The metabolic impact of hypoxia exposure in human obesity

THE METABOLIC IMPACT OF HYPOXIA EXPOSURE IN HUMAN OBESITY:
a translational approach

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CHAPTER 1

General Introduction

Obesity – a major public health issue

Obesity is considered a multifactorial disease, characterized by an imbalance between energy intake and energy expenditure resulting in excessive fat accumulation, and thereby presenting risks to human health. Various factors such as increasing economic growth, industrialization, urbanization, mechanized transport, and concomitant changes in behavior of people including a sedentary lifestyle, food preferences for processed foods, and the consumption of calorie-dense diets contribute to the rising prevalence of overweight and obesity (1). Collectively, the multifactorial causes of obesity plead for a multifactorial approach to prevent and treat obesity, as the obesity epidemic poses a major public health challenge.

Globally, obesity is a major cause of morbidity and mortality (2). Worldwide, more than 1.9 billion adults are overweight, defined by the World Health Organization (WHO) by a body mass index (BMI) ≥25 - 30 kg/m^2, of whom at least 650 million are obese (BMI ≥30 kg/m^2) (3). In the USA, it has been estimated that the age-adjusted prevalence of obesity is 42.1% (4). A similar trend is observed in Europe, in which estimations for the overall prevalence of overweight and obesity are approximated around 37.2 and 15.9% of the population, respectively (5). According to current estimates, the global prevalence of obesity will increase drastically in the near future (6). In the USA, it has been estimated that the obesity prevalence will increase to 51% of the population by 2030, whereas the prevalence of severe obesity (≥40 kg/m^2) potentially increases by 130% (7). European predictions forecast obesity prevalence to range from 13-43% by 2025 across different countries (8).

The high prevalence of overweight and obesity pose a major public health problem, as obesity is associated with a variety of complications and chronic diseases, including hypertension, dyslipidemia, type 2 diabetes, cardiovascular disease, cognitive decline, depression, obstructive sleep apnea syndrome, skin problems, asthma, and several types of cancer (9-14). Furthermore, recent analyses demonstrate that European countries spend on average 7% of their healthcare budget on obesity-related diseases, thereby substantially burdening on national budgets and economies, and hindering containment of healthcare costs (15).

Lifestyle intervention has been proven to be effective to reverse obesity progression and to decrease the risk of obesity-related complications. However, long-term maintenance of lifestyle changes (e.g. by adhering to dietary regimen and physical activity pattern) is very difficult for many people, which often hampers long-term maintenance of body weight loss and/or cardiometabolic improvements (16). Although lifestyle is a key factor in the etiology and consequences of obesity, multiple other aspects need to be considered in the management of obesity-related complications. Therefore, it is highly warranted to elucidate the underlying mechanisms and risk factors involved in obesity-related cardiometabolic complications to develop additional therapeutic approaches to prevent and treat overweight, obesity and its complications.

A putative strategy to improve the metabolic homeostasis in obesity is the application of hypoxia exposure, which has been investigated in the studies described in this thesis. Hypoxia exposure leads to a decrease in systemic oxygen saturation, thereby reducing the oxygen availability in metabolically active tissues. Interestingly, mechanistic studies have found that hypoxia exposure may improve glucose homeostasis, yet well-controlled human studies are lacking (17-19). In addition, underlying mechanisms are not fully understood and remain to be investigated.

Obesity-related metabolic impairments

Obesity is a well-established major risk factor for the development of insulin resistance, and subsequently type 2 diabetes mellitus. Obesity-induced insulin resistance is characterized by impaired insulin action in many processes and organs, amongst which are reduced insulin-stimulated glucose uptake by the adipose (AT) and skeletal muscle (SM) tissue, reduced insulin-mediated vasodilatation, diminished insulin-mediated suppression of hepatic glucose output, reduced insulin-mediated triglyceride (TAG) extraction and impaired insulin-mediated inhibition of endogenous lipolysis (20-22). In addition, AT dysfunction and related low-grade inflammation may disturb fatty acid metabolism, which plays a key role in the development of obesity-induced insulin resistance by excessive lipid deposition in non-adipose tissues due to inadequate containment of lipids by AT (23, 24). This process, often referred to as ectopic fat storage, has been described to manifest in the pancreas, liver, skeletal muscle and heart, and the accumulation of bioactive lipid metabolites interfere with insulin signaling (25). The decreased action of insulin with concomitant β-cell failure (i.e. insufficient insulin secretion) may ultimately result in hyperglycemia, which is characterized by elevated fasting glucose concentrations (impaired fasting glucose, IFG, 5.6 – 6.9 mmol/L) and/or elevated postprandial glucose concentrations (impaired glucose tolerance, IGT, 7.8 – 11.1 mmol/L; two-hour glucose concentration derived from an oral glucose tolerance test), and may further progress into overt type 2 diabetes (26). Current evidence indicates that various tissues are involved in the pathology of obesity-induced insulin resistance, amongst which are the liver, adipose and skeletal muscle tissue, and the gut, thereby emphasizing the complexity of the pathogenesis of obesity-induced insulin resistance and subsequent cardiometabolic disorders (24, 27, 28).

Adipose tissue dysfunction

AT is recognized for various physiological and functional properties, such as mechanical stress protection (29), thermal insulation and reproductive function (30, 31). In addition, AT is considered to store and provide long-term energy reserves,

which is illustrated by the storage of dietary TAG in the postprandial state, and mobilization of fatty acids under fasting conditions, in which energy expenditure exceeds intake for a substantial amount of time, or physical exercise (32). In the past decades, the view on AT has shifted from being inert, to a highly dynamic and metabolically active organ (33) (34). Indeed, the classical function of AT is to store triglycerides (TAG) and to liberate free fatty acids (FFA).

Besides its prominent role in lipid homeostasis, AT is recognized for its endocrine function. Indeed, factors secreted by AT (so called adipokines) regulate various physiological processes, such as energy homeostasis, blood pressure, inflammation, angiogenesis and food intake (35). Leptin, the first discovered adipokine, acts in an endocrine manner by regulating food intake and energy homeostasis in the hypothalamus and peripheral organs, respectively (36). To date, an enormous number of adipokines have been described, which can exert autocrine, paracrine and/or endocrine effects (37). Therefore, AT plays a key role in the regulation of many metabolic processes, and is not merely a passive energy depot, as originally thought.

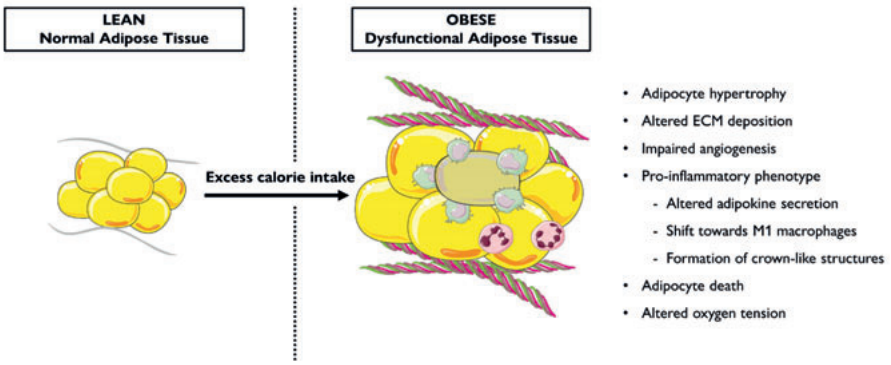

Figure 1. Obesity, characterized by excess caloric intake, contributes to the development of dysfunctional adipose tissue characterized by adipocyte hypertrophy, a proinflammatory phenotype (i.e., a shift toward more M1 macrophages), adipocyte cell death, and an altered adipokine secretion pattern. Surrounding necrotic adipocytes, crown-like structures can be observed, which consist of phagocytic cells that remove cell debris. Furthermore, dysfunctional adipose tissue shows disproportionate deposition of ECM components. Together with impaired angiogenesis, this may limit further expansion of adipose tissue in obese individuals. ECM, extracellular matrix. Adapted from van Meijel et al. (38)

Prolonged excess energy intake leading to obesity is accompanied by marked alterations in the structure and composition of AT. Obese AT is often associated with adipocyte hypertrophy, infiltration of various types of immune cells and an altered

composition of the basement membrane and extracellular matrix (39) (Figure 1). The enlarged AT mass is mainly generated by adipocyte hypertrophy, as well as hyperplasia. In general, AT mass expansion, and hence weight gain, are merely the net result of adipocyte hypertrophy. Yet, in the subcutaneous AT depot, hyperplasia may also play a key role due to higher adipocyte progenitor number and activity compared to visceral AT (40, 41). However, it has been postulated that the AT has a certain expansion limit, determined by both genetic and environmental factors (42, 43), hence lipid intake exceeding the AT storage capacity results in the deposition of lipids in non-adipose tissues (43). This phenomenon, referred to as ectopic fat deposition, may occur in various metabolically active organs such as liver, pancreas, heart, and SM, thereby interfering with various cellular processes, including insulin signaling. In addition, the presence of increased quantities of bioactive lipid intermediates in these tissues may provoke apoptosis and inflammation, impairs insulin signaling, hence compromising tissue function (44-46). The detrimental effects of impaired lipid buffering in AT are demonstrated by lipodystrophic animal models and humans. Indeed, lipodystrophic animals are characterized by ectopic fat accumulation, and hence demonstrate a variety of pathological symptoms such as insulin resistance, non-alcoholic hepatic steatosis and dyslipidemia (47). In line, patients with lipodystrophy are severely insulin resistant, and many of these patients eventually develop T2DM (48). Impaired TAG extraction from the circulation, caused by blunted insulin-stimulated LPL activity, may increase lipid spillover (24). Additionally, impaired suppression of lipolysis by insulin, together with increased AT mass, may result in increased FFA concentrations, and hence dyslipidemia, as often observed in obesity-induced insulin resistance (49). In addition to the lipolytic enzymes, the abundancy and conformation of lipid droplet integrity proteins, the so-called perilipins, play a key role in controlling lipolytic rate (46). People with obesity are often characterized by a deficiency in perilipin 1 expression (50), which may result in an increased basal rate of lipolysis (51). However, it has been found that FFA do not increase proportionally to fat mass in obese humans. In addition, resistance to catecholamine-induced lipolysis has been reported in obesity, which may ultimately contribute to a downregulation of lipolysis per unit of fat mass in insulin resistance (46, 52).

Taken together, impaired lipid homeostasis in obesity contribute to many adverse effects, in particular insulin resistance, impaired glucose tolerance, hepatic gluconeogenesis, increased VLDL-TAG production, and reduced insulin clearance by the liver (46, 53). Thus, AT dysfunction in obesity is associated with a variety of metabolic dysregulations in multiple organs involved in metabolic homeostasis.

Altered skeletal muscle metabolism

SM is recognized as being essential in physical movement, posture and breathing, however plays a key role metabolically active processes such as FFA and glucose handling, and protein synthesis, besides serving as a reservoir for amino acid, lipid and glucose storage. In the postabsorptive fasting state, SM predominantly uses FFA as energy source by ensuing fat oxidation. Postprandially, skeletal muscle tissue serves as the largest sink for glucose disposal in healthy individuals, taking up the majority of ingested glucose following the intake of a mixed meal (54). Indeed, it has been demonstrated that during hyperinsulinemic-euglycemic conditions, SM accounts for approximately 80% of glucose disposal (55). SM glucose uptake is primarily mediated via insulin-dependent mechanisms, involving GLUT4 translocation, hence facilitating glucose uptake into the muscle cells (54). This insulin-stimulated net increase in glucose flux results in a shift from fat oxidation during fasting conditions, towards oxidation of glucose during postprandial conditions, a phenomenon often referred to as metabolic flexibility (56, 57). The underlying mechanisms by which SM insulin resistance is caused involves the storage, localization and turnover of certain lipid intermediates such as diacylglycerol (DAG), fatty acyl-CoAs, and ceramides, as well as increased muscle TAG extraction during postprandial conditions, since these have been associated with insulin resistance (58-60). Under normal conditions, insulin binds the insulin receptor, resulting in IRS-1 phosphorylation followed by a downstream signaling cascade. Yet, DAG inhibits phosphorylation of IRS-1, hampering insulin signaling, thereby contributing to impaired insulin-stimulated responses. Interestingly, we have recently demonstrated increased VLDL-TAG extraction and higher saturation of the intramuscular FFA pool, rather than total DAG content, in obese individuals with severe insulin resistance (61). In addition, SM mitochondria may play a key role in preventing lipotoxicity, since they are essential for SM fatty acid oxidation (62, 63). More specifically, mitochondria consist of multi-subunit complexes, and play a major role in various metabolic homeostatic processes, amongst which are oxidative phosphorylation, reactive oxygen species (ROS) generation, and thermogenesis. Due to its prominent role in energy homeostasis, mitochondria may be implicated in states of energy imbalance, such as obesity. Indeed, mitochondrial dysfunction has been considered as a *sine qua non* in the progression of obesity and T2DM (64), as demonstrated in human and rodent studies (65, 66). Disruption of mitochondrial substrate utilization may evoke accumulation of lipid intermediates, eventually disrupting insulin signaling (67). In addition, obese insulin resistant individuals demonstrate accumulation of lipid-intermediates within SM, and show reduced capacity to oxidize fatty acid substrates. In contrast, overweight insulin sensitive individuals maintain lipid oxidation capacity with normal fat content. Yet, the causality of mitochondrial dysfunction in insulin resistance is still under debate (68). Interestingly, findings from a recent study have suggested that AT inflammation

is directly linked to obesity-induced insulin resistance, while mitochondrial respiratory dysfunction is not always required. Rather, changes in mitochondrial bioenergetics may reflect physiological consequences of high-fat feeding (69, 70). Furthermore, it has been demonstrated in both animals and humans that besides impaired insulin-stimulated glucose uptake, insulin resistance is characterized by diminished glycogen synthesis rate and glucose oxidation (71, 72), of which the exact mechanism is not fully understood (73).

Myokines

Similar to AT, SM secretes bioactive molecules, named myokines, which can act in an autocrine, paracrine and endocrine manner, and may exert a critical role in inter-organ crosstalk and, therefore, metabolic adaptations. Since the discovery of interleukin (IL)-6, which concentration could increase 100-fold whilst performing exercise (74), the endocrine function of SM has been recognized. Evidence suggests involvement of myokines in various metabolic processes, amongst which are glucose disposal, fatty acid oxidation and inflammation (75). Indeed, the pleiotropic effects of myokines released upon physical exercise, may mediate a variety of health benefits (76). For example, IL-6 is considered to play an important role in anti-inflammatory processes, triggering the release of IL-10, and IL-1 receptor antagonist, together reducing systemic low-grade inflammation (77, 78). Yet, the role of IL-6 in inflammation remains controversial, as postprandial IL-6 muscle release is increased in men with impaired glucose tolerance (79), and may induce pro-inflammatory responses through different signaling cascades (74, 80). IL-15, which is thought to play a role in regulation of fat mass in both rodents and humans, is negatively associated with several obesity parameters such as BMI (81). In line, overexpression of IL-15 reduced trunk fat mass in mice (81). Yet, the number of myokines which have been demonstrated to have implications in human metabolism is rather limited, including IL-6, -8, -13 and -15, angiopoietin-like 4 and others (78). Nevertheless, several novel myokines may play a role in glucose homeostasis, as assessed in preclinical models. Interestingly, secreted protein acidic and rich in cysteine (SPARC) improved glucose tolerance in a AMP-activated protein kinase (AMPK)-dependent manner in high-fat diet induced obese mice (82). Furthermore, fibroblast growth factor 21 (FGF21) prevented obesity and diabetes development by normalizing glucose and lipid metabolism in mice (83). Targeting myokines involved in glucose and lipid homeostasis might therefore be an attractive strategy in the prevention and therapy of obesity-induced insulin resistance and T2DM.

Gut microbiota in obesity

The gastrointestinal system consists of a highly complex microbial ecosystem. In general, six phyla dominate the intestinal community, amongst which are *Fusobacteria, Proteobacteria*, *Actinobacteria*, *Verrucomicrobia, Firmicutes* and *Bacteriodetes* (84), the latter two representing 90% of the gut microbiota (85). The gut microbiota have been linked to affect brain function (86), immune defense (87), and host energy metabolism (88, 89). The microbial system interacts with the host in several ways, including nutrient metabolism upon dietary intake, via generation, extraction and absorption of bile acids, lipids, amino acids, vitamins and short chain fatty acids (85). Furthermore, the gut microbiome influences immune function, xenobiotic metabolism and is involved in the maintenance of gut barrier function (27). The gut microbiome is acquired by birth, and may be influenced genetics, dietary and nutrient intake, pharmacological stimuli and other (host) factors (90, 91).

The gut microbiota, being predominantly present in the colon, ensure fermentation of proteins and indigestible fibers (92, 93). Indeed, important microbial fermentation products are the short-chain fatty acids (SCFAs), of which acetate, propionate and butyrate are the main metabolites produced. SCFA are being involved in epithelial energy metabolism, and have been associated with several gut-derived signaling molecules, body weight control, substrate and energy metabolism and insulin sensitivity (94, 95). The gut microbial composition is highly variable, inter- and intra-individually, changing drastically throughout the gastrointestinal tract, which seem to depend on factors such as local pH, O_2 tension, digestion flow rate and substrate availability (96).

Disturbances in gut microbiota composition, i.e. microbial dysbiosis, might contribute to altered substrate metabolism and energy expenditure, and may affect ectopic fat accumulation, the inflammatory profile, and fat oxidation in the liver, skeletal muscle and adipose tissue (93). Furthermore, microbial imbalance might decrease intestinal integrity, and may thereby induce metabolic endotoxemia, which may stimulate immune cell infiltration in various tissues, amongst which is AT (97). Previously, animal experiments provided evidence for a causal relationship between gut microbiota and development of obesity, low-grade inflammation and insulin resistance (98, 99). However, studies showing a causal relationship between gut microbiota composition and metabolic implications in humans are scarce (100). Nevertheless, several reports demonstrated pronounced alterations in gut microbial composition in obesity, characterized by increased *Firmicutes* and decreased *Bacteriodetes* as compared to lean individuals (89), yet conflicting findings have reported in humans (101, 102). Interestingly, calorie-restricted weight loss, and bariatric surgery (103) may increase the *Bacteroidetes* in obese participants, although conflicting findings have also been reported (89, 104). More recent reports demonstrate taxonomic signatures of obese individuals on the genus and species level, however, functional implications of the microbiota profiles are still poorly understood (105).

Since previous research suggests a link between obesity and the gut microbiota, targeting the gut microbiota may have potential therapeutic implications.

Clearly, a wide variety of tissues and processes are involved in the etiology of obesity and related cardiometabolic complications. A better understanding of these underlying mechanisms could contribute to a better understanding and targeted therapeutic strategies in obesity. Interestingly, tissue pO_2 might be one of the potential triggers of obesity-related alterations in the metabolic and inflammatory phenotype.

The impact of oxygen on whole-body metabolic homeostasis

Central metabolic systems are heavily dependent upon oxygen availability. The oxygen status, or oxygen tension (pO_2), of a certain tissue depends upon the delicate balance between oxygen supply and oxygen consumption. In order for oxygen to reach a target tissue, it is transported mainly within red blood cells (RBCs) bound to hemoglobin (106). The supply of oxygen is therefore dependent on several factors, including (*1*) the fraction of oxygen in inspired air (FiO_2), (*2*) the capacity of RBCs to transport oxygen throughout the circulation, and (*3*) local tissue blood flow. The oxygen consumption mainly reflects the use of oxygen by mitochondrial respiration, i.e. oxidative capacity and substrate metabolism.

Due to its central role in glucose and lipid metabolism, pO_2 may act as a metabolic regulator of whole-body and organ-specific metabolism (Figure 2). Indeed, several studies have suggested involvement of pO_2 in altered glucose and lipid homeostasis at high altitude.

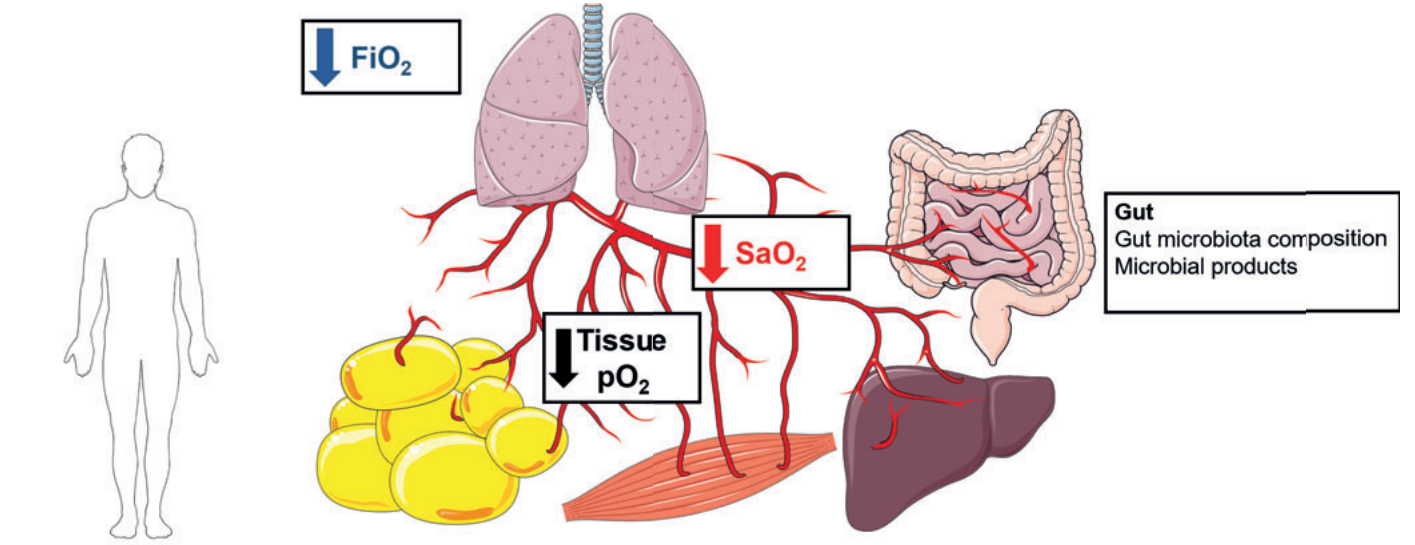

Figure 2. Potential impact of hypoxia exposure on whole-body physiology and adipose tissue, skeletal muscle, liver and gut tissue physiology. SaO_2, systemic oxygen saturation; FiO_2, fraction of inspired oxygen; pO_2, oxygen partial pressure. Figure is partially adapted from Lempesis et al. (19)

High altitude

Currently, it is estimated that a substantial proportion (approximately 7%) of the world population lives at high altitude (that is, >1500 m above sea level). Several environmental factors differ at high altitude compared to sea level, amongst which are low barometric pressure as well as low pO_2 relative to ambient air at sea level. As such, the FiO_2 remains constant at different altitudes, however, the increase in altitude results in a drastic reduction in atmospheric pressure, resulting in a pO_2 in inspired air (107). The reduction in atmospheric pressure and pO_2 reduce approximately linearly, to approximating 50% of sea level pO_2 at 5500 m of altitude (Table 1).

Table 1. Partial oxygen pressure (pO_2) at different altitudes and corresponding oxygen percentages under isobaric conditions (at sea level). Adapted from Kupper et al. (108)

(Corresponding) Altitude (m)	Atmospheric pressure (mm Hg)	pO_2 (mm Hg)	$\%O_2$, isobaric conditions, and sea level
0	760	158.8	20.9
500	716	149.6	19.7
1000	673.8	140.8	18.5
1500	634	132.5	17.4
2000	596	124.6	16.4
2500	560	117	15.4
3000	525.8	109.9	14.5
3500	493	103	13.6
4000	462	96.6	12.7
4500	432.6	90.4	11.9
5000	404.8	84.6	11.1
5500	378.6	79.1	10.4
6000	353.6	73.9	9.7
6500	330	69	9.1
7000	307.8	64.3	8.5
10 500	183	38.2	5
12 900	123.5	25.8	3.4

The human body's ability to adapt to these circumstances has been investigated thoroughly in previous decades. Well known adaptive mechanisms are an increased sympathetic nervous system activity, respiratory and hemodynamic alterations (109). Furthermore, epidemiological studies have pointed towards beneficial effects of

residing at high altitude on cardiovascular, respiratory and cancer-related mortality (110). Remarkably, accumulating evidence suggests that high altitude exposure affects glucose homeostasis as well (19, 111). Indeed, an inverse correlation has been demonstrated between altitude and fasting plasma glucose concentrations in a large cohort study in Peru, demonstrating significantly lower fasting plasma glucose concentrations at >3000 m (112). Furthermore, it has been estimated that people residing at high altitude have a 12% lower probability to develop T2DM compared to lowlanders (<500 m) (113). Noteworthy, participants living at these altitudes had similar age compared sea level, although BMI was significantly lower at high altitude. Nevertheless, several studies also found lower glycaemia in BMI-matched individuals at high altitude compared to sea level (114-119).

Besides investigating the long-term effects of high-altitude exposure in native highlanders, several studies focused on the acute effects of high altitude exposure in sea level residents. Following acute exposure to (very) high altitude, fasting plasma glucose concentrations have been demonstrated to increase initially in healthy humans (120). It has been proposed that increased catecholamine and/or cortisol release through altitude exposure might underlie these effects (121, 122), potentially through increased hepatic glucose output (123). Remarkably, the initial hyperglycemic response appears to be transient, demonstrating reductions in fasting plasma glucose to baseline, or even lower, after 1 week of exposure, illustrating the therapeutic potential of short-term high-altitude exposure on glucose homeostasis (118, 124).

Clearly, these high-altitude exposure studies demonstrate that exposure to lower oxygen availability may provoke beneficial effects on glucose homeostasis, and might therefore be an interesting alternative approach to improve metabolic status in metabolically compromised individuals. Importantly, however, a variety of confounding factors arise in high-altitude studies. Various studies examining the effects of high-altitude on glucose homeostasis included elements of physical activity (i.e. expedition and trekking), which induces improvements metabolic homeostasis (116). Furthermore, different dietary habits and physical activity patterns could greatly influence metabolic parameters in native highlanders (114). In addition, there is still debate whether hypobaric conditions (low atmospheric pressure) may induce alterations in metabolism and whether these effects are different from normobaric (i.e. isobaric) hypoxia exposure (125). Taken together, there is an unmet need to perform well-controlled studies to investigate the effects of normobaric hypoxia exposure on metabolic health in individuals at risk for obesity-related metabolic diseases, thereby exploring new therapeutic avenues to prevent and/or combat cardiometabolic diseases.

Several reports have investigated the potential of hypoxia exposure on various metabolic outcome parameters, amongst which are glucose and lipid

metabolism. However, hypoxia-mediated effects on metabolic regulation are still not fully understood. Indeed, some reports claim detrimental effects mediated by hypoxia exposure, whereas others demonstrate hypoxia-induced ameliorations of pathologies, such as hyperglycemia and hypertension (126). Varying factors regarding the design of the exposure regimens are (1) degree or severity of hypoxia (% O_2) exposure, (2) duration of the exposure, and (3) whether the exposure is continuous or intermittent.

Prolonged normobaric hypoxia

Simulated normobaric hypoxia exposure has been applied in several studies to investigate the potential effects of hypoxia on lipid and glucose homeostasis in lean and obese individuals at risk for developing type 2 diabetes mellitus. Overall, the oxygen level used in the majority of these studies is maintained within 14-16% O_2, reflecting the oxygen partial pressure at an altitude of 2000-3000 m. This O_2 exposure level is often referred to as '*mild*' or *'moderate'* hypoxia. It has been demonstrated that exposing obese men for 10 consecutive nights to moderate hypoxia (15% FiO_2) improved whole-body insulin sensitivity (127). Interestingly, the improvements in whole-body insulin sensitivity were greatest in individuals with the worst baseline insulin sensitivity, indicating that (pre)diabetic humans could potentially benefit from this intervention. In addition, it has been reported that exposure to normobaric 15% O_2 for 14 consecutive nights improved glucose tolerance, Matsuda-Index and Disposition Index in patients with T2DM (128). The latter two indices reflect estimations of whole-body insulin sensitivity and a composite index of beta-cell function and insulin sensitivity, respectively(129, 130). Interestingly, 7-day exposure (3h daily) to normobaric severe hypoxia (80% SpO_2) increased basal metabolic rate, and increased fat oxidation in sedentary overweight males, whereas it suppressed carbohydrate oxidation (131).

Taken together, prolonged normobaric hypoxia exposure might have beneficial effects on glucose homeostasis in individuals characterized by impaired glucose tolerance and T2DM. However, results from these studies should be interpreted cautiously, since the majority of studies did not include a control group.

Intermittent hypoxia

In contrast to continuous exposure, intermittent hypoxia is characterized by multiple hypoxic episodes per day of exposure. This is exemplified in obstructive sleep apnea syndrome (OSAS), in which patients are characterized by high frequential severe nocturnal hypoxic episodes, with concomitant systemic O_2 desaturation. This high-frequent hypoxic stimulus seems to be associated with the development of glucose intolerance and T2DM (101).

On the other hand, mild hypoxia exposure, often referred to as moderate hypoxia, characterized by 9-16% O_2 exposure, implemented as a low number of hypoxic episodes per day (3-15 episodes per day), may have beneficial effects. For example, it has been suggested to this may improve glucose and lipid metabolism (126). Indeed, 4-week MIH exposure (15% O_2, 1h per day, 6 sessions per week) improved fasting blood glucose as well as whole-body insulin sensitivity in diet-induced obese mice with T2DM, which was accompanied by increased Akt activity in muscle-derived protein lysates (73). Interestingly, 8-week exposure to MIH (14-15% O_2, 8h per day) improved glucose tolerance and insulin levels in Sprague-Dawley rats (132). In addition, Chiu et al. (133) found an improved glucose tolerance (as assessed by OGTT) subsequent to 4-week MIH exposure (14% O_2, 12h, per day) in a similar rat model. However, in both studies, rats showed reduced body weight and epididymal fat mass, hence improvements in glucose tolerance and insulin sensitivity found in these studies may not be directly related to MIH, but may be secondary to MIH-induced weight-loss.

In contrast to studies in rodents, human studies on the effects of MIH exposure on glucose and lipid metabolism are scarce. Interestingly, mild intermittent hypoxia (MIH) exposure to 12% O_2 for three consecutive weeks (consisting of 4 cycles of 5 minutes per day, 3 sessions per week) reduced fasting as well as 2h glucose in prediabetic individuals characterized with impaired fasting glucose and/or impaired glucose tolerance (134). Taken together, MIH might have beneficial effects on glucose homeostasis and insulin sensitivity in both humans and animal models, however, underlying mechanisms are not fully understood.

The impact of oxygen tension on adipose tissue, skeletal muscle and the gut

The role of oxygen in adipose tissue function

Due to the prominent role of AT dysfunction in obesity-induced insulin resistance and related disorders, an increasing number of studies have been conducted to identify potential triggers of AT dysfunction. Previously, it has been postulated that one of the key modulators in AT dysfunction is the oxygen tension of AT (135). Several reports have demonstrated that adipose tissue blood flow is compromised in both fasting and postprandial conditions in individuals with obesity (136, 137), which may reduce oxygen supply to the tissue. In addition, it has been postulated that during prolonged intake of excess calories leading to obesity, the adipose tissue vasculature is unable to keep pace with the expanding tissue, resulting in local hypoxia (138).

Indeed, experimental evidence indicates that AT pO_2 is reduced in obese mice (139). Several reports aimed to determine AT pO_2, using various measurements techniques (i.e. Clarke oxygen microelectrodes, optochemical measurement technique based on microdialysis), demonstrating that human AT pO_2 ranges between 3-11% O_2 (136, 140-142). However, whether adipose tissue hypoxia is a characteristic of human obesity remains controversial. Although it has previously been shown that AT pO_2 is lower in overweight and obese subjects (140), we have demonstrated a higher abdominal subcutaneous AT pO_2 in obese insulin resistant compared to lean insulin sensitive humans (136). In accordance with the latter, we also demonstrated that diet-induced weight loss decreased adipose tissue pO_2 in humans, which was paralleled by improved insulin sensitivity (141). More recently, we found that abdominal subcutaneous AT pO_2 is positively associated with insulin sensitivity in both men and women, independently of age and adiposity (143). Based these findings from our laboratory, it is tempting to postulate that AT pO_2 may be an interesting target to improve metabolic health in obesity. Therefore, numerous studies have been focusing to understand the metabolic adaptations in response to hypoxia exposure of the AT.

Prolonged exposure to hypobaric hypoxia (8848 m) substantially decreased lipolysis, and hence lipid mobilization in subcutaneous AT biopsies of healthy males (144). In agreement with these findings, 3T3-L1 adipocytes, which were repeatedly exposed to transient hypoxia (1% O_2, 2h per day, 4-8 days), demonstrated increased TAG accumulation, accompanied by improved insulin sensitivity (145). Interestingly, these metabolic adaptation involved 5' AMP-activated protein kinase (AMPK) activation, one of the key proteins involved in hypoxia-mediated effects on metabolism (146). In accordance, mild hypoxia exposure induced glucose transporter-1 (GLUT1) expression, a well-established AMPK target gene, facilitating basal glucose uptake (147). However, diminished insulin sensitivity has also been attributed to hypoxia exposure in adipocytes (148). Indeed, hypoxia exposure decreased insulin-stimulated glucose uptake, yet increased basal glucose uptake, potentially via increased GLUT1 expression in primary human adipocytes (148, 149). In line, increased GLUT1 gene and protein expression in response to hypoxia exposure have been demonstrated in both human and murine adipocyte models (19). Yet, the latter findings are based on acute hypoxia exposure (≤24h). Interestingly, prolonged (14 day) exposure to mild hypoxia (5% O_2) tended to increase insulin-independent glucose uptake in primary human femoral adipocytes (142). The findings in these studies might indicate that mild hypoxia induces glucose uptake in an insulin-independent manner, potentially via AMPK-dependent mechanisms.

Hypoxia exposure also appears to affect adipokine secretion. Indeed, primary human adipocytes exposed to 5% O_2 for 14 days demonstrated reduced leptin and increased IL-6 secretion (142). Furthermore, primary human adipocytes exposed to 1% O_2 for 24h demonstrated increased IL-6, vascular endothelial growth factor

(VEGF) and leptin release, but reduced adiponectin and monocyte chemoattractant protein-1 (MCP-1) secretion (150). In addition, several other adipokines, such as eotaxin and soluble tumor necrosis factor receptor-1 (sTNF-R1), which are both under the regulation of nuclear factor-kB (NF-kB), were downregulated by hypoxia exposure (150). Interestingly, it was found that tumor necrosis factor-α (TNF-α) induced NF-kB signaling, and eventually MCP-1 secretion were also reduced after hypoxia exposure (150). Altogether, these findings indicate that hypoxia exposure might exert beneficial anti-inflammatory effects in AT.

The impact of oxygen on skeletal muscle

It has been demonstrated that resting normoxic SM pO_2 values in humans are approximately 3% O_2, whereas strenuous physical exercise decreases pO_2 to ~1% O_2, as reviewed (151). Moreover, exposure to hypoxic air during exercise causes more pronounced reductions in systemic O2 saturation (that is, hypoxemia) relative to normal air, which ultimately also further reduces muscle oxygen availability and hence SM pO_2 (152). In addition, previous research demonstrated a reduction of resting SM pO_2 from 34 ± 6 mmHg (~4.5 % O_2) to 23 ± 6 mmHg (~3% O_2), in both healthy male and female volunteers acutely exposed to 10% O_2 (153).

Metabolic adaptations in response to hypoxia exposure have previously been studied *in vitro*. It has been demonstrated that hypoxia increased basal and insulin-stimulated glucose uptake compared to normoxia in muscle strips from *m. rectus abdominis* derived from lean, obese and T2DM individuals (154). In addition, prolonged continuous exposure of mice to 10% O_2 lowered plasma glucose and insulin levels, and improved insulin sensitivity as determined by homeostatic model assessment 2 for insulin resistance (HOMA2-IR) and increased Akt-phosphorylation in soleus muscle (155). In line with prolonged continuous hypoxia exposure, long-term MIH exposure (4 weeks, 1h per day, 15% O_2) normalized fasting blood glucose, and improved whole-body insulin sensitivity in mice with diet-induced obesity and T2DM (73). Interestingly, the magnitude of the effects of hypoxia exposure was similar to the effects induced by exercise during the same experimental time period (73). These findings led to the hypothesis that exercise, i.e. muscular contraction, and hypoxia might share similar underlying mechanisms in stimulating glucose uptake, potentially involving AMPK.

Several studies have provided evidence that muscle contraction enhances AMPK activity in both humans and animal models (156). AMPK becomes activated during cellular stress conditions, such as contraction, high cytosolic Ca^{2+} concentrations and an increased [AMP]/[ATP] ratio (157). Upon activation, AMPK acts directly on AS160 to stimulate GLUT4 translocation (18), whereas insulin phosphorylates AS160 via the PI-3K-Akt-signaling pathway (158). In line with this, hypoxia is known for its effects on cellular K^+ homeostasis, evoking depletion of

intracellular K^+ and accumulation of extracellular K^+ (159). This electrolyte imbalance causes membrane depolarization, hence releasing Ca^{2+} stored in the sarcoplasmatic reticulum, ultimately leading to high intracellular Ca^{2+} concentrations, similar to contraction (18). In addition, it has been proven that hypoxia induces intracellular AMP levels (160), and hence increases the [AMP]/[ATP] ratio (161), which results in AMPK activation (162). Interestingly, two-week severe intermittent hypoxia exposure (240 one-minute cycles of 5% O_2 per day) improved whole-body glucose tolerance in rodents, in an AMPK-dependent manner (163). Furthermore, these investigators found that hypoxia-induced improvements in glucose tolerance were diminished in muscle-specific AMPKα1α2 knockout mice, further highlighting the importance of muscle AMPK in hypoxia-induced improvements whole-body glucose homeostasis in rodents (163). In addition, it has been found that hypoxia-mediated effects on AMPK activation, and hence glucose tolerance, are muscle fiber-type specific. In slow-twitch fibers, hypoxia and contraction-mediated glucose transport seem entirely dependent upon Ca^{2+} dependent mechanisms (162). Yet, slow-twitch fibers are characterized by much lower expression of AMPKγ2 and γ3 subunits as compared to fast-twitch fibers (164). However, AMPK-dependent increases in glucose transport are mainly mediated by AMPKγ3, which may be mainly present in fast-twitch fibers, at least in mice (165). Remarkably, 5h acute intermittent hypoxia exposure (10.3% O_2, 480s-cycles) alters SM fiber type composition in rats, demonstrated by increased conversion towards fast-twitch fibers (166). In addition, it has been found that prolonged MIH (14-15% O_2, 8h per day, 6 days per week, 8 weeks) reduces muscle fiber cross-sectional area in rats, without changes in muscle weight, whilst improving glucose tolerance and insulin sensitivity (132). These alterations might be indicative of hypoxia-induced fiber-type replacement (132).

Taken together, hypoxia exposure appears to induce alterations in muscle fiber type composition, and enhances SM glucose uptake in an insulin-independent fashion, at least in rodents. The underlying mechanisms potentially involve Ca^{2+} and AMPK-dependent signaling pathways, similar to skeletal muscle contraction.

Synergistic effects of exercise and hypoxia exposure on muscle glucose uptake?

Since hypoxia and muscle contraction both induce glucose uptake, hypoxia exposure during exercise may have additive and/or synergistic effects on metabolic outcomes. Electrical pulse stimulation, which mimics SM contraction *in vitro*, induces glucose transport into the rat hindlimb muscle (167). Notably, concomitant local hypoxia exposure induced glucose transport to a higher extent, which was mainly observed in fast-twitch oxidative glycolytic muscle fibers (167). The additive effects of hypoxia and physical exercise have also been demonstrated in humans.

It has been shown that acute low-intensity exercise under mild hypoxic conditions (14.6% O_2) increased insulin sensitivity to a higher extent than normoxic exercise alone in patients with T2DM (168). In a follow-up study, it was shown that acute high-intensity exercise under mild hypoxic conditions (14.7% O_2) improved insulin resistance indices 24h and 48h after the exercise bout, whilst this was not observed following normoxic exercise (169).

However, results derived from studies investigating the effects of repeated hypoxic exercise sessions are inconclusive (170). In rats, physical exercise (swimming, 3h per day, 3 weeks) under MIH (14% O_2, 12h per day, 3 weeks) conditions increased GLUT4 mRNA and protein levels, and improved glucose tolerance to a higher extent than exercise under normoxic conditions (133). In healthy individuals, it was demonstrated that hypoxic (15% O_2) exercise (3 sessions per week, 4 weeks), consisting of 60 min running on a treadmill at an intensity corresponding to the heart rate at 3 mM lactate measured during the incremental workload test, improved HOMA-IR, whereas performing normoxic exercise did not (171). Furthermore, reductions in body fat and plasma TAG were more pronounced under hypoxic compared to normoxic conditions (171). In contrast, increased plasma glucose and insulin concentrations, and decreased glucose metabolic clearance rate have been found after long-term hypoxic (14.8% O_2) exercise (different cycling regimens, 3 sessions per week, 4 weeks) (172). In addition, eight weeks of hypoxic (15% O_2) training resulted in similar improvements in insulin sensitivity compared to normoxic exercise (173). Although it is difficult to compare hypoxic exercise studies due to differences in exposure time, severity of exposure, exercise type and duration of the intervention, it can be postulated that acute/short-term (mild) hypoxic exercise may improve glucose tolerance to a higher extent than performing exercise under normoxic conditions. However, results should be interpreted with caution, as the majority of these studies did not include a control group. Yet, evidence regarding the effects of long-term hypoxic exercise on glucose homeostasis in human studies is inconclusive. In addition, studies focusing on the effects of hypoxic exercise in humans at the level of SM are lacking, in particular in people with obesity at risk for developing T2DM. Therefore, further well-controlled studies are warranted to investigate the effects of hypoxic exercise on glucose and lipid homeostasis, and the potential application of hypoxia exposure to prevent or treat obesity-induced insulin resistance and T2DM.

The impact of oxygen on gut microbiota composition

Since the gut microbiota play a prominent role in metabolic health, many studies have investigated strategies (i.e. dietary interventions) to modulate the gut microbiome, thereby alleviating the progression of aforementioned disorders. While the vast majority of studies investigated the metabolic adaptations in response to

hypoxia exposure in organs such as AT and SM, less is known on the effects of hypoxia exposure on the gut. Interestingly, there is some evidence to suggest that modulation of pO_2 within the gut may impact the gut microbiota composition.

The vascularization, within the different layers of the intestines differs drastically, which is illustrated by a rather unique oxygenation profile. Indeed, the pO_2 of the outermost (serosal) layer of the ileum and sigmoid colon, has been demonstrated to range between 34-39 mmHg (4.5-5% O_2) in humans (174). However, the pO_2 reduces drastically towards the luminal side, characterized by pO_2 levels <10 mmHg (1.0-1.5% O_2) (175). In addition, moving across the longitudinal axis follows a similar pattern, with highest reported pO_2 levels at the level of the duodenum (32 mmHg), decreased in the ascending colon (11 mmHg), and lowest in the sigmoid colon (3 mmHg) in mice (176, 177). Interestingly, rats acutely exposed to 15% O_2 showed reductions in both serosal and mucosal pO_2 (178). However, only a few animal studies have focused on the effects of hypoxia exposure on microbiota composition. Mice exposed to intermittent hypoxia (5% O_2, 20s per cycle, 360 cycles per day) for 6 weeks, demonstrated increased α- and β-diversity (Figure 2) (179). More specifically, intermittent hypoxia induced a higher abundance of *Firmicutes*, whilst reducing the abundance of *Bacteriodetes* and *Proteobacteria* phyla (179). In line with previous findings (178), the latter study demonstrated that hypoxic exposure decreased pO_2 values from 50 mm Hg towards 10 mmHg as determined in close vicinity of the intestinal epithelium (179). In addition, rats exposed to chronic intermittent (hypobaric) hypoxia (5000 m, 28 days, 6h per day) demonstrated increased abundance of *Lactobacillus*, *Prevotella* and *Methylotenera* genera, whereas *02d06* genus was decreased subsequent to exposure (180). Interestingly, a decrease in the *Firmicutes* to *Bacteriodetes* ratio was found, possibly partially underlying the effects of improved glucose and lipid metabolic effects subsequent to chronic intermittent hypobaric hypoxia exposure that was also found in this study (180). Remarkably, high altitude exposure increased *Prevotella* abundance in healthy males (181), which is in line with previous animal studies, demonstrating increased abundance of mainly obligate anaerobes by hypoxia exposure (182).

Taken together, intermittent hypoxia exposure appears to lower gut pO_2, and seems to affect gut microbiota composition in rodents. Thus, gut pO_2 may mediate the beneficial effects of hypoxia- exposure on metabolism, and might be considered a target to improve metabolic homeostasis. Importantly, however, to the best of our knowledge, controlled human studies investigating the effects of normobaric hypoxia exposure on gut microbiota composition are lacking.

Outline of this thesis

The main objective of this thesis was to investigate the effects of MIH exposure on the metabolic phenotype in obese individuals at risk for developing cardiometabolic complications such as cardiovascular diseases and type 2 diabetes, thereby aiming to understand the effects of hypoxia exposure on the whole-body, organ and cellular levels in humans. To accomplish this, we integrated innovative human *in vivo* techniques, analyses in adipose tissue and skeletal muscle biopsies, and fecal samples, as well as mechanistic *in vitro* experiments using human primary adipocytes and myotubes. Secondly, this thesis aimed to elucidate the effects of exercise under mild hypoxia on glucose and lipid metabolism.

In **Chapter 2**, we provide an overview of the current literature on the determinants of adipose tissue oxygenation, and its putative role in glucose and lipid metabolism, as well as inflammation. Furthermore, the effects of experimental hypoxia on AT and whole-body metabolism are discussed, leading us to propose that lowering of pO_2 in the adipose tissue and skeletal muscle microenvironment may improve metabolic health in humans, which would have important implications for the management of metabolic impairments in people living with obesity. Therefore, in **Chapter 3**, we investigated the effects of MIH exposure for 7 consecutive days on AT and SM pO_2, tissue-specific insulin sensitivity, whole-body substrate metabolism, and SM mitochondrial function in a randomized, single-blind, crossover study. In addition, microarray analysis was performed on both SM and AT biopsies to determine the impact of MIH on gene expression profiles. Furthermore, the effects of hypoxia exposure at the cellular level were examined in primary human adipocytes and myotubes. Next, in **Chapter 4**, we explored the effects of MIH exposure on AT protein expression profiles in overweight and obese insulin resistant men using proteomics analysis. **Chapter 5** describes the *in vitro* and *in vivo* effects of hypoxia exposure on myokine secretion and plasma myokine concentrations in primary human myotubes and obese men with impaired glucose homeostasis, respectively. Moreover, the effects of MIH exposure on gut microbiota composition in obese insulin resistant men were examined in **Chapter 6**. In **Chapter 7**, the effects of moderate-intensity exercise under hypoxic conditions on 24h glucose profile and fasting/postprandial glucose and lipid metabolism in obese men with impaired glucose homeostasis was investigated in a randomized, single-blind, crossover study. Finally, the results generated by the studies described in this thesis are put in broader perspective, and directions for future studies are provided in **Chapter 8**.

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CHAPTER 2

Oxygenation of adipose tissue: a human perspective

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Abstract

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

Keywords: obesity, adipose tissue, oxygen, hypoxia, inflammation, metabolism

Introduction

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

Dysfunctional WAT is characterized by adipocyte hypertrophy, impairments in lipid metabolism (including a reduced capacity to buffer the daily influx of dietary lipids, thereby contributing to ectopic fat accumulation), decreased adipose tissue blood flow, and a state of chronic low-grade inflammation (Figure 1) (18, 20, 21). The presence of adipose tissue (AT) inflammation in obesity is well established, and several factors that contribute to the sequence of events leading to a pro-inflammatory phenotype of obese AT have been identified, as extensively reviewed elsewhere (10, 22-24). Interestingly, more recent findings have provided evidence that the amount of oxygen in the adipose tissue microenvironment may also impact AT metabolism and inflammation, and WAT oxygenation may therefore be a key factor in the pathophysiology of AT dysfunction and related chronic diseases (18, 25, 26).

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

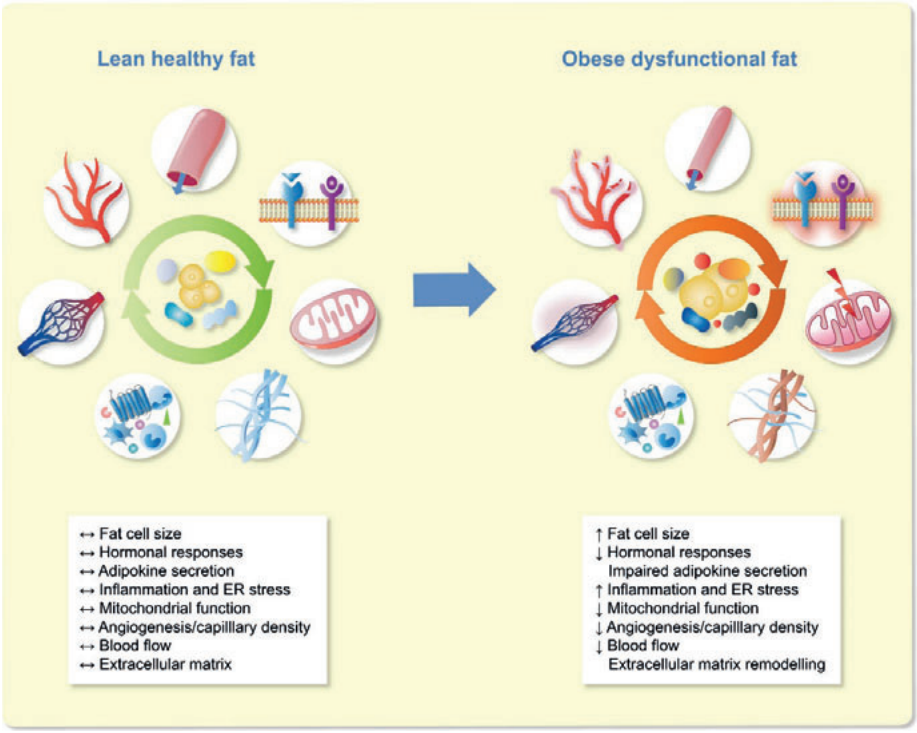

Figure 1. Characteristics of lean healthy and obese dysfunctional white adipose tissue. Adipose tissue dysfunction is characterized by adipocyte hypertrophy, impaired adipokine secretion, a chronic low-grade inflammation, apoptosis, extracellular matrix remodelling, hormonal resistance, vascular rarefaction, decreased adipose tissue blood flow and altered metabolic responses, together contributing to insulin resistance and related chronic diseases. ER, endoplasmic reticulum

Adipose tissue dysfunction in obesity

One of the main functions of WAT is the preservation of energy in the form of triacylglycerol (TAG) in response to a chronic positive energy balance (27). Adipose tissue has the capacity to expand at the cellular level by recruiting stem cells / pre-adipocytes from the stroma-vascular fraction (SVF) resulting in more adipocytes (hyperplasia), or by enlargement of existing adipocytes (hypertrophy)(28, 29). However, it has been suggested that there is a set number of pre-adipocytes that can be recruited, which seems to be genetically determined (30). Adipocytes can substantially increase in size but do have a certain expansion limit, implying that these cells have a maximum capacity of storing TAG(23, 25, 31, 32). What seems to be even more important than the maximal storage capacity is the ability to dynamically store lipids in the postprandial phase, the so called lipid buffering capacity, and to release fatty acids under fasting

conditions (33). Hypertrophic WAT has been shown to have an impaired capacity to store meal-derived fatty acids(34). As a consequence, more dietary lipids are diverted through the circulation to be stored in other tissues, which results in ectopic fat accumulation when lipid uptake exceeds lipid oxidation (35). The storage of excess lipids in non-adipose tissues in obesity has important metabolic consequences, since this is closely associated with insulin resistance(17, 23, 31). Furthermore, hypertrophic adipocytes are characterized by a pro-inflammatory phenotype, which may further aggravate insulin resistance(24, 36). Importantly, however, adipocyte inflammation also seems essential for healthy adipose tissue expansion and remodelling(37), suggesting that inflammation is not solely a pathological phenomenon. Noteworthy, medication used to treat type 2 diabetes may alleviate inflammation by reducing hyperglycaemia. However, the anti-inflammatory effects of these agents are inconsistent, and it remains to be established whether their beneficial metabolic effects are mediated via modulation of chronic low-grade inflammation (38).

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

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

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

Altered adipose tissue *oxygen partial pressure* in obesity

Since alterations in the oxygenation of WAT may contribute to WAT dysfunction, as will be discussed later in this review, adipose tissue oxygen partial pressure (AT pO_2) has been assessed in both rodents and humans. In addition to direct measurements of pO_2, indirect methods to estimate WAT oxygenation have been applied (Table 1). The direct studies on WAT oxygenation have yielded conflicting findings, which are summarised in Table 2 (25, 34, 59-66).

Table 1: Direct methods and surrogate markers used to determine adipose tissue oxygenation.

Methods applied to assess adipose tissue oxygenation
Direct
Silastic tonometer (69-72)
Polarographic micro clark-type electrode (60)
Optochemical, continuous monitoring via microdialysis (59, 73, 74, 116)
Combined oxygen and temperature probe (57)
Needle-type fibre-optic oxygen sensor (rodents) (64, 65, 85)
Indirect
Arterio-venous difference technique (34)
Gene expression of hypoxia-responsive genes / proteins (63)
Pimonidazole hydrochloride (63, 66)

The presence of hypoxia in obese adipose tissue was originally shown in murine models of obesity(18, 25). Direct measurements of pO_2 using needle-type O_2 electrodes showed that WAT oxygenation is lower in *ob/ob*, KKAy and diet-induced obese mice as compared to lean controls(18, 63-67). In line, gene expression of several hypoxia-related genes, including hypoxia-inducible factor-1 alpha (HIF-1α), were also increased. Moreover, using pimonidazole hydrochloride, which stains hypoxic areas, it has been demonstrated that hypoxic areas were more prevalent in WAT of obese rodents (18, 63-67). However, it is worth mentioning that these rodent models of obesity are characterized by a rapid and massive gain in adipose tissue mass due to genotype and/or the diet that these animals received, which is not comparable to the more gradual development of obesity in most humans (63-66, 68).

So far, not many human studies examining WAT pO_2 have been performed, and the results on WAT oxygenation are somewhat contradictory(18, 59). The first direct measurements of WAT pO_2 in humans were made in individuals undergoing surgery(69, 70). It was found that morbidly obese individuals had lower pO_2 levels in subcutaneous WAT (sWAT) of the upper arm as compared to lean subjects, determined the morning after surgery(69, 70). However, other studies in which sWAT

oxygenation has been measured both during and after surgery showed opposite results, with increased or no significant difference in WAT pO_2 between obese and lean individuals(71, 72). Notably, these initial studies assessed oxygenation in WAT of the upper arm, which is not of crucial importance for whole-body metabolism. Moreover, the O_2 levels measured in these studies could have been affected by the applied anaesthesia, and other factors related to morbid obesity.

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

The presence of hypoxia in sWAT in obesity has been challenged by recent studies in humans. We have demonstrated a higher rather than lower pO_2 in obese subjects with impaired glucose metabolism as compared to lean healthy, age-matched individuals, despite lower adipose tissue blood flow (oxygen supply) in obesity (59). These findings of higher abdominal sWAT pO_2 in obesity have been confirmed by very recent studies (73, 74). Abdominal sWAT pO_2 was found to be higher in obese insulin resistant as compared to lean and obese insulin sensitive men, with no significant differences in WAT oxygenation between obese insulin sensitive and lean insulin sensitive men (73). Furthermore, this study demonstrated that AT oxygenation was positively associated with insulin resistance, even after adjustment for age, sex and body fat percentage, suggesting that AT pO_2 may be more closely related to insulin sensitivity than obesity per se (73). To date, only one study investigated the effects of weight loss on sWAT pO_2 in humans. In this study, overweight and obese individuals underwent a dietary intervention, consisting of a 5-week very-low-calorie diet (VLCD, 500 kcal/day) and a subsequent 4-week weight stable diet. It was found that VLCD-induced weight loss markedly decreased abdominal sWAT pO_2, which was paralleled by improved whole-body insulin sensitivity (74).

The striking differences in findings on sWAT pO_2 between studies may be attributed to differences between study populations in terms of the onset and physical history (e.g. weight cycling) of obesity and other subjects' characteristics (e.g. age, sex, ethnicity, presence of type 2 diabetes), the sWAT depot studied, and variation in the methodology used (25, 59, 60).

In addition to direct measurements of sWAT pO_2 in humans, several studies have used alternative approaches to indirectly estimate tissue oxygenation, including metabolic profiling of sWAT in vivo and the assessment of hypoxia-responsive WAT gene expression. Hodson and co-workers (34) have measured

metabolic fluxes across abdominal sWAT in vivo in lean, overweight and obese humans, and their findings strongly argue against any functional consequences of WAT hypoxia in obesity; in fact, the opposite might be true. More specifically, these authors demonstrated that the fasting lactate-to-pyruvate ratio, which is a potential metabolic signature of 'hypoxia', in arterial blood, was inversely correlated with adiposity. Using arterio-venous difference methodology with selective venous catheterization of abdominal sWAT, no significant association was found between WAT-specific changes in lactate-to-pyruvate ratio and BMI. However, the proportion of glucose released as lactate and pyruvate in sWAT was strongly negatively correlated with BMI(34). Observational human studies examining hypoxia-related genes as surrogate markers of WAT oxygenation have shown increased HIF-1α expression in sWAT in humans with morbid obesity (57, 75, 76). Interestingly, HIF-1α expression was higher in the SVF than in adipocytes, which might imply that the SVF is more sensitive to changes in oxygenation (77). Importantly, however, HIF-1α mRNA expression seems not an appropriate marker for hypoxia(78). Also, upregulated genes in subcutaneous and visceral WAT of severely obese subjects that are under control of HIF were not responsive to hypoxia in adipocytes (79), which raises the question what pO_2 threshold is required for activation of the HIF pathway in adipose tissue (60). Furthermore, genome-wide association studies have shown a correlation between epigenetic methylation of the HIF3α gene in sWAT and BMI and WAT dysfunction markers(80-83). Following bariatric surgery, there was a reduction in HIF-1α mRNA expression in WAT (84). On the other hand, HIF-1α gene expression was upregulated during weight loss induced by a low caloric diet (74).

It is important to emphasize that a stronger mechanistic link exists between hypoxia and the spatial presence of HIF1α protein rather than its mRNA expression(85, 86). Further, HIF-1α is not only regulated by oxygen levels, but also by growth factors including insulin(87). Therefore, metabolic disturbances such as insulin resistance and/or hyperglycaemia may also have marked effects on HIF-1α protein stability(87), and may affect epigenetic modifications. This implies that one should be cautious when drawing conclusions about WAT oxygenation based on gene expression of classical hypoxia-responsive genes such as HIF1α, GLUT1 and VEGF(25).

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

Determinants of adipose tissue oxygenation in humans

WAT pO_2 is the result of a delicate balance between O_2 supply and consumption, which both seem to be altered in obesity. More specifically, differences in angiogenesis, capillary density and vascular function, together determining adipose tissue blood flow (ATBF), and the cellular demands affecting O_2 consumption contribute to changes in WAT pO_2 (18, 25, 68).

Adipose tissue oxygen supply

Both structural (i.e. capillary density) and functional (i.e. vascular tone) aspects of the vasculature determine ATBF and, therefore, oxygen supply to WAT. There is substantial evidence that there is insufficient angiogenesis in WAT depots in obesity. Obese individuals show decreased adipose tissue mRNA expression of VEGF, the master regulator of angiogenesis and a HIF-1α target protein(59, 60, 88). Pasarica and colleagues (60) showed that capillary density was lower in overweight/obese humans, and found a positive correlation between VEGF expression and capillary density. The lower capillary density in WAT of obese individuals has been confirmed by our laboratory (59). Furthermore, it has been shown that obese insulin resistant subjects had fewer capillaries and a greater number of large vessels in WAT as compared to lean individuals (89). Together, these findings are indicative of vascular rarefaction and decreased vascular remodelling in WAT in obese humans. Thus, the lower capillary density may reflect higher WAT oxygenation in obesity. Alternatively, if WAT oxygenation would be lower in obesity, the pro-angiogenic response is not effectively propagated (90).

In addition to a lower capillary density in WAT of obese individuals, an increased vascular tone may impair ATBF, which ultimately determines tissue oxygen delivery. It is well established that ATBF is impaired in human obesity. Fasting ATBF is lower in obese compared to lean individuals and has been linked to insulin resistance(59, 91-95). Furthermore, in the postprandial period as well as during insulin stimulation (i.e. hyperinsulinemic-euglycemic clamp), the increase in ATBF is blunted in obese versus lean subjects(59, 95, 96). These impairments seem to be related to impaired beta-adrenergic responsiveness and increased activity of the renin-angiotensin system in obesity(68, 94, 97, 98). We have previously shown that both pharmacological and physiological manipulation of ATBF induced concomitant alterations in WAT pO_2 in humans (59), suggesting that decreased ATBF in obesity indeed reduces AT oxygen supply. Importantly, however, WAT pO_2 is not only determined by oxygen supply to the tissue but is also dependent on WAT oxygen consumption, as discussed in more detail below.

Table 2: Summary of studies in which adipose tissue oxygenation has been directly measured in humans

Study	Site of sWAT	Technique used	Participants' characteristics	AT pO_2 (mmHg)
Kabon et al.(69) 2004 [a]	Upper arm	Silastic tonometer	*Non-obese*: n = 23 (12 M, 11 F); Age: 44 ± 9 y; BMI: 24 ± 4 kg/m²	Right arm: 54 (47, 64) [b] Left arm/wound: 62 (49, 68) [b]
			Obese: n = 23 (3 M, 20 F); Age: 44 ± 13 y; BMI: 51 ± 15 kg/m²	Right arm: 43 (37, 54) [b] Left arm/wound: 42 (36, 60) [b]
Fleischmann et al.(70) 2005	Upper arm	Silastic tonometer	*Non-obese*: n = 15 (10 M, 5 F); Age: 43 y (13) c; BMI: 24 (3) kg/m² c	57 (15) [c]
			Morbidly obese: n = 20 (4 M, 16 F); Age: 40 y (11) c; BMI: 46 (7) kg/m² c	41 (10) [c]
Hiltebrand et al. (71) 2008	Upper arm	Silastic tonometer	*Lean*: n = 7 (2 M, 5 F); Age: 31 ± 6y; BMI: 22 ± 2 kg/m²	52 ±10
			Obese: n = 7 F; Age: 37 ± 6y; BMI: 46 ± 4 kg/m²	58 ± 8
Pasarica et al.(60) 2009	Abdominal	Polarographic micro clark-type electrode	*Lean*: n = 9 (5 M, 4 F); Age: 22.6 ± 3.3 y; BMI: 22.1 ± 1.0 kg/m²	55.4 ± 9.1
			Overweight/obese: n = 12 (6 M, 6F); Age: 38.9 ± 15.8 y; BMI: 31.7 ± 1.9 kg/m²	46.8 ± 10.6
Goossens et al. (59) 2011	Abdominal	Optochemical, measurement system	*Lean*: n = 10 M; Age: 55.8 ± 4.1y; BMI: 23.4 ± 0.3 kg/m²	44.7 ±5.8
			Obese: n = 10 M; Age: 59.6 ± 3.1y; BMI: 34.2 ± 1.3 kg/m²	67.4 ±3.7
Lawler et al.(57) 2016	Abdominal	combined oxygen and temperature probe	*Obese Insulin Sensitive*: n=6 (4F/2M); Age: 36 ± 4; BMI: 32 ± 1 kg/m²	41.1 ± 1.2
			Obese Insulin Resistant: n=6 (6F); Age: 37 ± 3; BMI: 34 ± 2 kg/m²	37.7 ± 2.4
			Both obese groups: n=12 (10F/2M)	39.3 ± 1.5
			Lean: n=4 (3F/1M); Age: 31 ± 3y; BMI: 23 ± 1 kg/m²	53 ± 1.9

Study	Site of sWAT	Technique used	Participants' characteristics	AT pO_2 (mmHg)
Kaiser et al.(72) 2016	Right upper arm	Silastic tonometer	*Morbidly obese:* n=7 ; Age: 51 (35–55); BMI: 67 (57–71) kg/m²	Baseline (kPa): 6.8 (6.2–7.6 [4.4])
			Non-obese: n=7 ; Age: 62 (53–67); BMI: 26.5 (26–29) kg/m²	Baseline (kPa): 6.5 (6.1–7.5 [3.0])
Vink et al. (74) 2017	Abdominal	Optochemical, continuous monitoring via microdialysis	*Obese/overweight:* n=15 (9F/6M); Age: 50.9±2.1y; BMI: Baseline: 31.1 ±0.6 kg/m²	Baseline: 51.0±1.6
			End of WS : 27.9±0.5 kg/m²	End of WS: 41.3±3.1
Goossens et al.(73) 2018	Abdominal	Optochemical, continuous monitoring via microdialysis	*Lean Insulin Sensitive*: men n = 7; Age: 58.6 ± 2.6y; BMI 23.0 ± 0.3 kg/m²	40.4 ± 6.6
			Obese Insulin Sensitive: men n= 7; Age: 55.6 ± 2.8; BMI: 31.7 ± 0.8 kg/m²	56.1 ± 3.2
			Obese Insulin Resistant: men n=7; Age: 56.9 ± 4.0; BMI: 33.1 ± 1.3 kg/m²	68.5 ± 4.4
			Obese Insulin Sensitive: women n=7; Age: 50.6 ± 3.0; BMI: 30.5 ± 0.8 kg/m²	50.8 ± 2.5
			Obese Insulin Resistant: women n=7; Age: 51.0 ± 2.3; BMI: 32.9 ± 1.8 kg/m²	62.3 ± 5.3
Vogel M et al.(116) 2018	Abdominal & Femoral	Optochemical, continuous monitoring via microdialysis	*Obese/overweight:* n=8 (F); Age:52.5 ±1.8y; BMI 34.4±1.6 kg/m²	Abdominal : 62.7±6.6
				Femoral : 50.0±4.5

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

Adipose tissue oxygen consumption and mitochondrial function

In normal weight individuals, WAT oxygen consumption is relatively low as compared to other tissues, accounting for approximately 5% of whole-body oxygen consumption (34, 62, 99). It has been estimated that mitochondrial oxygen consumption accounts for up to 85%, while non-mitochondrial oxygen consumption may be responsible for 10–15% of total oxygen consumption in WAT under steady-state conditions(100, 101). Both mitochondrial and non-mitochondrial oxygen consumption may change during the marked WAT remodelling occurring in obesity and may induce alterations in WAT oxygenation.

It is well established that mitochondrial morphology, mass and function are impaired in multiple adipose tissue depots in obese rodents(102-106). Interestingly, it has been reported that early in the development of obesity, enhanced mitochondrial metabolism, biogenesis and reactive oxygen species (ROS) production seem critical to initiate and promote adipocyte differentiation (107, 108). In line with findings in animals, several human studies have reported impaired mitochondrial capacity and reduced expression of genes/proteins related to mitochondrial metabolism (e.g. peroxisome proliferator-activated receptor gamma coactivator 1-alpha and nuclear respiratory factor 1) in WAT in states of obesity, insulin resistance, and T2DM (34, 59, 109-112). Furthermore, it has been shown that mitochondrial proteins are downregulated not only at whole WAT level, but also in adipocytes from obese individuals(113, 114). In line, mitochondrial density and oxygen consumption rates are lower in adipocytes derived from obese versus lean subjects, independent of adipocyte size (113-115). Of note, there also appear to be sWAT depot-specific differences in oxygen consumption rates in obesity, since basal respiration was lower in abdominal as compared to femoral differentiated human multipotent adipose-derived stem cells (116). The latter finding may underlie the higher AT pO_2 in abdominal than femoral subcutaneous adipose tissue (116).

In accordance with impaired mitochondrial density and oxygen consumption in obese WAT in humans, there are indications that weight loss may evoke beneficial changes in WAT mitochondrial function. Following bariatric surgery, both mitochondrial respiratory capacity and biogenesis were increased in WAT(117, 118). We have recently shown that diet-induced weight loss increased WAT gene expression of mitochondrial biogenesis markers and non-mitochondrial oxygen consumption pathways in humans, which may have contributed to the reduction in WAT pO_2 following weight loss (74). In contrast, instead of improving WAT mitochondrial abnormalities, weight loss downregulated mitochondrial gene expression and density, and had neither effects on mitochondrial DNA transcripts nor OXPHOS proteins (119). Interestingly, the latter study showed that a higher initial mitochondrial number and gene expression was related to more successful weight loss after 12-month follow-up. Importantly, however, changes in gene expression do not necessarily translate into functional alterations. Taken together, it appears that oxygen consumption is impaired in obese WAT in humans, which may contribute to increased WAT pO_2 in human obesity.

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

In cell culture experiments investigating the molecular and cellular responses to hypoxia, cells are usually exposed to a substantially reduced level of oxygen (1% O_2 is frequently employed) as compared to 'normoxia' (ambient air, 21% O_2). The normal physiological range of AT pO_2 in human WAT is ~3-11% O_2 or ~23-84 mmHg(57, 59, 60, 73). Therefore, the outcomes of experiments comparing the effects of pO_2 below and well-above these physiological levels should be interpreted with caution, since results may not directly translate to the human in vivo situation. Moreover, it is important to distinguish between acute (<24h) and more prolonged exposure to different pO_2 levels, since this seems to have a major impact on the metabolic and inflammatory responses, as will be discussed later in this section.

The cellular response to low oxygen levels

As any other cell type, adipocytes must maintain and adjust their metabolic and physiological regulation in response to fluctuations in the local microenvironment, including variation in oxygen levels (25, 120). The main regulators of oxygen sensing are the oxygen sensitive HIFs. HIFs are transcription factors, binding to the DNA and changing gene expression in response to alterations in oxygen levels (121). HIFs consist of two subunits, α and β, with the former being the oxygen sensitive molecule and HIF-1β being constitutively expressed by cells (67). The HIF family consists of three members based on the three α-subunits, HIF-1α, HIF-2α and HIF-3α, with the predominant members being HIF-1α and HIF-2α (27, 120, 122). HIF-1α has received the most attention, and this transcription factor has been described as the master regulator of oxygen homeostasis. HIF-1α is continuously synthesized and rapidly degraded in the presence of oxygen but is stabilized when oxygen levels are low, and the functional HIF-1α transcription factor is then recruited. More specific, during sufficient oxygenation of the cells, HIF-1α is enzymatically degraded by prolyl-4-hydroxylases through the proteasome (121). During 'hypoxic' conditions, which are tissue-dependent, but usually defined as <1% of oxygen in most in vitro studies, the prolyl hydroxylase domain enzymes are inactivated, and HIF-1α is not subject to rapid degradation. Instead, HIF-1α then forms a heterodimer with the β subunit, acting on DNA binding areas called hypoxia-responsive elements, thus regulating gene expression of many different genes (10, 22, 25, 121, 123). These genes encode proteins involved in a multiplicity of cellular processes, including glucose and lipid metabolism, inflammation, ECM metabolism, and apoptosis (25). Thus, changes in tissue oxygenation seem to affect many physiological processes in WAT, and the metabolic and inflammatory effects will be discussed in more detail below (Figure 2).

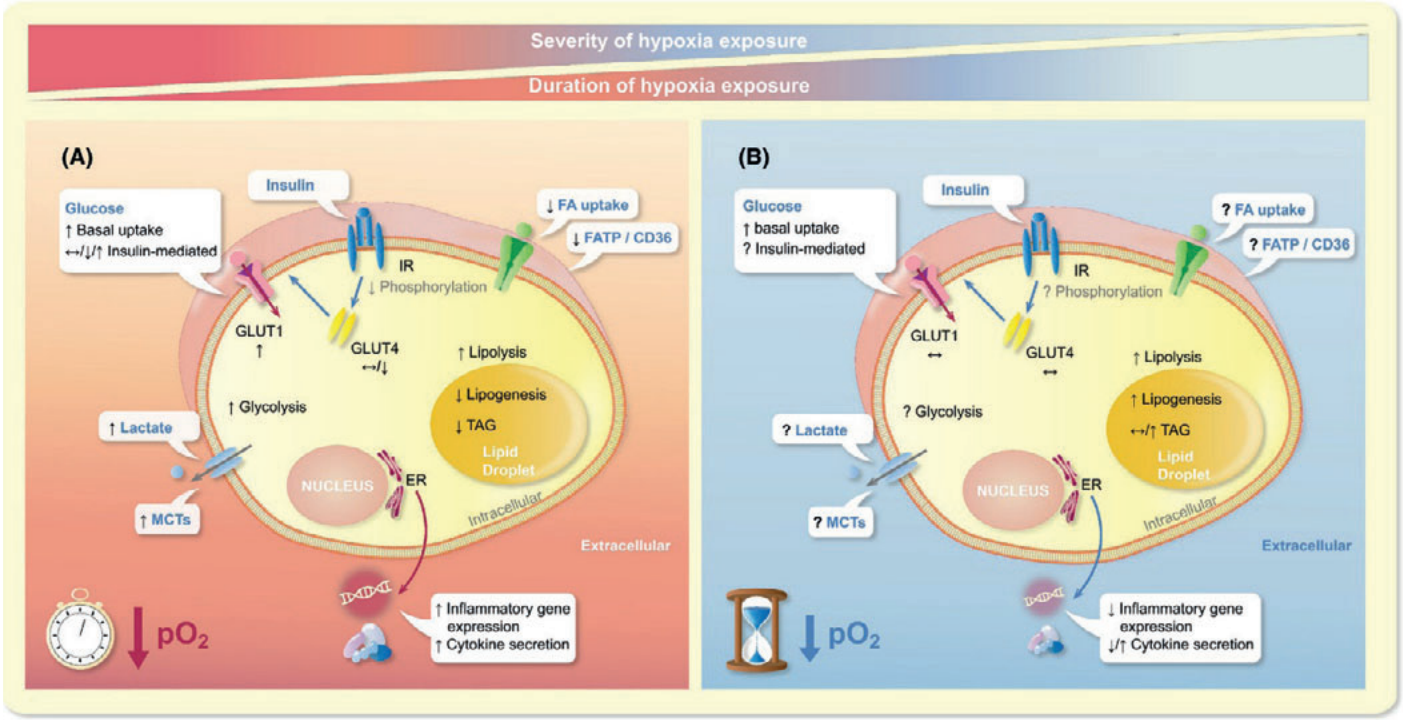

Figure 2. Adipocyte substrate metabolism, adipocyte gene expression and adipokine secretion are affected by alteration of oxygen partial pressure (pO_2). Both the severity and the duration of hypoxia exposure seem to impact cellular processes, as explained in more detail in the text. Panel A shows the effects of acute exposure to severe hypoxia (usually 1% O_2 for <24 hour), while panel B illustrates the putative effects of prolonged, mild hypoxia exposure (usually 5-10% O_2 for 7-14 days) on adipocyte biology. ER, endoplasmic reticulum; FA, fatty acids; FATP / CD36, fatty acid transporters; GLUT, glucose transporter; IR, insulin receptor; MCTs, monocarboxylate transporters; pO_2, oxygen partial pressure; TAG, triacylglycerol. , increase; , decrease; , unchanged; ?, not determined.

Metabolic effects of altered adipose tissue oxygenation

Glucose metabolism

Under hypoxic conditions a shift from aerobic to anaerobic metabolism occurs, with glucose becoming the major substrate for ATP generation(25, 67, 68, 121). In vitro studies have demonstrated an increase in basal glucose uptake in human and rodent adipocytes treated acutely, up to 24 hours, with 1% versus 21% O_2 (65, 124, 125). Furthermore, it has been shown that glucose uptake in human adipocytes is inversely related to O_2 levels (1, 3, 5, 10, 15 vs 21% O_2), peaking at 1% O_2 (126). In accordance with these findings, prolonged exposure (14 days) to low (5% O_2) but not high (10% O_2) physiological pO_2 levels tended to increase basal glucose uptake in differentiated human multipotent adipose-derived stem cells (116).

Conflicting findings, however, have been reported regarding the effects of pO_2 on insulin-mediated glucose uptake. Acute exposure to 1% O_2 (up to 24h) reduced insulin-mediated glucose uptake in human adipocytes (125), indicative of impaired insulin signalling, an effect that was reversible (125). This was further illustrated by decreased phosphorylation of the insulin receptor, IRβ, and IRS-1 proteins as well as protein kinase B (65, 125). In contrast, another study found that acute 1% O_2 exposure increased insulin-dependent and insulin-independent glucose uptake in 3T3-L1 adipocytes (127). Interestingly, it was shown that multiple exposures of differentiating 3T3-L1 adipocytes to transient hypoxia (1% O_2, 4h/day, 4-8 days) enhanced insulin signalling, illustrated by increased phosphorylation of Akt (T308 and S473 residues) and GSK3β (127).

Alterations in glucose uptake are due to changes in the expression and localization of the glucose transporters (GLUTs). GLUT-1 mRNA levels were increased following exposure to acute, severe hypoxia (1-2% pO_2, up to 24h) in both murine (3T3-L1) and human (pre)adipocytes (67, 126, 128-133). In contrast, insulin-dependent GLUT-4 mRNA expression in human adipocytes remained unchanged (124) or was significantly reduced by acute exposure to 1% O_2 (124, 126, 129, 132). In line with improved insulin-stimulated glucose uptake, GLUT-4 but not GLUT-1 expression was elevated in murine adipocytes exposed to transient hypoxia (127). During and after differentiation of human preadipocytes under low (5% O_2) and high (10% O_2) physiological pO_2 levels, basal GLUT-1 expression was not changed (134) or decreased (116), while GLUT-4 mRNA expression remained unchanged (116, 134).

Acute hypoxia exposure to 1% O_2 for 24h also increased gene and protein expression of enzymes involved in glycolytic metabolism in human adipocytes, including glucose phosphate isomerase, pyruvate kinase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (128, 133, 135-137). In accordance with these findings, the end-product of the glycolytic pathway, lactate, and the expression of genes encoding monocarboxylate transporters (MCT) mediating lactate transport were found to be increased in rodent and human adipocytes under hypoxic conditions (67, 138, 139).

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

Lipid metabolism

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

The amount of oxygen in the microenvironment also seems to impact adipocyte lipolysis. Several studies have shown that acute exposure to severe hypoxia (1% O_2) increased basal lipolysis in 3T3-L1 adipocytes(65, 68, 125). Moreover, prolonged exposure (14 days) to severe hypoxia modestly increased basal lipolysis, while low physiological pO_2 (4% O_2, 14 days) exposure increased lipolysis to a much greater extent in 3T3-L1 adipocytes (140). In theory, insulin resistance in adipocytes might explain the increased basal lipolytic rate due to reduced insulin-mediated suppression of lipolysis. However, since improved insulin sensitivity has also been found following hypoxia exposure, as discussed in the previous section, alternative mechanisms are likely involved in the pO_2-induced effects on basal adipocyte lipolysis. Furthermore, isoproterenol-induced lipolysis was also significantly elevated in human adipocytes differentiated at high (10% O_2) and low (5% O_2) physiological pO_2 as compared to exposure to ambient air (21% O_2), which was accompanied by increased protein expression of the lipolytic enzyme HSL and the lipid droplet-coating protein perilipin (134). In conclusion, hypoxia seems to increase lipolysis in rodent and human adipocytes, with more pronounced effects

found under physiological pO_2 (Figure 2). Clearly, more studies are required before strong conclusions can be drawn regarding the effects of oxygenation on lipid metabolism in human WAT, and to unravel underlying mechanisms.

Adipokines and inflammatory factors

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

As with other in vitro studies applying acute and severe hypoxia over 1-24 hours (25, 64, 66, 129, 132, 133), these findings should be interpreted with some caution, underlining the importance of applying more physiological conditions in cell culture experiments. Few in vitro studies have tried to better mimic physiological conditions in vivo in terms of oxygen partial pressure as well as the duration of exposure to altered pO_2 (116, 134). The effects of modest, rather than severe, hypoxia have also been investigated, showing a concentration-dependent change in adipokine expression and secretion in human adipocytes(126). Interestingly, prolonged exposure of human adipose tissue-derived mesenchymal stem cells to physiological pO_2 levels (i.e. 5% and 10% O_2) during differentiation towards mature adipocytes appears to elicit a different expression and secretion profile as observed following acute (severe) exposure to hypoxia. More specific, we have recently demonstrated that low physiological pO_2 decreased pro-inflammatory gene expression (i.e. IL-6, PAI-I, TNFα, MCP-1 and dipeptidyl-peptidase-4 (DPP-4)) in differentiated human adipocytes as compared to 21% and/or 10% O_2, whereas more heterogeneous effects on adipokine secretion were found (116). Exposure of these cells to low physiological pO_2 (5% O_2) for 14 days resulted in a reduced secretion of leptin and increased adiponectin and IL-6 secretion in these adipocytes, while no significant effects on DPP-4 and MCP-1 secretion were found (116). In contrast,

exposure to high physiological pO_2 (10% O_2) increased leptin and DPP-4, but reduced IL-6 and MCP-1 secretion (116). Famulla and colleagues (134) have shown increased DPP-4, adiponectin and IL-6 following prolonged exposure to high physiological pO_2 (10% O_2), while low physiological pO_2 (5% O_2) tended to reduce the secretion of adiponectin. These differences between studies suggest that donor characteristics may also influence the effects of pO_2 on the adipocyte secretory profile.

Taken together, oxygen levels and pattern of exposure seem to have a significant impact on adipocytokine expression and secretion (Figure 2). However, many aspects of exposure have not been examined in human cells, which is important to elucidate in future experiments.

Altered tissue oxygenation impacts whole-body physiology in humans

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

Living at high-altitude represents a condition of hypobaric hypoxic exposure (i.e. around 15% O_2 at ~3000 m) as oxygen partial pressure is relatively lower compared to sea-level (147). The impact of high-altitude habitation on chronic diseases is dependent on several factors such as ethnicity, environmental and behavioural factors that may vary across mountain dwellers (147, 153). It has been suggested that living at high-altitude is associated with improved cardiovascular and pulmonary function (154). Many studies have demonstrated a lower prevalence of obesity, cardiovascular diseases, T2DM, and cancer in populations living at high-altitude(147, 153, 155-157). For example, a cross-sectional study including 422,603 adults has shown an inverse relationship between elevation and obesity prevalence, after adjusting for temperature, diet, physical activity, smoking and demographic factors, in both males and females (158), which is in line with other studies demonstrating an inverse association between altitude and the prevalence of obesity (159-161). Interestingly, a lower prevalence of the metabolic syndrome,

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

Several intervention studies have been performed to elucidate the impact of exposure to altered pO_2 on body weight and metabolic homeostasis (Figure 3). We have previously demonstrated that chronic exposure to hypoxia (8% versus 21% O_2, 21 days) improved the WAT phenotype in C57Bl/6J mice, evidenced by decreased adipocyte size, decreased macrophage infiltration and inflammatory markers, and increased expression of mitochondrial function and biogenesis markers in visceral and subcutaneous AT (166). More recently, the same concept has been applied to humans. Exposure to moderate hypoxia (15% O_2) for ten subsequent nights increased whole-body insulin sensitivity in eight obese men (167). Since moderate hypoxia exposure also tended to reduce AT pO_2 (167) , these findings may imply that lowering of AT pO_2 by moderate hypoxia exposure may have contributed to improved insulin sensitivity (168). Furthermore, exposure to hypoxia under resting conditions increased energy expenditure and lipid metabolism, and reduced appetite and food intake (169, 170). Based on a recent systematic review, it was concluded that normobaric hypoxic conditioning, lasting from 5 days up to 8 months, may have beneficial effects on insulin levels, energy expenditure, body weight and blood pressure in rodents and humans, which may contribute to improved cardiometabolic health and body weight management in obesity (155). The putative effects of (severe) hypoxia exposure on orexigenic (i.e. ghrelin) and anorexigenic (i.e. leptin) peptides affecting appetite and food intake may, at least partially, underlie the effects on body weight and metabolic outcomes, as reviewed elsewhere (171, 172).

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

mechanisms. Importantly, the impact of hypoxia on cardiometabolic health may also be due to effects of altered pO_2 on other organs than adipose tissue, especially during exercise.

The beneficial effects of hypoxic exercise may be mediated to a large extent by alterations at the level of skeletal muscle. During contraction, glucose uptake in skeletal muscle is increased in an insulin-independent manner, likely involving independent effects of 5' AMP-activated protein kinase (AMPK), mechanical stress and Ca^{2+}/calmodulin-dependent protein kinase kinases (CaMKKs) (185). Interestingly, it has been demonstrated that hypoxia exposure increased glucose uptake in skeletal muscle cells through AMPK signalling. Therefore, hypoxia exposure during exercise might have additive or synergistic effects on peripheral glucose uptake. Indeed, exposing human myotubes to 7% O_2 in combination with electrical pulse stimulation (EPS), to mimic exercise, increased glucose uptake to a higher extent than EPS under 21% O_2, which seems at least partly due to an insulin-sensitizing effect of hypoxia (186). Taken together, hypoxia exposure may improve glucose homeostasis via insulin-dependent and insulin-independent effects, but more studies in humans on putative underlying mechanisms are needed.

Conclusions and future perspectives

The obesity epidemic presents a major public health challenge. Novel preventive measures and treatment alternatives are urgently needed to combat obesity and its comorbidities. Adipose tissue dysfunction in obesity is related to a plethora of metabolic and endocrine disturbances, contributing to impairments in lipid and glucose metabolism as well as immune homeostasis. It is well established that adipose tissue dysfunction has a central role in the aetiology of obesity-related comorbidities and chronic diseases, including T2DM and cardiovascular diseases. A reduced lipid buffering capacity of hypertrophic adipose tissue in obesity results in lipid accumulation in key metabolic organs such as the liver and skeletal muscle (i.e. ectopic fat storage), which is strongly associated with insulin resistance. Moreover, adipose tissue in obesity is characterized by a pro-inflammatory phenotype. This is reflected by a phenotypic shift towards a higher abundance of pro-inflammatory macrophages and other adaptive and innate immune cells in obese adipose tissue, leading to the production and secretion of a multitude of pro-inflammatory cytokines, which in turn may induce insulin resistance. Besides inflammation, a disproportionate deposition of ECM components during the development of obesity may contribute to adipose tissue fibrosis and insulin resistance (Figure 1).

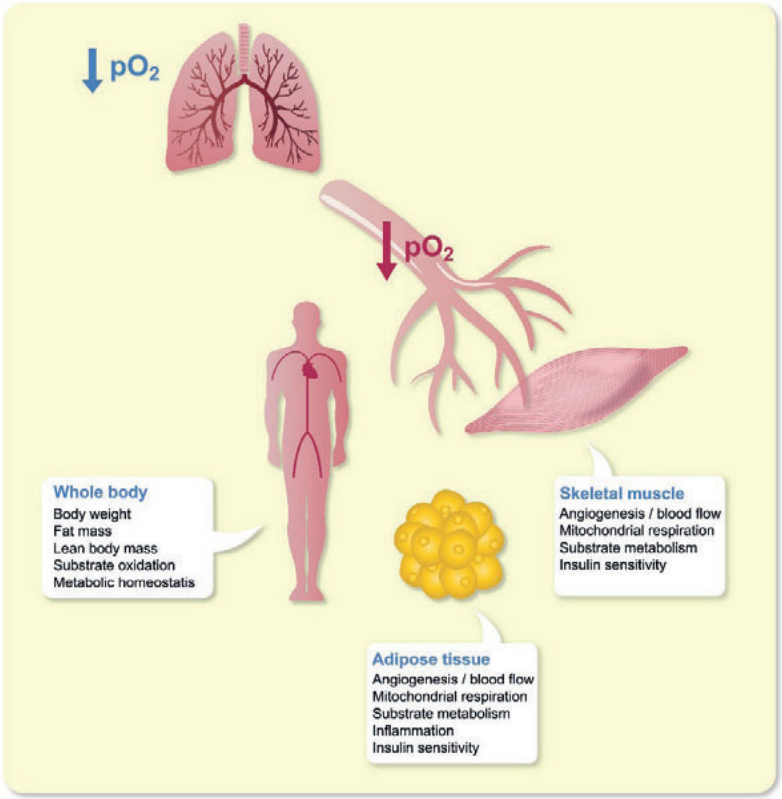

Figure 3. Putative impact of (moderate) hypoxia exposure on whole-body, skeletal muscle and adipose tissue physiology. O_2, oxygen; pO_2, oxygen partial pressure.

Adipose tissue oxygen partial pressure, determined by the balance between oxygen supply and consumption, may have a key role in the metabolic and inflammatory perturbations seen in most obese individuals. Animal models have shown lower pO_2 in obese WAT ('hypoxia'). Findings in humans are conflicting, which may be due to differences between study populations in terms of the onset and physical history (e.g. weight cycling) of obesity and other subjects' characteristics (e.g. age, sex, ethnicity, presence of type 2 diabetes), the WAT depot studied, and variation in the methodology used. Nevertheless, several studies performed in our laboratory indicate that AT pO_2 is higher in obese insulin resistant individuals, is positively related to insulin resistance (independently of adiposity), and is reduced after diet-induced weight loss, which is paralleled by improved insulin sensitivity. Adipose tissue mitochondrial dysfunction (i.e. reduced O_2 consumption) may contribute to higher AT pO_2 in obesity. There is no strong evidence to suggest that differences in pO_2 within the human physiological range (i.e. due to impaired

blood flow) have marked effects on mitochondrial respiration. Interestingly, many in vitro experiments have demonstrated that changes in oxygen levels impact the functionality of (pre)adipocytes and immune cells, leading to alterations in glucose and lipid metabolism, as well as inflammation in adipose tissue (Figure 2). Clearly, altered pO_2 may not only affect adipose tissue physiology but also whole-body metabolic homeostasis (Figure 3). In this respect, it remains to be elucidated whether AT pO_2 exerts a crucial role in the development and progression of obesity-related co-morbidities in humans. Although several lines of evidence suggest that exposure to lower levels of oxygen may enhance whole-body metabolic homeostasis and body weight regulation, intervention studies in humans are warranted to further investigate whether changes in tissue oxygenation may improve cardiometabolic health, thereby providing a novel strategy to combat chronic cardiometabolic diseases in obese humans.

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CHAPTER 3

Mild Intermittent Hypoxia Exposure Induces Metabolic and Molecular Adaptations in Obese Men

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Abstract

Recent studies suggest that hypoxia exposure may improve glucose homeostasis and insulin sensitivity, but well-controlled human studies are lacking. The primary objective of this study was to investigate whether mild intermittent hypoxia exposure (MIH) impacts tissue-specific insulin sensitivity in overweight and obese humans. We performed a randomized, single-blind crossover study, in which 12 overweight and obese participants ($HOMA_{IR}$ ≥2.2) were exposed to MIH (7-day exposure to 15%O_2, 3x2h/day vs. 21%O_2), and determined adipose tissue (AT) and skeletal muscle (SM) tissue oxygen partial pressure (pO_2), fasting and postprandial substrate metabolism, tissue-specific insulin sensitivity, and AT and SM gene/protein expression. Furthermore, we exposed primary human myotubes and adipocytes to physiological hypoxia to assess effects on glucose uptake and adipokine secretion. MIH decreased oxygen saturation, AT and SM pO_2, and increased whole-body carbohydrate oxidation, with concomitant elevation of plasma lactate concentrations compared to normoxia. Mechanistically, hypoxia exposure increased insulin-independent glucose uptake in primary human myotubes, but not in human adipocytes, through AMP-activated protein kinase. Moreover, MIH increased inflammatory/metabolic pathways and decreased extracellular matrix-related pathways in AT, but did not alter systemic inflammatory markers. Nevertheless, AT, hepatic and SM insulin sensitivity, as well as SM mitochondrial respiration/oxidative capacity, were not affected by MIH. We provide the first evidence that 7-day MIH reduces AT and SM pO_2, evokes a shift toward glycolytic metabolism, induces adaptations in AT and SM but does not alter tissue-specific insulin sensitivity in overweight and obese humans.

Introduction

Oxygen homeostasis, or the balance between oxygen supply and demand, is critical in cellular survival. Indeed, alterations in tissue oxygen partial pressure (pO_2) impact a variety of physiological responses. In 2019, the Nobel Prize in Physiology or Medicine was awarded to William Kaelin, Jr., Sir Peter Ratcliffe, and Gregg Semenza for their discoveries of how cells sense and adapt to oxygen availability. The response to oxygen in cells, tissues and organisms is one of the most central and important physiological adaptations.

Compelling evidence points towards an important role of altered oxygen availability (i.e. '*hypoxia*') in cardiometabolic perturbations (*1, 2*). Intriguingly, although metabolic adaptations to hypoxia are not fully understood, oxygen availability in key metabolic organs such as adipose tissue (AT) and skeletal muscle (SM) may play a major role in the pathophysiology of obesity-related cardiometabolic complications, as recently reviewed (*2*). We have recently demonstrated that AT pO_2 was increased in obese insulin resistant compared to lean and obese insulin sensitive individuals(*3, 4*). Indeed, AT pO_2 was positively associated with insulin resistance, independently of age, sex and adiposity (*3*). In line, diet-induced weight loss markedly decreased AT pO_2 in humans, which was accompanied by increased insulin sensitivity (*5*). These findings suggest that tissue oxygenation may be a target to lower the risk for cardiometabolic complications in people with obesity (*2, 6*).

However, conflicting findings on the effects of pO_2 on inflammation and insulin sensitivity have also been reported, which may be related to the severity, pattern and duration of hypoxia exposure (*2*). Indeed, acute exposure to severe hypoxia impaired insulin signaling both in murine and human adipocytes (*7*), while prolonged, repeated exposure of differentiating adipocytes to transient hypoxia was able to reprogram these cells for enhanced insulin sensitivity(*8*). Furthermore, exposure to severe hypoxia increased glucose uptake in murine and human myotubes, and human SM explants (*9, 10*). Interestingly, prolonged mild intermittent hypoxia (MIH) exposure, which is characterized by multiple hypoxic episodes per day, has been shown to improve glucose homeostasis in rodents (*11-14*). Moreover, a small, uncontrolled trial in obese men suggested that mild hypoxia exposure (15% O_2) may increase whole-body insulin sensitivity (*15*). Importantly, however, placebo-controlled trials to investigate the effects of prolonged exposure to MIH on tissue-specific insulin sensitivity and cardiometabolic risk factors in humans are lacking, and underlying mechanisms for the putative beneficial effects of MIH on glucose homeostasis remain to be established.

Based on these findings, we hypothesized that MIH improves insulin sensitivity and induces a shift towards increased glucose utilization in individuals with overweight and obesity. Here, we report on the first randomized, single-blind crossover trial that was designed to investigate the effects of MIH exposure (FiO_2

15% O_2, 3 times 2h/day vs. 21% O_2) for 7 consecutive days on tissue-specific insulin sensitivity (primary study outcome), AT and SM pO_2, and fasting and postprandial energy/substrate metabolism in overweight/obese insulin resistant men. The reason for this MIH protocol was that exposure to 15% O_2 improved insulin sensitivity in a previous uncontrolled human study (*15*), and that an intermittent pattern of mild hypoxia exposure (3-15 hypoxic episodes/day) might elicit more beneficial effects on cardiovascular risk profile (*16*), without inducing adverse effects. Moreover, we determined the impact of MIH on gene/protein expression in AT and SM, and performed functional mechanistic experiments using human primary myotubes and adipocytes that were cultured under oxygen levels mimicking the AT and SM microenvironments in humans.

Materials and Methods

Study design

Twelve overweight/obese men (BMI ≥28 kg/m^2, age 30-65 yrs) with $HOMA_{IR}$ ≥2.2 participated in the present study. Exclusion criteria were smoking, cardiovascular disease, type 2 diabetes mellitus, liver or kidney malfunction, use of medication known to affect body weight, glucose and/or lipid 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 design of the present randomized, single-blind, crossover study is depicted in Supplementary Figure 1. Study participants were exposed to normobaric mild intermittent hypoxia (FiO_2 15%; equivalent to ~2800m 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 (computer-generated randomization plan; block size, *n*=4), separated by a 3-6 week wash-out period. Individuals were asked to refrain from drinking alcohol and to perform no exercise 48h hours before the start and during the exposure regimens. Furthermore, participants performed standardized light-stepping activity (3x5 min, p. day, 15 steps/min) during days 1-7. Measurements performed on days 6 (AT and SM pO_2), day 7 (high-fat mixed-meal (HFMM) test), and day 8 (two-step hyperinsulinemic-euglycemic clamp), as explained in detail below, were performed after an overnight fast of at least 10 hours. Participants were kept under energy-balanced conditions, as described in detail in the Supplementary Methods. Assessment of hunger and satiety was performed on days 1, 3 and 7 by means of visual analog scale (VAS) questionnaires. Adverse events of MIH were monitored by means of the Lake Louise questionnaire for Acute Mountain Sickness (AMS). The study was approved by the Medical-Ethical Committee of Maastricht University, and performed according to the Declaration of Helsinki. All subjects gave their written informed consent before participation in the study. This study is registered at the Netherlands Trial Register (NTR7325).

Anthropometric measurements

Body weight was measured to the nearest 0.1 kg (model 220, Seca). Height was measured using a wall-mounted stadiometer (Seca). Blood pressure and heart rate were assessed (UA-789XL digital blood pressure monitor, A&D medical) on multiple occasions during the exposure protocol (days 1-5).

Systemic oxygen saturation, adipose tissue and skeletal muscle partial oxygen pressure

Systemic oxygen saturation levels were continuously monitored throughout the exposure regimens by pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcor).

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, as described previously (*4*) and explained in detail in the Supplementary Methods.

High-fat mixed-meal test

On day 7 of each exposure regimen, a high-fat mixed-meal challenge test was performed, fasting and postprandial blood samples were collected, and substrate oxidation was determined, as described in detail in the Supplementary Methods.

Two-step hyperinsulinemic-euglycemic clamp

A two-step hyperinsulinemic–euglycemic clamp combined with D-[6,6-$^2H_2$]-glucose tracer infusion (Cambridge Isotope Laboratories, no. DLM-349) was performed on day 8, under normoxic conditions, to determine hepatic, adipose tissue and peripheral insulin sensitivity, as described previously (*17*) and outlined in the Supplementary Methods.

Skeletal muscle and adipose tissue biopsies

Before the start of the hyperinsulinemic-euglycemic clamp, a SM (80-100 mg; *m. vastus lateralis*) and abdominal subcutaneous AT biopsy (~1 g; 6-8 cm lateral from the umbilicus) were collected under local anesthesia (2% lidocaine without epinephrine). Another muscle biopsy was collected at the end of the steady state of the high-insulin step of the clamp.

Part of the fasting muscle biopsy was immediately placed in ice-cold preservation medium (BIOPS, OROBOROS Instruments), to prepare intact permeabilized muscle fibers.. Permeabilized muscle fibers were used to determine mitochondrial oxidative capacity using an oxygraph (OROBOROS Instruments), as described previously (*18*). The remaining part of the SM biopsy was snap frozen in liquid nitrogen, and stored at -80˚C until analysis. The AT specimen was washed using sterile saline, visible blood vessels were removed using sterile tweezers, and the AT biopsy was snap frozen in liquid nitrogen and stored at -80˚C.

Plasma biochemistry

Blood was collected into pre-chilled tubes, centrifuged at 1,000g, and plasma was snap-frozen and stored at -80˚C until analysis. Plasma levels of glucose, FFA, TAG, insulin and lactate were measured according to manufacturer's guidelines, as explained in the Supplementary Methods. Plasma TNFα, IFNγ, IL-6 and IL-8 concentrations were determined in fasting plasma samples collected at day 8, subsequent to 7-d MIH exposure, using the V-PLEX Proinflammatory Panel I Human Kit (Mesoscale, 4-plex, no. K15052D), according to manufacturer's guidelines.

Adipose tissue and skeletal muscle gene expression

Microarray analysis was performed on SM and AT samples, as described previously (*19*). Gene set enrichment analysis was based upon FDR q-value <0.20 on the filtered data set (IQR > 0.2 (log2), intensity >20, >5 arrays, >5 probes per gene), using the databases Kyoto Encyclopedia of Genes and Genomes, Reactome, Wikipathways and Biocarta. The present data have been deposited in NCBI's Gene Expression Omnibus (*20*) and are accessible through GEO Series accession number (accession number: to be added after manuscript acceptance)

Skeletal muscle protein expression

To evaluate the effect of MIH on (p)-Akt, (p)-AMPK and OXPHOS complex protein expression in skeletal muscle, Western Blot analysis was performed on protein lysates derived from the biopsies, as described in the Supplementary Methods.

Human primary cell culture experiments

Cell culture of human primary satellite cells

Primary human satellite cells were obtained from *m. rectus abdominis* muscle of a lean, insulin sensitive male subject. Cell culture procedures are extensively described in the Supplementary Methods. After differentiation, myotubes were exposed to 1, 3 and 21% O_2 for 24h, as described earlier (*21*). Gas mixtures were refreshed every 8 hours. Subsequently, functional experiments were performed and medium, protein and RNA were collected and stored at -80˚C until further analysis.

Cell culture of human multipotent adipose-derived mesenchymal stem cells

Human multipotent abdominal subcutaneous adipose-derived mesenchymal stem cells (hMADS) were obtained from overweight/obese men with impaired glucose metabolism, were pooled and differentiated into the adipogenic lineage. Therefore, cells were seeded at a density of 2000 cells•cm^{-2} and cultured, as

described previously (*22, 23*) and described in detail in the Supplementary Methods. All cells were proliferated and differentiated under 21% O_2, and thereafter exposed to either 10% O_2 continuously (resembling physiological normoxia), or to MIH consisting of 3 x 2h cycles per day, alternating between 5-10% O_2, during the final 7 days of cell culture.

Glucose uptake in primary human myotubes and adipocytes

Basal and insulin-stimulated glucose uptake were determined in both differentiated hMADS and primary human myotubes following serum-starvation for 2h, as described previously (*23*) and explained in the Supplementary Methods.

To investigate the involvement of adenosine monophosphate-activated kinase (AMPK) in the effects of hypoxia on glucose uptake in human primary myotubes, cells were co-incubated with and without 1 mM AICAR (no. A9978, Sigma) as well as 10 μM Compound C (no. P5499, Sigma) during hypoxia exposure. Next, basal glucose uptake was measured as described in the Supplementary Methods.

Myotube protein expression

After exposure to 1, 3 and 21% O_2 for 24h, protein lysates were collected. Subsequently, protein concentrations were determined and p-AMPKαThr^{172}, AMPKα and OXPHOS complex protein expression was determined by Western Blotting. Detailed procedures as well as used antibodies are described in the Supplementary Methods.

Adipokine secretion

At the end of each exposure regimen, medium of the differentiated adipocytes and myotubes was collected to determine adipokine medium concentrations of IL-6, MCP-1, leptin, adiponectin and VEGF using ELISA, as described previously (*22*).

Adipocyte gene expression

To obtain RNA for gene expression analysis, TRIzol Reagent (Invitrogen) was added to the cells at the end of exposure, at day 14 of hMADS differentiation, as described previously (*22*).

Statistical analysis

Sample size was calculated based on a physiologically relevant 20% change of peripheral insulin sensitivity ($\alpha = 0.05$, $1-\beta = 0.9$). Data were checked for normality by means of the Shapiro-Wilk test. The effects of MIH as compared to normoxia

exposure were assessed by means of a paired Student's *t*-test, whilst nonparametric data was analysed using the Wilcoxon Signed-Rank test. Area under the curves were calculated using the trapezoidal method. *In vitro* experiments were analyzed using Wilcoxon Signed-Rank tests or the Friedman's test with *post-hoc* Dunn's test. 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. Figures were created using Graphpad Prism.

Results

Subject characteristics

Twelve overweight and obese men with homeostasis model assessment for insulin resistance ($HOMA_{IR}$) ≥2.2 and without any chronic disease or endocrine disorder participated in the present randomized, single-blind crossover study (Table 1). Study participants were exposed to normobaric MIH (FiO_2 15%; equivalent to ~2800m above sea level) and 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 (computer-generated randomization plan; block size, *n*=4), separated by a 3-6 week wash-out period (Supplementary Figure 1), and were kept in energy balance throughout the study. Hence body weight remained stable during MIH and normoxia exposure.

Table 1. Baseline characteristics of study participants.

	Baseline
Age (y)	61 ± 1
BMI (kg/m²)	30.8 ± 0.9
Hemoglobin (mmol•l⁻¹)	9.5 ± 0.5
HbA_{1c} (%)	5.6 ± 0.1
Fasting glucose (mmol•l⁻¹)	5.7 ± 0.2
2h-glucose (mmol•l⁻¹)	6.2 ± 0.4
$HOMA_{IR}$	3.7 ± 0.4

BMI, body-mass index; HbA_{1c}, glycated hemoglobin; $HOMA_{IR}$, Homeostatic Model of Assessment of Insulin Resistance. Values are represented as mean ± SEM (*n*=12).

Mild intermittent hypoxia exposure reduces systemic oxygen saturation and partial oxygen pressure in adipose tissue and skeletal muscle

To determine whether MIH exposure reduces oxygen availability in key metabolic organs, systemic oxygen saturation (SpO_2) as well as partial oxygen pressure (pO_2) in AT and SM were determined using a highly accurate, microdialysis-based optochemical measurement system to continuously monitor tissue pO_2 *in vivo* in humans(*4*). MIH exposure significantly reduced SpO_2 (normoxia: 97.1 ± 0.3 *vs*. hypoxia: 92.0 ± 0.5 %, $p<0.001$), and markedly decreased AT pO_2 (normoxia: 36.5 ± 1.5 mmHg versus hypoxia: 21.0 ± 2.3 mmHg, $p<0.001$) and SM pO_2 (normoxia: 15.4 ± 2.4 mmHg versus hypoxia: 9.5 ± 2.2 mmHg; $p=0.002$) (Figure 1).

Collectively, these findings provide the first proof-of-concept in humans that MIH exposure reduces not only systemic oxygen saturation but also consistently decreased AT and SM pO_2 (~40%). In addition, these data show that SM pO_2 is significantly lower than AT pO_2 (p<0.001), likely reflecting the higher metabolic rate (i.e. oxygen consumption rate) in SM. No adverse events were reported.

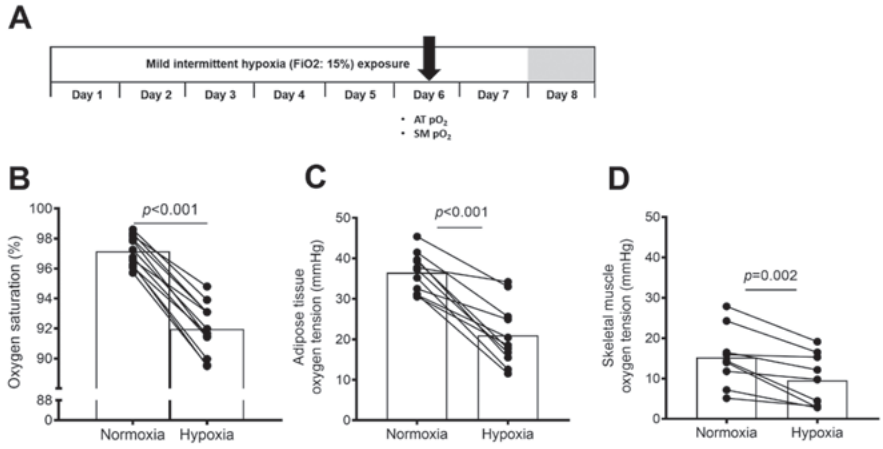

Figure 1. Mild intermittent hypoxia exposure decreases systemic oxygen saturation, adipose tissue and skeletal muscle oxygen tension. Adipose tissue and skeletal muscle oxygen tension pO_2 were determined during MIH exposure at day 6 (**A**). MIH exposure significantly decreased (**B**) systemic oxygen saturation, (**C**) adipose tissue and (**D**) skeletal muscle pO_2. Data are represented as mean (bars) and individual data points. Statistical analysis was performed using two-tailed Student's paired t-test. The exact p-values are shown.

Mild intermittent hypoxia exposure induces a shift towards glycolytic metabolism

To determine whether MIH exposure affects fasting and postprandial substrate utilization, we performed a high-fat mixed-meal test with blood sampling and indirect calorimetry measurements on day 7 of both exposure regimens (Figure 2A). Fasting and postprandial energy expenditure (Figure 2B) were not significantly altered by MIH, although fasting energy expenditure tended to be reduced (p=0.053). Interestingly, MIH induced a pronounced increase in fasting respiratory exchange ratio (RER, p=0.001) and carbohydrate oxidation (CHO, p=0.002), whereas fat oxidation was reduced (FAO, p=0.013) compared to normoxia exposure. Moreover, following meal intake, RER remained elevated (p_{AUC}=0.003, Figure 2C) during MIH compared to normoxia exposure due to a marked shift towards increased postprandial CHO (p_{AUC}=0.003, Figure 2D) and suppression of FAO (p_{AUC}=0.018, Figure 2E).

In addition, MIH exposure did not alter fasting and postprandial plasma glucose, insulin, free fatty acid, triacylglycerol and glycerol concentrations compared to normoxia exposure (Figure 3B-F). However, plasma lactate levels were significantly increased by MIH compared to normoxia (p_{AUC}=0.005, Figure 3G), reflecting the MIH-induced shift towards enhanced glycolytic metabolism. Notably, the MIH-induced effects on fasting substrate utilization and plasma lactate concentrations were maintained but not more pronounced during postprandial conditions, since MIH did not alter the incremental areas under the curves (iAUC•min^{-1}) for these parameters (Figure 3).

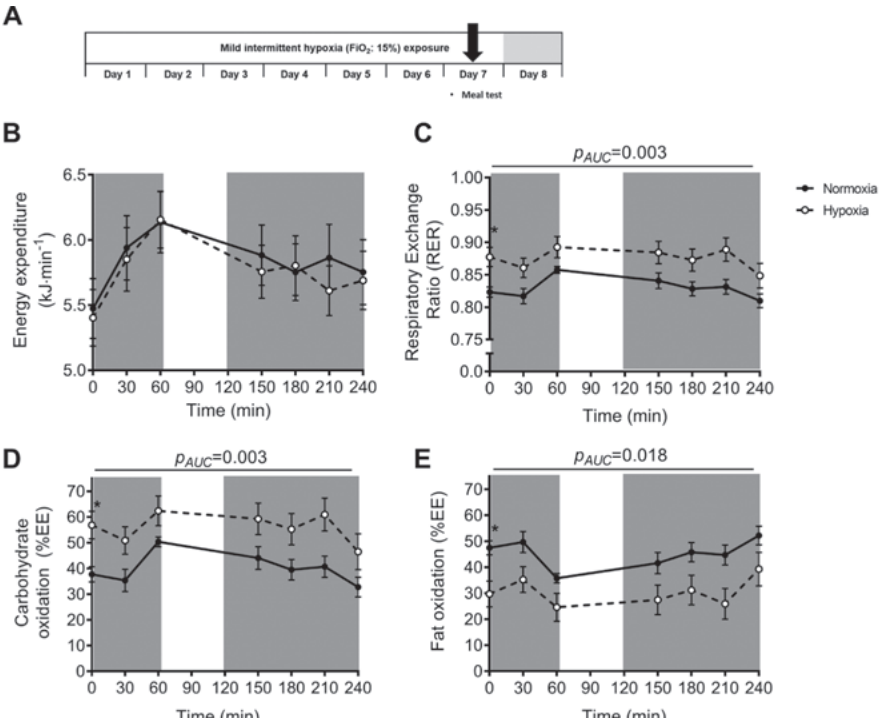

Figure 2. Mild intermittent hypoxia exposure alters fasting and postprandial substrate oxidation. Indirect calorimetry was performed before (t=0 min) and after ingestion of a high-fat mixed meal (t = 0 – 240 min) to determine energy expenditure substrate oxidation during mild intermittent hypoxia (MIH) exposure (**A**). MIH exposure did not alter (**B**) energy expenditure but significantly increased fasting and postprandial (**C**) respiratory exchange ratio and (**D**) carbohydrate oxidation (% of total energy expenditure: EE%), and decreased (**E**) fat oxidation compared to normoxia exposure. Total area under the curves (AUC) but not incremental AUCs (iAUC) were significantly different between conditions. Open circles / dashed line, MIH exposure; Closed circles / solid line, normoxia exposure. Grey areas indicate time periods when study participants resided in the hypoxic room. Data are represented as mean ± SEM. Statistical analysis was performed using two-tailed Student's paired t-test. The exact *p*-values are shown.

Mild intermittent hypoxia exposure increases basal glucose uptake in human primary myotubes via AMPK

To elucidate whether the MIH-induced increase in carbohydrate utilization may be mediated by effects of hypoxia exposure on glucose uptake in SM, we performed a series of mechanistic experiments using human primary myotubes. First, we exposed human primary myotubes to oxygen levels that we found *in vivo* during MIH and normoxia exposure (Figure 4A). More specific, myotubes were exposed to physiological hypoxia (1% O_2), physiological normoxia (3% O_2), and standard laboratory conditions (21% O_2) for 24h. Of note, more prolonged exposure to MIH was not feasible due to the relatively short lifespan of differentiated primary human myotubes. 1% O_2 markedly increased glucose uptake compared to 3% O_2

(p=0.019, Figure 4B) and 21% O_2 (p<0.001, Figure 4B). Similar findings were observed after 30 min of insulin stimulation, with significantly increased insulin-mediated glucose uptake after 1% O_2 compared to 21% O_2 (p=0.011, Figure 4C). Nevertheless, the insulin-induced increase in glucose uptake (insulin-stimulated – basal) did not significantly differ between exposure conditions, indicating that the hypoxia-induced increase in glucose uptake is mainly driven by insulin-independent glucose uptake in human primary myotubes.

To further explore the potential involvement of AMPK in the hypoxia-induced increase in glucose uptake in human primary myotubes, we next determined AMPK activity. Exposure to 1% O_2 increased p-AMPK^{Thr172}/AMPK ratios in human myotubes compared to 3% O_2 (p=0.077) and 21% O_2 (p=0.013) (Figure 4D, E), reflecting increased AMPK activity. To confirm functional involvement of AMPK in hypoxia-induced glucose uptake, we co-incubated primary human myotubes with an AMPK inhibitor. Indeed, inhibition of AMPK by Compound C during 1% O_2 exposure decreased glucose uptake (p=0.028) to levels found after 3% O_2 exposure (Figure 4F). Collectively, these experiments demonstrate that exposure of human primary myotubes to oxygen levels that mimic *in vivo* SM pO_2 values during MIH exposure ('*physiological hypoxia*') increases insulin-independent glucose uptake, which is at least partially mediated via AMPK.

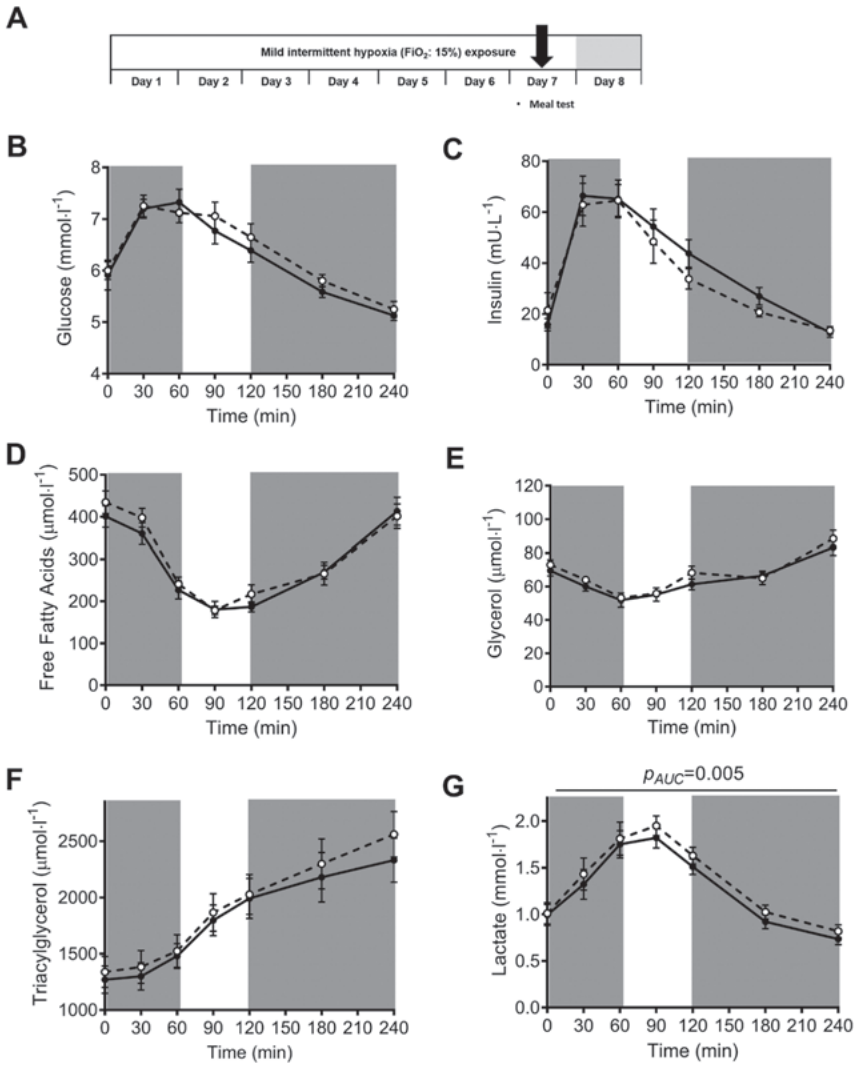

Figure 3. The effects of mild intermittent hypoxia exposure on fasting and postprandial plasma metabolites. Mild intermittent hypoxia exposure during the high-fat mixed meal challenge (**A**) did not significantly alter fasting (t=0 min) and postprandial (t=0 - 240 min) plasma concentrations of **(B)** glucose, **(C)** insulin, **(D)** free fatty acids, **(E)** glycerol, and **(F)** triacylglycerol but significantly increased **(G)** postprandial plasma lactate concentrations. Open circles / dashed line, MIH exposure; Closed circles / solid line, normoxia exposure. Grey areas indicate time periods when study participants were inside the hypoxic room. Data are represented as mean ± SEM. Statistical analysis was performed using nonparametric Wilcoxon's signed-rank test. The exact *p*-values are shown.

Mild intermittent hypoxia exposure does not affect tissue-specific insulin sensitivity *in vivo* but induces a metabolic shift towards increased carbohydrate oxidation.

To determine the prolonged effects of MIH exposure on adipose tissue, hepatic and peripheral insulin sensitivity (primary study outcome), we performed a two-step hyperinsulinemic-euglycemic clamp with [6,6-$^{2}H_{2}$]-glucose tracer infusion under normoxic conditions after cessation of the 7-day exposure regimens (at day 8, Figure 5A). MIH did not significantly alter peripheral insulin sensitivity (insulin-stimulated glucose rate of disappearance, Rd) compared to normoxia exposure (Figure 5E). Furthermore, AT insulin sensitivity (insulin-mediated suppression of free fatty acids during low-insulin infusion, 10 mU•m^{-2}•min^{-1}) and hepatic insulin sensitivity (suppression of endogenous glucose production (EGP) during low-insulin infusion, 10 mU•m^{-2}•min^{-1}) were not significantly affected by MIH (Figure 5C, D). In addition, non-oxidative glucose disposal (NOGD) remained unaltered following MIH compared to normoxia exposure.

Accordingly, we found that MIH did not induce changes in SM insulin signaling, determined using SM biopsies that were collected before the start of the hyperinsulinemic-euglycemic clamp as well as during the steady-state of the high-insulin infusion step (40 mU•m^{-2}•min^{-1}). More specific, the p-Akt^{Ser473}/total Akt protein expression ratio (insulin-stimulated ratio – basal ratio) was comparable following MIH and normoxia exposure (Figure 5F, G).

Since hypoxia might influence glucose homeostasis via insulin-independent mechanisms, possibly involving AMPK (*24*), we further investigated the effect of MIH exposure on AMPK phosphorylation in fasting SM biopsies. However, MIH did not change the p-AMPK^{Thr172}/AMPK ratio compared to normoxia (p=0.346) (Figure 5H, I).

Next, we assessed whether the impact of MIH on substrate utilization under hypoxic conditions (high-fat mixed-meal test on day 6) persists after 7-day MIH exposure. Therefore, we measured substrate oxidation rates before the start as well as during the steady-states of the two-step hyperinsulinemic-euglycemic clamp, which was performed under normoxic conditions after cessation of the MIH exposure regimen (day 8). Remarkably, fasting RER (p=0.050) and CHO (p=0.050) remained increased at day 8, whereas FAO remained reduced (p=0.049), indicating that MIH impacts substrate utilization for at least 16 hours after cessation of the last bout of hypoxia (Supplementary Figure 2). In line, following MIH, the RER (p=0.070) and CHO (p=0.095) tended to be increased, whereas FAO tended to be reduced (p=0.091) during high-insulin infusion. However, insulin-induced changes in substrate oxidation rates were not altered, suggesting that metabolic flexibility was not affected by MIH. Together, these data suggest that MIH exposure for 7 days induces metabolic reprogramming towards more glycolytic metabolism.

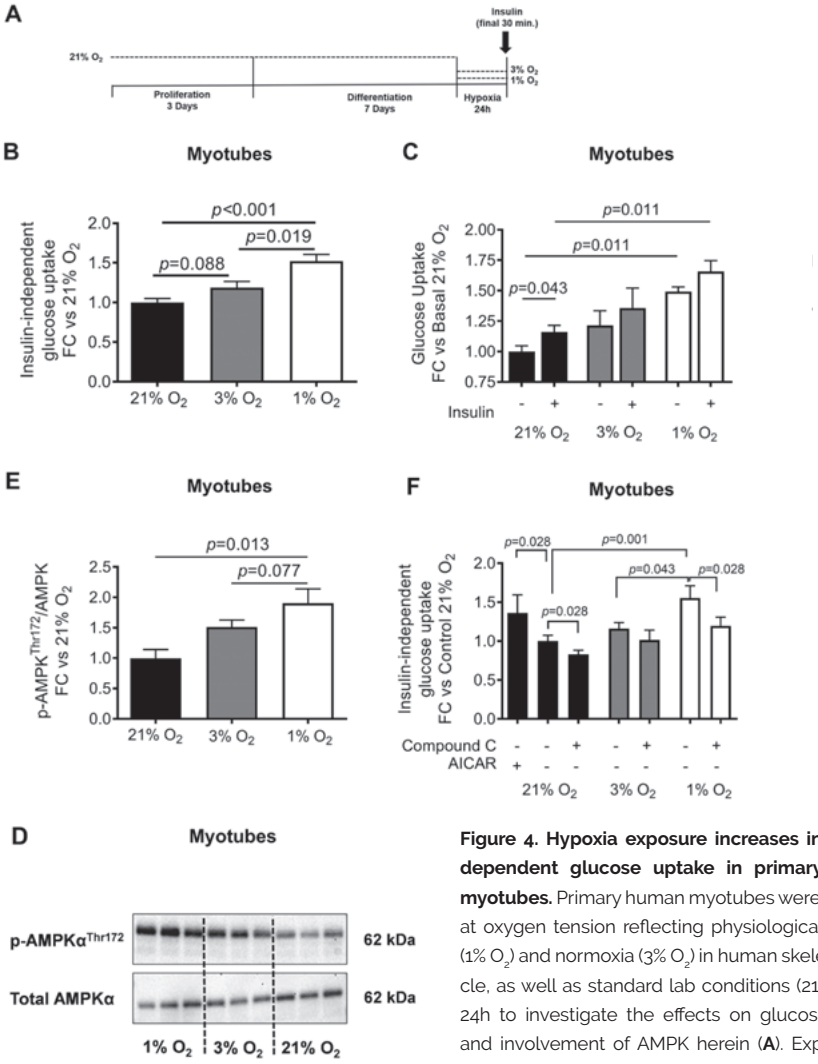

Figure 4. Hypoxia exposure increases insulin-independent glucose uptake in primary human myotubes. Primary human myotubes were cultured at oxygen tension reflecting physiological hypoxia (1% O_2) and normoxia (3% O_2) in human skeletal muscle, as well as standard lab conditions (21% O_2), for 24h to investigate the effects on glucose uptake and involvement of AMPK herein (**A**). Exposure to 1% O_2 increased (**B**) insulin-independent (basal) glucose uptake but did not alter (**C**) the increase in glucose uptake under insulin-stimulated conditions (100 nM insulin) in primary human myotubes compared to 3% and 21% O_2. (**D, E**) Hypoxia exposure increased p-AMPK^{Thr172}/AMPK protein expression compared to standard lab conditions and (**F**) co-incubation experiments using the AMPK inhibitor Compound C (dorsomorphin, 10 µM) and the AMPK agonist AICAR (1 mM; positive control under 21% O_2) demonstrated that the hypoxia-induced increase in insulin-independent glucose uptake is mediated through AMPK activation in human primary myotubes. Black bars, 21% O_2; grey bars, 3% O_2; white bars, 1% O_2 exposure. Data are represented as mean ± SEM, **(B)** n = 11, **(C)** n = 5, **(D, E)** n = 4, **(F)** n = 6. Statistical analysis was performed using nonparametric Wilcoxon's signed-rank test and Friedman's test using Dunn's *post-hoc* multiple comparison test. The exact p-values are shown. Compound C, dorsomorphin; AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide; FC, fold change.

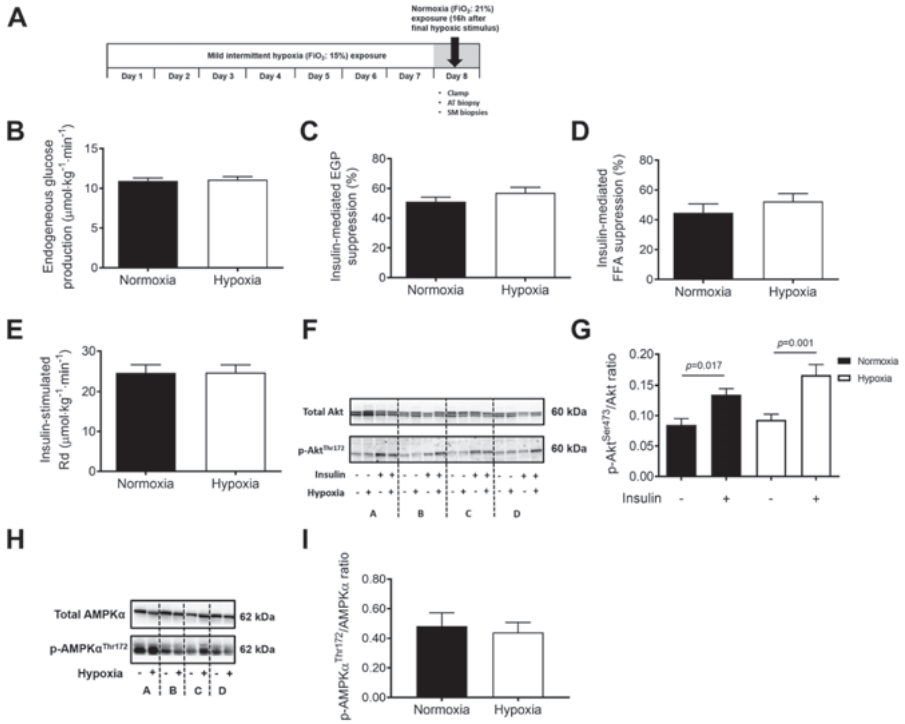

Figure 5. Mild intermittent hypoxia exposure does not alter hepatic, adipose tissue and peripheral insulin sensitivity. Subsequent to 7 days of MIH exposure, tissue-specific insulin sensitivity was determined by a two-step hyperinsulinemic-euglycemic clamp under normoxic conditions (**A**). MIH does not significantly affect (**B**) fasting liver endogenous glucose production (EGP), (**C**) the suppression of EGP (%) (**D**) and the suppression of plasma free fatty acid concentration (%) during the steady state of low-insulin infusion (10 mU•m^{-2}•min^{-1}), and (**E**) insulin-stimulated rate of glucose disposal during the steady state of high-insulin infusion (40 mU•m^{-2}•min^{-1}). (**F**) p-Akt^{Ser473} and Akt protein expression and (**G**) p-Akt^{Ser473}/Akt ratio in fasting and insulin-stimulated (40 mU•m^{-2}•min^{-1}) skeletal muscle biopsies (representative Western Blot of 4 participants indicated (participant A, B, C and D). **(H)** Fasting p-AMPK**α**^{Thr172} and AMPK**α** protein expression, and **(I)** ratio in fasting skeletal muscle biopsies. Data are represented as mean (bars) and individual data points. Statistical analysis was performed using two-tailed Student's paired t-test. The exact *p*-values are shown.

Mild intermittent hypoxia exposure does not alter skeletal muscle oxidative capacity

Since tissue pO_2 reflects the balance between oxygen supply and the metabolic rate, we also examined the effects of MIH on mitochondrial respiratory capacity in SM, a highly metabolically active organ accounting for 20-30% of resting EE (*25*). To this end, we performed high-resolution *ex vivo* respirometry using freshly permeabilized human SM fibers, derived from the same individuals that participated in the *in vivo* studies, using both carbohydrate- and lipid-derived substrates. MIH did not induce alterations in state 3 and maximally uncoupled mitochondrial respiration using carbohydrate- and lipid leads (Figure 6A-C). In agreement with these findings, protein expression of OXPHOS complex components was unchanged following MIH (Figure 6D, E). Taken together, these data indicate that MIH exposure has no significant effects on *ex vivo* mitochondrial respiration and oxidative capacity in human SM fibers. In line with these findings, Western Blot analysis revealed that mild hypoxia exposure did not affect OXPHOS complex protein expression in human primary myotubes (Figure 6F, G).

Mild intermittent hypoxia exposure impacts inflammatory, metabolic and ECM-related pathways in adipose tissue but not skeletal muscle

To provide further insight into MIH-induced adaptations at the level of AT and SM, we next performed gene expression analysis by Affymetrix micro-arrays. In total, 171 gene sets were positively, and 285 negatively enriched in AT following 7-day MIH compared to normoxia, whereas no significantly differentially expressed pathways could be identified in SM after gene set enrichment analysis (GSEA) at FDR q-value <0.2, p<0.05 (all affected pathways in AT and individual genes in SM are in Supplementary Table 1 and 2, respectively). In AT, pathways related to inflammation, and carbohydrate and lipid metabolism were significantly upregulated, reflected by positive normalized enrichment scores. Expression of gene sets related to cell cycle, mitochondrial translation and extracellular matrix components was downregulated, reflected by negative normalized enrichment scores (Figure 7). Interestingly, we found that MIH downregulated the collagen assembly pathway, which may reflect reduced ECM stress, possibly reflective of the initiation of AT remodeling. In contrast, it has been postulated previously that hypoxia in AT may be associated with increased deposition of various types of collagen, collagen-crosslinking and, hence, AT fibrosis, which in turn may limit adipocyte hypertrophy (*26*). Furthermore, we found that MIH upregulated pathways related to inflammation transcription factor nuclear factor-κB (NF-κB), and various downstream cytokine signaling pathways involving cytokines such as interleukin-1 (IL-1) and IL-2, and complement factors. However, systemic levels of inflammatory cytokines tumor necrosis factor α (TNFα), interferon-γ (IFN-γ), IL-6 and IL-8 were not affected by MIH exposure (Supplementary Figure 3). Taken together, we

demonstrate that MIH exposure increases gene expression of pathways related to inflammation and lipid/carbohydrate metabolism, and downregulates expression of genes involved in collagen assembly in human AT, yet does not alter systemic concentrations of inflammatory cytokines. These data may point towards initiation of AT remodeling and metabolic rewiring to adapt to lower oxygen availability.

Mild hypoxia exposure affects adipokine expression and secretion but not glucose uptake in primary human adipocytes

To further explore the impact of MIH exposure on primary human adipocytes, we exposed these cells to three different conditions: 1) O_2 levels that resemble those in obese AT under normoxic conditions (10% O_2), 2) MIH (3x2h 5% O_2) and 3) standard tissue culture conditions (21% O_2) for 7 consecutive days. First, we examined the effects of MIH on adipokine secretion. Therefore, we collected cell culture medium after exposure to the three different conditions, and measured IL-6, MCP-1, leptin, adiponectin, and VEGF concentrations. Interestingly, MIH exposure reduced VEGF (p=0.005) and leptin (p=0.034) secretion compared to 10% and 21% O_2, respectively (Supplementary Figure 4). In line with reduced leptin secretion, leptin mRNA expression was significantly reduced by MIH exposure compared to 21% O_2 (p=0.005) (Supplementary Figure 4). In contrast, MIH increased VEGFA mRNA expression compared to 10% O_2 (p=0.011).

Moreover, we determined the impact of MIH exposure on glucose uptake in primary human adipocytes. MIH did not significantly alter insulin-(in)dependent glucose uptake compared to 10% O_2 (Supplementary Figure 4). Taken together, these experiments indicate that MIH exposure affects VEGF and leptin expression and secretion but does not alter insulin-(in)dependent glucose uptake in primary human adipocytes.

Mild intermittent hypoxia induces slight hemodynamic adaptations but does not alter feelings of hunger and satiety

To assess the effects of MIH exposure on hemodynamic parameters, we assessed heart rate, systolic (SBP) and diastolic blood pressure (DBP). MIH exposure did not affect SBP and DBP, despite a slight but significant increase in mean heart rate (p=0.017, Supplementary Figure 5). Finally, since previous studies have shown that exposure to low environmental oxygen may reduce appetite and hence body weight (*27*), we examined feelings of thirst, hunger and satiety. As expected, based on the severity of hypoxia exposure in the present study, we found that feelings of hunger, satiety and thirst were not significantly affected by MIH exposure, determined by Visual Analogue Scales (VAS) that were completed by the participants before and after breakfast, lunch and dinner (Supplementary Figure 6).

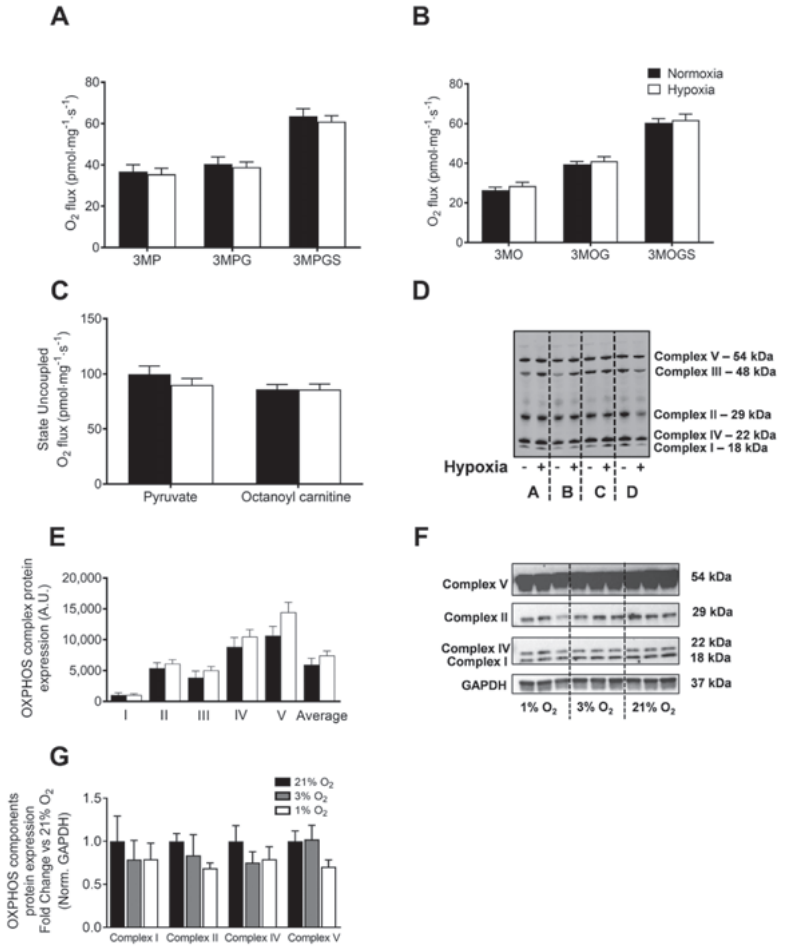

Figure 6. Mild intermittent hypoxia exposure does not affect skeletal muscle oxidative capacity. A skeletal muscle biopsy was obtained from the *m. vastus lateralis* under fasting conditions. *Ex vivo* mitochondrial respiration experiments were performed to determine (**A**) State 3 ADP-stimulated respiration upon a carbohydrate-like (pyruvate), (**B**) lipid-like (octanoyl-carnitine) substrate with parallel electron input into complex I and II, and (**C**) maximal uncoupled respiration upon stimulation with FCCP in both leads. (**D**) OXPHOS components complex (I-V) protein expression in skeletal muscle biopsies collected under fasting conditions (representative Western Blot of 4 participants, indicated by A-D). (**E**) Quantification of OXPHOS protein expression. (**F**) Primary human myotubes were cultured at oxygen tension reflecting physiological hypoxia (1% O_2) and normoxia (3% O_2) in human skeletal muscle, as well as standard lab conditions (21% O_2), for 24h to investigate the effects of hypoxia exposure on OXPHOS protein expression. (**G**) Quantification of OXPHOS protein expression in myotubes. Closed circles, normoxia exposure; open circles, MIH exposure. Data are represented as mean ± SEM. 3, state 3; M, malate; G, glutamate; S, succinate; P, pyruvate; O, octanoyl-carnitine; FCCP, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone. I-V; Complex I-V proteins; A.U., arbitrary units.

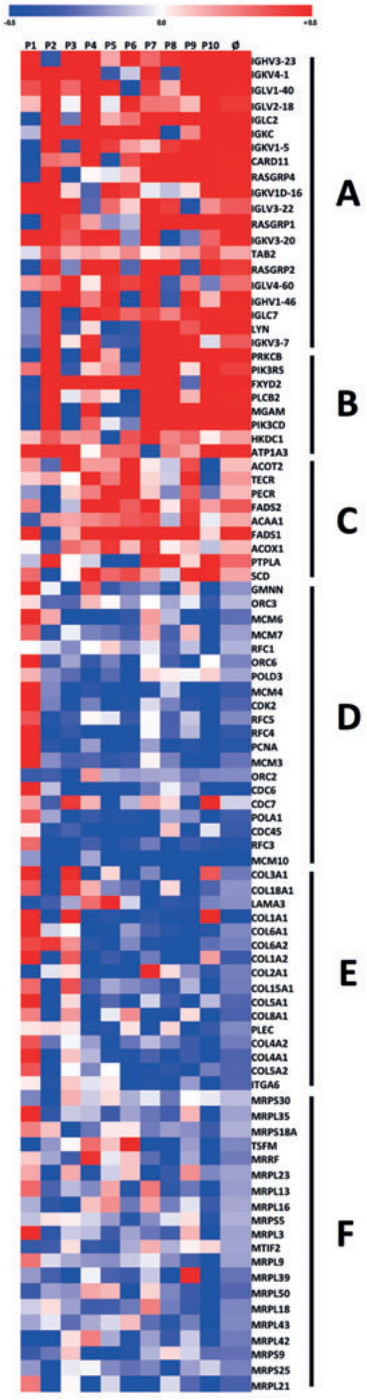

Figure 7. Mild intermittent hypoxia exposure alters gene expression in human adipose tissue.

Adipose tissue biopsies were collected on day 8 to determine the effects of MIH exposure as compared to normoxia exposure for seven consecutive days on adipose tissue expression. Gene set enrichment analyses was performed (signal log ratios: hypoxia – normoxia), and signal to log ratios are depicted per subject and as overall mean (Ø). Genes most significantly contributing to up- and downregulated pathways following MIH compared to normoxia exposure were selected (false discovery rate, *q*-value <0.2, $p<0.05$). Colors reflect the individual signal to log ratios, with red being upregulated and blue being downregulated compared to normoxia exposure. (**A**) FCERI-mediated NF-**K**B activation. (**B**) Carbohydrate digestion and absorption. (**C**) Biosynthesis of unsaturated fatty acids. (**D**) WP466: DNA replication. (**E**) WP2798: Assembly of collagen fibrils and other multimeric structures. (**F**) Mitochondrial translation. A list of all pathways significantly altered by MIH exposure is shown in Supplementary Table 2.

Discussion

Compelling evidence indicates that human tissues adapt to alterations in oxygen availability. Previous studies have suggested that tissue oxygenation may be a target to improve glucose homeostasis (*2, 6*). In the present randomized, single-blind crossover study, we demonstrate for the first time that MIH exposure for 7 consecutive days reduces systemic oxygen saturation, decreases AT and SM pO_2, evokes a shift toward glycolytic metabolism, induces adaptations in AT and SM but does not impact AT, hepatic and SM insulin sensitivity in obese men.

We demonstrated that MIH exposure markedly increased whole-body carbohydrate oxidation, an effect that persisted after cessation of the exposure regimen until at least the next day. This shift in substrate oxidation was also reflected by increased plasma lactate concentrations during the high-fat mixed-meal test under MIH, indicative of an increased glycolytic rate. In accordance with our findings, it has been demonstrated previously that short-term mild hypoxia exposure (15% O_2) augments fasting (*28*) and postprandial carbohydrate oxidation, but does not affect postprandial glucose and insulin levels (*29*). Additionally, increased reliance on carbohydrate oxidation during three weeks of chronic hypobaric hypoxia exposure has previously been shown in healthy males (*30, 31*).

Mechanistically, the present data demonstrate that hypoxia exposure increased glucose uptake via an AMPK-dependent mechanism in primary human myotubes but not adipocytes. In agreement with our findings, increased p-AMPKα expression has been found after chemically-induced hypoxia in C2C12 myotubes (*32*). In addition, exposure to 3% O_2 increased glucose uptake in L6 myotubes (*33*). Other studies that employed supraphysiological O_2 levels *in vitro* (i.e. 7% (*34*) and 15% O_2 (*15*)) found increased glucose uptake compared to standard laboratory conditions (21% O_2) in human myotubes. Since SM pO_2 values in resting skeletal muscle range between ~1 - 3% O_2 (*35*), as also demonstrated in the present study, the physiological relevance of the latter *in vitro* experiments can be questioned. In contrast to our *in vitro* findings and previous findings in rodent skeletal muscle (*36, 37*), we did not find altered p-AMPK^{Thr172}/AMPK protein expression in human SM following MIH exposure. Since the muscle biopsies were collected ~16h after the last hypoxic stimulus, we cannot exclude that AMPK was already dephosphorylated at the time of sample collection. On the other hand, the muscle cell protein lysates from the primary human myotube experiments were immediately harvested after 24h hypoxia exposure, and hence may reflect more acute effects of hypoxia on AMPK activity.

Importantly, the findings of the present randomized crossover study demonstrate that MIH for 7 days did not alter AT, hepatic and SM insulin sensitivity. In contrast, previous studies in rodents have shown that prolonged exposure to MIH improves insulin sensitivity and/or glucose homeostasis (*11-14*). Moreover, an

uncontrolled study found that exposure to mild hypoxia (FiO_2 15%) for 10 consecutive nights slightly but significantly increased whole-body insulin sensitivity in obese humans (*15*). However, is important to emphasize that the latter finding was based on a within-group comparison, since a control group was not included in the study design (*15*).

Moreover, our data show that MIH increased AT gene expression of inflammatory/metabolic pathways and decreased extracellular matrix-related pathways. However, MIH-effects on adipokine expression/secretion did not translate into changes in systemic low-grade inflammation, as plasma concentrations of the TNFα, IFN-γ, IL-6 and IL-8 remained unaltered. Conflicting findings have been reported on the effects of (severe) hypoxia on inflammatory processes, which seems to be related to the severity, pattern and duration of hypoxia exposure (*2*). We have recently shown that prolonged exposure to mild hypoxia reduced the expression of pro-inflammatory genes in human adipocytes (*22*). An alternative explanation for the upregulation of inflammation-related pathways in AT that we found in the present study may be hypoxia-induced lactate production, which may contribute to increased NF-κB related gene expression, as observed in L6 myocytes and macrophages (*38, 39*). On the other hand, the hypoxia-lactate axis might mitigate inflammation by suppressing macrophage activation and polarization towards anti-inflammatory M2-macrophages (*40, 41*).

Furthermore, *ex vivo* SM mitochondrial respiratory capacity as well as OXPHOS protein expression in SM was unaltered following MIH exposure. Hypoxia-induced activation of hypoxia-inducible factor (HIF)-1α activation might inhibit decarboxylation of pyruvate into acetyl-CoA by pyruvate dehydrogenase (*42*). This may then shunt pyruvate away from the mitochondria, resulting in reduction of mitochondrial respiration, hence enhancing glycolysis to ensure sufficient ATP production (*42*). Indeed, MIH increased plasma lactate concentrations during the high-fat mixed-meal test (Figure 5f). However, this response, potentially induced by HIF-1α, may be considered acute, since HIF1 transcripts are inversely proportional, and exponential to pO_2 only between *in situ* oxygen levels of 0.1 – 5% O_2 (*43-45*). Of note, in the present study, the SM biopsies used to isolate the SM fibers for determining oxygen consumption rates were collected ~16h after the last hypoxic stimulus of the exposure regimen, and therefore reflect the chronic rather than acute response of MIH on the oxidative machinery in human SM.

The strengths of the present study are that, to our knowledge, this is the first randomized crossover trial investigating the effects of MIH exposure on many cardiometabolic risk factors in humans. Importantly, measurements were performed under well-controlled conditions, whilst participants were kept in energy balance throughout the study. Furthermore, we studied the isolated effects of MIH under normobaric conditions, thereby excluding possible effects of different ambient

pressure, as present at high altitude (i.e. hypobaric hypoxia). Finally, we performed extensive measurements in tissue biopsies and human primary myotubes and adipocytes, which were cultured under oxygen levels that mimic the adipose tissue and skeletal muscle microenvironments in humans, to explore underlying mechanisms.

Noteworthy, several nuances have to be made with respect to the conclusions of the present randomized, single-blind crossover study. First, since we studied obese men with modest impairments in glucose homeostasis, we cannot exclude that MIH exposure may have differential effects in more metabolically compromised individuals or women. Secondly, the duration of exposure to MIH was relatively short (in total 42 hours), compared to previous studies in rodents (*11, 37*), and the uncontrolled study by Lecoultre and coworkers (*15*) that did find improved insulin sensitivity. The reason for this was that the implementation of MIH in clinical practice to improve metabolic health might be easier than long-term continuous hypoxia exposure. Therefore, we cannot exclude that more prolonged exposure to mild (intermittent) hypoxia, or exposure to more severe hypoxia, is required to induce beneficial effects on glucose homeostasis.

To conclude, the findings of the present randomized single-blind crossover trial demonstrate that 7 consecutive days of MIH exposure decreases AT and SM pO_2, induces a metabolic switch towards increased reliance on glycolysis to ensure ATP production, and evokes adaptations in AT and SM in humans with obesity. This, however, does not translate into alterations in AT, hepatic and SM insulin sensitivity. Further studies are warranted to investigate whether other hypoxia exposure regimens (longer intervention period and/or shorter but more severe hypoxic episodes) may elicit beneficial effects on glucose homeostasis in people at risk or living with type 2 diabetes mellitus, thereby providing a putative novel avenue to prevent and treat obesity-related metabolic perturbations. If hypoxia exposure proves effective in improving glucose homeostasis in humans, this may have direct clinical implications. For example, prolyl-hydroxylase (PHD) inhibitors, which like hypoxia exposure stabilize HIF, improved glucose tolerance, serum cholesterol and inflammation in rodents (*46, 47*). Interestingly, PHD inhibitors have recently been used in clinical trials to treat anemia in chronic kidney disease (*48*), but putative effects on glucose homeostasis in metabolically compromised individuals have to our knowledge not been examined yet.

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Author Contributions

G.G. supervised this work. R.V.M., M.V., E.B., and G.G conducted hypothesis generation, conceptual design, and data analysis. R.V.M., M.V., L.V., J.J, J.S. N.H., J.H., Y.E., P.S., H.S., S.K., and G.G. contributed to data acquisition. G.G. acquired funding for the study. R.V.M., M.V. and G.G. wrote the manuscript and G.G. had the primary responsibility for the final content. All authors revised the content of the manuscript and read and approved the final version of the manuscript for publication.

Competing interests

None.

Data and materials availability

The datasets generated by microarray analysis are deposited at the Gene Expression Omnibus (GEO) database (accession number <to be added when manuscript is accepted>). Furthermore, primer sequences used in this study are included in Supplementary Table 3.

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Supplementary Methods

Study design and setting

The diet was adjusted individually to match energy requirements and maintain energy balance throughout the study. Based on the estimated daily energy expenditure (basal metabolic rate (BMR) (Ventilated Hood, Omnical, Maastricht University) multiplied by 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 throughout the study.

Adipose tissue and skeletal muscle partial oxygen pressure

Microdialysis catheters (CMA60, CMA microdialysis AB) were inserted into abdominal subcutaneous AT 6-8 cm lateral from the umbilicus (skin anesthetized using EMLA cream) and SM (*m. gastrocnemius*; local anesthesia using 2% lidocaine, after which perfusion (Ringer solution) After insertion, both microdialysis catheters were perfused with Ringer solution (Baxter BV). at a flow rate of 2 µl/min (CMA400 microinfusion pump, CMA Microdialysis AB). The interstitial fluid was then directed towards a flow-through cell, containing an optochemical O_2-sensor (*49*). Within 2-3 hours after insertion of the microdialysis probes in AT and SM, pO_2 had reached stable values (change in pO_2 <2.0 mmHg over a 20-min period). Fasting AT and SM pO_2 values were calculated by averaging these 20-min periods with stable pO_2 readings.

High-fat mixed meal test

Following a 30-min baseline period under fasting conditions, individuals were asked to ingest a liquid test meal, providing 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), at t=0 within 5 min. Blood samples were collected from a superficial dorsal hand vein, which was arterialized by placing the hand into a hotbox (~55˚C). Blood samples were taken under fasting (t=0 min) and postprandial conditions (t=30, 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 hours after ingestion of the high-fat mixed-meal (*50*). Calculations of energy expenditure and substrate oxidation were performed according to the formulas of Weir (*51*) and Frayn (*52*). Nitrogen excretion was based on the assumption that protein oxidation represents ~15% of total energy expenditure (*53*).

Hyperinsulinemic-euglycemic clamp

First, a primed (bolus-injection of 2.4 mg•kg^{-1}) continuous infusion of D-[6,6-$^{2}H_{2}$]-glucose was started and continued throughout the measurement at 0.04 mg•kg^{-1}•min^{-1} 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^{-1}). 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 sampled every 15 min to determine steady-state kinetics.

Plasma biochemistry

Plasma glucose (ABX Pentra Glucose HK CP, Horiba ABX Diagnostics), FFA (WAKO NEFA-HR (2) ACS-ACOD method, WAKO Chemicals GmbH), TAG (ABX Pentra Triglycerides CP, Horiba ABX Diagnostics) and glycerol (Glycerol-kit UV-test, r-Biopharm) were determined. Plasma insulin was measured using a double-antibody radioimmunoassay (Millipore). Lactate was measured in plasma using standard enzymatic techniques automated on a Cobas Fara centrifugal spectrophotometer (Roche Diagnostics).

Adipose tissue and skeletal muscle gene expression

Total RNA was extracted from adipose tissue and skeletal muscle biopsies using the Trizol method (Qiagen, Venlo, Netherlands). Subsequently, 100 ng of intact total RNA was processed applying the GeneChip® WT PLUS Reagent Kit (Affymetrix) and Human Transcriptome Array (HTA) 2.0 GeneChip (Affymetrix) according to the manufacture's manual.

Skeletal muscle protein expression

Proteins were lysed as described previously (*54*), and were subsequently homogenized. Next, a BCA assay was performed to determine protein lysate concentrations. Equal amounts of protein were loaded ((p)AMPK and (p)Akt 25 µg, OXPHOS 7.5 µg). and separated on the different gels. Primary antibodies: mouse monoclonal antibodies cocktail directed against human OXPHOS (dilution: 1:10.000; no. ab110411/no. MS601, Abcam/MitoSciences), rabbit anti-human AMPKα (1:1000,

no. 2603, Cell Signaling) rabbit anti-human p-AMPKα^{Thr172} (1:1000, no. 2535, Cell Signaling), rabbit anti-mouse Akt (1:1000, no. 9272, Cell signaling), rabbit-anti mouse p-Akt^{Ser473} (1:1000, no. 9271, Cell Signaling). Secondary antibodies were donkey anti-mouse conjugated with IRDYe800 (OXPHOS), swine anti-rabbit HRP (AMPKα, p-Akt and Akt), goat anti-rabbit HRP (p-AMPKα^{Thr172}). Visualization and analysis was performed using a CLx Odyssey Near Infrared Imager (OXPHOS) or ChemiDoc XRS system (AMPK, Akt) and Quantity One software. Individual intensities were quantified and are expressed in arbitrary units, or derived ratios.

Cell culture of human primary satellite cells

Satellite cells were cultured using proliferation medium, consisting of low glucose (1000 mg/l) Dulbecco's Modified Eagle's Medium (DMEM, no. D6046, Sigma) supplemented with 0.05% bovine serum albumin (BSA, no. A4503, Sigma), 1 µM Dexamethasone (no. D4902, Sigma), 16% fetal bovine serum (FBS, no. BDC-6086, Bodinco BV) 0.5 mg/ml bovine fetuin (no. 10344-034/026, Invitrogen, Life Technologies), 1x Antibiotic-Antimycotic (no. 15240-62, Gibco, Thermo Fisher Scientific) and 0.01 µg/ml recombinant human epidermal growth factor (no. PHG0311, Gibco). At 70-80% confluence, differentiation medium was added containing MEM-α-Glutamax (no. 32561-029, Gibco) supplemented with 2% FBS (Bodinco BV), 0.5 mg/ml bovine fetuin (Invitrogen, Life Technologies) and 1x Antibiotic-Antimycotic (100 units•ml^{-1} penicillin, 100 µg•ml^{-1} of streptomycin and 0.25 µg•ml^{-1} Amphotericin B (no. 15240-62, Gibco)) until myotube formation was completed.

Cell culture of human multipotent adipose-derived mesenchymal stem cells

Human multipotent adipose-derived mesenchymal stem cells (hMADS) were kept in proliferation medium containing DMEM and Ham's F-12 (DMEM-Ham's F-12) Nutrient Mixture (no. 31330-095, Gibco), 10% FBS (Bodinco BV), 1x Antibiotic-Antimycotic (Gibco). At ~80% confluence, differentiation medium was added to the cells containing DMEM-Ham's F-12, 3% FBS (Bodinco BV), 1x Antibiotic-Antimycotic (Gibco), 33 µM D-Biotin (no. B4693, Sigma), 17 µM D-pantothenate (no. P5155, Sigma), 0.1 µM h-insulin (no. 91077C, Sigma), 1 µM dexamethasone (no. D4902, Sigma), 250 µM 3-isobutyl-1-methylxanthine (IBMX, no. I5879, Sigma) and 5 µM rosiglitazone (no. ALX-350-125-M025, Enzo Life Sciences). After 7 days, IBMX and rosiglitazone were removed from the medium.

Glucose uptake in primary human myotubes and adipocytes

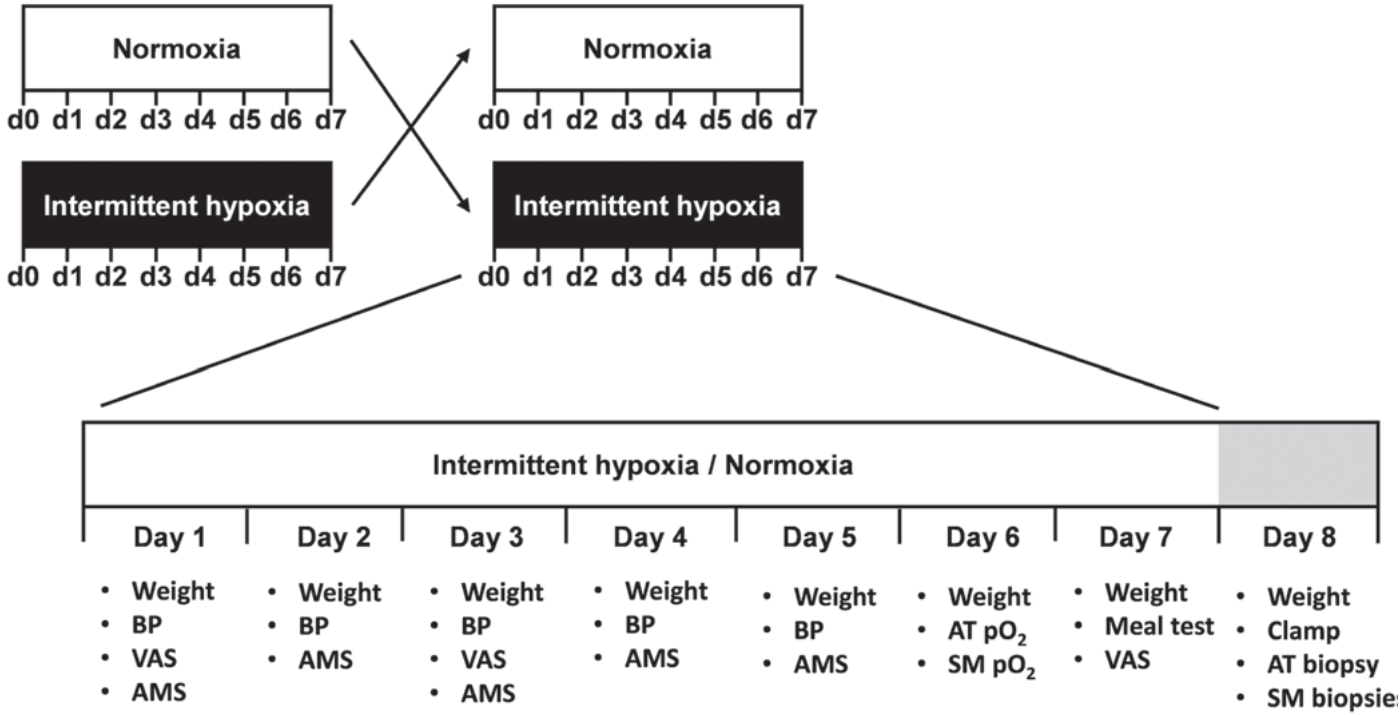
Briefly, hMADS and myotubes were serum-starved 2h prior to the glucose uptake experiment. After two washes in modified Krebs Ringer buffer (1.17 M NaCl, 26 mM KCl, 12 mM KH_2PO_4, 12 mM $MgSO_4$, 100 mM $NaHCO_3$, 100 mM HEPES, 0.1% BSA and 1mM $CaCl_2$) cells were incubated for 30 minutes in 20 µM 2-Deoxy-D-glucose and 55µM (0.44 µCi/ml) (^{3}H-)-2-Deoxy-D-glucose (#NET328A001MC, Perkin Elmer) at 37C. Subsequently, h-insulin was added and the cells were incubated for another 30 minutes. The cells were then scraped and lysed in 0.05 M NaOH. Subsequently, 200 µL of the cell lysate was transferred into glass scintillation vials containing 5 mL OptiFluor (Perkin Elmer). β-decay was measured using a liquid scintillation counter (Perkin Elmer), reflecting cellular uptake of (^{3}H-)-2-Deoxy-D-glucose.

Myotube protein expression

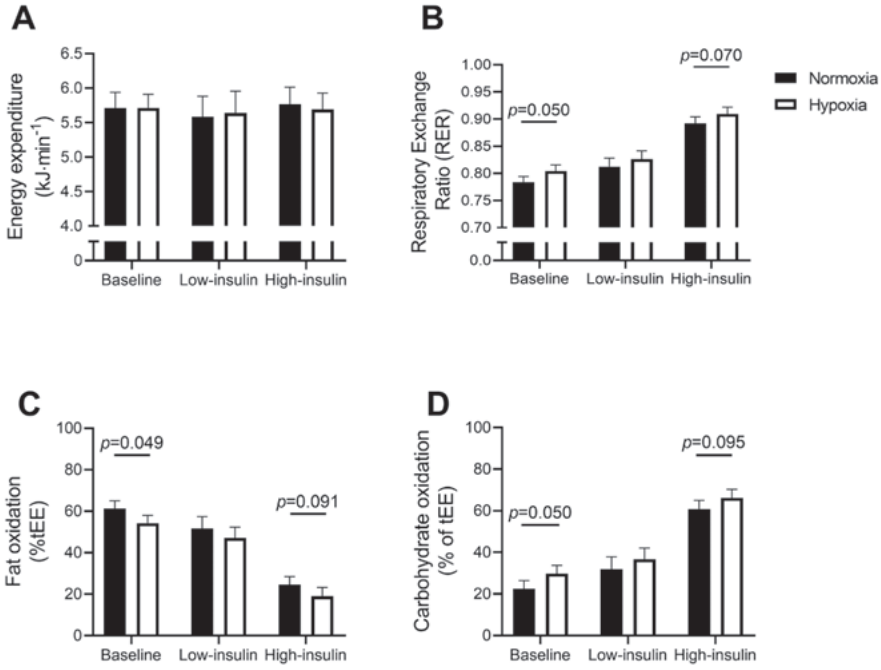
1x RIPA buffer supplemented with a protease/phosphatase inhibitor cocktail (Cell Signaling Technology Europe, Leiden, The Netherlands) was added to lyse the myotubes. After scraping the cells, the protein lysate was collected and protein concentration was determined (BCA Protein Assay kit; Santa-Cruz Biotechnology Inc.). 15 µg protein was separated on BioRad Criterion TGX gels (Bio-Rad Laboratories) (12% for OXPHOS, Any-kD for other targets) and subsequently blotted using Trans-Blot Turbo Transfer System (Bio-Rad; for AMPKα, OXPHOS, GAPDH) and Bio-Rad Criterion Blotter (Bio-Rad; for p-AMPKαThr^{172}, GAPDH), respectively. Membranes were incubated overnight 4˚C with primary antibodies in corresponding blocking buffers according to manufacturer's protocols. Subsequently, blots were incubated with secondary antibodies for 1h at room temperature. Primary antibodies used were: AMPKα (no. 2603, Cell Signaling), p-AMPKαThr^{172} (no. 2535, Cell Signaling), total OXPHOS antibody cocktail (no. MS601, Mitosciences) and GAPDH (no. 2118, Cell Signaling). Secondary antibodies used were swine-anti-rabbit HRP (no. P0339, DAKO), rabbit-anti-mouse HRP (no. P0161, DAKO) and goat-anti-rabbit HRP (no. PI-1000, Vector Laboratories,). Antigen-antibody complexes were visualized by chemiluminescence using SuperSignal™ West Femto and Dura extended Duration Substrates (Life Technologies). Visualization and analysis was performed using a ChemiDoc XRS system (Bio-Rad) and Quantity One software.

Adipocyte gene expression

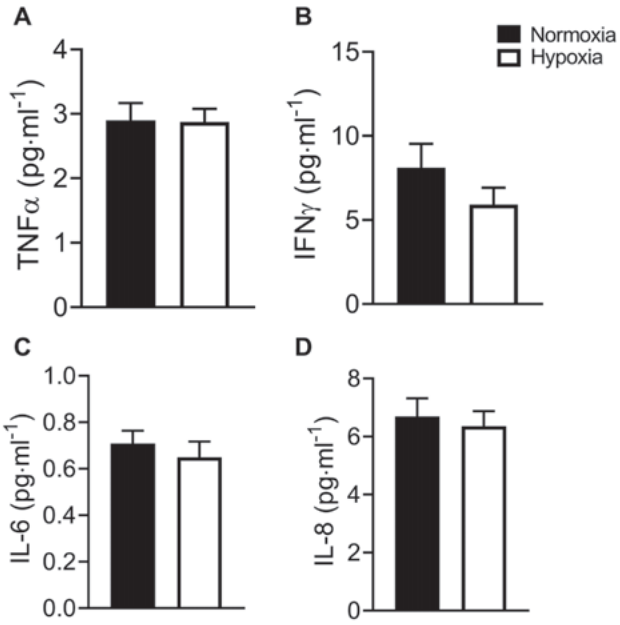
The extracted RNA was precipitated and purified according to manufacturer's protocol. Subsequently, cDNA was synthesized out of purified RNA, using RT-PCR (iScript cDNA synthesis kit, no. 170-8891, Bio-Rad). Next, SYBR-Green based real-time PCRs were performed using an iCycler (Bio-Rad; primer sequences are provided in Supplementary Table 3). Results were normalized to the geometric mean of 18S ribosomal RNA and RPL13A.



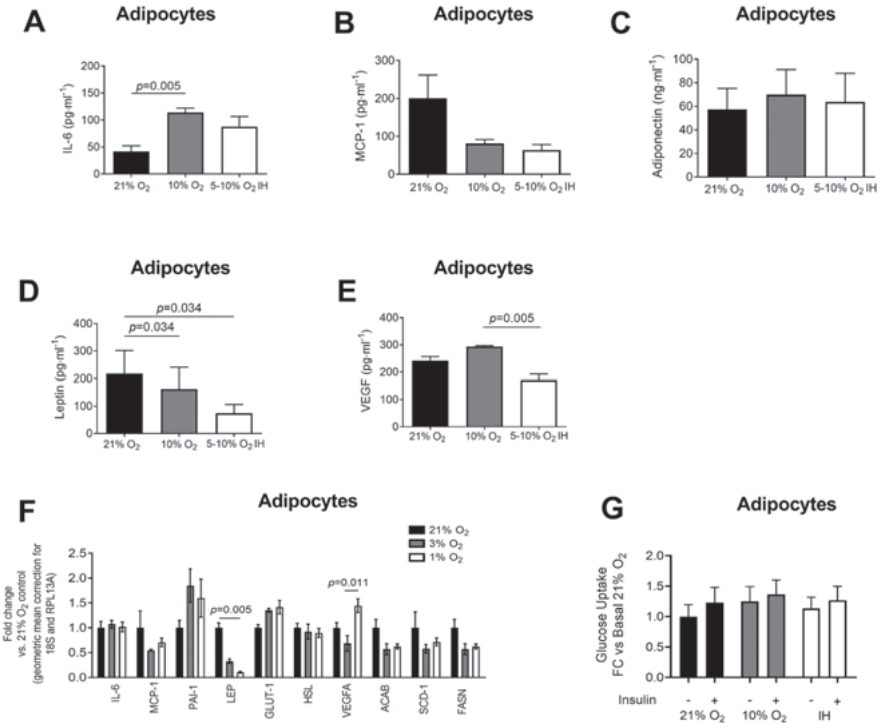
Supplementary Figure 1. Study flowchart of the randomized, single-blind crossover trial. Participants were exposed to normoxia (21% O_2) and mild intermittent hypoxia (15% O_2, 3x2h per day) with a 3-6 week washout period, in a randomized fashion. At day 8, a two-step hyperinsulinemic-euglycemic clamp was performed under normoxic conditions, during both exposure regimens. BP, blood pressure; VAS, visual analog scale questionnaire; AMS, acute mountain sickness questionnaire; AT pO_2, adipose tissue; SM, skeletal muscle; pO_2, oxygen tension.



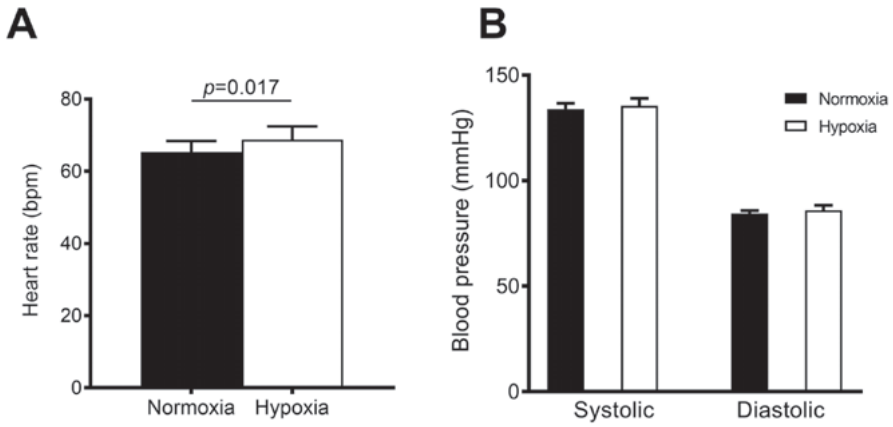
Supplementary Figure 2. Substrate oxidation during the hyperinsulinemic-euglycemic clamp (day 8). Fasting and insulin-stimulated (low-insulin: 10 mU•m^{-2}•min^{-1}; high-insulin: 40 mU•m^{-2}•min^{-1}) energy expenditure and substrate oxidation was measured by indirect calorimetry during the steady state phases of a two-step hyperinsulinemic-euglycemic clamp. MIH exposure did not alter energy expenditure **(A)** but increased fasting respiratory exchange ratio **(B)**, which was reflected by reduced fat oxidation **(C)** and increased carbohydrate oxidation **(D)** compared to normoxia exposure. Closed circles, normoxia exposure; open circles, MIH exposure. Values are represented as individual data (connected lines). Statistical analysis was performed using two-tailed Student's paired t-test. The exact *p*-values are shown. %EE, percentage of total energy expenditure.



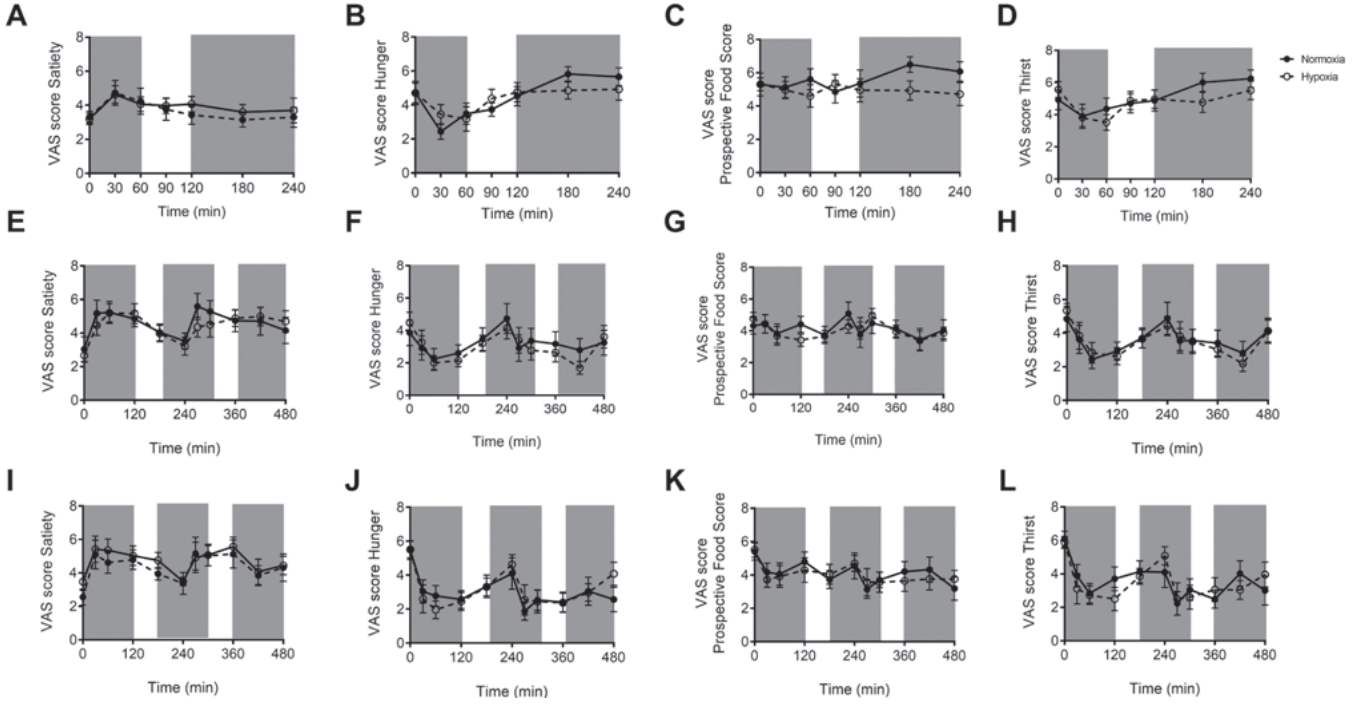
Supplementary Figure 3. The effect of mild intermittent hypoxia compared to normoxia exposure on systemic concentrations of inflammatory cytokines. Mild intermittent hypoxia (MIH) exposure did not significantly alter fasting plasma concentrations of **(A)** TNFα, **(B)** IFN-γ, **(C)** IL-6 and **(D)** IL-8, determined at day 8, subsequent to MIH exposure. Black and white bars represent normoxic and MIH conditions, respectively. Data are represented as mean ± SEM. TNFα, tumor necrosis factor α; IFN-γ, interferon-γ; IL, interleukin.



Supplementary Figure 4. The effect of mild intermittent hypoxia compared to normoxia exposure on adipokine secretion and gene expression in primary human adipocytes. Adipose tissue-derived mesynchymal stem cells were differentiated for 14 days. During days 7-14, the cells were exposed to mild intermittent hypoxia exposure (from 10% to 5% O_2, 3x2h per day) or continuous exposure to 10% O_2 and 21% O_2, resembling normoxia and standard lab conditions, respectively. Secretion of (**A**) IL-6, (**B**) MCP-1, (**C**) adiponectin, (**D**) leptin and (**E**) VEGF were measured after 7 days of exposure to the different oxygen levels. (**F**) Gene expression (mRNA) of *IL-6*, *DPP-4*, *MCP-1*, *PAI-1*, *LEP*, *GLUT-1*, HSL, *VEGFA*, *ACAB*, *SCD1* and *FASN* was quantified and expressed as fold change compared to 21% O_2, and corrected for the geometric mean of 18S and RPL13A expression. **(G)** Differentiated primary human adipocytes exposed to MIH for 7 days (alternating cycles of 10-5% O_2, 3x2h per day) did not alter basal and insulin-stimulated glucose uptake compared to a fixed oxygen level at 10% O_2 and 21% O_2, resembling normoxia in human adipose tissue and standard laboratory conditions, respectively. Black bars, 21% O2; grey bars 10% O2; white bars, 5-10% O2, intermittent hypoxia. Data are represented as mean ± SEM, **(A-E)** n = 4, **(F)** n = 4-5, **(G)** n = 4. Statistical analysis was performed using Friedman's test with Dunn's *post hoc* multiple comparison test. The exact p-values are shown. *IL-6*, Interleukin-6; *MCP-1*, monocyte chemoattractant protein-1; *PAI-1*, plasminogen activator inhibitor-1; *LEP*, leptin; *GLUT1*, glucose transporter 1; *HSL*, hormone sensitive lipase; *VEGF*, vascular endothelial growth factor; *ACAB*, acetyl-CoA acetyltransferase; *SCD-1*, stearoyl-CoA desaturase-1; *FASN*, fatty acid synthase; *18S*, 18S ribosomal RNA; *RPL13A*, ribosomal protein L13a.



Supplementary Figure 5. The effects of mild intermittent hypoxia compared to normoxia exposure on heart rate, systolic and diastolic blood pressure. Heart rate and blood pressure (average values on days 1-5) were measured under fasting conditions. (**A**) MIH exposure significantly increased HR but (**B**) did not induce changes in systolic and diastolic blood pressure. Closed circles, normoxia exposure; open circles, MIH exposure. Data are represented as mean ± SEM. Statistical analysis was performed using two-tailed Student's paired t-test. The exact *p*-values are shown.



Supplementary Figure 6. The effects of mild intermittent hypoxia exposure on thirst, hunger and satiety feelings. At days 1, 3, and 7, visual analog scale questionnaires were completed by the study participants to assess feelings of thirst, hunger and satiety, and prospective food consumption scores. (**A-D**) Feelings of thirst, hunger and satiety determined during the high-fat mixed meal challenge (day 7: meal intake at t=0 min) . Feelings of thirst, hunger and satiety determined before and after breakfast (t=0 min), lunch (t=240 min) and dinner (t=480 min) on day 1 (**E-H**) and day 3 (**I-L**). Satiety scores (**A**, **E, I**), hunger scores (**B**, **F**, **J**), prospective food consumption scores (**C, G, K**), and thirst scores (**D, H, L**) were not significantly different between MIH and normoxia exposure. Open circles / dashed line, MIH exposure; Closed circles / solid line, normoxia exposure. Grey areas indicate time periods when study participants were inside the hypoxic room. Data are represented as mean ± SEM.

Supplementary Tables

Supplementary Table 1. List of significantly upregulated (positive values) and downregulated (negative values) pathways by mild intermittent hypoxia exposure in abdominal subcutaneous adipose tissue identified with gene set enrichment analyses (n=10).

Gene set	NES	FDR q-value
Mild intermittent hypoxia exposure – upregulated		
FCGR.ACTIVATION	2,52	0
IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL	2,49	0
FCERI.MEDIATED.NF.KB.ACTIVATION	2,48	0
ROLE.OF.PHOSPHOLIPIDS.IN.PHAGOCYTOSIS	2,42	0
SCAVENGING.OF.HEME.FROM.PLASMA	2,34	0
KEGG_NATURAL.KILLER.CELL.MEDIATED.CYTOTOXICITY	2,3	0
WP1829.IMMUNOREGULATORY.INTERACTIONS.BETWEEN.A.LYMPHOID.AND.A.NON.LYMPHOID.CELL	2,3	0
CREATION.OF.C4.AND.C2.ACTIVATORS	2,25	0
KEGG_B.CELL.RECEPTOR.SIGNALING.PATHWAY	2,24	0
ANTIGEN.ACTIVATES.B.CELL.RECEPTOR.BCR.LEADING.TO.GENERATION.OF.SECOND.MESSENGERS	2,23	0
CLASSICAL.ANTIBODY.MEDIATED.COMPLEMENT.ACTIVATION	2,22	0
WP23.B.CELL.RECEPTOR.SIGNALING.PATHWAY	2,17	0
WP1927.TCR.SIGNALING	2,16	0
FCERI.MEDIATED.CA.2.MOBILIZATION	2,16	0
INITIAL.TRIGGERING.OF.COMPLEMENT	2,15	0
TCR.SIGNALING	2,14	0
WP69.TCR.SIGNALING.PATHWAY	2,08	0
FCERI.MEDIATED.MAPK.ACTIVATION	2,07	0
KEGG_OSTEOCLAST.DIFFERENTIATION	2,05	0
BIOC_NKCELLSPATHWAY	1,99	0,01
FCGAMMA.RECEPTOR.FCGR.DEPENDENT.PHAGOCYTOSIS	1,98	0,01
WP2732.INTERLEUKIN.2.SIGNALING	1,98	0,01
WP2694.DAP12.INTERACTIONS	1,97	0,01
REGULATION.OF.ACTIN.DYNAMICS.FOR.PHAGOCYTIC.CUP.FORMATION	1,96	0,01
KEGG_LEISHMANIASIS	1,93	0,02

Gene set	NES	FDR q-value
WP2291.DEREGULATION.OF.RAB.AND.RAB.EFFECTOR.GENES.IN.BLADDER.CANCER	1,92	0,02
KEGG_T.CELL.RECEPTOR.SIGNALING.PATHWAY	1,91	0,02
KEGG_PRIMARY.IMMUNODEFICIENCY	1,91	0,02
WP254.APOPTOSIS	1,9	0,02
BIOC_IL7PATHWAY	1,88	0,02
ARACHIDONIC.ACID.METABOLISM	1,88	0,02
WP167.EICOSANOID.SYNTHESIS	1,86	0,03
COMPLEMENT.CASCADE	1,85	0,03
WP2650.ARACHIDONIC.ACID.METABOLISM	1,84	0,03
WP2700.LATENT.INFECTION.OF.HOMO.SAPIENS.WITH.MYCOBACTERIUM.TUBERCULOSIS	1,84	0,03
BIOC_AMIPATHWAY	1,83	0,03
WP1742.TP53.NETWORK	1,83	0,03
RECYCLING.OF.BILE.ACIDS.AND.SALTS	1,82	0,03
WP127.IL.5.SIGNALING.PATHWAY	1,82	0,03
FC.EPSILON.RECEPTOR.FCERI.SIGNALING	1,82	0,03
BIOC_CSKPATHWAY	1,82	0,03
WP1840.INTERLEUKIN.3.5.AND.GM.CSF.SIGNALING	1,81	0,03
WP2849.HEMATOPOIETIC.STEM.CELL.DIFFERENTIATION	1,81	0,03
DOWNSTREAM.TCR.SIGNALING	1,81	0,03
KEGG_GRAFT.VERSUS.HOST.DISEASE	1,8	0,03
WP22.IL.9.SIGNALING.PATHWAY	1,8	0,03
WP286.IL.3.SIGNALING.PATHWAY	1,78	0,04
BINDING.AND.UPTAKE.OF.LIGANDS.BY.SCAVENGER.RECEPTORS	1,77	0,04
WP1826.GPVI.MEDIATED.ACTIVATION.CASCADE	1,77	0,04
GENERATION.OF.SECOND.MESSENGER.MOLECULES	1,77	0,04
SIGNALING.BY.INTERLEUKINS	1,76	0,05
KEGG_TUBERCULOSIS	1,75	0,05
WP2507.NANOMATERIAL.INDUCED.APOPTOSIS	1,75	0,05
PHOSPHORYLATION.OF.CD3.AND.TCR.ZETA.CHAINS	1,75	0,05
WP2775.TOLL.LIKE.RECEPTORS.CASCADES	1,73	0,05
INTERLEUKIN.3.5.AND.GM.CSF.SIGNALING	1,73	0,06
KEGG_STAPHYLOCOCCUS.AUREUS.INFECTION	1,73	0,06

Gene set	NES	FDR q-value
GPVI.MEDIATED.ACTIVATION.CASCADE	1,71	0,06
INTERLEUKIN.2.SIGNALING	1,71	0,07
WP205.IL.7.SIGNALING.PATHWAY	1,7	0,07
WP1772.APOPTOSIS.MODULATION.AND.SIGNALING	1,7	0,07
WP2759.FC.EPSILON.RECEPTOR.FCERI.SIGNALING.	1,69	0,07
BIOC_TOLLPATHWAY	1,66	0,09
WP2739.AMYLOIDS	1,65	0,09
KEGG_ARACHIDONIC.ACID.METABOLISM	1,65	0,09
KEGG_HEMATOPOIETIC.CELL.LINEAGE	1,65	0,1
LATENT.INFECTION.OF.HOMO.SAPIENS.WITH.MYCOBACTERIUM. TUBERCULOSIS	1,64	0,1
CHEMOKINE.RECEPTORS.BIND.CHEMOKINES	1,64	0,1
WP2112.IL17.SIGNALING.PATHWAY	1,64	0,1
KEGG_CHEMOKINE.SIGNALING.PATHWAY	1,64	0,1
TOLL.LIKE.RECEPTORS.CASCADES	1,64	0,1
WP585.INTERFERON.TYPE.I.SIGNALING.PATHWAYS	1,64	0,1
SYNTHESIS.OF.LEUKOTRIENES.LT.AND.EOXINS.EX.	1,63	0,1
ROLE.OF.LAT2.NTAL.LAB.ON.CALCIUM.MOBILIZATION	1,63	0,1
PHAGOSOMAL.MATURATION.EARLY.ENDOSOMAL.STAGE.	1,63	0,1
BIOC_KERATINOCYTEPATHWAY	1,63	0,1
WP619.TYPE.II.INTERFERON.SIGNALING.IFNG.	1,62	0,1
BIOC_CERAMIDEPATHWAY	1,61	0,11
TRANSLOCATION.OF.ZAP.70.TO.IMMUNOLOGICAL.SYNAPSE	1,61	0,11
WP1815.FACTORS.INVOLVED.IN.MEGAKARYOCYTE.DEVELOPMENT .AND.PLATELET.PRODUCTION	1,61	0,11
WP2708.DEGRADATION.OF.COLLAGEN	1,6	0,12
PHOSPHOLIPID.METABOLISM	1,59	0,13
PD.1.SIGNALING	1,59	0,13
TOLL.LIKE.RECEPTOR.4.TLR4.CASCADE	1,58	0,12
RNA.POLYMERASE.I.PROMOTER.OPENING	1,58	0,13
WP1433.NOD.PATHWAY	1,58	0,13
KEGG_LYSOSOME	1,58	0,13
KEGG_TYPE.I.DIABETES.MELLITUS	1,57	0,13
WP49.IL.2.SIGNALING.PATHWAY	1,56	0,14

Gene set	NES	FDR q-value
KEGG_FC.GAMMA.R.MEDIATED.PHAGOCYTOSIS	1,56	0,14
KEGG_ALLOGRAFT.REJECTION	1,56	0,14
KEGG_CARBOHYDRATE.DIGESTION.AND.ABSORPTION	1,55	0,14
BIOC_GLEEVECPATHWAY	1,55	0,14
KEGG_GLYCEROPHOSPHOLIPID.METABOLISM	1,54	0,16
TOLL.LIKE.RECEPTOR.TLR6.TLR2.CASCADE	1,52	0,17
REV.ERBA.REPRESSES.GENE.EXPRESSION	1,52	0,17
BIOC_BCRPATHWAY	1,52	0,17
WP2761.MYD88.MAL.CASCADE.INITIATED.ON.PLASMA.MEMBRANE	1,52	0,17
TOLL.LIKE.RECEPTOR.TLR1.TLR2.CASCADE	1,52	0,17
SYNTHESIS.OF.IP3.AND.IP4.IN.THE.CYTOSOL	1,52	0,17
MYD88.MAL.CASCADE.INITIATED.ON.PLASMA.MEMBRANE	1,51	0,17
WP24.PEPTIDE.GPCRS	1,51	0,17
KEGG_AUTOIMMUNE.THYROID.DISEASE	1,51	0,17
WP2447.AMYOTROPHIC.LATERAL.SCLEROSIS.ALS.	1,51	0,17
TOLL.LIKE.RECEPTOR.2.TLR2.CASCADE	1,5	0,18
INTERLEUKIN.1.SIGNALING	1,5	0,18
WP1449.REGULATION.OF.TOLL.LIKE.RECEPTOR.SIGNALING.PATHWAY	1,5	0,18
RORA.ACTIVATES.CIRCADIAN.GENE.EXPRESSION	1,5	0,18
KEGG_AFRICAN.TRYPANOSOMIASIS	1,49	0,19
WP455.GPCRS.CLASS.A.RHODOPSIN.LIKE	1,49	0,19
BIOC_TCRPATHWAY	1,49	0,19
WP1904.RIG.I.MDA5.MEDIATED.INDUCTION.OF.IFN.ALPHA.BETA.PATHWAYS	1,49	0,19
BIOC_STRESSPATHWAY	1,49	0,19
GLYCEROPHOSPHOLIPID.BIOSYNTHESIS	1,48	0,19
KEGG_FC.EPSILON.RI.SIGNALING.PATHWAY	1,48	0,19
DEFENSINS	1,48	0,19
WP384.APOPTOSIS.MODULATION.BY.HSP70	1,48	0,19
WP231.TNF.ALPHA.SIGNALING.PATHWAY	1,47	0,19
KEGG_INFLAMMATORY.BOWEL.DISEASE.IBD.	1,47	0,19
WP1799.COSTIMULATION.BY.THE.CD28.FAMILY	1,47	0,19
WP2203.TSLP.SIGNALING.PATHWAY	1,47	0,2
WP395.IL.4.SIGNALING.PATHWAY	1,46	0,2

Gene set	NES	FDR q-value
WP1794.CELL.SURFACE.INTERACTIONS.AT.THE.VASCULAR.WALL	1,46	0,2
KEGG_LEGIONELLOSIS	1,46	0,2
WP304.KIT.RECEPTOR.SIGNALING.PATHWAY	1,46	0,2
Mild intermittent hypoxia exposure – downregulated		
WP2652.MITOTIC.PROMETAPHASE	-2,46	0
MITOTIC.PROMETAPHASE	-2,4	0
WP466.DNA.REPLICATION	-2,39	0
DNA.STRAND.ELONGATION	-2,36	0
RESOLUTION.OF.SISTER.CHROMATID.COHESION	-2,34	0
KEGG_DNA.REPLICATION	-2,33	0
ACTIVATION.OF.ATR.IN.RESPONSE.TO.REPLICATION.STRESS	-2,31	0
KEGG_RIBOSOME.BIOGENESIS.IN.EUKARYOTES	-2,28	0
WP2446.RB.IN.CANCER	-2,26	0
WP2798.ASSEMBLY.OF.COLLAGEN.FIBRILS.AND.OTHER.MULTIMERIC. STRUCTURES	-2,26	0
G2.M.CHECKPOINTS	-2,24	0
WP2757.MITOTIC.METAPHASE.AND.ANAPHASE	-2,22	0
ACTIVATION.OF.THE.PRE.REPLICATIVE.COMPLEX	-2,21	0
MITOCHONDRIAL.TRANSLATION	-2,21	0
LAMININ.INTERACTIONS	-2,2	0
ASSEMBLY.OF.COLLAGEN.FIBRILS.AND.OTHER.MULTIMERIC. STRUCTURES	-2,2	0
E2F.MEDIATED.REGULATION.OF.DNA.REPLICATION	-2,19	0
CELL.CYCLE.MITOTIC	-2,19	0
MITOTIC.M.M.G1.PHASES	-2,18	0
MITOCHONDRIAL.TRANSLATION.ELONGATION	-2,17	0
MITOCHONDRIAL.TRANSLATION.TERMINATION	-2,17	0
DNA.REPAIR	-2,16	0
MITOCHONDRIAL.TRANSLATION.INITIATION	-2,14	0
SEPARATION.OF.SISTER.CHROMATIDS	-2,14	0
MITOTIC.METAPHASE.AND.ANAPHASE	-2,13	0
MITOTIC.ANAPHASE	-2,11	0
KEGG_MISMATCH.REPAIR	-2,1	0

Gene set	NES	FDR q-value
KEGG_CELL.CYCLE	-2,09	0
KEGG_AMINOACYL.TRNA.BIOSYNTHESIS	-2,09	0
RNA.POLYMERASE.I.PROMOTER.ESCAPE	-2,08	0
WP179.CELL.CYCLE	-2,07	0
BIOC_VEGFPATHWAY	-2,07	0
KEGG_AMINO.SUGAR.AND.NUCLEOTIDE.SUGAR.METABOLISM	-2,06	0
KEGG_ECM.RECEPTOR.INTERACTION	-2,04	0
WP411.MRNA.PROCESSING	-2,03	0
WP1938.TRNA.AMINOACYLATION	-2,03	0
TRANSPORT.OF.MATURE.TRANSCRIPT.TO.CYTOPLASM	-2,02	0
RNA.POLYMERASE.II.TRANSCRIPTION	-2,02	0
LAGGING.STRAND.SYNTHESIS	-2,01	0
WP2006.MIR.TARGETED.GENES.IN.SQUAMOUS.CELL.TARBASE	-2,01	0
TRANSPORT.OF.MATURE.MRNA.DERIVED.FROM.AN.INTRON. CONTAINING.TRANSCRIPT	-2,01	0
PHOSPHORYLATION.OF.THE.APC.C	-2,01	0
WP2715.METABOLISM.OF.NON.CODING.RNA	-2	0
TRANSCRIPTION.OF.THE.HIV.GENOME	-2	0
EXTENSION.OF.TELOMERES	-1,99	0
WP1785.ASPARAGINE.N.LINKED.GLYCOSYLATION	-1,99	0
INACTIVATION.OF.APC.C.VIA.DIRECT.INHIBITION.OF.THE.APC.C. COMPLEX	-1,99	0
TRNA.AMINOACYLATION	-1,99	0
WP1889.PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	-1,98	0
RNA.POLYMERASE.II.PRE.TRANSCRIPTION.EVENTS	-1,98	0
MITOTIC.SPINDLE.CHECKPOINT	-1,98	0
XBP1.S.ACTIVATES.CHAPERONE.GENES	-1,97	0
WP1807.DOUBLE.STRAND.BREAK.REPAIR	-1,97	0
FORMATION.OF.TUBULIN.FOLDING.INTERMEDIATES.BY.CCT.TRIC	-1,97	0
IRE1ALPHA.ACTIVATES.CHAPERONES	-1,96	0
METABOLISM.OF.NON.CODING.RNA	-1,96	0
M.PHASE	-1,96	0
CELL.CYCLE.CHECKPOINTS	-1,96	0,01

Gene set	NES	FDR q-value
INHIBITION.OF.THE.PROTEOLYTIC.ACTIVITY.OF.APC.C.REQUIRED. FOR.THE.ONSET.OF.ANAPHASE.BY.MITOTIC.SPINDLE.CHECKPOINT. COMPONENTS	-1,96	0
G1.S.TRANSITION	-1,95	0,01
RNA.POLYMERASE.II.TRANSCRIPTION.ELONGATION	-1,95	0,01
PROCESSING.OF.CAPPED.INTRON.CONTAINING.PRE.MRNA	-1,95	0,01
RNA.POLYMERASE.I.TRANSCRIPTION.INITIATION	-1,94	0,01
WP1980.NUCLEOTIDE.EXCISION.REPAIR	-1,94	0,01
DNA.REPLICATION	-1,94	0,01
S.PHASE	-1,94	0,01
SNRNP.ASSEMBLY	-1,93	0,01
SMOOTH.MUSCLE.CONTRACTION	-1,93	0,01
UNFOLDED.PROTEIN.RESPONSE.UPR.	-1,93	0,01
RNA.POLYMERASE.I.TRANSCRIPTION.TERMINATION	-1,93	0,01
COLLAGEN.FORMATION	-1,93	0,01
WP2667.ACTIVATION.OF.CHAPERONE.GENES.BY.XBP1.S.	-1,92	0,01
WP1859.MITOTIC.G2.G2.M.PHASES	-1,92	0,01
PREFOLDIN.MEDIATED.TRANSFER.OF.SUBSTRATE.TO.CCT.TRIC	-1,92	0,01
WP2772.S.PHASE	-1,91	0,01
MITOTIC.G2.G2.M.PHASES	-1,91	0,01
RNA.POLYMERASE.II.TRANSCRIPTION.INITIATION.AND.PROMOTER. CLEARANCE	-1,91	0,01
SIGNALING.BY.BMP	-1,91	0,01
LATE.PHASE.OF.HIV.LIFE.CYCLE	-1,91	0,01
RNA.POLYMERASE.II.PROMOTER.ESCAPE	-1,91	0,01
RNA.POLYMERASE.II.TRANSCRIPTION.PRE.INITIATION.AND.PROMOTER. OPENING	-1,91	0,01
FORMATION.OF.RNA.POL.II.ELONGATION.COMPLEX	-1,91	0,01
TAT.MEDIATED.ELONGATION.OF.THE.HIV.1.TRANSCRIPT	-1,91	0,01
G2.M.TRANSITION	-1,91	0,01
CYTOSOLIC.TRNA.AMINOACYLATION	-1,9	0,01
POST.CHAPERONIN.TUBULIN.FOLDING.PATHWAY	-1,9	0,01
MRNA.CAPPING	-1,9	0,01
G1.S.SPECIFIC.TRANSCRIPTION	-1,9	0,01

Gene set	NES	FDR q-value
RNA.POLYMERASE.II.HIV.PROMOTER.ESCAPE	-1,9	0,01
CYCLIN.A.B1.ASSOCIATED.EVENTS.DURING.G2.M.TRANSITION	-1,9	0,01
WP1861.MRNA.CAPPING	-1,9	0,01
HIV.TRANSCRIPTION.INITIATION	-1,9	0,01
RESPIRATORY.ELECTRON.TRANSPORT	-1,9	0,01
FORMATION.OF.HIV.ELONGATION.COMPLEX.IN.THE. ABSENCE.OF.HIV.TAT	-1,89	0,01
TRANSCRIPTION.COUPLED.NER.TC.NER.	-1,89	0,01
FORMATION.OF.HIV.1.ELONGATION.COMPLEX.CONTAINING. HIV.1.TAT	-1,89	0,01
O.GLYCOSYLATION.OF.TSR.DOMAIN.CONTAINING.PROTEINS	-1,88	0,01
WP1775.CELL.CYCLE.CHECKPOINTS	-1,88	0,01
RNA.POLYMERASE.II.TRANSCRIPTION.INITIATION	-1,88	0,01
KEGG_PROTEIN.EXPORT	-1,88	0,01
WP405.EUKARYOTIC.TRANSCRIPTION.INITIATION	-1,87	0,01
MRNA.3.END.PROCESSING	-1,87	0,01
INTERACTIONS.OF.REV.WITH.HOST.CELLULAR.PROTEINS	-1,87	0,01
NUCLEOTIDE.EXCISION.REPAIR	-1,86	0,01
MRNA.SPLICING.MAJOR.PATHWAY	-1,86	0,01
POST.ELONGATION.PROCESSING.OF.INTRON. CONTAINING.PRE.MRNA	-1,86	0,01
KEGG_NUCLEOTIDE.EXCISION.REPAIR	-1,86	0,01
TRANSPORT.OF.THE.SLBP.INDEPENDENT.MATURE.MRNA	-1,85	0,01
WP1906.RNA.POLYMERASE.II.TRANSCRIPTION	-1,85	0,01
HIV.TRANSCRIPTION.ELONGATION	-1,85	0,01
REV.MEDIATED.NUCLEAR.EXPORT.OF.HIV.RNA	-1,85	0,01
COOPERATION.OF.PREFOLDIN.AND.TRIC.CCT.IN.ACTIN. AND.TUBULIN.FOLDING	-1,85	0,01
SYNTHESIS.OF.DNA	-1,85	0,01
MRNA.SPLICING	-1,85	0,01
DOUBLE.STRAND.BREAK.REPAIR	-1,85	0,01
TELOMERE.C.STRAND.LAGGING.STRAND.SYNTHESIS	-1,85	0,01
WP2005.MIR.TARGETED.GENES.IN.MUSCLE.CELL.TARBASE	-1,85	0,01
WP1925.SYNTHESIS.OF.DNA	-1,84	0,01

Gene set	NES	FDR q-value
HIV.LIFE.CYCLE	-1,84	0,01
COLLAGEN.BIOSYNTHESIS.AND.MODIFYING.ENZYMES	-1,84	0,01
WP2654.MITOTIC.PROPHASE	-1,83	0,01
TRANSCRIPTION	-1,83	0,01
FANCONI.ANEMIA.PATHWAY	-1,83	0,01
MITOCHONDRIAL.TRNA.AMINOACYLATION	-1,83	0,01
ELASTIC.FIBRE.FORMATION	-1,81	0,01
TRANSPORT.OF.THE.SLBP.DEPENDANT.MATURE.MRNA	-1,81	0,01
PROTEIN.FOLDING	-1,8	0,01
EXTRACELLULAR.MATRIX.ORGANIZATION	-1,79	0,02
BIOC_PROTEASOMEPATHWAY	-1,79	0,02
NUCLEAR.IMPORT.OF.REV.PROTEIN	-1,79	0,02
ORGANELLE.BIOGENESIS.AND.MAINTENANCE	-1,79	0,02
KEGG_SPLICEOSOME	-1,78	0,02
INTERACTIONS.OF.VPR.WITH.HOST.CELLULAR.PROTEINS	-1,78	0,02
KEGG_FANCONI.ANEMIA.PATHWAY	-1,78	0,02
CHROMOSOME.MAINTENANCE	-1,77	0,02
GLOBAL.GENOMIC.NER.GG.NER.	-1,77	0,02
TRANSPORT.OF.MATURE.MRNAS.DERIVED.FROM. INTRONLESS.TRANSCRIPTS	-1,77	0,02
MITOTIC.G1.G1.S.PHASES	-1,76	0,02
WP2004.MIR.TARGETED.GENES.IN.LYMPHOCYTES.TARBASE	-1,76	0,02
KEGG_RNA.TRANSPORT	-1,75	0,02
CENTROSOME.MATURATION	-1,75	0,02
RECRUITMENT.OF.MITOTIC.CENTROSOME.PROTEINS. AND.COMPLEXES	-1,75	0,02
EPIGENETIC.REGULATION.OF.GENE.EXPRESSION	-1,75	0,02
WP2760.SIGNALING.BY.BMP	-1,74	0,02
KEGG_BASAL.TRANSCRIPTION.FACTORS	-1,74	0,02
KEGG_PURINE.METABOLISM	-1,74	0,02
GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION. IN.GG.NER	-1,74	0,02
WP1892.PROTEIN.FOLDING	-1,74	0,02
NUCLEAR.PORE.COMPLEX.NPC.DISASSEMBLY	-1,74	0,02

Gene set	NES	FDR q-value
HOMOLOGOUS.RECOMBINATION.REPAIR	-1,74	0,02
REPAIR.SYNTHESIS.OF.PATCH.27.30.BASES.LONG.BY. DNA.POLYMERASE	-1,73	0,02
HOMOLOGOUS.RECOMBINATION.REPAIR.OF.REPLICATION .INDEPENDENT.DOUBLE.STRAND.BREAKS	-1,73	0,03
NON.INTEGRIN.MEMBRANE.ECM.INTERACTIONS	-1,72	0,03
SCAVENGING.BY.CLASS.A.RECEPTORS	-1,72	0,03
RNA.POL.II.CTD.PHOSPHORYLATION.AND.INTERACTION. WITH.CE	-1,72	0,03
MRNA.SPLICING.MINOR.PATHWAY	-1,72	0,03
WP1858.MITOTIC.G1.G1.S.PHASES	-1,71	0,03
M.G1.TRANSITION	-1,71	0,03
G0.AND.EARLY.G1	-1,71	0,03
KEGG_OOCYTE.MEIOSIS	-1,71	0,03
WP1928.TELOMERE.MAINTENANCE	-1,71	0,03
KEGG_SELENOCOMPOUND.METABOLISM	-1,71	0,03
REPAIR.SYNTHESIS.FOR.GAP.FILLING.BY.DNA. POLYMERASE.IN.TC.NER	-1,71	0,03
WP2658.HIV.LIFE.CYCLE	-1,71	0,03
CONVERSION.FROM.APC.C.CDC20.TO.APC.C.CDH1.IN. LATE.ANAPHASE	-1,71	0,03
WP2002.MIR.TARGETED.GENES.IN.EPITHELIUM.TARBASE	-1,71	0,03
WP2785.M.G1.TRANSITION	-1,7	0,03
DNA.REPLICATION.PRE.INITIATION	-1,7	0,03
WP2672.ISG15.ANTIVIRAL.MECHANISM	-1,7	0,03
FORMATION.OF.TRANSCRIPTION.COUPLED.NER.TC.NER.REPAIR.COMPLEX	-1,7	0,03
TRANSPORT.OF.MATURE.MRNA.DERIVED.FROM.AN.INTRONLESS. TRANSCRIPT	-1,7	0,03
WP2679.MHC.CLASS.II.ANTIGEN.PRESENTATION	-1,7	0,03
RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY.CHEMIOSMOTIC. COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING.PROTEINS.	-1,7	0,03
KEGG_ONE.CARBON.POOL.BY.FOLATE	-1,7	0,03
APC.CDC20.MEDIATED.DEGRADATION.OF.NEK2A	-1,69	0,03
WP1816.FANCONI.ANEMIA.PATHWAY	-1,69	0,03
WP111.ELECTRON.TRANSPORT.CHAIN	-1,69	0,03

Gene set	NES	FDR q-value
FORMATION.OF.THE.EARLY.ELONGATION.COMPLEX	-1,69	0,03
CGMP.EFFECTS	-1,69	0,03
PURINE.METABOLISM	-1,68	0,03
GAP.FILLING.DNA.REPAIR.SYNTHESIS.AND.LIGATION.IN.TC.NER	-1,68	0,03
WP1902.RESPIRATORY.ELECTRON.TRANSPORT.ATP.SYNTHESIS.BY. CHEMIOSMOTIC.COUPLING.AND.HEAT.PRODUCTION.BY.UNCOUPLING. PROTEINS.	-1,68	0,03
VPR.MEDIATED.NUCLEAR.IMPORT.OF.PICS	-1,68	0,03
MOLECULES.ASSOCIATED.WITH.ELASTIC.FIBRES	-1,68	0,03
DUAL.INCISION.REACTION.IN.TC.NER	-1,68	0,03
BIOC_NO1PATHWAY	-1,67	0,03
KEGG_FOCAL.ADHESION	-1,67	0,03
CLEAVAGE.OF.GROWING.TRANSCRIPT.IN.THE.TERMINATION.REGION	-1,66	0,04
REGULATION.OF.GLUCOKINASE.BY.GLUCOKINASE.REGULATORY. PROTEIN	-1,66	0,04
RNA.POLYMERASE.II.TRANSCRIPTION.TERMINATION	-1,66	0,04
REGULATION.OF.PLK1.ACTIVITY.AT.G2.M.TRANSITION	-1,66	0,04
REGULATION.OF.MITOTIC.CELL.CYCLE	-1,65	0,04
WP2725.COLLAGEN.BIOSYNTHESIS.AND.MODIFYING.ENZYMES	-1,65	0,04
POST.TRANSLATIONAL.PROTEIN.MODIFICATION	-1,65	0,04
APC.C.CDC20.MEDIATED.DEGRADATION.OF.CYCLIN.B	-1,64	0,04
LOSS.OF.PROTEINS.REQUIRED.FOR.INTERPHASE.MICROTUBULE. ORGANIZATION.FROM.THE.CENTROSOME	-1,64	0,04
LOSS.OF.NLP.FROM.MITOTIC.CENTROSOMES	-1,64	0,04
POST.ELONGATION.PROCESSING.OF.THE.TRANSCRIPT	-1,64	0,04
PROCESSIVE.SYNTHESIS.ON.THE.LAGGING.STRAND	-1,64	0,04
APC.C.MEDIATED.DEGRADATION.OF.CELL.CYCLE. PROTEINS	-1,63	0,04
CHAPERONIN.MEDIATED.PROTEIN.FOLDING	-1,63	0,04
FORMATION.OF.THE.HIV.1.EARLY.ELONGATION.COMPLEX	-1,63	0,05
MISMATCH.REPAIR.MMR.DIRECTED.BY.MSH2.MSH6. MUTSALPHA.	-1,62	0,05
MISMATCH.REPAIR	-1,62	0,05
WP1782.APC.C.MEDIATED.DEGRADATION.OF.CELL.CYCLE. PROTEINS	-1,61	0,05

Gene set	NES	FDR q-value
WP45.G1.TO.S.CELL.CYCLE.CONTROL	-1,61	0,05
WP1873.NGF.SIGNALLING.VIA.TRKA.FROM.THE.PLASMA. MEMBRANE	-1,61	0,05
EPH.EPHRIN.SIGNALING	-1,61	0,05
KEGG_PROTEIN.PROCESSING.IN.ENDOPLASMIC.RETICULUM	-1,59	0,06
KEGG_PYRIMIDINE.METABOLISM	-1,59	0,06
WP2703.EXTRACELLULAR.MATRIX.ORGANIZATION	-1,59	0,06
GENERIC.TRANSCRIPTION.PATHWAY	-1,59	0,06
REGULATION.OF.APC.C.ACTIVATORS.BETWEEN.G1.S.AND.EARLY.ANAPHASE	-1,59	0,06
KEGG_PI3K.AKT.SIGNALING.PATHWAY	-1,59	0,06
WP244.ALPHA.6.BETA.4.SIGNALING.PATHWAY	-1,58	0,06
EPHB.MEDIATED.FORWARD.SIGNALING	-1,58	0,06
ASPARAGINE.N.LINKED.GLYCOSYLATION	-1,58	0,06
BIOC_ERYTHPATHWAY	-1,57	0,07
BIOC_EDG1PATHWAY	-1,57	0,07
TIE2.SIGNALING	-1,57	0,07
WP1539.ANGIOGENESIS	-1,56	0,07
MISMATCH.REPAIR.MMR.DIRECTED.BY.MSH2.MSH3.MUTSBETA.	-1,56	0,07
BIOC_ARFPATHWAY	-1,56	0,07
NEP.NS2.INTERACTS.WITH.THE.CELLULAR.EXPORT. MACHINERY	-1,56	0,07
NEGATIVE.EPIGENETIC.REGULATION.OF.RRNA. EXPRESSION	-1,56	0,07
BIOC_MTORPATHWAY	-1,56	0,07
WP241.ONE.CARBON.METABOLISM	-1,55	0,07
NUCLEAR.ENVELOPE.BREAKDOWN	-1,55	0,08
ECM.PROTEOGLYCANS	-1,54	0,08
NITRIC.OXIDE.STIMULATES.GUANYLATE.CYCLASE	-1,53	0,09
RNA.POLYMERASE.I.RNA.POLYMERASE.III.AND. MITOCHONDRIAL.TRANSCRIPTION	-1,53	0,09
ACTIVATION.OF.APC.C.AND.APC.C.CDC20.MEDIATED. DEGRADATION.OF.MITOTIC.PROTEINS	-1,52	0,09
KEGG_GAP.JUNCTION	-1,52	0,09
GLUTAMATE.NEUROTRANSMITTER.RELEASE.CYCLE	-1,51	0,1
NORC.NEGATIVELY.REGULATES.RRNA.EXPRESSION	-1,51	0,1

Gene set	NES	FDR q-value
WP2784.BINDING.AND.UPTAKE.OF.LIGANDS.BY.SCAVENGER.RECEPTORS	-1,51	0,1
WP1905.RNA.POLYMERASE.I.RNA.POLYMERASE.III.AND.MITOCHONDRIAL.TRANSCRIPTION	-1,51	0,1
SIGNALING.BY.FGFR2.MUTANTS	-1,51	0,1
METABOLISM.OF.NUCLEOTIDES	-1,5	0,11
ASSOCIATION.OF.TRIC.CCT.WITH.TARGET.PROTEINS.DURING.BIOSYNTHESIS	-1,49	0,11
BIOC_INFLAMPATHWAY	-1,49	0,11
EXPORT.OF.VIRAL.RIBONUCLEOPROTEINS.FROM.NUCLEUS	-1,48	0,12
EPHA.MEDIATED.GROWTH.CONE.COLLAPSE	-1,48	0,12
REMOVAL.OF.LICENSING.FACTORS.FROM.ORIGINS	-1,48	0,12
ISG15.ANTIVIRAL.MECHANISM	-1,48	0,12
HIV.INFECTION	-1,48	0,12
APC.C.CDC20.MEDIATED.DEGRADATION.OF.MITOTIC.PROTEINS	-1,47	0,12
WP2363.GASTRIC.CANCER.NETWORK.2	-1,47	0,13
KEGG_DILATED.CARDIOMYOPATHY	-1,47	0,13
REGULATION.OF.DNA.REPLICATION	-1,46	0,13
TELOMERE.MAINTENANCE	-1,46	0,13
WP1885.PLATELET.HOMEOSTASIS	-1,46	0,13
MITOTIC.PROPHASE	-1,46	0,13
ANTIVIRAL.MECHANISM.BY.IFN.STIMULATED.GENES	-1,46	0,13
WP107.TRANSLATION.FACTORS	-1,45	0,14
KEGG_PROGESTERONE.MEDIATED.OOCYTE.MATURATION	-1,45	0,14
AXON.GUIDANCE	-1,44	0,14
WP1898.REGULATION.OF.DNA.REPLICATION	-1,44	0,15
TRANSLATION	-1,44	0,15
WP2683.INFLUENZA.LIFE.CYCLE	-1,43	0,15
SIGNALING.BY.HEDGEHOG	-1,43	0,15
HEDGEHOG.OFF.STATE	-1,42	0,16
KEGG_VASCULAR.SMOOTH.MUSCLE.CONTRACTION	-1,42	0,16
O.LINKED.GLYCOSYLATION	-1,42	0,17
RNA.POLYMERASE.I.TRANSCRIPTION	-1,41	0,17
AUTODEGRADATION.OF.CDH1.BY.CDH1.APC.C	-1,41	0,17

Gene set	NES	FDR q-value
KEGG_PROTEIN.DIGESTION.AND.ABSORPTION	-1,4	0,18
CDC20.PHOSPHO.APC.C.MEDIATED.DEGRADATION.OF.CYCLIN.A	-1,4	0,18
MEIOSIS	-1,4	0,18
APC.C.CDH1.MEDIATED.DEGRADATION.OF.CDC20.AND.OTHER. APC.C.CDH1.TARGETED.PROTEINS.IN.LATE.MITOSIS.EARLY.G1	-1,39	0,18
SIGNALING.BY.VEGF	-1,38	0,19
WP306.FOCAL.ADHESION	-1,38	0,19

Supplementary Table 2. Top 100 genes significantly upregulated (positive values) and downregulated (negative values) by mild intermittent hypoxia compared to normoxia exposure in human skeletal muscle (*m. vastus lateralis*) (*n*=11).

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
Mild intermittent hypoxia exposure – upregulation				
CORT	cortistatin	1325	2,37	5,10E-05
APOC1	apolipoprotein C-I	341	1,9	3,10E-04
LINC01194	long intergenic non-protein coding RNA 1194	404663	1,9	7,30E-03
SPIN2B	spindlin family, member 2B	474343	1,81	3,70E-03
LOC101928236	uncharacterized LOC101928236	101928236	1,74	5,10E-03
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	2919	1,73	7,90E-03
SNHG4	small nucleolar RNA host gene 4 (non-protein coding)	724102	1,71	5,10E-03
C2orf27A	chromosome 2 open reading frame 27A	29798	1,69	1,30E-02
LCN2	lipocalin 2	3934	1,69	3,30E-04
OR2AT4	olfactory receptor, family 2, subfamily AT, member 4	341152	1,68	1,30E-02
TCTEX1D2	Tctex1 domain containing 2	255758	1,67	6,30E-03
S100A7A	S100 calcium binding protein A7A	338324	1,64	4,10E-03
GSTM5	glutathione S-transferase mu 5	2949	1,6	6,40E-03
ARHGEF33	Rho guanine nucleotide exchange factor (GEF) 33	100271715	1,59	1,40E-04

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
GOLGA8DP	golgin A8 family, member D, pseudogene	100132979	1,58	1,80E-03
SCGB3A1	secretoglobin, family 3A, member 1	92304	1,57	2,30E-03
ZNF285	zinc finger protein 285	26974	1,54	3,70E-03
LOC283683	uncharacterized LOC283683	283683	1,54	1,10E-03
LOC101927973	uncharacterized LOC101927973	101927973	1,54	1,00E-02
LINC01534	long intergenic non-protein coding RNA 1534	101927621	1,51	7,60E-03
EXTL3-AS1	EXTL3 antisense RNA 1	101929402	1,51	1,40E-02
LINC00263	long intergenic non-protein coding RNA 263	90271	1,51	3,70E-02
ACOT1	acyl-CoA thioesterase 1	641371	1,5	2,30E-02
SH3GL1	SH3-domain GRB2-like 1	6455	1,49	3,70E-03
LOC100131289	uncharacterized LOC100131289	100131289	1,48	2,40E-02
WASH1	WAS protein family homolog 1	100287171	1,48	1,00E-02
MIR3192	microRNA 3192	100422875	1,47	2,70E-02
FAM179A	family with sequence similarity 179, member A	165186	1,47	3,20E-03
LOC100507387	uncharacterized LOC100507387	100507387	1,47	8,90E-03
SLC5A6	solute carrier family 5 (sodium/ multivitamin and iodide cotransporter), member 6	8884	1,47	1,60E-02
LOC101927696	uncharacterized LOC101927696	101927696	1,47	2,00E-02
LOC146880	Rho GTPase activating protein 27 pseudogene	146880	1,45	7,30E-03
CHGB	chromogranin B (secretogranin 1)	1114	1,45	4,40E-03
ZNF613	zinc finger protein 613	79898	1,45	1,40E-02
CAPG	capping protein (actin filament), gelsolin-like	822	1,45	1,30E-02
MYZAP	myocardial zonula adherens protein	100820829	1,44	2,50E-02
IPO5P1	importin 5 pseudogene 1	100132815	1,44	1,90E-02
TRAPPC2	trafficking protein particle complex 2	6399	1,44	4,30E-03
OSR1	odd-skipped related transciption factor 1	130497	1,44	6,50E-03

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
LY6G5C	lymphocyte antigen 6 complex, locus G5C	80741	1,44	3,40E-02
TRMT112	tRNA methyltransferase 11-2 homolog (S. cerevisiae)	51504	1,44	1,40E-02
TRAJ35	T cell receptor alpha joining 35 (non-functional)	28720	1,44	2,90E-02
CASC22	cancer susceptibility candidate 22 (non-protein coding)	283854	1,44	4,40E-02
QPCT	glutaminyl-peptide cyclotransferase	25797	1,43	1,20E-02
TRIM16L	tripartite motif containing 16-like	147166	1,43	1,50E-02
LOC100506368	uncharacterized LOC100506368	100506368	1,43	1,90E-02
LOC101929592	uncharacterized LOC101929592	101929592	1,43	6,30E-03
OR2L8	olfactory receptor, family 2, subfamily L, member 8	391190	1,43	7,20E-03
CAMKMT	calmodulin-lysine N-methyltransferase	79823	1,42	1,30E-02
ARPIN	actin-related protein 2/3 complex inhibitor	348110	1,42	3,20E-02
OR2A5	olfactory receptor, family 2, subfamily A, member 5	393046	1,42	1,20E-02
FCGBP	Fc fragment of IgG binding protein	8857	1,42	3,90E-03
UBXN8	UBX domain protein 8	7993	1,42	1,70E-02
MIR3622B	microRNA 3622b	100500871	1,41	1,60E-02
FGF13-AS1	FGF13 antisense RNA 1	100129662	1,41	3,20E-02
SCARNA15	small Cajal body-specific RNA 15	677778	1,41	1,30E-02
BMP1	bone morphogenetic protein 1	649	1.4	3,80E-02
SLC13A3	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 3	64849	1.4	7,80E-03
IGHG3	immunoglobulin heavy constant gamma 3 (G3m marker)	3502	1.4	1,50E-02
C8orf76	chromosome 8 open reading frame 76	84933	1.4	5,70E-03
SNORD114-6	small nucleolar RNA, C/D box 114-6	767582	1.4	1,80E-02
NBPF15	neuroblastoma breakpoint family, member 15	284565	1,39	2,20E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
RNF216P1	ring finger protein 216 pseudogene 1	441191	1,39	1,80E-02
FAM107B	family with sequence similarity 107, member B	83641	1,39	6,30E-03
FAM90A10P	putative protein FAM90A10	441328	1,39	3,10E-02
NINJ1	ninjurin 1	4814	1,39	1,40E-02
STYXL1	serine/threonine/tyrosine interacting-like 1	51657	1,39	3,10E-03
BEGAIN	brain-enriched guanylate kinase-associated	57596	1,39	3,40E-02
TRAJ46	T cell receptor alpha joining 46	28709	1,39	1,70E-02
LOC100288637	OTU deubiquitinase 7A pseudogene	100288637	1,38	6,90E-03
ANLN	anillin, actin binding protein	54443	1,38	7,60E-03
GSTT2	glutathione S-transferase theta 2	2953	1,38	1,20E-02
HORMAD1	HORMA domain containing 1	84072	1,38	8,10E-03
NME9	NME/NM23 family member 9	347736	1,38	3,20E-02
HCAR2	hydroxycarboxylic acid receptor 2	338442	1,38	1,70E-02
LRRC38	leucine rich repeat containing 38	126755	1,38	3,40E-02
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	1577	1,38	1,10E-02
LOC101929634	uncharacterized LOC101929634	101929634	1,37	3,00E-03
SCNN1A	sodium channel, non-voltage-gated 1 alpha subunit	6337	1,37	1,40E-02
LINC01491	long intergenic non-protein coding RNA 1491	101928442	1,37	7,90E-03
CYSLTR1	cysteinyl leukotriene receptor 1	10800	1,37	2,80E-03
C8orf37-AS1	C8orf37 antisense RNA 1	100616530	1,37	1,90E-02
ADORA2A	adenosine A2a receptor	135	1,37	4,20E-03
TRAV8-1	T cell receptor alpha variable 8-1	28685	1,37	2,00E-03
OR2A1	olfactory receptor, family 2, subfamily A, member 1	346528	1,36	1,90E-02
TTC9	tetratricopeptide repeat domain 9	23508	1,36	2,60E-02
LOC101928916	uncharacterized LOC101928916	101928916	1,36	4,90E-02
IL32	interleukin 32	9235	1,36	6,40E-03
C8orf31	chromosome 8 open reading frame 31	286122	1,36	1,10E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
C9orf173-AS1	C9orf173 antisense RNA 1	100129722	1,36	1,90E-02
C21orf140	chromosome 21 open reading frame 140	101928147	1,35	2,80E-03
PCDHB13	protocadherin beta 13	56123	1,35	4,90E-02
KIAA0513	KIAA0513	9764	1,35	4,50E-03
UBE2Q2P1	ubiquitin-conjugating enzyme E2Q family member 2 pseudogene 1	388165	1,35	3,70E-02
LOC647859	occludin pseudogene	647859	1,35	2,30E-02
TBL1X	transducin (beta)-like 1X-linked	6907	1,35	9,00E-03
PDIA3	protein disulfide isomerase family A, member 3	2923	1,35	2,20E-02
LOC100130476	uncharacterized LOC100130476	100130476	1,35	2,20E-02
MIR670	microRNA 670	100313777	1,34	2,00E-02
OSTCP1	oligosaccharyltransferase complex subunit pseudogene 1	202459	1,34	4,20E-03
Mild intermittent hypoxia exposure - downregulation				
LOC149351	uncharacterized LOC149351	149351	-1,31	2,50E-02
LOC101927002	uncharacterized LOC101927002	101927002	-1,31	3,70E-02
MAS1L	MAS1 proto-oncogene like, G protein-coupled receptor	116511	-1,31	2,20E-02
FGF22	fibroblast growth factor 22	27006	-1,31	4,10E-03
PUS1	pseudouridylate synthase 1	80324	-1,31	2,70E-02
LINC01140	long intergenic non-protein coding RNA 1140	339524	-1,31	3,80E-02
LOC101928220	putative IQ motif and ankyrin repeat domain-containing protein LOC642574-like	101928220	-1,31	1,90E-02
PCOLCE2	procollagen C-endopeptidase enhancer 2	26577	-1,31	2,70E-02
NA	NA	100996474	-1,31	1,10E-02
MGC16142	uncharacterized protein MGC16142	84849	-1,31	1,30E-02
OR10P1	olfactory receptor, family 10, subfamily P, member 1	121130	-1,31	1,60E-02
LOC101929500	uncharacterized LOC101929500	101929500	-1,32	4,20E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
C17orf67	chromosome 17 open reading frame 67	339210	-1,32	1,20E-03
RBM12B-AS1	RBM12B antisense RNA 1	55472	-1,32	8,00E-03
MIR1246	microRNA 1246	100302142	-1,32	2,80E-02
CXCL2	chemokine (C-X-C motif) ligand 2	2920	-1,32	4,70E-02
SPACA6P	sperm acrosome associated 6, pseudogene	147650	-1,32	8,60E-03
MT1M	metallothionein 1M	4499	-1,32	2,90E-02
EDN1	endothelin 1	1906	-1,33	5,80E-03
GIPC2	GIPC PDZ domain containing family, member 2	54810	-1,33	2,10E-02
ZNF781	zinc finger protein 781	163115	-1,33	6,50E-03
RPL23AP64	ribosomal protein L23a pseudogene 64	649946	-1,33	4,90E-03
SPRR4	small proline-rich protein 4	163778	-1,33	9,20E-03
SURF2	surfeit 2	6835	-1,33	1,10E-02
IFIT3	interferon-induced protein with tetratricopeptide repeats 3	3437	-1,33	1,30E-02
MIR4252	microRNA 4252	100422975	-1,34	3,00E-02
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	1543	-1,34	2,30E-02
MIR376A2	microRNA 376a-2	664615	-1,34	6,60E-03
FAM109A	family with sequence similarity 109, member A	144717	-1,34	6,40E-03
BASP1P1	brain abundant, membrane attached signal protein 1 pseudogene 1	646201	-1,34	6,10E-03
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	5320	-1,34	1,40E-02
TAS2R50	taste receptor, type 2, member 50	259296	-1,34	2,30E-02
FLJ41278	uncharacterized LOC400046	400046	-1,35	3,50E-02
LOC101928264	uncharacterized LOC101928264	101928264	-1,35	6,80E-03
BTN3A1	butyrophilin, subfamily 3, member A1	11119	-1,35	4,70E-02
PAK3	p21 protein (Cdc42/Rac)-activated kinase 3	5063	-1,35	3,10E-02
LOC101927685	heat shock transcription factor, X-linked-like	101927685	-1,35	1,80E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
PRIM2B	primase, DNA, polypeptide 2 (58kDa) pseudogene	100996481	-1,35	1,50E-03
CSGALNACT1	chondroitin sulfate N-acetylgalactosaminyltransferase 1	55790	-1,36	6,40E-03
SEC24B-AS1	SEC24B antisense RNA 1	100533182	-1,36	3,90E-02
LINC00421	long intergenic non-protein coding RNA 421	100287114	-1,36	3,70E-03
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	3815	-1,37	1,30E-02
ZNF806	zinc finger protein 806	646915	-1,37	2,30E-02
LOC101928948	uncharacterized LOC101928948	101928948	-1,37	2,50E-02
REG3G	regenerating islet-derived 3 gamma	130120	-1,37	1,90E-02
TAS2R19	taste receptor, type 2, member 19	259294	-1,37	1,50E-02
LLPH	LLP homolog, long-term synaptic facilitation (Aplysia)	84298	-1,37	8,90E-04
KRTAP13-3	keratin associated protein 13-3	337960	-1,38	4,50E-03
CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1	9420	-1,38	5,50E-03
CALCB	calcitonin-related polypeptide beta	797	-1,38	1,50E-02
IGHV6-1	immunoglobulin heavy variable 6-1	28385	-1,38	1,20E-02
LOC100506127	putative uncharacterized protein FLJ37770-like	100506127	-1,38	3,00E-02
TTTY20	testis-specific transcript, Y-linked 20 (non-protein coding)	252951	-1,39	1,90E-02
MAGEC2	melanoma antigen family C, 2	51438	-1,39	1,40E-02
SNORA60	small nucleolar RNA, H/ACA box 60	677837	-1,39	4,60E-02
AKR7A2	aldo-keto reductase family 7, member A2 (aflatoxin aldehyde reductase)	8574	-1,39	1,40E-02
ZNF37A	zinc finger protein 37A	7587	-1,39	2,10E-02
CLP1	cleavage and polyadenylation factor I subunit 1	10978	-1,39	2,30E-02
LOC285191	uncharacterized LOC285191	285191	-1,4	2,90E-02
RRP7B	ribosomal RNA processing 7 homolog B (S. cerevisiae)	91695	-1,4	2,70E-03
ZNF563	zinc finger protein 563	147837	-1,4	2,00E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
CMC1	C-x(9)-C motif containing 1	152100	-1,4	2,80E-02
NA	NA	101927261	-1,4	1,30E-02
TRAV13-2	T cell receptor alpha variable 13-2	28670	-1,4	3,90E-03
KCNAB1	potassium voltage-gated channel, shaker-related subfamily, beta member 1	7881	-1,4	1,80E-02
NA	NA	101927091	-1,41	4,40E-02
CMIP	c-Maf inducing protein	80790	-1,42	1,30E-02
SPRNP1	shadow of prion protein homolog (zebrafish) pseudogene 1	399833	-1,42	4,10E-02
LINC00882	long intergenic non-protein coding RNA 882	100302640	-1,42	1,10E-03
PTENP1	phosphatase and tensin homolog pseudogene 1 (functional)	11191	-1,43	8,00E-05
MIR711	microRNA 711	100313843	-1,43	4,50E-03
RPL12	ribosomal protein L12	6136	-1,43	1,30E-03
OR4C46	olfactory receptor, family 4, subfamily C, member 46	119749	-1,44	1,60E-04
NA	NA	101929686	-1,44	4,80E-02
NBPF18P	neuroblastoma breakpoint family, member 18, pseudogene	441908	-1,45	1,40E-02
MIR30D	microRNA 30d	407033	-1,45	3,20E-02
LCE1E	late cornified envelope 1E	353135	-1,45	3,30E-03
SNORD113-8	small nucleolar RNA, C/D box 113-8	767568	-1,45	1,80E-02
LOC100506470	uncharacterized LOC100506470	100506470	-1,46	7,20E-03
ZNF304	zinc finger protein 304	57343	-1,47	2,50E-03
FAM182A	family with sequence similarity 182, member A	284800	-1,47	3,00E-02
KRT19P2	keratin 19 pseudogene 2	160313	-1,47	4,20E-02
CDC14C	cell division cycle 14C	168448	-1,47	2,80E-03
PRG4	proteoglycan 4	10216	-1,48	4,20E-02
MILR1	mast cell immunoglobulin-like receptor 1	284021	-1,49	8,10E-03
APOC4-APOC2	APOC4-APOC2 readthrough (NMD candidate)	100533990	-1,5	3,80E-02

Gene symbol	Gene name	Entrez ID	Mean fold change	IBMT regularized paired t-test raw p-value
LOC101928269	uncharacterized LOC101928269	101928269	-1,52	3,00E-02
CA14	carbonic anhydrase XIV	23632	-1,53	2,90E-02
MIR1273F	microRNA 1273f	100616156	-1,53	3,70E-02
LOC101929251	uncharacterized LOC101929251	101929251	-1,54	6,50E-03
MIR3928	microRNA 3928	100500901	-1,55	2,20E-02
LINC01489	long intergenic non-protein coding RNA 1489	101928340	-1,55	9,40E-03
GEMIN2	gem (nuclear organelle) associated protein 2	8487	-1,56	2,30E-04
LOC439933	uncharacterized LOC439933	439933	-1,57	1,30E-03
FOLH1	folate hydrolase (prostate-specific membrane antigen) 1	2346	-1,58	1,80E-02
SNORD114-4	small nucleolar RNA, C/D box 114-4	767580	-1,6	2,50E-02
OR5P2	olfactory receptor, family 5, subfamily P, member 2	120065	-1,64	1,40E-02
MAGOHB	mago-nashi homolog B (Drosophila)	55110	-1,65	4,80E-04
LOC101928722	flocculation protein FLO11-like	101928722	-1,7	3,80E-02
TRBJ2-6	T cell receptor beta joining 2-6	28623	-1,7	1,10E-02

Supplementary Table 3. Primer sequences used for gene expression analysis in primary human adipocytes.

		Sequence
IL-6	Forward	AAATTCGGTACATCCTCGACGG
	Reverse	GGAAGGTTCAGGTTGTTTTCTGC
MCP-1	Forward	CCCCAGTCACCTGCTGTTAT
	Reverse	TCCTGAACCCACTTCTGCTT
PAI-1	Forward	TCGTCCAGCGGGATCTGAA
	Reverse	GCCGTTGAAGTAGAGGGCATT
Leptin	Forward	GCTGTGCCCATCCAAAAAGTCC
	Reverse	CCCAGGAATGAAGTCCAAACCG
GLUT1	Forward	TTGCAGGCTTCTCCAACTGGAC
	Reverse	CAGAACCAGGAGCACAGTGAAG
HSL	Forward	GCGGATCACACAGAACCTGGAC
	Reverse	AGCAGGCGGCTTACCCTCAC
VEGFA	Forward	TTGCCTTGCTGCTCTACCTCCA
	Reverse	GATGGCAGTAGCTGCGCTGATA
ACAB	Forward	GCAAGAACGTGTGGGGTTACT
	Reverse	TCGCCTCGGATGGACAGTT
SCD1	Forward	CCTGGTTTCACTTGGAGCTGTG
	Reverse	TGTGGTGAAGTTGATGTGCCAGC
FASN	Forward	CCGAGACACTCGTGGGCTA
	Reverse	CTTCAGCAGGACATTGATGCC
18S	Forward	AGTTAGCATGCCAGAGTCTCG
	Reverse	TGCATGGCCGTTCTTAGTTG
RPL13A	Forward	CCTGGAGGAGAAGAGGAAAGAGA
	Reverse	TTGAGGACCTCTGTGTATTTGTCAA

CHAPTER 4

The effects of mild intermittent hypoxia exposure on the abdominal subcutaneous adipose tissue proteome in overweight and obese men: A first-in-human randomized, single-blind, cross-over study

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Ready to be submitted

Abstract

Adipose tissue (AT) oxygen tension (pO_2) has been implicated in AT dysfunction and metabolic perturbations in both rodents and humans. Compelling evidence suggests that hypoxia exposure alters metabolism, at least partly through effects on AT. However, it remains to be elucidated whether mild intermittent hypoxia (MIH) exposure impacts the AT proteome. We performed a randomized, single-blind, cross-over study to investigate the effects of seven consecutive days of MIH (FiO_2 15%, 3x2h/d) compared to normoxia (FiO_2 21%) exposure, on the AT proteome in overweight/obese men. AT insulin sensitivity was determined by a two-step hyperinsulinemic-euglycemic clamp, and abdominal subcutaneous AT biopsies were collected (n=11) under normoxic, fasting conditions following both exposure regimens. AT proteins were isolated and quantified using liquid chromatography-mass spectrometry. After correction for blood contamination, 1022 AT protein IDs were identified, of which 123 were differentially expressed following MIH ($p < 0.05$). These proteins were involved in redox systems, cell-adhesion, actin cytoskeleton organization, extracellular matrix composition and energy metabolism. We demonstrate for the first time that MIH exposure impacts the AT proteome. Moreover, differential AT TMOD3 expression is related to changes in AT insulin sensitivity, thereby linking MIH-induced effects on the AT proteome to metabolic changes in overweight/obese humans.

Introduction

The prevalence of obesity has increased drastically over the last decades, with nearly a third of the word population living with overweight or obesity(1). Obesity is a multifactorial disease, which is characterized by excess adipose tissue (AT) mass. AT is a metabolically active, endocrine organ, playing a central role in immunity, glucose and lipid homeostasis, angiogenesis, coagulation, vascular function, appetite regulation and body weight control(2). Thus, AT dysfunction is closely associated with an increased risk of cardiometabolic complications(3-5) and hence mortality(6).

AT oxygen tension (pO_2) has been implicated in AT dysfunction in both rodents and humans, as reviewed recently(7). Although AT hypoxia has been consistently shown in rodent models of obesity, conflicting findings have been reported in humans(7). We have previously demonstrated higher AT pO_2 in obese compared to lean individuals(8), decreased AT pO_2 following diet-induced weight loss(9), and a positive association between AT pO_2 and insulin resistance in humans, independently of adiposity(10). Many *in vitro* studies have been performed to investigate whether exposure to hypoxic environments affects AT glucose and lipid metabolism. Indeed, (intermittent) hypoxia exposure appears to have profound effects on mRNA expression of several genes related to glucose and lipid metabolism in murine and human adipocytes, as reviewed extensively(7, 11).

However, to investigate and better understand the effects of hypoxia exposure on a more functional level, proteomics analysis seems highly valuable. Indeed, previous studies have indicated that the proteomic profile of *3T3-L1* adipocytes during long-term mild hypoxia exposure, at physiological pericellular oxygen concentrations (4% O_2), affects various pathways involved in energy metabolism, suggestive of increased glycolytic metabolism and triacylglycerol synthesis(12). In addition, 24h exposure to 1% O_2 mainly increased expression of proteins related to the extracellular matrix (ECM) in human adipose stem cells(13). These *in vitro* findings suggest that hypoxia may induce ECM remodeling and induce metabolic changes in AT. Importantly, however, human *in vivo* studies that examined the effects of prolonged mild hypoxia exposure on the AT proteome are lacking.

In the present study, we investigated the impact of mild intermittent hypoxia (MIH) compared to normoxia exposure on the abdominal subcutaneous AT proteome in overweight and obese men, using untargeted liquid chromatography-mass spectrometry, to elucidate the physiological and functional adaptations in human AT evoked by MIH. Secondly, we explored the associations between MIH-induced alterations in AT protein expression and *in vivo* AT insulin sensitivity.

Materials and Methods

Subjects

Twelve overweight and obese (BMI ≥28 kg/m^2) male subjects (30-65 years) participated in the present 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. Participants were asked to refrain from drinking alcohol and perform no exercise 24h prior to the start and during exposure regimen. The study, registered at the Netherlands Trial Register (NTR7325), 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.

Study design

The study design has been described in detail elsewhere (Chapter 3). Briefly, participants enrolled in this randomized, single-blind, cross-over study were exposed to normobaric MIH (15% FiO_2) and normobaric normoxia (21% FiO_2) for 7 consecutive days (3 cycles of 2h/d with 1h of normoxia exposure between hypoxic cycles), separated by a 3-6 wk wash-out period. Systemic oxygen saturation was continuously monitored throughout the exposure regimens by finger pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcor). At day 6 of the exposure regimens, AT pO_2 was determined using an optochemical measurement system for continuous monitoring of tissue pO_2, as described previously(8). At day 8, following the 7-day MIH/normoxia exposure regimens, fasting abdominal subcutaneous adipose tissue biopsies were collected, and a two-step hyperinsulinemic-euglycemic clamp was performed to determine AT, hepatic and peripheral insulin sensitivity under normoxic conditions, as described previously(51).

Abdominal subcutaneous adipose tissue biopsy

Fasting abdominal subcutaneous AT biopsies were collected (approximately 1g) using needle aspiration under local anaesthesia (2% lidocaine), 6-8 cm lateral from the umbilicus. After thorough rinsing with sterile saline, visible blood vessels were removed using sterile tweezers. Subsequently, specimens were snap-frozen using liquid nitrogen and stored at -80°C for further analysis. Due to sampling issues, we could not collect enough material following hypoxia exposure in one participant. Therefore, paired adipose tissue biopsies (following both MIH and normoxia exposure) were available for analysis in n = 11 individuals.

Protein isolation and preparation for LC-MS

Frozen 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 total of 100 µg protein in 50 µl 50 mM ammonium bicarbonate (ABC) with 5 M urea was used. 5 µL of DTT solution (20 mM final) was added and incubated at room temperature for 45 minutes. The proteins were alkylated by adding 6 µL of IAA solution (40 mM final). The reaction was taken place at room temperature for 45 minutes in the darkness. The alkylation was stopped by adding 10 µL of DTT solution (to consume any unreacted IAA) and incubated at room temperature for 45 minutes. For the digestion 2 µg trypsin/lysC was added to the protein and incubated at 37°C for 2 hours. 200 µl of 50mM ABC was added to dilute the urea concentration and further incubate at 37°C for 18 hours. The digestion mixture was centrifuge at 2.500 g for 5 minutes and the supernatant, which contains the peptide mixture, collected for the use of LCMS analysis.

Protein identification and quantification using LC-MS

A nanoflow HPLC instrument (Dionex ultimate 3000) was coupled on-line to a Q Exactive (Thermo Scientific) with a nano-electrospray Flex ion source (Proxeon). Each sample was runed separately for label free quantification. 5 µl of the 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 240 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 70,000 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 17,500 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 and quantification

The MS data were searched using Proteome Discoverer 2.2 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 were set as a dynamic modification. Carbamidomethylation of cysteines were set as fixed modifications. Label free quantitation was conducted using the Minora Feature Detector node in the processing step and the Feature Mapper node combined with the Precursor Ions Quantifier node in the consensus step with default settings within Proteome Discoverer 2.2(52).

Protein signal normalization and adjustment for blood protein contamination

The inter-run variation was normalized using ppm fractional normalization. To reduce the influence of the blood protein contamination on the AT proteome, we retrieved information from the UniProt database and GeneCards to set up a blood protein exclusion list with known proteins exclusively expressed in blood, including all immunoglobulins(53, 54). The amount of signal contributed by these blood proteins in our AT sample were found with a mean value of 76%. We defined valid human AT proteins based on these quality criteria: 1) not exclusively expressed in blood, 2) identification Score Sequest HT >=2.5, 3) present in > 50% (>=6 of 11) samples in at least one treatment group. The final signal of a valid human AT protein was the protein relative ppm abundance in a fixed amount (76%) blood contaminated AT sample. This final signal (below referred to as 'signal') was used in data analysis.

Final signal [protein x, sample i]
= normalized signal [protein x, sample i] /(Σ normalized signal [sample i] – Σ Blood protein normalized signal [sample i])*1000000*(1-0.76)

Statistical analysis

Only valid human AT proteins were taken into statistical analyses. First, missing values were imputed using random forest algorithm with R missForest package. Thereafter, data were log2 transformed. The effects of MIH compared to normoxia exposure on abdominal subcutaneous AT protein expression were assessed using two-sided paired Student's t-tests. The fold change was expressed as back-transformed mean difference. 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. Subsequently, these differentially expressed proteins (p<0.05) were imported into *Cytoscape plug-in* ClueGO v2.5.7 for functional analysis(55), based on GO biological process terms and KEGG pathways dated on 08.05.2020. Spearman's rank correlation analysis was performed to determine correlations between changes in AT proteins and changes in AT insulin sensitivity (expressed as suppression (%) of FFA plasma concentration upon 10 mU•m^{-2} insulin infusion). All statistical analyses were performed in R environment, version 3.5, with various packages (stats, missForest, gplots and pheatmap) and SPSS.

Results

Characteristics of the study participants are shown in Table 1. All individuals were overweight or obese (BMI≥28 $kg \cdot m^{-2}$) and demonstrated a homeostatic model assessment for insulin resistance ≥ 2.2.

Table 1. Baseline characteristics of male study participants.

	Baseline
Age (y)	61 ± 1
BMI (kg/m^2)	30.8 ± 3.6
Hemoglobin ($mmol \cdot l^{-1}$)	9.5 ± 0.5
HbA1c (%)	5.6 ± 0.1
Fasting glucose ($mmol \cdot l^{-1}$)	5.7 ± 0.5
2h-glucose ($mmol \cdot l^{-1}$)	6.2 ± 1.3
HOMA-IR	3.7 ± 0.4

Hb, hemoglobin; Hb1Ac, glycated hemoglobin; HOMA-IR, homeostasis model of assessment of insulin resistance; 2h-glucose determined during a 75g oral glucose tolerance test (OGTT). Values are mean ± SEM (n=11)

Mild intermittent hypoxia exposure decreases adipose tissue oxygen tension

To provide the proof-of-concept in humans that MIH exposure reduces oxygen availability in abdominal subcutaneous AT, we determined both systemic oxygen saturation (finger pulse oximetry) and AT pO_2, using a highly accurate, microdialysis-based optochemical measurement system to continuously monitor AT pO_2 *in vivo* in humans(8). As previously reported (Chapter 3), MIH exposure reduced systemic oxygen saturation (normoxia: 97.1 ± 0.3 *vs.* hypoxia: 92.0 ± 0.5 %, p<0.001) and decreased AT pO_2 (normoxia: 36.5 ± 1.5 mmHg versus hypoxia: 21.0 ± 2.3 mmHg, p<0.001).

Mild intermittent hypoxia exposure impacts the adipose tissue proteome

We quantified 1091 accession IDs of 1074 proteins in the AT samples with HT score >2.5, which appeared in more than half of the samples in at least one of the conditions (MIH and/or normoxia). Several of these proteins were considered blood-specific (e.g. hemoglobins, serum albumins and erythrocyte proteins), explained by AT specimen contamination with some blood during sample collection, despite thorough cleaning using sterile saline, as described previously(14). Indeed, 69

identified IDs of 63 unique proteins were blood-specific, and attributed to 62-82% of the total protein signal (Figure 1). Therefore, we corrected the quantification after removal of these blood-specific proteins (Supplementary table 1). In the resulting 1022 AT protein IDs for 1011 proteins, 123 IDs of 123 unique proteins were differentially expressed ($p < 0.05$; Table 2 and Supplementary Table 2) following MIH compared to normoxia exposure (42 upregulated, 81 downregulated), as visualized in a heat-map (Figure 2). Differential expression of top 5 upregulated/downregulated (based on p-values) proteins per individual is visualized in Supplementary Figure 1. Changes of individual proteins were statistically non-significant when corrected for multiple testing at a FDR q-value < 0.05.

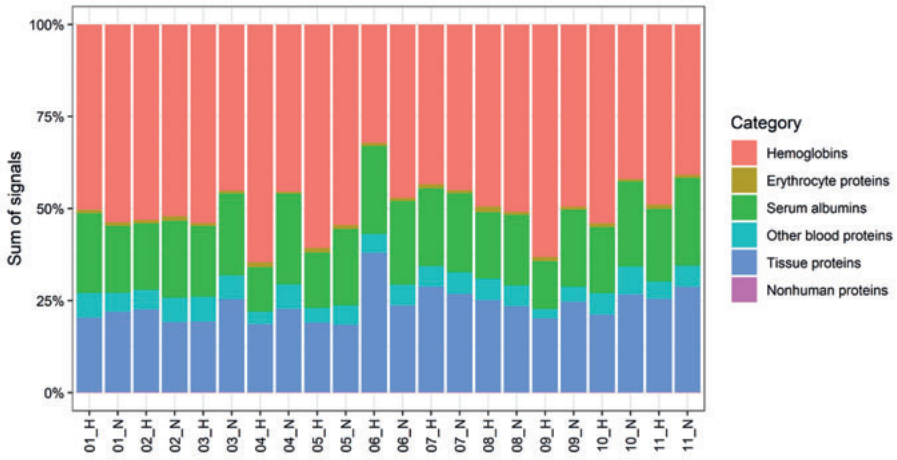

Figure 1. Contribution to the total proteome signals of AT by human abdominal subcutaneous adipose tissue proteins and different groups of blood-specific proteins. Each bar represents an adipose tissue biopsy, with number indicating the respective participant. H; biopsy after mild intermittent hypoxia exposure, N; normoxia exposure.

Mild intermittent hypoxia exposure alters adipose tissue expression of proteins involved in structural and metabolic pathways

All 123 differentially expressed proteins were functionally annotated. 11 Functional groups with a Bonferroni step-down corrected *p*-value <0.05 were identified (Figure 3). These functional groups cover 104 representative GO terms and pathways (Supplementary Table 3), to which 79 proteins are associated (Figure 3). The functional groups cover predominantly structural and metabolic-related GO terms and pathways. MIH induced AT expression of several proteins related to actin cytoskeleton organization, focal adhesion and myeloid development, whereas it reduced the expression of proteins related to collagen fibril organization (Table 3, Figure 3). In addition, MIH reduced the enrichment of several pathways related to oxidoreductase activity, regulation of lipolysis in adipocytes, polysaccharide biosynthetic and ADP metabolic processes. Moreover, MIH increased proteins involved in bicarbonate transport, iron ion homeostasis, as well as platelet degranulation (Table 3, Figure 3).

Mild intermittent hypoxia-induced effects on the adipose tissue proteome are related to *in vivo* adipose tissue insulin sensitivity

Since MIH exposure had a significant impact on several ECM- and cytoskeleton-related proteins, as well as proteins involved in energy metabolism, we next determined the associations between differentially expressed AT proteins and *in vivo* AT insulin sensitivity. Although AT insulin sensitivity did not significantly change following MIH exposure (data not shown), the increase in tropomodulin-3 (TMOD3) protein expression evoked by MIH exposure was positively associated with the change in AT insulin sensitivity ($r = 0.806$ $p = 0.005$, Figure 4).

Table 2. Top 20 up- and downregulated proteins by MIH compared to normoxia exposure in overweight and obese individuals (p-value<0.05, q-value>0.05).

Upregulated	Uniprot	Gene	Protein names	Fold-change (Hypoxia/ Normoxia)	*p*-value
1	P11166	SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1 (Glucose transporter type 1)	1,81	0,006
2	P28289	TMOD1	Tropomodulin-1 (Erythrocyte tropomodulin)	1,97	0,006
3	P16104	H2AX	Histone H2AX	1,29	0,007
4	O00194	RAB27B	Ras-related protein Rab-27B	2,16	0,008
5	P07451	CA3	Carbonic anhydrase 3	1,36	0,009
6	P29972	AQP1	Aquaporin-1 (Water channel protein for red blood cells and kidney proximal tubule)	1,63	0,013
7	P07384	CAPN1	Calpain-1 catalytic subunit	1,39	0,013
8	P02730	SLC4A1	Band 3 anion transport protein	1,65	0,015
9	P22061	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	1,21	0,015
10	P21333	FLNA	Filamin-A	1,38	0,015
11	O15511	ARPC5	Actin-related protein 2/3 complex subunit 5	1,30	0,015
12	P09493	TPM1	Tropomyosin alpha-1 chain	1,21	0,016
13	P31146	CORO1A	Coronin-1A	1,93	0,018
14	Q15942	ZYX	Zyxin	1,59	0,018
15	O60256	PRPSAP2	Phosphoribosyl pyrophosphate synthase-associated protein 2	1,50	0,018
16	P80188	LCN2	Neutrophil gelatinase-associated lipocalin	2,04	0,018
17	P48643	CCT5	T-complex protein 1 subunit epsilon	1,15	0,021
18	O14561	NDUFAB1	Acyl carrier protein, mitochondrial	1,43	0,022
19	P30626	SRI	Sorcin	1,17	0,022
20	P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	1,85	0,025

Downregulated	Uniprot	Gene	Protein names	Fold-change (Hypoxia/ Normoxia)	*p*-value
1	P05091	ALDH2	Aldehyde dehydrogenase, mitochondrial	0,81	8,7E-04
2	Q8WTS1	ABHD5	1-acylglycerol-3-phosphate O-acyltransferase ABHD5 (Lipid droplet-binding protein CGI-58)	0,70	0,003
3	Q99536	VAT1	Synaptic vesicle membrane protein VAT-1 homolog	0,78	0,004
4	P06737	PYGL	Glycogen phosphorylase, liver form	0,62	0,006
5	Q02952	AKAP12	A-kinase anchor protein 12	0,59	0,008
6	Q16851	UGP2	UTP-glucose-1-phosphate uridylyltransferase	0,65	0,008
7	P16403	H1-2	Histone H1.2	0,73	0,009
8	P07099	EPHX1	Epoxide hydrolase 1	0,64	0,014
9	P16083	NQO2	Ribosyldihydronicotinamide dehydrogenase [quinone]	0,33	0,014
10	Q9NVD7	PARVA	Alpha-parvin	0,51	0,015
11	P21695	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	0,56	0,016
12	P02511	CRYAB	Alpha-crystallin B chain	0,58	0,017
13	P10301	RRAS	Ras-related protein R-Ras	0,61	0,017
14	Q16836	HADH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0,51	0,018
15	O60240	PLIN1	Perilipin-1 (Lipid droplet-associated protein)	0,65	0,019
16	Q9BX66	SORBS1	Sorbin and SH3 domain-containing protein 1	0,53	0,019
17	Q14112	NID2	Nidogen-2	0,60	0,019
18	P08294	SOD3	Extracellular superoxide dismutase	0,47	0,020
19	P36871	PGM1	Phosphoglucomutase-1	0,48	0,020
20	Q9BX68	HINT2	Histidine triad nucleotide-binding protein 2, mitochondrial	0,46	0,021

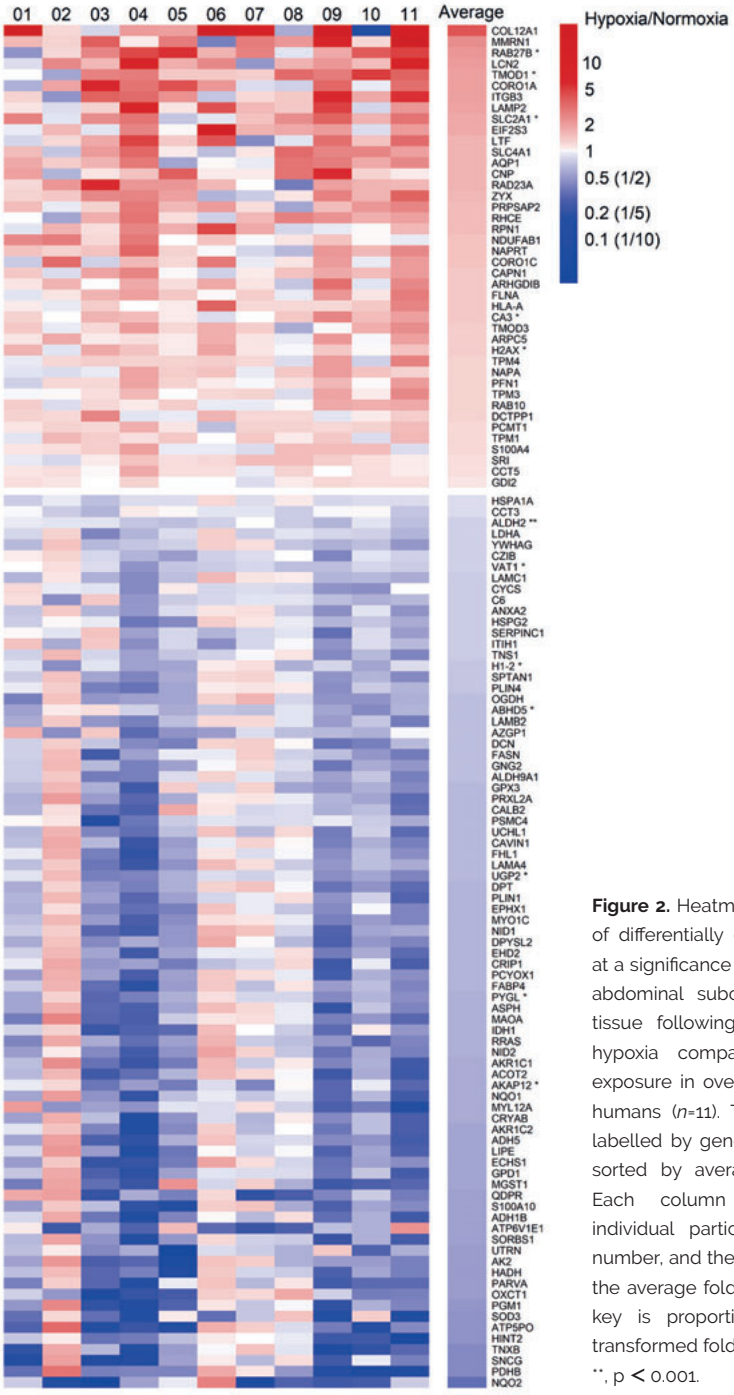

Figure 2. Heatmap of fold changes of differentially expressed proteins at a significance level of $p < 0.05$ in abdominal subcutaneous adipose tissue following mild intermittent hypoxia compared to normoxia exposure in overweight and obese humans (*n*=11). These proteins are labelled by gene symbols and are sorted by average fold changes. Each column represents one individual participant labelled by number, and the last column shows the average fold change. The color key is proportional to the log2 transformed fold change. *, $p < 0.01$; **, $p < 0.001$.

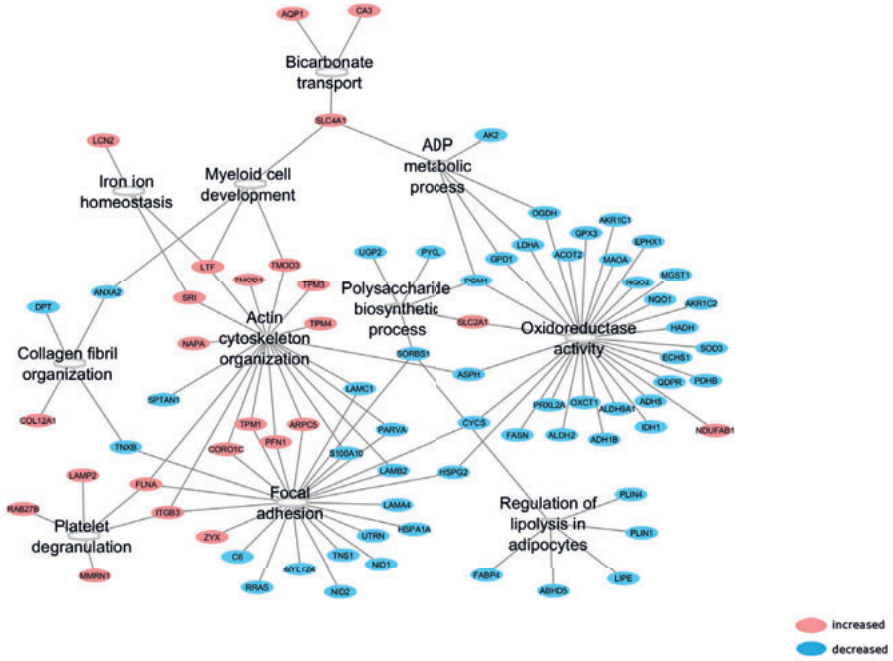

Figure 3. Functional groups affected by mild intermittent hypoxia exposure. After functional annotation of differentially expressed proteins, 11 functional groups were identified ($p<0.05$, corrected with Bonferroni step-down procedure) with associated proteins which were altered by mild intermittent hypoxia exposure. Red encircled proteins represent upregulated, whereas blue encircled proteins represent downregulated proteins subsequent to MIH exposure.

Table 3. Functional groups affected by mild intermittent hypoxia based on representative GO terms and KEGG pathways.

Functional group	Group adjusted *p*-value
Oxidoreductase activity	4.7E-10
Focal adhesion	2.8E-09
Actin cytoskeleton organization	1.0E-06
Collagen fibril organization	0.003
ADP metabolic process	0.005
Myeloid cell development	0.009
Bicarbonate transport	0.010
Platelet degranulation	0.010
Regulation of lipolysis in adipocytes	0.010
Iron ion homeostasis	0.012
Polysaccharide biosynthetic process	0.012

Bonferroni step-down corrected *p*-values. GO, Gene Ontology; KEGG, Kyoto Encyclopedia for Genes and Genomes.

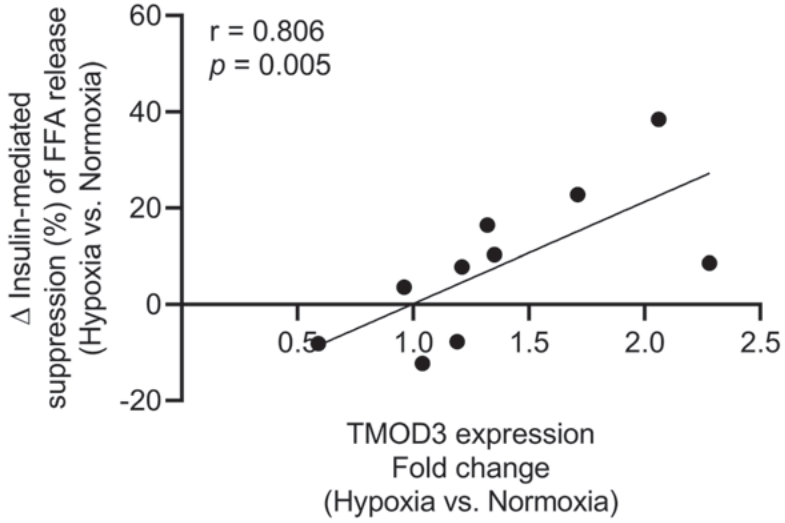

Figure 4. Association between TMOD3 and adipose tissue insulin sensitivity (insulin-mediated suppression of plasma free fatty acids (%)). The MIH-induced increase in TMOD3 expression was correlated with improved adipose tissue insulin sensitivity (n=10). TMOD3; tropomodulin-3.

Discussion

In the present randomized, single-blind cross-over study, we examined for the first time the effects of 7 consecutive days of MIH compared to normoxia exposure on the abdominal subcutaneous AT proteome in overweight and obese men. From the 1011 AT-specific proteins identified, 123 proteins were differentially expressed following MIH compared to normoxia exposure. MIH induced marked alterations of functional groups, mainly related to oxidoreductase systems, cell-adhesion, actin cytoskeleton and ECM organization, and energy metabolism. Moreover, the MIH-induced increase in TMOD3 expression was significantly related to improved AT insulin sensitivity, suggesting a link between MIH-induced effects on the AT proteome and metabolic changes in human AT.

We found that MIH exposure significantly downregulates oxidoreductase activity-related pathways in AT. More specifically, NAD(P) and NAD(P)H-dependent dehydrogenase activity-related pathways contributed to the changes in this functional group. Out of the 31 proteins associated with oxidoreductase activity, aldehyde dehydrogenase 2 (ALDH2), glycerol-3-phosphate dehydrogenase 1 (GPD1) and 3-hydroxyacyl-CoA dehydrogenase (HADH) were most significantly downregulated. The expression of ALDH2, which catalyzes the oxidation of aldehydes, has been found to be increased with adiposity(15). ALDH2 may counteract reactive oxygen species (ROS)-induced lipid aldehyde formation, which has been described in obesity and insulin resistance(16). HADH, a key enzyme involved in fatty acid oxidation, was also downregulated by MIH exposure. In agreement with our findings, it has recently been shown that exposure to severe hypoxia exposure markedly reduced fatty acid oxidation in murine adipocytes(17). Interestingly, we also found increased protein abundance of the mitochondrial complex I subunit NADH:Ubiquinone Oxidoreductase Subunit AB1 (NDUFAB1)(18) following MIH. Indeed, cardiac-specific deletion of NDUFAB1 in mice resulted in defective mitochondrial bioenergetics and excessive production of ROS, whereas NDUFAB1 overexpression enhanced mitochondrial functioning and limited ROS production(19), and prevented obesity-induced insulin resistance in mice fed high-fat diet(20). Of note, hypoxia decreases tissue NAD^+ content, and substantially increases the NADH/NAD+ ratio(21, 22). Most of these oxidoreductases are dependent upon NAD^+ as an electron acceptor. As such, this may explain reduced expression of these enzymes following prolonged *in vivo* MIH exposure. Notably, an increased NADH/NAD^+ ratio inhibits NAD+ -dependent processes(22), covering a large number of central metabolic pathways such as the tricarboxylic acid cycle and fatty acid catabolism (Supplementary Table 3). Taken together, our findings suggest that MIH reduced AT protein expression of oxidoreductases, thereby affecting the direction of redox processes, and may promote alternative pathways to yield energy under hypoxic conditions.

In addition, AT expression of several proteins related to focal adhesion-related processes were increased subsequent to MIH exposure. Focal adhesion between the basement membrane or the extracellular matrix (ECM) and the adipocytes regulates cytoskeletal changes as well as adipocyte differentiation(23). In addition, focal adhesions exert a key role in ECM-mediated integrin signaling, and may promote adipocyte survival and insulin sensitivity through focal adhesion kinase activity(24). Out of the 21 proteins associated with focal adhesion, zyxin (ZYX) was upregulated. ZYX is essential in localizing the focal adhesion sites and stress-fibers generated by mechanical cues, thereby regulating the adipocyte cytoskeleton(25). On the other hand, Ras-related (RRAS), which has been demonstrated to markedly enhance focal adhesion formation(26), was most significantly downregulated. In agreement with the present *in vivo* findings in humans, several reports have demonstrated predominant effects of hypoxia on cytoskeletal organization *in vitro*, in particular an increase in actin-stress fibers in various cell types(27, 28).

In line with the above, MIH exposure induced the expression of processes related to actin-cytoskeleton organization. Indeed, it has been suggested that actin remodeling is essential during adipocyte maturation, demonstrated by major changes in actin dynamics during adipocyte expansion(29). Furthermore, actin remodeling might enhance insulin signaling and glucose transporter-4 (GLUT4) translocation, thus improving glucose homeostasis(29, 30). Here, we found increased AT protein expression of actin related protein 2/3 complex subunit 5 (ARPC5) and tropomodulin-1 (TMOD1), which are involved in the regulation of actin polymerization and differentiation(31). Furthermore, MIH exposure increased TMOD3 protein expression, which is essential for insulin signal transduction(32). Interestingly, the MIH-induced increase in AT TMOD3 expression was correlated with improved AT insulin sensitivity. In agreement with this, we found that the expression of α-II spectrin (SPTAN1) in AT, which appeared to be higher in subcutaneous adipocytes of obese insulin resistant compared to insulin sensitive individuals(33), was reduced after MIH exposure. Collectively, these findings suggest that prolonged exposure to MIH has pronounced effects on dynamics of focal adhesion, as well as AT remodeling, which may contribute to improved AT insulin sensitivity.

Interestingly, we found that collagen fibril organization was reduced by MIH exposure. It has previously been suggested that hypoxia induces AT fibrosis, by increasing collagen type I deposition, resulting in increased stiffness of the ECM(34). The present findings, however, show that MIH reduced the expression of tenascin-X (TNXB) and dermatopontin (DPT), which are both implicated in collagen fibrillogenesis(35, 36). Of note, it has been demonstrated that circulating levels and visceral AT mRNA expression of DPT are increased in obese insulin resistant individuals(37). Furthermore, we found 4-fold increased AT protein expression of collagen type XII α 1 chain (COL12A1), which is a non-fibrillar type of collagen involved in adipocyte differentiation, and as such a putative marker of adipogenesis(38). Taken

together, our findings indicate that MIH may elicit ECM remodeling and reduce fibrillogenesis in AT, which may enhance the fat storage capacity of adipocytes, allowing safe storage of lipids in abdominal subcutaneous AT(24).

Furthermore, AT proteins associated with the regulation of lipolysis in adipocytes appeared to be reduced by MIH. Indeed, decreased expression of abhydrolase domain containing 5 (ABDH5), fatty acid binding protein 4 (FABP4), hormone-sensitive lipase (LIPE) and perilipin-1 (PLIN1), and PLIN4 were found. ABDH5 and PLIN1, which appeared to be most significantly downregulated, exert an important role in lipid catabolism, acting as activator for triacylglycerol hydrolases and coating protein surrounding lipid droplets in adipocytes, respectively(39, 40). It has been reported that long-term hypoxia (5% O_2) reduced ABDH5, and tended to reduce PLIN1 expression in *3T3-L1* adipocytes compared to standard laboratory conditions (21% O_2)(41). In agreement with our findings, reduced LIPE expression subsequent to long-term hypoxia exposure was also observed in that study(41). Thus, the present findings, together with results from previous *in vitro* studies, suggest that MIH exposure may reduce lipid turnover in human AT to adapt to the lower O_2 availability. Moreover, this may imply that MIH exposure evokes a shift towards glycolytic metabolism to provide energy for the cells.

Indeed, MIH decreased the expression of several proteins associated with biosynthesis of polysaccharides, and therefore suggest that functional alterations in glycogen metabolism are associated with cellular adaptations to hypoxia, as previously described(42). However, both UDP-glucose pyrophosphorylase 2 (UGP2), phosphoglucomutase 1 (PGM1) and glycogen phosphorylase l (PYGL) enzymes were downregulated subsequent to MIH exposure, suggestive of lower glycogen flux. In adipocytes, it has been demonstrated that hypoxia increases glycogen synthesis, which may induce autophagic flux(43). Furthermore, the present data also shows a ~2-fold increase in AT expression of glucose transporter 1 (SLC2A1) protein after MIH exposure, which may imply that hypoxia enhances insulin-independent glucose uptake. Indeed, it has been demonstrated that hypoxic treatment induces insulin-independent glucose transport in various cell types, mainly mediated by SLC2A1(17, 44). Thus, the present findings suggest that MIH exposure alters glucose homeostasis within AT.

Next, we found that MIH induced several AT proteins associated with iron ion homeostasis. In line with the present results, (hypobaric) hypoxia exposure has previously been implicated in iron homeostasis, affecting the regulation of several enzymes involved in iron absorption such as repression of hepcidin(45)(46). In addition, bicarbonate transport appeared to be upregulated by MIH, which has also been described previously in tumor hypoxia(47). The latter may be related to hypoxia-induced lactate production, thereby decreasing interstitial pH(48), which might explain increased bicarbonate transport subsequent to MIH.

Although we have filtered out proteins exclusively abundant in blood in our analysis, the present data analysis still covered some proteins enriched in blood such as lactotransferrin (LTF), and the altered AT expression of these proteins may be due to blood cells. We found that MIH exposure increased the functional groups 'platelet degranulation' and 'myeloid cell development', reflecting functional alterations in blood cells. In line, it has been shown that human platelets exposed to 5% O_2 were characterized by altered phenotype and enhanced activity(49). However, conflicting findings have also been reported in humans. Healthy individuals exposed to short-term severe hypoxia *in vivo* (8% O_2) did not show any differences in platelet activity, and hence blood coagulation(50). Therefore, the functional implications of MIH-induced alterations in the expression of proteins associated with platelet degranulation remains to be elucidated.

In conclusion, the present study demonstrates for the first time that human *in vivo* MIH exposure for seven consecutive days has pronounced effects on the abdominal subcutaneous AT proteome in overweight and obese men. Moreover, we found that the increased expression of TMOD3 was associated with improved AT insulin sensitivity following MIH exposure, thereby linking MIH-induced adaptations in the AT proteome to metabolic changes in human AT.

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Author Contributions

R.V.M., E.B. and G.G. designed the research. R.V.M and G.G. performed sample collection. N.H. and Y.E. contributed to sample preparation and data acquisition. F.B. performed LC-MS analysis. P.W. and R.V.M performed data analysis. R.V.M. wrote the manuscript. P.W., F.B., N.H., Y.E., E.B., E.M. and G.G. revised the manuscript. All authors contributed to data interpretation and approved the final version of the manuscript.

Competing Interests

The authors declare no competing interests.

Supplementary Material

Supplementary Table 1. Proteins exclusively expressed by blood and identified in human adipose tissue biopsies.

Category	Group	Uniprot	Name
Hemoglobins	Hemoglobins	P69905	Hemoglobin subunit alpha
Hemoglobins	Hemoglobins	P68871	Hemoglobin subunit beta
Hemoglobins	Hemoglobins	P69891	Hemoglobin subunit gamma-1
Hemoglobins	Hemoglobins	P69892	Hemoglobin subunit gamma-2
Hemoglobins	Hemoglobins	P02042	Hemoglobin subunit delta
Hemoglobins	Hemoglobins	P02008	Hemoglobin subunit zeta
Hemoglobins	Hemoglobins	P02100	Hemoglobin subunit epsilon
Hemoglobins	Hemoglobins	Q6B0K9	Hemoglobin subunit mu
Hemoglobins	Hemoglobins	P09105	Hemoglobin subunit theta-1
Serum albumin	Albumin	P02768	Serum albumin
Erythrocyte proteins	Erythrocyte	P02549	spectrin alpha chain, erythrocytic 1
Erythrocyte proteins	Erythrocyte	P11277	Spectrin beta chain, erythrocytic (Beta-I spectrin)
Erythrocyte proteins	Erythrocyte	P16157	Ankyrin-1
Erythrocyte proteins	Erythrocyte	P16452	Erythrocyte membrane protein band 4.2
Erythrocyte proteins	Erythrocyte	P11171	Erythrocyte membrane protein band 4.1
Erythrocyte proteins	Erythrocyte	Q00013	55 kDa erythrocyte membrane protein
Erythrocyte proteins	Erythrocyte	P27105	Erythrocyte band 7 integral membrane protein
Erythrocyte proteins	Erythrocyte	Q02094	Ammonium transporter Rh type A (Erythrocyte membrane glycoprotein Rh50)
Erythrocyte proteins	Erythrocyte	Q08495	Dematin (Dematin actin-binding protein) (Erythrocyte membrane protein band 4.9)
Other blood proteins	Prealbumins	P02766	Transthyretin
Other blood proteins	Prealbumins	P01009	Alpha-1-antitrypsin
Other blood proteins	Alpha 1 globulins	P02763	Alpha-1-acid glycoprotein 1
Other blood proteins	Alpha 1 globulins	P19652	Alpha-1-acid glycoprotein 2
Other blood proteins	Alpha 2 globulins	P00738	Haptoglobin
Other blood proteins	Alpha 2 globulins	P01023	α2-macroglobulin

Category	Group	Uniprot	Name
Other blood proteins	Alpha 2 globulins	P00450	Ceruloplasmin
Other blood proteins	Beta globulins	P00747	plasminogen
Other blood proteins	Beta globulins	P02787	Serotransferrin
Other blood proteins	Immunoglobins	P01871	Immunoglobulin heavy constant mu
Other blood proteins	Immunoglobins	P01876	Immunoglobulin heavy constant alpha 1
Other blood proteins	Immunoglobins	P01877	Immunoglobulin heavy constant alpha 2
Other blood proteins	Immunoglobins	P01860	Immunoglobulin heavy constant gamma 3
Other blood proteins	Immunoglobins	P01764	Immunoglobulin heavy variable 3-23
Other blood proteins	Immunoglobins	P01857	Immunoglobulin heavy constant gamma 1
Other blood proteins	Immunoglobins	P01859	Immunoglobulin heavy constant gamma 2
Other blood proteins	Immunoglobins	P23083	Immunoglobulin heavy variable 1-2
Other blood proteins	Immunoglobins	P01861	Immunoglobulin heavy constant gamma 4
Other blood proteins	Immunoglobins	P01780	Immunoglobulin heavy variable 3-7
Other blood proteins	Immunoglobins	P01766	Immunoglobulin heavy variable 3-13
Other blood proteins	Immunoglobins	P01880	Immunoglobulin heavy constant delta
Other blood proteins	Immunoglobins	P01743	Immunoglobulin heavy variable 1-46
Other blood proteins	Immunoglobins	P06331	Immunoglobulin heavy variable 4-34
Other blood proteins	Immunoglobins	P01619	Immunoglobulin kappa variable 3-20
Other blood proteins	Immunoglobins	P01834	Immunoglobulin kappa constant
Other blood proteins	Immunoglobins	P01593	Immunoglobulin kappa variable 1D-33
Other blood proteins	Immunoglobins	P01602	Immunoglobulin kappa variable 1-5
Other blood proteins	Immunoglobins	P06312	Immunoglobulin kappa variable 4-1
Other blood proteins	Immunoglobins	P01611	Immunoglobulin kappa variable 1D-12
Other blood proteins	Immunoglobins	P04430	Immunoglobulin kappa variable 1-16
Other blood proteins	Immunoglobins	P06310	Immunoglobulin kappa variable 2-30
Other blood proteins	Immunoglobins	P04433	Immunoglobulin kappa variable 3-11
Other blood proteins	Immunoglobins	P01701	Immunoglobulin lambda variable 1-51

Category	Group	Uniprot	Name
Other blood proteins	Immunoglobins	P01700	Immunoglobulin lambda variable 1-47
Other blood proteins	Immunoglobins	A0M8Q6	Immunoglobulin lambda constant 7
Other blood proteins	Immunoglobins	P80748	Immunoglobulin lambda variable 3-21
Other blood proteins	Immunoglobins	P01717	Immunoglobulin lambda variable 3-25
Other blood proteins	Immunoglobins	P01714	Immunoglobulin lambda variable 3-19
Other blood proteins	Immunoglobins	P01715	Immunoglobulin lambda variable 3-1
Other blood proteins	Immunoglobins	B9A064	Immunoglobulin lambda-like polypeptide 5
Other blood proteins	Immunoglobins	P04211	Immunoglobulin lambda variable 7-43
Other blood proteins	Immunoglobins	P0DOY2	Immunoglobulin lambda constant 2
Other blood proteins	Platelet	P02776	Platelet factor 4
Other blood proteins	Platelet	P13224	Platelet glycoprotein Ib beta chain

Supplementary Table 2. Proteins that were altered in adipose tissue by MIH compared to normoxia exposure in overweight and obese individuals at a significance level of $p<0.05$.

Uniprot	Gene	Name	Fold change (MIH / Normoxia)	*p*-value
Q99715	COL12A1	Collagen alpha-1(XII) chain	4,07	0,041
Q13201	MMRN1	Multimerin-1	2,34	0,029
O00194	RAB27B	Ras-related protein Rab-27B	2,16	0,008
P80188	LCN2	Neutrophil gelatinase-associated lipocalin	2,04	0,018
P28289	TMOD1	Tropomodulin-1	1,97	0,006
P31146	CORO1A	Coronin-1A	1,93	0,018
P05106	ITGB3	Integrin beta-3	1,92	0,027
P13473	LAMP2	Lysosome-associated membrane glycoprotein 2	1,85	0,025
P11166	SLC2A1	Glucose transporter type 1	1,81	0,006
P41091	EIF2S3	Eukaryotic translation initiation factor 2 subunit 3	1,8	0,044
P02788	LTF	Lactotransferrin	1,66	0,049
P02730	SLC4A1	Solute carrier family 4 member 1	1,65	0,015
P29972	AQP1	Aquaporin-1	1,63	0,013
P09543	CNP	2',3'-cyclic-nucleotide 3'-phosphodiesterase	1,61	0,038
P54725	RAD23A	UV excision repair protein RAD23 homolog A	1,6	0,037
Q15942	ZYX	Zyxin	1,59	0,018
O60256	PRPSAP2	Phosphoribosyl pyrophosphate synthase-associated protein 2	1,5	0,018
P18577	RHCE	CD antigen CD240CE	1,49	0,029
P04843	RPN1	Ribophorin-1	1,48	0,03
O14561	NDUFAB1	Acyl carrier protein, mitochondrial	1,43	0,022
Q6XQN6	NAPRT	Nicotinate phosphoribosyltransferase	1,42	0,033
Q9ULV4	CORO1C	Coronin-1C	1,41	0,048
P07384	CAPN1	Calpain-1 catalytic subunit	1,39	0,013
P21333	FLNA	Filamin-A	1,38	0,015
P52566	ARHGDIB	Rho GDP-dissociation inhibitor	1,38	0,037
P07451	CA3	Carbonic anhydrase 3	1,36	0,009
P04439	HLA-A	HLA class I histocompatibility antigen, A alpha chain	1,36	0,035
Q9NYL9	TMOD3	Tropomodulin-3	1,32	0,039
O15511	ARPC5	Actin-related protein 2/3 complex subunit 5	1,3	0,015

Uniprot	Gene	Name	Fold change (MIH / Normoxia)	*p*-value
P16104	H2AX	Histone H2AX	1,29	0,007
P67936	TPM4	Tropomyosin alpha-4 chain	1,27	0,047
P54920	NAPA	Alpha-soluble NSF attachment protein	1,26	0,025
P07737	PFN1	Profilin-1	1,25	0,027
P06753	TPM3	Tropomyosin alpha-3 chain	1,24	0,043
Q9H773	DCTPP1	dCTP pyrophosphatase 1	1,23	0,043
P61026	RAB10	Ras-related protein Rab-10	1,23	0,027
P09493	TPM1	Tropomyosin alpha-1 chain	1,21	0,016
P22061	PCMT1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase	1,21	0,015
P26447	S100A4	Protein S100-A4	1,2	0,05
P30626	SRI	Sorcin	1,17	0,022
P48643	CCT5	T-complex protein 1 subunit epsilon	1,15	0,021
P50395	GDI2	Rab GDP dissociation inhibitor beta	1,09	0,026
P0DMV8	HSPA1A	Heat shock 70 kDa protein 1A	0,88	0,022
P49368	CCT3	T-complex protein 1 subunit gamma	0,87	0,026
P05091	ALDH2	Aldehyde dehydrogenase, mitochondrial	0,81	0,001
P61981	YWHAG	14-3-3 protein gamma	0,8	0,047
P00338	LDHA	L-lactate dehydrogenase A chain	0,8	0,046
Q9NWV4	CZIB	CXXC motif containing zinc binding protein	0,79	0,032
P11047	LAMC1	Laminin subunit gamma-1	0,78	0,049
Q99536	VAT1	Synaptic vesicle membrane protein VAT-1 homolog	0,78	0,004
P99999	CYCS	Cytochrome c	0,77	0,04
P98160	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	0,76	0,041
P07355	ANXA2	Annexin A2	0,76	0,036
P13671	C6	Complement component C6	0,76	0,023
Q9HBL0	TNS1	Tensin-1	0,75	0,035
P19827	ITIH1	Inter-alpha-trypsin inhibitor heavy chain H1	0,75	0,043
P01008	SERPINC1	Antithrombin-III	0,75	0,037
P16403	H1-2	Histone H1.2	0,73	0,009
Q96Q06	PLIN4	Perilipin-4	0,72	0,036

Uniprot	Gene	Name	Fold change (MIH / Normoxia)	*p*-value
Q13813	SPTAN1	Spectrin alpha chain, non-erythrocytic 1	0,72	0,039
P49327	FASN	Fatty acid synthase	0,7	0,049
P07585	DCN	Decorin	0,7	0,046
P25311	AZGP1	Zinc-alpha-2-glycoprotein	0,7	0,04
P55268	LAMB2	Laminin subunit beta-2	0,7	0,026
Q8WTS1	ABHD5	1-acylglycerol-3-phosphate O-acyltransferase ABHD5	0,7	0,003
Q02218	OGDH	2-oxoglutarate dehydrogenase, mitochondrial	0,7	0,044
P49189	ALDH9A1	Aldehyde dehydrogenase family 9 member A1	0,69	0,022
P59768	GNG2	Guanine nucleotide-binding protein G(I)/G(S)/ G(O) subunit gamma-2	0,69	0,024
P43686	PSMC4	26S proteasome regulatory subunit 6B	0,68	0,049
P22676	CALB2	Calretinin	0,68	0,049
Q9BRX8	PRXL2A	Peroxiredoxin-like 2A	0,68	0,044
P22352	GPX3	Glutathione peroxidase 3	0,68	0,041
P09936	UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	0,67	0,044
Q16363	LAMA4	Laminin subunit alpha-4	0,66	0,034
Q13642	FHL1	Four and a half LIM domains protein 1	0,66	0,043
Q6NZI2	CAVIN1	Caveolae-associated protein 1	0,66	0,043
O60240	PLIN1	Perilipin-1	0,65	0,019
Q07507	DPT	Dermatopontin	0,65	0,026
Q16851	UGP2	UDP-glucose pyrophosphorylase	0,65	0,008
Q9UHG3	PCYOX1	Prenylcysteine oxidase 1	0,64	0,037
P50238	CRIP1	Cysteine-rich protein 1	0,64	0,039
Q9NZN4	EHD2	EH domain-containing protein 2	0,64	0,026
Q16555	DPYSL2	Dihydropyrimidinase-related protein 2	0,64	0,024
P14543	NID1	Nidogen-1	0,64	0,045
O00159	MYO1C	Unconventional myosin-Ic	0,64	0,022
P07099	EPHX1	Epoxide hydrolase 1	0,64	0,014
P15090	FABP4	Fatty acid-binding protein 4	0,63	0,034
Q12797	ASPH	Aspartyl/asparaginyl beta-hydroxylase	0,62	0,046
P06737	PYGL	Glycogen phosphorylase, liver form	0,62	0,006
P10301	RRAS	Ras-related protein R-Ras	0,61	0,017

Uniprot	Gene	Name	Fold change (MIH / Normoxia)	*p*-value
O75874	IDH1	Isocitrate dehydrogenase [NADP] cytoplasmic	0,61	0,033
P21397	MAOA	Monoamine oxidase type A	0,61	0,045
Q04828	AKR1C1	Aldo-keto reductase family 1 member C1	0,6	0,047
Q14112	NID2	Nidogen-2	0,6	0,019
Q02952	AKAP12	A-kinase anchor protein 12	0,59	0,008
P49753	ACOT2	Acyl-coenzyme A thioesterase 2	0,59	0,038
P02511	CRYAB	Alpha-crystallin B chain	0,58	0,017
P19105	MYL12A	Myosin regulatory light chain 12A	0,58	0,033
P15559	NQO1	NAD(P)H dehydrogenase [quinone] 1	0,58	0,022
P11766	ADH5	Alcohol dehydrogenase class-3	0,57	0,025
P52895	AKR1C2	Aldo-keto reductase family 1 member C2	0,57	0,024
P21695	GPD1	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	0,56	0,016
P30084	ECHS1	Enoyl-CoA hydratase, mitochondrial	0,56	0,027
Q05469	LIPE	Hormone-sensitive lipase	0,56	0,027
P09417	QDPR	Dihydropteridine reductase	0,55	0,034
P10620	MGST1	Microsomal glutathione S-transferase 1	0,55	0,042
P36543	ATP6V1E1	V-type proton ATPase subunit E 1	0,54	0,026
P00325	ADH1B	Alcohol dehydrogenase 1B	0,54	0,021
P60903	S100A10	Protein S100-A10	0,54	0,022
Q9BX66	SORBS1	Sorbin and SH3 domain-containing protein 1	0,53	0,019
P46939	UTRN	Utrophin	0,52	0,047
Q9NVD7	PARVA	Alpha-parvin	0,51	0,015
Q16836	HADH	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	0,51	0,018
P54819	AK2	Adenylate kinase 2, mitochondrial	0,51	0,045
P55809	OXCT1	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	0,5	0,034
P36871	PGM1	Phosphoglucomutase-1	0,48	0,02
P48047	ATP5PO	ATP synthase subunit O, mitochondrial	0,47	0,041
P08294	SOD3	Extracellular superoxide dismutase	0,47	0,02
Q9BX68	HINT2	Histidine triad nucleotide-binding protein 2, mitochondrial	0,46	0,021
P22105	TNXB	Tenascin-X	0,44	0,022

Uniprot	Gene	Name	Fold change (MIH / Normoxia)	*p*-value
O76070	SNCG	Gamma-synuclein	0,43	0,025
P11177	PDHB	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	0,42	0,03
P16083	NQO2	Ribosyldihydronicotinamide dehydrogenase [quinone]	0,33	0,014

Supplementary Table 3. Functional groups affected by mild intermittent hypoxia exposure. After functional annotation of differentially expressed proteins, 11 functional groups were identified ($p<0.05$, corrected with Bonferroni step-down procedure) covering 104 GO terms and KEGG pathways, which were altered by mild intermittent hypoxia exposure. *p*-values are corrected with Bonferroni step-down procedure. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

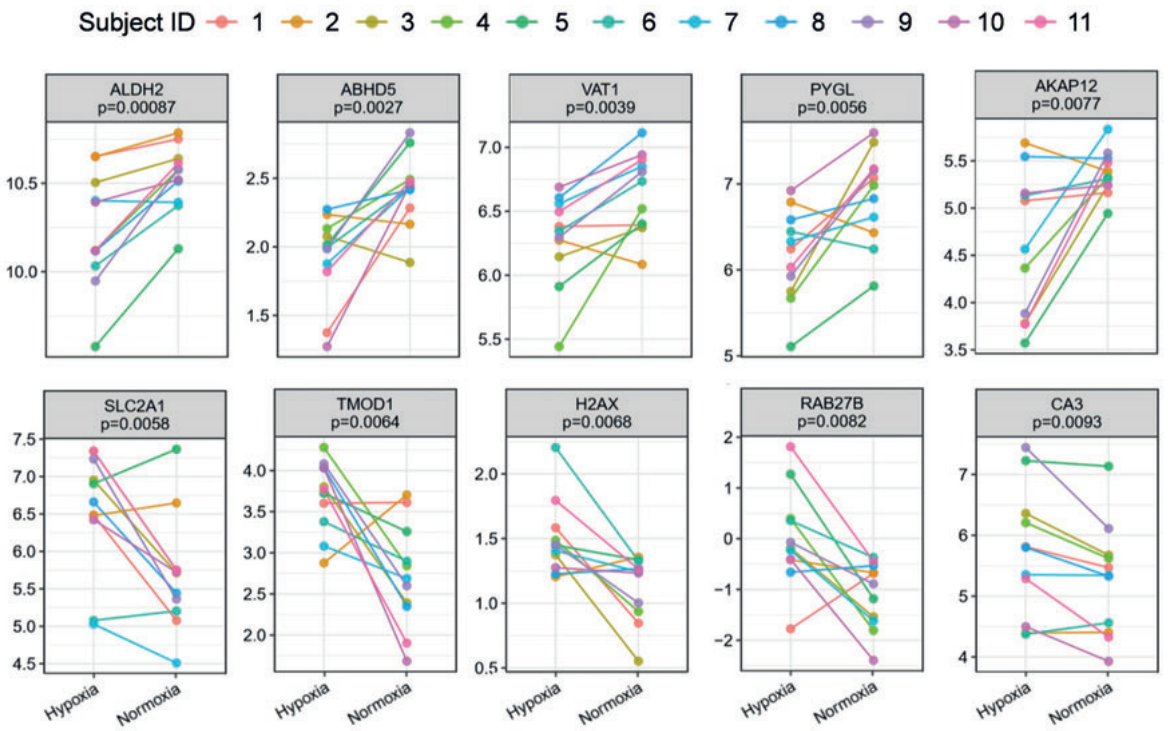
Functional group	GO Term	GO ID	GO Term *p*-value	Functional group *p*-value
cellular iron ion homeostasis	cellular iron ion homeostasis	GO:0006879	5,86E-02	1,17E-02
bicarbonate transport	bicarbonate transport	GO:0015701	9,01E-02	9,66E-03
platelet degranulation	platelet degranulation	GO:0002576	8,23E-02	1,03E-02
collagen fibril organization	collagen fibril organization	GO:0030199	2,41E-02	3,05E-03
myeloid cell development	myeloid cell development	GO:0061515	7,39E-02	9,48E-03
Regulation of lipolysis in adipocytes	Regulation of lipolysis in adipocytes	KEGG:04923	3,23E-02	1,03E-02
	PPAR signaling pathway	KEGG:03320	7,23E-02	1,03E-02
	neutral lipid catabolic process	GO:0046461	9,61E-02	1,03E-02
	glycerolipid catabolic process	GO:0046503	4,39E-02	1,03E-02
	acylglycerol catabolic process	GO:0046464	9,61E-02	1,03E-02
	triglyceride catabolic process	GO:0019433	7,43E-02	1,03E-02
ADP metabolic process	ADP metabolic process	GO:0046031	2,38E-02	4,91E-03
	purine nucleoside diphosphate metabolic process	GO:0009135	3,68E-02	4,91E-03
	ribonucleoside diphosphate metabolic process	GO:0009185	3,92E-02	4,91E-03
	ATP generation from ADP	GO:0006757	7,41E-02	4,91E-03

Functional group	GO Term	GO ID	GO Term *p*-value	Functional group *p*-value
	purine ribonucleoside diphosphate metabolic process	GO:0009179	3,68E-02	4,91E-03
	glycolytic process	GO:0006096	7,36E-02	4,91E-03
polysaccharide biosynthetic process	polysaccharide biosynthetic process	GO:0000271	2,03E-02	1,23E-02
	Starch and sucrose metabolism	KEGG:00500	7,40E-02	1,23E-02
	polysaccharide metabolic process	GO:0005976	8,86E-02	1,23E-02
	energy reserve metabolic process	GO:0006112	9,66E-02	1,23E-02
	cellular carbohydrate biosynthetic process	GO:0034637	9,96E-02	1,23E-02
	cellular polysaccharide metabolic process	GO:0044264	1,00E-01	1,23E-02
	glucan metabolic process	GO:0044042	8,21E-02	1,23E-02
	cellular glucan metabolic process	GO:0006073	8,21E-02	1,23E-02
	cellular polysaccharide biosynthetic process	GO:0033692	3,24E-02	1,23E-02
	glycogen metabolic process	GO:0005977	8,09E-02	1,23E-02
	glucan biosynthetic process	GO:0009250	1,04E-01	1,23E-02
	glycogen biosynthetic process	GO:0005978	1,04E-01	1,23E-02
Actin cytoskeleton organization	substrate adhesion-dependent cell spreading	GO:0034446	7,37E-04	1,04E-06
	Cardiac muscle contraction	KEGG:04260	8,47E-02	1,04E-06
	Hypertrophic cardiomyopathy (HCM)	KEGG:05410	9,26E-02	1,04E-06
	Dilated cardiomyopathy (DCM)	KEGG:05414	9,66E-02	1,04E-06
	actin-mediated cell contraction	GO:0070252	1,59E-03	1,04E-06
	muscle cell migration	GO:0014812	8,98E-02	1,04E-06
	actin-myosin filament sliding	GO:0033275	9,36E-03	1,04E-06
	regulation of ATPase activity	GO:0043462	8,21E-02	1,04E-06
	smooth muscle cell migration	GO:0014909	7,05E-02	1,04E-06
	muscle filament sliding	GO:0030049	9,36E-03	1,04E-06
	myofibril assembly	GO:0030239	6,77E-02	1,04E-06
	ruffle organization	GO:0031529	1,05E-01	1,04E-06
	positive regulation of ATPase activity	GO:0032781	9,55E-02	1,04E-06

Functional group	GO Term	GO ID	GO Term *p*-value	Functional group *p*-value
	negative regulation of protein polymerization	GO:0032272	7,28E-02	1,04E-06
	positive regulation of actin filament bundle assembly	GO:0032233	5,18E-02	1,04E-06
	negative regulation of actin filament polymerization	GO:0030837	4,63E-02	1,04E-06
Focal adhesion	Focal adhesion	KEGG:04510	6,87E-04	2,80E-09
	ECM-receptor interaction	KEGG:04512	2,27E-03	2,80E-09
	Prion diseases	KEGG:05020	6,98E-02	2,80E-09
	Toxoplasmosis	KEGG:05145	4,80E-02	2,80E-09
	Small cell lung cancer	KEGG:05222	8,98E-02	2,80E-09
	muscle cell migration	GO:0014812	8,98E-02	2,80E-09
	substrate adhesion-dependent cell spreading	GO:0034446	7,37E-04	2,80E-09
	cell-substrate junction organization	GO:0150115	9,14E-03	2,80E-09
	smooth muscle cell migration	GO:0014909	7,05E-02	2,80E-09
	extracellular matrix assembly	GO:0085029	9,27E-02	2,80E-09
	positive regulation of cell-matrix adhesion	GO:0001954	9,23E-02	2,80E-09
	cell-substrate junction assembly	GO:0007044	7,25E-03	2,80E-09
	neuromuscular junction development	GO:0007528	1,14E-01	2,80E-09
	basement membrane organization	GO:0071711	5,08E-03	2,80E-09
	glomerulus development	GO:0032835	8,67E-02	2,80E-09
	glomerular basement membrane development	GO:0032836	4,82E-03	2,80E-09
	positive regulation of actin filament bundle assembly	GO:0032233	5,18E-02	2,80E-09
Oxidoreductase activity	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	GO:0016616	2,72E-05	4,67E-10
	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	GO:0016620	5,95E-04	4,67E-10
	oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	GO:0016628	3,93E-02	4,67E-10

Functional group	GO Term	GO ID	GO Term *p*-value	Functional group *p*-value
	oxidoreductase activity, acting on NAD(P)H, quinone or similar compound as acceptor	GO:0016655	4,90E-03	4,67E-10
	tricarboxylic acid cycle	GO:0006099	8,73E-02	4,67E-10
	ethanol metabolic process	GO:0006067	2,54E-02	4,67E-10
	aerobic respiration	GO:0009060	9,96E-02	4,67E-10
	aldehyde dehydrogenase [NAD(P)+] activity	GO:0004030	1,28E-02	4,67E-10
	diterpenoid metabolic process	GO:0016101	7,68E-02	4,67E-10
	aldehyde dehydrogenase (NAD+) activity	GO:0004029	9,05E-03	4,67E-10
	acyl-CoA metabolic process	GO:0006637	1,11E-01	4,67E-10
	retinoid metabolic process	GO:0001523	9,96E-02	4,67E-10
	Glycolysis / Gluconeogenesis	KEGG:00010	3,23E-05	4,67E-10
	Citrate cycle (TCA cycle)	KEGG:00020	5,18E-02	4,67E-10
	Fatty acid elongation	KEGG:00062	4,09E-02	4,67E-10
	Fatty acid degradation	KEGG:00071	4,13E-05	4,67E-10
	Valine, leucine and isoleucine degradation	KEGG:00280	1,42E-03	4,67E-10
	Lysine degradation	KEGG:00310	4,09E-02	4,67E-10
	Arginine and proline metabolism	KEGG:00330	9,72E-02	4,67E-10
	Histidine metabolism	KEGG:00340	2,86E-02	4,67E-10
	Tyrosine metabolism	KEGG:00350	7,40E-02	4,67E-10
	Tryptophan metabolism	KEGG:00380	7,44E-04	4,67E-10
	beta-Alanine metabolism	KEGG:00410	5,47E-02	4,67E-10
	Glutathione metabolism	KEGG:00480	1,07E-01	4,67E-10
	Pyruvate metabolism	KEGG:00620	9,36E-03	4,67E-10
	Butanoate metabolism	KEGG:00650	4,48E-02	4,67E-10
	Metabolism of xenobiotics by cytochrome P450	KEGG:00980	1,18E-02	4,67E-10
	Drug metabolism	KEGG:00982	6,21E-02	4,67E-10
	Chemical carcinogenesis	KEGG:05204	1,60E-02	4,67E-10
	Central carbon metabolism in cancer	KEGG:05230	5,42E-02	4,67E-10
	Detoxification	GO:0098754	3,92E-02	4,67E-10

Functional group	GO Term	GO ID	GO Term *p*-value	Functional group *p*-value
	electron transport chain	GO:0022900	4,94E-03	4,67E-10
	thioester metabolic process	GO:0035383	1,11E-01	4,67E-10
	cellular detoxification	GO:1990748	1,61E-02	4,67E-10
	xenobiotic metabolic process	GO:0006805	8,23E-02	4,67E-10
	oxidoreductase activity, acting on CH-OH group of donors	GO:0016614	4,31E-05	4,67E-10
	oxidoreductase activity, acting on the CH-CH group of donors	GO:0016627	9,19E-02	4,67E-10
	oxidoreductase activity, acting on the aldehyde or oxo group of donors	GO:0016903	3,76E-03	4,67E-10
	antibiotic metabolic process	GO:0016999	2,19E-02	4,67E-10
	drug catabolic process	GO:0042737	9,84E-02	4,67E-10
	electron transfer activity	GO:0009055	1,40E-02	4,67E-10
	antibiotic catabolic process	GO:0017001	5,06E-02	4,67E-10
	primary alcohol metabolic process	GO:0034308	4,07E-03	4,67E-10
	ethanol oxidation	GO:0006069	4,82E-03	4,67E-10



Supplementary Figure 1. Differentially expressed proteins in abdominal subcutaneous adipose tissue following MIH and normoxia exposure. Log2 protein expression is depicted for each individual separately (*n*=11), with corresponding *p*-value for top five up/downregulated (based on *p*-value) protein. ALDH2, aldehyde dehydrogenase 2; ABHD5, abhydrolase domain containing 5; VAT1, vesicle amine transport 1; PYGL, glycogen phosphorylase l; AKAP12, a-kinase anchoring protein 12; SLC2A1, solute carrier type 2A1/glucose transporter-1; TMOD1, tropomodulin-1; H2AX, H2A histone family member X; CA3, carbonic anhydrase III.

CHAPTER 5

The impact of mild hypoxia exposure on myokine secretion in human obesity

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Ready to be submitted

Abstract

Aims/hypothesis: Diet and physical exercise modulate myokine secretion by skeletal muscle (SM). Compelling evidence indicates that myokines act in an autocrine, paracrine and endocrine manner to alter metabolic homeostasis. The mechanisms underlying exercise-induced changes in myokine secretion remain to be elucidated. Since exercise acutely decreases oxygen partial pressure (pO_2) in SM (*in situ* hypoxia), the present study was designed to test the hypothesis that 1) hypoxia exposure impacts myokine secretion in primary human myotubes and 2) exposure to mild hypoxia *in vivo* alters plasma myokine concentrations in humans.

Methods: Differentiated primary human myotubes were exposed to 1% O_2 (mimicking physiological hypoxia in SM), 3% O_2 (physiological normoxia in SM) and 21% O_2 (standard laboratory conditions) for 24h, and cell culture medium was harvested to determine myokine secretion. Furthermore, we performed a randomized single-blind crossover trial to investigate the impact of mild intermittent hypoxia exposure (MIH: 7-day exposure to 15% O_2, 3x2h/day vs. normoxia: 21% O_2; 3-6 week washout period) on *in vivo* SM pO_2 (assessed on day 6 using a microdialysis-based optochemical measurement system) and plasma myokine concentrations in 12 individuals with overweight and obesity (body mass index ≥ 28 kg/m²). Plasma concentrations of interleukin-6 (IL-6), interleukin-15 (IL-15), secreted protein acidic and rich in cysteine (SPARC), leukemia inhibitory factor (LIF), oncostatin M, osteocrin/musclin, brain-derived neurotrophic factor (BDNF), follistatin-related protein (FSTL1), fatty acid binding protein 3 (FABP3), apelin, fractalkine and erythropoietin were determined during a high-fat mixed-meal test under hypoxic conditions (day 7), and under normoxic conditions at day 8 (~16 hours after the final hypoxic stimulus).

Results: Hypoxia exposure (1% O_2) increased SPARC (p=0.043) and FSTL1 (p=0.021), and reduced LIF secretion (p=0.009) compared to 3% O_2 in primary human myotubes. In addition, 1% O_2 exposure increased IL-6 (p=0.004) and SPARC (p=0.021) secretion, whilst reducing FABP3 secretion (p=0.021) compared to 21% O_2. *In vivo*, hypoxia exposure markedly decreased SM pO_2 (≈40%, p=0.002) but did neither alter plasma myokine concentrations under fasting nor postprandial conditions. Moreover, no differences in plasma myokine concentrations were found following seven days of MIH exposure in individuals with overweight/obesity.

Conclusions: We demonstrate for the first time that hypoxia exposure alters the secretion of several myokines in primary human myotubes, revealing hypoxia as a novel modulator of myokine secretion. However, both acute and 7-day MIH exposure *in vivo* do not induce alterations in plasma myokine concentrations in individuals with overweight and obesity.

Keywords:

FSTL1; Hypoxia; Insulin resistance; LIF; Myotubes; Obesity; Oxygen tension; Skeletal muscle; SPARC.

Abbreviations

AUC:	Area under the curve
AMPK:	5'AMP-activated protein kinase
BDNF:	Brain-derived neurotrophic factor
GLUT4:	Glucose transporter 4
FABP3:	Fatty acid binding protein 3
FGF21:	Fibroblast growth factor 21
FSTL1:	Follistatin-related protein 1
HIF1α:	Hypoxia-inducible factor α
iAUC:	Incremental area under the curve
IL-6:	Interleukin-6
IL-15:	Interleukin-15
LIF:	Leukemia inhibitory factor
MIH:	Mild intermittent hypoxia
pO_2:	Oxygen partial pressure
SM:	Skeletal muscle
SPARC:	Secreted protein acidic and rich in cysteine

Research in context

What is already known about this subject?

- Exercise and muscular contraction induce myokine secretion, which might contribute to improvements in metabolic homeostasis.
- Exercise evokes hypoxia in skeletal muscle.

What is the key question?

- What is the impact of mild hypoxia exposure on 1) myokine secretion in primary human myotubes and 2) plasma myokine concentrations in individuals with overweight and obesity?

What are the new findings?

- The present data demonstrate that hypoxia exposure alters the secretion of several myokines in primary human myotubes, revealing hypoxia as a novel modulator of myokine secretion.
- Mild hypoxia exposure increases SPARC and FSTL1, and reduces LIF secretion by primary human myotubes.
- However, both acute and prolonged (7-day) exposure to mild intermittent hypoxia *in vivo* do not induce alterations in plasma myokine concentrations in individuals with overweight and obesity.

How might this impact on clinical practice in the foreseeable future?

- It is well established that physical activity improves metabolic homeostasis, which is at least partly mediated by the secretion of myokines. Hypoxia exposure might provide a novel strategy to induce myokine secretion, thereby eliciting beneficial metabolic effects.

Introduction

According to the WHO, approximately 2 million deaths per year are attributed to physical inactivity(1). A sedentary lifestyle, which characterizes 60-85% of the world population, drastically increases the risk of developing obesity and related complications such as type 2 diabetes mellitus (T2DM) and cardiovascular diseases. Exercise has been proven to alleviate detrimental metabolic processes induced by sedentary lifestyle. Indeed, compelling evidence indicates that exercise improves glycemic control and cardiovascular health in people living with obesity and insulin resistance(2, 3). Furthermore, physical exercise seems to improve mitochondrial function and induces adaptations in energy metabolism in key metabolic organs such as human skeletal muscle (SM)(4), adipose tissue(5-7) and the liver(8).

Whilst performing exercise, various myokines are produced and secreted by SM. These myokines can in turn exert autocrine, paracrine and endocrine effects, thereby contributing to crosstalk between SM and other organs(9). The endocrine function of SM has been widely recognized since the identification of interleukin-6 (IL-6), which systemic concentrations increase up to 100-fold after exercise(10). Paradoxically, IL-6 has been implicated in obesity-induced insulin resistance in metabolically comprised conditions, whereas exercise-induced release of IL-6 may contribute to the insulin-sensitizing effects of physical exercise(11). Indeed, contraction-induced myokine secretion has been implicated in the beneficial metabolic adaptations evoked by physical activity(9, 12), amongst which are enhanced SM glucose uptake and fatty acid oxidation(13). Therefore, myokines are promising targets for the prevention and treatment of obesity-induced insulin resistance, cardiovascular diseases and T2DM.

Exercise-induced contraction of SM is dependent upon generation of ATP, a process that is largely supported by oxidative phosphorylation. Previously, it has been estimated that high-intensity exercise reduces intracellular SM oxygen partial pressure (pO_2) from 25 mmHg (≈ 3% O_2) to 8 mmHg (≈1% O_2) in untrained individuals(14, 15), emphasizing the importance of oxygen in SM contraction. Interestingly, we recently found similar reductions in pO_2 in *m. gastrocnemius* during passive mild intermittent hypoxia (MIH) exposure (15% FiO_2) in overweight and obese men (Chapter 3). These findings imply that both SM contraction and passive MIH exposure markedly decrease SM pO_2 in humans, which in turn may elicit metabolic adaptations in SM(16). It is therefore tempting to postulate that passive mild hypoxia exposure might affect myokine secretion in humans. However, studies that have investigated the effects of hypoxia exposure on myokine secretion in humans are lacking.

The aims of the present translational studies were 1) to explore the impact of 24h mild hypoxia exposure on myokine secretion in differentiated primary human myotubes and 2) to investigate the effects of MIH exposure on plasma myokine concentrations in individuals with overweight and obesity. To address these

objectives, we performed *in vitro* hypoxia exposure experiments in primary human myotubes, and executed a randomized single-blind crossover trial in which 12 individuals with overweight and obesity were exposed to MIH for seven consecutive days under well-controlled conditions.

Methods

Hypoxia exposure in primary human myotubes

Primary human satellite cells were obtained from *m. rectus abdominis* muscle tissue of a lean, insulin sensitive male subject, and were cultured as described previously (Chapter 3). Briefly, satellite cells were cultured using proliferation medium, consisting of low glucose (1000 mg•l^{-1}) Dulbecco's Modified Eagle's Medium (DMEM, cat#D6046, Sigma) supplemented with 0.05% bovine serum albumin (BSA, cat#A4503, Sigma), 1 µM Dexamethasone (cat#D4902, Sigma), 16% fetal bovine serum (FBS, cat#BDC-6086, Bodinco BV) 0.5 mg•ml^{-1} bovine fetuin (cat#10344-034/026, Invitrogen, Life Technologies), 1x Antibiotic-Antimycotic (cat#15240-62, Gibco, Thermo Fisher Scientific) and 0.01 µg•ml^{-1} recombinant human epidermal growth factor (cat#PHG0311, Gibco). At 70-80% confluence, differentiation medium was added containing MEM-α-Glutamax (cat#32561-029, Gibco) supplemented with 2% FBS (Bodinco BV), 0.5 mg•ml^{-1} bovine fetuin (Invitrogen, Life Technologies) and 1x Antibiotic-Antimycotic (100 units•ml^{-1} penicillin, 100 µg•ml^{-1} of streptomycin and 0.25 µg•ml^{-1} Amphotericin B (cat#15240-62, Gibco)) until myotube formation was completed.

Next, to determine the effects of hypoxia exposure on myokine secretion, primary human myotubes were exposed to 1% O_2 (mimicking physiological hypoxia in SM), 3% O_2 (physiological normoxia in SM) and 21% O_2 (standard laboratory conditions) for 24h using in-house developed hypoxic chambers(17). Briefly, myotubes were incubated in sealed chambers with an inlet and outlet for gasses. Gasses were composed of 5% CO_2, 1%, 3% or 21% O_2, and the remainder N_2 under humidified conditions (2g/l H_2O). Composition and inlet of the gas mixture were controlled by mass flow controllers (Bronkhorst, Ruurlo, The Netherlands) and continuous measurements of oxygen levels (Servomex, Zoetermeer, The Netherlands). Gas mixtures were refreshed every 8 hours to assure oxygen levels remained constant throughout the experiment. Experiments were six times individually replicated (n=6). Finally, cell culture medium was harvested to determine myokine concentrations, as described in more detail below.

Prolonged mild hypoxia exposure in humans with overweight and obesity

Subjects

Twelve male individuals with overweight and/or obesity (BMI ≥28 kg/m^2), with a homeostatic model for assessment of insulin resistance (HOMA-IR) ≥2.2 (30-65 years) participated in a randomized single-blind crossover trial, which was designed to investigate the impact of MIH on tissue-specific insulin sensitivity and substrate metabolism in obese individuals (Netherlands Trial Register, NTR7325) (Chapter 3). 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. Participants were asked to refrain from drinking alcohol and perform no exercise 24h prior to the start and during exposure regimen. The study was performed according to the declaration of Helsinki (as revised in 2008) 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 this study has been described in detail previously (Chapter 3). Briefly, participants enrolled in this randomized, single-blind, crossover study were exposed to normobaric MIH (15% FiO_2) and normobaric normoxia (21% FiO_2) for 7 consecutive days (3 cycles of 2h/d with 1h of normoxia exposure between hypoxic cycles), separated by a 3-6 week washout period, in a randomized fashion. Systemic oxygen saturation levels were continuously monitored throughout the exposure regimens by pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcor).

At day 6 of the exposure regimens, SM pO_2 was determined using an optochemical measurement system for continuous monitoring of tissue pO_2, as described previously (18). At day 7, a high-fat mixed-meal test was performed, during which individuals were asked to ingest a liquid test meal, providing 2.6MJ (consisting of 61 E% fat, 33 E% carbohydrates and 6 E% protein), within 5 min. Blood samples were collected from a superficial dorsal hand vein, which was arterialized by placing the hand into a hotbox (~55˚C). Blood samples were taken under fasting (t=0 min) and postprandial conditions (t=30, 60, 90, 120, 180, and 240 min). At day 8, subsequent to the 7-day MIH/normoxia exposure regimens, fasting blood samples were collected again.

Myokine analyses

Myokine concentrations were determined in 1) human cell culture medium derived from the human myotube experiments, and 2) human plasma samples from the randomized, single-blind, crossover study using the MILLIPLEX MAP Human Myokine Magnetic Bead Panel kit (cat#HMYOMAG-56K, Merck, Kenilworth, NJ, USA), according to the manufacturer's guidelines. The concentrations of the following myokines were determined: apelin, brain-derived neurotrophic factor (BDNF), fatty-acid binding protein-3 (FABP3), fractalkine, follistatin-like protein-1 (FSTL1), interleukin-6 (IL-6), IL-15, leukemia inhibitory factor (LIF), oncostatin M, osteocrin and secreted protein acidic and rich in cysteine (SPARC). Results were

quantified in pg•ml^{-1}, except for SPARC that was expressed in ng•ml^{-1}. Human plasma samples (*n*=12) and *in vitro* samples (*n*=6) were measured in duplicate.

Statistical analyses

To investigate the effects hypoxia exposure on myokine secretion in human primary myotubes, a non-parametric Friedman's test with Dunn's post-hoc comparison test was performed. Data obtained from the human intervention study was checked for normality by means of the Shapiro-Wilk test. The impact of MIH exposure on plasma myokine concentrations was determined using two-tailed Student's paired *t*-test. Pearson's correlation analysis was performed to determine associations between MIH-induced alterations in SM pO_2 and plasma myokine concentrations. *p*-values <0.05 were considered to be statistically significant. Statistical analyses were performed using SPSS 24.0. Data are expressed as means ± SEM.

Results

Hypoxia alters myokine secretion in primary human myotubes

In primary human myotubes, hypoxia (1% O_2) exposure increased the secretion of SPARC (p=0.043, Figure 1A) and FTSL1 (*p*=0.021, Figure 1B), and reduced LIF secretion (*p*=0.009, Figure 1C) compared to 3% O_2. In addition, 1% O_2 exposure increased IL-6 (*p*=0.004, Figure 1D) and SPARC (*p*=0.021, Figure 1A), and reduced FABP3 secretion (*p*=0.021, Figure 1E) relative to 21% O_2. Hypoxia (1% O_2) exposure did not affect BDNF secretion (Figure 1F). In addition, 3% O_2 exposure increased LIF (*p*=0.009), but decreased FTSL1 (*p*=0.043) and FABP3 (*p*=0.043) secretion compared to 21% O_2 (Figure 1).

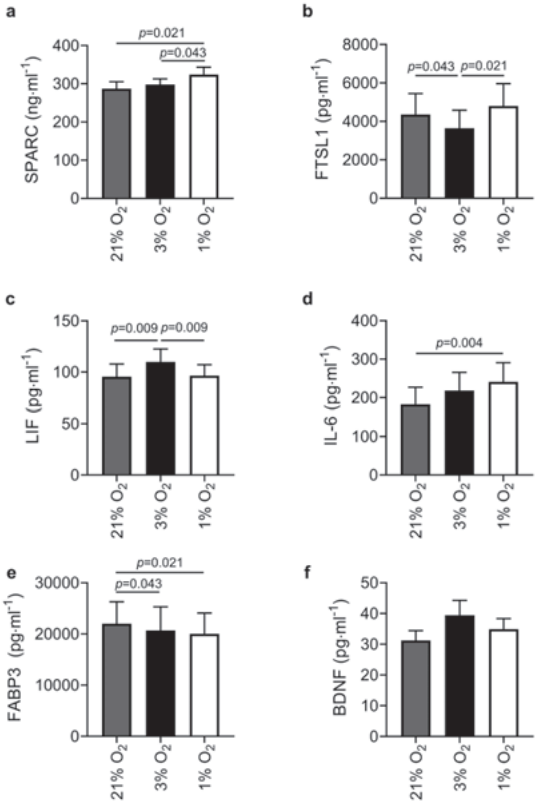

Figure 1. Primary human myotubes were exposed to 21% (standard lab conditions), 3% (normoxia), 1% O2 (hypoxia) for 24h, and subsequently (**A**) SPARC, (**B**) LIF, (**C**) FSTL1, (**D**) FABP3, (**E**) IL-6, (**F**) BDNF secretion in medium supernatant was measured. Data are expressed as mean ± SEM (*n*=6) in ng•ml^{-1} (SPARC) and pg•ml^{-1}. Statistical analysis was performed using nonparametric Friedman's test with Dunn's *post-hoc* multiple comparison test. SPARC, secreted protein acidic and rich in cystein; LIF, leukemia inhibitory factor; FSTL1, follistatin-related protein 1; FABP3, fatty acid binding protein 3; IL-6, interleukin-6; BDNF, brain-derived neurotrophic factor.

Mild intermittent hypoxia does not impact plasma myokine concentrations

The characteristics of the study participants are depicted in Table 1. By design, male participants were overweight/obese (BMI >28 kg/m²) and had a HOMA-IR ≥2.2. MIH exposure reduced SpO_2 (normoxia: 97.1 ± 0.3 *vs.* hypoxia: 92.0 ± 0.5 %, $p<0.001$) and induced a pronounced decrease in SM pO_2 (normoxia: 15.4 ± 2.4 mmHg *vs.* hypoxia: 9.5 ± 2.2 mmHg, $p=0.002$). These SM pO_2 values correspond with ≈2% O_2 (normoxia exposure) and ≈1.3% O_2 (MIH exposure), respectively. No adverse events were reported.

Table 1. Baseline characteristics of study participants.

	Baseline
Age (y)	61 ± 1
BMI (kg/m²)	30.8 ± 0.9
Hemoglobin (mmol•l^{-1})	9.5 ± 0.5
HbA_{1c} (mmol•mol^{-1})	37.9 ± 0.9
HbA_{1c} (%)	5.6 ± 0.1
Fasting glucose (mmol•l^{-1})	5.7 ± 0.2
2h-glucose (mmol•l^{-1})	6.2 ± 0.4
HOMA-IR	3.7 ± 0.4

BMI, body-mass index; HbA_{1c}, glycated hemoglobin; 2h-glucose, glucose concentration after 2h during oral glucose tolerance test; HOMA-IR, homeostatic model assessment for insulin resistance.

Fasting and postprandial plasma concentrations (Figure 2A) of apelin ($p_{fasting}$=0.515, p_{AUC}=0.973), BDNF ($p_{fasting}$=0.519, p_{AUC}=0.173), EPO ($p_{fasting}$=0.875, p_{AUC}=0.937), SPARC ($p_{fasting}$=0.653, p_{AUC}=0.352), LIF ($p_{fasting}$=0.648, p_{AUC}=0.676), FABP3 ($p_{fasting}$=0.813, p_{AUC}=0.481), FSTL1 ($p_{fasting}$=0.271, p_{AUC}=0.436), oncostatin M ($p_{fasting}$=0.610, p_{AUC}=0.978), IL-6 ($p_{fasting}$=0.804), IL-15 ($p_{fasting}$=0.580), osteocrin ($p_{fasting}$=0.908, p_{AUC}=0.456) and fractalkin ($p_{fasting}$=0.854, p_{AUC}=0.638) were not altered during acute exposure to hypoxia relative to normoxia. Furthermore, plasma myokine concentrations assessed under normoxic conditions at day 8 (that is ~16h after the final hypoxic stimulus of the 7-day exposure to either MIH or normoxia) were not significantly altered by MIH compared to normoxia exposure (Fig. 3A-L). Furthermore, we found an inverse correlation between the relative MIH-induced decrease in SM pO_2 and relative changes in LIF plasma concentration (r=-0.670, p=0.048). MIH-induced changes SM pO_2 were not significantly associated with alterations in the levels of other myokines following MIH (data not shown).

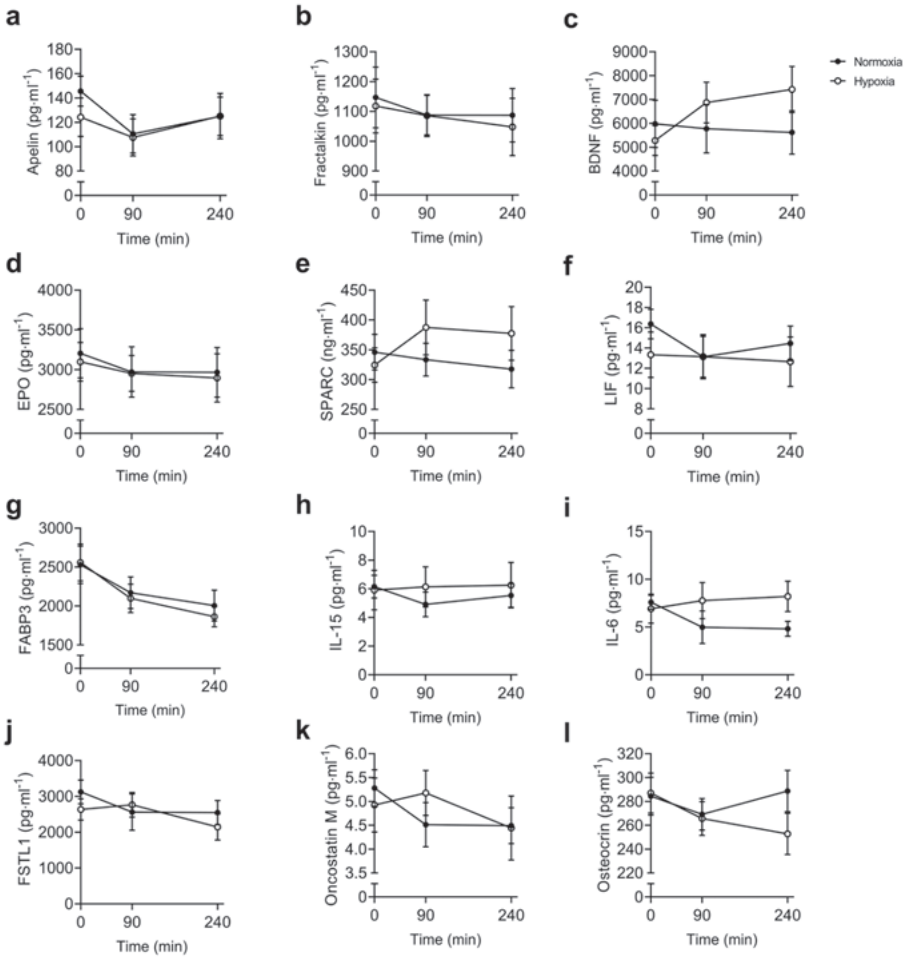

Figure 2. Plasma myokine concentrations in overweight and obese insulin resistant men during the high-fat mixed meal challenge, which was performed under mild intermittent hypoxia (3x2h per day, 15% O2) and normoxia. Plasma concentrations of (**A**) apelin, (**B**) fractalkine, (**C**) BDNF, (**D**) erytropoetin, (**E**) SPARC, (**F**) LIF, (**G**) FABP3, (**H**) IL-15, (**K**) IL-6, (**I**) FSTL1, (**J**) oncostatin M, and (**L**) osteocrin were determined. Data are expressed as mean ± SEM (n=12) in ng•ml-1 (SPARC) and pg•ml-1. (incremental) area under the curve values were determined using the trapezoidal method. Statistical analysis was performed using a two-tailed Student's paired t-test. Open circles, hypoxia; closed circles, normoxia. BDNF, brain-derived neurotrophic factor; SPARC, secreted protein acidic and rich in cystein; LIF, leukemia inhibitory factor; IL-15, interleukin-15; FABP3, fatty acid binding protein 3; FSTL1, follistatin-related protein 1; IL-6, interleukin-6.

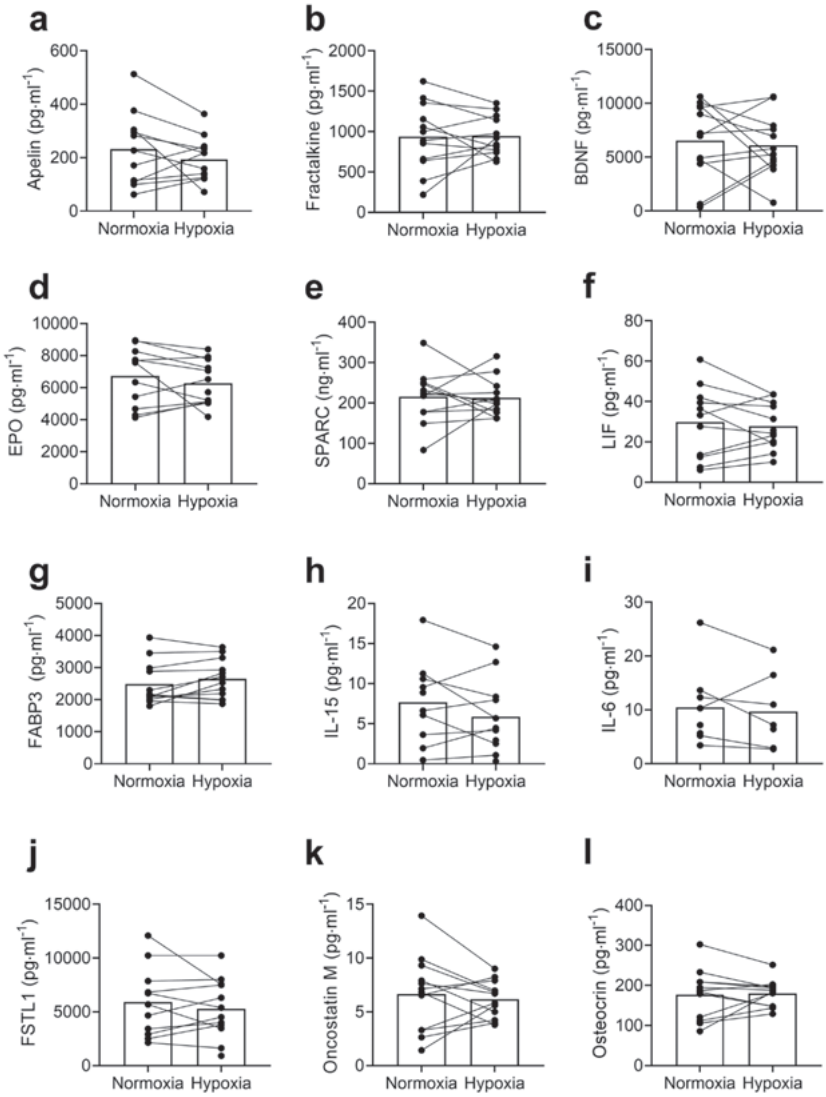

Figure 3. Overweight and obese insulin resistant men were exposed to mild intermittent hypoxia (3x2h per day, 15% O2) or normoxia for seven consecutive days and subsequently analysis took place to determine secretion of (**A**) apelin, (**B**) fractalkine, (**C**) BDNF, (**D**) erytropoetin, (**E**) SPARC, (**F**) LIF, (**G**) FABP3, (**H**) IL-15, (**K**) IL-6, (**I**) FSTL1, (**J**) oncostatin M, and (**L**) osteocrin in human plasma samples derived at day 8, under normoxic conditions. Data are expressed as individual data points (n=12) in ng•ml-1 (SPARC) and pg•ml-1. Bars represent mean per condition. Statistical analysis was performed using a two-tailed Student's paired t-test. BDNF, brain-derived neurotrophic factor; SPARC, secreted protein acidic and rich in cystein; LIF, leukemia inhibitory factor; IL-15, interleukin-15; FABP3, fatty acid binding protein 3; FSTL1, follistatin-related protein 1; IL-6, interleukin-6.

Discussion

In the present study, we investigated the impact of mild hypoxia exposure on *in vitro* myokine secretion in primary human myotubes and *in vivo* plasma myokine concentrations in individuals with overweight and obesity. Our findings demonstrate for the first time that hypoxia exposure alters the secretion of several myokines in primary human myotubes, thereby revealing hypoxia as a novel modulator of myokine secretion. Moreover, we found that both acute and 7-day MIH exposure *in vivo* do not induce alterations in plasma myokine concentrations in individuals with overweight and obesity.

Intriguingly, we found that mild hypoxia exposure for 24h increased SPARC secretion by human myotubes. SPARC is recognized for its role in extracellular matrix organization(19), and appears to be essential for maintenance of glucose homeostasis(20). Indeed, SPARC induces AMPK-mediated glucose uptake through regulation of GLUT4 expression in rodent myocytes(21). We have recently demonstrated that hypoxia exposure (1% O_2) increased insulin-independent glucose uptake through AMPK in primary human myotubes (Chapter 3). Interestingly, it has previously been demonstrated that an acute bout of moderate-intensity exercise, which lowers SM pO_2(22, 23) and activates AMPK(24), increased SPARC serum concentrations(25). Interestingly, we also found that mild hypoxia exposure dose-dependently induces IL-6 secretion in human myotubes. IL-6 is released upon exercise(26), and has been shown to improve glucose metabolism in skeletal muscle in rodents and humans(27). Thus, the present findings may imply that hypoxia is an upstream regulator of IL-6 and SPARC-induced glucose uptake in human myotubes. In the present study, however, we also found that neither acute hypoxia exposure during fasting and postprandial conditions subsequent to high-fat mixed meal challenge (measurements at day 7) nor 7-day MIH exposure (cumulative effects assessed at day 8) did alter SPARC and IL-6 plasma concentrations. Therefore, it might be that the hypoxic stimulus *in vivo* was not severe or long enough to detect changes in plasma myokine concentrations. Alternatively, myokines that are locally produced following hypoxia exposure may not reach the circulation to exert endocrine effects.

Moreover, we found that hypoxia exposure induced FTSL1 secretion in primary myotubes. Interestingly, exercise has been shown to increase serum FSTL1 concentrations in well-trained lean humans. Furthermore, it has previously been found that human myotubes indeed secrete FSTL1 upon IFNγ and IL-1β stimulation(28). In addition, FSTL1 knockdown results in ablation of basal Akt signaling in cardiac myocytes(29), and the expression of FSTL1 is positively correlated with brown adipose activation and hence thermogenesis in rodents(30). On the other hand, Fan and coworkers(31) showed that FSTL1 serum levels were higher in people with overweight and obesity as compared to lean controls, and

may induce adipose tissue inflammation and insulin resistance. Nevertheless, we did neither find changes in plasma FSTL1 concentrations during acute (day 7) nor following 7-day MIH compared to hypoxia exposure, which may suggest that a more severe or more prolonged hypoxic stimulus is needed to evoke changes in plasma myokine concentrations *in vivo*.

Furthermore, the present data demonstrate that human myotubes secreted less LIF subsequent to hypoxia compared to normoxia exposure. In line, we found an inverse correlation between the MIH-induced decrease in SM pO_2 and LIF plasma concentration. LIF treatment has been shown to diminish early myogenic differentiation in mouse C2C12 myoblasts(32). In addition, acute LIF treatment increased glucose uptake, independent of insulin, whereas chronic treatment induced insulin resistance, possibly through altered GLUT4 expression and reduced insulin-mediated Akt phosphorylation as has been shown in rat cardiomyocytes(33). Taken together, hypoxia exposure appears to reduce LIF secretion, which was further exemplified by an inverse association between the absolute reduction in SM pO_2 and MIH-induced alteration in LIF plasma concentration.

One of the strengths of the present study is that we for the first time exposed primary human myotubes to *in situ* O_2 levels that mimic (mild) hypoxic and normoxic conditions in human SM (Chapter 3) to determine the impact on myokine secretion. This approach enables assessment of a causal relationship between physiological relevant pO_2 levels and myokine secretion in primary human myotubes. Furthermore, we not only explored the impact of acute hypoxia exposure under fasting and postprandial conditions, but also investigated the cumulative effect of MIH exposure for 7 consecutive days in individuals with overweight and obesity. A limitation of our study is that we cannot exclude that MIH affects the production and secretion of myokines by SM, since several of the myokines we determined can also be secreted by various other organs such as the liver and adipose tissue(34). Thus, the effects of MIH on plasma myokine concentrations may be influenced by hypoxia-induced effects in other organs.

To conclude, our findings show that for the first time that hypoxia exposure alters the secretion of several myokines in primary human myotubes, thereby revealing hypoxia as a novel modulator of myokine secretion. Moreover, we found that both acute and prolonged MIH exposure do not induce alterations in plasma myokine concentrations in individuals with overweight and obesity. Further research is warranted to investigate whether more prolonged and/or more severe hypoxia exposure regimens impact myokine secretion in humans, and to elucidate the functional implications of MIH-induced alterations in SPARC, FSTL1 and LIF secretion in human SM.

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Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Ethics declaration

All authors declare no duality of interest associated with this manuscript.

Contributions

RVM, EB and GG were responsible for the conceptualization and design of the studies. RVM, LV, and SH conducted the experiments, and contributed to data acquisition and analysis of data. GG acquired funding for the study. RVM wrote the manuscript, and LV, SH, SL, HAH, EB and GG critically revised the manuscript. All authors approved the final version of the manuscript for publication. G.G. is guarantor of the work.

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CHAPTER 6

Mild intermittent hypoxia exposure alters gut microbiota composition in men with overweight and obesity

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Ready to be submitted

Abstract

Results from high altitude studies in humans and controlled animal experiments suggest that hypoxia exposure induces alterations in gut microbiota composition, which may in turn affect host metabolism. However, well-controlled studies investigating the effects of normobaric hypoxia exposure on gut microbiota composition in humans are lacking.

The aim of this study was to explore the impact of mild intermittent hypoxia (MIH) exposure on gut microbiota composition in men with overweight and/or obesity. We performed a randomized, single-blind crossover study, in which participants were exposed to MIH (FiO_2: 15%, 3x2h per day) and normoxia (FiO_2: 21%) for seven consecutive days. Following the exposure regimens (day 8), fecal samples were collected for determination of fecal microbiota composition using 16S rRNA gene amplicon sequencing. Furthermore, tissue-specific insulin sensitivity was determined using the gold-standard two-step hyperinsulinemic-euglycemic clamp.

MIH did not affect microbial alpha and beta-diversity but reduced the relative abundance of *Christensenellaceae* and *Clostridiaceae* bacterial families. MIH significantly increased the abundances of several obligate anaerobic bacterial genera including *Fusicatenibacter*, *Butyricicoccus* and *Holdemania*, whilst reducing *Christensenellaceae R-7 group* and *Clostridium sensu stricto 1*, although these findings were not statistically significant after correction for multiple testing. Furthermore, MIH-induced alterations in abundances of several genera were associated with changes in metabolic parameters such as adipose and peripheral insulin sensitivity, plasma levels of insulin, fatty acids, triacylglycerol and lactate, and substrate oxidation. In conclusion, we demonstrate for the first time that MIH exposure induces modest effects on fecal microbiota composition in humans, shifting several bacterial families and genera towards higher abundances of anaerobic butyrate-producing bacteria. Moreover, MIH-induced effects on fecal microbial composition were associated with parameters related to glucose and lipid homeostasis, supporting a link between MIH-induced alterations in fecal microbiota composition and host metabolism.

Keywords: Mild intermittent hypoxia, gut microbiota, anaerobic bacteria, metabolism, obesity.

Introduction

The gastrointestinal microbiota consists of a highly complex composition, which has been demonstrated to affect brain function (1), immune defense (2), and host metabolism (3). Disturbances in gut microbiota composition (that is, microbiome dysbiosis), might contribute to altered substrate metabolism and energy expenditure, and may affect inflammatory processes as well as metabolism in the liver, skeletal muscle (SM) and adipose tissue (AT) (4). Thus, microbial dysbiosis might contribute to several chronic diseases such as the inflammatory bowel diseases Crohn's disease and ulcerative colitis, cardiovascular diseases and type 2 diabetes, and may contribute to the pro-inflammatory profile of these diseases (5, 6). Since the gut microbiota may play a key role in metabolic health, recent studies focus on strategies to modulate the gut microbiome, with the aim to prevent or alleviate the progression of cardiometabolic diseases.

Intriguingly, the oxygenation of the different layers of the intestines may affect the gut microbiota composition. The vascularization within the different layers of the intestines differs considerably, which is illustrated by a rather unique oxygenation profile. The oxygen tension (pO_2) within the intestines reduces drastically moving across the longitudinal axis, with highest reported pO_2 levels at the level of the duodenum (32 mmHg), decreased levels in the ascending colon (11 mmHg), and lowest levels in the sigmoid colon (3 mmHg) in mice (7, 8). In line, the human colon is inhabited predominantly by anaerobic bacteria (9). Interestingly, rats acutely exposed to hypoxia (15% O_2) showed reductions in both serosal and mucosal pO_2 (10), providing proof-of-principle that hypoxia exposure is able to modulate intestinal pO_2.

Thus far, only a few human and animal studies have examined the effects of hypoxia exposure on gut microbiota composition. Mice exposed to severe, intermittent hypoxia (5% O_2, 20s per cycle, 360 cycles per day) for 6 weeks, demonstrated increased α- and β-diversity (11). More specifically, intermittent hypoxia exposure, which reduced pO_2 in close vicinity of the intestinal epithelium (10, 11), increased the abundance of *Firmicutes*, whilst reducing the abundance of *Bacteriodetes* and *Proteobacteria* phyla (11). In addition, rats exposed to chronic, severe intermittent (hypobaric) hypoxia (5000 m, 6h per day for 28 days) demonstrated increased abundance of the genera *Lactobacillus*, *Prevotella* and *Methylotenera*, whereas the *02d06* genus within the *Clostridiaceae* family was decreased subsequent to exposure (12). Furthermore, a decrease in the *Firmicutes* to *Bacteriodetes* ratio was found in the latter study, which may at least partially underlie the observed improvements in glucose and lipid metabolism (12). Remarkably, high altitude exposure (4300 m for 22 days) increased *Prevotella* abundance in healthy men (13). These findings in healthy men are in agreement with previous animal studies, demonstrating increased abundance of mainly obligate

anaerobes following hypoxia exposure (14). Taken together, intermittent hypoxia exposure appears to affect gut microbiota composition, which may contribute to hypoxia-mediated effects on host metabolism. However, controlled human studies that investigated the effects of normobaric hypoxia exposure on the gut microbiota composition, and related effects on host metabolism, are lacking.

Therefore, in the present randomized, single-blind crossover study, we exposed men with overweight and obesity to mild intermittent hypoxia (MIH; FiO_2: 15%, 3x2h per day) and normoxia (FiO_2: 21%) for seven consecutive days, to determine for the first time the effects of MIH exposure on gut microbiota composition in a well-controlled, laboratory setting. Furthermore, we examined whether hypoxia-induced effects on gut microbiota composition were associated with alterations in host metabolism.

Methods

Study design

The design of this study has been described in detail previously (Chapter 3). Briefly, participants enrolled in this randomized, single-blind, crossover study were exposed to normobaric MIH (15% O_2) and normobaric normoxia (21% O_2) for 7 consecutive days (3 cycles of 2h/d with 1h of normoxia exposure between hypoxic cycles), separated by a 3-6 week wash-out period. Systemic oxygen saturation levels were continuously monitored throughout the exposure regimens by pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcor). At day 6 of the exposure regimens, AT and SM pO_2 were determined using an optochemical measurement system for continuous monitoring of tissue pO_2, as described previously (15). At day 7, a high-fat mixed meal test was performed (2.6MJ, consisting of 61 E% fat, 33 E% carbohydrates and 6 E% protein). At day 8, a two-step hyperinsulinemic–euglycemic clamp was performed, under normoxic conditions, to determine hepatic, adipose tissue and peripheral insulin sensitivity.

Microbiota analysis

Genomic DNA extraction was performed using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (Zymo Research, Leiden, The Netherlands) according to the manufacturer's instructions. 16S rRNA gene amplicon libraries for Illumina 2 × 300 bp paired end sequencing were generated and sequenced on an Illumina MiSeq (Illumina, Eindhoven, The Netherlands), according to the standard Illumina protocols. Briefly, barcoded amplicons from the V3–V4 region of 16S rRNA genes were generated using a 2-step PCR. For this, 10–25 ng genomic DNA was used as the template for the first PCR using the 341F (50 -CCTACGGGNGGCWGCAG–3 0) and the 785R (50 -GACTACHVGGGTATCTAATCC–3 0) primers appended with Illumina adaptor sequences in a total volume of 50 µL. PCR products were purified (QIAquick PCR Purification Kit, Qiagen, Venlo, The Netherlands). Subsequently, the size of the PCR products was checked on a fragment analyzer (Advanced Analytical, Ankeny, IA, USA) and quantified by fluorometric analyses. These purified PCR products were used for the second PCR in combination with sample-specific unique barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer and quantified as described above. Then samples were multiplexed, clustered and sequenced on an Illumina MiSeq. The raw data were analyzed with the Illumina CASAVA pipeline (v1.8.3; Illumina) with demultiplexing based on the unique sample-specific barcodes. Sequences were converted into FASTQ files using BCL2FASTQ pipeline version 1.8.3. The quality cut was applied based on the quality level of the Phred (Phred quality score). The Quantitative Insights Into Microbial Ecology (QIIME) software package (1.9.0) was

used for microbial analyses (16). The sequences were classified using Greengenes (version 13.8) as a reference 16S rRNA gene database.

Statistical analysis

The effects of MIH exposure on alpha-diversity indices were analyzed using Wilcoxon-signed rank tests using SPSS version 24 (IBM), whereas effects on beta-diversity indices were tested using nonparametric permutational multivariate analysis of variance (PERMANOVA) with 999 permutations (17) in RStudio. In addition, to compare MIH-induced effects on microbiota composition, relative abundances of taxa were tested using nonparametric Wilcoxon-signed rank tests, and Benjamini-Hochberg FDR correction for multiple testing was also applied. Data are expressed as mean ± SEM. A p-value of <0.05 was considered statistically significant. Correlation analysis between changes in the relative abundances (Δrelative abundances: MIH relative abundance – normoxia relative abundance) and alterations in metabolic parameters (Δmetabolic parameter: MIH metabolic parameter – normoxia metabolic parameter) was performed using Spearman's rank-order correlation with multiple testing correction using the software package R (3.5.0) (R-Core team, http://www.R-project.org/).

Results

Participant characteristics

Characteristics of study participants in which stool samples were collected (*n*=5 paired samples) are depicted in Table 1. By design, participants had a BMI ≥ 28 kg/m² and HOMA-IR ≥ 2.2.

Table 1. Participant characteristics at baseline.

Age (y)	**62 ± 2**
BMI (kg/m²)	30.1 ± 1.1
Hemoglobin (mmol/L)	9.4 ± 0.4
Creatinine (µmol/L)	89 ± 3.7
ALAT (U/L)	29 ± 6.7
HbA_{1c} (%)	5.6 ± 0.2
Fasting glucose (mmol/L)	5.9 ± 0.3
2h-glucose (mmol/L)	5.6 ± 1.0
HOMA-IR	4.0 ± 0.6

BMI, body mass index; ALAT, alanine aminotransferase; HbA_{1c}, glycated hemoglobin; 2h-glucose, glucose concentration after 2h oral glucose tolerance test; HOMA-IR, homeostatic model assessment for insulin resistance.

MIH exposure alters gut microbiota composition

Samples were rarified to 10,000 sequences before determining α-diversity (within-sample diversity) and β-diversity (inter-sample similarity) indices. The rarefaction curves of observed operational taxonomic units (OTUs) demonstrated plateau phase around 6500 reads, which suggests that higher than 6500 reads per sample would not give a more distinctive catalogue of bacterial taxa. Shannon index (Fig 1A, p=0.374) and Faith's phylogenetic diversity (Fig 1B, p=0.929) were not different after seven days of MIH exposure relative to normoxia exposure. In addition, the observed OTUs (Fig 1C, p=0.536) and the evenness index (Fig 1D, p=0.624) were not different between MIH and normoxia exposure, suggesting that α-diversity was not affected by MIH exposure. In addition, unweighted (p=0.973) and weighted Unifrac (p=0.508), Jaccard distance (p=0.975) and Bray-Curtis dissimilarity (p=0.886) indices were not significantly different between MIH and normoxia exposure, indicating that MIH did not alter gut microbial β-diversity.

Furthermore, we found that MIH exposure for seven days did not significantly alter the relative abundances of taxonomical phyla, classes and orders. In

addition, the *Firmicutes/Bacteriodetes* ratio was not altered (p=0.500) by MIH exposure. However, MIH significantly reduced relative abundances of the families *Clostridiaceae* (1.6% MIH versus 4.7% normoxia, p=0.043) and *Christensenellaceae* (1.0% MIH versus 2.1% normoxia, p=0.043). At genus level, relative abundances of *Clostridium sensu stricto 1* (1.6% MIH versus 4.7% normoxia, p=0.043, Fig. 2A) and *Christensenellaceae R-7 group* (1.0% MIH versus 1.9% normoxia, p=0.043, Fig. 2B) were significantly reduced by MIH, whereas *Fusicatenibacter* (1.3% MIH versus 0.9% normoxia, p=0.043, Fig. 2C), *Butyricicoccus* (1.7% MIH versus 0.7% normoxia, p=0.043, Fig. 2D) and *Holdemania* (0.06% MIH versus 0.02% normoxia, p=0.043, Fig. 2E) were significantly increased subsequent to MIH exposure. These differences were non-significant after correction for multiple testing.

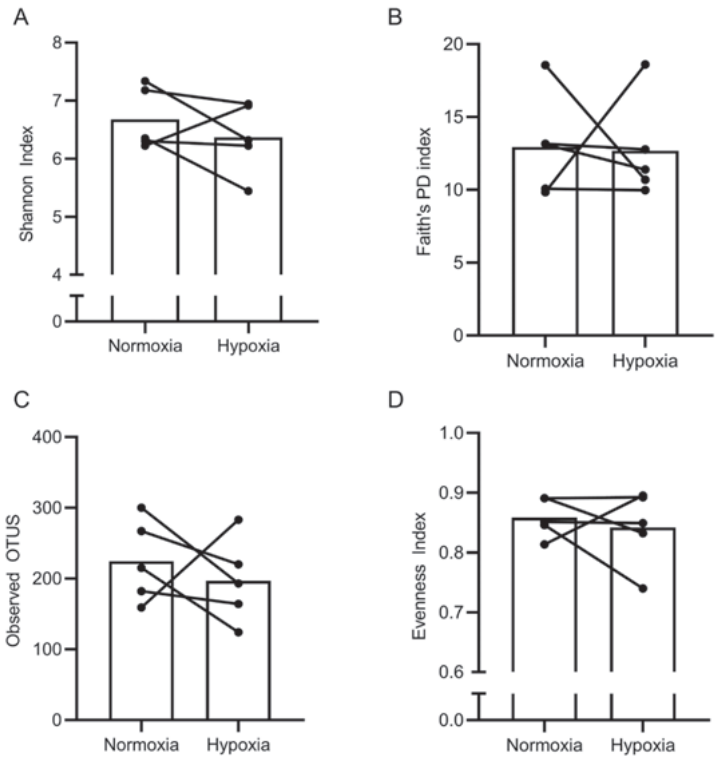

Figure 1. The effect of mild intermittent hypoxia compared to normoxia exposure on alpha-diversity indices. Alpha-diversity, determined by **(A)** Shannon index, **(B)** observed OTUS, **(C)** Faith's phylogenetic diversity and **(D)** evenness indices. The line and whiskers represent the median, and the lowest/highest values, respectively. Individual samples are plotted as circles (n=5 paired samples). Closed circles; normoxia exposure, open circles, hypoxia exposure. Statistical analysis was performed using nonparametric related-samples Wilcoxon-signed rank tests.

MIH-induced changes in levels of genera are associated with host metabolism

MIH-induced alterations (Δ) in the abundance of several genera were strongly associated with MIH-differences in several metabolic parameters (Figure 3, Supplementary Fig. 1). Δ*Barnesiella* was inversely associated with ΔAT insulin sensitivity (q=1.53•10^{-21}). Moreover, Δ*Butyricimonas* (q=1.08•10^{-22}), Δ*Odoribacter* (q=1.08•10^{-22}), Δ*Parabacteroides* (q=1.08•10^{-22}) and Δ*Ruminococcus torques group* (q=1.08•10^{-22}) were positively associated with Δperipheral insulin sensitivity. Furthermore, Δ*Alistipes* (q=5.4•10^{-22}) and Δ*Coprococcus 2* (q=5.4•10^{-22}) were associated with Δfasting and Δpostprandial triglycerides, respectively. In addition, Δ*Christensenellaceae R-7 group* (q=5.09•10^{-22}) and Δ*Marvinbryantia* (q=5.09•10^{-22}) were inversely, whereas Δ*Holdemania* (q=5.09•10^{-22}) was positively associated with Δpostprandial systemic free fatty acid (FFA) concentrations. Δ*Caproiciproducens* (q=5.4•10^{-22}) was associated with Δpostprandial plasma lactate levels. Δ*Eubacterium rectale group* (q=5.4•10^{-22}) was positively, whereas Δ*Ruminococcaceae UGC 014* (q=7.64•10^{-22}) was inversely associated with Δfasting plasma insulin levels. Lastly, Δ*Ruminococcacceae UGC 010* was positively associated with Δpostprandial carbohydrate oxidation (q=5.4•10^{-22}), whereas it inversely associated with Δfat oxidation (q=1.53•10^{-21}).

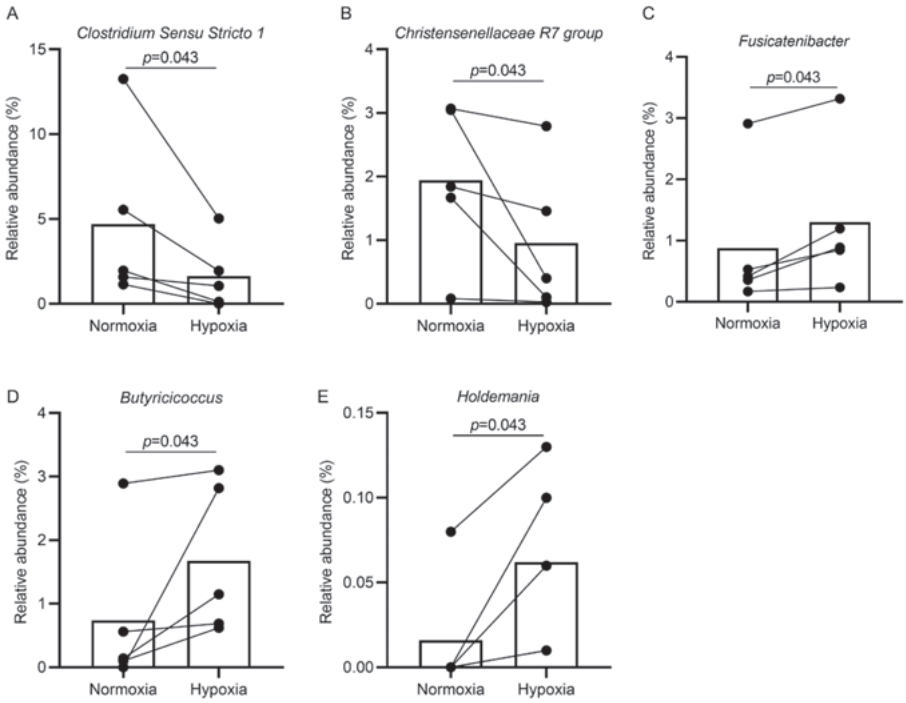

Figure 2. Relative abundances of genera affected by mild intermittent hypoxia exposure. Relative abundances of **(A)** Clostridium sensu stricto 1 and **(B)** Christensenellaceae R7 group genera were significantly reduced, whereas **(C)** Fusicatenibacter, **(D)** Butyricicoccus and **(E)** Holdemania genera were significantly increased subsequent to mild intermittent hypoxia exposure as compared to normoxia. The *p*-values are based on related-samples Wilcoxon-signed rank tests on paired samples (*n*=5), without false discovery rate (FDR) correction. Closed circles, normoxia exposure; Open circles, hypoxia exposure.

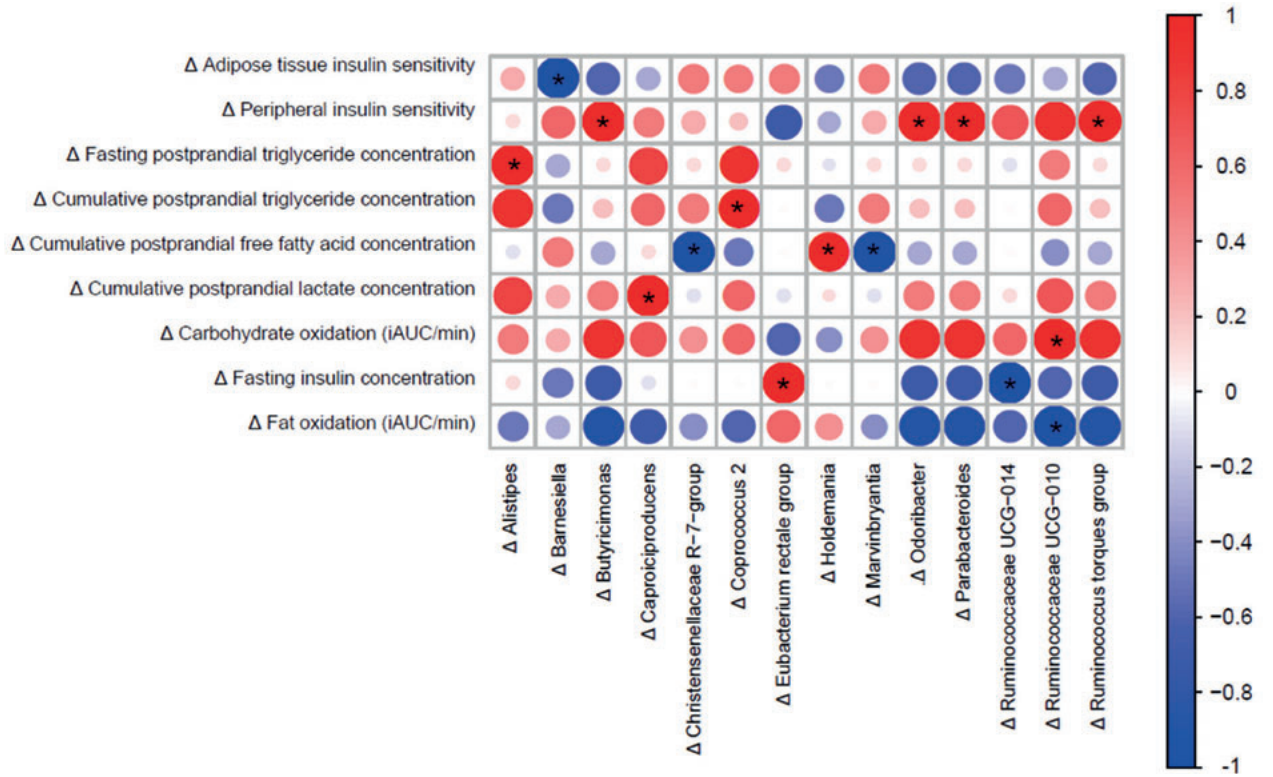

Figure 3. Heatmap of associations between mild intermittent hypoxia-induced alterations in the abundance of several genera and mild intermittent hypoxia-induced change in several metabolic parameters. Correlation analysis between changes in the relative abundances (Δrelative abundances: mild intermittent hypoxia (MIH) relative abundance – normoxia relative abundance) and alterations in metabolic parameters (Δmetabolic parameter: MIH metabolic parameter – normoxia metabolic parameter) was performed using Spearman's rank-order correlation with multiple testing correction. Asterisk, statistically significant correlation.

Discussion

To the best of our knowledge, the present randomized, single-blind crossover trial is the first well-controlled study investigating the effects of normobaric MIH exposure on fecal microbiota composition in overweight and/or obese humans. Here, we report that MIH has slight effects on gut microbial composition, with no effect on alpha- and beta-diversity indices. Interestingly, our findings indicate that MIH-induced alterations in relative abundances of fecal microbiota composition on genus level are associated with several alterations in metabolic outcome parameters, including AT and peripheral insulin sensitivity, as well as fasting and postprandial metabolite plasma levels in overweight and obese men.

In contrast to previous findings, we found no alterations in microbial composition on phylum, order and class levels. Indeed, animal studies demonstrated that intermittent hypoxia exposure, albeit under very severe and hypobaric conditions, decreased *Firmicutes*, whilst increasing *Bacteriodetes* (11, 12) and hence a reduced *Firmicutes/Bacteriodetes* ratio (12). Recently, it has been demonstrated that Tibetan highlanders, who are chronically exposed to hypobaric hypoxia, show reductions in *Prevotella* and *Bacteroides* genera compared to sea level residents (18). In the present study, MIH seemed to evoke a reduction in relative abundance on family and genus level of *Christensenelleceae* and *Clostridiaceae-1*, with concomitant decreased abundances of *Clostridium sensu stricto-1* and *Christensenelleceae R-7 group*. Our results are partially in contrast with previous findings, demonstrating increased *Christensenelleceae* abundance in healthy males when exposed to high altitude hypoxia (4300 m) (13). Interestingly, however, *Christensenelleceae R-7 group* was previously found to be negatively associated with VLDL, small-sized HDL-particles and triglycerides within medium-sized HDL particles (19), whereas *Clostridium sensu stricto-1* was associated with large-sized HDL particles (19). In line with these findings, we found a relationship of these families and genera with parameters of lipid metabolism, showing an inverse association between *Clostridiaceae-1* and *Christensenellaceae (and Christensenellaceae R-7 group)* and fasting and postprandial systemic FFA concentrations, respectively.

Moreover, we found that MIH increased the relative abundances of the obligate anaerobic genera *Holdemania*, *Butyricicoccus* and *Fusicatenibacter*. These findings are in agreement with previous studies in humans and rodents, demonstrating that hypoxia increased the abundance of anaerobic bacteria in the gut (11, 14, 18). Indeed, high altitude exposure has been demonstrated to increase abundance of *Holdemania* (13). In addition, *Holdemania*, *Butyricicoccus* and *Fusicatenibacter* are butyrate-producers, which plays an important role in gut homeostasis by ensuring mucus production and maintaining tight-junction integrity (20).

MIH-induced effects on fecal microbiota composition might affect host metabolism. Indeed, we found that MIH-induced alterations in the abundances of

Buytiricimonas, *Odoribacter*, *Parabacteroides* and *Ruminococcus torques group* were significantly associated with peripheral insulin sensitivity. In agreement with our findings, *Butyricimonas* and *Odoribacter*, both strictly anaerobic butyrate-producers with anti-inflammatory effects, were inversely correlated with glucose levels in people with morbid obesity (21), and positively associated with Matsuda index in non-diabetic humans (22). Previous studies demonstrate that the *Ruminococcus torques group* was decreased after diabetes remission (23). Yet, *Ruminococcaceae* are able to generate short-chain fatty acids, enhancing energy metabolism, attenuating inflammatory processes and decreasing gut permeability (21). Interestingly, we found that *Ruminococcaceae UGC-014* was inversely associated with plasma insulin levels, whereas *UGC-010* was positively associated with carbohydrate oxidation, and negatively with fat oxidation. Furthermore, *Caproiciproducens* was positively associated with MIH-induced lactate formation, suggesting a contribution of gut-derived lactate production to circulating lactate levels. Alternatively, MIH-induced peripheral lactate production may induce intestinal caproic acid generation via microbial chain elongation (reverse β-oxidation) (24, 25). Finally, we found an inverse association between MIH-induced alteration in the relative abundance of *Barnesiella* and changes in AT insulin sensitivity. Interestingly, *Barnesiella* was found to be less abundant in native highlanders, who are chronically exposed to hypobaric hypoxia, compared to sea level residents (18). In line, in mice fed an anti-diabetogenic diet (gluten-free diet), the abundance of *Barnesiella* decreased with concomitant reduced incidence of hyperglycemia (26). Taken together, our findings together with previous studies might support a putative role for *Barnesiella* in the modulation of insulin sensitivity in humans.

Although the present randomized, single-blind crossover study is the first well-controlled study to investigate the effects of MIH on fecal gut microbiota composition in humans, several limitations need to be taken into account. Firstly, our study population consisted of men with overweight/obesity and mild impairment in glucose homeostasis. Therefore, the effects of MIH exposure on fecal microbial composition in other subgroups of the population such as those with a different metabolic status or women remain to be elucidated. Furthermore, study participants were exposed to MIH for a relatively short duration (1 week, 42h mild hypoxia exposure in total) compared to previous animal studies (4-6 weeks) (11, 12) and observational studies in humans (high-altitude natives). Therefore, the impact of the severity as well as duration of hypoxia exposure on gut microbial composition requires further investigation. Finally, the present data should be interpreted with caution due to the small sample size and hence limited statistical power. Thus, further studies are warranted to confirm the present findings.

In conclusion, the present randomized, single-blind crossover study indicates for the first time that normobaric MIH exposure has slight effects on fecal microbial composition in men with overweight and obesity, inducing changes in several

bacterial families and genera such as a shift towards higher abundances of anaerobic butyrate-producing bacteria. Furthermore, we found that MIH-induced alterations in gut microbial composition are associated with changes in several parameters related to glucose and lipid homeostasis. Together, our findings imply that MIH-induced alterations in gut microbiota composition might affect host metabolism in humans.

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Conflicts of interest

The authors declare no conflict of interest

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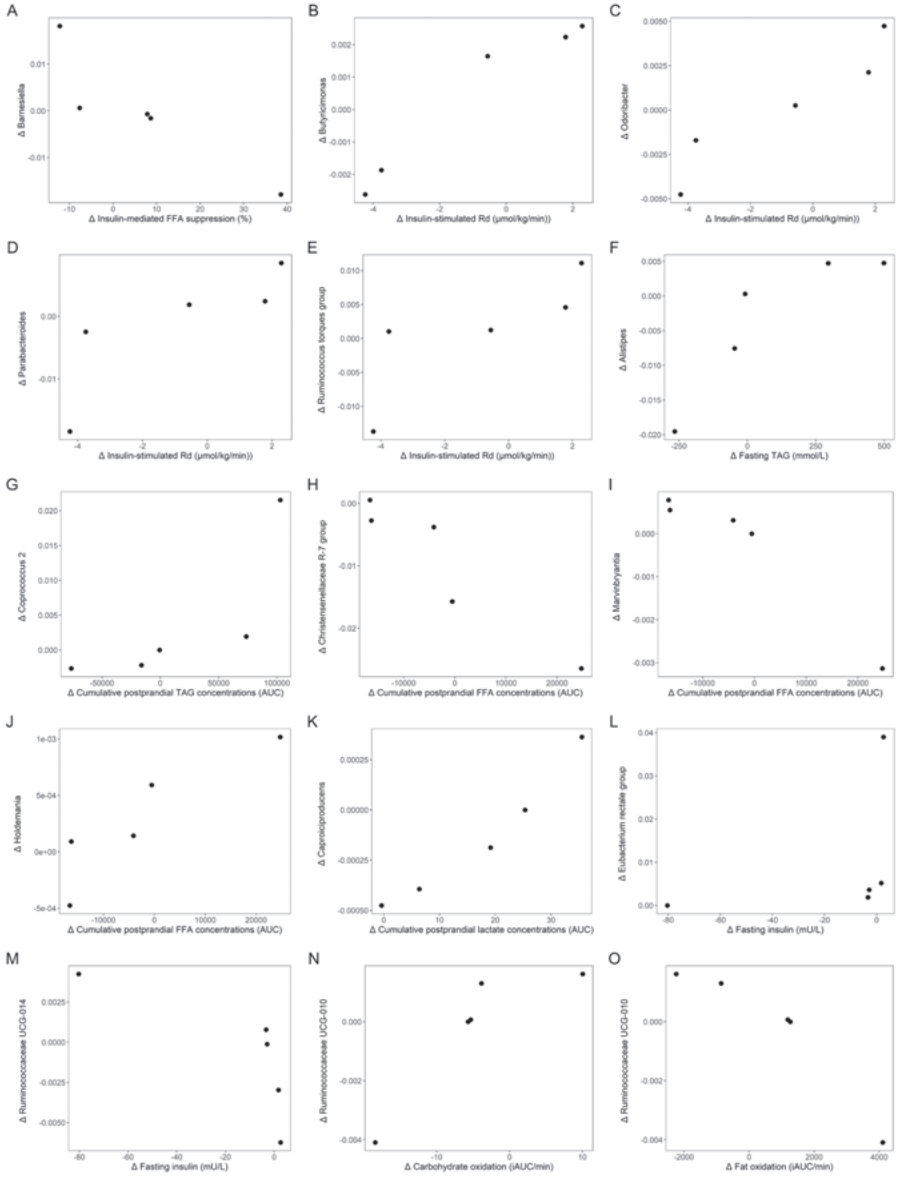
Supplementary Material



Supplementary Figure 1. Correlation analysis of mild intermittent hypoxia induced alterations in relative abundance of genera and alterations in metabolic outcome parameters. Spearman's correlation plots of **(A)** ΔBarnesiella and Δadipose tissue insulin sensitivity (% suppression of free fatty acid release upon 10 mU/m²/min insulin stimulation). **(B)** Δ*Butyricimonas* **(C)** Δ*Odoribacter* **(D)** Δ*Parabacteroides* **(E)** Δ*Ruminococcus torques group* and Δperipheral insulin sensitivity, expressed by insulin-stimulated rate of

glucose disposal upon 40 mU/m²/min insulin infusion. **(F)** Δ*Alistipes*, **(G)** Δ*Coprococcus 2* and Δfasting and Δcumulative postprandial TAG concentrations, respectively. **(H)** Δ*Christensenellaceae R7 group*, **(I)** Δ*Marvinbryantia*, **(J)** Δ*Holdemania* and Δcumulative postprandial free fatty acid concentrations. **(K)** Δ*Caproiciproducens* and Δcumulative postprandial lactate concentrations. **(L)** Δ*Eubacterium rectale group*, **(M)** Δ*Ruminococcaceae UCG 014* and Δfasting insulin concentrations. **(N, O)** Δ*Ruminococcaceae UCG 010* and Δcarbohydrate and Δfat oxidation incremental area under the curve. Δ: Mild intermittent hypoxia – normoxia. TAG, triglyceride.

CHAPTER 7

The effects of hypoxic exercise on 24-hour glucose profile and substrate metabolism in overweight and obese men with impaired glucose metabolism: a randomized, single-blind, cross-over study

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In preparation

Abstract

Introduction: Hypoxic exercise (HE) may have more pronounced effects on glucose homeostasis compared to exercise under normoxic conditions (NE), but effects on 24-h glucose profile and substrate utilization remain unclear. We investigated the effects of moderate-intensity HE compared to NE on 24-h glucose profile and substrate metabolism in overweight/obese individuals.

Methods: Ten overweight/obese men with impaired fasting glucose and/or impaired glucose tolerance participated in a randomized single-blind crossover trial. Participants performed moderate-intensity cycling exercise for 4 consecutive days under mild normobaric hypoxic (FiO_2: 15%) or normoxic (FiO_2: 21%) conditions at similar relative exercise intensity (2x30 min/d at 50% of maximal heart rate (HR_{MAX})), with a washout of 3-6 weeks. 24-h glucose levels and systemic oxygen saturation (SpO_2) were monitored throughout the study. At day 5, a mixed-meal test was performed under normoxic conditions, and plasma metabolites and substrate oxidation were determined.

Results: SpO_2 (91.6 vs. 96.3%, respectively, $p<0.001$) and absolute workload (-11.6%, $p<0.001$) were lower during HE than NE, whilst HR during exercise was comparable. HE did not significantly alter mean 24-h, daytime, and nighttime glucose concentrations, or measures of glycemic variability compared to NE. Nevertheless, the HE-induced decrease in SpO_2 was positively correlated with HE-induced alterations in mean 24-h and daytime glucose concentrations (r=0.683, p=0.042 and r=0.783, p=0.013, respectively).

Discussion: We demonstrate that 4-day HE at similar relative exercise intensity reduces SpO_2 and has similar effects on mean 24-h glucose concentration and glycemic variability than NE in overweight/obese men with impaired glucose metabolism. Yet, a more pronounced reduction in SpO_2 during HE was associated with lower 24-h glucose concentrations.

Introduction

It is well established that both an acute bout of exercise and exercise training increase peripheral glucose uptake and improve glycemic control (1, 2). Interestingly, several studies suggest that inhaling hypoxic air during exercise at a similar relative intensity (i.e. lower absolute workload) than exercise under normoxic conditions may have more pronounced effects on insulin sensitivity and glycemic control, although no differential effects on insulin sensitivity have also been reported (3-6). Thus, training under hypoxic conditions may yield maximal cardiometabolic benefit, while minimizing orthopedic injury risk in people with obesity due to lower absolute workload.

Indeed, it has previously been shown that an acute bout of exercise (cycling for 60 min at 90% of lactate threshold) under normobaric hypoxic conditions (14.6% FiO_2) increased glucose disappearance during a 4-h intravenous glucose tolerance test that was performed immediately after the exercise session compared to normoxic exercise in patients with type 2 diabetes (3). Interestingly, the beneficial effects on glucose homeostasis were still apparent 24h and 48h after the hypoxic exercise (HE) bout (3). In line, exercise training under hypoxic conditions (15% $FiO_{2;}$; 3 x 60 min/week for 4 weeks) decreased homeostatic model assessment of insulin resistance (HOMA-IR), fasting and postprandial insulin levels in healthy volunteers (6). Although the mechanisms underlying improved glucose homeostasis following HE are not fully elucidated, increased skeletal muscle glucose uptake and elevated carbohydrate oxidation may be involved (7). Importantly, however, several studies that investigated the effects of HE on glucose homeostasis did not include a control group (4, 8, 9).

Accumulating evidence indicates that glycemic variability is associated with obesity-related cardiometabolic complications (10-12). 24-h continuous glucose monitoring (CGM) provides important information about glucose levels as well as glycemic variability (i.e., frequency and amplitude of glucose fluctuations) throughout the day and night. Although exercise under mild hypoxic conditions may improve insulin sensitivity and glucose homeostasis as compared to normoxic exercise (NE), the impact of moderate-intensity exercise under mild hypoxic conditions on glucose profile and glycemic variability during daytime and nighttime remains to be established.

Therefore, we performed a randomized, single-blind crossover trial to investigate the effects of mild hypoxic, moderate-intensity exercise for 4 consecutive days (FiO_2 15%; 2 x 30-min cycling at 50% HR_{MAX} / day) on 24-h glucose profile in overweight and obese individuals with impaired glucose metabolism. Furthermore, we examined the effects of 4-day HE on fasting and postprandial substrate metabolism under normoxic conditions at day 5.

Methods

Study participants

Overweight and obese men (BMI≥28 kg/m^2) with impaired fasting glucose (defined as fasting glucose: 5.6 – 6.9 mmol/l) and/or impaired glucose tolerance (defined as 2-h glucose 7.8 – 11.0 mmol/l; determined by oral glucose tolerance test), aged 30-70 years, participated in the study. Exclusion criteria were smoking, cardiovascular diseases, type 2 diabetes mellitus, kidney or liver malfunction (determined by ALAT and creatinine), diagnosis of a disease with a life expectancy shorter than 5 years, lactose intolerance, substance abuse, following a hypocaloric diet, >3h per week of exercise, high dosage antioxidant use, use of medication known to affect glucose metabolism or inflammation. In addition, spirometry was performed to exclude any (undiagnosed) pulmonary diseases. 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. This study is registered at Clinicaltrials.gov (NCT04280991).

Experimental protocol

In this randomized, single-blind, cross-over study, participants were exposed to normobaric hypoxia (FiO_2 15%) and normoxia (FiO_2 21%) during the exercise sessions on days 1-4 (see '*Exercise regimens*' below), in a randomized fashion, with a wash-out period of 3-6 weeks. Randomization was performed through computer-generated randomization program with a block size of 4. The hypoxic and normoxic conditions were achieved in a clinical room in which O_2 levels were tightly controlled and maintained between 14.8-15.2% O_2 during hypoxia exposure. On day 5, a high-carbohydrate high-fat mixed meal test was performed under normoxic conditions (Figure 1).

Subjects were asked to refrain from drinking alcohol for a period of 24h before the first study day until the end of the 5-day period and were asked not to perform any strenuous exercise for a period of 72h before the start until the end of the exercise regimens. Subjects were asked to come to the research facility by car or public transport. After an overnight fast, participants were provided with a standardized breakfast, lunch and dinner with snacks in between on days 1-4. Meals were identical and isocaloric between both regimens. The standardized diet was based on the estimated daily energy expenditure (basal metabolic rate (BMR) (Ventilated Hood, Omnical, Maastricht University) multiplied by activity score of 1.55), consisting of 50% carbohydrate, 35% fat and 15% protein, thereby keeping participants in energy-balanced conditions throughout the study.

Exercise regimens

At least one week before the start of the exercise regimen, an incremental workload test was performed to determine maximal workload capacity (W_{MAX}) and heart rate (HR_{MAX}) under normoxic conditions. Participants arrived at the research facility after an overnight fast. Upon arrival on days 1-4, blood pressure and body weight were determined, and a standardized breakfast was provided. Thereafter, participants performed two 30-min exercise sessions on a cycle ergometer at 50% W_{MAX} during normoxic conditions, with a cadence of 60-70 rpm, in the morning with 1 hour rest between the two exercise sessions (Figure 1). To achieve similar relative exercise intensity, participants cycled at a heart rate corresponding to 50% W_{MAX}, as determined under normoxic conditions, during the HE sessions. Systemic oxygen saturation and heart rate of the participant were measured throughout the exercise sessions by finger pulse oximetry (Nellcore N-595 Pulse oximeter, Nellcore). After completion of both 30-min exercise sessions, a standardized lunch and dinner was provided.

Continuous glucose monitoring

On day 1, prior to the first exercise session, a glucose sensor to measure 24-h glucose profiles (Enlite-sensor, Medtronic, Northridge, CA, USA) was inserted subcutaneously at 5 cm lateral from the umbilicus, on the right side of the abdomen. This sensor was connected to a continuous glucose monitoring device (iPro2 Professional CGM MiniMed; Medtronic). Glucose concentrations were measured subcutaneously in the interstitial fluid, every 5 minutes, providing 288 data points per 24h. For calibration purposes, subjects were asked to measure blood glucose via a capillary glucose meter (Accu-Chek; Roche Diagnostics India, Mumbai, India) before breakfast, before lunch and before going to sleep. After arrival at the research facility on day 5, the glucose sensor was removed (Figure 1).

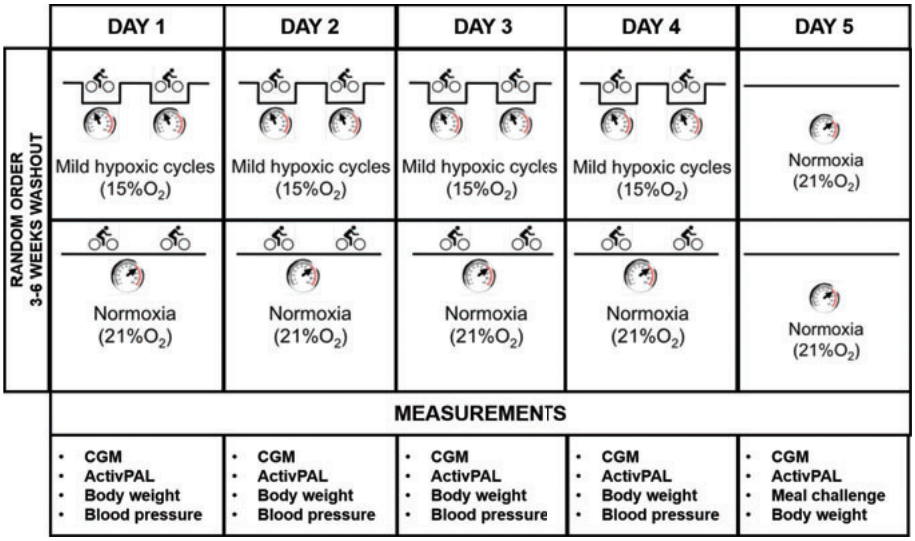

Figure 1. Study flowchart. Participants performed hypoxic exercise (HE, FiO_2: 15%) and normoxic exercise, with a 3-6 week washout period, in a randomized fashion. Exercise was performed at 50% HR_{MAX} (NE) or corresponding heart rate (HE) to achieve similar relative exercise intensity. Participants performed exercise for 30 min per session, twice a day, for 4 consecutive days. On day 1, a continuous glucose monitor and accelerometer was placed to monitor glucose concentration and physical activity levels, respectively, throughout the study. During the exercise regimens, participants received standardized breakfast, lunch and dinner, at fixed times, which were identical and isocaloric for both regimens. On day 5, under normoxic conditions, a high-carbohydrate mixed-meal challenge was performed to investigate the effects of HE on plasma metabolite concentrations and substrate oxidation. CGM, continuous glucose monitoring; ActivPAL, physical activity monitoring.

At the end of the study, data were retrieved using web-based iPro CareLink software, and analyzed using GlyVarT (Medtronic) software. Data were analyzed according to guidelines for summarizing CGM data (13) to determine mean 24-h glucose concentration (mmol/l) at day 4 (primary outcome). Furthermore, measures of glycemic variability including the standard deviation (mmol/l), coefficient of variation (%), interquartile range (difference between 75th and 25th percentiles, mmol/l), variability ((mean glucose variability around normoglycemia (5.55 mmol/l), mmol/l)) and mean amplitude of glycemic excursion (MAGE, mean of glycemic oscillations exceeding >1 SD of mean glucose, mmol/l) were analyzed at day 4. In addition, daytime (7 AM – 10 PM) and nighttime (10:01 PM – 06.59 AM) 24-h glucose concentrations as well as measures of glycemic variability at day 4 were calculated. Furthermore, the total area under the curve (tAUC) for glucose was determined for daytime, nighttime and total day 4 (24-h). Finally, the incremental area under the curve (iAUC) for the decrease of glucose concentration during and 30 min post-exercise (60 min glucose drop) at day 4 was calculated by using the trapezoidal method.

Physical activity monitoring

On day 1, an accelerometer (activPAL monitor, PAL Technologies, Glasgow, Scotland) was waterproofed using a small sleeve and subsequently wrapped in adhesive tape (Tegaderm, 3M, Saint Paul, MN, USA), and was attached to the anterior thigh, as described previously (14). Levels of physical activity were measured 24h/day throughout the study using the activPAL monitor (Figure 1). After arrival at the research facility on day 5, the activPAL was removed. At the end of the study, data were analyzed using PALanalysis tool (version 8). Data from the activPAL was used to determine activity score ($MET \cdot h^{-1}$), cycling time (min), sitting time (min), primary lying time (min), number of cycling steps and total number of steps, during both exercise regimens. In addition, these parameters were separately analyzed for free-living conditions (from lunch until breakfast on the subsequent day).

Mixed-meal challenge test

On day 5, a high-carbohydrate high-fat mixed meal test was performed under normoxic conditions following both exercise regimens (~20h after the final exercise bout) (Figure 1). Upon arrival, a catheter was inserted in the antecubital vein. After 30-min baseline measurement to determine fasting substrate metabolism, individuals were asked to consume a liquid test meal (Abbot Ensure Plus; 750 kcal (3.16MJ: 54 EN% carbohydrate, 29 EN% fat, 17 EN% protein)) within 5 minutes at t=0 min. Venous blood samples were collected under fasting (t=0 min) and postprandial conditions (t=30, 60, 90, 120, 180, 240, 300 and 360 min) for assessment of plasma metabolite concentrations (see '*Biochemical analyses*' below).

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 6 hours after ingestion of the high-carbohydrate mixed-meal. Calculations of energy expenditure and substrate oxidation were performed according to the formulas of Weir (15) and Frayn (16), respectively. Nitrogen excretion was based on the assumption that protein oxidation represents ~15% of total energy expenditure (17).

Biochemical analyses

Plasma FFA, TAG, glucose and lactate levels were determined using automated spectrophotometric enzymatic assays (Cobas Fara auto-analyzer, Roche, Basel, Switzerland). Plasma insulin was measured through ELISA (Human Insulin ELISA kit, Meso Scale Discovery, Gaithersburg, USA).

Statistical analyses

The calculated sample size (n=10) was based on a physiologically relevant difference of 0.4 mmol/l in mean 24-h glucose concentration (α = 0.05, 1-β = 0.8). The effects of HE were compared to NE using the Student's paired t-test, whilst nonparametric data was analyzed using the Wilcoxon Signed-Rank test. The time-effect in the reduction of systemic oxygen saturation was determined using repeated-measures ANOVA. Area under the curves (AUC) and incremental AUCs (iAUC) were determined using the trapezoidal method. Correlation analyses was performed using Spearman's rank correlation analysis. Data are represented as mean ± 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. Figures were generated using Graphpad Prism.

Results

Characteristics of the study participants are shown in Table 1. As expected, body weight remained constant and was not significantly different between both exercise regimens, as participants were kept in energy balance throughout the study.

Table 1. Baseline characteristics of study participants.

	Baseline
Age (y)	66 ± 0.9
BMI (kg/m^2)	29.6 ± 0.8
SBP (mmHg)	138 ± 3
DBP (mmHg)	90 ± 3
Resting HR (bpm)	66 ± 3
Creatinine (U/l)	89 ± 3
ALAT (U/l)	36 ± 2
Hb (mmol/l)	9.4 ± 0.2
Fasting glucose (mmol/l)	5.9 ± 0.2
2h-glucose (mmol/l)	8.0 ± 0.5
Resting SpO_2 (%)	97 ± 0.2
W_{MAX} (W)	202 ± 17
FEV1 (L)	3.62 ± 0.16
FEV1/VC (%)	73.7 ± 1.7

BMI, body-mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; ALAT, ; Hb, hemoglobin; 2h-glucose, plasma glucose concentration after 2h oral glucose tolerance test; SpO_2, systemic oxygen saturation; FEV1, forced expiratory volume in 1s; FEV1/VC, ratio of FEV1 to forced vital capacity. Values are represented as mean ± SEM (*n*=10).

Lower absolute workload during hypoxic exercise

To achieve similar relative intensity of exercise under both hypoxic and normoxic conditions, absolute exercise workload was significantly reduced under hypoxic conditions (-11.6%, p<0.001, Figure 2A), whereas mean heartrate was not different between HE and NE sessions (p=0.103, Figure 2B). In addition, physical activity level was comparable during both regimens. More specific, activity score, total number of steps, cycling time, upright time, standing time, sitting time and primary lying time did not differ between both exercise regimens (Table 2).

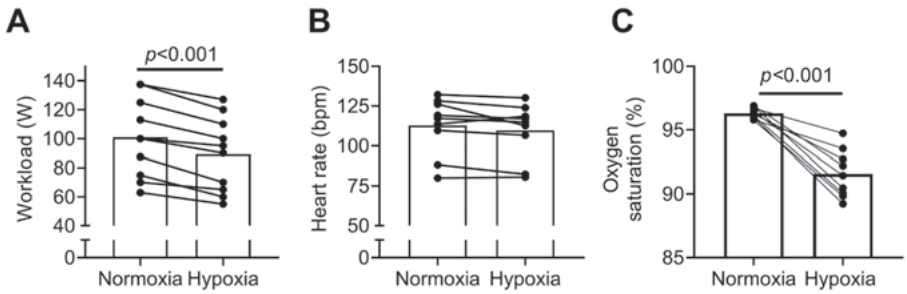

Figure 2. Hypoxic exercise decreased systemic oxygen saturation. (A) Exercise was performed at reduced absolute workload during hypoxic conditions to maintain similar **(B)** heartrate during the exercise sessions in both exposure regimens. **(C)** Hypoxic exercise reduced systemic oxygen saturation during the exercise bouts. Data are represented as individual data points for both exposure regimens (connected lines); boxes represent mean values per condition. Heart rate and systemic oxygen saturation were averaged for the 4 consecutive training days. Statistical analysis was performed using Student's paired t-test.

Hypoxic exercise induces a more pronounced reduces systemic oxygen saturation throughout the training regimen

Systemic oxygen saturation (SpO_2) was measured during each of the exercise sessions. HE significantly reduced SpO_2 during both exercise sessions, as found for both sessions at day 2 (92.0 vs. 96.5%, respectively, p=0.001), 3 (91.6 vs. 96.3 %, respectively, p<0.001) and 4 (91.5 vs. 96.3%, respectively, p<0.001) as compared to NE (Figure 2C). No alterations in SpO_2 were found during the exercise sessions over the course of the exercise days (days 1-4) in the hypoxic training regimen (time effect: p=0.509).

Table 2. Physical activity monitoring throughout the hypoxic and normoxic regimens.

	Hypoxic exercise	Normoxic exercise
Activity score (MET • h^{-1})	36.46 ± 0.46	36.42 ± 0.48
Total number of steps	15260 ± 1089	15294 ± 1114
Upright time (min)	383 ± 37	358 ± 35
Standing time (min)	226 ± 29	202 ± 25
Cycling time (min)	63 ± 6	65 ± 8
Sitting time (min)	520 ± 52	540 ± 45
Primary lying time (min)	511 ± 27	502 ± 17

MET • h^{-1}, metabolic equivalent task per hour. Data represent the mean values of days 2, 3 and 4.

Hypoxic exercise does not alter mean 24-h glucose concentration and glycemic variability

HE did not alter mean 24-h glucose concentration on day 4 as compared to normoxic exercise (6.1 mmol/l vs 6.2 mmol/l, p=0.678, Fig 3A, B). In addition, daytime (6.3 mmol/l vs. 6.2 mmol/l, p=0.678, Fig. 3C) and nighttime (5.7 mmol/l vs 6.0 mmol/l, p=0.325, Fig. 3D) mean glucose concentrations at day 4 were not different between the hypoxic and normoxic regimens, respectively. However, the hypoxia-induced decrease in SpO_2 was positively correlated with the hypoxia-induced difference in mean 24-h glucose concentration (ρ=0.683, p=0.042, Fig. 3E), as well as daytime mean glucose concentration (ρ=0.783, p=0.013, Fig. 3F) at day 4, but not nighttime mean glucose concentration. Besides, parameters related to glycemic variability (SD, CV, MAGE, IQR, variability and total AUC) were not significantly different following HE compared to NE (Table 3). To assess the acute effects of HE on glycemia, we determined iAUC of glucose concentration during and for 30 min after starting the exercise sessions at day 4. The 60-min reductions in glucose concentrations during exercise sessions 1 (p=0.943) and 2 (p=0.859) were similar between HE and NE (Fig. 3G).

Hypoxic exercise tends to increase postprandial carbohydrate oxidation but does not alter plasma metabolite concentrations

To determine the effects of HE on fasting and postprandial substrate metabolism, we performed a high-carbohydrate, high-fat meal challenge with blood sampling and indirect calorimetry measurements on day 5 of both exposure regimens. Fasting and postprandial energy expenditure was not different between HE and NE (Fig. 4A). In addition, fasting respiratory exchange ratio (p=0.450, Fig. 4B), carbohydrate (p=0.439, Fig. 4C) and fat (p=0.439, Fig. 4D) oxidation were not different between HE and NE. However, HE tended to increase the postprandial respiratory exchange ratio (p_{AUC}=0.110, Fig. 4B), reflected by a tendency towards increased postprandial carbohydrate oxidation (p_{AUC}=0.088, Fig. 4C) and reduced fat oxidation (p_{AUC}=0.099, Fig. 4D).

In addition, HE did not alter fasting and postprandial plasma glucose ($p_{fasting}$=0.404, p_{AUC}=0.372, Fig. 5A), insulin ($p_{fasting}$=0.332, p_{AUC}=0.811, Fig. 5B), free fatty acid ($p_{fasting}$=0.418, p_{AUC}=0.843, Fig. 4C), triacylglycerol ($p_{fasting}$=0.234, p_{AUC}=0.240, Fig. 5D) and lactate ($p_{fasting}$=0.836, p_{AUC}=0.091, Fig 5E) concentrations as compared to NE. HE did not alter the iAUC for plasma glucose, insulin, free fatty acids, triacylglycerol and lactate levels.

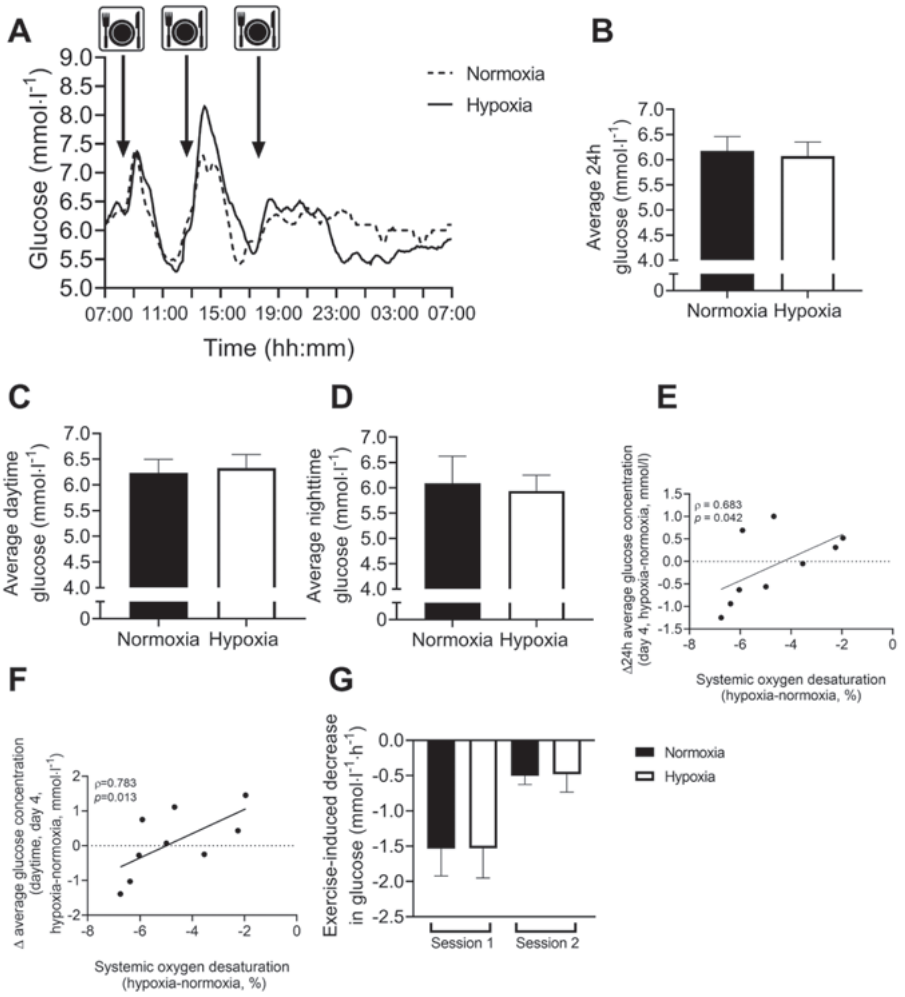

Figure 3. Hypoxic exercise did not alter mean glucose concentration and glycemic variability compared to normoxic exercise determined by continuous glucose monitoring. (**A**) mean 24h glucose concentration at day 4 (from 07:00 – 06:59h). (**B**) Mean 24h glucose profiles at day 4 for 9 individuals (*n*=9), with breakfast (08:30h), lunch (12:30h) and dinner (17:30h) at standardized times. (**C**) Mean glucose concentration during daytime (from 07:00 – 21:59h) and (**D**) nighttime (from 22:00 – 06:59h) at day 4. (**E**) Spearman's correlation analysis was performed to determine associations between hypoxic exercise-induced alterations in systemic oxygen saturation and mean 24-h glucose concentrations (day 4) and (**F**) mean daytime glucose concentrations (07:00 – 21:59h, day 4). (**G**) mean decrease in glucose concentration, calculated by the incremental area under the curve, during and 30 min after cessation of hypoxic and normoxic exercise sessions 1 and 2. Data are represented as mean ± SEM. Solid line, hypoxic exercise; dashed line, normoxic exercise.

Table 3. Summary of continuous glucose monitoring measures during study conditions.

	Hypoxic exercise	Normoxic exercise	*p*-value
24h day 4			
Mean glucose (mmol•l^{-1})	6.07 ± 0.28	6.17 ± 0.29	0.678
tAUC (mmol•h•l^{-1})	335.10 ± 36.30	337.30 ± 36.05	0.888
SD (mmol•l^{-1})	0.98 ± 0.11	0.92 ± 0.12	0.619
CV (%)	16.1 ± 1.6	14.6 ± 1.3	0.429
MAGE (mmol•l^{-1})	2.33 ± 0.30	2.05 ± 0.21	0.301
IQR (mmol•l^{-1})	1.03 ± 0.16	1.08 ± 0.15	0.859
Variability (mmol•l^{-1})	0.94 ± 0.21	0.99 ± 0.19	0.953
Daytime (07:00 – 21:59) day 4			
Mean glucose (mmol•l^{-1})	6.32 ± 0.26	6.23 ± 0.26	0.678
tAUC (mmol•h•l^{-1})	223.24 ± 20.70	217.33 ± 19.30	0.441
SD (mmol•l^{-1})	1.05 ± 0.14	0.97 ± 0.13	0.594
CV (%)	16.3 ± 1.7	15.3 ± 1.5	0.555
MAGE (mmol•l^{-1})	2.06 ± 0.36	2.28 ± 0.32	0.594
IQR (mmol•l^{-1})	1.32 ± 0.21	1.27 ± 0.15	0.782
Variability (mmol•l^{-1})	1.05 ± 0.23	1.03 ± 0.18	0.767
Nighttime (22:00– 06:59) day 4			
Mean glucose (mmol•l^{-1})	5.68 ± 0.37	6.02 ± 0.47	0.325
tAUC (mmol•h•l^{-1})	127.06 ± 8.32	136.24 ± 10.38	0.219
SD (mmol•l^{-1})	0.38 ± 0.09	0.38 ± 0.09	0.624
CV (%)	6.8 ± 1.8	5.8 ± 0.9	0.779
MAGE (mmol•l^{-1})	0.32 ± 0.09	0.45 ± 0.21	0.674
IQR (mmol•l^{-1})	0.40 ± 0.06	0.66 ± 0.19	0.237
Variability (mmol•l^{-1})	0.77 ± 0.26	0.92 ± 0.36	0.327

tAUC, total area under the curve; SD, standard deviation; CV, coefficient of variation; MAGE, mean amplitude of glucose excursions; IQR, interquartile range.

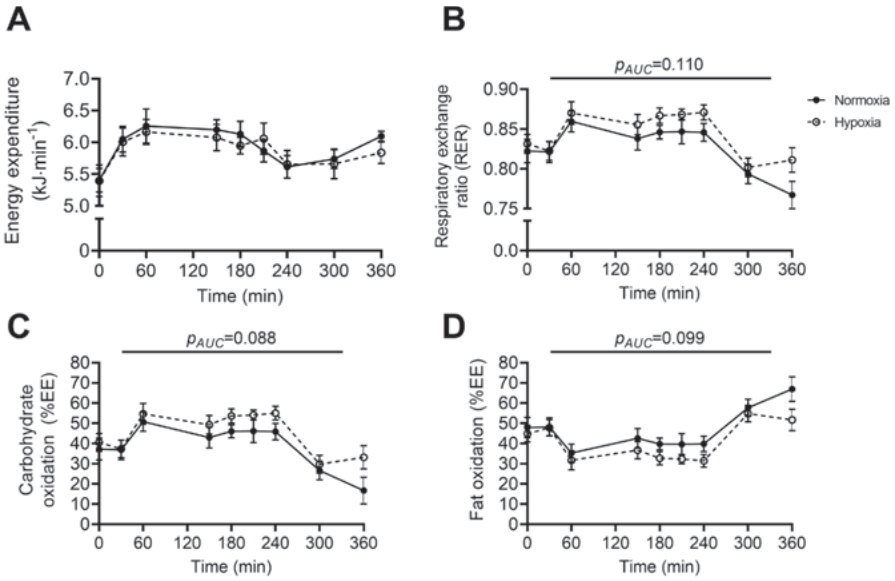

Figure 4. Hypoxic exercise tends to increase carbohydrate oxidation and reduce fat oxidation in response to a high-carbohydrate mixed-meal challenge. Indirect calorimetry was performed before (t=0 min) and after ingestion of a high-carbohydrate mixed meal (t = 0 – 360 min) to determine energy expenditure and substrate oxidation subsequent to 4-day hypoxic exercise. Hypoxic exercise did not alter (**A**) fasting and postprandial energy expenditure, but tended to increase the (**B**) respiratory exchange ratio and (**C**) carbohydrate oxidation (% of total energy expenditure: %EE), and tended to decrease (**D**) fat oxidation compared to normoxic exercise. Total area under the curves but not incremental area under the curves tended to be significantly different between HE and NE conditions. Open circles / dashed line, hypoxic exercise; closed circles / solid line, normoxic exercise. Data are represented as mean ± SEM.

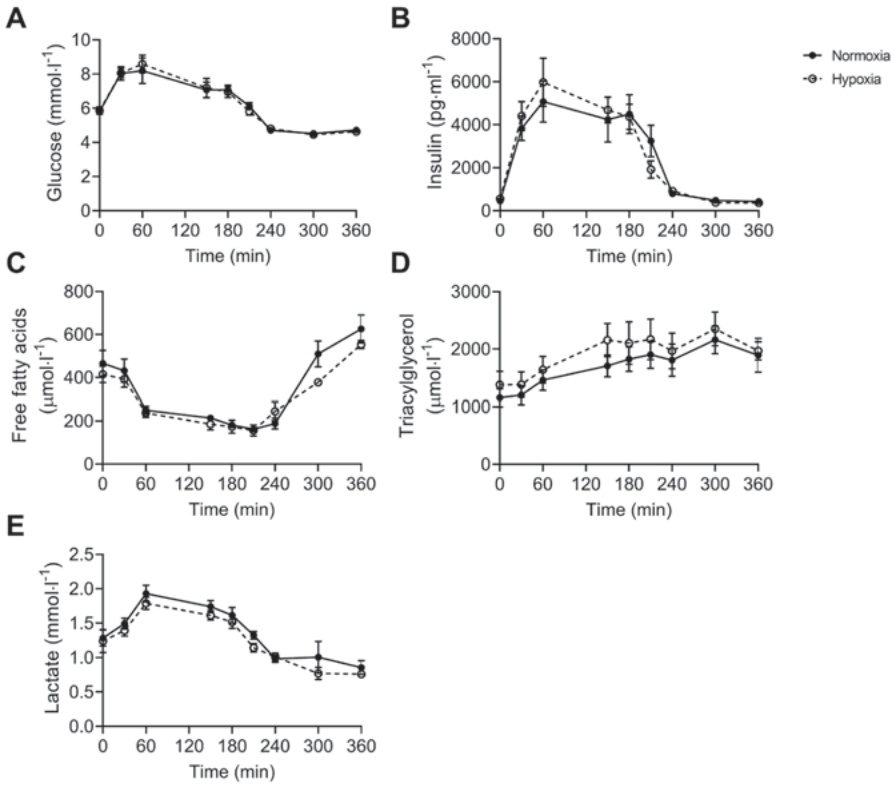

Figure 5. The effects of hypoxic exercise on fasting and postprandial plasma metabolite concentrations. Subsequent to 4-day hypoxic exercise (HE) regimen, a high-carbohydrate mixed-meal challenge was performed. HE did not alter fasting and postprandial plasma (**A**) glucose, (**B**) insulin, (**C**) free fatty acid, (**D**) triglyceride and (**E**) lactate concentrations as compared to normoxic exercise (NE). Open circles / dashed line, HE; Closed circles / solid line, NE. Data are represented as mean ± SEM.

Discussion

Accumulating evidence suggests that exercise under hypoxic conditions may improve glucose homeostasis to a greater extent than exercise under normoxic conditions, but the effects of HE on 24-h glucose profile have not been studied before. In the present randomized, single-blind crossover study, we demonstrated that moderate-intensity exercise under mild normobaric hypoxic conditions (HE, FiO_2: 15%) at a similar relative exercise intensity as NE, decreased SpO_2 and tended to elicit a more pronounced increase in carbohydrate oxidation in response to a mixed-meal challenge performed the day after the exercise regimens. Interestingly, although HE did not alter mean 24-h glucose concentration and glycemic variability in overweight and obese men with impaired glucose metabolism, the HE-induced decrease in systemic oxygen saturation was positively correlated with HE-induced alterations in mean 24-h and daytime glucose concentrations.

In the present study, we demonstrate for the first time in a controlled setting that moderate-intensity HE did neither alter mean 24-h glucose concentration and glycemic variability (day 4), nor fasting and postprandial glucose and insulin levels following a mixed-meal challenge test (day 5; performed ~20h after the last exercise session). Our results are in agreement with a previous studies, showing that both acute and prolonged moderate-intensity exercise under hypoxic conditions similar to those in the present study did not change plasma glucose, insulin (7) and HOMA-IR (18) in overweight and obese individuals. In contrast, acute and prolonged moderate-intensity exercise under hypoxic conditions have been demonstrated to induce beneficial effects on the insulin sensitivity index and fasting plasma insulin levels and HOMA-IR compared to NE in healthy volunteers (6) and individuals with T2DM (3). Taken together, the majority of the studies investigating the effects of hypoxic compared to normoxic exercise demonstrate increased (4-6) or unchanged (7, 18-21) insulin sensitivity (and related measures). The findings of the present study suggest that hypoxia exposure during exercise has no additional or synergistic effects on glucose homeostasis compared to NE in people with overweight/obesity and impaired glucose metabolism. Notably, our data indicate that comparable improvements in glucose homeostasis may be achieved at a significantly lower absolute workload under hypoxic conditions, thereby reducing mechanical strain, which may be beneficial for patients with obesity and orthopedic comorbidities (18).

Interestingly, we found positive associations between the reduction in SpO_2 induced by HE and the HE-induced changes in mean 24-h and daytime glucose concentrations. More specific, the hypoxia-induced reduction in SpO_2 varied between -2% to -7%, where the largest decrease in SpO_2 was associated with the greatest improvement in glucose concentrations. Interestingly, it has previously been proposed that using a fixed FiO_2 level may result in heterogeneity of

systemic oxygen desaturation, depending on inter-individual differences in hypoxic responses, thereby influencing metabolic adaptations induced by the intervention (22). Therefore, we cannot exclude potential beneficial effects of HE on glucose homeostasis, and it is tempting to postulate that exposure to more severe hypoxia during exercise and/or achieving a certain (lower) SpO_2 level for each individual may improve glycemia. Notably, HE at an individual SpO_2 target of 80% did not improve glucose homeostasis in overweight/obese individuals (20), although this might be explained by the fact that study participants in the latter study were already normoglycemic and had normal insulin sensitivity.

Additionally, we demonstrated that postprandial carbohydrate oxidation tended to be increased following the 4-day hypoxic training regimen. Interestingly, these effects were observed ~20h after the last hypoxic training stimulus and may thus reflect more chronic than acute effects of HE on substrate utilization. In accordance with our findings, it has been demonstrated previously that performing exercise whilst being exposed to (hypobaric) hypoxic conditions increased carbohydrate oxidation as compared to NE (23, 24). Indeed, hypoxia may enhance the reliance on glucose for energy yield, ultimately resulting in increased carbohydrate oxidation (25, 26). Previously, it has been found that carbohydrate oxidation is acutely increased during HE compared to NE in sedentary overweight men, whereas fasting plasma glucose and insulin levels remain unaltered (7). These findings suggest that the hypoxia-mediated increase in carbohydrate oxidation, as achieved during exercise under 15% O_2 in the present study, may not be sufficient to lower plasma glucose levels.

The strengths of the present study are that, to the best of our knowledge, this is the first randomized crossover study to investigate the effects of moderate-intensity exercise under mild hypoxic compared to normoxic conditions on 24-h glucose homeostasis in a controlled setting. The effects of HE on glucose homeostasis were examined using continuous glucose monitoring, which provides a more detailed assessment of glucose homeostasis as well as glycemic variability throughout daytime and nighttime as compared to single-meal studies (27). Importantly, participants were kept in energy balance by providing standardized breakfast, lunch and dinner at standardized time points during the day. In addition, physical activity was monitored throughout the study, and these measurements indicated no alterations in physical activity level during the individual experimental days (neither in the laboratory nor under free-living conditions) and between both regimens.

However, the present study also has some limitations. First, we studied overweight and obese men with impaired glucose tolerance and/or impaired fasting glucose. Therefore, we cannot exclude that HE may have different effects in other populations such as patients with type 2 diabetes, or women with impaired

glucose metabolism. Furthermore, we cannot exclude that more severe hypoxia exposure and/or a longer study duration (22) may induce differential effects on glucose homeostasis.

To conclude, the findings of the present randomized single-blind crossover study demonstrate that 4 consecutive days of moderate-intensity mild HE, at similar relative exercise intensity (lower absolute workload) than NE, reduces systemic oxygen saturation and tends to increase reliance on carbohydrate oxidation to yield energy in overweight and obese men with impaired glucose metabolism. Furthermore, HE has comparable effects on mean 24-h glucose concentration and glycemic variability than NE. In addition, we found that the HE-induced decrease in systemic oxygen saturation was positively associated with HE-induced changes in mean 24-h and daytime glucose concentrations. Future studies should consider targeting a set systemic oxygen level during HE by continuously adjusting FiO_2, thereby minimizing inter-individual differences in oxygen desaturation during hypoxia exposure, and hence on local tissue oxygen tension in metabolically active organs such as skeletal muscle. These insights might contribute to the development of alternative therapeutic strategies to prevent and treat obesity-induced impairments in glucose homeostasis.

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Author contributions

G.G. supervised this work. R.V.M., E.B., and G.G conducted hypothesis generation, conceptual design, and data analysis. R.V.M. contributed to data acquisition. G.G. acquired funding for the study. R.V.M. wrote the manuscript and G.G. had the primary responsibility for the final content. All authors revised the content of the manuscript and read and approved the final version of the manuscript for publication.

Competing interests

None.

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CHAPTER 8

General Discussion

The obesity epidemic is associated with an increased incidence of cardiovascular diseases, type 2 diabetes mellitus, and several types of cancer (1). Lifestyle intervention has been proven effective in prevention of obesity-related complications. However, large inter-individual differences exist in response to interventions, with some individuals responding not as good to a certain intervention as others. The alarming trend of increased prevalence of obesity and related complications warrants the development of alternative therapeutic strategies to alleviate obesity-related health complications.

One of the key characteristics in the development and progression of obesity-related complications is adipose tissue (AT) dysfunction (2, 3), which is characterized by various dysregulations including adipocyte hypertrophy, impaired adipokine secretion, decreased AT blood flow, low-grade inflammation, hormonal resistance, and impaired glucose and lipid homeostasis (4, 5). Since AT dysfunction plays a major role in obesity-induced insulin resistance and related disorders, an increasing number of reports has been focusing on the underlying mechanisms of these pathological manifestations. Recently, it has been postulated that one of the modulators of AT function is the amount of oxygen that is present in the tissue, the so-called oxygen partial pressure (pO_2) of AT (5, 6). Indeed, previous evidence suggests an increased AT oxygen tension in obese compared to lean individuals (7), which was inversely associated with insulin sensitivity in both men and women, independently of age and adiposity (8). In addition, diet-induced weight loss has been shown to reduce AT pO_2, accompanied by improved insulin sensitivity (9). In addition to AT dysfunction, disturbances in other key metabolic organs such as the skeletal muscle (SM), liver and gut play an important role in the development and progression of obesity and metabolic derangements (10, 11).

Interestingly, evidence suggests that local oxygen availability appears to be a determinant in modulating homeostasis in these tissues, thereby impacting metabolic health. Lowering ambient oxygen availability can be achieved by normobaric hypoxia exposure, which has previously been applied in continuous and intermittent protocols. Indeed, exposure to mild intermittent hypoxia (MIH) exposure demonstrated reduced fasting as well as 2h glucose concentrations in obese individuals with impaired glucose homeostasis (12) and improved glucose homeostasis in patients with type 2 diabetes (13).

In the present thesis, we investigated the potential of normobaric mild intermittent hypoxia (MIH) exposure to improve metabolic health in human obesity. Firstly, we performed a randomized, single-blind, cross-over study to investigate the effects of MIH exposure on peripheral, hepatic and AT insulin sensitivity, substrate metabolism, inflammation and mitochondrial function in overweight and obese men **(Chapter 3)**. Furthermore, we gained mechanistic insights in the effects of hypoxia exposure on glucose metabolism in both primary human

adipocytes and myotubes by exposing these cells to physiologically relevant pO_2 levels **(Chapter 3)**. Next, we explored the molecular adaptations induced by MIH exposure in abdominal subcutaneous AT using a proteomics approach **(Chapter 4)**, on alterations in myokine secretion in primary human myotubes and on circulating myokine concentrations *in vivo* **(Chapter 5)**, and on gut microbiota composition in overweight and obese men **(Chapter 6)**. Finally, to investigate the putative additive or synergistic effects of exercise and hypoxia exposure on glucose homeostasis, the effects of normobaric hypoxic relative to normoxic exercise on 24-h glucose concentrations and substrate metabolism were investigated in overweight and obese men with impaired glucose metabolism in a randomized, single-blind, cross-over study **(Chapter 7)**.

The impact of mild intermittent hypoxia exposure on systemic oxygen saturation and tissue oxygen partial pressure

In **Chapter 2**, we have described that hypoxia exposure may evoke pronounced effects on metabolic homeostasis. To investigate whether MIH exposure alters tissue oxygenation, insulin sensitivity and metabolic health, we determined in **Chapter 3** the systemic oxygen saturation (SpO_2), and pO_2 within abdominal subcutaneous AT and SM (*m. gastrocnemius*) using a microdialysis-based optochemical measurement system (7). We demonstrated that MIH exposure (15% O_2) significantly reduces SpO_2 from 97% to 92% in overweight and obese men. In line, in **Chapter 7**, we showed that hypoxic exercise decreases SpO_2 from 96% to 92% as compared to normoxic exercise in overweight and obese men with impaired glucose metabolism.

Interestingly, we show that under normoxic resting conditions, the pO_2 within SM was lower (~15 mmHg) than that in abdominal subcutaneous AT (37 mmHg), which is likely explained by the higher metabolic rate in SM than AT (14). In **Chapter 3**, we furthermore demonstrate that MIH exposure (15% O_2) reduced pO_2 within both AT and SM by ~ 40%. Taken together, we found that exposure to ambient MIH reduces systemic oxygen availability as well as oxygen availability at the level of AT and SM in vivo in humans. Therefore, lower tissue pO_2 due to MIH may potentially elicit (metabolic) adaptations in AT and SM.

The effect of mild intermittent hypoxia exposure on glucose metabolism

Evidence suggests that MIH exposure may beneficially influence glucose homeostasis and insulin sensitivity, as has been reviewed in **Chapter 2**, and elsewhere (15). However, well controlled studies investigating the effects of

hypoxia exposure on glucose homeostasis in people with overweight and obesity are scarce. To this end, in **Chapter 3**, we performed a randomized single-blind cross-over study, and demonstrated that normobaric MIH (15% O_2, 3x2h/day, 7 consecutive days, vs. 21% O_2) induces a shift towards glycolytic metabolism during both fasting and postprandial conditions, reflected by an increased respiratory exchange ratio and carbohydrate oxidation, with simultaneous elevation of plasma lactate concentrations. Thus, 7 consecutive days of MIH exposure appears to be a potent stimulus to increase glycolytic metabolism. Interestingly, carbohydrate oxidation was still significantly higher on day 8, that is 16h after the hypoxic exposure bout, under normoxic conditions. However, MIH did not alter fasting and postprandial glucose and insulin concentrations. In line with these findings, previous reports show increased reliance on carbohydrate oxidation under both fasting (16) and postprandial conditions in response to short-term (17) and long-term hypoxia exposure (18, 19), without alterations in systemic glucose and insulin levels (17). Interestingly, in **Chapter 7,** we demonstrate that a 4-day hypoxic training regimen tended to increase respiratory exchange ratio and carbohydrate oxidation as compared to normoxic exercise, an effect observed ~ 20h after the last exercise bout (day 5). In accordance with these findings, others have demonstrated that (hypobaric) hypoxic exercise increased carbohydrate oxidation as compared to normoxic exercise (16, 20, 21). Together, these findings indicate increased reliance on glucose for energy yield during hypoxic conditions.

The switch towards carbohydrate oxidation, and hence glycolytic metabolism, is favorable under (modest) oxygen deprivation to yield energy, since glucose oxidation required less oxygen then lipid oxidation (22) In accordance with this, in **Chapter 4**, we found that MIH induced alterations in the abdominal subcutaneous AT proteome, namely a downregulation of NAD^+-dependent pathways. Amongst the latter are central metabolic pathways such as the tricarboxylic acid cycle, thereby potentially promoting alternative (anaerobic) pathways to yield energy under hypoxic conditions.

Furthermore, in **Chapter 3**, we found that primary human myotubes exposed to mild hypoxic conditions for muscle (1% O_2), which resembles SM pO_2 values in humans during moderate-intensity exercise under normoxic conditions (and passive hypoxia exposure to 15% O_2), increased insulin-independent glucose uptake. The latter was at least partially driven by AMP-dependent kinase (AMPK). Interestingly, AMPK seems to regulate several pathways downstream of hypoxia-inducible factor (HIF). For example, AMPK upregulates glucose transporter (GLUT)-1, one of the key biomarkers for anaerobic glycolysis (23), and is responsible for insulin-independent effects on glucose homeostasis (24). In line, we found MIH-induced upregulation of GLUT1 protein expression in abdominal subcutaneous AT, as described in **Chapter 4**.

Similar to hypoxia, performing exercise induces a shift towards an increased [AMP]/[ATP] ratio, increasing AMPK-induced insulin-independent glucose uptake (25). Interestingly, since hypoxia and exercise share similar pathways in modulating glucose homeostasis, synergistic effects of both have been described previously in rodents (26). In line, it has previously been demonstrated that performing exercise during hypoxic conditions (hypoxic exercise, HE) may improve glucose homeostasis and insulin sensitivity to a greater extent as compared to normoxic exercise (NE) (27-30). In **Chapter 7**, we demonstrate that performing 4-day HE at similar relative intensity has comparable effects on mean 24-h glucose concentration and glycemic variability as compared to NE. Interestingly, however, the HE-induced decrease in systemic oxygen saturation was positively correlated with HE-induced reduction in average 24-h and daytime glucose concentrations. Indeed, we demonstrate that the HE-induced reduction in systemic oxygen saturation varied between -2% to -7%, where the largest decrease in systemic oxygen saturation was associated with the greatest improvement in glucose concentrations. Hence, based on these data it is tempting to speculate that HE may have beneficial effects on glucose homeostasis if a more severe hypoxic stimulus, inducing a lower systemic oxygen level, is applied. On the other hand, it has been reported that HE at a systemic oxygen saturation target of 80%, resembling severe hypoxia, did not improve parameters related to glycemia and insulin sensitivity as compared to NE (31). Notably, in the latter study, participants were normoglycemic and insulin sensitive (31) and it is possible that effects may be more pronounced in people with impaired fasting glucose, impaired glucose tolerance and/or patients with type 2 diabetes. Taken together, we demonstrate that HE induces on average similar effects on glucose homeostasis compared to NE, although our data suggest that more pronounced reduction in systemic oxygen saturation may improve glycemia. Importantly, our findings may have implications for obese individuals with orthopedic complications, since exercise under hypoxic conditions with a reduced absolute workload may lower stress on the joints (31, 32).

Insulin sensitivity

To further elucidate the role of hypoxia in glucose homeostasis, we explored the effects of MIH exposure on tissue-specific insulin sensitivity in obese men in **Chapter 3**. We found for that MIH did not significantly alter AT, hepatic and peripheral insulin sensitivity. Our findings seem in contrast with several previous studies in rodents (33-36) and obese humans (13, 28, 29, 32, 37) that demonstrated improved glucose homeostasis. Importantly, however, the human studies that did find an improved whole-body insulin sensitivity following hypoxia exposure did not include a control group (13, 28, 37) or the hypoxia-induced improvement in insulin sensitivity was not different from the control condition (32). In addition, the

duration of the hypoxic stimulus may be an important determinant of the effects of hypoxia exposure on insulin sensitivity. Indeed, several human studies used prolonged hypoxia exposure regimens (13, 37), whereas in our study the cumulative hypoxic exposure was in total 42 hours. Therefore, we cannot exclude that more prolonged MIH exposure is required to induce beneficial effects on insulin sensitivity and glucose homeostasis. In addition, in **Chapter 3**, we included obese men with modest impairments in glucose homeostasis (mean HOMA-IR: 3.7 ± 0.4; fasting glucose: 5.7 ± 0.2 mmol/l; 2-h glucose: 6.2 ± 0.4 mmol/l). Hence, we cannot exclude that MIH may elicit a differential response in individuals with more severe insulin resistance such as patients with type 2 diabetes. In line with unchanged glucose disposal during the hyperinsulinemic-euglycemic clamp following the 7-day MIH exposure protocol, MIH did not alter insulin signaling in SM (assessed by measurement of insulin-induced Akt phosphorylation in SM biopsies collected at baseline and during hyperinsulinemic conditions during the clamp). These *in vivo* findings, described in **Chapter 3**, are corroborated by the mechanistic experiments we performed using primary human myotubes and adipocytes exposed to hypoxia. Thus, we found similar *insulin-mediated* glucose uptake following hypoxia as compared to normoxia exposure, suggesting no alterations in insulin sensitivity upon mild hypoxia exposure.

Although AT insulin sensitivity remained unchanged upon MIH exposure, in **Chapter 4**, we demonstrate that the MIH-induced increase in tropomodulin-3 (TMOD3) protein expression in AT was positively associated with the change in AT insulin sensitivity in obese men. Interestingly, TMOD3 plays a key role in insulin signaling transduction, and may therefore be related to improved AT insulin sensitivity (38). In addition, we found other proteins involved in actin dynamics to be upregulated subsequent to MIH exposure. Notably, actin cytoskeleton reorganization is essential in insulin signaling and, therefore, has been shown to enhance glucose homeostasis (39, 40). Moreover, in **Chapter 4**, we found a hypoxia-induced upregulation of focal adhesion-related proteins in abdominal subcutaneous AT, which may promote insulin sensitivity through focal adhesion kinase activity (41). Together, these findings suggest that MIH evoked adaptations in abdominal subcutaneous AT that might contribute to improved AT insulin sensitivity, yet this remains to be established in studies with more prolonged hypoxia exposure.

Adipokine/myokine secretion and systemic low-grade systemic inflammation

Dysfunctional white adipose tissue (WAT) in obesity has been characterized by a state of chronic low-grade inflammation (2). Indeed, several events that may contribute to the pro-inflammatory state of WAT in obesity have been identified (42).

Yet, inflammation appears essential for AT remodeling, and is therefore not solely a pathological phenomenon (43). Recently, evidence suggests a role for hypoxia in WAT inflammation (44-47). Interestingly, hypoxia is linked to both anti-inflammatory and pro-inflammatory responses (48-51). The discrepancies in findings on the effects of hypoxia on inflammation are likely due to different study designs. Indeed, *in vitro* studies investigating the effects of acute, severe hypoxia report increased expression and secretion of pro-inflammatory factors (52, 53), whereas prolonged mild hypoxia exposure, thereby better mimicking the *in vivo* microenvironment, may decrease pro-inflammatory gene expression (48). In **Chapter 3**, we investigated the effects of MIH on AT and systemic low-grade inflammation. First, we performed microarray analysis on both AT and SM biopsies. Remarkably, we did not find any significantly altered pathways in SM upon MIH exposure. This might be explained by the fact that performing daily physical activity and exercise result in local hypoxia due to increased oxygen consumption (54). Indeed, increased HIF1α stabilization in SM has been found in response to an acute bout of exercise (54), suggesting that SM is generally more adapted to hypoxia compared to AT. However, in AT, we found an upregulation of several genes involved in pro-inflammatory pathways, with the most pronounced upregulation of the NF-kB-related pathway. One explanation for the induction of genes involved in the NF-kB pathway might be related to the hypoxia-induced increase in systemic lactate concentrations, as we observed in **Chapter 3**. In line, lactate also stimulated increased NF-kB activation in L6 myocytes and macrophages (55, 56). On the other hand, the hypoxia-lactate axis have been found to attenuate inflammation by suppressing macrophage activation and polarization towards M2-macrophages (57, 58).

The effects on AT gene expression did not translate into changes in systemic low-grade inflammation, as several markers of low-grade inflammation such as TNFα, IFNγ, IL-6 and IL-8 were not altered following MIH (**Chapter 3**) Additionally, in **Chapter 5** we found an increased secretion of IL-6 by primary human myotubes exposed to 1% O_2 as compared to 21% O_2 (standard lab conditions), but no effects of hypoxia exposure on plasma IL-6 concentrations were found in obese men (**Chapter 3 and 5**). However, the role of IL-6 is still under debate, and IL-6 is being recognized for both pro- and anti-inflammatory characteristics (59). Yet, in **Chapter 3**, mechanistic studies using primary human adipocytes demonstrated unchanged mRNA expression of several inflammatory genes, amongst which are IL-6, MCP1, PAI-1 and leptin, upon MIH exposure (cycles alternating from 10% to 5% O_2, 3x2h per day, total duration 7 days). In addition, MIH did not alter the secretion of IL-6, MCP-1 and leptin as compared to normoxic exposure in primary human adipocytes (**Chapter 3**). Taken together, these results suggest tissue-specific effects on differential secretion of inflammatory cytokines upon hypoxia exposure.

Substrate metabolism and skeletal muscle mitochondrial function

Since tissue pO_2 reflects the balance between supply of oxygen and oxygen consumption (i.e. metabolic rate), in **Chapter 3**, we investigated the effects of MIH exposure on mitochondrial oxygen consumption in SM. Indeed, SM is a highly metabolically active organ, accounting for 20-30% of total energy expenditure (60). It has been thought that hypoxia-induced HIF-1α expression potentially inhibits decarboxylation of pyruvate into acetyl-CoA by pyruvate dehydrogenase (61). This may then shunt pyruvate away from the mitochondria, resulting in reduced mitochondrial respiration, hence enhancing glycolysis to ensure sufficient ATP production (61). Therefore, we determined *ex vivo* SM mitochondrial respiration in permeabilized human SM fibers derived from the muscle biopsies collected from the study participants in **Chapter 3**. Mitochondrial respiration was determined using both lipid and carbohydrate substrates (i.e. octanoyl-carnitine and pyruvate, respectively). MIH did not induce any changes in oxygen consumption rates in permeabilized muscle fibers. In addition, MIH exposure did not alter SM protein expression of OXPHOS complex proteins in obese individuals. In accordance with these findings, we found that 1% O_2 exposure did not alter OXPHOS complex protein expression in primary human myotubes compared to normoxia exposure.

We demonstrate unaltered insulin sensitivity and oxidative capacity in obese humans, subsequent to MIH exposure. Interestingly, it has been demonstrated that hypoxia might refine plasticity of mitochondrial respiration, possibly through the switch from complex IV protein cytochrome c oxidase subunit 4 isoform 1 (COX4I1), towards COX4I2, which is characterized by higher oxygen affinity (62, 63). However, we could not find a switch towards increased COX4I2 gene expression in both AT and SM tissue induced by MIH on the gene and protein expression level **(Chapters 3 and 4)**. Nevertheless, we found decreased SM gene expression of pathways related to translation of mitochondrial proteins **(Chapter 3**). Indeed, it has been demonstrated that hypoxia inhibits the mitochondrial ribosomal proteins (64), which are responsible for translation of the mitochondrial proteins. Taken together, we demonstrated that MIH exposure does not affect oxidative capacity and protein expression of mitochondrial complex components in skeletal muscle tissue. However, MIH induces a downregulation of pathways related to translation of mitochondrial proteins in SM. Whether this differential expression might result in functional implications following longer-term hypoxia exposure remains to be elucidated.

Modulation of microbial composition

Interestingly, the gut microbiota has emerged as an integral factor in impacting host metabolism, and has been suggested to play a key role in regulating energy and substrate metabolism and maintaining insulin sensitivity (65). Indeed, it has been demonstrated that manipulation of gut microbiota composition using fecal transplantation studies with lean microbiota improved peripheral insulin sensitivity in males with metabolic syndrome (66), although data in this respect are not consistent (67). Remarkably, several studies suggest that hypoxia exposure may also alter gut microbiota composition, thereby potentially contributing to alterations in host metabolism. For instance, rats exposed to prolonged intermittent hypobaric hypoxia had a lower *Firmicutes* to *Bacteriodetes* ratio, which may at least partially underlie the improvements in glucose and lipid metabolism observed subsequent to the hypoxia exposure regimen (68). In addition, the abundance of strictly anaerobic bacteria was increased upon hypoxia exposure in rodents (68-70) and humans (71, 72). Taken together, hypoxia exposure may alter gut microbiota composition, thereby affecting host metabolism, yet well-controlled studies are lacking. In **Chapter 6**, we describe that 7-day MIH exposure has slight but significant effects on gut microbiota composition in overweight and obese men. In line with previous findings, we found an increased relative abundance of the anaerobic genera *Holdemania*, *Butyricicoccus* and *Fusicatenibacter*, which all are butyrate-producers. Interestingly, it appears that increased levels of butyrate-producing bacteria are associated with improved glucose metabolism in humans (65). In addition, we found that MIH-induced alterations in abundances of *Butyricimonas*, *Odoribacter*, *Parabacteroides* and *Ruminococcus torques group* were positively correlated with MIH-induced alterations in peripheral insulin sensitivity. Moreover, the MIH-induced change in the relative abundance of *Barnesiella* was inversely correlated with changes in AT insulin sensitivity. Previous reports have demonstrated reduced abundance of *Barnesiella* in native highlanders, being chronically exposed to hypobaric hypoxia (72). Also, it has been demonstrated that mice fed an anti-diabetogenic diet showed reduced abundance of *Barnesiella*, with concomitant decrease in hyperglycemia (73). Taken together, our findings might suggest a putative role for *Barnesiella Butyricimonas*, *Odoribacter*, *Parabacteroides* and *Ruminococcus torques group* in the modulation of glucose metabolism and insulin sensitivity in obese humans. However, our results should be interpreted with caution due to relatively small sample size and hence limited statistical power.

Main outcomes of this thesis

The main outcomes of this thesis are that MIH exposure (3x2h 15% O_2 for 7 consecutive days) did not alter AT, SM and hepatic insulin sensitivity in obese men. Yet, our studies demonstrate potent effects of hypoxia on substrate utilization in overweight/obese individuals *in vivo*, as well as in mechanistic *in vitro* studies using primary human myotubes. Interestingly, hypoxia exposure altered the expression of several myokines *in vitro*, which may underlie the hypoxia-mediated increase in glucose uptake in myotubes. Nevertheless, alterations in systemic myokine concentrations could not be detected. In addition, MIH increased gene expression of inflammatory pathways in AT in obese individuals, although no changes in low-grade systemic inflammatory markers were found. Furthermore, we demonstrated that MIH has pronounced effects on the abdominal subcutaneous AT proteome, mainly on proteins related to oxidoreductase systems, cell-adhesion, actin cytoskeleton and ECM organization, and energy metabolism, suggesting molecular adaptations in AT induced by hypoxia. Moreover, MIH had an impact on the gut microbiome, since MIH exposure increased the relative abundance of anaerobic butyrate-producing bacteria. The latter finding suggests that MIH-induced alterations in gut microbiota composition might affect host metabolism. Finally, we demonstrate that 4-day HE at similar relative intensity has comparable effects on mean 24-h glucose concentrations and glycemic variability, and tended to increase carbohydrate oxidation, as compared to NE in overweight and obese men with impaired glucose metabolism. Interestingly, however, the HE-induced decrease in systemic oxygen saturation was positively correlated with HE-induced reduction in average 24-h and daytime glucose concentrations.

Future perspectives

Future studies investigating the potential of hypoxia exposure to improve metabolic health should focus on various aspects:

The **hypoxic exposure regimen**. The hypoxia exposure protocol may greatly influence the metabolic adaptations induced by the hypoxic stimulus (15). Interestingly, previous intervention studies investigating the effects of mild hypoxia exposure on insulin sensitivity showed improved glucose tolerance and whole-body insulin sensitivity in obese men (13, 37). Although the O_2 exposure level was similar compared to our design **(Chapter 3)**, total exposure duration was much higher (100h (37) and 98-168h (13) vs. 42h (**Chapter 3**)). Notably, both studies did not include a control group (13, 37), so results should be interpreted cautiously. Not only the duration, but also severity of hypoxic exposure might be an important

determinant of metabolic adaptations (15). Indeed, in **Chapter 7** we found that performing HE at 15% O_2 resulted in an absolute difference of -2 to -7% in SpO_2 in the studied population. Remarkably, the HE-induced decrease in SpO_2 correlated with the improvement in mean 24-h and daytime glucose concentrations, with the largest reduction in SpO_2 associated with the greatest improvement in mean glucose concentration. Future research should focus on the exposure to more severe hypoxia during exercise and/or achieving a certain (lower) SpO_2 level for each individual and investigate the effects on glucose homeostasis.

The **effects of hypoxia exposure on the liver**. In **Chapter 3**, we describe the effects of MIH exposure on hepatic insulin sensitivity by determining basal endogenous glucose production as well as its suppression under insulin-stimulated conditions during the two-step hyperinsulinemic euglycemic clamp. Although we found that MIH exposure has no significant effects on hepatic insulin sensitivity, previous reports suggest that acute exposure to severe hypoxia may increase hepatic glucose output (74). However, prolonged (7-day) high altitude exposure resulted in similar hepatic glucose output as compared to baseline (75). Both the acute and short-term high altitude studies found an association between hepatic glucose output and plasma epinephrine concentrations (74, 75), which may point towards transiently increased sympathetic nervous system activity, which may lead to transient hyperglycemia. In addition, others have found selective depletion of hepatic glycogen content with concomitant decrease in glucose concentrations in rodents after prolonged severe hypoxia exposure (76). Furthermore, it would be interesting to investigate the *in vitro* effects of hypoxia exposure on the expression and secretion of hepatokines, as hepatokines may be involved in the induction and progression of metabolic impairments (77). Taken together, the liver may play an important role in hypoxia-mediated effects on glucose homeostasis, and the mechanism by which this occurs is little understood (78).

The **metabolic phenotype** of individuals. Indeed, it would be worthwhile to investigate the effects of long-term hypoxia exposure in obese individuals with more severe insulin resistance and/or more pronounced perturbations in glucose homeostasis such as patients with T2DM. Previous studies demonstrated that participants with the lowest insulin sensitivity at baseline improved most upon mild hypoxia exposure (37). In addition, individuals with T2DM performing acute HE showed improved insulin sensitivity (27, 28), whereas in lean endurance trained men, prolonged HE impaired glucose metabolism, as demonstrated by increased plasma glucose and insulin concentrations as compared to NE (79).

In addition, if hypoxia exposure regimens have proven to be effective in improving glucose homeostasis the application of both hypoxic tents (13, 37) and clinical rooms in which O_2 levels can be manipulated and monitored (**Chapter 3 and 7**) are not convenient in the clinical setting and for large-scale dissemination.

Indeed, one of the key aspects for future research should focus **on the manner, and applicability of administration of the hypoxic stimulus** for clinical use. Interestingly, several other model designs have been suggested for future therapeutic applications for inducing hypoxic exposure, amongst which are programmable hypoxic bedrooms via modification of heating and cooling units, and the development of nitrogen dilution capabilities in continuous positive airway pressure (CPAP) devices (13).

Furthermore, the underlying response upon hypoxic exposure (i.e. HIF-1α-mediated pathways) may alternatively be generated by **a pharmacological approach**. Remarkably, several small molecule inhibitors of prolyl-hydroxylase dehydrogenases (PHDs), resulting in the stabilization of HIFs, have been developed recently. These agents have already been applied in clinical trials for the treatment of anemia in chronic kidney disease (80, 81). Notably, inhalation of ambient hypoxic air induces a similar response as the pharmacological inhibition of PHD and subsequent stabilization of HIFs. Interestingly, effective inhibition of PHD2 results in erytropoetin (EPO) biosynthesis and secretion by the liver, which decreased gluconeogenesis and prevented high-fat diet induced glucose intolerance and hepatic inflammation in rodents (82). In addition, modest stabilization of HIF-2α appears to be protective against the development of T2DM in mice (83). Furthermore, FG-4497 treatment, stabilizing both HIF1α and 2α, resulted in improved diet-induced glucose tolerance, lowered hepatic steatosis and decreased visceral adiposity in rodents (84). In addition, FG-4497 administration resulted in a reduction of serum cholesterol, inflammation and the incidence of atherosclerosis in mice (85). Based on these observations, it would be highly interesting to elucidate the potential implications of PHD inhibitors in maintaining and improving metabolic homeostasis in humans at high risk of cardiometabolic complications.

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APPENDIX A

SUMMARY

The drastic increase in the obesity prevalence over the last decades, and the concomitant growth in the incidence of related complications such as cardiovascular diseases, type 2 diabetes mellitus and cancer, calls for alternative treatment strategies. Indeed, lifestyle interventions have been proven to be effective, but large inter-individual differences exist in treatment response and long-term compliance is an important issue, highlighting the need for alternative approaches.

Adipose tissue (AT) dysfunction is one of the hallmarks in the development of obesity-related insulin resistance. Interestingly, oxygen levels in tissues may play a key role in metabolic homeostasis in organs such as the AT, skeletal muscle (SM), liver and gut. Conflicting findings have been reported on AT pO_2 in obesity. We have recently demonstrated that AT pO_2 was higher in people with obesity, and was inversely associated with insulin sensitivity. The aim of this thesis was to investigate the effects of normobaric mild intermittent hypoxia (MIH) exposure on the metabolic phenotype of AT, SM, liver and the gut in overweight and obese men. Furthermore, we aimed to gain mechanistic insight in the putative metabolic and inflammatory effects of hypoxia using primary human adipocytes and myotubes. Finally, we examined the effects of hypoxic exercise on glucose homeostasis in overweight and obese men with impaired glucose homeostasis.

In **Chapter 2**, we reviewed the role of AT pO_2 on glucose and lipid metabolism, as well as inflammation. In addition, we discussed the putative effects of lowering pO_2 by means of hypoxia exposure on AT as well as whole-body metabolism. Interestingly, experimental hypoxia exposure impacts adipocyte function by altering glucose and lipid homeostasis, inflammation and by affecting the expression/secretion of adipokines. Furthermore, several human intervention studies provided evidence that hypoxia exposure may enhance glucose homeostasis and insulin sensitivity. However, well-controlled studies investigating the effects of hypoxia exposure in overweight and obese humans are scarce, since most studies did not include a control group.

Therefore, in **Chapter 3**, we performed a single-blind, randomized, cross-over study to investigate the effects of normobaric MIH on tissue-specific insulin sensitivity in 12 overweight and obese men. Participants were randomly exposed to MIH for 7 consecutive days (15% O_2 for 3x2h per day or 21% O_2 (control)), with a 3-6 weeks wash-out period. AT and SM pO_2, fasting and postprandial substrate metabolism, tissue-specific insulin sensitivity and AT/SM gene and protein expression were determined. Mechanistically, we investigated the effects of hypoxia exposure on glucose uptake, gene and protein expression in primary human adipocytes and myotubes. We showed that MIH exposure decreased systemic O_2 saturation and pO_2 in both AT and SM. In addition, we demonstrated that MIH exposure increased whole-body carbohydrate oxidation with concomitant increased plasma lactate concentrations compared to normoxia exposure. Furthermore, we found increased

gene expression of several pathways related to inflammation and carbohydrate/lipid metabolism in AT. MIH did not alter SM mitochondrial respiration/oxidative capacity, nor did it affect AT, hepatic and peripheral insulin sensitivity. Mechanistically, we demonstrated that hypoxia exposure markedly increased insulin-independent glucose uptake in primary human myotubes, but not in adipocytes, which appears to be mediated at least partially through AMP-dependent protein kinase (AMPK).

Since it has been demonstrated that experimental hypoxia exposure affects various pathways involved in energy metabolism, we next investigated the effects of MIH exposure on the AT proteome in **Chapter 4**. Abdominal subcutaneous AT biopsies were taken after 7 consecutive days of MIH exposure under normoxic, fasting conditions, in the randomized cross-over design study. After protein isolation, proteins were identified and quantified using liquid chromatography-mass spectrometry. After correction for blood protein contamination, 1022 AT proteins were identified, from which 123 were differentially expressed compared to normoxia. MIH exposure increased several proteins related to focal adhesion, actin cytoskeleton organization and iron homeostasis, whereas it reduced the expression of proteins related to the biosynthesis of polysaccharides, collagen fibril organization and NAD^+ dependent processes. Interestingly, amongst the latter are central metabolic pathways, such as the tricarboxylic acid cycle, thereby potentially promoting alternative pathways for energy yield under mild hypoxic conditions. Furthermore, we found that an increased tropomodulin-3 (TMOD3) expression was associated with MIH-induced alterations in AT insulin sensitivity. Indeed, TMOD3 has been previously implicated in insulin signal transduction in AT.

We demonstrate, in **Chapter 3**, that MIH exposure substantially decreased SM pO_2, a phenomenon which is also observed with SM contraction during physical exercise. Exercise-induced SM contraction promotes the secretion of myokines, which may enhance SM glucose uptake and fatty acid oxidation. Since both MIH exposure and SM contraction substantially decrease SM pO_2, we aimed to investigate whether MIH exposure affects myokine secretion in **Chapter 5**. Therefore, differentiated primary human myotubes were exposed to 1% O_2 (mimicking physiological hypoxia in SM), 3% O_2 (physiological normoxia in SM) and 21% O_2 (standard laboratory conditions) for 24h. Subsequently cell medium was harvested for myokine analysis. Furthermore, we determined plasma concentrations of several myokines in overweight and obese individuals exposed to normobaric MIH for 7 consecutive days during a high-fat mixed meal test under hypoxic conditions (at day 7), and under normoxic conditions (at day 8, approximately 16h after the final hypoxic stimulus), in a randomized cross-over fashion. Interestingly, we demonstrate that *in vitro* hypoxia exposure (1% O_2) altered the secretion of myokines suggested to be involved in glucose homeostasis, revealing hypoxia as a novel modulator of myokine secretion. More specific, *in vitro* hypoxia exposure increased secreted protein acidic and rich in cysteine (SPARC) and follistatin-related protein

(FSTL-1) expression, whereas it reduced the expression of leukemia inhibitory factor (LIF) as compared to 3% O_2. However, 7-day MIH did not alter plasma myokine concentrations during (at day 7) and subsequent to (at day 8) the exposure regimen in overweight and obese men.

High-altitude studies suggest that hypoxia exposure may induce alterations in gut microbiota composition, which may in turn affect host metabolism, but well-controlled studies are lacking. Therefore, in **Chapter 6**, we investigated the effects of normobaric MIH expression (15% O_2, 3x2h per day, 7 consecutive days) compared to normoxia (21% O_2) on gut microbiota composition in overweight and obese men. Using 16S rRNA gene amplicon sequencing, we demonstrated that MIH exposure induced modest changes in fecal microbiota composition in overweight and obese humans, shifting several bacterial families and genera towards higher abundances of strict anaerobic butyrate-producing bacteria. Furthermore, we demonstrated that several of these MIH-induced effects on microbial composition were associated with parameters of glucose and lipid metabolism.

Since hypoxia-induced effects on glucose homeostasis may share similar underlying mechanisms compared with physical exercise, we next explored the synergistic potential of hypoxia and exercise. Thus, we performed a single-blind, randomized, cross-over study to investigate the effects of moderate-intensity hypoxic exercise (HE) on glucose homeostasis in overweight and obese humans with impaired glucose homeostasis in **Chapter 7**. Ten participants performed moderate-intensity cycling sessions for 4 consecutive days under mild normobaric hypoxia (FiO_2: 15%) or normoxia (FiO_2: 21%) at similar relative exercise intensity (2x30 min/d at 50% of maximal heart rate (HR_{MAX}) or 50%W_{MAX}), with a wash-out period of 3-6 weeks. Throughout the study, 24h-glucose concentrations and systemic O_2 saturation was monitored. At day 5, a mixed-meal test was performed under normoxic conditions, and substrate metabolism was investigated. We demonstrated that HE performed at similar relative workload intensity as compared to normoxia reduced the systemic O_2 saturation compared to NE. Yet, HE did not alter mean 24-h, daytime and nighttime glucose concentrations, or any measures of glycemic variability. Nevertheless, the reduction in systemic O_2 saturation by HE was positively correlated with HE-induced change in mean 24-h and daytime glucose concentrations.

In conclusion, the main findings of this thesis are that MIH did not alter tissue-specific insulin sensitivity. However, both *in vivo* and *in vitro* studies demonstrate potent effects on substrate utilization, pointing towards increased glycolytic metabolism. Furthermore, hypoxic exercise did not alter glucose homeostasis in overweight and obese men with impaired glucose metabolism. Future studies are warranted to investigate the potential of different hypoxia exposure regimens, taken into the duration, frequency and severity of the hypoxic stimulus into account, to

induce metabolic adaptations. It is worthwhile to examine this in men and women separately, and to investigate the effects of hypoxia exposure in study populations with a different metabolic/inflammatory phenotype. If hypoxia exposure appears to elicit beneficial effects, it might provide a novel therapeutic strategy to prevent or treat obesity-related complications.

A

SAMENVATTING

Samenvatting

De drastische toename in de prevalentie van obesitas en de gelijktijdige toename van de incidentie van gerelateerde complicaties zoals hart- en vaatziekten, diabetes mellitus type 2 en kanker in de afgelopen decennia, onderlijnen de vraag om alternatieve behandelingsstrategieën. Heden ten dage is er bewijs dat leefstijlinterventies effectief zijn, maar er bestaan grote interindividuele verschillen in de respons op de behandeling en therapietrouw op de lange termijn, wat de behoefte aan alternatieve benaderingen benadrukt.

Vetweefseldysfunctie omvat een van de kenmerken van de ontwikkeling van obesitas-gerelateerde insulineresistentie. Zuurstofniveaus in weefsels zouden mogelijk een sleutelrol kunnen spelen binnen de metabole homeostase in organen zoals de vetweefsel, skeletspier, lever en darmen. Er zijn tegenstrijdige bevindingen gerapporteerd over vetweefsel zuurstofspanning (pO_2) bij obesitas. Onlangs is aangetoond dat de vetweefsel pO_2 hoger is bij mensen met obesitas, en tevens omgekeerd geassocieerd is met insulinegevoeligheid. Het doel van dit proefschrift was om te onderzoeken wat de effecten van blootstelling aan normobare milde intermitterende hypoxie (MIH) op het metabole fenotype van het vetweefsel, de skeletspier, lever en darmen bij mannen met overgewicht en obesitas. Bovendien wilden we mechanistisch inzicht verschaffen in de mogelijke metabole en inflammatoire effecten van hypoxie met behulp van primaire humane adipocyten en myotubes. Tenslotte onderzochten we de effecten van fysieke inspanning onder hypoxische condities op glucosehomeostase bij mannen met overgewicht en obesitas met verminderde glucosehomeostase.

In **Hoofdstuk 2** wordt de rol van vetweefsel pO_2 op het glucose- en lipidenmetabolisme, evenals op inflammatie toegelicht. Daarnaast beschrijven we de vermeende effecten van pO_2-verlaging middels hypoxie blootstelling op het vetweefsel en het metabolisme van het hele lichaam. Experimentele blootstelling aan hypoxie kan de functie van adipocyten beïnvloeden door de glucose- en lipidehomeostase en inflammatie te veranderen, tevens door de expressie en secretie van adipokines te beïnvloeden. Bovendien hebben verschillende humane interventiestudies aangetoond dat blootstelling aan hypoxie de glucosehomeostase en insulinegevoeligheid kan verbeteren. Gecontroleerde onderzoeken naar de effecten van blootstelling aan hypoxie bij mensen met overgewicht en obesitas zijn echter schaars, aangezien de meeste onderzoeken geen controlegroep omvatten.

Daarom hebben we in **Hoofdstuk 3** een enkelblinde, gerandomiseerde, cross-over studie uitgevoerd om de effecten van normobare MIH op weefselspecifieke insulinegevoeligheid bij 12 mannen met overgewicht en obesitas te onderzoeken. Deelnemers werden willekeurig blootgesteld aan MIH gedurende 7 opeenvolgende dagen (15% O_2 gedurende 3x2 uur per dag of 21% O_2 (controle)), met een uitwasperiode van 3-6 weken. Vetweefsel en skeletspier pO_2, nuchtere

en postprandiaal substraatmetabolisme, weefselspecifieke insulinegevoeligheid en gen- en eiwitexpressie werden bepaald. Mechanistisch onderzochten we de effecten van blootstelling aan hypoxie op glucoseopname, gen- en eiwitexpressie in primaire humane adipocyten en myotubes. We toonden aan dat MIH-blootstelling de systemische O_2-verzadiging en pO_2, in zowel vet- als skeletspierweefsel, verlaagde. Bovendien hebben we aangetoond dat blootstelling aan MIH de oxidatie van koolhydraten in het hele lichaam verhoogde met gelijktijdige verhoogde plasmalactaatconcentraties in vergelijking met blootstelling aan normoxie. Bovendien vonden we verhoogde genexpressie van verschillende routes die verband houden met inflammatie en het metabolisme van koolhydraten / lipiden in vetweefsel. MIH veranderde de mitochondriële respiratie / oxidatieve capaciteit van de SM niet, noch had het invloed op de vetweefsel, hepatische en perifere insulinegevoeligheid. Mechanistisch hebben we aangetoond dat blootstelling aan hypoxie de insuline-onafhankelijke glucoseopname in primaire humane myotubes aanzienlijk verhoogde, die op zijn minst gedeeltelijk lijkt te worden gemedieerd door AMP-afhankelijke proteïnekinase (AMPK). Dit effect werd echter niet geobserveerd in primaire humane adipocyten.

Omdat is aangetoond dat experimentele blootstelling aan hypoxie verschillende onderliggende routes beïnvloedt die betrokken zijn bij het energiemetabolisme, onderzochten we vervolgens de effecten van MIH-blootstelling op het vetweefsel-proteoom in **Hoofdstuk 4**. Abdominale subcutane vetweefselbiopsieën werden afgenomen na 7 opeenvolgende dagen van MIH-blootstelling onder normoxisch, gevaste condities, in de gerandomiseerde cross-over studie. Na proteïne-isolatie werden proteïnen geïdentificeerd en gekwantificeerd met behulp van vloeistofchromatografie-massaspectrometrie. Na correctie voor bloedeiwitverontreiniging werden 1022 AT-eiwitten geïdentificeerd, waarvan 123 differentieel tot expressie werden gebracht in vergelijking met normoxie. MIH-blootstelling verhoogde de expressie verschillende eiwitten gerelateerd aan focale adhesie, actine-cytoskelet-organisatie en ijzerhomeostase, terwijl het de expressie van eiwitten gerelateerd aan de biosynthese van polysachariden, collageenfibril-organisatie en NAD^+-afhankelijke processen verminderde. Interessant is dat onder de laatste centrale metabole routes zijn, zoals de citroenzuurcyclus, waardoor mogelijk alternatieve routes voor energieopbrengst onder mild hypoxische omstandigheden worden bevorderd. Bovendien vonden we dat een verhoogde tropomoduline-3 (TMOD3) -expressie geassocieerd was met MIH-geïnduceerde veranderingen in insulinegevoeligheid van het vetweefsel. Reeds is aangetoond dat TMOD3 betrokken is bij de transductie van insulinesignalen in het vetweefsel.

In **Hoofdstuk 3** laten we zien dat blootstelling aan MIH de skeletspier pO_2 substantieel verlaagde, een fenomeen dat ook wordt waargenomen bij SM contractie tijdens lichamelijke inspanning. Door inspanning geïnduceerde skeletspiercontractie bevordert de secretie van myokines, wat de opname van

glucose en vetzuuroxidatie in het skeletspierweefsel kan verbeteren. Aangezien zowel MIH-blootstelling als skeletspiercontractie de skeletspier pO_2 substantieel verlagen, wilden we onderzoeken of MIH-blootstelling de myokinesecretie beïnvloedt in **Hoofdstuk 5**. Daarom werden gedifferentieerde primaire humane myotubes blootgesteld aan 1% O_2 (nabootsing van fysiologische hypoxie in skeletspierweefsel), 3% O_2. (Fysiologische normoxie in skeletspierweefsel) en 21% O_2 (standaard laboratoriumomstandigheden) gedurende 24 uur. Vervolgens werd celmedium verzameld voor myokine analyse. Verder bepaalden we plasmaconcentraties van verschillende myokines bij personen met overgewicht en obesitas die 7 opeenvolgende dagen waren blootgesteld aan normobare MIH tijdens een maaltijdtest onder hypoxische omstandigheden (op dag 7) en onder normoxische omstandigheden (op dag 8, ongeveer 16 uur na laatste hypoxische stimulus), in een gerandomiseerde cross-over studie. We toonden aan dat *in vitro* blootstelling aan hypoxie (1% O_2) de secretie van myokines veranderde. Van deze myokines wordt gesuggereerd dat ze betrokken zijn bij glucosehomeostase, waarbij hypoxie mogelijk een rol speelt als modulator van myokinesecretie. *In vitro* blootstelling aan hypoxie verhoogde de secreted protein, acidic and rich in cysteine (SPARC) en follistatin-related protein-1 (FSTL-1) expressie, terwijl het de expressie van leukemia inhibitory factor (LIF) verminderde in vergelijking met 3% O_2. 7-daagse MIH veranderde de plasma myokine concentraties echter niet tijdens (op dag 7) en volgend op (op dag 8) het blootstellingsregime bij mannen met overgewicht en obesitas.

Studies op hoge hoogte suggereren dat blootstelling aan hypoxie veranderingen in de samenstelling van de darmflora zou kunnen veroorzaken, die op hun beurt het metabolisme van de gastheer kunnen beïnvloeden. Echter, gecontroleerde studies ontbreken in de huidige literatuur. Daarom onderzochten we in **Hoofdstuk 6** de effecten van normobare MIH-expressie (15% O_2, 3x2 uur per dag, 7 opeenvolgende dagen) vergeleken met normoxie (21% O_2) op de samenstelling van de darmflora bij mannen met overgewicht en obesitas. Met behulp van 16S rRNA-genamplicon-sequencing hebben we aangetoond dat blootstelling aan MIH bescheiden veranderingen in de samenstelling van de fecale microbiota veroorzaakte bij mensen met overgewicht en obesitas, waardoor verschillende bacteriële families en geslachten verschoven naar hogere abundantie van strikt anaerobe butyraat-producerende bacteriën. Bovendien hebben we aangetoond dat verschillende van deze door MIH geïnduceerde effecten op de microbiële samenstelling verband hielden met parameters van glucose- en lipidenmetabolisme.

Omdat hypoxie-geïnduceerde effecten op glucosehomeostase mogelijk vergelijkbare onderliggende mechanismen zou kunnen delen in vergelijking met lichaamsbeweging, hebben we vervolgens het synergetische potentieel van hypoxie en lichaamsbeweging onderzocht. Daarom hebben we een enkelblinde, gerandomiseerde, cross-over studie uitgevoerd om de effecten van hypoxische

inspanning (HI), op matige intensiteit, op glucosehomeostase bij mensen met overgewicht en obesitas met verminderde glucosehomeostase te onderzoeken in **Hoofdstuk 7**. Tien deelnemers fietsten op matige intensiteit, gedurende 4 opeenvolgende dagen, onder milde normobare hypoxie (FiO_2: 15%) of normoxie (FiO_2: 21%) bij vergelijkbare relatieve trainingsintensiteit (2x30 min / d bij 50% van de maximale hartslag (HR_{MAX}) of 50% W_{MAX}), met een uitwasperiode van 3-6 weken. Gedurende het onderzoek werden 24-uurs glucoseconcentraties en systemische O_2 saturatie gevolgd. Op dag 5 werd onder normoxische omstandigheden het substraatmetabolisme onderzocht. Middels deze studie hebben we aangetoond dat HI bij een vergelijkbare relatieve intensiteit van de inspanningsbelasting in vergelijking met normoxie, de systemische O_2 saturatie verminderde in vergelijking met normoxische inspanning. Echter, inspanning onder hypoxische condities veranderde de gemiddelde glucoseconcentraties over 24 uur, overdag en 's nachts niet. Tevens bleven verschillende parameters welke de glycemische variabiliteit omvatten ongewijzigd. Desalniettemin was de vermindering van de systemische O_2 saturatie door HI positief gecorreleerd met door HI geïnduceerde verandering in de gemiddelde glucoseconcentraties gedurende 24 uur en overdag

De belangrijkste bevindingen van dit proefschrift tonen aan dat MIH de weefselspecifieke insulinegevoeligheid niet veranderde. Zowel *in vivo* als *in vitro* onderzoeken tonen echter effecten aan op substraatmetabolisme, wijzend op een verhoogd glycolytisch metabolisme. Bovendien veranderde hypoxische inspanning de glucosehomeostase niet bij mannen met overgewicht en obesitas met een verstoord glucosemetabolisme. Toekomstige studies zijn essentieel om het potentieel van verschillende hypoxische blootstellingsregimes te onderzoeken, met inachtneming van de duur, frequentie en intensiteit van de hypoxische stimulus, teneinde metabole aanpassingen te induceren. Het loont de moeite om dit bij mannen en vrouwen afzonderlijk te onderzoeken, en om de effecten van blootstelling aan hypoxie te onderzoeken in studiepopulaties met een ander metabool en inflammatoir fenotype. Indien blootstelling aan hypoxie gunstige effecten lijkt te hebben, kan dit een nieuwe therapeutische strategie zijn om obesitas-gerelateerde complicaties te voorkomen of te behandelen.

IMPACT

This thesis describes the effects of mild hypoxia exposure as a potential strategy for improving metabolic health in obesity. We investigated the effects of mild intermittent hypoxia (MIH) exposure on metabolic health, in particular tissue-specific insulin sensitivity and glucose homeostasis, in overweight and obese men. In addition, we determined whether hypoxic exercise improved glucose homeostasis in overweight and obese men with impaired glucose metabolism. Finally, we investigated the effects of hypoxia exposure in at the molecular level by using adipose (AT) and skeletal muscle (SM) biopsies, as well as primary AT and SM *in vitro* cell models. In this section, the impact of the work described in this thesis will be discussed in terms of scientific and societal relevance, and further implications and applications of hypoxia exposure as a potential therapeutic strategy to improve metabolic health in obesity will be highlighted.

Societal relevance

Obesity is a major cause of morbidity and mortality worldwide, with more than 1.9 billion and 650 million people being overweight and obese, respectively (1, 2). In addition, the global prevalence of obesity will increase drastically in the near future (3). For instance, it is estimated that the prevalence of obesity in the USA will increase to 51% of the total population (4). Since obesity is often accompanied with complications such as cardiovascular diseases, type 2 diabetes mellitus, cognitive decline, depression, obstructive sleep apnea syndrome, skin problems, asthma and several types of cancer (5-10), a forecasted further increase in obesity prevalence will pose a major public health challenge. In addition, improving metabolic health in obesity may also be an interesting approach from a socioeconomic perspective. Indeed, in European countries, it has been stated that on average 7% of their national healthcare budgets is spent on obesity-related complications, which substantially burdens national economies (11). Furthermore, in 2012, €1.6 billion, i.e. 2.2% of total healthcare budget, was spend on overweight-related complications in the Netherlands (12), thereby hindering containment of healthcare costs. Therefore, alternative strategies to prevent or reverse obesity and related complications are urgently required.

Scientific impact

In the past decade, it has been postulated that tissue oxygen tension (pO_2) may be a key determinant in cardiometabolic health in obesity (13, 14). Indeed, it had been found that abdominal adipose tissue (AT) pO_2 was increased in obese (15), and appeared to be inversely correlated with peripheral insulin sensitivity, independent of adiposity and gender (16). Moreover, diet-induced weight loss

decreased AT pO_2 in humans with overweight/obesity, which was accompanied by improved insulin sensitivity (17). Lowering of pO_2 by mild hypoxia exposure may therefore be an alternative and effective approach for improving metabolic health in human obesity.

This thesis focused on the application of MIH exposure as an intervention to study the effects of lowering AT and SM pO_2 on various metabolic parameters like substrate utilization and insulin sensitivity. The outcomes of this study gained insight in the metabolic adaptations induced by MIH exposure in obesity. In addition, this thesis provides knowledge for the scientific community on the molecular adaptations induced by hypoxia exposure in AT, SM and the gut, as well as in vitro AT and SM models.

In addition, this thesis provides insight on the effects of hypoxic exercise in overweight and obese metabolically comprised individuals. Noteworthy, 4-day HE at lower absolute workload intensity had similar effects on mean glucose homeostasis as compared to normoxic exercise (NE). However, the reduced absolute workload results in less mechanical strain, and therefore may be a valuable approach for improving metabolic health in obese individuals with orthopedic comorbidities, such as elderly (18).

Furthermore, the findings in this thesis provide knowledge regarding the effects of different oxygen levels in mechanistic experiments using *in vitro* cell models. Indeed, hypoxia exposure greatly influenced glucose uptake in primary human myotubes experiments. Interestingly, exposure to physiologically relevant oxygen levels (*in situ* hypoxia or normoxia) substantially altered myotube and adipocyte function, and gene/protein expression as compared to standard laboratory conditions (21% O_2). This knowledge further underlines the importance of taking the physiological relevant oxygen levels of tissues from which cells originate into account, which may be implemented in a wide variety of *in vitro* cell culture models in different research areas.

The findings in this thesis are relevant for the scientific community studying the effects of hypoxia in different metabolically active tissues, such as AT, SM, gut and the liver. In addition, we provide molecular insight in the effects of hypoxia exposure on regulation of gene expression, and found a wide variety of pathways being affected by hypoxia, which may be relevant for scientists outside our field of study. The findings of this thesis were presented and discussed at (interdepartmental) research meetings and symposia (*Annual NUTRIM symposia*, 2016-2019, Maastricht) and national (*The Netherlands Association for the Study of Obesity, NASO spring meeting*, 2018, Utrecht, *Annual Dutch Diabetes Research Meeting, ADDRM*, 2017-2019, Oosterbeek) and international conferences (*European/International Congress on Obesity, ECOICO2020*, 2020, virtual meeting; *Diabetes and Metabolism Research Symposium*, 2018, Maastricht). The aim of presenting and discussing our findings

at these conferences was to increase knowledge transfer to both the scientific community and medical specialists (i.e. clinicians and dietitians) on the potential role of tissue oxygenation in metabolic health in human obesity. In addition, the outcomes of this thesis will be communicated with the scientific community by publication in international peer-reviewed journals.

The studies performed in this thesis are conducted in collaboration with several other research groups and institutes. For the *in vitro* cell culture experiments, we used the Roxybot technology developed by the Department of Radiotherapy (MAASTRO) of Maastricht University. In addition, we collaborated with Dr. Henrike Sell and Prof. Hadi Al-Hasani at the Institute for Clinical Biochemistry and Pathobiochemistry of the German Diabetes Center (Dusseldorf, Germany) to determine adipokine/myokine secretion in human and *in vitro* samples. Furthermore, we performed microarray and gene set enrichment analyses in collaboration with Prof. Sander Kersten (Division of Human Nutrition and Health, Wageningen University and Research). Lastly, we collaborated with Prof. Koen Venema, Centre for Healthy Eating & Food Innovation, Campus Venlo of Maastricht University, to determine the effects of hypoxia exposure on gut microbiota composition.

Further research is warranted to investigate variations in hypoxia exposure regimen, since it has been found that severity, frequency and duration of hypoxic exposure determine metabolic adaptations. In addition, a personalized strategy may be optimal for investigating the effects of hypoxia exposure on metabolism in obesity. In fact, we demonstrate that hypoxic exercise has the most pronounced effect on 24-h glucose levels in peole with obesity who demonstrated the greatest reduction in systemic saturation induced by hypoxia. Due to the inter-individual difference in tolerability of hypoxia, it would be interesting to personalize the intervention by adjusting FiO_2 according to the change in systemic oxygen saturation, thereby optimizing metabolic adaptations. Furthermore, it has previously been suggested that the metabolic phenotype may play a key role in the response to hypoxia, since obese individuals with worst baseline insulin sensitivity improved most upon mild hypoxia exposure (19).

Commercial exploitation

The present results may be of interest to pharmaceutical companies in the field of type 2 diabetes, cardiovascular and liver disease, since these chronic diseases are strongly associated with impairments in glucose homeostasis and inflammation. For example, a pharmacological approach using prolyl-hydroxylase dehydrogenase (PHD) inhibitors may be used to improve metabolic health, since both environmental hypoxia and PHD inhibitors stabilize hypoxia-inducible factor-1α (HIF-1α). Interestingly, several PHD inhibitors are currently used in clinical trials for

treatment of anemia in chronic kidney disease (CKD). The metabolic and molecular adaptations induced by MIH may therefore be interesting for the scientific community studying the use of PHD inhibitors in other clinical fields such as CKD.

In addition, a non-pharmacological approach may be of interest for commercial exploitation. For example, the commercialization of an air-tight room, with an airlock system, in which nitrogen dilution can regulate the oxygen content, thereby potentially influencing metabolic health, may be worthwhile to explore. However, this might be a costly innovation. Nevertheless, continuous positive airway pressure (CPAP) therapy, currently mainly used in the treatment of obstructive sleep apnea syndrome (OSAS), may provide a valuable alternative. OSAS is characterized by frequent hypoxic episodes during sleep, resulting in systemic oxygen desaturation. The positive airway pressure in CPAP prevents the upper airways from collapsing, thereby eliminating apnea and desaturation. Interestingly, if CPAP devices may be developed with nitrogen dilution capabilities, CPAP therapy may expose the individual to the desired oxygen content for inducing metabolic adaptations. In that way, hypoxia may be applied at home during the night.

To conclude, this thesis provides information about the metabolic and molecular effects of mild hypoxia exposure on metabolic health in obesity, based on human studies and human primary cell culture experiments. In addition, this thesis generates knowledge on the combined effects of hypoxia and moderate-intensity exercise on glucose homeostasis in obesity. The results of this thesis may also be used to further investigate variable hypoxic exposure regimens, as well as a personalized intervention using variable FiO_2. Therefore, the overall findings of this thesis may contribute to the development of alternative approaches for combatting obesity-related complications, other than traditional lifestyle interventions, and as such may be of interest for both academic and industrial scientists, as well as healthcare professionals.

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defended your thesis, you and your family returned to Indonesia, to continue your academic career. Unfortunately, due to the COVID19 circumstances, we never had a proper farewell. I hereby propose that **Manuel** and I will visit you in the near future, of course with a perfect tour across Semarang, Java and the rest of Indonesia. **Manuel** and **Adriyan**, thanks for everything, and all the best in your future careers. We keep in touch!

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LIST OF PUBLICATIONS

List of publications

van Meijel RLJ, Blaak E, Goossens G. Chapter 1 Adipose tissue metabolism and inflammation in obesity. In: Johnston R, Suratt B, editors. **Mechanisms and Manifestations of Obesity in Lung Disease. Cambridge, MA: Academic Press; 2019. p. 1 22.**

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van Meijel RLJ, Vliex LMM, Hartwig S, Lehr S, Al-Hasani H, Blaak EE, Goossens GH. The impact of mild hypoxia exposure on myokine secretion in human obesity. ***Ready to be submitted***

van Meijel RLJ, Venema K, Canfora EE, Blaak EE, Goossens GH. Mild intermittent hypoxia exposure alters gut microbiota composition in overweight and obese men. ***Ready to be submitted***

van Meijel RLJ, Blaak EE, Goossens GH. The effects of hypoxic exercise on 24-hour glucose profiles in overweight and obese men with impaired glucose metabolism: a randomized, single-blind, cross-over study. ***In preparation***

ABOUT THE AUTHOR

Curriculum Vitae

Rens van Meijel was born on the 23rd of November 1991 in Venray, The Netherlands. In 2010, he obtained his Gymnasium (VWO) diploma from the Raayland College in Venray. Thereafter, he started his B.Sc. studies in Biomedical Sciences at Radboud University Nijmegen, the Netherlands. In 2014, he continued to study at Radboud University for his M.Sc. Biomedical Sciences, with a specialization in Human Pathobiology, Toxicology and Clinical Chemistry. During his studies, he performed internships at several research groups in the Departments of Biochemistry, Pharmacology and Toxicology and the Translational Metabolic Laboratory of Radboud UMC. In addition, he performed an internship at the Department of Molecular Neurobiology at the University of Groningen. He was awarded the poster prize for best M.Sc. poster at the annual scientific conference of the Dutch Association of Toxicology. In 2016, he obtained his M.Sc. degree with honours (*cum laude*).

Upon graduation, he started as a PhD candidate at the Department of Human Biology (NUTRIM) at Maastricht University, the Netherlands, under the supervision of Dr. Gijs Goossens and Prof. Ellen Blaak. During his PhD program, he investigated the impact of mild hypoxia exposure in human obesity, by performing human intervention studies as well as mechanistic *in vitro* studies. He presented his findings at both national and international conferences such as the Annual Dutch Diabetes Research Meeting, the Annual meeting of the Netherlands Association for the Study of Obesity, and the European/International Congress on Obesity. After completing his PhD, he started as a Resident in Clinical Chemistry at Zuyderland MC in Sittard-Geleen and Heerlen.