Metabolic and immune system cross-talk in human adipose tissue

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METABOLIC AND IMMUNE SYSTEM CROSS-TALK IN HUMAN ADIPOSE TISSUE

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ABSTRACT

The overall aim of the work presented in this thesis was to further characterise aspects of metabolic and immune system cross-talk in human subcutaneous adipose tissue, with a particular emphasis on the potential role of T-lymphocytes in adipose tissue dysfunction and insulin resistance. Chapter 3 characterised macrophage and T-lymphocyte populations residing in adipose tissue from lean through to class I obese men. This work demonstrated that T-lymphocytes display increased activation with increased adiposity and that potential compensatory mechanisms may be present to help counteract adipose tissue inflammation. In Chapter 4, the same participants were exposed to a meal-based stimulus in order to examine the postprandial metabolic and inflammatory responses in blood and adipose tissue. Despite increased glucose and insulin responses in blood with obesity, there were no differences in inflammatory cytokine gene expression responses in adipose tissue. This suggests that mechanisms may be present to limit or dampen inflammatory output from adipose tissue after feeding in individuals with modestly increased adiposity. Chapter 5 examined metabolic and immune system changes to 50 % calorie restriction for 3 days, resulting in reduced serum leptin which was temporally associated with a reduction in blood T-lymphocyte activation. In adipose tissue, however, leptin gene expression/secretion was not reduced and neither was resident T-lymphocyte activation, indicating that there may be local tissue-specific responses of immune cells to caloric restriction. Chapter 6 characterised differences between obese individuals with either normal or impaired glucose tolerance, and their respective responses to 10 days of diet and activity modification. Overall, this thesis highlights key differences in properties of T-lymphocyte populations with increasing levels of adiposity and insulin resistance together with responses in adipose tissue and the immune system in times of feeding, severe calorie restriction and glucose lowering diet and activity.

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ABBREVIATIONS

AEE	Activity energy expenditure
ALT	Alanine transaminase
ANOVA	Analysis of variance
ATGL	Adipose triglyceride lipase
AUC	Area under the curve
BANES	Bath and North East Somerset
BMI	Body mass index
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA (deoxyribonucleic acid)
CLS	Crown like structure
CO_2	Carbon dioxide
CRP	C-reactive protein
Ct	Threshold cycle
CT	Computerised tomography
CV	Coefficient of variation
CVD	Cardiovascular disease
DEXA	Dual-energy X-ray absorptiometry
DIT	Diet induced thermogenesis
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
ECBM	Endothelial cell basal media
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FBS/FCS	Fetal bovine/calf serum
FMI	Fat mass index
FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
GCSF	Granulocyte colony–stimulating factor
GI	Glycaemic index

GL	Glycaemic load
GLUT4	Glucose transporter type 4
GP	General Practitioner
HDL	High-density lipoprotein
HOMA-IR	Homeostasis model assessment for insulin resistance
HSL	Hormone-sensitive lipase
IFNɣ	Interferon gamma
IGT	Impaired glucose tolerance
IL-1β	Interleukin-1 beta
IL-1Ra	Interleukin-1 receptor agonist
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-18	Interleukin-18
IP-10	Interferon gamma induced protein 10
IRS2	Insulin receptor substrate 2
ISI	Insulin sensitivity index
L1-L4	Lumbar regions L1-L4
LDL	Low-density lipoprotein
LN	Natural logarithm
LPL	Lipoprotein lipase
MCP-1	Monocyte chemotactic protein 1
MFI	Mean fluorescent intensity
MHC	Major histocompatability comlex
MIP-1β	Macrophage inflammatory protein 1 beta
mRNA	Messenger ribonucleic acid
NBCS	Newborn calf serum
NEFA	Non-esterified fatty acid
NGT	Normal glucose tolerant
NHS	National Health Service
NL	The Netherlands
OGTT	Oral glucose tolerance test
O_2	Oxygen
PAL	Physical activity level

PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCRN	Primary Care Research Network
PPARγ	Peroxisome proliferator-activated receptor gamma
PPIA	Peptidylpropyl isomerase A
RANTES	Regulation on activation, normal t cell expressed and secreted
REC	Research Ethics Committee
RMR	Resting metabolic rate
RNA	Ribonucleic acid
SAA	Serum amyloid A
sICAM1	Soluble intercellular adhesion molecule-1
sVCAM1	Soluble vascular cell adhesion molecule-1
SD	Standard deviation
SEM	Standard error of the mean
SVF	Stromavascular fraction
TBX21	T-box transcription factor 21
TEE	Total energy expenditure
Th	Helper T cell
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
UK	United Kingdom
WBC	White blood cell

CHAPTER 1
Review of Literature

1.1 Overview

Our knowledge of the many complex roles of adipose tissue in heath and disease is rapidly emerging and evolving. It is becoming widely acknowledged that in addition to being the body's primary store for surplus energy in the form of triglycerides, adipose tissue is a dynamic organ that secretes and responds to various metabolic and inflammatory mediators. Within the last decade it has become apparent that adipose tissue contains a whole host of other cell types including immune cells in addition to adipocytes. Differences in the secretion of inflammatory and metabolic mediators from adipose tissue have been observed between lean and obese individuals, as have differences in certain adipose tissue resident immune cell populations. Adipose tissue metabolic dysfunction and inflammation are implicated in the development of obesity related diseases, although it is not necessarily the total fat mass, but the properties and interactions of the various cells located within it which may important in adipose tissue pathophysiology. A greater understanding of this metabolic and immune system 'cross-talk' could ultimately lead to the development of a better means of prevention and treatment of obesity related disease.

1.2 Context

Overweight and obesity are caused by a positive imbalance between energy intake versus expenditure, resulting in excessive fat accumulation in adipose tissue. The latest statistics from the Health Survey for England shows that in 2008 nearly one in four adults and over one in ten children between the ages of 2-10 years are obese, as classified by a body mass index (BMI) >30 kg/m^2 (Department of Health, 2010). Without intervention, the incidence of obesity is predicted to increase to over 50 % of adults and 25 % of children by 2050 (Department of Health, 2010). The rapid rise in prevalence suggests environmental influences are key, with genetics playing a minor role in modifying susceptibility (de Ferranti & Mozaffarian, 2008). The exact aetiology in the development of obesity is multifactorial. Aspects of modern Western lifestyles such as consumption of large

portions of calorie rich foods combined with low physical activity and technological advances contributing towards more sedentary lifestyles, are likely to be major contributors (de Ferranti & Mozaffarian, 2008).

Given one of its primary roles, adipose tissue has evolved to have a great capacity for storing excess energy as triglycerides, and is capable of expansion to account for over 80 % body weight in obese individuals (Thompson *et al.*, 2012). The excess adiposity observed in obesity, however, has a severe impact on health, predisposing to a number of inflammatory related diseases such as type 2 diabetes, atherosclerotic cardiovascular disease (CVD), fatty liver disease, osteoarthritis, rheumatoid arthritis and cancer (Malnick & Knobler, 2006; Schelbert, 2009). Consequently, obesity poses a significant burden on the NHS and is estimated to cost £4.2 billion per year which could more than double by 2050 if the predicted rise in incidence occurs (Department of Health, 2010), with the majority of this being accounted for by type 2 diabetes and CVD. A key factor linking obesity to its associated complications is chronic low grade inflammation. The adipose tissue itself is a source of inflammatory molecules such as TNFα and IL-6, which are increased in blood and adipose tissue with its expansion and are associated with the development of insulin resistance (Hotamisligil *et al.*, 1993; Weisberg *et al.*, 2003). *In vivo* experiments in humans have shown that infusion of TNFα impairs skeletal muscle insulin signalling and decreases whole body glucose uptake in healthy humans (Plomgaard *et al.*, 2005) supporting the impact of inflammatory mediators on insulin resistance. Conversely, adipose tissue inflammation and whole body insulin resistance can be reduced by diet and exercise (Tuomilehto *et al.*, 2001; Li *et al.*, 2008). There is great controversy, however, regarding whether inflammation, particularly within the adipose tissue, is the cause or consequence of insulin resistance (Tam *et al.*, 2010).

The development of type 2 diabetes is a gradual process and it is the events occurring in the development of this particular obesity related complication that will be the focus of the work presented in this thesis. Throughout the course of this PhD, the underlying aim is to find out more about the potential role of the immune system in human adipose tissue and its involvement in the early stages of insulin resistance.

1.3 Insulin resistance, Pre-diabetes and Type 2 diabetes

In line with increases in obesity, the global prevalence of type 2 diabetes is also increasing. It is estimated that 330 million individuals will have type 2 diabetes by 2025 with a further 472 million of the global population having impaired glucose tolerance if current trends continue (Schrauwen, 2007). Type 2 diabetes accounts for approximately 90 – 95 % of cases of diabetes mellitus, the majority of whom are either overweight or obese (ADA, 2010).

The increased low grade inflammation typically observed with increasing adiposity is implicated in the development of insulin resistance by its interference with insulin signalling (Shah *et al.*, 2008). Insulin resistance is a pre-requisite to type 2 diabetes and refers to the resistance of various tissues including liver, muscle and adipose tissue, to the metabolic effects of insulin. The liver is important in maintaining fasting glucose concentrations in blood via regulation of glucose and glycogen synthesis. A fasting hyperglycaemia results from an insensitivity of the liver to the suppressive effects of insulin on gluconeogenesis and glycogenolysis (Lee *et al.*, 2009). Insulin resistance in other tissues such as adipose tissue and skeletal muscle manifests as impaired glucose uptake from the bloodstream. Adipose tissue insulin resistance is also characterised by elevated levels of non-esterified fatty acids (NEFA) in plasma due to decreased suppression of lipolysis (Campbell *et al.*, 1994; Jensen, 2008). In a vicious cycle, this elevation in NEFA exacerbates insulin resistance in liver and skeletal muscle (Abel *et al.*, 2001) and may also have toxic effects on pancreatic β-cells and contribute to vascular dysfunction (Jensen, 2008). The initial peripheral insulin resistance is compensated for by an increased drive of the pancreatic β-cells to produce more insulin until they ultimately fail to meet the demands required and blood glucose levels remain elevated. The consequences of a persistently elevated blood glucose concentration include both micro-vascular complications; nephropathy, neuropathy and retinopathy as well as macro-vascular complications: coronary artery disease, peripheral arterial disease and stroke (Fowler, 2008). In those with type 2 diabetes, levels of insulin produced in response to a given concentration of glucose are inappropriately low. Clinically, this is usually assessed using measures of fasting blood glucose and the glucose concentration at 2 hours after consumption of 75g glucose in an oral glucose

tolerance test (OGTT). Depending on the blood glucose test results (shown in Table 1.1), clinicians can assess the degree of insulin resistance/insulin sensitivity and whether treatment for type 2 diabetes or 'pre-diabetes' is required.

	Fasting plasma glucose	Plasma glucose 2h post 75g glucose drink	Interpretation
Target ranges	3.6-6.0 mmol/L	<7.8 mmol/L	Normal fasting glucose and normal glucose tolerance.
'Pre-diabetes'	6.1-6.9 mmol/L	7.8 - 11.1 mmol/L	Impaired fasting glycaemia and impaired glucose tolerance.
Probable diabetes	>7.0 mmol/L	>11.1 mmol/L	Diagnosis made if at least one of these criteria is met on 2 occasions

Table 1.1. Target, pre-diabetic and diabetic ranges for fasting and 2 hours post oral glucose tolerance test. (Diabetes UK).

1.3.1 Metabolic syndrome

It is important to note that insulin resistance seldom occurs in isolation. It is part of a cluster of risk factors for cardiovascular disease which occur together more often than by chance alone and collectively form the 'metabolic syndrome' (Grundy *et al.*, 2005). These risk factors include elevated fasting concentrations of glucose, triglycerides and insulin, reduced HDL cholesterol, raised blood pressure and increased waist circumference. In 2009, the American Heart Association and National Heart, Lung and Blood Institute and the International Diabetes Federation agreed a combined definition for the diagnosis of metabolic syndrome whereby individuals must meet a minimum of any 3 from the criteria shown in Table 1.2 (Alberti *et al.*, 2009). It is important to identify individuals clinically at increased risk to allow initiation of interventions to reduce risk factors. The criteria used to diagnose metabolic syndrome cannot determine absolute risk, however, because it does not include age, sex, cigarette smoking, and low-density lipoprotein cholesterol levels which also contribute. Despite this, patients diagnosed with metabolic syndrome are at twice the risk of developing CVD over

the next 5 to 10 years compared to individuals without the 'syndrome' (Alberti *et al.*, 2009).

Criterion	Categorical cut points
Increased waist circumference*	Population- and country-specific definitions
Hypertriglyceridemia	≥ 1.7 mmol/L
Hyperglycaemia	≥ 5.6 mmol/L fasting
Hypertension	Systolic ≥130 mm Hg and/or diastolic ≥85 mm Hg
Reduced HDL cholesterol	< 1.03 mmol/L in males < 1.29 mmol/L in females

Table 1.2. Definition of metabolic syndrome according to the American Heart Association/National Heart Lung and Blood Institute and the International Diabetes Federation 2009. *The World Health Organisation identifies 2 levels of abdominal obesity in Europeans depending on risk for metabolic complications. An increased risk occurs at waist circumferences of ≥94 cm in men and ≥80 cm in women, but risk is substantially higher at ≥102 cm in men and ≥88 cm in women. (*WHO, 2000).*

1.4 Cellular mechanisms of insulin resistance

Insulin resistance occurs due to impaired insulin signalling in various cell types. When insulin binds to the insulin receptor, the receptor becomes autophosphorylated and its tyrosine kinase is activated. Insulin signal transduction occurs via tyrosine phosphorylation of insulin receptor substrates (IRS) 1 and 2 at the cell membrane which subsequently activates PI3K. This generates a series of second messengers and activation/inactivation of downstream targets accordingly (Danielsson *et al.*, 2005). Downstream of PI3K, signalling via PKB and PKC is involved in translocation of GLUT4 from an intracellular pool to the cell membrane, resulting in glucose transport into the cell and stimulation of glycogen synthesis (Schenk *et al.*, 2008). Disruption to any part of this pathway can lead to insulin resistance. Insulin signalling is typically down-regulated by interference with the tyrosine phosphorylation or inhibitory serine phosphorylation of IRSs, both of which are increased with inflammation (Shah *et al.*, 2008). Insulin can

also activate the mitogen-activated protein kinase (MAPK) family of protein kinases (p38 MAPK, ERK-1/2, JNK), which are involved in controlling transcription factors and may therefore have a role in long term development of insulin resistance with prolonged stimulation. It is thought that the low grade inflammation in obesity contributes to insulin resistance by activation of these particular pathways, ultimately leading to either serine phosphorylation or reduced tyrosine phosphorylation of IRS 1/2. TNFα, for example, can cause inhibitory phosphorylation of IRS-1 at serine 307 via the JNK pathway (Hirosumi *et al.*, 2002). In adipocytes, activation of P38 MAPK by inflammatory cytokines interferes with insulin signalling on genes controlling GLUT4 (Magne *et al.*, 2010), further implicating this pathway in both insulin resistance and inflammation. PKC-θ activation also increases serine phosphorylation of IRS-1, stimulated by fatty acid metabolites (Shah *et al.*, 2008). NFκB is also an important kinase linking insulin resistance and inflammation, which is usually under inhibition from IκBα in the cytoplasm. With appropriate stimuli, particularly in the setting of obesity and high levels of dietary saturated fatty acids, this inhibition is removed (by the action of IκKβ and NFκB enters the nucleus where it stimulates transcription of inflammatory cytokines which further contribute to impaired insulin signaling (Shah *et al.*, 2008). PPARɣ is another key transcription factor highly expressed in adipose tissue and influences both insulin sensitivity and inflammation in addition to adipogenesis and lipid metabolism. Thiazolidinediones, used in the treatment of type 2 diabetes, are activators of PPARɣ to increase insulin sensitivity. However, due to the widespread expression of PPARɣ there are numerous side effects so research for better treatment options is still required (Ahmadian *et al.*, 2013).

1.5 Adipose tissue distribution

Although obesity is clearly a key risk factor for type 2 diabetes, it is not a simple matter of total fat mass that is important in the development poor metabolic health and obesity related disease. Since not all obese individuals exhibit poor metabolic health (Karelis *et al.*, 2005). Further, despite adipose tissue typically representing approximately 30 % body fat in females and 20 % in males (Thompson *et al.*, 2012), women are not found to be at increased risk of obesity related disease (Geer & Shen, 2009; Logue *et al.*, 2011). The anatomical location of adipose

tissue is an important factor and increased upper body fat is associated with higher disease risk than increased lower body fat, which holds true over a range of BMI (Kissebah & Krakower, 1994; Manolopoulos *et al.*, 2010). Specifically it is an increased central/abdominal adiposity which is considered more pathogenic compared to lower body fat (Jensen, 2008) and as such waist circumference can often be a better predictor of type 2 diabetes than BMI (Diabetes Prevention Program Research, 2006). An increased proportion of gluteo-femoral fat compared to upper body adipose tissue is actually associated with lower cardiovascular risk and increased insulin sensitivity (Manolopoulos *et al.*, 2010). The exact mechanisms that cause central fat to be more closely associated with metabolic and inflammatory complications are not known. Striking differences exist between the different adipose tissue depots particularly in their lipolytic activity, abilities to remove triglyceride from the circulation and their sensitivities to the suppressive effects of insulin on free fatty acid release. Upper body adipose tissue is considered to be more metabolically adverse since it has a higher basal rate of lipolysis (Jensen, 2008). Another important consideration is likely to be that the different adipose tissue depots within the body have depot and gender specific gene expression profiles and unique biological and inflammatory characteristics (Karelis *et al.*, 2005; Karastergiou *et al.*, 2013). The presence of increased visceral adipose tissue in particular is considered to be higher risk due to its elevated levels of inflammation and fatty acid output directly to the liver via hepatic portal vein (Jensen, 2008). However, abdominal subcutaneous adipose tissue usually represents a larger depot in comparison to the visceral depot (Hill *et al.*, 2007; Thompson *et al.*, 2012) making it is particularly important not to overlook its role in the development of obesity related complications.

1.6 Adipose tissue health and dysfunction

The last decade has shown a dramatic increase in our understanding of the roles of adipose tissue and how its dysfunction is involved in mediating the development of obesity related disease. Nevertheless, a major role of adipose tissue is storing energy in the form of triglycerides during periods of positive energy balance and secreting fatty acids during periods of fasting and long term energy depravation (Trayhurn *et al.*, 2011). Adipose tissue metabolism is dynamic and the balance between triglyceride storage and lipolysis is complex and affected by various

factors such as insulin, catecholamines, adipose tissue blood flow and inflammatory cytokines such as TNFα and IL-6; all of which are dysregulated in obesity related insulin resistance (Manolopoulos *et al.*, 2010; Frayn & Karpe, 2014). It is important to understand the various roles of adipose tissue in health and disease and aspects of metabolic dysfunction occurring with its expansion are outlined below.

1.6.1 The traditional role of adipose tissue

Adipocytes are cells which are highly specialised for the storage of energy as triglycerides; a combination of fatty acids and glycerol. Since the body's storage capacity for carbohydrate and protein is limited, excess energy is reflected as triglyceride stored in adipose tissue (Schrauwen, 2007). Fatty acid flux between tissues is largely regulated by insulin and therefore reflects the body's current nutritional state. Following ingestion of dietary fats in the small intestine, they enter the circulation as triglyceride-rich chylomicrons via the lymphatic system. Fatty acids are taken up into adipocytes (and other cells including myocytes and hepatocytes) via the action of lipoprotein lipase (LPL). LPL is synthesised in adipocytes and is up-regulated in the postprandial state, stimulated by insulin, and transported to capillary endothelial cells within adipose tissue. LPL hydrolyses triacylglycerol from chylomicrons and once the free fatty acids are taken up into adipocytes they become esterified with α-glycerol-phosphate to reform triglycerides. The remaining chylomicron 'remnant' particles are then taken up by the liver and processed to form low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL). In times of fasting and when insulin levels are low, NEFA, particularly as palmitate and oleate, are released into the circulation following hydrolysis of stored triglycerides by the action of hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). NEFA circulate bound to albumin and are taken up by the liver and skeletal muscle amongst other tissues where they are either used for β-oxidation or triglyceride synthesis (Frayn & Karpe, 2014).

The balance between uptake and release of triglycerides is highly regulated according to energy balance. In times of weight gain, adipocyte hypertrophy occurs, reflecting an increased storage of triglycerides, however large adipocytes

are linked to an increased production of inflammatory mediators known to affect insulin resistance and lipolysis (Skurk *et al.*, 2007). Although it is not a simple matter of adipocyte size being the link to insulin resistance since visceral adipose tissue is considered more inflammatory even though it is composed of smaller adipocytes (Cancello *et al.,* 2006). Disruption to the regulation of blood flow to the adipose tissue, sensitivity of HSL to the suppressive effects of insulin and responsiveness to glucocorticoid and catecholamines are all thought to contribute to the elevated NEFA observed with insulin resistance (Manolopoulos *et al.*, 2010). However adipose tissue is capable of adapting via down-regulation of HSL and ATGL as an attempt by the whole tissue to prevent increased output of NEFA per unit fat mass and normalise blood levels (Karpe *et al.*, 2011). Although insulin resistant individuals frequently show increased circulating NEFA, it is not clear whether this is due to impaired fat storage or dysregulation by inflammatory cytokines (Karpe *et al.*, 2011).

1.7 Adipose tissue as an endocrine organ

A significant turning point in obesity research was the discovery that white adipose tissue acts as an endocrine organ (Hotamisligil *et al.*, 1993; Zhang *et al.*, 1994). The hormones produced by adipose tissue are termed 'adipokines' and the expression of a number of these are altered with excess weight (Balistreri *et al.*, 2010). Adipokines have a key role in regulating inflammation, insulin sensitivity, glucose metabolism and a variety of other physiological processes. They can act locally in an autocrine and paracrine manner but also in an endocrine manner, eliciting effects on the rest of body (Balistreri *et al.*, 2010). Many adipokines have been linked to insulin resistance (by interference with insulin signalling pathways discussed in section 1.4); particularly pro-inflammatory cytokines including leptin, IL-6, TNFα and MCP-1, which are increased in adipose tissue with increasing levels of adiposity (Weisberg *et al.*, 2003; You *et al.*, 2005) but can be reduced with weight loss (Clement *et al.*, 2004; Cancello *et al.*, 2005). In lean individuals, however, production of anti-inflammatory cytokines in adipose tissue such as adiponectin, IL-10 and IL-1Ra predominates. It is therefore likely that the balance between the production of pro- and anti-inflammatory cytokines in adipose tissue is key to the development of insulin resistance.

The majority of studies investigating cytokine production from adipose tissue and their respective appearance in the blood circulation are conducted in the fasting state and therefore reflect chronic changes at a systemic level. However, this is not necessarily an accurate reflection of metabolic health and postprandial changes may also be important, particularly since we now spend the majority of waking hours within 8 hours of consuming food. It is possible that repeated acute inflammatory changes in the adipose tissue and circulation following consumption of a meal may be responsible for the chronic low grade inflammation seen with increased adiposity and it is not known how this varies with differing levels of adiposity. This hypothesis is addressed to some extent in Chapter 4, in which postprandial responses in adipose tissue across varying levels of adiposity were examined. There are over 50 cytokines produced by adipose tissue that may be implicated in the development of obesity related insulin resistance, and it is beyond the scope of this PhD to investigate each of them. The adipokines that will be measured during the studies presented in this thesis are described below and in Table 1.3.

1.7.1 Leptin

Leptin is the product of the *Ob* gene and was one of the first proteins identified to be secreted by adipose tissue (Zhang *et al.*, 1994). It is primarily produced by adipocytes in proportion to body fat mass and has a key role in regulating energy homeostasis by reducing food intake, stimulating energy expenditure and regulating glucose and lipid metabolism (Carbone *et al.*, 2012). Its production is therefore largely regulated by nutrient intake and insulin, but its levels can conversely be dramatically reduced during periods of fasting (Friedman & Halaas, 1998). Leptin and insulin overlap in their signalling pathways, implicating this hormone in the development of insulin resistance. Leptin shares structural homology with long-chain helical cytokines and the leptin receptor is widely expressed on immune cells. This hormone therefore represents an important link between metabolism and immune system function (Lord *et al.*, 1998).

1.7.2 Adiponectin

Adiponectin is predominantly produced by adipocytes and has an inverse relationship with fat mass. It is particularly reduced with visceral adiposity and

correlates with levels of insulin sensitivity (Tilg & Moschen, 2006). Adiponectin is itself considered anti-inflammatory since it can suppress production of pro-inflammatory TNFα and IFNɣ whilst increasing production of anti-inflammatory cytokines such as IL-10 and IL-1Ra (Tilg & Moschen, 2006). Obese animals treated with adiponectin show improvements in glucose control and reduced free fatty acids in blood (Berg *et al.*, 2002).

1.7.3 IL-6

IL-6 is a pleiotropic cytokine secreted by white adipose tissue, skeletal muscle and liver. In healthy individuals, adipose tissue contributes to approximately one third of IL-6 in the circulation (Mohamed-Ali *et al.*, 1997), with the majority of this being produced by macrophages present within adipose tissue (Weisberg *et al.*, 2003). IL-6 is typically thought of as a pro-inflammatory cytokine as its expression is increased in adipose tissue with obesity, especially from the visceral depot (McLaughlin *et al.*, 2008). Furthermore, IL-6 can stimulate production of the acute phase protein CRP from the liver and can induce insulin resistance in adipocytes (Rotter *et al.*, 2003). With weight loss, IL-6 is reduced (Bastard *et al.*, 2000). There are, however, a number of studies describing anti-inflammatory effects of IL-6 (Frisdal *et al.*, 2011) and that it may even have a role in improving insulin sensitivity in muscle (Carey *et al.*, 2006).

Cytokine	Main role	Effect of obesity on adipose tissue levels
Monocyte chemoattractant protein 1 (MCP-1)	Chemokine involved in the stimulation of monocyte/macrophage migration. Secreted by mature adipocytes and monocyte/macrophages for further monocyte recruitment. Expression of MCP-1 is sensitive to insulin, however, this chemokine can also interfere with insulin signalling (Sartipy & Loskutoff, 2003).	Increased expression with obesity in mice with diet induced obesity (Xu *et al.*, 2003). In humans, expression and secretion from adipose tissue are increased but not necessarily into the blood (Dahlman *et al.*, 2005).
Tumour necrosis factor α (TNFα)	First inflammatory cytokine identified in adipose tissue and increased with obesity (Hotamisligil *et al.*, 1993). Can cause insulin resistance in adipocytes by interference with IRS phosphorylation via IKK/JNK/S6K/mTOR (Hirosumi *et al.*, 2002). Promotes lipolysis and interferes with adipocyte differentiation (Zhang *et al.*, 2002).	In humans, increased in plasma and adipose tissue obese vs. lean, decreased with weight loss (Kern *et al.*, 1995). Correlates with measures of insulin resistance (Hivert *et al.*, 2008).
Macrophage inflammatory protein 1β (MIP-1β)	Pro-inflammatory, potent chemokine, recruit immune cells to sites of infection and inflammation (Ren *et al.*, 2010).	MIP-1 proteins upregulated in adipose tissue with diet induced adiposity in mice (Xu *et al.*, 2003).
Interleukin 10 (IL-10)	Anti-inflammatory cytokine associated with Th2 and T-regulatory CD4+ cells and M2 macrophages. Inhibits effects of proinflammatory cytokines on reduction of insulin signalling (Hong *et al.*, 2009)	Increased production by adipose tissue (mainly non-fat cells) with obesity, likely attempt to dampen inflammation (Fain, 2010).

Table 1.3. Overview of the cytokines measured in the studies presented in this thesis.

Cytokine	Main role	Effect of obesity on adipose tissue levels
Interferon ɣ (IFNγ)	Primarily produced by CD4+ helper (Th1) and cytotoxic CD8+ T-lymphocytes (Schroder *et al.*, 2004). Skews macrophage differentiation towards pro-inflammatory (M1) response, induces insulin resistance in mature human adipocytes via interference with insulin signaling pathway. Also interferes with triglyceride storage and pre-adipocyte differentiation into mature adipocytes (McGillicuddy *et al.*, 2009).	In human subcutaneous adipose tissue, waist circumference correlates with IFNɣ mRNA (Kintscher *et al.*, 2008).
Interleukin 8 (IL-8)	Pro-inflammatory chemokine, primarily inducing chemotaxis of neutrophils, however, a variety of immune cells are sensitive to this hormone including monocytes. Production can be stimulated by pro-inflammatory cytokines including TNFα and induces insulin resistance in adipocytes via PKB phosphorylation (Kobashi *et al.*, 2009).	IL-8 mRNA and secretion in obese adipose tissue is higher compared to lean and is mainly from non-fat cells in adipose tissue. IL-8 production is particularly elevated in visceral compared to subcutaneous adipose tissue (Bruun *et al.*, 2004).
Interleukin 18 (IL-18)	Pro-inflammatory cytokine, together with IL-12 can induce Th1 differentiation of CD4+ helper T-lymphocytes and is implicated in the development of insulin resistance (Vandanmagsar *et al.*, 2011). Product of NLRP3 inflammasome which is mainly expressed in monocytes/macrophages (Guarda *et al.*, 2011).	Production mainly from non-fat cells in adipose tissue. Elevated in obesity and correlates with measures of insulin resistance (Bruun *et al.*, 2007). Decreased following calorie restriction and exercise (Vandanmagsar *et al.*, 2011)

Table 1.3 continued. Overview of the cytokines measured in the studies presented in this thesis.

Cytokine	Main role	Effect of obesity on adipose tissue levels
Interferon gamma inducible protein 10 (IP-10)	Chemoattractant important in recruiting activated T-lymphocytes to site of inflammation, production regulated by IFNɣ. Widely expressed including immune cells and adipocytes (Herder *et al.*, 2007).	IP-10 may be increased in blood of obese compared to lean but not necessarily in adipose tissue (Herder *et al.*, 2007).
Regulated on activation, normal T cell expressed and secreted (RANTES)	Potent chemokine for T-lymphocytes, sustained production linked to other chronic inflammatory diseases including atherosclerosis. Particularly involved in trafficking and survival of inflammatory immune cells including M1 macrophages and Th1 CD4+ T-lymphocytes (Baturcam *et al.*, 2014).	Increased expression in obese adipose tissue and reduced with exercise (Baturcam *et al.*, 2014).
Inerleukin 1β (IL-1β)	Proinflammatory cytokine implicated in many chronic inflammatory diseases and linked to insulin resistance (Grant & Dixit, 2013). Along with IL-18, is a product of the NLRP3 inflammasome which is mainly expressed in monocytes/macrophages (Guarda *et al.*, 2011).	Adipose tissue expression increased with obesity and in type 2 diabetes, production is decreased following calorie restriction and exercise (Vandanmagsar *et al.*, 2011)
Interleukin-1Ra (IL-1Ra)	Anti-inflammatory cytokine, competes with cytokines from the IL-1 family thereby antagonising the effects of IL-1β to reduce pro-inflammatory signalling (Juge-Aubry *et al.*, 2003).	Adipose tissue is an important source of IL-1Ra, adipose tissue production and levels in blood are increased with adiposity. Increased expression as attempt to dampen inflammation (Juge-Aubry *et al.*, 2003).

Table 1.3 continued. Overview of the cytokines measured in the studies presented in this thesis.

1.8 Inflammatory cells in adipose tissue

In addition to adipocytes, adipose tissue contains a whole range of cell types, including progenitor cells, endothelial cells, fibroblasts, and immune cells such as macrophages and lymphocytes which are collectively referred to as the stromavascular fraction (SVF). Due to their size, adipocytes represent approximately 80-90 % of the volume of adipose tissue and 60-70 % of the total cell population, with the remainder being attributed to the SVF (Thompson *et al.*, 2012). These non-adipocyte cells may be responsible for the majority of cytokine secretions from adipose tissue (Fain, 2010). Current research suggests that the interaction between these different cell populations within adipose tissue and the balance of pro- and anti-inflammatory cytokines acting in a paracrine and/or endocrine manner may be a key determinant of an individual's risk of developing obesity related disease. Immune cells often reside within tissues and tissue macrophages have specific functions, for example; kupfer cells line sinusoids in the liver, multinucleated osteoclasts are present in the periosteum (bone), microglia are interspersed around neurons in the central nervous system, mesangial cells are present in the kidney (Gordon, 1995). The role of immune cells within adipose tissue, however, is not yet fully understood.

Despite observations that adipose tissue secretes and responds to pro-inflammatory molecules, the infiltration of adipose tissue with immune cells was not described until 2003. Xu *et al.,* (2003) and Weisberg *et al.,* (2003) independently discovered that in obesity, macrophages accumulate in adipose tissue (attracted by chemokines such as MCP-1) and produce inflammatory mediators (including TNFα) which could contribute to metabolic complications including insulin resistance. For a long time it was thought that increased weight gain simply resulted in greater accumulation of macrophages in adipose tissue which were responsible for the observed systemic inflammation and this was initially the focus of much research. Mouse models genetically deficient in macrophages however, showed only partial protection from diet induced obesity and insulin resistance, suggesting that other cells may be implicated in adipose tissue inflammation and insulin resistance (Weisberg *et al.*, 2003). It was not until 2007 that T-lymphocytes were also identified in adipose tissue, so consequently relatively little is known regarding the mechanisms for their recruitment,

involvement in adipose tissue inflammation and any influences on 'metabolic health'. A diagram summarising what is currently known about immune cell residence and recruitment into adipose tissue is shown in Figure 1.1. The studies contributing to this model will be discussed in the following sections.

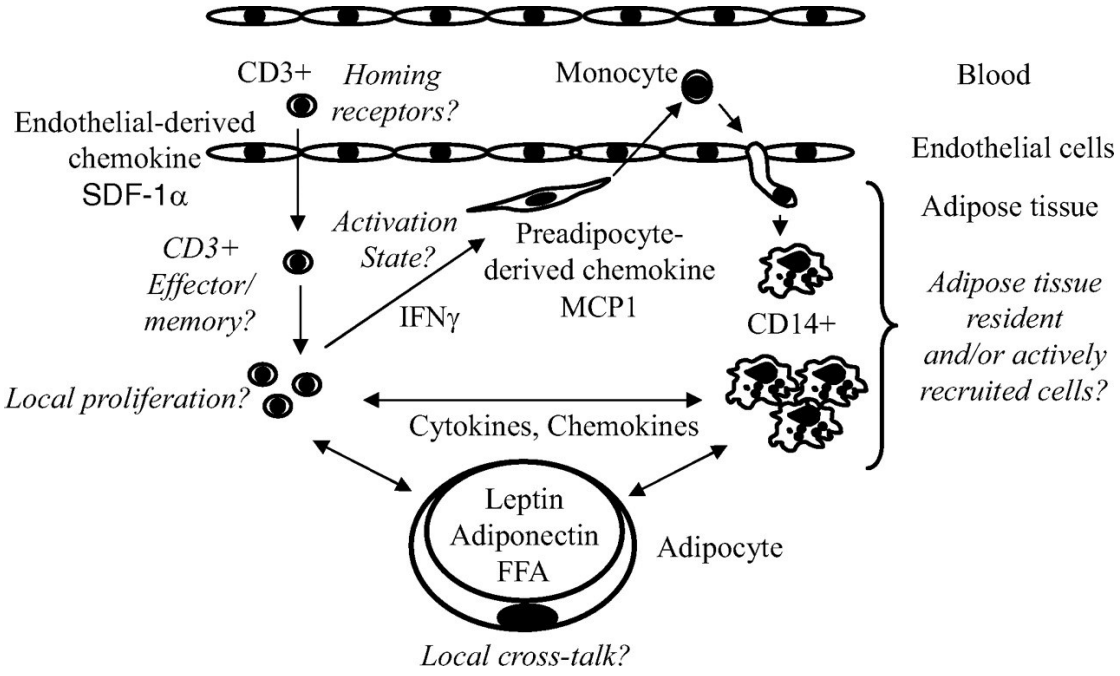


Figure 1.1. Putative processes involved the accumulation of immune cells within adipose tissue and their pathogenic roles in obesity and associated pathologies. Blood circulating lymphocytes might be attracted through the production of the SDF-1 produced by endothelial cells. Extravasation of T-lymphocytes and their accumulation within the fat mass might be the primary event involved in the further accumulation of macrophages through the IFNɣ-stimulated production of MCP-1 by the stromal pre-adipocytes. In italics are the open questions that remain to be answered. Figure taken from (Bouloumie *et al.*, 2008).

1.8.1 Macrophages

Macrophages typically have scavenger functions which are important in innate immune responses, where they recognise and remove infected cells/foreign material by phagocytosis. Through their secretions they attract further immune cells and also have a role in the adaptive immune system, whereby they can present foreign antigens to lymphocytes. Macrophages secrete various cytokines to regulate immune responses and wound repair/tissue remodelling. A number of these cytokines can also cause insulin resistance in adipocytes (Gordon, 1998).

The studies by Xu *et al.* (2003) and Weisberg *et al.* (2003) investigated progressive changes in gene expression occurring in various tissues of mouse models with the development of diet induced obesity. In addition to increases in expression of various inflammatory genes, a number of macrophage related genes were specifically up-regulated in white adipose tissue and occurred prior to increases in fasting blood insulin and glucose. Both groups confirmed the presence of macrophages in murine adipose tissue using immunohistochemistry. However, Weisberg *et al.* (2003) additionally performed immunohistochemistry on human subcutaneous adipose tissue and showed that the accumulation of cells expressing the macrophage marker CD68 was correlated with body mass and adipocyte size. The accumulation of macrophages in all human adipose tissue depots and their relationship with body mass has been reported subsequently in a variety of populations (Curat *et al.*, 2004; Cancello *et al.*, 2006; Curat *et al.*, 2006; Zeyda *et al.*, 2007). In insulin resistant obese populations, adipose tissue inflammation and infiltration of macrophages may also be increased compared to 'metabolically healthy' obese (Apovian *et al.*, 2008). Furthermore, calorie restriction or gastric bypass surgery reduces inflammation and macrophage content of adipose tissue (Clement *et al.*, 2004; Cancello *et al.*, 2005).

In mouse models of obesity there appears to be two separate polarisation states of macrophage with specialised functions that may be important in determining the inflammatory phenotype of lean and obese adipose tissue (Zeyda & Stulnig, 2007). M1 'classically activated' macrophages are increased in obese mice and release pro-inflammatory cytokines including TNFα and IL-6. M2 'alternatively activated' macrophages, however, produce anti-inflammatory cytokines; IL-10

and IL-1Ra and represent a greater proportion of macrophages in lean mice (Lumeng *et al.*, 2007). It was therefore suggested that a shift in cytokine profile with increasing obesity favours the conversion of M2 to M1 macrophages. In humans, the phenotype appears to be different, however, in that markers associated with each of these murine macrophage types are present on a single population of human macrophages but can modify their phenotype according to the local stimuli (Zeyda *et al.*, 2007; Bourlier *et al.*, 2008). It is suggested that the increased macrophages in human obesity are polarised towards an anti-inflammatory phenotype as an attempt to counteract the elevated levels of inflammation and they may also have an important role in the re-modelling of adipose tissue (Zeyda *et al.*, 2007; Bourlier *et al.*, 2008; Fjeldborg *et al.*, 2014). It may be the balance between the two phenotypes that is important in the development of obesity related complications. Species differences in macrophage phenotype are likely to be a factor in differences observed. The development of obesity is also much more acute for mouse models that are fed a high fat diet for a matter of weeks, as compared to in humans where obesity typically occurs over months/years, potentially allowing for adaptations to take place (Bourlier & Bouloumie, 2009).

1.8.2 The origin of macrophages in adipose tissue

The increased presence of macrophages in adipose tissue with obesity may either arise from blood monocytes recruited to the adipose tissue by changes in chemokines/cytokines or proliferation of resident tissue macrophages. Another potential source of macrophages is trans-differentiated pre-adipocytes, which share expression of a number of genes and have phagocytic properties (Weisberg *et al.*, 2003). Transmigration experiments have shown that blood monocytes can be recruited to adipose tissue via endothelial cells activated by conditioned media from human adipocytes (Curat *et al.*, 2004). Lipid depletion of the media used in these experiments had little effect on these observations, however protein degradation appeared to markedly reduce adhesion and diapedesis suggesting it is inflammatory mediators involved in the activation of endothelial cells and recruitment of monocytes rather than lipid. Within the adipocytes used to make the conditioned media, high expression of MCP-1, IL-8 and leptin was identified, all of which are increased with obesity. Leptin alone was able to induce the same

diapedesis of monocytes. However, since the Xu and Weisberg studies still reported macrophages in Leptin deficient mice models, other factors in addition to Leptin are therefore likely to be involved (Weisberg *et al.*, 2003; Xu *et al.*, 2003). Increased plasma concentrations of soluble cell adhesion molecules (E-selectin, VCAM-1, ICAM-1) are observed with increasing adiposity, which suggests that increased fat mass may be associated with increased endothelial activation. The work by Curat *et al.* (2004) supports the theory that factors released from adipocytes may be involved in activation of endothelial cells and further recruitment immune cells into adipose tissue.

Weight gain is associated with adipocyte hypertrophy and remodelling within the expanding adipose tissue (Curat *et al.*, 2004). It has been suggested that areas of local hypoxia as a result of this remodelling may promote adipocyte necrosis and could be important in the recruitment/proliferation of macrophages for their scavenger function (Villaret *et al.*, 2010). In obese adipose tissue, this is proposed as the primary stimulus for macrophages since they are often found in 'crown like structures' (CLS) surrounding dead adipocytes, as shown in Figure 1.2 (Cinti *et al.*, 2005). Macrophages are also implicated in the remodelling of the extracellular matrix, angiogenesis and regulation of adipocyte proliferation that occurs with adipose tissue expansion (Lumeng *et al.*, 2007; Sun *et al.*, 2011).

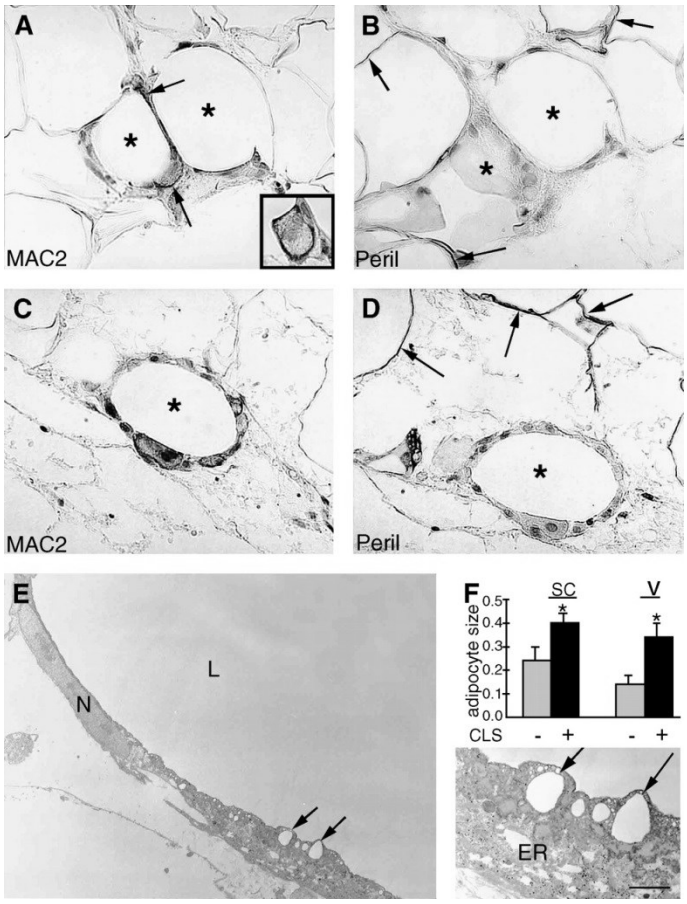


Figure 1.2. CLS formation at sites of adipocyte death in human adipose tissue and correlation between adipocyte death and adipocyte size. Human subcutaneous (A, B) and visceral (omental; C, D) fat depots demonstrating MAC-2-positive (brown) macrophages (A, C) organized exclusively in CLS surrounding degenerate (perilipin-free; B, D) lipid droplets (asterisks). E: Representative electron micrograph showing necrotic degenerative features of obesity-associated adipocyte death in human WAT. F: Adipocyte death is correlated with mean adipocyte size in human subcutaneous (SC) and visceral (V) adipose tissue. Fat biopsies from human subjects (body mass index range of 20–45) were scored for the presence (+) or absence (−) of CLS Values shown are means ± SD. * $P < 0.05$ for within-depot comparison. Bar = 27 μm for A–D, 3.7 μm for E, and 1.5 μm for E, inset. Image taken from Cinti *et al.* (2005).

Dysregulated lipolysis due to adipocyte insulin resistance may also contribute to the increase in macrophages, particularly seen with type 2 diabetes. This has become apparent since short term calorie restriction results in increased lipolysis and is associated with an increased infiltration of macrophages to clear the increased lipid in adipose tissue (Capel *et al.*, 2009; Kosteli *et al.*, 2010). Furthermore, macrophages can be activated via toll-like receptor 4 (TLR4) receptors which are stimulated by free fatty acids, such that mice lacking TLR4 are found to be protected from insulin resistance following lipid infusion and diet induced obesity (Shi *et al.*, 2006)

1.8.3 Adipose tissue depot differences

Cancello *et al.* (2006) demonstrated that in humans the accumulation of macrophages correlates with fat cell size in visceral and subcutaneous adipose tissue. However, greater macrophage accumulation is found in visceral adipose tissue despite subcutaneous fat cells being 40 % larger in diameter (Cancello *et al.*, 2006). This observed difference in cell size between the two depots is also supported by Villaret *et al.*, (2010). Visceral adipose tissue exhibits a more pro-inflammatory state, characterised by increased IL-6, IL-8, IL-1β and MCP-1 (Bouloumie *et al.*, 2005) and shows greater expression of hypoxia related genes (HIF-1α) compared to subcutaneous adipose tissue (Villaret *et al.*, 2010). It was suggested that these may be a consequence of 'hypo-perfusion' as a result of rapidly expanding adipose tissue mass and may play a role in recruiting a greater number of macrophages to this depot (Villaret *et al.*, 2010).

1.8.4 Lymphocytes

It is not surprising that macrophage accumulation and activation has been a main focus for research, especially since they can represent up to 40 % of cells in adipose tissue SVF (Weisberg *et al.*, 2003). Although T-lymphocytes represent a comparatively lower proportion (~6 %) of adipose tissue immune cells (Duffaut *et al.*, 2009b), they may have an important role in regulating macrophage differentiation, activation and insulin resistance, as in atherosclerosis (Witztum & Lichtman, 2014). T-lymphocytes are central to adaptive immunity and typically either reside in tissues to carry out 'immune monitoring' as resting memory T cells, or as effector T cells which are attracted to sites of inflammation. CD4+ T-

lymphocytes are 'helper T cells' and recognise MHC class II on antigen presenting cells such as dendritic cells or macrophages. Their role is to activate and direct immune cells, with Th1 cells releasing pro-inflammatory cytokines like IFNɣ, IL-12 and TNFα and Th2 cells releasing anti-inflammatory cytokines including IL-10 and IL-4. Another subset of CD4+ lymphocytes are T-regulatory cells which have a key role in regulating the immune response via their suppressor activity (Josefowicz *et al.*, 2012). CD8+ cells recognise antigens presented in MHC class I by antigen presenting cells. They are cytotoxic T-lymphocytes and typically have key roles in destroying virally infected cells and tumour cells.

Wu *et al.* (2007) were the first to demonstrate the presence of CD3+ T-lymphocytes in the visceral adipose tissue of diet-induced obese insulin-resistant mice, and morbidly obese humans together with the expression of the T cell specific cytokine; 'regulated on activation, normal T cell expressed and secreted' (RANTES) and its receptor CCR5. This was also one of the first investigations to suggest that T-lymphocytes may also have an important role in the development of insulin resistance in obesity. Mouse models have been used to monitor lymphocyte accumulation in adipose tissue and show that they increase at the onset of insulin resistance prior to the increase in macrophages; suggesting that macrophage infiltration may not be involved in the initiation of insulin resistance (Kintscher *et al.*, 2008; Nishimura *et al.*, 2009). Initially it was thought that it was the CD8+ T-lymphocytes that may accumulate at the initial onset of obesity (Nishimura *et al.*, 2009), although not all mouse models support this (Strissel *et al.*, 2010). In mice, it is thought that a loss of 'protective' T-regulatory cells and anti-inflammatory Th2 lymphocytes may be important in the development of obesity related insulin resistance (Feuerer *et al.*, 2009; Nishimura *et al.*, 2009). Furthermore, depletion of cytotoxic T-lymphocytes or increasing the proportion of T-regulatory cells can lead to an improvement in obesity induced insulin resistance (Feuerer *et al.*, 2009).

In humans, CD3+ mRNA in subcutaneous adipose tissue was initially found to correlate with waist circumference as well as expression of IFNɣ, suggesting an increase in pro-inflammatory T-lymphocytes with obesity (Kintscher *et al.*, 2008). Subsequently, the presence of CD4+ and CD8+ T-lymphocytes has been

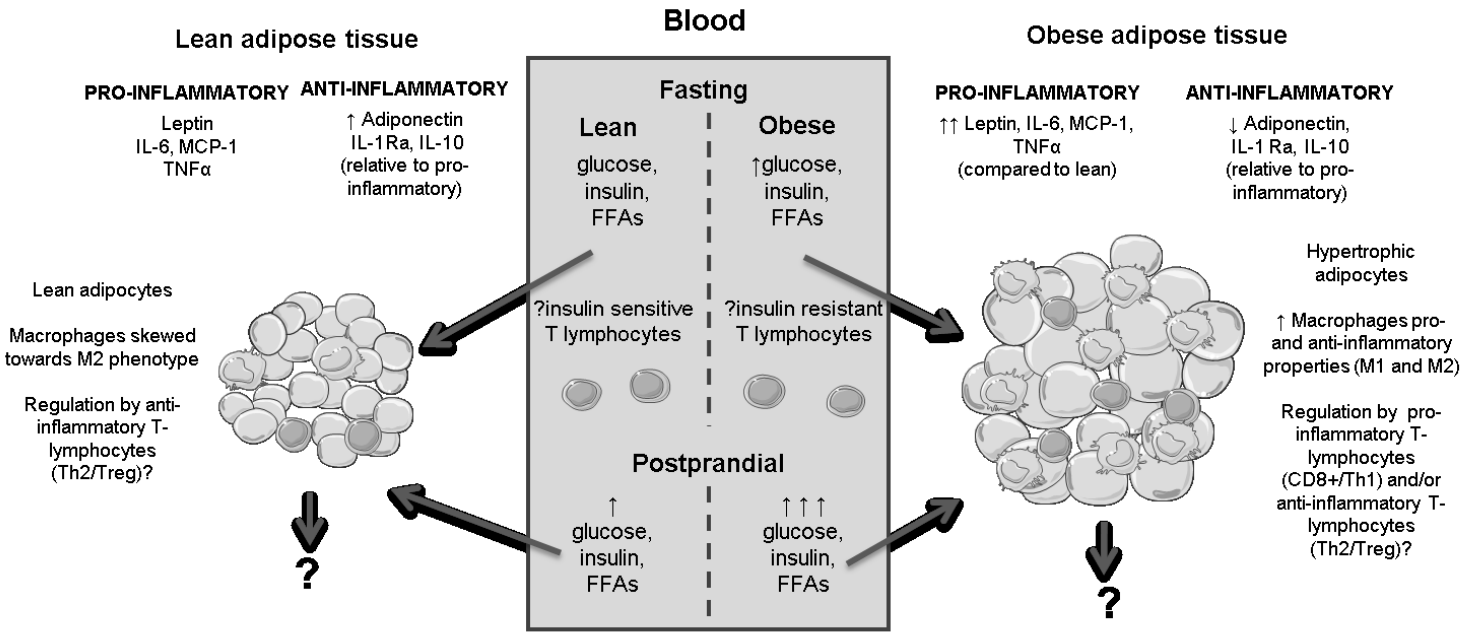
identified in human adipose tissue using gene expression and flow cytometry. Confocal microscopy suggests that the CD3+ cells may be present in clusters around adipocytes (Duffaut *et al.*, 2009b). The consensus appears to be that both CD4+ and CD8+ T-lymphocytes are increased in human obesity and that visceral tissue is more enriched with these cell types than subcutaneous adipose tissue (Kintscher *et al.*, 2008; Duffaut *et al.*, 2009b; Zeyda *et al.*, 2011).

Further human studies have aimed to characterise T-lymphocyte subsets present in human adipose tissue, but results are not always consistent. Zeyda *et al.* (2011) identified the presence of cytotoxic, Th1, Th2 and T regulatory lymphocytes using gene expression analysis and showed that the expression of each of these T-lymphocyte markers were higher in obese compared to well matched lean and overweight controls in both visceral and subcutaneous adipose tissue. In both visceral and subcutaneous adipose tissue, proportions of T-regulatory cells were increased, however, which is in contrast to the animal models of diet induced obesity. In visceral adipose tissue an increase in Th2 transcripts was also found, suggesting that there may be an increase in lymphocytes with an anti-inflammatory phenotype, perhaps as an adaptive mechanism to counteract increased visceral adipose tissue inflammation (Zeyda *et al.*, 2011). The work by Goossens *et al.* (2012) suggests that there may be an increased proportion of Th1 polarised CD4+ T-lymphocytes in glucose intolerant obese individuals compared to a lean control group. Furthermore, the group suggests that the proportion of Th1 T-lymphocytes may be directly related to adipose tissue inflammation and whole body insulin sensitivity (Goossens *et al.*, 2012). Some groups however, have reported an increase in CD4+ T-lymphocytes skewed towards a Th17 phenotype; another helper T-lymphocyte subset, typically involved in autoimmune diseases (Bertola *et al.*, 2012; Fabbrini *et al.*, 2013). These observed discrepancies may be a factor of the different populations being investigated.

Few functional studies have been performed to investigate T-lymphocyte accumulation in human adipose tissue. *In-vitro* studies using adipocyte conditioned media, suggest that leptin, IL-6 and CCL20 may be involved in the recruitment of T-lymphocytes (Duffaut *et al.*, 2009b) all of which are expressed to a greater extent with obesity. Moreover, conditioned media produced by adipose

tissue lymphocytes can down-regulate the insulin stimulated increase in lipogenesis (Duffaut *et al.*, 2009b). Pre-adipocyte and endothelial cell factors such as SDF-1α may also have a role in inducing T-lymphocyte migration (Kintscher *et al.*, 2008)

Since this is such a new and evolving area of research, these discrepancies warrant further investigation and little is known about the extent of their activation and whether it is influenced by adiposity and adipose tissue inflammation. It is the functional roles of the various subsets of T-lymphocytes in the development of obesity related diseases that will form one of the main foci of this thesis. A schematic summarising the main observations in adipose tissue across varying levels of adiposity and some of the primary unanswered questions that this thesis aims to cover is shown in Figure 1.3.



Unanswered questions

Are there adipose tissue inflammatory postprandial responses that are related to metabolic response in blood that may contribute to chronic adipose tissue inflammation?

Are there important differences in immune cell populations with increased levels of adiposity and are there specific differences in insulin resistant populations?

How are these properties of adipose tissue altered with diet and/or activity leading to weight loss?

Nature of the cross-talk between the different cells in adipose tissue?

Figure 1.3. Schematic summarising the main observations in adipose tissue across varying levels of adiposity and some of the primary unanswered questions that this thesis aims to cover.

1.8.5 Other immune cells in human adipose tissue

Other cell populations from both the innate and adaptive immune systems have been identified in adipose tissue, however they appear to represent a much lower proportion of cells in the SVF and relatively few studies have investigated their potential roles in obesity related insulin resistance. These cells together with their preliminary observations in obesity are shown in Table 1.4.

Immune cell	Typical role	Observations in adipose tissue expansion
Innate Immunity		
Neutrophils	Granulocytic cells, release contents (antimicrobial) and phagocytose invading pathogens/foreign material.	Increased early in diet induced obesity (at 3 days) in mouse models (Talukdar *et al.*, 2012).
Eosinophils	Roles in asthma, allergy, response to parasites and Th2 immunity. Anti-inflammatory.	Present in adipose tissue, express IL-4 which may maintain M2 polarisation of macrophages. May decrease in mice on high fat diet (Wu *et al.*, 2011).
Mast cells	Granulocytic cells, roles in allergic responses and wound healing.	Increased with obesity in humans (Liu *et al.*, 2009).
Adaptive immunity		
Dendritic cells	Professional antigen presenting cells.	CD1c+ dendritic cells may accumulate with insulin resistance in humans (Bertola *et al.*, 2012).
NKT cells	Recognise lipid antigens presented by CD1d.	Present in murine and human adipose tissue but not dependent on BMI (Duffaut *et al.*, 2009b; Nishimura *et al.*, 2009).
B-lymphocytes	Humoral immunity, produce antibodies.	Present in human adipose tissue (Duffaut *et al.*, 2009b). Accumulate in adipose tissue of diet induced obese mice (Winer *et al.*, 2011).

Table 1.4 Other immune cells present in adipose tissue SVF, in addition to macrophages and lymphocytes.

1.9 Aim

This review has examined the evidence relating metabolic dysfunction and inflammation occurring within adipose tissue to the development of obesity related diseases such as type 2 diabetes. In the last decade or so, a better understanding of adipose tissue inflammation in obesity has emerged, which is characterised by an increased infiltration of immune cells, in particular macrophages and T-lymphocytes. Macrophages have been the main focus of work with relatively little being known about the role of T-lymphocytes and their potential involvement in the development of obesity related disease.

The overall aim of the work presented in this thesis is to further characterise aspects of metabolic and immune system cross-talk in human adipose tissue with a particular interest in the role of T-lymphocytes and their potential implications in adipose tissue dysfunction and insulin resistance.

CHAPTER 2
General Methods

2.1 Introduction

This chapter will describe generic protocols that were used throughout the PhD. Methods which are specific to a particular study will be detailed in the relevant chapter. Experimental procedures involving subjects were conducted in the 'Resting Metabolic Laboratory' at the University of Bath. Each study received ethical approval from local NHS South West Research Ethics Committees (Frenchay/Southmead) prior to commencement. The analysis of blood and adipose tissue samples were carried out in the Applied Physiology Laboratory at the University of Bath and the Nutritional Immunology Laboratories within the Unilever Food and Health Research Institute (Vlaardingen, The Netherlands).

For all studies, participants were actively recruited by local advertisement. Participants in study 1 (Chapter 3) included male volunteers aged between 35-55 years, with adiposities ranging from lean through to obese based on waist circumference. These participants were well characterised according to various physical measures, metabolic/inflammatory profiles and immune cell subsets in blood and adipose tissue. These same participants then consumed a mixed meal, and metabolic and inflammatory responses were examined in blood and adipose tissue as detailed in Study 2 (Chapter 4). In study 3 (Chapter 5), overweight/obese males aged between 35-55 years underwent a short-term period of 50 % calorie restriction and the effects on resident immune cells and metabolic/inflammatory properties of blood and adipose tissue were examined. For study 4 (Chapter 6), overweight/obese males and postmenopausal females aged between 45-65 years were recruited and categorised into groups of 'normal glucose tolerance' and 'impaired glucose tolerance' following results of a preliminary screen including an oral glucose tolerance test. Study 4 received additional support for recruitment via the Primary Care Research Network (PCRN), allowing GP surgeries within the Bath and North East Somerset (BANES) area to act as patient identification centres. Following database searches by Practice staff, letters of invitation to

participate along with study information were sent out to potentially eligible participants.

In studies 1 and 2 females were excluded from the study since they are protected from chronic diseases to some extent until the menopause and also show significant metabolic differences to males, in particular with leptin concentrations, which may cause conflicting results within the otherwise more 'homogenous' group. In study 4, postmenopausal women were included in part due to the anticipated small numbers of people being identified with impaired glucose tolerance from the initial screen.

For each study, participants were provided with an information sheet giving details of the specific study requirements. Each participant gave written informed consent to participate and was asked to complete a questionnaire related to their medical history and lifestyle (Appendix 1). Individuals were excluded if they smoked or had not been weight stable for more than 3 months, i.e. no weight change > 3 % (Stevens *et al.*, 2006). Furthermore, no participants had any medical conditions or were taking any medications that are known to interfere with lipid/glucose metabolism or immune system function.

2.2 Anthropometry

Height was measured with the participant barefoot using a fixed stadiometer (Holtain Ltd, UK) to within 0.1 cm. Body mass was measured to the nearest 0.1 kg (post- void mass on the morning of the trial) using digital mass scales (TANITA corp. Japan). Body mass index was calculated for all subjects using the equation BMI = mass (kg) / height^2 (m^2). Waist and hip circumferences were measured in triplicate using a plastic non-stretch measuring tape (SECA, Germany) and mean value calculated. Waist circumference was measured as mid-way between the lowest rib and the iliac crest (WHO, 2008). The tape was passed around the waist parallel to the floor with the subject standing at the end of gentle expiration. Hip circumference was measured horizontally around the widest portion of the buttocks (WHO, 2008). For both circumference measurements, the participants were stood with feet close together, arms at sides and weight distributed evenly with the tape snug around the body but not pulled so tight that

it is constricting. Classification of participants based on their waist circumference measurements was according to the WHO criteria (2008) shown in Table 2.1.

	Lean Normal risk	Overweight Increased risk	Obese Higher risk
Men	< 94 cm	94-102 Cm	> 102 cm
Women	< 80 cm	80-88 cm	> 88 cm

Table 2.1. Classification ranges for waist circumference for lean, overweight and obese individuals according to sex. Based on WHO criteria (2008).

2.3 Blood Pressure

Blood pressure was measured using an automated blood pressure monitor (Alvita, MC101, UK) whilst participants were upright in bed after resting for at least 10 minutes. Three readings were taken and mean values of systolic and diastolic blood pressure calculated.

2.4 Dual Energy X-ray Absorptiometry

Participant body composition was characterised using dual energy X-ray absorptiometry (DEXA). Participants were asked to consume 1 pint of water to rehydrate upon waking on the morning of the scan, then void bladder immediately before the scan to minimise variations in hydration status between individuals because body water affects lean mass estimates (Lohman *et al.*, 2000). Participants were permitted to wear only lightweight clothing for the analysis. Before each scan, a quality control check was performed using material provided by the manufacturer. Descriptive statistics for each participant including date of birth, height and weight were entered into the QDR for Windows software (Hologic, Bedford, UK). Participants were asked to lay supine on the DEXA scanning table (Discovery, Hologic, Bedford, UK) and were positioned centrally with feet spread apart and arms with an even gap from the trunk. It was requested that participants remained as still as possible during the 7 minute scan. The scan was performed by an individual who had completed training in ionising radiation (medical exposure) regulations (IR(ME)R). Following completion of the scan, whole body composition analysis was performed with regions sectioned as recommended in the QDR for Windows manual (Hologic, Bedford, UK). Scans

were analysed for total and percentage fat mass and lean mass, and 'Central adipose tissue' (abdominal subcutaneous and visceral adipose tissue) was estimated from a central region between L1-L4. This region (L1-L4) has previously been shown to be comparable to estimates of central adipose tissue measured by computerised tomography (CT) (Glickman *et al.*, 2004) and correlates with measures of metabolic health (Paradisi *et al.*, 1999). Fat mass index (FMI) was calculated using the equation FMI = total fat mass (kg) / height^2 (m^2) and interpreted using the ranges shown in Table 2.2 that match the prevalence of WHO BMI classifications (Kelly, 2009).

	Fat deficit	**Normal**	**Excess fat**	**Obese**
Male	< 3	3 - 6	6 - 9	> 9
Female	< 5	5 - 9	9 - 13	> 13

Table 2.2. Classification ranges for fat mass index 'FMI'. Established by matching to prevalence at age 25 for each threshold of WHO classification of BMI. Ranges are sex specific but are not confounded by lean tissue unlike BMI (Kelly, 2009).

2.5 Physical activity assessment

Participants were fitted with a combined heart rate/accelerometer (Actiheart, Cambridge Neurotechnology Ltd., Cambridge, UK) to determine habitual physical activity. The monitor was worn for 7 whole consecutive days and subjects were asked to record a corresponding diary of their physical activity during the monitoring period to aid its interpretation. Subjects were asked to maintain their normal lifestyle habits/routines during the monitoring period. Data was downloaded and analysed using associated software (Actiheart 2.0 Cambridge Neurotechnology Ltd. Cambridge, UK) and was used to plot heart rate and accelerometry. The software estimates activity energy expenditure (AEE) using measurements of R waves detected in 60 second epochs whilst an internal accelerometer simultaneously senses intensity and frequency of the subject's torso movements. The Actiheart monitor has previously been validated using the branched model equations for estimating low-to-moderate intensity physical

activity energy expenditure which is likely to be typical of the 'general population' as included in these studies (Thompson *et al.*, 2006). Total energy expenditure (TEE) is then calculated as AEE + resting metabolic rate as measured using indirect calorimetry (General Methods, 2.6) + diet induced thermogenesis (estimated as a constant 10 % of TEE). Physical activity level (PAL) was then determined using the formula PAL = Total energy expenditure/basal energy expenditure.

2.6 Gas collection and analysis for indirect calorimetry

Resting metabolic rate (RMR) was determined using substrate oxidation under resting conditions as described by Frayn, (1983). Expired O_2 (1 d.p) and CO_2 (2 d.p) was analysed using a Servomex 1400 gas analyser with the participant lying in a bed at rest. The analyser was calibrated on the day of the trial using 100 % Nitrogen and mixed gas of 15.9 % Oxygen and 5.00 % Carbon Dioxide, and checked against atmospheric values 20.9 % Oxygen and 0.04 % Carbon Dioxide. During collections, participants were required to wear a nose clip and breathe into a mouthpiece with breathing valves to ensure all expired gas was collected into evacuated Douglas bags. During each gas collection, samples of ambient CO_2 and O_2 were measured to account for small changes observed throughout the day in an enclosed room rather than assuming outside atmospheric values (Betts & Thompson, 2012). The gas sampling tube was held as close as possible within 1 metre of the participant for the final 2 minutes of each separate gas collection (read after 1 minute 30 seconds of ambient sampling allowing sufficient time for values to stabilise). Participants were given the mouthpiece and nose clip at least 30 seconds before the collection began to flush the associated tubing of any atmospheric air and to settle any initial changes to breathing. The stopwatch was started as soon as the stopcock was turned, diverting expired gases into the Douglas bag. Expired gas samples were collected for 5 minutes and CO_2 and O_2 within the Douglas bag were sampled for 2 minutes at a flow rate of 2.1 L/min with values recorded after 1 minute 30 seconds of sampling when values had stabilised. The Douglas bag was then completely evacuated using a dry gas meter (Harvard Apparatus) and total volume of expired gas and its temperature (measured using Checktemp thermometer attached to the dry gas meter) were recorded. Barometric pressure and ambient laboratory temperature were also

measured and adjustments to temperature made as necessary to maintain it between 20 – 25 °C. Estimation of RMR was made using a minimum of 4 separate consecutive collections to ensure 3 readings within 100 kcal (Compher *et al.*, 2006; Betts *et al.*, 2011). The mean of the values within 100 kcal was used as RMR. Measurements were made early in the morning with as little participant exertion prior to gas sampling as possible. Time was given for participant recovery as necessary prior to sampling (minimum 10 minutes).

2.7 Dietary analysis

Subjects received a diary and set of digital weighing scales (model 1306, Salter, Kent, UK) to record their food and fluid intake during diet monitoring periods. Detailed nutritional analysis was performed using the software COMP-EAT Pro (version 5.8.0, Nutrition systems, UK) whereby users can input average and 'custom' food information for a more accurate dietary analysis. For study 2 it was anticipated that 3 days would be sufficient to capture a representative example of macronutrient intake without being too time consuming for participants (Whybrow *et al.*, 2008; Fyfe *et al.*, 2010). For studies 3 and 4 a full week of diet recording was required to be used in conjunction with activity monitoring or glucose monitoring data respectively to inform the specific changes to be made during the 2 interventions.

2.8 Restrictions for participants pre-trial day

Subjects were asked to refrain from performing any strenuous physical activity and consuming alcohol/caffeine for 48 hours and 24 hours before the main trial, respectively. Trial days were scheduled so participants had been free from any self-reported illness for a minimum of 2 weeks in order to reduce immune system disturbance. Subjects were asked to arrive at the laboratory after fasting for a minimum of 10 hours and to consume 1 pint of water upon waking on the day scheduled for testing.

2.9 Blood sampling

An appropriately sized cannula (18G-21G; BD Venflon Pro, Becton Dickenson & Co., Sweden) was inserted into an antecubital forearm vein and blood samples obtained via stopcock (BD connecta, Beckton Dickenson, Sweden) and syringe(s)

at the required time points. Venous blood samples were collected using syringes and dispensed into blood collection tubes (Sarstedt Ltd., Leicester, UK) containing either serum separator beads for serum collection or Potassium-ethylenediaminetera acetic acid (K3-EDTA) as an anticoagulant for plasma samples. Blood was dispensed into serum tubes first to avoid EDTA contamination which may affect some metabolite and enzyme measurements. Patency of the cannula was maintained by flushing with sterile isotonic saline (0.9 % sodium chloride solution (B Braun, UK) following blood sampling. Prior to obtaining the subsequent sample, 3 mL waste was taken from the cannula to remove this saline that would otherwise dilute the sample. For venepuncture sampling a single-use 21G needle (BD Valu-Set, Beckton Dickenson & Co., Plymouth) was inserted into antecubital forearm vein and syringes used to draw the blood sample.

2.10 Blood analysis

Following gentle mixing by inversion, EDTA tubes were immediately spun at 3465 g (5000 rpm) for 10 minutes at 4 °C (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., Bishops Stortford, UK). Venous blood added to the tube containing serum separation beads was mixed by inversion and allowed to clot for 45 minutes at room temperature prior to centrifugation. Samples were then transferred to 1.5 mL eppendorf tubes and frozen at -80 °C for later analysis. Whole blood differential leukocyte counts were obtained using an automated haematology system (SF300 Sysmex Ltd. Milton Keynes, UK). Glucose, total cholesterol, HDL-cholesterol, triglycerides, non-esterified free fatty acids (NEFA) and hs-CRP concentrations and ALT activity were measured using commercially available assay kits (Randox Laboratories, Crumlin, NI) with the Daytona automated analyser (Rx Series, Randox Laboratories, Crumlin, NI). LDL-cholesterol was calculated using Friedwald equation (Friedwald, 1972);

LDL-cholesterol = Total-cholesterol – HDL-cholesterol – (0.456 x triglycerides)

ELISA was used for the measurement of serum insulin (Mercodia, Sweden), Leptin and Adiponectin (both R&D Systems Inc., UK) according to manufacturer instructions. All samples for each participant (i.e. baseline and follow-up) were

included on the same ELISA plate or batch for analysis. Where samples were measured as below the measureable range, they were assigned the value of the lowest limit of detection for statistical analysis. Coefficients of variation and lower limits of detection for each ELISA are shown in Table 2.3.

Parameter	Sensitivity	Intra-assay precision (CV)	Inter-assay precision (CV)
Glucose	0.54 mmol/L	< 5 %	< 6 %
NEFA	0.04 mmol/L	< 5 %	<5 %
Triglycerides	0.134 mmol/L	< 4 %	< 4 %
Total-Cholesterol	0.865 mmol/L	< 4 %	< 2 %
HDL-Cholesterol	0.189 mmol/L	< 4 %	< 3 %
ALT	9.70 U/L	< 6 %	<5 %
CRP	0.477 mg/L	< 3 %	< 5 %
Insulin	1 mU/L	<4 %	<4 %
Adiponectin	0.246 ng/mL	<5 %	< 8 %
Leptin	7.8 pg/mL	< 3 %	< 5 %

Table 2.3. Sensitivity and coefficient of variation data for the assays used to measure blood parameters. Where there were multiple quality controls used to analyse CV, the highest of these values is shown (Manufacturers values).

2.11 Isolation of Peripheral Blood Mononuclear Cells

Viable peripheral blood mononuclear cells (PBMCs; lymphocytes and monocytes) were isolated from blood using a density separation medium Lympholyte-Mammal (Cedarlane Laboratories Limited, Burlington, Canada). The method results in a greater non-selective recovery of viable lymphocytes and monocytes whilst removing most granulocytes (including neutrophils) and dead cells by using Sodium Diatrizoate combined with Dextran to induce erythrocyte aggregation but reduce platelet aggregation (Lympholyte kit insert). Blood was first collected into tubes containing EDTA and diluted with an equal volume of saline (1:1) before carefully layering over Lympholyte (1.5 x volume of undiluted blood) with as little mixing as possible to ensure distinct interface formation in an RNase/DNase-free, non-pyrogenic, sterile centrifuge tube (Corning, NY). The sample was centrifuged for 20 minutes at 800 g at room temperature (22 °C) on the slowest acceleration setting. Using a Pasteur pipette, cells were carefully removed from the interface and transferred to a new centrifuge tube. The transferred cells were then diluted with PBS to reduce the density of the solution

and centrifuged for 10 minutes at 800 g (22 °C) using the fastest acceleration setting to pellet the lymphocytes. The supernatant was discarded and lymphocytes washed in this manner a further 3 times in PBS. Cells were stored in 1 mL PBS:FCS:DMSO in ratio 5:4:1 respectively and frozen at a rate of -1 °C/minute to –80 °C using a freezing container (Mr Frosty, Nalgene, Thermo Fisher Scientific Inc.) filled with isopropanol. Cells were stored at –80 °C until subsequent analysis by Flow cytometry (Materials and Methods sections 2.15 – 2.18).

2.12 Oral glucose tolerance test

In some studies, participants were required to consume a glucose drink consisting of 75 g (113 mL) anhydrous glucose (maltodextrin) solution (Polycal, Nutricia, UK), diluted to 200 mL with water within 5 minutes. Participants received a further 100 mL in the same glass to ensure the entire glucose solution had been consumed. Cannula blood samples were taken every 15 minutes for the following 2 hours for measurement of plasma glucose and serum insulin. The results of the OGTT were used to calculate total areas under the curve (tAUC) using the trapezium rule (Matthews *et al.*, 1990). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated using the equation: fasting glucose (mmol l^{-1}) × fasting insulin (mU l^{-1})/22.5 (Matthews *et al.*, 1985) and insulin sensitivity index (ISI comp/Matsuda index) was calculated as 10,000/√[fasting glucose (mg/dl) × fasting insulin (μU/mL) × mean OGTT glucose value (mg/dL) × mean OGTT insulin value (μU/mL)] (Matsuda & DeFronzo, 1999).

2.13 Adipose tissue sampling

Adipose samples were collected from the area around the waist, approximately 5 cm lateral to the umbilicus, using a well-established 'needle aspiration' technique. The area was thoroughly disinfected with Videne, before injection of anaesthetic (approximately 5 mL Lignocaine hydrochloride 1 %) into a small fan shaped area beneath the skin using a 27G needle (PrecisionGlide, Beckton Dickinson, USA). Five minutes later, a larger 14G needle (Kendall Monoject, Tyco Healthcare, USA) was inserted into the subcutaneous fat tissue around the waist in order to collect ~1-2 g of fat tissue. Swabs and pressure were then applied to the area once the sample has been collected and before a dressing is applied.

Visible connective tissue and blood vessels were removed from the adipose tissue with scissors before the remaining adipose tissue was washed with PBS over single-use sterile gauze membrane to remove any further blood, clots and connective tissue. All re-usable equipment used for tissue handling was pre-sterilised by autoclaving.

2.14 Whole adipose tissue culture

Adipose tissue was minced into approximately 5-10 mg explants using sterilised scissors and transferred to sterile culture plates (Nunclon surface, Nunc, Denmark) containing Endothelial cell Basal media (ECBM; Promocell, Germany) supplemented with 0.1 % fatty acid free BSA and 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma Aldritch, UK). Tissue was incubated at a final concentration of approximately 100 mg tissue per 1 mL (Fain *et al.*, 2004). Explants were then transferred to an incubator (MCO-18A1C CO_2 incubator, SANYO) at 37 °C, 5 % CO_2 and 95 +/-5 % relative humidity. After 3 hours, media was removed and transferred to sterile eppendorfs and stored at -80 °C. A short incubation period was chosen since relative proportions of cytokine production dramatically changes between 4 and 48 hours and not in a linear/predictable manner (Fain *et al.*, 2004). From preliminary work, 3 hours was shown to be sufficient time for measureable concentrations of cytokines of interest to be measured.

A fluorescent bead (Bio-Plex) multiplex system (Luminex, BIO-RAD) was used for the measurement of cytokines (GCSF, MCP-1, IP-10, IL-8, IL-6, IL-10, IL-1Ra, MIP-1β, TNFα, IL-18) secreted by adipose tissue (Chapters 3, 4 and 5) and present in serum (Chapter 5). Coefficients of variation and lower limits of detection for each cytokine measured by Luminex are shown in Table 2.4. Secretion of cytokines by 100 mg adipose tissue explants was multiplied by central fat mass (L1-L4) estimated using DEXA to predict total central adipose tissue secretion. This accounts for the profound differences between individuals in absolute levels of adiposity and is thus more representative of secretion *in-vivo*.

Parameter	Sensitivity (pg/mL)	Intra-assay precision (CV) %	Inter-assay precision (CV) %
GCSF	1.7	10	5
MCP-1	1.1	9	7
IP-10	6.1	11	9
IL-8	1.0	9	4
IL-6	2.6	7	11
IL-10	0.3	5	6
IL-1Ra	5.5	9	8
MIP-1β	2.4	8	8
TNFα	6.0	8	6
IL-18	0.2	4	5

Table 2.4. Sensitivity and coefficient of variation data for the cytokines measured in blood and adipose tissue by Luminex. (Manufacturers values; BIO-RAD).

2.15 Preparation of the stromavascular fraction

Adipocytes were isolated from the other cells contained within the adipose tissue comprising the stromavascular fraction by collagenase digestion based on a protocol by Curat *et al.*, (2004). Whole tissue was minced using sterilised scissors and digested using an equal volume (to tissue mass) of buffer containing type 1 collagenase with specific activity of 250 u/mL (Worthington Biochemical, NJ) in PBS / 2 % BSA at pH 7.4 in a shaking water bath (220 rpm) at 37 °C for approximately 45 minutes (or until digestion complete). The digestion was terminated by addition of 2 volumes 10 % NBCS in PBS. The suspension was filtered into a sterile plastic tube through a sterilised nylon mesh with pore size 400 μm to remove undigested connective tissue. Adipocytes were allowed to float by gravity for a minimum of 10 minutes at 37 °C to ensure all were at the top (Skurk *et al.*, 2007) before being harvested using a pipette with a wide bore tip to reduce cell lysis. Once the adipocytes were removed, the remaining sample was centrifuged at 300 g for 10 minutes at 4 °C to pellet the SVF. The supernatant was removed and the pellet re-suspended in Erythrocyte Lysis Buffer (155 mM NH_4Cl, 5.7mM K_2HPO_4, $3H_2O$, 0.1M EDTA H_2O, pH 7.3) for 10 minutes. The sample was then centrifuged at 300 g for 5 minutes at 4 °C, and the supernatant discarded. The SVF pellets were resuspended in 1 mL PBS:FBS:DMSO in a ratio of 5:4:1 respectively and frozen at a rate of -1 °C/minute to -80 °C using a

freezing container (Mr Frosty, Nalgene, Thermo Fisher Scientific Inc.). Samples were stored at -80 °C until subsequent analysis by flow cytometry.

2.16 Thawing and counting of SVF and PBMCs

Frozen SVF and PBMCs were rapidly thawed and added drop wise to 10 mL DMEM (Lonza) supplemented with 10 % FBS. Once thawed, samples were kept on ice at all times between each step in the procedure. Samples were centrifuged at 300 g for 5 minutes at room temperature and the supernatant removed using suction. Samples were then resuspended in 5 mL DMEM. SVF samples only, were filtered through a 100 μM mesh, rinsed with a further 10 mL DMEM and centrifuged at 300 g for 5 minutes. SVF samples were then re-suspended in 2 mL FACS buffer; 0.1 % (w/v) fatty-acid free BSA in PBS (Lonza) and transferred to 5 mL FACS tubes (Beckton Dickenson) for staining with a single mixture of antibodies. For PBMC samples, cells were counted using an automated cell counter and the appropriate volume for 250,000 cells dispensed into a 96 well plate, with samples dispensed in duplicate across separate rows for simultaneous staining with the 2 different antibody cocktails.

2.17 Staining of PBMCs for flow cytometry analysis

The SVF and PBMC samples were centrifuged at 350 g for 5 minutes at room temperature and supernatant discarded. Cells were blocked with 25 μL of mouse serum (20 %) and incubated for 10 minutes prior to addition of 25 μL of the appropriate mixture of fluorescently labelled antibodies (described below) and a further 10 minute incubation in the dark. Samples were then diluted with 225 μL FACS buffer and centrifuged at 330 g for 5 minutes at room temperature. This final step was repeated once more using 250 μL buffer, and samples were resuspended in a final 250 μL for analysis by flow cytometry.

2.18 Flow Cytometry

Flow cytometry (using the FACSverse, Beckton Dickenson) was used to identify CD4+/CD8+ T-lymphocytes (CD45+CD3+ cells) and macrophages/monocytes (CD45+CD14+/CD45+HLA-DR+CD16+ cells) in SVF and PBMCs together with respective levels of activation. Due to the limited size of SVF samples for analysis by flow cytometry, cells were labeled using a single cocktail comprising the

following antibodies; CD4-FITC, CD163-PE, CD14-PerCP, CD8-PE-Cy7, CD69-APC, CD25-APC-Cy7, CD3-V450, CD45-V500 (Beckton Dickenson). PBMCs were labeled using 2 separate cocktails to identify monocytes; CD45-V500, CD295-FITC, CD220-PE, CD14-PerCP, CD16-APC and T-lymphocytes; CD45-V500, CD3-V450, CD295-FITC, CD220-PE, CD25-APC-Cy7, CD69-APC, CD4-PerCP, CD8-PE-Cy7 (Beckton Dickenson) (Duffaut *et al.*, 2009b). Compensation settings were adjusted using CompBeads (Beckton Dickenson) prior to sample measurement. Monocytes/macrophages were identified using the CD45 versus side scatter (SSC) plot and a gate drawn to include all CD45+ cells. CD45 was then plotted against CD14 and gated on the CD45+ population previously identified. A gate was then drawn around the CD45+CD14+ cells and these were taken to be monocyte/macrophage populations. For an example of T-lymphocyte gating strategies – see Figure 2.1.

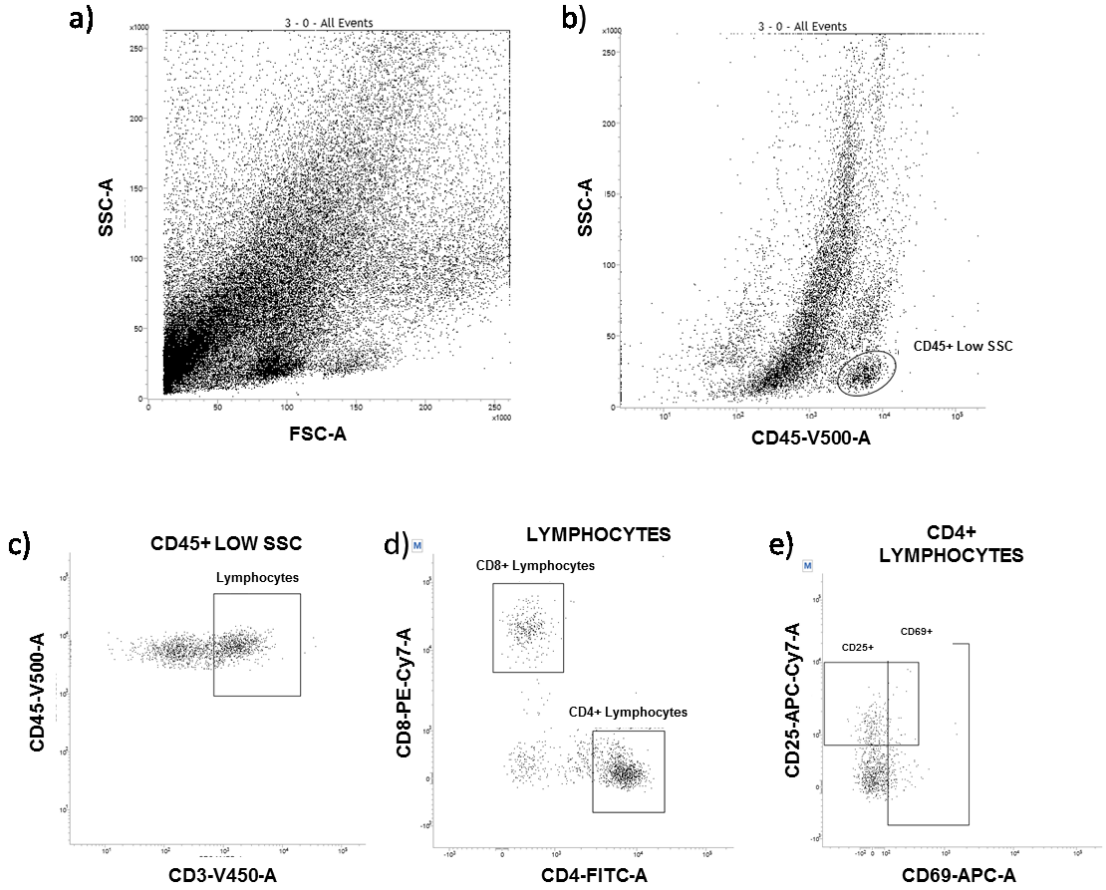


Figure 2.1. Gating strategy to identify T-lymphocytes (CD45+CD3+ cells). From the total SVF cells present (a), the CD45+Low-SSC population was identified (b) and lymphocytes (CD45+CD3+ cells) selected (c) for further analysis of CD4+ and CD8+ lymphocytes (d) and their respective activation (e).

2.19 Adipose mRNA; sample preparation and analysis

Approximately 200 mg whole adipose tissue was transferred to an RNase/DNase free sterile centrifuge tube, homogenised in 5 mL Trizol reagent (Invitrogen) and frozen on dry ice before being stored at – 80 °C (in study 1 only, however, samples were frozen immediately and homogenised in 5 mL Trizol before thawing). The homogeniser was washed with ethanol and then distilled water between samples. Once thawed, samples were centrifuged at 2500 g at 4 °C for 5 minutes. After removal of the lipid layer (top), the remaining supernatant was removed and transferred to a new RNase/DNase free sterile centrifuge tube and 200 µL of chloroform was added per 1 mL of Trizol. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 3 minutes before

centrifugation at 2500 g at 4 °C for 15 minutes. The aqueous (top) phase was removed and mixed with an equal volume of 70 % ethanol before being transferred to RNA extraction columns (RNeasy mini kit; Qiagen, Crawley, UK) and total RNA extracted according to manufacturer instructions. Samples were quantified (Qubit 2.0 fluorimeter, Life Technologies, UK) and 2 μg reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed using a StepOne™ (Applied Biosystems) with pre-designed primers and probes obtained from Applied Biosystems for measurement of macrophages (CD68; Hs02836816_g1), T-lymphocyte populations and subsets (CD3G; Hs00962186_m1, CD4; Hs01058407_m1, CD8A; Hs00233520_m1, FOXP3; Hs01085834_m1, GATA3; Hs00231122_m1 and TBX21; Hs00203436_m1) and for expression of GLUT4 (Hs00168966_m1), IRS2 (Hs00275843_s1), HSL (Hs00193510_m1), Leptin (Hs00174877_m1), Adiponectin (Hs00605917_m1), LPL (Hs Hs01012567_m1), PPAR-Ɣ (Hs01115513_m1), MCP-1 (Hs00234140_m1), RANTES (Hs00982282_m1), IP-10 (Hs01124251_g1), IL-6 (Hs00985639_m1), IL-8 (Hs99999034_m1), IL-10 (Hs00961619_m1), IL-1Ra (Hs00893626_m1), TNFα (Hs99999043_m1), IL-1β (Hs01555410_m1) and IL-18 (Hs00155517_m1). Peptidylpropyl isomerase Aα (PPIA) was used as an endogenous control (Neville *et al.*, 2011). Results were analysed using the comparative Ct method and expression normalized to an internal calibrator specific to each gene using the formula $2^{-\Delta\Delta C_T}$; where $\Delta\Delta C_T$ is [(C_T gene of interest – C_T PPIA) – lowest ΔC_T for gene of interest] and statistical analysis performed on LN transformed values (Livak & Schmittgen, 2001). Individuals were excluded from analysis where one or both samples were outside the detectable limit (Ct >35).

CHAPTER 3
The impact of adiposity on adipose tissue-resident lymphocyte activation in humans

3.1 Introduction

The presence of immune cells within human adipose tissue was only discovered a decade ago when macrophages were first identified in abdominal subcutaneous adipose tissue (Weisberg *et al.*, 2003). Since then, macrophages have taken centre stage in this field of research (Xu *et al.*, 2003; Curat *et al.*, 2004; Zeyda *et al.*, 2007; Bourlier *et al.*, 2008) whereas the roles of lymphocytes and other cells of the immune system have been largely overlooked in human studies. This is despite the fact that lymphocytes are key players in the initiation and regulation of immune responses within conditions such as atherosclerosis, asthma and rheumatoid arthritis (van Oosterhout & Bloksma, 2005; Cope *et al.*, 2007; Witztum & Lichtman, 2014). Most investigations of T-lymphocytes in human adipose tissue have used immunohistochemistry, gene expression and/or flow cytometry to identify the presence of these cells (Wu *et al.*, 2007; Kintscher *et al.*, 2008; Duffaut *et al.*, 2009b; Feuerer *et al.*, 2009; Nishimura *et al.*, 2009; Zeyda *et al.*, 2011; Goossens *et al.*, 2012). Indeed, other than the documented presence of activated T-lymphocytes in adipose tissue (Duffaut *et al.*, 2009b; Bertola *et al.*, 2012), little is known about the extent of lymphocyte activation and whether this is influenced by levels of adiposity and adipose tissue function.

Studies using rodent models of diet-induced obesity to investigate the time course of immune cell accumulation in adipose tissue suggest that T-lymphocytes precede macrophage infiltration/proliferation (Kintscher *et al.*, 2008; Nishimura *et al.*, 2009). Cytotoxic (CD8+) lymphocytes in particular may be key mediators of early adipose tissue inflammation, insulin resistance and macrophage migration, activation and differentiation (Nishimura *et al.*, 2009). These animal models provide extremely useful insights but require confirmation in humans, especially since obesity typically occurs over a much longer period than diet-induced obesity in rodents. Importantly, these rodent studies reinforce the hypothesis that lymphocytes play a central physiological role during the early stages of adipose

tissue expansion. Much of the available information about immune cells in human adipose tissue comes from morbidly obese individuals where the state of dysfunction is already profoundly apparent at a systemic level, characterised by insulin resistance/type 2 diabetes with adipose tissue showing a pro-inflammatory phenotype (Kintscher *et al.*, 2008; Duffaut *et al.*, 2009b; Zeyda *et al.*, 2011; Goossens *et al.*, 2012). Less is known however, about the role of adipose tissue resident immune cells during more 'common' forms of overweight and obesity. This is important from a mechanistic and clinical perspective since approximately 62 % adults in the UK are overweight with less than 3 % of people having a BMI >40 kg/m^2 (HSE tables, 2012). Additionally, interventions targeting people with modest obesity and 'early' metabolic dysfunction would yield the greatest rewards given the relative prevalence of modest obesity and the opportunity to prevent the development of future adiposity-related chronic disease.

Thus, the aim of the present study was to investigate human adipose tissue resident T-lymphocyte subsets and their activation in lean to moderately obese individuals who have been carefully characterised in terms of metabolic health. Furthermore, to put these findings into context, we also determined whether T-lymphocyte activation was related to either pro- or anti- inflammatory properties of adipose tissue and commonly-used clinical markers of metabolic health.

3.2Materials and Methods

3.2.1 Experimental design

Thirty men aged between 35 and 55 years were recruited by local advertisement and visited the laboratory for preliminary anthropometric measurements including waist circumference, which was used to classify participants as lean <94 cm, overweight >94 cm but <102 cm, and obese >102 cm (WHO, 2008). Recruitment continued until there was an equal distribution of 10 participants in each waist circumference category. Participants also attended the laboratory on one separate occasion for sampling of blood and adipose tissue. Blood samples were collected for measurement of clinical markers of metabolic health and isolation of peripheral blood mononuclear cells (PBMCs). Adipose tissue samples were divided into three portions for gene expression analysis, culture or isolation of the stromavascular fraction (SVF). The protocol was reviewed and given approval by the South West, Southmead NHS Research Ethics Committee (REC Reference: 11/SW/0193) and all participants provided written informed consent.

3.2.2 Participants

Participants were grouped according to waist circumference and individuals were excluded if they had any medical conditions or were taking any medication known to interfere with immune function or lipid/glucose metabolism. Individuals were also excluded if they smoked or had not been weight stable for more than 3 months (i.e., weight change >3 %).

3.2.3 Pre-trial requirements

Participants were asked to refrain from performing any strenuous physical activity and consuming alcohol/caffeine for 48 h and 24 h prior to testing respectively. Trial days were scheduled so participants had been free from any self-reported illness for a minimum of 2 weeks in order to reduce immune system disturbance. Participants arrived in the laboratory in the morning after an overnight fast (minimum 10 h) and consuming 1 pint of water upon waking and rested in the laboratory for 45 minutes prior to testing.

3.2.4 Body composition analysis

Participant waist circumference, body mass and height were measured as described in General Methods, 2.2. Body composition analysis was performed using DEXA with central adipose tissue (L1-L4) and fat mass index estimated as described in General Methods, 2.4.

3.2.5 Blood and adipose sampling and preparation

A venous blood sample was taken from an antecubital vein and dispensed into tubes containing either K_3EDTA or serum separation beads and processed as described in General Methods, 2.9-2.10. PBMCs were isolated by density gradient separation according to the protocol described in General Methods, 2.11.

Subcutaneous adipose tissue samples (~1 g) were obtained under local anaesthetic (1 % lidocaine) approximately 5 cm lateral to the umbilicus with a 14 gauge needle using an 'aspiration' technique and processed as described in General Methods, 2.13. Approximately 200 mg whole adipose tissue was transferred to an RNase/DNase free sterile centrifuge tube and frozen immediately on dry ice and later homogenised in Trizol (Invitrogen, UK). The remainder was used for adipose tissue culture (General Methods, 2.14), and preparation of SVF (General Methods, 2.15).

3.2.6 Real time-PCR

Total RNA was extracted from whole adipose tissue, quantified and 2 μg reverse transcribed as described in General Methods, 2.19. Real-time PCR was performed using a StepOne^{TM} (Applied Biosystems) with pre-designed primers and probes obtained from Applied Biosystems for measurement of macrophages (CD68), T-lymphocyte populations and subsets (CD3G, CD4, CD8A, FOXP3, GATA3 and TBX21) and for expression of GLUT4, IRS2, HSL, Leptin, Adiponectin, MCP-1, RANTES, IP-10, IL-6, IL-8, IL-10, IL-1Ra, TNFα, IL-1β and IL-18. Peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011). Results were analysed using the comparative Ct method and expression normalized to an internal calibrator specific to each gene using the formula $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is [(C_T gene of interest – C_T PPIA) – lowest ΔC_T for gene of interest] and statistical analysis performed on LN transformed values (Livak &

Schmittgen, 2001). Data for adipose tissue expression of GCSF, MIP-1β and IFNγ are not shown because they were only detectable in 4-8 individuals. Details of specific primer Hs numbers can be found in General Methods, 2.19.

3.2.7 Analysis of SVF and PBMCs by flow cytometry

Flow cytometry (using the FACSverse, Beckton Dickenson) was used to identify CD4+/CD8+ T-lymphocytes (CD45+CD3+ cells) and macrophages/monocytes (CD45+CD14+/CD45+HLA-DR+CD16+ cells) in SVF and PBMCs together with respective levels of activation. Due to the limited size of SVF samples remaining for analysis by flow cytometry, cells were labeled using a single cocktail comprising the following antibodies; CD4-FITC, CD163-PE, CD14-PerCy5.5, CD8-PE-Cy7, CD69-APC, CD25-APC-Cy7, CD3-V450, CD45-V500 (Beckton Dickenson). PBMCs were labeled using 2 separate cocktails to identify monocytes; CD45-V500, CD16-APC, HLA-DR-FITC, CD11b-PeCy5 and CD86-PE-Cy7 and T-lymphocytes; CD45-V500, CD3-V450, CD4-FITC, CD8-PE-Cy7, CD69-APC, CD25-APC-Cy7, CD220-PE (Beckton Dickenson) (Duffaut *et al.*, 2009b). Gating strategies are described in General Methods, 2.18.

3.2.8 Biochemical analysis

Plasma glucose and serum total cholesterol, HDL cholesterol and triglyceride concentrations and ALT activity were measured using commercially available assay kits and analyser (Daytona Rx, Randox). ELISA was used for the measurement of serum insulin (Mercodia, Sweden) and adiponectin (R&D Systems, UK) and both serum and adipose tissue leptin secretion (R&D systems). Adipose tissue secretion of GCSF, MCP-1, IP-10, IL-8, IL-6, IL-10, IL-1Ra, MIP-1β, TNFα, and IL-1β were measured using Luminex (BIO-RAD) and multiplied by central fat mass (L1-L4) estimated using DEXA to predict total central adipose tissue secretion.

3.2.9 Statistical analysis

All data are presented as mean and standard error of the mean (SEM). Comparisons were made between the lean, overweight and obese groups using one-way ANOVA irrespective of normality (Maxwell and Delaney, 1990, p. 109). Relationships between parameters were analysed using Pearson's correlation. Statistical analysis was performed using SPSS version 20 and $p<0.05$ was considered to be statistically significant.

3.3 Results

3.3.1 Participant characteristics

Participants in each group differed in terms of physical measures of adiposity and blood concentrations of glucose, insulin and leptin (Table 3.1).

Classification based on waist circumference	Lean n = 10	Overweight n = 10	Obese n = 10	ANOVA *p*-value
Physical Characteristics				
Age (y)	43.5 ± 1.7	48.0 ± 1.8	45.2 ± 1.9	0.218
Waist Circumference (cm)	87.0 ± 1.4	97.7 ± 0.8	109.4 ± 1.8	<0.001
Body Mass Index (kg/m^2)	23.6 ± 0.6	26.7 ± 0.4	30.7 ± 0.9	<0.001
Fat Mass Index (kg/m^2)	4.5 ± 0.3	6.9 ± 0.2	9.5 ± 0.6	<0.001
L1-L4 (%)	19 ± 1	30 ± 1	37 ± 2	<0.001
Fasting Metabolic Characteristics				
Leptin (ng/mL)	10.0 ± 1.6	26.6 ± 3.4	40.1 ± 4.4	<0.001
Adiponectin (µg/mL)	9.6 ± 1.3	8.7 ± 1.2	8.5 ± 1.3	0.834
Glucose (mmol/L)	4.4 ± 0.3	4.8 ± 0.3	5.3 ± 0.2	0.043
Insulin (pmol/L)	27.3 ± 4.8	39.6 ± 7.0	59.5 ± 10.1	0.020
HOMA-IR	0.9 ± 0.2	1.4 ± 0.3	2.4 ± 0.4	0.012
Total-Cholesterol (mmol/L)	4.6 ± 0.3	5.1 ± 0.3	4.4 ± 0.3	0.267
Triglycerides (mmol/L)	0.9 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	0.123
HDL-Cholesterol (mmol/L)	1.3 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.091
NEFA (mmol/L)	0.33 ± 0.04	0.48 ± 0.13	0.43 ± 0.05	0.469
ALT (U/L)	18.5 ± 1.3	27.4 ± 2.6	28.5 ± 5.0	0.087

Table 3.1. Participant descriptive statistics. Data presented as mean ± SEM. Effects of adiposity analysed by one-way ANOVA (*p*-values shown). L1-L4 = central fat mass within the lumbar region L1-L4 as determined by DEXA scan, HOMA-IR = homeostasis model assessment-established insulin resistance, HDL = high-density lipoprotein, NEFA = non-esterified fatty acids, ALT = alanine aminotransferase.

3.3.2 Lymphocyte numbers and activation in adipose tissue

Gene expression analysis of whole adipose tissue (n=30) revealed the presence of T-lymphocytes on the basis of CD3, CD4 and CD8 expression (Figure 3.1a). Relative expression of CD4+ was significantly increased with adiposity and further analysis using CD4+ lymphocyte lineage markers; FOXP3, (T-regulatory cells), GATA3 (Th2) and TBX21 (Th1) revealed an increase only in the relative expression of T-regulatory cell transcripts (Figure 3.1b).

There was sufficient adipose tissue from 17 of the 30 participants to perform flow cytometry of the SVF to characterise these T-lymphocyte populations by both proportion of total cells and activation status. Flow cytometry confirmed the presence of both CD4+ and CD8+ lymphocytes within the CD45+CD3+ population. As a percentage of total cells present in the SVF, CD4+ cells ranged from 0.3 to 4.7 % and CD8+ cells ranged from 0.5 to 5.6 % but there were no correlations between cell percentages and measures of adiposity (data not shown).

The lymphocyte subsets were further characterised using activation markers CD69 and CD25. Activated T-lymphocytes were assessed according to both the proportion of cells that were activated and their mean level of activation (mean fluorescent intensity of each activation marker). Proportions of activated CD4+ and CD8+ T-lymphocytes (CD25+ or CD69+) as a percentage of either total SVF cells or percentage CD4+/CD8+ T-lymphocytes (CD45+CD3+ cells) within the SVF were not related to measures of adiposity (Figure 3.1c). When examining the level of T-lymphocyte activation, however, significant correlations were found between central adiposity and the level of expression of CD69 and CD25 on activated CD4+ and CD8+ T-lymphocytes (Figure 3.1d i – iv).

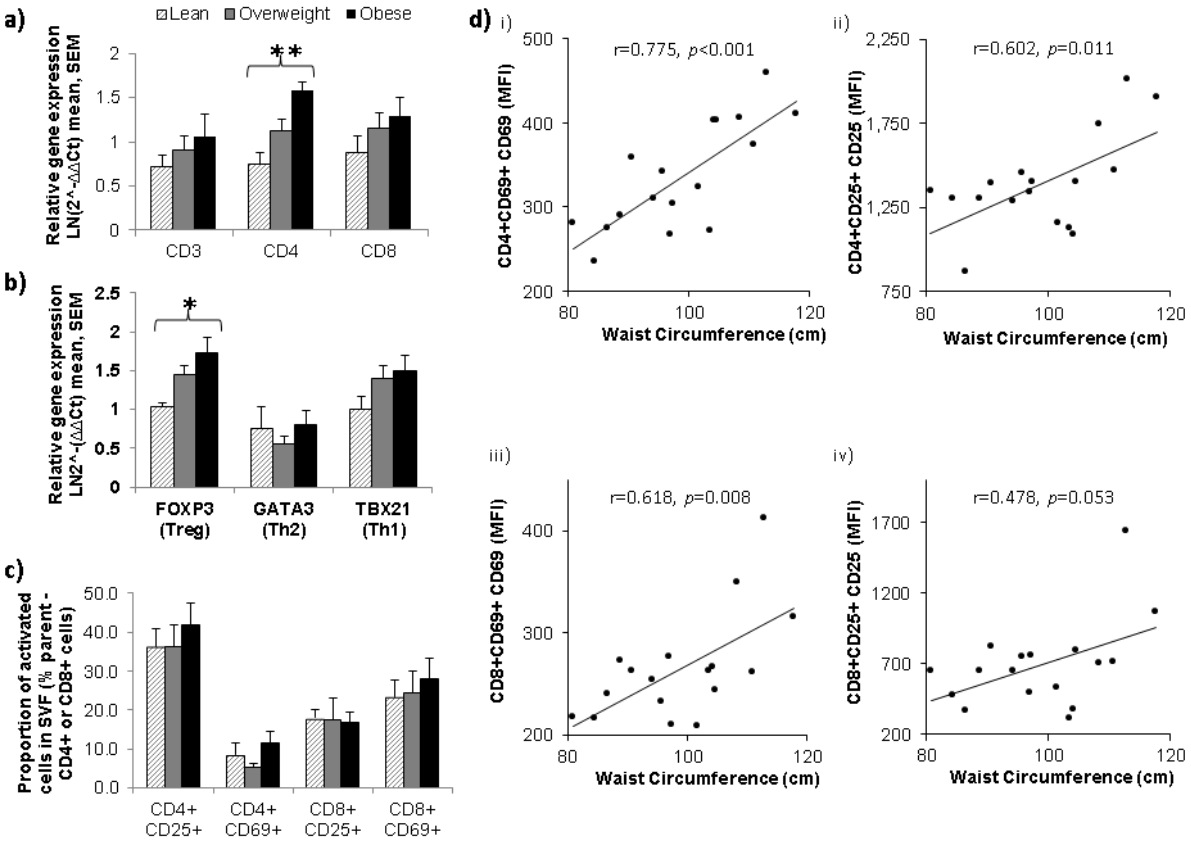


Figure 3.1. Lymphocyte phenotype and activation in adipose tissue according to levels of central adiposity. a) Relative gene expression of cluster differentiation markers to identify T-lymphocytes (n=30), b) CD4+ T-Lymphocyte subsets present within the adipose tissue (n=30), c) Proportions of activated T-lymphocytes in adipose tissue SVF as a percentage of CD4+ and CD8+ cells measured by flow cytometry (n=17). Gene expression data presented as mean $2^{-\Delta\Delta Ct}$ ± SEM. Effects of adiposity analysed by one-way ANOVA, *p<0.05, **p<0.001. MFI denotes mean fluorescence intensity. d i)-iv) Correlations between waist circumference and activation status of CD4+ and CD8+ T-lymphocytes in the adipose tissue (n=17). Pearson's (R) correlation coefficients shown along with significance values.

3.3.3 Macrophage numbers and activation in adipose tissue

Macrophages were identified using gene expression analysis of CD68 (n=30) which was significantly increased with greater levels of adiposity (Figure 3.2a). Using flow cytometry of the available samples (n=17), macrophages (CD45+CD14+) represented 2.9 to 15.5 % of total cells present in the SVF. The proportion of macrophages was positively related to central adiposity (Figure 3.2b). Flow cytometric analysis of CD163 expression on CD45+/CD14+ cells was used as a measure of macrophage 'alternative/anti-inflammatory' activation, but this marker was not affected by adiposity (Figure 3.2c).

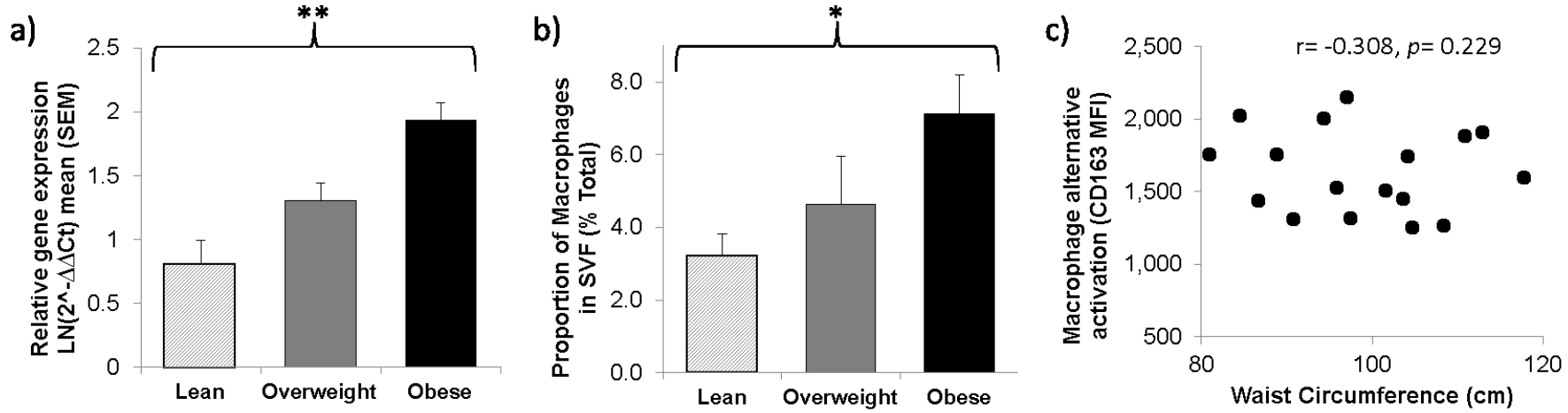


Figure 3.2. Macrophage numbers and activation in adipose tissue with varying levels of adiposity. a) Relative gene expression of CD68 used to identify macrophages (n=30), b) Proportions of macrophages in adipose tissue SVF as a percentage of the total cells (n=17). Data presented as mean $2^{-\Delta\Delta Ct}$ ± SEM. Effects of adiposity analysed by one-way ANOVA, *p<0.05, **p<0.001. c) Correlations between waist circumference and proportion of macrophages present in the adipose tissue stromavascular fraction (n=17) Pearson's (R) correlation coefficients shown, MFI denotes mean fluorescence intensity.

3.3.4 Metabolic and inflammatory properties of adipose tissue

To identify potential factors within the adipose tissue that may contribute to this increased T-lymphocyte activation and macrophage accumulation with increased adiposity, gene expression and protein secretion from whole adipose tissue was examined. With greater levels of adiposity, relative gene expression of the adiposity-related hormone leptin was increased as expected, with reduced expression of adiponectin, GLUT4 and HSL (Figure 3.3). The majority of adipose tissue inflammatory cytokines showed a trend towards increased expression with adiposity; however, this only reached statistical significance for the typically pro-inflammatory cytokine IL-18, anti-inflammatory IL-1Ra and MCP-1, a monocyte/macrophage chemo-attractant (Figure 3.3).

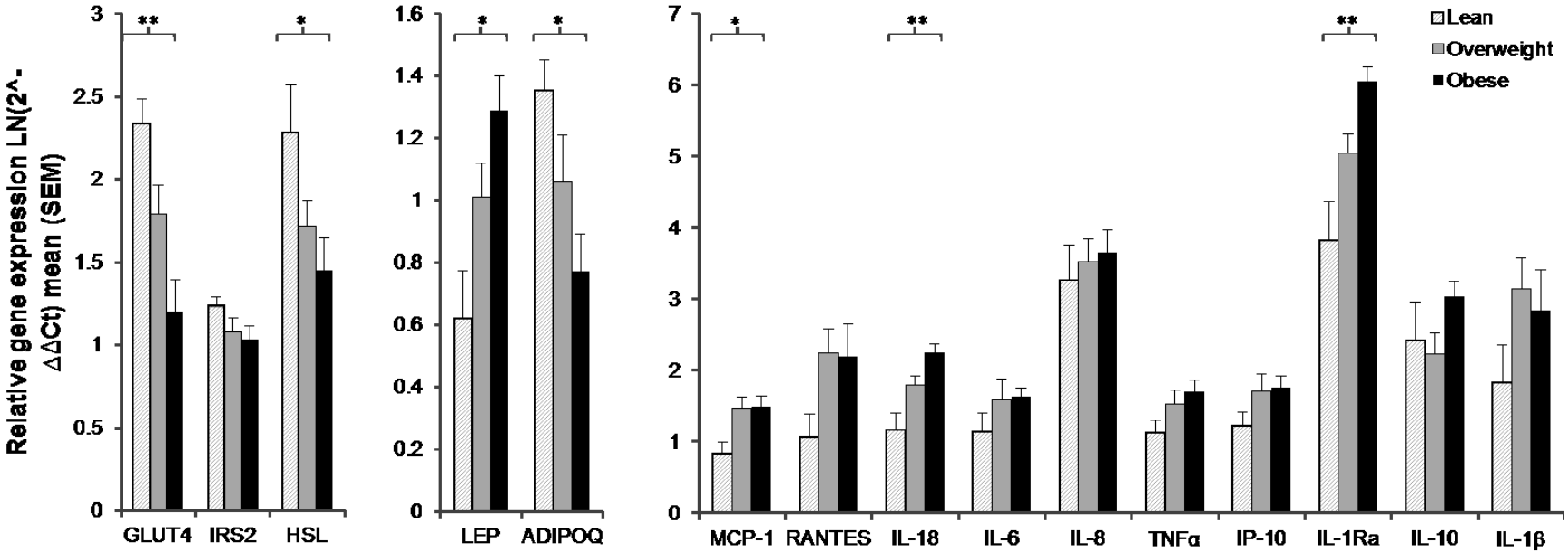


Figure 3.3. Relative gene expression of proteins related to metabolism, appetite/adiposity and inflammatory cytokines by whole adipose tissue samples with varying levels of adiposity. Data presented as mean $2^{-\Delta\Delta Ct}$ ± SEM with participants classified equally based on waist circumference (n=30). Effects of adiposity analysed by 1-way ANOVA, *p<0.05, **p<0.005. Note that IL-10 was expressed in 3 lean, 8 overweight and 7 obese individuals. IL-6, IL-8 and IL-1β were detected in all overweight and obese individuals but only 8, 6 and 9 lean individuals respectively.

Adipokine secretion from whole adipose tissue explants was determined per 100 mg cultured tissue (Figure 3.4a) and multiplied by L1-L4 fat mass to predict total central adipose tissue adipokine secretion (Figure 3.4b). Adjusted adipokine secretion accounts for the profound differences between individuals in absolute levels of adiposity and is thus more representative of secretion *in vivo*. Adjusted adipokine secretion increased with adiposity for the majority of measured adipokines (Figure 3.4b) and adjusted values better correlated with systemic concentrations (e.g. adipose tissue leptin secretion normalised to L1-L4 fat mass was more strongly correlated with serum leptin than unadjusted leptin secretion: r=0.9 vs. r=0.6 respectively (Figure 3.5).

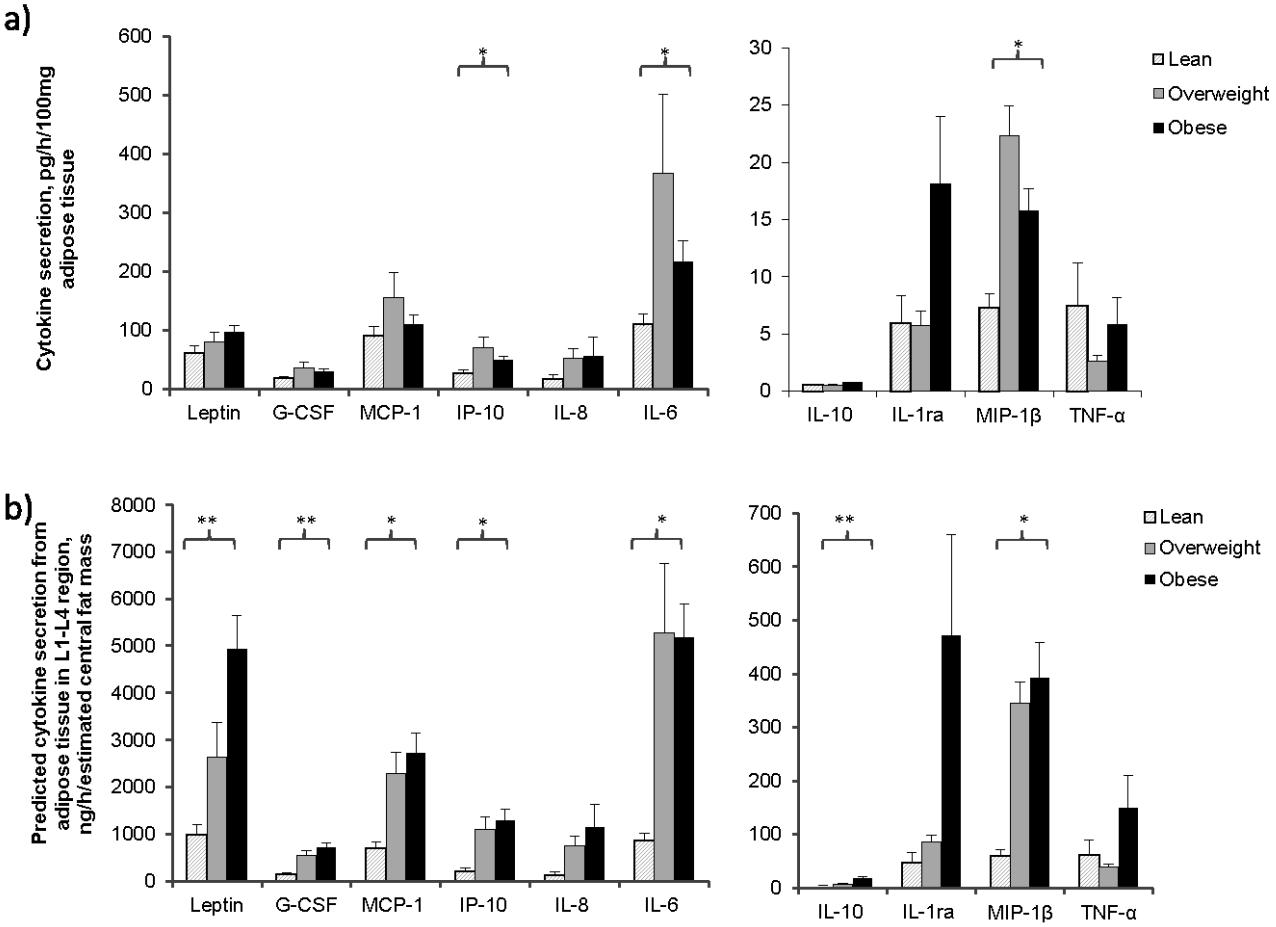


Figure 3.4. Cytokine secretion by whole adipose tissue explants cultured for 3h with varying levels of adiposity. a) Adipokine secretion normalised per 100 mg adipose tissue cultured, b) Adipokine secretion multiplied by L1-L4 fat mass to predict total central adipose tissue adipokine secretion. Mean and SEM values shown for groups based on waist circumference. Effects of adiposity analysed by 1-way ANOVA, *p<0.05, **p<0.005 (lean n =8, overweight n=6, obese n=10).

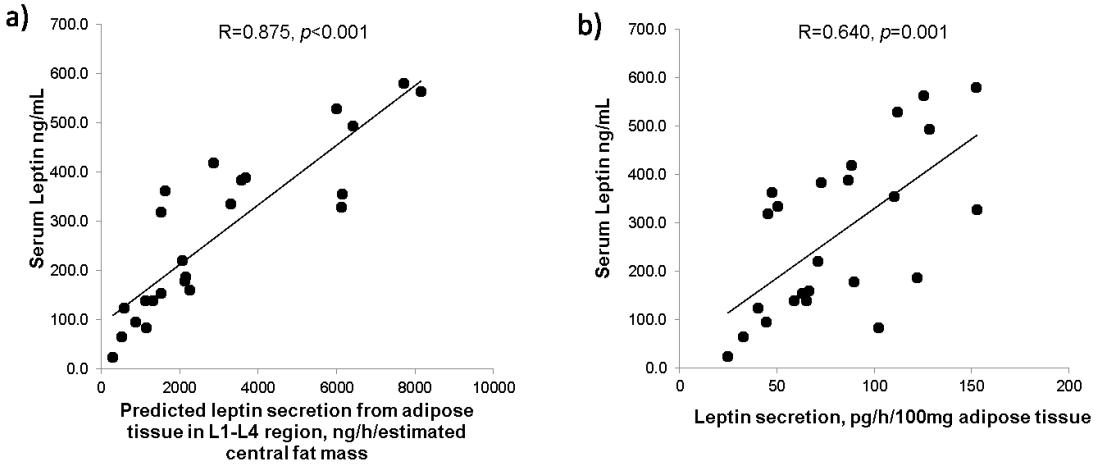


Figure 3.5. Improvement in correlation between serum leptin and adipose tissue when a) adjusted to central fat mass compared to b) secretion per 100mg adipose tissue. Pearson's (R) correlation coefficients and significance (p-values) shown, n = 24.

3.3.5 Relationships between adipose tissue resident immune cells, inflammatory cytokines and clinical systemic markers of metabolic health

A comprehensive unbiased approach was used to investigate relationships between immune cell properties and pro- and anti- inflammatory adipokines in adipose tissue. We also explored relationships between measures of metabolic health that are commonly used in clinical practice. A number of consistent significant positive correlations were observed between levels of T-lymphocyte activation and relative levels of gene expression and adipose secretion of IL-18, IL-10, IL-1Ra, leptin and MCP-1 and serum leptin (Table 3.2). Relationships between the percentage of macrophages in the SVF and adipokines were less consistent at the gene expression and secretion levels and, instead, appeared to be more closely related to leptin in serum and secretion from adipose tissue as well as a number of clinical blood markers of metabolic health (Table 3.2).

	Serum				mRNA							Whole AT protein secretion (3h) adjusted to central fat mass L1-L4 mass					
	Leptin (pg/mL)	HOMA-IR	Total:HDL Chol. Ratio	ALT (U/L)	Leptin	IL-6	TNFα	MCP-1	IL-10 (N=13)	IL-1Ra	IL-18	Leptin	IL-6	TNFα	MCP-1	IL-10	IL-1Ra
T-lymphocytes																	
CD4+CD69+ MFI	.557*	0.303	0.219	.273	.483*	.195	.127	.596*	.644*	.674**	.742**	.574*	.410	.403	.633**	.727**	.523*
CD4+CD25+ MFI	.522*	.409	0.375	.567*	.281	.086	.363	.729**	.576*	.697**	.744**	.645**	.370	.156	.674**	.596*	.288
CD8+CD69+ MFI	.472$	.291	0.264	.349	.313	.020	.067	.647**	.624*	.795**	.703**	.703**	.351	.036	.684**	.718**	.222
CD8+CD25+ MFI	.305	.200	0.238	.476$	.161	.121	.293	.757**	.587*	.836**	.609**	.447$	.233	.221	.536*	.450$	.360
Macrophages CD45+CD14+ %Total cells in SVF	.626**	.485*	0.539*	.621**	.449$	.024	.283	.383	.278	.519*	.667**	.769**	.346	.097	.479*	.671**	.205

Table 3.2. Associations between adipose tissue immune cell characteristics, blood markers of metabolic health and adipose tissue gene expression and secretion of pro- and anti- inflammatory adipokines. Data presented as Pearson's r value (n=17). MFI denotes mean fluorescent intensity. *p<0.05, **p<0.005, $ p=0.05-0.1.

3.3.6 Blood immune cell subsets

To investigate the specificity of these relationships to adipose tissue resident immune cells, paired blood samples were obtained and PBMCs isolated for characterisation according to cell subset and activation by flow cytometry. None of the correlations observed in the SVF regarding lymphocyte activation with waist circumference were found for paired PBMC samples, indicating that increased activation with increased adiposity is specific to adipose tissue (Figure 3.6). Furthermore, in contrast to findings for immune cells in SVF, there were no consistent relationships between PBMC activation and any blood markers of metabolic health.

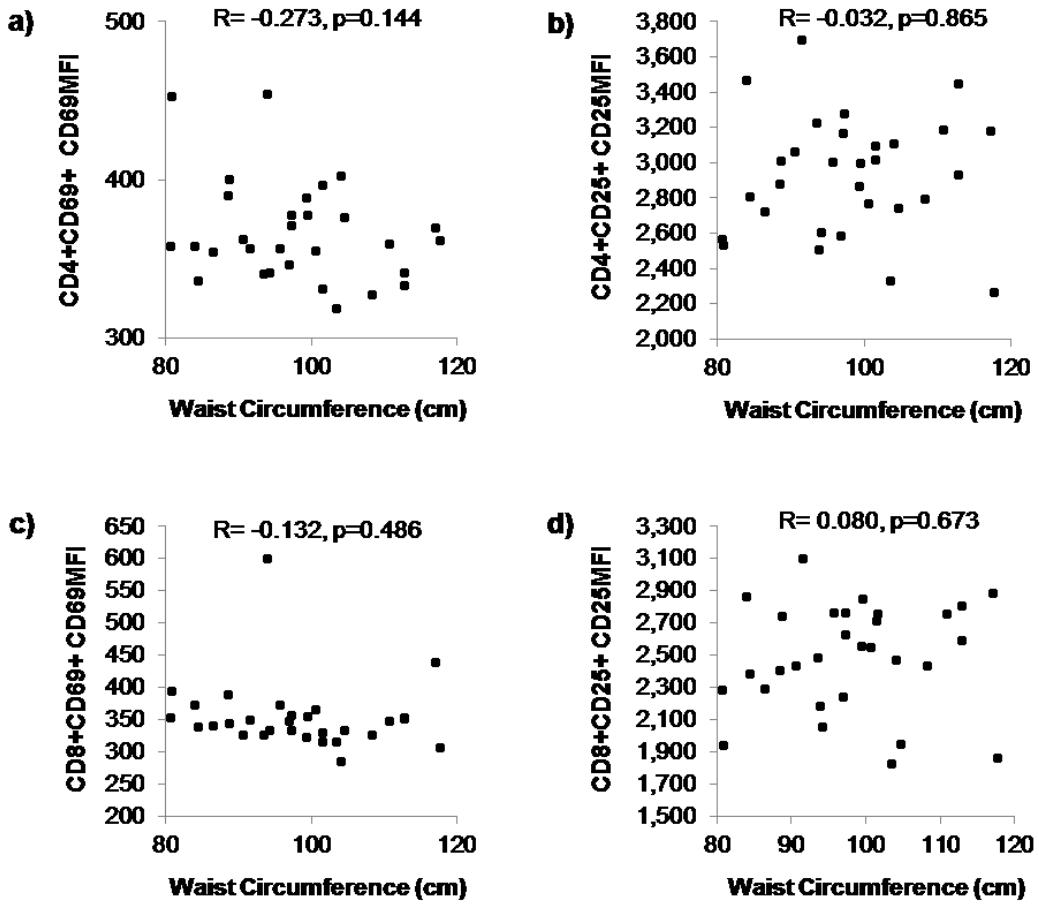


Figure 3.6. Correlations between waist circumference and activation status of blood CD4+ and CD8+ T-Lymphocyte populations a) – d). Pearson's (R) correlation coefficients and significance (p-values) shown, n=30. **MFI** denotes mean fluorescence intensity.

3.4 Discussion

This is the first study to demonstrate that in men with moderately increased adiposity typical of common overweight and obesity, there is increased activation of adipose tissue-resident T-lymphocyte populations. At the whole tissue level, an increase in FOXP3 gene expression with adiposity and reduced pro-inflammatory cytokine production per gram of fat in obese compared to overweight participants indicates the emergence of potential compensatory mechanisms, possibly through an increase in T-regulatory cells.

3.4.1 T-lymphocytes and their activation in human adipose tissue

Our results from both gene expression and flow cytometry document the presence of CD4+ and CD8+ T-lymphocyte populations together with the well described accumulation of macrophages in human subcutaneous adipose tissue SVF (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Curat *et al.*, 2004; Zeyda *et al.*, 2007; Bourlier *et al.*, 2008; Kintscher *et al.*, 2008; Duffaut *et al.*, 2009b; Feuerer *et al.*, 2009; Nishimura *et al.*, 2009; Zeyda *et al.*, 2011; Goossens *et al.*, 2012). Earlier rodent studies investigating the time course of immune cell infiltration with diet-induced obesity suggested an early increase in CD8+ T-lymphocytes may be important and linked to the development of systemic insulin resistance (Nishimura *et al.*, 2009). Our cross-sectional analysis of immune cells in human adipose tissue with varying levels of adiposity, however, showed no differences in levels of CD8+ T-lymphocytes by either gene expression or flow cytometry analysis. Instead our results showed an accumulation of CD4+ T-lymphocytes at the gene expression level. From the 30 participants, there were sufficient cells to perform further analysis by flow cytometry on 17 samples. A substantial fraction of both CD4+ and CD8+ T cells were in an activated state (CD25+ and/or CD69+) in the men recruited in the present study, as previously reported for women (Duffaut *et al.*, 2009b; Bertola *et al.*, 2012). Interestingly, although no differences were detected in the *proportion* of activated CD4 and CD8 T-lymphocytes within adipose tissue SVF, the level of CD25 and CD69 expression on activated T-lymphocytes showed a positive correlation with waist circumference. Importantly, the increased T-lymphocyte activation with overweight/obesity was not observed with lymphocytes isolated from paired blood samples, therefore indicating that

this finding is specific to adipose tissue and not a systemic or whole-body response.

In parallel, our gene expression data showed a gradual increase in FOXP3 transcripts with increasing adiposity. The limited number of cells in our SVF samples prevented intracellular staining for FOXP3 or cell sorting of CD25(hi) cells for subsequent *ex-vivo* suppression assay. Therefore, although the increase in FOXP3 transcripts cannot be formally linked to an increase in T-regulatory cells (Schmetterer *et al.*, 2012), this observation gives a strong indication of a possible increase in T-regulatory cells with increased levels of adiposity. The presence of T-regulatory cells in adipose tissue has been shown in other human studies (Duffaut *et al.*, 2009b; Feuerer *et al.*, 2009; Goossens *et al.*, 2012), and one other study reported increased T-regulatory cells in men and women with morbid class III obesity (Zeyda *et al.*, 2011). T-regulatory cells control both adaptive and innate immune responses via their suppressor activity and are therefore vital for immune function and homeostasis (Josefowicz *et al.*, 2012). Human obesity tends to occur over much longer periods of time compared to mouse models where diet-induced obesity develops over just a few weeks (Kintscher *et al.*, 2008). The more gradual adipose tissue expansion in humans may allow for compensatory mechanisms such as the accumulation of T-regulatory cells as an attempt to limit local inflammation. Indeed, in mouse models, a loss of T-regulatory cells in adipose tissue accompanies the development of insulin resistance, (Feuerer *et al.*, 2009; Nishimura *et al.*, 2009) with gain-of-function experiments improving insulin sensitivity, confirming the potential importance of T-regulatory cells in obesity-related insulin resistance (Feuerer *et al.*, 2009).

3.4.2 Adipose tissue genotype/phenotype and resident T-lymphocyte activation

The presence of relationships between leptin, IL-18, IL-10, IL-1Ra and MCP-1 with levels of CD8+ and CD4+ T-lymphocyte activation and proportion of macrophages suggests there may be complex interactions between the adaptive and innate immune system within adipose tissue. Our results suggest that leptin may be important in adipose tissue T-lymphocyte activation and indeed, *in-vitro* work has shown that leptin enhances T-lymphocyte homeostasis/function (Lord *et*

al., 1998) with dose-dependent increases in CD4+ and CD8+ expression of CD25 and CD69 (Martin-Romero *et al.*, 2000; Sanchez-Margalet *et al.*, 2003). Relationships between CD4+ activation and IL-18 and IL-10 adipose gene expression/secretion in the present study are also of particular interest. IL-18 is a pro-inflammatory cytokine produced by the NLRP3 inflammasome in response to 'danger signals' and is implicated in the differentiation of CD4+ T-lymphocytes into Th1 cells (Vandanmagsar *et al.*, 2011; Witztum & Lichtman, 2014). IL-10 conversely is typically anti-inflammatory and indicative of Th2/T-regulatory lymphocyte differentiation (Witztum & Lichtman, 2014). This supports the hypothesis that, in addition to pro-inflammatory changes in adipose tissue, there are potential protective compensatory responses involving T-lymphocytes (Zeyda *et al.*, 2011; Goossens *et al.*, 2012). The absence of relationships between IL-6 or TNFα with T-lymphocyte activation and macrophages may provide further evidence to support this contention. In mouse models of obesity, macrophages switch from an anti-inflammatory M2 phenotype (producing IL-10) to a pro-inflammatory M1 phenotype which overexpress inflammatory cytokines including IL-6 and TNFα (Lumeng *et al.*, 2007) and are likely to be a major contributing source of these cytokines in murine adipose tissue. Human data regarding macrophages suggests that with increased levels of adiposity, macrophage numbers are increased and exhibit an anti-inflammatory phenotype associated with tissue re-modelling (Zeyda *et al.*, 2007; Bourlier *et al.*, 2008; Fjeldborg *et al.*, 2014). In this context, the absence of relationships between T-lymphocyte activation and macrophage accumulation may not be so surprising, particularly given our focus on modest overweight and obesity. Together, these data suggest that the development of a pro-inflammatory phenotype as seen in obesity is associated with adaptive responses and an attempt to develop a protective, more anti-inflammatory profile which is presumably lost or overcome with either further increases in obesity or a further deterioration of metabolic health.

3.4.3 Adipose tissue compensation with increased adiposity

Adipose tissue is dynamic and undergoes adaptations in times of both calorie restriction and chronic over-nutrition. During chronic energy surplus, adipose tissue expands and regulates expression of proteins related to fatty acid trafficking at the cellular level to prevent increases in fasting blood free fatty acids (McQuaid

et al., 2011). Our observed down-regulation of adipose tissue HSL gene expression with increasing adiposity supports this suggestion. This ability of adipose tissue to adapt with its expansion may also extend to regulating secretion of a number of adipokines measured in this study. At the per unit fat mass level, secretion of some adipokines including IL-6, IP-10 and MIP-1b was actually reduced in obese individuals (relative to their overweight counterparts). Thus, in the context of modest overweight and obesity, adipose tissue appears to adapt per unit of tissue in an attempt to regulate overall adipokine output to the circulation, although whether this is in any way linked to the down-regulation of free fatty acid delivery is far from certain.

3.4.4 T-lymphocytes and systemic markers of metabolic health

In contrast to our findings for adipose tissue resident macrophages, there were no relationships between T-lymphocyte subsets and their activation with systemic measures of health that are routinely used in clinical practice (e.g., HOMA-IR). This supports findings from another study where no correlations were found between T-lymphocyte subsets in subcutaneous adipose tissue and HOMA-IR (Zeyda *et al.*, 2011). In the present study, the percentage of macrophages in adipose tissue was significantly related to HOMA-IR, Total:HDL-cholesterol ratio and ALT. This could indicate that macrophages have a more direct role than lymphocytes in obesity-mediated changes in systemic inflammation/insulin resistance. However, there may be temporal considerations to these comparisons which make such conclusions difficult. Macrophages may reside in tissues much longer than T-lymphocytes and therefore have time to influence/become influenced by changes in local and systemic metabolism and inflammation (Galli *et al.*, 2011). In contrast, effector T-lymphocytes can have relatively shorter life-spans and some lymphocyte subsets (e.g., memory T-lymphocytes) transit through tissue prior to their recirculation (Mora & von Andrian, 2006). The dynamic nature of lymphocytes in adipose tissue may make it difficult to draw conclusions about their role based on a snapshot but, may also indicate that these fast-changing cell populations represent an exciting opportunity for intervention.

These relationships have been demonstrated across lean to modestly obese middle-aged men, but whether these relationships hold true with further increases

in adiposity, in women or men of a different age and in people with metabolic complications such as insulin resistance warrants further investigation.

3.4.5 Conclusions

Following the discovery of immune cells in adipose tissue, macrophages have taken centre stage whereas other cells such as lymphocytes have been somewhat overlooked. The present study demonstrates for the first time that modest adipose tissue expansion is characterised, not by an increase in the proportion of activated T-lymphocytes but rather by a stronger state of activation in the T-lymphocytes already expressing CD69 and/or CD25. Importantly, this increased activation was not observed in circulating blood T-lymphocytes. In addition to positive relationships with pro-inflammatory cytokine production, we show that T-lymphocyte activation is also positively related to anti-inflammatory cytokine production at both the gene expression and secretion level – providing further evidence of attempts by adipose tissue and resident T-lymphocytes to limit pro-inflammatory output from adipose tissue at least with modestly increased levels of overweight/obesity. From our results, one of the possible mechanisms that could drive this anti-inflammatory compensation is an increased presence of T-regulatory cells. T-lymphocytes are therefore likely to play a key role in the regulation of adipose tissue inflammation and important adaptations seen even with modestly increased levels of adiposity.

CHAPTER 4

Metabolic and inflammatory responses to a mixed meal in lean, overweight and obese men

4.1 Introduction

Obesity is associated with adipose tissue expansion, low-grade chronic inflammation and deteriorating metabolic health, but the underlying pathogenesis linking these factors is not well understood (Esposito & Giugliano, 2004; Lumeng & Saltiel, 2011). Adipose tissue is a highly dynamic organ responsible for regulating the storage of surplus energy as it secretes and responds to various adipokines (Balistreri *et al.*, 2010). With its expansion, a more pro-inflammatory gene expression profile develops within adipose tissue (Shah *et al.*, 2008), along with increased secretion of inflammatory cytokines (Fain, 2010), which contribute to the low-grade inflammation and complications of obesity including systemic insulin resistance. These inflammatory changes come not only from the adipocytes but also from the repertoire of cells comprising the stromavascular fraction present within the adipose tissue including macrophages and T-lymphocytes (Fain, 2010).

Measures of metabolic health and inflammation in blood and adipose tissue across varying levels of adiposity are often investigated in the fasted state. However, the majority of the waking day is often spent in a fed (i.e., postprandial) state and vital information regarding the progression of obesity-related diseases may be gained from investigations conducted following the consumption of a meal. Obese individuals typically exhibit exaggerated postprandial responses in blood, with peak glucose, insulin and triglyceride concentrations that are higher and remain elevated for longer when compared to lean individuals (Manning *et al.*, 2008a; Jonk *et al.*, 2011). There is, however, much conflicting evidence regarding whether inflammatory mediators are acutely increased in the circulation following consumption of a meal (Nappo *et al.*, 2002; Poppitt *et al.*, 2008; Dixon *et al.*, 2009). It is possible that, even in the absence of changes in inflammatory cytokines at a systemic level, changes may occur at the level of adipose tissue and it is not known whether metabolic differences seen with increasing levels of

adiposity will reflect/dictate processes within adipose tissue. It is also possible that inflammatory responses in adipose tissue after the consumption of each meal could contribute to systemic inflammation.

The only studies to date investigating the presence of postprandial inflammation within adipose tissue in humans have either compared postprandial responses to differing qualities/quantities of lipids in people with metabolic syndrome (Meneses *et al.*, 2011) or compared responses in (non-obese) relatives of people with type 2 diabetes versus controls (Pietraszek *et al.*, 2011). In both studies, significant increases in adipose tissue gene expression of a number of inflammatory genes including MCP-1, TNFα, IL-1β and IL-6 were detected, suggesting that there may indeed be postprandial inflammatory responses in adipose tissue (Meneses *et al.*, 2011; Pietraszek *et al.*, 2011). Interestingly, no such work has been undertaken to compare such postprandial responses within adipose tissue between lean and obese individuals. Thus, it is unclear whether these changes in inflammatory markers in adipose tissue represent a 'normal' phenomenon or something that is influenced by increased levels of adiposity in line with other more systemic changes (e.g., postprandial insulin concentrations). This is of particular importance to understanding the pathology of obesity-related inflammation and chronic disease.

The main aim of this study was to investigate whether the greater postprandial metabolic response (i.e. hyperinsulinaemia) observed with increased adiposity leads to an increased inflammatory response within adipose tissue. In this study, postprandial metabolic and inflammatory responses were compared in the blood and adipose tissue following consumption of a high fat, high carbohydrate meal amongst carefully-characterised men covering a range of adiposities from lean through to Class I levels of obesity.

4.2 Materials and Methods

4.2.1 Experimental design

This study is based on the same participants that were recruited and described in Chapter 3. Thirty men aged between 35 and 55 years were recruited by local advertisement and visited the laboratory for preliminary anthropometric measurements including waist circumference, which was used to classify participants as lean <94 cm, overweight >94 cm but <102 cm, and obese >102 cm (WHO, 2008). Recruitment continued until there was an equal distribution of 10 participants in each waist circumference category. Participants were characterised according to body composition, diet and physical activity since each of these factors can independently affect metabolic health. Participants attended one main trial in which blood and adipose tissue samples were obtained before and 6 hours after consumption of a mixed meal. Blood samples were taken at regular intervals throughout. The protocol was reviewed and given approval by the South West, Southmead NHS Research Ethics Committee (REC Reference: 11/SW/0193) and all participants gave their written informed consent.

4.2.2 Participants and sample size determination

Participants were grouped according to waist circumference and individuals were excluded from participation if they had a history of/existing diabetes, cardiovascular disease or dyslipidaemia, were taking any medications known to interfere with immune function or lipid/carbohydrate metabolism, if they smoked, or had not been weight stable for >3 months (i.e. weight change >3 %; Stevens, 2006). In addition, participants were excluded if they reported food intolerances/allergies to any component of the meal (e.g. dairy or wheat) and habitually performed more than 6 hours of vigorous-intensity physical activity or 10 hours of moderate-intensity physical activity per week, assessed via a self-report questionnaire (as these individuals would not fit the meal standardisation procedure – see below for details).

The sample size for this study was determined using a clinically relevant difference in serum insulin response to a meal between lean (AUC 93 μU/mL (+/- 29 SD)) and obese (AUC 169μU/mL (+/- 51 SD)) individuals with an effect size of 1.8 (G*Power 3.1.5, Germany) (Hudgins *et al.*, 2000). Thus, with 95 % power

and 5 % alpha, 18 participants (9 lean and 9 obese), would be required to detect a statistically significant difference in serum insulin AUC between groups. An intermediate (overweight) group was also included for comparison and to account for participant re-distribution/allocation between groups based on other parameters (e.g., other measures of body composition which may provide a different picture to waist circumference alone), and to allow for more powerful correlations of adiposity against study outcome measures to be made. Participant characteristics within the categories lean, overweight and obese based on measurements of waist circumference are shown in Table 4.1.

Classification based on waist circumference	**Lean n = 10**	**Overweight n = 10**	**Obese n = 10**	**One-way ANOVA *p***
Age (y)	44 ± 2	48 ± 2	45 ± 2	0.218
Height (m)	1.78 ± 0.02	1.77 ± 0.02	1.81 ± 0.03	0.469
Body mass (kg)	74.8 ± 1.6	83.7 ± 1.8	100.2 ± 3.3	<0.001
Body Mass Index (kg/m^2)	23.6 ± 0.6	26.7 ± 0.4	30.7 ± 0.9	<0.001
Waist Circumference (cm)	87.0 ± 1.4	97.7 ± 0.8	109.4 ± 1.8	<0.001
Fat Mass Index (kg/m^2)	4.5 ± 0.3	6.9 ± 0.2	9.5 ± 0.6	<0.001
L1-L4 fat (%)	19 ± 1	30 ± 1	37 ± 2	<0.001
Resting Metabolic Rate (kcal/day)	1644 ± 65	1722 ± 55	1882 ± 74	0.045
PAL[a]	1.94 ± 0.10	1.66 ± 0.06	1.67 ± 0.10	0.066

Table 4.1. Descriptive statistics of participants classified according to waist circumference. Mean ± SEM values shown and statistical differences between the 3 groups were assessed by one-way ANOVA, *p*-values shown. Abbreviations used; L1-L4 = central fat estimated between Lumbar regions 1-4 using DEXA, PAL = Physical activity level which is the product of Total Energy Expenditure ÷ Basal Metabolic Rate ([a] lean n=9).

4.2.3 Preliminary tests

Participant waist circumference, body mass and height were measured as described in General Methods, 2.2. Participants were fitted with a combined heart rate and accelerometry monitor (Actiheart) for a period of 9 consecutive days to determine habitual physical activity level (PAL; total energy expenditure/basal metabolic rate; General Methods, 2.5). Participants were asked to maintain their

normal lifestyle habits/routines during this period with the first 2 days of activity monitoring being excluded from analysis to account for potential reactivity (Loney *et al.*, 2011). Participants received a diary and set of digital weighing scales to record their food and fluid intake during the 3 days prior to the trial day with nutritional analysis performed using COMP-EAT (General Methods, 2.7).

4.2.4 Pre-trial requirements

Participants were asked to refrain from performing any strenuous physical activity for 48 hours and consuming alcohol/caffeine for 24 hours prior to testing. Trial days were scheduled so participants had been free from any self-reported illness for a minimum of 2 weeks in order to reduce immune system disturbance. Participants arrived in the laboratory in the morning after an overnight fast (minimum 10 h) and after consuming 1 pint of water upon waking.

4.2.5 Body composition analysis

Body mass (post-void on the morning of the trial) was determined with participants wearing lightweight shorts. Body composition analysis was performed using DEXA with central adipose tissue (L1-L4) and fat mass index estimated as described in General Methods, 2.4.

4.2.6 Resting metabolic rate

Participants rested supine in bed for 10 minutes before estimation of resting metabolic rate (RMR) using the protocol described in General Methods, 2.6. This value was used to adjust estimates of total energy expenditure and PAL, and to calculate the energy requirements for the test meal given to participants.

4.2.7 Meal composition and energy requirements

Participants were given a breakfast meal relative to their resting metabolic rate comprising brioche, strawberry jam (both Sainsbury's, UK) margarine (Stork, Unilever), a milkshake (fresh chocolate milk and whipping cream; both Sainsbury's, UK, with added icing sugar; Silver Spoon) and a cup of decaffeinated tea (PG tips, Unilever) with semi skimmed milk (Sainsbury's, UK). The total energy content of the meal represented approximately 65 % RMR and comprised 39 % calories from carbohydrate, 54 % calories from fats and 7 %

calories from protein. The exact composition of the meal was designed to ensure that an average 80 kg man with an RMR of 1791 kcal/day (estimated from Schofield equation (Schofield, 1985)) received 1.5 g/kg carbohydrate (Betts *et al.*, 2011). A worked example showing exact proportions of each meal item is available in Appendix 2. During the trial, participants were free to consume water *ad-libitum* but consumed no further food until the end of the trial.

The standardised mixed meal with a high carbohydrate and fat content was intended to produce a more 'physiological' response compared to glucose or fat only challenges (Selimoglu *et al.*, 2009). The meal was given relative to each individual's resting metabolic rate to standardise energy intake for inter-individual differences in body weight/composition and thus resting energy requirements. In the general population, RMR is a good reflection of daily energy requirements, however, this would not hold true and would be an underestimate for people who perform very large amounts of physical activity (hence why people who engaged in greater than 6 hours of vigorous or greater than 10 hours moderate-intensity physical activity per week were excluded).

4.2.8 Blood and adipose sampling before and after meal consumption

A venous blood sample was taken from an antecubital forearm vein and dispensed into separate tubes containing either K_3EDTA or serum separation beads (Sarstedt Ltd., Leicester, UK) for analysis of plasma and serum measures of metabolism and inflammation (General Methods, 2.9 – 2.10). Subcutaneous adipose tissue samples (~1 g) were obtained under local anaesthetic (1 % lidocaine) approximately 5 cm lateral to the umbilicus using a 'needle aspiration' technique and processed as described in General Methods, 2.13.

After baseline blood and adipose tissue sampling, the meal was consumed within 15 minutes and blood samples taken from the cannula at 15, 30, 60, and 90 minutes and then at every hour until 6 hours after consumption of the meal. A second adipose sample was also taken at 6 hours on the opposite side of the umbilicus to the sample collected at baseline. At both sampling time points, approximately 200 mg adipose tissue was processed for gene expression analysis (General Methods, 2.19) and small portions minced (~5-10 mg explants) for

analysis of adipokine secretion (General Methods, 2.14). Additional blood samples were obtained at 2 and 6 hours post meal by venepuncture from the contralateral arm for analysis of inflammatory markers as there is evidence that the cannula can stimulate local inflammation (Dixon *et al.*, 2009).

4.2.9 RT-PCR

After extraction and quantification, 2 μg total RNA was reverse transcribed to cDNA and real-time PCR performed (General Methods, 2.19) to measure expression of GLUT4, IRS2, HSL, leptin, adiponectin, MCP-1, RANTES, IP-10, IL-6, IL-8, IL-10, IL1Ra, TNFα, IL-1β and IL-18. Data for adipose tissue expression of GCSF, MIP-1b and IFNɣ are not shown because they were only detectable in 4-8 individuals. Peptidylpropyl isomerase A (PPIA/Cyclophilin A) was used as an endogenous control (Neville *et al.*, 2011). Results were analysed using the comparative Ct method and expression normalised to an internal calibrator specific to each gene using the formula $2^{-\Delta\Delta C_T}$ (where $\Delta\Delta C_T$ is [(C_T gene of interest – C_T PPIA) – lowest ΔC_T for gene of interest] and statistical analysis performed on LN transformed values (Livak & Schmittgen, 2001). Details of specific primers used can be found in General Methods, 2.19.

4.2.10 Biochemical analysis

Plasma glucose and serum insulin, adiponectin, leptin, total cholesterol, HDL cholesterol and triglyceride concentrations and ALT activity were measured using techniques described in General Methods, 2.10. Plasma CRP, SAA, sVCAM-1, and sICAM-1 were measured as a 4-plex and IFNɣ, TNFα, IL-10, IL-12p70, IL-1β, IL-8 and IL-6 were measured as a 7-plex using an 'electrochemiluminescence detection and patterned array' technology (MULTI-ARRAY®, Meso Scale Discovery). This analysis was kindly performed by TNO Triskellon as part of the ADMIT study run in collaboration with Unilever Food and Health Research Institute, NL. Adipose tissue secretion of Leptin was measured by ELISA (R&D Systems) and GCSF, MCP-1, IP-10, IL-8, IL-6, IL-10, IL-1Ra, MIP-1b, TNFα, IL-1β were measured using Luminex (BIO-RAD).

4.2.11 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Total area under the curve (AUC) for insulin, glucose, triglycerides and NEFA and homeostasis model assessment for insulin resistance (HOMA-IR) were calculated using the formulae described in General Methods, 2.12. Comparisons were made between the lean, overweight and obese groups at baseline and for measures of AUC using one-way ANOVA. Adipose tissue responses to the meal, with only 2 time-points available, were compared between the 3 groups using 2-way mixed-model ANOVAs irrespective of normality (Maxwell and Delaney, 1990, p. 109). For blood measures of metabolism and inflammation where more than 2 time-points were available, mixed-model ANOVA was also used to compare responses between the 3 groups. The Greenhouse–Geisser correction was applied to intra-individual contrasts where $\varepsilon < 0.75$, however, for less severe asphericity the Huynh–Feldt correction was selected (Atkinson, 2002). Where mixed model ANOVA identified significant effects (and more than 2 timepoints available), multiple *t*-tests were applied to identify which specific time points were different from baseline and to identify the location of any variance between groups at level time points, with *p*-values subject to a Holm–Bonferroni correction (Atkinson, 2002). Statistical analysis was performed using SPSS version 20 and $p \leq 0.05$ was considered to be statistically significant. The symbols used to identify significant differences are as follows; * denotes a main effect of time, † denotes a main effect of adiposity and # denotes any adiposity x time interaction effects.

4.3 Results

4.3.1 Fasting and postprandial blood glucose and insulin

As reported in Chapter 3, measures of glucose control increased in line with adiposity as confirmed by fasting levels of glucose (lean 4.4 ± 0.2 mmol/L, overweight 4.8 ± 0.3 mmol/L and obese 5.3 ± 0.2 mmol/L, one-way ANOVA p=0.043) and insulin (lean 27.3 ± 4.8 pmol/L, overweight 39.6 ± 7.0 pmol/L and obese 59.5 ± 10.1 pmol/L, one-way ANOVA p=0.020). In addition, main effects of group revealed that postprandial responses for both glucose (p=0.030, F=3.998) and insulin (p=0.056, F=3.220) were affected by adiposity (Figures 4.1a and c respectively). Post-hoc t-tests (of mean responses of participants within each group) revealed that glucose (p=0.022) and insulin (p=0.038) responses were significantly higher in the obese group compared to lean. Total areas under the curve for glucose and insulin also increased in line with adiposity (Figures 4.1b and d respectively).

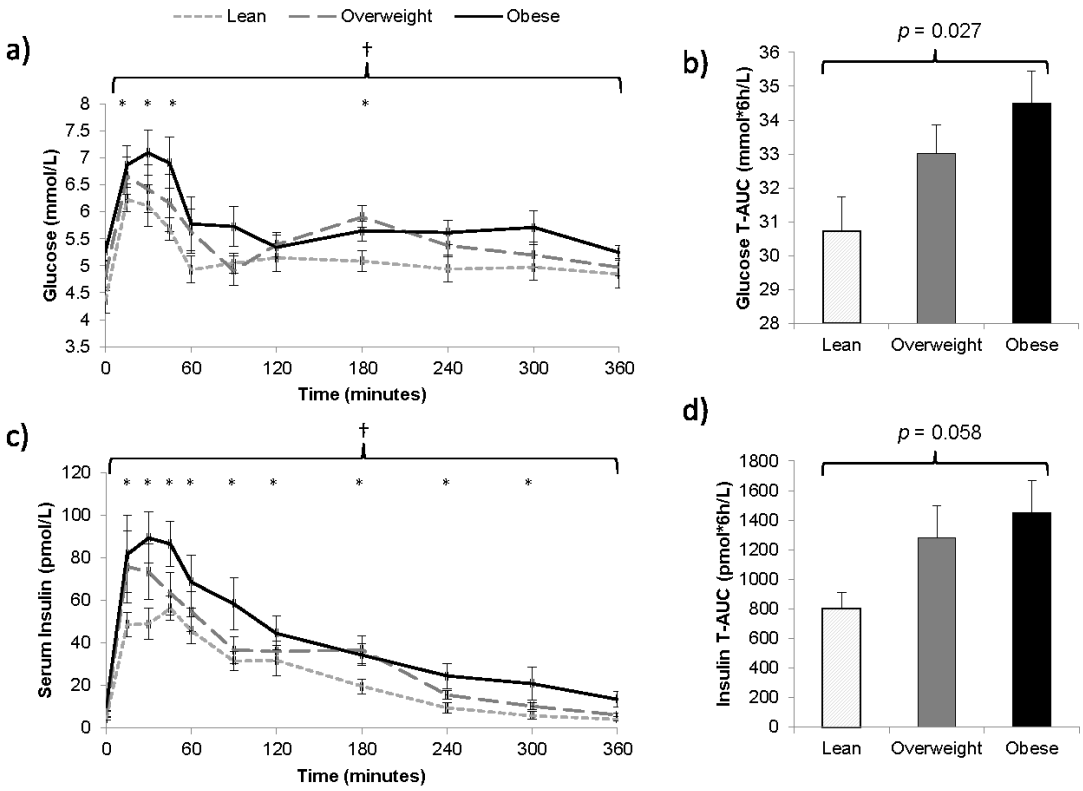


Figure 4.1. Blood glucose and insulin responses over the 6 hours following consumption of the meal for lean, overweight and obese individuals classified based on waist circumference. Measures of glucose following the meal according to a) mean group responses over time, and b) total AUC for glucose. Corresponding measures of c) mean insulin by group, and d) total AUC for insulin. Mixed-model ANOVAs were performed with post-hoc *t*-tests applied to identify which specific time points were different from baseline; * denotes effect of time ($p<0.05$) compared to t=0; † denotes main effect of adiposity ($p<0.05$), with no interaction effects identified (n=30). Total-AUC compared using one-way ANOVA and *p*-values shown.

4.3.2 Changes in adipose tissue gene expression at 6 hours following the meal

Levels of baseline gene expression in subcutaneous adipose tissue according to levels of adiposity have been presented in Chapter 3. Mixed-model ANOVA also identified main effects of adiposity for the gene expression of GLUT4 (F=9.9, p=0.001), IRS2 (F=5.2, p=0.012) and HSL (F=4.8, p=0.016), whereby each was reduced with increased levels of adiposity (Figure 4.2). At 6 hours after the meal only IRS2 gene expression was down-regulated relative to baseline in all groups (F=23.9, p<0.001; Figure 4.2b). No significant adiposity x time interactions were identified for the gene expression of GLUT4, IRS2 or HSL in subcutaneous adipose tissue.

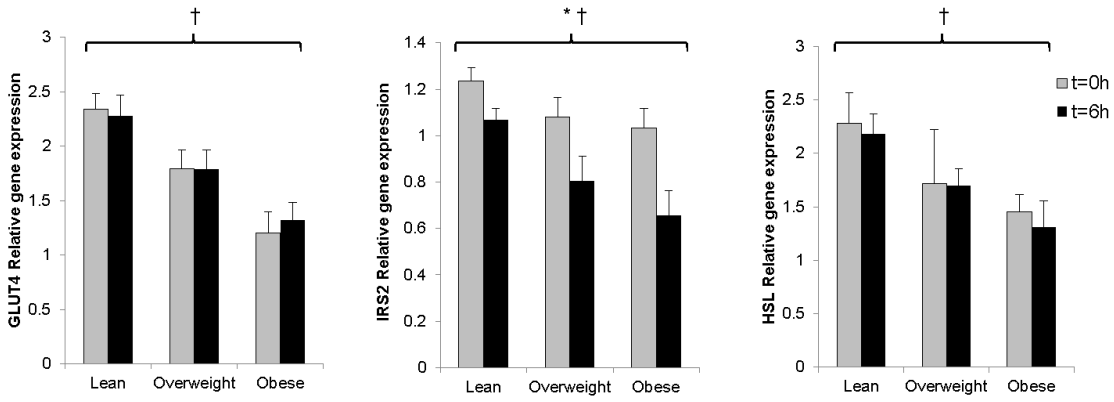


Figure 4.2. Relative expression of GLUT4, IRS2 and HSL by whole adipose tissue samples pre (0h) and post (6h) meal consumption. Data presented as LN $2^{-\Delta\Delta Ct}$ mean ± SEM with participants classified equally based on waist circumference (n=30). Mean and SEM values shown for groups based on waist circumference. Groups compared by mixed-model ANOVA; †denotes main effect of adiposity, *denotes significant main effect of time (p<0.05) across the 3 groups.

Gene expression responses for a number of adipokines related to inflammation following the meal were also examined and are shown in Figure 4.3 (again, results at baseline are presented in Chapter 3). With increased adiposity, gene expression in subcutaneous adipose tissue was elevated for pro-inflammatory IL-18 ($F=8.3$, $p=0.002$), anti-inflammatory IL-1Ra ($F=6.1$, $p=0.006$), monocyte chemo-attractant MCP-1 ($F=8.8$, $p=0.001$), and IP-10 ($F=4.1$, $p=0.029$). Significant time effects reflected an increase in gene expression of both IL-6 ($F=13.8$, $p=0.001$) and MCP-1 ($F=8.3$, $p=0.008$) for all groups as shown in Figure 4.3, however, no adiposity x time interactions were apparent for any gene.

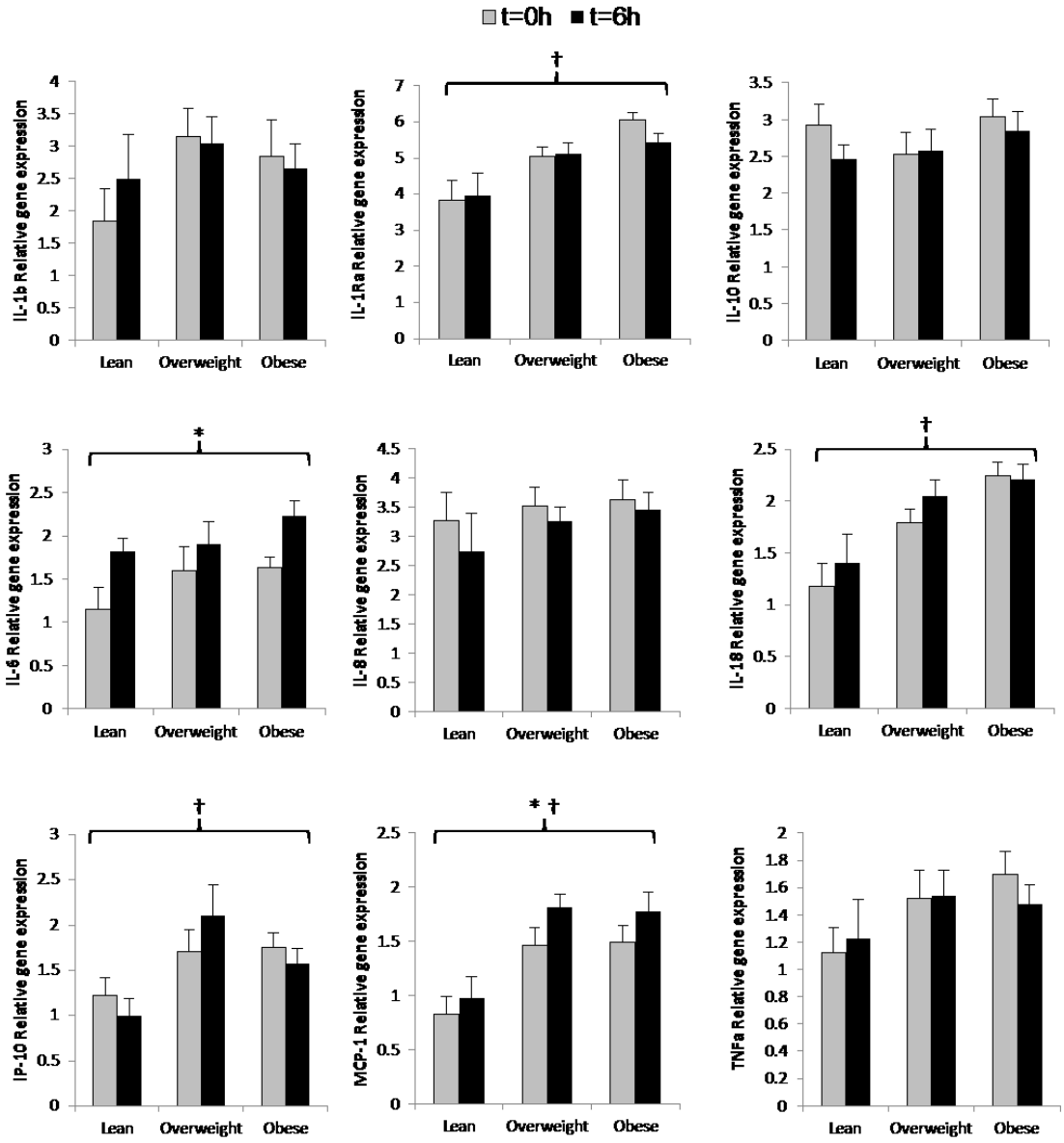


Figure 4.3. Relative gene expression of pro- and anti- inflammatory cytokines by whole adipose tissue samples pre (0h) and post (6h) meal consumption. Data presented as LN $2^{-\Delta\Delta Ct}$ mean ± SEM with participants classified as lean, overweight or obese based on waist circumference (n=30). Mean and SEM values shown for groups based on waist circumference. Groups compared by mixed-model ANOVA; † denotes main effect of adiposity, $p<0.05$, * denotes main effect of time, $p<0.05$. (Note; IL-1β n=29, IL-10 n=14, IL-6 n=28, IL-8 n=24).

4.3.3 Adipose tissue secretion before and after the meal

Adipokine secretion from subcutaneous adipose tissue explants was adjusted to central fat mass (L1-L4 from DEXA) as described in Chapter 3. The overall main effects of group identified by mixed-model ANOVA showed that secretion of leptin, MCP-1, IP-10, MIP-1b, IL-10, IL-6 and GCSF is elevated with increased levels of adiposity. These results are in line with the results presented at baseline in Chapter 3 indicating that these differences were maintained over time and from a different sample site in the abdominal subcutaneous adipose tissue. Despite changes in gene expression of IL-6 and MCP-1 at 6 hours following the meal, no such responses were found at the level of adipokine secretion from whole adipose tissue explants as shown in Table 4.2. The only significant adiposity x time interaction (after post-hoc *t*-tests and Holm-Bonferroni corrections) was found for IL-10, whereby levels of IL-10 secretion from the obese group was significantly reduced to a level that was similar to the overweight group at 6 hours ($p<0.05$). Due to the limited size of some adipose tissue samples obtained, there was only sufficient to culture tissue for 6 lean and 5 overweight individuals. Thus, these comparisons will be compromised to some extent.

		T=0h	T=6h	Main effect of adiposity	Main effect of time	adiposity*time interaction
		ng/3h/estimated central fat mass	**ng/3h/estimated central fat mass**	(*p*-value)	(*p*-value)	(*p*-value)
Leptin	Lean	2946 ± 713	2887 ± 371			
	Overweight	8217 ± 2658	9348 ± 3108	**0.004**	0.560	0.153
	Obese	14799 ± 2106	12373 ± 2472			
IL-6	Lean	2672 ± 543	6517 ± 1977			
	Overweight	16776 ± 5295	14035 ± 3222	**0.002**	0.764	0.345
	Obese	15573 ± 2091	15982 ± 1591			
MCP1	Lean	1985 ± 380	2381 ± 774			
	Overweight	7033 ± 1588	5260 ± 598	**0.002**	0.284	0.525
	Obese	8155 ± 1298	6969 ± 1242			
G-CSF	Lean	487 ± 112	987 ± 439			
	Overweight	1763 ± 320	1481 ± 232	**0.001**	0.954	0.311
	Obese	2133 ± 285	1876 ± 195			
IL-8	Lean	463 ± 208	1144 ± 505			
	Overweight	2479 ± 695	1481 ± 346	0.300	0.212	0.149
	Obese	3414 ± 1450	1614 ± 517			
IP-10	Lean	639 ± 183	1278 ± 578			
	Overweight	3746 ± 770	2238 ± 650	**0.014**	0.099	**0.029**
	Obese	3867 ± 709	3251 ± 631			
IL-1Ra	Lean	167 ± 73	161 ± 60			
	Overweight	279 ± 40	763 ± 417	0.159	0.661	0.086
	Obese	1418 ± 563	626 ± 172			
MIP-1b	Lean	220 ± 29	401 ± 167			
	Overweight	1119 ± 101	852 ± 143	**0.003**	0.874	0.469
	Obese	1180 ± 194	1341 ± 279			
TNFα	Lean	230 ± 110	180 ± 87			
	Overweight	130 ± 15	221 ± 107	0.451	0.692	0.253
	Obese	450 ± 182	355 ± 135			
IL10	Lean	14 ± 2	12 ± 3			
	Overweight	24 ± 2	35 ± 9	**0.004**	0.765	**0.017**
	Obese	55 ± 8	43 ± 8			

Table 4.2. Adipokine secretion by whole adipose tissue explants over 3 hours; pre and 6h post meal. Participants were classified as lean (n=6), overweight (n=5) or obese (n=10) based on waist circumference. Mean and SEM values shown with *p* values for effects of adiposity, time and adiposity x time interactions analysed by mixed-model ANOVA.

4.3.4 Fasting and postprandial blood measures of lipid metabolism and inflammation

With increasing levels of adiposity there were no differences between the groups when examining either fasting values or total areas under the curve for measures of lipid metabolism (NEFA or triglcerides), vascular markers of inflammation (CRP, SAA, s-ICAM1 and s-VCAM1), or cytokines IL-8, IL-6, and TNFα (Table 4.3). Furthermore mixed model ANOVA confirmed there were no main effects of adiposity or adiposity*time interactions for any of these parameters.

	Fasting			One-way ANOVA	Total-AUC (6h)			One-way ANOVA
	Lean	**Overweight**	**Obese**	***P***	**Lean**	**Overweight**	**Obese**	***p***
Markers of lipid metabolism								
Triglycerides (mmol/L)	0.9 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	0.123	8.8 ± 1.1	12.3 ± 1.9	10.5 ± 0.2	0.192
NEFA (mmol/L)	0.33 ± 0.04	0.48 ± 0.13	0.43 ± 0.05	0.469	1.99 ± 0.23	2.31 ± 0.39	2.74 ± 0.41	0.339
Markers of Inflammation								
CRP (mg/L)	716 ± 328	1234 ± 290	1897 ± 775	0.282	3851 ± 1711	7011 ± 1545	10935 ± 4866	0.288
SAA (ng/mL)	1416 ± 688	1119 ± 205	1248 ± 270	0.894	8094 ± 3863	6798 ± 1108	7339 ± 1529	0.934
s-ICAM1 (ng/mL)	245 ± 24	223 ± 17	235 ± 15	0.708	1378 ± 110	1370 ± 58	1324 ± 61	0.877
s-VCAM1 (ng/mL)	430 ± 35	342 ± 29	428 ± 29	0.087	2439 ± 148	2132 ± 87	2420 ± 142	0.186
IL-6 (pg/mL)	2.2 ± 0.7	1.6 ± 0.3	1.7 ± 0.4	0.614	14.6 ± 3.4	11.7 ± 1.7	12.5 ± 1.7	0.675
IL-8 (pg/mL)	3.4 ± 0.4	3.5 ± 0.2	3.7 ± 0.2	0.787	21.1 ± 2.7	20.6 ± 1.2	22.2 ± 1.6	0.833
TNFα (pg/mL)	8.0 ± 1.5	7.0 ± 1.1	9.7 ± 1.4	0.381	41.9 ± 8.5	38.3 ± 5.5	51.8 ± 7.3	0.406

Table 4.3. Fasting and total area under the curve (6h) measures for blood markers of lipid metabolism and inflammation following a mixed meal for participants classified based on waist circumference. Mean (SEM) values shown and one-way ANOVAs performed to identify significant differences; * denotes significant difference compared to lean $p<0.05$). Abbreviations used: AUC = area under the curve, NEFA = non-esterified free fatty acids.

Only SAA and TNFα showed significant changes in blood in the 6 hours following the meal, whereby SAA increased by approximately 27 % (p=0.008) above baseline at t=300 minutes in all groups (Figure 4.4a) and TNFα was significantly reduced at each of the measured time points following the meal as compared to baseline (all p<0.05) (Figure 4.4b). IL-6 was significantly increased at t=240 minutes (p=0.001) and t=360 (p<0.001) minutes compared to baseline (Figure 4.4c). Based on previous observations by our group, it was suspected that the rise in IL-6 may be due to the presence of local inflammation caused by the cannula which was confirmed in the separate venepuncture samples taken from a subset of 15 participants at 120 and 360 minutes (Figure 4.4d).

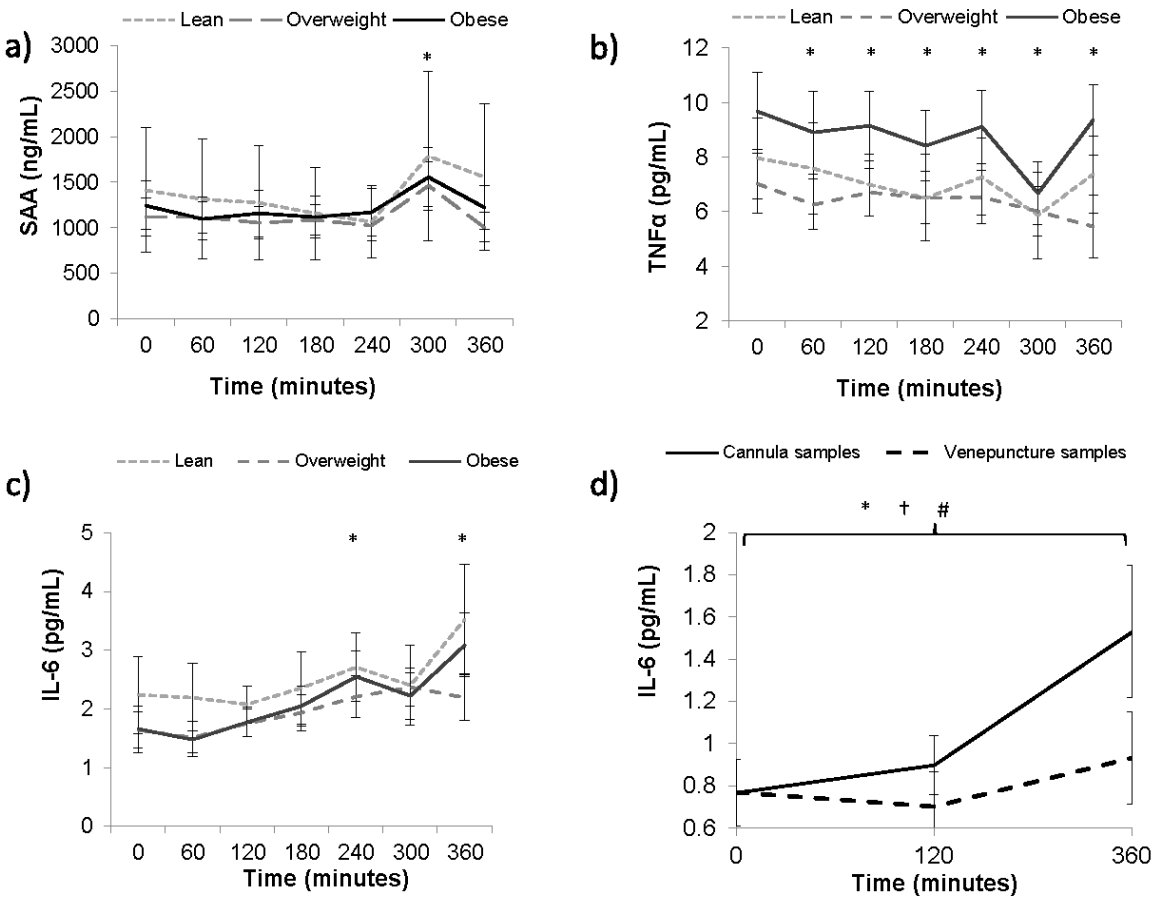


Figure 4.4. Changes in blood levels of a) SAA, b) TNFα and c) IL-6 for lean, overweight and obese individuals classified based on waist circumference at baseline and over the six hours following the meal (n=30). Mixed-model ANOVAs were performed to identify significant time, group and interaction effects with post-hoc *t*-tests applied; * denotes main effect of time ($p<0.05$) compared to t=0 (n=15). d) Analysis of venepuncture samples for IL-6 revealed that the increase was due to local inflammation at the cannula site since there was no change in samples taken by venepuncture from the contralateral arm; main effect of time (*) (F=7.714, p=0.011), condition (†) (F= 6.178, p=0.026) and time x condition (#) (F= 6.278, p=0.019) as analysed by 2-way repeated measures ANOVA**.**

4.4 Discussion

The present study investigated metabolic and immune responses to the consumption of a mixed meal in the circulation and adipose tissue between men with varying levels of adiposity. As anticipated, the glucose and insulin responses to the meal were increased in line with increased adiposity. However, in general, the adipose tissue responses were not influenced by adiposity, for example, the expression of IL-6 and MCP-1 RNA transcripts in subcutaneous adipose tissue were increased and expression of IRS2 decreased to a similar extent in lean through to obese men at 6 hours following consumption of the meal.

4.4.1 Postprandial responses in adipose tissue with adiposity and insulin resistance

The main aim of the present research was to determine whether an increased postprandial metabolic response typically seen with increased levels of adiposity corresponds with increased inflammatory responses in adipose tissue. Indeed with increased levels of adiposity from lean through to class I obesity, the anticipated elevated magnitudes of glucose and insulin responses following consumption of the meal were observed (Manning *et al.*, 2008a). Within adipose tissue, gene expression of IL-6 and MCP-1 were increased at 6 hours following consumption of the meal, which supports findings from Meneses *et al.* (2011) who examined postprandial responses in adipose tissue gene expression from participants with metabolic syndrome and work by Pietraszek *et al.* (2011) who compared responses in non-obese relatives of people with type 2 diabetes to a control group. The magnitude of changes in IL-6, MCP-1 and IRS2 gene expression observed in the present study was equal across all groups from lean to class I obese despite poorer glucose control/poorer insulin sensitivity with increasing levels of adiposity. This may suggest that an increase in these parameters is part of a 'normal' postprandial response within adipose tissue, at least within 6 hours following a meal.

4.4.2 Regulation of postprandial inflammation in adipose tissue

Despite changes in IL-6 gene expression in adipose tissue, there was no corresponding change in either adipose tissue secretion of IL-6 or levels in the blood, at least over the 6 hours following the meal. It is possible that there may be

important post-transcriptional/translational modifications to prevent increased systemic inflammation from adipose tissue each time a meal is consumed. It is interesting that no differences were found in the magnitude of responses at the level of gene expression/secretion following the meal, even with increased levels of adiposity and both insulin and glucose responses in blood. It may be argued that the greater postprandial glucose and insulin responses in blood may have been due to the obese group ingesting more calories compared to lean/overweight groups since the meal was given relative to RMR. However, this is likely to be closer to typical energy intake and thus preferable to the provision of an unadjusted standardised meal. Moreover, despite the larger meal being consumed, there were no differences in gene expression in adipose tissue between groups. This does not exclude systemic insulin and/or glucose concentrations as mediators of the postprandial response in adipose tissue, since the increased level of insulin resistance in obese adipose tissue (suggested by the chronic down-regulation of GLUT4, HSL and IRS2 as identified in this study) may counteract the increased insulin response resulting in the same overall effect as in lean 'insulin sensitive' tissue.

It could also be argued that the obese adipose tissue is adapted to cope with a greater caloric challenge and there is greater storage capacity for the increased triglycerides (and glucose). Given the increased relative mass of adipose tissue in obese individuals compared to lean, exposure of each gram of obese adipose tissue to insulin may actually be comparable to lean. It would be interesting to investigate whether there may be greater levels of postprandial inflammation/stress in times of weight gain/overfeeding compared to times of weight maintenance, especially since this study gave the meal relative to requirements. Greater postprandial inflammation and stress may instead occur in adipose tissue during times of rapid weight gain/consuming more than requirements, where there is insufficient adipose tissue to immediately deal with and buffer the challenge. This cannot be deduced from this study population since it was required that participants had been weight stable for at least 3 months for their inclusion. Perhaps if the lean group were given the same amount of the meal as the obese group, greater levels of inflammation may have been observed

compared to obese individuals, but this would have addressed a different research question.

4.4.3 Potential origin and roles of IL-6 and MCP-1

The meal used in this study was relatively high in both carbohydrates and fats. However, it cannot be determined from this study whether either of these or another factor related to the ingestion of either of these meal components (e.g. insulin) is the main stimulus for the increase in adipose tissue expression of IL-6 and MCP-1. Adipose resident immune cells, endothelial cells, preadipocytes (etc.) comprising the stromavascular fraction, rather than adipocytes, are likely to be the major sources of adipokines such as MCP-1 and IL-6 (Fain, 2010). MCP-1 and IL-6 are produced primarily by macrophages (Moro *et al.*, 2007; Bourlier *et al.*, 2008), however, it is estimated that mature adipocytes also contribute approximately one third of the IL-6 present in the circulation of patients who are obese (Fantuzzi, 2005) so their contribution cannot be excluded. Each of the components of the meal and the associated insulin response have been shown *in-vitro* to affect inflammation in both macrophages and adipocytes. For example, experiments using (murine) 3T3-L1 adipocytes have shown that insulin can dramatically enhance MCP-1 expression from these cells and may therefore be an important contributing factor driving the observed increase. MCP-1 can also induce insulin resistance in adipocytes (Sartipy 2003). The increase in MCP-1 may be a contributing factor to enhance macrophage recruitment to adipose tissue (via repeated stimulation) as is observed in adipose tissue of obese individuals (Chapter 3, and well documented in the literature (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Curat *et al.*, 2004; Zeyda *et al.*, 2007). *In-vitro* work has shown that IL-6 production can be induced in macrophages following high levels of glucose uptake via GLUT1 (Freemerman *et al.*, 2014). Fatty acids (and lipopolysaccharides (LPS) produced by gram-negative bacteria present within the intestine following ingestion of triglycerides) may also have a role in regulating infiltration of macrophages into adipose tissue and stimulating their production of pro-inflammatory IL-6 (Makowski *et al.*, 2001; Sampey *et al.*, 2011; Johnson *et al.*, 2012).

Over longer periods of time, with repeated stimulation, these temporal changes in gene expression seen after the consumption of a single meal may contribute to the chronic increases in adipose tissue inflammation, although it is also possible that levels of postprandial inflammation may continually adapt over the course of a day to meal components. It would be interesting to investigate postprandial inflammatory changes in adipose tissue following subsequent calorie intake, since blood glucose and insulin responses are known to be much lower after subsequent feeding (Staub-Traugott effect), a phenomenon which is preserved in normal glucose tolerance through to type 2 diabetes (Bonuccelli *et al.*, 2009; Jovanovic *et al.*, 2009a; Jovanovic *et al.*, 2009b) and could help identify whether glucose/insulin are indeed key drivers of the postprandial adipose tissue responses.

The effect of hyperglycaemia alone (in the absence of increased insulin) has been examined on adipose tissue gene expression and immediately after 3 hours of hyperglyaemia (Meugnier *et al.*, 2007). The majority (>80 %) of genes examined in that particular study actually showed down-regulation and neither IL-6 or MCP-1 were reported to be up-regulated (Meugnier *et al.*, 2007). If the meal was indeed the key stimulus for the inflammation, another component to consider are the triglycerides, which, like the changes in adipose tissue gene expression, showed no differences in magnitudes of response with increased levels of adiposity. High-fat meal challenges also induce inflammation in adipose tissue (Magne *et al.*, 2010; Meneses *et al.*, 2011) although composition/quality of fat (i.e. saturated or unsaturated fats) is not necessarily important in determining the levels of postprandial inflammation in adipose tissue (Meneses *et al.*, 2011). It is possible that adipose tissue may respond to glucose, insulin and triglycerides (and other associated stimuli not examined in this study, e.g. LPS) 'independently' of each other over the course of their respective postprandial responses and adipose tissue sampling at different time points may have produced a different pattern of results.

4.4.4 Inflammatory responses in circulation

No differences in fasting vascular markers of inflammation, cytokine concentrations or their respective postprandial responses were found between the

three groups. The only temporal changes observed were for TNFα, which was reduced at all time-points, and SAA which was increased only at 5 hours after the meal. These results are consistent amongst other studies covering ranges of adiposity (Blackburn *et al.*, 2006; Poppitt *et al.*, 2008). Both these previous studies found a transient decrease in TNFα and increase in IL-6 after the meal and similarly they also report no change in CRP. There is, however, much conflicting evidence regarding the role of acute inflammatory mediators in the circulation following consumption of a meal as several studies report increases in several inflammatory cytokines including IL-6 (Nappo *et al.*, 2002; Blackburn *et al.*, 2006; Manning *et al.*, 2008b; Poppitt *et al.*, 2008; Laugerette *et al.*, 2011), although this was most likely due to localised inflammation at the site of the cannula as identified by our results in this study and previously (Dixon *et al.*, 2009). Gene expression of TNFα in adipose tissue was not altered at 6 hours after the meal, neither was there any change in tissue secretion *ex-vivo*, suggesting that the adipose tissue may not be directly linked to these inflammatory changes in the circulation in the hours following the meal.

4.4.5 Limitations

The lack of a control group remaining fasted for the duration of the postprandial response means we cannot be certain that the meal directly caused any of the reported changes. Such comparisons have been made previously in rats, which demonstrated that gene expression of inflammatory markers IL-6 and NF-κB were significantly increased (at 2 hours) specifically following a high-fat meal compared to water (Magne *et al.*, 2010). Furthermore, since we only included 2 time points for adipose tissue sampling, this is clearly not a full reflection of what is occurring within adipose tissue over the entire duration following consumption of the meal. For example, there may not have been sufficient time for synthesis of RNA transcripts/proteins for secretion into the circulation by the 6 hour time-point and later sampling may have shown an increase in secretion in response to the meal. Conversely, 6 hours may have been too late to observe some changes since a greater number of changes had been identified at 4 hours following lipid challenges (Meneses *et al.*, 2011) and after only 3 hours of hyperglycaemia (Meugnier *et al.*, 2007).

4.4.6 Conclusions

Participants were recruited to cover a range of postprandial responses for glucose and insulin according to varying levels of adiposity. However, despite these differences and differences in adiposity, the adipose tissue responses were broadly similar in lean, overweight and obese middle-aged men. Thus, a single feeding exposure does not appear to provoke a greater acute inflammatory response in expanded subcutaneous adipose tissue. Based on these results, acute feeding will not be examined further in the present thesis.

CHAPTER 5

Impact of calorie restriction on leptin and T-lymphocyte activation in blood and adipose tissue in overweight and obese men

5.1 Introduction

Leptin is a cytokine-like hormone primarily produced by adipocytes in proportion to body fat mass. In humans, it is predominantly produced by subcutaneous adipose tissue due to the increased relative size and secretion rate of this depot (Margetic *et al.*, 2002; Fain *et al.*, 2004). Leptin concentrations in blood typically reflect energy stored in adipose tissue and this hormone acts centrally on the hypothalamus to regulate energy intake and expenditure (Friedman & Halaas, 1998). Leptin also influences a variety of peripheral physiological functions, however, including immune system function (La Cava & Matarese, 2004) and the leptin receptor (Ob-R) is expressed by neutrophils, monocytes, macrophages, T-lymphocytes, B-lymphocytes, mast cells, dendritic cells and NK cells (La Cava & Matarese, 2004). Leptin can (with co-stimulation) elicit dose dependent effects on T-lymphocyte expression of 'early' activation marker CD69 and 'late' activation maker CD25, *in-vitro,* after 12 hours and 48 hours respectively (Martin-Romero *et al.*, 2000). Results from our previous research investigating the properties of T-lymphocytes across differing levels of adiposity suggested that adipose tissue resident CD4+ and CD8+ T-lymphocytes show greater levels of activation with increasing adiposity and this activation (levels of CD69 and CD25 expression) was further related to the increased serum concentrations of leptin (Chapter 3). Whether leptin *per se* is a key factor in determining the levels of T-lymphocyte activation in adipose tissue in humans is unclear. Given the role of leptin on CD69 and CD25 expression (Martin-Romero *et al.*, 2000), there is the exciting possibility that leptin-related modifications to T-lymphocyte activation could influence (improve) inflammation in adipose tissue and in blood *in-vivo*.

Leptin can be dramatically reduced with short periods of 'severe' calorie restriction to levels much lower than the corresponding change in adiposity would

predict (Boden *et al.*, 1996; Dubuc *et al.*, 1998; Haluzik *et al.*, 2001; Mars *et al.*, 2006). Importantly, expression of T-lymphocyte activation markers CD69 and CD25 are also rapid to respond to stimulation, with changes being detectable after just a few hours (Hashemi *et al.*, 1999; Martin-Romero *et al.*, 2000). Thus, a short period of severe calorie restriction and a reduction in leptin may provide a route to test whether T-lymphocyte activation in adipose tissue and blood can be modified *in-vivo* in humans.

The main aim of this research was therefore to investigate whether calorie restriction and the associated reduction in leptin leads to a reduction in T-lymphocyte activation within blood and adipose tissue in overweight subjects.

5.2 Materials and methods

5.2.1 Experimental design

Twelve abdominally overweight/obese males aged between 35-55 years were recruited from the local community following ethical approval from the South West, Frenchay NHS Research Ethics Committee (REC Reference: 12/SW/0324). Each participant gave written informed consent. Only overweight and obese participants were recruited since results from our previous study suggested that these individuals showed greatest activation of T-lymphocytes within adipose tissue (Chapter 3). After a 1-week period of monitoring energy intake and expenditure to confirm a sufficient state of 'energy balance', participants reduced their caloric intake to 50 % of their normal intake for 3 consecutive days. Participants attended the Laboratory before and after this intervention for analysis of immune cell activation and markers of inflammation and metabolism in blood and adipose tissue. An oral glucose tolerance test was also performed to assess glucose control before and after the 3 day calorie restriction intervention.

5.2.2 Participants

Participants were abdominally overweight/obese with waist circumference >94 cm (Lean *et al.*, 1995) and had been weight stable for more than 3 months (no change in weight +/- 3 %) (Stevens *et al.*, 2006). Individuals were excluded from participation if they smoked, had personal history of/existing cardiovascular disease, metabolic disease or dyslipidaemia or were taking medications that may influence lipid or carbohydrate metabolism or immune system function.

5.2.3 Sample size determination

There are no data regarding changes in T-lymphocyte activation in human adipose tissue in response to calorie restriction. However, we observed significant differences in T-lymphocyte activation between lean and obese individuals in our previous study. A short period of calorie restriction can reduce serum leptin values by around 40 % ((Mars *et al.*, 2006) and from pilot work in Appendix 3), which is sufficient to reduce typical values for an obese person to those of a lean person. Thus, we anticipate a similar reduction in T-lymphocyte activation in response to caloric restriction. Our recent data indicate that the CD69 mean fluorescence intensity (MFI) for lean CD4CD69 cells is 288 (+/45 SD) and obese

is 411 (+/31 SD) with an effect size of 2.98 (G-Power). Taking into account our range of activation markers, with 95 % power and 5 % alpha, an average of 11 participants were required to detect a statistically significant change in T-lymphocyte activation in adipose tissue in response to caloric restriction. This was rounded to 12 participants to allow for withdrawal of participants from the study.

5.2.4 Monitoring of energy balance

Participants were weighed before and after a 1-week period of 'energy balance' monitoring to ensure weight stability during this period using a digital balance (Tanita Corp., Japan). Participants were fitted with a combined heart rate and accelerometry monitor (Actiheart™; General Methods, 2.5) to determine habitual total energy expenditure (TEE) (Thompson *et al.*, 2006). TEE was adjusted for measured resting metabolic rate (RMR) using the protocol described in General Methods, 2.6. A weighed food and fluid intake record was used during this period to estimate participants' energy intake (General Methods, 2.7). Participants were asked not to make any conscious changes to their normal lifestyle habits/routines during this period. Participants were not told that the activity monitoring and food records would be used to directly influence the diet prescribed/given during the 3 day calorie reduction period so as to avoid influencing their habitual routines. This analysis was then used to confirm that participants were in a state of 'energy balance' and to write a 'diet prescription' for the 3-day intervention. The aim was to ensure that participants receive 50 % of their 'normal' calorie requirements (i.e. average of energy intake and energy expenditure) using foods they would normally consume. Since there are errors associated with estimations of total energy expenditure (Thompson *et al.*, 2006) and recording dietary intake (in particular underreporting) (Poslusna *et al.*, 2009), we stipulated *a priori* that total energy intake and energy expenditure values had to be within 25 % of each other and, furthermore, that the prescribed calorie intake had to be within 40-60 % both total energy expenditure and dietary intake values. If either of these requirements were not met, participants were asked to repeat the monitoring phase.

5.2.5 Calorie restriction protocol

For 3 consecutive days, participants were instructed to follow a prescribed diet calculated to be 50 % of their 'normal' energy requirements. To determine the exact calorie intake required for the 3 days, an average of energy expenditure and dietary intake from the monitoring period was taken. This value was then divided in half to give the 'target' calorie value for each of the 3 days in the diet. Subsequently, three separate days from the participant's one-week diet record were selected and the weight of each item adjusted to meet this daily target kcal value whilst maintaining the overall relative proportions so that participants' typical diet composition remained unaltered. A worked example showing how the weights of food items were adjusted whilst maintaining overall proportions is shown in Appendix 4. During the 3 day intervention, participants were asked to record the timing of each food/fluid intake within the prescribed food diary and to confirm that they had consumed the correct amount of food to help improve compliance with diet instructions. Participants were asked not to make any conscious changes to habitual physical activity during the calorie restriction period.

5.2.6 Pre-trial requirements

Participants were asked to refrain from performing any strenuous physical activity for 48 hours and from consuming caffeine/alcohol for 24 hours before both trial days (i.e., pre- and post-intervention). Trial days were scheduled so participants had been free from any self-reported illness for a minimum of 2 weeks in order to reduce immune system disturbance.

5.2.7 Trial days

On both main trial days, participants arrived at the Physiology Resting Laboratory in the morning following a 10 hour fast (approximately 8 am) and after consuming 1 pint of water upon waking. Participants arrived in the lab at the same time on both trial days.

5.2.8 Body composition analysis

Measurements of height, waist circumferences and body mass (post- void using a digital balance (TANITA corp. Japan)) were determined on both trial days.

Participants' body composition was characterised at baseline using dual energy X-ray absorptiometry (DEXA) and estimates of total and central fat mass (L1-L4) and FMI determined as described in General Methods, 2.4.

5.2.9 Blood and adipose sampling

A cannula was inserted into an antecubital forearm vein and baseline blood sample(s) taken for analysis of plasma and serum metabolic/inflammatory markers and isolation of peripheral blood mononuclear cells (PBMCs) according to the protocols described in General Methods, 2.9 – 2.11.

Subcutaneous adipose tissue samples (~1g) were obtained under local anaesthetic (1% lidocaine) approximately 5 cm lateral to the umbilicus using a 'needle aspiration' technique and processed as described in General Methods, 2.13). Approximately 100 mg whole adipose tissue was transferred to an RNase/DNase free sterile centrifuge tube and frozen immediately on dry ice and later homogenised in Trizol reagent (Invitrogen). The remainder was used for adipose tissue culture (General Methods, 2.14) and preparation of the stromavascular fraction (SVF; General Methods, 2.15). Due to the limited size of some adipose tissue samples, priority was given to preparing tissue for analysis of SVF to address the main aim of this study. Lowest priority was given to gene expression analysis. Paired samples were available for 9 participants for secretion analysis and 7 participants for gene expression analysis.

5.2.10 Oral glucose tolerance test

Participants were asked to consume a glucose drink consisting of 75 g anhydrous glucose (maltodextrin) solution (Polycal, Nutricia, UK) and cannula blood samples were taken every 15 minutes for the following 2 hours for measurement of plasma glucose and serum insulin (General Methods, 2.12).

5.2.11 Analysis of SVF and PBMCs by flow cytometry

Flow cytometry (using the FACSverse, Beckton Dickenson) was used to identify CD4+/CD8+ T-lymphocytes (CD45+CD3+ cells) and macrophages/monocytes (CD45+CD14+ cells) in SVF and PBMCs together with respective levels of

activation using methods and antibody cocktails detailed in General Methods, 2.16 – 2.18.

5.2.12 RT-PCR

Total RNA was extracted from whole adipose tissue, quantified and 1 μg reverse transcribed to cDNA as described in General Methods, 2.19. Real-time PCR was performed using a StepOne™ (Applied Biosystems) with pre-designed primers and probes obtained from Applied Biosystems for measurement of leptin adiponectin, GLUT4, IRS2, HSL, LPL, PPAR-Ɣ, MCP-1, IL-6, IL-8, IL-1Ra, and IL-18 expression (details of specific primer Hs numbers can be found in General Methods, 2.19). Peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011). Results were analysed using the comparative Ct method and expression normalized to an internal calibrator specific to each gene using the formula $2^{-\Delta\Delta C_T}$; where $\Delta\Delta C_T$ is [(C_T gene of interest – C_T PPIA) – lowest ΔC_T for gene of interest] and statistical analysis performed on LN transformed values (Livak & Schmittgen, 2001).

5.2.13 Biochemical analysis

Plasma glucose, serum total cholesterol, HDL cholesterol, triglycerides and CRP and ALT activity were measured using commercially available assay kits and analyser (Daytona Rx, Randox). ELISA was used for the measurement of serum Insulin (Mercodia, Sweden), plasma IL-6, IL-8 and TNFα (R&D Systems, UK) and both serum and adipose tissue Leptin and Adiponectin secretion (R&D systems). A fluorescent bead (Bio-Plex) multiplex system (Luminex, BIO-RAD) was used for the measurement of serum and adipose tissue secretion of G-CSF, M-CSF, MCP-1, IP-10, IL-10, IL-1Ra, MIP-1β, IL-18. Luminex was also used for the measurement of IL-6, IL-8 and TNFα secreted by adipose tissue. Serum G-CSF, M-CSF, IL-10 and IL-1Ra were detectable in fewer than 3 individuals so results were not included in statistical analysis. Neither IL-18, IL-10 or TNFα were detected in media from adipose explants.

5.2.14 Statistical analysis

All data are presented as mean and standard error of the mean (SEM). Total area under the curve, homeostasis model assessment for insulin resistance (HOMA-IR)

and insulin sensitivity index (ISI comp/Matsuda index) were calculated using the formulae described in General Methods, 2.12). Comparisons were made between pre- and post- diet values using paired Student's T-tests. Statistical analysis was performed using Excel 2010 (Microsoft) and SPSS version 20 and $p<0.05$ was considered to be statistically significant.

5.3 Results

Energy intake was reduced from 2499 ± 119 kcal/day to 1320 ± 52 kcal/day during the 3-day calorie restriction period (50 % of mean [energy intake and TEE] from energy balance monitoring week). During the 3 day calorie restriction period, participants did not change their physical activity levels (PAL 1.54 ± 0.06 during monitoring period and 1.52 ± 0.06 during the intervention period, p=0.549). Changes in anthropometric data and blood markers of metabolism pre- and post- diet are shown in Table 5.1. There was a significant reduction in body mass, waist circumference, glucose at 2 hours post-OGTT, triglycerides and ALT; with significant increases in Matsuda-ISI, total- and LDL-Cholesterol and NEFA.

	Pre-diet	Post-diet	Sig. *p*-value
Physical characteristics			
Body mass (kg)	93.1 ± 2.8	91.5 ± 2.8	**<0.001**
Body mass index (kg/m^2)	29.0 ± 0.7	28.5 ± 0.7	**<0.001**
Waist circumference (cm)	104.6 ± 1.7	103.6 ± 1.8	**<0.001**
Height (m)	1.79 ± 0.03	ND	
L1-L4 fat (%)	33 ± 1	ND	
Fat mass index (kg/m^2)	8.2 ± 0.5	ND	
Resting metabolic rate (kcal/day)	1800 ± 30	ND	
PAL	1.54 ± 0.06	ND	
Blood related measures of metabolism			
Insulin (pmol/L)	46.7 ± 9.5	42.6 ± 9.1	0.114
Insulin Total-AUC[a] (pmol*2h/L)	187 ± 42	185 ± 57	0.952
Glucose (mmol/L)	5.1 ± 0.2	5.1 ± 0.1	0.623
Glucose Total-AUC[a] (mmol*2h/L)	14.7 ± 0.7	14.4 ± 0.9	0.742
Glucose at 2 h (mmol/L)	6.8 ± 0.5	6.0 ± 0.4	**0.002**
HOMA-IR	1.8 ± 0.4	1.6 ± 0.4	0.190
Matsuda-ISI[a]	6.21 ± 0.37	7.29 ± 1.8	**0.020**
ALT (U/L)	40.2 ± 6.0	34.9 ± 5.1	**0.051**
Triglycerides (mmol/L)	1.36 ± 0.19	1.05 ± 0.16	**0.005**
Non esterified fatty acids (mmol/L)	0.34 ± 0.03	0.55 ± 0.04	**<0.001**
Total-Cholesterol (mmol/L)	5.3 ± 0.2	5.6 ± 0.2	**0.046**
LDL-Cholesterol (mmol/L)	3.47 ± 0.17	3.86 ± 0.21	**0.013**
HDL-Cholesterol (mmol/L)	1.19 ± 0.05	1.22 ± 0.05	0.631

Table 5.1. Anthropometric measures and blood markers of metabolism before and after 3 days of 50% caloric restriction. Mean ± SEM shown and differences between the two days analysed by two-tailed, paired, Student's t-tests, *p*-values shown (n=12). [a]T-AUC for insulin and glucose and Matsuda-ISI represent n=11 due to incomplete data. All blood measures are fasting samples unless otherwise stated. L1-L4 = central fat estimated between lumbar regions L1-L4 using DEXA, PAL = physical activity level (total energy expenditure/basal metabolic rate), ALT = alanine transaminase. ND = not determined.

5.3.1 Changes in leptin and immune cell activation in blood

As shown in Figure 5.1a, there was a significant reduction in serum leptin of 31 % from baseline (range 3 % to 64 %), and the reduction in serum leptin was significantly correlated with the percentage improvement in insulin sensitivity (Matsuda-ISI; Pearson's r = -0.629, p=0.028). As a percentage of total cells isolated from the blood (PBMCs), CD4+ T-lymphocytes represented 20 ± 2 % cells and CD8+ T-lymphocytes represented 7 ± 1 % cells at baseline. Considering the activated CD4+ and CD8+ T-lymphocytes present, there was an 8 ± 10 % reduction (from 62 ± 3 % to 54 ± 5 %, p=0.016) in the percentage of CD4+CD25+ T-lymphocytes (as % total lymphocytes) present in the blood after 3 days of 50 % reduction in caloric intake (Figure 5.1b) and the level of CD25 expression (MFI) on these CD4+CD25+ lymphocytes was also reduced by 8 ± 4 % (from 3397 ± 139 to 3107 ± 126, p=0.058; Figure 5.1c). There were no such changes in the proportion or levels of activation of CD8+CD25+ T-lymphocytes (Figures 5.1b and 5.1c). The proportion of CD4+ and CD8+ cells expressing CD69 was much lower than those expressing CD25 (as a percentage of total lymphocytes) and there were no changes in either the proportion of cells expressing CD69 (Figure 5.1b) or its levels of expression after 3 days of 50 % reduction in calorie intake (Figure 5.1d).

Levels of expression of the leptin receptor (CD295) and insulin receptor (CD220) on activated T-lymphocyte populations were also examined. Although there were no changes in leptin receptor expression on activated CD4+ or CD8+ T-lymphocytes in blood, expression of the insulin receptor, however, was modestly increased on CD4+CD25+ (MFI; 86 ± 3 to 99 ± 5, p<0.01) and CD8+CD25+ (MFI; 123 ± 4 to 141 ± 8, p=0.06) T-lymphocytes following 3 days of 50 % caloric restriction.

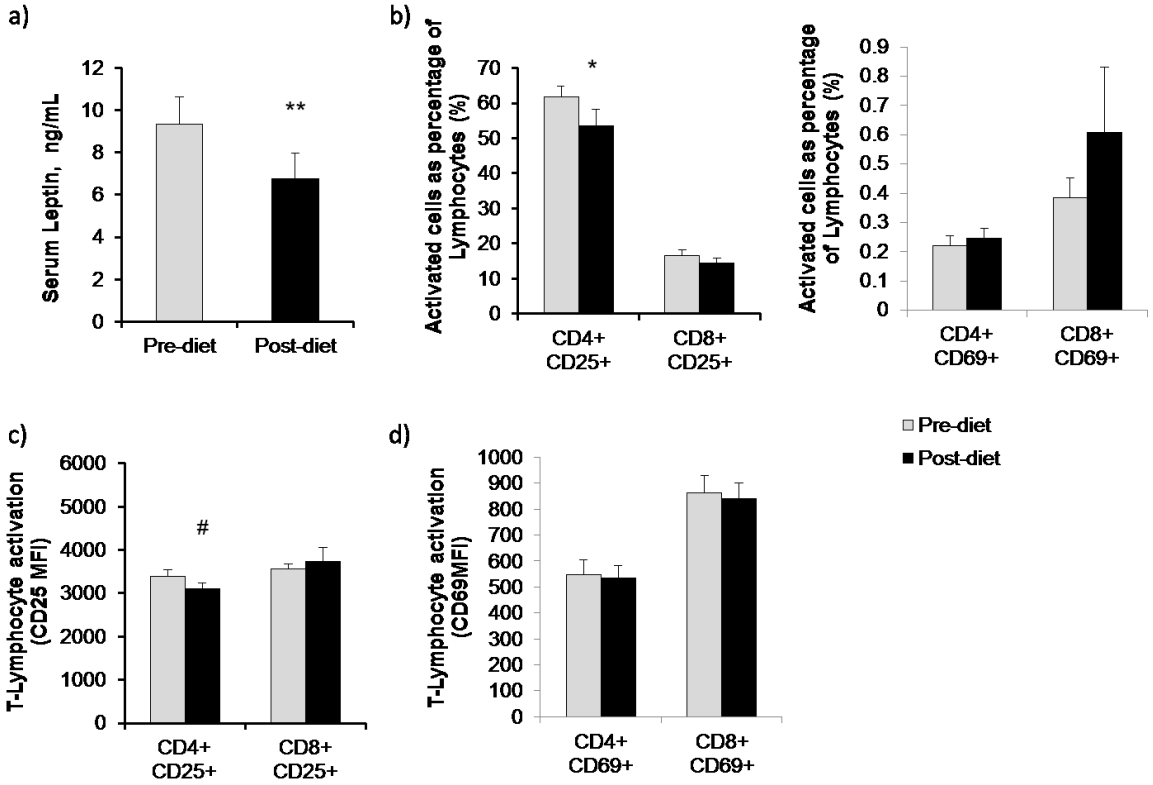


Figure 5.1. Changes in blood leptin and T-lymphocyte activation before and after 3 days of 50% calorie restriction. Changes in a) serum leptin concentration, b) percentage of T-lymphocytes expressing CD25 and CD69 activation markers, and levels of expression of c CD25 and d) CD69 on activated CD4+ and CD8+ T-lymphocytes (Mean fluorescence intensity) before and after 3 days of 50% caloric restriction. Mean ± SEM shown and differences between the two days analysed by two-tailed, Paired t-tests (n=12), *p<0.05, ** p<0.001, # p<0.06.

5.3.2 Changes in leptin and T-lymphocyte activation in subcutaneous adipose tissue

In the abdominal subcutaneous adipose tissue, resident CD4+ T-lymphocytes ranged from 1 – 7 % and CD8+ T-lymphocytes from 1 – 3 % as a percentage of total cells present in the SVF. Adipose tissue leptin gene expression and leptin secretion *ex-vivo* showed no significant reduction following 3 days of 50 % calorie restriction (Figures 5.2a and b respectively). This corresponded with a lack of change in the proportion of activated cells present (Figure 5.2c) and levels (MFI) of cell surface expression of activation markers CD25 and CD69 on adipose tissue resident CD4+ and CD8+ T-lymphocytes (Figure 5.2d and e).

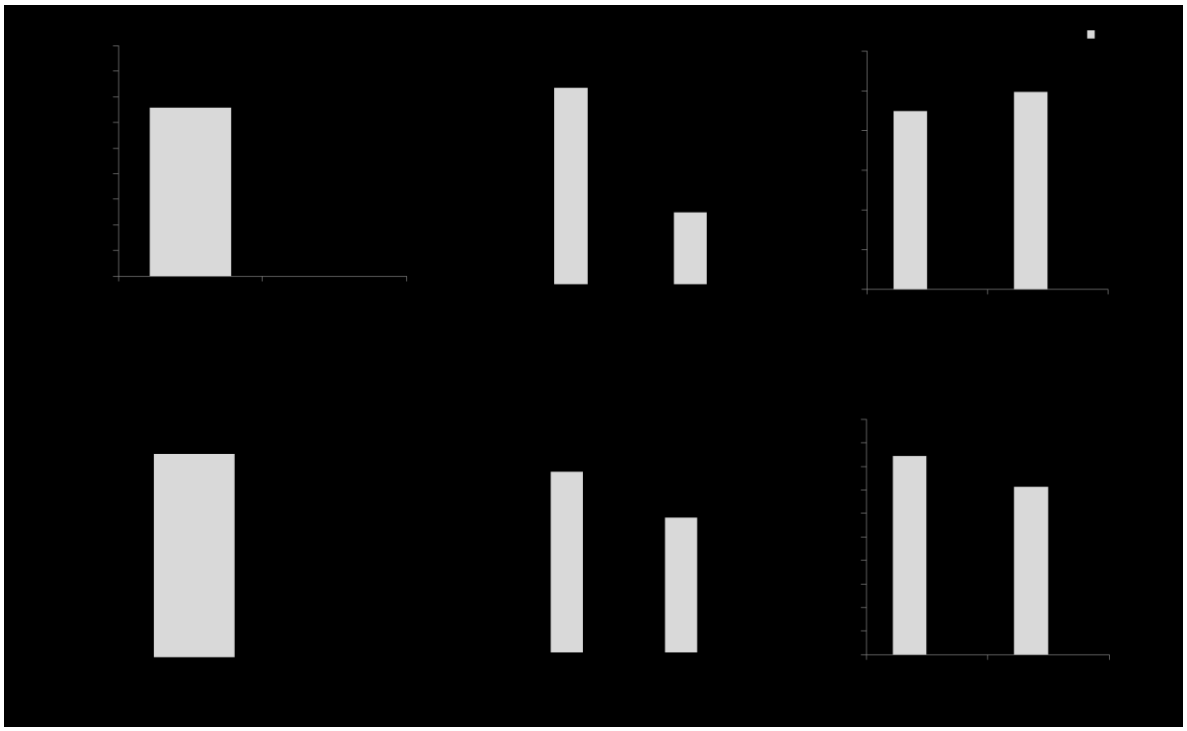


Figure 5.2. Changes in adipose tissue leptin and adipose resident T-lymphocyte activation before and after 3 days of 50% calorie restriction. Changes in adipose tissue a) leptin secretion (n=9) and b) relative leptin gene expression (normalised to cyclophilin A and internal housekeeper; n=7), c) proportions of activated adipose-resident T-lymphocytes and d) levels of expression of d) CD25 and e) CD69 on activated CD4+ and CD8+ T-lymphocytes before and after 3 days of 50% caloric restriction. Mean ± SEM shown and differences between the two days analysed by two-tailed, Paired t-tests (n=12 unless otherwise stated).

5.3.3 Changes to blood monocytes and adipose tissue resident macrophages following calorie restriction

Blood monocytes and adipose resident macrophages (CD45+CD14+ cells) were identified by flow cytometry and, at baseline, represented 3 to 9 % of total cells in blood (Figure 5.3a) and 2 to 15 % total cells present in the SVF (Figure 5.3c). There were no changes in proportions of either blood monocytes or adipose resident macrophages following 3 days of calorie restriction. Blood monocytes were examined by flow cytometry for their levels of cell surface expression of activation markers CD16 and CD14, and receptors for Leptin (CD295) and insulin (CD220). Only a reduction in CD14 expression was identified (Figure 5.3b). On adipose tissue resident macrophages, cell surface expression (MFI) of CD14 and CD163 were examined, however neither were affected following 3 days of calorie restriction (Figure 5.3d).

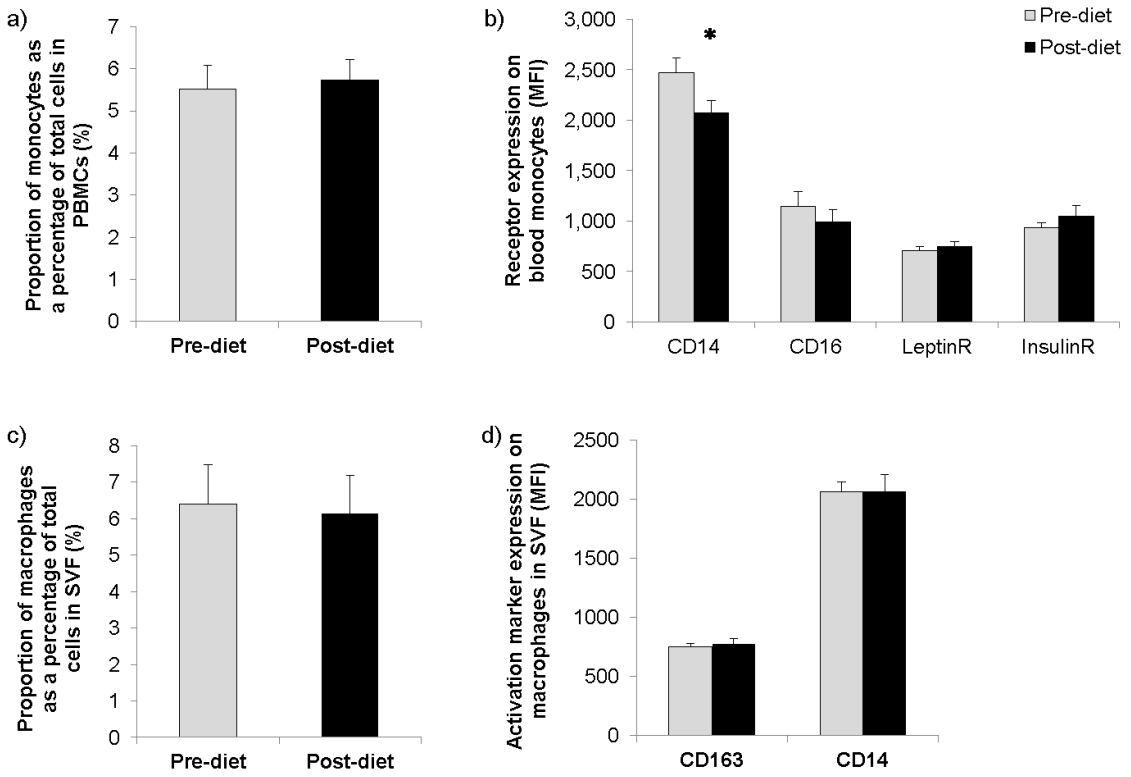


Figure 5.3. Proportions of blood monocytes and adipose tissue resident macrophages and their respective properties before and after 3 days of 50% calorie restriction. a) Proportions of monocytes as a percentage of total cells in blood (PBMCs), b) levels of expression of activation markers (CD14 and CD16) and receptors (for leptin and insulin) on blood monocytes, c) proportions of macrophages as a percentage of cells in adipose tissue SVF, d) Levels of activation markers CD163 and CD14 on adipose tissue resident macrophages before and after 3 days of 50% calorie restriction, mean ± SEM shown and differences between the two days analysed by two-tailed, Paired t-tests (n=12).

5.3.4 Changes in plasma adiponectin and measures of inflammation

Adiponectin and a number of inflammatory cytokines were measured in the blood before and after the 3 days of 50 % calorie restriction. Of those that were detected in the blood (shown in Table 5.2), only IL-18 and MCP-1 showed significant reductions after calorie restriction and the majority of cytokines remained unchanged.

	Pre-diet	***Post-diet***	***Sig. p-value***
Adiponectin µg/mL	6.65 ± 0.78	6.39 ± 0.69	0.206
CRP mg/L	1.54 ± 0.44	1.51 ± 0.32	0.914
IL-6 pg/mL	0.67 ± 0.11	0.75 ± 0.11	0.606
IL-8 pg/mL	5.61 ± 0.55	5.08 ± 0.25	0.408
TNFα pg/mL	1.21 ± 0.14	1.14 ± 0.12	0.267
MIP-1b pg/mL	69.6 ± 7.92	72.1 ± 8.4	0.261
IL-18 pg/mL	95.8 ± 11.9	88.4 ± 11.8	**<0.001**
IP-10 pg/mL	347.0 ± 55.5	340.3 ± 61.5	0.737
MCP-1 pg/mL	49.8 ± 6.3	43.4 ± 5.2	**0.016**

Table 5.2. Plasma adiponectin and markers of inflammation before and after 3 days of 50 % calorie restriction. Mean **±** SEM values shown and differences between the two days analysed by two-tailed, paired, Student's t-tests (n=12), *p*-values shown.

5.3.5 Gene expression and secretion of adipokines from subcutaneous AT

Changes in a selection of genes related to inflammation and glucose/lipid metabolism in adipose tissue were investigated. Data for adipose tissue gene expression and secretion are not available for all participants due to the limited size of some samples. Priority was given to preparing tissue for analysis of SVF to address the main aim of this study with lowest priority given to gene expression analysis. Paired samples were available for 9 participants for secretion analysis and 7 participants for gene expression analysis. Both adiponectin and IL-6 showed a significant modest increase and GLUT4 showed a reduction in expression 3 days after 50 % calorie restriction (Figure 5.4a). There were, however, no significant changes in secretion of any of the adipokines measured from whole adipose tissue following calorie restriction (Figure 5.4b).

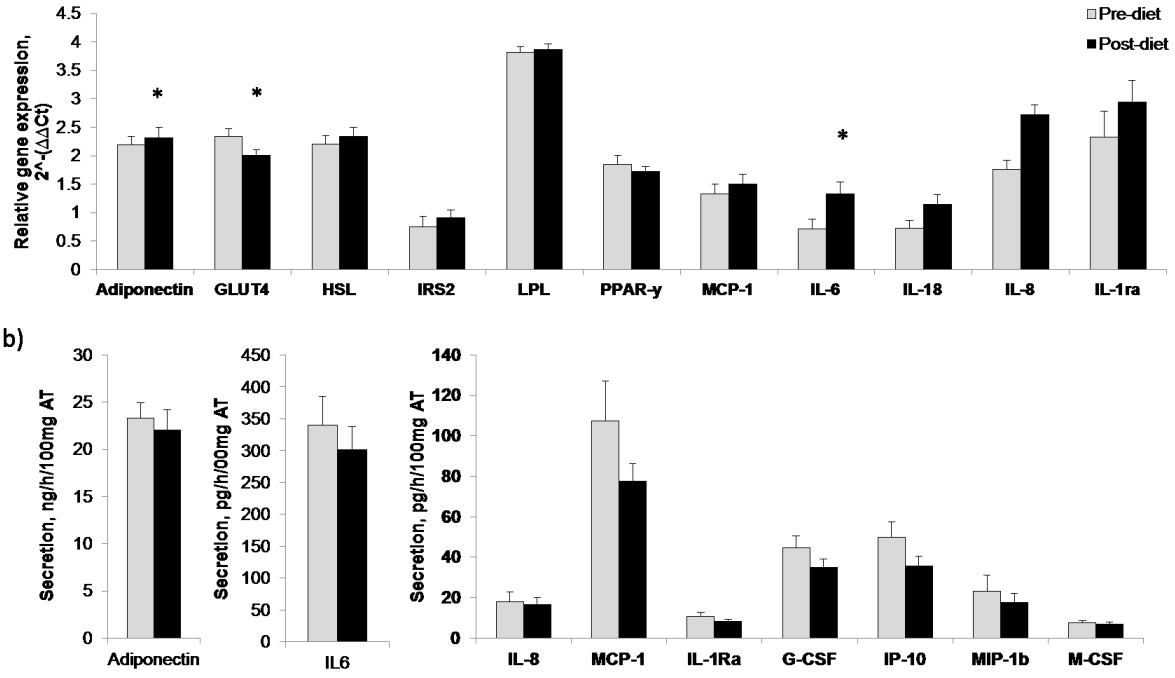


Figure 5.4. Gene expression and secretion changes in whole adipose tissue before and after 3 days of 50 % calorie restriction. a) Changes in relative gene expression of proteins related to metabolism and inflammatory cytokines within adipose tissue. Data presented as mean $2^{-\Delta\Delta Ct}$ ± SEM and differences between the two days analysed by two-tailed, Paired t-tests, *p<0.05 (n=7). b) Cytokine secretion by whole adipose tissue explants cultured for 3h. Mean and SEM values shown and differences between the two days analysed by two-tailed, Paired t-tests, *p<0.05 (n=9).

5.4 Discussion

In this study, we demonstrate that 3 days of 50 % calorie restriction reduced leptin concentrations in blood and this was temporally associated with a reduction in the proportion and levels of activation of blood CD4+CD25+ T-lymphocytes. In contrast, however, leptin secretion and gene expression in abdominal subcutaneous adipose tissue were not reduced and there was also no change in adipose tissue resident T-lymphocyte activation. These respective temporal associations with leptin in blood suggest this hormone may play a role in the *in-vivo* regulation of immune cell activation, however, the divergent changes in T-lymphocyte activation in blood and adipose tissue probably indicate that there are local tissue-specific responses of immune cells to calorie restriction. This study provides further novel insight into adipokine changes at the levels of subcutaneous adipose tissue and blood following short term 'severe' calorie restriction.

5.4.1 Changes in T-lymphocyte activation in blood following acute calorie restriction

The reduction in blood leptin was temporally associated with a reduction in CD4+ T-lymphocyte activation in blood. This observation is in-keeping with previous work by others showing a dose-dependent effect of leptin on CD4+ T-lymphocytes activation markers *in-vitro* (Lord *et al.*, 1998; Martin-Romero *et al.*, 2000; De Rosa *et al.*, 2007) although no changes in CD8+ T-lymphocyte activation were apparent in this study. Whether the reduction in blood leptin was the direct cause of this reduction in blood T-lymphocyte activation cannot be ascertained from the current study. However, leptin appears to be important in signaling whether sufficient energy is available to meet the demands for an immune response since leptin deficiency/starvation is associated with immunosuppression (Lord *et al.*, 1998; Saucillo *et al.*, 2014). Conversely, in obesity, high levels of leptin are implicated in the chronic low-grade inflammation observed, for example, via induction of proinflammatory Th1 T-lymphocytes and stimulation of inflammatory cytokine production (IL-6, TNFα) in adipose tissue (Paz-Filho *et al.*, 2012). With reduced levels of leptin and T-lymphocyte activation as observed with 3 days of calorie restriction, it is likely that there

would be a blunted immune response to immune challenges (e.g., infection) as is the case with more chronic starvation (Lord *et al.*, 1998; Paz-Filho *et al.*, 2012).

The reduction in CD4+ T-lymphocyte activation was specifically observed for 'late' activation marker CD25 (according to both proportion of cells expressing CD25 and levels of expression of this activation marker). This CD4+CD25+ T-lymphocyte population would include activated CD4+ effector cells, such as T-helper cells (Th1 and Th2) and T-regulatory cells. *In-vitro* work suggests that a reduction in leptin or blocking its action would favour proliferation of T-regulatory cells with a reduction in Th1 T-lymphocytes (Lord *et al.*, 1998; De Rosa *et al.*, 2007). It would have been interesting to see whether such skewing had taken place in either blood or adipose tissue if sufficient material had been available.

The change in leptin alone may not necessarily be the only factor driving the reduced activation of CD4+ T-lymphocytes. Leptin is essential for glucose uptake in activated effector T-lymphocytes, thus enabling them to perform their correct function when activated (Saucillo *et al.*, 2014). The exception to this is T-regulatory cells which are more dependent on lipid oxidation (Michalek *et al.*, 2011). Rather than being directly affected by leptin, the reduced levels of activation of CD4+CD25+ lymphocytes may also therefore be secondary to the reduced exposure to glucose and/or insulin as a consequence of calorie restriction. This is supported by the finding that insulin enhances levels of Th2 CD4+ T-lymphocyte proliferation (Viardot *et al.*, 2007). We also observed up-regulation of the insulin receptor (CD220), which is typically only up-regulated on activated compared to 'resting' T-lymphocytes (Viardot *et al.*, 2007). This could instead be a mechanism to maximize glucose intake in times of calorie restriction and/or result from reduced suppression/up-regulation of insulin signalling pathways within T-lymphocytes (Saltiel & Kahn, 2001).

5.4.2 Differing leptin responses in blood and adipose tissue to acute calorie restriction

Despite changes in leptin and T-lymphocyte activation in the blood following calorie restriction, there were no corresponding changes in adipose tissue. Based

on previous results, a change in T-lymphocyte activation in adipose tissue rather than blood may have been expected due to the relationship at baseline specifically between adipose tissue resident T-lymphocyte activation and leptin (Chapter 3), which indeed also held true even across the smaller and narrower range of adiposities included in the present study (see Appendix 5). The corresponding lack of response in leptin gene expression and secretion in adipose tissue is also of interest. In-keeping with our results, it appears that in times of short term calorie restriction/starvation, despite a significant reduction in blood leptin concentrations, adipose tissue leptin gene expression continues to reflect levels of adiposity (Vidal *et al.*, 1996; Liu *et al.*, 2003). Indeed, much longer periods of calorie restriction (e.g. >3 weeks) and weight loss are required to elicit changes in leptin gene expression (Bastard *et al.*, 1999; Behre *et al.*, 2007). In times of acute calorie restriction, the reduction in blood leptin serves as a signal to the brain and all systems in the body that there is an energy insufficiency irrespective of adiposity (Flier, 2004). It is not clear where or how this reduction in blood leptin occurs, and our results suggest there is no change in the secretion of leptin from adipose tissue, so one or a combination of specific post-translational modifications, clearance or degradation affecting half-life may be responsible for the reduced levels of leptin in blood. If the fall in blood leptin is the result of accelerated clearance/uptake or degradation, this could go some way to explain why there are no changes in adipose tissue T-lymphocyte activation despite an initial relationship at baseline.

It is also possible that other adipose tissue depots may respond differently to the abdominal subcutaneous adipose depot included in the present study, for example visceral and lower body subcutaneous adipose tissue depots have very different properties, and the latter depot will represent a significant proportion of total adipose tissue mass (Manolopoulos *et al.*, 2010). It is important to highlight that the media used for the adipose tissue culture in our *ex-vivo* model was not supplemented with glucose/insulin or other potential stimuli of leptin secretion from adipocytes (Cammisotto *et al.*, 2005). This was a deliberate decision but it is possible that this may have produced different results to those had the adipose tissue remained in situ. Future studies could therefore benefit from measuring

arterio-venous differences to see if the total output from the adipose tissue is reduced and whether there are depot-specific effects.

To further complicate matters, leptin secretion is pulsatile and has a diurnal pattern of secretion with a peak at night. Leptin can be affected by sleep patterns which are difficult to control even when scheduling follow up testing for the same time of day (Mantzoros *et al.*, 2011). Furthermore, obese individuals may also show a lower percentage reduction compared to lean individuals during calorie restriction (Haluzik *et al.*, 2001). These potential sources of variability may therefore have affected the responses observed in adipose tissue to the calorie restriction. Thus, a combination of factors could be responsible for the wide range of reductions in blood leptin (3 – 64 %), and divergent responses in blood and adipose tissue.

The absence of changes in levels of activation on adipose tissue resident T-lymphocytes may also be a reflection of the relative proportions of effector and T-regulatory cells making up the CD4+CD25+ population. We previously found an increase in FOXP3 transcripts with abdominal obesity suggesting an increase in T-regulatory cells (Chapter 3). Since the preferred fuel of T-regulatory cells is lipid (Michalek *et al.*, 2011; Saucillo *et al.*, 2014), these cells, if indeed present in increased number in adipose tissue may not be so sensitive to the reduction in glucose availability compared to other effector CD4+ T-lymphocytes.

5.4.3 Adipose tissue responses following acute caloric restriction

Several findings in the present study support the notion that a starvation-type response has been induced following 3 days of 50 % calorie restriction characterised by a profound reduction in triglycerides and an increase in NEFA seen in the blood. Adipose tissue gene expression of GLUT4 was reduced following calorie restriction as occurs in human skeletal muscle during starvation (Norton *et al.*, 2007). Following calorie restriction, adipose tissue gene expression of adiponectin and IL-6 were increased, however, there were no corresponding changes in blood concentrations or secretion of either of these parameters by adipose tissue explants. The increase in adiponectin gene expression, but not serum levels following acute calorie restriction is consistent with findings from

Liu *et al.* (2003) and serum changes may not be expected until greater weight loss (>5 % body weight) is achieved (Varady *et al.*, 2009). In the context of obesity, IL-6 is often regarded as a pro-inflammatory cytokine since blood concentrations and adipose tissue gene expression are elevated with increased levels of obesity (Vozarova *et al.*, 2001; Weisberg *et al.*, 2003) and are reduced following longer term calorie restriction (Bastard *et al.*, 2000). IL-6 can have a variety of effects in adipose tissue such as stimulation of lipolysis (van Hall *et al.*, 2003), affecting tissue remodelling (Ohsumi *et al.*, 1994) and inducing insulin resistance in adipocytes (Rotter *et al.*, 2003). Evidence also suggests that IL-6 can have anti-inflammatory properties (Scheller *et al.*, 2011). In the absence of a corresponding increase in secretion, however, it is difficult to determine whether this increase in IL-6 gene expression is meaningful or simply useful as a marker of a response to acute calorie restriction.

5.4.4 Conclusions

Short term severe calorie restriction causes a reduction in serum leptin which was temporally associated with a reduction in the number and levels of activation of CD4+CD25+ T-lymphocytes in the blood. This reduction in T-lymphocyte activation in blood seems appropriate given the limited nutrient availability, and serum leptin is likely to play an important role in conveying this information to the immune system. This confirms previous results seen *in-vitro* and in animal models. However, within subcutaneous adipose tissue there was no such reduction in either leptin or T-lymphocyte activation. These discrepancies may be due to differing responses of different tissues to calorie restriction and/or may depend on the specific mechanisms which explain the reduction in blood leptin.

CHAPTER 6
Effects of diet and activity modification on T-lymphocytes in adipose tissue

6.1 Introduction

Obesity, caused by adipose tissue expansion, is a major risk factor for type 2 diabetes and the latest Health Survey for England figures (2011) suggest that approximately one third of overweight or obese adults exhibit pre-diabetes (Mainous *et al.*, 2014). In addition to adipocytes, adipose tissue contains a whole range of other cell types which form the stromavascular fraction (SVF), including progenitor cells, endothelial cells and a range of immune cells (Weisberg *et al.*, 2003; Xu *et al.*, 2003; Curat *et al.*, 2004; Bourlier *et al.*, 2008; Duffaut *et al.*, 2009a). Current research suggests that the relative proportions of different immune cell populations and interactions between the different cell types within adipose tissue may be important in the development of type 2 diabetes. In particular, relationships have been proposed between an increased number and proportion of macrophages in adipose tissue and insulin resistance (Xu *et al.*, 2003; Apovian *et al.*, 2008), as also demonstrated in Chapter 3, where the proportion of macrophages positively correlated with HOMA-IR. The role of T-lymphocytes in the development of insulin resistance is less clear. In animal models, T-lymphocytes have been shown to increase prior to the onset of insulin resistance and infiltration of macrophages (Kintscher *et al.*, 2008; Nishimura *et al.*, 2009). In humans, differing populations and properties of T-lymphocytes have been identified depending on levels of adiposity and insulin resistance, however, results have not always been consistent. As suggested in Chapter 3 and by Zeyda *et al.* (2011) there may be an increase in anti-inflammatory T-lymphocytes such as T-regulatory cells with increasing levels of adiposity to limit/regulate adipose tissue inflammation. In obese participants with impaired glucose tolerance, however, T-lymphocytes may be skewed towards a proinflammatory Th1 phenotype (Goossens *et al.*, 2012). Another recent study, however, suggests there may be an increase in Th17 T-lymphocytes in 'metabolically abnormal' obese participants (Fabbrini *et al.*, 2013). These discrepancies may be partly due to the different study populations and control groups used for comparison, so it is

currently difficult to understand relationships between levels of glucose control and T-lymphocytes in human adipose tissue. One way to further understand the roles of T-lymphocytes is to compare people with different levels of glucose control with similar magnitude of adiposity. An additional way to investigate the effects of glucose control on T-lymphocytes is to experimentally manipulate glucose control.

Various acute diet and lifestyle changes have been shown to rapidly improve insulin resistance and sensitivity, including very low calorie diets, and low carbohydrate diets, either alone or in combination with increased levels of activity (Clement *et al.*, 2004; Yokoyama *et al.*, 2004; Boden *et al.*, 2005). However, it will be important to avoid a severe calorie deficit and starvation-type response, as observed in Chapter 5, since this may mask the effects of manipulating glucose control. There are, however, diet and activity modification strategies that can be used to rapidly improve glucose control as described below.

One way to manipulate systemic and tissue glucose concentrations is to change the type of carbohydrate according to glycaemic index (GI) and glucose load (GL). These are systems for classifying carbohydrates based on their relative effect on postprandial blood glucose (Jenkins *et al.*, 1981), with GL taking into account portion sizes (Willett *et al.*, 2002). Iso-caloric low GI/GL diets can reduce postprandial glucose and insulin compared to high GI/GL over the course of a day in a laboratory setting (Liu *et al.*, 2012). Further, they are capable of reducing fasting and postprandial glucose responses in free-living individuals with normal glucose tolerance and type 2 diabetes after just 1 week (Brynes A. 2005; Brynes A. 2003). Diet modifications to include lower GI/GL foods are therefore included in recommendations by Diabetes UK for people with type 2 diabetes and impaired glucose tolerance/fasting glycaemia (Diabetes UK, 2013). In the present study, it was anticipated that the manipulation of GI/GL would improve systemic (including adipose tissue) glucose exposure in people with and without IGT.

Another method for improving glucose control that has attracted recent interest is the introduction of low/moderate intensity ‘activity breaks’ throughout the day to reduce bouts of prolonged inactivity (Dunstan *et al.*, 2012; Latouche *et al.*, 2013;

Peddie *et al.*, 2013). These activity breaks are thought to improve glucose control by altering gene expression of proteins involved in carbohydrate metabolism in skeletal muscle, including those involved in glucose transport (GLUT4) (Latouche *et al.*, 2013). The addition of increased regular activity breaks could enhance improvements in glucose control if used alongside low GI/GL modifications to diet, thereby serving as a means of testing the effects of improved glucose control on T-lymphocyte activation in adipose tissue.

The main aim of this study was to determine whether there are important differences in adipose tissue lymphocyte populations and inflammatory properties between people with normal and impaired glucose tolerance who are well matched in terms of adiposity. Additionally, we aimed to determine whether a reduction in total glucose exposure brought about by diet and lifestyle modifications can reduce T-lymphocyte activation in blood and adipose tissue and whether glucose tolerance is a factor in the response.

6.2 Materials and Methods

6.2.1 Experimental design

A total of 19 abdominally overweight/obese males and post-menopausal females aged between 45–65 years with a waist circumference >94 cm, or >80 cm respectively, were recruited. Following a preliminary screen, participants were distributed between 2 groups with either normal glucose tolerance (NGT) or impaired glucose tolerance (IGT); based on the results of an oral glucose tolerance test (OGTT). Participants were recruited from the local community following ethical approval from the South West, Frenchay NHS Research Ethics Committee (REC Reference: 13/SW/0267). The study also received support from the Primary Care Research Network (Ref: 139443), which allowed GP Practices to perform database searches to help identify potentially eligible participants to whom letters could be sent with information about the study. After a 1 week period in which normal diet, daily activities and continuous glucose levels were monitored, participants modified their normal diet by substituting high GI for low GI foods and incorporating 15 additional activity breaks, each lasting 2 minutes, to their daily routine for 10 days to help reduce postprandial glucose levels. Participants attended the laboratory before and immediately after this diet and activity modification period for analysis of immune cell activation and measures of inflammation and metabolism in blood and adipose tissue. An OGTT was also performed at both laboratory visits to assess any changes in glucose metabolism following the diet and activity modification period.

6.2.2 Participants

Participants were abdominally overweight with waist circumference >94 cm (males) or >80 cm (females) (Lean *et al.*, 1995), and reported being weight stable for more than 3 months (no change in weight +/- 3 %) (Stevens *et al.*, 2006). Individuals were excluded from participation if they smoked, had personal history of/existing cardiovascular disease or diabetes or were taking medications that may influence lipid or carbohydrate metabolism or immune system function. Participants were also required to be sedentary i.e. not performing >150 minutes/week moderate intensity exercise for 3 months before the study since regular exercise can affect insulin sensitivity and therefore potential responses to

the new lifestyle modifications in this study may be reduced (Latouche *et al.*, 2013). This was assessed using the health screen questionnaire (Appendix 1).

6.2.2.1 Preliminary screen for eligibility

An OGTT was performed to determine into which group participants would be placed. NGT was defined by a plasma glucose concentration <7.8 mmol/L at 2 hours following a 75 g glucose drink. IGT was defined by a plasma glucose concentration >7.8 mmol/L but <11.1 mmol/L at 2 hours post 75 g glucose drink. Individuals would not be eligible to participate if their plasma glucose concentration was >11.1 mmol/L and would be advised to seek advice from their General Practitioner. (Letters were sent to all participants' GPs to inform them of the results). Glucose values at 2 hours can show great intra-individual variation (Christophi *et al.*, 2013). An average glucose concentration at 2 hours post 75 g glucose drink was therefore taken from the initial screen and the baseline OGTT (first main trial before the intervention) to confirm into which group the participants would be placed. No participants with known IGT identified in searches performed by the GP practices came forward to participate; therefore, all participants in this study with IGT were identified by the preliminary screen. Screening/recruitment continued until there were approximately equal numbers of participants in both groups.

6.2.3 Sample size determination

There are no data regarding changes in T-lymphocyte activation in human adipose tissue in response to diet and exercise modification. However, we observed significant differences in T-lymphocyte activation between lean and obese individuals in our previous study (Chapter 3). A short period of adhering to a low-GI diet can reduce fasting blood glucose levels in patients with type 2 diabetes from 8.0 (±1.0) mmol/L to 5.3 (±0.8) mmol/L and is therefore sufficient to reduce typical values for an obese person to those of a lean person (Brynes *et al.*, 2003). With the additional inclusion of activity breaks, an improvement in glucose control of at least this magnitude would therefore be anticipated. Thus, we anticipate a similar reduction in T-lymphocyte activation in response to diet and activity modification. Our recent data indicate that the CD69 mean fluorescence intensity (MFI) for lean CD4CD69 cells is 288 (+/45 SD) and obese is 411 (+/31

SD) with an effect size of 2.98. Taking into account our range of activation markers, with 95 % power and 5 % alpha, an average of 11 subjects in each group would be required to detect a statistically significant change in T-lymphocyte activation in adipose tissue in response to diet and activity modification. We therefore aimed to recruit 11 participants in each group (NGT and IGT). Descriptive statistics for the participants in NGT and IGT groups are shown in Table 6.1.

	NGT	IGT	*p*-value
Males/Females (n)	2/6	2/9	
Age (y)	56 ± 2	58 ± 1	0.415
Height (m)	1.64 ± 0.04	1.65 ± 1.16	0.868
BMI (kg/m^2)	32.9 ± 1.2	34.2 ± 1.4	0.486
Waist circumference (cm)	102.2 ± 3.6	106.8 ± 2.9	0.338
Hip circumference (cm)	116.4 ± 3.2	117.5 ± 3.3	0.803
FMI (kg/m^2)	14.2 ± 1.3	14.8 ± 1.2	0.750
L1-L4 body fat (%)	43 ± 2	44 ± 2	0.740
RMR (kcal/day)	1404 ± 41	1510 ± 63	0.178
Systolic blood pressure (mm Hg)[1]	122 ± 6	131 ± 6	0.338
Diastolic blood pressure (mm Hg)[1]	80 ± 3	83 ± 4	0.631

Table 6.1. Descriptive statistics of participants classified according to either NGT (n=8) or IGT (n=11) as defined using an OGTT. Mean ± SEM values shown and statistical differences between groups analysed using independent t-tests, *p*-values shown. Abbreviations used; BMI = body mass index, FMI = fat mass index, L1-L4 = central fat mass estimated between Lumbar regions 1-4 using DEXA, RMR = resting metabolic rate. [1]n=7 NGT and n=10 IGT.

6.2.4 Habitual diet, activity and continuous glucose monitoring

For 1 week, subjects were fitted with a combined heart rate and accelerometry monitor (Actiheart™) to determine habitual total energy expenditure (TEE) and physical activity levels (PAL) and were asked to record a corresponding diary of their physical activity during this period to aid its interpretation (General methods, 2.5). TEE was adjusted for measured resting metabolic rate (RMR) as described in General methods, 2.6, which was determined on the first main trial day.

Participants received a diary and a set of digital weighing scales to record their food and fluid intake (General methods 2.7). Diet analysis to estimate participants' energy intake and macronutrient composition was performed using Nutritics diet analysis software (Nutritics Ltd, Ireland). The weighted mean for GI and the total GL were determined using values in the DIOGENES database (Aston *et al.*, 2010). Participants were asked to maintain normal lifestyle habits/routines during this period and were not initially told that the activity monitoring and food diaries would be used to determine the diet during the 10 day diet modification period so as to avoid influencing their habitual routines. The participants' individual diet records were used to identify high GI foods that should be swapped for low GI foods for the diet and activity modification period.

Participants were also fitted with a continuous glucose monitor (CGMS; iPro™2, Medtronic, MiniMed, USA) to measure interstitial glucose levels in abdominal subcutaneous adipose tissue during the monitoring period. An electrochemical glucose oxidase sensor (Enlite®, Medtronic, MiniMed) was inserted using an automated insertion device (Enlite Serter™, Medtronic, MiniMed) and the monitor connected and secured in place with an adhesive plaster (IV3000, Smith and Nephew, UK). The monitor was worn for 6 days allowing the first 5 full days (midnight-midnight) to be recorded during the monitoring week. Participants were instructed to obtain four finger prick samples for glucose per day prior to meals using a point of care glucose monitoring device (XT Contour blood glucose meter, Beyer), and these were used to retrospectively calibrate the CGMS readings. The display of the glucose meter was covered to ensure that participant eating habits were not influenced by their perception of the blood glucose readings. Analysis was performed using the CareLink™ iPro™ Therapy

Management Software for Diabetes (Medtronic, MiniMed) for average daily glucose mean, standard deviation, peak and duration above 7.8 mmol/L.

6.2.5 Diet and activity modification protocol

For 10 consecutive days, participants were given instructions to consume a low GI/GL diet and to incorporate 15 activity breaks lasting 2 minutes throughout each day. During this period, participants wore an Actiheart for the full 10 days to measure changes in activity levels and assess compliance, and were fitted with a CGMS for 5 of the final 6 days of the intervention to measure any changes in tissue glucose concentrations as a result of the intervention.

In order to reduce dietary GI, participants were given their diets from the monitoring week with high GI food items highlighted. Participants were asked to replicate entire days, but to replace highlighted high GI foods with the same weight of lower GI foods and with no additional or higher calorie food items. Specifically they were instructed to swap the main carbohydrate at every meal during each of the 10 days; any cereals were to be swapped to All Bran (Kellogs), any breads to a soya and linseed bread (Vogels) and potatoes/rice/pizza/etc to either whole-wheat pasta or noodles (Sainsburys). Participants were given supplies of these food items to be swapped into the diet to help with compliance. This method had been successful in reducing fasting and postprandial responses in 'healthy' individuals and those with type 2 diabetes (Brynes *et al.*, 2003; Brynes *et al.*, 2005). Additional advice, adapted from www.diabetes.org.uk, was provided for information on further food swaps that could be made (but were not essential) to help reduce GI/GL, such as exchanging sweets/biscuits for chocolate, snacks/puddings for yogurt, dried fruit for fresh fruit, etc. For the first 5 days of the modification period participants recorded which food swaps had been made and, for the final 5 days, made a detailed record of food and fluid intake to interpret alongside the CGMS data.

In addition to the diet modification, participants were instructed to incorporate 15 'activity breaks', lasting 2 minutes during each of the 10 days. Due to the age range of the participants and range of physical capabilities, the activity intensity was self-selected, and participants were instructed that these could be something

as simple as a brisk level walk or walking up and down some stairs continuously for 2 minutes (Thompson et al., 2012). Importantly they were instructed that they did not need to do anything high impact or anything that would cause pain. Regarding the timing of these activity breaks, participants were instructed to take one break before consuming each meal during the day, with another 2 minute activity break 30-45 minutes after each meal, and a third break a further 30-45 minutes later to coincide with raised postprandial glucose vales. The remaining 6 activity breaks were to be spread evenly throughout the day and participants were to ensure that they never spent more than 90 minutes sat in any one place at any time throughout each day. The breaks had to be additional to usual activity and if a 'usual' activity coincided with a scheduled activity break, this would not be counted and they should schedule another break later in the day. Furthermore, participants were advised that fewer breaks lasting for a longer time would not count, even if the total duration added up to 30 minutes. Participants recorded the timing of each activity break during each of the 10 days in a chart found in the new diary. Regular activity breaks have been shown to be as effective at reducing blood glucose as 30 minutes continuous physical activity (Latouche *et al.*, 2013; Peddie *et al.*, 2013) and it was hoped that these alongside the diet modifications would result in the maximum improvement to postprandial glucose concentrations to reduce glucose/insulin exposure to the immune cells in blood and adipose tissue, whilst avoiding a starvation type response as observed in Chapter 4.

6.2.6 Pre-trial requirements

Participants were asked to refrain from performing any strenuous physical activity and consuming caffeine/alcohol for 48 and 24 hrs before trial days, respectively. Trial days were scheduled so participants had been free from any self-reported illness for a minimum of 2 weeks in order to reduce immune system disturbance.

6.2.7 Trial days

On both main trial days participants arrived at the Physiology Resting Laboratory in the morning following a 10hr fast and after consuming 1 pint of water upon waking. To minimise variation in timing of sample collection, participants arrived in the lab at the same time on both trial days (approximately 8.30 am).

Participants were also asked to wear the same clothes as on the main trial day 1 to minimise weight variations due to clothing differences.

6.2.8 Body composition analysis

Measurements of height, waist circumferences and body mass (post- void using a digital balance (TANITA corp. Japan)) were determined on both trial days. Participants' body composition was characterised at baseline using dual energy X-ray absorptiometry (General Methods, 2.4).

6.2.9 Blood and adipose sampling

A cannula was inserted into an antecubital forearm vein and baseline blood sample(s) taken for separation into plasma and serum for analysis of metabolic/inflammatory markers and isolation of peripheral blood mononuclear cells (PBMCs) as described in General methods, 2.9-2.11. A subcutaneous adipose tissue sample (~1 g) was then collected approximately 5 cm lateral to the umbilicus using a needle aspiration technique (General methods, 2.13) and approximately 200mg whole adipose tissue was processed for gene expression analysis (General Methods, 2.19). The remaining tissue was used for preparation of the SVF according to the protocol described in General Methods, 2.15. An OGTT was then performed as described in General Methods, 2.12.

6.2.10 Analysis of SVF and PBMCs by flow cytometry

Flow cytometry (using the FACSverse, Beckton Dickenson) was used to identify CD4+/CD8+ T-lymphocytes (CD45+CD3+ cells) and macrophages/monocytes (CD45+CD14 +) in SVF and PBMCs together with respective levels of activation using protocols and antibodies detailed in General Methods, 2.16-2.18.

6.2.11 RT-PCR

Total RNA was extracted from whole adipose tissue, quantified and 2 µg reverse transcribed to cDNA as described in General Methods, 2.19. Real-time PCR was performed using a StepOne^{TM} (Applied Biosystems) using pre-designed primers and probes obtained from Applied Biosystems for measurement of macrophages (CD68), T-lymphocyte populations and subsets (CD3G, CD4, CD8A, FOXP3, GATA3 and TBX21) and for expression of various metabolic and inflammatory

markers, further details of these are available in General Methods, 2.19. Peptidylpropyl isomerase A (PPIA) was used as an endogenous control (Neville *et al.*, 2011). Results were analysed using the comparative Ct method and expression normalized to an internal calibrator specific to each gene using the formula $2^{-\Delta\Delta C_T}$; where $\Delta\Delta C_T$ is [(C_T gene of interest – C_T PPIA) – lowest ΔC_T for gene of interest] and statistical analysis performed on LN transformed values (Livak & Schmittgen, 2001).

6.2.12 Biochemical analysis

Plasma glucose, serum total cholesterol, HDL cholesterol, triglycerides and CRP concentration and ALT activity were measured according to the methods described in General Methods, 2.10. This analysis was kindly performed by Enhad Chowdhury. Serum insulin was measured by ELISA (Mercodia).

6.2.13 Statistical analysis

All data are presented as mean and standard error of the mean (SEM). Comparisons of participant descriptive statistics available only at baseline, were made between the 2 groups using independent Student's T-tests. Total area under the curve (AUC), homeostasis model assessment for insulin resistance (HOMA-IR) and insulin sensitivity index (ISI comp/Matsuda index) were calculated using formulae described in General Methods, 2.12. Comparisons of repeated measurements of baseline blood and adipose tissue measures from both groups before and after the intervention were made using repeated measures, 2-way mixed-model ANOVAs irrespective of normality (Maxwell and Delaney, 1990, p109). A repeated measures 3-way mixed-model ANOVA was used to examine differences in glucose and insulin responses between the 2 groups at baseline and follow up and the nature of their responses. The Greenhouse–Geisser correction was applied to intra-individual contrasts where $\varepsilon < 0.75$, and for less severe asphericity the Huynh–Feldt correction was selected (Atkinson, 2002). Where the 3-way mixed-model ANOVAs identified significant interactions, multiple t-tests were applied to identify which specific time points were different from baseline and to identify the location of any variance between groups at level time points, with both methods subject to a Holm–Bonferroni correction (Atkinson, 2002). † denotes a main effect of group (i.e. glucose tolerance status), * denotes a main

effect of trial (i.e. baseline vs. follow-up) and # denotes an interaction effect (group x trial). Statistical analysis was performed using Excel 2010 (Microsoft) and SPSS version 20. A *p*-value of $p<0.05$ was considered to be statistically significant, however, other *p*-values close to 0.05 are included for information.

6.3 Results

6.3.1 Changes to activity, diet and continuous glucose monitoring during the intervention period

Measures of activity, diet composition and continuous glucose monitoring from the 1 week monitoring of habitual lifestyle are shown in Table 6.2 together with changes evoked during the 10 day diet and activity modification period. The increases in TEE and PAL during the diet and activity modification period were close to significance (p=0.069, F=3.808 and p=0.070, F=3.778 respectively) and both groups increased their activity by a similar number of calories (no interaction effects identified for either TEE; p=0.713 or PAL; p=0.683) with both groups reporting a similar average number of daily activity breaks (p=0.357).

There were no differences in dietary calorie and macronutrient intake between groups during the monitoring period. During the intervention period, participants in both groups successfully reduced the average daily GI ($p<0.001$, F=135) and total GL ($p<0.001$, F=104) of their diets. Additionally, participants unexpectedly reduced total calorie intake ($p<0.001$, F=55.00) with carbohydrate ($p<0.001$, F=71.77), fat ($p<0.001$, F=26.86) and sugar intake ($p<0.001$, F=44.05) being reduced by a similar amount in both groups.

For 7 participants in each group, CGMS data was available for both monitoring periods. The data set was incomplete either due to failure of participants to perform the finger-prick measurements to calibrate the CGMS monitor or, for 2 participants, due to failure of sensor insertion. CGMS did not detect any differences between the groups when examining average daily glucose mean, SD, peak and duration that glucose remained above 7.8 mmol/L. The increases in TEE/PAL and reduced GI/GL and calorie intake, did not lead to an improvement in glucose control assessed using CGMS during the diet and activity modification period (compared to the monitoring week) according to measures of mean glucose, peak glucose or duration that blood glucose remained above 7.8 mmol/L in either group. The reduction in standard deviation during the intervention period compared to the monitoring week was close to significance across both groups (trial; p=0.096, F=3.27).

	NGT		IGT		2-way Anova Effects
	Baseline	**Intervention**	**Baseline**	**Intervention**	
Measures of activity					
TEE kcal/day	2492 ± 153	2563 ± 116	2469 ± 102	2574 ± 125	
PAL	1.79 ± 0.08	1.84 ± 0.06	1.65 ± 0.06	1.72 ± 0.08	
Reported activity breaks		14 ± 1		13 ± 3	
Measures of dietary intake[1]					
Total kcal	2099 ± 159	1495 ± 172	2289 ± 108	1469 ± 92	*
Carbohydrate (g)	258 ± 23	161 ± 23	249 ± 18	135 ± 6	*
Fibre (g)	19 ± 2	25 ± 4	23 ± 2	24 ± 2	
Fats (g)	84 ± 8	59 ± 8	93 ± 8	61 ± 7	*
Protein (g)	83 ± 4	82 ± 8	93 ± 3	82 ± 3	
Sugars (g)	125 ± 17	87 ± 22	99 ± 9	64 ± 6	*
Total GL	147 ± 12	76 ± 16	132 ± 8	64 ± 4	*
Average GI	58 ± 1	48 ± 1	58 ± 1	49 ± 1	*
Continuous glucose monitoring (n=7 in each group)					
Mean glucose (mmol/L)	5.7 ± 0.1	5.4 ± 0.3	6.2 ± 0.2	6.0 ± 0.2	
SD glucose	0.67 ± 0.07	0.57 ± 0.08	0.79 ± 0.08	0.69 ± 0.09	
Peak glucose mmol/L	7.50 ± 0.21	7.49 ± 0.47	8.36 ± 0.46	7.92 ± 0.42	
Duration >7.8 mmol/L (% of day)	1.3 ± 0.6	2.6 ± 2.0	6.9 ±2.7	4.3 ± 2.2	

Table 6.2. Measures of activity, diet composition and continuous glucose monitoring from the 1 week monitoring of habitual lifestyle and changes evoked during the 10 day diet and activity modification period in participants with NGT (n=8) and IGT (n=11). Mean ± SEM values shown. Results compared using 2-way mixed-model ANOVA; * denotes a main effect of trial (i.e. baseline vs. follow-up); $p < 0.05$. [1] note NGT n=7 and IGT n=9 due to poor dietary records in one or both monitoring periods.

6.3.2 Changes in anthropometrics and clinical measures of glucose control

Baseline and follow-up anthropometric measurements after the 10 day diet and activity modification period, together with both fasting and 'summary' measures of glucose and insulin responses for participants with NGT and IGT are shown in Table 6.3. All participants showed a modest reduction in weight ($p<0.001$, F=75.2) and measurement of hip circumference ($p=0.037$, F=5.101) following the diet and activity modification period. Participants in the IGT group had significantly elevated fasting insulin ($p=0.043$, F=4.83), fasting glucose ($p<0.001$, F=19.14) and HOMA-IR ($p=0.019$, F=6.8) compared to those with NGT. At follow up, the reduction in fasting insulin was close to significant ($p=0.081$, F=3.46). In response to the OGTT, participants with IGT showed elevated glucose at 2 hours ($p=0.002$, F=14.69) which was reduced in both groups at follow-up ($p=0.037$, F=5.26). Matsuda-ISI was reduced in IGT compared to NGT participants ($p=0.003$, F=12.96) and was improved in both groups after the diet and activity modification period ($p=0.014$, F=7.77). Insulin at 2 hours ($p=0.018$, F=7.05), glucose peak ($p=0.001$, F=16.05) and insulin peak ($p=0.026$, F=6.10) were all elevated with IGT and showed no changes in either group after the diet and activity modification period.

The temporal glucose response and t-AUC following the OGTT for the NGT and IGT groups before and after the 10 day diet and activity modification period and are shown in Figures 6.1a and b respectively. The corresponding insulin responses and t-AUC are shown for the normal and impaired tolerance groups in Figure 6.1c and d respectively. Both parameters were increased in the IGT group compared to NGT (insulin $p=0.023$, F=6.44, glucose $p<0.001$, F=31.76) and remained elevated for longer in the IGT group (timepoint x group interaction, $p=0.038$, F=3.78 for insulin and $p=0.001$ and F=7.07 for glucose).

For the temporal glucose response the trial x group interaction was close to significant ($p=0.064$, F=4.01) and total area under the curve interaction was also close to significant; $p=0.072$, F=3.75).

	NGT		IGT		2-way Anova Effects
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	
Anthropometrics					
Mass (kg)	88.7 ± 4.3	87.2 ± 4.3	92.9 ± 3.9	91.6 ± 4.0	*
Waist circumference (cm)	102.2 ± 3.6	101.5 ± 3.6	106.8 ± 2.9	106.7 ± 2.9	
Hip circ. (cm)	116.4 ± 3.2	115.7 ± 3.4	117.5 ± 3.3	116.4 ± 3.4	*
Blood measures of glucose metabolism					
Fasting Insulin (pmol/L)	42 ± 7	37 ± 9	71 ± 10	62 ± 11	†
Fasting glucose (mmol/L)	5.34 ± 0.12	5.32 ± 0.12	6.26 ± 0.18	6.27 ± 0.17	†
Glucose at 2h (mmol/L)[1]	6.52 ± 0.27	6.28 ± 0.39	9.29 ± 0.38	8.04 ± 0.61	† *
Insulin at 2h (pmol/L)[1]	311 ± 68	216 ± 64	1026 ± 197	797 ± 229	†
HOMA-IR	1.66 ± 0.24	1.42 ± 0.34	3.28 ± 0.42	2.89 ± 0.53	†
Matsuda-ISI[1]	5.34 ± 0.81	7.66 ± 1.73	2.14 ± 0.24	2.71 ± 0.33	† *
Glucose peak (mmol/L)[1]	8.73 ± 0.44	9.28 ± 0.48	12.84 ± 0.71	12.31 ± 0.72	†
Insulin peak (pmol/L)[1]	610 ± 101	459 ± 117	1264 ± 207	1224 ± 277	†

Table 6.3. Anthropometric data and both fasting and summary statistics for glucose and insulin during the OGTT in NGT (n=8) and IGT (n=10) participants before and after the 10 day diet and activity modification period. Mean ± SEM values shown. Results compared using 2-way mixed-model ANOVA; † denotes a main effect of group (i.e. glucose tolerance status) and * denotes a main effect of trial (i.e. baseline vs. follow-up); $p < 0.05$. [1]n=7 NGT and 10 IGT due to incomplete data.

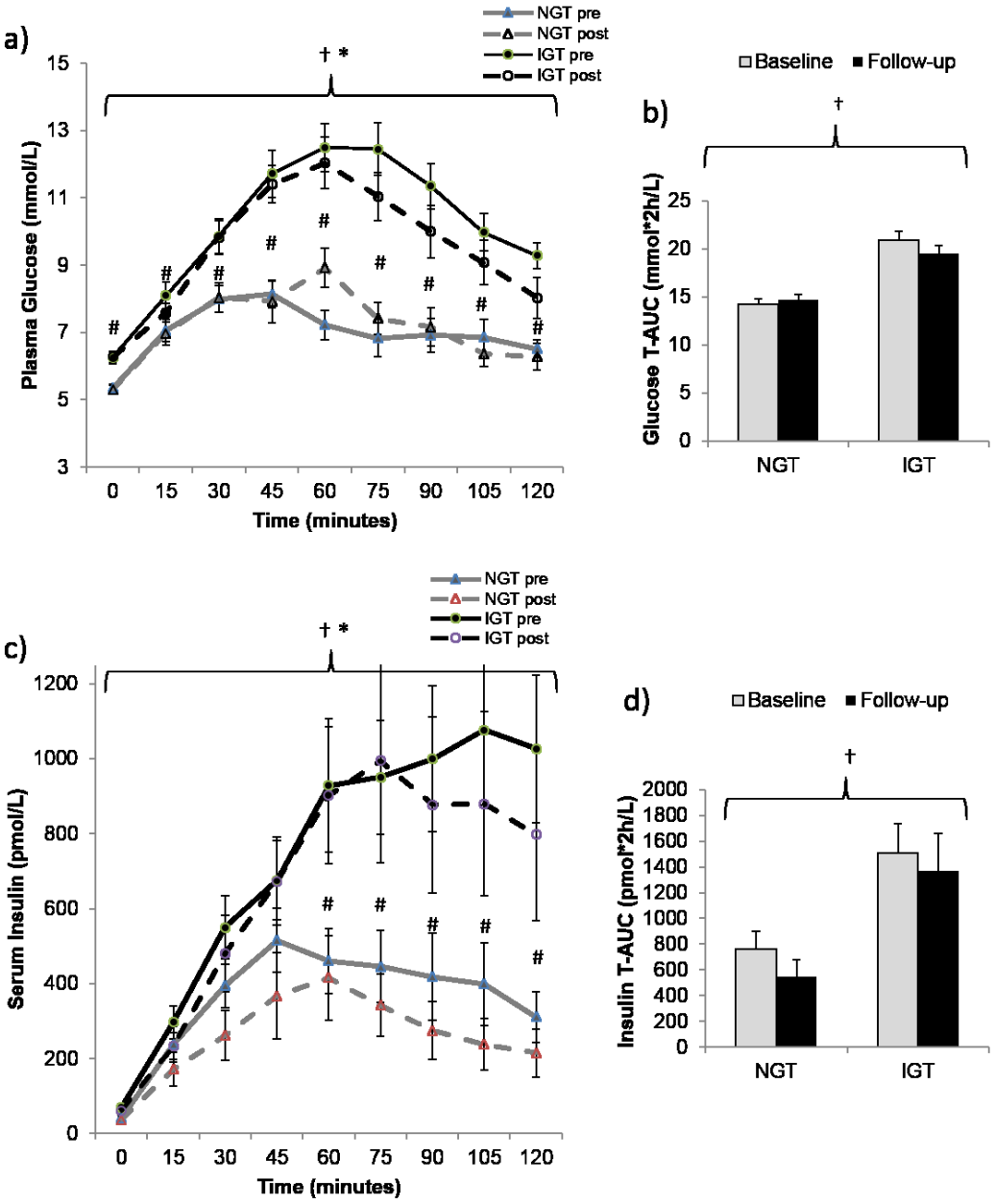


Figure 6.1. Glucose and insulin responses following a 2h oral glucose tolerance test in NGT (n=7) and IGT (n=10) participants before and after the 10 day diet and activity modification period. a) glycaemic responses, b) total area under the curve for glucose, c) insulinaemic responses and d) total area under the curve for insulin in NGT and IGT participants before and after the 10 day diet and activity modification period. Mean ± SEM values shown. Results compared using 2-way or 3-way mixed-model ANOVA; † denotes a main effect of group (i.e. glucose tolerance status) and * denotes a main effect of timepoint (i.e. compared to baseline); p <0.05.

6.3.3 Changes in blood measures of metabolic and cardiovascular risk

Clinical blood measures of metabolic health and cardiovascular risk were measured before and after the 10 day diet and activity modification period and are compared between NGT and IGT participants in Table 6.4. Across both groups there was a significant reduction in Total-Cholesterol (p<0.001, F=32.701), HDL-Cholesterol (p=0.006, F=10.239), LDL-Cholesterol (p=0.012, F=8.196) and triglycerides (p=0.002, F=14.91). There were no main effects of group for any of these 4 parameters and only HDL-Cholesterol showed a significant interaction effect (p=0.050, F=4.553). NEFA was significantly higher in the IGT group (p=0.006, F=10.15) and was increased in both groups in the follow-up trial (p=0.032, F=5.623). ALT showed no main effect of group (p=0.454), however a significant interaction effect was found (group x trial; p=0.050, F=4.404). Concentrations of CRP were not different between groups and did not show any response in either group to the intervention.

	NGT		IGT		2-way Anova Effects
	Baseline	**Follow-up**	**Baseline**	**Follow-up**	
Total Cholesterol (mmol/L)	5.63 ± 0.36	5.03 ± 0.35	5.60 ± 0.21	5.18 ± 0.24	*
HDL Cholesterol (mmol/L)	1.34 ± 0.11	1.32 ± 0.12	1.30 ± 0.05	1.20 ± 0.05	* #
LDL Cholesterol (mmol/L)	3.63 ± 0.30	3.29 ± 0.33	3.53 ± 0.21	3.39 ± 0.20	*
Triglycerides (mmol/L)	1.45 ± 0.29	0.90 ± 0.15	1.69 ± 0.20	1.30 ± 0.19	*
NEFA (mmol/L)	0.47 ± 0.05	0.62 ± 0.08	0.73 ± 0.05	0.83 ± 0.06	† *
ALT IU/L	32.5 ± 6.4	23.1 ± 2.8	31.0 ± 3.9	33.5 ± 4.4	#
CRP (mg/L)	1.86 ± 0.35	1.96 ± 0.39	2.86 ± 0.79	3.15 ± 0.88	

Table 6.4. Fasting blood measures of metabolic and cardiovascular risk before and after the 10 day intervention in participants with NGT (n=7) and IGT (n=10). Mean ± SEM values shown. Results compared using a 2-way mixed model ANOVA; † denotes a main effect of group (i.e. glucose tolerance status), * denotes a main effect of trial (i.e. baseline vs. follow-up) and # denotes in interaction effect (group x trial); p <0.05.

6.3.4 Adipose tissue resident immune cell populations in participants with normal and impaired glucose tolerance and their responses to diet and activity modifications

Gene expression analysis of whole adipose tissue (n=17 paired samples) revealed the presence of T-lymphocytes on the basis of CD3, CD4 and CD8 expression in both groups (Figure 6.2a). There were no differences in levels of expression of these lymphocyte markers according to group and there were no time or interaction effects following the intervention period. Further analysis using CD4+ lymphocyte lineage markers; FOXP3 (T-regulatory cells), GATA3 (Th2) and TBX21 (Th1) were also examined (Figure 6.2b). Each marker was expressed to a similar extent in NGT and IGT participants and GATA3 was significantly increased in both groups following the 10 day diet and activity modification period (p=0.006, F=9.985). Neither FOXP3 nor TBX21, however, changed at follow-up in either group.

There was sufficient adipose tissue from 15 of the 19 participants to perform flow cytometry of the SVF to characterise T-lymphocyte populations at baseline and following the 10 day intervention period. CD4+ and CD8+ T-lymphocyte subsets were further characterised using activation markers CD69 and CD25. The proportions of activated CD4+ and CD8+ T-lymphocytes, i.e. expressing CD25 and CD69 showed no differences according to group or trial following the 10 day diet and activity modification period (Figure 6.2c). Only the proportion of CD8+CD25+ lymphocytes showed a group x trial interaction effect that was close to significance (p=0.064, F=4.11). Furthermore, there were no differences in the level of expression of the 2 activation markers on CD4+ or CD8+ lymphocytes according to levels of glucose tolerance or following the intervention (Figure 6.2d).

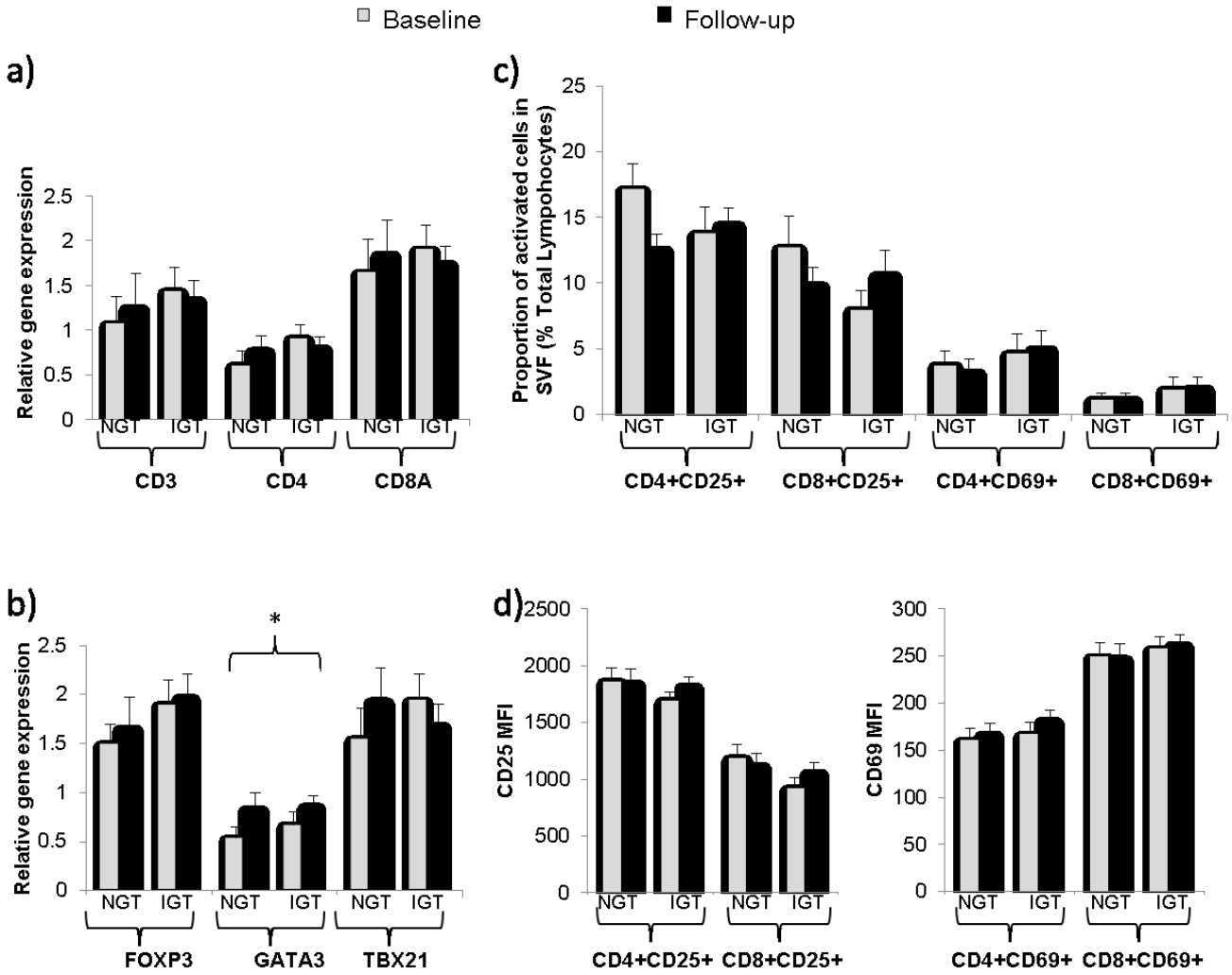


Figure 6.2. Adipose tissue resident T-lymphocyte phenotype and activation before and after diet and activity modification in participants with NGT (n=6) compared to impaired IGT (n=9). a) Relative gene expression of cluster differentiation markers to identify T-lymphocytes and b) CD4 lymphocyte population subsets present in adipose tissue. c) Proportions of activated cells as a percentage of total lymphocytes present and d) their respective levels of expression (mean fluorescent intensity; MFI) of activation markers CD25 and CD69. Results compared using 2-way mixed-model ANOVA, *denotes main effect of trial (i.e. baseline vs. follow-up) $p<0.05$.

6.3.5 Metabolic and inflammatory properties of adipose tissue

To further characterise the properties of adipose tissue from the NGT and IGT populations, the expression of a number of genes encoding proteins involved in insulin signalling were examined (Figure 6.3). No differences in relative gene expression of GLUT4, HSL, PPARɣ and IRS2 were found between the NGT and IGT groups. After the 10 day diet and activity modification period, there was a significant increase in adipose tissue gene expression of HSL in both groups (p=0.005, F=10.60) and the increase in IRS2 was close to significance (p=0.073, F=3.71).

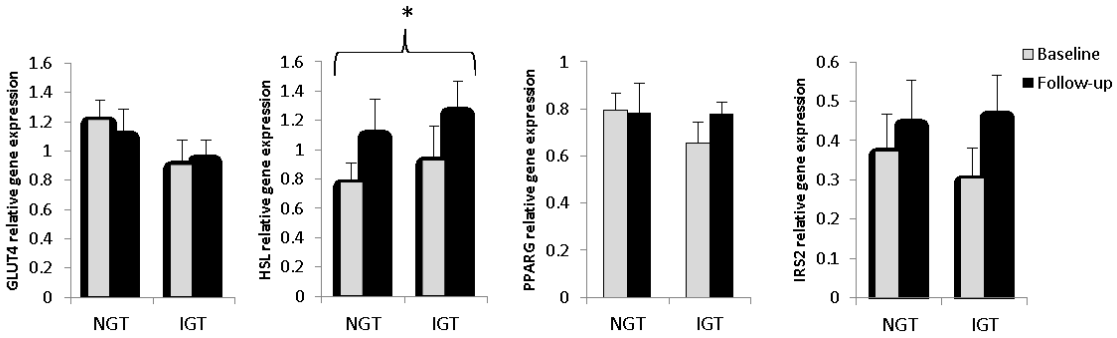


Figure 6.3. Expression of genes related to insulin signalling pathways in participants with NGT (n=7) and IGT (n=10) before and after the 10 day diet and activity modification period. Results compared using 2-way mixed-model ANOVA, *denotes main effect of trial (i.e. baseline vs. follow-up) p<0.05.

A number of pro- and anti- inflammatory cytokines were examined for their gene expression in the adipose tissue from the NGT and IGT populations, before and after the diet and activity modification period (Figure 6.4). Relative gene expression of IL-18 was increased in the IGT group compared to NGT (p=0.008, F=9.423) and the increased leptin gene expression with IGT was close to significance (p=0.06, F=4.14). After the 10 day diet and activity modification period, both groups showed increased gene expression of adiponectin (p=0.007, F=9.77) and IL-6 (p=0.019, F=6.91). The increase in MCP-1 in both groups was close to significance (p=0.09, F=3.29). A group x trial interaction was also identified for IL-1Ra (p=0.033, F=5.52).

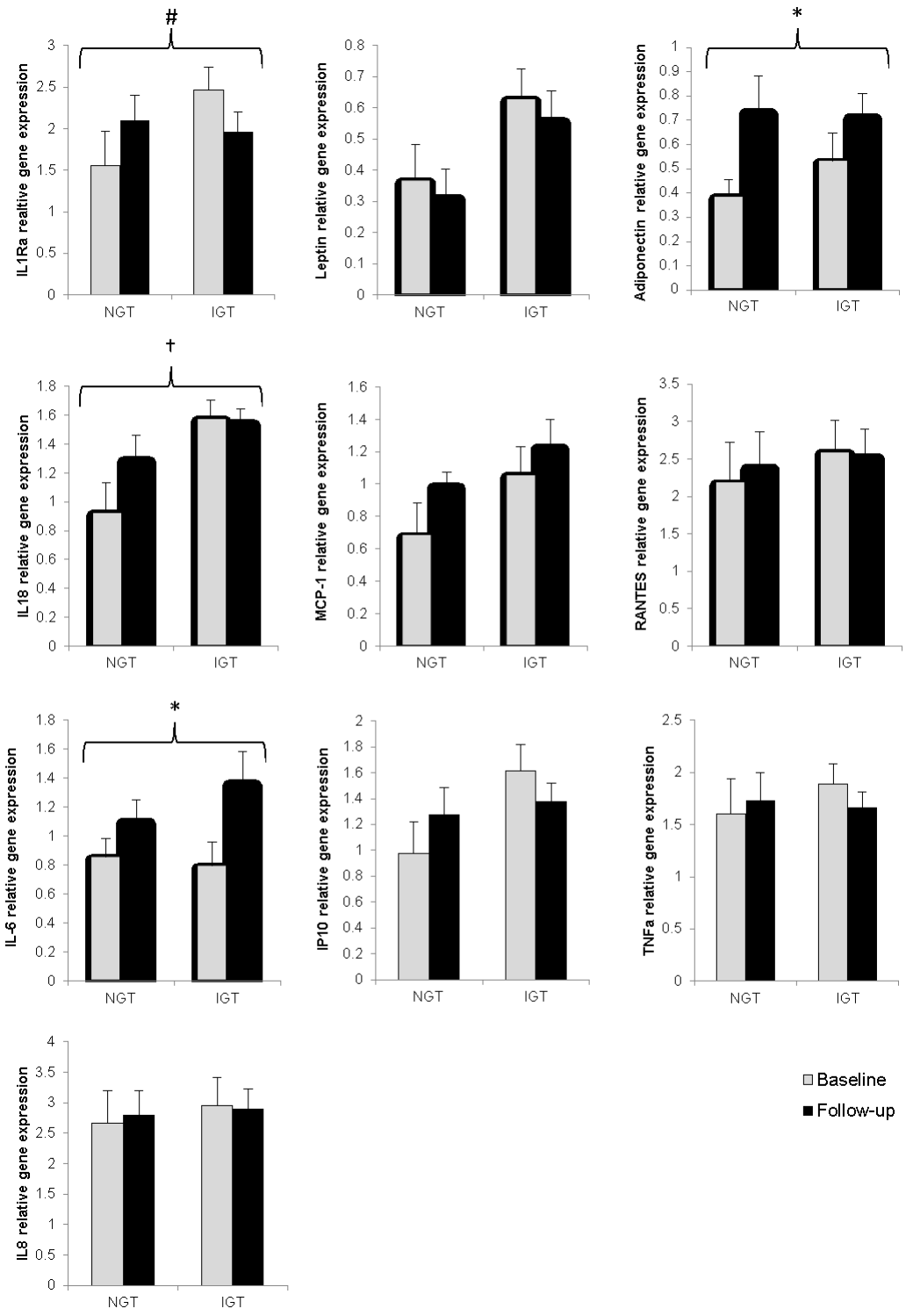


Figure 6.4. Relative gene expression of adipokines before and after the 10 day diet and activity modification period in participants with NGT (n=7) and IGT (n=10). Mean ± SEM values shown. Results compared using 2-way mixed-model ANOVA; † denotes a main effect of group (i.e. glucose tolerance status), * denotes a main effect of trial (i.e. baseline vs. follow-up) and # denotes in interaction effect (group x trial); $p < 0.05$.

6.3.6 Blood immune cell subsets

Blood T-lymphocytes and their activation were examined in participants with NGT and IGT before and after the 10 day diet and activity modification period (Figure 6.5). No significant group or trial effects were identified when examining the proportion of activated CD4+ or CD8+ T-lymphocytes (i.e. those expressing CD25 and CD69). Neither were there any significant group or trial effects for the levels of CD25 or CD69 expression (MFI) on either CD4+ or CD8+ T-lymphocytes. A significant group x trial interaction was identified for the proportion of CD8+CD25+ lymphocytes (p=0.011, F=8.56) and a group x trial interaction for CD25 expression on CD4+CD25+ cells was close to significance (p=0.069, F=3.89).

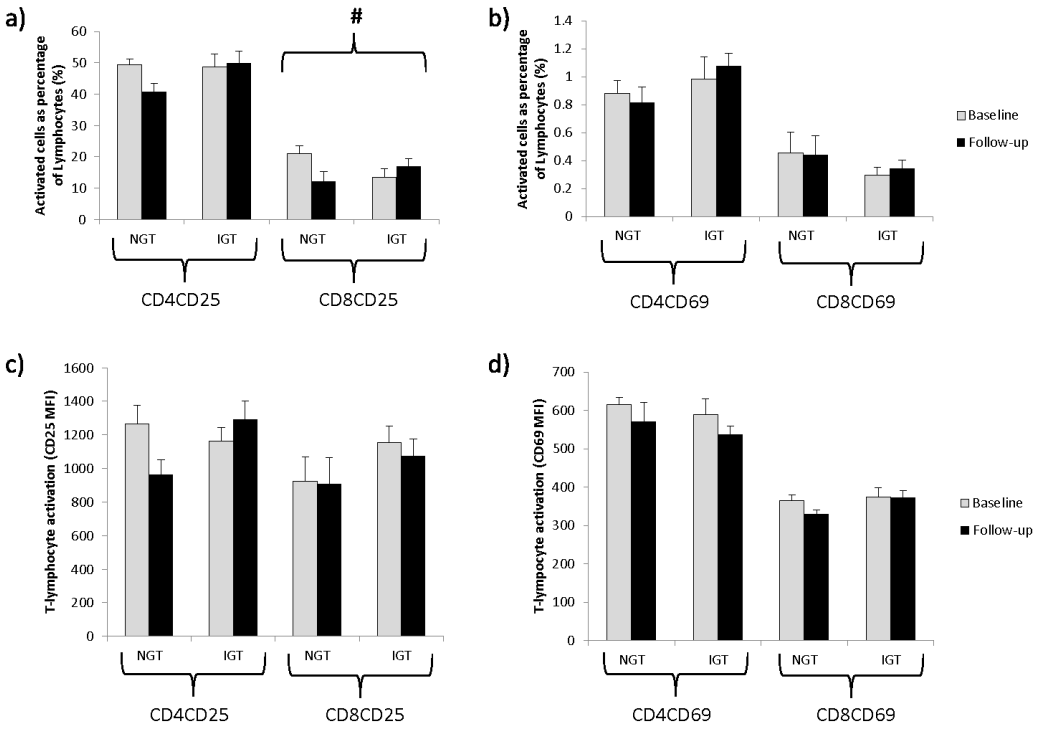


Figure 6.5. Blood immune cell populations in participants with NGT (n=7) and IGT (n=10) before and after the 10 day diet and activity modifications. Proportions of activated CD4+ and CD8+ T-lymphocytes according to expression of a) CD25 and b) CD69. Levels of expression (MFI) of activation markers c) CD25 and d) CD69 on CD4+ and CD8+ T-lymphocytes. Results compared using 2-way mixed-model ANOVA, # denotes trial x group interaction; p<0.05.

6.3.7 Adipose tissue resident macrophages and blood monocytes before and after the 10 day diet and activity modification period

Macrophages were identified in adipose tissue using gene expression analysis of CD68 (n=17 paired samples). No effects of group or trial were identified, however there was a significant group x trial interaction; p=0.029, F=5.86 (Figure 6.6a). At the baseline trial, CD68 expression showed a significant modest correlation with fasting plasma glucose (r=0.519, p=0.023) and total area under the curve for glucose (r=0.485, p=0.042).

Flow cytometry was used to identify adipose tissue macrophages and blood monocytes (CD45+CD14+ cells). Macrophages represented 5 to 21 % of the total cells present in the SVF and no differences in the proportions of macrophages (as a percentage of total cells present in the SVF) or their expression of CD163 or CD14 (MFI) were found based on glucose tolerance status or following the diet and activity modification period (Figures 6.6b-c).

There were also no differences in the proportions of monocytes in PBMCs as a percentage of total cells in the 2 groups or following the diet and activity modification period (Figure 6.6d). Monocyte expression of CD14, CD16, and the leptin and insulin receptors were also examined (Figure 6.6e). The insulin receptor expression on monocytes was significantly reduced in the IGT compared to NGT group (p=0.005, F=11.39) and a group x trial interaction effect was identified for monocyte CD14 expression (p=0.021, F=6.79).

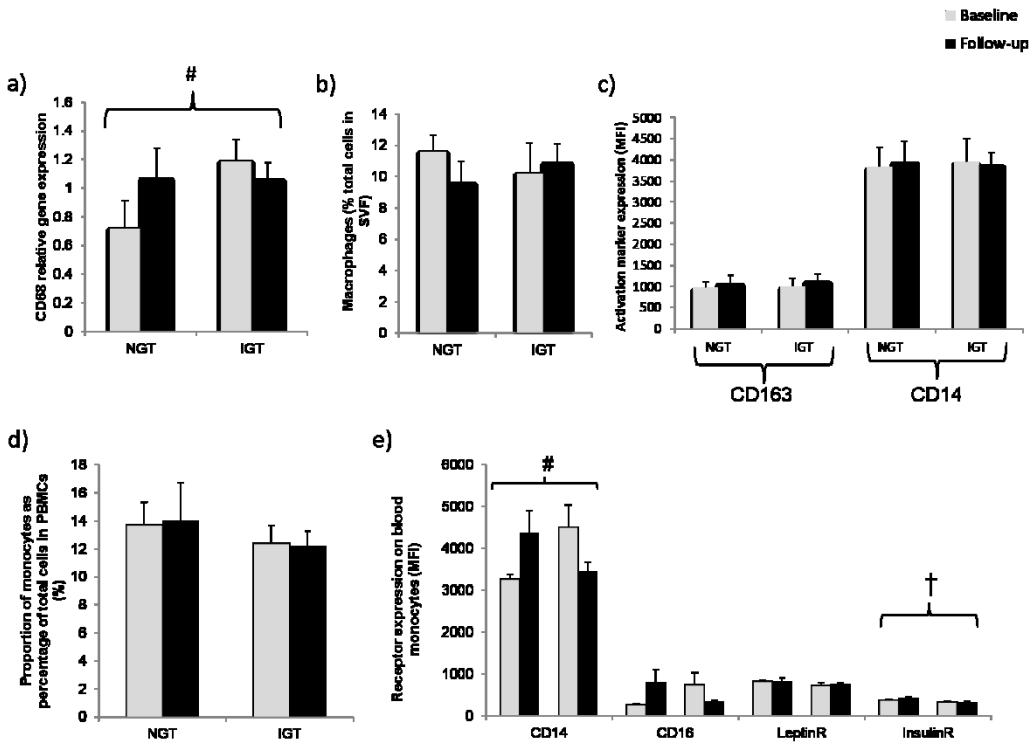


Figure 6.6. Adipose tissue macrophages and blood monocytes and measures of their activation before and after the 10 day diet and activity modification period in NGT and IGT participants. a) CD68 expression in subcutaneous adipose tissue, b) proportion of macrophages (CD45+CD14+ cells) in SVF and c) expression of CD163 and CD14 on adipose resident macrophages in participants with NGT (n=6) and IGT (n=9). d) Proportion of blood monocytes isolated from PBMCs and e) their expression of CD14, CD16 and the leptin and insulin receptors in participants with NGT (n=7) and IGT (n=10). Mean ± SEM values shown. Results compared using 2-way mixed-model ANOVA; † denotes a main effect of group (i.e. glucose tolerance status) and # denotes in interaction effect (group x trial); p <0.05.

6.4 Discussion

The aims of this study were to examine whether there are differences in T-lymphocyte populations and inflammatory profiles in adipose tissue from individuals with normal and impaired glucose tolerance. Further, we aimed to determine whether a reduction in glucose exposure (brought about by a 10 day diet and activity intervention) affects T-lymphocyte activation in blood and adipose tissue and whether glucose tolerance is a factor in the response.

6.4.1 Characterisation of adipose tissue lymphocytes in participants with NGT and IGT

In this study, the two groups of NGT and IGT participants were very well matched according to age, measures of adiposity and clinical blood markers of cardiovascular risk. However, as would be expected based on recruitment criteria, the two groups exhibited very different levels of glucose tolerance, as characterised by fasting measures of glucose and insulin and measurement of glucose and insulin responses during an OGTT. As such, the groups were ideal for examining glucose-related differences in immune cell subsets and activation at the level of abdominal subcutaneous adipose tissue.

Overweight and obese participants with NGT and IGT were very similar in terms of the proportion of CD4+ and CD8+ T-lymphocytes present in abdominal subcutaneous adipose tissue and their respective levels of activation. In Chapter 3, expression of T-lymphocyte markers CD4 and FOXP3 were elevated with increased levels of adiposity from lean through to class I obese, supporting findings from Zeyda *et al.* (2011). Furthermore, activated adipose tissue resident CD4+ and CD8+ T-lymphocytes showed greater expression of activation markers (CD25 and CD69) with increased adiposity and were related to 'anti-inflammatory' IL-1Ra and IL-10 expression. This suggested that there may be protective mechanisms to limit inflammation and insulin resistance with adipose tissue expansion. Notably, the two test groups in this study were very well matched in terms of adiposity and differed only in levels of glucose tolerance, perhaps suggesting that the proportions and activation of T-lymphocytes in adipose tissue may be influenced to a greater extent by adiposity rather than glucose tolerance status. In a study by Fabbrini *et al.* (2013) examining

lymphocyte populations in adipose tissue, a progressive increase in CD4 mRNA was observed across lean, 'metabolically normal obese' and 'metabolically abnormal obese' participants, supporting the findings presented in Chapter 3. They also reported that adipose tissue resident T-lymphocytes from metabolically abnormal obese were greatly elevated and skewed towards a Th17 and Th22 phenotype compared to both other groups. However, the subjects in this group had a significantly elevated BMI even compared to the metabolically normal obese participants, making it impossible to exclude adiposity as a key factor in T-lymphocyte accumulation to adipose tissue.

The mean adiposity of participants with IGT in this study is comparable to those in the study by Goossens *et al.* (2012) where an increased proportion of Th1 transcripts were found compared to lean controls. However, no differences in Th1 transcripts were observed between participants with IGT and NGT where levels of adiposity were similar in the present study. In addition to the fact that adiposity may be the primary factor as discussed above, it is possible that population differences between the studies may account for these discrepancies. Both men and women were included in the current study (~80 % women) as compared to only men being included in the study by Goossens *et al.* (2012). These authors also found that the expression of inflammasome related genes (NLRP3, IL-18 and CASPASE-1) were positively correlated with insulin resistance, which is consistent with the elevated expression of IL-18 in the adipose tissue from participants with IGT in this study. IL-18 is a product of NLRP3 inflammasome activation which contributes to obesity related inflammation and insulin resistance and can induce Th1 T-lymphocyte differentiation (Vandanmagsar *et al.*, 2011). The NLRP3 inflammasome is particularly active in monocytes and macrophages, which although not different in number between groups, may have had different inflammatory phenotypes. Macrophages are often skewed towards an M2 anti-inflammatory profile with obesity, but may represent more of a proinflammatory (M1) phenotype in participants with IGT. In the absence of data to fully characterise the macrophages we can only speculate that there may be an increase in some aspects of inflammation, possibly via the NLRP3 inflammasome, in the subcutaneous adipose tissue of participants with IGT.

Although the participants did not show differences in the proportion of adipose tissue macrophages when grouped according to glucose tolerance (plasma glucose at 2 hours during an OGTT), there was a correlation between the expression of macrophage marker CD68 with fasting blood glucose at baseline. This may suggest that it is the 'chronic' aspects of glucose control that are important in determining the abundance of macrophages in adipose tissue. This supports work presented in Chapter 3 and other published papers (Xu *et al.*, 2003; Apovian *et al.*, 2008), and suggests that macrophages, again, may have a more direct relationship with glucose control than T-lymphocytes.

6.4.2 Changes observed following diet and activity modification

Participants were instructed to exchange high GI foods for low GI foods and to incorporate 15 activity breaks, each lasting 2 minutes, into their daily routines for a 10 day period. Certain aspects of glucose control were found to improve in participants, including a reduction in glucose 2 hours after an OGTT, the Matsuda-ISI index as well as an improvement in fasting insulin that was close to significance. These responses were similar between NGT and IGT participants. There were no changes to the proportion of activated CD4+ or CD8+ T-lymphocytes, or their respective levels of activation. However, there was a significant increase in GATA3 expression (an indicator of Th2 T-lymphocytes) in both NGT and IGT groups along with significant increases in gene expression of adiponectin, IL-6 and HSL in adipose tissue which were also unaffected by glucose tolerance status. Measures of cardiovascular risk; Total-cholesterol, LDL-cholesterol and triglycerides were all reduced in both groups along with a reduction in HDL-cholesterol and increase in NEFA.

Rather than results at follow-up just reflecting effects of regular activity breaks and reduced GI as initially planned, participants also significantly reduced calorie intake over the 10 day modification period. The changes in glucose and lipid control in blood and metabolic and inflammatory changes in adipose tissue observed in the present study may therefore reflect adaptations to a modest calorie deficit. The changes to diet and activity were not sufficient to reduce the percentage of activated CD4+ T-lymphocytes in blood or to affect their expression of CD25 or the insulin receptor, as seen with the severe calorie

restriction employed in Chapter 5. Furthermore, the trend towards an increase in IRS2 expression may suggest an improvement in adipose tissue insulin sensitivity (Shea *et al.*, 2009) rather than a starvation-type response. However, in the absence of Western blot analysis to show either changes in IRS protein expression or its levels of phosphorylation, this cannot be confirmed. Similar increases in adiponectin and IL-6 as observed in Chapter 3, were seen for both NGT and IGT participants together with an increase in GATA3 (and a close to significant increase in MCP-1). These may reflect additional adaptations made by adipose tissue in an extended dynamic phase of weight loss compared to those observed after only 3 days of calorie restriction (Chapter 5). Indeed, the early phase of weight loss appears to be associated with adipose tissue remodeling and inflammation (Capel *et al.*, 2009; Kosteli *et al.*, 2010). The increase in GATA3 may be suggestive of an increased anti-inflammatory Th2 response, which may serve as a mechanism to dampen this inflammation. In the absence of flow cytometry data to support an increase in this specific population in adipose tissue, however, this hypothesis is purely speculative. The early phase of weight loss is also associated with an early increase in macrophage populations in adipose tissue (Capel *et al.*, 2009; Kosteli *et al.*, 2010), which are then reduced with further weight loss and weight stabilisation (Clement *et al.*, 2004; Capel *et al.*, 2009; Kosteli *et al.*, 2010). Murine models suggest that this is to remove excess free fatty acids, which are mobilised at an increased rate during an energy deficit (Kosteli *et al.*, 2010). The trend towards an increase in MCP-1 expression in adipose tissue may support these findings. The divergent changes in adipose tissue macrophages identified by the interaction effect for CD68 may reflect an element of reduced metabolic flexibility with IGT, since CD68 appeared to increase relative to the NGT group. The relative proportion of macrophages in adipose tissue, as assessed using flow cytometry, was not increased following the intervention, however, and perhaps this would have required a more pronounced calorie deficit.

The main purpose of this study was to examine the effects of reduced glucose exposure on adipose tissue resident immune cells and it was hoped that the addition of activity breaks to a low GI/GL diet would provide a good means of maximizing the effect. Due to the unexpected additional reduction in calorie

intake, it is not clear whether the observed effects are due to the reduction in GI/GL, the increased activity, the total calorie deficit or a combination of all three. It would take a great deal of future research to isolate these separate effects. Participants lost on average 1.5 kg over the course of the 10 days and this reflects the reported energy deficit of ~10,000 kcal (assuming 1 g adipose tissue = 7/8 kcal and assuming a similar calorie deficit throughout the intervention (Thompson *et al.*, 2012). It is interesting that there was not a greater improvement in glucose control despite the relatively good adherence to activity breaks, reduced GI/GL and the calorie deficit induced during the intervention. Other studies have reported changes in glucose control where only GI was changed and total calorie intake remained the same (Brynes *et al.*, 2003; Brynes *et al.*, 2005). In these studies, fasting glucose, mean glucose and 24 hour area under the curves were significantly reduced after just 1 week of participants adhering to a low GI diet and were assessed using only CGMS (Brynes *et al.*, 2003; Brynes *et al.*, 2005). These changes were assessed for a single 24 hour period following completion of a 1 week low GI diet by which time a larger improvement may have taken place. In the present study, it was important to measure glucose exposure to the adipose tissue for as long a duration as possible to assess whether glucose control had been improved throughout the 10 day period. It is also interesting that CGMS did not detect any significant differences between the NGT and IGT groups. Much of the literature reports that CGMS can be used to detect differences in participants with NGT and IGT, however, the majority of these studies have been conducted in specifically Asian populations with an average BMI < 26 kg/m^2 (Wang *et al.*, 2012; Chen *et al.*, 2013; Zhou *et al.*, 2013) or <30kg/m^2 (Madhu *et al.*, 2013). With greater levels of obesity as observed in the present study, adipose tissue expansion leads to reduced adipose tissue blood flow at the per gram level, and reduced responsiveness following consumption of a meal (Frayn & Karpe, 2014). This is particularly relevant since CGMS measures interstitial fluid in adipose tissue and this could represent a key issue and source of increased error in measuring differences between NGT and IGT participants and their responses to dietary intervention. This hypothesis is supported by a recent study from Salkind *et al.* (2014) who were also unable to detect differences between normal glucose tolerant and pre-diabetic morbidly obese participants. Further, this may lead to site specific differences in glucose measurements in expanded adipose tissue.

CGMS may therefore not be sensitive enough to detect subtle differences in NGT and IGT participants with BMI over 30 kg/m^2. Although the participants in the study by Brynes *et al.* (2003) had an average BMI over 30 kg/m^2 and were still able to detect changes following 1 week of low GI diet, these participants had type 2 diabetes and so may have been more responsive to a glucose lowering intervention. The participants in the present study still have some degree of ability to maintain glucose homeostasis and if possible it would have been interesting to measure corresponding continuous insulin concentrations for a full picture of their insulin sensitivities during the two monitoring periods.

6.4.3 Conclusions

In this study, participants were recruited to form 2 groups that were well matched in terms of adiposity, age and clinical blood markers of cardiovascular risk and differed only in their levels of glucose tolerance. Adipose tissue resident immune cell populations in participants with NGT and IGT displayed similar properties in terms of proportions of CD4+ and CD8+ T-lymphocytes and their respective levels of activation. The weight loss observed in this study may have masked any effects of low GI and activity breaks due to the added complexity of adipose tissue remodeling and other system adaptations to weight loss. As a result, this study has been unable to address whether glucose *per se* is a key factor in determining levels of T-lymphocyte activation and in turn their potential effects on glucose control. An alternative study design may have included a cross-over of high GI vs low GI diets, either prescribed or given according to energy requirements to produce a more extreme model.

CHAPTER 7
General Discussion

7.1 Overview

The overall aim of this thesis was to further characterise aspects of metabolic and immune system cross-talk in human subcutaneous adipose tissue, with a particular emphasis on the potential role of T-lymphocytes in adipose tissue dysfunction and insulin resistance. To address this aim, properties of T-lymphocyte populations across varying levels of adiposity and insulin resistance were examined together with the effects of acute interventions to examine adipose tissue metabolic and inflammatory responses to feeding, severe calorie restriction and glucose lowering diet and activity. Specifically, in Chapters 3 and 6, participants covering a range of adiposities and levels of glucose control were recruited and T-lymphocytes characterised together with activation status. Relationships were examined between T-lymphocyte properties with well-established physical and blood measures of metabolic health and measures of pro- and anti-inflammatory inflammation in adipose tissue. In Chapter 4 postprandial metabolic and inflammatory responses were examined across varying levels of adiposity to investigate whether the increased metabolic response in blood seen with obesity affects postprandial responses in adipose tissue. In Chapter 3, increased T-lymphocyte activation was identified with increased levels of adiposity. Since T-lymphocyte activation markers CD69 and CD25 can rapidly respond to stimulation in certain situations (Hashemi *et al.*, 1999; Martin-Romero *et al.*, 2000), this represented an exciting opportunity to examine whether the elevated T-lymphocyte activation seen with obesity could be modified through specific interventions. In Chapter 5, effects of a dramatic reduction in leptin, achieved by short-term calorie restriction on T-lymphocyte activation in adipose tissue and blood were examined. In Chapter 6, T-lymphocyte activation was compared between participants with normal and impaired glucose tolerance along with their responses to an intervention aiming to lower daily glucose concentrations.

7.2 Summary of findings

Chapter 3: The impact of adiposity on adipose tissue-resident lymphocyte activation in humans

- With levels of adiposity reflecting lean to common levels of obesity (Class I), adipose tissue expansion is characterised not by an increase in the proportion of activated T-lymphocytes but rather by a stronger state of activation in the T-lymphocytes already expressing CD69 and CD25.
- Adipose tissue resident T-lymphocyte activation was positively related to pro- and anti-inflammatory cytokine production both at the gene expression and secretion level, providing further evidence of attempts by adipose tissue and resident T-lymphocytes to limit pro-inflammatory output from adipose tissue (at least with modestly increased levels of overweight/obesity).
- One of the possible mechanisms that could drive this anti-inflammatory compensation is an increased presence of T-regulatory cells, suggested by increased FOXP3 expression with increased levels of adiposity.
- T-lymphocytes are therefore likely to play a key role in the regulation of adipose tissue inflammation and important adaptations seen even with modestly increased levels of adiposity.

Chapter 4: Metabolic and inflammatory responses to a mixed meal in lean, overweight and obese men

- The same participants from Chapter 3, covering a range of adiposities from lean through to class I obese, demonstrated postprandial responses for glucose and insulin that were increased in line with increased levels of adiposity.
- In adipose tissue, there were significant increases in MCP-1 and IL-6 gene expression and down-regulation of IRS2 at 6 hours after the meal. These responses were broadly similar in lean, overweight and obese middle-aged men in spite of differences in magnitudes of glucose and insulin responses to the meal.
- A single feeding exposure does not appear to provoke a greater inflammatory response in expanded subcutaneous adipose tissue.

Chapter 5: Impact of calorie restriction on leptin and T-lymphocyte activation in blood and adipose tissue in overweight and obese men

- Short-term severe calorie restriction causes a reduction in serum leptin which was temporally associated with a reduction in the number and levels of activation of CD4+CD25+ T-lymphocytes in the blood.
- Within subcutaneous adipose tissue, however, there was no such reduction in either leptin or T-lymphocyte activation.
- Adipose tissue gene expression of GLUT4 was decreased and IL-6 and adiponectin increased following the calorie restriction.
- These discrepancies may be due to differing responses of different tissues to calorie restriction and/or may depend on the specific mechanisms which explain the reduction in blood leptin (e.g., modulating the half-life of leptin in the blood).

Chapter 6: Effects of diet and activity modification on T-lymphocyte activation in adipose tissue

- Adipose tissue resident immune cell populations in participants with NGT and IGT who were well-matched for age and adiposity displayed similar properties in terms of proportions of CD4+ and CD8+ T-lymphocytes and their respective levels of activation.
- Following an intervention that aimed to reduce glucose concentrations and tissue exposure to glucose, T-lymphocyte activation did not appear to be affected in participants with either NGT or IGT.
- The only difference in adipose tissue between the groups was an increased expression of IL-18 in participants with IGT, but this was unaffected by the intervention.
- Following the intervention there was an increase in adipose tissue gene expression of GATA3, adiponectin, IL-6, HSL and a trend towards increased MCP-1 and IRS2 in participants with NGT and IGT.
- Whether adipose tissue resident T-lymphocyte activation is influenced by glucose exposure *per se* remains undetermined and the changes observed in adipose tissue may be due to an unexpected reduction in calorie intake and instead might reflect early changes in dynamic weight loss.

7.3 Impact of adiposity and glucose tolerance on immune cell populations in adipose tissue

The results presented in Chapters 3 and 6 characterised T-lymphocyte populations present across participants with varying levels of adiposity and levels of glucose control respectively. With obesity, the consensus in the current literature appears to suggest that CD4+ and CD8+ T-lymphocytes are increased in adipose tissue (Kintscher *et al.*, 2008; Duffaut *et al.*, 2009b; Zeyda *et al.*, 2011). These studies have typically included obese groups with BMI >40kg/m^2 which gives a good representation of differences in T-lymphocytes at two extremes. Our results presented in Chapter 3 suggest that, even with more modest levels of obesity, important changes in adipose resident lymphocyte populations may be taking place. Although no differences in proportions of CD4+ and CD8+ T-lymphocytes were found, levels of activation were increased in line with levels of adiposity and this activation was related to both pro- and anti-inflammatory properties of adipose tissue. Furthermore, an increase in FOXP3 transcripts was found suggesting there may be an increase in T-regulatory cells with increased levels of adiposity which is in line with findings by Zeyda *et al.* (2011). We did not, however, see any reduction in Th2 transcription with obesity, and this difference may be a factor of the modest levels of obesity in our cohort compared to those examined by Zeyda *et al.* (2011). Together, these results suggest that there are possible anti-inflammatory mechanisms in adipose tissue which may be mediated by T-lymphocytes in order to dampen inflammation with increasing obesity. This may be linked to observations that macrophages also appear to be increased but have an anti-inflammatory phenotype in obesity (Zeyda *et al.*, 2007; Bourlier *et al.*, 2008; Fjeldborg *et al.*, 2014).

Importantly, Chapter 3 captured differences in a 'metabolically healthy' obese population. Few studies have examined differences in T-lymphocyte populations specifically in pre-diabetic populations and it is not clear whether there may be an increase in pro-inflammatory Th1 T-lymphocytes (Goossens *et al.*, 2012) or Th17 T-lymphocytes (Fabbrini *et al.*, 2013). Neither of these studies had compared differences in participants with normal and impaired glucose tolerance but with similar levels of adiposity. This was an aim that we hoped to address in Chapter 6, and there did not appear to be a switch in T-lymphocytes in obese individuals with

IGT who are age and adiposity matched compared to those with NGT. These studies together suggest that adiposity may be more directly linked to the accumulation and properties of T-lymphocytes in adipose tissue than glucose control.

A consistent finding amongst the Chapters presented in this thesis is that the gene expression and proportion of macrophages identified by flow cytometry is more directly related to measures of metabolic heath compared to T-lymphocytes. In Chapter 3, the expression of CD68 and proportion of macrophages in the SVF was elevated with increased adiposity and related to measures of glucose control and clinical blood markers of cardiovascular disease risk. In Chapter 6, comparing immune cells present with NGT and IGT, CD68 expression was related to measures of blood glucose control. Whether macrophages are the cause or consequence of obesity-related insulin resistance is not clear. A short term (28 day) overfeeding study in humans by Tam *et al.* (2010) showed an increase in peripheral insulin resistance in the absence of any changes in adipose tissue macrophages. This suggests that, in humans, macrophages may contribute to the propagation of insulin resistance later in its development/progression. Due to the focus on T-lymphocytes and prioritisation of their markers, it was not possible to characterise whether the macrophages were skewed towards a pro- or anti-inflammatory phenotype and this may have been important, particularly in examining differences between the NGT and IGT populations in Chapter 6. Although the IGT and NGT groups had the same proportions of macrophages present within the SVF, these macrophages may have had very different properties.

It is widely described in the literature that adipose tissue gene expression and secretion of pro-inflammatory cytokines are increased with adiposity and this was observed in Chapter 3. Interestingly, IL-18 was related to both adiposity and T-lymphocyte activation in adipose tissue and its expression was increased in the adipose tissue of participants with IGT compared to those with NGT who exhibited similar levels of obesity (Chapter 6). IL-18 is a product of the NLRP3 inflammasome and is produced/activated mainly in monocytes and macrophages (Guarda *et al.*, 2011). The NLRP3 inflammasome is a large cytosolic protein

complex which includes Caspase-1; an enzyme required for the activation and secretion of pro-inflammatory IL-1β and IL-18 (Grant & Dixit, 2013). It is therefore key in the regulation of inflammation. The NLRP3 inflammasome is activated by DAMPS, ceramides, free/LDL cholesterol, ROS, hypoxia, necrosis which are all increased in obesity and related to the development of insulin resistance (Grant & Dixit, 2013). The work in Chapters 3 and 6 appear to support the involvement of the NLRP3 inflammasome in obesity related inflammation in adipose tissue and a potential involvement in insulin resistance. However, it is unknown whether the increased activity of the NLRP3 inflammasome is a cause or consequence of insulin resistance.

The cross-sectional relationships observed in Chapters 3 and 6 cannot be used definitively to establish whether there is a causal relationship or its direction. Thus, it is difficult to untangle precise mechanisms of cross-talk based on these observations and this requires more direct intervention and the assessment of specific physiological responses.

7.4 Metabolic and immune system adaptations – chronic and acute changes to interventions

To further investigate aspects of metabolic and immune system cross-talk in human adipose tissue, a series of acute interventions were conducted. These included examining metabolic and inflammatory responses in adipose tissue to feeding, short-term calorie restriction to achieve a dramatic reduction in serum leptin and responses of participants with NGT and IGT to glucose lowering diet and activity modification. There was a particular emphasis in the latter 2 studies to examine whether the increased T-lymphocyte activation observed in overweight and obese participants found in Chapter 3, could be modified/reduced to levels of activation observed in lean participants. In the context of relatively short term interventions, T-lymphocytes and levels of activation in adipose tissue appear resistant to change, even though expression of CD25 and CD69 can be rapid to respond to stimuli (Hashemi *et al.*, 1999; Martin-Romero *et al.*, 2000). The main difference compared to the work in this thesis is that these studies were conducted *in-vitro* and this may be a key factor in these discrepancies. With *in-vitro* work, conditions can be manipulated to change one component at a time. However, *in-*

vivo, there are many complex interactions to counteract and maintain homeostasis amongst other adaptations that may take priority and mask any effects.

Although there were no changes in T-lymphocyte activation, the expression of a number of genes encoding specific adipokines were altered following acute feeding and dietary interventions. At 6 hours following consumption of a meal in Chapter 4, increases in IL-6 and MCP-1 gene expression were observed in adipose tissue, although there were no corresponding changes to their secretion *ex-vivo*. Changes in gene expression and secretion may not always coincide with each other since there is regulation of protein translation, post-translational modifications and secretion as well as temporal differences introduced by the sampling framework and methodology. Therefore, the timing of increased gene expression may not coincide with an immediate increase in secretion. In Chapter 4, a further adipose tissue sample at a time-point greater than 6 hours after feeding, allowing time for synthesis and secretion, may have shown a corresponding increase in MCP-1 and IL-6 secretion and would further support these postprandial changes in adipose tissue. It is also possible that earlier changes may have been missed. In this context, it would be useful to examine arterio-venous differences to monitor changes in total adipose tissue output over time. This is particularly relevant since, in Chapter 3, results suggested that there may be a down-regulation of cytokine secretion at the per gram level to potentially limit total inflammatory output to the systemic circulation from the expanded adipose tissue.

Results from Chapters 5 and 6 may be suggestive of different acute immune system responses occurring with differing severity of calorie restriction. Chapter 5 suggested that, with severe calorie restriction, there may be an appropriate degree of 'immune suppression'. In both chapters 5 and 6, changes in adipose tissue gene expression were observed that were both related to metabolism and immune system function. Further alterations to adipose tissue gene expression observed in Chapter 6 may reflect the increased duration of less severe calorie restriction compared to Chapter 5, and may be involved in the dynamic remodelling of adipose tissue in the early stages of weight loss. There are very few studies examining acute changes in adipose tissue following calorie restriction in humans

for direct comparison. The potential roles of immune cells in these particular responses, however, are not clear. No changes in macrophage (CD68) gene expression was observed in Chapter 5 following 3 days of severe calorie restriction which is comparable to results from Clement *et al.* (2004) in participants after 2 days of very low calorie diet. However, 10 days of a more modest calorie deficit may lead to further changes in adipose tissue suggesting an increase in macrophages (Capel *et al.*, 2009; Kosteli *et al.*, 2010), possibly mediated by an increase in Th2 T-lymphocytes (Chapter 6). Several studies of longer duration show improvements in insulin resistance and adipose tissue inflammation, especially after period longer than 28 days (Clement *et al.*, 2004; Capel *et al.*, 2009). Interestingly, in mice, 'yoyo' dieting has suggested that subsequent weight gain following a period of weight loss results in increased levels of insulin resistance compared to those that maintained their increased weight (Anderson *et al.*, 2013). Furthermore, the adipose tissue of the weight-cycled mice contained an increase in both CD4+ and CD8+ T-lymphocytes in adipose tissue and specifically a significant increase in pro-inflammatory Th1 T-lymphocyte associated genes. Interestingly, there were no differences in macrophage accumulation (Anderson *et al.*, 2013). A personal history of weight cycling in humans may therefore also be a factor in inconsistencies observed between people. The most metabolically un-healthy state for adipose tissue may therefore be during dynamic phases of weight gain and weight loss whereby continual structural remodelling is taking place. When weight stability is reached, adaptations to counteract pro-inflammatory output maybe observed, possibly via an increase in anti-inflammatory activity of T-regulatory cells (Chapter 3 and Zeyda *et al.* (2011)) and M2 polarised macrophages (Zeyda *et al.*, 2007; Bourlier *et al.*, 2008; Fjeldborg *et al.*, 2014).

7.5 Limitations

Each of the studies reported in this thesis has tried to capture as much information as possible from the adipose tissue of the participants. The ideal study design would have as many sampling time-points as possible, however, this may yield greater number of artefacts due to changes in adipose tissue physiology induced by repeated biopsies. Often, the bruising following sample collection can be quite large so follow-up samples are always obtained from the opposite side, away from

the initial sample site. With further samples, there is the risk of results being affected by tissue damage and repair and infiltration of immune cells for remodelling and repair – and thus would not represent changes following an intervention.

Clearly one of the main limitations to examining T-lymphocytes in adipose tissue is the limited sample size of the material collected when using needle biopsy, particularly for work with flow cytometry. Especially when considering that the proportion of CD4+ and CD8+ T-lymphocytes represents such a small proportion of cells present within the SVF. Gene expression analysis is frequently used as a surrogate for identifying relative proportions of immune cells in adipose tissue, and are widely compared in the literature (Zeyda *et al.*, 2011; Goossens *et al.*, 2012). Harman-Boehm *et al.* (2007) showed that immunohistological staining for CD68+ cells highly correlates with CD68 mRNA by RT-PCR. Therefore, using gene expression as an indicator of the number of cells may be acceptable to correlate with the number of macrophages. Using flow cytometry, the relative proportions of cells were examined, however, if all cell numbers were equally increased, overall percentages may stay the same and not necessarily give the whole picture of changes occurring in adipose tissue. A recent study by Fabbrini *et al.* (2013) attempted to overcome the issues of small adipose tissue samples for flow cytometry analysis by expanding T-lymphocyte populations isolated from adipose tissue using phytohaemagglutinin and IL-2. However, this may change levels of activation and so may be better for identifying immune cell subsets rather than their properties.

Contamination of adipose tissue with immune cells from peripheral blood is always a possibility with these studies; particularly since there must be a fine balance between the time spent cleaning and processing the adipose samples and the risks of inducing changes to the integrity of the sample. However contamination is unlikely to be a significant factor. A consistent finding amongst the studies presented throughout this thesis and amongst other studies by other groups (Duffaut *et al.*, 2009b) is that the cells identified have very different levels of activation (i.e. MFI for the various markers) and different proportions of immune cells are identified in the 2 different tissue types.

In both intervention studies presented in Chapter 5 and 6, a definitive answer to whether leptin or glucose (respectively) can affect T-lymphocyte activation in human adipose tissue could not be reached. This is partly due to complex whole-body mechanisms that adapt to stresses on the body. In Chapter 6 in particular, despite the diet and activity modifications which should have improved glucose control, only certain aspects of insulin sensitivity assessed using an OGTT were improved. It is possible that the use of a 'hot box' to enable measurement of arterialised blood may have revealed greater differences in glucose concentrations (prior to glucose uptake by the tissues as measured in venous blood). However, it is arguably more useful to examine what is measured in a clinical setting for greater external validity.

7.6 Future work

The results presented in this thesis are all representative of differences observed in subcutaneous adipose tissue with differing levels of adiposity. Visceral adipose tissue is often considered to be more pro-inflammatory in terms of immune cell populations present and its cytokine output. Our results show that subcutaneous adipose tissue is highly dynamic and T-lymphocytes could be involved in important regulatory processes. The subcutaneous adipose tissue should therefore not be overlooked as an important depot when considering the pathology of obesity-related diseases. Comparatively little is known about immune cell populations in gluteo-femoral subcutaneous adipose tissue. Since this depot is associated with protection from obesity related insulin resistance, further investigation of T-lymphocyte populations within this depot may give greater insight into how the immune system may influence adipose tissue properties, particularly since T-regulatory and Th2 cells may have an important role in limiting inflammation in subcutaneous and visceral adipose tissue (Zeyda *et al.*, 2011).

The interesting changes in inflammatory gene expression in adipose tissue during acute periods of calorie restriction also warrant further investigation. There are many studies examining inflammatory changes with long term weight loss, however, our studies and other recent studies suggest there could be some evidence of increases in inflammation with dynamic weight loss (at least in the

short term). The finding in mice that subsequent regain in weight results in an increased accumulation of T-lymphocytes (Anderson *et al.*, 2013) is particularly relevant to investigate in humans since maintaining weight loss is often not achieved and 'yoyo dieting' in humans is associated with increased levels of insulin resistance (Waring *et al.*, 2010).

Gene expression analysis is currently a very useful tool to examine expression profiles relating to a variety of immune cells present in the small adipose tissue masses obtained from biopsy. However, these cells can only truly be confirmed using flow cytometry and immunohistochemistry. Further investigation into how lymphocyte expansion affects cell proportions and activation compared to baseline are warranted, since this would represent a means to potentially further characterise T-lymphocytes in adipose tissue, for example, using intracellular staining for flow cytometry.

Further work is required to address the nature of the 'cross-talk' involving immune cells and adipocytes in human adipose tissue. *In-vitro* work using conditioned media generated from adipocytes, lymphocytes and macrophages from participants covering ranges of adiposities and levels of insulin resistance may be useful to gain further insight into these relationships. Particularly experiments involving cross-over of lean and obese cells and conditioned media, and co-culture experiments will be useful to investigate this cross-talk. *In-vivo*, however, the complex interactions and mechanisms to maintain homeostasis may mean that these are not necessarily a true reflection of *in-vivo* interactions, but *in-vitro* work will certainly identify areas that warrant further investigation.

7.7 Conclusions

The work presented in this thesis has examined aspects of metabolic and immune system cross-talk in human adipose tissue and demonstrates that T-lymphocyte activation in adipose tissue may be an important factor in regulating the increased adipose tissue inflammation seen with modestly increased levels of adiposity. Glucose intolerance does not appear to induce a switch in T-lymphocyte phenotype or activation so the role of T-lymphocytes in insulin resistance is less clear. Interventions including short-term modest and severe calorie restriction did not result in a change in T-lymphocyte activation in adipose tissue. However, inflammatory changes were observed in adipose tissue suggesting that other aspects of the immune system are responsive to metabolic changes. Macrophages may have a more direct relationship with insulin resistance, although the potential involvement of T-lymphocytes in mediating this relationship is not clear.

In conclusion, T-lymphocytes are likely to have a key role in the regulation of adipose tissue inflammation seen with increased levels of adiposity but, based on the observations in this thesis, their role in the development of insulin resistance that often accompanies adipose tissue expansion remains unclear.

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APPENDICES

Appendix 1

HEALTH SCREEN

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continued well-being and (ii) to avoid the possibility of introducing bias into the study outcomes.

Please complete this brief questionnaire to confirm your eligibility to participate:

1. Age…….....

2. Do you smoke Yes ☐ No ☐

3. **A**t present, do you have any health problem for which you are:

(a) on medication, prescribed or otherwise..... Yes ☐ No ☐

(b) attending your general practitioner..... Yes ☐ No ☐

(c) on a hospital waiting list..... Yes ☐ No ☐

4. In the past two years, have you had any illness which require you to:

(a) consult your GP ..... Yes ☐ No ☐

(b) attend a hospital outpatient department..... Yes ☐ No ☐

(c) be admitted to hospital ..... Yes ☐ No ☐

5. Have you ever had any of the following:

(a) Convulsions/epilepsy..... Yes ☐ No ☐

(b) Asthma..... Yes ☐ No ☐

(c) Eczema ..... Yes ☐ No ☐

(d) Diabetes..... Yes ☐ No ☐

(e) A blood disorder..... Yes ☐ No ☐

(f) Head injury ..... Yes ☐ No ☐

(g) Digestive problems ..... Yes ☐ No ☐

(h) Heart problems ..... Yes ☐ No ☐

(i) Breathing problems ..... Yes ☐ No ☐

(j) Problems with bones or joints ..... Yes ☐ No ☐

(k) Disturbance of balance/coordination ..... Yes ☐ No ☐

(l) Numbness in hands or feet ..... Yes ☐ No ☐

(m) Disturbance of vision ..... Yes ☐ No ☐

(n) Ear / hearing problems ..... Yes ☐ No ☐

(o) Thyroid problems ..... Yes ☐ No ☐

(p) Kidney or liver problems ..... Yes ☐ No ☐

(q) Problems with immune system ……...Yes ☐ No ☐

(r) Allergies to any anaesthetics ..... Yes ☐ No ☐

(s) Porphyria ..... Yes ☐ No ☐

(t) Myasthenia gravis ..... Yes ☐ No ☐

(u) Adam's-Stokes Syndrome ..... Yes ☐ No ☐

(v) Wolff-Parkinson-White Syndrome ..... Yes ☐ No ☐

If YES to any question, or if there is any other information you think we should know, please describe briefly (eg to confirm problem was/is short-lived, insignificant or well controlled)

6. Has anyone in your family been diagnosed with any of the above? If so, please specify

7. During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number *–e.g. 2 hours total of moderate intensity exercise per week = 8 x 15 minutes, so write 8*):

a) STRENUOUS EXERCISE (HEART BEATS RAPIDLY)

_____

(e.g., running, jogging, hockey, football, soccer, squash, basketball, judo, roller skating, vigorous swimming, vigorous long distance bicycling)

b) MODERATE EXERCISE (NOT EXHAUSTING)

_____

(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, popular and folk dancing)

c) MILD EXERCISE (MINIMAL EFFORT)

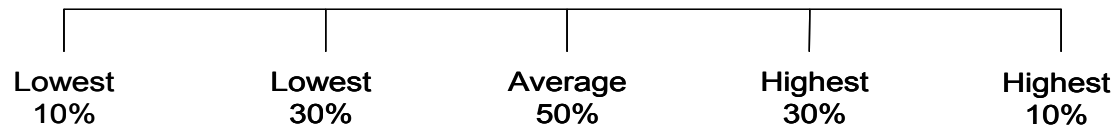
_____

(e.g., yoga, archery, fishing from river bank, bowling, golf, easy walking)

8. Has your weight been stable during the last 3 months.....

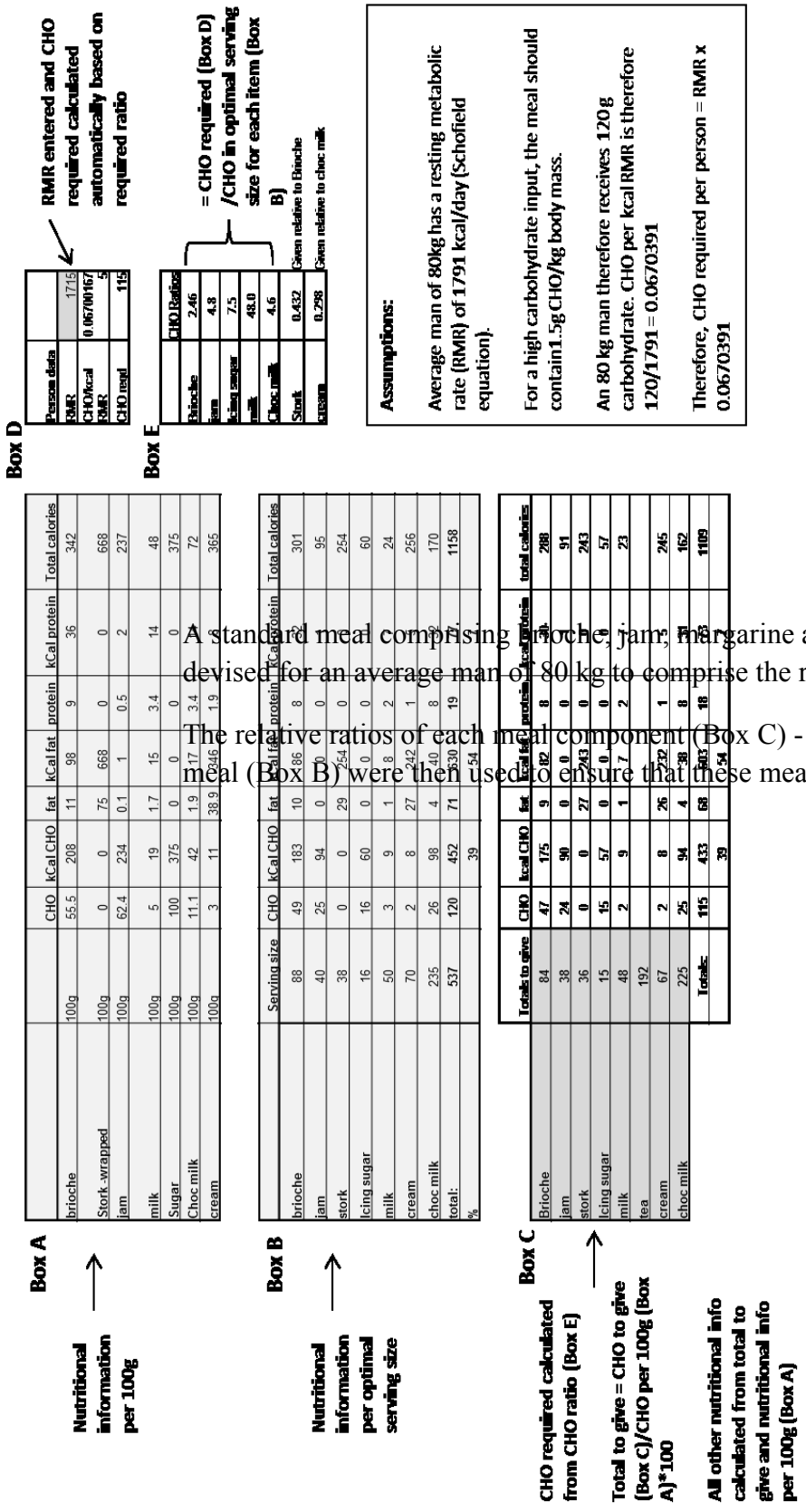
9. Do you know your birth weight? If so, please specify (approximate)…….....

10. When you were 16 years old, please indicate on this scale with an X where you think your weight was in comparison to other children your age:



Appendix 2

Examples of Excel Spreadsheet used to calculate meal composition relative to resting metabolic rate in Study 2 (Chapter 4).

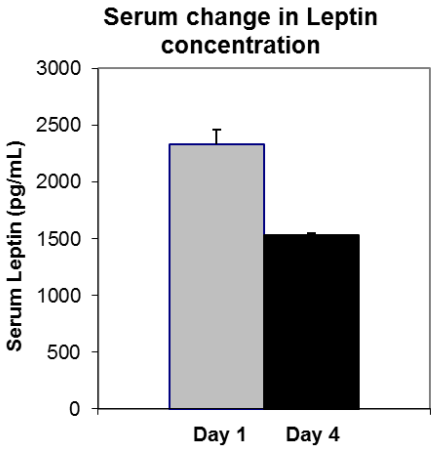


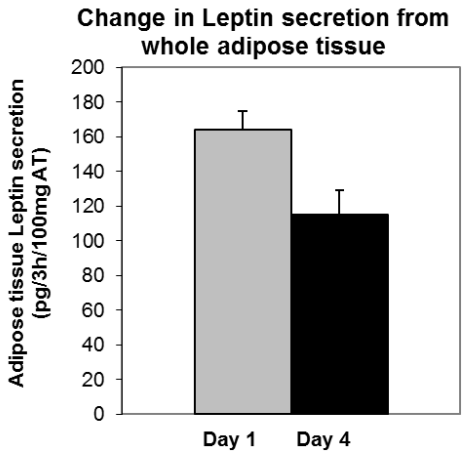
A standard meal comprising brioche, jam, margarine and milkshake (icing sugar, crea
devised for an average man of 80 kg to comprise the required total 120g CHO (1.5g/k

The relative ratios of each meal component (Box C) - i.e. how much each item contrib
meal (Box B) were then used to ensure that these meal components would be in the sa

Appendix 3

Pilot work showing reduction in serum leptin and adipose tissue secretion of leptin after 3 days of 50 % calorie restriction (n=1).





Appendix 4

Diet calculations for participants in Study 3 (Chapter 5).

1) Total energy intake (TEE) and Average energy intake entered and Target daily calories calculated.
2) Diet records entered into Excel with original weights consumed.
3) Conversion factor calculated for each diet record based on total calories in that diet option (3 options from participant's diet record selected for modification and they could choose from any of these during the 3 day 50 % calorie restriction period).
4) Weight of each item to be consumed based on target daily calorie intake (weight consumed in original diet x conversion factor).
5) Food/drink items and weights to be consumed entered into new diary for participants.

measured RMR	1761
TEE (actiheart)	3106
Avg energy In (diet records)	3123
Target daily calories:	1557.25

Target calories for each of the 3 days calculated as (TEE + Avg energy in)/4

conversion =target / calories in option — copy and paste — 0.50 below for all cells

Option 1

Day 1 calories	3100	0.50	1557.25
food/drink	weight	conversion	Weight to be consumed
Tesco gold coffee	4	0.50	2
sweetex x3		0.50	0
Hot water	252	0.50	127
skimmed milk	80	0.50	40
croissant	32	0.50	16
cafatiere coffee	240	0.50	121
sweetex x 3		0.50	0
skimmed milk	48	0.50	24
hovis white bread (toasted)	112	0.50	56
lurpak	28	0.50	14
marmite	4	0.50	2
coffee	250	0.50	126
skimmed milk	82	0.50	41
coffee	248	0.50	125
skimmed milk	80	0.50	40
mcvities chocolate digestives		0.50	4.5
carrot -raw	141	0.50	71
cafatiere coffee	270	0.50	136
skimmed milk	40	0.50	20
Etc…			

conversion =target / calories in option — copy and paste — 0.51 below for all cells

Option 2

Day 2 calories	3073	0.51	1557.25
food/drink	weight	conversion	Weight to be consumed
cafatiere coffee	290	0.51	147
skimmed milk	40	0.51	20
sweetex x3		0.51	0
hovis white bread (toasted)	132	0.51	67
lurpak	12	0.51	6
marmite	7	0.51	4
cafatiere coffee	270	0.51	137
skimmed milk	52	0.51	26
raisins	15	0.51	8
sandwich bread	41	0.51	21
butter	5	0.51	3
marmite	3	0.51	2
cheddar cheese	49	0.51	25
cappacino snackajack caramel	250	0.51	127
(total during day)	75	0.51	38
twix	22	0.51	11
ham	108	0.51	55
fried eggs	118	0.51	60
bread	164	0.51	83
Etc…			

conversion =target / calories in option — copy and paste — 0.41 below for all cells

Option 3

Day 3 calories	3799	0.41	1557.25
food/drink	weight	conversion	Weight to be consumed
coffee	290	0.41	119
skimmed milk	40	0.41	16
shreddies	82	0.41	34
skimmed milk	242	0.41	99
coffee	300	0.41	123
skimmed milk	50	0.41	20
celebrations	64	0.41	26
coke	500	0.41	205
cheddar	143	0.41	59
bread	202	0.41	83
onion	42	0.41	17
coffee	200	0.41	82
skimmed milk	40	0.41	16
tesco cherry low fat yogurt	450	0.41	184
pear	240	0.41	98
grapes	50	0.41	20
special k bars (total during day)	75	0.41	31
bread	100	0.41	41
cheese	49	0.41	20
Etc…			

Appendix 5

Correlations between levels of T-lymphocytes activation in adipose tissue, waist circumference and serum leptin in Study 3 (Chapter 5).

	Waist circumference	**Serum Leptin**
CD4+CD25+ CD25MFI	r=0.683, p<0.05	r=0.629, p<0.05
CD4+CD69+ CD69MFI	r=0.376, p=0.229	r=0.639, p<0.05
CD8+CD25+ CD25MFI	r=0.528, p=0.078	r=0.292, p=0.356
CD8+CD69+ CD69MFI	r=0.642, p<0.05	r=0.566, p=0.055