins, each of which recruits a distinct set of signalling proteins. To date, most attention has focussed on the IRS family of proteins, although it is also thought that the mitogen-activated protein (MAP) kinase plays an important role in insulin signalling. Mice lacking the IRS-1 protein are insulin resistant but not overtly diabetic (Tamemoto et al, 1994), and mice lacking the IRS-2 protein exhibit diabetes (Withers et al, 1998).

The insulin receptor catalyses the tyrosine phosphorylation of the IRS-family proteins, which generates docking sites for several SH2-containing proteins, including the p85 regulatory subunit of the type 1A phosphatidylinositol (PtdIns) 3-kinase (PI3-K). This ultimately results in translocation of a glucose transport protein,

GLUT4, to the cell membrane via a process of targeted exocytosis. GLUT4 endocytosis is simultaneously attenuated. The combination of translocation and reduction of endocytosis results in an accumulation of plasma-membrane-localised GLUT4, which allows inflow of glucose into the cell.

1.3.2 Measuring insulin resistance

Techniques for measuring insulin sensitivity in vivo are of varying complexity and range from simple fasting plasma insulin to the direct, but more time-consuming and labour-intensive, “gold standard” euglycaemic hyperinsulinaemic clamp. Advantages and disadvantages of these methods are outlined below.

Euglycaemic hyperinsulinaemic clamp (DeFronzo et al, 1979); A primed then constant exogenous insulin infusion maintains a steady state high physiological hyperinsulinaemia while suppressing endogenous β cell insulin secretion and hepatic glucose production. Glucose is infused at a variable rate in order to maintain blood glucose at a predetermined level (measured every five minutes at the bedside). At steady state the glucose infusion rate is equal to the rate of glucose uptake into tissues, i.e. insulin sensitivity, the assumption being made that hepatic glucose output is completely suppressed. Initial clamp studies used arterial blood samples for analysis of serum glucose levels, but hand warming is now widely used to “arterialise” venous blood (Petrie et al, 1996). Reproducibility is better when a three-hour rather than two hour clamp is performed (Morris et al, 1997).

Insulin suppression test; Glucose and insulin infusion rates are held constant, with steady state being achieved once plasma glucose reaches a plateau. Endogenous insulin release is suppressed using somatostatin. Steady state plasma glucose is used as an index of insulin sensitivity.

Frequently sampled intravenous glucose tolerance test with minimal model analysis; This technique, in which up to 22 glucose samples are obtained for the mathematical calculation of insulin sensitivity, has been reported as having a high degree of correlation with values of insulin sensitivity generated using the euglycaemic hyperinsulinaemic clamp in healthy volunteers (Anderson et al, 1995). In subjects with type 2 diabetes, however, the correlation is less strong, particularly when the number of glucose samples is reduced to twelve. As such, this technique is best employed in studies of non-diabetic populations.

Short insulin tolerance test; This is based on the rate at which plasma glucose falls during the first twenty minutes following an intravenous bolus of insulin.

Plasma insulin; Fasting plasma insulin has been used as a surrogate measure of insulin resistance, based on the assumption that compensatory hyperinsulinaemia occurs under normal circumstances in proportion to the degree of insulin resistance. The main difficulties in interpreting these values are variations in the performance of even the same assay kit across centres and possibly differences in specificity of insulin vs. its precursor molecules (Nagi et al, 1990; Petrie et al, 1997; Cotes et al, 1969).

Post-load insulin; Plasma insulin, sampled one or two hours after a glucose load, has been used in some prospective studies of hyperinsulinaemia and cardiovascular disease, such as the Busselton (Welborn et al, 1979) and Paris (Ducimetiere et al, 1980) cohorts.

Homeostasis Model Assessment (HOMA) and Fasting Insulin Resistance Index (FIRI); These simple indices are derived from fasting insulin and glucose levels. In non-diabetic populations, positive correlation with fasting insulin is almost unity. However, in datasets from which diabetic subjects have not formally been excluded, HOMA may provide a better surrogate measurement of insulin sensitivity than fasting insulin (Petrie et al, 1997).

1.3.3 Free fatty acids and insulin resistance

Associations between insulin resistance and obesity were noted as far back as 1965 (Randle et al 1965). Circulating concentrations of free fatty acid (FFA) are elevated in obesity because of increased production and reduced clearance of FFAs. In addition, elevated plasma FFA concentrations inhibit the anti-lipolytic action of insulin, further increasing the rate of FFA release into the circulation. .

In skeletal muscle, acute elevations in plasma FFA concentrations (by infusing heparinized lipid emulsions) reduces insulin-stimulated glucose uptake in individuals irrespective of gender and age (Roden et al, 1996). Under these conditions, insulin resistance develops 2–4 hours after elevation of plasma FFA concentrations and disappears 4 hours after normalization of FFA concentrations.

FFA-induced hepatic insulin resistance is more difficult to demonstrate because the liver is more insulin sensitive than skeletal muscle. Nevertheless, there is convincing evidence that physiological elevations of FFA, such as seen after a fat rich meal, inhibit insulin suppression of hepatic glucose production (HGP) resulting in an increase in HGP (Boden et al, 1997). In the acute setting, this rise in HGP is due to FFA-mediated inhibition of insulin suppression of glycogenolysis (Boden et al, 2002).

Chronically elevated plasma FFA concentration, are also associated with insulin resistance, and it has been demonstrated that short-term reduction of elevated plasma FFA concentrations improves insulin sensitivity. Normalization of plasma FFA concentrations resulted in significant improvements in insulin-stimulated glucose uptake in obese non-diabetic individuals and in patients with T2DM (Santomauro et al, 1999). Similar results have been reported in non-diabetic subjects genetically predisposed to T2DM (Cusi et al, 2007).

Further work has demonstrated the link between lipid accumulation in hepatic and skeletal muscle tissue, and the pathogenesis of insulin resistance (Savage et al 2007). The investigations of Savage et al demonstrated that infusion of lipids into the plasma led to significant accumulation of intracellular lipids especially diacylglycerol and fatty acyl-CoA in liver and muscle tissue. They revealed a negative correlation between intracellular lipids and rates of muscle glycogen synthesis and glucose oxidation i.e. insulin resistance in healthy human subjects, using the technique of nuclear magnetic resonance spectroscopy. Savage et al also demonstrated that

intracellular lipids inhibited the glucose transporter, GLUT4, the rate-limiting step in glycogen synthesis and glucose transport in skeletal muscle.

Choi et al (2007) subsequently investigated the molecular mechanisms whereby accumulation of lipid in liver and muscle would cause insulin resistance. Increased intracellular diacylglycerol (DAG) activates protein kinase C, and this initiates a serine / threonine phosphorylation cascade that phosphorylates insulin receptor substrate-1 (IRS-1). This inhibits tyrosine phosphorylation of IRS-1 and downstream activation of PI3 kinase, leading to reduced activation of v-akt murine thymoma viral oncogene homolog 2 (Akt2). This in turn affects translocation of GLUT4 and reduced glucose transport into cells.

1.3.4 Insulin resistance and adipocytokines

Proteins or “adipocytokines” secreted by adipose tissue appear to play an active role in metabolism including the modification of insulin sensitivity. The most important of these factors are described below.

a. Tumour necrosis factor alpha

Tumour necrosis factor- α (TNF- α) is an inflammatory cytokine, which until recently was thought to be produced mainly by macrophages. Hotamisligil et al, (1993) demonstrated increased levels in the adipose tissue of obese rodents, hypothesising that TNF α is also secreted by white adipose tissue. Subsequent works have

revealed similar findings in humans and $\text{TNF}\alpha$ levels correlate well with obesity and insulin resistance (Hotamisligil et al 1995; Kern et al 1995). Some data has also suggested that circulating concentrations of $\text{TNF}\alpha$ are increased in subjects with obesity or impaired glucose tolerance.

Whilst these studies suggest an association between $\text{TNF}\alpha$ and obesity, studies showing improved insulin sensitivity in rodent models of obesity with neutralisation of $\text{TNF}\alpha$, have been central to the hypothesis that this molecule plays an active role in insulin resistance (Hotamisligil et al 1993; Cheung et al 1998). There are a number of hypothesized mechanisms that could potentially explain the effects of $\text{TNF}\alpha$ on insulin resistance. Firstly, $\text{TNF}\alpha$ increases lipolysis, thus increasing circulating free fatty acid concentrations that affect insulin sensitivity. In addition, it has been demonstrated in vitro that high levels of $\text{TNF}\alpha$ can inhibit GLUT4 synthesis, thereby reducing insulin-mediated glucose transport (Stephens et al, 1996). Finally, $\text{TNF}\alpha$ is thought to have deleterious effects on insulin signalling by promoting serine phosphorylation of IRS-1 (Hotamisligil et al 2006).

b. Interleukin-6

Interleukin-6 (IL-6) is one of the mediators of the acute phase response and is secreted by many cell types, including adipocytes. Studies have shown that plasma IL-6 concentrations are elevated in obesity and the metabolic syndrome (Kern et al, 2001), and elevated plasma levels of IL-6 are predictive of the development of type 2 diabetes (Pradhan et al, 2001) and future myocardial infarction (Ridker et al, 2000).

Serum and adipose tissue levels of IL-6 are reduced by weight loss (Bastard et al, 2000), leading to the conclusion that IL-6 may contribute to insulin resistance in obesity.

c. Adiponectin

Adiponectin is made exclusively by adipocytes and was first reported in 1995. Unlike other adipocytokines, adiponectin mRNA is reduced in adipose tissue from obese mice and humans. Serum adiponectin levels correlate negatively with obesity (Arita et al, 1999), central obesity and insulin resistance (Weyer et al, 2001). In case-control studies, low plasma adiponectin has been shown to be an independent risk factor for future development of type 2 diabetes (Lindsay et al, 2002; Spranger et al, 2003)

Administration of adiponectin has been shown to reverse insulin resistance in rodent models of type 2 diabetes and obesity (Yamauchi et al, 2003). Adiponectin may ameliorate insulin resistance by decreasing circulating FFA by increasing fatty acid oxidation by skeletal muscle (Yamauchi et al, 2001). This leads to decreased triglyceride content of muscle, which has been associated with improved insulin sensitivity (Boden et al, 2002). The presence of adiponectin also has a similar impact on hepatic triglyceride content (Yamauchi et al, 2003), which also improves insulin sensitivity and hepatic glucose output. Therefore, adiponectin may of future therapeutic benefit in reversing some of the metabolic features of the insulin resistant state.

d. Leptin

Leptin is a 16 kDa protein secreted by adipocytes in proportion to adipocyte mass. The main role of the peptide hormone leptin is to regulate energy metabolism, predominantly by signalling to the hypothalamus and causing a reduction in food intake and subsequent weight loss. Leptin deficiency (Montague et al, 1997) and defects in the leptin receptor (Clement et al, 1998) are rare but are associated with extreme obesity and insulin resistance from an early age. However, the majority of human obesity is associated with high rather than low levels of leptin. This has indicated the possibility that common obesity is a leptin-resistant state, which may be due to a failure of leptin in crossing the blood-brain barrier. Administration of leptin to a group of obese subjects resulted in significant weight loss (Heymsfield et al, 1999). However, in order to achieve this, serum leptin concentrations 30-fold higher than normal were necessary.

1.3.5 Body fat distribution and insulin resistance

The gender differences in body fat distribution were first described in 1947 by Vague et al. who reported the phenomena of gynoid obesity in females and android obesity in men. It was also proposed that these phenotypes were associated with different cardiovascular risk profiles.

Since then it has become clear that visceral obesity is more closely associated with insulin resistance, hyperlipidaemia and type 2 diabetes. Positive correlations have been demonstrated between volume of visceral adipose tissue and fasting plasma

insulin concentrations (Despres et al, 1989). A number of studies have also documented the negative correlations between CT measured visceral fat mass and glucose disposal as measured by the hyperinsulinaemic-euglycaemic clamp technique (Rattarasarn et al, 2003).

1.4 The metabolic syndrome

1.4.1 Introduction

With the increasing availability of insulin assays in the 1960s, associations between hyperinsulinaemia and both hypertension (Welborn et al, 1996) and coronary heart disease (Stout et al, 1969) were noted. More than a decade later, data from relatively small cross-sectional studies (Modan et al, 1985; Zavaroni et al, 1989) sparked further interest in the role of hyperinsulinaemia and insulin resistance in CVD. In his Banting lecture of 1988, Reaven coined the term “Syndrome X” to describe clustering within subjects of cardiovascular risk factors thought to be underpinned by resistance to insulin-mediated glucose uptake. “Syndrome X” has been referred to as “metabolic syndrome X” by many authors in order to avoid confusion with “cardiac syndrome X” (microvascular angina). It has also been referred to as “the insulin resistance syndrome” (Haffner et al. 1992), and more simply, as “the metabolic syndrome” (Alberti et al. 1998).

1.4.2 Diagnostic criteria

Since Reaven’s Banting lecture, a number of other associations of insulin resistance have been suggested. The features of syndrome X, as proposed by Reaven (Reaven 1997) are numerous and varied depending on the defining body. In order to encourage an integrated rather than compartmentalised clinical approach to primary prevention of CVD, there have been three main attempts to define diagnostic criteria

for the metabolic syndrome. These definitions are compromised by the lack of standardisation across centres of insulin assays.

Though the constituents are similar for these sets of guidelines, there are notable differences that may have important effects on population prevalence. The World Health Organisation (WHO) (Alberti et al. 1998) criteria require either proven glucose intolerance or for a euglycaemic clamp to have been performed, while the Adult Treatment Panel (ATP III) criteria need no measurement of insulin sensitivity or abnormality of blood glucose if other criteria are met. In the European Group on Insulin Resistance (EGIR) (Balkau et al. 1999) and ATPIII definitions, the blood pressure thresholds are lower, while the diagnosis of central obesity does not require a measurement of hip circumference and microalbuminuria is not included.

1.4.3 Prevalence of the metabolic syndrome

When the WHO set of criteria is applied to the EGIR database of 1500 non-diabetic, normotensive European adults, a prevalence of 15.6% is derived (Beck-Nielsen et al. 1999). Subsequent application of diagnostic guidelines to other datasets has yielded a prevalence of between 7% and 30% using WHO criteria and between 1% and 22% using EGIR criteria, with only limited similarities in ranking of prevalence across studies ((Balkau et al, 1999). In the Botnia study, a large study of Finnish and Swedish families designed to identify early metabolic features in families with type 2 diabetes, the prevalence of the metabolic syndrome was 10% (females) and 15% (males) in subjects with normal glucose tolerance between the ages of 35 and 70 years. Insulin resistance was determined using $HOMA_{IR}$, with the metabolic

syndrome diagnosed if the subject had insulin resistance in the highest quartile of the HOMA_{IR} index in combination with two of the criteria detailed in table 4 (WHO criteria). In patients with type 2 diabetes, the prevalence of the metabolic syndrome was 78% (females) and 84% (males). A recently reported cross sectional study of more than 8000 members of the non-institutionalised civilian US population aged 20 years and over, the data having been collected as part of the National Health and Nutrition Examination Survey (NHANES) III survey between 1988 and 1994, demonstrated a prevalence of the metabolic syndrome, unadjusted for age, of 21.8%, using the ATP III criteria (NIH 2001). Perhaps most ominously, a prevalence of 6.7% was observed in subjects aged 20-29 years, a figure that is most likely greater now given the increasing problem of obesity, while application of these findings to census data from two years ago suggests that as many as 47 million US residents have the metabolic syndrome (Ford et al. 2002).

1.5 Obesity, insulin resistance and liver function tests

Recent research has suggested that a degree of liver disease may accompany the physical states of obesity, insulin resistance and type 2 diabetes. Non-alcoholic fatty liver disease (NAFLD) is a term used to describe a spectrum of pathological changes that may occur in the liver in association with obesity and insulin resistance. It is defined as the accumulation of intra-hepatic lipids, primarily in the form of triacylglycerols in individuals who do not consume significant amounts of alcohol (<20 g ethanol/day), and in whom other causes of steatosis have been excluded (McCullough et al, 2004). It is recognised that a spectrum of diseases may be present, ranging from steatosis alone (type 1), steatosis plus inflammation (type 2), steatosis plus hepatocyte injury (type 3), and steatosis plus sinusoidal fibrosis (type 4). The most severe forms of the condition are referred to as non-alcoholic steatohepatitis (NASH), and this can ultimately lead to cirrhosis or hepatocellular carcinoma.

The prevalence of NAFLD in type 2 diabetes and obesity is unknown. It has been estimated that the prevalence of NAFLD in the obese ranges from 50-90%, compared to 20-40% in the general population (Clark et al, 2002). Nonalcoholic steatohepatitis accounts for about 20% of NAFLD (estimated prevalence of NASH in western countries is 2% to 3%) and might be the cause of approximately 80% of cryptogenic cirrhosis (Ratziu et al, 2002).

In a recent study (Seppala-Lindroos et al, 2002) directly determined liver fat content was shown to correlate with several features of insulin resistance in normal weight

and moderately overweight subjects, independent of BMI and intra-abdominal or overall obesity. However, direct measurements of liver fat require ultrasound, computed tomography scan, or proton spectroscopy, and such techniques are unlikely to be recommended for this purpose in routine clinical practice. Fortunately, circulating concentrations of a number of variables appear to give insight into the extent of liver fat accumulation. Among these are γ -glutamyltransferase (GGT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST). Of these three, ALT is the most specific marker of liver pathology and appears to be the best marker for liver fat accumulation (Tiikainen et al, 2003). In addition, circulating concentrations of plasminogen activator inhibitor-1 may give insight into the extent of liver fat content (Festa et al, 2003) but, unlike ALT, its measurement is perhaps not as simple, standardized, or routinely available in laboratories.

1.6 ENDOTHELIAL DYSFUNCTION

1.6.1 Endothelial function and dysfunction – definitions

Vascular endothelial cells play a vital role in maintaining cardiovascular homeostasis in health. Until recently it was thought that these cells simply acted as an inert barrier between the vessel wall and lumen. Recent work has shown that this is not the case, the endothelium secreting a number of agents that are pivotal in the maintenance of vessel tone, platelet aggregation and coagulation (Stehouwer et al, 1997).

The key feature of endothelial dysfunction is reduced availability of vasoactive substances, with or without increased vasoconstricting agents, which leads to impairment of endothelium-dependent vasodilation (Lerman et al, 1992). Endothelial dysfunction coexists with insulin resistant states, and many of the mechanisms underlying acquired insulin resistance may also contribute to endothelial dysfunction. Endothelial dysfunction is also thought to be an important predisposing factor in the development of atherosclerosis and cardiovascular disease (Ross et al, 1993).

1.6.2 Mediators of endothelial function in health

One of the most important roles of the endothelium is its ability to facilitate vasodilatation. A number of factors are essential for endothelium-dependent vasodilatation, and their biological effects on vasculature have been extensively investigated.

a. Nitric Oxide (NO)

Nitric Oxide is synthesised within endothelial cells during conversion of L-arginine to L-citrulline (Vallance et al, 2001) by the endothelial form of nitric oxide synthase (NOS), termed eNOS. This process requires numerous cofactors including nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin and calmodulin.

The half-life of NO is less than 4 seconds and it is rapidly metabolised to nitrite and then nitrate before being excreted in the urine (Moncada et al, 1993). NO passes from the endothelial cell to the vascular smooth muscle cell by diffusion. Once in the smooth muscle cell it activates guanyl cyclase, leading to an increase in guanosine-3,5-monophosphate concentrations, and a reduction in smooth muscle tone and vasodilation (Vallance et al, 2001).

NO synthesis is blocked in the experimental setting by the use of L-arginine analogues. These act as competitive inhibitors of the metabolic pathway catalysed by eNOS.

Accelerated degradation of NO by reactive oxygen species generated by oxidised LDL-c or increased production of superoxide anion (Kojda et al, 1999), is probably the major mechanism impairing NO bioavailability in states of CVD (Behrendt et al, 2002). Decreased formation of NO as a result of inhibition of eNOS may also play a role.

b. Insulin

In addition to having multiple metabolic properties, insulin is also thought to have a direct role on the vascular endothelium. It has been suggested that insulin may have a direct effect on endothelial NO production and therefore on vascular tone. Initial observations demonstrated that insulin has a vasodilating effect within skeletal muscle and this effect was noted to be impaired in states of insulin resistance such as obesity and hypertension (Chen et al, 1996). Physiologically, skeletal muscle vasodilatation may be beneficial in that blood flow is diverted to target tissues, facilitating insulin delivery and consequent glucose disposal. An NO-mediated mechanism was proposed following studies investigating the effect of infusing the NOS inhibitor L-N^G-monomethyl arginine citrate (L-NMMA) on forearm blood flow pre and post euglycaemic hyperinsulinaemic clamp (Scherrer et al, 1994). It was noted that reductions in forearm vasodilatation were greater during hyperinsulinaemia, than at baseline, suggesting that blocking NO production has a negative effect on insulin-mediated vasodilatation. Similar results were achieved investigating the effect of L-NMMA on insulin-mediated increase in leg blood flow (Steinberg et al, 1994). Further studies in healthy volunteers demonstrated a correlation between insulin sensitivity and forearm vasoconstrictor responses to infused L-NMMA (a surrogate for basal NO production) (Petrie et al, 1996). Mechanistically, studies on cultured human aortic endothelial cells indicate that there are similarities between post-receptor signalling pathways utilised by insulin to upregulate both glucose transport into cells, and endothelial nitric oxide synthase activity. Briefly, PKB is phosphorylated following recruitment of PI3-kinase to IRS-1. Activated PKB phosphorylates endothelial nitric oxide synthase, upregulating NO production (Salt et al, 2003).

c. Endothelium derived hyperpolarising factor (EDHF)

While NO has been shown to be an important endothelium-dependent mediator of vascular tone in relatively large arteries and large arterioles, there are certain arteries in which endothelium-mediated vasodilatation is predominantly effected by endothelium-dependent hyperpolarisation of vascular smooth muscle cells. This hyperpolarisation and endothelium-dependent vasodilatation persists in the presence of inhibitors of eNOS (Garland et al, 1992). These findings suggest that a separate mechanism is responsible for endothelium-dependent vasodilatation in these arteries. The exact identity of the chemical involved in this mechanism is unclear at present. The relative importance of the EDHF mediated mechanisms alters with vessel size, and appears to increase as vessel size decreases (Shimokawa et al, 1996; Urakami-Harasawa et al, 1997).

A key feature of the EDHF mechanism is hyperpolarisation of the of the smooth muscle cell membrane mediated by the vascular endothelium. In the smooth muscle cell, hyperpolarisation of this membrane reduced both the open probability of voltage-dependent Ca channels, leading to a decrease in intracellular Ca concentration, and a reduction in smooth muscle tone, producing vasodilatation (Nelson et al, 1990).

1.6.2 Assessment of endothelial function

As greater emphasis has been placed on the role of endothelial function in the pathogenesis of disease, a number of methods have evolved for the assessment of

endothelial function in humans. The main aims of these developing techniques are the detection of endothelial dysfunction and the prevention of clinical disease processes.

Endothelium-dependent vasomotion is the most widely used clinical endpoint for assessment of endothelial function. This involves stimulation of endothelial release of NO and other vasoactive compounds, and often a comparison of vascular responses to endothelium independent dilators such as nitroglycerine.

The techniques for measurement of endothelial function fall into four main categories: coronary circulation; endothelial function in other conduit vessels; microvascular endothelial function (in resistance vessels); and the analysis of circulating factors.

a. Endothelial function in the coronary vascular bed

Initial clinical studies of endothelial function were performed in the coronary circulation. Patients with angiographically proven CAD displayed a paradoxical vasoconstrictor response to locally infused acetylcholine (Ludmer et al 1968; Vita et al, 1990). It has also been shown that shear stress induced by increases in coronary blood flow lead to reduced FMD in patients with established CAD (Cox et al 1989; Drexler et al 1999). Although the presence of endothelial dysfunction in the coronary circulation has prognostic implications for the development of CAD, these investigations are only suitable to perform on persons who require angiographic

studies for clinical reasons, and are not suitable techniques for repeated evaluations of vascular function.

b. Endothelial function in conduit vessels

The term “conduit vessel” is used to refer to larger arteries, such as the coronary, brachial, and femoral arteries. These vessels are most prone to atherosclerotic changes leading to cardiovascular disease.

Flow mediated dilatation; The limited clinical use of coronary vascular studies necessitated the development of alternative methods of assessing the endothelium. As a result, it has been demonstrated that impaired coronary endothelial responses found in those with cardiovascular risk factors are mirrored in the peripheral vasculature (Panza et al 1990; Linder et al 1990). Measurement of flow-mediated dilatation using ultrasonography has therefore been developed. This technique is based on the principle that increases in tissue blood flow caused by transient ischaemia leads to shear stress, and in turn increases in the release of NO and other vasoactive substances. The ischaemic stimulus is created by placing a blood pressure cuff, inflated to >50 mmHg above the systolic pressure, on the forearm. Subsequent release of the cuff leads to reactive hyperaemia and transient brachial artery dilatation. This non-invasive, relatively simple technique is useful in large populations, and for repeated assessments of endothelial function. However, there are a number of mechanistic and biological factors that can affect these measurements, and limit the use of this technique (Corretti et al, 2002).

Assessment of the pulse wave; Although initially described and used in clinical practice since the 19th century, assessment of the pulse wave as a measure of vascular health has undergone something of a renaissance in recent years. Technology now allows estimation of the ascending aortic pressure wave from that assessed peripherally in the radial artery.

The central arterial pulse is composed of two major components; a forward travelling wave generated by left ventricular systolic contraction, and a reflected wave arriving from the periphery. The reflected wave adds to the amplitude of the forward travelling wave by the principle of superimposition. The properties of the forward travelling wave are dependent on the physical and mechanical properties of the central large arteries. The properties of the reflected wave are dependent on the physical and mechanical properties of the whole arterial tree, the transmission velocity of the incident wave, and the distance to the major reflecting sites.

An increase in the stiffness of arterial walls causes an increase in the transmission velocity of both the incident pressure wave and the reflected wave. Arterial stiffness is a term used to describe overall rigidity of the arterial walls, and can be measured by different methods.

Pulse wave velocity (PWV): This measurement is determined by the time taken for the pulse wave to traverse the distance between two fixed measuring points. The PWV is derived as:

$$\text{Distance} / \text{Time in m/s}$$

where m is metres and s is seconds.

There are a number of potential confounding factors to be considered when measuring the PWV. The measurement required is of the velocity of the forward wave, and reflected waves may interfere with the accuracy of the calculation. Measurements must therefore be taken from the foot of the waveform (the upstroke of the pressure wave), which is generated entirely by the forward wave.

Another potential problem is the accuracy of the distance between measuring points. Out of necessity, the measurement (using standard techniques) is an approximation of the actual distance, as the actual length of the artery cannot be measured. This particular problem is addressed with new techniques for the measurement of PWV, using magnetic resonance imaging (Bradlow et al, 2007).

A simple and well-established method of measurement of the PWV is to use a semi-automated device for analysis of the pressure waveforms at the two measurement sites. The upstroke of the wave is identified at each of these sites, and the time between the two upstroke points is measured. Once the distance between measuring points has been identified and entered, the velocity of the pulse wave is calculable by the software. An average of multiple measures is made (between 10 and 25). An advantage of this method is that the pressure waves measured at each point stem from the same ventricular systolic contraction. This eliminates potential errors arising from beat-to-beat variability of cardiac output and consequent variability of the pulse wave morphology.

Pulse wave analysis: The pulse pressure wave is formed from the combination of the incident wave and waves reflected back from the periphery, and this can be

measured and analysed using applanation tonometry and appropriate software. The augmentation index (Aix) is a measure of the effect of wave reflection on the second systolic peak, and is thus one measure of the additional load to which the left ventricle is subject as a result of wave reflection. It is calculated as the increment in pressure from the first shoulder in the ascending aortic pressure wave to the peak of this wave expressed as a percentage of the ascending aortic pressure wave. The time of arrival of the reflected waves is heavily dependent on the PWV, which is influenced mostly by arterial stiffness. Early arrival of the reflected waves, which occurs with arterial stiffness, coincides with systole placing greater pressure on the left ventricle. Alternatively, if the arrival of the pulse wave coincides with diastole (as is the case with an elastic aorta) this can enhance coronary arterial filling.

A number of studies have been performed to assess the reproducibility of performing SphygmoCor Pulse Wave Analysis measurements. These studies have included healthy subjects (Siedenhofer et al, 1999; Filipovsky et al, 2000), hypertensive (Wilkinson et al, 1998) patients and renal pre-dialysis and dialysis (Savage et al, 2002) patients. The inter-observer variation and temporal variation was satisfactory in all groups.

c. Endothelial function in the microvasculature

Ex vivo tests – Wire myography: In 1972 Bevan and Osher first suggested a technique for the investigation of the properties of small arteries with diameters down to approximately 100µm. Mulvany and Halpern developed this technique, with the

method for investigation of a single vessel of diameter 100-1000um being published in 1977. Over subsequent years this technique has been developed to allow investigation of initially two and then four vessels simultaneously. This technique allows vessels to be mounted as cylinder or ring preparations, using a relatively atraumatic technique to preserve vascular endothelial functional integrity. The technique allows measurement of isometric responses and is termed Mulvany-Halpern (wire) myography. A detailed description of the practical aspects of this technique can be found in section 2.8.

Venous occlusion plethysmography (Whitney et al, 1953; Greenfield et al, 1963): This is a well-established technique for the study of the microcirculation. With a catheter placed in the brachial artery, drugs are infused into the forearm circulation, and blood flow is measured non-invasively by means of strain gauge plethysmography. The technique is based on the principle that obstruction of venous outflow in the setting of non-disturbed arterial flow, leads to an increase in limb volume that is directly proportional to the rate of arterial inflow. This highly reproducible technique has shown that many traditional cardiovascular risk factors such as hypertension (Panza et al, 1990), diabetes (Creager et al, 1990), and smoking (Heitzer et al, 1996) are associated with reduced forearm blood flow. The drawbacks of this technique are that it is invasive, which limits its use in large-scale studies or those requiring repeated measurements.

Laser Doppler techniques: Laser Doppler techniques, which measure skin blood flow, have also emerged as methods for assessing microvascular function. These

techniques have evolved as the need for non-invasive tools for the assessment of vascular function has increased.

Laser Doppler is based on the principle that light undergoes changes in wavelength when it hits moving blood cells. The magnitude and frequency distribution of these changes in wavelength are related to the number and velocity of red blood cells. Therefore, cutaneous microvascular blood flow can be measured as it is related to the properties of the reflected light.

There are two techniques available for measuring cutaneous blood flow. Laser Doppler flowmetry uses a single probe which records blood flow in a small volume of 1mm or less. The main drawback of this technique is the inherent variability in different areas of a tissue, i.e. spatial variation. Laser Doppler imaging can measure blood flow over a larger tissue area, as the laser beam is reflected over a given surface area by a computer driven mirror.

Laser Doppler techniques are useful for assessing microvascular responses to certain stimuli such as postocclusive hyperaemia, local thermal hyperaemia and acetylcholine iontophoresis. These measurements have been shown to be reproducible, but a number of issues have to be standardized to ensure reproducibility. It has been suggested recently however, that rather than assessing specific markers of endothelial function such as NO, these techniques allow assessments of global microvascular function (Berghoff et al, 2002).

d. Peripheral markers of endothelial health

Markers of thrombosis: Under physiological conditions, the endothelium prevents thrombus formation. However, endothelial damage leads to the transformation of the endothelium from an anti-coagulant to a procoagulant environment, due to changes in the expression of a variety of molecules, such as tissue factor, plasminogen activator inhibitor (PAI-1) increased secretion of von Willebrand factor (vWf). There is evidence to suggest that serum concentrations of vWf are associated with risk of future MI and stroke (Folsom et al, 1997; Smith et al, 1997)

Markers of inflammation: Atherosclerotic disease is known to be associated with systemic inflammation, which is a key player in the development and progression of atherosclerosis (Ross et al, 1999). Baseline plasma levels of intercellular adhesion molecule-1 (ICAM-1) and E-selectin have been shown to be associated with increased cardiovascular risk in healthy populations (Hwang et al, 1997), and adverse outcomes in those with established CAD (Blankenberg et al, 2001). C-reactive protein, an acute phase reactant synthesized by the liver and vascular smooth muscle cells has emerged as a potential marker for cardiovascular risk (Ridker et al, 2003; Pearson et al, 2003).

1.6.3 Endothelial dysfunction and cardiovascular risk

Endothelial dysfunction is associated with classical cardiovascular risk factors such as hypertension, dyslipidaemia and diabetes (Busse et al, 1996; Panza et al, 1993;

De Vriese et al, 2000). A strong association between insulin resistance, obesity and endothelial dysfunction has also been demonstrated (Steinberg et al, 1996).

A number of small studies have evaluated the prognostic value of endothelial dysfunction. Three studies have assessed the association between endothelial dysfunction in the coronary circulation and CVD, and in each of these cohorts endothelial dysfunction predicted the occurrence of CVD events (Halcox et al, 2002; Al Suwaidi et al, 2001; Schachinger et al, 2000). In contrast, Heitzer et al (2001) observed that forearm bloodflow responses, rather than coronary artery responses to intra-arterial acetylcholine infusion was an independent predictor of cardiovascular events. Neunteufl et al (2000) observed a similar association when examining brachial artery FMD using ultrasound.

In patients with risk factors for but no pre-existing CVD, associations were observed between endothelial dysfunction (assessed in the brachial artery) and subsequent cardiovascular events (Perticone et al 2001; Modena et al, 2002).

1.6.4 Endothelial dysfunction and insulin resistance – underlying shared pathophysiology

A robust association between insulin resistance and endothelial dysfunction in animals and human studies has therefore been demonstrated. Whether the two states are linked directly or are simply manifestations of an underlying pathology remains to be seen, but there are a number of factors common to both states, which

may contribute to the mechanistic link between them. Molecular and pathophysiological mechanisms underlying reciprocal relationships between insulin resistance and endothelial dysfunction result in a vicious cycle reinforcing the link between metabolic and cardiovascular disorders. There are three main factors associated with type 2 diabetes and insulin resistance which also predispose individuals to endothelial dysfunction: glucotoxicity and hyperinsulinaemia, lipotoxicity, and adipocytokines and inflammation.

a. Glucotoxicity and hyperinsulinaemia

Hyperglycaemia causes insulin resistance by three methods. Firstly, hyperglycaemia increases the production of reactive oxygen species (ROS) which are associated with the activation of various serine / threonine kinases. This leads to serine phosphorylation of IRS-1, which impairs its ability to bind and activate PI3 kinase. This in turn leads to diminished activation of downstream events, leading to reduced GLUT4 translocation and glucose transport (Furukawa et al, 2004, Hirosumi et al, 2002). In parallel, this increased oxidative stress has a number of effects on the endothelium, including reducing NO bioavailability, and increasing proinflammatory molecules, all of which increase the likelihood of endothelial dysfunction.

Advanced Glycation End Products (AGEs) also inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 and 2, leading to impaired activation of PI3 kinase and reduced glucose transport. AGEs also produce ROS, which increase oxidative stress, impairing the function of endothelial proteins, and enhancing the formation of foam cells from macrophages, which lead to atherosclerosis.

Hyperinsulinaemia is a feature of insulin resistant states and is also an independent risk factor for coronary artery disease (Despres NEJM 1996). Insulin acts as a vasodilator at physiological doses mediated by the production of NO. However, insulin also stimulates the release of the vasoconstrictor endothelin-1 (ET-1) from endothelial cells. Increased serum levels of ET-1 have been demonstrated in insulin resistant subjects. In such individuals, it is hypothesised that there may be an imbalance between PI3 kinase and MAP kinase mediated insulin signalling pathways. This overdrive of MAP-kinase subcellular pathways may lead to increased endothelin-1 and reduced NO expression, both of which are characteristic features of endothelial dysfunction.

The metabolic and vascular functions of insulin have been shown to be parallel both in healthy and diseased states. At the cellular level the signalling pathways by which insulin mediates glucose uptake and NO production are similar. After binding to its receptor, insulin promotes GLUT4 translocation in skeletal muscle and increases eNOS activity in the vascular endothelium. The subcellular signalling pathway employed to mediate this effect of insulin is the same as that central to glucose uptake, both featuring PI-3 kinase and Akt. This has led to the hypothesis that that disruption of PI3 kinase may be the unifying mechanism in insulin resistant states. Down regulation of PI3 kinase has been observed in skeletal muscle taken from insulin resistant humans (Cusi et al, 2000).

b. Lipotoxicity

Elevated FFA levels in insulin resistant states are hypothesized as being another factor linking this with endothelial dysfunction. Again, increased FFA levels have been shown to cause mitochondrial dysfunction (Lowell et al, 2005; Savage et al, 2005), which in turn increases the generation of ROS, having detrimental effects on insulin-mediated glucose transport and endothelium-dependent vasodilatation.

FFAs have also been shown to Activate NF- κ B, which stimulates the production of proinflammatory cytokines including IL-6 and TNF α . In addition, accumulation of ceramide, a product derived from long chain fatty acids, inhibits GLUT4 translocation and also increases ROS, further scavenging NO.

c. Cytokines and inflammation

Both endothelial dysfunction and insulin resistance are widely known as proinflammatory states, and are associated with increased levels of inflammatory cytokines (Berg et al, 2005). The most extensively studied proinflammatory cytokine implicated in insulin resistance is TNF α . TNF α activates a variety of serine kinases such as JNK (Nguyen et al, 2005), which increase serine phosphorylation of IRS-1/2, leading to decreased activity of PI-3 kinase and Akt. TNF α and IL-1 β also lead to the activation of NF- κ B, which inhibits insulin-stimulated activation of eNOS (Kim et al, 2001). TNF α also stimulates the expression of other inflammatory proteins such as CRP and IL-6, thereby further promoting a proinflammatory environment.

1.7 Morbidity and mortality associated with obesity

It is now well established that obesity is an independent risk factor for the development of CVD (Kannel et al, 1991), as well as being associated with many of the more traditional risk factors for atherosclerosis such as type 2 diabetes and hypertension (Chan et al, 1994). It is accepted that the obese state can also predispose to malignant disease and musculoskeletal conditions.

1.7.1 Obesity and mortality

Direct associations between obesity and disease processes are well documented, but the relationship between mortality and body weight is more controversial. Initial observational studies in males confirmed increased mortality risk with weights above a certain threshold, but described J-shaped curves, with increased mortality figures being noted in the very lean (Lew et al, 1979, Lee et al, 1993). However, after correction for smoking and chronic illness, no association between leanness and mortality was observed. These results were replicated in a female population in the Nurses Health Study (Manson et al, 1995). A further study (Adams et al, 2006) has also demonstrated that simply being overweight (BMI 25.0 to 29.9) rather than obese is also associated with a 20 to 40% increase in risk of death from all causes.

1.7.2 Obesity and type 2 diabetes

Many cross sectional and prospective studies have confirmed the association between obesity and type 2 diabetes. Biennial questionnaires were sent to more than 50,000 male health professionals in the US for 6 years to assess the effect of different anthropometric variables on development of type 2 diabetes. This study revealed that men with a BMI > 35 had a relative risk of developing diabetes of 42, compared to men with BMI < 23 (Chan et al, 1994). The study also showed that BMI at the age of 21, and absolute weight gain during adulthood predicted the development of type 2 diabetes. Similar findings have been reported for females from data again from the Nurses Health Study (Carey et al, 1997). The risk of developing diabetes, in this cohort of Caucasian women aged 30-55 years at baseline, was 49 times higher in those with a BMI >35 than amongst women whose baseline BMI <22.

The pattern of body fat distribution also plays a role in the development of type 2 diabetes. A prospective study to assess risk factors for coronary heart disease looked at 792 men aged 54 years. This Swedish study revealed a positive and significant correlation between increasing waist-hip ratio and development of type 2 diabetes, even when BMI was corrected for (Ohlson et al, 1985). The Iowa Women's Health Study, also a prospective questionnaire study, aimed to look at the association between body fat distribution and disease incidence in a large cohort of older women. Although the study was limited in that the diagnoses of diabetes were self-reported and prevalence may therefore be underestimated, this study did show that BMI, WC and WHR were significantly and positively correlated with the development of type 2 diabetes (French et al, 1997).

1.7.3 Obesity and hyperlipidaemia

Obesity also has a number of effects on lipoprotein metabolism. The typical pattern of dyslipidaemia associated with obesity tends to be that of elevated triglycerides, reduced HDL and small dense atherogenic LDL. The Framingham Offspring Study examined a cohort of 4260 male and female subjects, and revealed an inverse relationship between HDL cholesterol and obesity in all age groups (Garrison et al, 1980). The study was unable to demonstrate such a clear correlation between obesity and LDL cholesterol. The third round of the Framingham Offspring Study in 1996 supported the linear association between BMI and VLDL, LDL and total cholesterol, although the linear and inverse relationship between BMI and HDL cholesterol was noted to be the strongest (Lamon-Fava et al, 1996).

Body fat distribution also appears to play a role in the complex dyslipidaemic picture that is associated with obesity. A number of small studies have demonstrated this association. A study of 303 white male and female subjects which measured body fat distribution using densitometry, revealed a positive association between centripetal fat and serum triglycerides, and a negative association between central adiposity and HDL cholesterol in female subjects (Baumgartner et al, 1987). This association was not demonstrated in males in this particular study. A further study which recruited 52 premenopausal obese females, and measured abdominal obesity using CT scanning, demonstrated a similar association between central adiposity and serum HDL cholesterol concentrations (Depres et al, 1989). A study of 512 thirty eight year-old European males with a wide range of anthropometric measurements, revealed a positive association between BMI, WC and serum total cholesterol, LDL

cholesterol and a negative association between anthropometric measurements and HDL cholesterol.

1.7.4 Obesity and coronary artery disease

Obesity has traditionally been associated with coronary artery disease. Until recently, however the role of obesity as an independent risk factor remained controversial due to its co-existence with other traditional risk factors such as diabetes, hypertension and hyperlipidaemia. Several long-term studies have more recently demonstrated that obesity is an independent risk factor for CVD, leading the American Heart Association to reclassify obesity as a modifiable risk factor for coronary heart disease.

A cohort of 5029 male and female participants of the Framingham Heart Study was followed for 26 years, and results revealed obesity to be an independent predictor of CHD (Kannel et al, 1991). In addition, a prospective cohort study followed 115,886 female patients in the US for 8 years and detected a positive correlation between higher BMI and all categories of coronary artery disease. Lastly, a post-mortem study published in 2002, revealed an association between central obesity and the development of coronary atheroma in young males (McGill et al, 2002).

1.7.5 Obesity and hypertension

According to data from the NHANES III report in the US, the prevalence of hypertension was 2.5 times higher in men and 3 times higher in women (aged <55 years) with a BMI 30-34.9 than in age-matched subjects with a normal BMI. In this cohort the prevalence of hypertension rose from 15% in the normal weight category to 40% in obese subjects (Brown et al, 2000). In addition the Health Survey for England 2003 found that mean systolic blood pressure was about 6mmHg higher in obese men and women than in those with a BMI 18.5 to 25.

1.7.6 Obesity and cancer

Cancer is one of the less recognised complications of obesity, but excess body fat is now known to be a significant risk factor for malignant disease. Recent studies have suggested that 3.4% of cancers in men and 6.4% of cancers in women are attributable to obesity. In both men and women obesity was associated with higher mortality rates from cancer of the oesophagus, colon, pancreas, gallbladder and kidney. Obesity has also been associated with increased risk of death from cancers of the uterus, ovary and breast in females, and of the stomach and prostate in men (Calle et al, 2003).

The underlying cause of this increased risk is thought to be multifactorial. Increased concentrations of hormones such as insulin-like growth factor 1 (IGF1) and insulin promote growth and differentiation of cells. In addition, low levels of chronic inflammation such as those that accompany obesity also play a role in the development of malignant cells (McMillan et al, 2006).

1.8 Effects of weight loss on health

The long-term effects of intentional weight loss on mortality and health benefits of weight loss remain incompletely understood, and numerous small studies have attempted to examine this issue. The most comprehensive evaluation of this information in the UK to date seems to come from a Health Technology Assessment (HTA) review (Avenell et al, 2004). This extensive meta-analysis included cohort and prospective studies that looked at the effects on intentional weight loss in obese or overweight (BMI > 28) adults. There were a number of outcome measures examined, and subjects in the 37 trials included in the analysis were followed up for a minimum of two years.

1.8.1 Weight loss and mortality

The meta-analysis included 5 studies that looked at the effects of weight loss on mortality (Chaturvedi et al 1995; Williamson et al 1995; Williamson et al 1999; Williamson et al 2000; Rumpel et al 1993). All cause mortality was noted to be reduced in females with obesity related illness, regardless of the degree of weight loss, and in males and females with diabetes mellitus. There was no reduction in all cause mortality in any other group. Unintentional weight loss seemed to be associated with higher mortality rates, probably due to undiagnosed illness.

CVD-related mortality was reduced after intentional weight loss in subjects with diabetes (relative risk reduction of 28%), and if weight was lost within 1 year. Interestingly if weight loss was slower (> 1 year), there was an increased risk of

CVD-related mortality. Mortality related to type 2 diabetes was reduced in males and females, irrespective of the timing, or the amount of weight lost.

1.8.2 Diabetes

It is now incontrovertible that weight loss can prevent or delay the onset of type 2 diabetes. Subjects enrolled in the Diabetes Prevention Study (DPS) were randomised to a control or intervention group, the intervention being multifactorial lifestyle modifications aimed at weight loss. Modest weight loss in the active group was associated with a 58% relative risk reduction in the incidence of type 2 diabetes over a 3-year period (Tuomilehto et al 2001).

Similarly, the Diabetes Prevention Program (DPP) Research Group demonstrated that lifestyle interventions, aimed at weight reduction, led to a significant reduction in type 2 diabetes in a cohort of 3234 obese male and female patients, followed up for a period of 2.8 years (Knowler et al 2002).

1.8.3 Lipid profile

Thirteen studies included in the HTA review examined the effects of weight loss on lipid profiles (Davidson et al 1999; Hauptman et al 2000; Teupe et al 1991; Wing et al 1998; Hess et al 1998; O'Leary 1980; Karason et al 1999; Gleysteen et al 1992). The majority of the studies were relatively small with numbers of participants ranging from 7 to 323 subjects. Weight loss was achieved by diet, exercise or drugs in eight

of the 13 studies, and by surgical interventions in the other five studies. Overall, weight loss was associated with significant reductions in LDL-c, triglycerides and total cholesterol, with greatest reductions in lipid parameters observed in association with maximum weight loss.

1.8.4 Hypertension

There were 14 studies in the review that looked at the effects of weight loss on hypertension. Regression analysis of these results would suggest that a reduction in body weight of 10 kg leads to a drop of 3.6mmHg in diastolic blood pressure. Although the results for systolic blood pressure did not reach statistical significance, there was a trend towards lower systolic blood pressure with reduced body weight.

1.8.5 Cardiovascular Disease

Although short-term weight loss has been demonstrated to ameliorate obesity-related metabolic abnormalities and CVD risk factors, no prospective randomised studies have examined the long-term consequences of intentional weight loss in obese populations. The primary hypothesis of the Look AHEAD (Action for Health in Diabetes) study is that an intensive lifestyle intervention to reduce weight and increase physical activity will reduce cardiovascular morbidity and mortality. Primary outcome measures will include death from CVD, non-fatal myocardial infarction and non-fatal stroke. The planned follow-up period for this multi-centre randomised controlled trial is 11.5 years, with follow-up due to end in 2012 (Ryan et al, 2003).

1.8.6 Adverse effects of weight loss

The beneficial effects of intentional weight loss on health are numerous. There are some concerns however that weight loss may be associated with adverse health consequences. The main issue complication which can result from rapid weight loss is the development of gallstones. This has been reported following weight loss as a result of both lifestyle intervention and bariatric surgery.

1.9 EFFECTS OF WEIGHT LOSS ON METABOLIC AND VASCULAR PARAMETERS

1.9.1 Metabolic effects of intentional weight loss in obese, non-diabetic populations

A number of groups have examined the effects of intentional weight loss in obese populations. The vascular and metabolic effects of weight loss using diet, exercise, pharmacological measures, surgery, and a combination of the above have been studied. The results to date are variable, with study cohorts being heterogeneous. A number of studies, using a variety of methods to achieve weight loss, have assessed the effects of intentional weight loss on serum levels of adipocytokines (Table 1.1).

a. Diet only protocols

The association of adipocytokines with obesity has been a relatively recent discovery, but over the past ten years there is a growing body of work looking at the effect of weight loss on serum and tissue levels of these substances. Bastard et al (2000) demonstrated reductions in serum and adipose tissue IL-6 and leptin following three weeks of a very low calorie diet (VLCD), in association with mean weight loss of 3kg, and increased insulin sensitivity in a group of obese postmenopausal women. The study did not reveal any change in TNF α levels following weight loss.

In addition, a group of obese subjects were studied by Raitakari et al (2003). These 67 male and female subjects lost an average of 11kg after a 6-week period of VLCD

(580 kcal/day). Significant reductions in total cholesterol, LDL-c, oxidised LDL and CRP were achieved following weight loss, in combination with significant increases in serum adiponectin levels.

No change in serum adiponectin levels was observed following a 12-week period of low calorie diet (1200kcal/day) in a group of 15 females with an android pattern of obesity (Manigrasso et al, 2005). This was despite a median reduction in BMI of 8% and significant increase in insulin sensitivity as measured by the HOMA index.

A further study examined the effects of 6 months of calorie reduction in 12 male and female subjects. Significant reductions in body weight (mean reduction 6.8kg) and body fat percentage were associated with statistically significant improvements in total and LDL cholesterol, and insulin sensitivity. (Dengel et al, 2006)

b. Combined diet and exercise protocols

The effects of a combination of diet and exercise on the metabolic abnormalities associated with obesity have also been studied. Janssen et al (2002) compared the effects of a diet only approach versus diet and aerobic exercise or diet and resistance exercise in 38 postmenopausal women. A mean weight loss of 10kg after a 16-week programme in all three groups was associated with significant reductions in total and LDL cholesterol levels, with no obvious additional benefits conferred to those in the exercise groups.

In contrast, 6 months of weight reduction using diet and exercise led to no improvements in LDL cholesterol, but increased HDL cholesterol concentrations in

24 obese male and female subjects. This also resulted in significant reductions in serum ICAM-1, but not in vWf or vCAM (Hamdy et al, 2003).

However, a randomised single-blind trial (Esposito et al, 2003), which led to a mean weight loss of 14kg, did not demonstrate significant changes in lipid profile in 60 postmenopausal females following 2 years of diet and exercise. Compared to the placebo arm of this study, weight loss in this setting was associated with significant reductions in serum concentrations of IL-6, and CRP, whilst adiponectin levels were significantly increased.

c. Diet and pharmacological agents

Two studies have looked at the effect of a combination of diet and orlistat on weight loss and its associated adverse metabolic profile. Bergholm et al (2003) studied the effects of 3-6 months of dietary with and without the addition of orlistat on weight, metabolic and vascular parameters in 47 obese subjects. Reductions in weight were 7.3kg and 7.4 kg in the orlistat and diet only groups respectively. Interestingly there was a significant reduction in serum LDLc concentration of 0.48 ± 0.15 mmol/l in the orlistat group but not in the placebo group. A similar study by Brook et al (2004) also revealed significant reductions in plasma LDL-c, leptin and CRP concentrations after a mean body weight reduction of 6.6%.

d. Multidisciplinary approach

A multidisciplinary approach to achieve weight loss is often used in clinical practice. A combination of diet, exercise and, in some cases, local liposuction was employed

to achieve an average weight loss of 9.8kg in 56 obese female subjects (Ziccardi et al, 2002). This led to significant reductions in levels in serum levels of $\text{TNF}\alpha$, IL-6, p-selectin, ICAM and VCAM. There were no observed differences in cholesterol profile in these subjects after weight loss. Another multidisciplinary programme consisting of diet, exercise and liposuction surgery over one year resulted in a 10% reduction in body weight in 20 pre-menopausal women (Nicoletti et al, 2003). This was associated with significant reductions in cytokine concentrations.

1.9.2 Vascular effects of weight loss in obese non-diabetic subjects

A number of the studies described above also examined the effect of weight loss on measures of vascular function. The results of these are outlined below.

a. Diet only

Only two studies employing solely dietary methods for weight loss looked at the effects on vascular function. Raitakari et al (2004) looked at the effect of weight loss on two parameters of vascular function. This study demonstrated that reduction of body weight in 67 obese men and women was associated with significant improvements in brachial artery flow-mediated dilation (FMD). In contrast, there were no observed differences in endothelium independent function as assessed by vascular responses to sublingual GTN spray.

These results contrast with those of Dengel et al. where there was no noted improvement in brachial artery FMD or endothelium independent vasodilatation (EID)

after weight loss. In addition, no significant differences in carotid intima media thickness (IMT) were noted after a period of weight loss.

b. Diet and exercise

Only one study has examined the combined effects of diet and exercise on vascular function. A study by Hamdy et al (2003) revealed that a 6-month programme of diet and exercise caused significant improvements in macrovascular endothelial function as measured by flow-mediated dilatation in 24 subjects. However, microvascular reactivity measured by laser Doppler imaging remained unchanged.

c. Diet and pharmacological agents

Dietary measures and orlistat have been used in 2 studies to assess vascular responses to weight loss. Brook et al. studied the effect of diet with or without these two weight loss modalities in 43 otherwise healthy male and female subjects. Mean weight reduction of 6.6kg was not associated with any improvement in brachial artery FMD.

Assessment of forearm resistance artery endothelial function was measured by quantifying bloodflow responses to intraarterial acetylcholine and SNP (endothelium-dependent and independent responses respectively) by Bergholm et al (2003). Despite similar weight losses in both arms of the study (diet and placebo vs diet and orlistat), significant improvements in forearm bloodflow were seen in the group receiving orlistat, but not in the placebo treated group.

d. Multidisciplinary approach

Ziccardi et al (2002) achieved significant weight loss after a year-long multidisciplinary approach in a cohort of obese women. This led to significant improvements in blood pressure responses and blood viscosity following an intravenous infusion of L-arginine. These results were mirrored by Nicoletti et al (2003), who revealed similar improvements in endothelial function following one year of weight loss.

1.9.3 Metabolic and vascular effects of intentional weight loss in type 2 diabetes

The majority of studies designed to examine the effects of weight loss have, until recently, focused on subjects without diabetes. However, over the past few years, a number of groups have studied the effects of weight loss in patients with type 2 diabetes. The majority of these studies have looked at the effects of intentional weight loss on insulin sensitivity and circulating markers in type 2 diabetes, rather than dynamic vascular function testing.

In 1999, Halle et al demonstrated the effects of weight loss in 20 obese males with type 2 diabetes. A four-week intervention comprising calorie restriction to 1,000kcal day, and 30 minutes of aerobic exercise daily, led to significant reductions in weight, glycaemic control and fasting insulin levels. These changes were associated with reductions in serum total and LDL cholesterol and serum leptin concentrations. The changes in serum leptin concentration correlated with reductions in total cholesterol and triglycerides, but not with changes in glycaemic control, LDL or HDL cholesterol.

The results of a similar study were published in 2003. Monzillo et al demonstrated that a 6-month programme of diet and exercise lead to a mean reduction in weight of 6.9kg, in association with significant reductions in serum leptin, and IL-6. Of the 24 participants in this study, eight had type 2 diabetes, and weight loss lead to increased adiponectin levels only in this subgroup. In contrast, weight loss of 4.5 +/- 0.6kg in 33 postmenopausal females, following a regime of diet only or diet and exercise for 14 weeks, led to reductions in serum leptin and CRP levels (Giannopoulou et al, 2005). This intervention was not however, associated with any increase in serum adiponectin levels.

Interestingly, a randomised double blind placebo-controlled parallel comparison of sibutramine and placebo carried out in 48 patients with type 2 diabetes, showed similar results. Six months treatment with sibutramine was associated with an average weight reduction of 2.5 kg. Insulin sensitivity was enhanced (assessed using the steady-state plasma glucose level) but no changes in serum CRP or adiponectin were observed after a period of weight loss (Hung et al, 2005).

Barinas Mitchell et al (2006) observed the effect of a yearlong programme of diet, exercise and orlistat or placebo in a cohort of subjects with type 2 diabetes. Weight loss was similar in both groups, with significant improvements in PWV and reduced serum concentrations of TNF α , IL-6, PAI-1, and CRP.

Table 1.1: Weight loss intervention studies

Study	n	Age (yrs) M/F	BMI (kg/m ²)	T2DM (Y/N)	Intervention	Insulin sensitivity measurement	Endothelial function measurements	Metabolic measurements	Body composition measurements	Mean reduction body weight (%)	Main findings
Barinas-Mitchell et al. 2006	38	20 - 70 M+F	34 ± 5.2 37 ± 8	Y	Diet, exercise, placebo vs. orlistat (1 year)	Fasting plasma insulin	PWV, IL-6, TNF α , CRP, PAI-1, Fibrinogen	FPG, HbA1c, HDL-c, LDL-c,	Anthropometry	7.8	Improved PWV. Reduced TNF α , IL-6, PAI-1, CRP. Improved LDL-c and HbA1c
Bastard et al. 2000	14	45 ± 4 F	39.5 ± 1.1	N	VLCD (3 weeks)	FIRI	Leptin, IL-6, TNF α , CRP	FPG	BMI, WHR, DXA	5	Significant reductions in body fat mass, leptin and IL-6 after weight loss; increased insulin sensitivity
Bergholm et al. 2003	47	39 ± 1 F	32.3 ± 0.4	N	Diet + orlistat / placebo 3-6 months	Fasting insulin	Forearm blood flow responses to Ach and SNP	FPG, HDL-c, LDL-c, triglycerides, FFAs	BMI, WC, body fat estimation by bioimpedance	8.3	Improved forearm responses to Ach and reduced LDL-c in orlistat group but not in placebo group.
Brook et al. 2004	43	18 - 50	>27	N	Diet and orlistat (12 weeks)	Fasting insulin	Brachial artery FMD, CRP, leptin	FPG, TC, LDL-c, HDL-c	BMI, WC, HC, body fat estimation from skin fold thickness measurements	6.6	Reductions in LDL-c, fasting insulin and leptin. No improvement in brachial FMD

Dengel et al. 2006	12	54.9 ± 3.9 M + F	30.3 ± 3.7	N	Diet (6months)	IVGTT		TC, LDL-c, HDL-c, FPG, TGs	BMI, DXA	7.9	Significant reductions in BMI and body fat %; improved TC, LDL-c, TGs; improved insulin sensitivity. No improvements in endothelial function
Esposito et al. 2003	120	34.2 ± 4.8 F	35.0 ± 2.3	N	Diet and exercise (2 years)	Fasting insulin HOMA	Il-6, IL-18 Adiponectin, CRP	FPG, TC, HDL-c, trigs, FFAs	BMI, WHR	15	Mean weight reduction 14kg; significant improvements in all measurements.
Giannopoulou et al. 2003	33	50- 70 F	>30	Y	Diet alone (D), exercise alone (E), diet and exercise (D+E)	Fasting insulin	TNF α , IL-6, Adiponectin, leptin, resistin, CRP	HbA1c, TC, HDL-c, LDL-c, TGs	ADP, MRI (VAT, SAT)	6	Reductions in leptin and CRP in in D and D+E groups.
Halle et al. 1999	20	48 ± 8 M	32.9 ± 3.1	Y	Diet and exercise (4weeks)	Fasting insulin	leptin	FPG, fructosamine, TC, trigs, LDL-c, HDL-c, FFAs	BMI	4.4	Significant reductions in serum leptin and cholesterol profile; reduced leptin associated with reductions in cholesterol and trigs.
Hamdy et al. 2003	24	49.3 ± 1.9 M + F	36.7 ± 0.94	Y	Diet and exercise (6 months)	Frequently sampled IVGTT	FMD to shear stress and SLGTN. Laser Doppler iontophoresis; vWF, sICAM, sVCAM, PAI-1, t-PA	N/A	BMI, WHR	6.6	Significant improvements in insulin sensitivity, FMD. No change in LDI responses to Ach or SNP.
Hung et al. 2005	48	30- 75 M+F	>27	Y	Diet + sibutramine / placebo (6 months)	Modified insulin suppression test	Adiponectin, CRP	HbA1c, TC, LDL-c, HDL-c, TGs, FPG	BMI, WHR	7	Improved insulin sensitivity; no improvements in CRP or adiponectin

Janssen et al. 2002	38	> 30 F	>27	N	Diet only (DO), diet and aerobic exercise (DA), diet and resistance exercise (DR) (16 weeks)	OGTT insulin sensitivity index	N/A	FPG, TC, trigs, HDL-c, LDL-c	BMI, WC, WHR, MRI measures of SAT, VAT, and skeletal muscle fat	10	Significant reductions in body fat, fasting insulin, TC and LDL-c
Keogh et al. 2008	99	50.0 ± 8.3 M + F	33.7 ± 4.1	N	Low-CHO vs. standard CHO diet (8/52)	Fasting insulin	FMD, Aix, PWV, ICAM-1, E-selectin, P-selectin, VCAM-1, PAI-1, tPA, adiponectin, CRP	FPG, TC, HDL-c, LDL-c, triglycerides	BMI, DXA	8	No change in FMD with either diet. Improved PWV and endothelial markers with both diets.
Manigrasso et al. 2005	15	47.2 ± 6.7 F	39 ± 7.3	N	Diet and exercise (12 weeks)	HOMA	Adiponectin, IL-10	N/A	BMI, WHR, WC	8	Significant improvement in insulin sensitivity; no change in adiponectin or IL-10.
Monzillo et al. 2003	24	49.3 ± 1.9	36.7 ± 0.9	Y	Diet and exercise (6 months)	Frequently sampled IVGTT	Leptin, adiponectin, Resistin, TNF, IIL-6, CRP	FPG	BMI, WHR	8	Significant improvements in insulin sensitivity; reductions in leptin; increased adiponectin only in diabetic subjects
Pierce et al. 2008	40	49.5 ± 2.5 MF		N	Diet (12/52)	HOMA	FMD, responses to IA infusion of Ach, leptin, CRP, IL-6, TNF α , adiponectin	N/A	BMI, WC, HC, WHR	10.5	Improved FMD and forearm blood flow responses to IA Ach, correlating with degree of reduction in visceral fat.
Plat et al. 2007	11	59.0 ± 9	31.3 ± 2.1	N	Diet (6/52) and fish oil supplements (6/52)	HOMA	ICAM-1, E-selectin, CRP, MCP-1	FPG, TC, LDL-c, HDL-c, trigs	BMI, WHR	10.5	Reduced fasting and post-prandial CRP and ICAM-1 following weight loss but not fish oil supplements.

Raitakari et al. 2004	67	46 ± 7 M + F	35.2 ± 5.4	N	VLCD (6 weeks)	Fasting insulin	FMD, EID, CRP, adiponectin	FPG, TC, LDL-c, HDL-c, Trigs	BMI, WC, HC, WHR	14	Improvement in FMD which correlated with change in FPG
Sharman et al. 2004	15	33.2 ± 11.3 M	34.3 ± 5.6	N	Randomised crossover trial: low fat diet vs. v low CHO diet (6 weeks)	N/A	CRP, TNF α , IL-6, ICAM-1, P-selectin	N/A	BMI	7	Both diets led to reduced CRP, IL-6, TNF α , and ICAM-1.
Wycherly et al. 2008	29	52.4 1.4 M+F	34.2 0.9	Y	Calorie-reduction exercise (12 weeks)	HOMA	Brachial artery FMD,	FPG, HbA1c, lipids	BMI, WHR	6	Improved metabolic parameters but no improvements in FMD after weight loss.
Xydakis et al. 2004	80	47.1 0.9 M + F	38.3 0.7	N	VLCD (4-6 weeks)	HOMA	Leptin, adiponectin, CRP, TNF α	FPG, TC, HDL-c	BMI, WHR	7.6	Improved insulin sensitivity, lipoproteins, leptin. No change in TNF α or adiponectin
Ziccardi et al. 2002	56	35.3 4.8 F	37.2 2.2	N	Diet and exercise (1 year)	Fasting insulin	Platelet aggregation and BP responses to L-arginine infusion; TNF, IL-6 p-selectin, ICAM-1, VCAM-1	FPG, TC, LDL-c, HDL-c, Trigs	BMI, WHR	10	Improved vascular responses, reduced cytokines.

CHAPTER 2

METHODS AND MATERIALS

2.0 Summary

This chapter outlines general protocols and methods, both clinical (physiological) and laboratory-based used in the studies described in this thesis.

2.1 Subjects

2.1.1 General details

Clamp studies, arterial compliance studies, gluteal biopsies and collection of blood for analysis were carried out in the Clinical Investigation and Research Unit (CIRU) of the Western Infirmary, Glasgow. Body composition measurements were carried out in the Department of Human Nutrition at Glasgow Royal Infirmary. Anthropometric measurements and dietary follow-up took place at the Diabetes Centre, Glasgow Royal Infirmary

The North Glasgow Universities Trust Ethics Committee approved the study design and protocols. All subjects gave fully informed written consent for their inclusion within the study.

2.1.2 Recruitment

Initially subjects were recruited from diabetes clinics at Glasgow Royal Infirmary. It became apparent that patients attending secondary care diabetes clinics did not fit the inclusion criteria for this study. Therefore, subsequent recruitment was via advertisements in local press. The process of recruitment and randomisation is outlined in Figure 2.1. A total of four adverts were run over an 18-month period in two local newspapers (“The Metro”, “The Evening Times”). One-hundred and thirty two people responded to these advertisements. Eighty potential volunteers were excluded on the basis of a follow-up telephone call. These exclusions were mainly due to cigarette smoking, age, being insulin-treated, or having a past history of vascular disease. A further 30 were excluded following a health-screening visit. Nineteen of these volunteers were excluded on the basis of poor glycaemic control (HbA1c >9.0%), seven on the basis of hypertension, and four volunteers were lost to follow-up at this stage.

22 male and female patients (subjects) were recruited for this study. All subjects had type 2 diabetes diagnosed more than three months earlier. Subjects were treated with diet therapy or metformin. Subjects were recruited if they were obese (BMI >30kg/m²). Within the intervention group, six subjects were taking no glucose-lowering medication, seven subjects were prescribed metformin and three were prescribed a sulphonylurea. Nine of the fifteen subjects within the intervention group were had been prescribed cholesterol lowering drugs. No medication was discontinued or altered during the study. Subjects had no history of cardiovascular disease or microvascular complications related to diabetes.

Exclusion criteria included:

Cigarette smoking

Hb A1c > 9.0%

Total serum cholesterol > 8.0mmol/l

Insulin therapy

History of microvascular or macrovascular complications

History of delayed wound healing

Pregnancy or breast-feeding

Blood pressure >160/90

At screening, volunteers completed confidential health history questionnaires, had their weight and height measured, had their blood pressure checked and had screening blood samples taken in the fasting state to check for inclusion / exclusion criteria.

2.1.3 Randomisation

Subjects were randomised to a six-week period of low calorie diet or usual care using a permuted block randomisation technique, with block sizes of 6 and an allocation basis of 2:1. This was combined with stratified randomisation to ensure equal numbers of subjects with controlled hypertension in each group. Randomisation was performed by Dr L Murray, Biostatistician, Department of Medicine, Western Infirmary, Glasgow. Fifteen subjects were randomised to low calorie diet and seven were randomised to usual care.

2.2 Study protocol

On each attendance subjects attended fasting having been instructed to avoid food or fluids other than water for at least 12 hours prior to the study. All examinations and investigations were performed in the morning. An attempt was made to start each session between 0830 and 0900.

In order to reduce anxiety and delay, all subjects were offered transport to and from the CIRU and their home address. A light lunch was provided following each investigation session.

All subjects were issued with contact details for the principle investigator and advised that contact could be made at any time if required.

Subjects attended for three visits in a week to have initial tests performed. Thereafter all subjects had an introductory one-hour session with the dietician, for baseline measurements and to discuss dietary issues specific to their randomisation. Subjects who were randomised to the low calorie diet group visited the dietician once weekly for the next six weeks; subjects randomised to the usual care group visited the dietician on one occasion at the start of the six week period and once at the end.

All subjects had a one-week dietary washout after the six-week diet period. Another three visits then took place in order that anthropometric, metabolic and vascular measurements could be repeated. This protocol is illustrated in Figure 2.2.

Figure 2.1: Recruitment and randomisation process

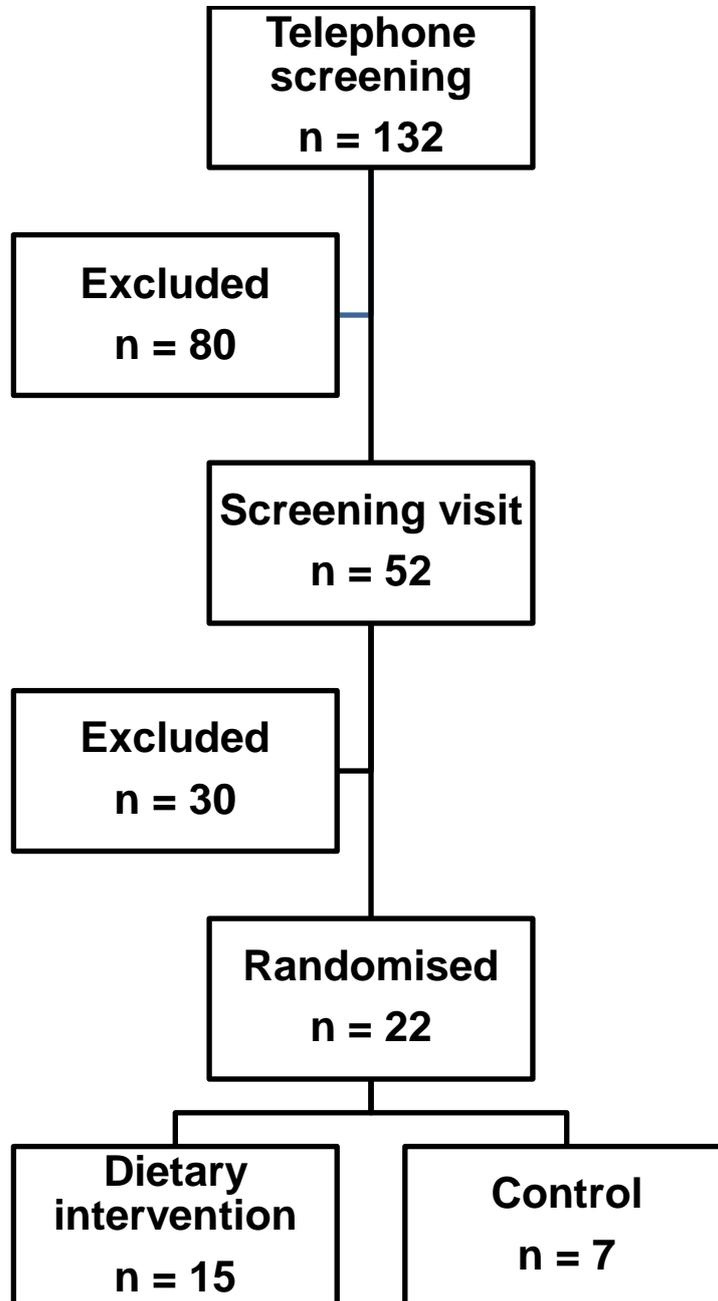
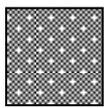
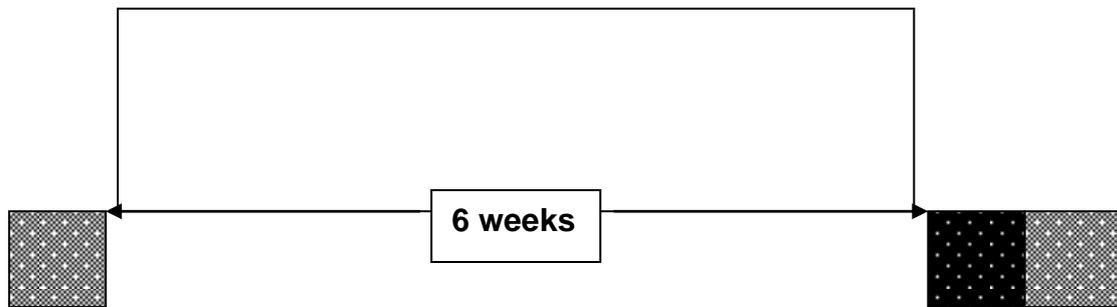
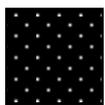


Figure 2.2: Study design



Vascular and metabolic assessments



1 week dietary washout

2.3 Dietary intervention

2.3.1 Intervention subjects

A dietician specialising in diabetes management reviewed subjects who were randomised to the low calorie diet on a weekly basis for six weeks. The purpose of these visits was to encourage dietary compliance and perform basic anthropometric measurements. All dietary assessments and anthropometric measurements were carried out by the same trained observer.

Dietary composition was based mainly on liquid foodstuffs such as cereals, soups, yoghurts, milky puddings and fruits. Calorie intake was restricted to 1200kcal/day. Some “solid” foodstuffs were introduced after four weeks in several patients. Patients were supplied with booklets containing detailed information on calorie content of a number of foodstuffs and were asked to keep accurate daily food and calorie intake diaries.

Following six weeks of low calorie diet, subjects were advised to adhere to a calorie intake of 2000kcal/day for weight maintenance purposes for one week, prior to repeat vascular and metabolic assessments.

No advice was given to subjects regarding exercise at any point during the study.

2.3.2 Control subjects

The dietician saw these subjects on two occasions: once at the start of the six-week period and once at the end. Basic anthropometric measurements and a “diet history” were taken. These subjects were given advice about healthy eating, but were not given specific instructions about a low calorie diet.

2.4. Clinical and morphometric measurements

2.4.1 History and Examination

A standard clinical history was taken and clinical examination made. Particular attention was made to symptoms and signs of cardiovascular disease. A record was made of history and examination findings, and of any regular medication taken. In the case of subjects recruited from diabetic clinic, the subject's general practitioner (GP) was informed of their inclusion in the study, along with details of the investigations involved.

2.4.2 Blood pressure

A standard technique was employed throughout the course of the study. Supine systolic and diastolic blood pressure were recorded after a standardised 15 minutes rest by an oscillometric technique using an semi-automatic sphygmomanometer (Dinamap Critikon, Johnson and Johnson Professional Products Ltd., U.K.) maintained by the Department of Clinical Physics, Western Infirmary.

2.4.3 Body Mass Index

A standard technique was employed throughout the course of the study. A single trained observer made measurements. Height and weight were measured without shoes and in indoor clothes. The same measuring scales were used throughout

(Seca, Germany). Height was measured, as far as was possible, to the nearest 0.005m. Weight was measured to the nearest 0.5kg.

Body mass index (BMI kg/ m²) was calculated according to the following standard equation:

$$\text{BMI} = (\text{body weight [kg]}) / (\text{height [m}^2\text{]})$$

2.4.4 Waist-hip ratio

A standard technique was employed throughout the course of the study. A single trained observer made measurements. Waist circumference was taken as the minimum value between iliac crests and costal margin, with hip circumference taken as the maximum value over the buttocks. A 1cm wide tape measure was used.

2.4.5 Skinfold thickness measurements

These measurements were performed using Holtain Tanner/Whitehouse Skinfold callipers (Holtain Ltd., Crymych, UK). Skinfold thickness was measured at the following sites on the right side of the body with subject standing:

Biceps: With the elbow flexed at an angle of 90°, the mid point between the lateral projection of the acromial process and the inferior margin of the olecranon process was marked. The arm was allowed to hang loosely with the palm facing forward and

skinfold measurement was made at the level of the mark over the belly of the biceps muscle above the centre of the cubital fossa.

Triceps: This measurement was at the same level as the biceps measurement on the midline of the posterior aspect of the arm.

Subscapular: This measurement was made along the natural cleavage line of the skin just inferior to the inferior angle of the scapula with subjects' arms hanging loosely by the sides of the body.

Suprailiac: This skinfold was measured vertically in the mid-axillary line, half way between the costal margin and the superior iliac crest.

Each skinfold was lifted between the thumb and index finger of the investigator's right hand, 1 cm above the site of measurement. Skinfold callipers were applied and the measurement taken after 5-8 second of calliper pressure.

The sum of skinfold measurements was used to estimate body density using the predictive equations derived by Durnin and Womersley (1974):

$$d = 1,1714 - 0.063 * \log (S) - 0.000406*A$$

Where d is body density, S is sum of all four skinfolds, and A is age in years.

Body fat percentage was then estimated using the Siri equation:

$$\%BF = [(4.95/BD) - 4.5] \times 100$$

Where BD is body density.

2.5 Body Composition measurements

2.5.1 Air displacement plethysmography

The air-displacement plethysmograph used in this study was the BOD POD body-composition system (model 2000A; Life Measurement Instruments, Concord, CA). The device consists of a computer-integrated, dual chambered air-plethysmograph, digital weighing scale and BOD POD software system. The system is located in the University Department of Human Nutrition at Glasgow Royal Infirmary (Figure 2.3). Measurements were carried out in a temperature-controlled room. The BOD POD device was initially calibrated using a 50-litre cylinder prior to each measurement.

For measurements, subjects were asked to change into a tight-fitting swimming costume and swimming cap, and were asked to remove all jewellery. Subjects' height, sex and age were entered into the computer. Body weight was then measured on a calibrated electronic scale. Subjects were asked to sit quietly with an erect posture and normal respiration, with their hands folded in their laps and their feet firmly on the floor of the device. A minimum of two 50-second tests was performed to ensure reliability of the measures. The body volume measurement was repeated if the two measurements were not within 150ml of each other. The device then estimated the subject's thoracic gas volume.

Once measurements were completed, the system's computer with the following equation calculated body density:

$$D_{b(AP)} = \text{mass}/V_{b(AP)}$$

where $V_{b(AP)}$ is body volume determined by air-displacement plethysmography (AP).

Percentage body fat by AP ($\%BF_{AP}$) was derived by using Siri's formula:

$$\text{Siri: } \%BF_{AP} = (4.95/D_b) - 4.50$$

2.5.2 Deuterium dilution technique

Total body water (TBW) was calculated from the distribution of a dose of deuterium oxide ($^2\text{H}_2\text{O}$ or sometimes referred to as D_2O) in body water. Urine, saliva or plasma can be sampled.

a. Collection of samples and administration of deuterium labelled water

A pre-dose urine sample was collected and an aliquot transferred to a clearly labelled sterile universal container for storage and deuterium analysis. ~20ml samples were stored and the remaining urine was discarded. A prepared deuterium dose, giving 0.05g $^2\text{H}_2\text{O}$ per kg body weight, was taken orally. Drinking water was used to rinse the vessel, which contained the dose and this was swallowed. The first three urine samples passed after taking the dose were collected and stored in labelled universal containers. The total volume and time urine was passed was logged. The urine samples were stored in a freezer at -20°C until analysis.

b. Analysis of deuterium

The deuterium contents of urine, diluted dose and tap water samples were determined by continuous-flow isotope ratio mass spectrometry (Hydra, PDZ Europa) at the University Department of Child Health, Royal Hospital for Sick

Children, Glasgow. Samples were analysed in triplicate after equilibration with a reference gas (5% hydrogen in helium) over a platinum catalyst. The mass spectrometer was calibrated using gravimetric standards of known deuterium content, which were prepared and analysed with each batch of samples. Total body water was calculated by isotope dilution of the deuterium dose after correction for non-aqueous hydrogen exchange and for deuterium passed in the urine. Lean body mass was calculated assuming this is 73.2% water. Body fat is calculated by difference: weight minus lean body mass.

c. Calculation of total body water from urine deuterium enrichment

Enrichment is the abundance of deuterium in a sample above a baseline level and is expressed in units of parts per million excess (ppm excess). A number of equations are needed to calculate total body water.

$$(1) \text{ ppm excess D} = (\text{ppm D in enriched sample}) - (\text{ppm D in the baseline sample})$$

Urinary losses (mmol) are calculated from the deuterium content of each sample.

$$(2) \text{ D content (mmol)} = (\text{Sample D ppm excess} / 10^6) \times (\text{Urine volume (ml)} / \text{MW H}_2\text{O}) \times 1000$$

where MW H₂O = molecular weight water = 18.0153

The cumulative loss is the sum of the D content of each sample.

$$(3) \text{ Apparent TBW (mol) in each sample, taking into account non-aqueous H exchange, but not urinary losses} = 10^6 / (\text{D ppm excess}) \times \text{Dose (mmol)} / (1000 \times 1.04)$$

where,

$$(4) \text{ Dose (mmol)} = \text{Wt dose consumed (g)} / \text{MW D}_2\text{O} \times 1000 \times \text{Dose D enrichment} / 100$$

MW D₂O = molecular weight deuterium oxide = 20.0274

$$(5) \text{ Dose D enrichment} = \text{Dose D abundance} - \text{D natural abundance} \\ = 99.9 - 0.015 = 99.885 \text{ atom \% D}$$

and 1.04 is the non-aqueous H exchange factor, the proportion of the dose sequestered into lipid and protein pools in the body.

$$(6) \text{ TBW (kg or L)} = \text{mol TBW} \times \text{MW H}_2\text{O} / 1000 \\ \text{where MW H}_2\text{O} = 18.0153$$

True TBW (mol) uses equation with the addition of the cumulative urinary loss.

$$(7) \text{ True TBW (mol)} \\ = 10^6 / (\text{D ppm excess}) \times (\text{Dose (mmol)} - \text{Urinary loss (mmol)}) / (1000 \times 1.04)$$

True moles TBW are converted to kg or L TBW as equation (6)

The mean TBW of all the plateau samples is used to calculate LBM and body fat.

The first sample is usually not included, except for calculation of urinary loss.

2.6 Hyperinsulinaemic isoglycaemic clamp

2.6.1 Preparation

Whole body glucose disposal was measured using the gold standard hyperinsulinemic isoglycemic clamp, utilising a technique modified from Defrozo et al. (1979). This technique has been shown to be accurate and reproducible by Morris et al (1997).

Subjects attended for the investigation fasting. Two 18-gauge PTFE intravenous cannulae (Venflon; Viggo, Helsingborg, Sweden) were inserted. The first was inserted in a retrograde fashion into a dorsal vein of the non-dominant hand (Figure 2.4). The second was inserted into an antecubital vein of the contralateral arm. A three-way tap was attached to each cannula, to allow co-infusion of insulin and glucose from the cannula sited in the antecubital fossa, and for ease of blood sampling from the retrograde cannula. Patency of the retrograde cannula was maintained by the use of a slow infusion of 0.9% saline.

2.6.2 Insulin Infusion

The infusion of soluble insulin (Actrapid; NovoNordisk A/S, Bagsvaerd, Denmark) was prepared in 45 mls 0.9% saline and 5 mls (10% vol / vol) of the subject's own blood. The saline / blood mixture was used to prevent absorption of the soluble insulin to the plastic of the infusion syringe and lines. The infusion was administered using a Braun Perfusor pump (Braun Medical Inc, Carrollton, Texas, USA). A

primed, constant rate infusion was used using a standard priming protocol (table 2.1), with the aim of achieving a steady-state serum insulin concentration approximately 120 μ U/ml above the fasting level (i.e., a high physiological level).

Time	Insulin infusion rate
0-4 minutes	4.5 mU/kg/min
4-7 minutes	3.0 mU/kg/min
7-180 minutes	1.5 mU/kg/min

Table 2.1. Insulin infusion priming protocol

2.6.3 Arterialisation of venous blood

Early experiments using hyperinsulinemic clamps measured glucose in blood drawn from the arterial circulation, and adjusted the glucose infusion rate accordingly. In order to avoid the use of arterial cannulation and its attendant risks, 'arterialisation' of the venous circulation in the hand and arm was used. This was achieved by heating the hand cannulated with the retrograde venous cannula in a cylindrical Perspex heated box (Department of Physiology and Pharmacology, University of Nottingham, Nottingham, U.K.), commencing immediately after cannulation of the dorsal hand vein. Under thermostatic conditions the heater maintains the ambient temperature surrounding the hand at 55° C.

2.6.4 Glucose infusion and blood sampling

20 minutes of supine rest were allowed following cannula placement. Following this period of rest, baseline blood samples were withdrawn and blood pressure was measured. Blood pressure was automatically measured every 15 minutes from this point, with automated recording of results.

Insulin infusion was commenced at 0 minutes ($t = 0$ min) as described above. A 20% dextrose (Baxter Healthcare, Norfolk, U.K.) infusion was administered via an IMED infusion system (IMED, Abingdon, U.K.), commencing at $t = 2$ min and continuing until $t = 180$ min. Serum glucose concentrations were maintained at fasting plasma glucose levels. The infusion rate was adjusted according to body weight and recorded as milligrams of glucose infused per kg body weight per minute (mg/kg/min). This was calculated as shown, for a 70kg man (20% glucose infusion):

$$(60 \text{ minutes} / 200\text{g glucose}) \times (70\text{kg}) = 21\text{ml} / \text{hour}$$

Arterialised blood samples were withdrawn at 10-minute intervals throughout the clamp. 1 ml samples were withdrawn from the retrograde cannula and spun in a centrifuge. Serum glucose analysis was performed at the bedside. The rate of glucose infusion was adjusted according to the serum glucose concentration to maintain target concentration at the level of fasting plasma glucose concentrations.

2.6.5 Calculation of insulin sensitivity

During the period of steady-state serum glucose concentrations (usually taken as the last 60 minutes of the clamp), the rate of glucose infusion equals the rate of glucose removal from the glucose space (equivalent to glucose metabolised, M). It is assumed the endogenous glucose production is suppressed, thus, the M value is an estimate of total body glucose metabolism, and reflects enhancement of tissue glucose disposal by insulin.

In practice, the glucose infusion rate must be modified by two factors before it approximates M. The following equation is used to incorporate these two factors:

$$M = I - UC + SC$$

where:

I = glucose infusion rate (mg/kg/min)

UC = correction for urinary glucose loss [usually negligible during a clamp]

SC = space correction (mg/kg/min) [for the inevitable deviations from isoglycemia]

SC, the space correction is calculated as follows ():

$$SC = (FPG - G) \times 17.86 \times 0.095$$

where:

G = ambient glucose concentration over last 40 min of clamp (mmol/l)

17.86 = unit conversion factor (mmol/l to mg/dl)

0.095 = glucose space constant

2.6.6 Calculation of MCR

This was done to take into account changes in fasting plasma glucose before and after diet. MCR is calculated from the following equation:

$$\text{MCRg (ml/kg/min)} = \text{M} / \text{FPG} \times 100.$$

2.7 Analysis of blood samples

2.7.1 General protocols

Venous blood samples for laboratory assay were withdrawn at baseline and during clamp studies from the retrograde cannula and collected into plain (electrolytes, lipids, liver function tests) and sodium citrate (endothelial markers and CRP) tubes. All samples were immediately placed in crushed ice prior to centrifugation (3000rpm, 4°C), decanting and storage at -20°C.

2.7.2 Routine biochemistry

Routine biochemical analyses were performed on an Olympus AU5200 autoanalyser.

2.7.3 Lipids and CRP

Total cholesterol, triglyceride, HDL-c and sensitive CRP assays were performed by the staff of the Routine Lipids Section (Miss C Gourlay), Biochemistry Department of Glasgow Royal Infirmary, using a Hitachi 917 analyser

VLDL and LDL Cholesterol results were calculated using the FRIEDWALD EQUATION

LDL cholesterol (mmol/l) = total Cholesterol-(HDL Cholesterol+Triglyceride/2.19)

VLDL Cholesterol (mmol/l)=total cholesterol-LDL Cholesterol – HDL Cholesterol.

2.7.4 Endothelial markers

Dr Lynne Cherry and Miss Pauline Watt of the Metabolic Medicine Group, of the Department of Vascular Biochemistry, Glasgow University, carried out ICAM, IL-6 and Adiponectin analysis. A Multiskan Ascent Plate reader and Ascent Software were used for calculation of results.

2.7.5 Serum and plasma glucose concentrations

Serum glucose was measured at 10-minute intervals at the bedside by the glucose oxidase method during the clamp studies. A Beckman 2 glucose analyser (Beckman Instruments, Fullerton, CA, USA; inter-assay coefficient of variation of 2%) was used for this purpose. Results were expressed in mmol/l. The machine was then calibrated prior to each study and then re-calibrated hourly with a control solution with a glucose concentration of 8.3mmol/l.

2.8 Wire Myography

Wire myography is an in vitro technique that is used to study the function of vascular smooth muscle. It is a technique pioneered by Mulvaney and Aalkjaer (1990) whereby the properties of small resistance arteries (<500um) can be investigated.

2.8.1 Tissue acquisition - Human gluteal fat biopsy

The subject was positioned prone, and the upper outer quadrant of the buttock was exposed. An aseptic technique was used. The area identified for biopsy was sterilised with iodine-based solution (Betadine; Perdue Pharma LP, Stamford, Connecticut, USA). The area was anaesthetised with lidocaine 1% to a depth of approximately 2cm. An elliptical incision was made and a segment of tissue approximately 2.5cm in length, 1cm in width and 1cm in depth was removed. Subcutaneous tissue was sutured with 2 deep sutures to adipose and 3 – 5 interrupted sutures to skin. A dry dressing was applied. Subjects returned at 7 days for wound inspection and suture removal.

The dissected biopsy was placed immediately in cold (4° C) 0.9% saline for transport to the myography laboratory and further dissection.

2.8.2 Preparation and dissection of human biopsies

Gluteal biopsies were transported to the laboratory in 0.9% saline, as above. On arrival in the laboratory, they were transferred to cold physiological salt solution (PSS).

Biopsies were transferred from PSS to an agar-filled Petri dish. Dissection of the biopsy was undertaken using a stereoscopic dissecting microscope. Dissection proceeded from the adipose tissue surface of the biopsy, towards the dermal surface. Arteries were localised and were removed from the biopsy and placed into cold PSS, before being further dissected to remove adherent adipose and connective tissue.

Once dissected, vessels were divided into segments approximately 2mm in length, and were stored in cold PSS at 4°C overnight prior to experimentation.

2.8.3 Mounting of resistance arteries

One stainless steel wire (40µm diameter) was attached at one end to a jaw of the Mulvany-Halpern four-channel myograph (Danish Myo Technologies) by means of a screw fitting. This jaw is attached to a sensitive strain gauge. One 2mm segment of resistance artery was threaded onto this wire and the free end of the wire was secured (Figure 2.4). A second 40µm stainless steel wire was passed into the lumen of the artery and secured at both ends to the second jaw of the myograph which, in turn, was attached to a micrometer (Figure 2.5). The jaws of the myograph were placed in near approximation (Figures 2.6, 2.7). This arrangement of jaws and

transducer allows the radial forces generated by the vessel in constriction and relaxation to be measured as it is held between the wires (Figure 2.8).

The 5ml myograph chamber was then filled with PSS and the unit containing the bath and jaws placed upon the heated block of the myograph (37°C). A 5% CO₂ / 95% O₂ mixture was bubbled through the PSS in the chamber.

Data acquisition was performed using a Dell Latitude notebook PC2.7.5 interfacing with the myograph via an RS232 port. Powerlab data recording software (DMT) was used to record and analyse myographic data obtained.

2.8.4 Normalisation

After a rest period of 45 minutes, each artery was stretched at 1-minute intervals to determine the passive exponential wall tension / internal circumference (L) relationship. According to the Laplace equation ($P=T/r$, where P is effective pressure, T is wall tension, and r is internal radius), the equivalent circumference (L_{100}) for a transmural pressure of 100 mm Hg was calculated for each vessel with an iterative computer method. Each vessel was then set to the normalized internal diameter, $L_1=0.9 \times L_{100}/\pi$, at which contraction is thought to be optimum. After normalization, the vessels were left for an additional hour and then exposed to a high concentration (123 mmol/L) of potassium (KPSS) for a series of 5-minute periods until repeatable maximal contractions were achieved.

2.8.5 Incubation

If incubations were required, the substance would be added to the myography chamber after vessels had returned to a baseline tension following any previous contraction. All substances were incubated for a period of 30 minutes prior to curve generation.

2.8.6 Vasodilator protocols

Vessels were precontracted with norepinephrine at a concentration of $3 \times 10^{-7} \text{M}$. Following addition of the constrictor, a plateau of response was awaited before agonist added. In order to produce vasodilatation, logarithmically increasing concentrations of agonist were added. In each case, a minimum of 2 minutes was allowed between additions of further concentrations of agonist. If a vasodilator response was noted, further agonist was not added until no further dilation was occurring (i.e. a plateau phase had been achieved).

2.8.7 Vasoconstrictor protocols

Following incubation if required, logarithmically increasing concentrations of vasoconstrictor were added. After each concentration addition, the plateau phase of response was awaited before further constrictor added. Additions were continued until no further increase in tone was noted after two consecutive concentrations, or until the greatest concentration available had been added.

2.9 Laser Doppler Iontophoresis

2.9.1 Laser Doppler Imaging

Measurements of peripheral cutaneous microvascular function were performed non-invasively using laser Doppler imaging (LDI) combine with iontophoresis of the vasoactive substances acetylcholine (ACh, endothelium dependent) and sodium nitroprusside (SNP, endothelium independent). The method was discussed and validated previously (Ramsay et al. 2002; Ferrell et al. 2002) Measurements were conducted in a quiet temperature controlled room, with subjects allowed to acclimatize for 30 minutes (Figure 2.10).

2.9.2 Iontophoresis

A battery-powered constant-current iontophoresis controller (MIC-1e; Moor Instruments Ltd., Axminster, U.K) was used to deliver the vasoactive drugs. The chambers used for iontophoresis (ION 6; Moor Instruments Ltd.) were made of Perspex (internal diameter, 22 mm; area, 3.8 cm²) with an internal platinum wire electrode. Doubled-sided adhesive disks were used to attach the chambers to the skin of the volar aspect of the forearm, avoiding scratches, hair, broken skin, and superficial veins. The chambers were connected to the anode and cathode connections on the iontophoresis controller. Voltage across the chambers was monitored using a voltage-sensing device (MIC-OP, Moor Instruments Ltd). Because a constant current source was used, resistance values were calculated from the recorded voltages using Ohm's Law.

Control of current delivery was programmed into the software for the laser Doppler imager such that current was switched on at the beginning of a scan and remained on throughout the scan until the start of the following scan. The current was then either left on for the next scan or was switched off once the total charge had been delivered. Current duration was determined by the time taken to complete each scan (50s) multiplied by the total number of scans programmed. To limit the iontophoresis dose, resulting from relatively long scan times, low currents were used: the protocol involved incremental current delivery with four scans at 5 μ A, four at 10 μ A, four at 15 μ A, and two at 20 μ A, giving a total charge of 8 mC. Each frame is associated with the current delivery during that scan, although the resulting vascular response could be delayed owing to the time required for chemical factors to initiate it. A 2.5-ml volume of 1% acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) was introduced into the anodal chamber with 2.5 ml of 1% sodium nitroprusside (Sigma) into the cathodal chamber. Thus both agents were delivered simultaneously during each period of current administration. Fluid was prevented from escaping by placing circular 32-mm cover slips over the chambers. The vehicle for these drugs was 0.5% NaCl.

2.9.3 Perfusion measurements

Non-invasive measurement of skin perfusion was performed by means of a laser Doppler imager (Moor Instruments Ltd.) equipped with a red laser (wavelength, 633 nm; power, 1 mW; beam diameter, 1 mm). The technique is based on the Doppler shift imparted by moving blood cells in the underlying tissue to the backscattered light. The laser is scanned in a raster fashion over both chambers and through the

cover slips. The backscattered light is collected by photo detectors and converted into a signal proportional to perfusion in arbitrary perfusion (flux) units (PU) that is displayed as a colour-coded image on a monitor. Perfusion measurements were obtained using the imager manufacturer's image analysis software by outlining a region of interest (ROI) around the internal circumference of the chamber. Statistical analysis of the ROI was subsequently performed offline to yield the median flux value across approximately 700 measurement points. Twenty repetitive scans were taken, the first being a control (before current administration), followed by the incremental current protocol described above (14 scans), and followed by five further scans with no current administration.

2.9.4 Terminology and units used in laser Doppler iontophoresis

As the volume of tissue sampled is not known, laser Doppler imaging does not permit measurement of blood flow in absolute terms, but rather changes in tissue perfusion, measured in arbitrary units called flux or perfusion units (PU). The Moor LDI has a wide dynamic range, with perfusion measurable across 0-5000 PU.

Flux: perfusion in arbitrary units (perfusion units; PU)

AUC: area under the curve

Corrected AUC: area under the curve in which flux is corrected for skin resistance using Ohm`s Law.

Perfusion can be corrected for variations in skin resistance between subjects thereby reducing measurement variability (Ramsay et al. 2002), and this is expressed as

corrected perfusion units which equals PU times the area under the resistance.time
integral (PU x M Ω .min)

Charge is expressed in milli Coulombs (mC).

2.10 Measures of arterial compliance

2.10.1 General details

Measurements of arterial stiffness were made using a Sphygmocor system (Atcor Medical, Sydney, Australia), which uses the principle of applanation tonometry to record a peripheral arterial waveform, and applying a generalized transfer function to obtain a derived central aortic waveform. Flattening (applanation) of a curved surface of a pressure-containing structure under the detecting device allows direct measurement of the pressure within.

SphygmoCor utilizes a small pencil sized tonometer (pressure transducer) that is placed on the skin over the radial and carotid arteries. The radial and carotid pulses were located by palpation with the optimal position selected as the position that yielded the quality signal output. The tonometer was placed at an angle that was perpendicular or directly above the artery with the appropriate pressure applied.

The SphygmoCor PWV was determined by using the intersecting tangent algorithm to identify the foot of the waveform using gating to the R-wave of the electrocardiogram (ECG) for the two sites. The tonometer was placed at the same point of the arteries during all testing. Recordings were analysed when a reproducible signal was obtained (usually two screens or 10 consecutive beats to cover a complete respiratory cycle are needed for subsequent analysis).

The SphygmoCor machine comes with two assessment facilities, pulse wave analysis (PWA) to calculate augmentation index (AI) and pulse wave velocity (PWV).

2.10.2 Pulse wave analysis

Measurements were carried out in a temperature-controlled room with the subject lying prone. ECG leads were attached to both arms and the left leg. The distances from the sternal notch to the patient's right radial artery and carotid artery were measured in mm. These measurements were added to the Sphygmocor database along with the patient's blood pressure, age, sex, weight and height.

Measurements of augmentation index were made by placing a tonometer over the most prominent area of pulsation of the right radial artery. Augmentation is probably the most important index derived from PWA. Because of the relationship between HR and AI, the software provides an AI value corrected for a HR of 75 beats/min and referred to as heart rate corrected –AI (c-AI).

2.10.3 Pulse wave velocity

Measurements were carried out in a temperature-controlled room with the subject lying prone.

SphygmoCor 2000 uses the principle of foot-to-foot method for calculating PWV. With time delay (transit time) measured between the foot of the pressure waves recorded at 2 different points (carotid-femoral) for aortic PWV and (carotid-radial) for brachial PWV, ECG gating allows the time lapse between pulse waves at the carotid and (radial/femoral) sites to be calculated from sequential rather than simultaneous measurements. PWV is calculated as the superficially measured distance divided by the transit time and expressed in metres / second (m/s). PWV is inversely related to the distensibility of the arterial walls.

2.11 Statistics

All statistical analyses were performed using a Minitab version 15 software package. Results are expressed as mean and the standard error of the mean (SEM). Comparisons of anthropometric measurements and circulating markers were made using the Student's *t* test. Correlation data was expressed as a Pearson's correlation coefficient. Comparisons of pD_2 and maximal response values for the myography studies were made using the Student's *t* test. LDI mean flux data was compared using 2 way ANOVA, and AUC data using Student's *t* test. Augmentation index and pulse wave velocity were compared using the Student's *t* test.



Figure 2.3: BODPOD equipment for the measurement of body composition.



Figure 2.4: Retrograde cannulation of a dorsal hand vein.

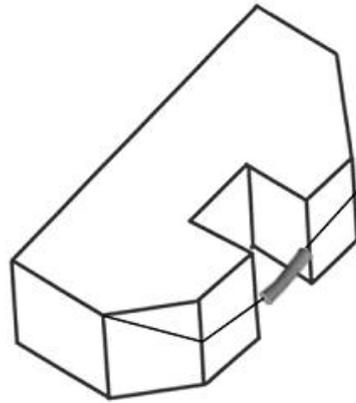


Figure 2.5 Vessel secured with first 40 μ m stainless steel wire.

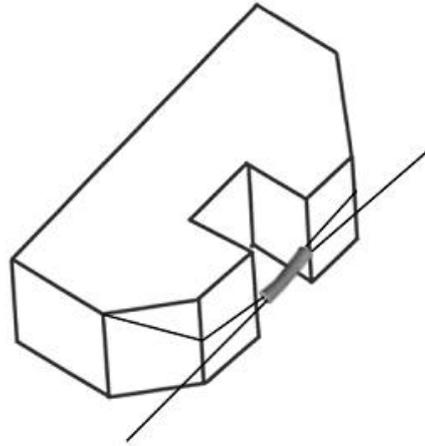


Figure 2.6 Second wire inserted

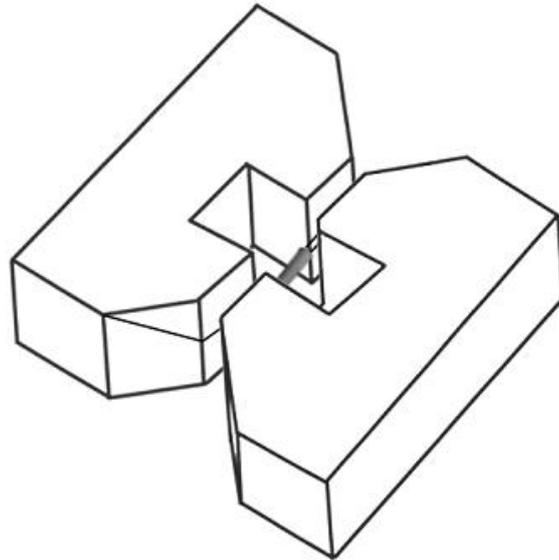


Figure 2.7: Both jaws of myograph opposed with wires mounted

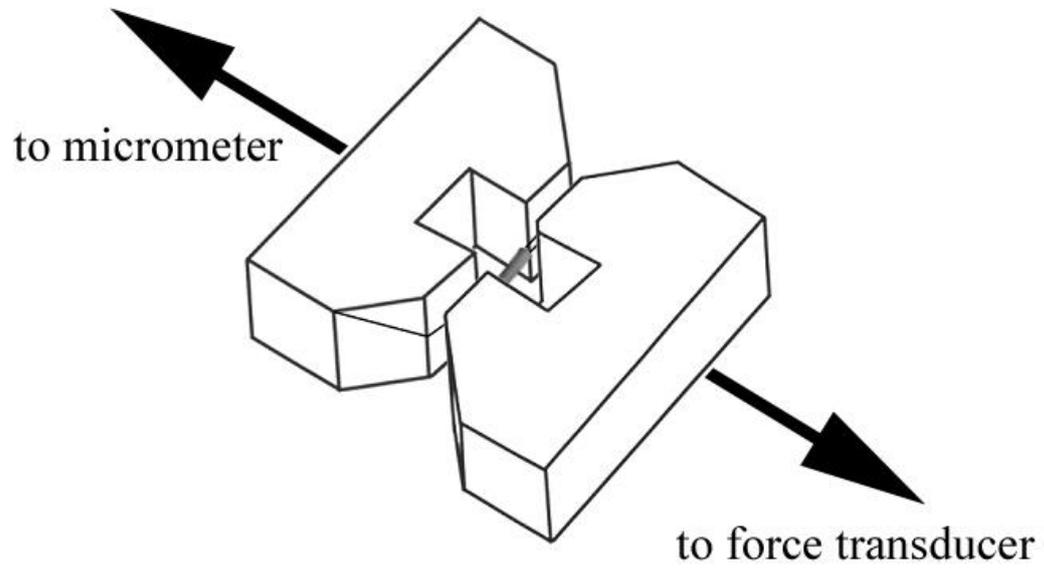


Figure 2.8 Jaws attached to a force transducer and a micrometer

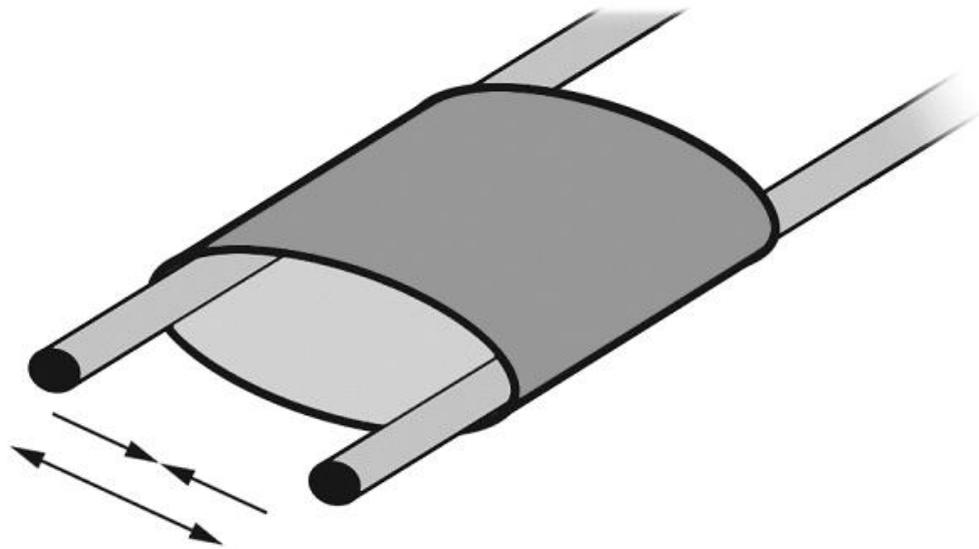


Figure 2.9 Measurement of radial force generated by vessel



Figure 2.10: Laser Doppler Imager and iontophoresis set-up

- a Drug chambers
- b Laser Doppler Imager
- c Iontophoresis Controller
- d Voltage monitor (Sensing Device)
- e Perfusion images
- f Thermometer probe

CHAPTER 3

EFFECT OF DIETARY INTERVENTION ON ANTHROPOMETRIC VARIABLES

3.1 Summary

The effect of dietary manipulation on body weight is well established. Although measures of BMI and body weight are useful for screening purposes, measures of body composition are necessary to determine the effects of diet on total body fat and fat-free mass (FFM). This study employed dietary means aiming for both reductions in body weight and in fat mass, with relative preservation of FFM.

Subjects were weighed prior to, and every week throughout the 6-week dietary intervention. In addition, skinfold thickness measurements, waist circumference and hip circumference were obtained at weekly intervals. Body fat percentage was measured at the start of and following the dietary intervention period using two methods – air displacement plethysmography, and measurement of total body water using the deuterium dilution technique.

The mean reduction in body weight following the intervention was 7.66 ± 0.64 kg ($p < 0.001$). Mean reductions in waist and hip circumferences were 7.67 ± 1.17 cm ($p < 0.001$) and 4.93 ± 0.0554 cm ($p < 0.001$) respectively. WHR dropped from 0.96 to 0.93 ($p = 0.095$). Body fat percentage was also significantly reduced when measured by air displacement plethysmography, total body water and when estimated from skinfold thickness.

In summary, a six-week 1200 kcal/day diet leads to significant weight loss, reductions in other anthropometric measurements, and reduced body fat percentage.

3.2 Introduction

There is an increasing prevalence of obesity and type 2 diabetes throughout the world, however the relationship between adiposity, insulin resistance and type 2 diabetes, and cardiovascular disease has not yet been fully elucidated. However, there is evidence that weight loss, and in particular reduced body fat is associated with improvements in metabolic parameters. The main objective of this study was to assess the effect of weight loss on vascular function, but it was important to ensure that the imposed dietary measures led to weight loss and changes in body composition.

BMI and weight are simple yet important means of assessing the effects of a dietary regimen, and patients easily understand changes in these measurements. They are probably less useful from the point of view of assessing body composition, which is why other simple anthropometric measurements such as waist and hip circumference were employed in this study. In addition, changes in waist circumference are likely to be more accurate in estimating body fat loss than changes in weight or BMI.

In addition to anthropometric measurements, body composition was also measured using two modalities – total body water measurement and air displacement plethysmography. Both of these techniques have been shown to be reliable and reproducible in the measurement of body fat mass in adults (Noreen et al 2006).

3.3 Methods

3.3.1 Subjects

Twenty-two male and female subjects were recruited and randomised to a six-week period of dietary intervention or control, as outlined in section 2.1. Fifteen subjects were allocated to the intervention group. Due to poor attendance of the seven control subjects, the dataset for this subgroup is very small and is therefore not presented here.

Written informed consent was obtained from each subject. The local hospital ethical review committee approved the study and study protocols.

3.3.2 Study protocol

Subjects attended for investigations before, during and after the six-week dietary intervention period as outlined in section 2.2.

3.3.3 Anthropometric measurements

a. Weight and BMI

Height and weight measurements were obtained and BMI was calculated as per section 2.4.1/2. These measurements were undertaken on a weekly basis throughout the intervention period.

b. Waist and hip circumference measurements

Waist and hip measurements were obtained, and WHR calculated as per section 2.4.3.

c. Skinfold thickness measurements

These measurements were obtained at baseline and after six weeks of dietary intervention as per section 2.4.4.

3.3.4 Measures of body composition

a. Air displacement plethysmography

Body composition was assessed using the BODPOD system (described in section 2.5.1) at baseline and after the intervention period.

b. Total body water

Body composition was also assessed using the three-compartment technique of total body water as described in section 2.5.2.

3.3.4 Statistics

Results are expressed as mean and the standard error of the mean (SEM). Comparisons were made using the Student's *t* test. Correlation data was expressed as a Pearson's correlation coefficient.

3.4 Results

3.4.1. Effect on body weight and body mass index

There was a significant reduction in body weight following the dietary intervention. (Mean weight at randomisation 100.01 ± 2.34 kg vs. mean weight at study completion (six weeks) weight 92.42 ± 2.14 kg, $n=15$. $p<0.0001$ (Figure 3.1)).

There was a significant reduction in BMI following the intervention. (Mean BMI at randomisation 35.75 ± 0.86 kg/m² vs. mean weight at study completion 33.21 ± 0.92 kg/m², $n=15$. $p<0.0001$ (Figure 3.2)).

3.4.2. Effect on waist circumference

There was a significant reduction in waist circumference following the dietary intervention. (Mean waist circumference at randomisation 109.47 ± 2.16 cm vs. mean waist circumference at study completion (six weeks) 101.43 ± 1.72 cm, $n=15$. $p<0.0001$ (Figure 3.3))

3.4.3 Effect on hip circumference

There was a significant reduction in hip circumference following the intervention. (Mean hip circumference at randomisation 114.23 ± 2.87 cm vs. mean hip circumference at study completion (six weeks) 109.3 ± 3.04 cm, $n=15$. $p<0.0001$ (Figure 3.4)).

3.4.4. Effect on waist-hip ratio

There was no significant difference in waist-hip ratio following the intervention. (Mean waist-hip ratio at randomisation 0.96 ± 0.03 vs. mean waist-hip ratio at study completion (six weeks) 0.93 ± 0.02 , $n=15$. $p=0.095$).

3.4.5. Effect on body fat percentage estimated from skinfold thickness measurements

Percentage body fat estimated from skinfold thickness measurements was significantly lower following the intervention. (Mean percentage body fat at randomisation $33.18 \pm 0.82\%$ vs. mean percentage body fat at study completion $32.17 \pm 0.83\%$. $n=15$, $p<0.05$ (Figure 3.5)).

3.4.6. Effect on body fat percentage measured by BODPOD

Percentage body fat measured by air displacement plethysmography (ADP) was significantly lower following the intervention. (Mean percentage body fat (as measured by ADP) at randomisation $38.83 \pm 1.91\%$ vs. mean percentage body fat at study completion $32.79 \pm 2.62\%$, $n=9$. $p=0.001$ (Figure 3.6)).

3.4.7. Effect on body fat percentage measured by total body water.

Mean body fat percentage as measured by the total body water method was significantly lower after the intervention than at baseline. (Mean percentage body fat

(as measured by TBW) at randomisation $38.25 \pm 2.46\%$ vs. mean percentage body fat at study completion $36.04 \pm 3.03\%$, $n=5$. $p<0.05$ (Figure 3.7)).

3.5 Discussion

This study has shown that adherence to a 1200kcal/day liquid-based diet leads to significant reductions in body weight and BMI in subjects with type 2 diabetes. The prescribed diet consisting mainly of soups, cereals, puddings and fruit, led to an 8% reduction in body weight. It has been shown that body weight reduction of 5-10% has health benefits, and weight loss similar to that seen in our study has been associated with reduced mortality (Williamson et al 2000).

Unsurprisingly, weight reduction was associated with reduction in waist circumference. Waist circumference correlates closely with body weight and BMI and also with abdominal obesity (Balkau et al, 2007). In this study the degree of reduction in body weight was highly correlated with the change in waist circumference (Figure 3.8). This was encouraging as it suggests benefit conferred by weight loss in terms of reduction in abdominal obesity. Unfortunately, we did not employ another modality for the measurement of abdominal obesity and if the study was to be repeated then it may be useful to utilise CT or MRI scanning in order to properly assess changes in abdominal and visceral obesity.

More surprisingly perhaps was the highly significant reduction in hip circumference associated with weight reduction. In contrast to waist circumference, hip circumference has been shown to correlate negatively with the metabolic syndrome and type 2 diabetes (Lissner et al, 2001). However, significant reductions in hip circumference were demonstrated in a study examining the effects of weight loss in a cohort of females with PCOS, using dietary manipulation that was similar to the approach adopted in this study (Croisignani et al, 2003).

In this cohort, no significant reduction in waist-hip ratio was observed after the dietary intervention. Other weight loss intervention studies have observed contrasting results in terms of the effect of calorie reduction on WHR. Bergholm et al (2003) observed a significant reduction in WHR in combination with mean weight loss of 7.3kg in a cohort of females with previous gestational diabetes, following 3-6 months of diet and orlistat or placebo. In contrast however, weight loss of around 3kg following six months treatment with sibutramine in a cohort of patients with type 2 diabetes was not associated with any change in WHR (Hung et al, 2005). In addition, Janssen et al (2002) assigned 38 obese females to diet alone or diet with aerobic or resistance exercise for a period of 14 weeks. This was associated with weight loss of around 10kg (10% of body weight) in all three groups but no changes in WHR.

There were significant reductions in body fat percentage in this study, as measured by three modalities. Mean body fat percentage was lower after the intervention when measured by the techniques of air displacement plethysmography, total body assessment and estimations derived from skinfold thickness. All three of these techniques are reliable and reproducible. However, none of these methods take into account the heterogeneous nature of fat-free mass i.e. the variable degrees of bone mineralisation and hydration, and this incurs a degree of error in estimating fat-free mass. This is a further limitation of this study that could have been overcome by measuring body fat percentage with a four-compartment model method such as DXA.

In addition, there was no correlation between the reductions in body fat percentage as measured by three methods in this study. This is likely due to small sample size and missing data.

Therefore, this study has demonstrated that significant reductions in body weight, waist and hip circumference, and body fat percentage can be achieved following a relatively short period of dietary intervention.

	Pre diet	Post diet	p value
Weight (kg)	100.01 (2.34)	92.42(2.14)	p<0.0001
BMI (kg/m)	35.75 (0.86)	33.21 (0.91)	p<0.0001
Waist circumference (cm)	109.47(2.16)	101.43(1.72)	p<0.0001
Hip circumference (cm)	114.23(2.87)	109.3(3.04)	p<0.0001
WHR	0.96(0.03)	0.93(0.02)	ns
% Body fat (skinfolds)	33.18 (0.82)	32.17 (0.32)	p<0.05
% Body fat (TBW)	38.25 (2.46)	36.04 (3.03)	p<0.05
% Body fat (BODPOD)	38.18 (1.91)	32.79 (2.62)	p<0.05

Table 3.1: Anthropometric variables at baseline and following intervention.

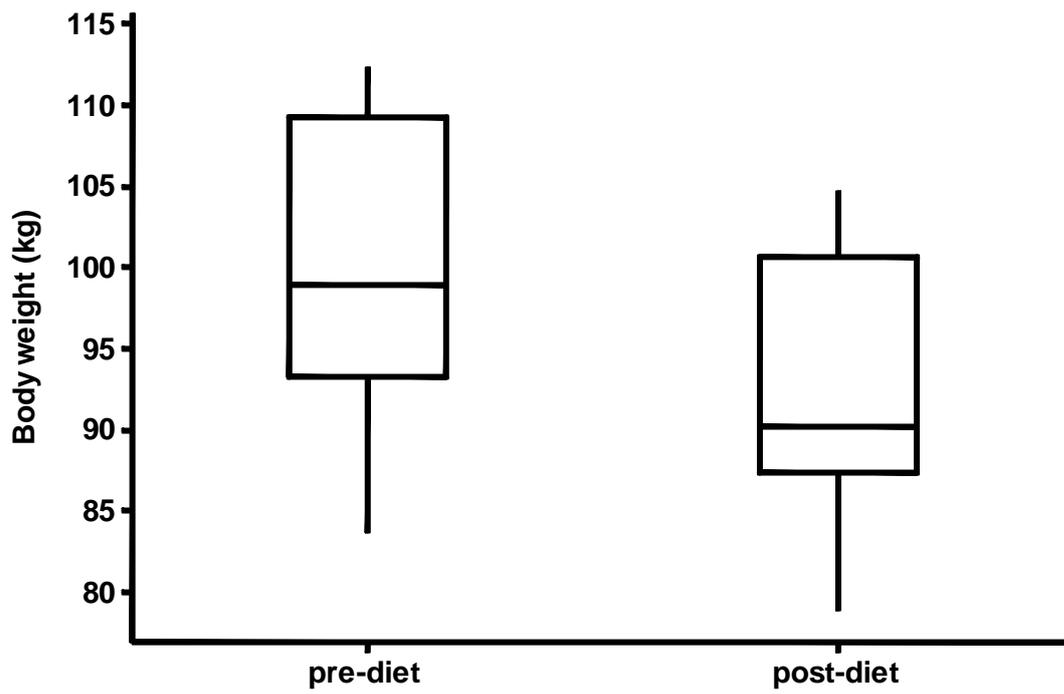


Figure 3.1: Body weight at baseline and following dietary intervention.

n=15, p<0.0001

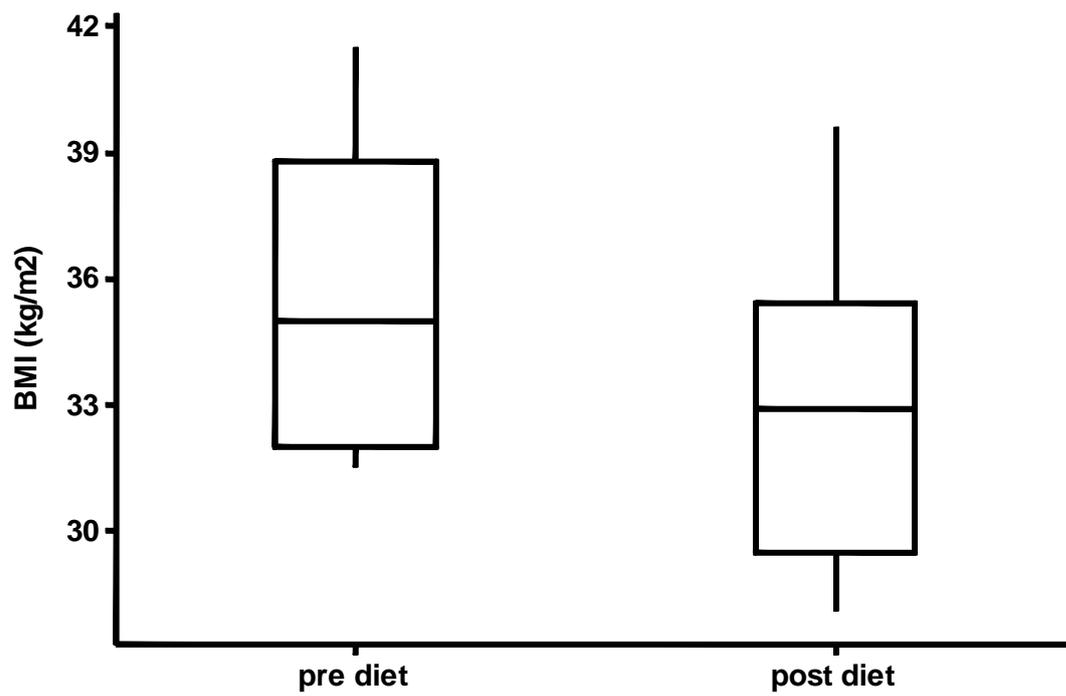


Figure 3.2: BMI at baseline and following intervention

n=15, p<0.0001

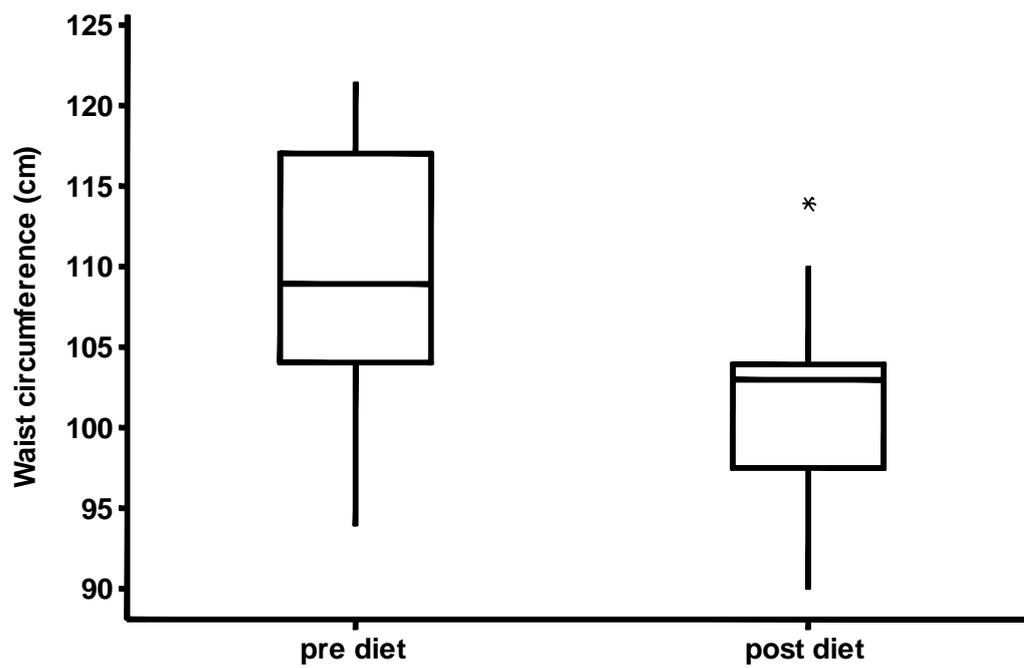


Figure 3.3: Waist circumference (cm) at baseline and following dietary intervention.

n=15, $p < 0.0001$

* outlier

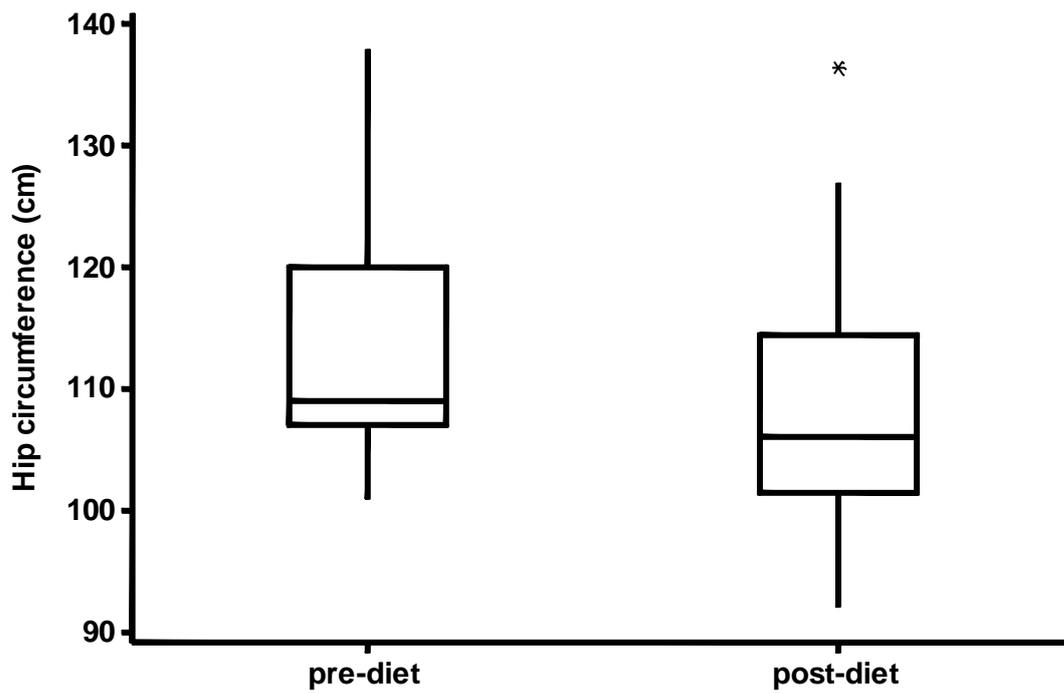


Figure 3.4: Hip circumference at baseline and following dietary intervention

n=15, $p < 0.0001$

* outlier

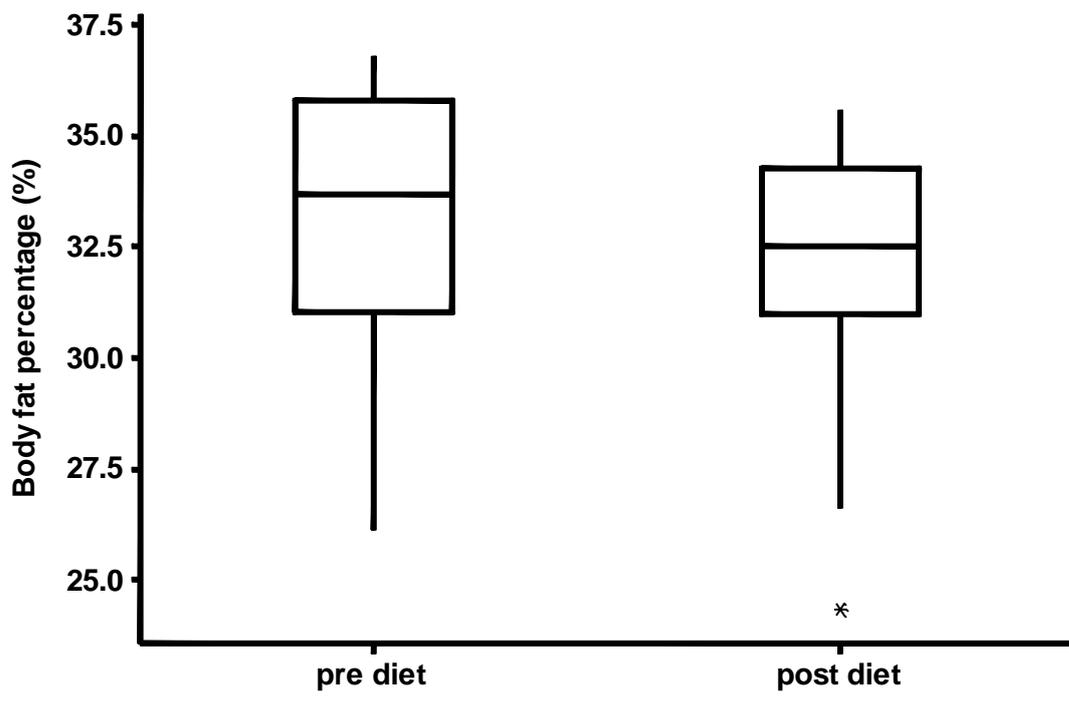


Figure 3.5 Body fat percentage estimated from skinfold thickness at baseline and following intervention.

n=15, p<0.05.

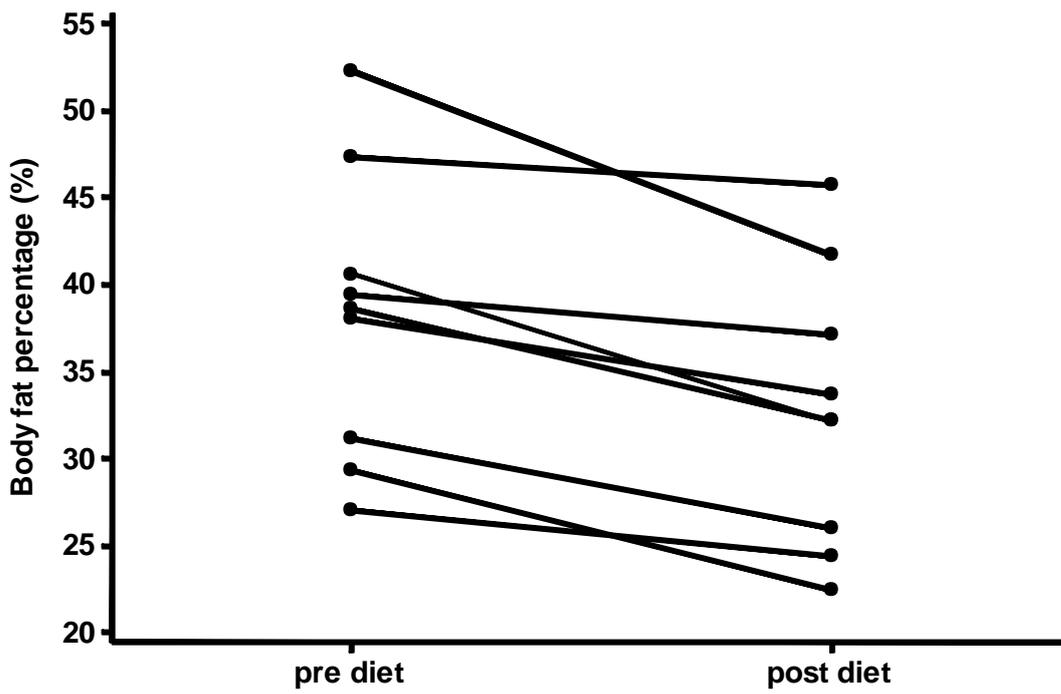


Figure 3.6: Body fat percentage as measured by ADP method at baseline and following intervention.

n=9, p=0.001

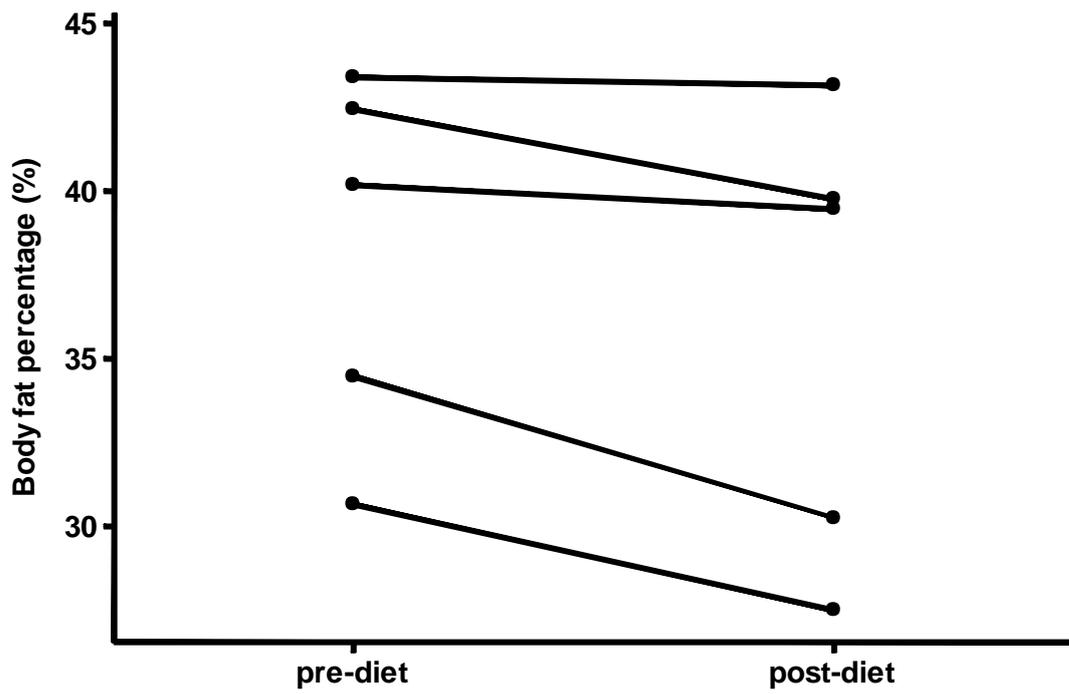


Figure 3.7: Body fat percentage as measured by deuterium dilution method at baseline and following intervention.

n=5, p<0.05

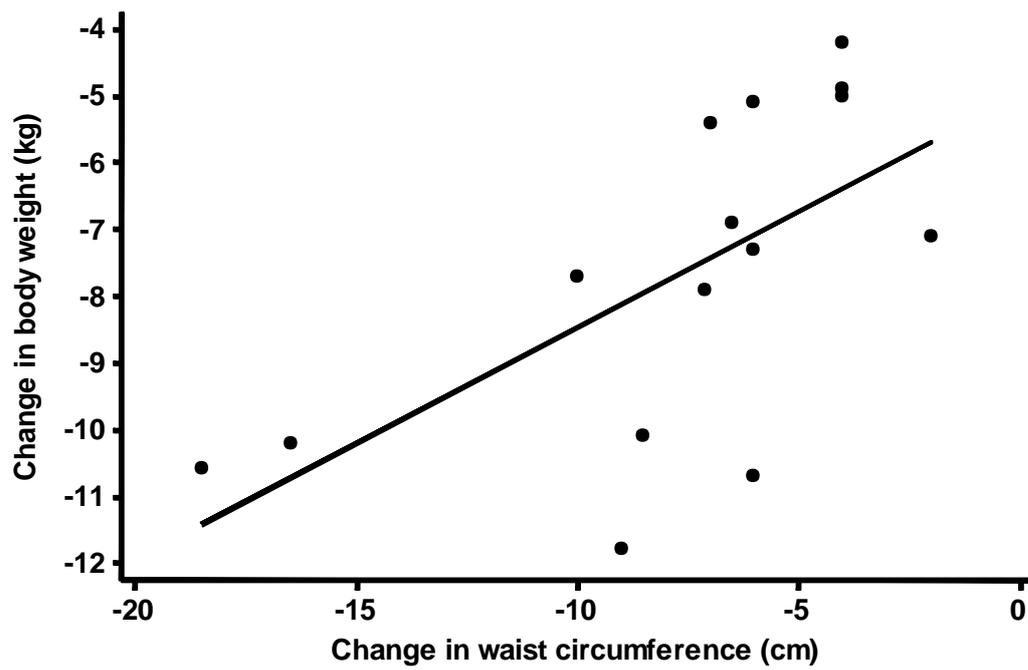


Figure 3.8 Correlation between change in body weight and change in waist circumference.

$p=0.01$, $r=0.633$

CHAPTER 4

EFFECTS OF DIETARY INTERVENTION ON METABOLIC PARAMETERS

4.1 Summary

The effect of weight loss on glucose tolerance and insulin sensitivity is established. It is clear that IGT and diabetes can be modified by changes in lifestyle. Insulin resistance and hyperglycaemia are associated with endothelial dysfunction and increases in the concentrations of inflammatory cytokines, both of which may contribute to the increased vascular risk seen in association with diabetes. The aim of these particular studies was to measure the effect of weight loss on other metabolic and vascular parameters in a cohort of subjects with type 2 diabetes, and to assess whether the anticipated improvement in metabolic markers was also associated with an improvement in circulating markers of endothelial function and inflammation.

Methods: Subjects attended for investigations before and after the intervention period. Baseline samples were obtained prior to the clamp study. Other samples were obtained during the clamp. Samples were stored and subsequently analysed for a number of metabolic and vascular markers.

Results: There were significant reductions in fasting plasma glucose, HbA1c and total cholesterol following the intervention. Serum ICAM concentration was reduced following the intervention. There were no significant changes in other vascular

markers. Significant improvements in AST, ALT and GGT were observed following the intervention. Changes in liver function and lipids correlated with changes in parameters of glucose tolerance.

Conclusion: Intentional weight loss in obese subjects with type 2 diabetes leads to improvements in glycaemic control, cholesterol and liver function. No significant changes in inflammatory cytokines were observed.

4.2 Introduction

The prevalence of obesity is increasing in both the developed and developing worlds and is threatening to be a major burden on health care resources. The main reason for this is essentially the metabolic perturbations associated with increased body weight, especially with regards to central abdominal obesity. The association between central obesity and insulin resistance is driving the current pandemic of type 2 diabetes (Gregg et al, 2004). Associated with this is a constellation of other biological changes such as hypertension and deranged lipoprotein metabolism, collectively known as the metabolic syndrome (Alberti et al, 1998). This combination of risk factors promotes a pro-atherogenic environment within the vasculature, and ultimately leads to the development of atherosclerotic cardiovascular disease (Gami et al, 2007).

Weight loss through means of lifestyle changes, pharmacotherapy and bariatric surgery has been shown to improve these metabolic and vascular factors. The majority of these studies have been carried out in patient groups without type 2 diabetes but who do have established risk factors such as insulin resistance and hyperlipidemia. In fact, lifestyle interventions have been shown to be very effective in reducing the risk or delaying the onset of type 2 diabetes in large randomised controlled trials (Tuomilehto et al, 2001; Knowler et al, 2002). The metabolic and vascular effects of weight loss are less well documented. Although lifestyle interventions in the form of diet and exercise improve insulin sensitivity and glycaemic control (Scheen et al, 1998), there is little evidence to suggest that weight

reduction is also associated with improvements in circulating markers in patients with type 2 diabetes.

Therefore, a further hypothesis investigated in this study was that weight loss by dietary means leads to improvements in circulating metabolic and vascular markers in subjects with obesity and type 2 diabetes.

4.3 Methods

4.3.1 Subjects

Twenty-two male and female subjects were recruited and randomised to a six-week period of dietary intervention or control, as outlined in section 2.1. Fifteen subjects were allocated to the intervention group. Due to poor attendance of the seven control subjects, the dataset for this subgroup is very small and is therefore not presented here.

Written informed consent was obtained from each subject. The local hospital ethical review committee approved the study and study protocols.

4.3.2 Study protocol

Subjects attended for investigations before, during and after the six-week dietary intervention period as outlined in section 2.2.

4.3.3 Hyperglycaemic isoglycaemic clamp

Clamp studies were performed as outlined in section 2.6

4.3.4 Analysis of blood samples

a. General Protocols

Blood samples were obtained as outlined in section 2.7.1

b. Routine biochemistry

Electrolytes and liver function tests were analysed as outlined in section 2.7.2.

c. Lipids and CRP

Lipids and CRP analyses were carried out as outlined in section 2.7.3.

d. Endothelial markers

Endothelial marker analyses were carried out as outlined in section 2.7.4.

e. Plasma and serum glucose concentrations

Plasma glucose concentrations were analysed as outlined in section 2.7.5.

4.3.5 Statistics

Results are expressed as mean \pm standard error of the mean (SEM). Comparisons were made using the Student's *t* test. Correlation data was expressed as a Pearson's correlation coefficient.

4.4 Results

4.4.1 Effect on fasting plasma glucose

There was a significant reduction in fasting plasma glucose (FPG) concentration following the dietary intervention. (Mean FPG at randomisation 9.3 ± 1.92 mmol/l vs mean FPG at study completion (six weeks) 7.0 ± 1.26 mmol/l, $n=15$, $p<0.0001$ (Figure 4.1)).

4.4.2 Effect on Haemoglobin A1c

There was a significant reduction in HbA1c concentration following the dietary intervention. (Mean HbA1c at randomisation $7.13 \pm 0.19\%$ vs mean HbA1c at study completion (six weeks) $6.36 \pm 0.16\%$, $n=15$, $p<0.0001$ (Figure 4.2)).

4.4.3 Effect on serum lipids

a. Total cholesterol

There was a significant reduction in serum total cholesterol (TC) concentration following the dietary intervention. (Mean TC at randomisation 4.55 ± 0.23 mmol/l vs mean TC at study completion (six weeks) 4.18 ± 0.19 mmol/l, $n=14$, $p<0.05$ (Figure 4.3)).

b. Low density lipoprotein cholesterol (LDL-c)

There was no significant difference in calculated LDL-c concentration following the dietary intervention. (Mean LDL-c at randomisation 2.53 ± 0.21 mmol/l vs mean LDL-c at study completion (six weeks) 2.27 ± 0.21 mmol/l, $n=13$, $p=0.093$).

c. High density lipoprotein cholesterol (HDL-c)

There was no significant difference in HDL-c concentration following the intervention. (Mean HDL-c at randomisation 1.07 ± 0.07 mmol/l vs mean HDL-c at study completion (six weeks) 1.07 ± 0.05 mmol/l, $n=13$, $p=0.90$).

d. Very low density lipoprotein cholesterol (VLDL-c)

There was no significant difference in VLDL-c concentration following the intervention. (Mean VLDL-c at randomisation 0.94 ± 0.16 mmol/l vs mean HDL-c at study completion (six weeks) 0.88 ± 0.19 mmol/l, $n=13$, $p=0.74$).

e. Triglycerides

There was no significant difference in triglyceride concentration following the intervention. (Mean triglyceride concentration at randomisation 2.11 ± 0.32 mmol/l vs mean triglyceride concentration at study completion (six weeks) 1.91 ± 0.39 mmol/l, $n=13$, $p=0.56$).

4.4.4 Effect on circulating factors

a. Adiponectin

There was no significant difference in serum adiponectin concentration following the intervention. (Mean adiponectin concentration at randomisation 2725 ± 379 ng/ml vs mean adiponectin concentration at study completion (6 weeks) 3004 ± 359 ng/ml, $n=13$, $p=0.276$).

b. Interleukin-6 (IL-6)

There was no significant difference in serum interleukin-6 concentration following the intervention. (Mean IL-6 concentration at randomisation 3.07 ± 0.45 pg/ml vs mean IL-6 concentration at study completion (6 weeks) 4.22 ± 1.03 pg/ml, $n=13$ $p=0.23$).

c. Intercellular adhesion molecule 1 (ICAM-1)

There was a significant reduction in serum ICAM-1 concentration after the intervention. (Mean ICAM-1 concentration at randomisation 243.1 ± 11.50 ng/ml vs mean ICAM-1 concentration at study completion (6 weeks) 226.6 ± 11.70 ng/ml, $n=13$ $p=0.002$. (Figure 4.4)).

d. High sensitivity c-reactive protein (hsCRP)

There was no significant change in serum hsCRP concentration following the intervention. (Mean hsCRP at randomisation 4.86 ± 1.54 mg/l vs mean hsCRP at study completion (6 weeks) 5.40 ± 1.66 mg/l, $n=13$ $p=0.696$).

4.4.5 Effect on insulin sensitivity

There was a significant improvement in insulin sensitivity following the intervention. (Mean MCR at baseline 6.36 ± 0.97 mg/kg/min vs mean MCR at study completion 8.83 ± 1.45 mg/kg/min, n=9, p<0.05 (Figure 4.5)).

4.4.6 Effect on Liver Enzymes

a. Aspartate aminotransferase (AST)

There was a significant reduction in AST concentrations following the intervention. (Mean AST concentration at baseline 32.15 ± 4.57 U/l vs mean AST concentration at study completion 25.92 ± 3.63 U/l n=13. p<0.05 (Figure 4.6).

b. Alanine aminotransferase (ALT)

There was a significant reduction in ALT concentration following the intervention. (Mean ALT concentration at baseline 53.5 ± 12.50 U/l vs mean ALT concentration at study completion (6 weeks) 36.4 ± 7.00 U/l, n=13, p<0.05, Figure 4.7)).

c. Gamma-glutamyl peptidase (GGT)

There was a significant reduction in GGT concentration following the intervention. (Mean GGT concentration at baseline 49.0 ± 14.70 U/l vs mean GGT concentration at study completion 39.7 ± 14.40 U/l. n=13. p< 0.05, Figure 4.8.)

4.4.7 Correlations between changes in metabolic parameters before and after intervention

a. IL-6 and hsCRP

Changes in IL-6 concentrations correlated significantly with changes in hsCRP concentrations.

$r=0.89$, $p<0.0001$ (Figure 4.9)

b. LDL-c and FPG

Changes in LDL cholesterol concentrations correlated significantly with changes in FPG concentration

$r=0.56$, $p<0.05$ (Figure 4.10)

c. AST and HbA1c

Changes in AST concentrations correlated significantly with changes in HbA1c levels.

$r=0.74$, $p=0.004$ (Figure 4.11)

d. ALT and HbA1c

Changes in ALT concentrations correlated significantly with changes in HbA1c levels.

$r=0.75$, $p=0.003$ (Figure 4.12)

f. ALT and FPG

Changes in ALT concentrations correlated significantly with changes in FPG concentrations.

$r=0.73$, $p=0.005$ (Figure 4.13)

g. ICAM and ALT

Changes in ICAM concentrations correlated significantly with changes in ALT concentrations.

$r=0.57$, $p<0.05$ (Figure 4.14)

h. LDL-c and GGT

Changes in LDL-c concentrations correlated significantly with changes in GGT concentrations.

$r=0.74$, $p<0.005$ (Figure 4.15)

i. Waist circumference and LDL-c

Changes in waist circumference correlated significantly with changes in LDL-c concentrations.

$r=0.67$, $p<0.02$ (Figure 4.16)

4.5 Discussion

4.5.1 Changes in FBP, HbA1c and insulin sensitivity

This study has demonstrated that lowering body weight by dietary intervention leads to significant improvements in fasting plasma glucose and HbA1c. It has been recognised for a few decades that marked calorie restriction resulting in weight loss can significantly improve glycaemic control in diabetic and non-diabetic subjects (Henry et al 1986). A number of other studies have confirmed these findings (Goodpaster et al, 1999; Kelley et al, 1993).

The mechanisms underlying improvements in glycaemic control following weight loss are unclear. Goodpaster et al (1999) demonstrated that weight loss is associated with reduced visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT), in combination with improved whole body insulin sensitivity. However, while changes in total body fat and SAT did not correlate with change in insulin sensitivity, change in VAT did (Petersen et al, 2005). The results of this study did not demonstrate correlation between changes in glycaemic control parameters and changes in measures of body fat and weight, but this may simply be because the study was not powered to do so.

4.5.2 Liver function tests and association with glycaemic control and insulin sensitivity

We have demonstrated that weight loss leads to reduced serum concentrations of liver enzymes in individuals with type 2 diabetes. Mean weight loss of 8% was associated with reduced serum concentrations of ALT, AST and GGT. Obesity and the metabolic syndrome are associated with fat accumulation in the liver and this can lead to a number of clinical scenarios (as outlined in chapter 1). Liver fat is closely correlated with serum ALT and AST, and weight loss has been shown to improve these parameters in insulin resistant states (Park et al, 1995; Palmer et al, 1990; Ueno et al, 1997).

This is the first study to demonstrate significant changes in liver enzymes following weight loss induced by diet alone in patients with type 2 diabetes. If these changes are indicative of reduced hepatic steatosis in this group of patients, then this adds further support for the use of lifestyle intervention in management of type 2 diabetes and the metabolic syndrome. Imaging methods used to assess changes in hepatic steatosis such as ultrasound (Engl et al, 2008) and magnetic resonance imaging (Cowin et al 2008) were not used in this study.

Here, improvements in liver enzymes were correlated with changes in parameters of glycaemic control. Changes in serum concentrations of ALT were closely associated with changes in FPG and HbA1c, whilst change in serum AST correlated with changes in HbA1c. GGT was correlated with change in FPG. Petersen et al (2005) demonstrated that weight loss (of a similar magnitude to this cohort) in a small group of patients with type 2 diabetes was associated with significant improvements in

hepatic but not peripheral insulin sensitivity. Weight loss of 8% of body weight led to normalisation of insulin-induced suppression of hepatic glucose output, with little effect on peripheral glucose uptake. These changes were associated with reduction in intrahepatic lipid content, but little or no change in intramyocellular lipid content. There were no significant changes in liver enzymes in the Petersen cohort, but baseline concentrations of ALT and AST were lower than in the current study. A further group has also recently shown that ALT is one of the best predictors for the development of type 2 diabetes in males (Sattar et al, 2004; Sattar et al 2007), also raising the possibility that intrahepatic fat deposition may be one of the main factors at play in the development and control of type 2 diabetes.

4.5.3 Changes in plasma lipid concentrations

The changes in plasma lipids observed in this study are not surprising. A significant reduction in mean total cholesterol concentration of around 0.38mmol/l, and a reduction in mean LDL-c concentration of 0.26mmol/l are fairly typical for weight loss of approximately 8% of body weight. A meta-analysis of seventy trials (Dattilo et al, 1992) has suggested that total cholesterol falls by 0.05mmol/l per kg reduction in body weight. In addition, LDL-c and triglyceride concentrations fall by around 0.02mmol/l and 0.015mmol/l respectively per kg of body weight lost. Surprisingly, HDL-c concentrations also fall during the active stage of weight reduction, and this only increases during subsequent weight stabilisation. The changes in lipid concentrations observed in this study are of a similar magnitude to those noted above.

In addition, this study has also demonstrated a positive association between change in serum LDL-c concentration and change in waist circumference. This is similar to results of a study of 110 obese (non-diabetic) women, where falls in LDL-c and total cholesterol were closely associated with a reduction in waist circumference, but not WHR (Han et al 1997).

4.5.4 Changes in circulating markers

Despite moderate reductions in body weight there were few changes in circulating factors after the dietary intervention in this study. With the exception of ICAM, all other cytokines were essentially unchanged after the period of weight loss. The available evidence on the changes in adipocytokines following weight loss also seems to be variable.

4.5.5 Significant reduction in serum ICAM-1

Our results demonstrated a significant reduction in serum ICAM-1 following weight reduction. Intracellular adhesion molecules are found on the surface of the endothelium and stimulate the adhesion of monocytes to the endothelium, this being one of the initial stages in the formation of the atherosclerotic plaque. It has been shown that circulating levels of ICAM-1 are elevated in diabetes (Wagner et al, 1997), suggesting that hyperglycaemia is associated with an increase in the adhesive properties of endothelial cells, and it has also been demonstrated that levels of circulating ICAM-1 correlates with degree of hyperglycaemia in patients with

type 2 diabetes (Bluher et al, 2002). ICAM-1 and other cellular adhesion markers are also associated with obesity.

Although it has been demonstrated repeatedly that weight loss is associated with reductions in ICAM-1 (Hamdy et al, 2003; Keogh et al, 2008; Sharman et al, 2004) there is no evidence that improved glycaemic control leads to significant changes in serum concentrations of cellular adhesion markers (Yudkin et al, 2000; Bagg et al, 2001). However, weight loss and improved glycaemic control often occur simultaneously. Pontiroli et al (2004) assessed the individual effects of each of these on serum ICAM-1 concentrations. A cohort of morbidly obese patients both with and without diabetes was treated with gastric banding surgery or dietary advice. Serum ICAM-1 concentrations were associated with hyperglycaemia and increased body weight. Although similar reductions in HbA1c were seen in both groups, weight loss was only observed in the group that had undergone bariatric surgery. Reductions in serum ICAM-1 levels were only seen in this subgroup.

TNF α is produced by adipocytes and can stimulate ICAM-1 production from the endothelium. This has been postulated as a potential mechanism for the changes observed with weight loss. In this study, TNF α was not measured and therefore correlations between changes in these two circulating markers cannot be made.

4.5.6 Lack of changes in other circulating markers

Results from studies examining the effects of dietary interventions on circulating markers in non-diabetic populations seem to suggest that weight loss leads to

amelioration in the proinflammatory environment associated with insulin resistance (Sharmann et al, 2004; Esposito et al, 2003; Ziccardi et al, 2002). However data from cohorts of diabetic subjects is scarce. Only three other small studies have assessed the effects of dietary induced weight loss in patients with type 2 diabetes. Barinas-Mitchell et al (2006) demonstrated significant reductions in IL-6, TNF α , CRP, PAI-1 and fibrinogen in 38 volunteers following average weight loss of around 10%. A one-year programme of diet, exercise and either orlistat or placebo led to these results. These results are obviously contrasting to the present study, but the duration, nature and timescale of the intervention was very different from the approach we adopted. In contrast, Giannopoulou et al (2005) looked at the effects of diet and exercise on 40 female patients with type 2 diabetes over a 14-week period. This cohort lost an average of 5kg, and although there were reductions in CRP and leptin concentrations, serum concentrations of resistin, IL-6 and TNF α remained unchanged. Similarly, four weeks of diet and exercise in 20 male volunteers led to mean weight reduction of around 4kg and improved glycaemic control (Halle et al, 1999). These changes were associated with reduced total cholesterol and leptin concentrations. However, no other circulating markers were measured.

There are a number of possible reasons why these results did not demonstrate significant changes in inflammatory markers despite weight loss. One explanation is that weight loss alone (without exercise) is insufficient to achieve significant alterations in serum concentration of inflammatory cytokines. Certainly it has been demonstrated that exercise is associated with reductions in serum IL-6 (Dekker et al, 2007) and resistin (Kadoglou et al, 2007) concentrations in subjects with type 2

diabetes. However, Giannopoulou et al were unable to demonstrate these findings in any of their three subgroups (diet alone, diet and exercise, exercise alone).

A further reason for the lack of change in inflammatory cytokines in this study could be the duration of the dietary intervention. Subjects lost an average of 8kg over a 6-week period, followed by a 1-week period of dietary washout. Although weight loss was of a similar magnitude in the Bariinas-Mitchell study (7.8% body weight), the length of time taken to achieve these losses was much longer (approximately one-year). Calorie reduction in our cohort was obviously more dramatic than in other studies. There is evidence that short-term starvation actually increases skeletal muscle IMLC and promotes peripheral insulin resistance. Perhaps relative starvation / calorie reduction has a similar effect. Although total body insulin resistance was reduced in our study, this could be due to significant reduction in hepatic insulin resistance with minimal change or an increase in skeletal muscle insulin resistance. One of the limitations of this study is that no assessment of the relative contributions of peripheral and hepatic insulin resistance were made. There are no studies that assess the effects of starvation on adipocytokine concentrations.

4.5.7 Correlation between IL-6 and CRP

We noted a very strong correlation between the changes in IL-6 and hsCRP concentrations. Although neither of these changed significantly after the intervention, it was clear there was a marked association between concentrations of these two substances. IL-6 has been shown to up-regulate hepatic production of CRP and

correlation between changes in these two substances has been demonstrated previously in a weight loss intervention study (Esposito et al, 2003).

In summary, weight loss of approximately 8 kg was associated with improvements in glycaemic control, insulin sensitivity, total cholesterol and serum concentrations of liver enzymes in this cohort of subjects with type 2 diabetes. There was also a significant reduction in serum ICAM-1 noted. On the other hand, no changes in circulating levels of adiponectin, CRP or IL-6 were observed.

	Pre intervention	Post intervention	p value
FPG (mmol/l)	9.3 ± 1.92	7.0 ± 1.26	p<0.0001
HbA1c (%)	7.13 ± 0.18	6.36 ± 0.15	p<0.0001
TC (mmol/l)	4.55 ± 0.22	4.18 ± 0.18	p<0.05
LDL-c (mmol/l)	2.53 ± 0.21	2.27 ± 0.21	ns
HDL-c (mmol/l)	1.07 ± 0.07	1.07 ± 0.05	ns
VLDL-c (mmol/l)	0.94 ± 0.15	0.88 ± 0.19	ns
Triglycerides (mmol/l)	2.11 ± 0.32	1.91 ± 0.39	ns
Adiponectin (ng/ml)	2725 ± 379	3004 ± 359	ns
IL-6 (pg/ml)	3.07 ± 0.45	4.22 ± 1.03	ns
ICAM (ng/ml)	243.1 ± 11.50	226.6 ± 11.70	p=0.002
HSCRP (mg/l)	4.86 ± 1.54	5.40 ± 1.66	ns
AST (IU/l)	32.15 ± 4.57	25.92 ± 3.63	p<0.05
ALT (IU/l)	53.5 ± 12.50	36.4 ± 7.00	p<0.05
GGT (IU/l)	49.0 ± 14.70	39.7 ± 14.40	p< 0.05

Table 4.1: Metabolic and vascular parameters at baseline and following dietary intervention.

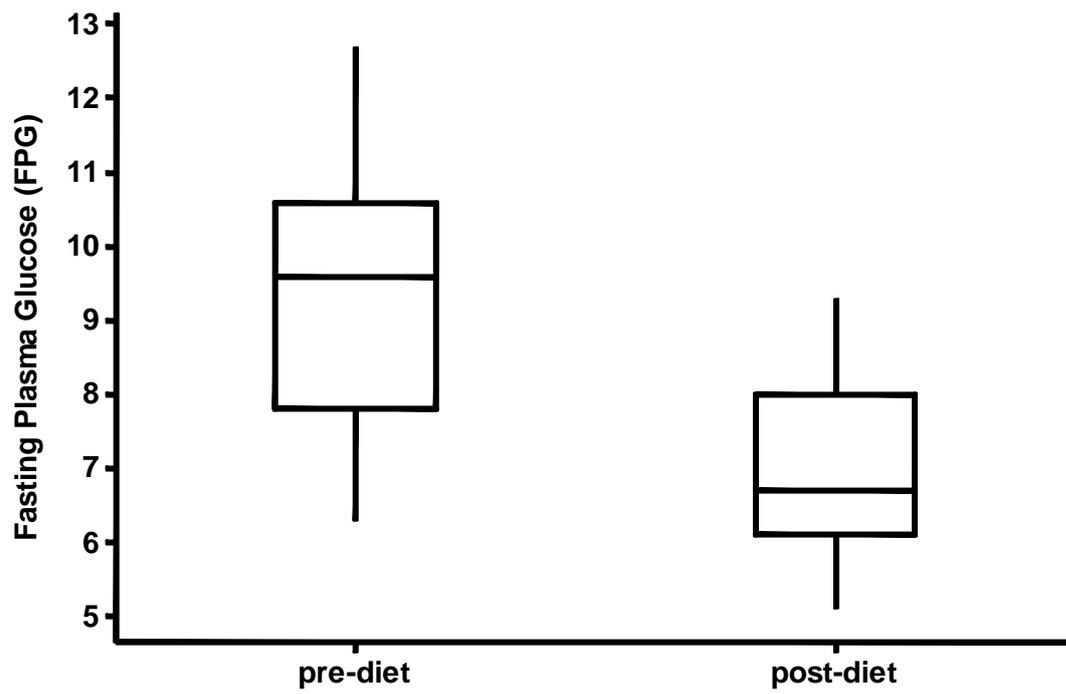


Figure 4.1 Fasting plasma glucose concentration before and after dietary intervention.

$p < 0.0001$, $n=15$

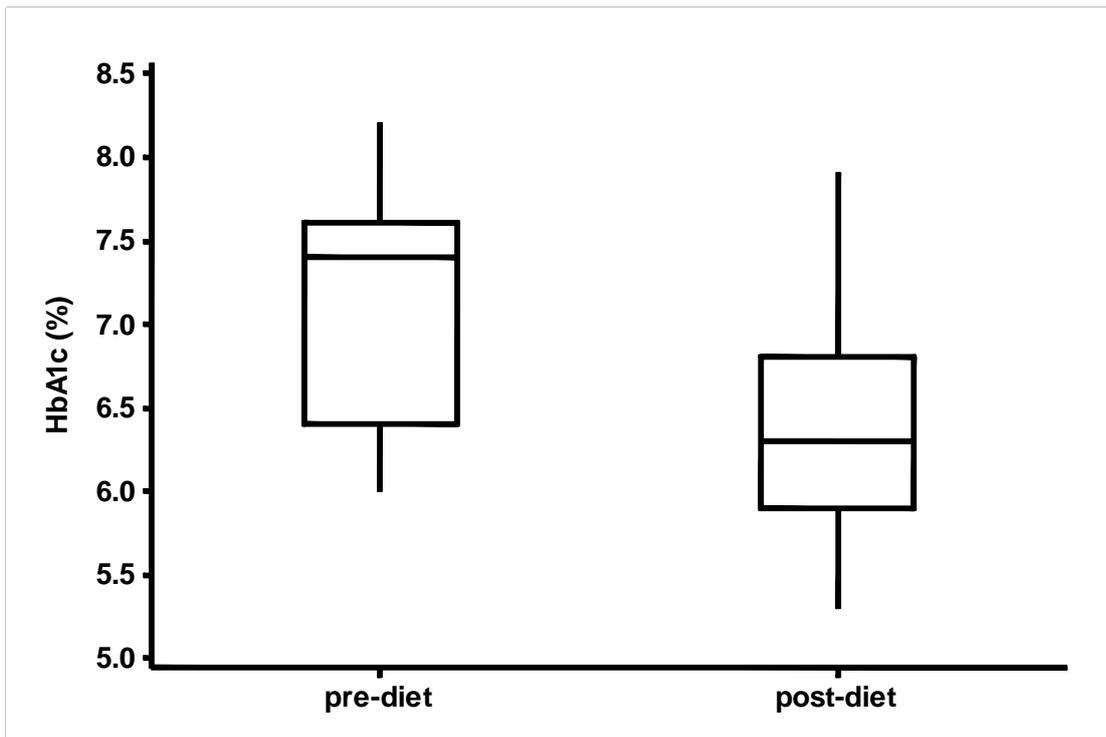


Figure 4.2: Serum (Haemoglobin A1c) HbA1c concentration before and after dietary intervention.

$p < 0.0001$, $n = 15$

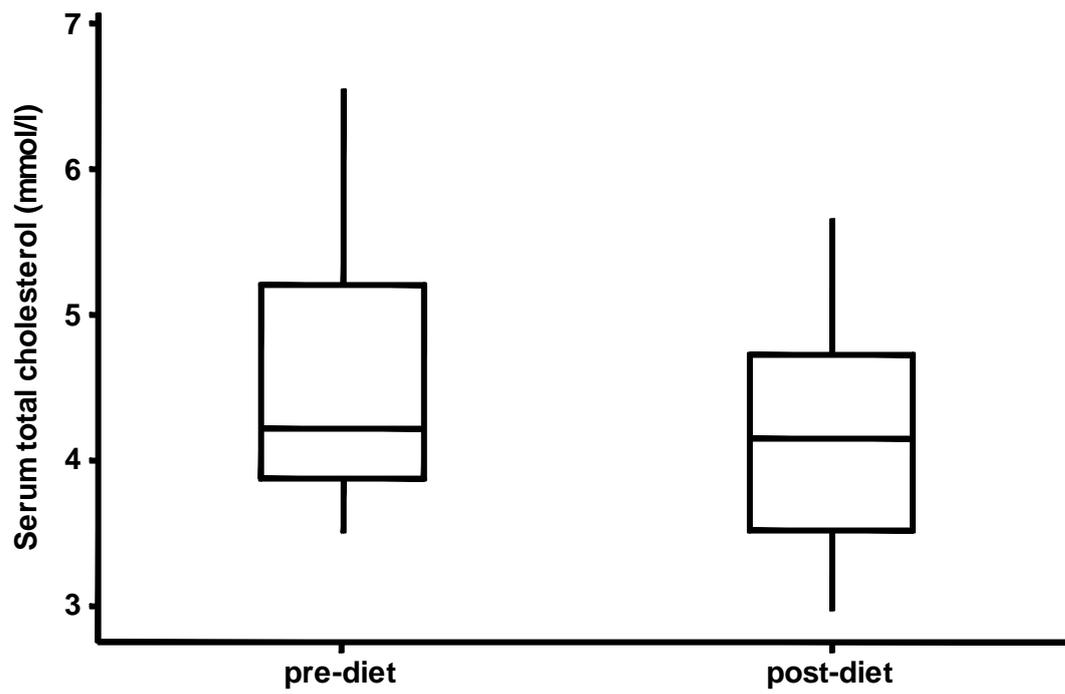


Figure 4.3: Serum total cholesterol concentration at baseline and following intervention.

$p < 0.05$, $n = 13$

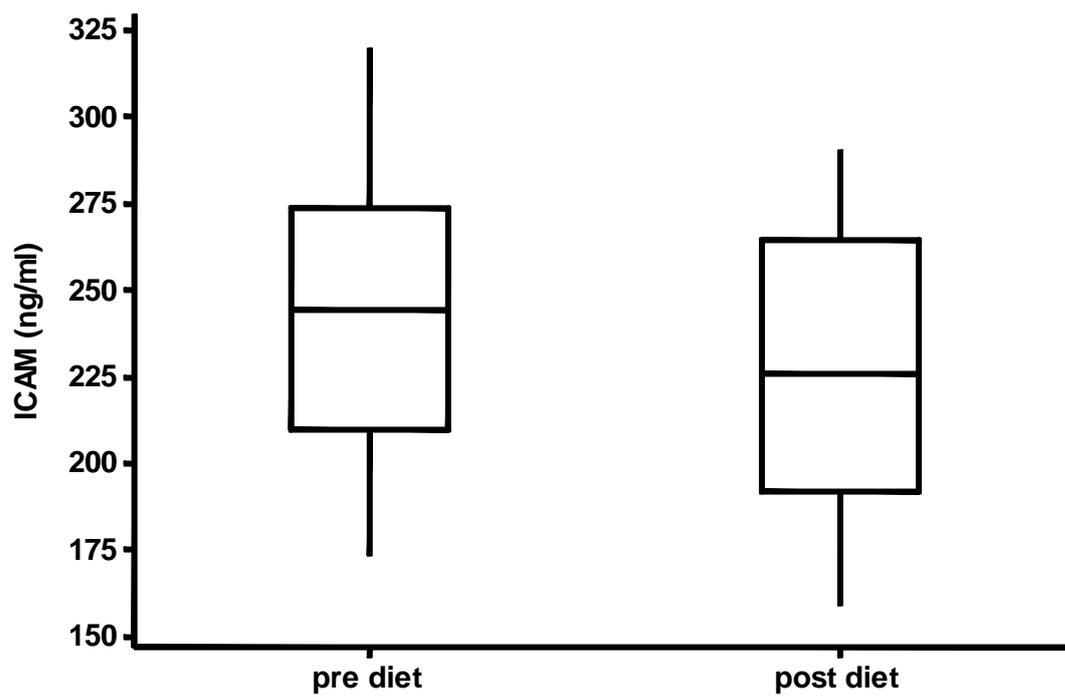


Figure 4.4 Serum intracellular adhesion molecule 1 (ICAM-1) concentration at baseline and following dietary intervention.

n=13. p=0.002

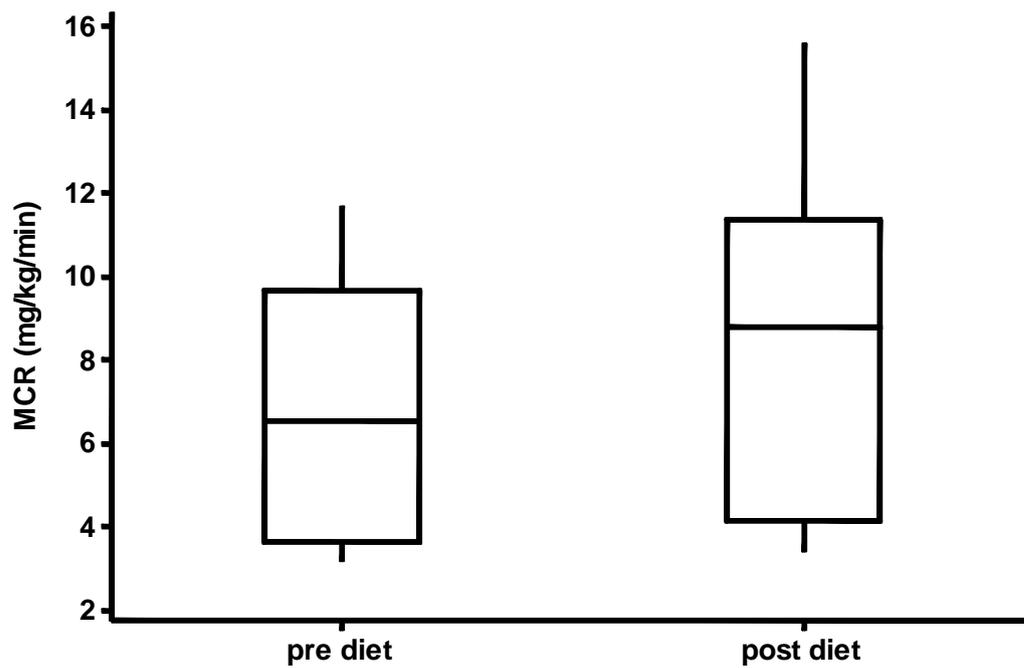


Figure 4.5: Metabolic clearance rate (MCR) of glucose at baseline and following dietary intervention.

n=9, p<0.05

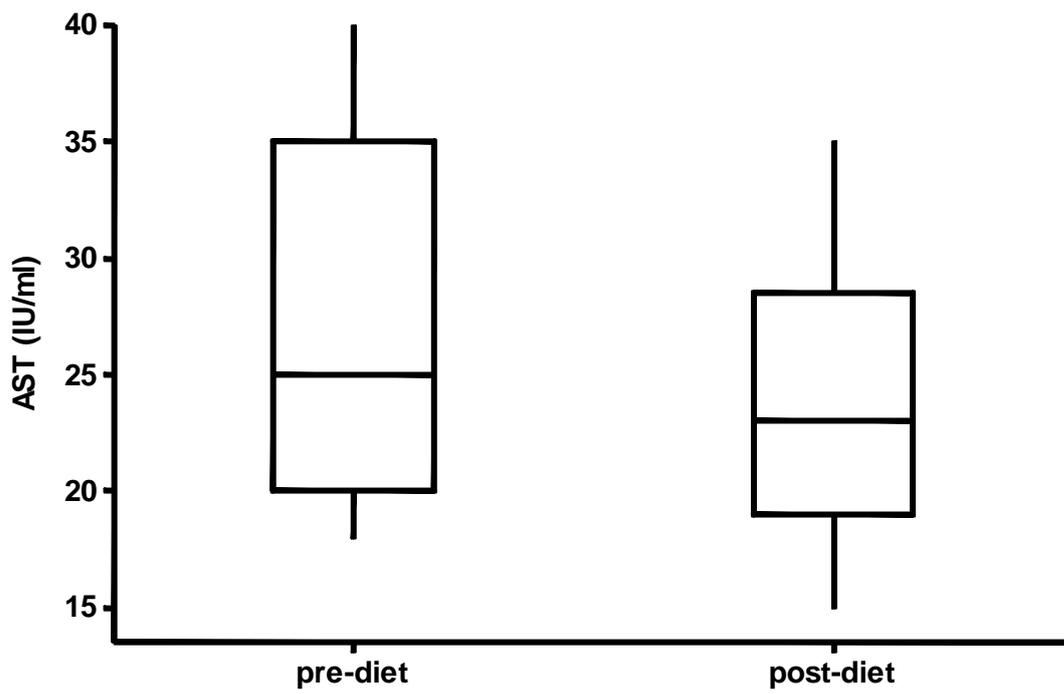


Figure 4.6 Serum aspartame transferase (AST) concentration at baseline and following dietary intervention.

n=13. p<0.05

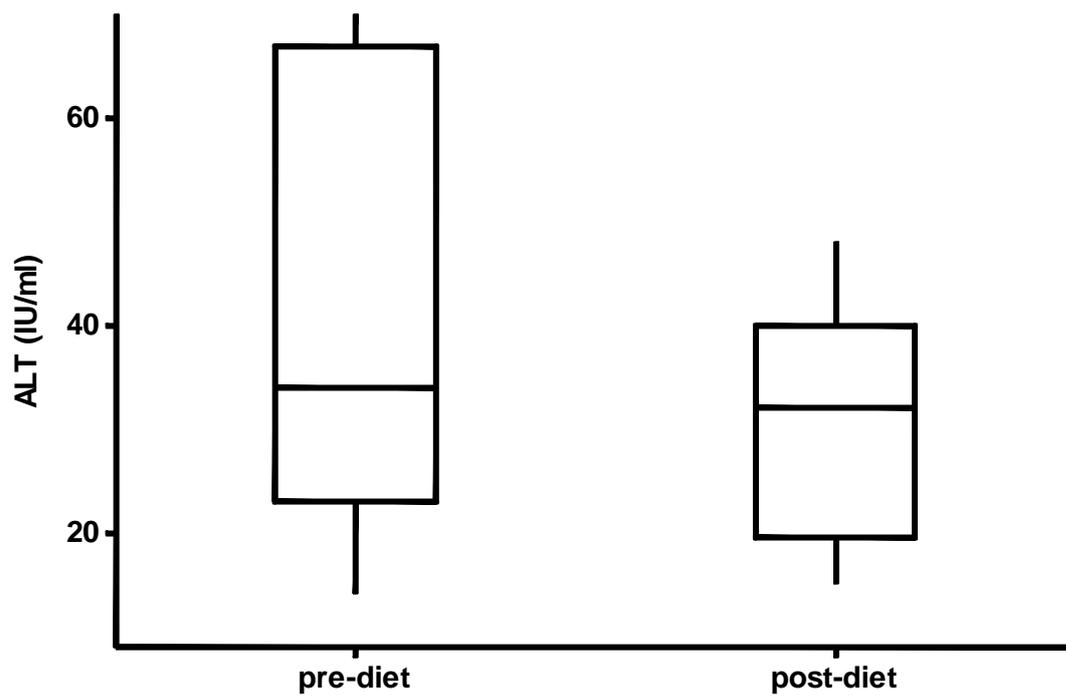


Figure 4.7 Serum alanine transferase (ALT) concentration at baseline and following dietary intervention.

n=13. p<0.05

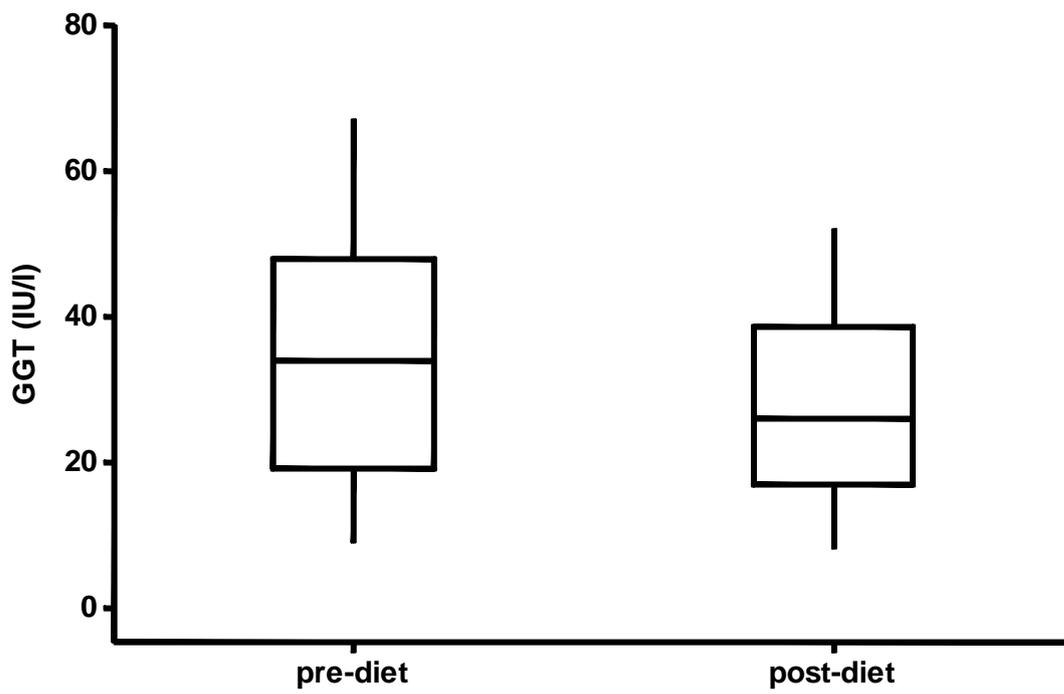


Figure 4.8 Serum gamma-glutamyl transferase (GGT) concentration at baseline and following dietary intervention.

n=13. p<0.05

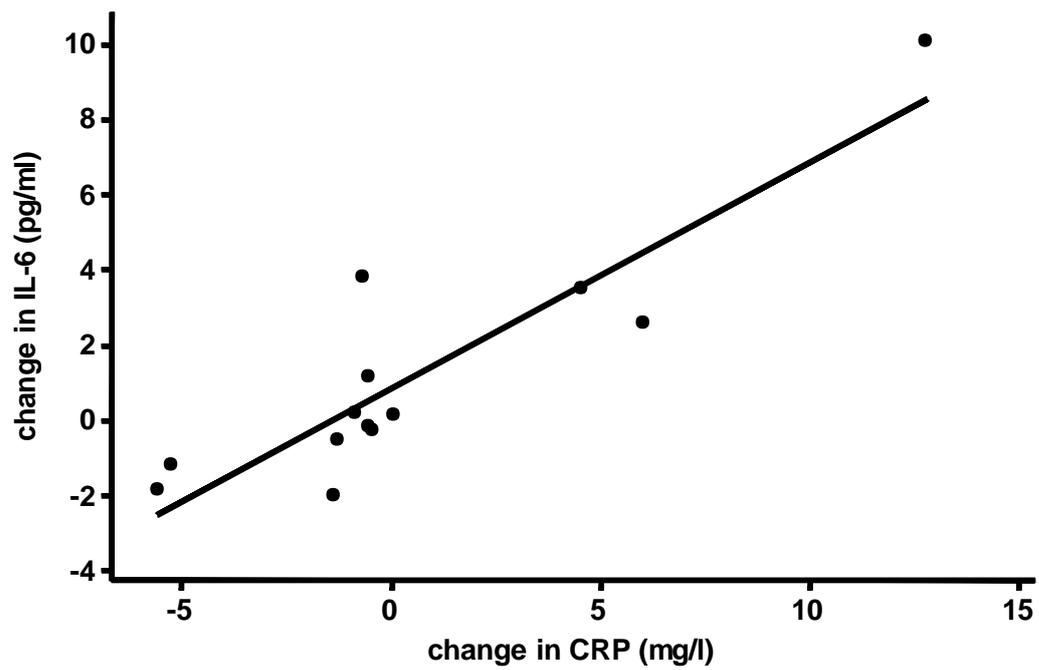


Figure 4.9: Relationship between change in interleukin-6 (IL-6) and c-reactive protein (CRP) concentrations following dietary intervention.

$r=0.89$, $p<0.0001$

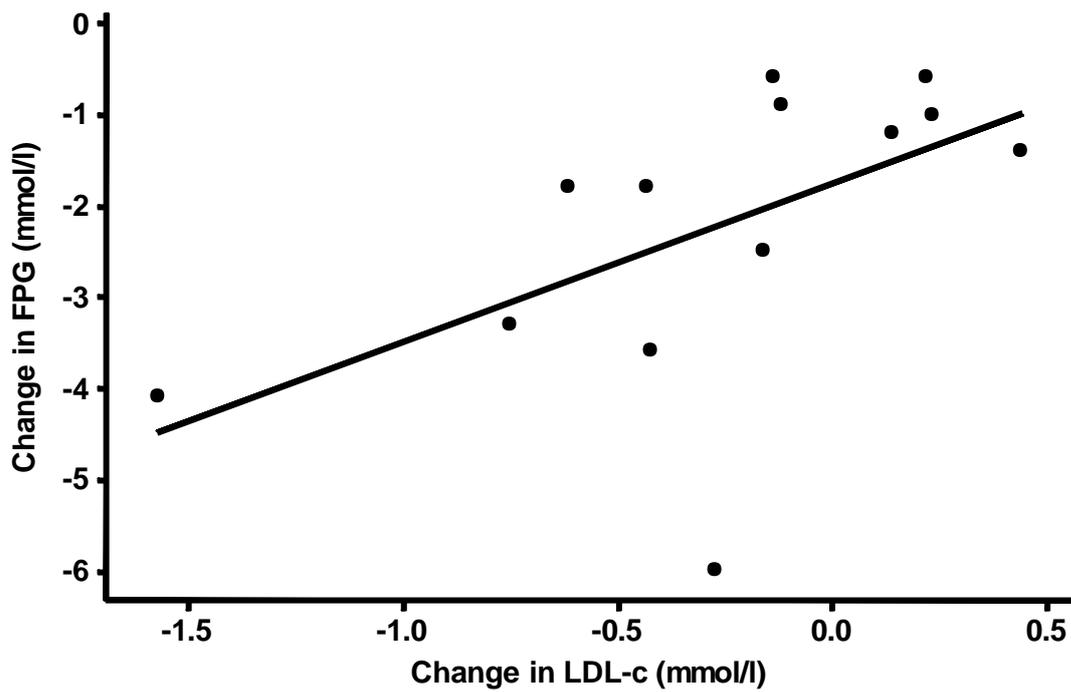


Figure 4.10: Relationship between change in fasting plasma glucose (FPG) and low density lipoprotein cholesterol (LDL-c) concentration following dietary intervention.

$r=0.56$, $p<0.05$

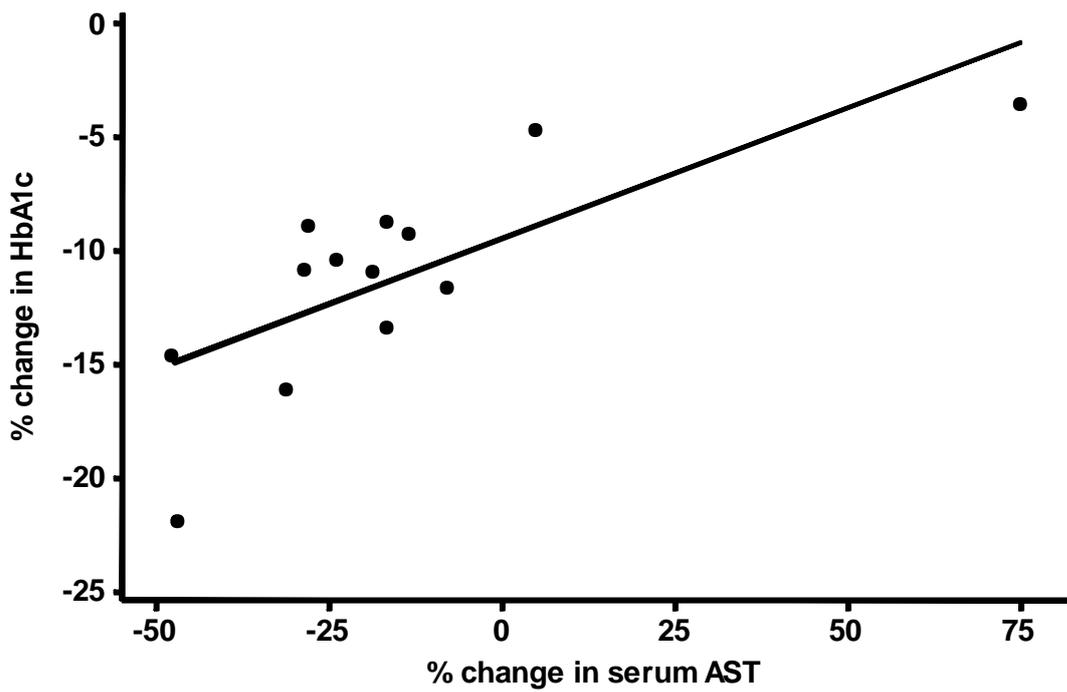


Figure 4.11: Relationship between change in serum aspartame transferase (AST) and haemoglobin A1c (HbA1c) concentration following dietary intervention.

$r=0.743$, $p=0.004$

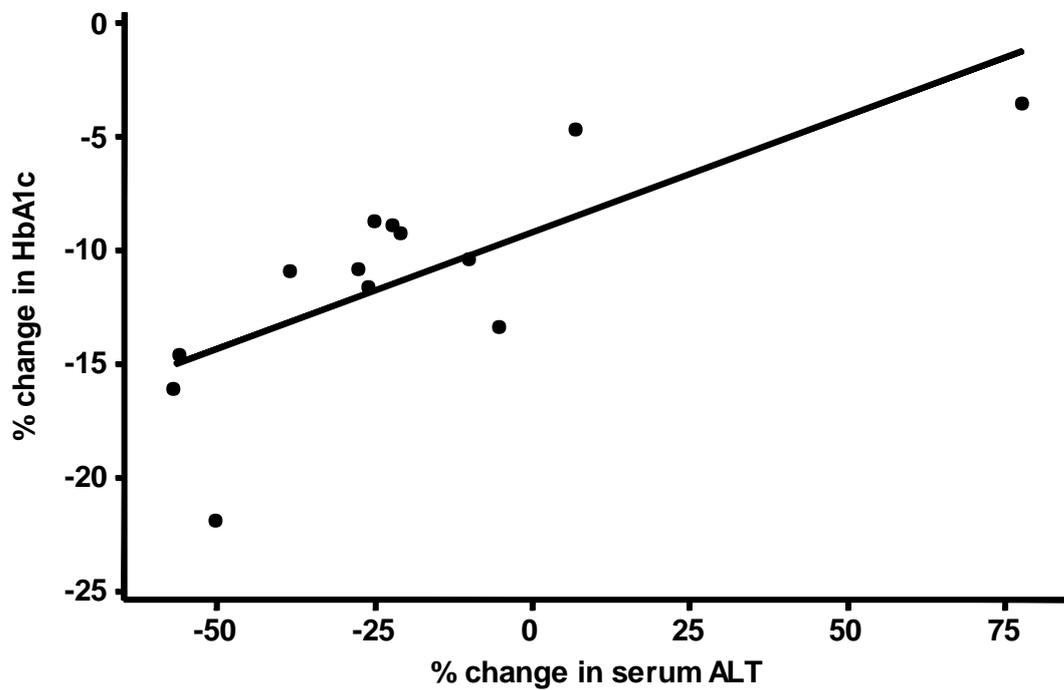


Figure 4.12: Relationship between change in alanine transferase (ALT) and haemoglobin A1c (HbA1c) concentrations following intervention.

$r=0.751$, $p=0.003$

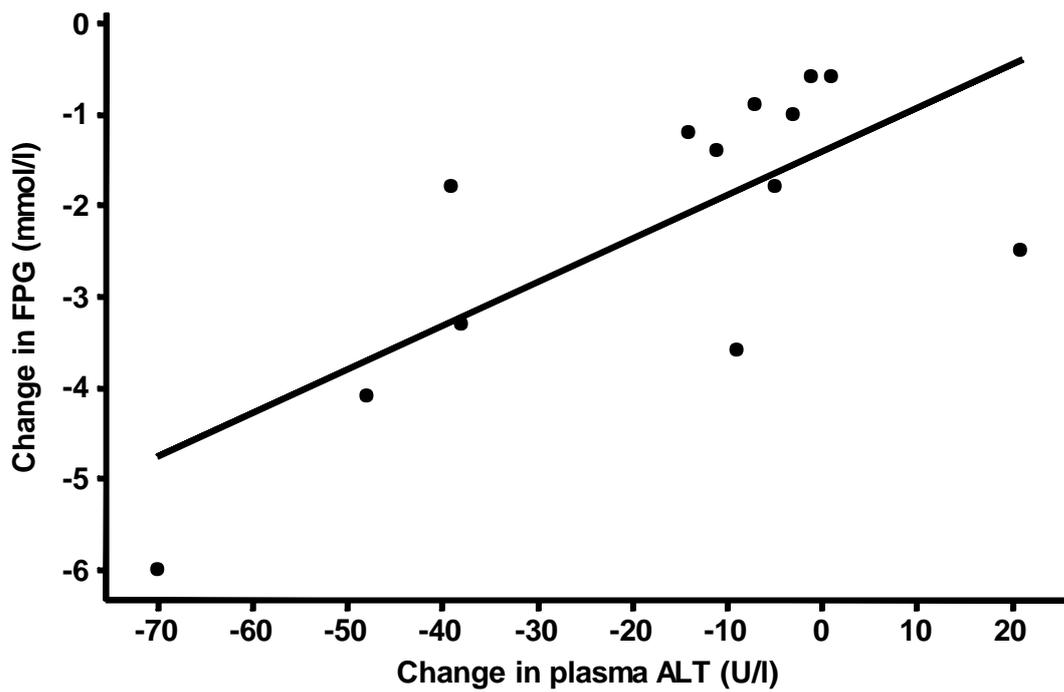


Figure 4.13: Relationship between change in fasting plasma glucose (FPG) and alanine transferase (ALT) concentrations following intervention.

$r=0.729$, $p=0.005$

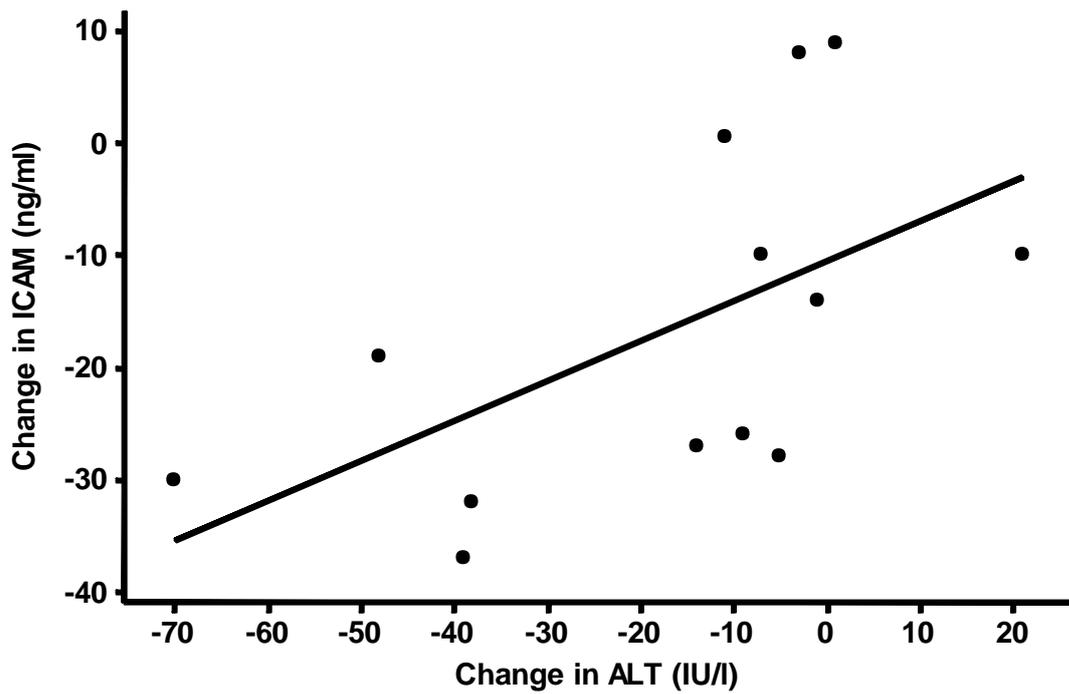


Figure 4.14: Relationship between change in intracellular adhesion molecule-1 (ICAM) and alanine transferase (ALT) concentrations following intervention. $r=0.57$, $p<0.05$

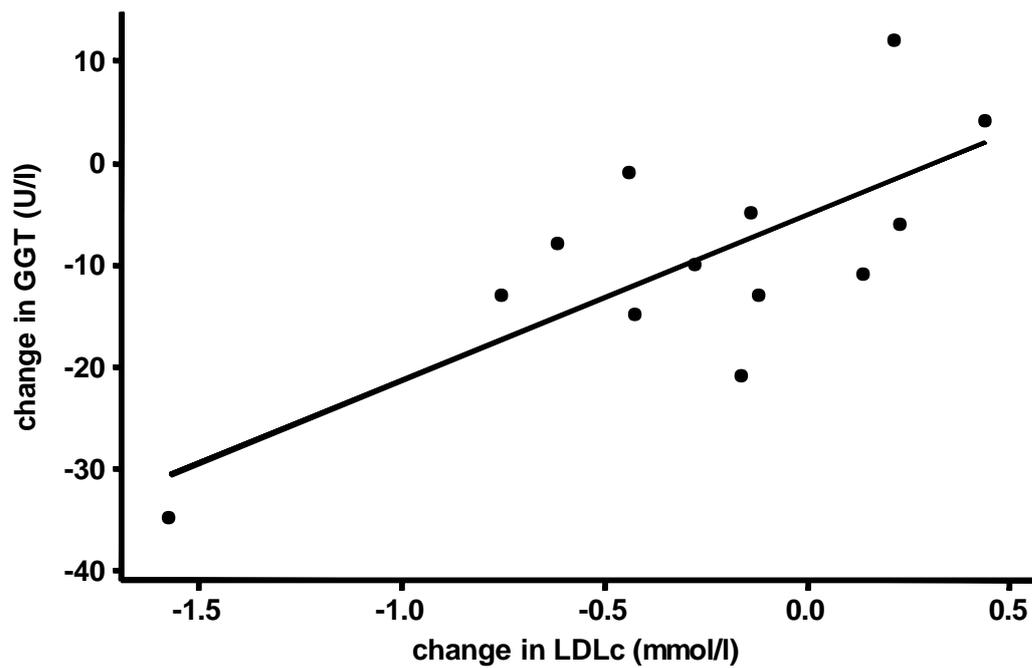


Figure 4.15: Relationship between change in serum low density lipoprotein cholesterol (LDL-c) and gamma-glutamyl transferase (GGT) concentrations following intervention.

$r=0.742$, $p<0.005$

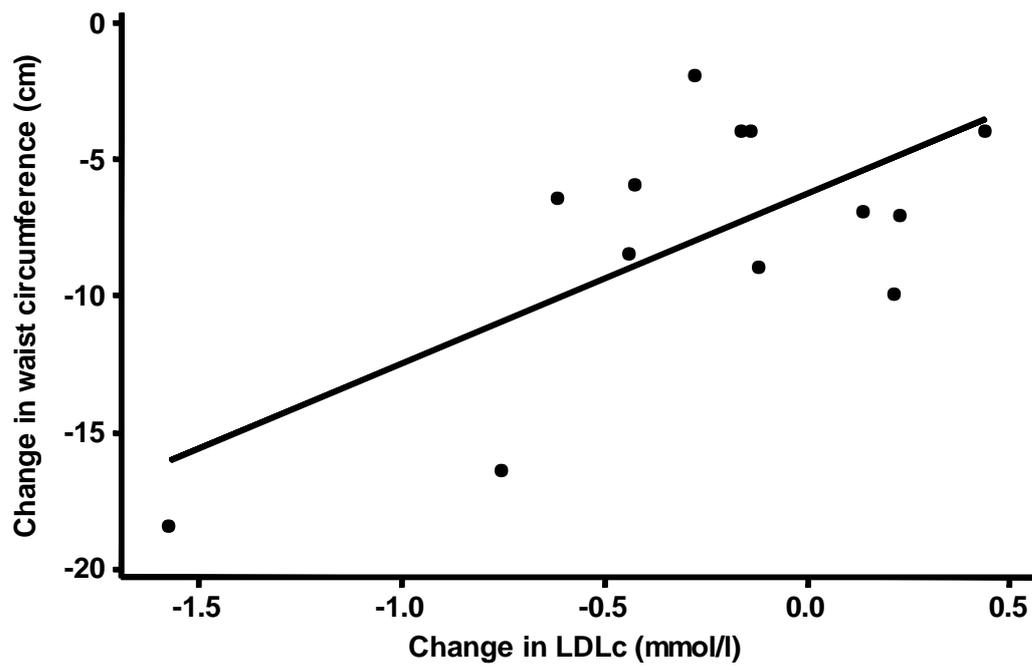


Figure 4.16: Relationship between change in waist circumference and serum low density lipoprotein cholesterol (LDL-c) concentration following intervention .
R=0.674, p<0.02

CHAPTER 5

EFFECT OF DIETARY INTERVENTION ON VASCULAR PARAMETERS

5.1 Summary

Introduction: Obesity has significant effects on morbidity and mortality, especially from cardiovascular causes. Associations between obesity, diabetes and endothelial dysfunction are established. The exact effect of weight loss on vascular function is unclear, with results conflicting. This is especially true in the case of type 2 diabetes where there is a lack of published evidence.

Methods: Each subject underwent vascular assessments before and after dietary intervention.

Results: Results from the wire myography studies showed either no change or a significant deterioration following weight loss. In addition, laser Doppler studies suggested deterioration in endothelium-dependent vasodilatation after the intervention. There was no significant change in augmentation index, but a significant improvement in pulse wave velocity after dietary-induced weight loss.

Conclusions: The results of these studies are also conflicting. There would appear to be deterioration in small vessel endothelial function following the intervention, with a significant improvement in one parameter of arterial stiffness. The reasons for these discrepancies are unclear and further studies in this area are warranted.

5.2 Introduction

The links between obesity, diabetes and vascular disease have been well established. Evidence suggests that diabetes increases the risk of cardiovascular mortality by two to six times that of the general population (Haffner et al, 1998), whilst obesity also has a significant impact on cardiovascular morbidity and mortality.

It has been notoriously difficult to ascertain the effects of weight loss on health, with significant difficulties undertaking studies and interpreting results. As endothelial function is one of the initial stages in the development of cardiovascular disease, and it has been shown to coexist with cardiovascular disease, it would seem logical to use this surrogate marker to determine whether improvements in cardiovascular risk factors are associated with changes in endothelial function.

A number of groups have tested this hypothesis and have confirmed the association between weight loss and improved endothelial function. However, the evidence that weight loss improves vascular parameters in subjects with type 2 diabetes remains scarce. The main aim of this study therefore, was to demonstrate that intentional weight loss improves measurements of endothelial function in subjects with type 2 diabetes.

5.3 Methods

5.3.1 Subjects

Twenty-two male and female subjects were recruited and randomised to a 6-week period of dietary intervention or control, as outlined in section 2.1. Fifteen subjects were allocated to the intervention group. Due to poor attendance of the seven control subjects, the dataset for this subgroup is very small and is therefore not presented here.

Written informed consent was obtained from each subject. The local hospital ethical review committee approved the study and study protocols.

5.3.2 Study protocol

Subjects attended for investigations before, during and after the six-week dietary intervention period as outlined in section 2.2.

5.3.3 Vascular measurements

i. Wire Myography

Tissue acquisition, preparation of arteries and vasoconstrictor and vasodilator protocols were carried out as described in sections 2.8.1 – 2.8.7.

ii. Laser Doppler iontophoresis

Laser Doppler imaging and iontophoresis were performed as described in section 2.9.1 – 2.9.3.

iii. Measures of arterial compliance

Pulse wave velocity and pulse wave analysis were carried out as described in section 2.10.1 – 2.10.2.

5.3.4 Statistical Analysis

Results are displayed as mean \pm SEM. Comparisons of pD₂ and maximal response values for the myography studies were made using the Student's *t* test. LDI mean flux data was compared using 2 way ANOVA, and AUC data using Student's *t* test. Augmentation index and pulse wave velocity were compared using the Student's *t* test.

5.4 RESULTS

5.4.1 Wire Myography

i. Effect of insulin on norepinephrine-mediated contraction pre-intervention

Analysis of maximal responses and pD_2 showed no significant difference in norepinephrine-mediated vasoconstrictor responses in the presence and absence of insulin ($10^{-9}M$) in vessels at baseline (Figure 5.1).

ii. Effect of insulin on norepinephrine-mediated contraction post-intervention

Analysis of maximal responses and pD_2 showed no significant difference in norepinephrine-mediated vasoconstrictor responses in the presence and absence of insulin ($10^{-9}M$) in vessels following the intervention (Figure 5.2).

iii. Comparison of insulin's effects on norepinephrine-mediated vasoconstriction before and after intervention

There was no significant difference in pD_2 values of norepinephrine-mediated vasoconstrictor concentration response before and after intervention when curves were compared, although there was a trend towards significance. (Mean pD_2 pre-intervention 6.24 ± 0.11 vs. mean pD_2 post-intervention 6.73 ± 0.19 ; $n=6$ $p=0.06$)

There was however, a significant difference when maximal contraction of norepinephrine-mediated vasoconstrictor response curves were compared. (Mean maximal contraction before the intervention $92.5 \pm 2.3\%$ vs. mean maximal contraction following the intervention $104.16 \pm 3.9\%$; $n=6$, $p<0.05$). (Figure 5.3).

5.4.2 Laser Doppler Iontophoresis

There was a significant deterioration in microvascular responses to iontophoresed acetylcholine following the intervention.

($p < 0.0001$, two-way ANOVA, $n = 10$, Figure 5.4)

5.4.3 Measures of arterial compliance

i. Pulse wave velocity

There was a significant reduction in pulse wave velocity after the intervention. (Mean PWV at baseline 10.00 ± 0.53 m/s vs. mean PWV following the intervention 6.95 ± 0.450 m/s, $n=4$, $p<0.0001$) (Figure 5.6)).

ii. Pulse wave analysis

There was no change in augmentation index following the intervention. (Mean AI at baseline $24.5 \pm 6.05\%$ vs. mean AI following intervention $27.17 \pm 6.13\%$, $n=6$, $p=0.56$) (Figure 5.7)).

5.5 Discussion

This study has demonstrated deterioration in microvascular function following dietary-induced weight loss. Results from wire myography and laser Doppler iontophoresis studies, both measuring endothelial function in small blood vessels, show a reduction in microvascular responsiveness following the intervention. Although the numbers in this study are small, making any statistical comparison difficult, there is a clear suggestion of poorer endothelial function following diet-induced weight loss. It is unfortunate that vessel yield from biopsies was insufficient to allow any meaningful assessment of acetylcholine-induced vasodilatation in the wire myography studies. However, this was demonstrated in the LDI studies.

The results of the studies of arterial stiffness were different again, with a significant improvement in pulse wave velocity following the intervention, but no change in augmentation index. Unfortunately, due to technical considerations, the numbers available for analysis were small, and as a result the significant results may simply have been due to statistical error.

To date, the results of weight loss studies in non-diabetic cohorts have been variable. Three studies employing solely dietary methods for weight loss examined the effects on vascular function. Raitakari et al (2004) looked at the effect of weight loss on two parameters of vascular function. This study demonstrated that reducing body weight in 67 obese men and women was associated with significant improvements in brachial artery flow-mediated dilation (FMD). In contrast, there were no observed differences in endothelium-independent function as assessed by vascular responses to sublingual GTN spray. Keogh et al (2007) carried out a

randomised parallel design of a low- versus a high-carbohydrate diet, observing weight loss of approximately 5% in each group. In contrast to the previous group, there were no significant changes in flow-mediated dilatation in either group. Furthermore, Dengel et al (2006) observed no improvement in brachial artery FMD or endothelium-independent dilatation after weight loss. In addition, no significant differences in carotid intima media thickness (IMT) were noted after a period of weight loss. There was an improvement in brachial artery compliance in this study.

One study has examined the combined effects of diet and exercise on vascular function. Hamdy et al (2003) revealed that a 6-month programme of diet and exercise resulted in significant improvements in macrovascular endothelial function as measured by flow-mediated dilatation in 24 subjects. However, microvascular reactivity measured by laser Doppler imaging remained unchanged.

Dietary measures combined with orlistat therapy have been used in two studies to assess vascular responses to weight loss. Brook et al (2004) studied the effect of dietary-induced weight loss with or without orlistat in 43 otherwise healthy male and female subjects. Mean weight reduction of 6.6kg was not associated with improvement in brachial artery FMD in either group. Assessment of forearm resistance artery endothelial function was measured by quantifying blood flow responses to intra-arterial acetylcholine and SNP (endothelium-dependent and independent responses respectively) by Bergholm et al (2003). Despite similar weight losses in both arms of the study (diet and placebo vs. diet and orlistat), significant improvements in forearm blood flow were seen in the subgroup receiving orlistat, but not in the placebo-treated subgroup.

The majority of studies designed to examine the metabolic and vascular effects of weight loss have, until recently, focused on subjects without diabetes. However, over the past few years, a number of groups have studied the effects of weight loss in patients with type 2 diabetes. Most of these studies have only observed changes in metabolic factors and circulating vascular markers (Halle et al, 1999; Monzillo et al, 2003; Boudou et al; Giannopoulou et al). Two groups have examined vascular function in this setting. Barinas-Mitchell et al (2006) observed significant improvements in pulse wave velocity in a cohort of 38 volunteers with type 2 diabetes, following a year long programme of weight loss including either orlistat or placebo. Wycherley et al (2008) observed the effects of weight loss with or without exercise training in a cohort of subjects with type 2 diabetes. Weight loss was associated with improvements in metabolic parameters but no change in vascular function measured by flow mediated dilatation.

The reasons for these varying results are unclear. It has to be noted that the baseline characteristics of the cohorts were variable. Some of the groups were simply obese but otherwise healthy (Brook et al, 2004), whilst some had other significant risk factors for CVD such as hypertension or the insulin resistance syndrome (Sasaki et al, 2002; Hamdy et al, 2003). This heterogeneity between groups suggest that baseline vascular function between cohorts may differ, and thus they may have variable responses to the chosen intervention.

It has also been noted that there are few studies where the degree of weight loss correlates with the degree of change in vascular function (Brook, 2006), suggesting that there are other factors which affect changes in vascular function rather than

weight loss *per se*. Weight loss has been correlated with changes in other traditional risk factors for CVD, such as glycaemic control (Raitakari et al, 2003) and LDL-c (Bergholm et al, 2006) although these observations are not universal. In addition, as per the previous chapter, there were no significant reductions in CRP or IL-6 following dietary-induced weight loss. Changes in endothelial function following dietary intervention may be due to reduction in inflammatory cytokines, although such a relationship has not been demonstrated in any weight loss intervention study to date.

Nutrient composition of the various dietary prescriptions could also play a role in the results. There is evidence that Mediterranean style diets without weight loss can improve endothelial function (Esposito et al, 2004; Fuentes et al, 2001), thought to be due to increased levels of antioxidants that have been shown to improve endothelial function (Plotnick et al, 1997) and fibre, which may have anti-inflammatory properties (Antoh et al, 1999). The diet prescribed in the current study may have been deficient in certain essential nutrients or low in antioxidants, which contributed to the lack of improvement in endothelial function.

A further possibility is that the quantity of salt in the prescribed diet was too high. Although this has not been formally analysed, salt content of processed food (which was a significant component of the diet in the form of “tinned soup”) is known to be high. There is a suggestion that salt may have blood pressure-independent adverse effects on vasculature. Tzemos et al (1998) demonstrated that oral salt loading in a cohort of healthy individuals led to reduced forearm blood flow responses to acetylcholine when compared with a control group. Recent *in vitro* work has

suggested that increases in dietary salt can inactivate eNOS, thereby reducing NO availability and vasodilatation (Li et al, 2009).

No other group has used the techniques of wire myography or laser Doppler iontophoresis in the assessment of small vessel endothelial function following weight loss in subjects with type 2 diabetes. However, Hamdy et al (2003) demonstrated the effects of a 6-month programme of diet and exercise in an obese cohort, which led to significant improvements in macrovascular endothelial function as measured by flow-mediated dilatation. However, microvascular reactivity measured by laser Doppler imaging remained unchanged. These conflicting results mirror those observed in the current study. This suggests that conduit vessels may have different properties from resistance arteries and microcirculation.

Previous groups have shown correlation between endothelial function in resistance arteries and conduit vessels. Park et al (2001) demonstrated an association between endothelial dysfunction in the brachial artery using flow-mediated dilatation, and resistance arteries using wire myography in a hypertensive cohort. Agewall et al (2006) carried out a similar study in a cohort of subjects with established CHD and observed similar associations. Pierce et al (2008) carried out a study to assess the effects of weight loss in an obese, nondiabetic cohort. Twelve weeks of dietary manipulation led to approximately 10% loss of body weight and significant improvements in large vessel and small vessel endothelial function, as measured by FMD and peak forearm blood flow in response to intra-arterial infusion of acetylcholine respectively. However, results from Hamdy et al (2003) do not show correlation between large and small blood vessels.

Possible reasons for the discrepancy between large and small blood vessel function in this study are unclear. Correlation between endothelial function and pulse wave velocity has been demonstrated (Nigam et al, 2003; McEniery et al, 2006), but no study to date has looked at the relationship between measures of arterial stiffness and endothelial function as measured by either wire myography or laser Doppler iontophoresis. The comparative effects of exercise intervention on conduit and resistance vessels have been assessed and the changes in each of these measurements did not correlate (Green et al, 2004). A number of reasons have been postulated for the differences in response to exercise interventions between the two types of vessel.

There was a clear reduction in vascular insulin sensitivity as assessed by the wire myography studies. Surprisingly the vasodilating effect of insulin on norepinephrine-induced vasoconstriction was less marked following the intervention, suggesting a reduction in vascular insulin sensitivity after weight loss. This is contrary to the results from isoglycaemic clamp studies, which show increased metabolic sensitivity to weight loss following the same intervention. Again, the numbers are too small to gain any meaningful correlation data, but the results are obviously conflicting. Some studies from our own group have suggested that insulin's vascular and metabolic actions are coupled in both healthy and hypertensive cohorts, and may share underlying molecular pathogenesis (Cleland et al, 1999; Cleland et al 2000). More recently this hypothesis has been challenged by Perry et al (2003) who demonstrated that glucocorticoid supplementation in healthy individuals led to reduced metabolic effects of insulin but no change in vascular insulin sensitivity.

In summary therefore, this study has demonstrated impairment in small vessel endothelial function following dietary-induced weight loss. Similar results were observed using both in vivo and ex vivo techniques. In contrast, pulse wave velocity, a method of measuring arterial stiffness, was improved following the intervention. The reasons for these surprising results, especially with regards to small vessel function are unclear, but are not dissimilar to previous results in cohorts both with and without type 2 diabetes. Potential reasons for the deterioration in vascular function could be related to the duration of the dietary intervention, or the composition of the diet. The discrepancy between the observed metabolic and vascular sensitivity to insulin therapy could add further weight to the argument that these two main roles of insulin are not coupled as previously thought. Further larger studies are required in order to clarify these issues.

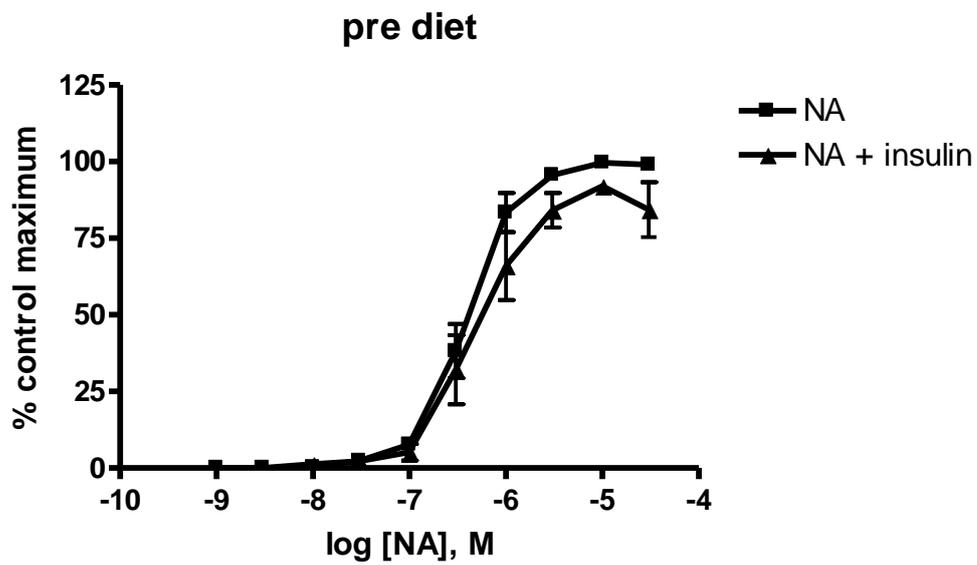


Figure 5.1 Effect of insulin on norepinephrine-mediated contraction before intervention.

n=6.

Comparison of pD_2 = ns. Comparison of maximal response = ns.

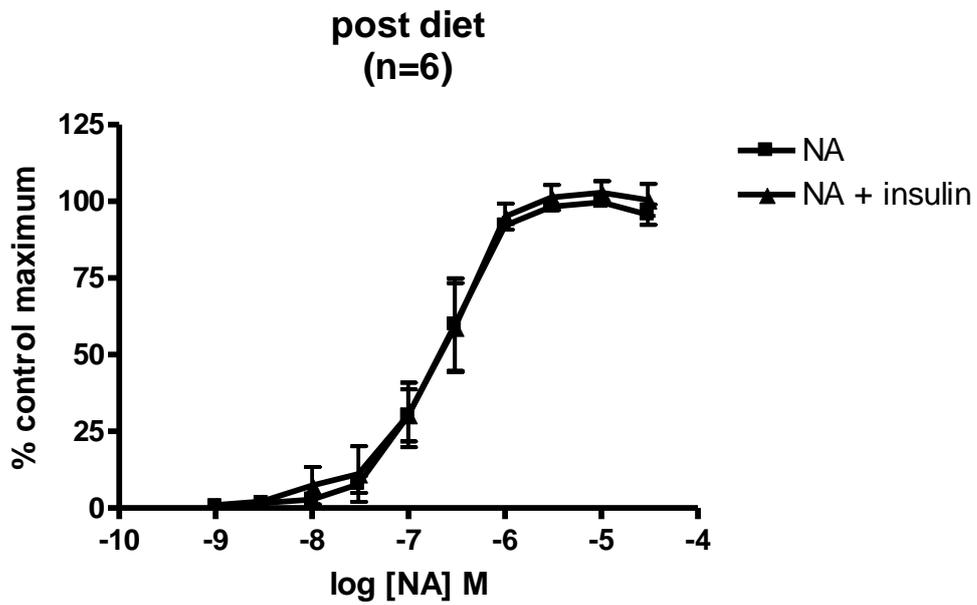


Figure 5.2 Effect of insulin on norepinephrine-mediated contraction following intervention.

n=6.

Comparison of pD_2 = ns. Comparison of maximal response = ns.

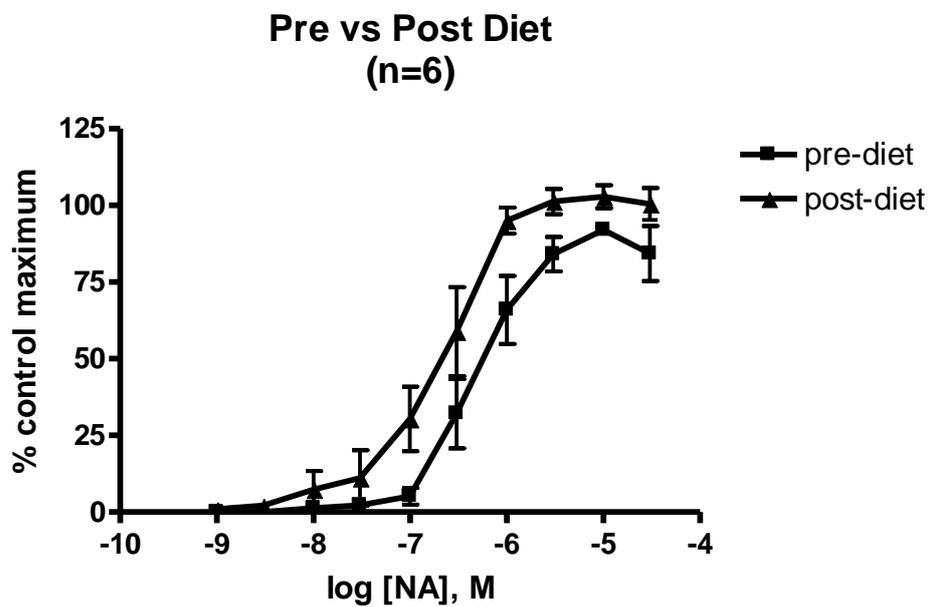


Figure 5.3 Effect of insulin on norepinephrine-mediated contraction before and following intervention.

n=6.

Comparison of pD_2 = ns. Comparison of maximal response $p < 0.05$.

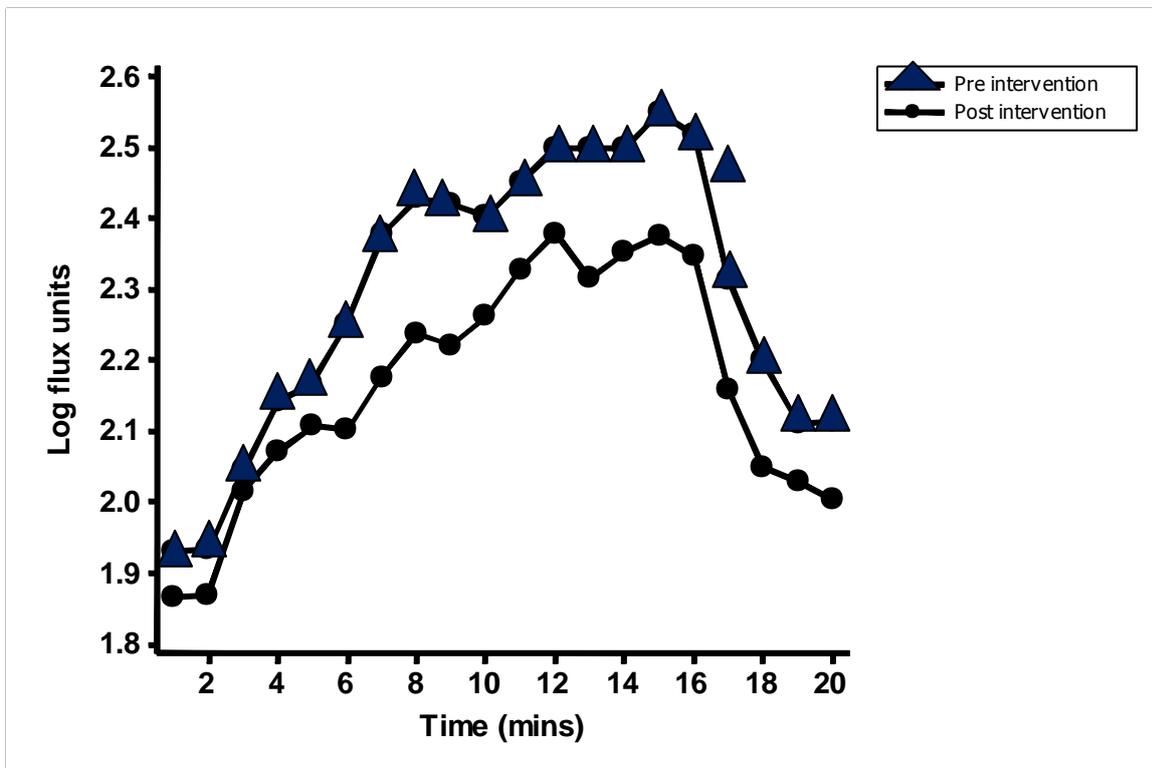


Figure 5.4 Endothelium-dependent vasodilatation measured by laser Doppler iontophoresis of acetylcholine before and after dietary intervention. n=10. $p < 0.0001$, two-way ANOVA.

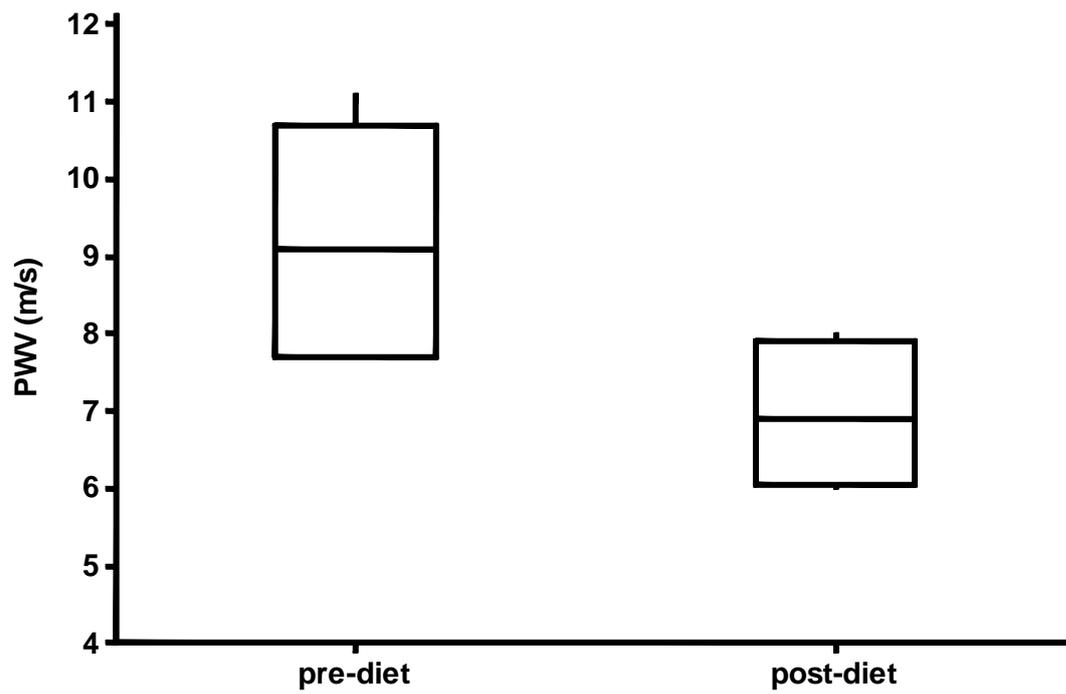


Figure 5.5: Pulse wave velocity (m/s) at baseline and following intervention.

n=4, $p < 0.0001$.

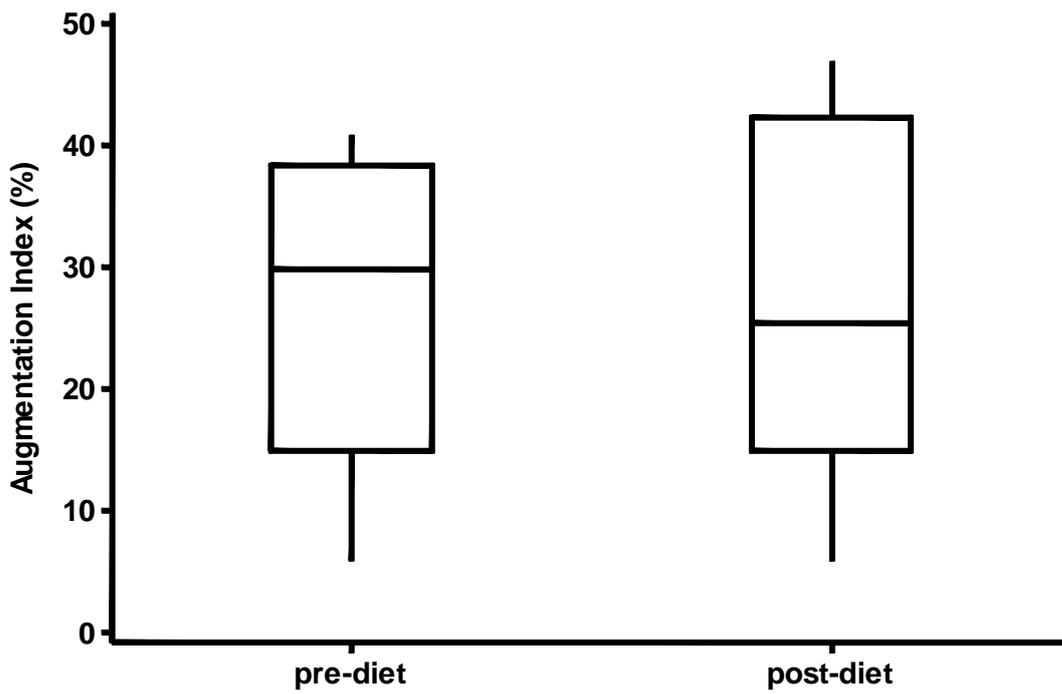


Figure 5.6: Augmentation index (%) at baseline and following intervention.

n=6, p=ns

Chapter 6

Discussion

6.1 Introduction

One of the major challenges to health-care systems throughout the world is to reduce mortality and morbidity associated with cardiovascular disease, particularly coronary artery disease and cerebrovascular disease. These disease processes are mediated by progressive atherosclerosis. This pathological process and its clinical sequelae are closely associated with a number of physiological parameters such as hypertension, insulin resistance and abnormal lipid profiles, and obesity, collectively termed the metabolic syndrome. There is good evidence that lowering cholesterol (Collins et al, 2003) and improved glycaemic control in type 2 diabetes results in reduced mortality from cardiovascular disease (UKPDS Group, 1998). However, it has been more difficult to demonstrate that reduced centripetal obesity leads to reduced morbidity or mortality from CVD. Although it is clear that weight loss can improve glycaemic control in type 2 diabetes, and thus indirectly affect CVD, no study has been able to prove that weight loss *per se* reduces cardiovascular risk, although work is ongoing (Ryan et al, 2003).

Endothelial dysfunction is known to be a precursor to atherosclerotic disease, and has been shown to be a surrogate marker of this process (Ross et al, 1993). Over the past two decades, a number of tools have been developed and validated to measure endothelial function. It has been demonstrated repeatedly that endothelial dysfunction is a precursor to clinical disease processes, and is associated with

poorer prognoses (Heitzer et al, 2001; Neunteufl et al, 2000). It seems reasonable therefore that the measurement of endothelial function would be a useful surrogate for observing the possible effects of weight loss on vascular disease processes.

As is the case with type 2 diabetes, hypertension, and cardiovascular disease, obesity is closely associated with endothelial dysfunction (Steinberg et al, 2006). A number of studies have attempted to demonstrate that weight loss is associated with improved endothelial function, using a variety of methods. The results of these studies have varied, probably as a result of the heterogeneous nature of the study cohorts, and the duration and nature of the intervention adopted. In addition, a variety of different methods to assess endothelial function have been used as described in Table 1.1.

This study attempted to demonstrate that weight loss is beneficial in terms of vascular health in a particularly at risk group. Patients with type 2 diabetes are generally overweight and insulin resistant, and have a much higher risk of CVD mortality than the general population (Haffner et al, 1998). Numerous studies have revealed the benefits of a number of interventions such as improved glycaemic control (UKPDS Group, 1998) and lipid-lowering therapy (Collins et al, 2003) on cardiovascular disease. Although one of the main driving forces behind the development of type 2 diabetes is increased body weight, there is little evidence that weight reduction has a significant impact on cardiovascular disease. Very few of the previous studies looking at the effects of weight loss on vascular function have included subjects with type 2 diabetes, despite their significantly higher

cardiovascular morbidity and mortality rates. Therefore, the main objective of this study was to demonstrate that intentional weight loss was associated with improved endothelial function in subjects with type 2 diabetes.

Initially, attempts were made to recruit diabetic patients treated with diet alone, and naïve to statin and angiotensin converting enzyme therapies. It became apparent, however after nine months of recruitment from primary and secondary care that there were very few patients with type 2 diabetes who were not taking oral medication for primary prevention of cardiovascular disease. It was therefore necessary to broaden the inclusion criteria to include such patients. In addition, given the intensive nature of the study, both in terms of the dietary intervention and the invasive nature of the investigations, many potential subjects were unable to commit to this. Both of these reasons made recruitment for this study very labour intensive and often fruitless. Data collection was also incomplete for many of the subjects, usually because of constraints on their time and availability, especially in the second phase of investigations following the dietary intervention. This was especially true for the control subjects, who were less motivated to attend for follow-up.

6.2 The effects of weight loss on anthropometric measurements

This study demonstrated that dietary manipulation leads to a reduction in body weight. Six weeks of replacing usual diet with a 1200kcal/day liquid based diet led to a mean reduction in body weight of 8kg, equivalent to a reduction of 5-10% in body weight. In addition, significant reductions in waist circumference and body fat mass

suggested that weight loss was mainly due to reduced central fat mass with preservation of fat-free mass. Unfortunately methods to assess the degree of change in visceral rather than subcutaneous fat, such as CT or MRI scanning were not employed.

Using three different methods, significant reductions in body fat percentage were observed following weight loss. Despite this however, there was no correlation between the changes in body fat obtained from any of the three techniques used, namely skinfold thickness measurements, air displacement plethysmography or total body water. The main reason for this is due to the relatively small numbers involved.

6.3 Effect of weight loss on metabolic parameters

Weight loss has a beneficial effect on many metabolic parameters. It was unsurprising therefore that weight loss in this study was associated with improved glycaemic control both in terms of HbA1c and fasting plasma glucose concentrations. In addition there were significant improvements in whole body insulin sensitivity as measured by the hyperisulinaemic isoglycaemic clamp technique in this cohort.

This intervention also led to significant improvements in liver function tests which are markers of hepatic steatosis, a common finding in individuals with the metabolic syndrome and type 2 diabetes. Unfortunately no formal measurement of liver fat content (such as CT or MRI scanning) was actually made. However, it has been shown that plasma levels of liver enzymes especially ALT are suitable surrogate markers of liver fat content (Tiikkainen et al, 2003). Interestingly there were

significant correlations between changes in liver function and changes in glycaemic control. This has supported the hypothesis that hepatic insulin resistance and the development of type 2 diabetes is closely related to liver fat accumulation.

6.4 Effect of weight loss on circulating markers of inflammation and endothelial function

The absence of any significant change in serum concentrations of adipocytokines and markers of inflammation following weight loss was surprising. Despite a significant reduction in sICAM-1, there were no significant changes in serum adiponectin, IL-6 or CRP following the intervention. Despite numerous studies demonstrating the benefits of weight loss in terms of reduced cytokine levels in non-diabetic cohorts, only one study has demonstrated such improvements in a diabetic cohort (Barinas-Mitchell et al, 2006). That particular trial adopted a year-long multi-disciplinary approach to weight loss including an exercise intervention, which has been demonstrated as being beneficial in reducing inflammatory markers in type 2 diabetes (Zoppini et al, 2006; Kadoglou et al, 2007; Dekker et al, 2007).

Potential reasons for the lack of change in inflammatory cytokines are numerous, including the duration of the intervention, the amount of weight lost, and the composition of the prescribed dietary modifications.

6.5 Effect of weight loss on vascular function

The results of the vascular function studies were also unexpected. There was a significant deterioration in endothelium-dependent vasodilatation of the microcirculation as measured by laser Doppler iontophoresis of acetylcholine. In addition, *ex vivo* studies in the form of wire myography also demonstrated that insulin-mediated vasodilation deteriorated after weight loss. In contrast, however there was a significant improvement in pulse wave velocity measurements following the intervention.

These findings are similar to those of the few studies available which have examined the effects of weight loss on vascular function in both diabetic and non-diabetic groups. Raitakari et al (2004) did demonstrate improved endothelial function as measured by brachial artery FMD following mean weight loss of 11 kg. In contrast, neither Keogh nor Dengel were able to demonstrate improved endothelium-dependent vasodilation following weight reduction. In addition, Wycherley et al (2008) were unable to demonstrate improvements in brachial artery FMD in a diabetic cohort despite changes in metabolic parameters following weight loss. However, Barinas-Mitchell et al (2006) and Halle et al (1999) demonstrated improved pulse wave velocity following weight loss in diabetic cohorts following weight loss induced by diet and exercise.

Therefore, it seems clear that weight loss in subjects both with and without diabetes leads to significant improvements in metabolic parameters. The effects of weight reduction on endothelial function in type 2 diabetes remains unclear and further research into this important subject would seem warranted.

6.6 Missed opportunities and future work

Whilst the number of investigations performed on subjects in this study was already high, there were opportunities missed. Undertaking a direct assessment of liver fat content would have been useful and correlation of liver fat loss with changes in insulin sensitivity would have been helpful. Other inflammatory cytokines could also have been measured. Given the deterioration in endothelial function which was observed thought to be due to relative starvation, it would have been useful to retest subjects after a further 6 weeks to see if these changes reversed although this would have entailed a further invasive biopsy.

The exact role of weight loss on the endothelium remains unclear. Although a number of studies have tried to address this issue, results are varied. This is probably due to variations in the intervention used and in the method of assessing endothelial function, with there being no current accepted gold standard for this measurement. There also appears to be degree of heterogeneity in the type of obesity that people have with some being more metabolically dangerous than others. Until these basic issues are teased out the answer remains unclear. What is clear however is that weight loss has undoubtedly a beneficial effect on insulin resistance and can only be in the long term good for people with T2DM.

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