Body fat distribution and obesity

Body fat distribution and obesity: a comparison of upper and lower body adipose tissue biology in humans

Ioannis G. Lempesis MD

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Abstract

Obesity is a chronic disease that is linked to increased cardiometabolic disease risk, partially attributable to upper body fat accumulation and adipose tissue (AT) dysfunction. AT dysfunction is characterised by impaired adipokine expression/ secretion, chronic low-grade inflammation, decreased AT blood flow (ATBF), mitochondrial dysfunction, and altered oxygen partial pressure.

This thesis investigated the biology of upper and lower body AT in humans with normal weight or obesity, focusing on potential differences in ATBF, the inflammatory phenotype and the oxidative machinery between abdominal and femoral subcutaneous AT. Furthermore, the impact of exposure to various oxygen levels on the inflammatory phenotype of abdominal and femoral adipocytes was examined.

First, we discussed the possible role of AT oxygen partial pressure (pO_2) in the metabolic and inflammatory perturbations associated with obesity. Next, we determined the feasibility to measure abdominal and femoral ATBF with intravascular percutaneous Doppler ultrasound in humans and performed a series of studies to investigate the inflammatory and oxidative phenotypes of abdominal and femoral subcutaneous AT in postmenopausal women with normal weight or obesity. We found that upper and lower body AT and adipocytes have distinct inflammatory signatures. Furthermore, *in vivo* AT oxygen fractional extraction and adipocyte oxygen consumption rates were lower in abdominal compared to femoral AT and adipocytes in postmenopausal women. Although lower in obesity, no significant depot-differences in oxidative phosphorylation (OXPHOS) protein expression and mitochondrial (mt)DNA content were apparent. Finally, we investigated the impact of prolonged exposure (14 days) to various pO_2 levels on adipokine expression and secretion in differentiated adipose tissue-derived mesenchymal stem cells, demonstrating that low physiological pO_2 (5%) decreased gene expression and secretion of several pro-inflammatory factors in both abdominal and femoral adipocytes derived from individuals with obesity but not normal weight.

Overall, this thesis provides important insights into the differences between upper and lower body AT biology, and the impact of oxygenation on the inflammatory phenotype of human adipocytes.

To my parents.

To Time.

"Time

I submit to you my dissertation

Its subject essentially being you

As what I am now

You got it here".

Kiki Dimoula *(prominent Greek poetess and academic) -*

"Greenhouse's Grass", Ikaros Publications, 2005

Translated from Greek by Ioannis Lempesis

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List of definitions/abbreviations

^{133}Xe: $^{133}Xenon$
ABD: abdominal
AT: adipose tissue
ATBF: adipose tissue blood flow
ATGL: adipose triglyceride lipase
ATP: adenosine triphosphate
AUC: area under the curve
BMI: Body mass index
BNIP3: Bcl-2 interacting protein 3
C/EBPα: CCAAT-enhancer binding protein α
CVD: cardiovascular diseases
DAG: diacylglycerol
DNL: *de novo* lipogenesis
DPP-4: dipeptidyl-peptidase 4
DXA: dual X-Ray absorptiometry
ECM: extracellular matrix
EFSD: European Foundation for the Study of Diabetes
EPS: electrical pulse stimulation
ER: endoplasmic reticulum
FAS: fatty acid synthase
FATP/CD36: fatty acid transporters
FE: fractional extraction
FEM: femoral
FFA: fatty acids
FR: fractional release
GIP: glucose-dependent insulinotropic polypeptide
GLP-1: glucagon-like peptide-1
HIF-1α: hypoxia-inducible factor-1 alpha
hMADS: human multipotent adipose-derived stem cells
HOMA: homeostatic model assessment
HSL: Hormone-sensitive lipase
iAUC: incremental area under the curve
IL-6: interleukin 6
IR: insulin receptor
LPL: lipoprotein lipase
MCP-1: monocyte chemoattractant protein 1
MCT: monocarboxylate transporters
mtDNA: mitochondrial DNA
NEFA: non-esterified fatty acids
OXPHOS: Oxidative phosphorylation
PAI-1: plasminogen activator inhibitor 1
PET: Positron emission tomography
PLIN1: Perilipin 1
pO_2: oxygen partial pressure

PPARγ: peroxisome proliferator-activated receptor γ
sAT: subcutaneous adipose tissue
SVF: stroma-vascular fraction
T2DM: type 2 diabetes mellitus
TAG: triacylglycerol
TNF-α: tumor necrosis factor α
VEGFA: vascular endothelial growth factor A
VLCD: very-low-calorie diet

Main Chapters

1 General introduction

1.1 The prevalence and impact of obesity

Obesity is a complex, multifactorial chronic disease, and is characterised by excessive fat accumulation (1). The global increase in the prevalence of obesity embodies a major public health problem, with more than 700 million children and adults impacted worldwide (2-5). Obesity is associated with several complications, including insulin resistance states, type 2 diabetes mellitus (T2DM), cardiovascular diseases (CVD), and various types of cancer (1, 3, 6-10). The obesity-related complications may affect quality of life as well as life expectancy, implying the need to better understanding the pathophysiology of obesity (4, 11). The biological – environmental interaction is likely to be key in the development in obesity, where a wide variety of environmental factors impact energy homeostasis in biologically predisposed individuals(4, 12-15). Extensive research has led to the view that the pathophysiology of obesity and its complications is not only determined by the amount of adipose tissue (AT), but rather by body fat distribution, AT function and disease stage (1, 16-19). These observations have resulted in an increased interest in understanding AT function during the previous decades, unveiling many important properties of a remarkable, previously overlooked organ (20).

1.2 Adipose tissue (dys)function in obesity

A normal function of AT is important for whole-body homeostasis, and impairments in AT function are often present in obesity and associated with obesity-related chronic cardiometabolic diseases (16, 17, 20, 21). Dysfunctional AT in obesity is characterised among others by hormonal/ adipokine dysregulation and a state of chronic low-grade inflammation, adipocyte hypertrophy, and impairments in lipid metabolism (reduced capacity to buffer the daily influx of dietary lipids, impaired regulation of endogenous lipolysis), resulting in ectopic fat accumulation (18, 21-23). Additionally, decreased adipose tissue blood flow (ATBF), mitochondrial dysfunction, and altered oxygenation are aspects of AT dysfunction that are frequently present in obesity, as will be discussed in detail in **Chapter 2** of this thesis.

1.2.1 Adipose tissue cellularity and remodelling

AT, along with other metabolic tissues, facilitates the human body to adapt to various situations, including changes in nutritional status, due to the dynamic changes and remodelling of the tissue (24). Different types of AT have been characterized based on differences in certain adipocyte properties, like mitochondrial abundancy and lipid droplet number into three main categories: white (unilocular/ single lipid droplet and less mitochondria), brown (many smaller lipid droplets/multilocular and more mitochondria) and beige/bright (intermediate phenotype) (20). White AT can be further anatomically divided into various depots, possessing different characteristics. The major fat depots

are the subcutaneous (abdominal and femoral) and visceral (intra-abdominal) AT (16, 25). These AT depots are linked to differential health profiles, as will be discussed in section 3 of this chapter. In brief, abdominal fat accumulation is associated with an increased incidence of obesity-related complications like hypertension and risk of T2DM (19, 26-29). On the contrary, accumulation of fat in the lower body (gluteofemoral AT) is associated with decreased cardiometabolic disease risk (26). AT plays a pivotal role in glucose and lipid metabolism, at least partly via the secretion of a spectrum of hormones (30, 31). In addition to adipocytes, AT consists of multiple cells. Cells that are present in the stroma-vascular fraction (SVF) include stem cells / pre-adipocytes, endothelial cells and immune cells (20).

One of the main functions of AT is the long-term preservation of energy in the form of triacylglycerols (TAG) (32). AT has the capacity to expand, which at the cellular level is achieved by recruiting stem cells / pre-adipocytes from the SVF, resulting in a greater number of adipocytes (hyperplasia), and/or by the enlargement of existing adipocytes (hypertrophy) (33, 34). Interestingly, it has been suggested that there is a set number of pre-adipocytes that can be recruited, which seems to be genetically determined (35). Adipocytes can substantially increase in size but do have a certain expansion limit, implying that these cells have a maximum capacity to store TAG (20, 36-38). Adipocytes are enlarged in most people with obesity, and adipocyte hypertrophy appears to play a key role in AT dysfunction (39). What seems to be equally or even more important than the maximal storage capacity is the ability to dynamically store lipids in the postprandial phase, the so-called lipid buffering capacity, and to release fatty acids under fasting conditions or exercise (40-42).

1.2.2 Adipose tissue as a metabolic organ

1.2.2.1 Adipose tissue lipid and glucose uptake and storage in obesity

Adipocytes are capable of storing lipids via two main processes (43). The first, lipogenesis, is occurring during well-fed / postprandial conditions when adipocytes are taking up dietary lipids from the circulation (43). This process is mediated by the action of lipoprotein lipase (LPL), which is secreted by the adipocytes, and regulated by insulin (44, 45). LPL is further transported to neighbouring capillaries' luminal surface, where it catalyses the hydrolysis of TAG, contained in meal-derived chylomicrons (from the gut) and liver-derived very-low-density lipoproteins, to non-esterified fatty acids (NEFA) (44-46). NEFA enter the adipocytes either by diffusion or via a specialized transporter protein, the fatty acid translocase (also known as CD36) (47). Glucose is also taken up by the adipocytes through glucose transporter 1 (GLUT1) and the insulin-dependent GLUT4 transporters, and can be used to form intracellular TAG (43, 48, 49). Diacylglycerol acyltransferase catalyses the final step for TAG synthesis, the re-esterification of circulating NEFA (50). The second process, likely occurring at a lesser extent in human than in rodent AT, is the *de novo* lipogenesis (DNL). DNL is a complex and highly regulated metabolic pathway in which excess carbohydrate, from the form of acetyl-CoA, is converted into fatty acids that are then esterified to form TAG (43, 48, 49, 51).

Perturbations in adipose tissue lipid uptake and release exist in people with obesity. Postprandial systemic concentrations of NEFA, largely associated with total AT mass, tend to be slightly higher in individuals with obesity (52-55). Impaired NEFA kinetics and lower storage of meal-derived lipids have been reported in people with obesity as compared to individuals with normal weight (52, 55, 56). These observations can be partly explained by lower insulin-mediated LPL activity per unit fat mass in obesity (52, 57). In addition, impaired glucose metabolism appears to be present in obesity as in T2DM due to insulin resistance which leads to decreased AT glucose uptake, which parallels the downregulation of AT GLUT4 production (58-60). However, an *in vivo* study has shown an inverse relationship between fat mass in abdominal subcutaneous and visceral AT depots and the corresponding regional glucose uptake rates per mass unit but showed no differences between individuals with or without obesity (61). Similar findings were shown in women with BMI over 40 kg/m^2, although increased fat mass appears to provide substantial storage for the excess glucose (62).

1.2.2.2 Adipose tissue lipid mobilization in obesity

During the postabsorptive period (i.e., after an overnight fast) and during exercise conditions AT releases fatty acids through activation of lipolysis. This is the result of low circulating concentrations of anti-lipolytic hormones, predominantly insulin, during the fasting state and increased catecholamine concentrations during exercise (48, 63, 64). The main enzyme responsible for TAG hydrolysis to diacylglycerol (DAG) is adipose triglyceride lipase (ATGL) (49). Hormone-sensitive lipase (HSL), displays some TAG hydrolysis activity but primarily hydrolyses DAG (49). The final step of lipolysis, hydrolysis of monoacylglycerol to glycerol and a fatty acid, is catalysed by monoglyceride lipase (49).

AT lipolysis in obesity appears to be impaired, largely due to blunted whole-body catecholamine-induced lipolysis in AT and during fasting state (reduce lipase activity possibly due to elevated insulin) (64-66). This observation may be due to a decreased number and function of β2-adrenoceptors or decreased HSL (but not ATGL) expression and activity (64, 65, 67, 68). A lower expression of aquaporin 7, which facilitates glycerol transport, in obesity may contribute to the reduced lipolytic potential (67, 69). However, it remains unclear whether the blunted AT lipolysis observed in individuals with obesity is the primary defect leading to increased AT storage or, alternatively, if this is an adaptational response to the insulin resistant state often present in obesity (64).

1.2.2.3 Adipose tissue mitochondrial (dys)function in obesity

Mitochondria are crucial organelles that influence many properties of AT, including adipocyte differentiation, adipokine secretion, insulin sensitivity, oxidative capacity, and glucose and lipid homeostasis (70-72). Indeed, mitochondria are involved in many of the key metabolic functions including ATP production through β-oxidation, the tricarboxylic acid cycle, oxidative phosphorylation (OXPHOS) and fatty acid synthesis (generating substrates for DNL), thereby regulating whole-body metabolism (71, 73-

76). Many essential mitochondrial proteins (over one thousand), including OXPHOS proteins, are encoded in nuclear DNA. However, mitochondria possess their own DNA (71, 77). This circular mitochondrial (mt)DNA encodes 13 proteins crucial to OXPHOS (71, 78).

Various levels of evidence show that mitochondrial oxidative metabolism in white AT is altered in obesity (21, 71), and this will be discussed in more detail in **Chapter 2**. In brief, it has been shown that mitochondrial morphology, mass, and function are altered in different AT depots in rodent models of obesity (74, 79-82). Human studies have also reported blunted mitochondrial capacity and reduced expression of genes and proteins related to mitochondrial biogenesis and oxidative metabolic pathways in abdominal AT in obesity, insulin resistant conditions and T2DM (83-90). Reduced expression of these factors has been shown in abdominal adipocytes from individuals with obesity (89). Finally, lower mitochondrial density and oxygen consumption rates were found in abdominal adipocytes derived from individuals with obesity as compared to those with normal weight, independent of adipocyte size (91).

1.2.2.4 Metabolic impairments, lipotoxicity and insulin resistance in obesity

As a response to energy excess and to store the surplus of energy ingested through the diet, AT can expand. However, it has been hypothesised that there is a limit to which it can expand (92). AT adipocytokine derangements associated with low-grade chronic inflammation, mitochondrial dysfunction, and impaired ATBF, amongst other factors, may contribute to alterations in AT metabolism (i.e., lipid metabolism/lipolysis) and the storage capacity of dietary lipids in AT (93-95). As obesity progresses, the daily influx of dietary lipids may exceed storage capacity of AT, resulting in ectopic deposition of lipids in the myocytes, cardiomyocytes, hepatocytes, pancreatic cells, and other cells/organs (96, 97). This deposition of excessive lipids and toxic lipid intermediates (such as ceramides and DAG) in these organs, interferes with cellular homeostasis (97). For example, ectopic fat storage, and more specifically, the accumulation, composition, and localization of bioactive lipid metabolites may interfere with insulin signalling and provoke apoptosis and inflammation, thereby compromising tissue function (97, 98). Insulin resistance may also contribute in worsening ectopic fat accumulation (93). Hepatic lipid accumulation for instance seems to be the result of increased DNL driven by hyperinsulinemia, present in insulin resistance states, and by increased NEFA supply deriving from AT (93, 99, 100). Additionally, skeletal muscle metabolic inflexibility, defined as the reduced ability to respond or adapt according to changes in metabolic or energy demand as well as the prevailing conditions or activity, is often described in individuals with obesity and insulin resistance (93, 94, 101, 102). In this case reduced capacity to oxidize fatty acids upon increased supply, potentially accompanied by mitochondrial dysfunction, contributes to lipid accumulation in muscle and associated metabolic derangements (93).

1.2.3 The secretory function of adipose tissue and the adipocytokine dysregulation and inflammation in obesity

It is well known that adipocytes, and other cells in the AT, express and secrete a variety of signalling molecules, known as adipokines (16, 103). These may act at both the local (autocrine and/or paracrine) and systemic (endocrine) level (16). These factors among others include hormones like leptin, adiponectin, growth factors, components of the renin-angiotensin system and chemoattraction/inflammatory factors, like plasminogen activator inhibitor-1 (PAI-1), interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), dipeptidyl peptidase 4 (DPP-4), and monocyte chemoattractant protein-1 (MCP-1) (16, 20, 103-105). Adipokine expression and/or secretion is altered in states of AT dysfunction and may contribute to obesity-associated diseases, as will be further discussed in Chapter 2 (16, 103).

Classical hormones that are secreted from AT and possess important physiological functions are leptin and adiponectin. For example, leptin is a key regulator of body weight acting as a signal of fat mass to the brain (106-110). Leptin circulating concentrations are elevated in people with obesity, and strongly and positively correlated to white AT mass (109). Leptin is circulating both in free form and bound to proteins (103), and its actions are mediated via binding to leptin receptors (a cytokine family receptor), found in the central nervous system and various peripheral tissues (109, 110). It is an important regulator of food intake and energy expenditure, providing important feedback in relation to energy storage in the body through the hypothalamus, but also poses pleotropic actions in relation to immunity, peripheral metabolism, and among other processes, fertility (103, 111-113). Another example of an adipokine that plays an important physiological role is adiponectin, which is almost exclusively produced by mature adipocytes (103, 114). This adipokine mediates insulin sensitizing, anti-inflammatory and antiapoptotic processes (103, 115, 116). Additionally, it enhances insulin secretion and acts via central mechanisms in the brain to increase energy expenditure, potentially mediating weight loss (103, 117). Interestingly, adiponectin circulating levels are negatively associated with the degree of overweight/obesity and metabolic syndrome, underlying differential regulation of adipokines in relation to adipocyte size (116, 118).

Leptin concentrations are strongly positively correlated to white AT mass, so are increased in obesity (109). As aforementioned, at the same time hypoadiponectinemia is another hallmark of obesity, suggesting a loss of the positive insulin-sensitizing and anti-inflammatory properties (116, 118, 119). It has been further shown that several pro-inflammatory molecules are increased in obesity and related to aspects of the metabolic syndrome, including TNF-α, IL-6, PAI-1, DPP-4, and MCP-1 (16, 20, 103-105).

TNF-α is a classical, pleiotropic pro-inflammatory cytokine, associated with obesity-linked insulin resistance and T2DM (120, 121). Another cytokine potentially involved in insulin resistance is IL-6, which is secreted by many cells, including adipocytes and adipose stromal cells. IL-6 is also linked to disease progression as it may predict T2DM (16, 121, 122). PAI-1 is a physiological inhibitor of activation of plasminogen to plasmin

and increased circulating concentrations can lead to hypo-fibrinolysis and may augment a pro-thrombotic status (103, 123, 124). It has recently been shown that PAI-1 mediates downregulation of adiponectin at least in patients with T2DM and those with the metabolic syndrome (125). DPP-4 is expressed in many tissues including AT and degrades the incretin hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (103, 104, 126). These incretin degrading properties may in part contribute to diabetes pathophysiology (103, 126, 127). Additionally, DPP-4 is expressed in higher levels in AT in obesity and seems to be involved and reflect the AT dysfunction (103, 126, 127). Additionally, DPP-4 may inhibit insulin signalling in skeletal muscle (104). Finally, MCP-1 is a chemokine which plays a role in monocyte and lymphocyte recruitment to sites of injury and infection (16, 128). It is produced by macrophages, endothelial cells, and adipocytes (129, 130), and its expression is closely related to the number of macrophages present in AT (131). It appears that MCP-1 expression increases the expression of macrophage markers (16, 132).

Various factors may contribute to AT inflammation, including saturated fatty acids, endoplasmic reticulum (ER) stress, cell death, and infiltration of certain immune cells (17, 20, 133, 134). Lipids acting as signalling moieties on innate receptors (Toll-like receptors), inflammasomes, or nuclear receptors (peroxisome proliferator-activated receptor – PPARs), and alterations in gut microbiota composition/functionality may link nutrient abundance with inflammation in obesity (17, 20, 133, 134). More recently, evidence has emerged that the amount of oxygen that is present in AT may affect the inflammatory phenotype of AT. Tissue oxygen levels are defined by a delicate balance between oxygen delivery, largely determined by the ATBF, and oxygen demand, primarily determined by mitochondrial oxygen consumption (18, 21, 36). As discussed in detail in **Chapter 2**, the oxygen levels in people of various BMIs and metabolic health status (e.g. with or without type 2 diabetes), as evaluated by a limited number of human *in vivo* studies, range from 3% to 11% O_2 (18, 21, 36). Oxygen levels seem to impact gene expression and secretion of many adipokines and inflammatory factors, and several studies have shown that AT oxygen tension may be altered in obesity (18, 21, 36). The latter will be extensively discussed in **Chapter 2** as there is still uncertainty around this question and the findings are inconsistent regarding the physiological levels of oxygen in AT in individuals with normal weight versus obesity.

1.3 Body fat distribution and impact on cardiometabolic health

Apart from total fat mass, the location where the lipids are stored in is an important determinant of total health status of individuals (19, 26, 135). Even though BMI is highly valuable, especially in epidemiology, it can be considered as a surrogate measure of adiposity. BMI is not always a good predictor of morbidity and mortality risk at an individual level (19, 136). A UK Biobank analysis, including data of >130,000 individuals, underlined the limitation of BMI in defining adiposity, particularly abdominal adiposity, as it has been shown that an increased waist-to-hip ratio was positively associated with excess mortality in individuals with normal weight or overweight (25, 137).

Several studies have presented a clear association between abdominal excess fat accumulation and increased incidence of obesity-related complications like hypertension, and increased plasma TAG concentrations and risk of T2DM development (19, 26-29). Both abdominal subcutaneous AT dysfunction and visceral fat accumulation seem to contribute to the adverse cardiometabolic risk associated with obesity (26). Compared to abdominal subcutaneous AT, ectopic and visceral fat (which can also be considered an ectopic fat depot) appear to be the most harmful, given that ectopic fat and visceral fat are closely associated with insulin resistance and cardiometabolic complications, potentially due an impaired storage capacity of subcutaneous AT (19, 26, 138, 139).

Nonetheless, it is noticeable that lower body (gluteofemoral) fat accumulation possesses protective functional properties and inverse cardiometabolic disease risk (19, 26, 140). Gluteofemoral fat, usually determined by either hip or thigh circumference, or leg adipose tissue mass is associated with an improved lipid profile and positively associated with insulin sensitivity (26, 141-144). The protective functions of lower-body fat are partly explained by differences in adipocyte metabolism (26, 135). Abdominal fat depots have a higher lipid turnover and react vigorously to stress hormones via lipolysis, while gluteofemoral fat has a greater buffering capacity and resistance to catecholamines, reducing lipid overflow and ectopic fat distribution (63, 135, 145-148). Lower lipolysis and higher lipogenesis appear to be present in gluteal and femoral AT depots *in vivo*, suggesting that lower body AT stores fat in a long-term manner (26, 146, 149). Additionally, adipokine expression/secretion between upper and lower body fat depots appears to be different with lower leptin expression in gluteofemoral fat and increased adiponectin levels (26, 135, 150, 151). Finally, it was also shown that some pro-inflammatory cytokines are either negatively associated with the amount of gluteofemoral fat, or are produced less (e.g., IL-6) in gluteofemoral fat depot as compared to abdominal AT (26, 145, 152). Even though it is apparent that differences in metabolic, endocrine, and inflammatory signatures of upper-body fat compared to lower-body fat exist, the exact mechanisms underlying the protective cardiometabolic role of lower-body AT remain to be clarified.

Furthermore, what determines where the excessive calories from the diet are stored, and the mechanisms that underlie inter-individual differences in body fat distribution are complex and remain to be elucidated (19). However, evidence indicates that use of glucocorticoids, genetic make-up, epigenetic mechanisms, and sex hormones are important determinants of body fat distribution (19, 153-156). Importantly, sex differences in body fat distribution are apparent. Women store excess calories predominantly in the subcutaneous lower body AT (gynoid / "pear-shaped" pattern) (25, 157-159). This sequestration of lipids in lower body AT depots in women seems to explain the protection against the development of cardiometabolic diseases (25, 135, 160). However, the reduction in oestrogens' levels during menopause is associated by changes in AT mass and lipid sequestration, pointing towards a key role for sex hormones in the regulation of body fat distribution (25). A shift in fat distribution from a predominant subcutaneous / lower body distribution, towards the android / 'apple-shaped' pattern (typically seen in men) with more visceral AT is seen during menopause transition (161-164). These changes induced by the menopause may explain the

increased cardiometabolic disease risk in postmenopausal compared to premenopausal women (25, 165, 166).

1.4 Metabolically healthy obesity

Even though obesity is related to metabolic complications, a subset (10% to 30%) of individuals with obesity does not present with metabolic impairments (167). There is no formal definition how to clinically distinguish these individuals. However, most studies define this phenotype as the absence of any metabolic abnormality like T2DM, dyslipidaemia, and hypertension in an individual with a BMI > 30 kg/m^2 (168). Nevertheless, this phenotype, often referred to as metabolically healthy obesity (17, 167-169), carries an increased risk to develop CVD and T2DM later in life as compared to individuals with normal weight (170-174). It could be claimed that metabolically healthy obesity is a transient phenotype to "unhealthy" obesity in about 30 - 50% of these individuals (19, 173). In people with metabolically healthy obesity, a more favourable inflammatory profile is observed as compared to metabolically unhealthy individuals with obesity, matched for age, sex, BMI, and fat mass (19, 168, 169, 174). Additionally, less ectopic fat accumulation in liver and skeletal muscle, smaller (supposedly more insulin sensitive) adipocytes, less macrophage infiltration and inflammation in (visceral) adipose tissue seem to be present in the metabolically healthy individuals (19, 168, 175). Finally, people with metabolically healthy obesity show a more favourable body fat distribution pattern, with more abdominal subcutaneous AT and less visceral fat mass (19).

1.5 Main thesis hypotheses and objectives

The overall aim of this thesis was to investigate the differences between upper and lower body AT biology, focusing on blood flow, the oxidative machinery, and inflammatory signatures of abdominal and femoral subcutaneous AT in humans with normal weight and obesity. A second aim was to investigate the effects of prolonged exposure to varying oxygen levels within the physiological oxygen range of human AT on the inflammatory phenotype of abdominal and femoral adipocytes. The specific study objectives and hypotheses related to the studies described in this thesis are explained in the next section.

1.5.1 ATBF Study hypotheses and objectives (Chapter 3)

This was a non-invasive pilot study in healthy volunteers. The aim was to explore intravascular Doppler ultrasound as a proxy, alternative, non-invasive method for measuring ATBF in humans, by establishing the technical feasibility, reproducibility, and ability of the method to detect ATBF changes in response to an oral glucose drink. The main hypotheses were that intravascular Doppler ultrasound is a suitable method to

detect a) an increase in postprandial ATBF and b) higher abdominal than the femoral ATBF. To test these hypotheses, we examined basal and postprandial (after the ingestion of an oral glucose drink) blood flow in abdominal and femoral subcutaneous adipose tissue in healthy men and women.

1.5.2 AdipO$_2$ Study hypotheses and objectives (Chapters 4, 5, and 6)

AdipO$_2$ was a human cross-sectional study to investigate differences in AT physiology between upper and lower body adipose tissue in humans. The study was carried out in well-phenotyped postmenopausal women with normal weight or obesity. The analysis focused on (intra-individual) depot-differences and between-group (obesity vs normal weight) comparisons.

Hypothesis and Objective 1 – inflammatory signatures (Chapter 4)

We hypothesized that the production of inflammatory factors in human AT is higher in upper body as compared to lower body AT. To test this hypothesis, we compared the *in vivo* release of adipokines across abdominal and femoral subcutaneous AT and differentiated adipocytes, investigated AT depot-specific adipocyte morphology and adipokine expression, and examined the expression and secretion of adipokines *in vitro* using differentiated human multipotent adipose-derived stem (hMADs) cells derived from abdominal and femoral AT from the same individuals.

Hypothesis and Objectives 2 – oxidative phenotype (Chapter 5)

We hypothesized that AT oxygen extraction and adipocyte mitochondrial respiration are lower in upper body as compared to lower body AT. To test this hypothesis, we investigated oxygen metabolism *in vivo* by comparing oxygen consumption between upper and lower body subcutaneous AT, by comparing gene/protein expression of oxidative metabolism markers between these AT depots, and by comparing mitochondrial respiration and oxidative metabolism markers in differentiated adipocytes from abdominal and femoral AT derived from the same donors.

Hypothesis and Objectives 3 – impact of oxygen levels on adipocytokines *in vitro* (Chapter 6)

We hypothesized that prolonged (14 days) exposure to low physiological oxygen levels (i.e., 5% O_2) reduce inflammatory gene expression in differentiated human multipotent adipose-derived stem (hMADS) cells, and that this response might be different in differentiated hMADS derived from postmenopausal women with normal weight compared to obesity, and in differentiated hMADS derived from upper and lower body AT depots.

1.6 Thesis outline

In **Chapter 2**, an extensive review of the literature concerning AT pO_2 is provided. Specifically, this chapter discusses the determinants of AT pO_2 as well as the effects of (alterations in) AT pO_2 on glucose homeostasis, lipid metabolism and inflammation. In **Chapter 3** we explored a novel non-invasive Doppler ultrasound technique for quantifying ATBF in abdominal and femoral AT in humans. Next, in **Chapter 4**, we investigated the potential differences in the inflammatory phenotype between abdominal and femoral AT as well as in differentiated adipocytes of postmenopausal women. Therefore, *in vivo* adipokine release was determined across abdominal and femoral subcutaneous AT using the arterio-venous balance technique in postmenopausal women with normal weight or obesity. Furthermore, adipokine expression/ secretion was measured in AT biopsies and differentiated primary human adipocytes derived from the same individuals. In **Chapter 5**, we used a similar integrated approach to investigate *in vivo* oxygen fractional extraction and carbon dioxide fractional release across abdominal and femoral AT in postmenopausal women. Additionally, oxidative signatures were determined in AT biopsies and differentiated primary human abdominal and femoral adipocytes. Finally, we performed *ex vivo* functional experiments to measure mitochondrial oxygen consumption in these adipocytes. In **Chapter 6**, the effects of prolonged physiological oxygen levels exposure on differentiated primary human adipocytes inflammatory signatures are presented. Finally, **Chapter 7** will reflect on the results generated by the studies described in this thesis, discuss the impact of our findings in a broader perspective, and provide directions for future research.

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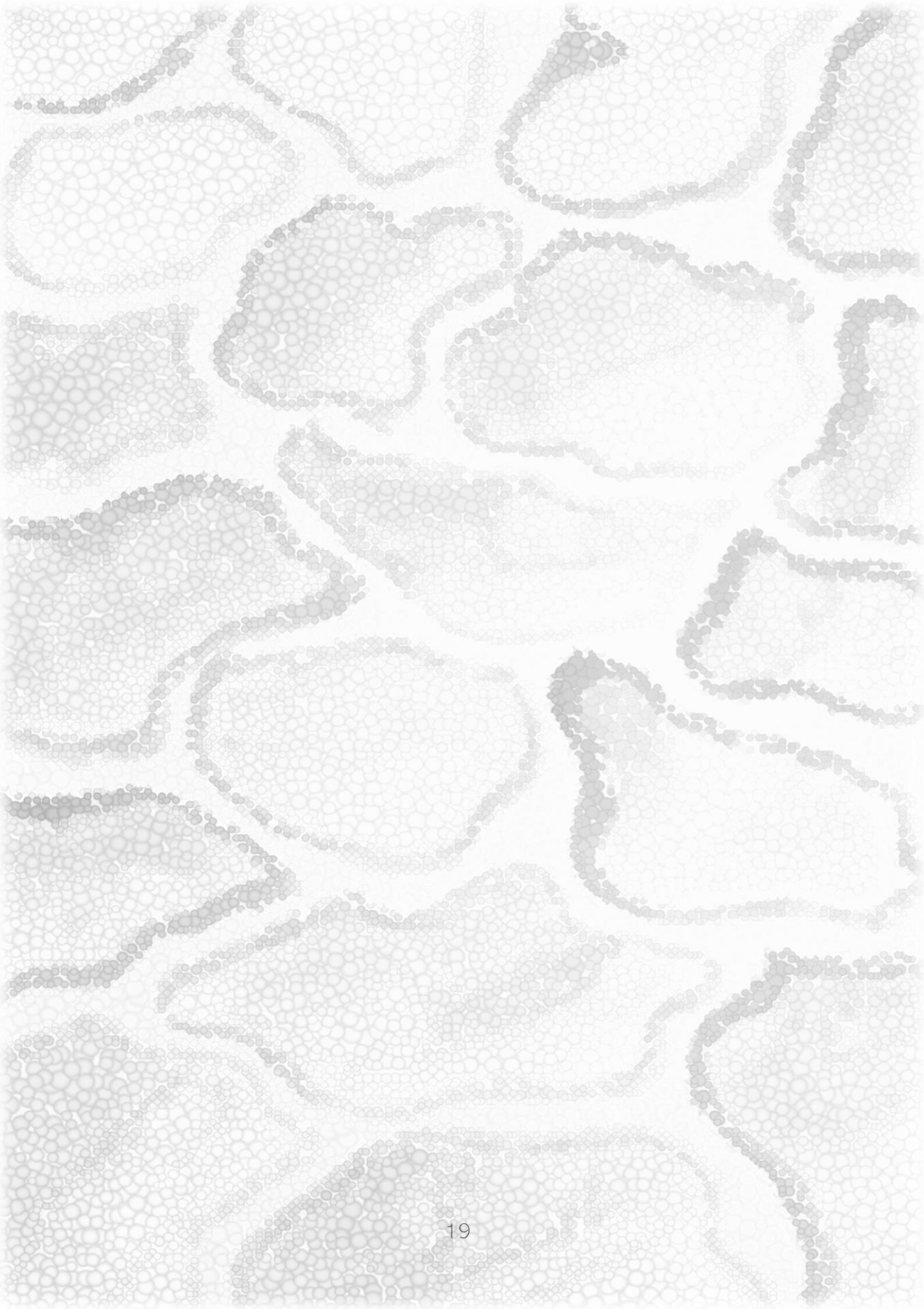

2 Oxygenation of adipose tissue: A human perspective

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Abstract

Obesity is a complex disorder of excessive adiposity and is associated with adverse health effects such as cardiometabolic complications, which are to a large extent attributable to dysfunctional white adipose tissue. Adipose tissue dysfunction is characterized by adipocyte hypertrophy, impaired adipokine secretion, a chronic low-grade inflammatory status, hormonal resistance and altered metabolic responses, together contributing to insulin resistance and related chronic diseases. Adipose tissue hypoxia, defined as a relative oxygen deficit, in obesity has been proposed as a potential contributor to adipose tissue dysfunction, but studies in humans have yielded conflicting results. Here, we will review the role of adipose tissue oxygenation in the pathophysiology of obesity-related complications, with a specific focus on human studies. We will provide an overview of the determinants of adipose tissue oxygenation, as well as the role of adipose tissue oxygenation in glucose homeostasis, lipid metabolism and inflammation. Finally, we will discuss the putative effects of physiological and experimental hypoxia on adipose tissue biology and whole-body metabolism in humans. We conclude that several lines of evidence suggest that alteration of adipose tissue oxygenation may impact metabolic homeostasis, thereby providing a novel strategy to combat chronic metabolic diseases in obese humans.

2.1 Introduction

Obesity is defined as a BMI of 30 kg/m^2 or above and is characterised by excessive expansion of white AT mass. The global trend in the prevalence of obesity represents a major public health problem, with more than 700 million children and adults affected worldwide (1-3). Obesity predisposes to multiple comorbidities, like insulin resistance and T2DM, CVD, and various types of cancer (2, 4-8), although 10% to 30% of the obese individuals will not present with a pathological metabolic profile (9). Nevertheless, this phenotype, often referred to as metabolically healthy obesity (9-12), carries an increased risk to develop CVD and T2DM later in life as compared to normal weight individuals(13-16). This has led to the view that the pathophysiology of obesity and its complications is driven by AT dysfunction rather than an increase in AT mass only (10, 17-19).

Dysfunctional AT is characterized by adipocyte hypertrophy, impairments in lipid metabolism (including a reduced capacity to buffer the daily influx of dietary lipids, thereby contributing to ectopic fat accumulation), decreased adipose tissue blood flow, and a state of chronic low-grade inflammation (Figure 2.1) (18, 20, 21). The presence of AT inflammation in obesity is well established, and several factors that contribute to the sequence of events leading to a pro-inflammatory phenotype of obese AT have been identified, as extensively reviewed elsewhere (10, 22-24). Interestingly, more recent findings have provided evidence that the amount of oxygen in the adipose tissue microenvironment may also impact AT metabolism and inflammation, and AT oxygenation may therefore be a key factor in the pathophysiology of AT dysfunction and related chronic diseases (18, 25, 26).

In this review article, we will consider the role of AT oxygenation in AT dysfunction and its putative impact on the pathophysiology of obesity-related metabolic and inflammatory diseases, with a focus on human studies. First, we will present a brief overview of the different aspects of AT dysfunction in obesity. Thereafter, the oxygenation of AT in obesity as well as the determinants of AT oxygenation will be discussed. Next, the effects of AT oxygenation on tissue (dys)function will be described, particularly in relation to inflammation and substrate metabolism. Finally, we will explore the effects of moderate hypoxia exposure on whole-body physiology in humans.

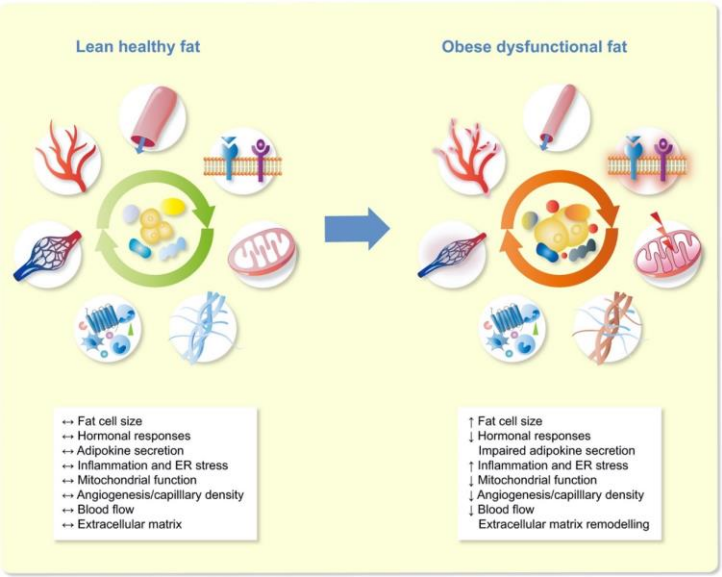

Figure 2.1: Characteristics of lean healthy and obese dysfunctional white adipose tissue. Adipose tissue dysfunction is characterized by adipocyte hypertrophy, impaired adipokine secretion, a chronic low-grade inflammation, apoptosis, extracellular matrix remodelling, hormonal resistance, vascular rarefaction, decreased adipose tissue blood flow and altered metabolic responses, together contributing to insulin resistance and related chronic diseases. ER, endoplasmic reticulum.

2.2 Adipose tissue dysfunction in obesity

One of the main functions of AT is the preservation of energy in the form of TAG in response to a chronic positive energy balance (27). Adipose tissue has the capacity to expand at the cellular level by recruiting stem cells / pre-adipocytes from the SVF resulting in more adipocytes (hyperplasia), or by enlargement of existing adipocytes (hypertrophy) (28, 29). However, it has been suggested that there is a set number of pre-adipocytes that can be recruited, which seems to be genetically determined (30). Adipocytes can substantially increase in size but do have a certain expansion limit, implying that these cells have a maximum capacity of storing TAG (23, 25, 31, 32). What seems to be even more important than the maximal storage capacity is the ability to dynamically store lipids in the postprandial phase, the so called lipid buffering capacity, and to release fatty acids under fasting conditions (33). Hypertrophic AT has been shown to have an impaired capacity to store meal-derived fatty acids (34). As a consequence, more dietary lipids are diverted through the circulation to be stored in other tissues, which results in ectopic fat accumulation when lipid uptake exceeds lipid oxidation (35). The storage of excess lipids in non-adipose tissues in obesity has important metabolic consequences, since this is closely associated with insulin resistance (17, 23, 31). Furthermore, hypertrophic adipocytes are characterized by a pro-inflammatory phenotype, which may further aggravate insulin resistance (24, 36). Importantly, however, adipocyte inflammation also seems essential for healthy adipose

tissue expansion and remodelling (37), suggesting that inflammation is not solely a pathological phenomenon. Noteworthy, medications used to treat type 2 diabetes may alleviate inflammation by reducing hyperglycaemia. However, the anti-inflammatory effects of these agents are inconsistent, and it remains to be established whether their beneficial metabolic effects are mediated via modulation of chronic low-grade inflammation (38).

AT inflammation is not only caused by secretion of pro-inflammatory factors by adipocytes, but is also determined by infiltration of various populations of specialised, pro-inflammatory immune cells (39, 40) such as macrophages (27, 41-44). In rodents, macrophages can be divided into two major phenotypes, the pro-inflammatory M1 and anti-inflammatory M2 macrophages (45). M1 macrophages are activated by damage-associated molecular patterns, cytokines such as interferon-γ, and free fatty acids (FFA), acting as a major source of pro-inflammatory cytokines, including TNF-α, interleukin (IL)-1β, IL-6, IL-12 and IL-23 (44, 46-49). In contrast, M2 macrophages play a role in tissue remodelling, and it seems that the M1/M2 ratio in AT is critical in the pathophysiology of obesity, since M2 macrophages act as regulators and suppressors of inflammation, counterbalancing the pro-inflammatory effects of M1 macrophages (23, 50-53). Noteworthy, the macrophage phenotypes seem more complex, especially in humans where no clear division in M1/M2 macrophages is apparent (54, 55).

In obesity, changes occur not only in the inflammatory cell population, but also in the extracellular matrix (ECM) of adipose tissue. The ECM consists of collagens, glycoproteins, and proteoglycans, providing mechanical support and protection (27, 56). At the same time, the ECM interacts directly with the adipocytes' signalling pathways in a dynamic way, affecting differentiation and expansion of the tissue (22, 57). The latter requires remodelling and alterations in the ECM composition, which has been associated with fibrosis and adipose tissue dysfunction in individuals with insulin resistance (57, 58).

More recently, evidence has emerged that the oxygenation of AT is altered in obesity, which may impact several aspects of AT function and whole-body physiology.

2.3 Altered adipose tissue oxygen partial pressure in obesity

Since alterations in the oxygenation of AT may contribute to AT dysfunction, as will be discussed, AT oxygen partial pressure (pO_2) has been assessed in both rodents and humans. In addition to direct measurements of pO_2, indirect methods to estimate AT oxygenation have been applied (Table 2.1). The direct studies on AT oxygenation have yielded conflicting findings, which are summarised in Table 2.2 (25, 34, 59-66).

Table 2.1: Direct methods and surrogate markers used to determine adipose tissue oxygenation.

Methods applied to assess adipose tissue oxygenation
Direct
Silastic tonometer **(67-70)**
Polarographic micro clark-type electrode **(60)**
Optochemical, continuous monitoring via microdialysis **(59, 71-73)**
Combined oxygen and temperature probe **(57)**
Needle-type fibre-optic oxygen sensor (rodents) **(64, 65, 74)**
Indirect
Arterio-venous difference technique **(34)**
Gene expression of hypoxia-responsive genes / proteins **(63)**
Pimonidazole hydrochloride **(63, 66)**

The presence of hypoxia in obese adipose tissue was originally shown in murine models of obesity (18, 25). Direct measurements of pO_2 using needle-type O_2 electrodes showed that AT oxygenation is lower in *ob/ob*, KKAy and diet-induced obese mice as compared to lean controls (18, 63-66, 75). In line, gene expression of several hypoxia-related genes, including hypoxia-inducible factor-1 alpha (HIF-1α), were also increased. Moreover, using pimonidazole hydrochloride, which stains hypoxic areas, it has been demonstrated that hypoxic areas were more prevalent in AT of obese rodents (18, 63-66, 75). However, it is worth mentioning that these rodent models of obesity are characterized by a rapid and massive gain in adipose tissue mass due to genotype and/or the diet that these animals received, which is not comparable to the more gradual development of obesity in most humans (63-66, 76). So far, not many human studies examining AT pO_2 have been performed, and the results on AT oxygenation are somewhat contradictory (18, 59). The first direct measurements of AT pO_2 in humans were made in individuals undergoing surgery (67, 68). It was found that morbidly obese individuals had lower pO_2 levels in subcutaneous AT (sAT) of the upper arm as compared to lean subjects, determined the morning after surgery(67, 68). However, other studies in which sAT oxygenation has been measured both during and after surgery showed opposite results, with increased or no significant difference in AT pO_2 between obese and lean individuals (69, 70). Notably, these initial studies assessed oxygenation in AT of the upper arm, which is not of crucial importance for whole-body metabolism. Moreover, the O_2 levels measured in these studies could have been affected by the applied anaesthesia, and other factors related to morbid obesity.

Table 2.2: Summary of studies in which adipose tissue oxygenation has been directly measured in humans

Study	Site of sAT	Technique used	Participant'' characteristics	AT pO_2 (mmHg)
Kabon et al.(67) 2004 [a]	Upper arm	Silastic tonometer	*Non-obese*: n = 23 (12 M, 11 F); Age: 44 ± 9 y; BMI: 24 ± 4 kg/m^2	Right arm: 54 (47, 64) [b] Left arm/wound: 62 (49, 68) [b]
			Obese: n = 23 (3 M, 20 F); Age: 44 ± 13 y; BMI: 51 ± 15 kg/m^2	Right arm: 43 (37, 54) [b] Left arm/wound: 42 (36, 60) [b]
Fleischmann et al.(68) 2005	Upper arm	Silastic tonometer	*Non-obese*: n = 15 (10 M, 5 F); Age: 43 y (13) c; BMI: 24 (3) kg/m^2 c	57 (15) [c]
			Morbidly obese: n = 20 (4 M, 16 F); Age: 40 y (11) c; BMI: 46 (7) kg/m^2 c	41 (10) [c]
Hiltebrand et al. (69) 2008	Upper arm	Silastic tonometer	*Lean*: n = 7 (2 M, 5 F); Age: 31 ± 6y; BMI: 22 ± 2 kg/m^2	52 ±10
			Obese: n = 7 F; Age: 37 ± 6y; BMI: 46 ± 4 kg/m^2	58 ± 8
Pasaric a et al.(60) 2009	Abdominal	Polarographic micro clark-type electrode	*Lean*: n = 9 (5 M, 4 F); Age: 22.6 ± 3.3 y; BMI: 22.1 ± 1.0 kg/m^2	55.4 ± 9.1
			Overweight/obese: n = 12 (6 M, 6F); Age: 38.9 ± 15.8 y; BMI: 31.7 ± 1.9 kg/m^2	46.8 ± 10.6
Goossens et al. (59) 2011	Abdominal	Optochemical, measurement system	*Lean*: n = 10 M; Age: 55.8 ± 4.1y; BMI: 23.4 ± 0.3 kg/m^2	44.7 ±5.8
			Obese: n = 10 M; Age: 59.6 ± 3.1y; BMI: 34.2 ± 1.3 kg/m^2	67.4 ±3.7
Lawler et al.(57) 2016	Abdominal	combined oxygen and temperature probe	*Obese Insulin Sensitive*: n=6 (4F/2M); Age: 36 ± 4; BMI: 32 ± 1 kg/m^2	41.1 ± 1.2
			Obese Insulin Resistant: n=6 (6F); Age: 37 ± 3; BMI: 34 ± 2 kg/m^2	37.7 ± 2.4
			Both obese groups: n=12 (10F/2M)	39.3 ± 1.5
			Lean: n=4 (3F/1M); Age: 31 ± 3y; BMI: 23 ± 1 kg/m^2	53 ± 1.9
Kaiser et al.(70) 2016	Right upper arm	Silastic tonometer	*Morbidly obese:* n=7 ; Age: 51 (35–55); BMI: 67 (57–71) kg/m^2	Baseline (kPa): 6.8 (6.2–7.6 [4.4])
			Non-obese: n=7 ; Age: 62 (53–67); BMI: 26.5 (26–29) kg/m^2	Baseline (kPa): 6.5 (6.1–7.5 [3.0])
Vink et al. (71) 2017	Abdominal	Optochemical, continuous monitoring via microdialysis	*Obese/overweight:* n=15 (9F/6M); Age: 50.9±2.1y; BMI: Baseline: 31.1 ±0.6 kg/m^2 End of WS : 27.9±0.5 kg/m^2	Baseline: 51.0±1.6 End of WS: 41.3±3.1

Goossens et al.(72) 2018	Abdominal	Optochemical, continuous monitoring via microdialysis	*Lean Insulin Sensitive*: men n = 7; Age: 58.6 ± 2.6y; BMI 23.0 ± 0.3 kg/m^2	40.4 ± 6.6
			Obese Insulin Sensitive: men n= 7; Age: 55.6 ± 2.8; BMI: 31.7 ± 0.8 kg/m^2	56.1 ± 3.2
			Obese Insulin Resistant: men n=7; Age: 56.9 ± 4.0; BMI: 33.1 ± 1.3 kg/m^2	68.5 ± 4.4
			Obese Insulin Sensitive: women n=7; Age: 50.6 ± 3.0; BMI: 30.5 ± 0.8 kg/m^2	50.8 ± 2.5
			Obese Insulin Resistant: women n=7; Age: 51.0 ± 2.3; BMI: 32.9 ± 1.8 kg/m^2	62.3 ± 5.3
Vogel M et al.(73) 2018	Abdominal & Femoral	Optochemical, continuous monitoring via microdialysis	*Obese/overweight:* n=8 (F); Age:52.5 ±1.8y; BMI 34.4±1.6 kg/m^2	Abdominal : 62.7±6.6 Femoral : 50.0±4.5

[a]: measurements were done the morning after surgery [b]: median with 25th–– 75th percentile [c]: results presented as means (SDs) AT, adipose tissue; BMI, body mass index; pO_2, oxygen partial pressure (mmHg, if not indicated otherwise); kPa: kilopascal; sAT, subcutaneous white adipose tissue; WS, weight stable period after diet-induced weight loss.

Pasarica and colleagues (60) were the first to measure abdominal sAT pO_2 in humans, using a polarographic micro-Clark-type electrode. Overweight and obese participants, including patients with T2DM, had a lower AT pO_2 compared to lean controls, which is in line with findings in rodents (25). Furthermore, it has been found that abdominal sAT pO_2 was higher in obese insulin sensitive and obese insulin resistant as compared to lean subjects, with no significant differences between the obese groups (57). Noteworthy, only four lean individuals were included in the latter study.

The presence of hypoxia in sAT in obesity has been challenged by recent studies in humans. We have demonstrated a higher rather than lower pO_2 in obese subjects with impaired glucose metabolism as compared to lean healthy, age-matched individuals, despite lower adipose tissue blood flow (oxygen supply) in obesity (59). These findings of higher abdominal sAT pO_2 in obesity have been confirmed by very recent studies (71, 72). Abdominal sAT pO_2 was found to be higher in obese insulin resistant as compared to lean and obese insulin sensitive men, with no significant differences in AT oxygenation between obese insulin sensitive and lean insulin sensitive men (72). Furthermore, this study demonstrated that AT oxygenation was positively associated with insulin resistance, even after adjustment for age, sex and body fat percentage, suggesting that AT pO_2 may be more closely related to insulin sensitivity than obesity per se (72). To date, only one study investigated the effects of weight loss on sAT pO_2 in humans. In this study, overweight and obese individuals underwent a dietary intervention, consisting of a 5-week very-low-calorie diet (VLCD, 500 kcal/day) and a subsequent 4-week weight stable diet. It was found that VLCD-induced weight loss markedly decreased abdominal sAT pO_2, which was paralleled by improved whole-body insulin sensitivity (71).

The striking differences in findings on sAT pO_2 between studies may be attributed to differences between study populations in terms of the onset and physical history (e.g. weight cycling) of obesity and other subjects' characteristics (e.g. age, sex, ethnicity,

presence of T2DM), the sAT depot studied, and variation in the methodology used (25, 59, 60).

In addition to direct measurements of sAT pO_2 in humans, several studies have used alternative approaches to indirectly estimate tissue oxygenation, including metabolic profiling of sAT *in vivo* and the assessment of hypoxia-responsive AT gene expression. Hodson and co-workers (34) have measured metabolic fluxes across abdominal sAT *in vivo* in lean, overweight and obese humans, and their findings strongly argue against any functional consequences of AT hypoxia in obesity; in fact, the opposite might be true. More specifically, these authors demonstrated that the fasting lactate-to-pyruvate ratio, which is a potential metabolic signature of 'hypoxia', in arterial blood, was inversely correlated with adiposity. Using arterio-venous difference methodology with selective venous catheterization of abdominal sAT, no significant association was found between AT-specific changes in lactate-to-pyruvate ratio and BMI. However, the proportion of glucose released as lactate and pyruvate in sAT was strongly negatively correlated with BMI (34). Observational human studies examining hypoxia-related genes as surrogate markers of AT oxygenation have shown increased HIF-1α expression in sAT in humans with morbid obesity (57, 77, 78). Interestingly, HIF-1α expression was higher in the SVF than in adipocytes, which might imply that the SVF is more sensitive to changes in oxygenation (79). Importantly, however, HIF-1α mRNA expression seems not an appropriate marker for hypoxia (80). Also, upregulated genes in subcutaneous and visceral AT of severely obese subjects that are under control of HIF were not responsive to hypoxia in adipocytes (81), which raises the question what pO_2 threshold is required for activation of the HIF pathway in adipose tissue (60). Furthermore, genome-wide association studies have shown a correlation between epigenetic methylation of the HIF-3α gene in sAT and BMI and AT dysfunction markers (82-85). Following bariatric surgery, there was a reduction in HIF-1α mRNA expression in AT (86). On the other hand, HIF-1α gene expression was upregulated during weight loss induced by a low caloric diet (71).

It is important to emphasize that a stronger mechanistic link exists between hypoxia and the spatial presence of HIF-1α protein rather than its mRNA expression (74, 87). Further, HIF-1α is not only regulated by oxygen levels, but also by growth factors including insulin (88). Therefore, metabolic disturbances such as insulin resistance and/or hyperglycaemia may also have marked effects on HIF-1α protein stability (88), and may affect epigenetic modifications. This implies that one should be cautious when drawing conclusions about AT oxygenation based on gene expression of classical hypoxia-responsive genes such as HIF-1α, GLUT1 and VEGF (25).

Taken together, recent cross-sectional and intervention studies that we have performed in our laboratory demonstrate higher rather than lower AT pO_2 in obese insulin resistant individuals, but findings on sAT oxygenation (markers) in humans with obesity are conflicting. Thus, further investigation of determinants of sAT oxygenation may help to better understand these discrepant findings.

2.4 Determinants of adipose tissue oxygenation in humans

AT pO_2 is the result of a delicate balance between oxygen supply and consumption, which both seem to be altered in obesity. More specifically, differences in angiogenesis, capillary density, and vascular function, together determining ATBF, and the cellular demands affecting O_2 consumption contribute to changes in AT pO_2 (18, 25, 76).

2.4.1 Adipose tissue oxygen supply

Both structural (i.e. capillary density) and functional (i.e. vascular tone) aspects of the vasculature determine ATBF and, therefore, oxygen supply to AT. There is substantial evidence that there is insufficient angiogenesis in AT depots in obesity. Obese individuals show decreased adipose tissue mRNA expression of VEGF, the master regulator of angiogenesis and a HIF-1α target protein (59, 60, 89). Pasarica and colleagues (60) showed that capillary density was lower in overweight/obese humans and found a positive correlation between VEGF expression and capillary density. The lower capillary density in AT of obese individuals has been confirmed by our laboratory (59). Furthermore, it has been shown that obese insulin resistant subjects had fewer capillaries and a greater number of large vessels in AT as compared to lean individuals (90). Together, these findings are indicative of vascular rarefaction and decreased vascular remodelling in AT in obese humans. Thus, the lower capillary density may reflect higher AT oxygenation in obesity. Alternatively, if AT oxygenation would be lower in obesity, the pro-angiogenic response is not effectively propagated (91).

In addition to a lower capillary density in AT of obese individuals, an increased vascular tone may impair ATBF, which ultimately determines tissue oxygen delivery. It is well established that ATBF is impaired in human obesity. Fasting ATBF is lower in obese compared to lean individuals and has been linked to insulin resistance (59, 92-96). Furthermore, in the postprandial period as well as during insulin stimulation (i.e. hyperinsulinemic-euglycemic clamp), the increase in ATBF is blunted in obese versus lean subjects (59, 96, 97). These impairments seem to be related to impaired beta-adrenergic responsiveness and increased activity of the renin-angiotensin system in obesity (76, 95, 98, 99). We have previously shown that both pharmacological and physiological manipulation of ATBF induced concomitant alterations in AT pO_2 in humans (59), suggesting that decreased ATBF in obesity indeed reduces AT oxygen supply. Importantly, however, AT pO_2 is not only determined by oxygen supply to the tissue but is also dependent on AT oxygen consumption, as discussed in more detail below.

2.4.2 Adipose tissue oxygen consumption and mitochondrial function

In normal weight individuals, AT oxygen consumption is relatively low as compared to other tissues, accounting for approximately 5% of whole-body oxygen consumption (34, 62, 100). It has been estimated that mitochondrial oxygen consumption accounts for up to 85%, while non-mitochondrial oxygen consumption may be responsible for 10–15% of total oxygen consumption in AT under steady-state conditions (101, 102). Both mitochondrial and non-mitochondrial oxygen consumption may change during the marked AT remodelling occurring in obesity and may induce alterations in AT oxygenation.

It is well established that mitochondrial morphology, mass and function are impaired in multiple adipose tissue depots in obese rodents (103-107). Interestingly, it has been reported that early in the development of obesity, enhanced mitochondrial metabolism, biogenesis, and reactive oxygen species production seem critical to initiate and promote adipocyte differentiation (108, 109). In line with findings in animals, several human studies have reported impaired mitochondrial capacity and reduced expression of genes/proteins related to mitochondrial metabolism (e.g. peroxisome proliferator-activated receptor gamma coactivator 1-alpha and nuclear respiratory factor 1) in AT in states of obesity, insulin resistance, and T2DM (34, 59, 110-113). Furthermore, it has been shown that mitochondrial proteins are downregulated not only at whole AT level, but also in adipocytes from obese individuals (114, 115). In line, mitochondrial density and oxygen consumption rates are lower in adipocytes derived from obese versus lean subjects, independent of adipocyte size (114-116). Of note, there also appear to be sAT depot-specific differences in oxygen consumption rates in obesity, since basal respiration was lower in abdominal as compared to femoral differentiated human multipotent adipose-derived stem cells (73). The latter finding may underlie the higher AT pO_2 in abdominal than femoral subcutaneous adipose tissue (73).

In accordance with impaired mitochondrial density and oxygen consumption in obese AT in humans, there are indications that weight loss may evoke beneficial changes in AT mitochondrial function. Following bariatric surgery, both mitochondrial respiratory capacity and biogenesis were increased in AT (117, 118). We have recently shown that diet-induced weight loss increased AT gene expression of mitochondrial biogenesis markers and non-mitochondrial oxygen consumption pathways in humans, which may have contributed to the reduction in AT pO_2 following weight loss (71). In contrast, instead of improving AT mitochondrial abnormalities, weight loss downregulated mitochondrial gene expression and density, and had neither effects on mitochondrial DNA transcripts nor OXPHOS proteins (119). Interestingly, the latter study showed that a higher initial mitochondrial number and gene expression was related to more successful weight loss after 12-month follow-up. Importantly, however, changes in gene expression do not necessarily translate into functional alterations. Taken together, it appears that oxygen consumption is impaired in obese AT in humans, which may contribute to increased AT pO_2 in human obesity.

2.5 Altered adipose tissue oxygenation may contribute to tissue dysfunction and metabolic impairments

In cell culture experiments investigating the molecular and cellular responses to hypoxia, cells are usually exposed to a substantially reduced level of oxygen (1% O_2 is frequently employed) as compared to 'normoxia' (ambient air, 21% O2). The normal physiological range of AT pO_2 in human AT is ~3-11% O_2 or ~23-84 mmHg (57, 59, 60, 72). Therefore, the outcomes of experiments comparing the effects of pO_2 below and well-above these physiological levels should be interpreted with caution, since results may not directly translate to the human *in vivo* situation. Moreover, it is important to distinguish between acute (<24h) and more prolonged exposure to different pO_2 levels, since this seems to have a major impact on the metabolic and inflammatory responses, as will be discussed later in this section.

2.5.1 The cellular response to low oxygen levels

As any other cell type, adipocytes must maintain and adjust their metabolic and physiological regulation in response to fluctuations in the local microenvironment, including variation in oxygen levels (25, 120). The main regulators of oxygen sensing are the oxygen sensitive HIFs. HIFs are transcription factors, binding to the DNA and changing gene expression in response to alterations in oxygen levels (121). HIFs consist of two subunits, α and β, with the former being the oxygen sensitive molecule and HIF-1β being constitutively expressed by cells (75). The HIF family consists of three members based on the three α-subunits, HIF-1α, HIF-2α and HIF-3α, with the predominant members being HIF-1α and HIF-2α (27, 120, 122). HIF-1α has received the most attention, and this transcription factor has been described as the master regulator of oxygen homeostasis. HIF-1α is continuously synthesized and rapidly degraded in the presence of oxygen but is stabilized when oxygen levels are low, and the functional HIF-1α transcription factor is then recruited. More specific, during sufficient oxygenation of the cells, HIF-1α is enzymatically degraded by prolyl-4-hydroxylases through the proteasome (121). During 'hypoxic' conditions, which are tissue-dependent, but usually defined as <1% of oxygen in most *in vitro* studies, the prolyl-hydroxylase domain enzymes are inactivated, and HIF-1α is not subject to rapid degradation. Instead, HIF-1α then forms a heterodimer with the β subunit, acting on DNA binding areas called hypoxia-responsive elements, thus regulating gene expression of many different genes (10, 22, 25, 121, 123). These genes encode proteins involved in a multiplicity of cellular processes, including glucose and lipid metabolism, inflammation, ECM metabolism, and apoptosis (25). Thus, changes in tissue oxygenation seem to affect many physiological processes in AT, and the metabolic and inflammatory effects will be discussed in more detail below (Figure2.2).

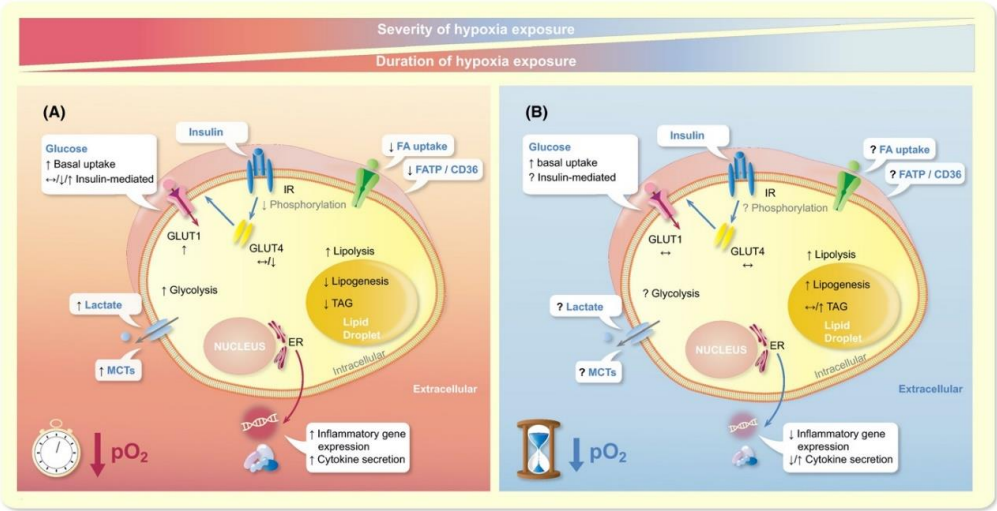

Figure 2.2: Adipocyte substrate metabolism, adipocyte gene expression and adipokine secretion are affected by alteration of oxygen partial pressure (pO_2). Both the severity and the duration of hypoxia exposure seem to impact cellular processes, as explained in more detail in the text. Panel A shows the effects of acute exposure to severe hypoxia (usually 1% O_2 for <24 h), while panel B illustrates the putative effects of prolonged, mild hypoxia exposure (usually 5%-10% O_2 for 7-14 d) on adipocyte biology. ER, endoplasmic reticulum; FA, fatty acids; FATP/CD36, fatty acid transporters; GLUT, glucose transporter; IR, insulin receptor; MCTs, monocarboxylate transporters; pO_2, oxygen partial pressure; TAG, triacylglycerol. ↑, increase; ↓, decrease; ↔, unchanged; ?, not determined.

2.5.2 Metabolic effects of altered adipose tissue oxygenation

2.5.2.1 Glucose metabolism

Under hypoxic conditions a shift from aerobic to anaerobic metabolism occurs, with glucose becoming the major substrate for ATP generation (25, 75, 76, 121). *In vitro* studies have demonstrated an increase in basal glucose uptake in human and rodent adipocytes treated acutely, up to 24 hours, with 1% versus 21% O_2 (65, 124, 125). Furthermore, it has been shown that glucose uptake in human adipocytes is inversely related to O_2 levels (1, 3, 5, 10, 15 vs 21% O_2), peaking at 1% O_2 (126). In accordance with these findings, prolonged exposure (14 days) to low (5% O_2) but not high (10% O_2) physiological pO_2 levels tended to increase basal glucose uptake in differentiated human multipotent adipose-derived stem cells (73).

Conflicting findings, however, have been reported regarding the effects of pO_2 on insulin-mediated glucose uptake. Acute exposure to 1% O_2 (up to 24h) reduced insulin-mediated glucose uptake in human adipocytes (125), indicative of impaired insulin signalling, an effect that was reversible (125). This was further illustrated by decreased phosphorylation of the insulin receptor, iRβ, and IRS-1 proteins as well as protein kinase B (65, 125). In contrast, another study found that acute 1% O_2 exposure increased insulin-dependent and insulin-independent glucose uptake in 3T3-L1 adipocytes (127). Interestingly, it was shown that multiple exposures of differentiating 3T3-L1 adipocytes

to transient hypoxia (1% O_2, 4h/day, 4-8 days) enhanced insulin signalling, illustrated by increased phosphorylation of Akt (T308 and S473 residues) and GSK3β (127).

Alterations in glucose uptake are due to changes in the expression and localization of the GLUTs. GLUT1 mRNA levels were increased following exposure to acute, severe hypoxia (1-2% pO_2, up to 24h) in both murine (3T3-L1) and human (pre)adipocytes (75, 126, 128-133). In contrast, insulin-dependent GLUT4 mRNA expression in human adipocytes remained unchanged (124) or was significantly reduced by acute exposure to 1% O_2 (124, 126, 129, 132). In line with improved insulin-stimulated glucose uptake, GLUT4 but not GLUT1 expression was elevated in murine adipocytes exposed to transient hypoxia (127). During and after differentiation of human preadipocytes under low (5% O_2) and high (10% O_2) physiological pO_2 levels, basal GLUT1 expression was not changed (134) or decreased (73), while GLUT4 mRNA expression remained unchanged (73, 134).

Acute hypoxia exposure to 1% O_2 for 24h also increased gene and protein expression of enzymes involved in glycolytic metabolism in human adipocytes, including glucose phosphate isomerase, pyruvate kinase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (128, 133, 135-137). In accordance with these findings, the end-product of the glycolytic pathway, lactate, and the expression of genes encoding monocarboxylate transporters (MCT) mediating lactate transport were found to be increased in rodent and human adipocytes under hypoxic conditions (75, 138, 139).

In conclusion, *in vitro* findings indicate that exposure to severe hypoxia (1-2% O_2), and likely also low physiological pO_2 (5% O_2), increases basal glucose uptake and induces a switch towards glycolytic metabolism in rodent and human adipocytes, while effects on insulin-mediated glucose uptake are conflicting (Figure 2.2).

2.5.2.2 Lipid metabolism

Few studies examined whether and how pO_2 influences lipid metabolism in AT, yielding conflicting results. FFA uptake and oxidation were significantly reduced by acute, severe hypoxia exposure (1% O_2, 24 hours) in 3T3-L1 adipocytes (65, 127). Reduced uptake may be explained by reduced expression of fatty acid transport proteins, as illustrated by decreased expression of FATP and CD36 in these cells (65). Lipid storage, assessed by TAG accumulation, was reduced both by chemically-induced hypoxia with $CoCl_2$ and prolonged severe hypoxia exposure in 3T3-L1 adipocytes (1% O_2 for 14 days) (140, 141). In accordance with these observations, 1% O_2 exposure for 14 days decreased lipogenesis in 3T3-L1 adipocytes (140, 141). However, 14 days of exposure to mild hypoxia exposure (4% O_2), which reflects low physiological pO_2, markedly increased lipogenesis and the formation of large lipid droplets in 3T3-L1 adipocytes (140). Furthermore, another study has shown that exposure of differentiating human adipocytes to high (10% O_2) but not low (5% O_2) physiological pO_2 for 14 days increased TAG accumulation (134). Taken together, it seems that exposure of adipocytes to severe hypoxia may reduce lipogenesis, while prolonged exposure to physiological pO_2 may increase lipogenesis, but these effects need to be studied in more detail to better understand the opposing results (Figure2.2).

The amount of oxygen in the microenvironment also seems to impact adipocyte lipolysis. Several studies have shown that acute exposure to severe hypoxia (1% O_2) increased basal lipolysis in 3T3-L1 adipocytes (65, 76, 125). Moreover, prolonged exposure (14 days) to severe hypoxia modestly increased basal lipolysis, while low physiological pO_2 (4% O_2, 14 days) exposure increased lipolysis to a much greater extent in 3T3-L1 adipocytes (140). In theory, insulin resistance in adipocytes might explain the increased basal lipolytic rate due to reduced insulin-mediated suppression of lipolysis. However, since improved insulin sensitivity has also been found following hypoxia exposure, as discussed in the previous section, alternative mechanisms are likely involved in the pO_2-induced effects on basal adipocyte lipolysis. Furthermore, isoproterenol-induced lipolysis was also significantly elevated in human adipocytes differentiated at high (10% O_2) and low (5% O_2) physiological pO_2 as compared to exposure to ambient air (21% O_2), which was accompanied by increased protein expression of the lipolytic enzyme HSL and the lipid droplet-coating protein perilipin (134). In conclusion, hypoxia is likely to increase lipolysis in rodent and human adipocytes, with more pronounced effects found under physiological pO_2 (Figure 2.2). Clearly, more studies are required before strong conclusions can be drawn regarding the effects of oxygenation on lipid metabolism in human AT, and to unravel underlying mechanisms.

2.5.3 Adipokines and inflammatory factors

Several studies have demonstrated that the expression and secretion of many adipokines are sensitive to pO_2 levels. Most *in vitro* studies have shown that acute exposure to severe hypoxia (1% O_2, up to 24 hours) induces a pro-inflammatory expression and secretion profile in (pre)adipocytes, with increased levels of TNF-α, IL-1, IL-6, MCP-1, PAI-1, macrophage-migration-inhibition factor, and inducible-nitric oxide synthase, in both adipocytes and SVF cells derived from human adipose tissue, as well as in murine adipose tissue resident macrophages (25, 41, 64, 142). Furthermore, several studies found that acute exposure to severe hypoxia decreased adiponectin and increased leptin expression and secretion in human and murine (pre)adipocytes (25, 63-66, 129, 132, 133, 143). Adiponectin, which is often reduced in individuals with obesity, is an important adipokine that has beneficial metabolic and anti-atherogenic properties (144, 145). Leptin, the concentrations of which are strongly positively correlated to adipose tissue mass, is an important regulator of food intake and energy expenditure, providing important feedback in relation to energy storage in the body (146).

As with other *in vitro* studies applying acute and severe hypoxia over 1-24 hours (25, 64, 66, 129, 132, 133), these findings should be interpreted with some caution, underlining the importance of applying more physiological conditions in cell culture experiments. Few *in vitro* studies have tried to better mimic physiological conditions *in vivo* in terms of oxygen partial pressure as well as the duration of exposure to altered pO_2 (73, 134). The effects of modest, rather than severe, hypoxia have also been investigated, showing a concentration-dependent change in adipokine expression and secretion in human adipocytes (126). Interestingly, prolonged exposure of human adipose tissue-derived mesenchymal stem cells to physiological pO_2 levels (i.e. 5% and

10% O_2) during differentiation towards mature adipocytes appears to elicit a different expression and secretion profile as observed following acute (severe) exposure to hypoxia. More specific, we have recently demonstrated that low physiological pO_2 decreased pro-inflammatory gene expression (i.e. IL-6, PAI-I, TNFα, MCP-1 and DPP-4) in differentiated human adipocytes as compared to 21% and/or 10% O_2, whereas more heterogeneous effects on adipokine secretion were found (73). Exposure of these cells to low physiological pO_2 (5% O_2) for 14 days resulted in a reduced secretion of leptin and increased adiponectin and IL-6 secretion in these adipocytes, while no significant effects on DPP-4 and MCP-1 secretion were found (73). In contrast, exposure to high physiological pO_2 (10% O_2) increased leptin and DPP-4, but reduced IL-6 and MCP-1 secretion (73). Famulla and colleagues (134) have shown increased DPP-4, adiponectin and IL-6 following prolonged exposure to high physiological pO_2 (10% O_2), while low physiological pO_2 (5% O_2) tended to reduce the secretion of adiponectin. These differences between studies suggest that donor characteristics may also influence the effects of pO_2 on the adipocyte secretory profile.

Taken together, oxygen levels and pattern of exposure seem to have a significant impact on adipocytokine expression and secretion (Figure 2.2). However, many aspects of exposure have not been examined in human cells, which is important to elucidate in future experiments.

2.6 Altered tissue oxygenation impacts whole-body physiology in humans

As indicated in the previous section, the cellular response to altered oxygen levels seems to depend to a large extent on the severity and duration of exposure. Not surprisingly, the effects of changes in oxygenation on whole-body homeostasis also seems to be determined by these factors, next to the oxygenation pattern (147). The clinical consequences of severe chronic hypoxia, as observed in patients with severe chronic obstructive pulmonary disease, and severe intermittent hypoxia as seen in patients with obstructive sleep apnoea syndrome are outside the scope of this review and have been discussed elsewhere (148-152). In this section, we will provide a brief overview of findings on the effects of altered (adipose) tissue oxygenation through physiological or experimental conditions on body weight and parameters related to cardiometabolic health.

Living at high-altitude represents a condition of hypobaric hypoxic exposure (i.e. around 15% O_2 at ~3000 m) as oxygen partial pressure is relatively lower compared to sea-level (147). The impact of high-altitude habitation on chronic diseases is dependent on several factors such as ethnicity, environmental and behavioural factors that may vary across mountain dwellers (147, 153). It has been suggested that living at high-altitude is associated with improved cardiovascular and pulmonary function (154). Many studies have demonstrated a lower prevalence of obesity, CVD, T2DM, and cancer in populations living at high-altitude (147, 153, 155-157). For example, a cross-sectional study including 422,603 adults has shown an inverse relationship between elevation and obesity prevalence, after adjusting for temperature, diet, physical activity, smoking and demographic factors, in both males and females (158), which is in line with other studies demonstrating an inverse association between altitude and the prevalence of

obesity (159-161). Interestingly, a lower prevalence of the metabolic syndrome, lower reduced fasting glucose levels and diabetes incidence have been found among highlanders(156, 162-165). Noteworthy, from most of these observational studies it cannot be concluded that exposure to lower pO_2 levels has beneficial health effects, since many potential confounders such as the diet and physical activity level may have affected these findings.

Several intervention studies have been performed to elucidate the impact of exposure to altered pO_2 on body weight and metabolic homeostasis (Figure 2.3). We have previously demonstrated that chronic exposure to hypoxia (8% versus 21% O_2, 21 days) improved the AT phenotype in C57Bl/6J mice, evidenced by decreased adipocyte size, decreased macrophage infiltration and inflammatory markers, and increased expression of mitochondrial function and biogenesis markers in visceral and subcutaneous AT (166). More recently, the same concept has been applied to humans. Exposure to moderate hypoxia (15% O_2) for ten subsequent nights increased whole-body insulin sensitivity in eight obese men (167). Since moderate hypoxia exposure also tended to reduce AT pO_2 (167) , these findings may imply that lowering of AT pO_2 by moderate hypoxia exposure may have contributed to improved insulin sensitivity (168). Furthermore, exposure to hypoxia under resting conditions increased energy expenditure and lipid metabolism, and reduced appetite and food intake (169, 170). Based on a recent systematic review, it was concluded that normobaric hypoxic conditioning, lasting from 5 days up to 8 months, may have beneficial effects on insulin levels, energy expenditure, body weight and blood pressure in rodents and humans, which may contribute to improved cardiometabolic health and body weight management in obesity (155). The putative effects of (severe) hypoxia exposure on orexigenic (i.e. ghrelin) and anorexigenic (i.e. leptin) peptides affecting appetite and food intake may, at least partially, underlie the effects on body weight and metabolic outcomes, as reviewed elsewhere (171, 172).

Interestingly, the combination of hypoxia exposure and exercise may have additive beneficial health effects in humans (147, 173). A greater decrease in total body weight, body fat mass and waist/hip ratio was found when exercise was performed under hypoxia compared to normoxia(170, 174-176), and appeared to be maintained following the intervention (177). Interestingly, hypoxia exposure also seems to exert effects on substrate oxidation but findings are conflicting, with some studies showing increased fat oxidation (178, 179), while others demonstrating increased carbohydrate oxidation both during and post-exercise (180, 181). Furthermore, exercise training under hypoxic conditions induced a more pronounced increase in adiponectin levels compared to normoxic exercise (182). Moreover, hypoxic exercise decreased insulin levels in obese individuals, and acutely improved insulin sensitivity in T2DM patients compared to normoxic exercise (175, 183, 184). The mechanisms underlying improvements in glucose homeostasis following hypoxia exposure remain to be elucidated but may involve insulin-independent mechanisms. Importantly, the impact of hypoxia on cardiometabolic health may also be due to effects of altered pO_2 on other organs than adipose tissue, especially during exercise.

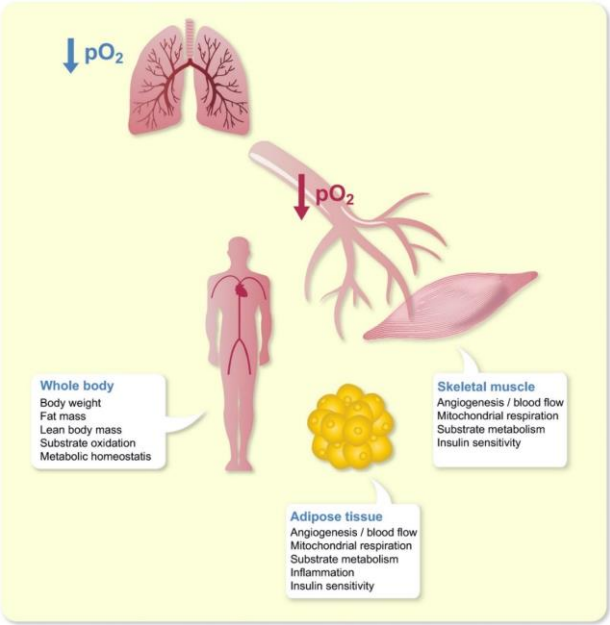

Figure 2.3: Putative impact of (moderate) hypoxia exposure on whole-body, skeletal muscle and adipose tissue physiology. O_2, oxygen; pO_2, oxygen partial pressure

The beneficial effects of hypoxic exercise may be mediated to a large extent by alterations at the level of skeletal muscle. During contraction, glucose uptake in skeletal muscle is increased in an insulin-independent manner, likely involving independent effects of AMP-activated protein kinase (AMPK), mechanical stress and Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) (185). Interestingly, it has been demonstrated that hypoxia exposure increased glucose uptake in skeletal muscle cells through AMPK signalling. Therefore, hypoxia exposure during exercise might have additive or synergistic effects on peripheral glucose uptake. Indeed, exposing human myotubes to 7% O_2 in combination with electrical pulse stimulation (EPS), to mimic exercise, increased glucose uptake to a higher extent than EPS under 21% O_2, which seems at least partly due to an insulin-sensitizing effect of hypoxia(186). Taken together, hypoxia exposure may improve glucose homeostasis via insulin-dependent and insulin-independent effects, but more studies in humans on putative underlying mechanisms are needed.

2.7 Conclusions and future perspectives

The obesity epidemic presents a major public health challenge. Novel preventive measures and treatment alternatives are urgently needed to combat obesity and its comorbidities. Adipose tissue dysfunction in obesity is related to a plethora of metabolic and endocrine disturbances, contributing to impairments in lipid and glucose metabolism as well as immune homeostasis. It is well established that adipose tissue dysfunction has a central role in the aetiology of obesity-related comorbidities and chronic diseases, including T2DM and CVD. A reduced lipid buffering capacity of

hypertrophic adipose tissue in obesity results in lipid accumulation in key metabolic organs such as the liver and skeletal muscle (i.e. ectopic fat storage), which is strongly associated with insulin resistance. Moreover, adipose tissue in obesity is characterized by a pro-inflammatory phenotype. This is reflected by a phenotypic shift towards a higher abundance of pro-inflammatory macrophages and other adaptive and innate immune cells in obese adipose tissue, leading to the production and secretion of a multitude of pro-inflammatory cytokines, which in turn may induce insulin resistance. Besides inflammation, a disproportionate deposition of ECM components during the development of obesity may contribute to adipose tissue fibrosis and insulin resistance (Figure 2.1).

Adipose tissue oxygen partial pressure, determined by the balance between oxygen supply and consumption, may have a key role in the metabolic and inflammatory perturbations seen in most obese individuals. Animal models have shown lower pO_2 in obese AT ('hypoxia'). Findings in humans are conflicting, which may be due to differences between study populations in terms of the onset and physical history (e.g. weight cycling) of obesity and other subjects' characteristics (e.g. age, sex, ethnicity, presence of T2DM), the AT depot studied, and variation in the methodology used. Nevertheless, several studies performed in our laboratory indicate that AT pO_2 is higher in obese insulin resistant individuals, is positively related to insulin resistance (independently of adiposity), and is reduced after diet-induced weight loss, which is paralleled by improved insulin sensitivity. Adipose tissue mitochondrial dysfunction (i.e. reduced O_2 consumption) may contribute to higher AT pO_2 in obesity. There is no strong evidence to suggest that differences in pO_2 within the human physiological range (i.e. due to impaired blood flow) have marked effects on mitochondrial respiration. Interestingly, many *in vitro* experiments have demonstrated that changes in oxygen levels impact the functionality of (pre)adipocytes and immune cells, leading to alterations in glucose and lipid metabolism, as well as inflammation in adipose tissue (Figure 2.2). Clearly, altered pO_2 may not only affect adipose tissue physiology but also whole-body metabolic homeostasis (Figure 2.3). In this respect, it remains to be elucidated whether AT pO_2 exerts a crucial role in the development and progression of obesity-related co-morbidities in humans. Although several lines of evidence suggest that exposure to lower levels of oxygen may enhance whole-body metabolic homeostasis and body weight regulation, intervention studies in humans are warranted to further investigate whether changes in tissue oxygenation may improve cardiometabolic health, thereby providing a novel strategy to combat chronic cardiometabolic diseases in obese humans.

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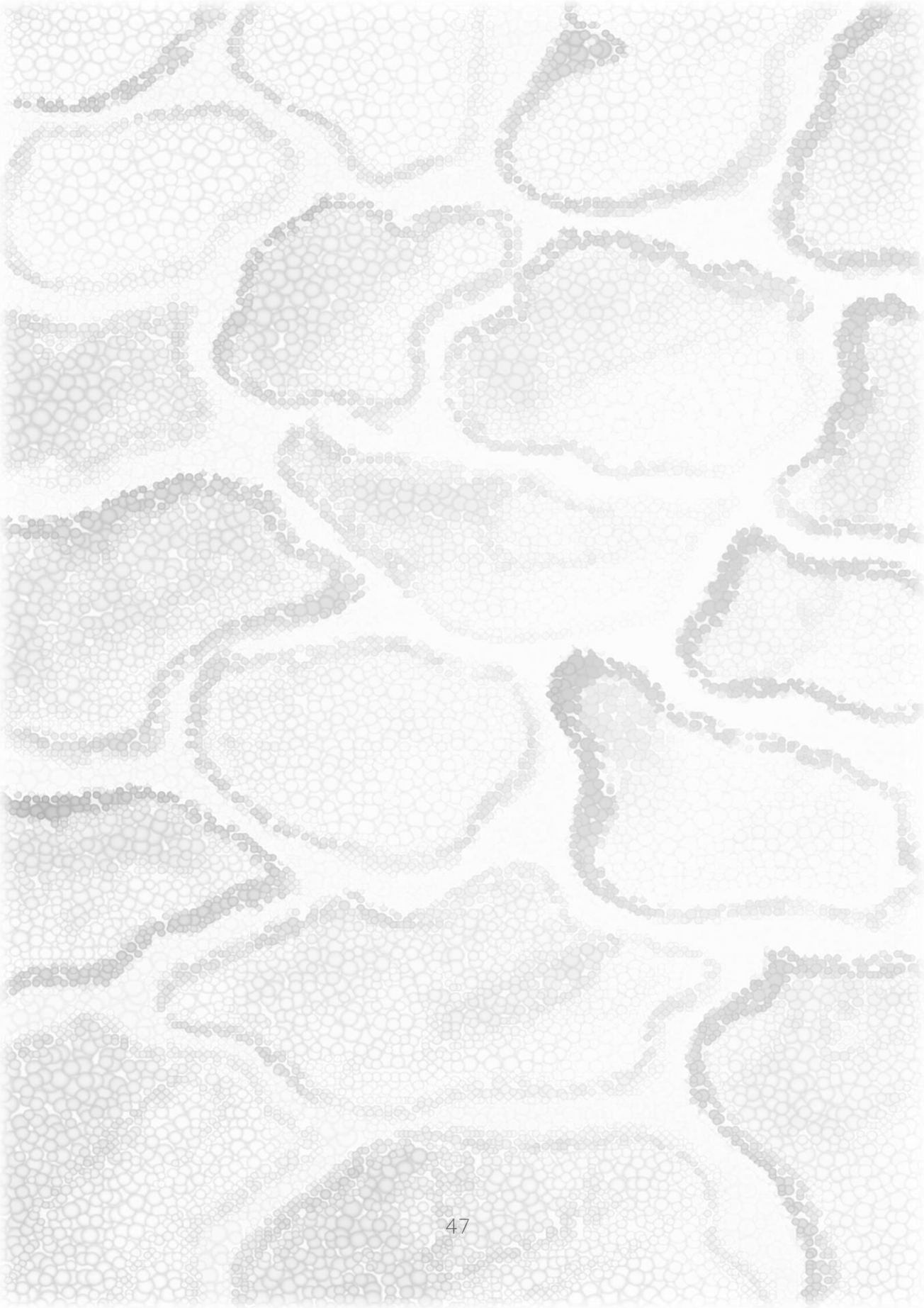

3 Measurement of human abdominal and femoral intravascular adipose tissue blood flow using percutaneous Doppler ultrasound

Lempesis I. G., Goossens G. H., Manolopoulos K. N. *Adipocyte. 2021;10(1):119-123*.

Abstract

ATBF is an important determinant of AT function. ^{133}Xenon wash-out technique is considered the gold-standard for human ATBF measurements. However, decreasing ^{133}Xenon clinical use and costly production and preservation, make alternative (non-invasive) methods necessary. Here, we explored percutaneous Doppler ultrasound as a proxy method to quantify intravascular subcutaneous abdominal and femoral ATBF in humans (n=17). Both fasting ATBF and the postprandial increase in ATBF were significantly higher in abdominal compared to femoral AT. Although anatomical variations in vein location and depot thickness may impact feasibility, we demonstrate that Doppler ultrasound detects the expected depot-differences and postprandial increase in ATBF in healthy individuals. This method warrants further investigation in other populations and metabolic conditions.

3.1 Introduction

Tissue-specific regulation of blood flow is needed to meet local physiological demands and to allow proper functioning of organs. ATBF is an important determinant of AT function, as it delivers nutrients and oxygen to AT, exerts a key role in fatty acid trafficking, and distributes adipokines and metabolites into the circulation (5, 6, 14). Under fasting conditions, nitric oxide, adrenergic regulation and the renin-angiotensin system determine ATBF (1, 10), while the increase in ATBF following ingestion of a glucose drink or mixed meal is mainly under beta-adrenergic control (5). Many studies have demonstrated that both fasting and the postprandial enhancement of ATBF are impaired in obesity and insulin-resistant conditions (8, 12, 17), thereby contributing to the metabolic perturbations in insulin-resistant individuals with obesity (4, 5, 9, 12, 14, 17). In line with an important role of ATBF in metabolic regulation, several, but not all, studies have demonstrated a lower ATBF in lower-body compared to upper-body AT (15, 18, 19).

The gold-standard method for measuring ATBF is the ^{133}Xenon (^{133}Xe) wash-out technique, first described by Larsen and colleagues in 1966 (13). The rate of disappearance of the lipid-soluble radioactive isotope ^{133}Xe, of which a small volume is administered in an area of subcutaneous AT, is used as an indicator of ATBF (9). Alternative methods to assess subcutaneous ATBF include measurement of ethanol wash-out using microdialysis, laser Doppler flowmetry, and contrast-enhanced ultrasound (2, 7, 9, 11, 20). The current reduced availability of ^{133}Xe due to its costly production and declining clinical use, as well as the invasive nature of some of the other techniques implies the need for an alternative, non-invasive method for the measurement of ATBF that can be used in physiological human *in vivo* studies. The aim of the present study, therefore, was to explore intravascular Doppler ultrasound as a proxy method for measuring ATBF in humans, by establishing technical feasibility, reproducibility, and sensitivity of the method to detect ATBF changes in response to an oral glucose drink.

3.2 Research Design and Methods

3.2.1 Study design

Seventeen healthy individuals with no known medical conditions who were non-smokers and not taking any medication participated in the present study. Participants arrived at the Clinical Research Facility in the morning around 08:30 a.m. after an overnight fast (at least 10h fasting). They were advised to abstain from coffee, tea and alcohol, and sports or intense physical activity one day prior to the measurements, but to keep on their usual activities. Following 30 min of rest, two ATBF measurements in abdominal and femoral AT, separated by 5 min, were acquired under fasted conditions. Next, subjects were asked to ingest 75 g of glucose in the form of a pre-made drink (113ml of Polycal Liquid, Nutricia Ltd, Trowbridge, Wiltshire, UK). Postprandial ATBF responses in abdominal and femoral AT were determined for 120 min at 10 min intervals.

Measurements were taken by a single operator and, depending on feasibility, repeated up to three times at each time-point for both AT depots. Femoral ATBF was measured in 11 individuals due to a near-parallel to the skin anatomical course of femoral AT veins resulting in poor Doppler signal. The study was approved by the University of Birmingham Ethics committee and the UK Health Research Authority. All participants provided written informed consent before taking part in the study procedures.

3.2.2 Doppler ultrasound technique

Vessels that specifically drain abdominal subcutaneous AT are branches of the superficial epigastric vein (*V. epigastrica superficialis*), that are located above the inguinal ligament, as determined by using the anterior superior iliac spine and the projected pubic symphysis as reference points (3). In the present study, the subcutaneous AT areas lateral of the umbilicus and between the lower end of the rib cage and the inguinal ligament were scanned on each side in order to identify suitable veins for ATBF measurements in the abdominal subcutaneous AT depot. In the femoral depot, the great saphenous vein and its branches drain mostly femoral subcutaneous AT (16). Suitable femoral veins for ATBF measurements were identified by scanning the inner aspect of the thigh, approximately half-way between the groin and the knee. A Philips CX50 ultrasound system (Philips Ultrasound, 22100 Bothell-Everett Highway, Bothell, WA 98021-8431, USA) with two different transducers was used for the measurements: Depending on the anatomical location, course, and size of the identified veins, a L15-7io broadband compact linear array transducer (Frequency range: 15-7 MHz) and a L12-3 (Frequency range: 12-3 MHz) were used.

The sequence of measurement involved the following steps. One or two suitable subcutaneous veins draining the respective AT depot were identified using a live grayscale imaging mode (2D Mode; Figure 3.1A), and their location was marked on the skin with a suitable marker for easy identification for measurements later during the study. The insonation angle was checked visually between two projected lines, one corresponding to the skin level and the other running horizontally across the vessel lumen, aiming for it to be less than 70 degrees to ensure an adequate Doppler signal. Flow within the selected vein was visualized using colour or colour power angio (CPA) mode (Figure 3.1B), before switching to pulsed waved (PW) Doppler to obtain the measurement. The quality of the Doppler signal was optimized using proprietary functions of the ultrasound system (iScan Intelligent Optimization on the system used in this study). Following the conduction of the PW Doppler measurement, the live image was frozen and flow volume calculations were performed using the ultrasound system's internal algorithms to obtain a time-averaged flow. Importantly, this included measurement of the vessel diameter (Figure 3.1C). During the PW Doppler measurement, great care was taken to apply the lowest possible pressure to the skin with the ultrasound transducer to avoid compression of the vessel, which could affect ATBF.

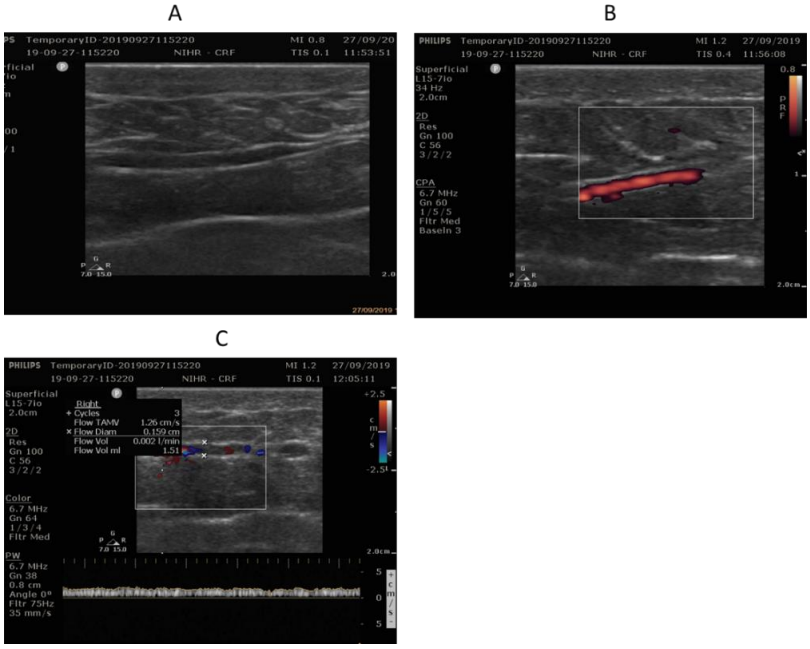

*Figure 3.1: Representative images from the workflow of obtaining an ATBF measurement using Doppler ultrasound. Identification of a suitable subcutaneous adipose tissue vein (**A**), confirmation of flow signal in colour power angio (PCA) mode (**B**), followed by measurement of vessel diameter and calculation of ATBF (**C**).*

3.2.3 Statistical analyses

For each time point, mean ATBF was determined from the available repeated measurements. Fasting ATBF was calculated as the mean of time points t= -5- and 0-min. Peak ATBF was defined as the highest value recorded during the measurements' timeframe. Time-averaged area under the curve (iAUC/min) was calculated following the trapezoid rule. Since parameters were not normally distributed (based on Shapiro-Wilk test), the Wilcoxon-Signed Rank test was used to compare fasting and postprandial ATBF within the same AT depot, and to compare ATBF between abdominal and femoral AT. SPSS version 25 and GraphPad Prism version 8 were used to perform statistics, and $p<0.05$ was considered as statistically significant.

3.3 Results

The participant characteristics are shown in Table 3.1.

Table 3.1: Participants' characteristics

Sex	5 Males / 12 Females
Age (years)	37 (24-58)
Height (m)	1.73 (1.58-1.88)
Weight (kg)	69.1 (49.5-111.4)
BMI (kg/m^2)	22.9 (19.8-33.8)
Waist circumference (cm)	83.4 (68-119)
Hip circumference (cm)	99.4 (89.5-117)
Waist/Hip ratio	0.84 (0.69-1.01)

BMI: Body Mass Index

Briefly, seventeen individuals (12 females, 5 males) with BMI range 19.8-33.8 kg/m^2 and age range 24-58 years were included in the present study. Of note, only one study participant had obesity (BMI, 33.8 kg/m^2) with the BMI range of all other participants being 19.8-24.8 kg/m^2. Fasting abdominal ATBF was 2.9 ± 0.8 mL/min, which increased to peak levels at 6.9 ± 1.7 mL/min at t=90 min after glucose ingestion (p=0.002 vs. fasting ATBF) (Figure 3.2). Femoral ATBF increased from 1.1 ± 0.3 mL/min under fasting conditions to peak ATBF values of 2.2 ± 0.6 mL/min at t=70 min after glucose ingestion (p=0.047 vs. fasting ATBF).

Fasting ATBF was higher in abdominal compared to femoral AT (2.9 vs. 1.1 mL/min, respectively, p=0.033, *n*=11 paired measurements). Furthermore, the postprandial increase in ATBF (iAUC/min) was significantly higher in abdominal than femoral AT (2.0 vs. 0.3 mL/min, respectively, p=0.033, *n*=11 paired measurements). The iAUCs for both abdominal and femoral ATBF were not significantly correlated with BMI (abdominal: r=-0.018, p=0.945; femoral: r=-0.388, p=0.237) or waist/hip ratio (abdominal: r=0.076, p=0.773; femoral: r=0.582, p=0.065). When examining the individual participants where a femoral ATBF measurement was not possible, there were no notable predictors of poor signal apart from the anatomical considerations outlined above. The coefficient of variation of repeated measurements in abdominal AT was 33 ± 6% for fasting ATBF and 20 ± 5% for peak ATBF. For femoral AT, the coefficient of variation of repeated measurements was 39 ± 7% for fasting ATBF and 51 ± 11% for peak ATBF.

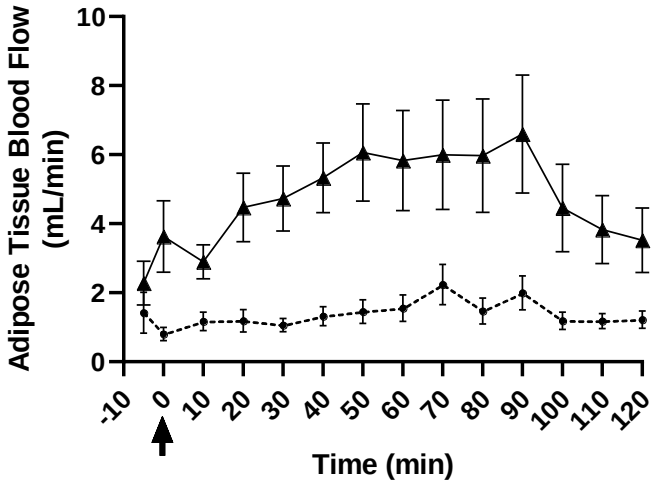

Figure 3.2: Abdominal (solid line with triangles, n=17) and femoral (dashed line with circles, n=11) adipose tissue blood flow measurements with Doppler ultrasound. A standardized 75g glucose drink was given at time 0 min (black arrow). ATBF follows the expected postprandial increase for the abdominal adipose tissue depot, peaking at 6.9 ± 1.7 mL/min (t=90 min post-glucose ingestion) (p=0.002 vs. fasting ATBF, n=17). The femoral AT depot showed a less pronounced increase in ATBF to 2.2 ± 0.6 mL/min (peak value at t=70 min post-glucose ingestion) (p=0.047 vs. fasting ATBF, n=11). The postprandial enhancement (iAUC) in abdominal ATBF was more pronounced than in femoral AT (p=0.033, n=11).

3.4 Discussion

The present study demonstrated that measurement of abdominal and femoral intravascular ATBF with percutaneous Doppler ultrasound is technically feasible. This non-invasive method is able to detect the expected increase in blood flow following oral glucose ingestion in both abdominal and femoral AT in healthy individuals (5, 6, 14). Furthermore, we found that abdominal ATBF was significantly higher than femoral ATBF under fasting conditions. Moreover, the postprandial increase in abdominal subcutaneous ATBF was significantly higher than the ATBF increase in femoral AT. These findings are in agreement with previous studies, in which ATBF was quantified using the gold-standard ^{133}Xe wash-out technique (5, 15). As expected, we found that there is large inter- and intra-individual variability in ATBF, which is commonly observed with ATBF (17) measurements, even when performed using the ^{133}Xe wash-out technique. However, intra-individual coefficients of variation with intravascular Doppler ultrasound seem larger than found with ^{133}Xe wash-out (17).

Important limitations of the method employed in the present study are those inherent to using ultrasound and relate to the quality of the ultrasound image that can be obtained in individual participants. Appropriate operator training paying special attention to the identification of suitable veins that allow acquisition of a high-quality PW Doppler signal is of paramount importance. Low blood flow in small AT veins may further hamper the

accuracy of ATBF measurement. These issues become especially noticeable when assessing ATBF in (very) lean participants due to thin subcutaneous fat layers and smaller blood vessels. While the anatomical properties of AT veins, especially in the femoral AT depot, may limit the ability of obtaining a good Doppler signal in every participant of a given study, it is important to note that other available methods also have intrinsic limitations such as exposure to radiation (i.e. Positron emission tomography (PET)-tracers, ^{133}Xe wash-out), thereby restricting their applicability. This also holds true for repeated measurements during a clinical study, which is often not possible with PET scans, for example. Although intravascular Doppler ultrasound may be an alternative method that could be applied to calculate metabolite fluxes across AT in physiological *in vivo* studies in humans, it is important to note that Doppler ultrasound provides data on intravascular blood flow in relatively large AT veins. In contrast, the ^{133}Xe wash-out technique on which the original calculations of metabolic fluxes were based provides ATBF values at the capillary level. Due to the global production stop of medical ^{133}Xe it was not possible to validate the present Doppler measurements against the gold-standard ^{133}Xe wash-out technique. A further limitation of our study is that we did not obtain information on the actual volume of the AT depot measured, e.g., through magnetic resonance imaging or dual x-ray absorptiometry, which could allow for the modelling of whole-depot blood flow values. Finally, we did not measure postprandial glucose and insulin concentrations in this study, although we would not expect to find any different postprandial glucose and insulin responses to previously published data given that all our participants were healthy volunteers (12).

To summarize, the present study demonstrates that AT Doppler ultrasound is a non-invasive method for measuring abdominal and femoral ATBF, that is sensitive enough to detect differences between AT depots as well as the expected postprandial increase in ATBF in healthy individuals. Future studies should explore the feasibility and sensitivity of this method in determining ATBF in other populations, for example individuals with obesity and patients with type 2 diabetes, establish inter-operator variability, and assess the usefulness of the method in combination with volumetric AT measurements as a basis of mathematical models allowing calculation of substrate fluxes across different AT depots.

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Declaration of interest statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Contribution statement

K.N.M. conceived and designed research; I.G.L. performed experiments; I.G.L. analyzed data; I.G.L., G.H.G. and K.N.M interpreted results of experiments; I.G.L. prepared figures and drafted manuscript; I.G.L., G.H.G and K.N.M. edited and revised manuscript; I.G.L., G.H.G and K.N.M. approved final version of manuscript.

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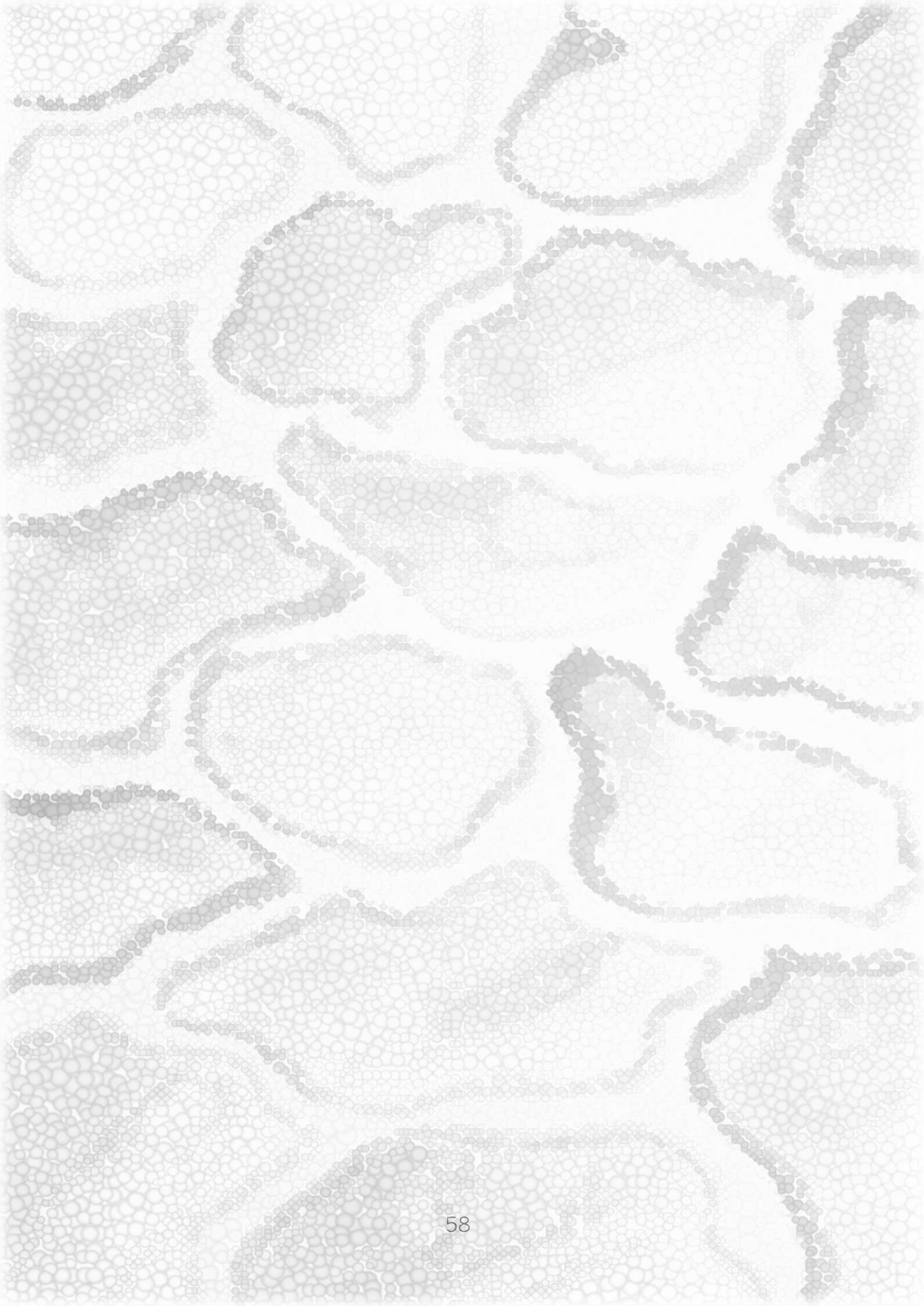

4 Distinct inflammatory signatures of upper and lower body adipose tissue in women with normal weight or obesity

Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Dineen R., Blaak E. E., Manolopoulos K. N., Goossens G. H. *Front Endocrinol 2023. Accepted*.

Abstract

Introduction*:* Upper and lower body fat accumulation poses opposing obesity-related cardiometabolic disease risk. Depot-differences in subcutaneous adipose tissue (SAT) function may underlie these associations. We aimed to investigate the inflammatory signatures of abdominal (ABD) and femoral (FEM) SAT in postmenopausal women with normal weight or obesity.

Methods: We included 23 postmenopausal women with normal weight (n=13) or obesity (n=10). *In vivo* secretion of adipokines from ABD and FEM SAT was measured using the arterio-venous balance technique. Adipokine gene expression and adipocyte morphology were examined in ABD and FEM SAT. Furthermore, adipokine expression and secretion were investigated *in vitro* using differentiated human primary ABD and FEM subcutaneous adipocytes derived from the study participants.

Results: Plasma leptin and PAI-1 concentrations were higher, and ABD and FEM adipocytes were larger in women with obesity than normal weight. No differences in adipocyte size and blood flow were apparent between ABD and FEM SAT. We found significant release of leptin and MCP-1 from ABD and FEM SAT, with higher fractional release of MCP-1 from ABD than FEM SAT. Gene expression of leptin, PAI-1 and TNF-α was lower in ABD than FEM SAT, and higher in women with obesity than normal weight. In ABD adipocytes, IL-6, PAI-1, and leptin gene expression was higher, while adiponectin and DPP-4 gene expression were lower than in FEM adipocytes. Finally, ABD adipocytes secreted less MCP-1 compared to FEM adipocytes.

Discussion: These findings demonstrate that upper and lower body SAT and adipocytes are characterized by distinct inflammatory signatures in postmenopausal women, which seem independent of adipocyte size.

4.1 Introduction

Obesity is characterized by excessive accumulation of white adipose tissue (AT), which contributes to the development of insulin resistance and related cardiometabolic diseases (1-4). Body fat distribution is an important determinant of cardiometabolic derangements (5). Abdominal (ABD) obesity (upper body fat accumulation) is associated with an increased risk for insulin resistance, type 2 diabetes mellitus, cardiovascular disease, and all-cause mortality, while gluteofemoral (lower body) fat storage is associated with a more beneficial cardiometabolic risk profile for a given body mass index (BMI) in both men and women (6-10).

ABD obesity is characterized by subcutaneous AT (SAT) and visceral AT accumulation, which both are related to cardiometabolic risk factors, dependent on factors such as sex and ethnicity (11-13). However, next to differences in body fat distribution, AT dysfunction is tightly linked to obesity-related complications (1, 2, 14). AT dysfunction is characterized by adipocyte hypertrophy, impaired lipid metabolism, decreased adipose tissue blood flow (ATBF), mitochondrial dysfunction, altered oxygenation, a state of chronic low-grade inflammation, and impaired adipokine expression/secretion (1-3, 15-17). Together, these impairments contribute to lipid spill-over in the circulation, ectopic fat deposition and low-grade systemic inflammation, collectively aggravating cardiometabolic disease development (1, 4, 5, 18-20). The predominant sequestration of lipids in lower body AT depots in premenopausal women seems to confer protection against the development of cardiometabolic diseases (4, 8, 21-23). In addition to AT depot-differences in lipid metabolism, differences in the inflammatory signatures between upper and lower body AT may contribute to the disease risk associated with a certain body fat distribution pattern (8).

Studies that have compared the inflammatory phenotype of upper and lower body SAT are scarce. Although no major differences in gene expression of inflammatory markers were previously found between abdominal and gluteal SAT (23, 24), recent findings suggest that *in vivo* IL-6 release from gluteofemoral SAT may be lower than from abdominal SAT in healthy men with normal body weight (23, 25). The latter findings might indicate that lower body SAT is characterized by a more beneficial inflammatory phenotype. Importantly, it remains to be established whether differences in the SAT depot-specific expression and secretion of (anti-)inflammatory factors exist in women as well as between people with normal weight and obesity.

Therefore, the present cross-sectional study aimed to investigate whether the expression and secretion of several well-known (anti-)inflammatory adipokines differ between upper and lower body SAT in postmenopausal women with normal weight or obesity. We hypothesized that the expression and secretion of pro-inflammatory factors are higher in upper body as compared to lower body SAT and adipocytes. To test our hypothesis, we compared the *in vivo* release of several adipokines across abdominal (ABD) and femoral (FEM) SAT, and investigated SAT depot-specific adipocyte morphology and adipokine expression in well-phenotyped postmenopausal women with normal weight or obesity. Furthermore, the expression and secretion of

adipokines was examined *in vitro* using differentiated human multipotent adipose-derived stem (hMADS) cells derived from ABD and FEM SAT from the same individuals.

4.2 Materials and methods

4.2.1 Study design

Twenty-three healthy postmenopausal women (aged 50 – 65 years) with normal weight (BMI 18-25 kg/m^2) or obesity (BMI 30-40 kg/m^2) were recruited. All subjects underwent a medical evaluation during the screening visit (see Supplementary Materials Methods – Study design for details). The *in vivo* measurements were conducted at the University of Birmingham/Queen Elizabeth Hospital Birmingham (Birmingham, UK). The University of Birmingham Ethics committee and the UK Health Research Authority National Health System Research Ethics Committee approved the present study (approval no. 18/NW/0392). The study was performed according to the Declaration of Helsinki, and all participants provided written informed consent before taking part in the study procedures. The *in vitro* experiments and sample analyses were performed at Maastricht University Medical Center+ (Maastricht, the Netherlands).

Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, any chronic medical condition requiring the use of medication known to affect body weight, glucose and/or lipid metabolism, use of anti-inflammatory agents (e.g., non-steroidal anti-inflammatory drugs, steroids) within 14 days prior to study start, planned blood donation two months prior to or after study completion, and marked alcohol consumption (>14 alcoholic units/week). Premenopausal or perimenopausal women, defined as either regular periods or a period within the last 12 months from screening date, were also excluded. Finally, individuals were excluded from the study if blood vessels were unsuitable for cannulation (i.e., too small veins or arterial plaques).

Participants were asked to arrive at the Clinical Research Facility after an overnight fast, having avoided strenuous exercise and alcohol for at least 24 hours, on three occasions. Each of these study visits took place within one week of the previous visit, separated by at least two days. Briefly, during the first visit participants were screened, and an oral glucose tolerance test (OGTT) was performed. During the second visit, arterio-venous concentration differences across ABD and FEM SAT were assessed and blood flow in these fat depots was determined. During the third visit, a dual-energy x-ray absorptiometry (DXA) scan was performed to determine body fat percentage and body composition, and ABD and FEM SAT biopsies were collected. These measurements are explained in more detail below.

4.2.2 *In vivo* measurements

4.2.2.1 Screening

Body weight, height, waist (measured midway between the lower margin of the last palpable rib and the top of the iliac crest) and hip circumferences (measured at the level of the greater trochanters) were determined. Blood pressure and heart rate were measured using a standard oscillometric blood pressure monitor with an upper arm cuff. Next, we screened blood vessels in ABD and FEM SAT using ultrasound to determine whether veins would be suitable for cannulation. Finally, an oral glucose tolerance test (OGTT) was performed to exclude individuals with type 2 diabetes mellitus.

4.2.2.2 Body composition

A dual X-Ray Absorptiometry (DXA) scan was performed after an overnight fast to determine body composition and body fat percentage (Lunar iDXA, GE Healthcare) (26).

4.2.2.3 Arterio-venous concentration differences

Arterio-venous concentration differences of adipokines across the ABD and FEM SAT depots were assessed, as described previously (27, 28). Briefly, selective venous catheterization of one the branches of the superficial epigastric veins (draining ABD SAT) was performed (28-30). Next, a superficial branch of the great saphenous vein (draining FEM SAT) was cannulated (31). Finally, an arterial catheter was inserted into the radial artery. Sixty minutes after the cannulation procedures (allowing participants to relax), blood samples were taken simultaneously from the three sites (arterial, ABD and FEM) at two different time-points, separated by 30 minutes, under fasting conditions

For the ABD SAT depot, veins located above the inguinal ligament, as determined by using the anterior superior iliac spine and the projected pubic symphysis as reference points were identified. The SAT areas lateral of the umbilicus and between the lower end of the rib cage and the inguinal ligament were scanned with ultrasound (Philips CX50 Ultrasound, Bothell, USA) on each side to identify suitable veins for cannulation and adipose tissue blood flow (ATBF) measurements in the ABD SAT depot. After application of local anesthetic (Lidocaine hydrochloride 1%), a 20-gauge central venous catheter was inserted with the Seldinger technique. Veins in FEM SAT that were suitable for cannulation and ATBF measurements were identified by scanning the inner aspect of the thigh, approximately half-way between the groin and the knee. A catheter (Venflon®) was placed and secured in place. Finally, an arterial catheter was inserted into the radial artery of the non-dominant hand using local anesthetic (1% lidocaine) and ultrasound guidance.

After completion of sample collection and blood flow measurements, all catheters were removed, and the study participants were given a meal. Due to the technical difficulties

to cannulate the small veins in these SAT depots and to collect blood samples, we successfully completed the measurements and sample collection for 9 women with normal weight and 6 women with obesity. Due to the limited number of paired blood samples draining ABD and FEM SAT for the individuals with normal weight and obesity, we decided to pool the data for all study participants per SAT depot to achieve sufficient statistical power to detect SAT depot-differences in adipokine release.

4.2.2.4 Adipose tissue blood flow

Fasting ATBF was measured in ABD and FEM SAT using a Doppler Ultrasound technique, as previously described (32). Briefly, the SAT areas lateral of the umbilicus and between the lower end of the rib cage and the inguinal ligament were scanned on each side to identify suitable veins for ATBF measurements in the ABD SAT depot. In the FEM depot, the great saphenous vein and its branches drain mostly femoral SAT. Suitable FEM veins for ATBF measurements were identified by scanning the inner aspect of the thigh, approximately half-way between the groin and the knee.

4.2.3 Biochemical analyses

During screening, blood samples were drawn to determine electrolytes, liver enzymes, full blood count, thyroid hormones, glucose, insulin and HbA1c. Blood samples were collected into heparinized tubes, centrifuged at 4°C at 1,000g, and plasma was snap-frozen and stored at −80°C until analysis. Adipokine concentrations were determined using high-sensitive ELISAs [adiponectin and plasminogen activator inhibitor (PAI)-1 from Biovendor, interleukin (IL)-6 and monocyte chemoattractant protein (MCP)-1 from Diaclone, and leptin and dipeptidyl-peptidase (DPP)-4 from R&D Systems, insulin MSD].

4.2.4 Adipose tissue biopsies and adipocyte morphology

ABD and FEM SAT biopsies and adipocyte morphology were collected and assessed, respectively, as described before (33). ABD SAT needle biopsy specimens (up to ~1 g) were collected 6 to 8 cm lateral from the umbilicus and from the FEM region (anterior site of the upper leg), respectively, under local anesthesia (1% lidocaine) after an overnight fast. Biopsy specimens were immediately rinsed with sterile saline and visible blood vessels were removed with sterile tweezers. A small part of the SAT sample was fixed overnight in 4% paraformaldehyde and embedded in paraffin for histology. Another part was used for isolation of human multipotent adipose-derived stem (hMADS) cells, as described before (33). The remaining tissue was snap-frozen in liquid nitrogen and stored at −80°C for gene/protein expression analysis.

Histological sections (8 µm) were cut from paraffin-embedded tissue, mounted on microscope glass slides, and dried overnight in an incubator at 37°C. Sections were

stained with hematoxylin and eosin. Digital images were captured with the use of a Leica DFC320 digital camera (Leica, Rijswijk, Netherlands) at ×20 magnification (Leica DM3000 microscope; Leica). Computerized morphometric analysis (Leica QWin V3, Cambridge, England) of individual adipocytes was performed by measuring at least 200 adipocytes per sample.

4.2.5 Calculations

Adipokine release across ABD and FEM adipose tissue was assessed using the arteriovenous difference technique. Fractional release [FR = ((venous - arterial concentration)/arterial concentration) * 100%] was calculated for each adipokine using the concentration from SAT depot-specific blood samples. A positive FR value reflects the release of adipokines from SAT. All calculations were performed as described previously (28-30).

Indexes of pancreatic β-cell function and insulin resistance were calculated using the updated computer model-based homeostatic model assessment (HOMA) method (34).

4.2.6 Human primary adipocyte experiments

Human multipotent adipose-derived stem (hMADS) cells, an established human white adipocyte model (35), were obtained from ABD and FEM subcutaneous SAT. . Cells were seeded at a density of 2000 cells/cm^2 and kept in proliferation medium for seven days and thereafter in differentiation medium for 14 days. All experiments were performed on day 14 of adipogenic differentiation. Paired ABD and FEM adipocyte samples derived from 9 women with normal weight and 9 women with obesity were used for these experiments.

4.2.7 Adipose tissue and adipocyte gene expression analysis

Total RNA was extracted from all frozen SAT specimens (~150 mg) and hMADS cells using TRIzol reagent (Invitrogen, Breda, Netherlands), and SYBR-Green–based real-time PCRs were performed using an iCycler (Bio-Rad, Veenendaal, Netherlands; primer sequences are shown in Supplemental Table 1). Results were normalized to the mean of 18S ribosomal RNA.

4.2.8 Adipocytokine secretion measurement

The medium of the hMADS cells was collected over 24 hours to determine adipokine secretion using high-sensitive ELISA. If necessary, samples were diluted with a provided

dilution buffer from the manufacturer prior to the assay, which was performed in duplicates, according to the manufacturer's instructions.

4.2.9 Statistical analyses

To assess whether there was significant release of adipokines from ABD and/or FEM SAT, we compared the fractional release value for each adipokine against zero release (that is, no net release). AT depot-differences in the secretion of adipokines and gene expression within women with normal weight and obesity were analyzed using Student's paired t-tests (Wilcoxon signed rank tests in case data were not normally distributed), while differences between individuals with normal weight and obesity were determined using unpaired t-tests (Mann Whitney test in case data were not normally distributed). GraphPad Prism version 8 for Windows was used to perform statistics, and $p < 0.05$ was considered as statistically significant. Data are presented as mean ± SEM.

4.3 Results

4.3.1 Subject characteristics

Participants' characteristics are shown in Table 4.1. By definition, BMI was higher in women with obesity compared to normal weight (both $p<0.001$). Furthermore, waist and hip circumferences were significantly higher in women with obesity, while waist-to-hip ratio was not statistically different between groups ($p=0.443$). The sizes of all AT depots examined (visceral, abdominal and leg fat) were higher in women with obesity (all $p<0.001$). In addition, women with obesity tended to have higher fasting insulin concentrations ($p=0.053$). In line, HOMA2 IR was higher in women with obesity compared with normal weight ($p=0.050$).

Table 4.1 Anthropometric characterisation and metabolic profile of participants

	Normal weight (*n*=13)	Obesity (*n*=10)	p Value
Age (years)	56.6 ± 1.5	56.6 ± 1.1	0.994
BMI (kg/m^2)	22.9 ± 0.4	34.5 ± 0.9	<0.001
Waist circumference (cm)	78.6 ± 2.2	105.1 ± 4.2	<0.001
Hip circumference (cm)	95.0 ± 2.1	125.2 ± 7.3	<0.001
Waist-to-Hip Ratio	0.83 ± 0.02	0.86 ± 0.05	0.538
Visceral fat mass (g)	350 ± 88	1,272 ± 140	<0.001
Abdominal fat mass (kg)	9.91 ± 0.98	23.22 ± 1.91	<0.001
Leg fat mass (kg)	7.57 ± 0.60	15.29 ± 1.18	<0.001
Fasting glucose (mmol/l)	4.97 ± 0.10	5.16 ± 0.20	0.404
2-Hour Glucose (mmol/l)	5.09 ± 0.20	4.99 ± 0.30	0.775
Fasting insulin (pmol/l)	25.40 ± 4.00	48.60 ± 12.90	0.053
HOMA2-IR	0.47 ± 0.1	0.92 ± 0.3	0.050
SBP (mmHg)	120.9 ± 3.9	131.3 ± 4.3	0.098
DBP (mmHg)	76.2 ± 3.1	80.6 ± 3.1	0.356

BMI, body mass index; DBP, diastolic blood pressure; HOMA2-IR, Homeostasis Model Assessment 2 – Insulin Resistance; SBP, systolic blood pressure. Data are mean ± SEM.

4.3.2 Plasma adipokine concentrations

Arterial plasma concentrations of adipokines were measured after an overnight fast (Figure 4.1). Plasma leptin concentrations were significantly higher in women with obesity compared to normal weight (46.6 ± 3.1 vs. 9.8 ±1.8 ng/mL, respectively, p<0.001) (Figure 4.1A). Furthermore, PAI-1 concentrations were higher in individuals with obesity than normal weight (39.8 ± 4.3 vs., 24.8 ± 2.4 ng/mL, respectively, p=0.036) (Figure 4.1B). No significant differences were found for circulating DPP-4 (412.0 ± 30.1 vs. 469.4 ± 15.4 ng/mL, p=0.272) and MCP-1 concentrations (339.6 ± 31.6 vs. 287.4 ± 17.1 ng/mL, respectively, p=0.299) between women with obesity and normal weight (Figure 4.1C and D). Finally, a tendency for lower circulating adiponectin concentration in women with obesity compared to normal weight was found (6.8 ± 0.9 vs. 12.1 ± 1.6 µg/mL, respectively, p=0.088) (Figure 4.1E). IL-6 concentrations were below the detection limit for most individuals and are therefore not reported.

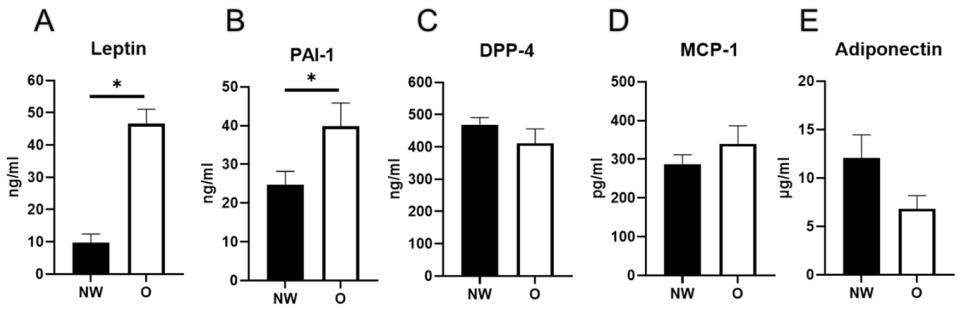

*Figure 4.1: Plasma adipokine concentrations in arterial blood from postmenopausal women with normal weight (n=9) and obesity (n=6). A) Leptin B) PAI-1, C) DPP-4, D) MCP-1, E) Adiponectin; NW, normal weight; O, obesity. Data are expressed as mean ± SEM. *p<0.05.*

4.3.3 *In vivo* secretion of adipokines from abdominal and femoral subcutaneous adipose tissue

To explore whether *in vivo* adipokine release is different across ABD and FEM SAT, we directly measured the fractional release (FR) of several adipokines in women with obesity or normal weight using the arterio-venous balance technique (Figure 4.2). Significant FR was only found for leptin and MCP-1 (both p=0.001 vs. zero release). Leptin FR was similar between ABD and FEM depots (30.7 ± 2.6 vs. 44.1 ± 11.4%, respectively, p=0.383) (Figure 4.2A). The FR of MCP-1 across ABD SAT was significantly higher than that across FEM SAT (31.6% ± 4.4% vs. 24.2% ± 4.5%, respectively, p=0.023) (Figure 4.2D).

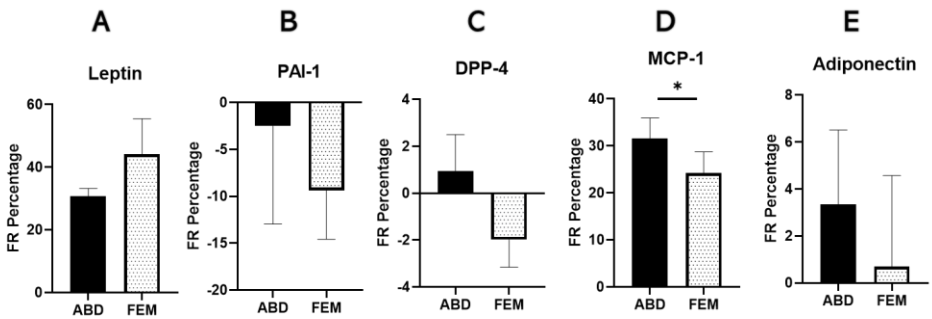

*Figure 4.2: Fractional release of adipokines across subcutaneous abdominal (ABD) and femoral (FEM) subcutaneous adipose tissue (SAT) in postmenopausal women with normal weight (n=9) and obesity (n=6). A) Leptin B) PAI-1, C) DPP-4, D) MCP-1, E) Adiponectin; Paired data from ABD and FEM SAT are shown. Data are expressed as mean ± SEM. *p<0.05.*

4.3.4 Abdominal and femoral subcutaneous adipose tissue blood flow

Pooled data from women with normal weight and obesity demonstrated that fasting ATBF was not significantly different between ABD and FEM SAT (9.3 ± 2.1 versus 5.8 ± 1.8 mL/min, p=0.296). More specific, there were also no significant differences between fasting ABD and FEM ATBF in women with normal weight (p=0.641, *n*=8) and obesity (p=0.313, *n*=6). Furthermore, ABD ATBF (8.4 ± 2.1 vs. 11.2 ± 2.9 mL/min, respectively, p=0.459) and FEM ATBF (5.3 ± 1.5 vs. 6.4 ± 2.1 mL/min, respectively, p=0.755) were not significantly different between women with normal weight and obesity.

4.3.5 Abdominal and femoral adipocyte morphology

Adipocytes from women with normal weight were significantly smaller compared to adipocytes from women with obesity, both for ABD (p=0.014) and FEM SAT (p=0.001) (Figure 4.3A). The smaller mean adipocyte size of ABD and FEM SAT in normal weight individuals was explained by a lower frequency of very large adipocytes and a higher frequency of very small adipocytes as compared to women with obesity (Figure 4.3B). Pooled data from women with normal weight and obesity showed that adipocyte size was not different between ABD and FEM SAT (68.5 ± 1.9 versus 68.4 ± 1.6 µm, p=0.791). In line, no significant differences in adipocyte size were found between ABD and FEM SAT in women with normal weight (p=0.730) and obesity (p=1.000).

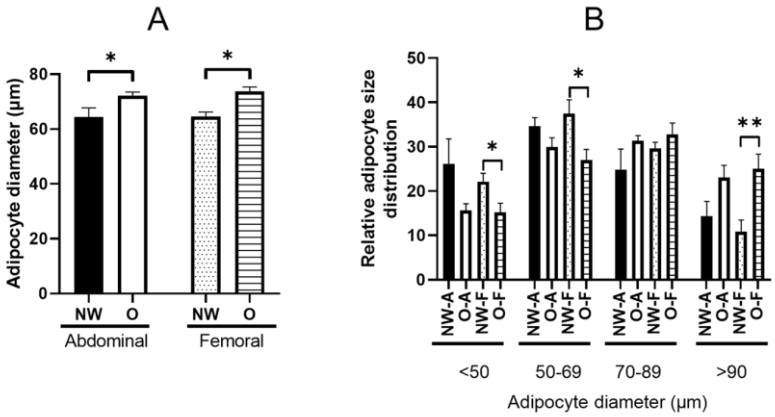

*Figure 4.3: A: Morphology of subcutaneous adipocytes from individuals with normal weight (n = 11) and obesity (n = 8). A: Fat cell size; B: Relative adipocyte size distribution. NW-A: Normal weight abdominal, O-A; Obese abdominal, NW-F; Normal weight femoral, O-F; Obese femoral. NW, normal weight; O, obesity. Data are expressed as mean ± SEM. *p<0.05, **p<0.001.*

4.3.6 Abdominal and femoral subcutaneous adipose tissue gene expression

Next, we assessed the adipokine gene expression profile in ABD and FEM SAT (Figure 4.4A-G). Pooled data from women with normal weight or obesity showed that gene

expression of leptin (p=0.010) and MCP-1 (p=0.027) was significantly lower in ABD than FEM SAT, while a tendency for lower PAI-1 (p=0.080) and tumor necrosis factor (TNF-α (p=0.090) gene expression in ABD compared to FEM was found. No significant differences in gene expression of IL-6, DPP-4, and adiponectin were found between ABD and FEM SAT.

Furthermore, we compared adipokine gene expression in women with normal weight and obesity separately (Figure 4.4H-N). ABD (p=0.002) and FEM (p=0.046) SAT gene expression of leptin were significantly higher in women with obesity than normal weight. Furthermore, ABD (p=0.095) and FEM SAT (p=0.014) gene expression of PAI-1 were higher in obesity. In addition, ABD SAT gene expression of IL-6 (p=0.053) tended to be higher in women with obesity than normal weight. When examining SAT depot-differences in normal weight and obese groups separately, we found a significantly lower PAI-1 gene expression in ABD than FEM SAT in women with obesity (p=0.008). Moreover, leptin (p=0.052) and MCP-1 (p=0.075) gene expression tended to be lower in ABD than FEM SAT in individuals with normal weight. No significant SAT depot-differences in adiponectin, DPP-4 and TNF-α gene expression were found in individuals with normal weight and obesity.

We found significant SAT depot-specific correlations between fat cell size and gene expression levels. Leptin gene expression was positively correlated with fat cell size both in ABD (r=0.657; p=0.024) and FEM SAT (r=0.515; p=0.024), while PAI-1 gene expression in FEM SAT was positively correlated with FEM fat cell size (r=0.690; p=0.001) but PAI-1 gene expression in ABD AT not significantly associated with ABD fat cell size (r=0.385, p=0.218). No significant correlations between fat cell size and gene expression levels of IL-6, TNF-α, DPP-4, MCP-1 and adiponectin were found (data not shown).

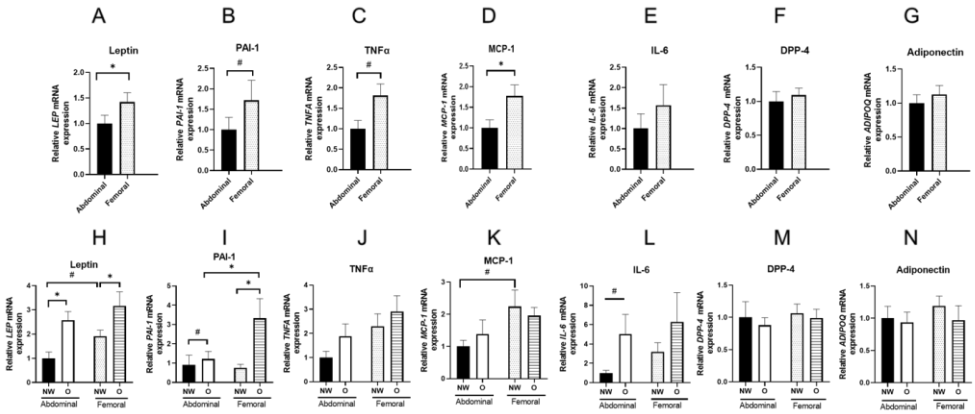

*Figure 4.4: Gene expression of adipokines in abdominal and femoral subcutaneous adipose tissue. Data are shown for the total group of women with normal weight and obesity (panels A – G; pairs n=18) as well as for the normal weight and obese groups separately (Panels H – N; Abdominal NW, n = 10, abdominal O n = 9, femoral NW n = 11, femoral O n = 9). NW, normal weight; O, obesity. Data are expressed as mean ± SEM. *p<0.05, # p<0.01.*

4.3.7 Gene expression in differentiated hMADS from abdominal and femoral subcutaneous adipose tissue

Since AT consists of multiple cells types (3), including immune cells, we next specifically examined gene expression in differentiated hMADS derived from ABD and FEM SAT (Figure 4.5A-G) obtained from the same individuals with normal weight or obesity that underwent the *in vivo* measurements and SAT biopsies (Figure 4.5). Pooled data from women with normal weight or obesity showed that gene expression of leptin (p=0.009, Figure 4.5A), PAI-1 (p<0.001, Figure 4.5B) and IL-6 (p<0.001, Figure 4.5E) were significantly higher in ABD compared to FEM adipocytes. ABD adipocytes showed a lower gene expression of DPP-4 (p=0.035, Figure 4.5F) and adiponectin (p=0.029, Figure 4.5G). No significant differences between ABD and FEM adipocytes were observed for TNF-α (p=0.284, Figure 4.5C) and MCP-1 (p=0.712, Figure 4.5D) gene expression. Gene expression of the adipocyte differentiation markers PPARγ, C/EBPα, PLIN1 and FAS was not significantly different between abdominal and femoral adipocytes. (Supplementary Materials – Figure 4.7).

Furthermore, we compared adipokine gene expression in differentiated adipocytes from women with normal weight or obesity separately (Figure 4.5H-N). We found a higher gene expression of IL-6 and PAI-1 in ABD compared to FEM adipocytes derived from individuals with normal weight (p=0.006 and p=0.068, respectively) and obesity (p=0.018 and p=0.002, respectively) (Figure 4.5L, I). Furthermore, ABD adipocytes derived from normal weight women showed lower adiponectin (p=0.005, Figure 4.5N) and higher leptin (p=0.098) gene expression compared to FEM adipocytes (Figure 4.5H). In addition, DPP-4 gene expression (Figure 4.5M) was significantly lower in ABD than FEM adipocytes derived from women with obesity (p=0.043). No adipocyte depot-differences were found for TNF-α and MCP-1 gene expression (Figure 4.5J, K). Finally, IL-6 gene expression tended to be higher in both ABD (p=0.054) and FEM (p=0.069) adipocytes derived from women with normal weight compared to obesity (Figure 4.5L).

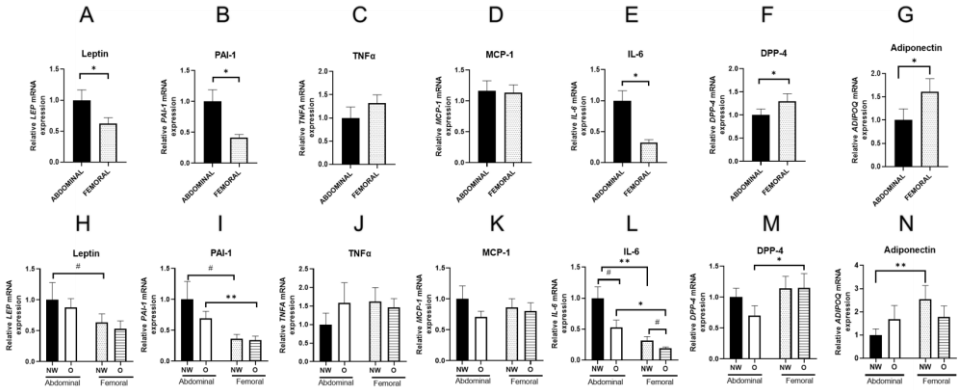

*Figure 4.5: Adipokine gene expression in adipose tissue-derived mesenchymal stem cells that were differentiated for 14 days. Data are shown for the total group of women with normal weight and obesity (panels A – G; n = 18) as well as for both groups separately (Panels H – N; abdominal NW, n = 9; abdominal O, n = 9; femoral NW, n = 9; femoral O, n = 9). NW, normal weight; O, obesity. Data are expressed as mean ± SEM. *p<0.05, **p<0.001, # p<0.01*

4.3.8 Adipokine secretion from differentiated abdominal and femoral hMADS

Finally, we investigated the secretion of adipokines from human primary ABD and FEM adipocytes (Figure 4.6). Pooled data from women with normal weight or obesity showed significantly lower secretion of MCP-1 from ABD compared to FEM adipocytes (198.5 ± 39.1 pg/mL versus 337.6 ± 58.5 pg/mL, p=0.004) (Figure 4.6C). No significant depot-differences in secretion rates of leptin, PAI-1, IL-6, and DPP-4, between ABD and FEM adipocytes were present.

When comparing adipokine secretion from ABD and FEM adipocytes from women with normal weight or obesity separately, we found a significantly lower secretion of MCP-1 from ABD compared to FEM adipocytes derived from women with obesity (165 ± 44 vs. 340 ± 85 pg/mL, p=0.013) (Figure 4.6H). No significant depot-differences in secretion rates of leptin, PAI-1, IL-6, DPP-4 and MCP-1 between abdominal and femoral adipocytes were present. In addition, the secretion of IL-6 from abdominal adipocytes tended to be higher in cells derived from women with normal weight compared to obesity (80.5 ±15.2 pg/mL versus 41.8 ± 9.4 pg/mL, respectively, p=0.063) (Figure 4.6D). Adiponectin secretion was below the detection limit, and this data is therefore not reported.

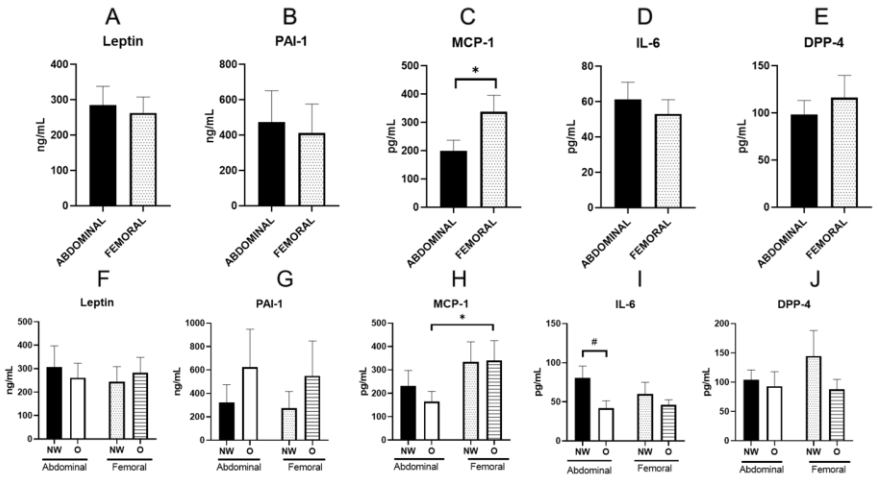

*Figure 4.6: Adipokine secretion from adipose tissue-derived mesenchymal stem cells that were differentiated for 14 days. Data are shown for the total group of women with normal weight and obesity (panels A – E; n = 18) as well as for both groups separately (Panels F – J); abdominal NW, n = 9; abdominal O, n = 9; femoral NW, n = 9; femoral O, n = 9). NW, normal weight; O, obesity. Data are expressed as mean ± SEM. *p<0.05, # p<0.01.*

4.4 Discussion

In the present study, we investigated the inflammatory signatures of ABD and FEM SAT in postmenopausal women with normal weight and obesity. More specifically, we compared the *in vivo* release of adipokine from ABD and FEM SAT in both groups, examined adipocyte morphology and gene expression of adipokines in these SAT depots, and determined gene expression and secretion of adipokines *in vitro* using differentiated human primary ABD and FEM subcutaneous adipocytes derived from the same study participants. The present findings demonstrate for the first time that upper and lower body adipose tissue as well as adipocytes are characterized by distinct inflammatory signatures in postmenopausal women with normal weight and obesity.

In the present study, we assessed leptin and adiponectin as classical adipokines altered in obesity (36-39), and also determined the expression and secretion of several well-known pro-inflammatory molecules (TNF-α, IL-6, PAI-1, DPP-4, and MCP-1) that have been linked to obesity and cardiometabolic disease risk (2, 3, 40-49). We found significant fractional release of leptin and MCP-1 from ABD and FEM subcutaneous SAT, with similar fractional release of leptin from both SAT depots and higher release of MCP-1 from ABD compared to FEM SAT. The comparable release of leptin from FEM and ABD SAT is in line with a previous report (23). No release of other adipokines, including PAI-1, DPP-4 and adiponectin, across ABD and FEM SAT was detectable. The latter is in line with previous studies, showing no significant release of adiponectin, IL-6, and DPP-4 across human ABD SAT in people with normal weight and obesity (48, 50). One study that did report *in vivo* DPP-4 release across human ABD SAT only found significant release in few individuals with low (<288 ng/ml) plasma DPP-4 concentrations (48), while mean DPP-4 concentrations were much higher (>400 ng/ml) in the present study. The lack of detectable adiponectin release across SAT may be explained by a low release rate and long half-life, reflected by relatively constant circulating concentrations of these adipokines (8). We found higher arterial concentrations of leptin and PAI-1 in women with obesity. Since no differences in the *in vivo* fractional release of these factors from ABD and FEM SAT were found between individuals with normal weight and obesity, the higher circulating leptin and PAI-1 concentrations are likely explained by the higher total fat mass in obesity.

Differences in the functional properties between AT depots may underlie the cardiometabolic disease risk associated with a certain body fat distribution pattern. Indeed, functional differences between ABD and FEM SAT seem to emerge from adipocytes having distinct properties (4, 9). Many studies have demonstrated a close relationship between adipocyte morphology and AT function, with hypertrophic adipocytes (as often seen in people with obesity) showing impairments in lipid metabolism and a more pro-inflammatory phenotype, which may aggravate insulin resistance (1, 3, 4, 51). In the present study, women with obesity had larger adipocytes than individuals with normal weight, both in ABD and FEM SAT. However, adipocyte size did not differ between ABD and FEM SAT in both groups. This is in agreement with some (23, 52, 53) but not all previous reports comparing upper and lower body SAT (33, 54-56), and may relate to characteristics of the study populations investigated (i.e. age and metabolic status). Our study participants did not have severe obesity and had,

by definition for inclusion in the study, a relatively healthy metabolic profile. In line with adipocyte hypertrophy in women with obesity, we found higher SAT gene expression of leptin, PAI-1, and IL-6 (only in ABD SAT) in the people with obesity. Few studies, however, have directly compared upper and lower body SAT inflammation. Intriguingly, despite similar fat cell sizes in both SAT depots, the present findings demonstrate lower gene expression of leptin, MCP-1, PAI-1 and TNF-α in ABD than FEM SAT. Previous reports indicated that lower body SAT shows a similar (24) or more pro-inflammatory profile compared to ABD SAT (57). Moreover, global transcriptional profiling of men and women failed to identify differentially expressed clusters of inflammation-specific genes between ABD and gluteal SAT, although stronger associations between the expression of pro-inflammatory factors and several obesity-related traits were found for ABD SAT (23).

Since whole-AT gene expression profiles are determined by gene expression in multiple adipose-derived cell types such as adipocytes and immune cells, we also specifically investigated gene expression profiles in differentiated human primary ABD and FEM subcutaneous adipocytes derived from the participants that underwent the *in vivo* measurements and SAT biopsies. Interestingly, we observed that hMADS derived from ABD and FEM SAT that have been differentiated *in vitro* (and therefore been exposed to the same experimental microenvironment) show different gene expression patterns. Indeed, we demonstrate higher gene expression of the pro-inflammatory factors IL-6 and PAI-1 in ABD compared to FEM adipocytes derived from women with both normal weight and obesity. Furthermore, the expression of leptin was higher and that of adiponectin lower in ABD compared to FEM adipocytes derived from women with normal weight. These findings highlight intrinsic differences in the inflammatory signatures of human abdominal and femoral adipocytes, which are already present in cells derived from a healthy ('non-obese') AT microenvironment (i.e., normal weight individuals). In addition, DPP-4 gene expression was lower in abdominal than femoral adipocytes derived from women with obesity. Adipocyte differentiation markers were not significantly different between ABD and FEM adipocytes, suggesting that these differences in adipocyte gene expression are not due to differences in adipocyte differentiation between abdominal and femoral adipocytes. The fact that inflammatory gene expression was not higher in differentiated human primary abdominal and femoral adipocytes derived from women with obesity compared to normal weight provides further support for the notion that adipocyte hypertrophy and/or the contribution of the inflammatory cell component are key factors determining the *in vivo* AT inflammatory signature. The differences in adipocyte gene expression did, however, not translate into functional differences in the secretion of adipokines. Specifically, we only found a lower secretion of MCP-1 from ABD compared to FEM adipocytes derived from both women with normal weight or obesity, but no differences in the secretion rates of leptin, PAI-1, IL-6, and DPP-4 between ABD and FEM adipocytes were apparent. The discrepancy between MCP-1 fractional release *in vivo* being higher from ABD versus FEM SAT, while ABD SAT MCP-1 gene expression as well as ABD adipocyte MCP-1 secretion were lower compared to FEM SAT/adipocytes might be explained by depot-differences in post-transcriptional regulation and secretory pathways influencing the release of adipokines from these fat depots. Notably, gene expression of IL-6 was higher in ABD than FEM adipocytes, while no significant differences in IL-6 gene expression were

found between ABD and FEM AT. This might be explained by depot-differences in IL-6 expression due to the presence of other cells than adipocytes such as immune cells (58), which warrants further investigation.

A strength of the present study is that we, for the first time, combined paired *in vivo* measurements across ABD and FEM SAT, analyses in ABD and FEM SAT biopsies, and *in vitro* experiments using differentiated human primary ABD and FEM subcutaneous adipocytes derived from the study participants. Furthermore, we did not perform the experiments using a pool of stem cells from normal weight and obese donors (risking those outcomes are influenced/masked by strong effects seen in a specific donor) or a single donor, as often done, but performed the *in vitro* experiments with cells from many donors with normal weight and obesity separately.

Noteworthy, the present study also has some limitations. First, a formal power calculation was not performed, given the exploratory nature of the study. The number of participants we aimed to include in our study to detect differences was based on previous studies using the arterio-venous balance technique to investigate group differences in adipokines/metabolites across upper-body versus lower-body AT (25). The data obtained from this study might be useful for power calculations of future larger studies. Third, due to the technical difficulties to cannulate and collect blood samples from the small veins in ABD and FEM, we were able to successfully complete sample collection for 9 women with normal weight and 6 women with obesity. Due to the limited number of paired blood samples draining ABD and FEM SAT for the individuals with normal weight and obesity, we could unfortunately not analyze data separately for both groups due to limited statistical power. Secondly, we determined ATBF using Doppler ultrasound, which provides data on intravascular blood flow in relatively large SAT veins rather than at the capillary level (32). Unfortunately, it was not possible to utilize the ^{133}Xe wash-out technique due to the global production stop of medical ^{133}Xe (32). Consequently, we could not quantify absolute fluxes of adipokines per unit AT, and data on *in vivo* release of adipokines should therefore be interpreted with some caution. Nevertheless, calculation of fractional release of adipokines also yields valuable insights into adipokine release across different AT depots, especially since ATBF was not significantly different between ABD and FEM SAT in the present study. Finally, we only studied the superficial layer of SAT. Previous studies have shown different functional properties when comparing adipocytes derived from the superficial and the deep subcutaneous layer (59).

In conclusion, our findings demonstrate that upper and lower body SAT are characterized by distinct inflammatory signatures in postmenopausal women with normal weight and obesity, which seem independent of adipocyte size. Future studies with a larger sample size are warranted to investigate functional differences of upper and lower body SAT in different populations, taking age, sex, metabolic status, body composition, obesity duration and weight cycling, as well as differential immune cell populations into account, and relate these to metabolic health at the whole-body level.

Data Availability Statement

The original data of the present study are included in the article/supplementary material. Further inquiries related to raw data can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the University of Birmingham Ethics committee and the UK Health Research Authority National Health System Research Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Konstantinos N. Manolopoulos and Gijs H. Goossens acquired funding, conceived, and designed research, interpreted data, and revised the manuscript; Ioannis G. Lempesis performed experiments, analyzed data, interpreted data, prepared figures, and drafted the manuscript; Nicole Hoebers, Yvonne Essers, Johan W.E. Jocken, and Rosemary Dineen performed experiments and analyzed data. Ellen E. Blaak, and Johan W.E. Jocken interpreted data and revised the manuscript. All authors contributed to the article and approved the submitted version..

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Supplemental materials

Human primary adipocyte experiments

We determined gene expression of several adipocyte differentiation markers: peroxisome proliferator-activated receptor γ (PPARγ), CCAAT-enhancer binding protein α (C/EBPα), fatty acid synthase (FAS), and Perilipin 1 (PLIN1). Total RNA was extracted from hMADS cells using TRIzol reagent (Invitrogen, Breda, Netherlands), and SYBR-Green–based real-time PCRs were performed using an iCycler (Bio-Rad, Veenendaal, Netherlands). Results were normalized to the mean of 18S ribosomal RNA.

Table 4.2: Primer sequences

Target	Direction	Sequence
TNF-α	Forward	CCGAGTGACAAGCCTGTAGC
	Reverse	GAGGACCTGGGAGTAGATGAG
IL-6	Forward	AAATTCGGTACATCCTCGACGG
	Reverse	GGAAGGTTCAGGTTGTTTTCTGC
DPP-4	Forward	AGTGGCGTGTTCAAGTGTGG
	Reverse	CAAGGTTGTCTTCTGGAGTTGG
PAI-1	Forward	TCGTCCAGCGGGATCTGAA
	Reverse	GCCGTTGAAGTAGAGGGCATT
MCP-1	Forward	CCCCAGTCACCTGCTGTTAT
	Reverse	TCCTGAACCCACTTCTGCTT
Adiponectin	Forward	TGGTGAGAAGGGTGAGAA
	Reverse	GTTCAGTCCACAGTGTCGCAGA
Leptin	Forward	GCTGTGCCCATCCAAAAAGTCC
	Reverse	CCCAGGAATGAAGTCCAAACCG
18S	Forward	AGTTAGCATGCCAGAGTCTCG
	Reverse	TGCATGGCCGTTCTTAGTTG
PPARγ	Forward	TACTGTCGGTTTCAGAAATGCC
	Reverse	GTCAGCGGACTCTGGATTCAG
FAS	Forward	CCGAGACACTCGTGGGCTA
	Reverse	CTTCAGCAGGACATTGATGCC
PLIN1	Forward	CTCTCGATACACCGTGCAGA
	Reverse	TGGTCCTCATGATCCTCCTC
VEGFA	Forward	TCCGGGCTCGGTGATTTA
	Reverse	GACTCCGGCGGAAGCAT
GLUT1	Forward	GATTGGCTCCTTCTCTGTGG
	Reverse	TCAAAGGACTTGCCCAGTTT
BNIP3	Forward	ATCAAAAGGTGCTGGTGGAG
	Reverse	ACCCTCAGCATGAGGAACAC
C/EBPα	Forward	AAGAAGTCGGTGGACAAGAACAG
	Reverse	GCGGTCATTGTCACTGGTCA

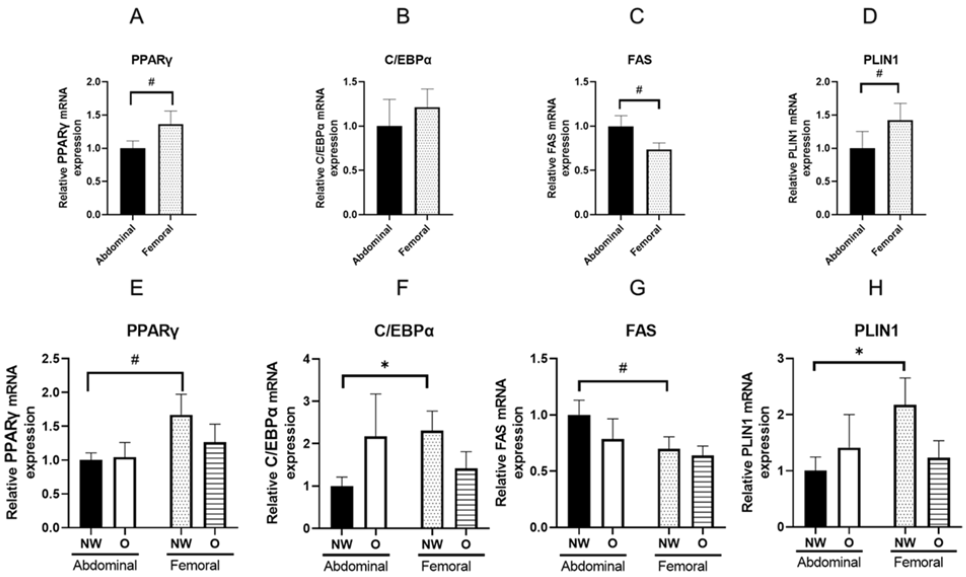


*Figure 4.7: Gene expression of adipocyte differentiation markers in adipose tissue-derived mesenchymal stem cells that were differentiated for 14 days. Data are shown for the total group of women with normal weight and obesity (panels A – D; n = 18) as well as for both groups separately (Panels E – H; abdominal NW, n = 9; abdominal O, n = 8; femoral NW, n = 9; femoral O, n = 9). C/EBPα, CCAAT-enhancer binding protein α; FAS fatty acid synthase; NW, normal weight; O, obesity; PLIN1, Perilipin 1; PPARγ, peroxisome proliferator-activated receptor γ. Data are expressed as mean ± SEM. *p<0.05, #p<0.10*

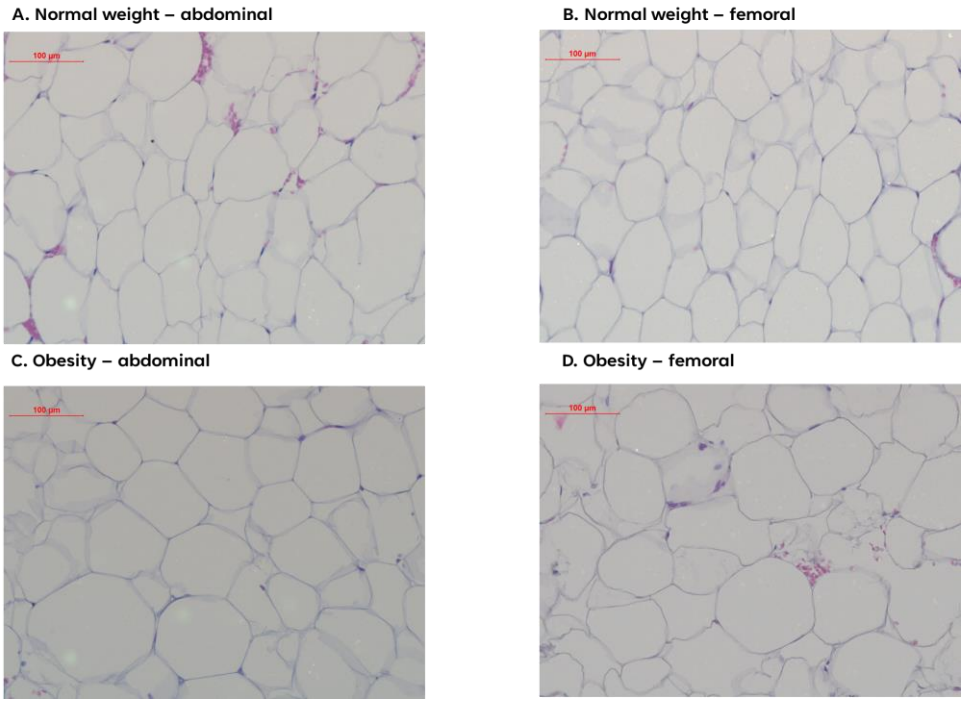
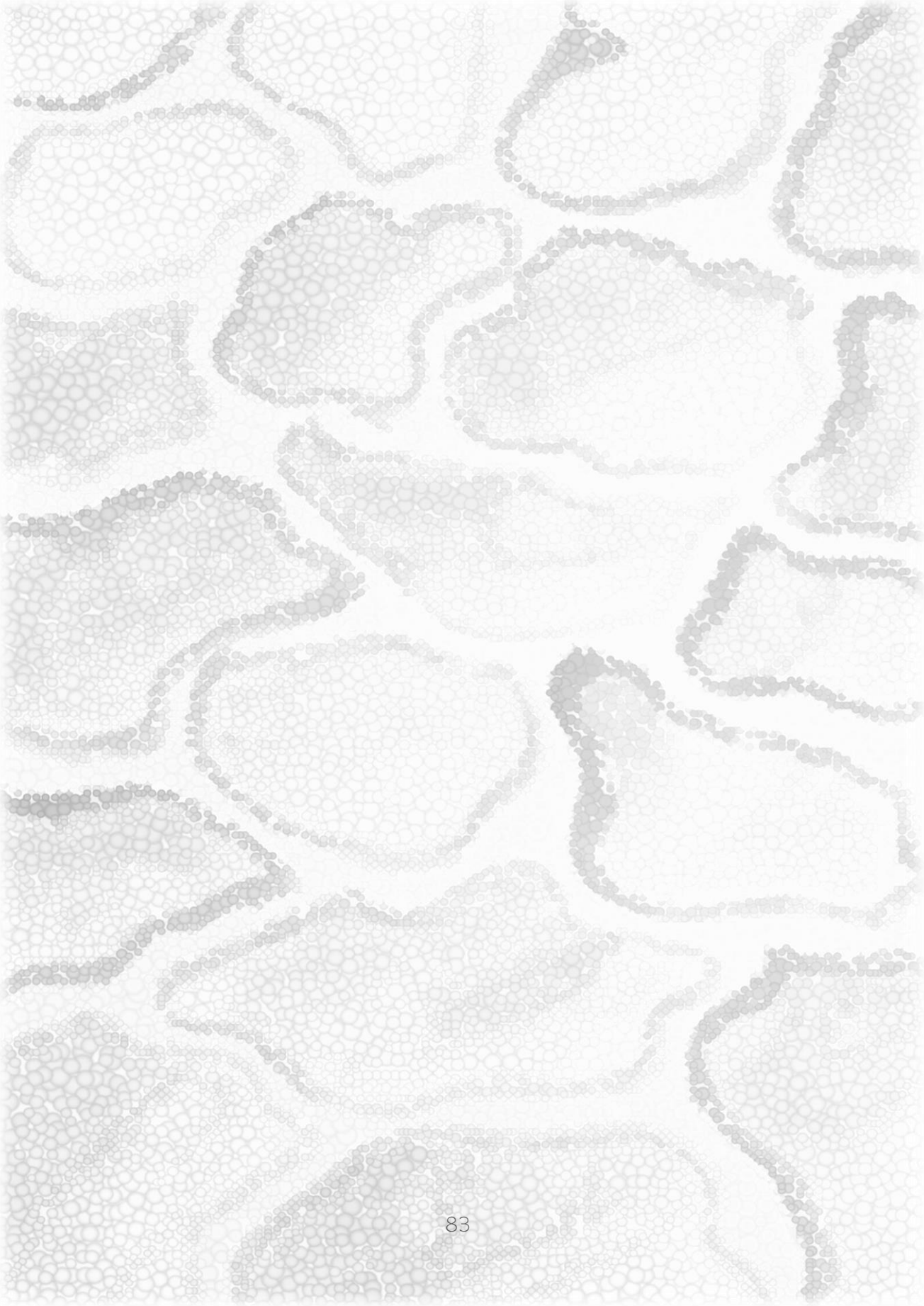
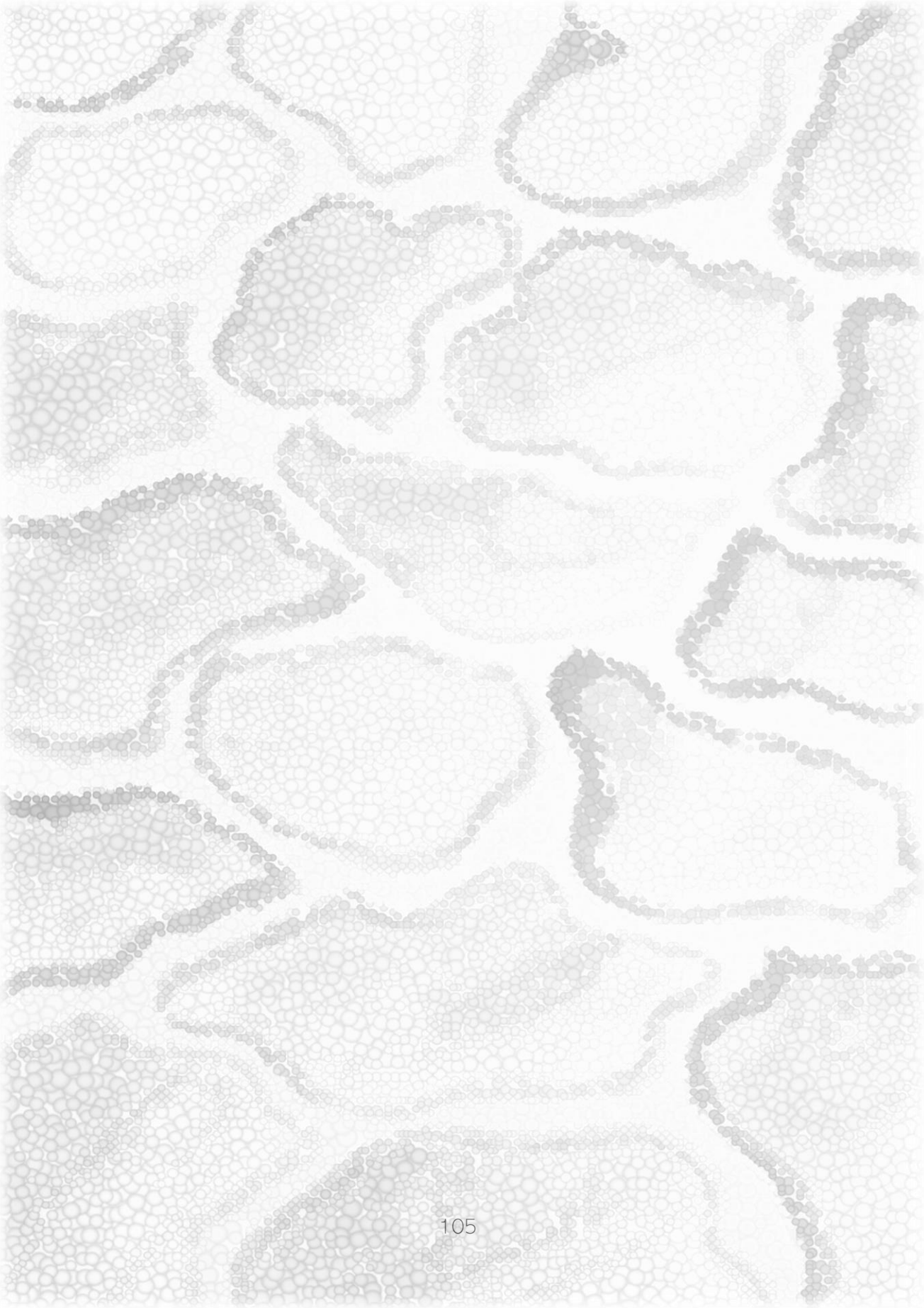


Figure 4.8: Representative images with Haemotoxylin and Eosin (H&E) staining from adipose tissue biopsies from individuals with normal weight (panels A and B) or obesity (panels C and D) from abdominal subcutaneous adipose tissue (panels A and C) and femoral subcutaneous adipose tissue (B and D). Images indicate the differences between BMI groups with simple visual inspection. Nuclei are stained purple/blue while collagen, cell cytoplasm, erythrocytes shades of pink red. The adipocyte area is represented by the empty-like white space, where the lipids accumulate.



5 Impaired mitochondrial respiration in upper compared to lower body differentiated human multipotent adipose-derived stem cells and adipose tissue in humans

Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Dubois L.J., Blaak E. E., Manolopoulos K. N., Goossens G. H. *Submitted*.



6 Physiological oxygen levels differentially regulate adipokine production in abdominal and femoral adipocytes from individuals with obesity versus normal weight

Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Rouschop K. M.A., Blaak E. E., Manolopoulos K. N., Goossens G. H. *Cells 2022;11(22): 3532*

Abstract

Adipose tissue (AT) inflammation may increase obesity-related cardiometabolic complications. Altered AT oxygen partial pressure (pO_2) may impact the adipocyte inflammatory phenotype. Here, we investigated the effects of *physiological* pO_2 levels on the inflammatory phenotype of abdominal (ABD) and femoral (FEM) adipocytes derived from postmenopausal women with normal weight (NW) or obesity (OB). Biopsies were collected from ABD and FEM subcutaneous AT in eighteen postmenopausal women (aged 50–65 years) with NW (BMI 18–25 kg/m^2, n = 9) or OB (BMI 30-40 kg/m^2, n = 9). We compared the effects of prolonged exposure to different *physiological* pO_2 levels on adipokine expression and secretion in differentiated human multipotent adipose-derived stem cells. Low *physiological* pO_2 (5% O_2) significantly increased leptin gene expression/secretion in ABD and FEM adipocytes derived from individuals with NW and OB compared with high *physiological* pO_2 (10% O_2) and standard laboratory conditions (21% O_2). Gene expression/secretion of IL-6, DPP-4, and MCP-1 was reduced in differentiated ABD and FEM adipocytes from individuals with OB but not NW following exposure to low compared with high *physiological* pO_2 levels. Low *physiological* pO_2 decreases gene expression and secretion of several proinflammatory factors in ABD and FEM adipocytes derived from individuals with OB but not NW.

6.1 Introduction

Excess fat mass in obesity poses a major health risk (1). Research of the past decades has clearly demonstrated that body fat distribution is a better predictor of cardiometabolic complications than total fat mass, with abdominal obesity increasing and lower-body fat (gluteofemoral) accumulation conferring relative protection against chronic cardiometabolic diseases (2-6) This seems related to distinct functional properties of these different AT depots. Many studies in rodents and humans have shown that AT dysfunction in obesity is characterised by adipocyte hypertrophy, mitochondrial dysfunction, reactive oxygen species (ROS) production, impaired lipid metabolism, reduced blood flow, and inflammation, together contributing to increased risk for developing cardiometabolic diseases and cancer (6-11).

The AT microenvironment impacts metabolic and inflammatory processes (8, 9). We, and others, have previously demonstrated that AT pO_2, which is determined by the balance between local oxygen supply (determined by adipose tissue blood flow) and oxygen consumption (primarily mitochondrial oxygen consumption), may be an important determinant of the AT phenotype and whole-body insulin sensitivity (9, 12-14). Interestingly, differences in AT blood flow and/or AT oxygen consumption between individuals with normal weight and obesity, and between upper and lower body AT depots, have previously been demonstrated (9, 10, 13, 15, 16). Although AT pO_2 is reduced in rodent models of obesity (17-19), conflicting findings on AT pO_2 have been reported in humans (9, 20-24). We have previously shown that AT pO_2 was higher individuals with obesity and was positively associated with AT gene expression of pro-inflammatory markers and whole-body insulin resistance (22, 25). Moreover, we found that AT pO_2 was lower in femoral compared to abdominal subcutaneous AT in women with obesity (16).

The normal physiological range of AT pO_2 in human AT is ~3-11% O_2 (~23-84 mmHg) (9, 21-23, 25). Therefore, the outcomes of experiments comparing the effects of pO_2 below and well-above these physiological levels should be interpreted with caution, since results may not directly translate to the human *in vivo* situation (9).Several *in vitro* studies have demonstrated that the expression and secretion of many adipokines are sensitive to changes in pO_2 levels, as extensively reviewed (9, 26). Most of these studies have shown that acute exposure to severe, non-physiological hypoxia (1% O_2 for 1-24 hours) induces a pro-inflammatory expression and secretion profile in (pre)adipocytes, while prolonged exposure to mild *physiological* hypoxia (5% O_2 for 14 days) seems to elicit a different adipokine expression/secretion profile (9, 16, 27). Recently, we found that prolonged exposure to low physiological hypoxia decreased pro-inflammatory gene expression in abdominal and femoral adipocytes derived from women with obesity (16). The metabolic and inflammatory responses to changes in the AT microenvironment may differ between individuals and AT depots. Thus, oxygen levels might exert distinct effects on AT function in people with different adiposity and in different AT depots. Importantly, however, studies that investigated the impact of altered pO_2 levels on the inflammatory phenotype of adipocytes derived from people with normal weight and obesity are lacking.

Therefore, the aim of the present study was to investigate the impact of prolonged exposure to various *physiological* oxygen levels on gene expression and secretion of inflammatory factors within upper and lower body differentiated human multipotent adipose-derived stem (hMADS) cells derived from women with normal weight or obesity.

6.2 Materials and methods

6.2.1 Upper and lower body adipose tissue biopsies

Paired abdominal (ABD) and femoral (FEM) subcutaneous AT needle biopsies were obtained from eighteen postmenopausal women (aged 50 – 65 years) with normal weight (NW: BMI 18-25 kg/m^2, n = 9) or obesity (OB: BMI 30-40 kg/m^2, n = 9) (Table 6.1). The UK Health Research Authority National Health System Research Ethics Committee approved the present study (approval no. 18/NW/0392). Briefly, the biopsy specimens (up to ~1 g) were collected 6 to 8 cm lateral from the umbilicus (abdominal AT) and from the anterior aspect of the upper leg (femoral AT) under local anaesthesia (1% lidocaine) after an overnight fast. Samples were immediately rinsed with sterile saline and visible blood vessels were removed with sterile tweezers. Isolation of hMADS cells followed, as described before (16).

Table 6.1: Subjects' characteristics

	Normal weight (*n*=9)	Obesity (*n*=9)	p Value
Age (years)	56.7 ± 1.8	56 ± 1.3	0.566
BMI (kg/m^2)	22.8 ± 0.4	34.8 ± 1.3	<0.001
Waist circumference (cm)	79.4 ± 3.1	105.2 ± 3.8	<0.001
Hip circumference (cm)	94.4 ± 2.8	119.9 ± 4.8	<0.001
Waist-to-Hip Ratio	0.84 ± 0.02	0.88 ± 0.04	0.127
Visceral fat mass (g)	402.5 ± 118	1,325 ± 153.3	0.003
Abdominal fat mass (*k*g)	10.01 ± 1.48	24.4 ± 2.37	<0.001
Leg fat mass (*k*g)	7.67 ± 0.86	16.03 ± 1.43	0.001
Fasting glucose (mmol/l)	4.91 ± 0.10	5.10 ± 0.23	0.416
2-Hour Glucose (mmol/l)	4.90 ± 0.34	4.70 ± 0.33	0.684
Fasting insulin (pmol/l)	28.40 ± 5.80	43.30 ± 10.20	0.202
HOMA2 IR	0.46 ± 0.10	0.72 ± 0.20	0.187
SBP (mmHg)	119.6 ± 4.4	133.0 ± 3.1	0.039
DBP (mmHg)	73.6 ± 4.3	81.7 ± 1.9	0.153

BMI, body mass index; DBP, diastolic blood pressure; HOMA2 IR, Homeostasis Model Assessment 2 – Insulin Resistance; SBP, systolic blood pressure. Data are mean ± SEM

6.2.2 Human primary adipocyte experiments

Human multipotent abdominal and femoral adipose-derived stem cells, an established human white adipocyte model (28), were seeded at a density of 2000 cells/cm^2 and kept in proliferation medium for seven days. Thereafter, these cells were differentiated under different physiological O_2 levels (10% O_2, high physiological pO_2; 5% O_2, low physiological pO_2) (16, 22, 29) as well as standard laboratory conditions (room air, 21% O_2) for 14 days. Gas mixtures were refreshed every 8 hours (to maintain variation <0.1% O_2), whereas medium was refreshed three times per week.

6.2.3 Adipocyte gene expression

Total RNA was extracted from hMADS cells using TRIzol reagent (Invitrogen, Breda, Netherlands), and SYBR-Green–based real-time PCRs were performed to assess gene expression of leptin, DPP-4, IL-6, PAI-1, adiponectin, TNF-α and MCP-1, the adipocyte differentiation markers peroxisome PPARγ, C/EBPα, FAS and PLIN1, as well as the hypoxia markers GLUT1, Bcl-2 interacting protein 3 (BNIP3), and vascular endothelial growth factor A (VEGFA) using an iCycler (Bio-Rad, Veenendaal, Netherlands). Results were normalized to 18S ribosomal RNA.

6.2.4 Adipokine secretion

The medium of the hMADS cells was collected over 24 hours, from day 13 (after replacement of medium) to day 14 of differentiation, to determine the secretion of adipokines using high-sensitive ELISAs (leptin and DPP-4 from R&D Systems, Inc., 614 McKinley Place NE, Minneapolis, MN 55413, USA; IL-6 and MCP-1 from Diaclone SAS, 1 Boulevard A.Fleming, 25020 Besancon Cedex, France; adiponectin and PAI-1 from BioVendor– Laboratorni medicina a.s. Karasek 1767/1 621 00 Brno Czech Republic). If necessary, samples were diluted with a provided dilution buffer from the manufacturer prior to the assay, which was performed in duplicates according to the manufacturer's instructions.

6.2.5 Statistical analyses

Data are presented as mean ± SEM. The effects of exposure to different oxygen levels on adipocyte gene expression and adipokine secretion were analysed using one-way ANOVA or the Friedman test when data were not normally distributed, followed by post-hoc comparison using Student's paired t-tests or the Wilcoxon signed-rank test in case of skewed data. GraphPad Prism version 8 for Windows (GraphPad Software, 2365 Northside Dr., Suite 560, San Diego, CA 92108)was used to perform statistical analyses. $P < 0.05$ was considered as statistically significant.

6.3 Results

6.3.1 The effects of oxygen partial pressure on adipocyte gene expression

Exposure of differentiated hMADS cells derived from abdominal and femoral AT to different pO_2 levels induced distinct gene expression patterns. Specifically, exposure to low physiological pO_2 (5% O_2) increased leptin expression compared to exposure to high physiological pO_2 (10% O_2) or room air (21% O_2) in differentiated abdominal and femoral hMADS derived from individuals with NW as well as OB (all $p < 0.01$,Figure 6.1A). Furthermore, low physiological pO_2 markedly reduced gene expression of the pro-inflammatory factors DPP-4 and IL-6 in both abdominal and femoral differentiated hMADS derived from donors with OB (all $p < 0.01$) but not NW compared to high physiological pO_2 (Figure 6.1B-C). Low physiological pO_2 levels did not significantly alter gene expression of PAI-1, TNF-α, and MCP-1 in differentiated abdominal and femoral hMADS derived from NW and OB individuals (Figure 6.1D-G), except for a modest but significant ($p = 0.041$) increase in adiponectin gene expression in FEM differentiated hMADS derived from individuals with obesity (Figure 1E). In addition, high physiological AT pO_2 (10% O_2) increased PAI-1 ($p = 0.005$) and reduced adiponectin expression ($p = 0.010$) in femoral differentiated hMADS derived from individuals with OB compared to 21% O_2 exposure. As expected, exposure to physiological oxygen levels, i.e., lower oxygen levels as compared to standard laboratory conditions, increased gene expression of the classical hypoxia markers GLUT1 and VEGFA and to a lesser extent BNIP3 (Figure 6.3A-C). Furthermore, exposure to low physiological oxygen levels (5% O_2) did not alter gene expression of adipocyte differentiation markers compared to room air (21% O_2) in differentiated hMADS derived from individuals with NW as well as OB (Figure 6.3 D-G). In differentiated hMADS derived from individuals with OB, gene expression of PPARγ, C/EBPα and FAS was lower, and expression of PLIN1 higher, following exposure to 5% compared to 10% O_2.

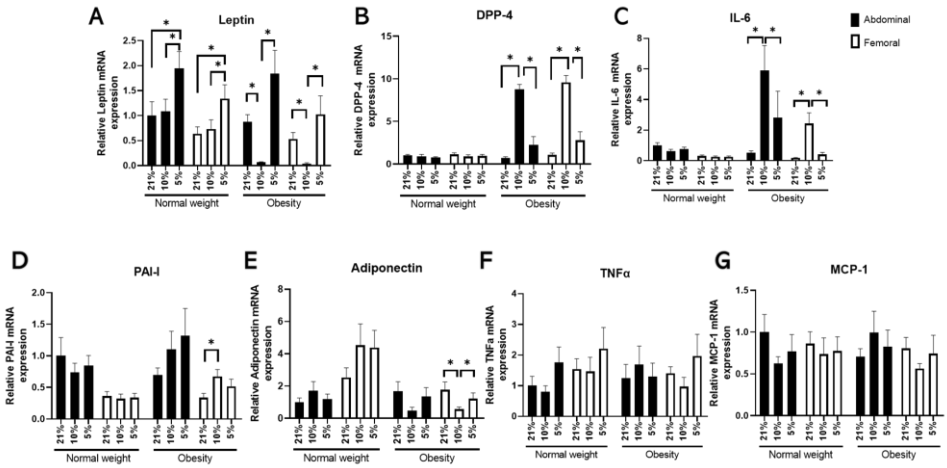


*Figure 6.1: Adipokine and inflammatory markers gene expression in hMADS cells, following differentiation under different pO_2s (21% vs 10% vs 5% O_2) (n = 9 paired samples). Panel A: Leptin, B: Dipeptidyl-peptidase (DPP)-4, C: Interleukin (IL)-6, D: Plasminogen activator inhibitor (PAI)-1, E: Adiponectin, F: Tumour necrosis factor (TNF)α, G: Monocyte chemoattractant protein (MCP)-1. Data are expressed as mean ± SEM. *p<0.05*

6.3.2 The effects of oxygen partial pressure on adipokine secretion

Next, we investigated whether exposure to different pO_2 levels elicits functional changes in adipokine secretion from differentiated abdominal and femoral hMADS. We found that adipokine secretion from both differentiated abdominal and femoral hMADS was significantly affected by changes in oxygen availability (Figure 6.2). Specifically, low physiological pO_2 (5% O_2) exposure increased leptin secretion in differentiated abdominal and femoral hMADS derived from individuals with OB compared to exposure to high physiological pO_2 (10% O_2: ABD, $p = 0.009$; FEM, $p = 0.021$), and in differentiated ABD and FEM hMADS derived from individuals with NW compared to exposure to room air (21% O_2: ABD, $p = 0.014$; FEM, $p = 0.006$) (Figure 6.2A). Furthermore, DPP-4 secretion was significantly lower following exposure to low (5% O_2) compared to high (10% O_2) physiological pO_2 in differentiated ABD ($p = 0.027$) and FEM hMADS ($p = 0.004$), and IL-6 secretion in differentiated FEM hMADS only ($p = 0.007$), derived from donors with OB but not NW (Figure 2B, C). Moreover, low physiological pO_2 (5% O_2) reduced MCP-1 secretion ($p = 0.030$) but did not alter PAI-1 secretion from differentiated abdominal hMADS derived from individuals with OB compared to 10% O_2 (Figure 6.2D, E). Finally, low physiological pO_2 (5% O_2) reduced both MCP-1 ($p = 0.028$) and PAI-1 ($p = 0.003$) secretion from differentiated femoral hMADS derived from donors with NW compared to 21% O_2 (Figure 6.2D, E). Adiponectin secretion was not detectable, and this data is therefore not reported.

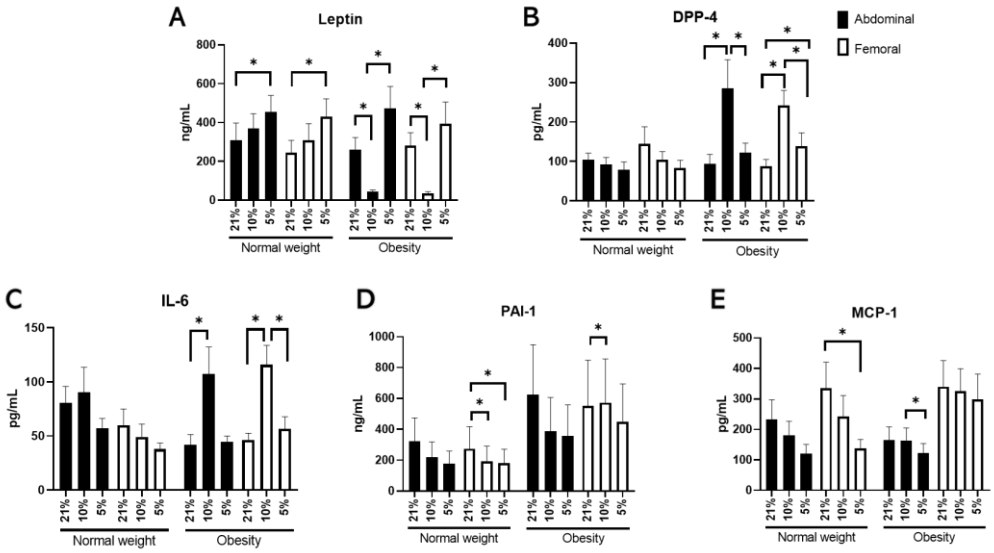


*Figure 6.2: Adipokine and inflammatory markers secretion in hMADS cells, following differentiation under different pO_2s (21% vs 10% vs 5% O_2) (n = 9 paired samples). Panel A: Leptin, B: Dipeptidyl-peptidase (DPP)-4, C: Interleukin (IL)-6, D: Plasminogen activator inhibitor (PAI)-1, E: Monocyte chemoattractant protein (MCP)-1. Data are expressed as mean ± SEM. *p<0.05*

6.4 Discussion

In the present study, we investigated the impact of oxygen tension on adipokine gene expression and secretion in differentiated human multipotent abdominal and femoral adipose-derived stem cells from female individuals with NW or OB. Here, we demonstrate that low physiological pO_2 decreases gene expression and secretion of pro-inflammatory factors DDP-4 and IL-6 in both differentiated abdominal and femoral hMADS derived from individuals with OB, while these responses were not present in differentiated hMADS cells from NW individuals. Our findings highlight those changes in pO_2 within the human physiological range in the adipocyte microenvironment contribute to alterations in the AT inflammatory phenotype, and that these effects may differ between individuals with normal weight and obesity.

To determine whether the amount of oxygen present in the AT microenvironment affects gene expression of adipokines, we exposed differentiating hMADS cells from abdominal and femoral AT to low (5%) and high (10%) physiological pO_2 levels in human AT (9, 16, 21-24). As expected, low physiological pO_2 levels increased gene expression of several hypoxia markers. Strikingly, we show for the first time that low physiological pO_2 during adipogenesis consistently decreased the expression and secretion of the pro-inflammatory markers IL-6 and DPP-4 in both differentiated femoral and abdominal hMADS derived from individuals with OB, but not NW. Thus, these findings suggest that a lower AT pO_2 may exert anti-inflammatory effects in adipocytes in women with obesity. Moreover, the present data suggest that these cells maintain a memory of origin (i.e., a normal weight or obesity microenvironment) *in vitro*, even after 14 days of exposure to the same experimental conditions. In agreement with our findings, we have previously reported that *in vivo* abdominal AT pO_2 was positively associated with AT gene expression of several pro-inflammatory markers (22), and that low physiological pO_2 exposure reduced gene expression of IL-6 and DPP-4 in adipocytes derived from women with obesity (16). In addition, the present results show that low physiological pO_2 levels consistently increased leptin gene expression and secretion in differentiated abdominal and femoral hMADS derived from donors with NW or OB. Leptin is an important regulator of appetite and energy expenditure, providing important feedback in relation to energy storage in the body through the hypothalamus, and is involved in multiple physiological processes such as the regulation of immunity (9, 30-32). Changes in leptin secretion due to altered oxygen tension in the AT microenvironment may thus affect these processes. Notably, pO_2-induced alterations in adipokine gene expression were paralleled by comparable changes in adipokine secretion. Importantly, the modest effects of pO_2 levels on adipocyte differentiation, if present at all, do not seem to explain the observed changes in adipokine expression and secretion, exemplified by the opposing effects of low pO_2 on the expression and secretion of leptin and the pro-inflammatory markers Il-6 and DPP-4. Famulla and colleagues (27) have previously shown increased DPP-4, adiponectin and IL-6 secretion following prolonged exposure to high physiological pO_2 (10% O_2), while low physiological pO_2 (5% O_2) tended to reduce the secretion of adiponectin. These differences between studies may at least partly be explained by differences in donor characteristics.

A strength of the present study is the paired comparisons between differentiated adipose-derived multipotent stem cells derived from abdominal and femoral AT of individuals with NW and OB. Previous studies examining the effects of pO_2 levels on adipocyte inflammation have either used cell lines, adipose-derived multipotent stem cells from a single donor, or a pool of stem cells obtained from different donors. Since our findings demonstrate that the impact of changes in the AT microenvironment (i.e., different physiological pO_2 levels) on adipokine expression and secretion depends on the characteristics of the donors, future studies in the field of AT biology should take this '*memory-of-origin effect*' into account. Secondly, in contrast to many studies showing that acute exposure to severe (non-physiological) hypoxia evokes a pro-inflammatory response in murine and human (pre)adipocytes (12, 14), we aimed to mimic physiological *in vivo* conditions in terms of pO_2 levels as well as the prolonged exposure duration in the present study. This study also has some limitations. We examined the effects of various oxygen levels in cells derived from postmenopausal women. Therefore, our findings cannot be translated to other subgroups of the population such as men or individuals of different age. Furthermore, we used a targeted approach to examine gene expression and secretion of several adipokines. Future studies using an untargeted approach (e.g., microarray analysis, RNA sequencing, proteomics) are warranted.

6.5 Conclusion

In conclusion, the present findings demonstrate that *physiological* oxygen levels regulate adipokine expression and secretion in differentiated abdominal and femoral hMADS. Differentiated hMADS cells derived from women with obesity display lower expression and secretion of several (pro-inflammatory) adipokines at low (5% O_2) compared to high (10% O_2) physiological oxygen tension. Except for effects on leptin expression, no significant effects of low compared to high physiological oxygen levels were observed in differentiated hMADS cells derived from individuals with NW. Our findings thus indicate that pO_2 levels alter the expression and secretion of several adipokines in differentiated human ABD and FEM hMADS, and that donor characteristics determine experimental outcomes. This has important implications for future mechanistic *in vitro* studies in the field of AT biology. For example, the outcomes of studies in which the effects of certain interventions on adipocyte inflammation and related biological mechanisms are investigated may depend on the microenvironmental oxygen tension. Furthermore, our findings highlight that it is important to report detailed characteristics of the cell donor(s) in studies examining human adipocyte biology.

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Disclosures

No conflicts of interest are declared by the authors.

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Author contributions

Konstantinos N. Manolopoulos and Gijs H. Goossens acquired funding, conceived, and designed research, interpreted data, and revised the manuscript; Ioannis G. Lempesis performed experiments, analyzed data, interpreted data, prepared figures, and drafted the manuscript; Nicole Hoebers, Yvonne Essers, and Johan W.E. Jocken performed experiments and analyzed data. Kasper M.A. Rouschop and Ellen E. Blaak interpreted data and revised the manuscript. All authors approved the final version of the manuscript.

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Supplemental results

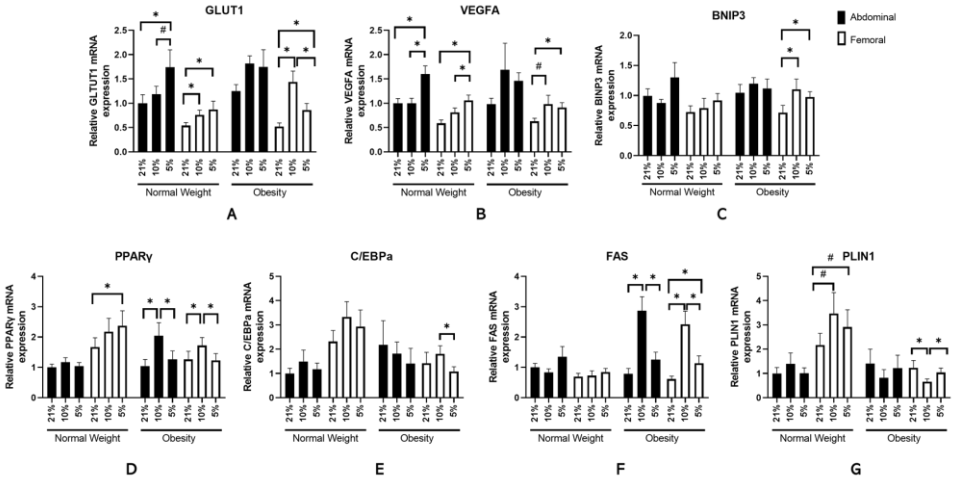
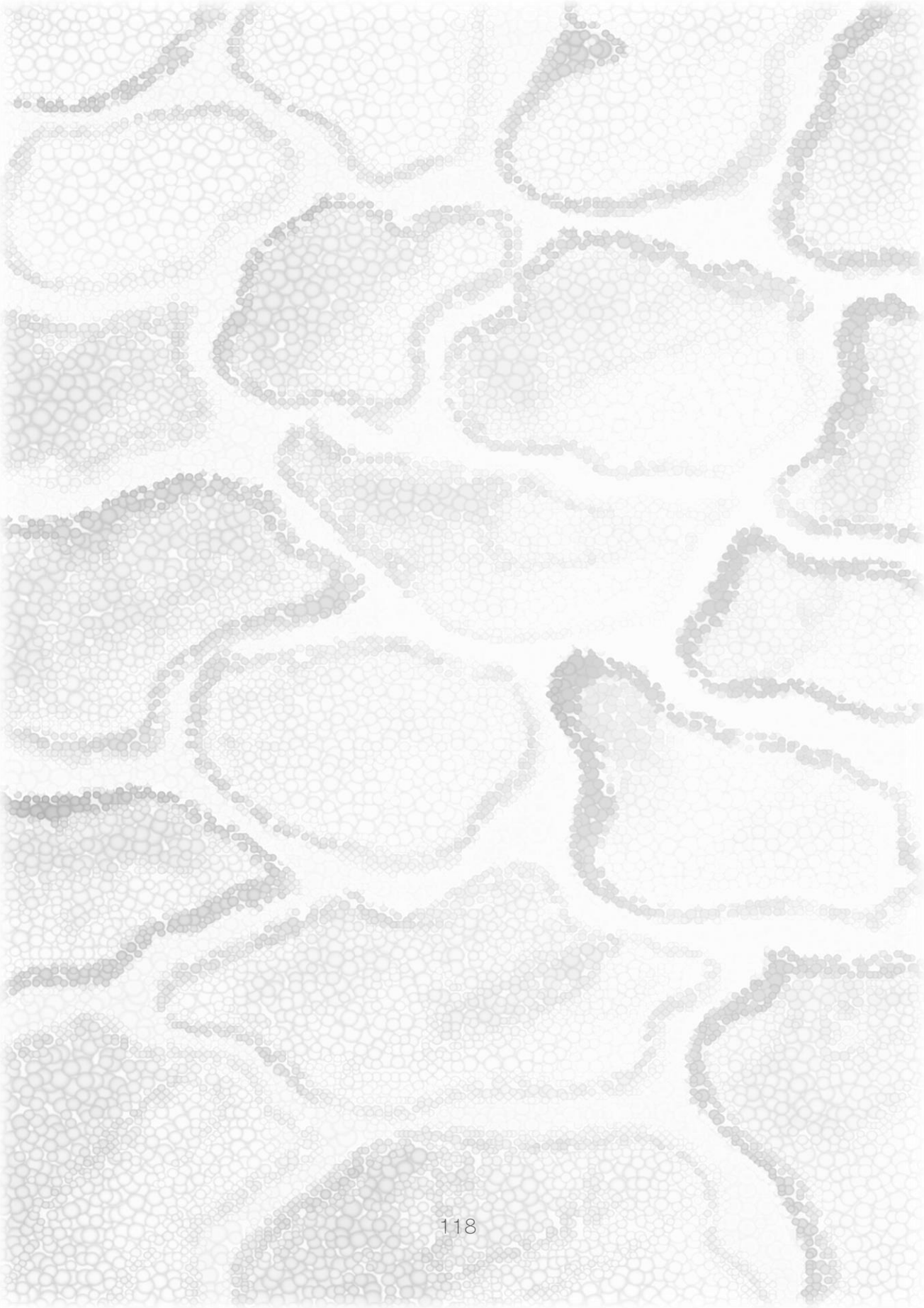


*Figure 6.3: Adipocyte differentiation and hypoxia markers gene expression in adipose tissue-derived mesenchymal stem cells following differentiation under different pO_2s (21% vs 10% vs 5% O_2). Panel A: PPARγ, peroxisome proliferator-activated receptor γ; B C/EBPα, CCAAT-enhancer binding protein α; C: FAS fatty acid synthase; D: PLIN1 Perilipin 1; E: GLUT1 glucose transporter 1; BNIP3 Bcl-2 interacting protein 3; G: VEGFA vascular endothelial growth factor A. Data are expressed as mean ± SEM. *p<0.05, # p<0.01*



7 General discussion

The obesity epidemic is a major global public health challenge (1-4). Obesity is a complex multifactorial disease linked to increased risk of developing insulin resistance, T2DM, CVD, and various types of cancer (2, 5-10). Life expectancy and quality are largely impaired and the need to better understand and treat obesity are of paramount importance. This is further underlined during the COVID-19 pandemic, as obesity has been linked to increased risk of infection and the severity of illness in patients with COVID-19 (3, 11). The major risk factors for obesity development, which in combination eventually lead to a positive energy balance, include among others genetic susceptibility, behavioural and societal factors, and easy access to low-quality, energy-rich food (3, 12-15).

The pathophysiology of obesity and its complications is determined by excess adiposity and, in particular, AT dysfunction and body fat distribution (10, 16-19). Dysfunctional AT is characterised by adipocytokine dysregulation, chronic low-grade inflammation, adipocyte hypertrophy, impaired lipid metabolism (reduced capacity to buffer the daily influx of dietary lipids), decreased ATBF, mitochondrial dysfunction, and altered oxygenation, together resulting in fat storage in non-adipose tissues (ectopic fat accumulation) and related complications (18, 20-22). The location where the lipids are stored is an important determinant of an individual's overall health status (19, 23, 24). Abdominal (upper body) fat accumulation is associated with an increased incidence of obesity-related complications like hypertension and risk of T2DM (19, 23, 25-27). On the contrary, accumulation of fat in the lower body (gluteofemoral AT) is associated with decreased cardiometabolic disease risk (23, 28-31).

Consequently, an improved understanding of the perturbations in metabolic and inflammatory processes in the various AT depots, and unveiling of their properties, can help us better understand the pathophysiology of obesity-related complications, and develop better prevention and treatment strategies (32).

Thus, the **main aim of this thesis** was to examine the differences between upper and lower body AT biology focusing on blood flow, the oxidative machinery, and inflammatory signatures of abdominal (upper-body) and femoral (lower-body) subcutaneous AT in humans with normal weight or obesity. A second objective was to investigate the influence of prolonged exposure to various physiological oxygen levels on the inflammatory phenotype of abdominal and femoral adipocytes.

In **Chapter 2** the importance of AT oxygenation was discussed based on an extensive review of the literature. Further, in **Chapter 3**, we explored a novel non-invasive Doppler ultrasound technique for quantifying ATBF in abdominal and femoral AT in humans. In **Chapters 4 and 5** we used an integrated approach to investigate AT and adipocyte physiology *in vivo* and *in vitro*, respectively. Specifically, in **Chapter 4** *in vivo* adipokine release was determined across abdominal and femoral subcutaneous AT using the arterio-venous balance technique in postmenopausal women with normal weight or obesity. Additionally, adipokine expression and/or secretion were measured in AT biopsies and differentiated primary human adipocytes derived from the same individuals. In **Chapter 5**, a similar approach was used to investigate *in vivo* oxygen fractional extraction and carbon dioxide fractional production across abdominal and femoral AT. Furthermore, the oxidative machinery was examined in AT biopsies and

differentiated primary human abdominal and femoral adipocytes. Finally, we performed *ex vivo* functional experiments to measure mitochondrial oxygen consumption in these adipocytes. In **Chapter 6**, the effects of prolonged exposure to various physiological oxygen levels on differentiated primary human adipocytes' inflammatory signatures were presented. In this final chapter **(Chapter 7)** we will summarize the main findings of this thesis, reflect on the results generated by the studies described in this thesis, discuss the impact of these findings in a broader perspective, and provide directions for future research.

7.1 Examining adipose tissue physiology *in vivo* – Methodological considerations

As already described in **Chapter 1**, the interest for understanding regional fat physiology has remarkably increased in the previous decades. However, investigating AT *in vivo* in humans remains challenging (33). The main techniques to examine *in vivo* AT physiology include isotopes/tracer methodology, the microdialysis technique and the arterio-venous concentration balance technique (33, 34). Many tracer studies have explored primarily NEFA and glycerol production, based on the principle of the tracer dilution and appearance rates in the circulation (33, 35). Microdialysis is based on the measurement of substrate diffusion across a semipermeable membrane at the tip of a microdialysis probe and, apart from allowing the measurement of metabolite concentrations, can provide methods of measuring and manipulating of ATBF also (33-35).

7.1.1 Arterio-venous difference technique

The arterio-venous difference technique, which has been used and described in more detail in **Chapters 4 and 5** of this thesis, is a valuable, direct, quantitative method used to assess AT metabolism and physiology *in vivo* in humans (33, 36). The technique was initially used by Gordon *et al.*(37), to assess fatty acid metabolism and further adapted for abdominal and femoral subcutaneous AT assessment (38, 39). It has been widely used for the *in vivo* characterisation of AT, with the benefits of examining homogenous parts of different depots, thus allowing direct comparisons between AT depots (33). The technique was further used for the study of adipokine release from AT, including leptin, DPP-4, IL-6, TNF-α and components of the renin-angiotensin system (40-43). Additionally, this methodology has been used to examine oxygen metabolism in abdominal AT (44). In this thesis, we aimed to further examine *in vivo* adipocytokine secretion and oxygen metabolism in abdominal as well femoral AT using the technique.

The simple logic of the technique is based on that the concentration difference of a selected molecule (e.g. lipids, glucose, a hormone) in blood derived from the artery supplying and the vein draining the tissue reflects the net uptake or release of this molecule (36). Cannulation of the radial artery in combination with selective venous catheterisation of (an) adipose vein(s) is crucial for the success of the technique, as

discussed in **Chapters 4 and 5** (33). By incorporating ATBF to the arterio-venous difference, the flux of metabolites or adipokines can be calculated (33, 36). The net release or uptake of a particular molecule can be expressed as fractional extraction (FE= [arterio-venous difference / arterial concentration]*100%) (36). A positive arterio-venous difference would represent net uptake of a substance, while a negative value is indicative of net release from the tissue. The later applies also in case of greater release than uptake of a substance, when both processes occur within the same tissue (45). Multiplying arterio-venous difference with ATBF provides information on the absolute net extraction (or release) per unit of AT (AE= arterio-venous difference x ATBF) and/or net clearance (FE x ATBF)(36).

7.1.2 Measuring adipose tissue blood flow

As discussed in **Chapters 1, 2 and 3** ATBF is an important determinant of AT function, as it delivers nutrients and oxygen to AT, exerts a key role in fatty acid trafficking, and distributes adipokines and metabolites into the circulation (16, 21, 22). Many studies have demonstrated that both fasting and the postprandial enhancement of ATBF are impaired in obesity and insulin-resistant conditions (46-48), thereby contributing to the metabolic perturbations in insulin-resistant individuals with obesity (21, 22, 34, 47-49). In line with an important role of ATBF in metabolic regulation, several, but not all, studies have demonstrated a lower ATBF in lower-body compared to upper-body AT (50-52).

The gold-standard method for measuring ATBF is the ^{133}Xe wash-out technique, first described by Larsen and colleagues in 1966 (53). Alternative methods to assess subcutaneous ATBF include measurement of ethanol wash-out using microdialysis, laser Doppler flowmetry, and contrast-enhanced ultrasound (34, 54-57). During the start of the studies of this thesis, reduced availability of ^{133}Xe due to its costly production and declining clinical use led us to develop and implement an alternative, non-invasive method for the measurement of ATBF that could be used in physiological human *in vivo* studies. In **Chapter 3** we have demonstrated that measurement of abdominal and femoral intravascular ATBF with percutaneous Doppler ultrasound is technically feasible. Moreover, we showed that this non-invasive method is likely able to detect the expected increase in blood flow following oral glucose ingestion in both abdominal and femoral AT in healthy individuals (16, 21, 22). Furthermore, we found that abdominal ATBF was significantly higher than femoral ATBF under fasting conditions in young, healthy individuals with normal body weight.

7.2 Strengths and limitations of the *in vivo* methods of this thesis

A strength of the approach we have taken in the execution of the translational studies presented in **Chapter 4 and 5** is that we phenotyped the study participants in detail, and for the first time combined paired *in vivo* measurements across different AT depots, gene expression analyses in abdominal and femoral AT biopsies and adipocytes, and *in vitro* functional experiments and measurements in differentiated human primary

abdominal and femoral adipocytes in the same individuals with both normal weight and obesity. Furthermore, we did not perform the experiments using a pool of stem cells from donors with normal weight and obesity (risking outcomes would be influenced/masked by strong effects seen in a specific donor) or a single donor, as often done, but performed the *in vitro* experiments with cells from many donors with normal weight and obesity separately.

However, there are also some important limitations of the methods utilized in the present thesis. For the ATBF measurement, the limitations are those inherent to using any kind of ultrasound methodology and relate to the quality of the ultrasound image that can be obtained in individual participants. Appropriate operator training paying special attention to the identification of suitable veins that allow acquisition of a high-quality Doppler signal is of paramount importance. This can be a limitation for researchers without any prior medical or clinical training, as it requires training from experienced healthcare professionals. Low blood flow in small AT veins may further hamper the accuracy of ATBF measurement. These issues become especially noticeable when assessing ATBF in (very) lean participants due to thin subcutaneous fat layers and smaller blood vessels. While the anatomical properties of AT veins, especially in the femoral AT depot, may limit the ability of obtaining a good Doppler signal in every study participant, it is important to note that other available methods also have intrinsic limitations such as exposure to radiation (i.e. PET-tracers, ^{133}Xe wash-out), thereby restricting their applicability. This also holds true for repeated measurements during a clinical study, which is often not possible with PET scans, for example. Although intravascular Doppler ultrasound may be an alternative method that could be applied to calculate metabolite fluxes across AT in physiological *in vivo* studies in humans, it is important to note that Doppler ultrasound provides data on intravascular blood flow in relatively large AT veins. In contrast, the ^{133}Xe wash-out technique on which the original calculations of metabolic fluxes were based provides ATBF values at the capillary level. Due to the global production stop of medical ^{133}Xe it was not possible to validate the present Doppler measurements against the gold-standard ^{133}Xe wash-out technique, as precisely that was the reason for developing the Doppler technique.

An important limitation of the study is that for validation purposes a method of assessment should ideally be compared to the gold-standard technique. However, as mentioned above this was unfortunately not feasible. Moreover, repeated measurements, i.e., acquiring measurements in the same participants over more than one visit in a short time frame such as one week, would have provided more information about the reproducibility of the Doppler measurements to assess ATBF. Obviously, this technique needs to be further validated.

A further limitation of the study presented in **Chapter 3** is that we did not obtain information on the actual volume of the AT depot measured, e.g., through magnetic resonance imaging or dual x-ray absorptiometry, which might allow estimating whole-depot blood flow values. This was not included in the protocol as the original plan was to use the ^{133}Xe washout technique. However, it was required to develop a new technique, as ^{133}Xe became unavailable during the early stages of the PhD project. Not

acquiring information about the AT volume when performing Doppler measurements limits quantifying blood flow per unit AT mass, which can be done when utilising the ^{133}Xe washout technique. Finally, we did not measure postprandial glucose and insulin concentrations in this study (**Chapter 3**), although this would not have changed the conclusions since we performed intra-individual comparisons.

The results described in **Chapter 3** are not fully in agreement with **Chapters 4 and 5** where we have shown no significant differences in blood flow between abdominal and femoral AT depots. However, these discrepancies could be due to the different macronutrients that were ingested, i.e., oral glucose drink in **Chapter 3** versus a high-fat mixed-meal shake in **Chapter 5**. The postprandial increase in ATBF is postulated to be driven not only by insulin but also gut hormones' increase, with a well-studied role of GIP (21, 58, 59) The ingestion of carbohydrates, like glucose alone can drive a substantial increase in gut hormones, however it has been shown that the ingestion of lipids as compared to protein produces reduced increase in gut hormones (60). This could be a reason for the discrepancy among the two stimuli, and because of other differences in characteristics of the study populations that have been investigated (i.e., phenotypic characteristics such as age, sex, and metabolic status).

We studied post-menopausal women only, to control for differences in depot-specific AT function between men and women, and to ensure feasibility of the femoral AT biopsy. Moreover, post-menopausal women are metabolically more compromised than pre-menopausal women and, therefore, differences between upper and lower-body AT may be more pronounced (61). Although not feasible within the current study, follow-up studies are clearly needed to elucidate whether sex-differences in AT function are present and may underlie the difference in cardiometabolic disease risk between men and women, as well as the role of the menopause transition in women in terms of upper and lower-body AT function. From that perspective these findings can neither be generalised to the general population nor to premenopausal women.

Another limitation of the studies is that a formal power calculation for each of the primary outcomes of the different studies was not performed, since the studies were exploratory in nature (discussed in more detail in the appendix "Methodological considerations, impact of COVID-19, and reflection on a joint PhD program"). Moreover, no adjustments were made for multiple testing and post-hoc statistical analysis was performed. Failing to account for multiplicity (also known as multiple comparisons) increases the likelihood of false positive results, commonly known as Type I errors (62, 63). This is due to the increased likelihood of finding at least one statistically significant result by chance alone when several comparisons are conducted within the same study (62).

Noteworthy, there were also some other limitations regarding the arterio-venous difference technique in **Chapters 4 and 5**. First, the sample size for the *in vivo* measurements across AT was relatively small, due to the technical difficulties to cannulate the small veins in these fat depots, as well as recruitment issues encountered due to the COVID-19 pandemic. The technical difficulties to cannulate and collect blood samples from the small veins in abdominal and femoral AT, meant that we were able to successfully complete sample collection for 9 women with normal weight and 6 women

with obesity. Due to the limited number of paired blood samples draining abdominal and femoral AT for the individuals with normal weight and obesity, we could not analyse data separately for individuals with normal weight and obesity due to limited statistical power. This resulted in pooling the measurements from the individuals with obesity and normal weight in order to compare *in vivo* adipose tissue uptake/release between abdominal versus femoral AT, rather than examining this separately for people with normal weight and obesity. This applies to the production/release of adipokines under fasting conditions in **Chapter 4,** and the fractional extraction or release of blood gases and metabolites under fasting conditions in **Chapter 5.** This could potentially impact the observations. Secondly, we determined AT blood flow using Doppler ultrasound, which provides data on intravascular blood flow in relatively large AT veins rather than at the capillary level (64). Consequently, we could not quantify absolute fluxes of adipokines and blood gases per unit AT, and data on *in vivo* release of adipokines should therefore be interpreted with some caution. Nevertheless, calculation of fractional extraction of oxygen and fractional release of adipokines and carbon dioxide also yields valuable insights into adipokine release and metabolic activity across different AT depots, especially since ATBF was not significantly different between abdominal and femoral AT in our study.

7.3 Phenotypic differences between abdominal and femoral adipose tissue

As discussed in **Chapter 1** and elsewhere (23, 61, 65), even though differences in functional properties of upper-body fat compared to lower-body fat exist, the precise mechanisms underlying the protective cardiometabolic role of lower-body AT remain to be elucidated. Functional differences between AT depots may explain the cardiometabolic disease risk associated with a certain body fat distribution pattern.

7.3.1 Adipocyte morphology

The functional differences between abdominal and femoral AT seem to emerge from adipocytes having distinct properties (61, 66). Many studies have shown a link between adipocyte morphology and AT function, with adipocyte hypertrophy (as frequently seen in people with obesity) associated with impairments in lipid metabolism and a more pro-inflammatory adipocyte phenotype, which have been shown to contribute to the development and worsening of insulin resistance (22, 32, 61, 67). In **Chapter 4** we have shown that women with obesity had larger adipocytes than women with normal weight, both in abdominal and femoral AT. However, adipocyte size did not differ between abdominal and femoral AT in either group. This is in agreement with some (68-70) but not all the previous reports (52, 71-73), and may relate to characteristics of the study populations investigated (i.e., age and metabolic status). The study participants did not have severe obesity and had a relatively healthy metabolic profile.

7.3.2 Depot-differences in adipokine expression and release

In line with adipocyte hypertrophy in women with obesity, we have found in **Chapter 4** higher AT gene expression of leptin, PAI-1, and IL-6 (only in abdominal AT) in individuals with obesity. Few studies, though, have directly compared upper and lower body AT inflammation. Intriguingly, despite similar fat cell size in both AT depots, the present findings demonstrate lower gene expression of leptin, MCP-1, PAI-1 and TNF-α in abdominal than femoral AT. These observations of increased gene expression of inflammatory markers in the femoral depot seem to be opposite to the initial hypothesis. However, since inflammatory processes also play a role in healthy AT expansion and response to lipid accumulation (74, 75), the higher gene expression level of these inflammatory markers in femoral AT does not necessarily imply that this AT depot has a more detrimental phenotype in this study population. A previous study did not find differences in the expression levels of macrophage markers and cytokines between gluteal and abdominal AT (76). Moreover, global transcriptional profiling of the AT of men and women failed to identify differentially expressed clusters of inflammation-specific genes between abdominal and gluteal AT (70). Importantly, whole-AT gene expression profiles are determined by gene expression in multiple adipose-derived cell types apart from adipocytes, including immune cells, endothelial cells and preadipocytes (32). Therefore, these observations cannot be directly and accurately linked to adipocyte morphology only.

In **Chapter 4** we have also examined, using the arterio-venous difference technique, *in vivo* adipocytokine production. We found significant fractional release of leptin and MCP-1 from abdominal and femoral subcutaneous AT, with similar fractional release of leptin from both AT depots and higher release of MCP-1 from abdominal compared to femoral AT. The comparable release of leptin from femoral and abdominal AT is in line with a previous report (70). No release of other adipokines, including PAI-I, DPP-4, and adiponectin, across abdominal and femoral AT was detectable. The latter is in line with previous studies, showing no significant release of adiponectin, IL-6, and DPP-4 across human abdominal AT in people with normal weight and obesity (46, 77). One study that did report *in vivo* DPP-4 release across human abdominal AT only found significant release in few individuals with low (<288 ng/ml) plasma DPP-4 concentrations (77), while mean DPP-4 concentrations were much higher (>400 ng/ml) in the present study. The lack of detectable adiponectin release across AT may be explained by a low release rate and the rather constant circulating concentrations, which may represent a near-steady state (24, 78). We found higher arterial concentrations of leptin and PAI-1 in women with obesity. Since no differences in the *in vivo* fractional release of these factors from abdominal and femoral AT were found between individuals with normal weight and obesity, the higher circulating leptin and PAI-I concentrations are likely explained by the higher total fat mass in the women with obesity compared to normal weight.

As mentioned earlier, whole-AT gene expression profiles are determined by gene expression in multiple adipose-derived cell types such as adipocytes and immune cells. Therefore, we also specifically investigated gene expression profiles in differentiated human primary abdominal and femoral subcutaneous adipocytes derived from the

participants that underwent the *in vivo* measurements and AT biopsies. Interestingly, it was observed that hMADS derived from abdominal and femoral AT that have been differentiated *in vitro* (and therefore been exposed to the same experimental microenvironment for 21 days; 7 days proliferation followed by 14 days differentiation) show marked differences in gene expression. Indeed, we demonstrated higher expression of the pro-inflammatory factors IL-6 and PAI-I in abdominal compared to femoral adipocytes derived from women with both normal weight and obesity. Furthermore, the expression of leptin was higher whereas adiponectin expression was lower in abdominal compared to femoral adipocytes derived from women with normal weight. These observations are not directly in line with the results from the AT biopsies where leptin expression was higher in the femoral depot. The discrepancies between adipokine expression in AT and adipocytes may be due to differences in the presence of other cells in the tissue which could either directly express and secrete these molecules or act in a paracrine manner to affect adipocyte gene expression. Clearly, the present findings highlight intrinsic differences in the inflammatory signatures of human abdominal and femoral adipocytes, which are already present in cells derived from a healthy ('non-obese') AT microenvironment (i.e., normal weight individuals). In addition, DPP-4 gene expression was lower in abdominal than femoral adipocytes derived from women with obesity. The fact that inflammatory gene expression was not higher in differentiated human primary abdominal and femoral adipocytes derived from women with obesity compared to normal weight provides further support for the notion that adipocyte hypertrophy and the contribution of the inflammatory cell component are key factors determining the *in vivo* AT inflammatory signature. Gene expression of the adipocyte differentiation markers PPARγ, C/EBPα, PLIN1 and FAS was not significantly different between abdominal and femoral adipocytes suggesting that the observations are not due to differences in adipocyte differentiation. The differences in adipocyte gene expression did, however, not translate into functional differences in the secretion of adipokines. Specifically, we only found a lower secretion of MCP-1 from abdominal compared to femoral adipocytes derived from both women with normal weight or obesity, but no differences in the secretion rates of leptin, PAI-I, IL-6, and DPP-4 between abdominal and femoral adipocytes were apparent. This could be due to low secretion rates and/or the detection limit of the assays that were used.

7.3.3 Depot-differences in oxidative signatures

Applying the same translational approach, we examined the oxidative machinery of upper and lower body subcutaneous AT in **Chapter 5**. Human *in vivo* data on AT oxygen consumption remain sparse (22, 79), which is at least partially due to the technical challenges related to the execution of these measurements in humans. In **Chapter 5** we demonstrated for the first time that the *in vivo* fractional extraction of oxygen is lower in abdominal compared to femoral subcutaneous AT. In line, we found lower carbon dioxide fractional release in abdominal versus femoral AT. As previously reported (52), we found no significant depot-differences in ATBF, as determined using Doppler ultrasound (64), between abdominal and femoral AT depots. Together, these findings suggest that abdominal AT is consuming less oxygen *in vivo*, which may reflect a lower

metabolic activity of abdominal compared to femoral subcutaneous AT. Interestingly, recent data from our research group in Maastricht demonstrated that pO_2 is higher in abdominal than femoral AT in postmenopausal women (52). In agreement with the present findings, in the latter study no significant differences in blood flow (i.e. oxygen supply) were found between abdominal and femoral AT in postmenopausal women (52). Together, these findings suggest that a lower oxygen consumption rate in abdominal AT may underlie the higher AT pO_2 in abdominal compared to femoral AT, which in turn might contribute to functional differences between upper and lower body AT in humans (22, 79).

Additionally, we found no significant differences in the *in vivo* fractional extraction or release of glucose, NEFA and lactate between abdominal and femoral AT. Previous studies have also shown similar (51) or higher NEFA release across abdominal compared to femoral subcutaneous AT in healthy, younger individuals (50, 80). Of note, NEFA fractional release was higher, though not significantly, in the abdominal as opposed to the femoral depot. This could be explained by the fact that femoral AT represents a larger storage depot in women, with lower rates of lipolysis, as opposed to the abdominal AT depot (50, 65).

We next investigated whether depot-differences in the metabolic rate of human adipocytes may underlie the lower oxygen fractional extraction in abdominal AT. Indeed, we found that the mitochondrial oxygen consumption rates (OCR) and mitochondrial respiratory capacity were lower in differentiated human multipotent adipose-derived stem cells derived from abdominal compared to femoral AT. Specifically, we found significantly lower basal respiration, ATP production, maximal respiration, and spare capacity in abdominal versus femoral adipocytes. Strikingly, these findings suggest that functional depot-differences in *in vivo* oxygen extraction are maintained *in vitro* after differentiation of hMADS derived from the same individuals from these different fat depots. As also described in Chapter 4, adipocyte differentiation markers were not significantly different between abdominal and femoral adipocytes, suggesting that these findings are not due to differences in the differentiation of the abdominal and femoral adipocytes. In line, it has previously been shown that (maximal) respiratory capacity was impaired in abdominal versus femoral adipocytes in women with insulin resistance and obesity (52). Collectively, the data presented in this thesis demonstrate that both human abdominal adipocyte oxygen consumption and *in vivo* oxygen fractional extraction across abdominal AT are lower than in femoral adipocytes and AT, respectively, in women with normal weight and obesity.

To examine the AT oxidative signatures in more detail, OXPHOS protein expression and mtDNA copy numbers in abdominal and femoral AT as well as adipocytes were also determined. Interestingly, we found no significant differences in the expression of the different OXPHOS complexes and mtDNA content between abdominal and femoral AT and adipocytes, although mtDNA content tended to be lower in abdominal adipocytes. These data suggest that expression of OXPHOS and mtDNA copy number are not directly associated with functional measurements of oxygen consumption in human AT and adipocytes. Indeed, a comparable dissociation between mitochondrial respiration (OCR) and mitochondrial content has been shown in mature abdominal subcutaneous

adipocytes isolated from AT biopsies obtained from individuals across a broad BMI range (from normal weight to severe obesity) (81).

In addition to AT depot-differences in the total study population, we also investigated depot-differences in the AT oxidative machinery between individuals with normal weight and obesity. In these studies, we demonstrate for the first time that protein expression of OXPHOS complexes was significantly lower in femoral AT (complexes I, III) and abdominal and femoral adipocytes (complexes III, V) derived from postmenopausal women with obesity compared to normal weight. This is in line with previous observations showing lower OXPHOS expression (82-84) and a tendency for lower *in vivo* abdominal subcutaneous AT oxygen consumption in obesity (44). In agreement with most (83-85) but not all (81, 84) findings, we also found that abdominal AT mtDNA content was significantly lower in individuals with obesity than normal weight. In contrast, no group differences in mtDNA content were found for femoral AT. Surprisingly, we did not observe any differences in OCR between differentiated hMADS derived from individuals with normal weight and obesity. The latter finding seems in contrast with previous studies showing impaired OCR in adipocytes from individuals with obesity compared to normal weight, independent of fat cell size (81, 84). However, it is important to emphasize that previous studies measured OCR in mature adipocytes isolated from fresh AT biopsies. Therefore, it cannot be excluded that these conflicting findings are due to differences in experimental conditions compared to the present study.

It would be of interest to determine whether the observed differences of inflammatory markers in AT and adipocyte level (Chapter 4) are linked to the differences in the oxidative machinery and mitochondrial respiration (Chapter 5). Indeed, mitochondrial dysfunction has been linked to inflammation in a bi-directional manner (86), and further studies to investigate the timeline of changes in these processes are warranted.

Finally, based on fasting insulin concentrations and HOMA2-IR, the individuals with obesity tended to be more insulin resistant, although no significant differences were found in circulating metabolites between the normal weight and obesity groups under fasting conditions. Furthermore, the postprandial decrease in arterial NEFA concentrations was more pronounced in the normal weight group, indicative of higher AT insulin sensitivity. It has been speculated that mitochondrial dysfunction may be a central cause of insulin resistance (87). However, studies that specifically targeted mitochondrial function in adipocytes indicated dissociation between impaired mitochondrial oxidative capacity and systemic insulin sensitivity (88). It would be of interest to see whether phenotypic or functional differences could be associated with different insulin sensitivity levels in other populations.

7.4 Importance of oxygen availability in the tissue microenvironment on adipocyte function

As extensively discussed in Chapter 2, the amount of oxygen that is present in the local AT microenvironment seems to impact tissue physiology. It remains unclear which are

the actual oxygen levels in obesity compared to normal weight status however, they appear to be altered. Animal models have shown lower pO_2 in obese adipose tissue ('hypoxia') (18, 89-93). Data in rodents and humans cannot be directly compared as the rates of expansion may differ in mice and humans; findings on AT pO_2 in humans are not always in line with the murine models and are conflicting. This may be attributable to differences between study populations in terms of the onset and physical history of obesity (e.g., weight cycling, establishment of excess adiposity in younger and for longer duration as opposed to cases of older individuals) and other individuals' characteristics (e.g., age, sex, ethnicity, presence or not of T2DM), the adipose tissue depot studied, and variation in the methodology used (18, 56, 94-96) (Chapter 2). The oxygen levels in AT seem to impact AT tissue inflammation, metabolism and adipokine expression/secretion. Importantly, the duration of exposure to various oxygen levels impacts the cellular response, as discussed in Chapter 2 and confirmed by a recent study (97). Interestingly, the latter study found that hypoxic conditioning impacts the adipocyte lipid storage processes, by reducing lipoprotein lipase activity and triacylglycerol content under acute and chronic hypoxic conditions. In order to further investigate the impact of various oxygen levels and especially the acute and severe hypoxic events as seen in obstructive sleep apnea (OSA), a common complication in individuals with obesity, it requires a different experimental approach compared to that applied in the present work, which is based on stable, albeit low, oxygen levels. This is because in OSA brief hypoxic episodes of various degree (usually rather severe hypoxia) and duration are observed, and the severity and frequency of hypoxia exposure play important roles in the biology of adipocytes, as extensively discussed in Chapter 2.

Oxygen tension (pO_2) is determined by a fine balance between oxygen delivery (determined by ATBF and vascular density) and demands (determined primarily by mitochondrial respiration). In Chapters 3 and 4 we have examined ATBF and in Chapter 5 the fractional oxygen extraction across AT and oxygen consumption in human adipocytes.

Next, in Chapter 6, we investigated the impact of changes in pO_2 levels on adipokine gene expression and secretion in differentiated human multipotent abdominal and femoral adipose-derived stem cells from the same female individuals with normal weight or obesity that participated in the studies described in Chapters 4 and 5. Therefore, we exposed differentiating hMADS cells from abdominal and femoral AT to low (5%) and high (10%) physiological pO_2 levels in human AT (22, 52, 56, 94, 98, 99), as also applied in previous *in vitro* experiments (52, 100). Of note, previous studies have reported that oxygen tension in human adipose tissue ranges from ~3% to ~11% (52, 56, 96, 98).

We demonstrated that low physiological pO_2 (5%) decreases gene expression and secretion of pro-inflammatory factors in both abdominal and femoral adipocytes derived from individuals with obesity, while these responses were not present in adipocytes derived from donors with normal weight. Specifically, we found that low physiological pO_2 during adipogenesis consistently decreased the expression and secretion of the proinflammatory markers IL-6 and DPP-4 in both femoral and abdominal adipocytes derived from individuals with obesity, but not normal weight. Thus, these findings

suggest that a lower AT pO_2 may exert anti-inflammatory effects in AT in women with obesity. This is in line with several previous studies from the Goossens laboratory suggesting that oxygen levels are higher in the AT of individuals with obesity and relate to AT inflammatory gene expression (52, 56). These data demonstrate that these cells maintain a *memory of origin* (i.e., a normal weight or obese microenvironment) *in vitro*, even after 21 days (7 days of proliferation plus 14 of differentiation) of exposure to the same experimental conditions. This *memory of origin* may be related to the existence of depot-specific human fat cell progenitors which are shown to have distinct expression profiles (101-103). Of note, it was postulated that differences in developmental gene expression may influence adipose tissue expansion and body fat distribution, implying an inherent tendency for biological differences independently of lifestyle or weight cycling (104). In agreement with the present findings, it was previously reported that *in vivo* abdominal AT pO_2 was positively associated with AT gene expression of several pro-inflammatory markers (56), and that low physiological pO_2 exposure reduced gene expression of IL-6 and DPP-4 in adipocytes derived from women with obesity (52). In addition, the present results show that low physiological pO_2 levels consistently increased leptin gene expression and secretion in abdominal and femoral adipocytes derived from donors with normal weight or obesity. Notably, pO_2-induced alterations in adipokine gene expression were paralleled by comparable changes in adipokine secretion. Interestingly, at 21% and 5% O_2, leptin and adiponectin appear to be expressed at comparable levels in both groups. However, at high physiological O_2 levels their expression levels are markedly lower in the cells derived from individuals with obesity Importantly, the modest effects of pO_2 levels on adipocyte differentiation, if present at all, does not seem to explain the observed changes in adipokine expression and secretion, exemplified by the opposing effects of low pO_2 on the expression and secretion of leptin and the pro-inflammatory markers Il-6 and DPP-4. Famulla and colleagues (100) have previously shown increased DPP-4, adiponectin and IL-6 secretion following prolonged exposure to high physiological pO_2 (10% O_2), while low physiological pO_2 (5% O_2) tended to reduce the secretion of adiponectin. These differences between studies may at least partly be explained by differences in donor characteristics.

Taken together, these findings highlight that changes in AT pO_2 within the human physiological range may contribute to alterations in the AT inflammatory phenotype, and that these effects may differ between individuals with normal weight and obesity.

A strength of the approach taken in **Chapter 6** is the paired comparisons between differentiated adipose-derived multipotent stem cells derived from abdominal and femoral AT of individuals with normal weight and obesity. Previous studies examining the effects of pO_2 levels on adipocyte inflammation have either used cell lines, adipose-derived multipotent stem cells from a single donor or a pool of cells obtained from different donors. Since these findings demonstrate that the impact of changes in the AT microenvironment (i.e., different physiological pO_2 levels) on adipokine expression and secretion depends on the characteristics of the donors, future studies in the field of AT biology should take this '*memory-of-origin effect*' into account. Secondly, in contrast to many studies showing that acute exposure to severe (non-physiological) hypoxia evokes a pro-inflammatory response in murine and human (pre)adipocytes (105, 106).

We aimed to mimic physiological *in vivo* conditions in terms of pO_2 levels as well as the prolonged exposure duration in the present study.

This study also has some limitations. HIF-α protein levels were not assessed in the *in vitro* experiments in Chapter 6 or in the AT biopsies described in Chapters 4 and 5. In a recent study, Todorčević and colleagues (107) showed that exposure to various degrees of low oxygen levels (from 0.5 to 5% for 24h) *in vitro* yielded inconsistent gene expression results for direct HIF1-α targets, and HIF1-α mRNA measurements are considered of limited value due to highly instable gene expression. Furthermore, in Chapter 6, gene expression of adipocyte differentiation markers gene expression were determined, and visual inspection of the cells was regularly performed. Additional assessment of lipid storage (i.e. Oil red O staining) would have been of added value to assess adipocyte maturation.

7.5 Main outcomes of this thesis

The main conclusions of the experimental studies described in this thesis are summarised below and in figure 7.1:

- Adipose tissue Doppler ultrasound is a non-invasive method for measuring abdominal and femoral ATBF, that is likely able to detect differences between adipose tissue depots as well as the expected postprandial increase in ATBF in healthy individuals. (Chapter 3)
- Upper and lower body adipose tissue are characterized by distinct inflammatory signatures in postmenopausal women with normal weight and obesity. (Chapter 4). Leptin and MCP-1 are released from abdominal and femoral AT, with no significant depot-differences. Gene expression of leptin, PAI-1, and TNF-α was lower in abdominal as compared to femoral AT, and higher in abdominal AT of women with obesity as compared to normal weight. In abdominal adipocytes, IL-6, PAI-1, and leptin gene expression was higher, while adiponectin and DPP-4 gene expression were lower as compared to femoral. Finally, abdominal adipocytes secreted less MCP-1 compared to femoral adipocytes.
- Upper and lower body adipose tissue are characterized by distinct oxidative signatures in postmenopausal women with normal weight and obesity, which seem independent of adipocyte size. Adipose tissue oxygen extraction and adipocyte oxygen consumption are lower in abdominal than femoral adipose tissue in postmenopausal women, with no significant depot-differences in OXPHOS protein expression and mtDNA content. In addition, we found lower OXPHOS protein expression in adipose tissue and adipocytes in women with obesity versus normal weight. (Chapter 5)
- Physiological oxygen levels regulate adipokine expression and secretion in abdominal and femoral adipocytes. Adipocytes derived from women with obesity display lower expression and secretion of pro-inflammatory factors at lower physiological pO_2 levels (5% O_2) as compared to high physiological pO_2 levels (10% O_2). Except for effects on leptin expression, no significant effects of low compared

to high physiological oxygen levels were observed in adipocytes derived from individuals with normal weight. These findings support that pO_2 levels impact the inflammatory signature of adipocytes, and that donor characteristics determine experimental outcomes. (Chapter 6)

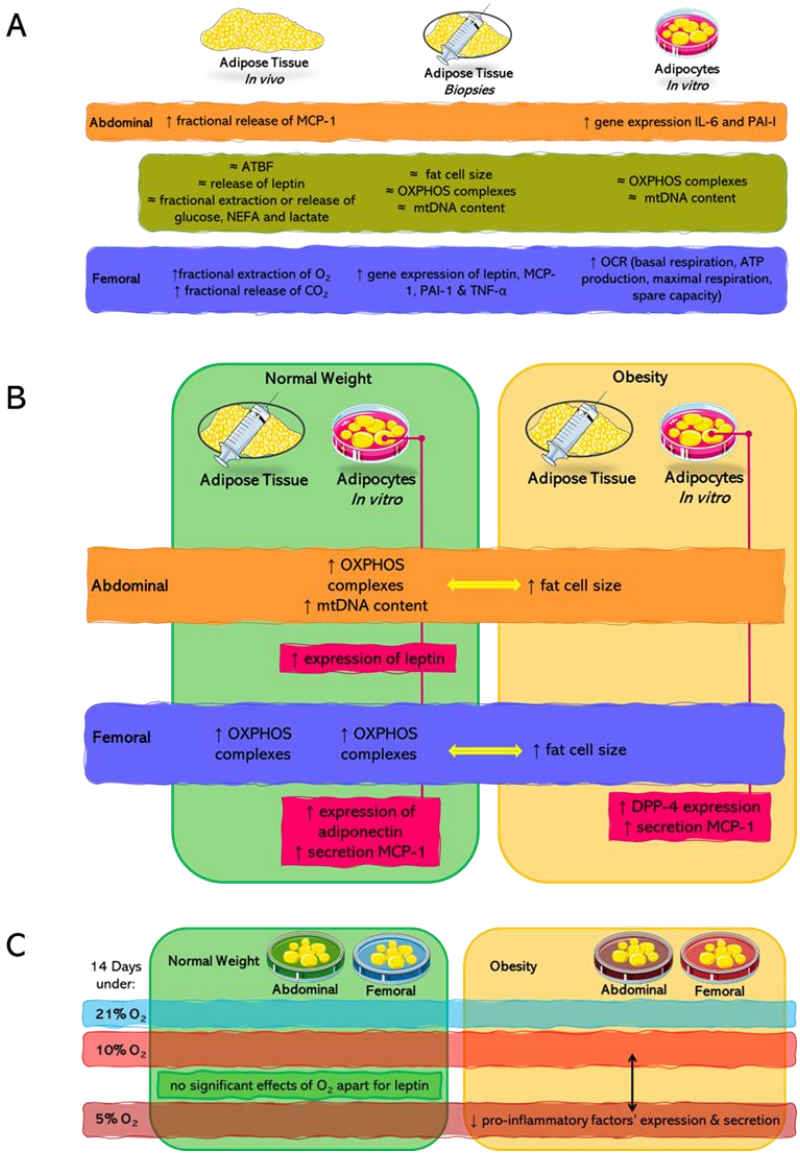


Figure 7.1: Graphical summary of main thesis findings. ***Panel A*** *focuses on adipose tissue depot differences, summarising the in vivo measurements, the analyses in adipose tissue biopsies and the mechanistic in vitro experiments described in chapters 4 and 5.* ***Panel B*** *presents the main findings related to the comparison between adipose tissue biopsies and cells derived from individuals with obesity and normal weight also described in chapters 4 and 5.* ***Panel C*** *summarizes the main findings from chapter 6. ↑ increased, ↓ decreased, ≈ no difference Parts of the figure were drawn by using pictures from Servier Medical Art (available from: smart.servier.com) licensed under a Creative Commons Attribution 3.0 Unported License.*

7.6 Future research directions

- Future studies need to unravel in more detail the key characteristics that determine the functionality of different AT depots such as visceral, abdominal, and femoral subcutaneous AT to better understand the disease risk associated with a certain body fat distribution and sex differences independent of body composition. Of interest would be to determine the immune cell populations of abdominal and femoral AT with Fluorescence activated cell sorting (FACS) analysis, a side project that we aimed to execute at the end of the recruitment period which was not eventually feasible due to COVID-19. Further characteristics could be metabolites, hormones, and inflammatory mediators' production.
- Adipose tissue depot-differences in the inflammatory phenotype and oxidative machinery should be examined in different subgroups of the general population. For example, in people who are more metabolically compromised (i.e., in individuals with T2DM or patients with severe obesity), as well as populations varying in age, sex, metabolic status, menopausal status, ethnicity and obesity duration and different body fat distribution patterns (android versus gynoid) for comparisons. Of interest would be to investigate the timeline of changes in these processes. For instance, measuring the mitochondrial function, inflammatory phenotype, and hormonal changes in a long (years) follow-up period in people with obesity or overweight.
- It would be important to investigate the effects of lifestyle and/or pharmacological interventions on the inflammatory phenotype and oxidative machinery in metabolically compromised individuals living with obesity.
- Finally, investigating the effects of *in vivo* hypoxia exposure on the inflammatory and oxidative phenotype of adipose tissue (different fat depots) in relation to whole-body metabolic alterations in humans would be of interest.

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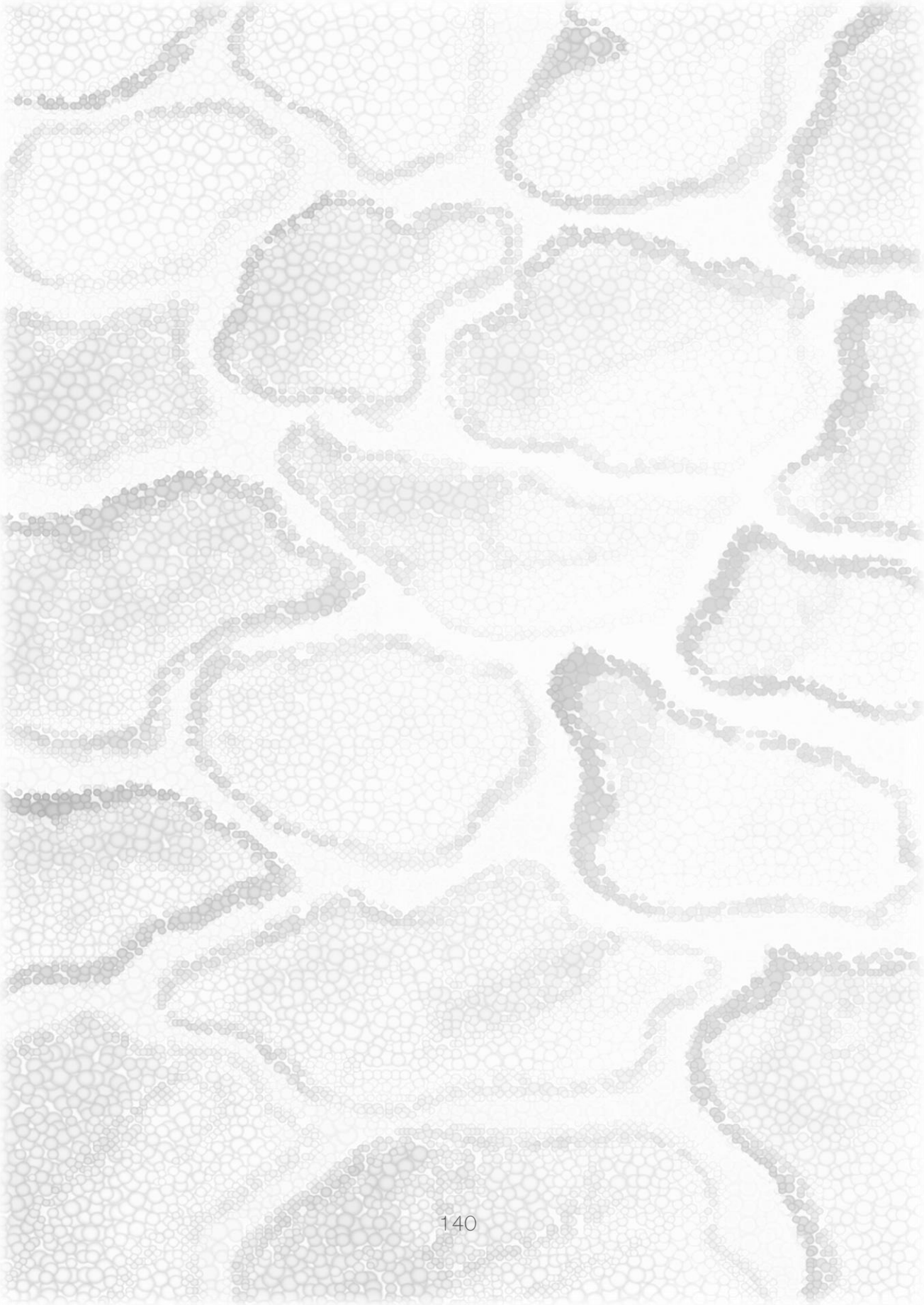

8 Appendices

8.1 MEMORANDUM

The collation, analysis, and interpretation of the work presented in this thesis are my original work carried out under the supervision of Dr. Konstantinos N. Manolopoulos [Institute of Metabolism and Systems Research (IMSR), College of Medical and Dental Sciences, University of Birmingham, and NIHR/Wellcome Trust Clinical Research Facility at Queen Elisabeth Hospital Birmingham, both in Birmingham, UK], Dr. Gijs H. Goossens and Prof. Dr. Ellen E. Blaak (Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands).

In relation to the *in vivo* studies, I obtained ethical approval, including the writing of all relevant ethics forms and committee-related procedures. I was responsible for the recruitment and screening process for volunteers. I performed all *in vivo* measurements, including adipose tissue blood flow measurements, cannulation of the radial artery, superficial epigastric, and great saphenous vein. I collected blood samples and adipose tissue biopsies, prepared the high-fat mixed-meal, and was responsible for all other practical study aspects. Various members of the staff assisted with the clinical studies, particularly the nursing staff of the NIHR/Wellcome Trust Clinical Research Facility at Queen Elisabeth Hospital Birmingham, especially research nurses Nula Kelly, Samantha Timmis, Katie Deans, Hafwen Thornhill, and Claire Brown. Valuable assistance was offered by the research fellow Dr. Rosemary Dineen. The processing and biochemical analysis of blood samples were done by members of the laboratory staff at the Queen Elisabeth Hospital Birmingham and Wendy Sluijsmans (Department of Human Biology, Maastricht University Medical Center+, the Netherlands).

In relation to the *in vitro* experiments, I performed cell isolation and cell proliferation/expansion, cultured hMADS, exposed these cells to different oxygen environments, collected the cell culture medium, and collected the cells for RNA isolation. The laboratory technicians Nicole Hoebers and Yvonne Essers, and Dr. Johan J.W. Jocken assisted with some experimental techniques when it was required. BCA and Seahorse experiments were executed with help from Nicole Hoebers. Molecular biology methods, PCRs, histology, and fat cell size measurements were performed by Yvonne Essers. ELISAs and OXPHOS Western Blots were performed by Nicole Hoebers. I was responsible for the collation, analysis, visualisation (tables and figures) and interpretation of all data.

8.2 Valorisation addendum

Obesity is an ever-increasing global health problem, that needs to be tackled to reduce the risk for other chronic diseases related to excess body adiposity and associated healthcare costs. The present thesis describes a series of studies that were performed to investigate (functional) differences between abdominal and femoral subcutaneous AT and adipocytes, as well as the impact of oxygen tension on the inflammatory phenotype of abdominal and femoral adipocytes. The valorisation potential of the work described in this thesis will be discussed in terms of socioeconomic relevance, the scientific implications for specific target groups, and potential applicability for commercial exploitation.

Societal relevance

Obesity is a complex chronic disease linked to increased risk of developing insulin resistance, T2DM, CVD, and various types of cancer (1-7). Furthermore, it appears to be linked to risk of nearly every chronic condition, ranging from osteoarthritis to poor mental health (8). Quality of life and life expectancy are significantly impaired in people living with obesity. Therefore, the need to better understand and treat obesity are of paramount importance. This has become even more apparent during the COVID-19 pandemic, as obesity is associated with an increased risk of infection and worse clinical outcomes in people with obesity that encounter COVID-19 (9, 10). The economic burden of obesity is associated with increased hospitalisation and healthcare-related costs (8, 11, 12). Obesity has also been linked to decreased productivity per person (8, 13). Taking these factors into account, obesity clearly is an important socio-economic problem that needs to be prevented and treated to increase population health and reduce future health care costs.

In this thesis, we have examined the properties of AT (dys)function in people with normal weight or obesity. The results may not be immediately applicable to tackle obesity. However, any attempt to expand our understanding of the pathophysiology associated with this complex condition, from a molecular and physiological approach as done in this thesis, may contribute to the development of novel and/or more personalized prevention and treatment avenues.

Scientific impact, innovation, and exploitation

Differences between distinct AT depots, like abdominal and femoral AT, seem to explain differences in chronic cardiometabolic disease risk in people with a different body fat distribution pattern. The present work underlines the presence and importance of these AT depot differences, indicating that abdominal and femoral AT differ in blood flow, the oxidative machinery, and inflammatory signatures. However, further characterisation of these (and other) AT depots is necessary, in combination with cardiometabolic risk assessment in humans.

Since data on AT depot differences in humans is scarce, better knowledge of the properties of abdominal and femoral subcutaneous AT using a translational approach is of great scientific value. In this thesis, we showed slight differences in the inflammatory

signatures of AT of the abdominal and femoral depot. However, for the first time we have presented important differences of the oxidative machinery, both *in vivo* and in primary human adipocytes.

The results described in this thesis have been presented at national (Society for Endocrinology BES 2019) and international conferences (European Congress of Endocrinology 2022, and 58th Annual Meeting of the EASD 2022) to scientists, health care professionals, physicians, and dietitians working in the field of endocrinology, obesity, and diabetes. Moreover, the results have been and will become available to the scientific community through publications in international peer-reviewed journals, with the aim to increase knowledge in the research area of obesity and AT function, inflammation, tissue oxygenation, oxidative metabolism, insulin resistance and T2DM.

I was privileged to be enrolled in a joint PhD program between the University of Birmingham (UK) and Maastricht University (the Netherlands). The nature of this joint PhD program also had a direct scientific impact. Collaborations between two countries and institutions were initiated and stimulated, which is a great example of innovation within the international scientific community. Interdisciplinary teams of clinical and basic scientists, research nurses, laboratory technicians and support staff have been involved in the studies described in this thesis.

The findings presented in this thesis are mainly focusing on the better understanding by detailed phenotyping and investigation of AT biology and *in vivo* physiology. The use of the Doppler ultrasound as a proxy method for ATBF measurement could be used by other research groups. The physiological and biological findings highlight the importance of examining (changes in) body fat distribution pattern and AT function in future clinical trials, and may give leads for the development of novel treatments (i.e. targeting AT oxygenation). In order to understand the implications of the depot-specific signatures, a deeper understanding of each adipose tissue depot's phenotype is needed. In the future, depot-specific signatures could serve as biomarkers for monitoring and/or predicting clinically relevant outcomes e.g., related to successful weight management and obesity-related risk reduction. Whether treatments can be developed based on these findings will need to be examined in future research as well, given the obstacle posed by having to address depot-specific targets rather than a systemic pathophysiological mechanism. Altogether, the scientific findings described in this thesis may be of value for academia, industry, and health care professionals.

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8.3 Methodological considerations, impact of COVID-19, and reflection on a joint PhD program

8.3.1 Study design and statistical issues

8.3.1.1 Adipose tissue blood flow study (Chapter 3)

8.3.1.1.1 Study design

The aim of this study [UK Health Research Authority approval number 19/HRA/0565 from the Health and Care Research Wales (HCRW)] was to measure adipose tissue blood flow (ATBF) by a novel ultrasound Doppler technique. We examined fasting ATBF as well as postprandial ATBF after the ingestion of an oral glucose drink in healthy men and women with a broad range of age and BMI.

8.3.1.1.2 Study Population and Sample Size

We aimed to recruit 16 individuals (8 female) with a BMI of 18-25 kg/m^2 (normal weight group), and 16 individuals (8 female) with a BMI of 30-40 kg/m^2 (people with obesity).

A formal power calculation was not performed since this was a pilot study to establish a new methodology and assess its ability to detect AT depot-differences in blood flow and differences between fasting and postprandial ATBF. Based on the known effect size in blood flow changes after glucose previously measured with the ^{133}Xenon techniques, the proposed sample size should provide sufficient power if it is assumed that the same sensitivity between the two methods.

8.3.1.2 'AdipO$_2$' Study (Chapters 4, 5 and 6)

The Adipose Oxygen ('AdipO$_2$') study was a human experimental study, in which we combined *in vivo* measurements, analyses in AT biopsies and *in vitro* experiments to compare oxygen metabolism, expression/secretion of inflammatory markers, adipokines and metabolites between upper-body and lower-body adipose tissue in postmenopausal women with normal weight or obesity.

8.3.1.2.1 Analysis of Outcome Measures and Power Calculation

The main aim was to compare differences in oxygen metabolism, inflammatory markers, adipokines and metabolites like non-esterified fatty acids and glucose between abdominal subcutaneous (upper-body) and femoral subcutaneous (lower-body) AT in postmenopausal women with normal weight or obesity. AT depot-differences in oxygen consumption rates and the secretion of inflammatory factors within women with normal weight or obesity were tested using Student's paired *t*-tests (or Wilcoxon signed rank tests in case of skewed data, as determined by the Shapiro-Wilk test), while AT differences between people with normal weight and obesity were determined using unpaired *t*-tests (Mann Whitney test in case data were not normally distributed).

A formal power calculation was not performed, given the exploratory nature of the study, which is a limitation. The number of participants that as a team aimed to include in

these studies to detect differences in the secretion of inflammatory factors and oxygen consumption across upper-body versus lower-body adipose tissue was based on previous studies using the arterio-venous balance technique to investigate group differences in adipokines/metabolites across AT. This is exemplified on pilot data obtained using the arterio-venous balance technique and ^{133}Xenon wash-out to determine ATBF that showed an average IL-6 release of 12.6 $pg.ml^{-1}.100g\ tissue^{-1}.min^{-1}$ (SD 11.1) from abdominal subcutaneous adipose tissue and 1.1 $pg.ml^{-1}.100g\ tissue^{-1}.min^{-1}$ (SD 1.7) from femoral adipose tissue (1). For these IL-6 release values across upper-body and lower body adipose tissue depots, the calculated effect size (*d*) was 1.45, and a sample size of 14 subjects in each group would be needed to detect a difference between means with a power of 95% and a significance level (alpha) of 0.05 (two-tailed). Based on this, a recruitment target of 16 subjects within each group was set, which included a 15% technical failure and/or dropout. Due to the COVID-19 pandemic, it was unfortunately not feasible to include the targeted number of participants, as explained in more detail below.

A further limitation of the study is that we did not correct for multiple testing. Failing to account for multiplicity (multiple comparisons), which can be done using Bonferroni correction, the Holm-Bonferroni method, and False discovery rate control (2, 3), increases the likelihood of false positive results, commonly known as Type I errors (2, 3). This is due to the increased likelihood of finding at least one statistically significant result by chance alone when several comparisons are conducted within the same research study (2). In the setting of clinical trials this can lead to false conclusions regarding the efficacy of a therapy or the significance of a certain biomarker. Furthermore, failing to account for multiplicity can lead to exaggerated estimates of a certain observation, leading to overestimation of the results of a study. This should be taken into consideration as a limitation within Chapters 4 to 6 (2, 3). Therefore, this study was largely explorative in nature, and future studies are needed to replicate the findings.

8.3.1.2.2 Justification for participant population

We studied post-menopausal women only to control for differences in (depot-specific) AT function between men and women, and to ensure feasibility of the femoral AT biopsy and cannulation, because men often have less femoral adipose tissue. Moreover, post-menopausal women are metabolically more compromised than pre-menopausal women and, therefore, differences between upper and lower-body AT may be more pronounced. Although not feasible within the current study, follow-up studies are clearly needed to elucidate whether sex-differences in AT function are present and may underlie the difference in cardiometabolic disease risk between men and women, as well as the role of the menopause transition in women in terms of upper and lower-body AT function.

8.3.2 Recruitment strategies

8.3.2.1 Adipose tissue blood flow Study (Chapter 3)

Participants were recruited from the wider University and Queen Elizabeth Hospital area, using print and electronic advertising (e.g., University social media, website, and mailing lists), by poster in local cafes, pubs, libraries, other communal areas and the hospitals, university departments, and in the publications (print and electronic) of these institutions. The study was advertised via the University of Birmingham social media (Facebook and Twitter accounts), a dedicated area on the University website and via mailing lists of different University and Queen Elizabeth Hospital Birmingham departments. Posters were displayed on departmental notice boards at the University and Queen Elizabeth Hospital Birmingham. Advertisements were also included in the University's and Queen Elizabeth Hospital Birmingham print media for public information.

8.3.2.2 AdipO$_2$ Study (Chapters 4, 5 and 6)

Healthy volunteers were recruited via advertisement by electronic means, in print, and with posters. Electronic advertisement included the University of Birmingham and Queen Elizabeth Hospital social media and the University and Queen Elizabeth Hospital websites, and via mailing lists of different University and Queen Elizabeth Hospital Birmingham departments following appropriate permissions from the mailing list administrator. Advertisements were also included in the University's and Queen Elizabeth Hospital Birmingham print media for public information. The study was also advertised in local newspapers and other suitable publications, online and social media outlets. Posters and study flyers were displayed and distributed on departmental notice boards and other suitable areas at the University of Birmingham, the Queen Elizabeth Hospital Birmingham, and the Birmingham Women's Hospital, as well as in suitable places in the community (e.g. local cafes, pubs, libraries, other communal areas and community centres).

Because of lower-than-expected interest in the study, potentially due to its invasive nature, and relatively low eligibility rate, we used additional ways of recruitment following a protocol amendment. Specifically, Primary Care sites were utilised as Participant Identification Centres (PICs). For this purpose, we worked together with the NIHR Clinical Research Network (CRN) West Midlands, who facilitated recruitment via suitable Primary Care PICs. Recruitment strategies included display of study advert posters within General Practice (GP) waiting rooms and pharmacies, as well as the development of a GP patient database search, based on coded inclusion/exclusion criteria. Eligible patient records were reviewed for suitability by their GP, before being sent a copy of the Participant Invitation letter and Participant Information Sheet. The development of the search was driven by the Primary Care Recruitment Optimisation Support Team (ROST) and the recruitment via Primary Care PICs was driven by the CRN West Midlands Primary Care Team. We were also planning to use, before the COVID-19 pandemic, the NIHR BioResource service, which holds a large database of

research volunteers who have consented to be approached for clinical research studies.

People responding to advertisements by contacting the research team (via email or telephone) were provided with the participant invitation letter and study participant information sheet (PIS), after which they could consider participating in the study. The participant would be given at least 48 hours, usually more, to read the PIS and to discuss their participation with others outside of the site research team. Those responding and wishing to take part were contacted (over the phone or via email) to discuss any initial questions and obtain consent to attend the screening visit. At the beginning of the screening visit there were further opportunities to discuss the study in detail and to answer any remaining questions, before obtaining written informed consent for participating in the study. The screening visit involved taking a medical history, a fasting blood sample and performing an oral glucose tolerance test (OGTT). All procedures took place after getting written informed consent. The results of the screening visit were checked against the inclusion and exclusion criteria, and together with the medical expert for the study (Dr. K. Manolopoulos), we would check eligibility for each study participant.

8.3.3 Impact of COVID-19 pandemic on PhD project

As any complex project, and especially a joint (double-degree) PhD, numerous tasks are required to be accomplished in given timelines. This PhD project was severely impacted by the COVID-19 pandemic, which resulted in all clinical research activities being ceased after March 2020. The restrictions made it impossible to recruit additional participants, just as the additional avenues of recruitment were implemented and started to become successful. Therefore, the initial recruitment target for the $AdipO_2$ study could not be accomplished. At that time, it was impossible to foresee how long it would take to return to any normal research activity, especially for a study like mine that was not treating a critical illness, given the risk of contracting COVID-19 by bringing healthy volunteers to a hospital environment. Also, apart from these practical reasons, the high NHS demands during the pandemic rerouted the highly skilled nurses and other members of the research team to the frontline, making it impossible to continue with any study days. All this resulted in great uncertainty and risk of extending the PhD timeline beyond reasonable limits. Therefore, in conjunction with my supervisors a decision was taken the stop further recruitment for the *in vivo* study prematurely and begin sample analysis and the *in vitro* work, using the material obtained up to then.

For sample analysis and the execution of all *in vitro* experiments, I relocated to Maastricht. The experimental work in Maastricht was also impacted by the restrictions applied to the laboratories at several times during lockdowns. The lockdowns meant that access to the specialised molecular biology laboratories at the Department of Human Biology and to the Maastricht University campus in general was restricted to essential staff only, and not PhD students. In addition, when PhD students were allowed to use the facilities, they had to have been trained in the techniques and facilities they wanted to use, which for several methods did not apply to me initially. A rule of one

person per room at the time was making it impossible for me to be adequately trained in all aspects, or to contribute and execute several experimental analyses myself, requiring therefore the assistance and support from the amazing laboratory technicians in Maastricht. As a result, I missed out on important research training opportunities that otherwise would have been a core part of my PhD.

Besides the very important point of recruitment and study disruption and the missed research training opportunities, the pandemic had many other important negative impacts. The usual PhD face-to-face supervision and live day-to-day interaction with supervisors switched to virtual online meetings, complicating the avenues of obtaining quick and timely feedback as everything required scheduling meetings etc. This was notable especially as before the pandemic the "open door" policy that both my supervisors had and was simplifying the resolution of any queries that could arise ceased to exist. Moreover, daily interactions with peers and other research group members that usually are extremely helpful far beyond the academic and research input (for instance humane, ethical, and psychological support) were also absent, intensifying the already demanding and stressful momentum that was brought by the overall disruptions. Another negative impact of the pandemic was also regarding attending and presenting at conferences, which is usually a highly rewarding PhD experience. Even though the involvement and presentation of these data at conferences became eventually feasible (i.e. an oral presentation at the European Association for the Study of Diabetes Annual meeting in 2022), this was not at the potential degree of what one could expected or want for a PhD. The absence of regular participation in conferences and scientific meetings also meant missed opportunities to practice presenting data to a scientific audience, interacting with fellow researchers and networking for future career steps.

Finally, unrelated to the pandemic, obtaining ethical approvals for the PhD studies, whilst a useful part of the training, in my case, where the joint PhD required spending specified time at each institution, was a significant drawback as it contributed to delays given very long timelines (several months up to half-a-year) for getting approval in the UK. This was especially obvious given that in the Netherlands for instance obtaining ethical approval is usually a matter of 3-4 months (referring to pre-COVID-19 timelines). The delays in moving on with the *in vivo* study were further impacted by the need to pass ethical amendments for dealing with the low recruitment rates after the first months of recruitment. It could be speculated that if as a team we had been slightly quicker to implement changes and alternative recruitment avenues, before the pandemic hit, the result might have been somewhat different, although again these were unforeseen factors and beyond our control.

8.3.4 Procedural and other insights from the joint PhD scheme

I was privileged to be enrolled in a joint PhD program executed between two leading institutions in the fields of Endocrinology, Metabolism, and in particular adipose tissue physiology/biology, the University of Birmingham (IMSR) in the UK, and Maastricht University (NUTRIM) in the Netherlands. This allowed me experiencing different working

environments, several different cultures and mindsets as especially Birmingham is one of the most multicultural cities in the world and this was clearly depicted in everyday life within and beyond the University. Likewise, Maastricht, being in the heart of Europe Union and the borders of three countries, has a rich (multi)cultural history present in most of its corners.

The nature of this joint PhD program also had a direct scientific impact as I have experienced how (dis)similar scientific approaches can be. The Dutch way can be sometimes favourably direct and efficient as opposed to the UK which requires several levels of bureaucracy, for instance in bioethics. The laboratory conditions can also differ, and these experiences lead to a broad and expanded scientific background. Even in ways of formulating presentations or dealing with practical trivial issues experiences from more than one institution, especially from different countries, creates a remarkable armoury of soft and practical skills.

Further benefits are including a broad scientific network. Collaborations between two countries and institutions were initiated and stimulated, which is a great example of innovation within the international scientific community. I have had the opportunity to be part of several multidisciplinary teams. These were including but not limited to scientists of several clinical and basic scientific (and cultural) backgrounds, dietitians, bioinformaticians, research nurses, laboratory technicians and support staff that also have been involved in the studies described in this thesis. These connections are multiplied in comparison of doing a standard PhD.

Of course, there were some additional struggles related to thesis writing that must fit several guidelines for meeting both Universities' requirements and the requirement of having a dual examination / viva / defence. In my view, a uniform thesis and single viva procedure accepted by both universities would be a great improvement of the joint PhD programme.

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8.4 Representative images

8.4.1 Cell culture images

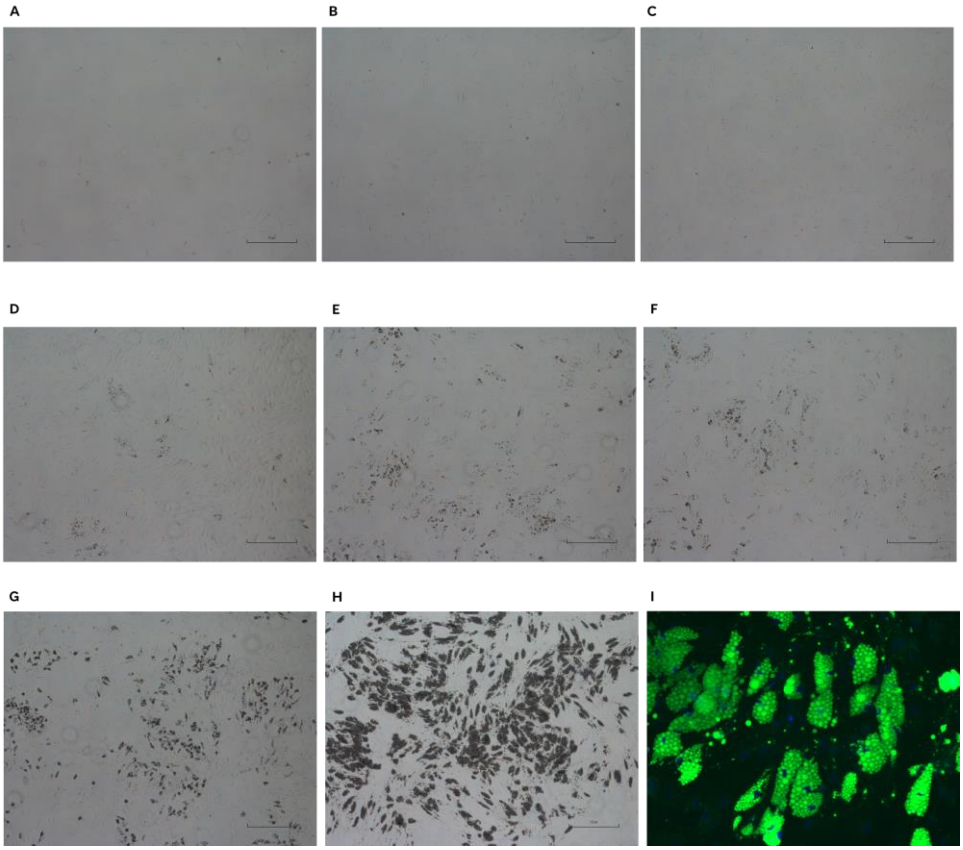

Figure 8.1: Representative microscopy images from the cultivation / differentiation of human multipotent adipose-derived stem cells (hMADS) used for the in vitro experiments. Panels A – H: direct light microscopy. Panel I: FLOID microscopy with 1ug/ml Hoechst 34580 (nuclei – blue stain) and 1:2000 dil. 10 mg/ml Bodipy 493/503 (fat droplet – green stain). hMADS morphology is fibrobrlast-like while undifferentiated (panels A, B, C – days of cultivation 1, 4 , and 7); amid on differentaiation process gradually lipid accumulation (droplets in cytoplasm) is observed (panels D to I; D: Day 11 / Day 4 of Differentiation; E: Day 12 / Day 5 of Differentiation; F: Day 14 / Day 7 of Differentiation; G: Day 15 / Day 8 of Differentiation; H: Day 19 / Day 12 of Differentiation; I: Day 21 / Day 14 of Differentiation)

8.5 Summary

Obesity is a complex chronic disease linked to increased risk of developing chronic cardiometabolic diseases, especially type 2 diabetes mellitus. Obesity-related complications are result of white adipose tissue (AT) dysfunction and are linked to body fat distribution. Dysfunctional AT is characterised by adipocyte hypertrophy, adipokine dysregulation, chronic low-grade inflammation, altered lipid metabolism, decreased AT blood flow (ATBF), mitochondrial dysfunction, and altered oxygenation. Abdominal (upper body) fat accumulation is associated with an increased incidence of obesity-related cardiometabolic complications. In contrast, fat accumulation in the lower body (gluteofemoral) is associated with decreased cardiometabolic disease risk. In this thesis we investigated the differences between upper and lower body AT biology, focusing on blood flow, the oxidative machinery, and inflammatory signatures of abdominal and femoral subcutaneous AT in humans with normal weight or obesity. A second aim was to investigate the influence of prolonged exposure to various oxygen levels on the inflammatory phenotype of abdominal and femoral adipocytes. In Chapter 2 the importance of AT oxygenation is discussed based on an extensive review of the relevant literature, concluding that AT oxygen partial pressure (pO_2) may play a key role in the metabolic and inflammatory perturbations seen in most individuals with obesity. In Chapter 3 we demonstrated that measurement of abdominal and femoral intravascular ATBF with percutaneous Doppler ultrasound is technically feasible and that fasting abdominal ATBF was significantly higher than femoral ATBF. Moreover, the postprandial increase in abdominal subcutaneous ATBF was significantly higher than the ATBF increase in femoral AT. In Chapter 4 we investigated the inflammatory signatures of abdominal and femoral subcutaneous AT in postmenopausal women with normal weight and obesity. We compared the *in vivo* fractional release of adipokines from abdominal and femoral AT in both groups, examined adipocyte morphology and gene expression of adipokines in these AT depots. Furthermore, we determined gene expression and secretion of adipokines *in vitro* using differentiated human primary abdominal and femoral subcutaneous adipocytes derived from the same study participants. The findings demonstrate that upper and lower body AT are characterized by distinct inflammatory signatures in postmenopausal women with normal weight or obesity. In Chapter 5 we examined the oxidative signatures of abdominal and femoral subcutaneous AT and adipocytes in the same group by investigating *in vivo* fractional O_2 extraction and CO_2 release across these AT depots, OXPHOS protein expression and mtDNA copy number in AT and adipocytes, and the oxygen consumption rates in differentiated abdominal and femoral adipocytes. AT oxygen extraction and adipocyte oxygen consumption were lower in abdominal than femoral AT in postmenopausal women, with no significant depot-differences in OXPHOS protein expression and mtDNA content. In addition, we found lower OXPHOS protein expression in AT and adipocytes in women with obesity versus normal weight. In Chapter 6 we investigated the impact of changes in pO_2 levels on adipocyte gene expression and secretion in differentiated human multipotent abdominal and femoral adipose-derived stem cells from the same individuals. Low physiological pO_2 (5%) decreases gene expression and secretion of pro-inflammatory factors in both abdominal and femoral adipocytes derived from individuals with obesity, while these responses were not present in adipocytes derived from individuals with normal weight. In conclusion, the studies described in this thesis provide important insights into the differences between upper and lower body AT

biology, in particular blood flow, the oxidative machinery, and inflammatory signatures of abdominal and femoral subcutaneous AT in humans with normal weight or obesity. Furthermore, the present work has contributed to a better understanding of the impact of prolonged exposure (14 days) to various oxygen levels (as present in human AT) on the inflammatory phenotype of abdominal and femoral adipocytes.

8.6 Acknowledgments

One of my most favourite poets, T.S. Eliot, in his magnum opus "*Four Quartets*" wrote in a way of metaphysical aphorism "*In my beginning is my end*" but also elsewhere "*In my end is my beginning*". This thesis is the end and the beginning of a long journey, literal and metaphorical. It was the typical goal and the transformative crucible. Like the essence of metabolism, a Greek word containing the constant change and transformation of things, my work and I have been metabolised; changed, transformed, improved. In an adaptation of a Nietzschean aphorism "*I am becoming what I am supposed to be*". I am grateful and thankful for this great journey, and for that, I have met so many people that the instances, fait, or good timing brought to my path.

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To arrive where you are, to get from where you are not,
You must go by a way wherein there is no ecstasy.
In order to arrive at what you do not know
You must go by a way which is the way of ignorance.
In order to possess what you do not possess
You must go by the way of dispossession.
In order to arrive at what you are not
You must go through the way in which you are not.
And what you do not know is the only thing you know
And what you own is what you do not own
And where you are is where you are not.

It was true indeed, the way through the PhD without results until what would seem too late, comparable with a state of loss of faith, but still here we are, and you were always positive, comforting, and encouraging.

I am honoured to also be supervised by **Prof. Dr. Ellen Blaak**. I am grateful for your sometimes unseen support, kindness, and feedback. As head of the Department of Human Biology, Maastricht University, you have created a supportive and positive environment, which is fundamental for making other people flourish.

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I apologise if I am not mentioning all the amazing people with whom I have been blessed with their company, memory sometimes fails, or, as Jorge Luis Borges could say, *memory sometimes is creative*. I want to thank my amazing friends and housemates that I met at Wesley House, BISH, Birmingham, which has truly become my home. And special thanks to **Chris, Paul**, and **Gemma** former and current staff that were so helpful on many occasions and created a friendly environment to live in. I am still grateful.

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And lastly, the most valuable thing in my life, my family. Thanks to my family, especially my parents Kiki and Giorgos, for supporting my dreams and giving me everything they could to achieve them. My great late cousin Maria to her beloved memory, remembering the times I have been mentioning my dreams for a PhD in the UK and her love and support. My beloved cousin Penelope is one of my greatest personal heroes, an admirer, and a supporter. Thank you so much for all your help. Also, I would like to thank my Greek – British family that helped in the first era of my English saga; without them, the first steps would have been more challenging. Dear Simon, Sheila, Sarah, and my great late auntie Margaret, I will always be grateful to you.

Finishing this part of the journey with a poetic licence and paraphrasing a lyric from the poem "Ithaca" by C. Cavafy, *the PhD gave me the nice journey*. And after all, as T. S. Eliot, (who else?) wrote, "*only through time, time is conquered*": only through PhD time is PhD time conquered.

8.7 About the author

Ioannis Lempesis was born on Good Friday, April 13th, 1990, in Marousi, in north Athens, Greece. He was raised on the island of Salamina (Athens' metro area). In 2014, he graduated as MD valedictorian of his class from the Department of Medicine, School of Health Sciences, University of Thessaly in Greece. As the top student, he received several scholarships and awards for academic excellence from various funds, including "IKY" (State Scholarships Foundation of Greece). As an undergraduate exchange student, he has spent a month at Leiden University Medical Centre Leiden, Netherlands. Following graduation, he worked as a resident in Internal Medicine (a compulsory part of training in Endocrinology) in the Third Internal Medicine Clinic in Evangelismos Hospital in Athens, Greece. After a year, he moved to the UK as a Visiting Clinical Research Fellow at the Division of Diabetes, Endocrinology, and Metabolism, Department of Medicine at Imperial College London, where he was trained in hyperinsulinemic-euglycemic clamps. Following that period, he started his joint Maastricht - Birmingham PhD programme, performed at the Department of Human Biology (NUTRIM, Maastricht University, NL), and at the Institute of Metabolism and Systems Research (University of Birmingham, UK – leading university). He has been involved in human *in vivo* physiology and molecular biology research, small group teaching, and public engagement, among other activities. During free time appreciates the adventure called life: to travel, discover cosy cafés and under-the-radar cinemas, meet new people and cultures, explore nature and urban landscapes, read literature, poetry, philosophy, and explore his potential in abstract painting.

8.8 Publications and presentations

Articles

1. Lempesis I. G. , van Meijel R. L.J., Manolopoulos K. N., Goossens G. H. Oxygenation of Adipose Tissue: a Human Perspective. *Acta Physiol.* 2020;228:e13298. .
2. Lempesis I. G., Goossens G. H., Manolopoulos K. N. Measurement of human abdominal and femoral intravascular adipose tissue blood flow using percutaneous Doppler ultrasound. *Adipocyte. 2021;10(1):119-123*.
3. Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Rouschop K. M.A., Blaak E. E., Manolopoulos K. N., & Goossens G. H. Physiological oxygen levels differentially regulate adipokine production in abdominal and femoral adipocytes from individuals with obesity versus normal weight. *Cells 2022;11(22): 3532*
4. Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Dineen R., Blaak E. E., Manolopoulos K. N., & Goossens G. H. Distinct inflammatory signatures of upper and lower body adipose tissue in women with normal weight or obesity. *Front Endocrinol 2023, Accepted.*
5. Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Dubois L.J., Blaak E. E., Manolopoulos K. N., & Goossens G. H. Impaired mitochondrial respiration in upper compared to lower body differentiated human multipotent adipose-derived stem cells and adipose tissue in humans. *Submitted.*

Posters presentations at international congresses

1. Lempesis I., Goossens G. H., Manolopoulos K. N. Intravascular subcutaneous adipose tissue blood flow measured with Doppler ultrasound for experimental medicine studies. In: *Endocrine Abstracts* (2019) (Vol. 65). Bioscientifica. Society for Endocrinology BES 2019, Brighton, United Kingdom, 11 - 13 November 2019 – Recipient of Travel Grant from the Society for Endocrinology.
2. Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W. E., Blaak E. E., Manolopoulos K. N., Goossens G. H. Distinct inflammatory signatures of upper-and lower-body adipose tissue in postmenopausal women with normal weight and obesity. In *Endocrine Abstracts* (2022) (Vol. 81); EP355. Bioscientifica. European Congress of Endocrinology 2022, Milan, Italy, 21 - 24 May 2022.

Oral presentations at international congresses

1. Lempesis I. G., Hoebers N., Essers Y. Jocken, J. W., Dubois L.J., Blaak E. E., Manolopoulos K. N., Goossens G. H. “The oxidative phenotype of abdominal and femoral adipose tissue in women with normal weight or obesity”. In *Diabetologia* (2022) 65 (Suppl 1): S73; 58th Annual Meeting of the European Association for the Study of Diabetes (EASD) 2022, Stockholm, Sweden, 19 - 23 September 2022.