TISSUE OXYGENATION IN HUMAN OBESITY

Role in adipose tissue dysfunction and metabolic health

Max A.A. Vogel

The studies presented in this thesis were performed within NUTRIM School for Nutrition and Translational Research in Metabolism, which participates in the Graduate School VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences.

Financial support by the Netherlands Association for the Study of Obesity (NASO) and the Dutch Heart Foundation for the publication of this thesis is gratefully acknowledged.

The studies described in this thesis were partly supported by a grant from the European Foundation for the Study of Diabetes (EFSD) and the Dutch Diabetes Research Foundation (DFN) (granted to Dr. G.H. Goossens).

Cover design:	Max Vogel
Layout:	Max Vogel
Printed & Published by:	Gildeprint
ISBN:	978-94-9301-436-7

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Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. dr. Rianne M. Letschert,
volgens het besluit van het College van Decanen
in het openbaar te verdedigen
op vrijdag 19 oktober 2018 om 12:00 uur

door

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Geboren op 5 december 1989 te Weert

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Voor papa en mama

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CHAPTER 1

GENERAL INTRODUCTION

Obesity

The prosperity of the last century has led to an increased prevalence of several chronic diseases. With enormous increase in the availability and innovation in the food sector, there is an abundance of brands and different foods to choose from. Physical inactivity and an excessive nutrient intake are very important factors with a negative effect on health status in our current population. Together with environmental and genetic factors these are the main causes for the increased prevalence of obesity, which is characterized by an accumulation of fat in the body that is accompanied by an elevated cardiometabolic risk and an increased mortality of cardiometabolic diseases.[1]

Obesity, which is the result of an imbalance between energy intake and energy expenditure, is diagnosed by body-mass index (BMI, kg/m^2) according to the WHO definition. A BMI of 18.5-24.9 kg/m^2 is considered as normal weight, overweight is classified when BMI is between 25.0-29.9 kg/m^2 and obese when it is above 30.0 kg/m^2.[2, 3]

The current cut-off values for BMI, used to classify an individual as underweight, normal weight, overweight or (morbidly) obese, do not consistently reflect adiposity in all individuals. These criteria do not take into account the body composition (skeletal muscle mass), which also varies according to ethnicity, sex, and age. For these reasons, BMI is somewhat controversial as a general indicator of metabolic health.[4, 5] Therefore, it is recommended to measure central adiposity (abdominal fat mass) together with BMI.

With every five-unit increment in a BMI above 25 kg/m^2 the overall mortality increases by about 29%. For vascular and diabetes-related mortality this is even higher, 41% and 120% respectively.[6] Since current anthropometric systems of classifying obesity have limitations, the Edmonton obesity staging system has been proposed, looking at risk factors (like hypertension, impaired glucose metabolism, elevated liver enzymes), physical symptoms (like occasional pains, fatigue), and psychological symptoms (like depression, eating disorders, anxiety, quality of life).[7]

According to the last WHO report, 38.3% of men and 39% of women are overweight while 10.8% of men and 14.9% of women are obese in the global adult population.[2] These numbers are still rising every year indicating that obesity-related complications will also increase, causing a larger social and financial burden for society.[3] Obesity is associated with many comorbidities, including depressive symptoms.[8] Although obesity predisposes towards depression, depression itself may also predict increased risk of later weight gain and onset of obesity.[8, 9] Indeed, mild psychosocial stress can lead to psychological and metabolic abnormalities in humans, and is associated with more abdominal body fat, including increased risk for type 2 diabetes (T2D) and cardiovascular disease.[10]

Lifestyle adaptations are able to reverse the increased risk of obesity-related comorbidities to a great extent.[11, 12] Unfortunately, however, the efforts to get high-risk individuals to eat less and be more physically active in general do not meet expectations. Moreover, it is very difficult to maintain a healthy lifestyle and a certain body weight. Prevention and reversal of obesity requires involvement and commitment of all involved stakeholders at multiple levels like politicians, policy makers, health professionals, clinicians, the food industry etc. In this respect, it is very important that we gain more insight in risk factors and underlying mechanisms to target interventions and prevent cardiometabolic diseases more effectively. In summary, new strategies and insights are needed to improve the current prevention and treatment strategies to combat obesity and related chronic diseases.

Insulin resistance and impaired glucose homeostasis

Obesity is a major risk factor for the development of insulin resistance and T2D. Obesity is characterized by impairments in lipid metabolism, and it is well established that this leads to excessive fat storage in non-adipose tissues e.g. ectopic fat storage, which in turn is closely linked to insulin resistance. This insulin resistance results in concert with progressive ß-cell failure, leading to increased blood glucose levels in the non-diabetic range classified as impaired fasting glucose (IFG, fasting glucose >5.6 mmol/l) or impaired glucose tolerance (IGT, 2h oral glucose tolerant test (OGTT)-derived glucose concentration >7.8 mmol/l). These states of impaired glucose metabolism precede the development of T2DM, although the insulin resistant phenotype may differ in individuals with IFG or IGT.[13, 14] Individuals with IFG predominantly have hepatic insulin resistance and normal or slightly lower peripheral insulin sensitivity. Where IGT individuals have a normal or slightly lower hepatic insulin sensitivity but a moderate to severe peripheral insulin resistance.[13-15] A recent analysis showed that IGT individuals have a higher prevalence of insulin secretion failure (83%) compared to IFG individuals (68%).[16] There is robust evidence that impairments in adipose tissue (AT) metabolism contribute to whole-body insulin resistance and, therefore, exert a central role in the pathophysiology of obesity-related cardiometabolic complications.[17-20]

Dysfunctional adipose tissue and insulin resistance

Research of the past decade has increased our understanding on the role AT metabolism in health and disease. AT is now recognized as a highly active metabolic and endocrine organ.[18, 21-23] Adipocytes are of importance in buffering the daily influx of dietary fat and exert autocrine, paracrine and/or endocrine effects by secreting a variety of adipokines. The normal function of AT is known to be disturbed in obese subjects and type 2 diabetic

individuals, and accumulating evidence suggests that an impaired function of AT, rather than the total fat mass, plays a crucial role in the development and progression of chronic metabolic diseases, including cardiovascular disease and T2D.[18]

AT dysfunction is characterized by enlarged adipocytes,[18, 24, 25] increased secretion of pro-inflammatory adipokines,[18, 26] impaired adipogenesis,[27] decreased AT blood flow,[18] and an impaired AT glucose and lipid metabolism.[28]

The expansion of AT occurs due to a misbalance between energy intake and energy expenditure, where the excess energy is stored in the adipocytes. For energy to be stored in adipocytes hypertrophy or hyperplasia needs to occur making sure there is enough capacity for storage.[29] If the storage capacity of adipocytes reaches its limit of expansion, adipocytes become saturated with lipids resulting in a reduced buffering capacity of the AT. Consequently, lipids flow towards other tissues like skeletal muscle, liver and heart, a phenomenon referred to as the lipid overflow hypothesis.[30, 31] This accumulation of ectopic fat and, in particular, the increased accumulation, altered localization and/or altered composition of bioactive lipid metabolites can decrease insulin sensitivity by interfering with the insulin signaling pathway.[32] Importantly, deposition of lipids in skeletal muscle, liver and heart have been linked to insulin resistance.[33-35]

Contribution of non-adipocyte cells

AT consists of different forms of adipocytes but also of non-adipocyte cell types, like fibroblasts, endothelial cells, macrophages, mast cells, and dendritic cells. These non-adipocyte cells, together with pre-adipocytes, are often referred to as the AT stromal vascular fraction (SVF). The SVF significantly contributes to the pro-inflammatory phenotype of AT. In obese individuals, the AT SVF may underlie the systemic low-grade inflammation that is frequently observed, which in turn may impair insulin sensitivity.[18, 36, 37] Secretion of pro-inflammatory adipokines/cytokines by AT, can cause immune cell infiltration of macrophages, leading to dysfunctional AT characteristics.[23, 38] Moreover, secretion of adipokines can also directly affect other tissues and whole-body metabolism. For example, it has been shown that human AT secretes DPP-4, which can directly impair insulin signaling at the level of skeletal muscle and AT.[39]

Adipose tissue function is more important than adipose tissue mass

AT has been shown to become dysfunctional when mass increases. However, not all individuals with a higher than average AT mass show signs of an impaired AT function and unhealthy metabolism.[40] With the increase in AT mass, which can be observed during the progression from a lean to an obese state, hyperplasia (increase in number of adipocytes) as well as hypertrophy (increase in adipocyte size) occurs to increase its capacity for

triglyceride storage. With this the AT phenotype changes, as mentioned before. Interestingly, obese individuals that are insulin sensitive have less visceral (intra-abdominal) fat as compared to their insulin resistant counterparts.[41] These insulin sensitive obese individuals also have less ectopic fat storage in liver and skeletal muscle, smaller adipocytes (in abdominal subcutaneous AT), and a more beneficial inflammatory profile (including less macrophage infiltration) (Figure 1).[42-45]

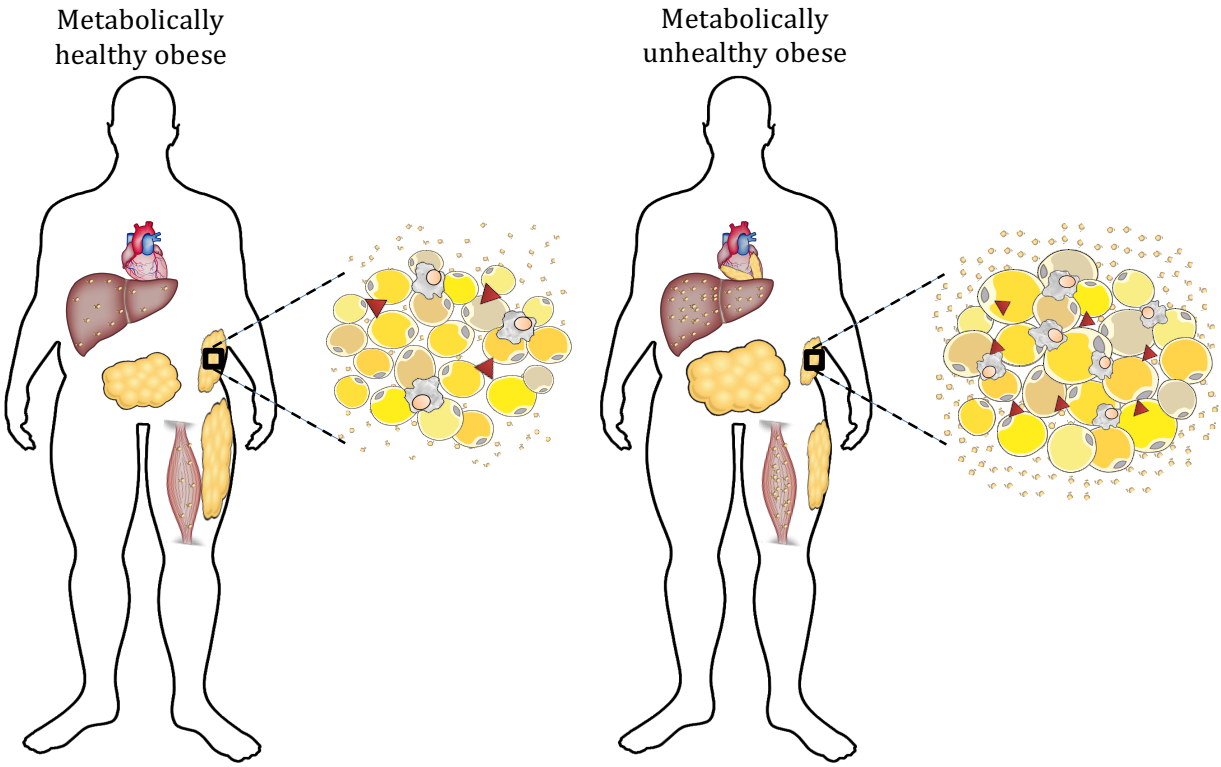

Figure 1. Characteristics of metabolically healthy as compared to metabolically unhealthy obese individuals.

Dysfunctional AT is characterized by adipocyte hypertrophy, infiltration of inflammatory cells (the grey cells in the figure), and altered abundance and secretion of pro-inflammatory cytokines (red triangles) and adipokines (yellow dots in between adipocytes). These characteristics, contribute to the development of insulin resistance and chronic cardiometabolic diseases. A subgroup of obese individuals is referred to as metabolically healthy, and are protected against development of cardiometabolic diseases. Metabolically unhealthy obese subjects have more ectopic fat storage (yellow dots in the liver, muscle, and heart) and have more visceral AT as compared to abdominal or femoral subcutaneous AT). AT dysfunction and body fat distribution are suggested to be the key factors underlying the metabolic health difference in obesity (*figure adapted from Goossens*[46]).

The importance of AT function over mass can be exemplified by the consequences of liposuction and thiazolidinedione (TZD) treatment. Liposuction leads to a very rigorous reduction of abdominal AT mass, but not to an improved metabolic health in part due to the fact that the dysfunction of AT and the enlarged adipocytes are not resolved.[47] On the contrary, TZDs do improve metabolic health (more specifically, insulin sensitivity), while also increasing fat mass.[48] The increase in fat mass by TZD can have multiple causes like an increased adipogenesis, increased appetite, and decreased leptin production.[48] This further underlines that expansion of AT does not necessarily have to result in a detrimental metabolic phenotype. Moreover, it is indeed the case that not all obese individuals are metabolically unhealthy, a substantial proportion of obese individuals (10-30%), are phenotyped 'metabolically healthy obese' (MHO).[40] These individuals do not have dyslipidemia, insulin resistance, impaired glucose metabolism, or T2D.[25, 40, 44, 45] But whether these individuals are really healthy remains controversial, since it has been shown that most MHO subjects have an increased risk of developing T2D[49] and cardiovascular disease as compared to normal weight individuals,[50, 51] and therefore may also have a higher mortality risk.[51] Nevertheless, MHO individuals have a significantly lower health risk compared to metabolically unhealthy obese individuals.[51] , which may partly be explained by a more favorable AT function.

Improving adipose tissue function

Even though AT function might be more important than AT mass, they are closely related (just not to the same extent in all individuals). Therefore, one of the most effective interventions to improve AT function and metabolic health is losing weight. With weight loss interventions it is often thought that individuals reduce the number of fat cells, however a decade ago it became clear that the number of fat cells is relatively stable during adulthood.[52] Following weight loss, the volume of fat cells decreases significantly but not the number of cells.[53, 54] These findings show that there must be a strong regulation of the cell turnover in AT, defined by adipogenesis and apoptosis/autophagy.[52]

The importance of body fat distribution in cardiometabolic health

Not only the total amount of AT but also the location where the lipids are stored is an important determinant of the health status of individuals. Excessive abdominal fat is associated with deleterious cardiometabolic consequences.[55] Ectopic and visceral fat storage are seen as most detrimental, clearly associated with insulin resistance and cardiometabolic complications.[56, 57] Strikingly, lower-body fat exerts specific *protective* functional properties that are associated with an improved metabolic and cardiovascular risk profile in humans.[58-61]

Sex differences in body fat distibution

Next to having a higher percentage of body fat, women also have a body fat distribution with more AT around the hips and thighs compared to men.[62] Also a greater amount of subcutaneous AT has been observed in women compared to men, when adjusted for age and BMI.[63] Sex hormones are crucial players in these differences in distribution patterns.[64] Lower-body fat is positively associated with insulin sensitivity, and with protection from hypertriglyceridemia.[65, 66] Independent of sex, elevated waist circumference and abdominal subcutaneous fat have negative consequences on metabolic health.[67, 68]

Protective properties of lower body fat

Evidence arising from several *in vitro* and *in vivo* studies suggests that the protective functions of lower-body fat are partly explained by differences in adipocyte metabolism and adipokine expression/secretion between upper (i.e. abdominal AT) and lower body fat depots (i.e. gluteal and femoral AT), as reviewed recently.[60] Lower body fat mass is positively associated with adiponectin and leptin levels, which are known to exert beneficial metabolic and cardiovascular effects in humans.[18]

Lower-body fat has a greater buffering capacity for dietary fat compared to upper-body fat preventing lipid overflow and subsequently ectopic fat distribution, where abdominal AT seems to be involved in more rapid uptake and storage of lipids in humans.[60, 69-72] Since lipogenesis is elevated and lipolysis is lower in the gluteal and femoral fat depot *in vivo* it is likely that this depot provides fat storage on long-term.[60, 69, 73] It has also been shown that leptin expression is lower in gluteal and femoral as compared to abdominal fat,[60] whereas serum levels of adiponectin are higher in individuals with more lower-body fat.[74, 75] These hormones play a crucial role in satiety, energy expenditure, angiogenesis, insulin sensitivity, and glycaemic control.[56, 60, 76, 77] Leptin, has the potential to improve insulin resistance by improving fat oxidation and energy expenditure, and reducing energy intake, although obese individuals have higher leptin levels, however because they also have leptin resistance they do not benefit from its effects.[78] Due to chronically elevated leptin levels, leptin resistance occurs. Therefore, it may be suggested that the lower production/secretion of leptin by lower-body fat contributes to the protective and/or beneficial role of lower-body AT. It has also been shown that some pro-inflammatory cytokines might be negatively associated with the amount of gluteofemoral fat, strengthening its proposed beneficial effects.[60, 79] The human in vivo studies described above revealed differences in metabolic, endocrine, and inflammatory regulation of the upper-body fat depot compared to the lower-body fat depot. Nevertheless, the exact mechanisms underlying the protective cardiometabolic role of lower-body fat remain to

be elucidated. AT characteristics seem to be determined not only by its size, the dysfunction of AT seems to have other underlying triggers. One of the proposed triggers for AT dysfunction is the oxygenation of AT. In this thesis, we will focus on the metabolic implications of oxygen tension in a key metabolic organ, namely the AT.

Microenvironmental oxygen in adipose tissue

AT oxygen tension (AT pO_2) is the result of the balance between oxygen supply to the tissue (AT blood flow) and oxygen consumption (metabolic rate) (Figure 2).

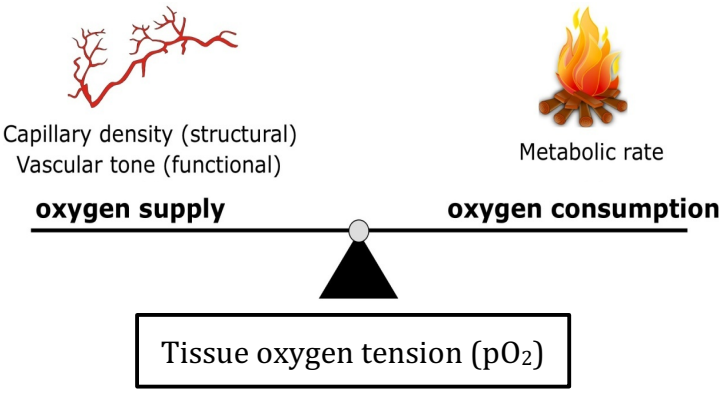

Figure 2. *Tissue oxygen tension is determined by the balance between oxygen supply (blood flow towards the tissue) and oxygen consumption (metabolic rate of the tissue) (figure adapted from Goossens et al.[80]).*

Perturbations in this delicate balance may occur in pathophysiological conditions that are characterized by disturbances in AT blood flow and/or metabolic rate.[80] AT possesses a relatively dense capillary network that ensures adequate delivery of nutrients and oxygen to the tissue. We and others have previously shown that both fasting AT blood flow and the increase in AT blood flow after meal intake are decreased in obese, insulin resistant and type 2 diabetic subjects compared with healthy controls.[81-84] Based on these findings, it was hypothesized in 2004 that the expansion of AT mass during the progressive development of obesity may lead to a relative oxygen deficit in certain parts of AT, because angiogenesis is insufficient to maintain normoxia in the entire AT depot.[85] Indeed, rodent studies have shown hypoxia in AT of obese compared to lean animals. For example. staining of AT against pimonidazole hydrochloride has shown that more hypoxic areas are present in the white AT of obese versus lean mice.[86] Another study demonstrated that hypoxic areas were co-localized with macrophages.[87] Furthermore, a

reduction of body weight by calorie restriction was associated with reduced inflammation and improved oxygenation in dietary-induced (high-fat diet) obese mice.[88]

In line with these animal studies, the first human study measuring pO_2 in the AT of lean and obese individuals showed lower oxygen levels in the obese AT.[89] Importantly, however, these results should be interpreted with some caution, since the lean and overweight/obese groups were not matched for age, sex, ethnicity, and presence of T2D. Further investigation of human AT (by the arterio-venous balance technique) showed that obese individuals have a lower oxygen delivery to AT.[90] Furthermore, this study found that AT oxygen consumption correlated negatively with BMI, whilst a group comparison between obese, overweight and lean groups showed a trend towards a lower oxygen consumption in subcutaneous abdominal AT in obese versus lean individuals.[90] Moreover, the metabolic signature of obese AT did not support a condition of local hypoxia.[90] More specific, the lactate/pyruvate ratio is seen as a metabolic signature of hypoxia, reflecting the cytosolic redox state, however within subcutaneous AT the ratio was not associated with BMI.[90, 91] The second human study measuring pO_2 in the AT of lean and obese individuals, performed in our laboratory, showed that pO_2 was higher, rather than lower, in obese men with impaired glucose metabolism as compared to lean men with normal glucose tolerance.[83] Follow-up studies in our laboratory demonstrated that diet-induced weight loss in humans reduced oxygen tension in abdominal subcutaneous AT, which was accompanied by an increased insulin sensitivity.[92]

Oxygen consumption in adipose tissue

The metabolic rate of a tissue is determined by the functioning of mitochondria in the cells. These organelles produce energy from dietary substrates using oxygen. Mitochondria have as main function the production of energy from carbohydrates, fatty acids and amino acids, a process called oxidative phosphorylation. Through the electron transport chain the substrates are oxidized where protein complexes are present on the inner mitochondrial membrane. In the end, energy is produced in the form of adenosine triphosphate (ATP). Cellular need for energy determines the rate of production of ATP by the mitochondria. In adipocytes, these organelles are not abundantly present, but in obesity it is known that excess fat accumulation is associated with dysfunctional mitochondria.[93-95] Next to dysfunction of mitochondrial biogenesis also mitochondrial mass was found the be reduced in obese animal models leading to lower oxygen consumption in AT.[96-98]

Thus, a possible explanation for increased AT pO_2, as found in obese AT compared to lean AT,[83] may be a lower consumption of oxygen, i.e. a lower metabolic rate. Indeed, oxidative capacity (oxygen consumption rate values, and citric synthase activity) of both

visceral (omental) and subcutaneous AT is negatively correlated with BMI, both expressed per unit AT and per mitochondrion within AT.[99] Furthermore, the latter study investigated AT biopsies from lean and obese non-diabetic men and women, where it was shown that adipocytes from obese AT have a reduced mitochondrial respiratory capacity.[99] Interestingly, this lower mitochondrial function in obesity was observed independent of fat cell size and mtDNA content.[99] In line, it has been reported that obese subjects have a reduced expression of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) in subcutaneous abdominal AT compared to lean subjects.[83, 100] This reduced expression of PGC-1α in AT was also found to be associated with insulin resistance.[101] In line, in insulin-resistant obese individuals markers of mitochondrial metabolism (expression of PGC-1α and COX1, citrate synthase activity and protein levels of complex I and III) were significantly lower compared to insulin-sensitive obese individuals.[102] Improving insulin sensitivity in these individuals by moderate exercise (6 weeks, 4x 40min/week of 55-70% VO_{2max}) did increase aerobic fitness but did not change mitochondrial markers, suggesting additional mechanisms to improve insulin sensitivity after exercise training in obese men.[102]

Down regulation of mitochondrial biogenesis was confirmed and further investigated in monozygotic weight-discordant twins, where one of the twins was significantly heavier than the co-twin.[95] Additionally, the reduced AT mitochondrial oxidative metabolism was associated with whole-body insulin resistance ($HOMA_{IR}$) and increased plasma CRP concentrations (a marker of systemic inflammation).[95] Next to the insulin resistance and inflammation, the mitochondrial pathways were negatively correlated with other measures of adiposity (subcutaneous AT, visceral AT, liver fat, and adipocyte volume), and positively correlated with plasma adiponectin levels.[95] Moreover, also metabolic parameters may influence mitochondrial density and function, for example high-fat feeding, glucose and free fatty acid concentrations.[103, 104]

Consequences of altered adipose tissue oxygenation

Recent studies have indicated that microenvironmental pO_2 in AT is related to metabolic and inflammatory processes in AT. Although most *in vitro* studies have shown a pro-inflammatory response to extreme hypoxia (1% O_2), some conflicting results have also been found, as reviewed.[28, 80] For example, exposure to supraphysiological pO_2 (95% O_2) also induced a proinflammatory gene expression pattern, increased reactive oxygen species content and reduced glucose uptake in *3T3-L1* adipocytes.[105] Human and 3T3-L1 adipocytes exposed to extreme (1% O_2) hypoxia developed insulin resistance by inhibition of the insulin-signaling pathway.[106]

Whereas the latter two studies showed a negative effect of hypoxia on glucose uptake and insulin sensitivity, also beneficial effects have been observed. Adipocytes exposed to hypoxia by either a chemical (cobalt chloride or desferrioxamine) or ambient air (5% O_2) for 24h, showed no signs of toxicity and enhanced lactate release and glucose uptake in all hypoxic conditions.[107] Also different levels of hypoxia (1-15% O_2) dose-dependently increased glucose uptake in human adipocytes.[108] Moreover, acute exposure to sodium sulfite (chemically-induced hypoxia) for 2 and 4 hours increased basal glucose uptake, and decreased fatty acid oxidation in *3T3-L1* adipocytes.[109] In line with these findings, exposing *3T3-L1* cells to transient chemically-induced hypoxia (1 time 4h/day for 4 or 8 days) enhanced lipid synthesis with increased triglyceride accumulation, and increased glucose uptake under insulin-stimulated conditions.[109] This was accompanied by increased protein levels of GLUT4 and pAMPK, and improved insulin signaling.[109]

In human adipocytes, lipid oxidation and lipolysis related genes were down-regulated following acute, severe hypoxia exposure (24h, 1% O_2).[110] In 3T3-L1 adipocytes, it has been shown that basal lipolysis was increased after acute hypoxia (24h, 1% O_2),[111] whereas another study with similar cells showed no effects of hypoxia (both chemically-induced hypoxia and exposure to 5% O_2 for 24h) on lipolysis, measured by glycerol release.[107] Overall, it seems that hypoxia can induce changes in glucose and lipid metabolism of adipocytes, although results are somewhat conflicting.

Effect of oxygen on SVF

Next to adipocytes, the stromavascular cells, of which macrophages are most abundantly present, are an important part of AT and may also be affected by altered O_2 availability in the microenvironment. Indeed, macrophages have shown to respond to acute hypoxia (24 hours of 1% O_2) by regulating the secretion of inflammatory cytokines, but also by increasing UCP-1 expression after hypoxia exposure.[112] These studies should be interpreted with some caution, since these cells were exposed to extremely low (usually 1% O_2) or high (95% O_2) pO_2. Two independent laboratories, including ours, have recently provided evidence that human abdominal subcutaneous AT pO_2 ranges between 3-11%,[83, 89] indicating that cell culture experiments to investigate the effects of *chronic* exposure to more *physiological* pO_2 are urgently warranted.

Microenvironmental oxygen in skeletal muscle

Skeletal muscle is one of the main organs involved in the regulation of glucose homeostasis. Oxygen levels in the skeletal muscle play a crucial role in energy-substrate usage and muscle function.

Oxygen tension in skeletal muscle

The extracellular pO_2 in skeletal muscle under normoxia has been measured in several studies using different techniques (catheters with polarographic probes), reporting values ranging from 3.0-4.5% O_2 (22.8-34.2 mmHg).[113-116] One study investigated intracellular oxygen levels in human skeletal muscle (measured indirectly by calculating deoxymyoglobin using nuclear magnetic resonance spectroscopy), and found ~4.5% oxygen levels under normoxia exposure and ~3% O_2 under ambient hypoxia of 10%.[117] However, it is known that the skeletal muscle shows more fluctuations in oxygen levels compared to AT, possibly due to higher, more variable oxygen consumption of skeletal muscle.[118]

Effects of oxygen on glucose homeostasis in skeletal muscle

Next to influencing AT metabolism, it is also suggested that hypoxia could beneficially affect glucose homeostasis in skeletal muscle. Indeed, studies have shown that acute hypoxia exposure may increase skeletal muscle glucose uptake in isolated muscle fibers derived from rats and humans, and increase mitochondrial biogenesis in rats preconditioned with $CoCl_2$ (a hypoxic mimetic).[119-121] Also, glucose uptake was increased to the same extent in muscle strips from lean, obese and obese type 2 diabetic subjects upon exposure to severe hypoxia (0% vs. 95% O_2), suggesting that in insulin resistance the hypoxia-induced glucose uptake pathway is still functioning.[120] This is strengthened by findings in rodents, showing that hypoxia stimulates glucose transport through AMPK- and Ca^{2+}/calmodulin protein kinase (CAMK)-dependent pathways, similar to the effect of exercise.[119, 122]

Exercise mimicking effects of hypoxia in skeletal muscle

Interestingly, the beneficial effects of hypoxia on skeletal muscle appear similar to the improvements that are seen during physical exercise.[124] Moreover, hypoxia (14.6% O_2) exposure during an acute bout of exercise (60 min) caused a more pronounced increase in insulin sensitivity than exercise alone in men with T2D.[123] It has been proposed that acute hypoxia and contraction stimulate glucose transport in skeletal muscle via the same mechanism, which is different from insulin-mediated glucose uptake.[119] This mechanism stimulates glucose uptake through the AMPK pathway, where calcium might be

involved.[119, 122, 124, 125] This is supported by the findings that there was no additive effect on glucose transport activity by the maximal effects of hypoxia and exercise, whereas *in vitro* these effects have shown to be additive to that of insulin.[119, 126-128] In both hypoxia and exercise, it has been shown that the increase in glucose uptake could be explained by increased GLUT4 translocation to the plasma membrane of skeletal muscle.[119, 129] Strikingly, hypoxia (0% O_2 for 60 min) stimulated glucose uptake to a similar magnitude as contraction or insulin in lean human skeletal muscle (compared to supraphysiological 95% O_2 for 60 min).[120]

Skeletal muscle has a strong dependence on oxygen availability for the generation of energy by oxidizing substrates, suggesting possible effects of hypoxia on mitochondrial biogenesis in myocytes. Indeed, when C2C12 myotubes were exposed to extreme hypoxia, the expression of PGC-1α, which is the master regulator of mitochondrial biogenesis and function, was increased.[130] However, experimental research on the role of hypoxia in the skeletal muscle oxidative phenotype has yielded inconsistent results.[131-133] Therefore, it is tempting to propose that, similar to the adaptation of skeletal muscle in response to exercise, low tissue pO_2 may increase muscle glucose uptake, mitochondrial biogenesis and function via the AMPK-SIRT1-PGC-1α pathway.

Exposing cells to hypoxia induces hypoxia-inducible factors (HIF's), which are proteins regarded as the master regulators of oxygen homeostasis, functioning as transcription factors in adapting the cellular response to low oxygen levels.[134] HIF-1 has two subunits (α and β), with the HIF-1β being expressed continuously and not responsive to levels of oxyen.[134, 135] HIF-1α is synthesized in the presence of oxygen and rapidly degraded by the proteasomal system. As the molecular O_2 sensor of a cell, this α-subunit needs stabilization upon recruitment, which occurs in low oxygen conditions.[136] Hypoxia causes rapid accumulation of HIF-α, leading to many responses including increased glycolysis (to compensate for reduced oxidative phosphorylation (energy loss)), and more efficient oxygen utilization (by upregulating erythropoiesis and angiogenesis).[137, 138] These α-subunits undergo hydroxylation on specific proline residues by prolyl-hydroxylases.[138] The hydroxylated subunits are recognized by von Hippel-Lindau protein of the E3 ubiquitin ligase complex and are degraded via polyubiquitination/proteasomal degradation pathway.[136, 139-141] More than 70 genes are known to be expressed by direct regulation of HIF-1, of which GLUT1, VEGF, and leptin are well-known examples.[134]

Moderate hypoxia exposure as a novel treatment strategy

At sea level, there is 21% (160 mmHg) oxygen present in the air, where the other main component is nitrogen (78%). Importantly, when we ascend to higher grounds (e.g. mountains), the oxygen levels in the air decrease due to pressure reduction, which is called hypobaric hypoxia. At the highest mountain, the Mount Everest with its top on 8,848 meters, oxygen levels are around 7% (53 mmHg). As highly adaptive organisms, humans can acclimatize to a certain level of lower oxygen availability and are therefore able to live at certain higher altitudes (up to 5,000m). The oxygen availability at 2,500-3,000 altitude meters is approximately 15% (about 114 mmHg) oxygen (table 1).

Table 1: Effective amount of oxygen at different altitudes and their interpreted severity.

ALTITUDE (METERS)	EFFECTIVE OXYGEN (%)	OXYGEN LEVEL (mmHG)	SEVERITY
0	20.9	158.8	-
500	19.6	149.0	Low
2000	16.3	123.9	Moderate
2500	15.3	116.3	Moderate
3000	14.4	109.4	Moderate
5000	11.2	85.1	High
7000	8.7	66.1	Extreme
9000	6.8	51.7	Extreme

The body is able to cope with these changed conditions in several ways. First of all, ventilatory adaptations (deeper breaths) increase the amount of oxygen into the lungs thereby utilizing unused parts of the lungs (increased inspiratory reserve volume). Secondly, reduced environmental oxygen may increase heart rate, thereby increasing blood flow and oxygen delivery to the tissues. Thirdly, reduced environmental oxygen may increase red blood cell number thereby improving oxygen transport. These processes are part of the acclimatization and make it possible for humans to ascend to the parts of the earth above 3,000m, which is approximately one-fortieth of total land surface. This acclimatization can take place within minutes (for heart rate and hyperventilation), days (for haemoglobin concentration and capillary density) or years (for advanced adaptation like hypoventilation).[142] How an individual responds is largely determined by the severity of hypoxia, the duration of exposure and/or frequency of hypoxic cycles, which will be further explained in one of the following sections.

Individuals living at high altitude showed to have a lower prevalence of impaired fasting glucose and T2D compared with individuals living at low altitude.[143] At high altitude it was

found that fasting glucose levels were lower compared to sea level.[144]

Environmental hypoxia decreases arterial pO_2, and as such reduces oxygen supply to peripheral tissues. Indeed, it has been demonstrated in rodents that lowering environmental oxygen availability to 5% in an intermittent manner (60 or 12 times/h) decreased pO_2 in AT, the liver and skeletal muscle.[118] Reducing tissue oxygen tension might be a very interesting therapeutic avenue to reduce glucose levels and improve glucose homeostasis.

Acute hypoxia exposure

As introduced above, human AT and skeletal muscle undergo alterations when exposed to different levels of oxygen. In skeletal muscle, these effects seem to mimic the effects of exercise, and are distinct from the effects of insulin. This suggest that environmental oxygen may affect or alter whole body substrate oxidation and energy metabolism.

Healthy males exercising for 60 minutes under normobaric hypoxia (15% O_2) increased lipid oxidation and tended to decrease carbohydrate oxidation 40-60 min post-exercise, whilst this shift in substrate oxidation was still present the next morning.[145] It has also been suggested that hypoxic exercise could change body weight through increases in the myokine apelin, known to play a key role in regulating energy metabolism in skeletal muscle of obese mice.[146] These results may have important consequences for treatment when they would translate to human in vivo conditions for overweight individuals at risk for developing T2D.

A possible explanation for the effects of hypoxia on glucose metabolism could be the Warburg effect, which describes an increased reliance on anaerobic ATP production (anaerobic glycolysis and lactate production) during exposure to hypoxia, firstly described in tumour biology.[147] The Warburg effect can occur even though adequate levels of oxygen are still available.[148] It was described that a key enzyme in glycolysis, 6-phosphofructokinase-1 (PFK-1) can be induced by hypoxia through the activation of the HIF-1 complex. Also, at high altitude (hypobaric hypoxia) shifts towards CHO oxidation have been seen, where fat oxidation was significantly reduced.[149, 150] The latter study showed that lactate levels are increased acutely when arriving at high altitude, suggesting that hypoxia stimulates epinephrine release, which in turn, stimulates muscle glycogenolysis, glycolysis, and muscle lactate production.[151]

Prolonged hypoxia exposure

Interestingly, we have recently found that chronic exposure to hypoxia (8% vs. 21% O_2 for 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.[152] Therefore, it is tempting to postulate that lowering AT pO_2 not only reverses AT inflammation but at the same time may induce mitochondrial biogenesis and improve mitochondrial function in AT. More recently, the same concept has been applied to humans. Interestingly, exposure to moderate hypoxia (15% O_2) for ten subsequent nights increased whole-body insulin sensitivity in eight obese men.[153] Although not commented upon by the authors, moderate hypoxia exposure also tended to reduce AT pO_2.[153] Therefore, these findings may imply that the decreased AT pO_2 after moderate hypoxia exposure has contributed or even driven the improved peripheral insulin sensitivity, as recently proposed.[154] All together these findings strongly suggest an insulin-sensitizing effect of hypoxia.

Intermittent hypoxia exposure

Interestingly, evidence suggests that *'low-dose'* or *mild intermittent hypoxia* (MIH) (9-16% O_2, 3-15 cycles/day) may have beneficial effects on the respiratory, cardiovascular, immune and central nervous system, and, importantly, lipid and glucose metabolism in rodents.[155] Intermittent hypoxia training in rats showed that 13.6% O_2 and training decreased total cholesterol and HDL levels, without significantly changing LDL, triglycerides and FFA in the plasma.[156] A similar treatment in obese rats showed that in skeletal muscle the gene expression of pathways related to glucose metabolism and mitochondrial biogenesis was improved, and, interestingly, that there was no difference between the trained group living in normoxia and the sedentary group under hypoxia.[157] Furthermore, after 4 weeks of exposure to MIH (12 hours of 14% O_2), glucose tolerance and GLUT4 protein expression were enhanced in skeletal muscle from rats.[158] More cycles were also shown to induce beneficial effects, where 40 days exposure to 14.6% O_2 for 8 times 15 min/day (with 5 min intervals) reduced body weight gain in high-fat diet-induced obese mice.[159] Interestingly, these mice also showed reductions in blood glucose and cholesterol concentrations.[159]

Hypoxia exposure: a beneficial add-on treatment opportunity?

The term hypoxia is used in a different context in many studies, of which several have been described earlier in this chapter. Dependent on the number of cycles, exposure level and duration, oxygen exposure can have different effects. It is tempting to speculate that beneficial effects may be observed when individuals are exposed to MIH, as discussed in the previous section.

Noteworthy, this is clearly different from severe intermittent hypoxia as observed during the frequent hypoxic episodes due to collapse of the upper airways during sleep in

patients with obstructive sleep apnea syndrome (OSAS), which is associated with impaired glucose homeostasis and increased cardiovascular risk, as will also be discussed in Chapter 2.[160] Furthermore, MIH is different from the chronic hypoxemia that can be observed in patients with severe chronic obstructive pulmonary disease (COPD).[131] Loss of skeletal muscle oxidative fiber types and mitochondrial capacity is a hallmark of chronic obstructive pulmonary disease (COPD). Muscles of COPD patients are characterized by a change of muscle oxidative phenotype, shifting type 1 to type 2 fibres, as compared to healthy individuals.[161] The reduced proportion of type 1 fibres, a reduction in aerobic metabolism and impairments in the oxidative capacity of skeletal muscle may support a role for hypoxia in patients with (severe) COPD.[162-164] Importantly, however, findings in COPD patients may be confounded by reduced physical activity levels that are frequently observed in these patients.

Taken together, exposure to normobaric *moderate* IH may be a promising strategy to reverse AT dysfunction, improve peripheral glucose disposal, and insulin resistance in obese individuals. However, this has not been investigated in humans yet, and underlying mechanisms for the potential insulin-sensitizing or glucose-lowering effect of *moderate* IH remain to be elucidated.

Outline of this thesis

The main aim of this thesis was to investigate the role of oxygen in AT dysfunction, with focus on different AT depots. In addition, a second aim was to investigate the effects of moderate hypoxia exposure on the metabolic phenotype in humans, taken into account effects on both AT and skeletal muscle metabolism. **Chapter 2** provides an overview of available studies that have examined the link between oxygen tension, inflammation and glucose homeostasis. It was concluded that both *in vivo* clinical studies in well-phenotyped humans and *in vitro* studies that better reflect human physiology by exposing primary human cells to physiological oxygen levels, are needed to elucidate the effects of different moderate hypoxia exposure regimens on insulin sensitivity, lipid and glucose homeostasis. Next, in **Chapter 3**, we investigated the difference in oxygen tension between abdominal subcutaneous and femoral AT in postmenopausal females. Additionally, the depot-specific effect of oxygen tension on the metabolic and endocrine function of AT was studied *in vitro* using human primary adipocytes from the same subjects. Differences between upper and lower-body AT protein expression profiles in post-menopausal women were examined using a proteomics approach in **Chapter 4**. **Chapter 5** describes the relation between abdominal subcutaneous AT oxygenation and insulin sensitivity in obese men and women.

With lower environmental oxygen levels as a possible novel therapeutic avenue, lowering systemic and thereby tissue oxygenation, could improve metabolic health in humans. Therefore, the effects of 7 days exposure to moderate intermittent hypoxia were investigated in a single-blind randomized cross-over study in **Chapter 6**. The effect of different exposure regimens on adipocyte functionality is examined in **Chapter 7**. Finally, the results described in this thesis are put into a broader perspective, and directions for future studies in this field of research are given in **Chapter 8**.

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CHAPTER 2

Moderate hypoxia exposure: a novel strategy to improve cardiometabolic profile in humans?

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Published in:
EMJ Diabetes, 2015;3[1]:73-79

Abstract

The obesity epidemic calls for novel strategies to prevent and treat obesity and its comorbidities. Several studies have indicated that the amount of oxygen to which tissues are exposed may substantially impact cardiometabolic health. Interestingly, living at high altitude (hypobaric hypoxia) seems to be associated with improved glucose homeostasis and a decreased prevalence of Type 2 diabetes. Furthermore, normobaric hypoxia exposure has been shown to exert beneficial effects on glucose homeostasis and insulin sensitivity in rodents and humans. This may, at least in part, be explained by altered adipose tissue and skeletal muscle oxygen tension. In contrast, patients with obstructive sleep apnoea syndrome, which is characterised by episodes of severe intermittent hypoxia due to periodic collapse of the upper airway during sleep, show impairments in glucose homeostasis and are at increased cardiovascular risk. These discrepancies may be explained by the severity, duration, and pattern (number of cycles) of hypoxic episodes, but underlying mechanisms have not yet been studied in detail. The purpose of this review is to provide an overview of available studies on the link between oxygen tension, inflammation, and glucose homeostasis. Detailed studies to elucidate the effects of moderate hypoxia exposure on whole-body and tissue-specific insulin sensitivity in humans are clearly warranted.

Introduction

The current obesity epidemic is accompanied by an increased prevalence of Type 2 diabetes (T2D)[1] and cardiovascular disease (CVD).[2] Insulin resistance, which may be present in multiple metabolic organs such as adipose tissue, skeletal muscle, and the liver, is one of the key processes in the development of T2D. Weight gain during the development of obesity is accompanied by adipose tissue dysfunction, which in turn contributes to excessive lipid accumulation in non-adipose tissues (ectopic fat deposition) when fat oxidative capacity is insufficient.[3, 4] It has been known for many years that an impaired function of adipose tissue and skeletal muscle is strongly related to peripheral insulin resistance and T2D.[5] Lifestyle interventions have been shown to be effective in the prevention of T2D and cardiometabolic complications,[6, 7] but there is large variability in the response to these interventions. This creates the need for additional strategies to improve cardiometabolic health in individuals at increased risk of developing CVD and T2D. Interestingly, there is evidence to suggest that modulation of oxygen availability may be a novel therapeutic avenue to prevent and treat cardiometabolic diseases, as will be discussed in more detail below.

Living at high altitude, ambient oxygen tension, and glucose homeostasis

Epidemiological data on the effects of living at high altitude on mortality from chronic diseases are somewhat conflicting, in part due to differences in ethnicity, behavioural factors, and complex interactions with the environment. Nevertheless, the majority of evidence indicates that living at high altitude, where oxygen partial pressure is relatively low (hypobaric hypoxia), seems to be associated with reduced mortality from CVD, stroke, and certain types of cancer.[8] The underlying mechanisms that may explain these observations are largely unexplored, but increased physical activity, decreased air pollution, and hypoxia at high altitude may be involved.[8] On the other hand, available evidence suggests that long-term residence at high altitude is a potential problem for chronic obstructive pulmonary disease (COPD) patients, since mortality from COPD and infections of the lower respiratory tract seem rather elevated. It seems that living at high altitude could adversely affect mortality when diseases progress.[8] It may be argued that moderate altitudes are more protective than high or even very high altitudes,[8] which can partly be attributed to chronic mountain sickness arising at higher altitudes (>3,000

m).[9]

A lower prevalence of impaired glucose tolerance and T2D has been found in individuals living at high altitude, namely the population of rural Aymara in Northern Chile, compared with those living at lower altitude, despite a relatively high occurrence of obesity.[10] In addition to a high level of physical activity (e.g. due to dependence on agriculture and time spent travelling) and possible differences in food intake, the lower ambient oxygen tension may play an important role in the lower prevalence of T2D in individuals living at high altitude.[10]

The supply of oxygen to organs is essential for living organisms. Importantly, available evidence indicates that alterations in ambient oxygen partial pressure, leading to changes in tissue oxygenation, may affect the metabolic profile. Interestingly, it has been demonstrated in humans that exposure to normobaric hypoxia during exercise reduces fasting glucose concentration and improves the insulin sensitivity index.[11-13] In line with this, exposure to moderate hypoxia (15% versus 21% O_2) for 10 subsequent nights increases peripheral insulin sensitivity in obese men.[14] The effects of environmental hypoxia exposure seem to be mediated, at least in part, via alterations in adipose tissue and skeletal muscle metabolism.

Oxygen tension and adipose tissue function

Adipose tissue oxygen tension (AT pO_2) is determined by the balance between oxygen supply via the vasculature and oxygen-consuming processes within adipose tissue.[15] Previous studies have clearly shown that fasting and postprandial adipose tissue blood flow (ATBF) is decreased in obese, insulin resistant individuals compared with those who are lean and insulin-sensitive.[16, 17] Moreover, the decrease in ATBF occurring in obesity induces a reduction in oxygen delivery to adipose tissue.[16, 18] Therefore, it has been postulated that insufficient angiogenesis in expanding adipose tissue may lead to a relative oxygen deficit during the development of obesity.[19]

This hypothesis has been confirmed by several animal studies showing an increased expression of hypoxia-responsive genes, a higher abundance of hypoxic areas, and lower oxygen tension in white adipose tissue in obese versus lean animals.[20-22] Importantly, it should be emphasised that these studies were performed in animal models of obesity, which are characterised by rapid and massive expansion of body fat mass. In human

pathophysiology, on the other hand, fat mass gain is certainly not as rapid, which implies that the reduction in oxygen supply to adipose tissue may be less severe in humans than in rodents.[5, 15] Pasarica et al.[23] reported lower AT pO_2 in overweight and obese individuals compared with lean controls, although these findings have not been replicated thus far. In contrast, we have demonstrated an increased AT pO_2 in obese compared with lean, well-phenotyped individuals matched for age and sex. This higher AT pO_2 was observed despite the obese displaying a lower ATBF, and was associated with adipose tissue inflammation and peripheral insulin resistance.[16] Importantly, both studies found that physiological AT pO_2 values range from approximately 3–11%,[16, 23] as assessed using either a polarographic micro Clark-type electrode[23] or an optochemical measurement system to continuously monitor AT pO_2.[16] The advantage of the optochemical measurement system (range: 0–300 mmHg; accuracy: 1 mmHg), which we have recently developed,[16, 24] is that it allows prolonged measurements of tissue pO_2 over a relatively large tissue area (approximately 3–4 cm2) and it can be applied to measure pO_2 in any tissue (e.g. skeletal muscle) as long as insertion of a microdialysis catheter is feasible.

Because oxygen tension is determined by oxygen supply and consumption, our findings of increased AT pO_2 in obese individuals[16] suggest the presence of reduced adipose tissue oxygen consumption. Indeed, impaired mitochondrial biogenesis, morphology, and function in white and brown adipose tissue has been described in mouse models of obesity and T2D.[25] Furthermore, human data indicate that adipose tissue oxygen consumption *in vivo* was lower in obese than lean individuals.[16, 18] Moreover, it has recently been demonstrated that mitochondrial biogenesis, oxidative metabolic pathways, mitochondrial oxidative phosphorylation protein levels, and mitochondrial oxygen consumption were decreased in adipose tissue and isolated white adipocytes of obese individuals.[26-28] Of note, adequate mitochondrial function is essential to maintain adipose tissue function, and it protects against insulin resistance and T2D.[25]

These findings challenge the concept of adipose tissue hypoxia in human obesity and provide preliminary evidence that increased AT pO_2 may elicit adipose tissue dysfunction and consequently insulin resistance in humans. Therefore, we recently exposed mice to chronic hypoxia or normoxia (8% versus 21% O2, respectively) for 21 days. We found that chronic hypoxia exposure improved visceral and subcutaneous adipose tissue function,[29] which was evidenced by decreased adipocyte size, decreased macrophage infiltration and gene expression of inflammatory markers, and increased expression of mitochondrial function and biogenesis markers.[29] These findings suggest that reducing

AT pO_2 may exert beneficial effects on adipose tissue function and, consequently, insulin sensitivity. However, these findings need to be confirmed in humans.

Oxygen tension and skeletal muscle glucose uptake

In addition to the effects of oxygen tension on adipose tissue function, there is evidence that hypoxia may also affect skeletal muscle glucose uptake and mitochondrial biogenesis. As such, hypoxia exposure might mimic the effects of exercise. More specifically, it has been shown that acute hypoxia exposure stimulates glucose transport in isolated muscle strips from insulin resistant humans.[30] Interestingly, the hypoxia-induced stimulation of glucose uptake in these muscle strips was comparable between those from lean and obese individuals, as well as those from obese patients with T2D.[30] However, it should be noted that 'normoxia' and 'hypoxia' reflected non-physiological conditions (95% versus 0% O_2, respectively). Furthermore, acute hypoxia exposure during exercise improved the effect of exercise on glucose tolerance compared with exercise under normoxic conditions.[11] In the insulin resistant muscle, the major defect is in insulin-mediated glucose uptake. However, the ability of hypoxia to induce skeletal muscle glucose uptake to the same extent in insulin-sensitive and insulin resistant muscle[30] indicates that the hypoxia-induced glucose uptake pathway is still intact in the insulin resistant muscle. Interestingly, hypoxia appears to stimulate skeletal muscle glucose transport through adenosine monophosphate-activated protein kinase and Ca2+/calmodulin-dependent protein kinase-dependent pathways in rodents.[31, 32] In line with this, it has recently been shown that exposure of human myotubes to 15% O_2 increased basal but not insulin-stimulated glucose uptake compared with 21% O_2.[14] Furthermore, hypoxia exposure increased the expression of the master regulator of mitochondrial biogenesis and function, peroxisome proliferator-activated receptor gamma coactivator-1α, in C2C12 myotubes.[33] In conclusion, several rodent and human studies have indicated that hypoxia may improve skeletal muscle glucose uptake, mitochondrial biogenesis and function, and whole-body glucose homeostasis.

Beneficial effects of moderate hypoxia exposure

Weight loss and increased physical activity are recommended to reduce cardiometabolic risk in obese humans. However, this is not easily achieved by all individuals and, therefore, alternative or additional strategies to improve cardiometabolic health are warranted. Although the effects of the severity and duration of oxygen exposure have

not been studied extensively, it seems that moderate hypoxia exposure (9–16% O_2) using a limited number of cycles (3–15 cycles per day) may have beneficial effects on neurodegenerative diseases, the immune system, body weight, CVD, exercise performance, and, importantly, lipid and glucose metabolism.[34]

Several studies have examined the effect of a combined hypoxia and exercise intervention on body weight. Interestingly, a larger decrease in body fat content was found when patients were exposed to moderate hypoxia rather than normoxia during the exercise sessions.[35-38] In line with this, it has been shown that hypobaric moderate hypoxia exposure induces a reduction in body weight together with increased metabolic rate in obese patients.[38] In addition, exercising under hypoxia (approximately 14–15% O_2) evoked a more pronounced improvement in insulin sensitivity and glucose tolerance compared with normoxia.[12, 13] More recently, it has been found that an 8-month exercise intervention programme reduces body weight, body mass index, and waist–hip ratio, and improves performance peak and systolic blood pressure to the same extent in those who completed the exercise sessions under moderate hypoxia as those who exercised under normoxic conditions.[39] However, it cannot be excluded that the effects of exercise per se may have masked beneficial effects of moderate hypoxia exposure. Furthermore, metabolic parameters, including glycosylated haemoglobin, glucose, triacylglycerol, and cholesterol concentrations, were not significantly altered in either group after the training programme.[39]

Importantly, moderate hypoxia exposure may have beneficial effects on glucose homeostasis. For example, glucose disposal was increased after acclimatisation to high altitude (4,300 m) compared with sea level in healthy humans.[40] In addition, normobaric intermittent hypoxia exposure decreased plasma glucose concentrations in rodents.[41] More recently, the effect of moderate hypoxia exposure on insulin sensitivity has been studied in obese humans. Interestingly, 10 consecutive nights of moderate hypoxia exposure (approximately 10 hours exposure/night to approximately 15% O_2) significantly improved peripheral insulin sensitivity and tended to reduce AT pO_2.[14] Therefore, it is tempting to postulate that the decrease in AT pO_2 observed in this study may have contributed to improved peripheral insulin sensitivity after moderate hypoxia exposure.[42] Furthermore, *in vitro* exposure of human myotubes derived from these individuals to 15% O_2 improved basal but not insulin-stimulated glucose uptake compared with normoxia exposure, supporting direct effects of moderate hypoxia exposure on skeletal muscle glucose uptake. Notably, acute mountain sickness symptoms (e.g. headache, nausea) may occur above approximately 2,500 m (<15% O_2) and adverse events should be carefully monitored when exposing individuals to (moderate) hypoxia.

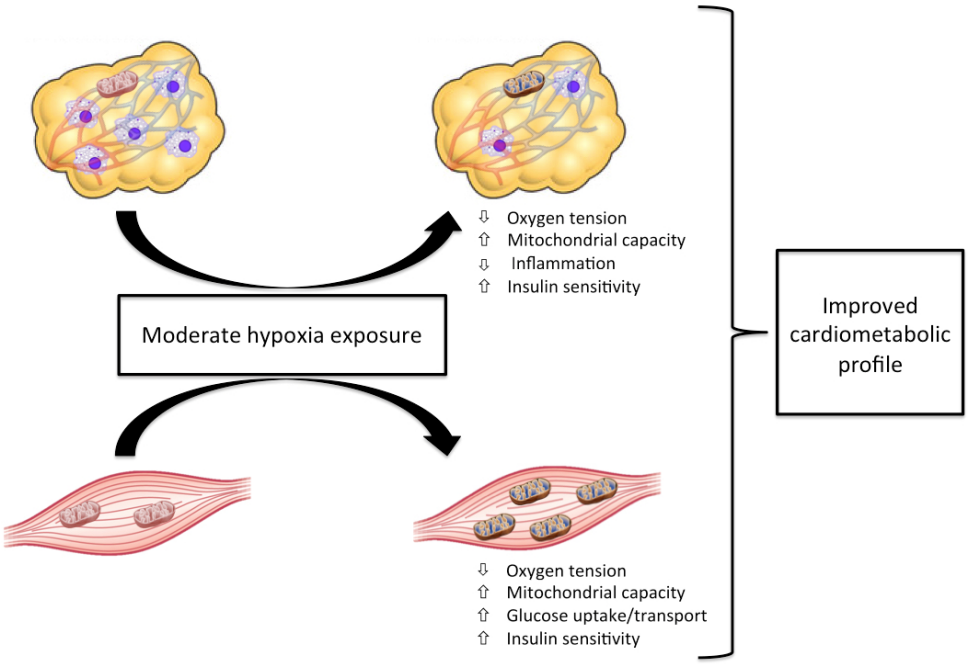

Figure 1: Proposed effects of moderate hypoxia exposure on adipose tissue and skeletal muscle that may contribute to improved glucose homeostasis. A pro-inflammatory phenotype, impaired mitochondrial capacity, and insulin resistance characterise dysfunctional adipose tissue in human obesity. Furthermore, skeletal muscle mitochondrial capacity, glucose uptake, and insulin sensitivity are decreased in obese insulin resistant humans. Moderate hypoxia exposure may decrease oxygen tension in adipose tissue and skeletal muscle, thereby increasing mitochondrial capacity, glucose uptake, and local insulin sensitivity, and reducing adipose tissue inflammation. Proposed molecular pathways that may mediate the effects of moderate hypoxia exposure in adipose tissue and skeletal muscle include PGC-1α, AMPK/CAMK-dependent pathways, and VEGF. Together, this may contribute to improved glucose homeostasis in humans. PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1α; AMPK: adenosine monophosphate-activated protein kinase; CAMK: Ca^{2+}/calmodulin-dependent protein kinase; VEGF: vascular endothelial growth factor.

Taken together, these studies suggest that normobaric moderate hypoxia exposure may elicit beneficial effects on glucose homeostasis (Figure 1). Nevertheless, most human *in vivo* studies performed thus far have either examined the effects of acute hypoxia exposure,[12] used surrogate markers of insulin sensitivity,[12] or did not include a control group,[14] and information on underlying mechanisms in relevant organs is very limited.[10, 12, 14, 40] Therefore, this promising treatment avenue needs to be explored in more detail in humans.

Detrimental effects of severe intermittent hypoxia in obstructive sleep apnoea syndrome patients

Obstructive sleep apnoea syndrome (OSAS) is a condition characterised by periodic collapse (obstruction) of the upper airway during sleep, resulting in episodes of severe hypoxia. OSAS affects 4–24% of men and 2–9% of women in the USA.[43, 44] However, OSAS prevalence is >50% in the obese population.[43, 44] Indeed, obesity is a major risk factor for OSAS, which results in severe intermittent hypoxia (SIH) as it promotes enlargement of the tissue surrounding the airway, leading to narrowing of the airway.[43, 45] It is well known that OSAS is a risk factor for the development and progression of cardiometabolic diseases, and exacerbates the metabolic syndrome. This is exemplified by the findings that obese OSAS patients have an increased risk of CVD, sympathetic activation, systemic inflammation, and endothelial dysfunction compared with obese individuals without OSAS.[46, 47] Furthermore, epidemiological studies have shown that an increased severity of OSAS is associated with progressive worsening of insulin resistance and other characteristics of the metabolic syndrome.[48, 49]

It has been proposed that impairments in lipid and glucose metabolism substantially contribute to the adverse clinical outcomes related to OSAS.[50] Interestingly, it has been demonstrated that SIH exposure reduces liver, muscle, and AT pO_2 *in vivo*, and impairs glucose homeostasis in lean mice.[51] In line with this, SIH has been found to acutely induce insulin resistance due to decreased skeletal muscle glucose utilisation in rodents.[52] Of note, lean mice exposed to intermittent hypoxia for several days do not show induction of insulin resistance, in contrast to genetically or diet-induced obese mice.[53]

Systemic low-grade inflammation is increased in OSAS patients.[54] Furthermore, SIH may increase reactive oxygen species.[55, 56] In addition to oxidative stress and inflammation, SIH leads to sympathetic system activation, which stimulates gluconeogenesis in the liver and may thereby contribute to impaired glucose homeostasis.[57] Although it cannot be excluded that other factors, including sleep fragmentation, play an important role in the adverse effects of OSAS, SIH is thought to be a major determinant of the detrimental metabolic and cardiovascular effects.

Continuous positive airway pressure (CPAP) is the first-line treatment for OSAS. CPAP, which delivers a stream of compressed air via a mask in order to keep the airway open under air pressure and thereby reduce or prevent nocturnal oxygen dips,[58] may have beneficial effects on lipid profile and glucose homeostasis. Strikingly, 3 months of CPAP treatment reverses several metabolic abnormalities in OSAS patients,[59] but the underlying the mechanisms are not fully understood.

Taken together, OSAS is associated with increased metabolic and cardiovascular risk, and several studies have suggested that this is related to the severity of the hypoxic episodes to which these patients are exposed.

Conclusion and perspective

The prevalence of obesity, CVD, and T2D is increasing at an alarming rate. Adopting a healthy lifestyle (e.g. healthy diet, increasing physical activity) can help to prevent or delay the onset of CVD and T2D. However, additional strategies are needed to mitigate the development of these chronic diseases in high-risk individuals.

There is substantial evidence that altered AT pO_2 is related to impaired adipose tissue function.

Likewise, hypoxia seems to improve skeletal muscle glucose metabolism. Therefore, modulation of ambient oxygen partial pressure, thereby affecting oxygen supply to key metabolic organs, may have positive cardiometabolic effects. Indeed, it has been demonstrated that *moderate* hypoxia exposure may improve adipose tissue function and skeletal muscle glucose uptake. Therefore, it is tempting to postulate that exposure to moderate hypoxia may be a promising strategy to reverse insulin resistance and to improve cardiometabolic health in obese individuals (Figure 1). However, OSAS patients are at increased metabolic and cardiovascular risk, which seems to be related to episodes of SIH that occur during sleep due to airway obstruction. The exposure regimen (e.g. severity and pattern of exposure) may therefore be critical regarding effects on metabolic health, and the underlying mechanisms responsible for the potential insulin-sensitising effect of moderate hypoxia exposure remain to be elucidated. Clinical studies in well-phenotyped humans are needed to further investigate the effects of different moderate hypoxia exposure regimens on insulin sensitivity, and lipid and glucose homeostasis. In addition, we need to obtain a better understanding of the underlying mechanisms in key metabolic organs, including adipose tissue and skeletal muscle.

Acknowledgements

The authors would like to thank all colleagues who have contributed to the studies performed by our research group, and would like to thank the Dutch Diabetes Research Foundation (Innovative Pilot Grant to G.H.G.) and European Foundation for the Study of Diabetes (Clinical Research Grant to G.H.G.) for financial support.

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CHAPTER 3

Differences in upper and lower-body adipose tissue oxygen tension contribute to the adipose tissue phenotype in humans

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Published in:
J Clin Endocrinol Metab. 2018, in press (doi 10.1210/jc.2018-00547)

Abstract

Context and Objectives: Upper and lower-body adipose tissue (AT) exhibit opposing associations with obesity-related cardiometabolic diseases. Recent studies have suggested that altered AT oxygen tension (pO_2) may contribute to AT dysfunction. Here, we compared *in vivo* abdominal (ABD) and femoral (FEM) subcutaneous AT pO_2 in overweight and obese women, and investigated the effects of physiological AT pO_2 on human adipocyte function.

Design: ABD and FEM subcutaneous AT pO_2 and AT blood flow (ATBF) were assessed in 8 overweight and obese (BMI 34.4±1.6 kg/m^2) post-menopausal women with impaired glucose metabolism. ABD and FEM AT biopsies were collected to determine adipocyte morphology and AT gene expression. Moreover, the effects of prolonged exposure (14d) to physiological AT pO_2 on adipokine expression/secretion, mitochondrial respiration and glucose uptake was investigated in differentiated human multipotent adipose-derived stem cells.

Results: AT pO_2 was higher in ABD than FEM AT (62.7±6.6 versus 50.0±4.5 mmHg, *P*=0.013), whereas ATBF was comparable between depots. Maximal uncoupled oxygen consumption rates were substantially lower in ABD than FEM adipocytes for all pO_2 conditions. Low physiological pO_2 (5% O_2) exposure decreased pro-inflammatory gene expression, increased basal glucose uptake and altered adipokine secretion in ABD and FEM adipocytes.

Conclusions: We demonstrated for the first time that AT pO_2 is higher in ABD than FEM subcutaneous AT in overweight/obese women, partly due to a lower oxygen consumption rate in ABD adipocytes. Moreover, low physiological pO_2 decreased pro-inflammatory gene expression in human adipocytes. Our findings suggest that altered microenvironmental pO_2 may modulate AT function in human obesity.

Introduction

Adipose tissue (AT) dysfunction in obese, insulin resistant subjects is characterized by adipocyte hypertrophy, an impaired AT blood flow (ATBF), an increased production of pro-inflammatory cytokines, and metabolic impairments.[1-3] These disturbances contribute to chronic low-grade inflammation, peripheral insulin resistance and the development of chronic cardiometabolic diseases.[1-3] However, the inciting event(s) underlying the metabolic and endocrine derangements in human AT are still not fully understood.

Recent evidence indicates that AT oxygen tension (pO_2) may affect AT function.[4] Although the importance of oxygen availability in key metabolic organs has not been investigated extensively, alterations in AT pO_2 have been linked to metabolic impairments.[4-6] We have previously shown that AT pO_2 is higher in obese (~9% O_2) than in lean (~6% O_2) individuals, and is positively associated with insulin resistance,[7] independently of adiposity in obese men and women.[8] More recently, we demonstrated that diet-induced weight loss decreased AT pO_2 in humans, which was accompanied by improved insulin sensitivity.[9] Noteworthy, findings on AT oxygenation in human obesity are conflicting.[10, 11]

Several studies have shown that acute exposure of (pre)adipocytes to hypoxia, especially severe hypoxia (1% O_2), evokes a pro-inflammatory phenotype.[12-15] In contrast, chronic exposure to severe hypoxia increased triacylglycerol accumulation and improved insulin sensitivity in *3T3-L1* adipocytes.[16] Moreover, exposure of differentiating abdominal adipocytes to human physiological pO_2 levels altered adipokine expression/secretion and adipocyte lipolysis as compared to 21% O_2,[17] suggesting that pO_2 in the AT microenvironment determines adipocyte functionality.

Next to total AT mass and function, body fat distribution is an important determinant of cardiometabolic health.[18, 19] Abdominal obesity is associated with an increased risk of developing type 2 diabetes and cardiovascular diseases.[20] In contrast, lower body fat has protective properties that are associated with an improved cardiometabolic risk profile in men and women.[21-23] However, underlying mechanisms for the apparent functional differences between upper and lower-body AT remain elusive. It is tempting to postulate that depot-differences in AT pO_2 contribute to distinct metabolic and inflammatory signatures of these AT depots and, therefore, the metabolic risk associated with a certain body fat distribution.

Here, we compared *in vivo* abdominal (ABD) and femoral (FEM) subcutaneous (sc) AT pO_2 and ATBF in well-phenotyped, overweight and obese post-menopausal women with

impaired glucose metabolism. Mechanistically, we investigated depot-specific effects of prolonged physiological AT pO_2 exposure on adipokine expression and secretion, oxygen consumption rate and glucose uptake in differentiated human multipotent adipose-derived stem cells (hMADS) derived from ABD and FEM scAT from the same individuals.

Materials and Methods

Subjects

Eight overweight and obese (BMI ≥28kg/m^2) post-menopausal women (40-65 yrs) with impaired glucose metabolism were recruited by public advertisements. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney disease, use of medication known to affect body weight and glucose metabolism, and marked alcohol consumption (>14 alcoholic units/wk). Furthermore, subjects had to be weight stable (weight change <3.0kg) for at least three months prior to the start of the study.
The study was performed according to the declaration of Helsinki and approved by the Medical-Ethical Committee of Maastricht University. All subjects gave written informed consent before participation in the study.

Anthropometric measurements

On the first visit, body weight, height, waist (top of the iliac crest) and hip (widest portion of the buttocks) circumferences were measured, and blood pressure was assessed (Intellisense, Omron Model M6 comfort; Omron Healthcare Inc., Vernon Hills, Illinois, US). Body composition and body fat distribution were determined by Dual Energy X-Ray Absorptiometry (DEXA) (Hologic QDR 4500-A, Waltham MA, USA).

Continuous monitoring of ABD and FEM subcutaneous AT pO_2

ABD and FEM scAT pO_2 were simultaneously monitored *in vivo* under fasting conditions on the first visit using a validated two-channel optochemical measurement system.[7, 24] Briefly, the system consists of microdialysis catheters for the extraction of interstitial fluid, miniaturized flow-through cells containing an O_2-sensitive membrane, and a two-channel optochemical measuring unit, which is connected to a computer for data collection.[24] On arrival, a microdialysis catheter (CMA60, CMA microdialysis AB, Stockholm, Sweden) was inserted 6-8 cm lateral from the umbilicus, as described previously.[7] A second catheter was inserted in the femoral scAT (anterior site). After insertion, both catheters were perfused with Ringer solution (Baxter BV, Utrecht, Netherlands) at a flow rate of 2 µl/min (CMA400 microinfusion pump, CMA Microdialysis AB, Stockholm, Sweden). AT pO_2 was calculated by averaging a 30-min period with stable pO_2 readings (change in AT pO_2 <2.0 mmHg).

ABD and FEM subcutaneous AT blood flow

ABD and FEM scATBF was measured under fasting conditions on the first visit using ^{133}Xe

wash-out.[7, 25] More specific, ATBF was continuously monitored 6-8 cm lateral from the umbilicus (ABD) and at the anterior site of the upper leg (FEM), contralateral to the sites of insertion of the microdialysis probes. Quantitative values of ATBF were calculated as described previously.[25]

Hyperinsulinemic-euglycemic clamp

A hyperinsulinemic-euglycemic clamp was performed on the second visit to assess whole-body insulin sensitivity.[26] Insulin was infused at a primed continuous infusion rate of 40mU•m^{-2}•min^{-1}. The mean glucose infusion rate during the steady-state (last 30 min) of the clamp was used to determine insulin sensitivity.

Adipose tissue biopsies

ABD and FEM scAT needle biopsies (~1g) were collected 6-8 cm lateral from the umbilicus and from the femoral region (anterior site of the upper leg), respectively, under local anaesthesia (2% lidocaine) after an overnight fast on the second visit. Biopsies were immediately rinsed with sterile saline and visible blood vessels were removed with sterile tweezers. AT was fixed overnight in 4% paraformaldehyde and embedded in paraffin for histology. Another part was used for isolation of hMADS, as described before.[27] The remaining tissue was snap-frozen in liquid nitrogen and stored at -80°C for gene expression analysis.

Adipocyte morphology

Histological sections (8µm) were cut from paraffin-embedded tissue, mounted on microscope glass slides, and dried overnight in an incubator at 37°C. Sections were stained with haematoxylin and eosin. Digital images were captured with the use of a Leica DFC320 digital camera (Leica, Rijswijk, Netherlands) at X20 magnification (Leica DM3000 microscope, Leica, Rijswijk, Netherlands). Computerized morphometric analysis (Leica QWin V3, Cambridge, United Kingdom) of individual adipocytes was performed by measuring approximately 400 adipocytes per sample.

Adipose tissue and adipocyte gene expression analysis

Total RNA was extracted from frozen AT specimens (~150mg) and hMADS using TRIzol reagent (Invitrogen, Breda, Netherlands), and SYBR-Green based real-time PCRs were performed using an iCycler (Bio-Rad, Veenendaal, NL, USA; primer sequences shown in Supplementary Table S1). Results were normalized to the geometric mean of 18S ribosomal RNA and RPL13A.

Human primary cell culture experiments

hMADS, an established human white adipocyte model,[27] were obtained from ABD and FEM scAT of the same subjects that also underwent the *in vivo* measurements. Cells were seeded at a density of 2000 cells/cm^2 and kept in proliferation medium under 21% O_2, and thereafter differentiated under 21%, 10% or 5% O_2 for 14 days, as described previously.[28] Gas mixtures were refreshed every 8 hours (to maintain variation <0.1% O_2), while medium was refreshed 2-3 times/wk. In our hands, 10% and 5% O_2 approximate the average ABD scAT pO_2 values in obese and lean subjects, respectively.[7] Since the potential differences in pO_2 between obese and lean FEM scAT are currently unknown, we decided to expose hMADS derived from FEM and ABD scAT to similar O_2 levels (21% O_2, room air; 10% O_2, high physiological pO_2; 5% O_2, low physiological pO_2) for 14 days.

All experiments were performed under 21% O_2 on day 14 of the adipogenic differentiation. For gene expression analyses and measurement of adipokine secretion, the individual hMADS from all donors were used. For the oxygen consumption and glucose uptake experiments, a hMADS pool (consisting of three donors from this study) from both ABD and FEM scAT was used.

Adipokine secretion measurement

The medium of the hMADS was collected over 24h to determine adipokine secretion using high-sensitive ELISA (ELISA kits were purchased: Adiponectin from Biovendor, IL-6 and MCP-1 from Diaclone, Leptin and DPP-4 from R&D Systems). 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.

Abdominal and femoral adipocyte oxygen consumption

Oxygen consumption rate (OCR), reflecting mitochondrial respiration, was measured in differentiated hMADS using a XF96 analyzer (Seahorse Bioscience, North Billerica, MA, USA). Just before the measurement, cells were washed with unbuffered DMEM.

A bioenergetics profile was determined using a four-step analysis: (*i*) basal OCR was measured in medium containing (17.5mM) glucose (Sigma) (calculated by the average respiration at the end of step *i* subtracted by the respiration after step *iv*); (*ii*) ATP production rate and respiration driving proton leak were determined by measuring OCR after inhibition of ATP synthase by oligomycin (1.4µM, Sigma) (calculated by the average of respiration at the end of step *i* subtracted by the respiration after step *ii*); (*iii*) maximal mitochondrial respiratory capacity was assessed by measuring OCR after stimulation

with the uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.3µM, Sigma) (calculated by the respiration at the end of step *iii* subtracted by the respiration after step *iv*); and (*iv*) finally, non-mitochondrial respiration was assessed by measuring OCR after addition of complex I inhibitor: Antimycin A/Rotenone (1µM, Sigma) (calculated by the average respiration at the end of step *iv*).

Glucose uptake in abdominal and femoral adipocytes

Basal glucose uptake was measured in differentiated hMADS using 2-deoxy-d-glucose following serum starvation for 18h in DMEM low-glucose, as described before.[27]

Statistical analyses

Data are presented as mean ±SEM. AT depot differences were analyzed using Student's paired t-test. The effects of different oxygen exposure levels on adipocyte gene expression, adipokine secretion, adipocyte oxygen consumption and adipocyte glucose uptake were analyzed using the Kruskal-Wallis test, followed by post-hoc comparison using the Wilcoxon signed-rank test. Calculations were performed with SPSS version 24 for Macintosh (Chicago, IL). $P<0.05$ was considered statistically significant.

Results

Subjects' characteristics are shown in Table 1. By design, the overweight/obese (BMI, 34.4±1.6 kg/m^2) subjects included in the present study had an impaired glucose metabolism.

Table 1. Subject characteristics.

	Study start
Age (yrs)	52.5 ±1.8
Weight (kg)	99.6 ±6.0
BMI (kg/m^2)	34.4 ±1.6
Body fat (%)	43.5 ±1.1
Trunk fat mass (kg)	20.4 ±1.9
Leg fat mass (kg)	15.9 ±1.0
Waist circumference (cm)	115.9 ±6.2
Waist/hip ratio	1.00 ±0.03
Fasting glucose (mmol/L)	5.8 ±0.1
2-h glucose (mmol/L)	6.1 ±0.8
Glucose Infusion Rate (μmol·kg bw^{-1}·min^{-1})	21.5 ±3.8

Oxygen tension is higher in abdominal than femoral adipose tissue

ABD AT pO_2 was significantly higher than FEM AT pO_2 (62.7±6.6 vs. 50.0±4.5 mmHg, P=0.013) (Figures 1A). ATBF was not significantly different between ABD and FEM AT (1.8±0.3 vs. 2.8±0.5 ml 100g $tissue^{-1}$ min^{-1}, respectively, P=0.122) (Figure 1B), indicating that a differential oxygen consumption rate between depots underlies the *in vivo* depot-differences in AT pO_2.

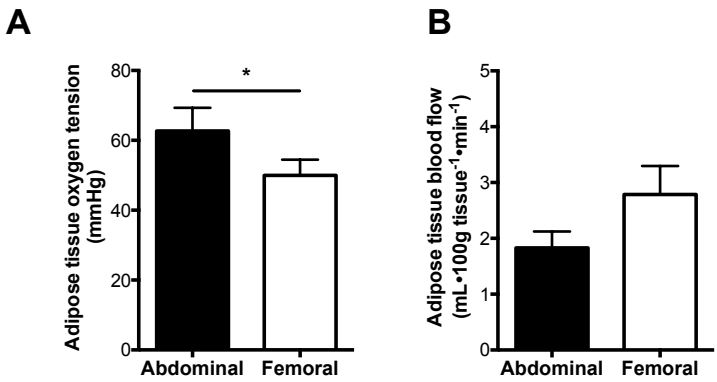

Figure 1. *In vivo* adipose tissue oxygen tension (*n*=8) and adipose tissue blood flow (*n*=6) in overweight/obese post-menopausal women. (**A**) Mean AT pO_2 was lower in femoral adipose tissue as compared to abdominal adipose tissue (*P*=0.013*)*. (**B**) ATBF was not significantly different between abdominal and femoral adipose tissue (*P*=0.122). Values are mean ± SEM. AT pO_2, adipose tissue oxygen tension; ATBF, adipose tissue blood flow. **P*<0.05.

Adipose tissue depot-differences in adipocyte morphology

ABD adipocytes were significantly smaller as compared to FEM adipocytes (Figure 2A). Moreover, the smaller mean adipocyte size in ABD AT was explained by a shift towards a lower frequency of very large adipocytes and a higher frequency of very small adipocytes as compared to FEM AT (Figure 2B).

Abdominal and femoral adipose tissue gene expression

Next, we assessed the gene expression profile of metabolic and inflammatory markers in ABD and FEM AT. Gene expression of the pro-inflammatory markers TNF-α, IL-6, DPP-4, PAI-1, MCP-1 and CD68 showed no significant differences between both AT depots (Figure 2C). However, ABD AT, characterized by smaller adipocytes, was accompanied by a significantly higher gene expression of adipogenic (adiponectin, PPARγ), mitochondrial (PGC-1α) and lipolytic (ATGL, HSL) markers as compared to FEM AT (Figure 2C).

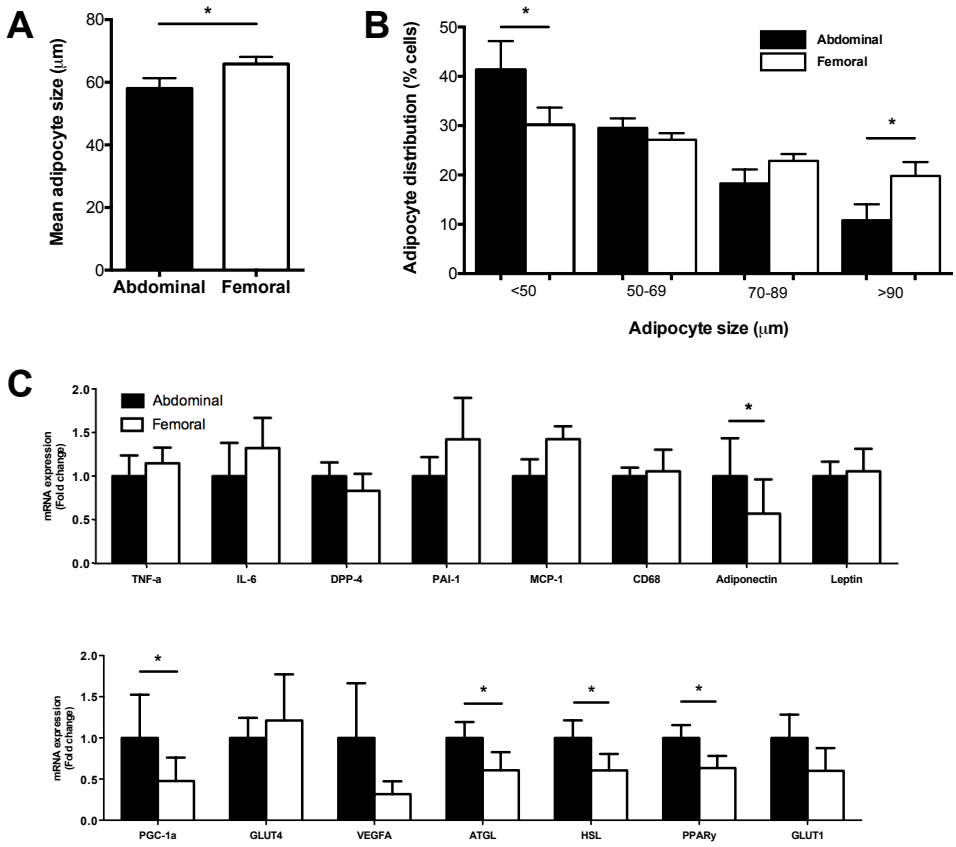

Figure 2. Mean adipocyte size and adipocyte size distribution of abdominal and femoral sc adipose tissue (*n*=8). (**A**) Mean adipocyte size was higher in femoral as compared to abdominal AT, with (**B**) a shift towards a higher proportion of large and a lower proportion of small adipocytes. (**C**) Gene expression in abdominal (black bars) and femoral (white bars) adipose tissue of inflammatory (top panel) and metabolic markers (bottom panel) (*n*=8). Data are expressed as fold change compared to abdominal AT. Values are mean ± SEM. **P*<0.05.

Low physiological pO_2 decreases pro-inflammatory gene expression in adipocytes

To investigate whether microenvironmental pO_2 affects gene expression in human adipocytes, we exposed hMADS derived from ABD and FEM scAT to low and high physiological pO_2 (5 and 10% O_2, respectively). Adipocyte differentiation in hMADS was induced under all pO_2 conditions, as reflected by increased PPARγ expression at days 3, 7 and 14 versus day 0 (Figure 3A). Moreover, exposure to low and high physiological pO_2 did not significantly alter PPARγ expression as compared to 21% O_2, suggesting no difference in adipogenic differentiation between pO_2 conditions (Figure 3A).

Low physiological AT pO_2 (5% O_2) markedly decreased gene expression of several pro-inflammatory markers in both ABD and FEM adipocytes (Figure 3). More specific, gene expression of the pro-inflammatory markers IL-6 (Figure 3C), DPP-4 (Figure 3D), and PAI-1 (Figure 3E), as well as VEGFA (Figure 3J) and GLUT1 (Figure 3K) expression, were significantly decreased in both ABD and FEM adipocytes following exposure to 5% O_2 as compared to 21% and/or 10% O_2. Moreover, 5% O_2 exposure reduced gene expression of the pro-inflammatory factor leptin (Figure 3G) in ABD adipocytes as compared to 21% and 10% O_2.

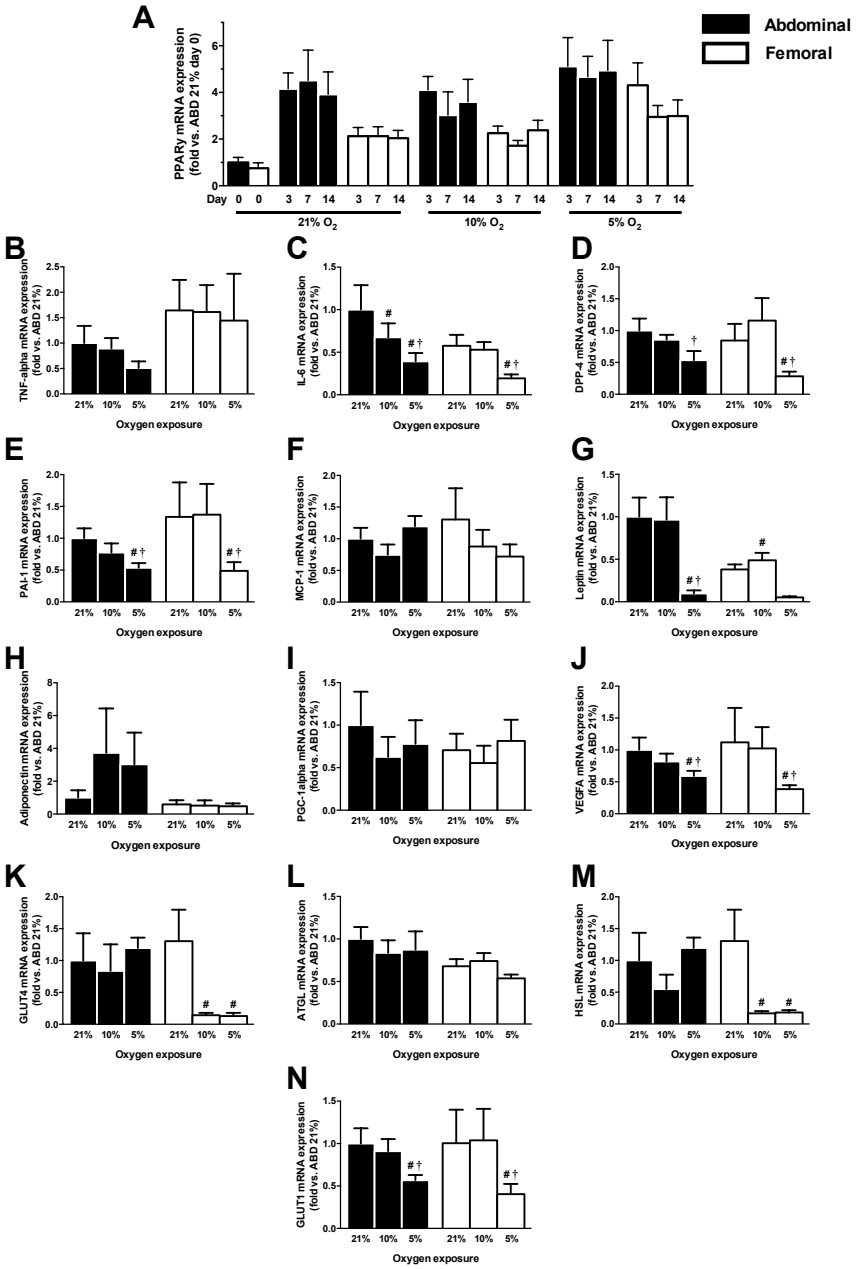

Figure 3. Gene expression in hMADS from abdominal (black bars) and femoral origin (white bars) following differentiation under different oxygen tensions (21% vs. 10% vs. 5% O_2) (*n*=4-8 paired samples). (**A**) PPARγ expression at days 0, 3, 7 and 14. (**B**) TNF-α, (**C**) IL-6, (**D**) DPP-4, (**E**) PAI-1, (**F**) MCP-1, (**G**) leptin, (**H**) adiponectin, (**I**) PGC-1α, (**J**) VEGFA, (**K**) GLUT4, (**L**) ATGL, (**M**) HSL, and (**N**) GLUT1. Values are mean ± SEM. Bars reflect the mean values of available data for pairwise comparison. $^{\#}P<0.05$ *vs.* 21% O_2 exposure (same depot), $^{\dagger}P<0.05$ *vs.* 10% O_2 (same depot).

Microenvironmental pO_2 affects adipokine secretion in human adipocytes

Next, we investigated whether the changes in ABD and FEM adipocyte gene expression following exposure to physiological pO_2 translate into functional changes in adipokine secretion. Indeed, adipokine secretion from ABD and FEM adipocytes was substantially affected by oxygen availability (Figure 4). Specifically, exposure to 5% O_2 evoked a reduction of leptin (Figure 4A) and an increase in IL-6 secretion (Figure 4B) as compared to 10% and 21% O_2 in a AT depot-independent manner. Moreover, low physiological pO_2 (5% O_2) tended to increase the secretion of the insulin-sensitizing factor adiponectin in FEM adipocytes as compared to 21% and 10% O_2 (both p=0.068, Figure 4C). No significant differences in the secretion of DPP-4 (Figure 4D) and MCP-1 (Figure 4E) from ABD and FEM adipocytes were found following the different oxygen exposure regimens.

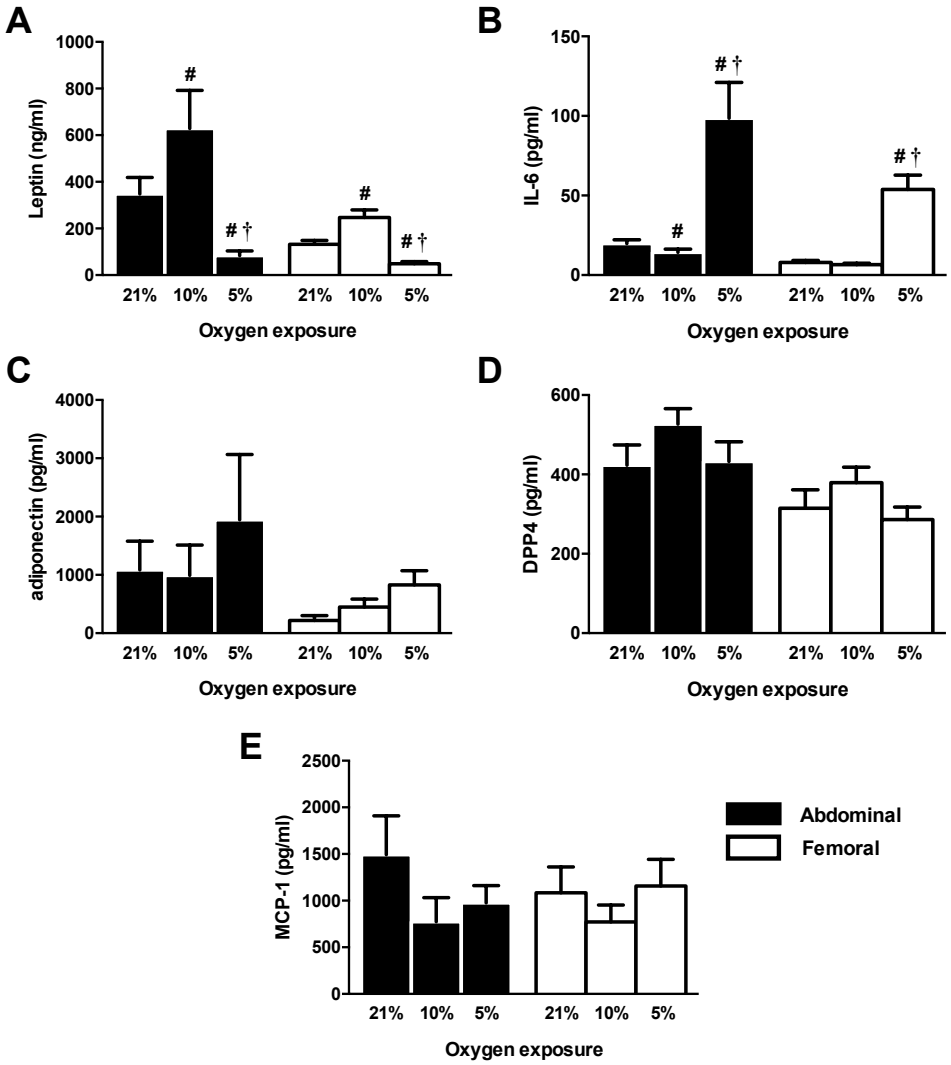

Figure 4. Secretion of adipokines from differentiated hMADS derived from abdominal (black bars) and femoral AT (white bars) following different oxygen exposure regimens (21% vs. 10% vs. 5% O_2) (*n*=4-8 paired samples). (**A**) Leptin, (**B**) IL-6, (**C**) adiponectin, (**D**) DPP-4 and (**E**) MCP-1. Values are mean ± SEM, Bars reflect the mean values of available data for pairwise comparison. $^{\#}P<0.05$ *vs.* 21% O_2 exposure (same depot), $^{\dagger}P<0.05$ *vs.* 10% O_2 exposure (same depot).

Oxygen consumption rate (OCR) is lower in abdominal than femoral adipocytes

Since ATBF, which determines AT oxygen supply, was not significantly different between ABD and FEM AT, we next investigated whether differences in the metabolic rate of adipocytes underlie the higher AT pO_2 in ABD than FEM scAT. Indeed, OCR was lower in ABD as compared to FEM adipocytes, where most pronounced effects are shown under lower physiological oxygen (5% O_2) (Figures 5A-F). Moreover, ABD adipocytes showed reduced ATP turnover (measured by basal-oligomycin), maximal respiratory capacity and non-mitochondrial respiration as compared to FEM adipocytes following exposure to physiological pO_2 (5 and 10% O_2) (figure 5C-F), where in the 21% O_2 condition only maximal respiratory capacity was significantly lower in ABD adipocytes (Figure 5A and B).

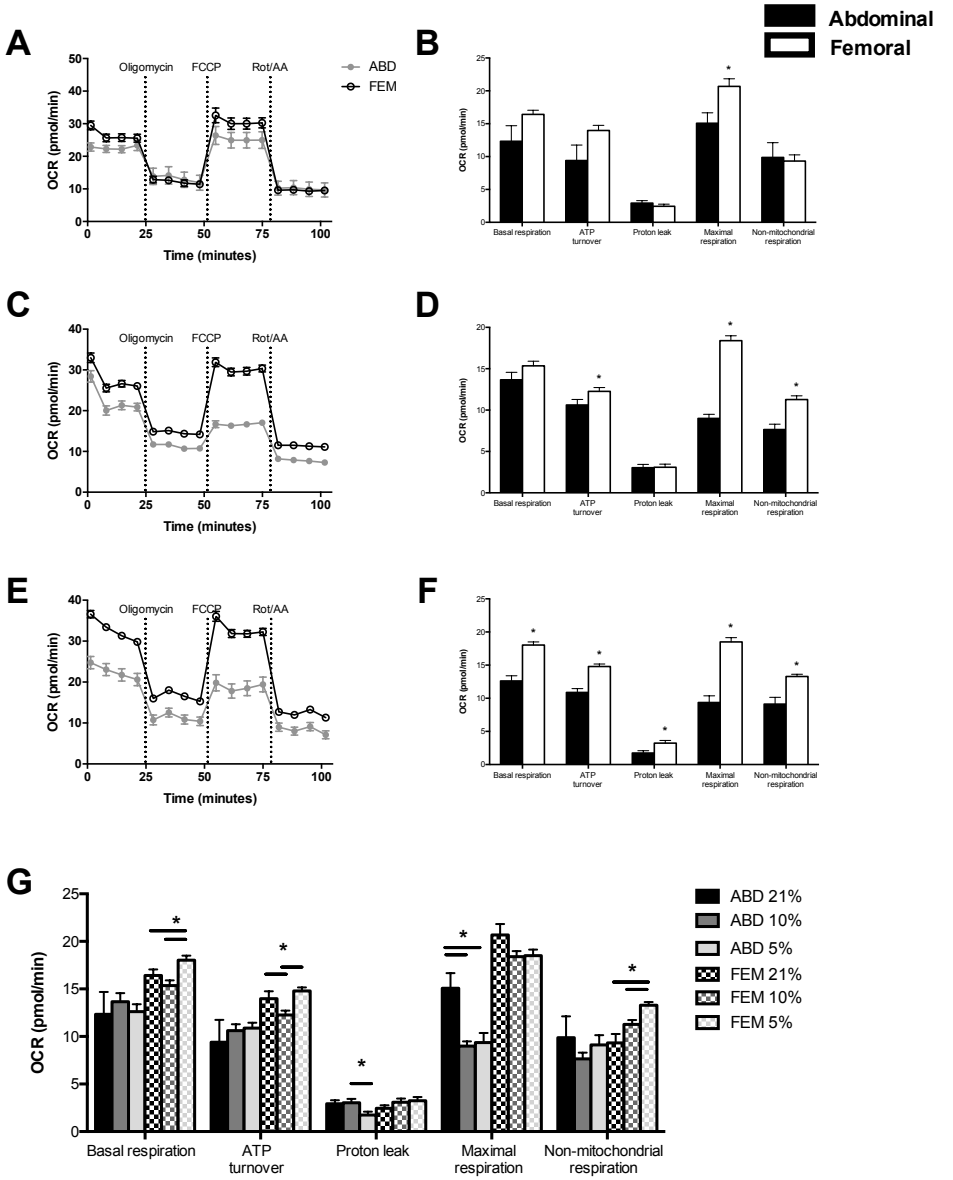

Figure 5. Oxygen consumption rates in hMADS from abdominal (black bars) and femoral origin (white bars) following differentiation under different oxygen tensions (21% vs. 10% vs. 5% O_2). Oxygen consumption rates following 14 days exposure to (**A**) 21% O_2, (**C**) 10% O_2 and (**E**) 5% O_2. The average values of the last two time points of basal respiration and last three time points following addition of the substrates Oligomycin, FCCP and Rotenone/Antimycin A (Rot/AA) were used to calculate the bioenergetics profile following 14 days exposure to (**B**) 21% O_2, (**D**) 10% O_2 and (**F**) 5% O_2. (**G**) Overview of oxygen consumption rates in ABD and FEM adipocytes per bioenergetics profile for the different oxygen exposure regimens. Values are mean ± SEM. (**A-F**) **P*<0.05 abdominal *vs.* femoral adipocytes. (**G**) **P*<0.05 between conditions.

Low physiological pO_2 increases glucose uptake in femoral adipocytes

Next, we assessed whether prolonged exposure of differentiating hMADS to physiological pO_2 induces alterations in basal glucose uptake in ABD and FEM adipocytes. Although exposure of adipocytes to 10% O_2 did not significantly alter basal glucose uptake as compared to 21% O_2, low physiological pO_2 (5% O_2) showed a trend towards an increased basal glucose uptake in FEM adipocytes but not in ABD adipocytes. Moreover, after exposure of hMADS to low physiological pO_2 (5% O_2), FEM adipocytes showed significantly higher basal glucose uptake as compared to ABD adipocytes (Supplemental Figure 1).

Discussion

In the present study, we compared *in vivo* ABD and FEM scAT pO_2 in overweight/obese women with impaired glucose metabolism, and performed mechanistic experiments to investigate the effects of prolonged physiological AT pO_2 exposure on ABD and FEM adipocyte metabolism and inflammation. Here, we demonstrate for the first time that *in vivo* AT pO_2 is higher in ABD as compared to FEM AT, which seems to be explained by a lower oxygen consumption rate in ABD than FEM adipocytes. Moreover, prolonged exposure to low physiological AT pO_2 altered gene expression, adipokine secretion, mitochondrial oxygen consumption rate and glucose uptake in human adipocytes, demonstrating that microenvironmental oxygen availability in AT affects human adipocyte function.

Differences in the functional properties between AT depots may underlie the cardiometabolic risk associated with a certain body fat distribution pattern. The functional differences between ABD and FEM AT seem to emerge from adipocytes having distinct properties.[21] In the present study, we found that ABD AT constitutes of smaller adipocytes than FEM AT in obese post-menopausal women, which is in agreement with most[29-31] but not all[32, 33] previous reports. Lower body AT is characterized by a lower lipid mobilization than upper body AT.[34, 35] A higher lipolytic rate in ABD AT may thus contribute to the smaller adipocytes in this depot. In line, we found that gene expression of ATGL and HSL was significantly higher in ABD than FEM AT. Furthermore, adiponectin, PGC-1α, and PPARγ were expressed at a higher level in ABD AT. Although hypertrophic adipocytes are characterized by a pro-inflammatory phenotype, we did not find differences in inflammatory gene expression between ABD and FEM AT depots. Moreover, we found a tendency for increased expression of VEGFA, the master regulator of vasculogenesis, angiogenesis and remodeling of blood vessels[36] in ABD AT, although this did not translate into differences in ATBF. Taken together, ABD AT is characterized by smaller adipocytes and a distinct pattern of gene expression with higher expression of adipogenic, lipolytic and mitochondrial genes as compared to FEM AT, while no differences were observed in inflammatory gene expression.

The local AT microenvironment may have a profound impact on the adipocyte phenotype. Accumulating evidence suggests that AT oxygenation may affect AT function.[4] Interestingly, we found that *in vivo* AT oxygenation was significantly higher in ABD than FEM AT in overweight/obese postmenopausal women. We have previously demonstrated that ABD AT pO_2 was significantly higher in obese insulin resistant as compared to lean insulin sensitive individuals, and was inversely associated with *in vivo*

whole-body insulin sensitivity,[7] independently of adiposity in obese men and women.[8] This raises the question whether the differences in ABD and FEM AT pO_2 that we found in the present study may underlie the different functional properties of these AT depots. First, to determine whether AT pO_2 affects gene expression of metabolic markers and adipokines, we exposed differentiating hMADS from ABD and FEM AT to low (5%) and high (10%) physiological pO_2. Interestingly, we demonstrated that low physiological pO_2 during adipogenesis consistently reduced the expression of the pro-inflammatory markers IL-6, DPP-4 and PAI-1 in both FEM and ABD adipocytes, and leptin expression in ABD adipocytes. Thus, the lower *in vivo* pO_2 in FEM as compared to ABD scAT, which reduces adipocyte gene expression of pro-inflammatory markers, may protect against a pro-inflammatory phenotype of hypertrophic FEM scAT in obese women. Noteworthy, we have previously found that *in vivo* ABD scAT pO_2 was positively associated with AT gene expression of several pro-inflammatory parameters.[7]

Next, we assessed whether the pO_2-induced alterations in adipocyte gene expression translate into changes in adipokine secretion. Interestingly, low physiological pO_2 decreased leptin secretion in both ABD and FEM adipocytes, and tended to increase the secretion of the insulin-sensitizing factor adiponectin in FEM adipocytes. In contrast to the decrease in IL-6 gene expression, we found that IL-6 secretion was increased following exposure to 5% O_2 during adipogenic differentiation in both depots. In addition, Famulla and colleagues[17] have shown opposite results of leptin and IL-6 secretion in differentiating adipocytes derived from healthy lean women, suggesting that donor characteristics may also influence the effects of pO_2 on the adipocyte phenotype.

Next, we questioned why AT pO_2 is higher in ABD than FEM AT. Since ATBF was not significantly different between ABD and FEM AT (if anything lower in ABD AT), it seems that lower O_2 consumption may underlie the higher AT pO_2 in ABD as compared to FEM AT. Indeed, maximal respiratory capacity was significantly lower in ABD than FEM adipocytes, independent of O_2 exposure level, where physiological O_2 also induced lower ATP turnover and non-mitochondrial respiration in ABD compared to FEM adipocytes. Moreover, cells differentiated at low physiological pO_2 (5% O_2) showed a most robust increase in oxygen consumption for the different states in FEM adipocytes. These data indicate that human primary adipocytes retain, at least to a certain extent, intrinsic differences in the metabolic rate of adipocytes *in vitro*. In line, pre-adipocytes from different AT depots exert distinct functions, and maintain their memory of origin *in vitro*.[37] Moreover, associations between the *in vivo* and *in vitro* phenotype of adipocytes were found, indicating inherent characteristics of adipocytes.[38, 39] Although it is likely that other cell types (e.g. immune cells) within AT contribute to oxygen consumption in

AT, the present in vitro data clearly indicate differences in ABD and FEM adipocytes. Depot-differences in adipocyte size do not seem to underlie the differences in oxygen consumption rate, since it has previously been shown that inter-individual differences in adipocyte oxygen consumption are independent of adipocyte size.[40]

Finally, we investigated whether prolonged exposure of differentiating hMADS to low and high physiological pO_2 exerts effects on glucose handling in ABD and FEM adipocytes. We found that low physiological pO_2 (5% O_2) tended to increase basal glucose uptake as compared to 21% and 10% O_2 in FEM adipocytes. Moreover, basal glucose uptake was higher in FEM than ABD adipocytes after exposure of hMADS to low physiological pO_2 (5% O_2). Thus, AT oxygenation seems to affect glucose uptake in differentiated human FEM adipocytes.

A strength of the present study is the detailed, paired comparisons between abdominal and femoral scAT as well as the differentiated hMADS derived from these AT depots. Future studies should investigate whether AT depot-differences in oxygenation also exists in other populations than obese post-menopausal women. A limitation is that measurements of oxygen consumption rate and glucose uptake following prolonged exposure (14d) to 5%, 10% or 21% O_2 were performed under 21% O_2, which might have blunted the effects of physiological oxygen exposure to a certain extent.

In conclusion, we demonstrated for the first time that AT pO_2 is higher in ABD than FEM scAT in overweight/obese post-menopausal women, which seems to be explained by a lower oxygen consumption rate in ABD adipocytes. Moreover, low physiological pO_2 decreased the expression of pro-inflammatory markers and improved the metabolic phenotype in human adipocytes, whereas more heterogeneous effects on adipokine secretion were found. Together, the present findings suggest that altered microenvironmental pO_2 may modulate AT function in human obesity. Therefore, AT oxygenation may be a putative target to improve AT dysfunction and related cardiometabolic complications in obese individuals. Future studies are warranted to investigate whether modulation of AT pO_2 may improve the cardiometabolic risk in human obesity.

Acknowledgements

The authors would like to thank the study participants for their invaluable contribution to the study.

Funding

This work was supported by the European Foundation for the Study of Diabetes (EFSD) Clinical Research Grant (to G.H.G.).

Duality of interest

The authors declare no conflict of interest.

Contribution Statement

M.A.A.V, J.W.E.J, E.E.B and G.H.G. designed the study and analyzed the data; M.A.A.V, J.W.E.J, H.S, N.H, Y.E, K.M.A.R, M.C and G.H.G. contributed to data acquisition; M.A.A.V wrote the manuscript, and G.H.G. had the primary responsibility for the final content. All authors revised the content of the manuscript and read and approved the manuscript for publication.

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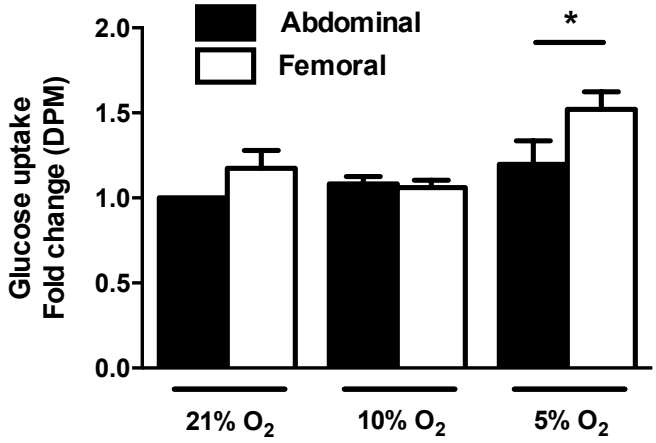
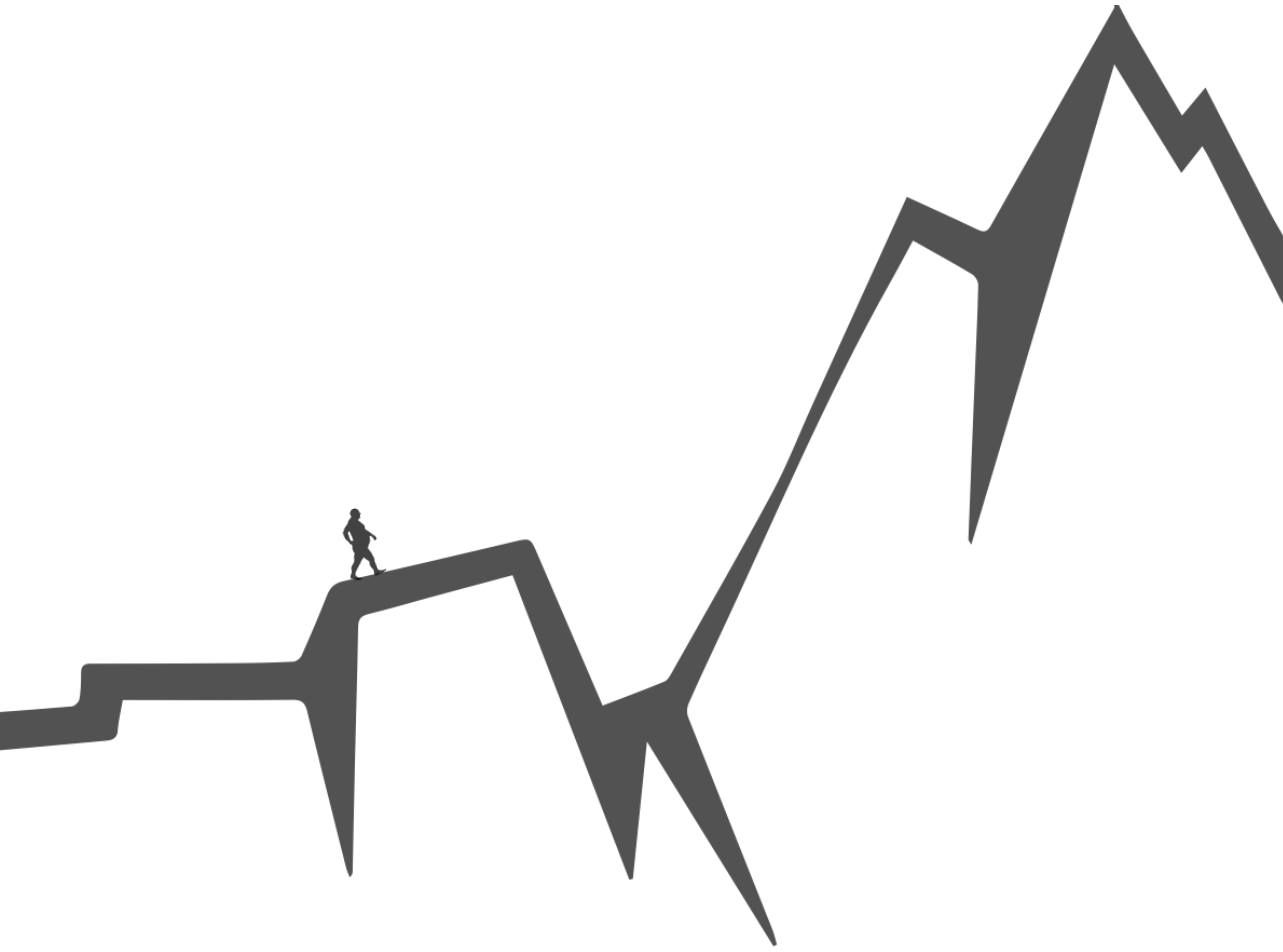
Supplemental Table 1. Primer sequences.

		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
CD68	Forward	CCCTATGGACACCTCAGCTTT
	Reverse	GAAGGACACATTGTACTCCACC
Adiponectin	Forward	TGGTGAGAAGGGTGAGAA
	Reverse	GTTCAGTCCACAGTGTCGCAGA
Leptin	Forward	GCTGTGCCCATCCAAAAAGTCC
	Reverse	CCCAGGAATGAAGTCCAAACCG
PGC-1α	Forward	TCTGAGTCTGTATGGAGTGACAT
	Reverse	CCAAGTCGTTCACATCTAGTTCA
GLUT4	Forward	TGGGCGGCATGATTTCCTC
	Reverse	GCCAGGACATTGTTGACCAG
VEGFA	Forward	TTGCCTTGCTGCTCTACCTCCA
	Reverse	GATGGCAGTAGCTGCGCTGATA
ATGL	Forward	GTGTCAGACGGCGAGAATG
	Reverse	TGGAGGGAGGGAGGGATG
HSL	Forward	GCGGATCACACAGAACCTGGAC
	Reverse	AGCAGGCGGCTTACCCTCAC
PPARγ	Forward	TACTGTCGGTTTCAGAAATGCC
	Reverse	GTCAGCGGACTCTGGATTCAG
GLUT1	Forward	TTGCAGGCTTCTCCAACTGGAC
	Reverse	CAGAACCAGGAGCACAGTGAAG
RPL13A	Forward	CCTGGAGGAGAAGAGGAAAGAGA
	Reverse	TTGAGGACCTCTGTGTATTTGTCAA
18S	Forward	AGTTAGCATGCCAGAGTCTCG
	Reverse	TGCATGGCCGTTCTTAGTTG



Supplemental Figure 1. Glucose uptake in hMADS from abdominal (black bars) and femoral origin (white bars) following differentiation under different oxygen tensions (21% vs. 10% vs. 5% O_2) (*n*=4). Values are mean ± SEM. Bars reflect the mean values of available data for pairwise comparison. **P*<0.05, †*P*<0.05 *vs.* 10% O_2 exposure (same depot).



CHAPTER 4

A comparison between the abdominal and femoral adipose tissue proteome of overweight and obese women

M.A.A. Vogel, P. Wang, F.G. Bouwman, N. Hoebers, E.E. Blaak, J. Renes, E.C. Mariman, G.H. Goossens

Submitted

Abstract

Background: Body fat distribution is an important determinant of cardiometabolic health. Lower-body adipose tissue (AT) has protective characteristics as compared to upper-body fat, but the underlying differences between these AT depots remain to be elucidated. Here, we compared the proteome and morphology of abdominal and femoral AT, and investigated which proteins and molecular pathways may contribute to putative functional differences between these fat depots.

Methods: Paired biopsies from abdominal and femoral subcutaneous AT were taken from eight overweight and obese (BMI ≥28 kg/m^2) women after an overnight fast for assessment of fat cell size and the adipose tissue proteome. Proteins were isolated and quantified using liquid chromatography-mass spectrometry (LC-MS), and AT protein expression in abdominal and femoral subcutaneous AT was compared. Moreover, correlations between fat cell size and the proteome of abdominal and femoral adipose tissue were determined.

Results: In total, 651 proteins were identified, of which 41 proteins were blood-specific proteins. Only 22 proteins tended to be differentially expressed between abdominal and femoral AT after removal of blood protein signals ($p<0.05$). Proteins involved in cell structure organization and energy metabolism seemed to be differently expressed between abdominal and femoral subcutaneous AT. Fat cell size, which was higher in femoral adipose tissue, was significantly correlated with ADH1B, POSTN and LCP1.

Conclusions: The present findings suggest that there are only slight differences in protein expression between abdominal and femoral subcutaneous AT after an overnight fast. However, it remains to be determined whether these differences, as well as differences in protein activity, may contribute to functional and/or morphological differences between these fat depots.

Introduction

Obesity is related to cardiometabolic disorders that contribute to increased morbidity and mortality.[1, 2] Being a highly active metabolic and endocrine organ,[3] adipose tissue (AT) is involved in the regulation of many physiologic processes, like immune responses, energy balance, blood pressure regulation, and glucose homeostasis.[4] The expansion and remodeling of AT during excessive weight gain renders the tissue dysfunctional.[5] AT dysfunction in obesity is strongly linked to metabolic dysregulation and increased risk of cardiometabolic diseases.[5, 6]

In addition to total AT mass, the location where lipids are stored seems an important determinant of the cardiometabolic consequences.[7, 8] Contrary to central obesity, accumulation of lower-body fat appears protective against metabolic derangements and hypertension,[9] and is associated with a reduced incidence of type 2 diabetes mellitus and cardiovascular disease when adiposity is comparable.[10, 11] However, the underlying mechanisms for the differences in disease risk associated with a certain body fat distribution remain elusive. We have recently demonstrated that abdominal subcutaneous adipose tissue is characterized by smaller adipocytes and a distinct pattern of gene expression compared to femoral adipose tissue in overweight/obese women, which may contribute to functional differences between these fat depots.[12]

Omics methodology provides excellent opportunities to investigate putative differences between AT depots. Microarray analysis of gluteofemoral (GFAT) and abdominal AT revealed that expression of energy-generating metabolic genes was inversely, and of inflammatory genes was positively associated with obesity.[13] Interestingly, for GFAT, the association between AT inflammation and BMI was weaker as compared to abdominal AT, which was confirmed by a lower secretion of interleukin-6 from lower-body AT. Moreover, markers of macrophage infiltration were not enriched in GFAT but increased in abdominal AT with obesity.[13]

To investigate AT depot-differences at a more functional level, proteomics analysis may be highly valuable. It has previously been shown that proteins related to metabolic processes such as glucose and lipid metabolism, lipid transport, protein synthesis, protein folding, response to stress and inflammation were differentially expressed in abdominal subcutaneous as compared to omental AT in humans.[14] Furthermore, proteome differences in either subcutaneous or visceral AT in relation to BMI or metabolic health have been investigated in humans.[15-18] In this respect, it has previously been found that several proteins related to AT remodeling, including several keratin and annexin proteins, and proteins related to oxidative stress were more abundant in the

abdominal AT of obese and overweight as compared to lean individuals, both in men and women.[16] However, a direct comparison of the proteome of upper- and lower-body human AT has not been performed.

In the present study, we compared for the first time, to our knowledge, the proteome of abdominal and femoral subcutaneous AT in overweight and obese women using untargeted quantitative liquid chromatography-mass spectrometry to obtain insights in the physiological differences between these AT depots in humans.

Materials and Methods

Subjects

Eight overweight and obese (BMI ≥28 kg/m^2) women with an impaired fasting glucose (IFG: fasting plasma glucose 5.6-7.0 mmol/l) or impaired glucose tolerance (IGT: 2h plasma glucose 7.8-11.1 mmol/l) participated in the present study, since AT depot-differences may be more pronounced in metabolically compromised individuals. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney disease, use of medication known to affect body weight and glucose metabolism, marked alcohol consumption (>14 alcoholic units/wk). Furthermore, subjects had to be weight stable (weight change <3.0 kg) for at least three months prior to the start of the study.

The study was performed according to the declaration of Helsinki and was approved by the Medical-Ethical Committee of Maastricht University. All subjects gave their written informed consent before participation in the study.

Anthropometric measurements

Body weight was measured to the nearest 0.1 kg (Seca, Hamburg, Germany). Height was measured using a wall-mounted stadiometer (model 220; Seca, Hamburg, Germany). Waist (top of the iliac crest) and hip (widest portion of the buttocks) circumferences were measured. Body composition and body fat distribution were determined by DEXA (Hologic QDR 4500-A, Waltham MA, USA).

Adipose tissue biopsies

Abdominal and femoral subcutaneous AT biopsies (~1 g) were collected using needle aspiration under local anaesthesia (2% lidocaine), 6-8 cm lateral from the umbilicus and from the lateral site of the upper leg, respectively, after an overnight fast. Biopsies were immediately rinsed with sterile saline and visible blood vessels were removed with sterile tweezers. The tissue was snap-frozen in liquid nitrogen and stored at -80°C until analysis.

Adipocyte morphology

Histological sections were cut from paraffin-embedded AT, and stained with haematoxylin and eosin. Fat cell size was measured using digital images that were captured and analyzed using a Leica DFC320 digital camera (Leica, Rijswijk, Netherlands) and software (Leica QWin V3, Cambridge, United Kingdom), as described before.[12]

Protein isolation and preparation for LC-MS

Fozen AT (~100 mg) was ground in a mortar with liquid nitrogen. Per microgram of grounded powder, 2 µl of 50 mM ammonium bicarbonate with 5 M urea was added to dissolve the powder. The solution was freeze-thawed in liquid nitrogen 3 times after which it was vortexed for 5 min. The homogenate was centrifuged at 20,000 g for 30 min at 10°C. The supernatant was carefully collected and protein concentrations were determined with a Bradford-based protein assay (Bio-Rad, Veenendaal, the Netherlands). A control sample was prepared from a pool of 10 µl of each sample.

Samples were digested with Trypsin (Promega) and peptides from 100 µg protein were labelled with TMT isobaric mass tagging labelling reagent (10-plex; Thermo Scientific, West Palm Beach, FL, USA) according to the manufacturer's protocol and as described before,[19] using 100 µg of protein for each sample. Equal amounts of the 8 samples from 4 subjects and one control were combined in a new vial and analyzed by LC-MS.

Protein quantification using LC-MS

A nanoflow HPLC instrument (Dionex ultimate 3000) was coupled on-line to a Q Exactive HF (Thermo Scientific) with a nano-electrospray Flex ion source (Proxeon). One µg of TMT labeled peptide mixture was loaded onto a C18-reversed phase column (Thermo Scientific, Acclaim PepMap C18 column, 75-µm inner diameter x 15 cm, 2-µm particle size). The peptides were separated with a 150 min linear gradient of 4-50% in buffer A (100% water with 0.1% TFA) with buffer B (80% acetonitrile and 0.08% formic acid) at a flow rate of 300 nL/min.

MS data was acquired using a data-dependent top-10 method, dynamically choosing the most abundant precursor ions from the survey scan (280–1400 m/z) in positive mode. Survey scans were acquired at a resolution of 60,000 and a maximum injection time of 120 ms. Dynamic exclusion duration was 30 s. Isolation of precursors was performed with a 1.8 m/z window and a maximum injection time of 200 ms. Resolution for HCD spectra was set to 30,000 and the Normalized Collision Energy was 30 eV. The under-fill ratio was defined as 1.0%. The instrument was run with peptide recognition mode enabled, but exclusion of singly charged and charge states of more than five.

Database search

The MS data were searched using Proteome Discoverer 2.1 Sequest HT search engine (Thermo Scientific), against the UniProt human database. The false discovery rate (FDR) was set to 0.01 for proteins and peptides, which had to have a minimum length of 6 amino acids. The precursor mass tolerance was set at 10 ppm and the fragment

tolerance at 0.02 Da. One miss-cleavage was tolerated, oxidation of methionine was set as a dynamic modification and carbamidomethylation of cysteines, TMT reagent adducts (+229.162932 Da) on lysine and peptide amino termini were set as fixed modifications.

Data quantification and normalization

The MS-acquired data were first analyzed with Thermo Scientific Proteome Discoverer software version 2.1. Relative quantitation of peptides from mixed samples was extracted by comparing the signal to noise ratio (S/N) of the TMT reporter ions peak in the MS/MS spectrum. The S/N signal of multiple distinct peptides from each protein was summed to report the protein signal.

The intra-run variation for each sample in the mix was normalized with the Proteome Discoverer software to get the total protein signal of each sample the same as the highest one in the run. The inter-run variation was normalized based on the identical control samples in each run, performed in R environment (Supplemental File 1).

Then, low-quality proteins with identification Score Sequest HT <5 were removed from the data set.

Adjustment for blood protein contamination

To reduce the influence of the blood protein contamination on the AT proteome, we retrieved information from the UniProt database to set up a blood protein exclusion list (Supplemental Table 1) with known abundant blood-specific proteins,[20] including all immunoglobulins. The final signal was the protein abundance in a blood protein-free AT sample.

Final signal *[protein x, sample i]*= normalized signal *[protein x, sample i]* * Σ normalized signal *[sample i]* /(Σnormalized signal *[sample i]* - Σ Blood protein normalized signal *[sample i]*)

This final signal (below referred to as 'signal') was used in data analysis.

Univariate statistics

For statistical analyses, missing values in proteome data were imputed with the half of the lowest positive signal in the dataset. Thereafter, data were log2 transformed. Paired Student's t-test was used to test AT depot-differences for fat cell size and each protein. False discovery rate (FDR) q-value was calculated to adjust proteomics data for multiple testing. Proteins with a p-value <0.05 were regarded as differentially expressed, and were selected for further biological annotation and analysis.

A heat-map was generated based on these proteins using their scaled data (mean-centered value divided by standard deviations per protein). The Euclidean method was

used to compute distance, and the ward.D method was used to compute the hierarchical clusters of proteins.
The association between fat cell size and protein expression was analyzed by Spearman's rank correlation for both abdominal and femoral subcutaneous AT.

Multivariate analysis
Hotelling's T^2-test for two dependent samples is the multivariate extension of the two-group paired Student's t-test.[21, 22] The proteome profile data of 610 proteins Log2 transformed expression values were first downscaled to 7 principle components (the maximal degree of freedom for 8 paired samples) by multidimensional scaling, and then entered the Hotelling's T^2-test. A p-value <0.05 was considered to be statistically significant.
All statistical analyses were performed in R environment, version 3.4.2, with various packages (stats, gplots, limma, ICSNP).

Results

Subjects' characteristics are shown in Table 1. By design, the overweight/obese (BMI, 34.4±1.6 kg/m^2) subjects included in the present study had an impaired glucose metabolism. Fat cell size was smaller in abdominal as compared to femoral subcutaneous AT (58.0±3.3 vs. 65.9±2.3 μm, p=0.011).

Table 1. Subject characteristics (*n*=8).

	Baseline Mean ± SEM
Age (yrs)	52.5 ±1.8
Weight (kg)	99.6 ±6.0
BMI (kg/m^2)	34.4 ±1.6
Body fat (%)	43.5 ±1.1
Trunk fat mass (kg)	20.4 ±1.9
Leg fat mass (kg)	15.9 ±1.0
Waist circumference (cm)	115.9 ±6.2
Waist/hip ratio	1.00 ±0.03
Fasting glucose (mmol/L)	5.8 ±0.1
2-h glucose (mmol/L)	6.1 ±0.8
Abdominal fat cell size (μm)	58.0 ±3.3
Femoral fat cell size (μm)	65.9 ±2.3

In total, 651 proteins with sufficient HT score were identified in the AT samples, and were subsequently quantified. Several of the identified proteins were blood-specific proteins (Supplemental Table 1) due to the presence of some blood in the whole-AT biopsies, despite thorough cleaning of the biopsies with sterile saline. We found that about one third (range 18-38%) of the total protein signals were attributable to blood-specific proteins, warranting a correction for blood contamination (Figure 1). Data cleaning by removal of these blood-specific protein signals resulted in 610 identified AT proteins.

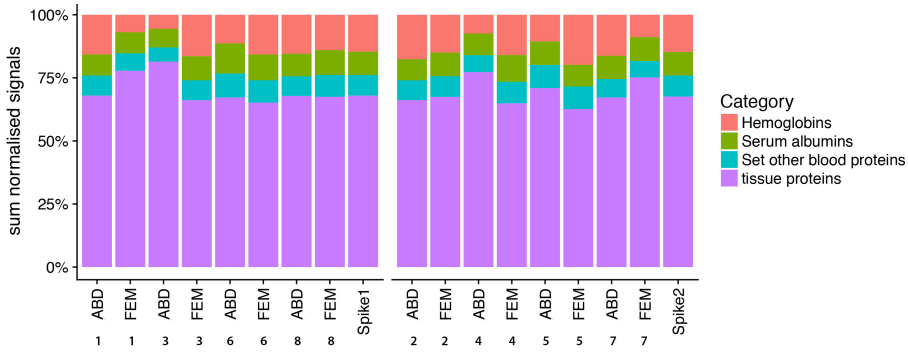


Figure 1: Percentage of signal belonging to tissue or different blood-specific protein groups. Each bar represents an adipose tissue biopsy, with the number representing a certain subject. ABD; abdominal, FEM; femoral. Spike refers to the control (pooled sample) in each run.

The total proteome with 7 principle components, which explained 92% of total variation of the 610 AT proteins, tended to be different between abdominal and femoral subcutaneous AT ($p=0.053$) by multivariate Hotelling's T^2-test.

Comparison of protein expression revealed that 22 of the 610 identified AT proteins tended to be differentially expressed between both AT depots ($p<0.05$, but all $q>0.05$, Table 2). A heat-map was constructed to visualize the pattern in protein expression between abdominal and femoral subcutaneous AT for these 22 proteins (Figure 2). Individual differences in protein expression between these AT depots are shown in Supplemental Figure 1.

Table 2: Proteins that tended to be differently expressed between abdominal and femoral adipose tissue (p-value < 0.05).

Accession	Gene symbol	Full protein name	Fold-change ABD vs FEM	p-value
Q15063	POSTN	Periostin	0.80	<0.001
P13796	LCP1	Plastin-2	0.88	0.049
O14950	MYL12B	Myosin regulatory light chain 12B	0.89	0.047
P37802	TAGLN2	Transgelin-2	0.92	0.011
P00338	LDHA	L-lactate dehydrogenase A chain	0.92	0.010
P06396	GSN	Gelsolin	0.92	0.029
P00441	SOD1	Superoxide dismutase	0.93	0.028
P67936	TPM4	Tropomyosin alpha-4 chain	0.93	0.036
P40939	HADHA	Trifunctional enzyme subunit alpha, mitochondrial	0.93	0.019
P13489	RNH1	Ribonuclease inhibitor	0.93	0.021
P13639	EEF2	Elongation factor 2	0.94	0.010
P60709	ACTB	Actin, cytoplasmic 1	0.95	0.006
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	1.05	0.003
O95865	DDAH2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	1.08	0.047
P54727	RAD23B	UV excision repair protein RAD23 homolog B	1.09	0.037
P62805	HIST1H4A	Histone H4	1.11	0.004
P08133	ANXA6	Annexin A6	1.12	0.031
P07355	ANXA2	Annexin A2	1.13	0.043
P04899	GNAI2	Guanine nucleotide-binding protein G(i) subunit alpha-2	1.14	0.006
Q16851	UGP2	UTP--glucose-1-phosphate uridylyltransferase	1.19	0.032
P00325	ADH1B	Alcohol dehydrogenase 1B	1.22	0.012
P00167	CYB5A	Cytochrome b5	1.25	0.039

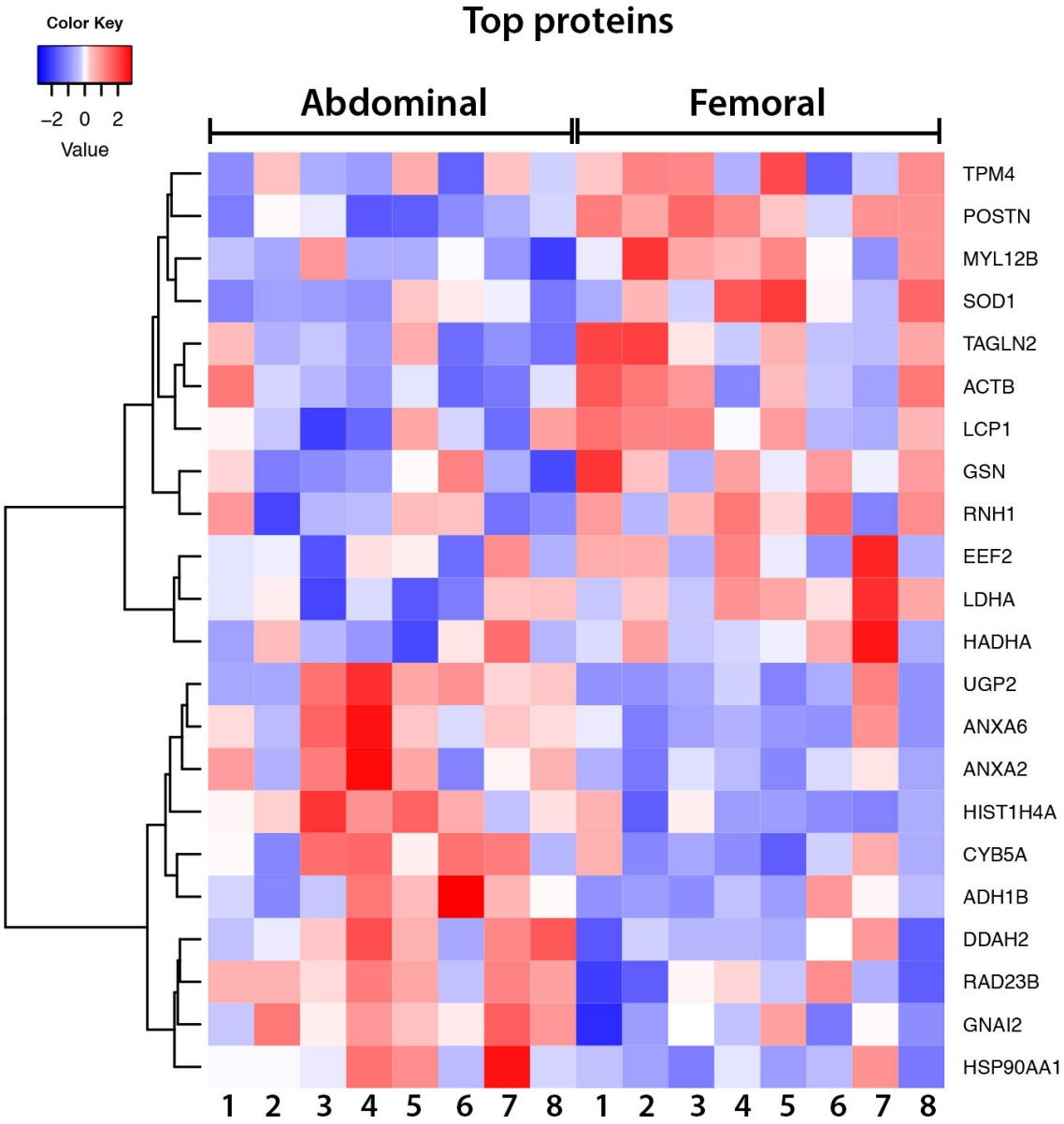


Figure 2: Heatmap of proteins that were differentially expressed between abdominal and femoral subcutaneous adipose tissue. Each cell represents one protein expression standardized score in one subject (organized by column, subjects are labelled by number). The color key is proportional to the protein expression standardized score.

Several of the 22 identified proteins that were differentially expressed between femoral and abdominal AT were related to cellular structure, including the extracellular matrix (ECM). More specific, protein expression of periostin (POSTN), myosin regulatory light chain 12B (MYL12B), gelsolin (GSN), tropomyosin alpha-4 chain (TPM4), actin cytoplasmic 1 (ACTB), and actin-binding protein lymphocyte cytosolic protein 1 (LCP1) was higher in femoral as compared to abdominal AT. Moreover, some proteins related to energy metabolism were expressed at a higher level in femoral than abdominal AT, including mitochondrial hydroxyacyl-coenzyme A dehydrogenase (i.e. trifunctional enzyme subunit alpha, HADHA) and L-lactate dehydrogenase A chain (LDHA). In contrast, certain other proteins related to energy metabolism such as alcohol dehydrogense 1B (ADH1B), and cytochrome b5 (CYB5A), were expressed at a lower level in femoral versus abdominal AT. We also found a higher expression of protein synthesis complex component elongation factor 2 (EEF2) and redox superoxide dismutase (SOD1)) in femoral AT. Furthermore, chaperone heat shock protein HSP 90-alpha (HSP90AA1), nitric oxide production related enzyme dimethylarginine dimethylaminohydrolase 2 (DDAH2), and glycogen synthesis enzyme UTP-glucose-1-phosphate uridylyltransferase (UGP2) were also expressed at a lower level in femoral as compared to abdominal subcutaneous AT.

Since the differentially expressed proteins seems to be related to processes of cellular structure and energy metabolism, we next investigated the association between fat cell size and protein expression of these differentially expressed proteins. Fat cell size in abdominal and femoral AT was negatively associated with ADH1B (p=0.004 and p=0.028, respectively), while fat cell size was positively correlated with LCP1 (p=0.028) and POSTN (p=0.028) in femoral AT (Figure 3).

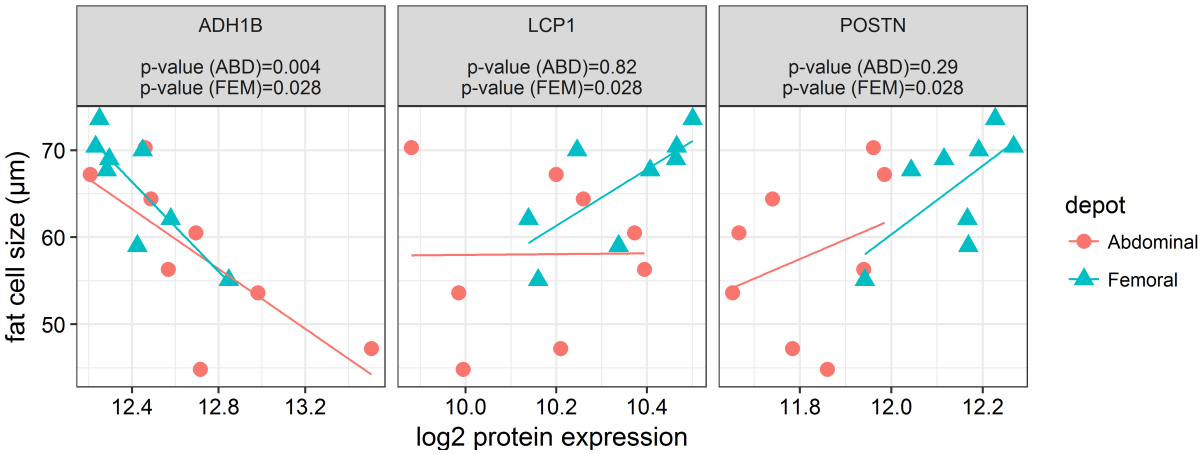


Figure 3: Correlation between proteins (AHDH1, LCP1, and POSTN) and fat cell size (µm) in abdominal and femoral subcutaneous adipose tissue.

Discussion

The aim of the present study was to investigate differences in the proteome of abdominal and femoral subcutaneous AT in overweight and obese humans, and to examine the associations between fat cell size and the AT proteome, since this may yield mechanistic insight into functional differences between these AT depots. For this purpose, quantitative LC-MS analysis of paired biopsies was performed to identify and quantify proteins. Here, we identified and quantified 610 proteins, and demonstrated that no major differences exist between the proteome of abdominal and femoral subcutaneous AT following an overnight fast. Nevertheless, 22 proteins seemed to be differentially expressed between abdominal and femoral AT, and fat cell size was significantly correlated with ADH1B, POSTN and LCP1.

We found that, using Panther (panther.org), the 22 differentially expressed proteins were related to 'protein binding' (8/22) and 'catalytic activity' (8/22) in the category 'molecular function'. Moreover, in the category 'biological processes', the most prominent differences were related to 'metabolic processes' (13/22) and 'cellular processes' (11/22). In line with this global analysis, a more detailed examination of the expression of these proteins showed that differences in structural proteins may exist between abdominal and femoral AT, indicated by the lower abundance of the ECM protein POSTN and the cytoskeleton-related proteins MYL12B, TPM4, ACTB, GSN, TAGLN2, and LCP1 in abdominal as compared to femoral subcutaneous AT. Interestingly, POSTN was the only protein that showed consistent AT depot differences in all subjects, and also showed the most pronounced difference (~25% higher expression in femoral AT). This protein functions as a cell adhesion component,[23] which may stimulate the maturation of ECM (by similarity, Uniport). POSTN is also highly expressed in collagen-rich connective tissue and has previously been associated with obesity[24] and weight regain in females.[25] These findings suggest that POSTN may be involved in lipid storage in adipocytes, and repair and/or expansion of AT. POSTN is able to bind to integrins and can transduce external signals into cells via the focal adhesion kinase pathway.[26] Intracellularly, focal adhesions are attached to the actin filaments, which undergo a structural reorganization during the differentiation of adipocytes.[27] In this regard, both POSTN and ACTB may be involved in AT expandability or, in other words, the fat storage capacity of AT. Indeed, we found POSTN to be positively correlated with femoral but not abdominal fat cell size. In addition, the protein abundances of EEF2 and RNH1, a translation elongation factor and an inhibitor of mRNA turnover, were also lower in abdominal AT, which may be indicative of differences in protein synthesis accompanying

tissue expansion. In agreement with these findings, fat cell size was significantly smaller in abdominal than femoral subcutaneous AT, as we have also reported previously.[12] The latter is in line with earlier studies showing larger femoral than abdominal adipocytes in obese men and women.[28, 29] Adipocyte expansion by fat storage may lead to cell stress.[30] In this respect, a lower abundance of HSP90AA1 in femoral AT would be in line with larger expansion capacity of femoral adipocytes, without being hampered by cell stress. Altogether, the present proteome analysis may point towards key proteins involved in human AT remodelling.

Moreover, many of the proteins that had a lower abundance in abdominal subcutaneous AT are functionally related to actin filaments. GSN binds to the positive end of actin monomers and filaments, thereby preventing monomer exchange. TPM4 forms dimers, which interact with the actin filaments and controls the access of actin-associated proteins to the filaments.[31, 32] MYL12B is a component of myosin II, which can form contractile structures in connection with actin filaments.[33] Its phosphorylation triggers formation of myosin II filaments but also actin polymerization. Both TAGLN2 and LCP1 are proteins which regulate actin filament polymerization.[34, 35] Notably, both of these proteins may be involved in the formation of immunological synapses between leukocytes and target cells.[36, 37] Taken together, the differences in protein expression levels of structural proteins may indicate that differences exist in tissue structure between upper- and lower-body AT depots in humans, and suggest a different tendency for the interaction of immune cells inside AT. This is further strengthened by the positive correlation between fat cell size and LCP1 in femoral AT.

Furthermore, we found that protein expression of cytoplasmic SOD1 was lower in abdominal than femoral AT. This protein plays a role in scavenging naturally occurring oxygen radicals. In accordance, RNH1, previously found to be implicated in protection against oxidative stress,[38] was also expressed at a lower level in abdominal AT. Together, these findings suggest that abdominal AT may have less protection against oxidative stress. The lower abundance in HADHA and LDHA proteins in abdominal versus femoral AT further suggests lower metabolic activity in abdominal as compared to femoral AT, since these proteins are involved in mitochondrial beta-oxidation and anaerobic glycolysis, respectively. However, other metabolic enzymes (ADH1B and CYB5A) appeared to be higher expressed in abdominal AT, implying pathway-specific differences in the expression of proteins involved in metabolic activity between both fat depots. Interestingly, ADH1B was negatively correlated with abdominal and femoral AT fat cell size. Moreover, ADH1B has also been implicated in body weight regulation,[39] and was negatively correlated with waist circumference, BMI and fasting plasma insulin.[40] Taken together, it is tempting to suggest that ADH1B may be involved in AT expandability.

The strength of the present study is that we, for the first time to our knowledge, compared the proteome of human abdominal and femoral AT. Moreover, the paired comparisons that were made between both AT depots for each individual further strengthen this study. Thus far, very few proteomic analyses of human whole-AT have been performed. Despite thorough cleaning, the AT biopsies still contained residual blood, which resulted in the identification of several blood proteins that accounted for ~20-30% of the total AT protein signal. Blood contamination is not surprising, since AT is a relative highly vascularized tissue. Unavoidable blood contamination might be one of the barriers to obtain a real 'AT proteome', but we adjusted for blood proteins in the present analyses. A limitation of the present study is the relatively small number of participants. Furthermore, our proteomic analysis covered only a small amount of proteins of the AT proteome, based on human adipose tissue transcriptome data (https://www.proteinatlas.org/humanproteome/adipose). Thus, our findings may reflect only part of the AT depot proteome differences. Furthermore, AT depot differences in protein abundance did not yield significant findings following adjustment for multiple testing in the present study, which might be due to limited power and/or subtle differences in protein expression between these AT depots. Therefore, the differences reported here should be confirmed in future studies. Finally, AT biopsies were collected following an overnight fast. Therefore, it may well be that under challenged conditions such as after physical exercise, prolonged fasting or a dietary intervention, proteome differences are more outspoken. Noteworthy, protein abundance may not always be a valid surrogate for protein activity and, as such, our observations warrant follow-up research.

In conclusion, comparison of human abdominal and femoral subcutaneous AT using non-targeted, quantitative proteomics revealed slight but specific differences in protein expression between these AT depots in overweight/obese women, and indicated that fat cell size was significantly correlated with ADH1B, POSTN and LCP1. These differences in protein expression may reflect depot-differences in adipocyte morphology, adipocyte expandability, immune cell interaction and energy metabolism. Importantly, it cannot be excluded that differences in protein activity, particularly under challenged state, contribute to divergent functioning of these AT depots.

Funding

This work was partly supported by the European Foundation for the Study of Diabetes (EFSD) (Clinical Research Grant to G.H.G.).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank the study participants for their invaluable contribution to the study.

Author contributions

M.A.A. Vogel: (i) study design, (ii) sample collection, (iii) interpretation of data, (iv) writing of manuscript, and (v) final approval of manuscript. P. Wang: (i) statistical analysis and interpretation of data, (ii) revision of manuscript, and (iii) final approval of manuscript. F.G. Bouwman: (i) sample preparation and data acquisition. N. Hoebers: (i) sample preparation and data acquisition. E.E. Blaak: (i) interpretation of data, (ii) revision of manuscript, and (iii) final approval of manuscript. J. Renes: (i) interpretation of data, (ii) revision of manuscript, and (iii) final approval of manuscript. E.C. Mariman: (i) interpretation of data, (ii) revision of manuscript, and (iii) final approval of manuscript. G.H. Goossens: (i) study design, (ii) sample collection, (iii) interpretation of data, (iv) revision of manuscript, and (v) final approval of manuscript.

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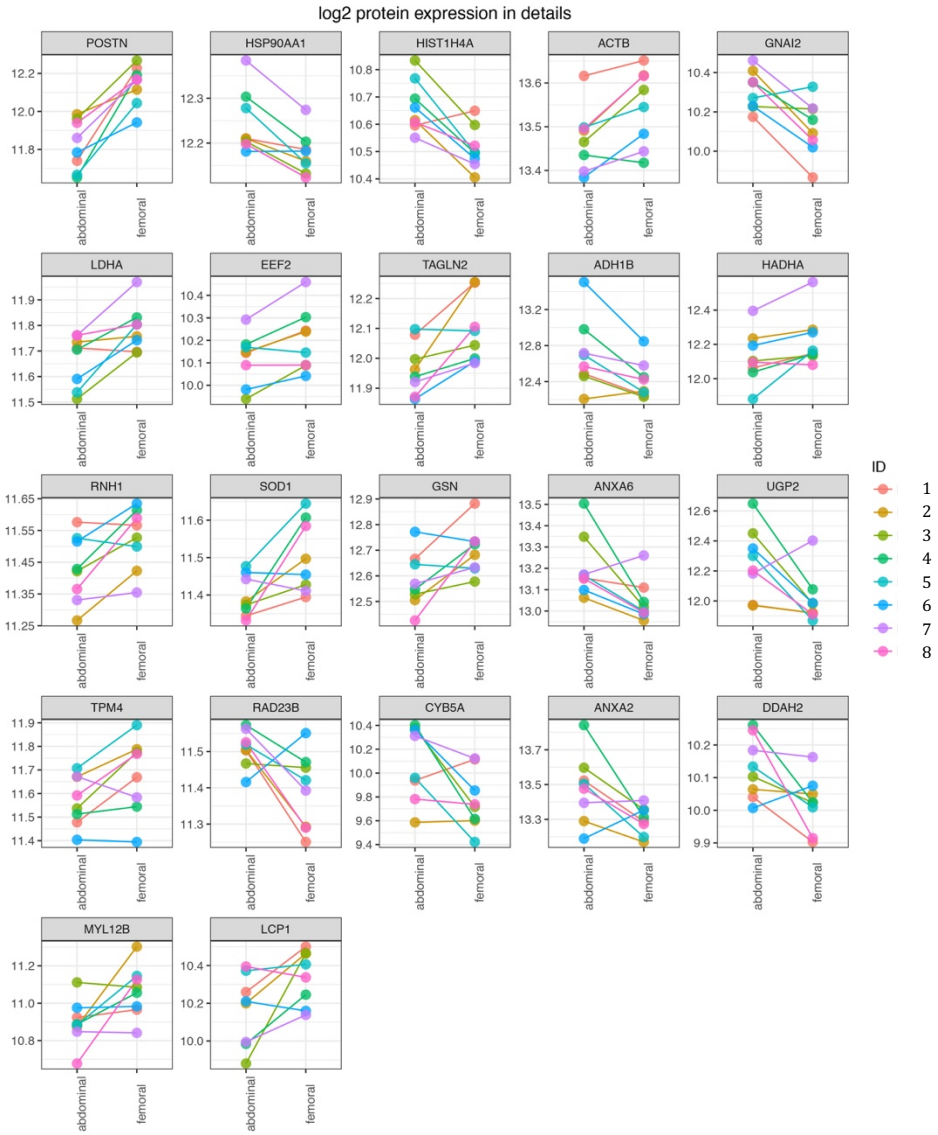
Supplemental Table 1: Identified blood proteins in the AT biopsies.

Accession	Gene symbol	Full protein name
P02768	ALB	Serum albumin
P69905	HBA1	Hemoglobin subunit alpha
P68871	HBB	Hemoglobin subunit beta
P02549	SPTA1	Spectrin alpha chain, erythrocytic 1
P02787	TF	Serotransferrin
P01023	A2M	Alpha-2-macroglobulin
P01009	SERPINA1	Alpha-1-antitrypsin
P0DOX5		Immunoglobulin gamma-1 heavy chain
P00738	HP	Haptoglobin
P02042	HBD	Hemoglobin subunit delta
P00450	CP	Ceruloplasmin
P01871	IGHM	Immunoglobulin heavy constant mu
P69891	HBG1	Hemoglobin subunit gamma-1
P01876	IGHA1	Immunoglobulin heavy constant alpha 1
P01860	IGHG3	Immunoglobulin heavy constant gamma 3
P0DOY2	IGLC2	Immunoglobulin lambda constant 2
P02763	ORM1	Alpha-1-acid glycoprotein 1
P0DOX7		Immunoglobulin kappa light chain
P01834	IGKC	Immunoglobulin kappa constant
P01859	IGHG2	Immunoglobulin heavy constant gamma 2
P00747	PLG	Plasminogen
P19652	ORM2	Alpha-1-acid glycoprotein 2
P02766	TTR	Transthyretin
P01861	IGHG4	Immunoglobulin heavy constant gamma 4
P0DOX8		Immunoglobulin lambda-1 light chain
P01780	IGHV3-7	Immunoglobulin heavy variable 3-7
A0A0C4DH34	IGHV4-28	Immunoglobulin heavy variable 4-28
P01768	IGHV3-30	Immunoglobulin heavy variable 3-30
P09105	HBQ1	Hemoglobin subunit theta-1
A0A0C4DH25	IGKV3D-20	Immunoglobulin kappa variable 3D-20
P01619	IGKV3-20	Immunoglobulin kappa variable 3-20
A0A0C4DH68	IGKV2-24	Immunoglobulin kappa variable 2-24
P01764	IGHV3-23	Immunoglobulin heavy variable 3-23
P69892	HBG2	Hemoglobin subunit gamma-2
P01824	IGHV4-39	Immunoglobulin heavy variable 4-39
P01591	JCHAIN	Immunoglobulin J chain
A0A0B4J1X5	IGHV3-74	Immunoglobulin heavy variable 3-74
P01877	IGHA2	Immunoglobulin heavy constant alpha 2
P04220		Ig MU heavy chain disease protein
P02100	HBE1	Hemoglobin subunit epsilon
P80748	IGLV3-21	Immunoglobulin lambda variable 3-21



Supplemental Figure 1. Top 22 identified proteins that were differentially expressed between abdominal and femoral subcutaneous adipose tissue ($p < 0.05$). Expression data (n=8) are illustrated for each individual (n=8) separately.

Supplemental File 1: Inter-run data normalization

The inter-run variation was normalized per protein based on the identical control samples spiked in each run, performed in R environment v3.4.

- If signals existed in multiple runs and also in control samples: correction is based on the signal of this specific protein.

Normalized signal *[protein x, sample i, run j*]= signal [*protein x, sample i, run j*]/
(signal [*protein x, control, run j*]/median (signal [*protein x, controls, run 1:m*]))

- If signals existed in multiple batches but not in (one of the) control samples: correction is based on total signal of the sample.

Normalized signal [protein x, sample i, run j]= signal [protein x, sample i, run j]/
(Σ signal [control, run j]/median (Σ signal [controls, run 1:m]))

- If no signal existed in the sample, it is left as empty.

R code

```
# normalized signal=raw signal/batch correction factor
my.normalization <- function(x, y) {
      obj<-try(x/y, silent=TRUE)
      if (is(obj, "try-error")) return(NA) else return(obj)
}

# dat.raw is a dataframe of all raw signals of all samples including control and real sample
# with columns of "accession" , "sample",  "signal",     "Batch",    "sample.type", "Quality"

library(reshape2)
dat<-dcast(dat.raw, Batch+sample.type+sample~accession,       value.var="signal",fun.aggregate = sum)
dat[dat==0]<-NA
dat$Batch<-as.factor(dat$Batch)
dat.control<-dat[dat$sample.type=="control",]
sums<-as.data.frame(rowSums(dat.control[,c(4:ncol(dat.control))],na.rm = TRUE))
names(sums)<-"sum"
if(nrow(sums)>1 & length(levels(dat$Batch))>1){
 # exist multiple batch and control samples
 dat.control<-cbind(dat.control,sums)
 summedian<-median(dat.control$sum, na.rm=TRUE)
 dat.control$sumcoefficient<-dat.control$sum/summedian # overal batch ratio coefficient
 for (i in 4:ncol(dat)){
  # normalize protein one by one
  protmedian<-median(dat.control[,i], na.rm=TRUE)
for (b in 1: length(dat.control$Batch)){
    if(!is.na(dat.control[dat.control$Batch==levels(dat.control$Batch)[b],i])){
     # protein is present in this batch control
     # batch correction factor for this protein
     c=dat.control[dat.control$Batch==levels(dat.control$Batch)[b],i]/protmedian
     } else {
     # protein is absent in the batch control
     # batch correction factor for this protein
     c=dat.control$sumcoefficient[dat.control$Batch==levels(dat.control$Batch)[b]]
     }
    dat[dat$Batch==levels(dat.control$Batch)[b],i]<-
my.normalization(dat[dat$Batch==levels(dat.control$Batch)[b],i],c)
     }
 }
 dat<-dat[order(dat$sample.type),]
 dat.melt<-melt(dat[,c(3:ncol(dat))], id.vars ="sample" )
 dat.melt$proteinID<-interaction (dat.melt$variable,dat.melt$sample)

# signals after normalization
dat.norm<-dat.raw
dat.norm$proteinID<-interaction (dat.norm$accession,dat.norm$sample)
dat.norm$signal<-dat.melt$value[match(dat.norm$proteinID,dat.melt$proteinID)]
}
```

CHAPTER 5

Adipose tissue oxygenation is associated with insulin sensitivity independently of adiposity in obese men and women

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Published in:
Diabetes Obes Metab. 2018, in press (doi 10.1111/dom.13329)

Abstract

Adipose tissue (AT) dysfunction contributes to the pathophysiology of insulin resistance and type 2 diabetes. Previous studies have shown that altered AT oxygenation affects adipocyte functionality, but it remains to be elucidated whether altered AT oxygenation is more strongly related to obesity or insulin sensitivity. In the present study, we tested the hypothesis that AT oxygenation is associated with insulin sensitivity rather than adiposity in humans. Thirty-five lean and obese individuals (21 men and 14 women, aged 40-65 years) with either normal or impaired glucose metabolism participated in a cross-sectional single-centre study. We measured abdominal subcutaneous AT oxygenation, body composition and insulin sensitivity. AT oxygenation was higher in obese insulin resistant as compared to obese insulin sensitive (IS) individuals with similar age, body mass index and body fat percentage, both in men and women. No significant differences in AT oxygenation were found between obese IS and lean IS men. Moreover, AT oxygenation was positively associated with insulin resistance ($r = 0.465$; $P = .005$), even after adjustment for age, sex and body fat percentage (standardized $\beta = 0.479$; $P = .005$). In conclusion, abdominal subcutaneous AT oxygenation is associated with insulin sensitivity both in men and women, independently of adiposity. AT oxygenation may therefore be a promising target to improve insulin sensitivity.

Introduction

Adipose tissue (AT) dysfunction, rather than total adiposity, plays a central role in the pathophysiology of insulin resistance and type 2 diabetes.[1, 2] This is exemplified by the finding that the expansion of AT does not necessarily translate into metabolic abnormalities. A subset (~10-30%) of obese individuals seems to be relatively protected against worsening of metabolic health, including insulin resistance,[3, 4] which can at least partly be explained by maintenance of a normal AT function.[2, 3] Elucidating differences in AT biology between obese insulin sensitive (IS) and obese insulin resistant (IR) individuals will provide mechanistic insight into early factors involved in the etiology of type 2 diabetes.

Recent studies suggest that AT oxygenation may relate to obesity-associated AT dysfunction and insulin resistance, as reviewed.[5, 6] Although reduced AT oxygen partial pressure (AT pO_2) has consistently been demonstrated in rodent models of obesity, findings in humans are controversial.[5, 7, 8] We have shown that AT pO_2 was higher in obese IR as compared to lean IS men, which seemed to be explained by lower AT oxygen consumption in obesity.[9] More recently, it was demonstrated that diet-induced weight loss in overweight/obese humans decreased AT pO_2, which was paralleled by improved insulin sensitivity.[10] However, it remains unclear whether AT oxygenation is related to obesity and/or insulin resistance. Moreover, studies examining AT oxygenation in obese men and women separately are currently lacking. Here, we hypothesized that AT oxygenation is associated with insulin sensitivity rather than adiposity. More specific, we tested the hypotheses that 1) abdominal subcutaneous AT oxygenation is higher in obese IR than obese IS and lean IS men and women, and that 2) AT oxygenation is positively associated with insulin resistance, independently of age, sex and adiposity.

Materials and Methods

Subjects

Thirty-five Caucasian individuals (21 men, 14 women) aged 40-65 years with normal or impaired glucose metabolism participated in this study. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, use of medication known to affect body weight and glucose metabolism, marked alcohol consumption (> 14 alcoholic units/week) or untreated hypertension. Subjects had to be weight stable (weight change <3.0 kg) for at least three months prior to the study.

Study design

Study participants came to the university twice after an overnight fast of at least 10 hours within a period of one week, with at least two days between each visit. During the first visit, blood samples were collected under fasting conditions, and anthropometric measurements were performed. During the second visit, an abdominal subcutaneous AT biopsy was collected, AT blood flow (ATBF) was determined using ^{133}Xe wash-out, and abdominal subcutaneous AT pO_2 was measured using a validated microdialysis-based optochemical measurement system, as described previously.[9] Within 2-3 hours after insertion of the microdialysis probe, AT pO_2 had reached stable values (change in AT pO_2 < 2.0 mmHg within a 20-minute period). AT pO_2 was calculated by averaging the 20-min period with stable pO_2 readings. Subjects were asked to refrain from drinking alcohol and to perform no exercise for a period of 24 hours before the test days. The Medical Ethical Committee of Maastricht University has approved this study. All subjects gave their written informed consent prior to their inclusion in the study, which has been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2008.

Laboratory analyses

Blood samples were collected into ice-chilled EDTA-tubes and centrifuged at 1000*g* at 4°C for 10 minutes. Plasma was immediately frozen in liquid nitrogen and subsequently stored at -80°C until analysis. Plasma glucose was analysed with standard enzymatic methods (ABX Pentra Glucose HK CP, Radiometer, Copenhagen, Denmark). Plasma insulin was determined by a radioimmunoassay for human insulin (Human insulin specific RIA, Millipore Corporation, Billerica, MA, USA).

Statistics

Data are presented as means SEM. Variables with a skewed distribution (homeostatic model assessment of insulin resistance [HOMA-IR]) were log-transformed before analysis. Men were divided into a lean (body mass index [BMI] <25 kg/m2) IS (n = 7), obese (BMI > 30 kg/m2) IS (n = 7) and obese IR (n = 7) group, while women were divided into an obese IS (n = 7) and obese IR (n = 7) group. The division into obese IS or obese IR groups was based on the median of HOMA-IR for obese men (3.80) and women (2.31) separately.

Differences between lean IS, obese IS and obese IR men were assessed using 1-way ANOVA, with post hoc testing in case of significance. Obese IS and obese IR women were compared using the independent-samples t test. Pearson correlation analysis was used to investigate associations between AT pO2 and logHOMA-IR. Multiple regression analyses were performed with age, sex and AT pO2 as independent variables and logHOMA-IR as a dependent variable (model 1), and with further adjustment for body fat percentage (model 2) and waist:hip ratio (model 3). P values <.05 (2-tailed tests) were taken to indicate statistical significance. Analyses were performed using SPSS version 23 for Macintosh (Chicago, Illinois).

Results

Study population

Participants' characteristics are summarized in Table 1. By design, HOMA-IR was significantly higher in the obese IR than obese IS and lean IS individuals, with no significant differences in age, BMI and body fat percentage between the obese groups in both men and women. Fasting glucose, fasting insulin and HOMA-IR did not significantly differ between obese IS and lean IS men (Table 1).

TABLE 1 Characteristics of the study participants (N = 35)

	Lean IS men	Obese IS men	Obese IR men	*P* values (ANOVA)	Obese IS women	Obese IR women	*P*
n	7	7	7		7	7	
Age, years	58.6 ± 2.6	55.6 ± 2.8	56.9 ± 4.0	.909	50.6 ± 3.0	51.0 ± 2.3	.913
Weight, kg	73.0 ± 2.4	101.6 ± 3.1‡	103.8 ± 5.7‡	**<.001**	87.6 ± 2.8	93.9 ± 5.4	.259
BMI, kg/m^2	23.0 ± 0.3	31.7 ± 0.8‡	33.1 ± 1.3‡	**<.001**	30.5 ± 0.8	32.9 ± 1.8	.226
Body fat, %	23.0 ± 2.4	32.0 ± 1.6†	32.6 ± 1.8†	**.001**	45.5 ± 1.5	43.1 ± 1.3	.230
Waist, cm	89.1 ± 1.8	107.5 ± 3.0‡	116.2 ± 2.9‡	**<.001**	98.6 ± 3.1	106.2 ± 7.1	.297
Waist:hip ratio	0.96 ± 0.01	1.00 ± 0.02	1.07 ± 0.01‡,§	**<.001**	0.86 ± 0.03	0.94 ± 0.05	.126
SBP, mm Hg	112.8 ± 8.0	139.9 ± 5.0*	141.0 ± 7.5*	**.023**	119.9 ± 5.4	124.4 ± 6.2	.591
DBP, mm Hg	71.4 ± 5.2	90.7 ± 1.7†	82.3 ± 2.4§	**.002**	84.7 ± 4.3	83.7 ± 3.4	.857
Fasting glucose, mmol/L	5.22 ± 0.13	5.44 ± 0.24	5.58 ± 0.11	.333	4.71 ± 0.17	5.54 ± 0.18	**.005**
Fasting insulin, mU/L	11.2 ± 0.7	9.0 ± 0.9	22.6 ± 2.6†,¶	**<.001**	7.9 ± 0.6	14.1 ± 1.7	**.002**
HOMA-IR	2.6 ± 0.1	2.2 ± 0.3	5.6 ± 0.7†,¶	**<.001**	1.5 ± 0.2	3.5 ± 0.4	**.001**
ATBF, mL/100 g tissue per min	2.3 ± 0.5	1.0 ± 0.3	1.6 ± 0.2	.060	1.6 ± 0.2	1.5 ± 0.2	.779

Abbreviations: ATBF, adipose tissue blood flow; BMI, body mass index; DBP, diastolic blood pressure; HOMA-IR, homeostatic model assessment of insulin resistance; IR, insulin-resistant; IS, insulin-sensitive; SBP, systolic blood pressure. *P<.05, †P<.01, ‡P<.001 vs lean IS men. §P<.05, ¶P<.01 vs obese IS men. Values are means ± SEM. Bold values are statistically significant.

Adipose tissue oxygenation is higher in obese insulin resistant individuals

Abdominal subcutaneous AT pO_2 was significantly different between obese IR, obese IS and lean IS men (P_{ANOVA}=0.003). Obese IR men demonstrated significantly higher AT pO_2 values than obese IS men (68.5±4.4 vs 56.1±3.2 mmHg, respectively, *P*=0.041) with comparable age, BMI and body fat percentage, and lean IS men (40.4±6.6 mmHg, *P*<0.004). Obese IS men had intermediate AT pO_2 values that tended to be higher than those in lean IS men (*P*=0.053) (Figure 1A). In women, there was a tendency for a higher abdominal subcutaneous AT pO_2 in obese IR than obese IS individuals (62.3±5.3 vs 50.8±2.5 mmHg, respectively, *P*=0.074) with comparable for age, BMI and body fat percentage (Figure 1B).

ATBF, regulating oxygen supply to the tissue, was not significantly associated with AT pO_2 (*r*=0.015, *p*=0.934).

Adipose tissue oxygenation is associated with insulin sensitivity

We found a positive correlation between abdominal subcutaneous AT pO_2 and HOMA-IR (*r*=0.465, *p*=0.005, *n*=35) (Figure 1C), indicating that higher levels of AT pO_2 were related to insulin resistance. This association was present in both men (*r*=0.506, *p*=0.019, *n*=21) and women (*r*=0.536, *p*=0.048, *n*=14). Moreover, AT pO_2 remained significantly associated with HOMA-IR after adjustment for age and sex (standardized β=0.488, *p*=0.002, *n*=35) using multiple regression analysis. Further adjustment for body fat percentage did not change (the strength of) this association (standardized β=0.479, *p*=0.005, *n*=35). Finally, further inclusion of waist:hip ratio in the model reduced the strength of the association between AT pO2 and HOMA-IR (standardized β=0.322; *p*=0.058).

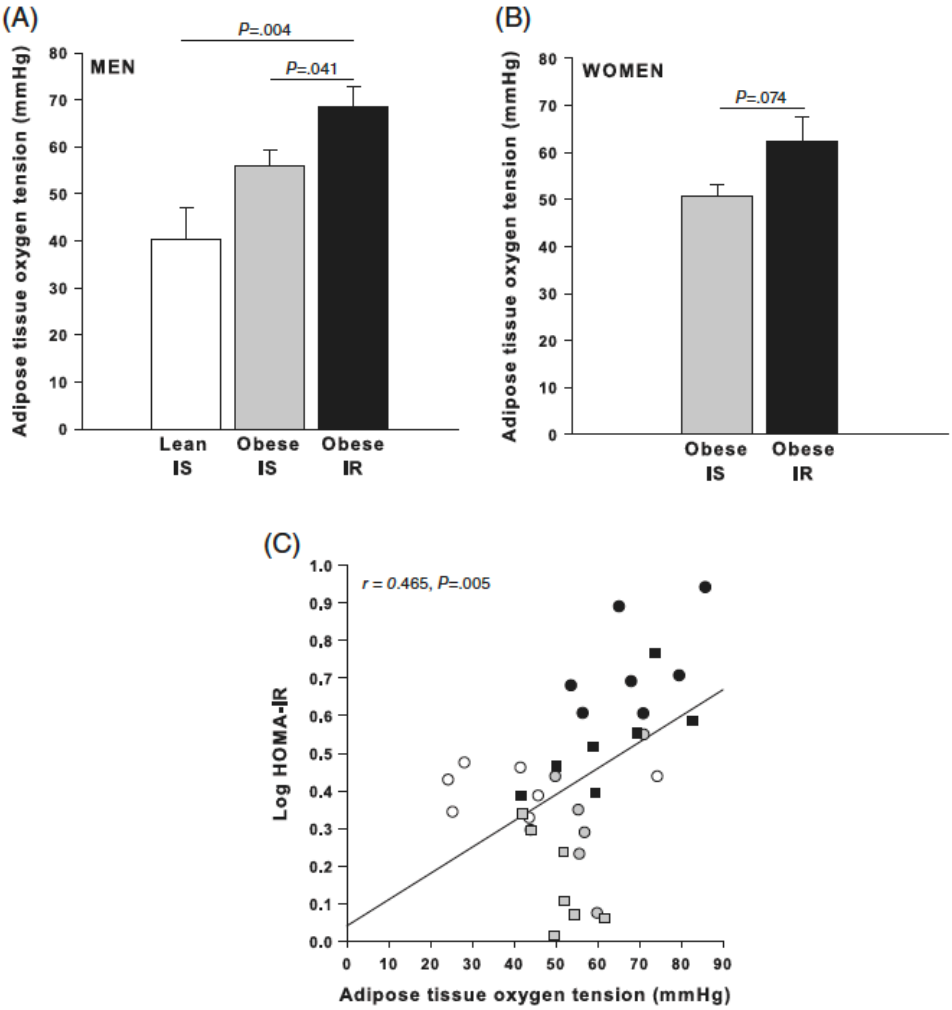

Figure 1 A, Adipose tissue (AT) oxygenation in lean insulin sensitive (IS), obese IS and obese insulin resistant (IR) men and **B**, obese IS and obese IR women. **C**, AT oxygenation was positively associated with insulin sensitivity. White symbols, lean IS individuals; grey symbols, obese IS individuals; black symbols, obese IR individuals. Women are represented by squares and men by circles. HOMA-IR, homeostatic model assessment of insulin resistance.

Discussion

The present study demonstrated that abdominal subcutaneous AT pO_2 was significantly higher in obese IR than obese IS and lean IS individuals. Moreover, AT pO_2 was positively associated with insulin resistance both in men and women, and this relationship was independent of age, sex and adiposity.

We have previously demonstrated that AT pO_2 was higher in obese IR than lean IS men, which seemed to be explained by lower AT oxygen consumption in obesity, and was inversely correlated with insulin sensitivity.[9] In line, we have recently shown that diet-induced weight loss markedly decreased abdominal subcutaneous AT pO_2 in overweight/obese humans, which was paralleled by improved whole-body insulin sensitivity.[10] Until now, however, it remained unclear whether AT oxygenation is related to adiposity and/or insulin sensitivity. Here, we demonstrated for the first time that AT oxygenation is higher in obese IR than obese IS and lean IS individuals, and is an independent determinant of whole-body insulin sensitivity. We have previously demonstrated that abdominal subcutaneous AT pO_2 was positively associated with AT gene expression of several pro-inflammatory factors.[9] Thus, it is tempting to speculate that the association between AT pO_2 and insulin resistance may be mediated at least in part by adipokines. In accordance with our findings, it has previously been shown that moderate hypoxia exposure improves insulin sensitivity in obese humans,[11] which has been hypothesized to be at least partly mediated through reduced AT oxygenation.[12] Since ATBF was not significantly associated with AT pO_2, it is tempting to speculate that the difference in AT pO_2 between IS and IR individuals may be explained by altered AT oxygen consumption rather than oxygen supply to AT. In line, it has previously been found that abdominal subcutaneous adipocytes from obese individuals show a lower oxygen consumption rate as compared to those derived from lean individuals, independently of adipocyte size,[13] although it it remains to be established whether this difference in adipocyte oxygen consumption is related to insulin sensitivity.

The strengths of this study are that AT pO_2 was investigated in Caucasian men and women who were phenotyped with respect to body fat mass, body fat distribution and insulin sensitivity. Moreover, age was comparable across groups, and the obese groups had comparable BMI and body fat percentage. Thus, the differences in AT pO_2 that we found between obese IS and obese IR subjects cannot be explained by differences in age, ethnicity, BMI and body fat percentage. This is further strengthened by the outcomes of the multiple regression analyses, demonstrating that AT pO_2 was an independent determinant of insulin sensitivity. The strength of the association between

AT pO_2 and HOMA-IR, however, was reduced after further adjustment for waist/hip ratio (standardized β=0.322, p=0.058), suggesting that body fat distribution may influence the relationship between AT pO_2 and insulin sensitivity to a certain extent. This study also has several limitations. First, insulin sensitivity was determined using HOMA-IR, which is a surrogate marker of whole-body insulin sensitivity, rather than a hyperinsulinaemic-euglycaemic clamp. Secondly, a causal relationship between AT pO_2 and insulin sensitivity cannot be inferred from the present cross-sectional study. Moreover, it was unfortunately not possible to measure oxygenation in visceral AT or other key metabolic organs. Thus, it remains to be established whether differences in oxygenation of other tissues than abdominal subcutaneous AT contribute to differences in whole-body IR. Finally, the sample size was relatively small. In particular, the number of individuals in each subgroup was limited. It would be interesting to investigate the relationship between AT pO_2 and insulin sensitivity in individuals with a different metabolic phenotype in future studies. However, we were able to perform these measurements in a unique population of obese IS and obese IR male and female individuals, who had a comparable age, BMI and body fat percentage, yielding novel and potentially clinically relevant data.

In conclusion, we demonstrate for the first time that abdominal subcutaneous AT oxygenation is higher in obese IR as compared to obese IS and lean IS humans. Moreover, AT oxygenation is associated with insulin sensitivity, independently of adiposity. Future clinical studies in humans should investigate whether modulation of AT oxygenation may improve insulin sensitivity and related complications.

Acknowledgments

The authors would like to thank Nadia Roumans (Maastricht University, The Netherlands) for practical support during the experiments, and Merima Čajlaković and Michael Suppan (Joanneum Research Forschungsgesellschaft mbH, Weiz, Austria) for technical support. We thank the participants for their contribution to the study.

Conflict of interest

None declared.

Author contributions

G.H.G. designed the study, collected data, performed the statistical analysis, interpreted data and wrote the manuscript. M.A.A.V. and R.G.V. contributed to the acquisition and interpretation of data, and revised the manuscript. E.C.M., M.A.V.B., E.E.B. contributed to the interpretation of data and revised the manuscript. All authors have read and approved the final version of this manuscript. G.H.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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CHAPTER 6

The effects of mild intermittent hypoxia exposure on tissue oxygenation, substrate metabolism and insulin sensitivity in overweight and obese men: a randomized, single-blind, placebo-controlled, cross-over study

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*these authors contributed equally

In preparation

Abstract

Background: Adipose tissue (AT) dysfunction and impairments in skeletal muscle (SM) metabolism contribute to the pathophysiology of cardiometabolic diseases. Recent studies suggest that mild hypoxia exposure (MIH) may improve glucose homeostasis through effects on the functioning of these organs. Here, we investigated the effects of MIH exposure on AT and SM oxygenation, SM mitochondrial respiration, substrate metabolism and insulin sensitivity in obese individuals.

Methods: Eight overweight/obese (BMI>28 kg/m^2) men participated in a randomized, single-blind, placebo-controlled, cross-over study. Subjects were exposed to 1) normobaric MIH (FiO_2 15%; equivalent to ~3000m above sea level) and 2) normoxia (21% O_2) for 7 consecutive days (3 cycles of 2h exposure/d) in a randomized fashion, separated by a wash-out period of 3-6 weeks. We determined AT and SM oxygen partial pressure (pO_2) (day 6), fasting and postprandial substrate metabolism (day 7, high-fat mixed-meal), and insulin sensitivity (day 8, two-step hyperinsulinemic-euglycemic clamp with D-[6,6-$^2H_2$]-glucose tracer under normoxia). AT and SM biopsies were collected on day 8 for gene and protein expression analyses, and measurement of *ex vivo* SM mitochondrial respiration.

Results: MIH reduced systemic oxygen saturation (SpO_2: 92.4 ± 0.6 vs. 97.6 ± 0.2%, p<0.001), AT pO_2 (18.2±1.9 vs. 37.0±2.7 mmHg, p=0.001) and SM pO_2 (6.4±2.1 vs. 11.4±1.8 mmHg, p=0.04) as compared to normoxia exposure. MIH increased postprandial lactate concentrations (p=0.034), while circulating glucose and lipid concentrations remained unchanged. Moreover, fasting and postprandial carbohydrate oxidation increased (p<0.05 and p=0.043, respectively) and fat oxidation decreased (p<0.05 and p=0.043, respectively), resulting in a significant increase in respiratory exchange ratio (RER) during MIH versus normoxia exposure, whilst energy expenditure was unchanged. Fasting and insulin-stimulated RER were still elevated one day after cessation of MIH (p=0.042 and p=0.035, respectively), despite unchanged whole-body insulin sensitivity.

Conclusions: These preliminary data demonstrate for the first time that MIH effectively decreased AT and SM oxygenation, and induced a shift in substrate metabolism towards increased carbohydrate oxidation under fasting and postprandial conditions in overweight/obese individuals, as reflected by increased circulating lactate concentrations. This shift in substrate oxidation seemed to persist after cessation of MIH and was not accompanied by a change in insulin sensitivity.

Introduction

The obesity epidemic calls for novel strategies to prevent and treat obesity and its comorbidities. Skeletal muscle (SM), liver and adipose tissue are key organs in the pathogenesis of obesity-related insulin resistance and type 2 diabetes. Adipose tissue (AT) dysfunction and impaired SM substrate metabolism are hallmarks of obesity and type 2 diabetes (T2DM).[1] The enlarged adipocytes,[1, 2] impaired AT blood flow,[1, 3-5] an increased production of pro-inflammatory cytokines by adipose tissue,[3, 6] together with impaired substrate handling in SM, result in lipid accumulation and insulin resistance in metabolic tissues such as SM and the liver.[7, 8]

Populations living at high altitude, which are exposed to lower levels of oxygen due to lower air pressure (hypobaric hypoxia), are characterized by a lower type 2 diabetes prevalence as compared with individuals living at sea level.[9] Although the latter findings may be confounded by many other factors such as altered lifestyle at high altitude, several studies have indicated that the amount of oxygen to which tissues are exposed may impact cardiometabolic health.

We have previously demonstrated that AT oxygen tension (pO_2) was increased in obese insulin resistant as compared to lean insulin sensitive individuals,[5] which opposes previous observations in rodents[10-12] and humans[13]. Moreover, AT pO_2 was positively associated with AT inflammation and peripheral insulin resistance in humans.[5] More recently, we have shown that diet-induced weight loss decreased AT pO_2 (~20%) in humans, which was accompanied by increased insulin sensitivity.[14] Indeed, several in vitro studies demonstrated a link between pO_2 and AT dysfunction, albeit with conflicting results, as reviewed.[8, 15] Interestingly, hypoxia exposure may also affect SM metabolism. Hypoxia exposure increased glucose uptake in murine and human SM *in vitro* via insulin-independent mechanisms.[16, 17] In accordance with these findings, mild hypoxia exposure (15% O_2) for ten consecutive nights increased whole-body insulin sensitivity in obese individuals.[18] Moreover, T2DM patients exercising under hypoxic (14.7% O_2) as compared to normoxic conditions showed greater improvements in glucose metabolism and insulin sensitivity.[19] Taken together, exposure to normobaric mild hypoxia may be a promising strategy to reverse metabolic impairments and peripheral insulin resistance in obese individuals, as recently postulated.[20]

It has been suggested that the exposure regimen (i.e. severity, pattern and duration of exposure) may significantly impact experimental outcomes.[8] For example, acute exposure to (severe) hypoxia impaired insulin signaling in adipocytes from human and murine origin,[21] while it has recently been shown that prolonged hypoxia exposure to similar pO_2 improved adipocyte insulin sensitivity.[22] The effects of prolonged exposure to mild

intermittent hypoxia (MIH), however, have not been investigated in humans, and underlying mechanisms for the potential beneficial effects of MIH on insulin sensitivity and glucose homeostasis remain to be elucidated. To this end, we here investigated the effects of MIH exposure (FiO_2 15%, 3 times 2h/day, 7 consecutive days) on AT and SM pO_2, substrate metabolism and insulin sensitivity in overweight/obese insulin resistant men. Furthermore, adipose tissue and SM biopsies were taken to determine gene and protein expression, and to perform functional mechanistic experiments.

Materials and Methods

The findings reported in this manuscript are an interim analysis of an ongoing trial. For this Chapter, data obtained from 8 subjects who completed the study were used. Twelve subjects will be included in the final analyses.

Subjects

Eight overweight/obese (BMI ≥28 kg/m^2) male subjects (30-65 yrs) participated in the study. Subjects needed to be insulin resistant, defined as $HOMA_{IR}$ index ≥2.2. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, use of medication known to affect body weight and glucose metabolism, and marked alcohol consumption (>14 alcoholic units/wk). Furthermore, subjects had to be weight stable (weight change <3.0 kg) for at least three months prior to the start of the study. The study was performed according to the declaration of Helsinki and was approved by the Medical-Ethical Committee of Maastricht University. All subjects gave their written informed consent before participation in the study.

Experimental protocol

The design of the present randomized, single-blind, placebo-controlled, cross-over study is depicted in Figure 1. Participants were exposed to normobaric mild intermittent hypoxia (FiO_2 15%; equivalent to ~3000m above sea level) and normobaric normoxia (FiO_2 21%) for 7 consecutive days (3 cycles of 2h exposure/day, with 1h of normoxia exposure between hypoxic cycles) in a randomized fashion, separated by a wash-out period of 3-6 weeks. Subjects were asked to refrain from drinking alcohol and to perform no exercise 24 hours before the start and during the exposure regimens. Blood collection and measurements on days 1, 3, 6 (tissue oxygenation), 7 (high-fat mixed-meal (HFMM) test), and 8 (hyperinsulinemic-euglycemic clamp and biopsies) were performed after an overnight fast of at least 10 hours.

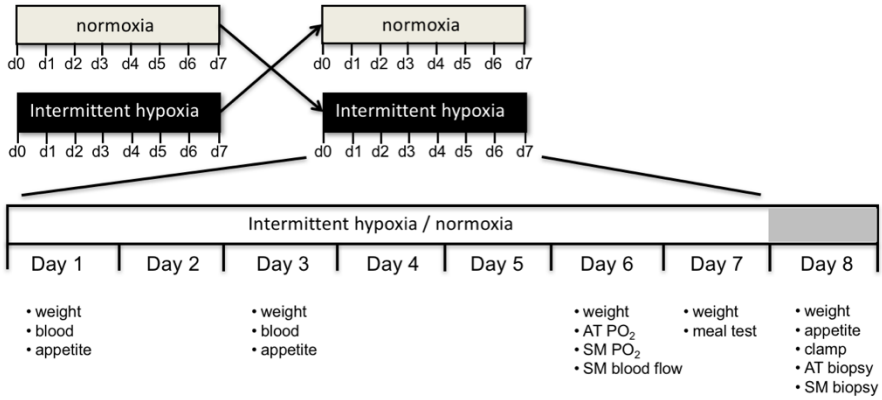

Figure 1: Study design. Subjects were exposed to normobaric mild intermittent hypoxia (FiO_2 15%) or normoxia (FiO_2 21%) for 7 consecutive days (days 1-7, 3 cycles of 2h/day) in a randomized, cross-over fashion, with a 3-6 week wash-out period. The specific measurements that were performed on days 1-8 are indicated at the bottom of the figure. AT, adipose tissue; pO_2, oxygen tension; SM, skeletal muscle.

During the first 5 days, individuals were exposed to the mild intermittent hypoxia (MIH) protocol while either sitting or lying on a bed during the 3 cycles, and were exposed to normoxia (21% O_2) while sitting (Figure 2). Furthermore, participants performed standardized activity (3 x 5 minutes of stepping activity per day, stepping frequency 15 steps/min) during days 1-5. Importantly, diet was adjusted individually to match energy expenditure and maintain energy balance throughout the study. Based on the estimated daily energy expenditure (basal metabolic rate (BMR) (Ventilated Hood, Omnical, Maastricht University, The Netherlands) * activity score of 1.55), subjects received a standardized diet consisting of 50% carbohydrate, 35% fat and 15% protein to maintain a stable body weight during while in the study. All foods and drinks were prepared and provided by the investigators.

Assessment of hunger and satiety was performed on days 1, 3 and 7 by means of visual analog scale (VAS) questionnaires.[23] Moreover, the overall appetite score (OAS) was calculated as the average of individual scores ((satiety + fullness + (100-prospective food consumption) + (100-hunger))/4.[24] Additionally, adverse events of MIH were monitored and recorded on days 1-5 by means of the Lake Louise questionnaire for Acute Mountain Sickness (AMS).[25]

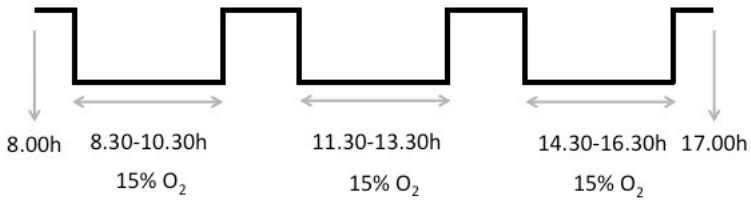

Figure 2: Time-line of the hypoxia exposure regimen during one day of the MIH protocol. During the normoxic control period, individuals were continuously exposed to 21% O_2.

Anthropometric and cardiovascular measurements

Body weight was measured to the nearest 0.1 kg (Seca, Hamburg, Germany). Height was measured using a wall-mounted stadiometer (model 220; Seca, Hamburg, Germany). Body composition and body fat distribution were determined by DEXA (Hologic QDR 4500-A, Waltham MA, USA). Blood pressure and heart rate were assessed (UA-789XL digital blood pressure monitor, A&D medical, USA) on multiple occasions during the exposure protocol (days 1-5). We continuously monitored oxygen saturation levels by pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcor Puritan Bennett, USA) for safety purposes.

Abdominal subcutaneous adipose tissue and skeletal muscle oxygenation

On day 6 of each exposure regimen, AT and SM pO_2 were measured by means of an optochemical measurement system for the continuous monitoring of tissue pO_2 *in vivo* in humans, which we have applied in humans previously.[5] Briefly, microdialysis catheters (CMA60, CMA microdialysis AB, Stockholm, Sweden) were inserted into abdominal subcutaneous AT 6-8 cm lateral from the umbilicus (skin anesthetized using EMLA cream) and SM (*m. gastrocnemius*; anesthetized using 2% lidocaine). After insertion, both catheters were perfused with Ringer solution (Baxter BV, Utrecht, The Netherlands) at a flow rate of 2 µl/min (CMA400 microinfusion pump, CMA Microdialysis AB, Stockholm, Sweden). The interstitial fluid was then directed towards a flow-through cell, containing a highly accurate O_2-sensor.[5] Within 2-3 hours after insertion of the microdialysis probe, pO_2 had reached stable values (change in pO_2 <2.0 mmHg over a 20-min period). Fasting pO_2 was calculated by averaging this 20-min period with stable pO_2 readings.

Forearm blood flow

Since tissue pO_2 reflects the balance between oxygen supply and consumption,[26] forearm blood flow was measured under fasting conditions on day 6 using venous occlusion plethysmography.[27] Briefly, total forearm blood flow (TBF) was measured with a mercury strain gauge (Periflow 0699; Janssen Scientific Instruments). First, the wrist was occluded (220 mmHg by a sphygmomanometer), and after 30 seconds the cuff on the upper arm was inflated (47 mmHg) for 7 seconds. These steps were repeated every 7 seconds until at least 3 good readings were obtained.

High-fat mixed-meal test

On day 7 of each exposure regimen, subjects received a high-fat mixed-meal (ingested at t=0 within 5 min) following a 30-min baseline period under fasting conditions (Figure 3). The liquid test meal provided 2.6MJ, consisting of 61 E% fat (35.5 E% saturated fat, 18.8 E% monounsaturated fat, and 1.7 E% polyunsaturated fat), 33 E% carbohydrates and 6.3 E% protein.

Blood was sampled from a superficial dorsal hand vein, which was arterialized by placing the hand into a hot-box (~55°C). Blood samples were taken under fasting (t0 min) and postprandial conditions (t30, 60, 90, 120, 180, and 240 min). Energy expenditure and substrate oxidation were assessed using indirect calorimetry (open-circuit ventilated hood system, Omnical, Maastricht University) under fasting conditions (t-30 - 0 min) and for 4-hr after meal ingestion postprandial state (Figure 3).[28] Calculations of energy expenditure and substrate oxidation were performed according to the equations of Weir[29] and Frayn[30]. Nitrogen excretion was based on the assumption that protein oxidation represents ~15% of total energy expenditure.[31]

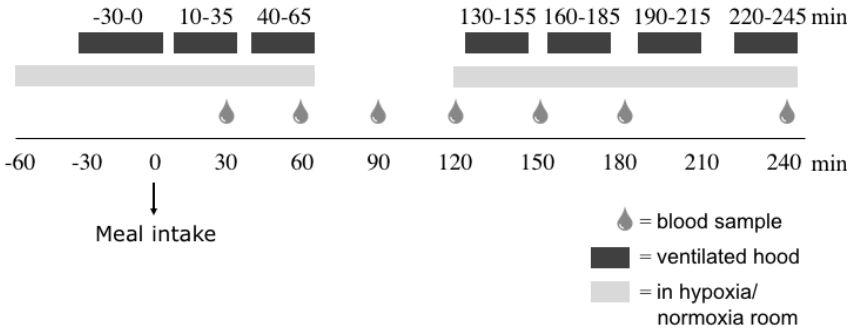

Figure 3: Protocol of the high-fat mixed-meal test.

Hyperinsulinemic-euglycemic clamp

A two-step hyperinsulinemic–euglycemic clamp combined with a D-[6,6-$^2H_2$]-glucose tracer (Cambridge Isotope Laboratories) was performed on day 8 to determine hepatic, adipose tissue and peripheral insulin sensitivity, as described previously.[32] First, a primed (bolus-injection of 2.4 mg/kg) continuous infusion of D-[6,6-$^2H_2$]-glucose was started and continued throughout the measurement at 0.04 mg/kg/min to determine baseline endogenous glucose production (EGP), glucose rate of appearance (Ra) and glucose disposal (Rd). After 2 hr, insulin was infused at a primed continuous low rate of 10 mU*m^{-2}*min^{-1} for 3 hr to assess hepatic insulin sensitivity (% suppression of EGP) and adipose tissue insulin sensitivity (% suppression of free fatty acids (FFA)), followed by insulin infusion at a high rate of 40 mU*m^{-2}*min^{-1} for 2.5 hr to determine peripheral insulin sensitivity (Rd). Arterialized blood samples (hot-box at ~55°C) were frequently taken from a superficial dorsal hand vein and blood glucose concentration directly determined. By variable co-infusion of a 17.5% glucose solution, enriched by 1.1% tracer, euglycemia was maintained (5.0 mmol/l). During the last 30-min of each step (0, 10, and 40 mU*m^{-2}*min^{-1} insulin), substrate oxidation was measured using indirect calorimetry and blood was samples every 15 min. Steele's single pool non-steady state equations will be used to calculate Ra and Rd. Since the study was not completed at the time of submission of this thesis, the tracer concentrations in the samples have not yet been determined and EGP, Ra and Rd could therefore not be calculated. Instead, the M-value was calculated using the average glucose infusion rate during the steady-state (late 30 min) of the high-inulin infusion step.

Skeletal muscle biopsies and high-resolution respirometry

Before the start of the hyperinsulinemic-euglycemic clamp a SM biopsy (80-100 mg; *m. vastus lateralis*) was collected under local anesthesia (2% lidocaine without epinephrine). Part of the muscle biopsy was immediately placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments, Innsbruck, Austria). Subsequently, intact permeabilized muscle fibers (~2.5-3 mg wet weight) were prepared, as described elsewhere[33, 34], and used to determine mitochondrial oxidative capacity using an oxygraph (OROBOROS Instruments, Innsbruck, Austria). The respiration chambers were hyper-oxygenated up to ~500 µmol/l O_2, to prevent oxygen limitation. Subsequently, two different multi-substrate/inhibition protocols were used in which substrates and inhibitors were added consecutively in saturating concentrations. State 2 respiration was measured after the addition of malate (4 mmol/l) plus octanoyl-carnitine (1 mmol/l) or malate (4 mmol/l) plus pyruvate (5 mmol/l). Coupled (state 3) respiration was measured by adding an excess of ADP (2 mmol/l), after which maximal coupled respiration was initiated through addition of

saturating concentration of succinate (10 mmol/l). Next, the integrity of the outer membranes of the mitochondria was tested by addition of cytochrome C (20 µmol/l). State U, the maximal uncoupled state, was induced by titration of FCCP (steps of 0.25 µmol/l). The remaining part of the SM biopsy was snap frozen in melting isopentane, followed by liquid nitrogen, and stored at -80°C until further analysis.

Biochemical analyses

Blood was collected into pre-chilled tubes, centrifuged at 1000g, and plasma was snap-frozen and stored in -80°C until analysis. Plasma levels of glucose (ABX Pentra Glucose HK CP, Horiba ABX Diagnostics, Montpellier, France), FFA (WAKO NEFA-HR (2) ACS-ACOD method, WAKO Chemicals GmbH), TAG (ABX Pentra Triglycerides CP, Horiba ABX Diagnostics, Montpellier, France) and glycerol (Glycerol-kit UV-test, r-Biopharm, Damstadt) were determined. Plasma insulin was measured using a double-antibody radioimmunoassay (Millipore, MA, USA). Lactate was measured in plasma using standard enzymatic techniques automated on a Cobas Fara centrifugal spectrophotometer (Roche Diagnostics, Basel, Switzerland).

Statistics

The effects of MIH as compared to normoxia exposure were assessed by means method of a paired Student's *t*-test. For data derived from the HFMM and appetite ratings (hunger and satiety), the areas under the curve (AUCs) were calculated using the trapezoidal rule. Data are expressed as means ± standard error of the mean (SEM), with a two-sided significance level of $P<0.05$. Statistical analysis was performed using SPSS 24.0 for Macintosh.

Results

Subject characteristics are shown in Table 1. None of the subjects reported signs of acute mountain sickness upon MIH exposure, assessed using the Lake Louise questionnaire, which was completed by the subjects throughout the exposure regimen (4 times a day on days 1-5) (data not shown).

Table 1: Subject characteristics (n=8)

Age (yrs)	60.6 ± 1.5
Body weight (kg)	101.7 ± 6.7
BMI (kg/m^2)	31.5 ± 1.5
Body fat (%)	27.6 ± 1.4
Total fat mass (kg)	25.7 ± 2.5
Lean mass (kg)	64.1 ± 1.9
Systolic BP	138.9 ± 6.5
Diastolic BP (mmHg)	89.5 ± 3.9
HbA1c (%)	5.6 ± 0.1
Fasting glucose (mmol/l)	5.46 ± 0.10
2-hour glucose (mmol/l)	6.06 ± 0.56
HOMA-IR	3.42 ± 0.43

BMI, body mass index; BP, blood pressure; HbA1c, glycated hemoglobin; HOMA-IR, homeostatic model assessment for insulin resistance. Values are mean ± SEM.

Mild intermittent hypoxia did not affect body weight and heart rate

During the exposure of 7 days no significant differences in body weight were observed on both exposure regimens (Figure 4A), indicating that individuals were successfully kept in energy balance during the study. Moreover, heart rate (p=0.348) and blood pressure (SBP p=0.367 and DBP p=0.709) were not significantly affected by MIH as compared to the normoxia exposure regimen (Figure 4B-C). The AUCs for hunger, satiety and overall appetite score were not significantly different between MIH and normoxia exposure (Supplemental Figure 1).

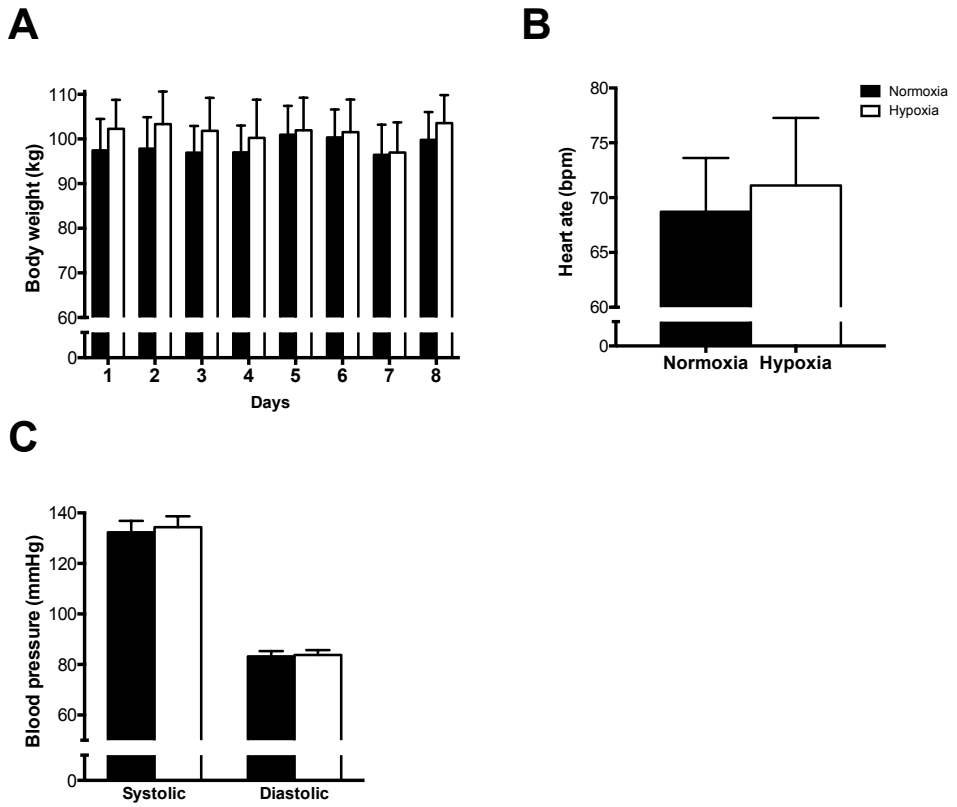

Figure 4: Body weight (A), heart rate (B), and blood pressure (C) during hypoxia (FiO_2 15%) and normoxia (FiO_2 21%) exposure. Values are shown as mean ± SEM (n=7-8).

Mild intermittent hypoxia exposure decreased oxygen saturation and tissue oxygenation

MIH significantly decreased systemic oxygen saturation as compared to normoxia exposure (92.4±0.6 vs. 97.6±0.2 %, p<0.001). Hemoglobin concentrations (9.8±0.7 vs. 10.0±0.7 mmol/L, p=0.60) and hematocrit levels (48.4±2.2 vs. 48.3±2.9 %, p=0.87) were not significantly altered by MIH as compared to normoxia. Oxygen saturation, hemoglobin and hematocrit values remained constant, with no significant differences over time throughout the entire exposure period (data not shown).

Next, to assess whether lowering of systemic oxygen saturation by MIH induced significant reductions in tissue oxygenation, oxygen partial pressure (pO_2) was determined in both abdominal subcutaneous AT and SM. MIH markedly and consistently decreased AT pO_2 as compared to normoxia exposure (37.0 ±1.9 mmHg vs. 18.2 ±2.7 mmHg,

p<0.001) (Figure 5B and 5C). Moreover, MIH exposure evoked a significant reduction of SM pO_2 (MIH: 11.4 ±1.8 mmHg vs. normoxia: 6.4±2.1 mmHg, p=0.040) (Figure 5B and 5D). These findings demonstrate proof-of-concept, showing that MIH exposure reduced systemic O_2 saturation and pO_2 in AT and SM. Furthermore, total forearm blood remained unchanged during MIH (Figure 5E), suggesting that the lower systemic and SM pO_2 were not compensated by an increased SM blood flow.

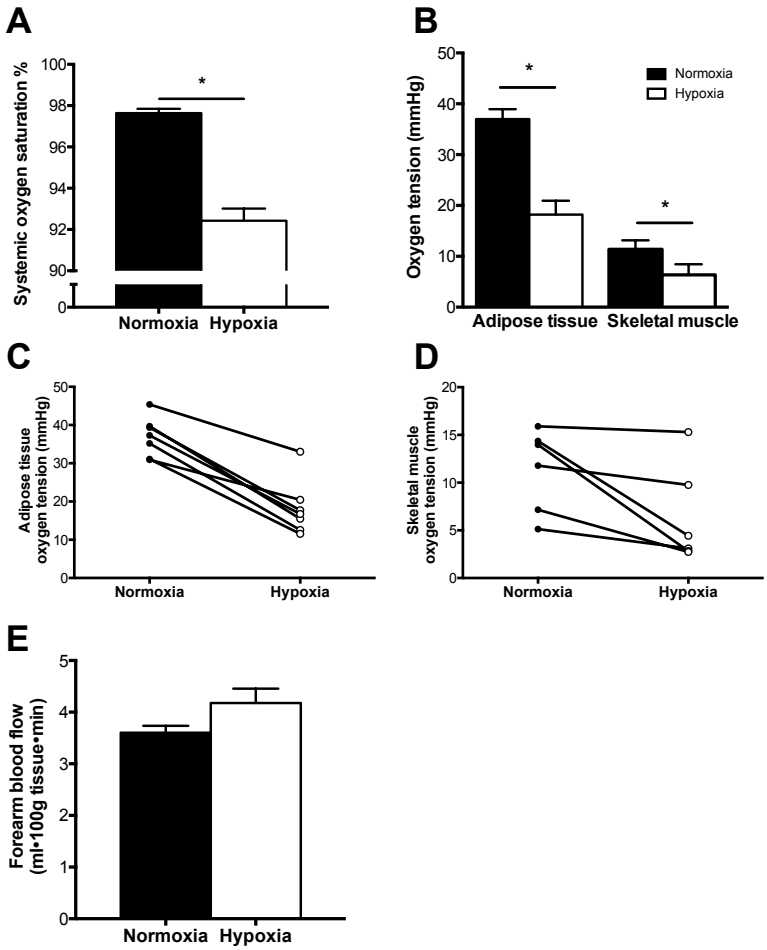

Figure 5: Mean oxygen saturation (n=8) (A) and AT and SM pO_2 during exposure to MIH (FiO_2 15%) and normoxia (FiO_2 21%, control) (B), individual data for AT (n=7) (C) and SM (n=6) (D), and forearm blood flow (n=7) (E). Data are mean ± SEM. *P<0.05.

The effects of mild intermittent hypoxia on plasma metabolite concentrations

On day 7, a HFMM test was performed to assess the effects of MIH on fasting and postprandial plasma metabolite concentrations. MIH did neither significantly affect the postprandial increase in glucose, insulin and TAG concentrations, nor the postprandial suppression of plasma FFA and glycerol levels as compared to normoxia exposure (Figure 6A-E). Postprandrial insulin concentrations tended to be slightly higher after MIH as compared to normoxia ($AUC_{0\text{-}240min}$, p=0.084). However, postprandial lactate concentrations were significantly higher during MIH as compared to normoxia exposure ($AUC_{0\text{-}240min}$, p=0.034) (Figure 6F).

Effects of mild intermittent hypoxia on substrate oxidation and energy expenditure

During the HFMM test that was performed on day 7, we also assessed the effects of MIH on energy expenditure and substrate utilization. MIH did not significantly affect energy expenditure (p=….), but markedly increased the respiratory exchange ratio (RER) as compared to normoxia exposure ($AUC_{0\text{-}240min}$, p=0.042) (Figure 7A and 7B, respectively). Carbohydrate oxidation (CHO) was significantly increased, while fat oxidation was significantly decreased under fasting conditions by MIH as compared to normoxia ($AUC_{0\text{-}240min}$, both p=0.043) (Figure 7C and 7D, respectively). Moreover, these differences in substrate oxidation persisted throughout the entire postprandial phase.

On day 8, one day after cessation of the exposure regimen, there was a significant increase in energy expenditure during the low insulin infusion step after MIH compared to normoxia exposure (Mean ± SEM, p=0.003) (Figure 7E). RER was significantly higher after MIH exposure under fasting and high insulin-stimulated conditions (Mean ± SEM, p=0.042 and p=0.035, respectively) (7F). CHO was significantly increased (similar to day 7) while fat oxidation was significantly decreased under high insulin-stimulated conditions after 7 days of MIH compared to normoxia exposure (Mean ± SEM, p=0.036 and p=0.034, respectively) (Figure 7G and 7H).

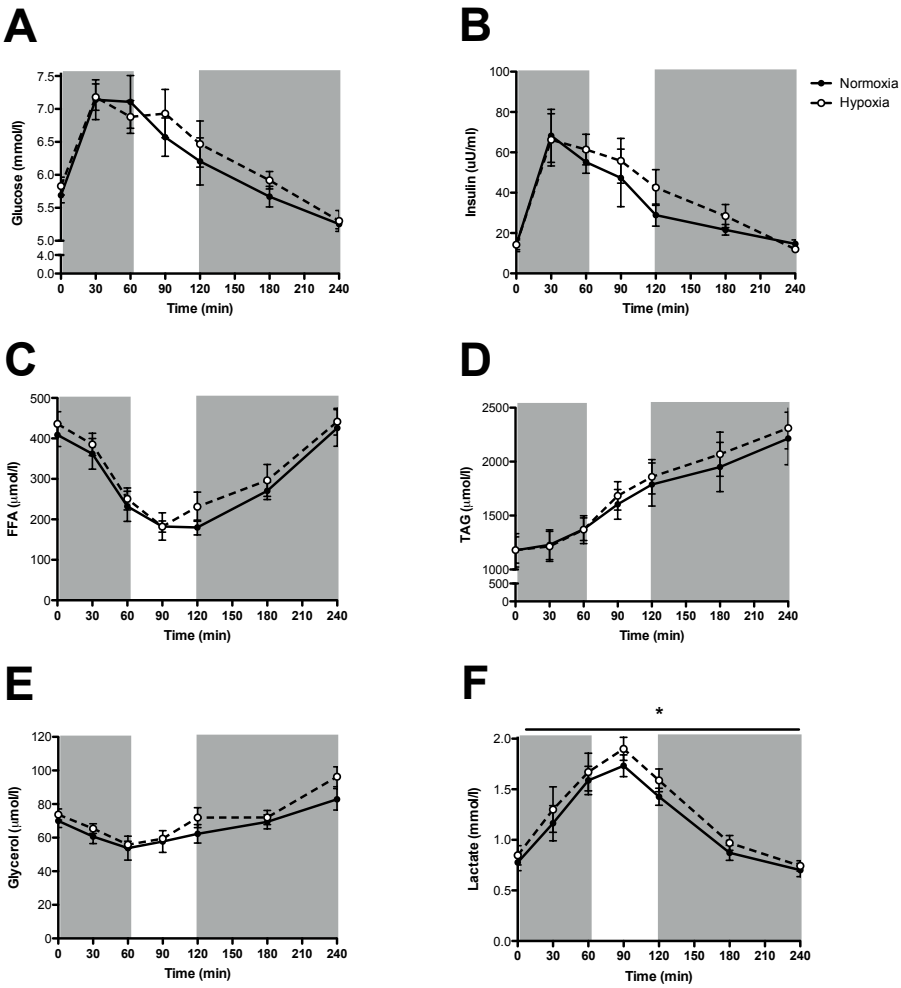

Figure 6: Fasting and postprandial plasma metabolite concentrations during the HFMM (day 7). At several time points during the HFMM, glucose (A), insulin (B), FFA (C), TAG (D), glycerol (E), and lactate (F) were determined. The grey area represents the time that individuals resided inside of the hypoxic/normoxic room, and stayed in another room (normoxic conditions). Data is shown as mean ± SEM (n=7). * P<0.05.

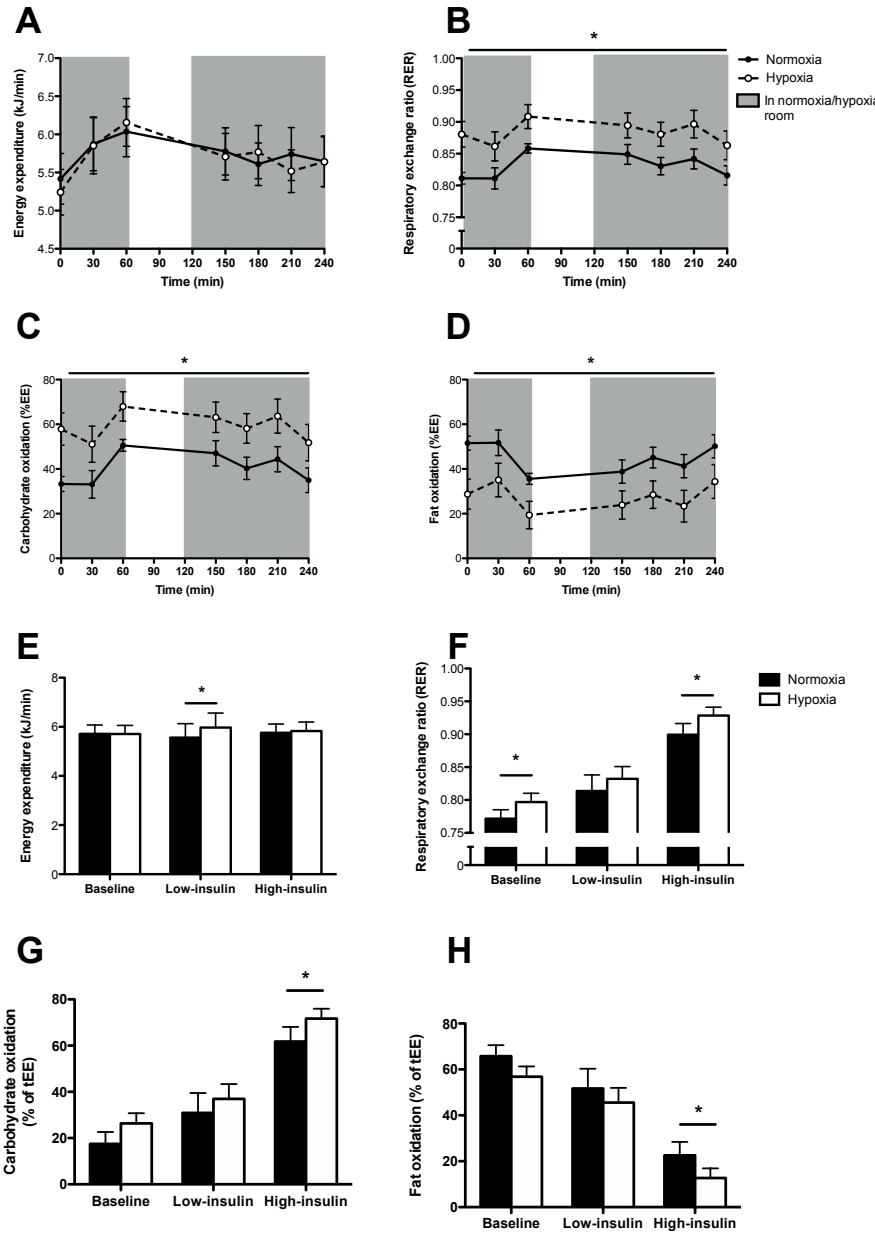

Figure 7: Energy expenditure and substrate oxidation during the HFMM (day 7) under hypoxia (FiO_2 15%) and normoxia FiO_2 21%) and clamp (day 8, normoxia). Energy expenditure (A and E), respiratory quotient (B and F), carbohydrate oxidation (C and G), and fat oxidation (D and H). Data is shown as mean ± SEM (HFMM n=8, clamp n=6). * P<0.05.

The effects of mild intermittent hypoxia on insulin sensitivity

On day 8, the cumulative effect of MIH exposure for seven consecutive days on insulin sensitivity was determined. We found no significant differences in insulin sensitivity between MIH and normoxic exposure, based on the glucose infusion rates (GIR) during the low (10 $mU*m^{-2}*min^{-1}$) and the high insulin infusion step (40 $mU*m^{-2}*min^{-1}$) (Figure 8A). Insulin-stimulated suppression of FFA during the low insulin infusion step, reflecting AT insulin sensitivity, was also not significantly different between both conditions (Figure 8B). Moreover, MIH had no significant effects on fasting plasma glucose (MIH: 5.58 ± 0.31, normoxia: 5.52 ± 0.31) and insulin concentrations (MIH: 13.52 ± 4.69, normoxia: 13.49 ± 4.31) on day 8 (the day following the 7d exposure regimen) (Figure 8C and 8D). In line, $HOMA_{IR}$ remained unchanged following MIH (MIH: 3.37 ± 1.26, normoxia: 3.28 ± 0.97) (Figure 8E).

Skeletal muscle mitochondrial oxidative capacity

At day 8, *ex vivo* SM mitochondrial oxidative capacity was determined. MIH did not significantly affect mitochondrial oxidative capacity as compared to normoxia exposure, irrespective of the substrates added. Both pyruvate and octanoyl carnitine, showed no significant differences for state 2 (figure 9A and 9B), state 3 (figure 9C and 9D), or state U (figure 9E and 9F).

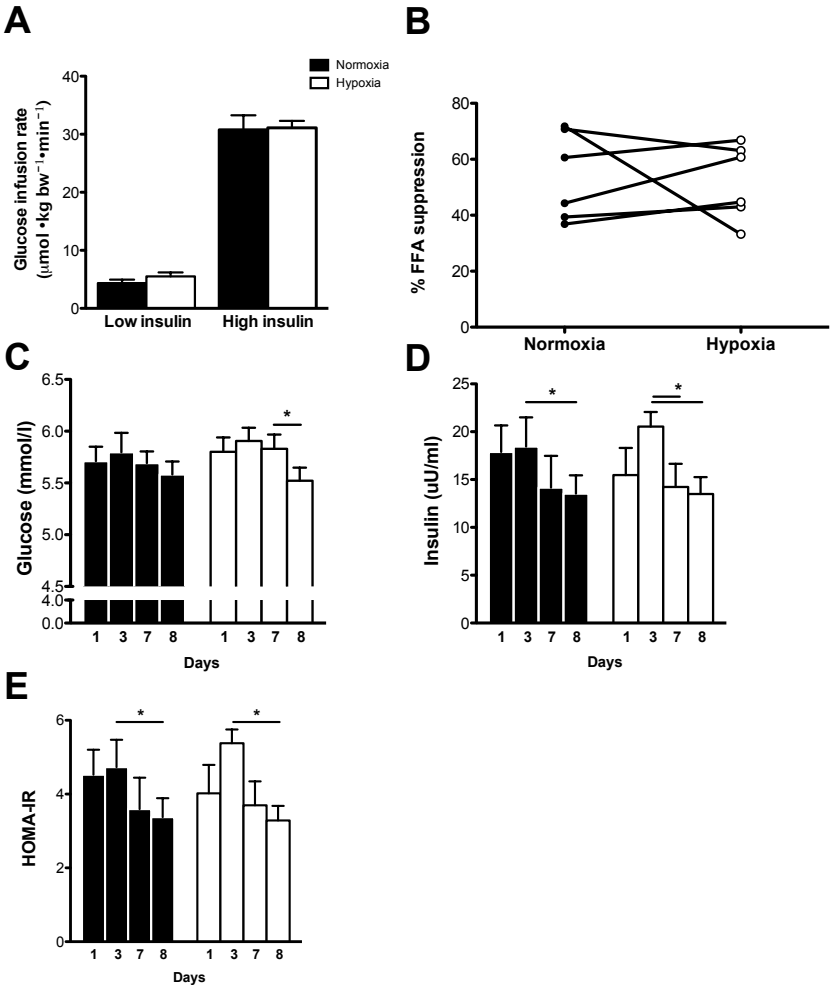

Figure 8: The glucose infusion rate (reflecting whole-body insulin sensitivity) (n=7) (A), percentage suppression of plasma FFA concentrations (reflecting adipose tissue insulin sensitivity) (n=6) (B), plasma glucose (n=6) (C) and insulin concentrations (n=6) (D), and HOMA-IR values (n=6) (E) on day 8, following 7-days MIH and normoxia exposure. Data is shown as mean ± SEM. * P<0.05.

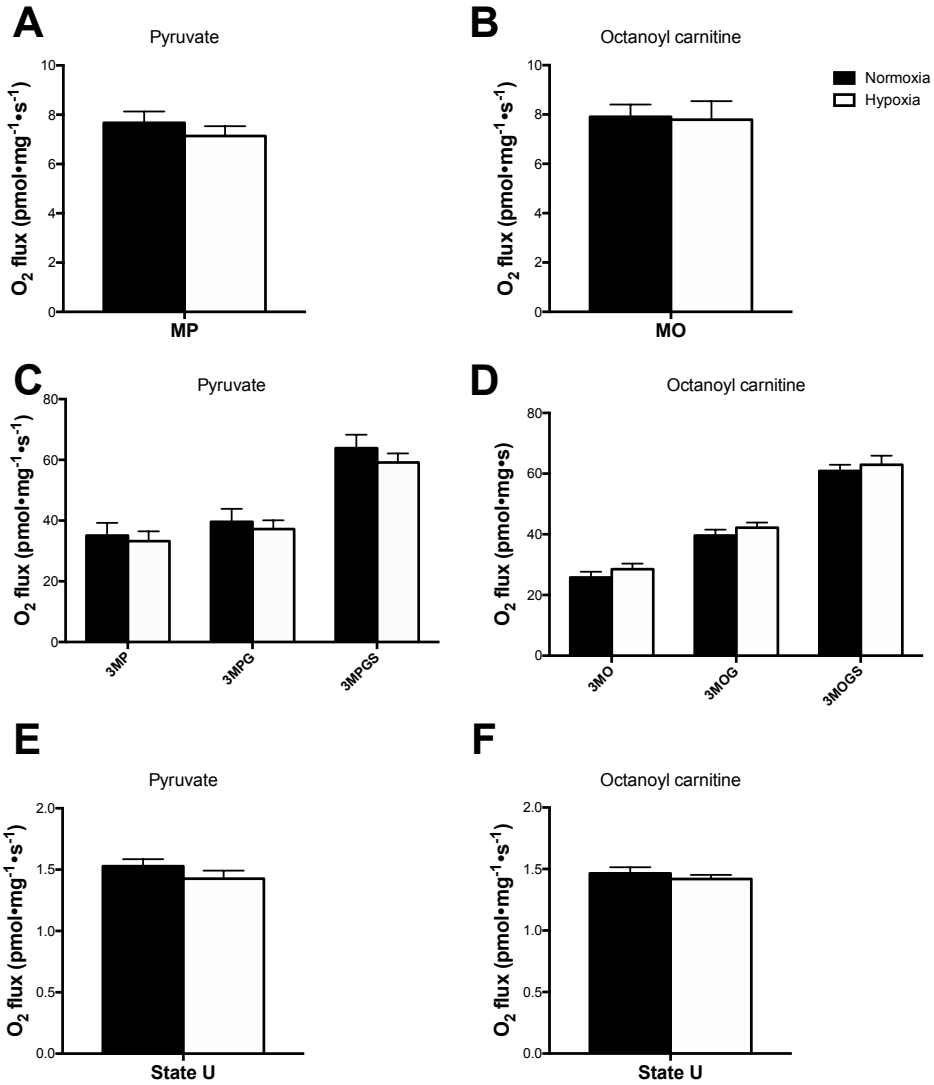

Figure 9: Mitochondrial oxygen flux measured in permeabilized muscle fibers following exposure to MIH (FiO_2 15%, white bars) and normoxia (FiO_2 21%, black bars). Mitochondrial respiration upon the substrates pyruvate (A) and octanoyl carnitine (B) without ADP present. ADP-stimulated respiration (state 3), state 3 respiration fueled by complex I-linked substrates and parallel electron input into complex I and II upon a glucose (C) or lipid substrate (D). Maximal uncoupled respiration upon FCCP (state U) for a glucose (E) and lipid trace (F). Data represents oxygen consumption per mg wet weight per second, and is depicted as mean ± SEM (n=8). M, malate; P, pyruvate; O, octanoyl-carnitine; G, glutamate; S, succinate.

Discussion

This ongoing study investigated the effect of MIH exposure for 7 consecutive days on AT and SM pO_2, substrate metabolism and insulin sensitivity in overweight/obese insulin resistant men. Here, we demonstrated that MIH exposure reduced systemic oxygen saturation and decreased AT and SM pO_2 in humans. Moreover, MIH significantly increased carbohydrate oxidation, while decreasing fat oxidation. This shift in substrate oxidation, which was maintained one day after cessation of the exposure regimen, was reflected by increased plasma lactate concentrations following MIH exposure. Despite changes in substrate oxidation, MIH exposure did not induce significant changes in whole-body insulin sensitivity. Also, SM *ex vivo* mitochondrial oxidative capacity was not altered by MIH as compared to normoxia exposure.

MIH exposure did not affect blood pressure and heart rate, despite the decrease in systemic oxygen saturation that was induced. A hypoxia-induced decrease in systemic oxygen partial pressure may lead to activation of arterial chemoreceptors and stimulation of the sympathetic nervous system, while suppressing the activity of the parasympathetic nervous system.[35] Indeed, previous studies have found that hypoxia exposure increased heart rate and blood pressure due to the secretion of norepinephrine.[36-38] However, conflicting results on the effects of MIH on heart rate and blood pressure have been reported, which may be explained by differences in exposure protocols (i.e. severity of hypoxia, hypobaric versus normobaric conditions).[39, 40] Moreover, the present MIH exposure protocol did not significantly affect hunger and satiety. Previous studies have shown that exposure to low environmental oxygen may reduce appetite, thereby reducing body weight.[41-43] Taken together, the MIH exposure regimen that we applied in the present study did not elicit any adverse events (i.e. on cardiovascular parameters), while significantly reducing systemic oxygen saturation.

As a proof-of-concept, we next demonstrated that the decrease in systemic oxygen saturation during MIH consistently reduced AT and SM pO_2. MIH did not significantly affect hemoglobin and hematocrit levels, suggesting that systemic adaptations were not induced by the MIH protocol that we applied. Thus, the MIH exposure protocol evoked a significant hypoxic stimulus in both AT and SM, which may induce metabolic alterations.

Interestingly, MIH exposure induced a pronounced shift in substrate utilization. More specific, we found that MIH increased fasting and postprandial CHO oxidation by about 50%, while at the same time suppressing fat oxidation. Strikingly, this shift towards CHO oxidation seemed to be maintained after cessation of MIH exposure under normoxic conditions (day 8), suggesting that MIH exposure is able to induce a shift in human substrate utilization also after stopping the exposure. In agreement with our findings,

increased reliance on CHO oxidation during three weeks of chronic hypobaric hypoxia (4,300m) exposure has previously been shown in healthy males.[44, 45] Hypoxia may trigger glucose utilization via AMPK-related pathways, as demonstrated in human and rat soleus muscle.[16, 46] Indeed, increased glucose uptake after hypoxia exposure (1% O_2) has been found both in rodent-derived adipocytes and myotubes.[17, 22, 46] The marked effects of MIH exposure on CHO oxidation, however, did not translate into significant effects on plasma glucose and insulin levels, though it significantly increased circulating lactate concentration. Furthermore, MIH did not alter fasting or postprandial FFA and TAG plasma levels. In contrast, intermittent hypoxia training in mice reduced plasma glucose and cholesterol levels, body weight and prevented steatosis in liver cells.[47] Since we assesses substrate metabolism at the whole-body level, we cannot exclude that MIF affects metabolism at the tissue level.

Based on our previous findings of higher AT pO_2 in obese men[5], and the positive association between AT pO_2 and insulin resistance in humans[5], we hypothesized that lowering of AT pO_2 may contribute to an improved whole-body insulin sensitivity. However, these preliminary data demonstrated that MIH did not significantly alter insulin sensitivity, assessed one day after cessation of the exposure regimen using the gold standard hyperinsulinemic-euglycemic clamp. The unchanged insulin sensitivity together with the increase in CHO oxidation may suggest that non-oxidative glucose disposal (NOGD) (i.e. glycogen storage) was decreased following MIH exposure. Decreased NOGD seems to be the main component of SM insulin resistance in patients with T2DM.[48] Therefore, it can be questioned whether this is a detrimental effect or a normal physiological response to MIH exposure. It has previously been shown that prolonged (10 consecutive days, 10 hours/d,) exposure to 15% O_2 improved insulin sensitivity as compared to normoxia exposure (21% O_2) in obese individuals.[18] Interestingly, intermittent hypoxia exposure (60 cycles/h, 30s of 5% O_2 per cycle, 8h/day for 2 weeks) in mice decreased insulin sensitivity but improved glucose tolerance, which was, at least in part, due to SM-specific activation of adenosine monophosphate-activated protein kinase (AMPK).[49] Noteworthy, in the latter study a severe protocol was applied with respect to both the level of hypoxia (FiO_2 5%) and the number of hypoxic cycles (480 cycles/d), which reflects the situation seen in patients with obstructive sleep apnea syndrome. Strikingly, this effect was maintained for several weeks after cessation of hypoxia exposure.[49] Thus, it may be that a more prolonged exposure to MIH is needed to induce beneficial effects on glucose tolerance and, possibly, adverse effects on insulin sensitivity.

To further elucidate the effects of MIH on substrate oxidation, we investigated mitochondrial function in human SM. We found that MIH had no effects on *ex vivo* SM mitochondrial function, irrespective of the substrates (pyruvate and octanoyl carnitine)

used. Hypoxia has been proposed as a determinant of the muscle oxidative phenotype.[50] It has been previously shown that acute hypoxia (4% O_2) exposure increased HIF-1α, inhibited the PPAR/PGC-1α pathway, and decreased the expression of mitochondrial components in cultured muscle cells, whilst increasing the expression of slow-oxidative type 1 myosin.[51] Moreover, prolonged hypoxia (8% O_2) exposure decreased the muscle oxidative phenotype in aged mice, whereas young mice showed the most prominent change in fiber-type composition.[52] Interestingly, chronic hypoxia exposure (8% O_2) in mice increased gene expression of markers involved in oxidative metabolism in AT.[53] Based on these in vitro and animal data, we cannot exclude that mitochondrial function in our study would have been altered after a more severe hypoxia exposure and/or a longer exposure duration.

The strengths of the present study are that all measurements were performed under well-controlled conditions, while subjects were fed according to energy requirements and were in energy balance throughout the study. Furthermore, we studied the isolated effect of MIH under normobaric conditions, thereby excluding possible effects of different ambient pressure (hypobaric hypoxia) as present at high altitude. A limitation is the number of subjects (n=8) included in the present analyses. In addition, although we aimed to study the effects of prolonged rather than acute MIH exposure, the study duration was still relatively short. Therefore, future studies should investigate the cardiometabolic effects of exposure duration, the amount of cycles and severity of hypoxic in more detail.

In conclusion, these preliminary findings demonstrate that exposure to MIH decreases systemic oxygen saturation, leading to a pronounced reduction in AT and SM pO_2. MIH exposure for seven days evoked a shift in substrate utilization towards increased carbohydrate oxidation, which was still present the day following cessation of the MIH regimen, but did not significantly alter glucose homeostasis and whole-body insulin sensitivity in overweight/obese men. Future studies are warranted to investigate the potential of different hypoxia exposure regimens to affect human substrate metabolism and insulin sensitivity, thereby possibly providing an alternative strategy to prevent obesity-related metabolic complications.

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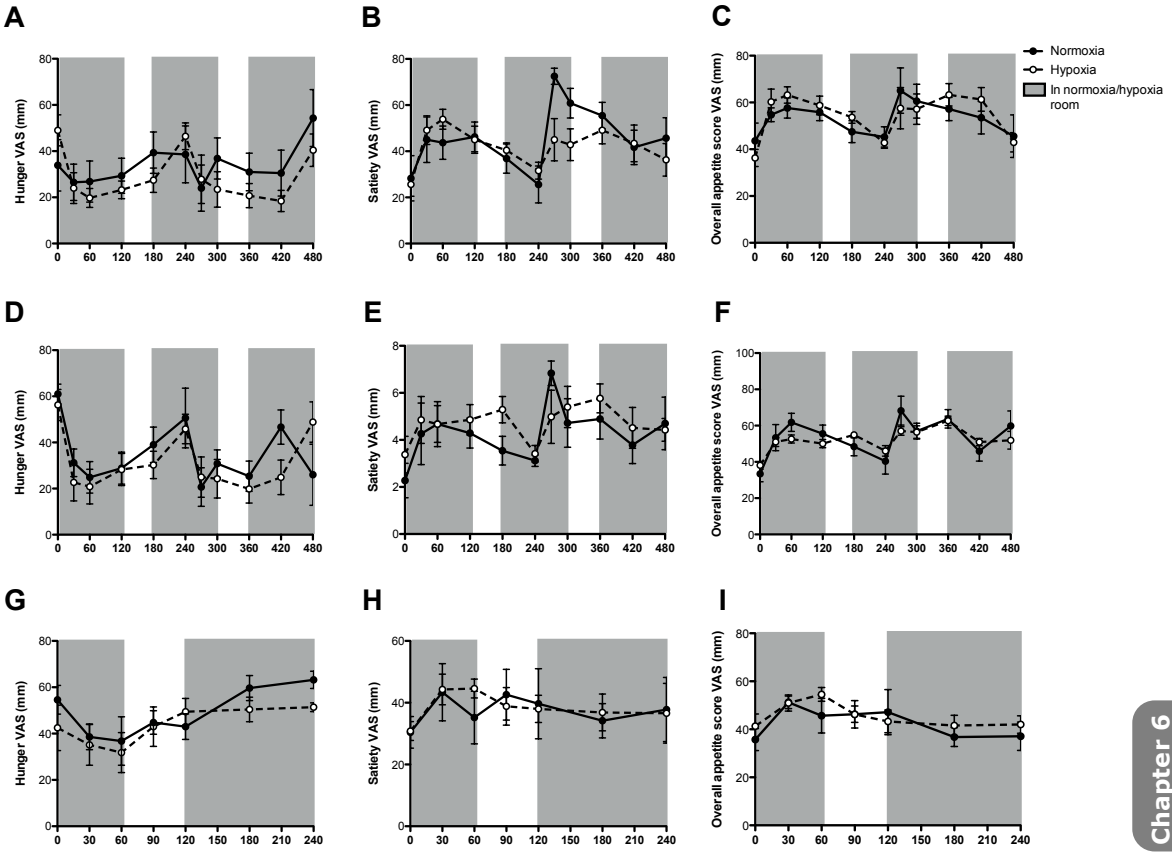
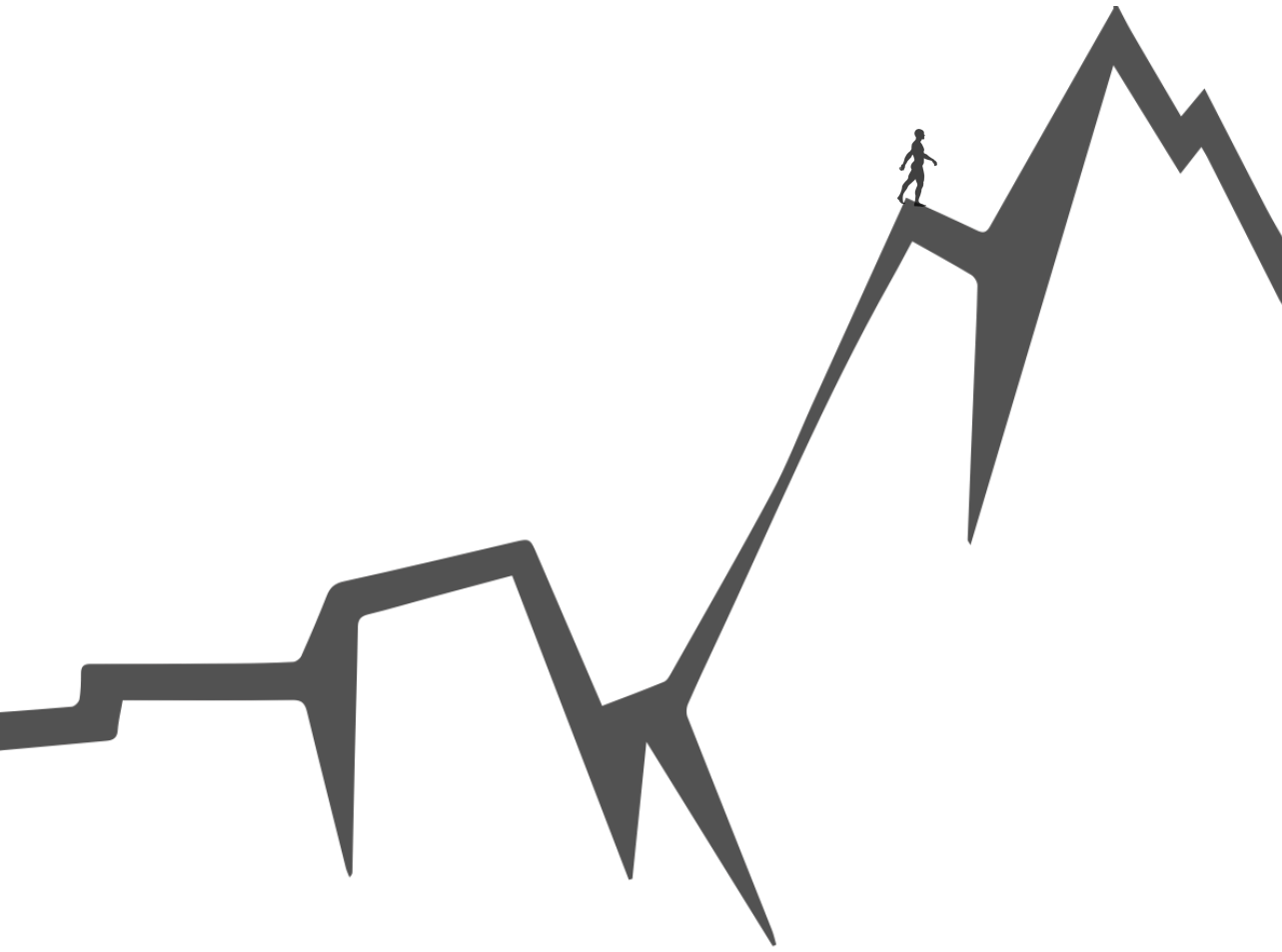
Supplemental Figure 1



Supplemental Figure 1: Appetite ratings (assessed by VAS) for hunger and satiety during day 1 (A-C), 3 (D-F) and 7 (G-H) (HFMM). At several time points during the day, a VAS questionnaire was taken for hunger (A, D and G), satiety (B, E and H), and the overall appetite score (OAS) was calculated (C, F, I). The grey area shows the time that individuals resided inside of the hypoxic (FiO_2 15%) or normoxic (FiO_2 21%) room. Data is shown as mean ± SEM (n=6-8).



CHAPTER 7

The effects of mild intermittent hypoxia exposure on adipocyte function

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In preparation

Abstract

Background: Obesity is characterized by adipocyte dysfunction, which contributes to impairments in glucose and lipid metabolism. Recent studies suggest that mild hypoxia exposure (MIH) may improve glucose homeostasis. Here, we investigated the effect of MIH exposure on abdominal subcutaneous adipocyte function.

Methods: Human abdominal subcutaneous adipose tissue-derived mesenchymal stem cells (hMADS) were isolated from biopsies obtained from four overweight and obese men. Adipocytes were exposed to different MIH regimens, varying in the duration (7 or 14 days) and pattern (3 or 6 cycles of 2h per day) of exposure, or were continuously exposed to high physiological pO_2 (10% O_2) or normal environmental conditions (21% O_2) for 14 days during their differentiation phase. In the MIH protocols, oxygen levels cycled from 10% to 5% O_2, resembling high and low physiological values in human adipose tissue, respectively. Following exposure, gene expression, adipokine secretion and glucose uptake were assessed in differentiated hMADS.

Results: Exposure to the different MIH protocols did not induce pronounced effects on adipocyte gene expression of markers of inflammation, lipid and glucose metabolism, mitochondrial function and browning as compared to continuous exposure to 10% O_2. The secretion of IL-6 was decreased after all MIH exposure protocols as compared to continuous exposure to 10% O_2, with no clear differences between MIH regimens. Both continuous exposure to 10% O_2 and all MIH regimens increased basal but not insulin-stimulated glucose uptake versus 21% O_2, but no additional effects of MIH exposure on glucose uptake were found as compared to continuous 10% O_2 exposure.

Conclusion: Exposure of human abdominal subcutaneous adipocytes to MIH (5% O_2 cycles of 2h) reduced IL-6 secretion but did not induce different effects on glucose uptake and gene expression of metabolic and inflammatory markers as compared to continuous 10% O_2 exposure. These findings suggest that MIH has minor effects on adipocyte function.

Introduction

The adipose tissue (AT) plays an important role in the etiology of obesity-related comorbidities. Obese insulin resistant individuals and patients with type 2 diabetes are characterized by AT dysfunction,[1] which is hallmarked by enlargement of adipocytes,[1, 2] an impaired AT blood flow,[1, 3-5] an increased production of pro-inflammatory cytokines,[3, 6] and impairments in lipid and glucose metabolism.[1, 6, 7] It has been suggested that the amount of oxygen to which tissues are exposed may impact metabolism and cardiometabolic health.[8] In line, we found that AT oxygen tension (AT pO_2) was higher in obese insulin resistant (~9% O_2) as compared to lean insulin sensitive individuals (~6% O_2), and that AT pO_2 was positively associated with AT inflammation and whole-body insulin resistance.[5] Moreover, diet-induced weight loss induced a marked and consistent decrease in AT pO_2 (~20% decrease) in obese individuals that was accompanied by a reduction in adipocyte size and an increased insulin sensitivity.[9] These data support the hypothesis that reducing AT pO_2 might be a potential strategy to improve AT function and metabolic health.[8] A reduction in AT pO_2 may be achieved by lowering the oxygen content of inhaled air, thereby reducing oxygen delivery to peripheral tissues. However, it remains to be established which oxygen levels and what exposure duration may affect adipocyte function.

It has previously been reported that continuous exposure of differentiating abdominal subcutaneous adipocytes to 5% O_2 (low physiological pO_2) induced a lower expression of IL-6 and reduced secretion of DPP-4 as compared to 10% O_2 exposure (high physiological pO_2),[10] indicating a possible beneficial effect of low physiological pO_2 on adipocyte functioning. Moreover, continuous exposure to physiological pO_2 (5-10% O_2) has been shown to alter gene expression, lipid droplet size, adipokine secretion and lipolytic rate as compared to 21% O_2 exposure (i.e. normal laboratory conditions) in human abdominal subcutaneous adipocytes.[10] Recently, we have demonstrated that continuous exposure of human mesenchymal stem cells derived from abdominal subcutaneous and femoral AT to low physiological oxygen levels (5% O_2) during differentiation reduced the expression of pro-inflammatory genes and altered adipocyte physiology (Chapter 3). Moreover, acute exposure to severe hypoxia (1% O_2) has been shown to increase insulin-dependent and -independent glucose uptake, while multiple exposures to transient hypoxia during differentiation enhanced insulin signaling in *3T3-L1* adipocytes.[11] Taken together, prolonged exposure to hypoxia may induce beneficial metabolic adaptations in adipocytes. Interestingly, evidence suggests that intermittent hypoxia exposure (9-16% O_2 in environmental air (FiO_2), 3-15 cycles/day) may also have

substantial effects on lipid and glucose metabolism, as reviewed.[12] The advantages of intermittent as compared to continuous hypoxia exposure may be that the exposure duration is shorter, and certain systemic adaptations that could blunt the hypoxic stimulus and related metabolic effects may be prevented or delayed.

Here, we hypothesized that exposure to normobaric *mild intermittent hypoxia* (MIH) improves human adipocyte function. Therefore, we investigated the effects of different MIH exposure protocols, varying in the duration and pattern of exposure, on gene expression of metabolic and inflammatory markers, adipokine secretion, and glucose uptake in differentiated human abdominal subcutaneous AT-derived mesenchymal stem cells (hMADS).

Materials and Methods

Human primary adipocyte cell culture experiments

Human abdominal subcutaneous adipose tissue-derived mesenchymal stem cells (hMADS), an established human white adipocyte model,[13, 14] were obtained from four male overweight and obese men with impaired glucose metabolism (subject characteristics are shown in Supplemental Table 1). Next, a pool of hMADS from these individuals were differentiated into the adipogenic lineage. Therefore, cells were seeded at a density of 2000 cells/cm^2 and kept in proliferation medium (Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture (Gibco, Blijswijk, NL, USA), 10% fetal bovine serum (Bodinco BV, Alkmaar, NL, USA) and 50 units ml^{-1} penicillin (Gibco), 50 µg ml^{-1} of streptomycin (Gibco)). At ~80% confluence, IBMX (Sigma, St Louis, MI, USA) and rosiglitazone (Enzo Life Sciences, Raamsdonksveer, NL, USA) were added to induce adipogenic differentiation. All cells were proliferated under 21% O_2, and thereafter the cells were exposed to different protocols under normobaric conditions (Figure 1):

1) Continuous exposure to 21% O_2 (Protocol 1)
2) Continuous exposure to 10% O_2 (Protocol 2)
3) Continuous exposure to 10% O_2 for 7 days, MIH exposure, 3x2h (Protocol 3) for 7 days
4) Continuous exposure to 10% O_2 for 7 days, MIH exposure, 6x2h (Protocol 4) for 7 days
5) MIH exposure, 3x2h (Protocol 3) for 14 days
6) MIH exposure, 6x2h (Protocol 4) for 14 days

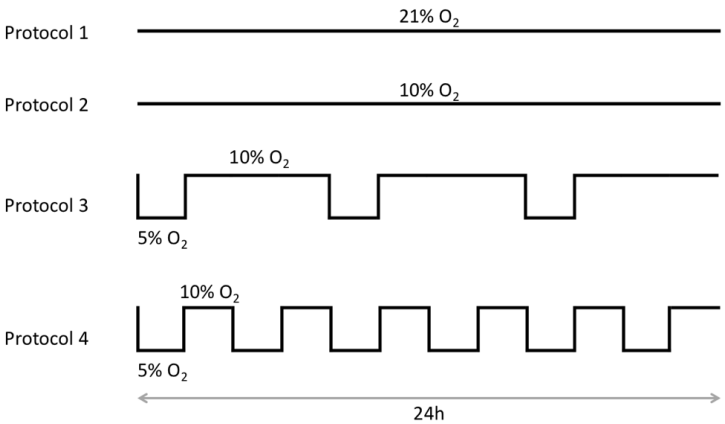

Figure 1: hMADS were exposed to continuous oxygen levels (21% and 10% O_2) or mild intermittent hypoxia (5% O_2 cycles of 2 hours) under normobaric conditions. The MIH regimen varied in the number of hypoxic cycles (3 or 6 cycles/d) and the duration of exposure (7 or 14 days).

Thus, the cells were exposed to continuous exposure to 21% and 10% O_2, or mild intermittent hypoxia exposure (10%-to-5% O_2 cycle) for 7 or 14 days, with 3x2h or 6x2h cycles per day. For the 7 days MIH protocols, cells were first exposed to continuous 10% O_2 for 7 days during differentiation, after which they were exposed to the MIH protocol for the last 7 days of differentiation. For the 14 days MIH protocols, cells were exposed to MIH for 14 days (during the entire differentiation period). In our hands, 10% and 5% O_2 levels approximate the average AT pO_2 values of obese insulin resistant and lean insulin sensitive subjects, respectively.[5]
Gas mixtures were refreshed every 8 (protocols 1 and 2) or every 2 hours (protocols 3 and 4), while medium was refreshed 2-3 times/wk. The cells were exposed to hypoxia using an in-house developed system, as described before.[15] All experiments and material collection, which are described in detail below, were carried out on day 14 of the adipogenic differentiation.

Adipocyte gene expression
Total RNA was extracted from hMADS at day 14 of the adipogenic differentiation using TRIzol reagent (Invitrogen, Breda, The Netherlands), and SYBR-Green based real-time PCRs were performed using an iCycler (Bio-Rad, Veenendaal, NL, USA; primer sequences are provided in Supplemental Table 2). Results were normalized to the geometric mean of 18S ribosomal RNA and RPL13A.

Adipokine secretion
The medium surrounding the differentiated adipocytes was collected over 24h (at day 13-14) to determine the secretion of IL-6, MCP-1, and adiponectin using high-sensitive ELISA (ELISA kits were purchased: Adiponectin from Biovendor, IL-6 and MCP-1 from Diaclone). If necessary, samples were diluted with a provided dilution buffer from the manufacturer prior to the assay, which was performed in duplicate, according to the manufacturer's instructions.

Glucose uptake
Basal and insulin-stimulated glucose uptake was measured in differentiated hMADS using 2-deoxy-D-glucose, as described before.[13] Cells were serum-starved for 2h in DMEM low-glucose. After two washes in Krebs Ringer buffer, cells were incubated for 30 min with or without insulin (100 nM). Then, 5 µM 2-deoxy-D-glucose and 0.1 µCi 2-deoxy-D-[^{3}H] glucose per well were added for 30 min at 37 °C. Cells were then scraped in

0.05 M NaOH, and 2-deoxy-D-[^{3}H] glucose uptake was measured by liquid scintillation counting of the cell lysates.

Statistical analyses

Data are presented as mean ± SEM. The effects of MIH on adipocyte gene expression, adipokine secretion, and adipocyte glucose uptake were analyzed using Student's paired *t*-test. More specific, we compared differences between 21% versus 10% O_2 exposure, and between the MIH regimens versus continuous 10% O_2 exposure using Student's paired *t*-tests. Calculations were performed with SPSS version 24 for Macintosh (Chicago, IL). $P<0.05$ was considered statistically significant.

Results

Abdominal subcutaneous adipocyte gene expression

To investigate the effects of MIH exposure on human adipocyte function, the gene expression of several markers related to AT inflammation, glucose and lipid metabolism, mitochondrial function and browning were determined.

Effect of continuous exposure to 10% O_2 on gene expression

Leptin gene expression was reduced after continuous 10% O_2 as compared to 21% O_2 exposure (p=0.005, Figure 2A). Moreover, PPARγ, GLUT-1, DPP-4 and PAI-1 were significantly increased after continuous 10% O_2 as compared to 21% O_2 exposure (Figure 2C, D, F, G). Gene expression of the pro-inflammatory markers IL-6 and MCP-1 (Figure 2H-I), markers of lipolysis (HSL and ATGL; Figure 2J-K), lipogenesis (ACC2, FASN and SCD1; Figure 2L-N), angiogenesis (VEGF-A; Figure 2O), mitochondrial function and biogenesis (PGC-1α; Figure 2P) and browning (UCP-1 and PRDM16; Figure 2Q-R), did not show significant differences between continuous exposure to 10% and 21% O_2.

Effect of MIH on gene expression

The MIH protocol, 7d 3x2h, decreased leptin gene expression as compared to continuous 10% O_2 (p=0.014), while leptin gene expression was significantly increased in the other MIH protocols (7d 6x2h: p=0.014, 14d 3x2h: p=0.032, and 14d 6x2h: p=0.091). Overall, no clear effects of the MIH exposure protocols on gene expression were found as compared to continuous exposure to 10% O_2, except for some minor differences. Thus, MIH does not seem to induce pronounced differences in abdominal subcutaneous adipocyte gene expression of inflammatory, lipid metabolism, mitochondrial function/biogenesis, and browning markers as compared to continuous 10% O_2 exposure.

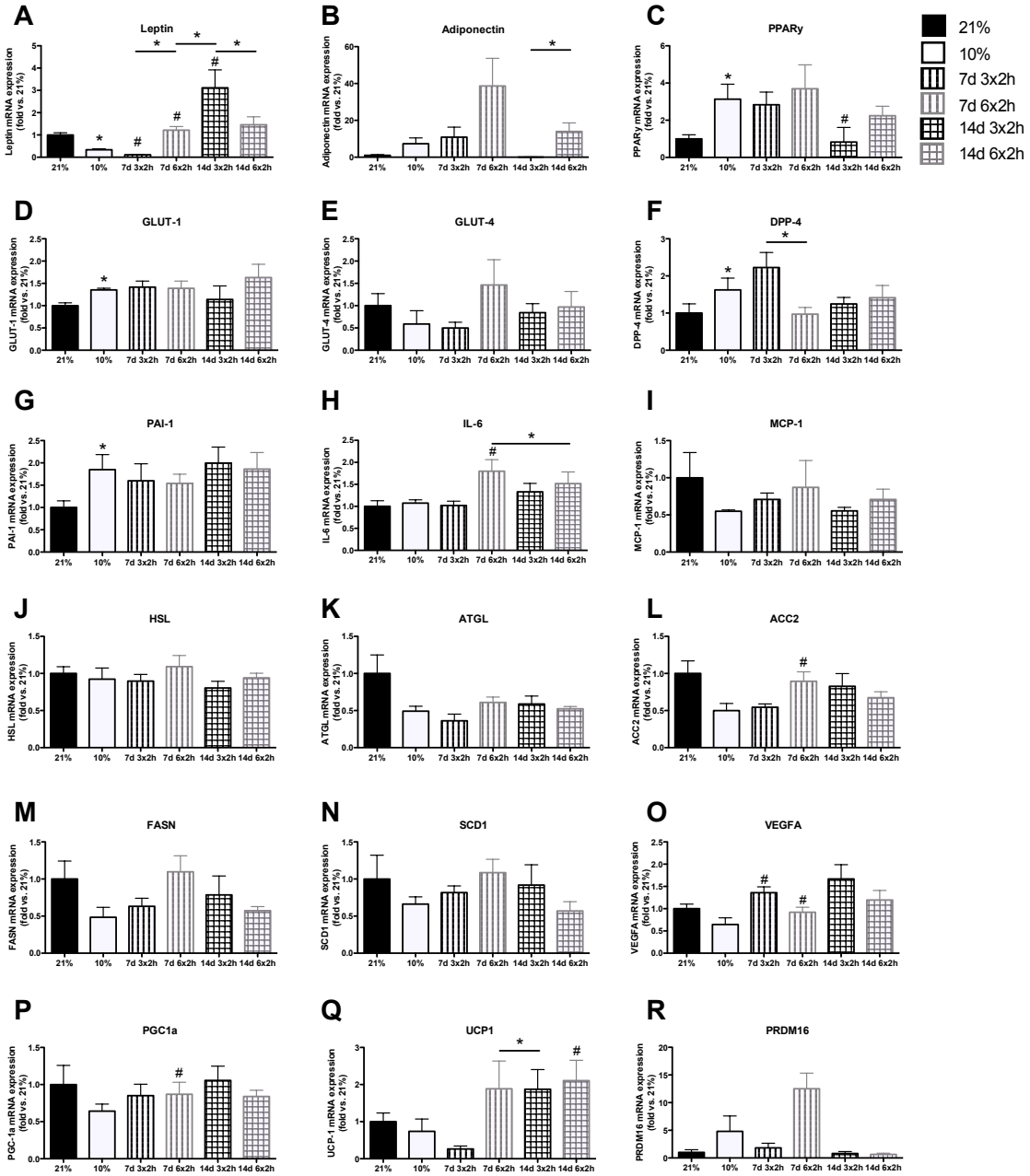

Figure 2. *Gene expression in hMADS following differentiation under continuous oxygen exposure (21 vs 10% O_2) and under distinct MIH protocols, varying in duration (7 or 14 days) and number of hypoxic 10-to-5% O_2 cycles (n=4-6 paired samples). (**A**) leptin, (**B**) adiponectin, (**C**) PPARγ, (**D**) GLUT1, (**E**) GLUT4, (**F**) DPP-4, (**G**) PAI-1, (**H**) IL-6, (**I**) MCP-1, (**J**) HSL, (**K**) ATGL, (**L**) ACC2, (**M**) FASN, (**N**) SCD1, (**O**) VEGFA, (**P**) PGC1a, (**Q**) UCP1, and (**R**) PRDM16. Values are mean ± SEM. * p<0.05 vs. 21% or between MIH exposure protocols, # p<0.05 vs. 10%.*

Adipokine secretion

Effect of continuous exposure to 10% O_2 on adipokine secretion

Next, we investigated the effects of continuous exposure of 10 versus 21 % O_2 on adipokine secretion in differentiated hMADS. IL-6 secretion was increased after continuous 10% O_2 exposure as compared to 21% O_2 (p=0.003, Figure 3A), despite unchanged IL-6 gene expression (Figure 2F). No significant differences were found in MCP-1 and adiponectin secretion between continuous 10% and 21% O_2 exposure (Figure 3B and 3C).

Effect of MIH on adipokine secretion

When adipocytes were exposed to MIH, the secretion of IL-6 was decreased as compared to continuous 10% O_2 exposure (7d 3x2h: p=0.040, 7d 6x2h: p=0.043, 14d 3x2h: p=0.053, and 14d 6x2h: p=0.033). No clear differences in IL-6 secretion were present between the different MIH protocols, except for lower IL-6 secretion following 7d 6x2h versus 7d 3x2h (p=0.015). No significant differences were found in MCP-1 and adiponectin secretion between the MIH regimens and continuous 10% exposure (Figure 3B and 3C).

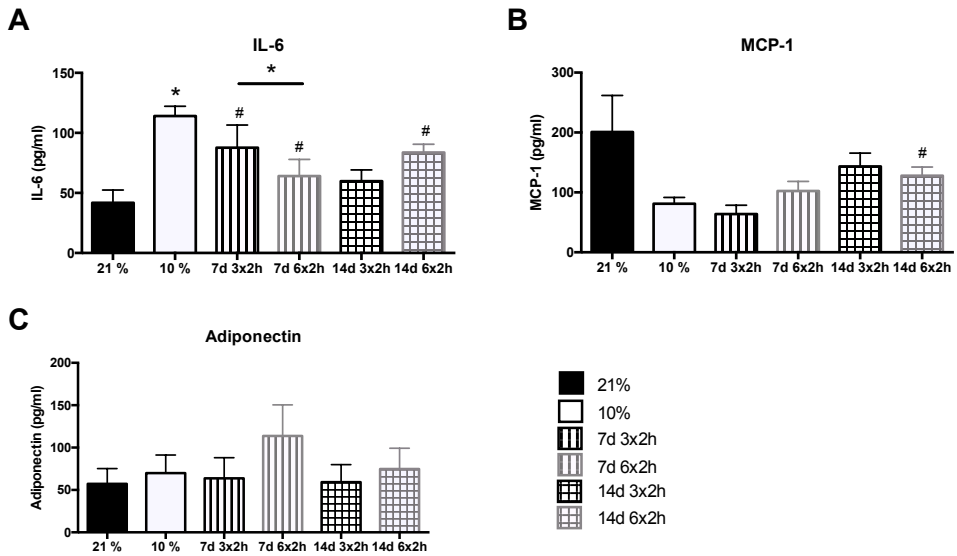

Figure 3. *Secretion of adipokines from hMADS derived from abdominal AT following differentiation continuous oxygen exposure (21 vs 10% O_2) and under distinct MIH protocols, varying in duration (7 or 14 days) and number of hypoxic 10-to-5% O_2 cycles (n=4-6 paired samples). (**A**) IL-6, (**B**) MCP-1, and (**C**) adiponectin. Values are mean ± SEM. * p<0.05 vs. 21% or between MIH exposure protocols, # p<0.05 vs. 10%.*

Basal and insulin-stimulated glucose uptake

Exposure of adipocytes to 10% O_2 continuously, significantly increased basal glucose uptake (p=0.035) as compared to 21% O_2 (Figure 4). Insulin stimulation resulted in an increase in glucose uptake after continuous 21% (p=0.033) and 10% O_2 (p=0.023), with no significant increase after MIH protocols (Figure 4).

The more prolonged MIH exposure protocols, 14d 3x2h and 14d 6x2h, significantly increased basal glucose uptake as compared to 21% O_2 (p=0.028 and p=0.041, respectively), while both shorter MIH exposure regimens, 7d 3x2h and 7d 6x2h, showed a trend for an increased basal glucose uptake as compared to 21% O_2 (p=0.052 and p=0.080, respectively) (Figure 4). Basal glucose uptake was not significantly different between the MIH exposure regimens and continuous exposure to 10% O_2.

Insulin-stimulated glucose uptake was only significantly increased as compared to basal glucose uptake following continuous exposure to 10% (p=0.023) and 21% O_2 (p=0.033). The insulin-induced increase in glucose uptake (insulin-stimulated – basal uptake), however, was not significantly different between exposure protocols (data not shown) (Figure 4).

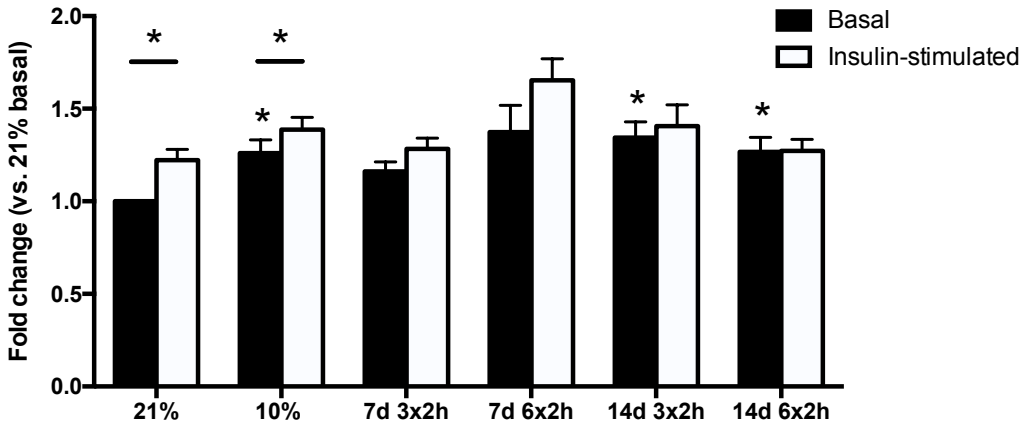

Figure 4. *Glucose uptake in hMADS derived from abdominal AT following differentiation according to continuous oxygen exposure (21 and 10% O_2) and different MIH protocols, varying in duration (7 or 14 days) and number of hypoxic 10-to-5% O_2 cycles (n=4). Values are mean ± SEM. * p<0.05 vs. 21% or between basal and insulin-stimulated.*

Discussion

Adipocyte dysfunction in obesity is characterized by inflammation, impairments in lipid metabolism, and mitochondrial dysfunction.[1, 6, 7, 16, 17] The aim of the present study was to investigate whether MIH exposure alters adipocyte functionality in hMADS derived from overweight/obese men as compared to continuous exposure to high physiological pO_2 exposure (10% oxygen) under normobaric conditions. We demonstrated that IL-6 secretion was reduced after exposure to MIH as compared to continuous 10% O_2 exposure, irrespective of the number of cycles and duration of exposure. Furthermore, basal but not insulin-stimulated glucose uptake was increased to a similar extent following MIH and continuous 10% O_2 exposure as compared to 21% O_2. However, MIH exposure did not significantly affect gene expression of markers related to inflammation, glucose and lipid metabolism, mitochondrial function/biogenesis and browning. Collectively, these data suggest that MIH, with pO_2 fluctuating from high to low physiological values, does not induce major alterations in the abdominal subcutaneous adipocyte phenotype as compared to continuous exposure to 10% O_2.

In the present study, we found no significant effects of MIH exposure on adipocyte gene expression of the pro-inflammatory markers IL-6, DPP-4, MCP-1, and PAI-1**.** In contrast, others[10] and we (Chapter 3) have previously shown that prolonged exposure (14 days) to continuous mild hypoxia (5% O_2) altered adipocyte gene expression of inflammatory markers as well as adipokine secretion.
MIH also had no significant effects on markers related to lipolysis, lipogenesis, browning of white adipocytes, and mitochondrial function/biogenesis. More specific, MIH did not significantly affect the expression of ATGL, HSL, SCD1, ACC2, FASN, UCP-1, PRDM16, and PGC-1α. Previous studies have demonstrated that continuous physiological O_2 exposure may alter AT lipid metabolism, possibly mediated through perilipin that was found to be elevated after prolonged exposure to 5 and 10% O_2.[10] Interestingly, overexpression of perilipin reduced lipid droplet size in *3T3-L1* adipocytes and also shifted the cells towards a brown-like adipocyte phenotype, evidenced by increased expression of adipocyte browning markers such as PGC-1α and PRDM16, and reduced expression of genes related to lipid synthesis.[18] In line with our present findings, Famulla et al.[10] did not observe any differences in UCP-1 mRNA expression after continuous exposure to physiological pO_2 (5% and 10% O_2) as compared to 21% O_2.[10] In contrast to our findings of unchanged gene expression of ATGL and HSL, continuous exposure to physiological

pO_2 increased lipolysis in the latter study.[10] Thus, it might be that MIH exposure has no effects on lipolysis, although care has to be taken in interpreting this since gene expression of lipolytic markers does not necessarily reflect the lipolytic rate.

The present findings showed that adipocyte gene expression of leptin was decreased whilst DPP-4 expression was increased after continuous 10% as compared to 21% O_2 exposure. These results are in line with previous observations that the secretion of leptin was decreased and DPP-4 protein expression as well as secretion was higher after 10% versus 21% O_2 exposure.[10] A reduction in DPP-4 production by adipocytes may be beneficial, since DPP-4 was found to positively correlate with BMI and is upregulated by TNF-α.[19] Moreover, DPP-4 inhibition reduced inflammatory markers and infiltration of inflammatory cells in obese mice.[20, 21] Importantly, however, in the present study we found that MIH had no significant effects on leptin and DPP-4 gene expression as compared to continuous exposure to 10% O_2.

Moreover, in the present study, we found that MIH exposure had no significant effects on MCP-1 and adiponectin secretion as compared to continuous exposure to 10% O_2 and 21% O_2. Interestingly, however, MIH decreased the secretion of IL-6 as compared to continuous 10% O_2 exposure. This may reflect a beneficial effect on the inflammatory phenotype, yet no differences were observed for MCP-1 secretion and gene expression of pro-inflammatory markers.

Furthermore, the expression of PPARγ was increased after continuous 10% as compared to 21% O_2 exposure, but we did not find a clear effect of MIH exposure on PPARγ expression in human adipocytes, except for a reduction of PPARγ expression following 14 days of exposure to 3x2h hypoxia per day versus continuous exposure to 10% O_2. Since PPARγ is an important regulator of adipocyte differentiation, these findings may suggest that MIH has no pronounced effects on adipocyte differentiation. Previous findings on the effects of continuous hypoxia on adipocyte differentiation are conflicting, with a study showing no effects on adipocyte differentiation after continuous physiological O_2 exposure (5% and 10% O_2),[10] whilst others have reported either induction (2% O_2, 4-7 days)[22] or inhibition of adipocyte differentiation (2% O_2, during differentiation)[23]. The reason for these discrepancies in study outcomes remains to be elucidated.

In addition, we found that continuous exposure to 10% O_2 increased basal glucose uptake as compared to 21% O_2 exposure. In accordance, it has been shown that acute, severe hypoxia (1% O_2) exposure increased both basal and insulin-stimulated glucose uptake in *3T3-L1* adipocytes, which may at least partly be explained by elevated AMPK activity.[11] Although we hypothesized that MIH may be a more potent stimulus to trigger metabolic adaptations, we did neither find significant differences in basal nor insulin-

induced glucose uptake between MIH and continuous 10% O_2 exposure in human adipocytes. Interestingly, insulin stimulation did not further increase basal adipocyte glucose uptake following the different MIH exposure regimens. It may be that the maximal capacity for glucose uptake was already reached under basal conditions following MIH exposure, and that stimulation with insulin had no additional effect on GLUT4 translocation in these adipocytes. The latter is underscored by the observation that absolute insulin-stimulated glucose uptake did not differ between exposure conditions. Alternatively, one could argue that MIH evoked adipocyte insulin resistance, since it has previously been found that severe hypoxia (1% O_2) exposure impaired insulin signaling in both murine and human adipocytes.[24] In contrast, repeated exposure of differentiating *3T3-L1* adipocytes to transient severe hypoxia (1% O_2) was able to reprogram the cells, leading to enhanced insulin sensitivity.[11] More recently, it has been shown in mice that two weeks of prolonged exposure to severe intermittent hypoxia *in vivo* (60 cycles/hour, FiO_2 5%, 8 hours/day) induced systemic insulin resistance due to impairments in insulin signaling in skeletal muscle, the liver and adipose tissue. Interestingly, however, glucose tolerance was markedly improved, which seemed to be due to enhanced AMPK signaling.[25] Future studies need to investigate the effects of prolonged MIH on insulin signaling in human adipocytes.

Importantly, until now, no studies have investigated the effects of exposing adipocytes to intermittent physiological oxygen. Since there is a pronounced discrepancy between MIH and continuous hypoxia exposure, it could be that these protocols yield different results, where also exposure time can be important factor. Indeed, an important difference between this study and previous studies (i.e. Famulla et al.[10] and Chapter 3 of this thesis) is the total hypoxia exposure time. It may well be that more prolonged exposure is needed to induce changes in adipocyte gene expression.

In conclusion, the present study demonstrated that exposure of human abdominal subcutaneous adipocytes to MIH (5% O_2 cycles of 2h) reduced IL-6 secretion but did not have any additional impact on adipokine secretion, adipocyte glucose uptake and gene expression of markers related to inflammation, lipid metabolism, and mitochondrial function/biogenesis, and browning of white adipocytes as compared to continuous 10% O_2 exposure. Future studies should investigate whether MIH exposure alters insulin signaling in human adipocytes, may affect the functionality of other cell types in adipose tissue (i.e. immune cells), and whether the effects of MIH on adipocytes are related to the metabolic phenotype of the tissue donors.

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Supplemental *Table 1*. *Characteristics of hMADS donors (n=4, *n=3).*

	Average ±SEM
Age (yrs)	*62.8 ±2.7*
Weight (kg)	*92.3 ±3.5*
BMI (kg/m^2)	*29.2 ±0.7*
Waist circumference (cm)	*107.5 ±3.9*
Waist/hip ratio	*1.05 ±0.02*
Fasting glucose (mmol/L)	*8.6 ±1.8**
Fasting insulin (mU/L)	*11.4 ±2.0*
HbA1c (%)	*6.2 ±0.5*
HOMA-IR	*4.9 ±1.6**

Supplemental Table 2. Primer sequences.

		Sequence
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
PGC-1α	Forward	TCTGAGTCTGTATGGAGTGACAT
	Reverse	CCAAGTCGTTCACATCTAGTTCA
GLUT4	Forward	TGGGCGGCATGATTTCCTC
	Reverse	GCCAGGACATTGTTGACCAG
VEGFA	Forward	TTGCCTTGCTGCTCTACCTCCA
	Reverse	GATGGCAGTAGCTGCGCTGATA
ATGL	Forward	GTGTCAGACGGCGAGAATG
	Reverse	TGGAGGGAGGGAGGGATG
HSL	Forward	GCGGATCACACAGAACCTGGAC
	Reverse	AGCAGGCGGCTTACCCTCAC
PPARγ	Forward	TACTGTCGGTTTCAGAAATGCC
	Reverse	GTCAGCGGACTCTGGATTCAG
GLUT1	Forward	TTGCAGGCTTCTCCAACTGGAC
	Reverse	CAGAACCAGGAGCACAGTGAAG
SCD1	Forward	CCTGGTTTCACTTGGAGCTGTG
	Reverse	TGTGGTGAAGTTGATGTGCCAGC
ACC2	Forward	GCAAGAACGTGTGGGGTTACT
	Reverse	TCGCCTCGGATGGACAGTT
FASN	Forward	CCGAGACACTCGTGGGCTA
	Reverse	CTTCAGCAGGACATTGATGCC
UCP1	Forward	AGTTCCTCACCGCAGGGAAAGA
	Reverse	GTAGCGAGGTTTGATTCCGTGG
PDRM16	Forward	CAGCCAATCTCACCAGACACCT
	Reverse	GTGGCACCTGAAAGGCTTCTCC
RPL13A	Forward	CCTGGAGGAGAAGAGGAAAGAGA
	Reverse	TTGAGGACCTCTGTGTATTTGTCAA
18S	Forward	AGTTAGCATGCCAGAGTCTCG
	Reverse	TGCATGGCCGTTCTTAGTTG

CHAPTER 8

GENERAL DISCUSSION

The alarming increase in prevalence of obesity, cardiovascular diseases (CVD) and type 2 diabetes (T2D) warrants the need for novel insights that can pave the way to alternative treatment strategies. A key organ involved in pathophysiology of these cardiometabolic diseases is the adipose tissue (AT).[1, 2] In fact, the increased fat mass in obesity predisposes towards several chronic diseases.[3] Besides increased AT mass, the distribution of body fat is a major determinant of cardiometabolic diseases.[3] Interestingly, excessive abdominal fat is associated with deleterious metabolic consequences,[4] while lower-body AT seems to have certain protective characteristics.[5] Thus, different fat depots apparently exert distinct functions. Importantly, the normal function of AT is disturbed in obesity, and accumulating evidence suggests that an impaired function of AT, rather than the total fat mass, plays a crucial role in the development and progression of chronic diseases, including CVD and T2D.[1] AT dysfunction in obese insulin resistant subjects is characterized by enlargement of adipocytes,[1, 6] an impaired AT blood flow,[1, 7-9] an increased production of pro-inflammatory cytokines,[10, 11] and impairments in lipid and glucose metabolism.[1, 10, 12-14] These disturbances in AT may contribute to chronic systemic low-grade inflammation, peripheral insulin resistance and T2D.[10] Although several factors have been linked to the development of AT dysfunction, the trigger that instigates AT dysfunction in obesity remains elusive.

Recent cell culture experiments and animal studies have shown that AT oxygen tension (AT pO_2) may be involved in AT dysfunction.[15-17] Human studies so far yielded conflicting results.[18-20] A lower AT pO_2 ('hypoxia') has been shown in obese as compared to lean animals,[15-17] whereas we have demonstrated that AT pO_2 was increased in obese individuals with impaired glucose tolerance, and was associated with AT inflammation and peripheral insulin resistance.[19]

Therefore, the present thesis investigated the possible involvement of tissue oxygenation in metabolism and inflammation in human obesity. At first, we investigated whether differences exist in pO_2 between lower- and upper-body AT depots, since fat accumulation in these lipid storage sites is associated with distinct metabolic risk profiles, and examined the effects of AT pO_2 on femoral and abdominal adipocyte function using differentiated AT-derived mesenchymal stem cells (**Chapter 3**). Next, we aimed to obtain insight into differences in the proteome of lower- and upper-body fat depots of obese women to better understand functional differences between these fat depots (**Chapter 4**). Furthermore, we examined whether differences in abdominal AT pO_2 in obese individuals are related to AT mass or insulin sensitivity, independent of body fat percentage (**Chapter 5**). In addition, a randomized, single-blind, placebo-controlled, cross-over study was performed to investigate the effects of mild intermittent hypoxia (MIH) exposure on tissue oxygenation, insulin sensitivity and substrate metabolism in obese

individuals (**Chapter 6**). Finally, we investigated the effects of different MIH exposure protocols on human primary adipocyte gene expression, adipokine secretion and glucose uptake (**Chapter 7**).

Phenotypic differences between lower- and upper-body adipose tissue

Evidence arising from *in vivo* studies suggests that the cardiometabolic risk associated with a certain body fat distribution may be due to differences in AT metabolism, including altered function of other cells than adipocytes.[5] For example, lower-body fat mass is positively associated with adiponectin and leptin levels, adipokines known to exert beneficial metabolic and cardiovascular effects in humans.[1] Importantly, however, studies that examined differences between lower- and upper-body AT are scarce.

In **Chapter 3**, we found that the adipocytes were larger in lower-body than upper-body subcutaneous AT in obese postmenopausal women. The larger adipocytes in femoral as compared to abdominal subcutaneous AT may be explained by a lower lipid mobilization.[21, 22] Indeed, we found that gene expression of the lipolytic markers ATGL and HSL was lower in femoral versus abdominal subcutaneous AT. Since hypertrophic adipocytes in general are associated with a pro-inflammatory phenotype, the larger adipocytes in femoral AT may be counterintuitive in view of the protective effects of lower-body AT. However, we did not find differences in gene expression of inflammatory markers between femoral and abdominal AT (**Chapter 3**), suggesting that the increased femoral adipocyte size did not lead to increased inflammation in this depot as compared to abdominal AT. It is tempting to hypothesize that this may be explained by a higher expandability of femoral adipocytes than abdominal adipocytes. In other words, for a given fat cell size, femoral adipocytes may experience less cell stress than abdominal adipocytes. In agreement with the latter, when we examined the AT proteome using liquid chromatography-mass spectrometry (LC-MS) on paired biopsies from abdominal and femoral subcutaneous AT to assess differences in protein expression between upper- and lower-body AT, we found that proteins related to remodelling and extracellular matrix were more abundant in femoral AT (**Chapter 4**).

Thus, it could be speculated that larger adipocytes and a higher abundance of proteins related to remodelling and ECM in femoral AT may contribute to an enhanced capacity of lower-body AT to store lipids, thereby possibly reducing lipid overflow into the circulation and, consequently, ectopic fat deposition. However, the functional difference in expandability between abdominal and femoral AT cannot be derived from the present study, and care has to be taken in interpreting the proteome data since we cannot

exclude that the increased abundance of these proteins in femoral AT might also depict a compensation for a decreased capacity to expand.
Since AT biopsies still contain blood after thorough cleaning with sterile saline due to small blood vessels present, in chapter 4 blood-specific proteins were identified and subsequently excluded from analysis. Of the 610 proteins that were identified, 22 proteins were differentially expressed between abdominal and femoral AT. In addition to the higher abundance of proteins related to ECM remodelling and extracellular matrix in femoral AT, we found that the abundance of proteins related to immune cell interaction and energy metabolism was different between femoral and abdominal subcutaneous AT. These insights may provide leads for future studies to better understand the cardiometabolic disease risk associated with a certain body fat distribution pattern.

Measuring tissue oxygenation

In this thesis, we made use of a microdialysis-based optochemical measurement system that we have recently developed to continuously, and simultaneously, measure AT pO_2 in femoral and abdominal AT (**Chapter 3**). In **Chapter 6**, this technique was also used to assess the effects of mild intermittent hypoxia exposure on pO_2 in abdominal AT and, for the first time, human skeletal muscle. Compared to other techniques to measure tissue oxygenation, like the commercially available Clark electrodes, this system uses a microdialysis probe of about ~2 cm with a semi-permeable membrane (20 kDa cut-off). The pores in the microdialysis catheter allow small molecules like oxygen to diffuse over this membrane. This requires a low perfusion speed in order to allow equilibration, thereby yielding a very high recovery (about 99% for oxygen). In other words, the pO_2 that is measured reflects the true *in vivo* interstitial pO_2. The oxygen sensors and the *in vivo* experimental set-up have previously been validated.[19, 23] One of the advantages of this system is that tissue pO_2 is measured over a relatively large tissue area (approximately 2-3 cm^3) as compared to measurements that are made at the tip of the needle ('point measurement') when using needle-type electrodes. This is important, since it is likely that certain heterogeneity in tissue oxygenation exists. Furthermore, a disadvantage of the Clark electrode is that a needle needs to remain inserted into the tissue during the measurement. In contrast, the microdialysis probe allows measurements without significant discomfort.

Abdominal and femoral subcutaneous adipose tissue oxygen tension

AT possesses a relatively dense capillary network that ensures adequate delivery of nutrients and oxygen to the tissue. AT oxygenation is the result of the balance between oxygen supply to the tissue (ATBF) and oxygen consumption (metabolic rate).

Perturbations in this delicate balance may occur in pathophysiological conditions that are characterized by disturbances in ATBF and/or local metabolic rate.[2] Likewise, differences in blood flow and/or the metabolic rate in lower- as compared to upper-body AT would result in distinct AT pO_2 between these fat depots. Interestingly, in **Chapter 3**, we demonstrated that AT pO_2 was lower in femoral compared to abdominal subcutaneous AT. This is likely not explained by differences in oxygen supply, since ATBF was not significantly different between femoral and abdominal AT. Therefore, these data suggest that a differential oxygen consumption rate exists between these fat depots. Indeed, we found that the oxygen consumption rate, as measured by Seahorse, was higher in femoral than abdominal human adipocytes (**Chapter 3**). Thus, these data suggest that the oxygen consumption rate, rather than ATBF, is the major determinant of depot-differences in AT pO_2. Interestingly, these findings furthermore indicate that differentiated human primary adipocytes retain, at least to a certain extent, intrinsic AT depot-differences. In line, it has previously been shown that pre-adipocytes from different AT depots exert distinct functions, and are able to maintain their *in vivo* memory of origin during *in vitro* culture.[24] Moreover, certain associations between the *in vivo* and *in vitro* phenotype of adipocytes were found, indicating inherent characteristics of adipocytes.[25, 26] Although it is likely that other cell types (e.g. immune cells) within AT contribute to oxygen consumption in adipose tissue, the present data at least indicate that oxygen consumption rates differ between abdominal and femoral subcutaneous adipocytes. Depot-differences in adipocyte size do not seem to underlie these differences, since it has previously been shown that inter-individual differences in adipocyte oxygen consumption are independent of adipocyte size.[27]

In addition, as described in **Chapter 4,** the proteins EEF2 and RNH1, a translation elongation factor and an inhibitor of mRNA turnover, respectively, were higher in femoral AT. Moreover, the proteins HADHA and LDHA, which are involved in mitochondrial beta-oxidation and anaerobic glycolysis, respectively, were found to be higher in femoral versus abdominal AT. The different abundance in these proteins between depots could suggest differences in protein synthesis, beta-oxidation and anaerobic glycolysis, which may be related to a different oxygen consumption rate, as found in **Chapter 3**. Noteworthy, from these data we cannot conclude whether these proteins are also functionally active, and the difference in their abundance may also reflect a compensatory mechanism for inadequate functioning of these processes.

Effects of microenvironmental oxygen tension on adipocyte function

The lower pO_2 in the femoral as compared to abdominal AT may be physiologically relevant since oxygen availability in the tissue microenvironment seems to affect

metabolism and/or inflammatory processes. Interestingly, in **Chapter 3**, we found that exposing adipocytes to physiological low pO_2 (5% O_2) reduced the expression levels of inflammatory markers in adipocytes originating from femoral and abdominal AT. Moreover, low physiological pO_2 altered adipokine secretion in differentiating femoral and abdominal adipocytes, and increased basal glucose uptake in femoral adipocytes. Increased glucose uptake in adipocytes exposed to hypoxia (acute, 1% O_2) has previously been shown,[28, 29] and appeared to be pO_2-dependent,[30] suggesting that hypoxia exposure may indeed affect glucose metabolism. We did, however, not found marked differences in gene expression levels of metabolism markers, except for VEGFA and GLUT1, which were both decreased after low physiological pO_2 in abdominal and femoral adipocytes. Since the effects of physiological oxygen levels (5-10% O_2) on femoral and abdominal adipocyte gene expression and adipokine secretion were comparable, the different pO_2 levels found in lower- versus upper-body AT may contribute to the distinct functional properties of these AT depots.

Previous *in vitro* studies that assessed the inflammatory response of human adipocytes exposed to hypoxia mainly applied extremely low pO_2 (1% O_2), showing that that severe hypoxia evokes a pro-inflammatory phenotype,[8, 9, 11, 14, 30] as extensively reviewed.[2, 31] Interestingly, not only extremely low (1% O_2) but also very high pO_2 (95% O_2)[32] induced inflammation, oxidative stress, and insulin resistance in adipocytes. These data, together with our findings described in **Chapter 3**, highlight the importance of using more physiologically relevant pO_2 to better reflect *in vivo* physiology. Current *in vitro* models often use non-physiological conditions (e.g. high glucose concentration in the medium and exposure of cells to room air). These conditions, together with the lack of interaction with the different cells types present in AT and the absence of circulatory factors (i.e. no blood flow), likely results in modification of differentiation, metabolism, adipokine secretion, and gene expression.[33] Future cell culture studies, therefore, should try to better mimick *in vivo* physiology.

Collectively, our findings show that microenvironmental oxygen availability affects adipocyte function, and suggest that AT depot-differences in pO_2 may contribute to distinct functional properties of lower- and upper -body AT in human obesity

Adipose tissue oxygen tension, obesity and insulin resistance

An important question is whether AT pO_2 is altered in obesity, since results thus far are conflicting. AT hypoxia has recently been demonstrated after a very rapid and massive increase in body fat mass in animal models of obesity.[15-17] Importantly, this does not reflect human pathophysiology, where obesity develops gradually over a period of many years, and the reduction of oxygen supply to AT is less severe than in rodents.[2] We have

previously reported that AT pO_2 was higher rather than lower in obese as compared to lean subjects, despite lower ATBF, which may be explained by lower AT oxygen consumption in obese humans.[19] In line, impaired mitochondrial oxygen consumption in AT is present in mouse models of obesity and type 2 diabetes,[34] and mitochondrial oxidative pathways are downregulated in obese human AT.[35-37]

We have previously demonstrated that increased AT pO_2 in obese individuals was associated with local inflammation and peripheral insulin resistance *in vivo*.[19] More recently, we have also shown that diet-induced weight loss markedly decreased abdominal subcutaneous AT pO_2 in overweight/obese humans, paralleled by an improved whole-body insulin sensitivity.[38] However, it remains to be established whether abdominal AT pO_2 is related more to adiposity or insulin sensitivity.

In **Chapter 5**, we investigated whether abdominal subcutaneous AT pO_2 is different in obese insulin resistant compared to obese insulin sensitive men and women. Interestingly, AT oxygenation was higher in obese insulin resistant as compared to obese insulin sensitive individuals, both in men and women. Furthermore, AT pO_2 was comparable between lean and obese insulin sensitive men. Moreover, we found that AT pO_2 was positively associated with insulin resistance, even after adjustment for age, sex and body fat percentage. The strength of the association between AT pO_2 and insulin sensitivity, however, was reduced after further adjustment for waist/hip ratio, suggesting that body fat distribution may influence the relationship between AT pO_2 and insulin sensitivity to a certain extent. These findings suggest that abdominal subcutaneous AT pO_2 may be an important factor in the pathogenesis of insulin resistance. Based on our findings in **Chapter 5** and earlier studies from our group,[19, 38] it is tempting to hypothesize that lowering of AT oxygenation may improve whole-body insulin sensitivity.

Modulation of tissue oxygenation: a novel avenue to improve metabolic health?

There is evidence that mild hypoxia exposure (MIH) may be a potential treatment strategy for individuals with an impaired glucose homeostasis, as reviewed in **Chapter 2**. In **Chapter 6,** we conducted a randomized, single-blind, placebo-controlled trial to investigate the effects of MIH exposure (FiO_2 15%, 3 times 2h/day, 7 consecutive days) on AT and skeletal muscle pO_2, substrate metabolism and tissue-specific insulin sensitivity in overweight/obese insulin resistant men. We found that MIH exposure reduced systemic oxygen saturation, which led to a marked reduction (~40-50%) in both AT and skeletal muscle pO_2, suggesting that the hypoxic stimulus was present in both AT and skeletal muscle, and not compensated for by changes in tissue blood flow. Interestingly, we found that MIH exposure increased carbohydrate oxidation (CHO), not only during MIH exposure on day 7 but also one day after cessation of MIH exposure (~18 hours after the

last exposure). In line with this shift towards glycolytic metabolism, fasting and postprandial plasma lactate concentrations were increased during MIH exposure. Indeed, it is well-established that with an increased glycolytic flux lactate production increases.[39]
The pronounced change in substrate oxidation was, however, not paralleled by alterations in plasma glucose, insulin, FFA and TAG levels. Moreover, no significant effect of MIH on whole-body insulin sensitivity was found. Noteworthy, the present data on insulin sensitivity are preliminary, since glucose tracer data needed to calculate tissue-specific insulin sensitivity were not yet available. Nevertheless, the increased CHO oxidation, together with unchanged whole-body insulin sensitivity, may suggest that non-oxidative glucose disposal (NOGD) (i.e. glycogen storage) was decreased following MIH exposure. Since the main defect in skeletal muscle glucose metabolism in type 2 diabetic individuals seems to be an impaired NOGD,[40] this might not be a beneficial effect of MIH.
In contrast to our preliminary findings in **Chapter 6**, it has previously been demonstrated that mild hypoxia exposure (FiO_2 15%, 10 nights, 10h/night continuous exposure) improved glucose disposal rate at low and high insulin infusion rates in overweight individuals, likely due to improvements both in hepatic and peripheral insulin sensitivity.[41, 42] Insulin sensitivity was improved by approximately 20% on average, but individuals with more pronounced insulin resistance at baseline showed the greatest improvements.[41]
In **Chapter 6**, we included individuals with mild insulin resistance (HOMA-IR 3.4±0.4) with and without an impaired glucose metabolism. Differences in metabolic phenotype may have contributed to the differential effects in both studies.
A second factor explaining the discrepancy between the latter study and our study in chapter 6 may be related to differential effect on body weight since in study of Lecoultre et al.[41] participants in the latter study lost 1.2 kg body weight.[41] Indeed, Hypoxia may reduce appetite, which in turn affects food intake, possibly inducing a decrease in body weight, thereby improving metabolic outcomes like insulin sensitivity.[43, 44] In our study (**Chapter 6)**, we controlled food intake, and subjects were kept in energy balance throughout the study. Although MIH exposure did not affect feelings of hunger and satiety, we can conclude that the effects of MIH on substrate metabolism were not explained by changes in body weight. Thus, the decrease in body weight in the study by Lecoultre et al.[41] may possibly contribute to the discrepant findings with our study. Nevertheless, the improvement in insulin sensitivity in the former study was not associated with body weight change.[41, 42, 45]
A third factor explaining the difference between our study (**Chapter 6**) and that by Lecoultre et al.[41] may be related to the intermittent character and total duration of hypoxia exposure (three 2-h hypoxic cycles per day for 7 days (total exposure of 42h)

versus one 10-h hypoxic cycle per day for 10 days (total exposure of 100h), respectively). Moreover, in contrast to the study by Lecoultre et al.,[41] we included a control condition (normoxia exposure). Future studies need to investigate whether the total exposure duration and/or the amount of hypoxic cycles impact improvements in insulin sensitivity and glucose metabolism in individuals with an impaired glucose homeostasis.

In mice, intermittent hypoxia exposure (60 cycles/h, 30s of 5% O_2 per cycle, 8h/day for 2 weeks) decreased insulin sensitivity but improved glucose tolerance. This improvement, could at least partially be explained by SM-specific activation of adenosine monophosphate-activated protein kinase (AMPK).[46] Despite a reduction in body weight, these mice developed insulin resistance, illustrated by increased fasting plasma insulin levels and a reduced insulin-induced phosphorylation of PKB in the liver, skeletal muscle and AT,[46] confirming that insulin signalling was impaired in all main metabolic tissues by severe intermittent hypoxia (SIH).[46] Despite inducing insulin resistance, no signs of inflammation were found in white AT.[46] Although HIF-1α protein expression was increased by SIH, it appeared that HIF-1α is not the key player in SIH-induced insulin resistance.[46] Noteworthy, the severity of hypoxia exposure with respect to both the level of hypoxia (5% O_2) and the number of hypoxic cycles (480 cycles/d) in the latter study reflects the situation seen in patients with obstructive sleep apnea syndrome (OSAS). Strikingly, the beneficial effect on glucose tolerance was maintained for several weeks after cessation of hypoxia exposure.[46] In **Chapter 6**, we found no effect of MIH on insulin sensitivity or glucose homeostasis, suggesting that a more prolonged exposure to MIH might be needed to induce beneficial effects on glucose tolerance and, possibly, adverse effects on insulin sensitivity.

We have recently hypothesized that hypoxia may have exercise-mimicking effects on glucose homeostasis and insulin sensitivity.[47] Interestingly, exposure of human skeletal muscle cells to 'hypoxia' (7% O_2, 24h) during contraction (electrical pulse stimulation, (EPS)) induced a more pronounced increased in insulin-stimulated glucose uptake as compared to EPS alone. However, exposure of cells to 7% O_2 without EPS did not affect glucose uptake.[48] These data suggest that hypoxia and exercise might have additive or synergistic effects.

In accordance with these findings, a previous study showed that type 2 diabetic individuals did improve their insulin sensitivity after intermittent exercise with and without hypoxia exposure (FiO_2 14.7%), with more pronounced improvements in insulin sensitivity following hypoxia exposure.[49] Interestingly, it was found that hypoxia exposure during exercise also improved glucose tolerance, which could be attributed to improvements in peripheral insulin sensitivity.[50]

In **Chapter 6**, we found that MIH decreased skeletal muscle oxygenation to a similar extent than in AT (~40-50%). In order to investigate if, in addition to whole-body substrate metabolism, skeletal muscle mitochondrial function might be affected by MIH, we performed *ex vivo* mitochondrial respiration measurements. Muscle fibers were isolated from a muscle biopsy (*m. vastus lateralis*), permeabilized, and exposed to different substrates (i.e. pyruvate and octanoyl-carnitine), representing glucose and fatty acid substrates, respectively. Irrespective of the substrates used, no differences between normoxia and MIH exposure were found in mitochondrial oxygen consumption rates. Since we performed these measurements under normoxic conditions, ~18 hours after the last hypoxic cycle, we cannot exclude that mitochondrial respiration is altered under hypoxic conditions. It has previously been shown that hypoxia may inhibit the PPAR/PGC-1α pathway and the expression of mitochondrial components in cultured muscle cells, although hypoxia stimulated the expression of slow-oxidative type I myosin through HIF-1α, suggesting that hypoxia may differentially regulate contractile and metabolic components of muscle oxidative phenotype.[51] Hypoxia did induce decreased expression of markers of mitochondrial metabolism and shifted muscle fibers to less oxidative types in rodents.[52] The severity of hypoxia exposure and/or the duration of exposure may both determine the effects of hypoxia on skeletal muscle oxidative metabolism.

In **Chapter 7**, we investigated whether the duration and number of hypoxic cycles affect the functionality of adipocytes derived from overweight/obese, relatively insulin resistant males. We have previously found that the average *in vivo* AT pO_2 in obese insulin resistant individuals is ~10%.[19] To mimick the *in vivo* changes in AT pO_2 following MIH, as described in **Chapter 6**, we reduced oxygen availability by approximately 50%, using different intermittent protocols (cycling from 10% to 5% O_2, 3x2h or 6x2h, 7 or 14 days) (**Chapter 7**). Continuous exposure to 10% O_2 significantly increased basal glucose uptake as compared to 21% O_2. MIH exposure, however, had no additional impact on glucose uptake and adipocyte gene expression of markers related to inflammation, lipid metabolism, and browning/mitochondrial biogenesis, adipokine secretion as compared to continuous 10% O_2 exposure. These findings suggest that effects of MIH on adipocytes (**Chapter 7**) might not contribute to possible effects of MIH on substrate metabolism.

Main outcomes of this thesis

1. AT pO_2 is higher in abdominal as compared to femoral subcutaneous adipose tissue in overweight/obese post-menopausal women, which seems to be explained by a higher oxygen consumption rate in femoral adipocytes. **Chapter 3**

2. Continuous exposure to low physiological pO_2 reduces pro-inflammatory gene expression in abdominal and femoral human adipocytes. **Chapter 3**

3. Comparison of human abdominal and femoral subcutaneous AT using non-targeted, quantitative proteomics revealed slight differences in protein expression between these AT depots in overweight/obese women. These differences were mainly related to cell structure organization and energy metabolism, which may contribute to functional differences between upper- and lower-body AT depots. **Chapter 4**

4. Abdominal subcutaneous AT pO_2 is higher in obese insulin resistant as compared to obese and lean insulin sensitive individuals. In line, AT pO_2 is associated with insulin sensitivity, independent of age, sex and adiposity. **Chapter 5**

5. Exposure to MIH decreases systemic oxygen saturation, leading to a pronounced reduction in AT and SM pO_2. MIH exposure for seven days evoked a shift in substrate utilization towards increased carbohydrate oxidation, which was still present the day following cessation of the MIH regimen, but did not alter glucose homeostasis and whole-body insulin sensitivity in overweight/obese men. **Chapter 6**

6. MIH exposure (5% O_2 cycles of 2h) did not induce different effects on adipocyte gene expression, adipokine secretion and glucose uptake as compared to continuous 10% O_2 exposure in human adipocytes. **Chapter 7**

Future directions

1. Based on proteome differences, upper- and lower-body AT depots seem to have slight but possibly important differences related to AT function. 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 fat to better understand the disease risk associated with a certain body fat distribution.

2. Hypoxia exposure seems to have the potential to alter substrate metabolism and affect adipocyte biology. We found that continuous hypoxia exposure *in vitro* alters adipocyte function, although MIH showed no additional effects on adipocyte gene expression, adipokine secretion and glucose uptake. Moreover, MIH exposure *in vivo* in obese humans induced marked changes in whole-body substrate oxidation. The effects of both continuous and intermittent long-term hypoxia exposure remain to be elucidated. Furthermore, future *in vivo* and mechanistic *in vitro* studies (i.e. myotubes) are warranted to investigate the potential synergistic metabolic effects of hypoxia and exercise.

3. In the present thesis, we investigated the effects of MIH with a relatively low total exposure time and amount of hypoxic cycles. The importance of the severity of hypoxia exposure, the amount of hypoxic cycles, and the duration of hypoxia exposure should be investigated in more detail at the whole-body and tissue level (i.e. adipose tissue, skeletal muscle and the liver).

4. Due to possible adaptations induced by hypoxic exposu*re,* like ventilatory adaptations, effects can vary between individuals.[53] It should be investigated whether a more personalized approach, i.e. adjusting FiO_2 according to its effects on systemic oxygen saturation (SpO_2), may increase metabolic outcomes.

5. The effects of mild intermittent and continuous hypoxia exposure may vary depending on the metabolic phenotype. Therefore, it should be examined whether effects of hypoxia exposure may be more pronounced in individuals with more severe impairments in glucose homeostasis such as patients with type 2 diabetes.

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SUMMARY

Summary

The prevalence of obesity has increased rapidly over the last decades and reached epidemic proportions. Since obesity is strongly linked to the development of insulin resistance and cardiometabolic diseases, it is important to increase knowledge about underlying mechanisms and investigate alternative strategies to improve cardiometabolic health in obese individuals. The development of insulin resistance is caused by a complex interplay of different organs, including key metabolic organs such as the liver, skeletal muscle, and adipose tissue (AT). In this thesis, we aimed to investigate the role of oxygen tension (pO_2) in human AT function, with focus on different AT depots. In addition, we examined the effects of mild intermittent hypoxia exposure on metabolic health in overweight/obese humans, taken into account effects on both AT and skeletal muscle.

Chapter 2 provides a literature overview of studies that have examined the effects of pO_2 on inflammation and glucose homeostasis. There is substantial evidence that changes in pO_2 in the AT microenvironment may impair AT function. Moreover, studies suggest that hypoxia may improve skeletal muscle glucose metabolism. We concluded, however, that more *in vivo* clinical studies in humans and *in vitro* studies that better reflect human physiology, by exposing primary human cells to physiological pO_2, are needed to elucidate the effects of different (mild) hypoxia exposure regimens on energy and substrate metabolism.

Upper and lower-body AT exhibit opposing associations with obesity-related cardiometabolic diseases. We hypothesized that differences in pO_2 between these AT depots may contribute to distinct metabolic and inflammatory signatures of these AT depots. Therefore, in **Chapter 3**, we compared *in vivo* abdominal (ABD) and femoral (FEM) subcutaneous (sc) AT pO_2 and AT blood flow in well-phenotyped, overweight and obese post-menopausal women with impaired glucose metabolism. Mechanistically, we investigated depot-specific effects of prolonged physiological AT pO_2 exposure on adipokine expression and secretion, oxygen consumption rate and glucose uptake in differentiated human multipotent adipose-derived stem cells (hMADS) derived from ABD and FEM sc AT from the same individuals. Together, our findings described in **Chapter 3** show for the first time, to our knowledge, that AT pO_2 was higher in ABD than FEM sc AT in overweight/obese women, partly due to a lower oxygen consumption rate in ABD adipocytes. Moreover, low physiological pO_2 (5% O_2) decreased pro-inflammatory gene expression and improved the metabolic phenotype in differentiated human adipocytes, whereas more heterogeneous effects on adipokine secretion were found.

Body fat distribution is an important determinant of cardiometabolic health and FEM AT has protective characteristics as compared to ABD AT, but the underlying differences between these AT depots remain to be elucidated. In **Chapter 4**, we compared the proteome of ABD and FEM AT, and investigated which proteins may contribute to putative functional differences between these AT depots. Therefore, paired biopsies from ABD and FEM sc AT were taken from eight overweight and obese post-menopausal women, proteins were isolated and quantified using liquid chromatography-mass spectrometry (LC-MS), and protein expression in ABD and FEM sc AT was compared. We identified and quantified 651 proteins in total, of which 22 proteins were differentially expressed between both AT depots after adjustment for blood protein signals. Interestingly, proteins involved in cell structure organization and energy metabolism seemed to be differently expressed between ABD and FEM sc AT, which may reflect depot-differences in adipocyte expandability, immune cell interaction and energy metabolism. Our findings in **Chapter 4** suggest that there are only slight differences in protein expression between ABD and FEM sc AT after an overnight fast, although it cannot be excluded that differences in protein activity, particularly under challenged conditions, may be more pronounced.

Although previous studies have shown that altered AT pO_2 may affect AT function, it remained to be investigated whether changes in AT pO_2 are more strongly related to obesity or insulin sensitivity. In **Chapter 5**, we tested the hypothesis that AT oxygenation is associated with insulin sensitivity rather than adiposity in humans. Therefore, we measured ABD sc AT pO_2, body composition and insulin sensitivity in thirty-five lean and obese individuals (21 men and 14 women, aged 40-65 years) with either normal or impaired glucose metabolism. Indeed, AT pO_2 was higher in obese insulin resistant as compared to obese insulin sensitive individuals with similar age, body mass index and body fat percentage, both in men and women. Moreover, no significant differences in AT pO_2 were found between obese insulin sensitive and lean insulin sensitive men. Finally, we found that AT pO_2 was positively associated with insulin resistance, even after adjustment for age, sex and body fat percentage. Therefore, findings described in **Chapter 5** suggest that AT pO_2 may be a promising target to improve whole-body insulin sensitivity.

Based on the conclusions derived from a literature search (**Chapter 2**), and our findings described in **Chapter 3 and 5**, we hypothesized that lowering of AT pO_2 may have beneficial effects on AT inflammation and substrate metabolism. This may be achieved by lowering the environmental oxygen levels, thereby possibly lowering pO_2 levels in different tissues, including AT and skeletal muscle. Therefore, the effects of 7 days

exposure to mild intermittent hypoxia (MIH) were investigated in **Chapter 6**. In this ongoing study, we exposed eight overweight/obese individuals to normobaric MIH (FiO_2 15%; equivalent to ~3000m above sea level) and normoxia (FiO_2 21%) for 7 consecutive days (3 cycles of 2h exposure/d) in a randomized fashion, separated by a wash-out period of 3-6 weeks. Our preliminary data demonstrated that MIH effectively decreased systemic oxygen saturation, AT pO_2 and SM pO_2, and induced a shift in substrate metabolism towards increased carbohydrate oxidation under fasting and postprandial conditions, as further illustrated by increased circulating lactate concentrations. This shift in substrate oxidation seemed to persist after cessation of MIH. However, MIH did neither induce changes in plasma glucose and lipid levels nor insulin sensitivity.

Since it could be that different duration and amount of cycles in MIH have distinct effects on metabolic and inflammatory processes, we examined the effect of different hypoxia exposure regimens on the function of human adipocytes in **Chapter 7**. Thus, we exposed adipocytes to 4 different low oxygen protocols (7 or 14 days, 3x2h or 6x2h exposure per day). We hypothesized that lowering oxygen levels from 10% to 5% (as we have previously found in obese and lean human AT, respectively), induces beneficial effects on adipocyte inflammation and glucose uptake. Exposure of adipocytes to low oxygen (5% O_2 cycles of 2h) reduced the secretion of the pro-inflammatory cytokine IL-6. However, this exposure did not induce different effects on the uptake of glucose by the adipocytes and expression of genes related to metabolism and inflammation as compared to continuous 10% O_2 exposure. These findings suggest that MIH has minor effects on adipocyte function.

This thesis provides important insights into the effects of pO_2 on AT, adipocyte function, and human metabolism. Moreover, also differences between AT depots were examined. Based on findings in this thesis, future studies should be performed to further examine the effects of tissue oxygenation on several pathophysiological processes involved in the development of chronic cardiometabolic diseases, since mild intermittent hypoxia exposure may turn out to be an alternative strategy to combat these diseases.

SAMENVATTING

Samenvatting

De afgelopen decennia is de prevalentie van obesitas enorm toegenomen en heeft epidemische vormen aangenomen. Aangezien obesitas sterk gerelateerd is aan de ontwikkeling van insulineresistentie en cardiometabole ziekten, is het belangrijk om onderliggende mechanismen beter te begrijpen en alternatieve therapieën te onderzoeken met als doel de cardiometabole gezondheid in personen met obesitas te verbeteren. De ontwikkeling van insulineresistentie wordt veroorzaakt door een complexe interactie tussen verschillende organen, waaronder de lever, skeletspieren en het vetweefsel, die een belangrijke rol spelen bij het metabolisme. In dit proefschrift hebben we getracht de rol van zuurstofspanning bij het functioneren van het vetweefsel te onderzoeken in de mens, met nadruk op de verschillende opslagplaatsen van vetweefsel. Daarnaast hebben wij ook de effecten van milde intermitterende hypoxie blootstelling op insulinegevoeligheid en metabole gezondheid onderzocht in personen met overgewicht/obesitas. Hierbij hebben wij de effecten op zowel het vetweefsel als de skeletspieren in acht genomen.

In **Hoofdstuk 2** geven we een overzicht van de literatuur waarin de effecten van zuurstofspanning op inflammatie en glucose homeostase zijn beschreven. Er is substantieel bewijs dat veranderingen in zuurstofspanning in het vetweefsel de functie hiervan zou kunnen verstoren. Verder suggereren verschillende onderzoeken dat hypoxie het glucosemetabolisme van de skeletspieren kan verbeteren. Onze conclusie was dat meer klinisch onderzoek en *in vitro* experimenten, die beter de humane fysiologie reflecteren, nodig zijn om de effecten van verschillende (milde) hypoxie blootstellingsprotocollen op het energie- en substraatmetabolisme te onderzoeken.

Het vetweefsel in het boven- en onderlichaam hebben tegenovergestelde relaties met obesitas-gerelateerde cardiometabole ziekten. Wij veronderstelden dat verschillen in zuurstofspanning tussen deze vetweefsel opslagplaatsen bijdragen aan de verschillende metabole en inflammatoire eigenschappen van deze vetdepots. Om die reden hebben we in **Hoofdstuk 3** de verschillen in zuurstofspanning en vetweefseldoorbloeding onderzocht in abdominaal en femoraal subcutaan vetweefsel van postmenopauzale vrouwen met overgewicht/obesitas en verstoringen in het glucosemetabolisme. Om inzicht te krijgen in onderliggende mechanismen, hebben we de effecten van langdurige blootstelling aan fysiologische zuurstofspanning op de expressie en secretie van

adipokines, zuurstofconsumptie, en glucose opname onderzocht in gedifferentieerde humane stamcellen afkomstig van abdominaal en femoraal subcutaan vetweefsel van dezelfde individuen. Op basis van onze bevindingen in **Hoofdstuk 3** tonen we voor de eerste keer aan dat de zuurstofspanning hoger is in abdominaal ten opzichte van femoraal vetweefsel in vrouwen met overgewicht/obesitas. Dit wordt gedeeltelijk verklaard door een lagere zuurstofconsumptie in abdominale adipocyten. Verder vonden we dat een lage fysiologische zuurstofspanning de inflammatoire genexpressie verlaagde, en het metabole fenotype in gedifferentieerde humane adipocyten verbeterde, waarbij minder eenduidige resultaten werden gevonden met betrekking tot de secretie van adipokines.

Lichaamsvetverdeling is een belangrijke determinant van cardiometabole gezondheid, waarbij femoraal vetweefsel beschermende eigenschappen heeft ten opzichte van abdominaal vetweefsel. De oorzaak van de verschillen tussen deze vetweefseldepots is nog niet duidelijk. In **Hoofdstuk 4** hebben we het proteoom van abdominaal en femoraal vetweefsel vergeleken en onderzochten we welke eiwitten bijdragen aan de vermeende functionele verschillen tussen deze vetdepots. Hiervoor gebruikten we biopten van abdominaal en femoraal vetweefsel van acht postmenopauzale vrouwen met overgewicht/obesitas. Uit deze biopten werden de eiwitten geïsoleerd en gekwantificeerd met behulp van vloeistofchromatografie-massaspectrometrie (LC-MS), en werd de eiwitexpressie tussen abdominaal en femoraal vetweefsel vergeleken. In totaal identificeerden en kwantificeerden we 651 eiwitten, waarvan 22 eiwitten verschillend tot expressie kwamen in beide vetdepots na correctie voor eiwitsignalen afkomstig uit het bloed. Eiwitten betrokken bij celstructuur organisatie en het energie metabolisme bleken verschillend tot expressie te komen, hetgeen mogelijke verschillen in de capaciteit van vetcellen om te vergroten, immuuncel interactie en energie metabolisme tussen de vetweefsels reflecteert. Onze bevindingen in **Hoofdstuk 4** suggereren dat er kleine verschillen in eiwitexpressie bestaan tussen abdominaal en femoraal vetweefsel onder gevaste omstandigheden, hoewel het niet kan worden uitgesloten dat verschillen in eiwitactiviteit, in het bijzonder tijdens gevoede condities, groter kunnen zijn.

Hoewel eerdere studies hebben aangetoond dat modulatie van de zuurstofspanning in het vetweefsel mogelijk het functioneren van dit weefsel beïnvloedt, was het nog niet eerder onderzocht of veranderingen in zuurstofspanning in het vetweefsel sterker gerelateerd zijn aan obesitas of aan insulinegevoeligheid. In **Hoofdstuk 5** hebben we bekeken of de zuurstofspanning in het vetweefsel geassocieerd is met

insulinegevoeligheid of obesitas in mensen. Hiervoor hebben we de zuurstofspanning in het abdominale subcutane vetweefsel, de lichaamssamenstelling en insulinegevoeligheid gemeten in vijfendertig slanke en obese personen (21 mannen en 14 vrouwen, 40-65 jaar oud) met een normaal of verstoord glucosemetabolisme. Zoals verwacht, zagen we dat de zuurstofspanning in het buikvet hoger was in obese, insulineresistentie personen in vergelijking met obese, insulinegevoelige personen (zowel mannen als vrouwen) met een vergelijkbare leeftijd, BMI en lichaamsvetpercentage. Bovendien vonden we geen significante verschillen tussen de zuurstofspanning in het buikvet van obese insulinegevoelige mannen en insulinegevoelige mannen met een normaal gewicht. Tenslotte toonden we aan dat de zuurstofspanning in het buikvet positief geassocieerd was met insulineresistentie, zelfs na correctie voor leeftijd, geslacht en lichaamsvetpercentage. De bevindingen beschreven in **Hoofdstuk 5** suggereren daarom dat de zuurstofspanning in het vetweefsel met name gerelateerd is aan insulinegevoeligheid, en deze daarom een veelbelovend doelwit is om insuline gevoeligheid te verbeteren.

Op basis van de conclusies uit ons literatuuronderzoek (**Hoofdstuk 2**) en onze bevindingen beschreven in **Hoofdstuk 3 en 5**, was de hypothese dat verlaging van de zuurstofspanning in het vetweefsel en de skeletspieren mogelijk gunstige effecten heeft op de ontsteking in vetweefsel, op het metabolisme en op de insulinegevoeligheid. Deze verlaging zou kunnen worden bereikt door het verlagen van de hoeveelheid zuurstof in de omgevingslucht, waarbij mogelijk de zuurstofspanning in verschillende weefsels daalt, waaronder het vetweefsel en de skeletspieren. In **Hoofdstuk 6** hebben we de effecten van 7 dagen blootstelling aan milde intermitterende hypoxie (MIH) onderzocht. In dit onderzoek, dat momenteel nog gaande is, hebben we acht personen met overgewicht/obesitas blootgesteld aan normobare MIH (FiO_2 15%; gelijkwaardig aan ~3000m boven zeeniveau) en normoxie (FiO_2 21%) voor 7 achtereenvolgende dagen (3 cycli van 2h blootstelling/dag) op een gerandomiseerde manier, met een wash-out van 3-6 weken tussen beide periodes. Onze voorlopige resultaten lieten zien dat MIH effectief de systemische zuurstofsaturatie evenals de zuurstofspanning in het buikvet en de skeletspier verlaagd. Daarnaast induceerde MIH een verschuiving in het substraatmetabolisme richting een verhoogde koolhydraatverbranding onder gevaste en postprandiale omstandigheden, hetgeen tevens gereflecteerd werd in verhoogde circulerende lactaat concentraties in het bloed. Deze verschuiving in substraatmetabolisme leek aan te houden na het beëindigen van MIH. MIH induceerde echter geen veranderingen in plasma glucose en lipiden concentraties en had ook geen duidelijk effect op de insulinegevoeligheid.

Aangezien het mogelijk is dat de totale blootstellingduur en het aantal MIH cycli van invloed kan zijn op metabole en inflammatoire processen, onderzochten we de effecten van verschillende hypoxie blootstellingsprotocollen op de functie van humane adipocyten in **Hoofdstuk 7**. We stelden adipocyten bloot aan 4 verschillende protocollen waarbij ze aan lage zuurstof blootgesteld werden (7 of 14 dagen, 3x2h of 6x2h blootstelling per dag). Onze hypothese was dat het verlagen van de hoeveelheid zuurstof van 10% naar 5% (zoals voorheen gevonden in vetweefsel van obese en slanke personen, respectievelijk), gunstige effecten heeft op ontstekingsparameters en de glucose opname in adipocyten. Blootstelling van adipocyten aan lage zuurstof (5% O_2 cycli van 2h) reduceerde de secretie van de pro-inflammatoire cytokine IL-6. Desalniettemin, in vergelijking met continue blootstelling aan 10% O_2, had deze blootstelling geen ander effect op de opname van glucose in deze adipocyten en de expressie van andere genen gerelateerd aan het metabolisme en ontsteking. Deze bevindingen suggereren dat MIH geen duidelijke effecten heeft op de functie van adipocyten.

Dit proefschrift biedt belangrijke inzichten in de effecten van zuurstofspanning op vetweefsel, adipocyte functie en humaan metabolisme. Daarnaast zijn ook de verschillen tussen vetdepots onderzocht. Op basis van de bevindingen beschreven in dit proefschrift zouden toekomstige onderzoeken uitgevoerd worden om de effecten van de hoeveelheid zuurstof in organen op verschillende pathofysiologische processen die betrokken zijn in de ontwikkeling van chronische cardiometabole ziekten te onderzoeken, aangezien milde intermitterende hypoxie een alternatieve strategie zou kunnen zijn om deze ziekten te bestrijden.

VALORIZATION

Valorization

Societal and economic relevance

Obesity is an increasing worldwide problem, warranting innovative strategies to reduce the risk for chronic diseases related to excess body weight. The present thesis describes the effects of tissue oxygenation, and alterations thereof, on adipose tissue function, substrate metabolism and glucose homeostasis in humans. The valorization potential of the work described in this thesis will discussed in terms of societal and economic relevance, the implications for specific target groups, future research perspectives and potential relevance for industry.

The 2016 report of the World Health Organization (WHO) indicated that the worldwide prevalence of obesity is still increasing, with more than 39% of the adults being overweight (>1.9 billion adults, >50% in Europe) and 13% clinically obese (>600 million, >20% in Europe).[1] It is especially alarming that 42 million children under the age of 5 years were overweight or obese in 2013, and numbers are still rising. Obesity is often accompanied by many comorbidities such as cardiovascular disease, type 2 diabetes, certain forms of cancers, psychological abnormalities, osteoarthritis, respiratory diseases, gynaecologic abnormalities and skin problems. Currently, overweight/obesity is the 5th leading cause of death and the increasing prevalence has besides public health issues also major socio-economic consequences.[2]

A positive energy balance usually causes overweight/obesity, where the intake of energy exceeds energy expenditure for a prolonged period of time. This leads to storage of excess energy as body fat, also leading to lipid overflow in the circulation, ectopic fat storage and chronic low-grade inflammation. It appears to be very difficult to counteract the positive energy balance for overweight/obese individuals. Over time, this problem will only increase, partly due to the increased access and exposure to all kinds of foods. Moreover, the sedentary lifestyle of most individuals promotes obesity and increases disease risk. Fortunately, a modest weight loss can already lead to considerable beneficial health effects. The US Diabetes Prevention Program indicated that every kilogram of weight loss within the context of a lifestyle intervention focussed on diet and physical activity, reduced the risk of developing diabetes by 16%. Interestingly, recent data from the DIRECT trial demonstrated that more pronounced weight loss leads to higher remissions rates in type 2 diabetic individuals.[3]

Scientific community

The results described in this thesis have been presented at national and international conferences to colleagues inside and outside the field with the purpose to increase the awareness of the medical, societal and economic consequences of overweight/obesity and to highlight the potential role of tissue oxygenation for scientists, health care professionals, physicians and dietitians. Moreover, the results will become available to the scientific community through publication in peer-reviewed journals, with the aim to increase knowledge in the research area of obesity and adipose tissue and skeletal muscle oxygenation and human substrate metabolism.
Within the scientific community we have collaborated with the Maastricht Radiation Oncology (MaastRO) Laboratory, providing the use of their roxybot allowing us to incubate our human adipose-derived stem cells under different levels of oxygen. In addition, we also collaborated with the German Diabetes Center, who measured the adipokines secreted in the adipocyte medium. Finally, the Joanneum Research institute has produced and provided the oxygen-sensitive membranes ('oxygen sensors'), needed to continuously monitor oxygen tension in adipose tissue and skeletal muscle using the optochemical measurement device.

The findings presented in this thesis, may give leads for the development of treatment strategies including moderate hypoxia exposure. The duration and severity of oxygen exposure can be varied to obtain optimal effects in different populations. Moreover, a combination of treatments should be explored, since hypoxia exposure, exercise and dietary interventions might work synergistically. However, this should be investigated in future research. Altogether, the work described in this thesis may be of value for industry and health care professionals (e.g. dieticians, physiotherapists and physicians).

Activities and products

In this thesis, we applied several techniques, combining state-of-the-art in vivo methodology (hyperinsulinemic-euglycemic clamp, substrate metabolism, adipose tissue blood flow, and oxygen tension measurements) with mechanistic human cell culture experiments (gene expression, adipokine secretion, protein abundance, oxygen consumption, and glucose uptake), giving important insights for prevention and treatment of obesity related diseases.

Previous research from our group showed that abdominal subcutaneous adipose tissue oxygen tension is higher in obese individuals and is related to a metabolic profile. Interestingly, in Chapter 5 we found an inverse correlation between abdominal adipose tissue oxygen tension and insulin sensitivity, independently of adiposity, suggesting that lowering adipose tissue oxygen tension may contribute to improved insulin sensitivity in humans. We subsequently examined whether mild intermittent hypoxia (MIH) exposure may affect insulin sensitivity and metabolic health in a randomized placebo-controlled cross-over trial in overweight and obese men with a relative low insulin sensitivity (chapter 6). We found that treatment with MIH is a potent way to reduce adipose tissue and skeletal muscle oxygenation by ~45% and ~50%, respectively. However, this was not accompanied by beneficial effects of MIH on insulin sensitivity, although marked changes in substrate metabolism were observed. It is evident from these findings that MIH, as used in the current protocol, may not be the optimal strategy in improving metabolic health and insulin sensitivity. Subsequently, we studied in chapter 7 the impact of continuous oxygen exposure (10% O_2) versus different intermittent hypoxia protocols (10% to 5% O_2) on adipocytes *in vitro*, and could not demonstrate differential effects of MIH as compared to continuous exposure.

In summary, our research has provided insight in the role of adipose tissue oxygen tension in insulin sensitivity as well as leads to further investigate the impact of environmental hypoxia exposure on adipose tissue function and metabolic health. Future studies may include the combination of hypoxia and exercise, the exposure to different continuous and intermittent hypoxia protocols as well as a more targeted approach based on the initial metabolic phenotype.

Innovation

In this thesis, we measured for the first-time oxygen tension in both abdominal and femoral subcutaneous adipose tissue of overweight and obese adults simultaneously using the recently developed optochemical measurement system.[4, 5] These measurements showed that we can continuously monitor oxygen tension in different adipose tissue depots *in vivo* over a longer time period. Moreover, for the first time we used our optochemical measurement system to accurately measure oxygen tension in skeletal muscle. The use of this novel technique provided insight in physiological tissue oxygenation of key metabolic organs, and provided insight in the role of oxygenation in adipose tissue and muscle metabolism. The continuous monitoring of oxygen tension *in*

vivo, allowed us to show that depot differences in oxygen tension might be related to adipose tissue function.

Since data on depot differences is scarce, especially human studies, the comparison of the proteome of abdominal and femoral subcutaneous adipose tissue is of great scientific value. In this thesis, we showed only slight differences in the adipose tissue proteome of the abdominal and femoral depot.

We have built a hypoxic room, allowing lowering of oxygen levels from 21% to 15%. The room was made as airtight as possible, with an air-lock system to prevent direct airflow when entering or leaving the room. Due to these adaptations, the room remained constant in oxygen level varying between 15+/- 0.5% O_2. This room has allowed us to show that MIH treatment reduced systemic oxygen saturation and tissue oxygenation, altered substrate oxidation but did not significantly affect insulin sensitivity in overweight/obese men.

Planning and realization

In the prevention of obesity and type 2 diabetes, changes in lifestyle are usually the main part of the treatment. Interventions with a combination of physical activity and diet have been shown effective, reducing the incidence of diabetes by ~50% over 3-6 years.[6] In these interventions, healthcare professionals play an important role in the implementation and adherence to these lifestyle changes. Although a profound knowledge has been gathered in the studies described in this dissertation with respect to the effects of oxygen on human metabolism (mainly in adipose tissue), translation into guidelines for healthcare professionals requires more research.

Differences between distinct adipose tissue depots (i.e. upper versus lower-body adipose tissue) are important in the cardiometabolic risk of individuals. This is underlined in our research, indicating that abdominal and femoral adipose tissue differ in oxygen tension, adipocyte size, and proteome. However, further characterisation of these (and other) adipose tissue depots is necessary in combination with cardiometabolic risk assessment in humans.

In Chapter 6 and 7, we demonstrated that on a relatively short-term, mild intermittent hypoxia exposure had no pronounced effects on adipocyte and whole-body metabolism and insulin sensitivity. Future studies should investigate the effects of both continuous

and intermittent long-term hypoxia regimens and should investigate the potential synergistic metabolic effects of hypoxia and exercise. In these kind of studies, the importance of the severity of hypoxia exposure, the amount of hypoxic cycles, and the duration of hypoxia exposure should be taken into account at the whole-body and tissue level (i.e. adipose tissue, skeletal muscle and the liver).
In chapter 6 and 7, we investigated insulin resistant obese men (in chapter 7, cells were derived from individuals with these characteristics). Since inter-individual differences in the effect of MIH exposure on systemic oxygen saturation (SpO_2) are present, future research has to show whether these results can be extrapolated to other metabolic phenotypes. Based on these outcomes, it may be an option to personalise the treatment, i.e. to adjust FiO_2 according to its effects on SpO_2, in order to achieve a more beneficial metabolic effect. Moreover, effects of MIH should also be investigated in individuals with more severe impairments in glucose homeostasis such as patients with type 2 diabetes.

In the upcoming years, these types of research are needed to further elucidate the true potential of exposure to hypoxia (also in combination with other inventions, such as exercise and diet) in the prevention and treatment of chronic metabolic disease.

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ACKNOWLEDGEMENTS

Acknowledgements

Dit is het dan! Het einde van het promoveren, iets waar veel mensen bij betrokken zijn geweest, ik veel heb mogen leren en waarbij velen een onmisbare bijdrage hebben geleverd ofwel direct in de onderzoeken, ofwel in de nodige ontspanning daarnaast. Het is tijd om een aantal mensen te bedanken die mij geholpen hebben deze top te bereiken.

Allereest wil ik mijn promotieteam, Prof. **Ellen Blaak** en Dr. **Gijs Goossens** bedanken voor alle ondersteuning bij dit traject. Vanaf het eerste moment heb ik mij thuis gevoeld binnen de afdeling humane biologie. Ellen, ik heb de meetings altijd als zeer prettig ervaren, je stond altijd klaar als dat nodig was en hebt onmisbare feedback gegeven om alle manuscripten naar een hoger niveau te brengen.
Gijs, bij jou kon ik altijd binnen lopen voor allerlei zaken, je nam altijd ruim de tijd om alles uit te leggen en mij zo veel wetenschappelijke inzichten bij te brengen. Ondanks dat dit soms erg uitgebreid was, heb ik dit altijd enorm gewaardeerd. Ook bij testdagen, kon ik waar nodig altijd op jou rekenen en heb ik enorm veel van je geleerd. Ik bewonder de manier waarop jij met situaties omgaat, je laat altijd iedereen in zijn waarde en geeft goede constructieve feedback. Door jou toewijding, enthousiasme en positieve instelling zijn de afgelopen jaren voorbijgevlogen.

Ellen en **Gijs**, ik had me geen beter promotieteam kunnen wensen. Jullie stonden altijd voor me klaar en zijn altijd eerlijk en direct geweest. Als er een proefpersoon had afgezegd, een meting was mislukt, een uitkomst niet zo was als we hadden verwacht of als een manuscript werd afgewezen, kon ik altijd rekenen op jullie positieve instelling. Dit heeft mij steeds gestimuleerd om verder te gaan en een volgende poging te wagen, met deze thesis als resultaat. Kortom, bedankt voor de prettige samenwerking en ik hoop met jullie in contact te blijven!

Next, I would like to thank the members of the assessment committee, **Prof. A. Schols**, **Prof. K. Schalkwijk**, **Prof. L. van Loon**, **Dr. K. Manolopoulos**, and **Dr. B. Guigas** for taking the time and effort to review my thesis and being present at the official defence of this dissertation.

Verder wil ik natuurlijk alle co-auteurs bedanken voor hun bijdrage aan de mooie studies die mede dankzij jullie mogelijk zijn gemaakt! Ik heb hier enorm veel van geleerd en vond het leuk om met jullie samen te werken.

Humaan onderzoek is natuurlijk onmogelijk zonder proefpersonen. Ik moet zeggen dat ik mede hierdoor een ontzettend leuke tijd heb gehad, waar we flink hebben kunnen lachen maar ook hele mooie goede metingen hebben kunnen doen. Sommige verhalen en momenten zal ik dan ook nooit vergeten. Dit is cruciaal geweest in het succes van dit proefschrift.

Rudi, met jou heb ik het grootste deel van mijn promotie een werkkamer gedeeld. Ik had me geen betere kamergenoot kunnen wensen. Ik zal ons experiment met bier koelen in droogijs nooit vergeten, waarbij we met filters het bier nog hebben proberen te redden. Ook de gezamenlijke interesse in horloges en whisky maakte dat we over veel konden praten en het een hele mooie tijd was. Ook tijdens de verschillende congressen, het NVDO, EASD en de summer school in Lissabon hebben we veel gelachen en meegemaakt.

Rens, wij hebben in het laatste jaar van mijn promotie de HYPEX-studie samen uitgevoerd. Hier hebben we ontzettend veel gelachen samen met de proefpersonen en in korte tijd veel meegemaakt. Het was heel prettig om met jou samen te werken en uiteraard blijf ik nog zeker zo nu en dan inchecken hoe het met de HYPEX-studie en vervolgstudies gaat. Ik denk dat ik het nog het beste zo kan samenvatten: 'Ja, wel gelachen hoor'.

Dan zijn er natuurlijk enorm veel collega's, zoals de onmisbare secretaresses **Cleo**, **Desiree**, en **Claudia** die altijd klaar stonden om te helpen. **Paul**, **Marc** en **Loek**, die alle technische problemen tijdens testen, software, en vele andere zaken altijd feilloos hebben weten op te lossen. En **Paul** en **Marc**, jullie natuurlijk speciaal aangezien jullie het mogelijk hebben gemaakt om de HYPEX study uit te kunnen voeren waarbij jullie een kamer speciaal hebben omgebouwd om de zuurstof te kunnen verlagen.

Tijdens de humane studies heb ik gelukkig heel veel fijne hulp gekregen.
Joey, bedankt dat jij ons hebt geholpen met het nemen van alle spierbiopten en het includeren van de proefpersonen. Wij konden altijd op je rekenen en daarnaast ben je een zeer leuke collega die altijd bereid is om te helpen.

Johan en **Nicole**, jullie hebben alle oxygraafmetingen voor jullie rekening genomen, dit heeft veel tijd en energie gekost waar ik jullie enorm dankbaar voor ben! **Joris**, bedankt voor de hulp bij de kwaliteitscontrole en het analyseren van de oxygraafmetingen. **Joan** en **Antoine**, op jullie kon ik altijd rekenen voor last-minute hulp bij het zetten van infusen, enorm bedankt hiervoor! En natuurlijk bedankt voor alle analyses in de plasma samples, **Wendy**, **Hasibe**, en **Jos.**

Jos, humane biologie zal nooit meer hetzelfde zijn zonder jou. Je wordt gemist!

Roel, **Marlies**, **Charlotte**, **Dirk**, **Niels**, **Alexandre**, **Cyril**, **Yvo**, **Jan**, **Dennis**, and **Emmani, aka the Nerds**, even though I was not the most active participant in this privileged group, I had some awesome moments with you guys! Loved all the memes passing by in the app-group. Japanese night changed so many things, the weirdness we saw during this evening will make it so difficult to encounter something weirder. But, the racing event, board/card games (especially exploding kittens), and the nice Raclette dinners, were truly amazing!

Peter, **Guy**, **Roel**, **Dirk** en **Cyril**, bedankt voor de gezellige pokeravonden met heerlijke speciaal bieren.

Dan mijn **NUTRIM-council** collega's, **Charlotte**, **Pauline**, **Mattea**, **Mirjam**, **Elisa** en **Jacqueline**. Bedankt voor de leuke en leerzame tijd. Ik vond het ontzettend leuk om samen met jullie in dit council te zitten met als hoogtepunt toch wel het 25-jarig jubileum van NUTRIM, wat een groot succes was.

En uiteraard ook alle (ex)collega's binnen onze onderzoeksgroep, **Rudi**, **Rens**, **Emanuel**, **Dorien**, **Jasper**, **Birgitta**, **Ines**, **Suzanne**, **Adriyan**, **Manuel**, **Qing**, **Qi**, **Mattea**, **Kenneth**, **Ruth**, **Johan**, **Nicole**, **Yvonne**, **Gijs**, **Ellen**, en ook al was het niet direct hoorde jullie er toch bij **Roel** en **Nadia**. Bedankt voor alle hulp, discussies en vooral ook al het plezier dat we hebben gehad de afgelopen jaren. Ik heb het enorm fijn gevonden dat iedereen altijd voor elkaar klaar stond! De congressen, dagjes uit of feesten waren altijd memorabel en zal ik dan ook zeker gaan missen.

Nicole en **Yvonne**, bedankt voor al jullie hulp op het lab, en het uitvoeren van verschillende analyses. Ik vond het altijd heel leuk en gezellig om samen met jullie experimenten uit te voeren en heb enorm veel aan jullie hulp gehad!

Aan **alle collega's** binnen de vakgroep Humane biologie en natuurlijk ook de collega's van de vakgroep Voedings- en bewegingswetenschappen, bedankt voor de leuke borrels, uitjes, feesten en geweldige weekendjes. Het zijn te veel activiteiten en herinneringen om hier allemaal te benoemen, maar ik zal ze nooit vergeten ☺.

Niet te vergeten natuurlijk, mijn MLW-vrienden. **Niels**, **Alex**, **Joel**, **Camiel**, **Thomas**, **Mick**, **Mandy**, **Johanna**, **Carmen**, **Svenja** en **Leslie**, wat hebben we al enorm veel onvergetelijke tijden samen beleefd. Alle weekendjes weg, festivals, armin nights, en noem het maar op. De ontspanning die dit heeft gebracht waren heel belangrijk en ik ben dan ook heel blij dat we elkaar nog steeds regelmatig zien!

Een van mijn uitlaatkleppen is toch wel hockey geweest. De eerste jaren in Weert, waarbij **Heren 3** een geweldig leuk team was waar ik met het grootste plezier heb gehockeyd. Ook de wintersport was een zeer belangrijke vakantie die ik ieder jaar heb mogen meemaken, heren bedankt daarvoor! Daarna werd het toch tijd om wat dichter bij huis te gaan hockeyen, Maastricht, hier kwam ik in **Herren zwei** terecht. Dit was een jonger team maar hier heb ik twee geweldige seizoenen op leuk niveau kunnen spelen, bedankt voor deze twee topseizoenen! En **Laurens**, ik hoop dat we nog veel formule 1 wedstrijden samen mogen kijken in de toekomst!

Wil, **Anja**, **Loes** en **Willem**, vanaf het begin heb ik mij altijd welkom gevoeld bij jullie. Bedankt voor alle steun, interesse en de vele leuke momenten die ik al met jullie heb mogen meemaken.

Ries en **Maud**, bedankt voor jullie interesse, steun en vooral voor de gezellige en leuke momenten die we altijd samen hebben. Leuk dat we straks weer allemaal in Weert wonen, dan kunnen we elkaar hopelijk wat vaker zien!

Lieve **Papa** en **Mama**, jullie hebben mij altijd gesteund in alles wat ik doe! Ondanks dat ik jullie niet genoeg kan bedanken, wil ik deze thesis heel graag aan jullie opdragen als teken van dank. Ik ben enorm dankbaar voor alle kansen die jullie me al gegeven hebben en dat ik altijd op jullie kan rekenen of het nou is voor advies of om mijn (soms erg enthousiaste) verhalen aan te horen. Ik heb het voor een groot deel aan jullie te danken dat ik dit heb kunnen bereiken en jullie hebben me altijd alle vrijheid en ruimte hiervoor gegeven. Ik kan me dan ook geen betere ouders wensen!

Lieve **Claudia**, jij hebt alles van dichtbij meegemaakt. Ik ben ontzettend blij en dankbaar voor al jouw steun, interesse en vooral jouw liefde. Of je nu een stuk moest nalezen/verbeteren, advies moest geven over een situatie of dat ik druk met iets bezig was, je bent altijd een stabiele steun geweest en hebt nooit ergens over geklaagd. Zonder jou was het allemaal een stuk zwaarder geweest.
Nu gaan we allebei de volgende fase in en ik kan niet wachten op alle avonturen die we samen gaan beleven. Ik kijk enorm uit naar onze toekomst, bedankt voor alle liefde, steun en geduld. Ik hou van jou!

Bedankt allemaal!

LIST OF PUBLICATIONS

List of publications

Differences in Upper and Lower-body Adipose Tissue Oxygen Tension Contribute to the Adipose Tissue Phenotype in Humans. **Vogel MAA**, Jocken JWE, Sell H, Hoebers N, Essers Y, Rouschop KMA, Čajlaković M, Blaak EE and Goossens GH. ***J Clin Endocrinol Metab*. 2018, in press (doi 10.1210/jc.2018-00547)**

Adipose tissue oxygenation is associated with insulin sensitivity independently of adiposity in obese men and women. Goossens GH, **Vogel MAA**, Vink RG, Mariman EC, van Baak MA and Blaak EE. ***Diabetes Obes Metab*. 2018, in press (doi 10.1111/dom.13329)**

Diet-induced weight loss decreases adipose tissue oxygen tension with parallel changes in adipose tissue phenotype and insulin sensitivity in overweight humans. Vink RG, Roumans NJ, Čajlaković M, Cleutjens JPM, Boekschoten MV, Fazelzadeh P, **Vogel MAA**, Blaak EE, Mariman EC, van Baak MA and Goossens GH. ***Int J Obes (Lond)*. 2017;41:722-728**

Moderate hypoxia exposure: a novel strategy to improve glucose metabolism in humans? **Vogel MAA**, Blaak EE and Goossens GH. ***EMJ Diabetes*. 2015;3:73-79**

A comparison between the abdominal and femoral adipose tissue proteome of overweight and obese women. **Vogel MAA**, Bouwman FG, Hoebers N, Blaak EE, Renes J, Mariman EC and Goossens GH. **Submitted**

The effects of mild intermittent hypoxia exposure on tissue oxygenation, substrate metabolism and insulin sensitivity in overweight and obese men: a single-blind, randomized, placebo-controlled, cross-over study. **Vogel MAA***, van Meijel RLJ*, Jocken JWE, Hoebers N, Hoeks J, Schoffelen PFM, Blaak EE and Goossens GH. **In preparation**
*shared first authorship

The effects of mild intermittent hypoxia exposure on adipocyte function. **Vogel MAA***, van Meijel RLJ*, Jocken JWE, Sell H, Rouschop KMA, Blaak EE and Goossens GH. **In preparation**
*shared first authorship

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About the author

Max Vogel was born on the 5th of December 1989 in Weert, the Netherlands. In 2008, he obtained his Gymnasium (VWO) diploma from het Bisschoppelijk College in Weert. In the same year, he started his studies Molecular Life Sciences at Maastricht. The study was later renamed to Biomedical Sciences. For his master degree, he performed an internship at the department of Toxicology, Maastricht University, in which he studied the role of the NRF protein on carbon nanotube induced lung fibrosis. During the second year he performed an internship and Molecular Genetics, Maastricht University and the National Institute of Health (NIH) in Bethesda, USA investigating the role of Parkin in the fat metabolism of the heart.

He graduated in 2013, and started his PhD at the department of Human Biology at Maastricht University under the supervision of Prof. dr. Ellen Blaak and Dr. Gijs Goossens. During his PhD, he studied the role of tissue oxygenation in human metabolism, with a focus on adipose tissue and metabolic health. In addition, he presented his research findings at several national and international conferences, including the yearly European Congress on Obesity (ECO) meetings. In 2015, he was awarded with the NUTRIM poster prize for the best clinical research poster. Furthermore, he was awarded the best abstract prize by the Netherlands Association for the Study of Obesity (NASO) in 2016.