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Effects of pharmacological and lifestyle interventions

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door

Rudi Michel Elisabeth Stinkens

Voor papa en mama

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CHAPTER 1

GENERAL INTRODUCTION

OBESITY – THE BURDEN OF A BIG PLAYER

Over the last decades, the worldwide prevalence of obesity has increased enormously and reached pandemic proportions. According to the World Health Organization, 39% of adults over 18 years of age (38% of men and 40% of women) were overweight, while 13% of the world's adult population (11% of men and 15% of women) was obese in 2014 [1]. Furthermore, globally, approximately 6.3% of the children under the age of 5 years were overweight [2] and on their way to become obese. Clearly, not only the Western society is affected by obesity, also many low- and middle-income countries have increased morbidity and mortality rates due to the increasing prevalence of obesity [1]. Nowadays, the amount of people suffering from obesity exceeds the number of people suffering from malnutrition [3], making obesity the 5th leading cause of death worldwide [1].

Obesity is associated with an increased risk for developing insulin resistance [4] and type 2 diabetes [5]. Peripheral insulin resistance is defined as a reduced ability of insulin to stimulate glucose uptake in peripheral tissues, mainly skeletal muscle [6], while type 2 diabetes is characterized by insulin resistance accompanied with pancreatic beta-cell failure (inability of the pancreatic beta-cells to adequately secrete insulin) [7]. Furthermore, obesity is also related to the development of hypertension and cardiovascular diseases [8], fatty liver disease [9], mental disorders (e.g. depression) [10], musculoskeletal disorders (e.g. osteoarthritis) [11] and certain types of cancer [12, 13].

Since obesity is a major public health issue and one of the most important risk factors for the development of cardiometabolic diseases, it is needless to say that the increasing obesity prevalence has major socioeconomic consequences [3]. The World Health Organization defines overweight as a body mass index (BMI) between 25-30 kg/m^2 and obesity as a BMI of 30 kg/m^2 or greater [1]. However, the use of BMI gives an underestimation of the amount of people suffering from obesity and the related health complications globally [14, 15] since it does not take into account the body composition (i.e. skeletal muscle mass) and body fat distribution (i.e. abdominal and gluteofemoral fat depot) [16]. Also, the use of universal BMI cut-off points does not consistently reflect adiposity in different ethnic populations. South Asian populations, displaying a greater proportion of body fat for a given BMI than Caucasians [17], have a higher susceptibility to develop type 2 diabetes and coronary artery disease, despite lower BMI values [18]. Therefore, BMI is only an approximation and should be considered as a rough indicator to determine obesity and cardiometabolic disease risk [19]. Nowadays, more sensitive techniques are available and assessment of a patient's metabolic phenotype is needed to enhance diagnosis, prognosis and optimization of interventions [20]. Metabolic phenotyping generates detailed information regarding a patient's (patho)physiological state and makes it possible to stratify subjects into different subgroups. This stratification at baseline may improve the effectiveness of a particular intervention in a specific subgroup of the population [21]. Therefore, detailed metabolic phenotyping is necessary to identify individuals or subgroups of a population that have an increased risk for developing metabolic diseases and to optimize prevention and treatment strategies.

OBESITY AND CARDIOMETABOLIC HEALTH

The etiology of obesity is complex and multifactorial. However, the fundamental cause of overweight and obesity is an imbalance between energy intake (i.e. energy-dense diet) and energy expenditure (i.e. physical inactivity), which depends on the interaction between biological, genetic, behavioral, social and environmental factors [22].

The development of obesity is accompanied by a substantial increase in adipose tissue mass. While adipose tissue used to be considered a passive organ, only involved in the storage and release of energy, it has become clear over the last two decades that adipose tissue is an active metabolic and endocrine organ. Moreover, adipose tissue mass *per se* may not be the most important contributor to the development of obesity-related disorders. For example, abdominal liposuction, which is the surgical removal of abdominal subcutaneous adipose tissue, did not significantly improve obesity-related disturbances like insulin resistance [23]. Furthermore, pharmacological treatment with insulin-sensitizing thiazolidinediones (PPARγ agonists) is accompanied by a significant increase in subcutaneous adipose tissue mass [24]. Additionally, patients with lipodystrophy, who are characterized by a partial or complete lack of adipose tissue, have pronounced insulin resistance and a high incidence of type 2 diabetes [25]. Collectively, these findings indicate that total adipose tissue mass *per se* is not the predominant contributor to the development of obesity-related complications. Body fat distribution and adipose tissue (dys)function seem to play a more prominent role in cardiometabolic health [26, 27]. Adipose tissue accumulation in the upper body (abdominal region) is associated with the development of obesity-related comorbidities and all-cause mortality, while fat accumulation in the lower body (gluteofemoral region) is considered to be even protective against metabolic and cardiovascular disturbances [28, 29]. Apparently, the functional properties of these adipose tissue depots, seem to play an important role in the disease risk [16]. Adipose tissue dysfunction, which is characterized by an impaired capacity to store lipids in combination with low-grade inflammation, seems to contribute to the development of insulin resistance and impaired glucose metabolism by promoting excessive fat storage in non-adipose tissues (ectopic fat deposition), such as the liver, skeletal muscle, pancreas and heart [26, 30-33]. Furthermore, functional impairments in either of these organs may further contribute to the development of impaired glucose metabolism, type 2 diabetes and cardiovascular disease, as extensively described in chapter 2 of this thesis [34].

Interestingly, around 10-30% of the obese population seems to be relatively protected against the development of cardiometabolic complications and are therefore referred to as 'metabolically healthy obese' (MHO) [35, 36]. The reason why these obese individuals are protected from cardiometabolic complications is currently not completely understood, but may be caused by several mechanisms, including a beneficial body fat distribution (low visceral and ectopic fat storage compared to subcutaneous fat depots), normal adipose tissue function (a normal adipose tissue inflammatory phenotype and adipokine secretion pattern) as well as high physical activity and fitness levels, together contributing to preserved insulin sensitivity [37-39]. Importantly, at a given BMI, there are obese individuals who are either very insulin sensitive or extremely insulin resistant. Thus, there is no clear

BMI-cutoff separating these distinct obese subgroups [35]. It is also important to acknowledge that the criteria to define MHO have only been established recently [36, 40] and studies performed thus far characterized MHO as the absence of metabolic disorders, such as dyslipidemia, insulin resistance, impaired glucose metabolism and type 2 diabetes. Interestingly, the concept of MHO may be misleading, since several meta-analyses of prospective cohort studies showed an increased risk of developing type 2 diabetes [41] and cardiovascular disease [42, 43] over time when compared to healthy normal weight subjects. The duration of a MHO phenotype for 5.5-10.3 years of follow-up was not associated with an increased risk to develop diabetes and cardiovascular disease, compared to metabolically healthy normal weight subjects, but in about one-third of the MHO subjects, the healthy phenotype was transient [44]. However, a 24% increased mortality and cardiovascular risk was observed in the MHO group compared to healthy normal weight subjects, when the MHO phenotype persisted over 10 years of follow up [42]. Clearly, the MHO individuals may develop a metabolically 'unhealthy' phenotype [45, 46] over time and therefore MHO should not be considered a harmless condition [47, 48]. Major efforts should be made to prevent obesity and obesity-related comorbidities to maintain or reach a metabolically healthy phenotype.

There is substantial evidence to suggest that targeting the renin-angiotensin system and the natriuretic peptide system, either via pharmacological treatment or lifestyle intervention, may beneficially affect cardiometabolic health. In the next paragraphs, these two systems and their metabolic effects will be described in more detail. Thereafter, the potential of physical exercise interventions to enhance metabolic health will be addressed.

THE RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is known as an important regulator of systemic blood pressure and electrolyte homeostasis [49]. It has also been shown that several RAS components are increased in obesity and insulin resistance and are present in different tissues, including the adipose tissue [50-53]. Furthermore, an increased RAS activity has been linked to the development of type 2 diabetes and cardiovascular disease [54, 55].

In the classical (systemic) RAS, renal-derived renin converts circulating angiotensinogen (AGT), mainly produced in the liver, into angiotensin I (ANG I), which is then converted to angiotensin II (ANG II) by the action of angiotensin-converting enzyme (ACE) found in the lung capillaries. ANG II is the main effector hormone of the RAS and binds to the ANG II type 1 (AT_1) and ANG II type 2 (AT_2) receptors to exert its biological functions. Most of the (patho)physiological effects, such as vasoconstriction, aldosterone production, sodium reabsorption and nervous system activation are induced by binding of ANG II to AT_1. In contrast, binding of ANG II to AT_2 counteracts the AT_1-mediated effects and promotes vasodilation, natriuresis and anti-inflammatory responses. Clearly, AT_1 and AT_2 receptors induce opposite physiological and metabolic effects [54].

Metabolic effects of the renin-angiotensin system

Different RAS components have been identified in several tissues, such as the adipose tissue [50, 51], skeletal muscle and pancreas [52, 53] and a role for the local RAS in obesity-related metabolic disturbances has been suggested [54, 56]. The metabolic effects of RAS on adipose tissue, skeletal muscle and pancreatic function or metabolism will be addressed below.

The renin-angiotensin system and adipose tissue function

The adipose tissue of obese, insulin resistant and type 2 diabetic patients is characterized by enlarged (hypertrophic) adipocytes, which can be attributable to a prolonged positive energy balance and impaired adipocyte differentiation. On the one hand, ANG II has been shown to promote adipocyte growth and differentiation via several mechanisms [57, 58], but on the other hand it has also been shown to inhibit differentiation of cultured human preadipocytes, mediated via the AT_1 receptor [59] and to reduce insulin-induced adipocyte differentiation in human preadipocytes [60]. Long-term RAS blockade, with the angiotensin receptor blocker (ARB) valsartan, reduced abdominal subcutaneous adipocyte size and increased the amount of small adipocytes in obese individuals with impaired glucose metabolism [61].

Furthermore, ANG II has been shown to increase lipid synthesis and storage in both rodent and human adipocytes [58] and to reduce adipose tissue lipolysis [62-64], while blockade of the RAS reversed these effects [65]. However, conflicting results have also been reported, with ANG II increasing adipose tissue lipolysis in lean, but not in obese men [66] and RAS blockade not affecting adipose tissue lipolysis in obese subjects [67].

Adipocyte size plays an important role in adipokine expression and secretion, with a more pro-inflammatory phenotype of hypertrophic adipocytes. It has been shown that ANG II increased, while RAS blockade decreased adipose tissue gene expression of pro-inflammatory markers in rodents, as reviewed [50, 51]. In human adipose tissue, long-term RAS blockade with valsartan reduced the expression of macrophage infiltration markers [61] and several studies observed that ARB treatment altered circulating adipokine concentrations, although conflicting results have been reported [50].

Another important contributor to adipose tissue lipid handling is adipose tissue blood flow (ATBF), which is reduced in obese, insulin resistant and type 2 diabetic individuals, in both the fasting and postprandial state, as reviewed elsewhere [68]. While ANG II reduces ATBF [62, 69], local RAS blockade with losartan increased human abdominal subcutaneous ATBF [69]. In line, long-term RAS blockade with valsartan increased both fasting and postprandial ATBF [61] and may have contributed to the previously observed valsartan-induced increase in insulin sensitivity [70].

Taken together, the RAS may affect adipose tissue function by altering adipocyte size and lipid metabolism, inflammation and adipose tissue blood flow, thereby contributing to the development of both insulin resistance and type 2 diabetes. However, the metabolic effects of the RAS go beyond the adipose tissue and also affect skeletal muscle and the pancreas.

The renin-angiotensin system and skeletal muscle metabolism

An increased RAS activity may cause impairments in skeletal muscle metabolism by affecting tissue perfusion, insulin signaling and mitochondrial function. The vascular system delivers nutrients and hormones to the skeletal muscle and local tissue perfusion may regulate skeletal muscle metabolism and contractile performance. Local infusion of ANG II in the gastrocnemius muscle reduced skeletal muscle blood flow in both lean and obese individuals [62], while chronic RAS blockade, with the ACE inhibitor (ACEi) captopril, increased postprandial forearm blood flow in subjects with type 2 diabetes [71]. However, an increased basal and insulin-stimulated total forearm blood flow was not observed after 2 weeks of ACEi treatment in obese insulin resistant subjects [72]. Thus, it is not completely clear whether the RAS plays a major role in the regulation of total skeletal muscle blood flow and/or microvascular function under physiological conditions.

As reviewed elsewhere [50, 52], evidence suggest that the RAS may contribute to impaired insulin signaling in skeletal muscle, either directly or indirectly via the induction of oxidative stress. Importantly, most results are obtained from rodent studies and need further investigation in humans.

An impaired mitochondrial function in skeletal muscle has been suggested to contribute to the development of insulin resistance and type 2 diabetes, but whether reduced mitochondrial function is a cause or consequence of these disturbances remains to be established [73]. Nevertheless, chronic ANG II infusion in mice increased mitochondrial reactive oxygen species, decreased expression of genes involved in mitochondrial biogenesis and reduced mitochondrial content in C2C12 myocytes [74]. Furthermore, ANG II reduced muscle mitochondrial content, increased intramuscular triacylglycerol concentrations and reduced glycemic control in mice, while RAS blockade partially reversed these effects, leading to an increased fat oxidation, a decreased intramuscular triacylglycerol concentration and an improved glucose tolerance [74]. In contrast, treatment with the ACEi ramipril for two weeks had no significant effects on whole-body substrate oxidation, intramuscular triacylglycerol content and insulin sensitivity in obese insulin resistant men [72].

The renin-angiotensin system and pancreatic beta-cell function

Evidence suggests that elevated RAS activity may also contribute to an impaired insulin secretion [50]. In rodents, RAS activation reduced pancreatic islet blood flow, induced pancreatic islet fibrosis, oxidative stress, inflammation and impaired insulin secretion, whereas RAS blockade, with ACEi or ARB, improved pancreatic islet functionality and morphology and increased glucose tolerance, as reviewed [50]. Furthermore, in patients with impaired glucose metabolism, long-term RAS blockade, with valsartan, showed beneficial effects on pancreatic insulin secretion [70].

Taken together, the RAS may affect adipose tissue, skeletal muscle and pancreatic function and metabolism via different mechanisms, thereby contributing to the development of insulin resistance, an impaired insulin secretion and type 2 diabetes. Therefore, blockade of the RAS may exert protective effects against the development of metabolic disturbances.

Pharmacological modulation of the renin-angiotensin system

Meta-analyses of comparative outcome trials have shown that RAS blockade, with either ACEi or ARB, reduced the incidence of new-onset type 2 diabetes by 20-30% in populations at high risk for developing type 2 diabetes [75, 76]. More recently, the prospective NAVIGATOR trial also showed that ARB treatment with valsartan, in addition to lifestyle modification, reduced the incidence of type 2 diabetes by 14% in subjects with impaired glucose homeostasis after a median follow-up of 5.3 years [77]. However, less pronounced improvements in glucose metabolism have been shown by the prospective DREAM trial [78]. In this trial, ACE inhibition, compared to placebo, non-significantly reduced the incidence of type 2 diabetes by 9% after a median follow-up of 3 years in subjects with impaired glucose homeostasis but without cardiovascular disease. Nevertheless, ACEi treatment significantly reduced 2-hour glucose concentrations and increased regression to normoglycemia [78]. Differences in study design, population and treatment duration may underlie the somewhat different outcomes in the DREAM trial and NAVIGATOR trials. Taken together, most randomized clinical trials indicate that RAS blockade may protect against the development of type 2 diabetes [75, 76].

THE NATRIURETIC PEPTIDE SYSTEM

Although data are not entirely consistent [79, 80], several community-based cohort studies have shown a lower activity of the natriuretic peptide (NP) system in obese subjects, which has been shown to be related the development of type 2 diabetes and chronic metabolic diseases [81-83]. As described below, modulation of the NP system can be protective against the development of insulin resistance and type 2 diabetes via multiple mechanisms, making it an interesting target to combat metabolic diseases.

The natriuretic peptide system contains several hormones of which Atrial NP (ANP), Brain-type NP (BNP) and C-type NP (CNP) are the most abundant ones. ANP and BNP are secreted from the cardiac atria [84] and ventricles [79], respectively, in response to cardiac wall stress, but also via other factors, such as weight loss [85], exercise [86], cold exposure [87] and hypoxia [88]. They bind to guanylyl cyclase-coupled receptors: type A (NPRA) and type B (NPRB), which are expressed in several tissues, including the adipose tissue [89]. While NPRA mediates the majority of the metabolic effects of ANP and BNP, type-C NP receptor (NPRC) is known as a membrane-bound clearance receptor that binds and incorporates circulating NP into cytoplasm where NP are inactivated [90, 91]. In addition to the NPRC-mediated degradation, NP are also degraded via extracellular proteases, such as the insulin-degrading enzyme [92] and neprilysin (NEP: neutral endopeptidase 24.11) [93] of which the latter is mainly expressed in the kidneys, but also present in adipocytes [94].

The lower NP effects in obesity and type 2 diabetes can be explained by a reduced cardiac NP secretion, a reduced tissue NPRA signaling and/or an increased systemic and tissue clearance, leading to reduced circulating NP concentrations. These reduced NP concentrations have been implicated in the development of insulin resistance and type 2 diabetes [83, 95-98], while higher NP concentrations

are associated with a lower prevalence of new-onset type 2 diabetes [99, 100]. Beside changes in circulating NP concentrations, changes in the NP-receptors have also been observed. A decreased NPRA receptor expression at the level of abdominal subcutaneous adipose tissue, together with an increased NPRC receptor expression at both abdominal subcutaneous and omental adipose tissue has been observed in obese subjects with or without type 2 diabetes as compared to lean individuals, thereby altering the NPRA/NPRC ratio [80, 101-104]. This altered receptor expression, in combination with an increased NEP expression in adipose tissue of obese and insulin resistant subjects [94], may cause an increased NP clearance, leading to reduced NP bioavailability, thereby contributing to the development of cardiometabolic disturbances.

Metabolic effects of the natriuretic peptide system

The NP system was primarily known for the regulation of blood pressure and its physiological effects on the cardiovascular system, body fluid and electrolyte homeostasis [105, 106]. Nowadays, it is established that the NP system also exert effects on several key metabolic organs such as the adipose tissue and skeletal muscle, as reviewed elsewhere [107-109].

The natriuretic peptide system and adipose tissue function

The natriuretic peptides have been shown to stimulate lipolysis in human adipocytes, with ANP as the strongest effector, followed by BNP and CNP [110]. ANP and BNP stimulated *in vitro* lipolysis as much as the non-selective β-adrenergic receptor agonist isoproterenol, while *in situ* microdialysis experiments confirmed these NP-mediated lipolytic effects in abdominal subcutaneous adipose tissue of healthy young men [110]. Even under local α_2- and $\beta_{1/2}$-adrenergic blockade, a substantial non-adrenergic-mediated lipolysis was observed in subcutaneous adipose tissue of healthy young lean [111] and overweight men [112]. Intravenous infusion of ANP also acutely increased plasma concentrations of glycerol and free fatty acids (FFA) in young healthy lean and obese men, independently of the activation of the sympathetic nervous system [113]. Beside lipid mobilization, intravenous infusion of ANP also rapidly increased lipid oxidation in healthy normal weight men, both in the fasted [114, 115] and postprandial state [116]. When directly infused into the human subcutaneous adipose tissue of young lean men, ANP increased extracellular glycerol concentration and enhanced adipose tissue blood flow [110], together contributing to an increased lipid mobilization. This NP-mediated lipolysis revealed to be a cyclic guanosine monophosphate/protein kinase G (cGMP/PKG) dependent pathway, which induces the phosphorylation of perilipin 1 and hormone sensitive lipase [117].
ANP-treated human adipocytes also showed an increased activation of AMP-protein kinase (AMPK), a major metabolic energy sensor and master regulator of metabolic homeostasis [118]. Indeed, ANP increased energy expenditure and oxidative capacity and also increased mitochondrial biogenesis and function in differentiated human adipocytes [87, 118].
Beside effects on adipose tissue lipid mobilization and oxidation, NP have also been shown to possess anti-inflammatory properties [119, 120], to induce expression and secretion of the insulin-sensitizing factor adiponectin in both

chronic heart failure patients [121] and healthy men [122], and to reduce systemic leptin concentrations in healthy men [123].

The natriuretic peptide system and skeletal muscle metabolism

In skeletal muscle, a NP-induced increase in peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) and proliferator-activated receptor-delta (PPAR-δ) mRNA expression as well as an increase in mitochondrial mass has been observed in C2C12 myocytes [124]. In line, in human myotubes, ANP caused an increase in PGC-1α mRNA expression, which was paralleled by an upregulation of several genes and proteins involved in oxidative phosphorylation (OXPHOS), although mitochondrial proliferation and mitochondrial mass were not affected by ANP treatment [125].
Taken together, these findings suggest that NP may enhance the oxidative capacity of skeletal muscle.

The natriuretic peptide system and pancreatic beta-cell function

At the level of the pancreas, intravenous infusion of ANP increased insulin secretion in human subjects [114, 116, 126] and improved pancreatic β-cell function in rodents [127]. ANP directly enhanced glucose-stimulated insulin secretion in cultured pancreatic islets of Langerhans [127] and induced β-cell growth in isolated rat pancreatic islets [128].
Interestingly, NP treatment may also induce changes at the level of the gastrointestinal tract. Intravenous BNP infusion, in young healthy lean men, showed a fasting-induced increase in total and acylated ghrelin concentrations, decreased the subjective rating of hunger and increased the feeling of satiety, without significantly changing circulating peptide YY, glucagon-like peptide 1, oxyntomodulin, pancreatic polypeptide, leptin and adiponectin concentrations [129]. Furthermore, intravenous infusion of BNP showed a decreased gastric emptying in a rodent model [130]. Also a novel gut-heart crosstalk has been described in rodents in which ANP mediates the blood pressure-lowering effects of glucagon-like peptide-1 (GLP-1) agonists [131]. The GLP-1 agonist, liraglutide, induced ANP secretion from the cardiac atrium and the blood pressure lowering effect of liraglutide was abrogated in *Nppa* (ANP) knockout mice.
Taken together, these data clearly show the involvement of the NP system in metabolic homeostasis and indicate that modulation of the NP system can be protective against the development of insulin resistance and type 2 diabetes via multiple mechanisms. Targeting the NP system may therefore serve as an effective strategy to combat these metabolic diseases.

PHARMACOLOGICAL MODULATION OF THE RENIN-ANGIOTENSIN SYSTEM AND THE NATRIURETIC PEPTIDE SYSTEM

As discussed above, obesity, type 2 diabetes and cardiovascular diseases are interrelated and may be characterized by a reduced NP system activity, an increased RAS activity as well as similar metabolic impairments. Recently, a novel dual-acting drug (sacubitril/valsartan) has been developed that combines ARB and NEP inhibition and facilitates beneficial effects of NP and other neprilysin substrates, while inhibiting the detrimental effects of the RAS [132]. In this respect, the latter cardiovascular medication, targeting the RAS and NP system, may also have positive metabolic effects. Moreover, the dual-acting mechanism of sacubitril/valsartan may have synergistic beneficial effects on several metabolic parameters. As described earlier in this thesis, the ARB valsartan improved insulin sensitivity [70] and beneficially affects adipocyte size and lipid metabolism, inflammation and adipose tissue blood flow [61]. The additional effects of the neprilysin inhibitor, sacubitril, may further enhance metabolic health (i.e. insulin sensitivity) via the increased NP concentrations, which also beneficially affect lipid mobilization from adipose tissue [114], postprandial lipid oxidation [116], adiponectin release and muscular oxidative capacity [125, 133]. Combined, sacubitril/valsartan facilitates beneficial effects of NP and other neprilysin substrates, while inhibiting the detrimental effects of the RAS. However, until recently, the metabolic effects of sacubitril/valsartan have not been investigated. In chapters 3, 4 and 5 of this thesis, the effects of sacubitril/valsartan on metabolic parameters, such as peripheral insulin sensitivity, whole-body and local subcutaneous adipose tissue lipolysis as well as energy expenditure and substrate oxidation were investigated in obese hypertensive patients [134].

LIFESTYLE INTERVENTION

The management and treatment of obesity and obesity-related disorders is more complex than only achieving weight loss. The rational should not only focus on the maintenance of fat mass loss, but also on prolonged cardiometabolic risk reduction and health improvement. Lifestyle characteristics such as diet, physical inactivity, smoking, alcohol consumption and stress are important factors that influence the development of obesity and related comorbidities, and guidelines recommend changes in these lifestyle characteristics for both prevention and management of metabolic disease [135].
Manipulation of diet and physical activity levels are the first-choice interventions to reverse metabolic disturbances. Even though several large lifestyle intervention trials did not show significant beneficial effects of intensive exercise or combined diet and exercise lifestyle interventions on reducing the risk for cardiovascular outcomes [136, 137], other trials did observe beneficial effects on modifiable risk factors for cardiovascular disease [138], type 2 diabetes incidence and the metabolic syndrome [139]. This indicates that lifestyle interventions do have beneficial effects. Several important large lifestyle intervention trials that used dietary advice, exercise strategies and/or combinations have been performed to prevent type 2 diabetes. In this respect, the Malmö-study [140, 141], the Chinese

Da Qing IGT and Diabetes study [142], the Finnish Diabetes Prevention Study (DPS) [143], the Diabetes Prevention Program in the USA (DPP) [144] and the Study on Lifestyle intervention in Impaired glucose tolerance Maastricht (SLIM) [145] indicated that the incidence of type 2 diabetes was reduced by 40-60% following intensive (combined) diet and exercise intervention program. Importantly, long-term follow-up of these trials revealed that diabetes risk reduction still existed after 3 to 14 years after cessation of the intervention program [146-149]. Thus, lifestyle interventions clearly show improvements in a variety of health outcomes related to cardiometabolic health and can reduce the incidence of diseases [150]. However, the exact effects of the different components of the lifestyle program are not fully elucidated. While one study revealed that the combination of diet and exercise was more beneficial in reducing insulin resistance [151], another study showed that diet and exercise were equally effective compared to exercise alone to prevent the progression towards type 2 diabetes [142]. Moreover, another study found that the combination of diet and exercise was more effective than either treatment alone [145]. Nevertheless, combined dietary and exercise interventions have been shown to be most effective in reducing body weight [152].

Clearly, both prevention and management of disease progression can be achieved by strategies such as increased physical activity as well as dietary manipulation via a hypocaloric diet or an improved nutritional composition [153]. Interestingly, several dietary components, such as fatty acids, polyphenols and fibers may modulate fatty acid metabolism in tissues like skeletal muscle, liver and pancreas, both acute (i.e. postprandial phase) and more long-term, ultimately improving glucose metabolism, as extensively reviewed in chapter 2 [34]. Physical activity strategies include increased habitual physical activity (e.g. sitting less) and physical exercise levels (e.g. cycling) which lead to increased energy expenditure, improved physical fitness [154, 155] and contribute to an improved metabolic health. The exercise-induced improvements in metabolic risk profile have largely been attributed to changes in skeletal muscle metabolism and function, but physical exercise is likely to induce alterations in almost all metabolically active tissues, including the adipose tissue, as will be discussed below.

Physical activity-induced effects on adipose tissue metabolism

While physical activity is defined as any movement exerted by the skeletal muscle to increase resting energy expenditure, exercise is usually defined as a part of physical activity that is planned and/or structured [156]. When other aspects of energy expenditure or energy intake are not changed, increased physical activity and exercise induce a negative energy balance, resulting in a reduced fat mass [157]. This reduced fat mass depends on the accumulated effect of each bout of exercise and is caused when adipose tissue lipolysis and oxidation exceeds fat storage. Even though, physical activity and exercise-induced effects on adipose tissue metabolism are still not completely understood, several rodent studies suggest that exercise training may improve adipose tissue metabolism and function [158]. However, human studies that investigated the effects of exercise training on the adipose tissue function are scarce and need further investigation [157-159].

The exercise-induced fatty acid mobilization from adipose tissue is influenced by fatty acid re-esterification, adipose tissue lipolysis and adipose tissue blood flow

(ATBF). A part of the exercise-induced increase of fatty acid mobilization can be attributed to a decreased rate of fatty acid re-esterification [160, 161] and an increased ATBF [162]. The exact mechanism for the increased ATBF is currently unknown and could be explained by an increased cardiac output or by other factors such as increases in circulating catecholamines and natriuretic peptides concentrations [163-165]. This increased ATBF contributes to an increased supply of hormones and signaling molecules (e.g. catecholamines, myokines) to the adipose tissue as well as the supply of mobilized FFA and adipokines to other tissues, such as skeletal muscle [166].

Adipose tissue lipolysis is mostly stimulated by low-intensity exercise and does not increase further at higher exercise intensities [167-169]. This process depends on both the adrenergic pathway, in which catecholamines (adrenalin and noradrenalin) bind to the adrenergic receptors, and the non-adrenergic pathways, that consist of the natriuretic peptide system, insulin, growth hormone and cortisol. In obesity, a blunted catecholamine-mediated lipolysis has been observed in subcutaneous adipose tissue [170, 171], in which the anti-lipolytic α_2-adrenergic receptor becomes more predominant compared to the lipolytic $\beta_{1,2}$-adrenergic receptors [172, 173]. Also, as described earlier in this thesis, the natriuretic peptide concentrations are reduced in obesity and may contribute to an altered exercise-induced lipolysis. Interestingly, after blocking the β-adrenergic receptor in subcutaneous adipose tissue during exercise in overweight men, it has been shown that adrenalin is not the primary lipolytic stimulus and that ANP might be of more importance in overweight men [112]. Noteworthy, several cross-sectional studies and exercise intervention studies have shown an increased lipolytic response in isolated adipocytes of active individuals compared to their sedentary counterparts [174-176] or as result of exercise training [177-179], reflecting an increased capacity for lipolysis at the cellular level. Furthermore, there is some evidence that exercise-induced adipose tissue lipolysis is increased with training [157], which is reflected by a similar or even greater lipolysis in spite of lower circulating concentrations of lipolytic hormones during exercise [180-182]. In line, exercise training has been shown to improve the β-adrenergic responsiveness in overweight [164] and obese men [183] and to improve the natriuretic peptide sensitivity in overweight men [164], which ultimately leads to an increased lipolysis. However, results are not always consistent and are complicated by confounding factors such as recent energy balance [157]. Nevertheless, in general, there seems to be an improved adipose tissue lipolytic sensitivity after training, which causes an increased fat mobilization and may reduce fat mass when the mobilized FFA are oxidized.

Several rodent studies have shown that exercise training may increase adipose tissue mitochondrial biogenesis [184, 185] and function [186-188] and induce browning of white adipose tissue [184, 185, 187, 189, 190], leading to an increased energy expenditure. In line, exercise training in human studies has been shown to increase white adipose tissue gene expression of PGC-1α [191] and oxidative metabolism markers [192], suggesting a higher mitochondrial capacity to oxidize fatty acids.

This increased mitochondrial oxidation can contribute to a reduced fat mass and reduced adipocyte size, which has beneficial effects on disease progression [193] and may lead to an altered adipokine expression [194]. Several rodent studies

have shown that exercise training may beneficially alter adipokine expression [195, 196], but human data are conflicting. Although there is consistent evidence that exercise increases adipose tissue interleukin-6 (IL-6) expression and secretion after a single bout of exercise [197-199], results of other adipokines, such as adiponectin, leptin, TNF-α after a single bout of exercise or after exercise training are currently limited and conflicting [181, 200-206].

Collectively, these data may suggest that exercise improves adipose tissue metabolism and function and can ultimately contribute to a reduced disease progression and improved peripheral insulin sensitivity. Indeed, transplantation of white adipose tissue from trained animals to untrained recipients markedly improved skeletal muscle glucose uptake [187], suggesting that improvement of adipose tissue function may contribute to the increased peripheral insulin sensitivity after exercise training.

In chapters 6 and 7 of this thesis, the exercise training-induced effects on abdominal subcutaneous adipose tissue metabolism and lipolysis were investigated in obese and lean subjects.

THESIS OUTLINE

This thesis describes the effects of a pharmacological intervention as well as physical exercise interventions to improve metabolic health in obese individuals, with a focus on adipose tissue metabolism.

As described earlier in this thesis, adipose tissue dysfunction contributes to the development of insulin resistance and impaired glucose metabolism. By targeting fatty acid metabolism in the adipose tissue, liver, skeletal muscle or even the pancreas and the intestine, insulin sensitivity and glucose homeostasis may be improved. In **Chapter 2**, an extensive overview is provided of the fatty acid metabolism-related pathways in several metabolically active organs that can be targeted by dietary interventions, thereby improving whole-body glucose metabolism and insulin sensitivity.

Targeting the renin angiotensin system and the natriuretic peptide system by cardiovascular medication may improve adipose tissue and metabolic dysfunction, next to its effect on the cardiovascular system and hypertension. To investigate this, we conducted in **Chapter 3**, a multicenter, randomized, double-blind, double-dummy, parallel-group study to examine the metabolic effects of sacubitril/valsartan, which is a first-in-class angiotensin receptor neprilysin inhibitor, in obese hypertensive patients. By means of a hyperinsulinemic-euglycemic glucose clamp, we investigated the effects on peripheral insulin sensitivity, while whole-body lipolysis was determined using a stable isotope tracer ([1,1,2,3,3-^{2}H]-glycerol) and abdominal subcutaneous adipose tissue lipolysis was measured with the microdialysis technique. In addition to the collection of adipose tissue biopsies, we also performed indirect calorimetry measurements to assess energy expenditure and substrate utilization in these patients.

Chapter 4 extends the outcomes from the previous chapter and describes the effects of the 8-weeks treatment with sacubitril/valsartan compared to amlodipine on whole-body and adipose tissue lipolysis and lipid oxidation during a single bout of exercise.

To obtain more detailed insight into possible mechanisms underlying the findings described in chapters 3 and 4, we assessed adipose tissue gene expression patterns using microarray analysis and protein expression profiles of enzymes involved in lipolysis, the natriuretic peptide signaling pathway and mitochondrial oxidative phosphorylation complexes. The results from these analyses are provided in **Chapter 5**.

There is some evidence that exercise training may improve adipose tissue function, which may contribute to the reduced risk for developing obesity-related insulin resistance and other comorbidities. However, human studies that investigated the effects of exercise training on the adipose tissue function are limited. **Chapter 6** addresses the results of a supervised, progressive, combined endurance and resistance exercise training intervention for 12 weeks in well-phenotyped, obese subjects. In this study, we investigated exercise training-induced effects on adipose tissue by measuring abdominal subcutaneous adipocyte morphology, gene and protein expression of markers related to adipose tissue function. Moreover, we determined the exercise training-induced effects on *ex vivo* adipocyte lipolysis.

The aim of the study, described in **Chapter 7**, was to elucidate the physiological role of ANP-mediated lipolysis in abdominal subcutaneous adipose tissue of middle-aged obese insulin sensitive, obese insulin resistant and age-matched lean insulin sensitive men. By means of local combined blockade of the α- and β-adrenergic receptors (using a microdialysis approach), abdominal subcutaneous adipose tissue lipolysis was investigated during a single bout of low-intensity endurance exercise. In addition, we examined whether a combined endurance and resistance exercise training intervention for 12 weeks could improve abdominal subcutaneous adipose tissue lipolysis in obese insulin resistant individuals.

The main conclusions from the studies described in this thesis are discussed in **Chapter 8** and placed into a broader perspective, accompanied by suggestions for further research.

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CHAPTER 2

Targeting fatty acid metabolism to improve glucose metabolism

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ABSTRACT

Disturbances in fatty acid metabolism in adipose tissue, liver, skeletal muscle, gut and pancreas play an important role in the development of insulin resistance, impaired glucose metabolism and type 2 diabetes mellitus. Alterations in diet composition may contribute to prevent and/or reverse these disturbances through modulation of fatty acid metabolism.

Besides an increased fat mass, adipose tissue dysfunction, characterized by an altered capacity to store lipids and an altered secretion of adipokines, may result in lipid overflow, systemic inflammation and excessive lipid accumulation in non-adipose tissues like liver, skeletal muscle and the pancreas. These impairments together promote the development of impaired glucose metabolism, insulin resistance and type 2 diabetes mellitus. Furthermore, intrinsic functional impairments in either of these organs may contribute to lipotoxicity and insulin resistance. The present review provides an overview of fatty acid metabolism-related pathways in adipose tissue, liver, skeletal muscle, pancreas and gut, which can be targeted by diet or food components, thereby improving glucose metabolism.

INTRODUCTION

Obesity is considered a global health problem, since it is closely associated with the development of chronic metabolic diseases, including cardiovascular disease, type 2 diabetes mellitus (T2D) and certain types of cancer [1]. The pathogenesis of T2D is characterized by the development of both insulin resistance in peripheral tissues and pancreatic β-cell failure [2, 3]. Disturbances in fatty acid metabolism play a crucial role in the development of an impaired glucose metabolism and diabetes. Combined dietary and physical activity intervention may reduce the incidence of T2D by 30-60% [4, 5], which may for a considerable part be explained through modulation of fatty acid metabolism [6]. A better understanding of the interaction between diet, fatty acid metabolism, insulin resistance and β-cell dysfunction is needed to develop novel strategies to prevent impairments in glucose metabolism and, consequently, the development of T2D.

A tight interplay between adipose tissue, skeletal muscle, liver, pancreas and the gut regulate fatty acid metabolism in the human body. Besides an increased fat mass, adipose tissue dysfunction, characterized by an altered capacity to store lipids and low-grade inflammation, plays a major role in the development of insulin resistance and impaired glucose metabolism by promoting excessive fat storage in non-adipose tissues like liver, skeletal muscle, pancreas, the heart and kidneys [7-10] (Figure 1).

Furthermore, intrinsic functional impairments in either of these organs may contribute to lipotoxicity and insulin resistance. Skeletal muscle is generally considered the most important organ in peripheral insulin resistance [11]. Beside an increased fatty acid (FA) supply [12], a reduced skeletal muscle oxidative capacity [13, 14] may contribute to the accumulation of triacylglycerol (TAG) and bioactive lipid metabolites [15-17]. An increased lipid supply to the liver may result in an increased hepatic TAG content and very low-density lipoprotein (VLDL-TAG) output [18], a higher glucose production and output [19-21] and a reduced insulin clearance by the liver [22-24], leading to hyperlipidemia, insulin resistance and glucose intolerance [25]. Furthermore, hyperglycemia may, together with hyperlipidemia-related lipotoxicity in the pancreas, result in decreased glucose-stimulated insulin secretion by the pancreatic β-cells [26].

Recent evidence also indicates that the gut microbiota and its products may contribute to the development of insulin resistance and a disturbed glucose metabolism (Figure 1). There is accumulating evidence that alterations in the gut microbiota composition and function may affect adipose tissue, liver and skeletal muscle lipid and glucose metabolism, at least partly through effects on bile acid metabolism [27-29].

Energy intake and diet composition (i.e. dietary fatty acids, polyphenols, fibers) may have a significant impact on many aspects of fatty acid metabolism in different tissues. These effects can be both acute (i.e. postprandial phase) and more long-term, ultimately affecting health status. The aim of the present review is to provide an overview of fatty acid metabolism related pathways in adipose tissue, liver, skeletal muscle, pancreas and gut that can be targeted by diet or food components and, as a consequence, improve whole-body glucose metabolism and insulin sensitivity. The focus of the present review will be on the major tissues involved in inter-organ substrate metabolism and lipid-induced insulin resistance. Associations

between an increased myocardial triglyceride content or epicardial fat volume with insulin resistance, T2D and cardiac dysfunction [30-32], and the beneficial effects of diet on these deleterious parameters have been described elsewehere [33-35]. In addition, recent evidence points out that lipid accumulation in the kidneys [36, 37] and bones [38-40] may contribute to disturbances in whole-body glucose metabolism. These findings may be promising areas of future research but are beyond the scope of the present review.
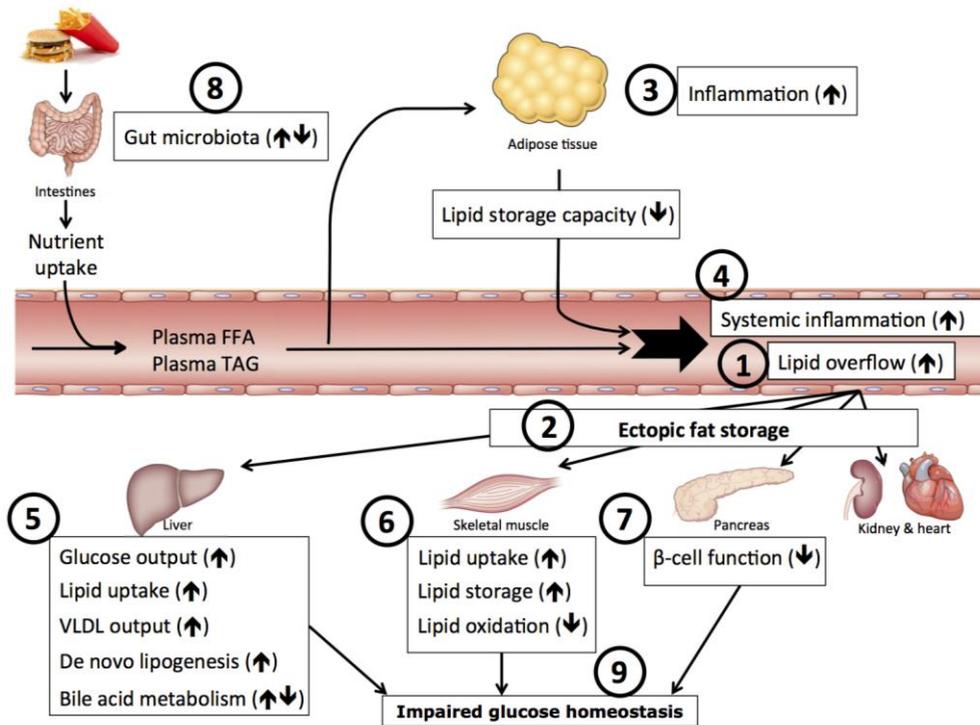
Figure 1. Inter-organ crosstalk in fatty acid metabolism and insulin resistance

An impaired adipose tissue lipid metabolism, as observed in obesity, is characterized by a decreased lipid storage capacity, which contributes to lipid overflow in the circulation **(1)**, resulting in excessive fat storage in peripheral tissues such as skeletal muscle, liver, pancreas, kidney and heart (ectopic fat storage) **(2)**. Furthermore, adipose tissue dysfunction is characterized by an altered expression and secretion of adipokines, inducing a state of chronic low-grade inflammation **(3)**. This inflammatory state may on the one hand affect local adipose tissue lipid metabolism and on the other hand contribute to systemic inflammation **(4)**, which together may affect lipid handling in peripheral tissues such as liver and skeletal muscle and promote insulin resistance through interference with insulin signaling. An increased lipid supply to the liver may result in a higher glucose production, an increased hepatic TAG content and VLDL-TAG output and a reduced insulin clearance by the liver **(5)**. The increased insulin concentration stimulates de novo lipogenesis and, together with an increased VLDL-TAG output, results in hypertriglyceridemia, ultimately leading to insulin resistance and glucose intolerance. In skeletal muscle, besides an increased fatty acid supply and uptake, an impaired muscle lipid turnover may contribute to accumulation of TAG and bioactive lipid metabolites (DAG, LCFA-CoA and ceramides), which may interfere with insulin signaling **(6).** Hyperglycemia may, together with the formation of hyperlipidemia-related toxic metabolites and lipid accumulation in the pancreas, result in decreased glucose-stimulated insulin secretion by the pancreatic β-cells **(7)**. Finally, alterations in gut microbiota composition and function **(8)** may affect adipose tissue, liver and skeletal muscle lipid and glucose metabolism, possibly through effects on SCFA production and bile acid metabolism. Ectopic fat storage is associated with impaired function of the liver, skeletal muscle and pancreas, leading to derangements in whole-body glucose homeostasis and, consequently, type 2 diabetes **(9)**.
Symbols: (↑↓): Altered; (↑): Increased; (↓): Decreased

ADIPOSE TISSUE DYSFUNCTION AND IMPAIRED GLUCOSE METABOLISM

Adipose tissue is highly important in buffering the daily influx of dietary lipids. The buffering capacity of adipose tissue refers both to its ability to suppress the release of free fatty acids (FFA) into the circulation and to increase lipid uptake, which is most pronounced in the postprandial state [41].

Firstly, disturbances in the adipose tissue lipid buffering capacity, like impairments in lipid uptake, lipolysis and fatty acid storage, adipocyte differentiation, adipose tissue expandability and adipose tissue mitochondrial function will be discussed. Subsequently, the browning of white adipose tissue (WAT) in relation to thermogenesis will be adressed. Thirdly, lipid-induced inflammation in relation to impaired glucose metabolism will be described, as depicted in Figure 2. Finally, putative nutritional targets that may improve adipose tissue dysfunction will be addressed.

Lipid uptake in adipose tissue

In the postprandial period, VLDL- and chylomicron-TAG are hydrolyzed in the process of intravascular lipolysis by lipoprotein lipase (LPL) [42]. The FFA that are liberated by this process can be taken up by the adipose tissue but a significant proportion will also spillover in the circulation and enter the plasma FFA pool [43]. LPL is regulated in a tissue-specific manner by nutrients and hormones [44]. In adipose tissue, the major activator of LPL activity is insulin [45, 46]. Therefore, adipose tissue LPL activity is high in the fed state and low when fasted [47, 48].

Furthermore, research has shown that dexamethasone (a synthetic glucocorticoid) decreased *in vitro* and *in vivo* LPL mRNA expression and activity in isolated rat and mice adipose cells and tissue, resulting in increased circulating TAG concentrations [49, 50]. In contrast, dexamethasone increased LPL mRNA and activity in human adipose tissue cultures [51, 52]. Whether these differences in LPL response reflect species-specific differences remains unclear. Another feeding-related hormone that could be responsible for changes in LPL expression and activity is glucose-dependent insulinotropic polypeptide (GIP), which increases LPL activity and mRNA in isolated human and rodent adipocytes [53, 54], although the regulation of adipose LPL activity by GIP *in vivo* remains to be established.

Recent studies also showed that LPL activity is altered by glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1), which binds LPL [55] in the interstitial space and transports it to the capillary lumen across endothelial cells [56, 57]. Fasting and peroxisome proliferator-activated receptor (PPAR)-γ agonists increased GPIHBP1 expression in rodents, while PPAR-α and PPAR-δ agonists had little or no effect [58]. Furthermore, hyperglycemia altered GPIHBP1 expression and activity in rodent and bovine endothelial cells [59]. Whether nutritional status or PPAR-γ agonists alter GPIHBP1 expression in humans is currently unknown, but several GPIHBP1 mutations have been discovered in humans [60] and result in severe hypertriglyceridemia [55, 56, 61]. Therefore, modulation of GPIHBP1 expression or activity might provide a strategy to treat hypertriglyceridemia, cardiovascular disease and T2D.

A considerable part of the variation in LPL activity during fasting, exercise and in the postprandial state may be explained by post-translational regulation by apolipoproteins (APO-C1, -2, -3, -5, APO-E) and the angiopoietin-like proteins (ANGPTL-3, -4, -8), as reviewed in detail elsewhere [62]. ANGPTL4, in particular, has been identified as an inhibitor of LPL activity and may be regulated by diet and fasting, since its expression and secretion changes in parallel with circulating FFA concentrations [62-66].

In obese conditions, basal LPL activity has been reported to be increased, whereas FA spillover from LPL-mediated TAG hydrolysis across adipose tissue during hyperinsulinemia is less suppressed as compared to lean healthy individuals [67, 68]. Indeed, the removal of TAG across adipose tissue was found to be impaired in obesity, insulin resistance and T2D, due to a reduced insulin-mediated stimulation of LPL activity [68-73], suggesting less efficient removal of dietary lipids by adipose tissue in these subjects. In line, a recent study has demonstrated that the relative quantity of meal fat stored in adipose tissue after the intake of subsequent meals was significantly reduced in (abdominally) obese versus lean individuals [69], which in turn may contribute to increased lipid spillover and ectopic fat deposition. To define nutritional targets affecting the regulation of LPL activity, which may subsequently reduce lipid spillover, ectopic fat deposition and improve insulin sensitivity, additional studies to determine the role of different LPL modulators in the regulation of its activity *in vivo* are clearly needed.

In addition to impaired LPL action in obese and insulin resistant conditions, disturbances in the uptake of the liberated FFAs (lipid spillover) may contribute to impaired lipid buffering. FAs are able to cross the endothelial barrier via passive diffusion, which is dependent on the concentration gradient of FAs across the membrane of the adipocyte, or via active transport [41, 43, 74, 75]. Active FA transport is facilitated by several enzymes and proteins, of which numerous FA-transport proteins have been identified in adipocytes [76], including fatty acid translocase/CD36 (CD36), membrane-bound and cytosolic fatty acid binding protein (FABPpm and FABPc, respectively) and fatty acid transporter protein (FATP) [77]. It has been shown that the translocation of CD36 from the intracellular stores to the plasma membrane is regulated by various stimuli, including insulin and increased activation of AMP-activated protein kinase (AMPK) [78-81]. Although CD36 protein expression is higher in the subcutaneous adipose tissue of obese, overweight and T2D compared to lean subjects [82], the net removal of TAG and FAs by adipose tissue is reduced in the former groups, suggestive of impairments in intracellular CD36 trafficking. Taken together, strategies to reduce spillover and increase FFA trapping in adipose tissue, mainly in the postprandial phase, will lead to a reduced lipid spillover and may consequently prevent or at least reduce ectopic fat deposition. On the one hand, this may be achieved by an increased FFA concentration gradient across the adipocyte membrane and a subsequent decreased intracellular FFA concentration. This might be accomplished by increasing FA reesterification and/or oxidation, which will be discussed later in this section. On the other hand, an increased facilitated diffusion by fatty acid transporters like CD36 may possibly increase fatty acid trapping, thereby reducing FFA spillover. Indeed, it has been shown that tissue-specific CD36 transcripts differentially influence fatty acid homeostasis and insulin sensitivity [83]. Adipocyte CD36 appears to be metabolically protective, and its selective upregulation might

have therapeutic potential in insulin resistance. Nevertheless, it has been shown that CD36 only facilitates fatty acid transport in adipose tissue and muscle when extracellular concentrations are low, suggesting no major regulatory role when circulating FFA concentrations are equal or above those seen in the overnight postabsorptive state [84].
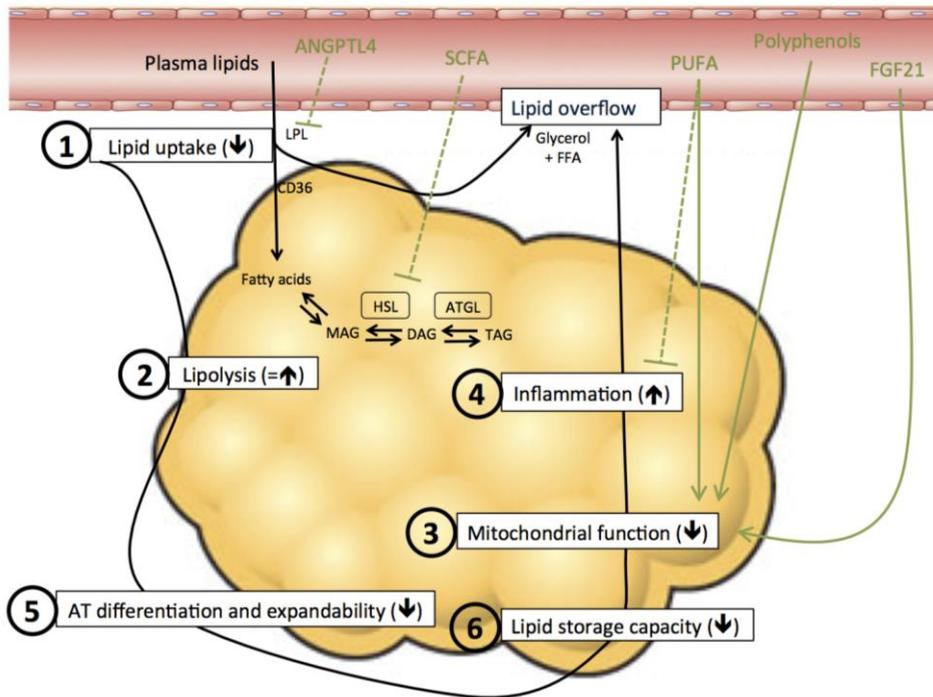
Figure 2. Disturbances in adipose tissue fatty acid metabolism

Adipose tissue lipid uptake **(1)** is impaired in obesity, insulin resistance and T2D, due to a reduced insulin-mediated stimulation of LPL activity and, possibly, decreased facilitated FFA uptake via CD36 in adipocytes, which contributes to lipid overflow in the circulation. TAG and DAG are broken down by the lipases ATGL and HSL. Fasting lipolysis, expressed per unit fat mass, may be decreased in obese subjects **(2)**. Nevertheless, insulin-mediated suppression of adipose tissue lipolysis per unit fat mass is attenuated in obese individuals, resulting in an increase in whole-body lipolysis during postprandial conditions. A decreased adipose tissue mitochondrial function **(3)** may lead to impaired scavenging of fatty acids, resulting in increased intracellular FFA concentrations, reducing FFA trapping and an enhanced release of FFA into the circulation. Furthermore, dynamic changes occur in the adipose tissue immune cell populations during the development of obesity, causing a shift from an anti-inflammatory towards a more pro-inflammatory phenotype, resulting in a state of low-grade inflammation **(4)**, affecting insulin sensitivity through different mechanisms. Finally, a reduced adipose tissue differentiation and expandability **(5)**, leading to a reduced lipid storage capacity **(6)**, contributes to lipid overflow into the circulation.

Abbreviations: LPL: Lipoprotein lipase; FFA: Free fatty acid; CD36: Fatty acid translocase CD36; TAG: Triacylglycerol; DAG: Diacylglycerol; MAG: monoacylglycerol; ATGL: Adipose triglyceride lipase; HSL: Hormone sensitive lipase; ANGPTL4: Angiopoietin-like protein 4; PUFA: Poly unsaturated fatty acid; SCFA: Short chain fatty acid; FGF21: Fibroblast growth factor 21.

Dashed lines indicate inhibition. Solid lines indicate stimulation. Green lines indicate beneficial effects. Symbols: (=): unchanged; (↑): Increased; (↓): Decreased.

Fatty acid release from adipose tissue

When energy is required (e.g. during exercise and fasting), the lipids that have been stored in intracellular lipid droplets (LD's) as TAG, are hydrolyzed via activation of the intracellular lipolytic pathway, which involves different lipases and lipid droplet-associated proteins that are under hormonal control [85].

Catecholamines and natriuretic peptides (NPs) act as lipolytic hormones, while insulin acts as the major anti-lipolytic hormone in human adipose tissue. The net lipolytic effect of catecholamines is determined by the ratio between lipolytic beta (β1,2 and 3)- and anti-lipolytic alpha (α2)-receptors, and subsequent protein kinase A (PKA) activation. NPs act through natriuretic peptide receptor type A, B and C (NPRA, NPRB, NPRC) to increase protein kinase G (PKG) activity [86-88]. Stimulation of intracellular lipolysis is dependent on PKA and PKG-mediated phosphorylation of LD-associated proteins, including perilipin 1 (PLIN-1), hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), as extensively reviewed elsewhere [89, 90].

Whole-body lipolysis under fasting conditions (basal lipolysis) may be increased in obesity because of the increased total adipose tissue mass. However, if adipose tissue would release FFA at the same rate in obese and lean subjects, then circulating FFA would be much higher than observed in obesity (only 20-30% higher), suggesting that FFA concentrations are not elevated in proportion to fat mass in obese individuals. Indeed, others and we have demonstrated that fasting lipolysis expressed per unit fat mass is rather reduced in obesity [91-93]. This was accompanied by downregulation of the expression of the key lipolytic enzymes HSL and ATGL [91, 94, 95]. Data on PLIN-1 are inconclusive, being reduced [96-98] or even elevated [99] in obese adipose tissue. Since humans are in the postprandial state most of the day, insulin-mediated inhibition of adipose tissue lipolysis is a major regulator of lipolytic rate. Insulin-mediated suppression of adipose tissue lipolysis per unit fat mass is attenuated in obese individuals, suggesting that chronic hyperinsulinemia cannot overcome the increase in whole-body lipolysis. Therefore, inhibition of adipose tissue lipolysis might be a therapeutic strategy to limit excess FFA release, thereby alleviating the development of insulin resistance and obesity-associated metabolic abnormalities. Although data are scarce, reduced plasma FFA and glucose levels have been demonstrated in diabetic rats treated with a selective HSL inhibitor for 3-8 hours [100]. Recently, Girousse *et al.* [101] have shown that mice treated with the same pharmacological HSL inhibitor for 7 days and haploinsufficient HSL^{+/-} mice were paradoxically resistant to diet-induced obesity due to a reduction in FFA uptake and reesterification, suggestive of reshaping the FFA flux in peripheral tissues [101]. Furthermore, in that study, systemic glucose tolerance was improved through induction of *de novo* lipogenesis in murine and human adipocytes. In addition to selective inhibition of HSL, recent data report about the development of a selective inhibitor of ATGL, Atglistatin [102], and about the mechanisms of inhibition of ATGL by long-chain acyl-coenzyme A [103], highlighting the increasing interest in selective lipase inhibition to correct defects in lipid metabolism for the treatment and prevention of obesity and obesity-associated metabolic diseases.

In contrast to an elevated basal lipolysis, others and we have clearly shown that *in vitro* and *in vivo* catecholamine-induced lipolysis is blunted in subcutaneous

adipose tissue of obese subjects, which persists after significant weight loss [89, 94, 95]. These data suggest that impaired cathecholamine-induced lipolysis may be an important primary factor in the development of obesity. Data are limited with respect to NP-induced lipolysis. However, reduced circulating NP levels [104] and a defective ANP-mediated lipolytic response in subcutaneous adipocytes and adipose tissue from obese subjects have been observed [105]. Therefore, in contrast to the anti-lipolytic approach with selective lipase inhibition, improving catecholamine- and NP-sensitivity has been extensively investigated for the treatment of obesity and obesity-related complications but has, so far, not shown promising results due to cardiovascular side effects and receptor desensitization [106-108].

In addition to classical lipolysis, three recent studies have implicated autophagy, a homeostatic mechanism functioning as a 'self-digestion' system, in selective lipid hydrolysis under basal and catecholamine-stimulated conditions in adipocytes, termed lipophagy [109-111]. These data indicate that activation of cytosolic lipases (i.e. ATGL and HSL) is no longer the sole molecular mechanism to liberate FA from adipocyte TAG stores. Since ATGL and HSL are expressed at much higher level in adipocytes than other cell types it is plausible that under normal physiological conditions adipocytes rely mainly on classical cytosolic lipolysis, while the alternative pathway for lipid breakdown, lipophagy, may become more important in pathophysiological conditions, with a reduced ATGL and HSL activity, to maintain lipid homeostasis. In line, autophagy markers and fluxes appear to be elevated in adipose tissue of obese insulin resistant and T2D subjects [112-115]. In addition, nutritional and hormonal regulation of adipose tissue autophagy is impaired in obese rodents [116].

To summarize, obesity is characterized by an increased basal and a blunted catecholamine and NP-stimulated lipolysis in subcutaneous adipocytes. This altered lipid turnover may be an early factor in the development of increased fat stores and obesity-associated metabolic complications. Modulation of classical lipolysis recently regained interest in the treatment of obesity-related insulin resistance, indicated by the development of selective ATGL and HSL inhibitors. However, to prevent excessive gain in body weight, tissue FFA turnover (uptake, esterification and oxidation) should be adapted accordingly. Furthermore, lipohagy might be increased in adipose tissue of obese subjects as compensatory mechanism to deal with the increased lipid availability due to an attenuated classical lipolysis. Importantly, before considering manipulation of the classical and/or alternative pathway of adipose tissue lipolysis for therapeutic purposes, a better insight into its role in pathophysiology as well as hormonal and nutritional regulation is warranted.

Lipid droplet formation and fatty acid storage in the adipose tissue

In humans, adipocytes, the major cell type in white and brown adipose tissue (BAT), are specialized for storing lipids in LD. Several proteins bind the LD surface and regulate LD size, fusion and number. They include PAT proteins (i.e. perilipin 1, perilipin 2/adipophilin/ADRP, perilipin 3/TIP47 and perilipin 4/S3-12), CIDE (Cell Death inducing DNA Fragmentation Factor) proteins, Soluble NSF Attachment

Protein Receptor (SNARE), caveolins and several lipases. LDs are dynamic organelles, constantly forming, growing or shrinking. In recent years, our knowledge of LD dynamics and biogenesis has increased, as reviewed extensively elsewhere [117-123]. Impaired LD expansion and TAG storage capacity may play a role in lipid storage diseases, including obesity and T2D. In obese conditions, the storage capacity of the LD in adipocytes is exceeded. CIDE-C/FSP27 (fat-specific protein 27) and PLIN-1 largely regulate TAG storage in human adipocytes by facilitating lipid transfer from smaller to larger LDs and by regulating intracellular lipolysis. Studies examining the expression of CIDE proteins and PLIN-1 as a function of insulin sensitivity found that mRNA levels of these LD-associated proteins correlate positively with insulin sensitivity in subjects with similar body mass index [124, 125]. These findings in humans contrast with findings in mice, in which the lack of CIDEC/FSP27 or PLIN-1 protected against high fat diet-induced obesity and insulin resistance [126-128], highlighting the difficulties in extrapolating results from mice to human pathologies.

In summary, although human data are limited, high levels of protein that promote TAG storage, including CIDEC/FSP27 and PLIN-1, might help to sequester lipids in the adipose tissue and to protect against insulin resistance. Furthermore, impaired LD expandability may prevent the recruitment of new adipocytes by either initiating a pro-inflammatory response or by preventing the secretion of yet unidentified factors that promotes recruitment of adipocyte progenitors.

Adipocyte differentiation and adipose tissue expandability

A unique property of adipose tissue is its capacity to change its dimensions. This can be achieved by fat cell enlargement (hypertrophy) or by recruitment of new adipocytes from the resident pool of progenitor cells (hyperplasia).

During periods of chronic excessive energy intake, the adipose tissue mass expands first by hypertrophy to the point that the maximal fat cell expandability is achieved. Then, signals are released by the adipocyte to stimulate proliferation and/or differentiation of preadipocytes (adipogenesis) [129], leading to an increased number of mature adipocytes. This process is necessary to store the excessively available FAs and to protect cells from detrimental effects of high concentrations of circulating FAs [130]. Interestingly, there seems to be adipose tissue depot-specific patterns in adipose tissue expansion. Overfeeding evokes adipocyte hypertrophy in abdominal subcutaneous adipose tissue, whereas adipocyte hyperplasia, but not hypertrophy, occurs in the femoral subcutaneous fat [131]. Adipogenesis is also accompanied by specific changes in the adipocyte extracellular matrix (ECM), as reviewed elsewhere [132-134], and this ECM remodeling seems essential in adipose tissue expansion. Research showed that ECM processes are disrupted in obesity and impair metabolic function and fat mass expansion [135, 136]. Furthermore, diet induced weight gain by overfeeding, resulted in fat mass expansion and upregulation of genes involved in lipid metabolism and storage, angiogenesis and ECM remodeling [137].

The inability of the adipose tissue to store excess lipids in newly differentiated adipocytes results in enlargement of existing adipocytes [138]. These hypertrophic adipocytes are less sensitive to the action of insulin and have impaired lipid

buffering capacity [139]. The adipogenic potential of adipose-derived stem cells (ASCs) depends on their depot specific origin and host characteristics such as age, sex and metabolic status [140]. ASCs from subcutaneous fat have been reported to differentiate better into mature adipocytes than those from visceral fat [141]. In contrast to what was thought in the past, the expandability of the adipose tissue is not an unlimited process. Importantly, impaired adipose tissue expandability has been linked to metabolic derangements. For example, lipodystrophic patients with severely reduced adipose tissue mass, show a substantial reduction in fat storage capacity. Hypertrophic adipocytes in obese individuals, on the other hand, are overloaded with stored TAG. In both conditions, the storage capacity of adipose tissue seems insufficient, resulting in ectopic fat storage and, consequently, insulin resistance [7].

Kim *et al.* [138] have shown that ectopic fat storage and insulin resistance can be ameliorated when adipose tissue mass can properly expand to accommodate excess calories. Involvement of ASCs in the impaired adipose tissue expandability is only beginning to be explored. It has been reported that the number of mature adipocytes is set during childhood and stays constant throughout adulthood regardless of fat mass changes in humans [142], but it is not yet clear how adipose progenitor cells contribute to this process. Thus, strategies to increase adipose tissue expandability may reduce ectopic fat storage, thereby improving insulin sensitivity, despite possible body weight gain.

Adipose tissue mitochondrial function and browning

Mitochondria play a central role in the catabolism of nutrients to provide energy that is required for numerous cell functions. During periods of energy requirement, FFAs are liberated to fuel mitochondrial β-oxidation. There is substantial evidence that adipose tissue mitochondrial mass and function determine metabolic health. Rodent studies have suggested that mitochondria in white adipose tissue contribute to overall fat oxidation [143]. Impaired mitochondrial oxygen consumption in adipose tissue is present in mouse models of obesity and T2D [144], and mitochondrial oxidative pathways are downregulated in human adipose tissue [145]. Importantly, adequate mitochondrial function is essential to maintain adipose tissue function, glucose homeostasis [146] and protects against insulin resistance and T2D [144]. This is further highlighted by a recently large-scale microarray analysis using >1000 human abdominal subcutaneous adipose tissue biopsies, revealing that mitochondrial oxidative pathways were markedly downregulated, whereas inflammatory pathways were upregulated, in morbidly obese insulin resistant as compared to morbidly obese insulin sensitive patients [147]. In accordance, mitochondrial mass and electron transport chain genes in adipose tissue are lower in obese and T2D subjects [148, 149]. A recent study has demonstrated that both human adipocyte mitochondrial content and *ex vivo* respiration (oxygen consumption) were significantly reduced in obese as compared to lean individuals, independent of adipocyte size [150]. A decreased adipose tissue mitochondrial function may lead to an incorrect scavenging of fatty acids, resulting, on the one hand, in increased intracellular FFA concentrations reducing FFA trapping and, on the other hand an enhanced release of FFA, liberated by

intravascular or intracellular lipolysis into the circulation, contributing to lipid overflow [151]. Interestingly, the 'browning' of white adipocytes, which will be further discussed below, likely involves lipase-generated PPAR ligands, whereby lipolysis may be an initiating factor (e.g. recruiting adult adipocyte progenitor cells into a brown adipocyte lineage) [152].

Traditionally, fat cells have been divided into unilocular white and multilocular brown adipocytes. Brown adipocytes are specialized heat producing cells, and transfer energy from lipid and carbohydrate substrates into heat through the actions of uncoupling protein-1 (UCP-1). In contrast to infants, who have substantial amounts of BAT to maintain body temperature, it was thought for many years that healthy adult humans living under normal environmental temperatures lack BAT. However, ^{18}F-fluoro-D-2-deoxy-D-glucose (FDG) positron emission tomography (PET)-positive areas have recently been identified in lean healthy adults, indicating that substantial amounts of BAT are present in humans [153-155]. Furthermore, it has been demonstrated that BAT is inversely related to body fat percentage and BMI [153], and BAT activity was increased 1 year after bariatric surgery-induced weight loss in morbidly obese subjects [156]. Recently, BAT has been implicated to improve whole body glucose homeostasis and insulin sensitivity in humans [157]. Moreover, a third category of fat cells has been introduced, namely "beige/brite" adipocytes, which have a brown fat-like morphology within white fat depots [158-161]. Beige adipocytes seem to be programmed to be bifunctional, meaning they are suitable for energy storage in the absence of thermogenic stimuli but capable of switching on heat production [158]. It is not entirely clear yet what distinguishes brown from beige adipocytes, and the relative importance of BAT in energy homeostasis also requires further investigation. Nevertheless, it has been estimated that BAT may contribute to 3-5% of basal metabolic rate in humans [162]. Therefore, an intriguing question is how BAT can be activated and whether this may induce weight loss in humans.

In addition to cold-induced BAT activity, several factors have been studied that affect activity and recruitment of brown adipocytes. Briefly, these include adrenergic stimulation, natriuretic peptides, irisin, capsinoids, and insulin in humans [162, 163]. In rodents, it has been suggested that, in addition to the above-mentioned factors, thyroid hormone, bile acids, fibroblast growth factor 21 (FGF21) [164, 165] and bone morphogenetic proteins (BMP) [166] may also play a role. Future studies will likely elucidate whether strategies to increase browning of white adipose tissue hold promise to prevent and/or treat obesity and related impairments in lipid and glucose metabolism.

Lipid-induced inflammation and impaired glucose metabolism

Adipose tissue used to be considered as a passive fat storage organ. However, it is clear for more than two decades that adipose tissue is an active endocrine organ by the release of a variety of lipids (lipokines) [167] and signaling molecules (adipokines). These factors can both act locally (autocrine and/or paracrine) and at the whole-body level (endocrine function) to exert a wide range of biological effects and to regulate systemic metabolic homeostasis [7]. The endocrine function of adipose tissue is impaired in obese, insulin resistant individuals, which may

contribute to chronic low-grade inflammation and, consequently, obesity-related insulin resistance and other complications [7]. In addition to adipocytes, adipose tissue contains a wide variety of stromal vascular cells, including endothelial cells, fibroblasts, preadipocytes and immune cells. Although mature adipocytes make up >90% of adipose tissue volume, they account for only 20-40% of the cellular content. Interestingly, immune cells present in adipose tissue have recently been shown to play an important role in adipose tissue biology [164]. The low-grade inflammatory state in obesity is the result of an imbalance between the production of pro-inflammatory and anti-inflammatory factors. It was shown about 10 years ago that many proinflammatory factors are produced by macrophages that infiltrate hypertrophic adipose tissue [168, 169]. More recent evidence indicates that also other innate and adaptive immune cells are present in adipose tissue, as reviewed elsewhere [170]. Furthermore, it seems that dynamic changes occur in the adipose tissue immune cell populations during the development of obesity, causing a shift from an anti-inflammatory towards a more pro-inflammatory phenotype. It has been demonstrated that there is a relative abundance of M1 polarized macrophages, Treg cells and eosinophils in lean visceral adipose tissue, whereas the proportion of M2 polarized macrophages, Th1 cells, mast cells, dendritic cell, neutrophils and natural killer T cells is increased in obesity. Intriguingly, many of the latter proinflammatory immune cells have been linked to impairments in glucose homeostasis [171, 172] and decreasing the inflammatory status may improve insulin resistance in rodents and humans [173].

An important but still unanswered question is what triggers adipose tissue inflammation in obesity? Lipids act as important signal moieties regulating both metabolism and immune responses. This is exemplified by the induction of hepatic and peripheral insulin resistance [174] and pro-inflammatory responses in adipose tissue [175] by lipids. Various mechanisms seem to be involved in the induction and progression of inflammatory responses in adipose tissue and consequently insulin resistance in obesity. Briefly, lipid-induced activation of innate receptors (Toll-Like Receptors (TLR)) and the inflammasome, death of hypertrophic adipocytes and resulting macrophage infiltration, lipid-induced endoplasmic reticulum stress (ER stress) and the unfolded protein response and, more recently, the gut microbiota have been linked to adipose inflammation and consequently insulin resistance in obesity [176]. On the other hand, activation of PPAR-γ by polyunsaturated fatty acids (PUFA) [177] may reduce adipose tissue inflammation [178]. However, it remains to be established to what extent the above-mentioned mechanisms contribute to systemic inflammation and whole-body insulin resistance in humans. Over the last decade, evidence has emerged that ER stress in several organs, including adipose tissue, plays a direct (i.e. as a negative modulator of the insulin signaling pathway) and indirect role (i.e. by promoting lipid accumulation) in the onset of insulin resistance, as reviewed elsewhere [179].

Adipose tissue inflammation may in turn exert detrimental effects on insulin sensitivity through different mechanisms. For example, tumor necrosis factor alpha (TNF-α) and Interleukin 6 (IL-6) inhibit adipocyte differentiation [180-182] and TNF-α induces adipocyte apoptosis in pre- and mature adipocytes [183], which could lead to the enlargement of the remaining fat cells and consequently, reduced adipose tissue lipid buffering. Furthermore, TNF-α and IL-6 both stimulate adipocyte lipolysis [184-187], thereby contributing to systemic FA release, which

may in turn lead to ectopic fat storage and insulin resistance [188]. In addition, lipid-induced impairments in adipokine secretion may also exert direct effects on peripheral insulin sensitivity [189]. It has been demonstrated that adiponectin increases skeletal muscle fat oxidation and therefore, the decreased adiponectin concentrations in obese conditions may impact fat oxidation, and as such, affect lipid accumulation and insulin sensitivity [190, 191].

As reviewed by Ohashi *et al* [192], several anti-inflammatory adipokines, such as adiponectin, the family of the C1q/TNF-related Proteins (CTRP3, -6, -9), adipolin and omentin-1 may exert beneficial effects on obesity-related complications. CTRP3 is a novel adipokine, expressed at the AT and found in circulating plasma [193, 194], which regulates hepatic glucose output [195], suppresses chemokine production in response to lauric acid, LPS or TLR stimulation in macrophages and adipocytes [196] and stimulates the expression of adiponectin in primary human adipocytes and cultured 3T3-L1 adipocytes [197]. CTRP6 has been found to increase the expression of the anti-inflammatory cytokine IL-10 in human monocyte-derived macrophages [198], to stimulate activation of AMPK and enhance fatty acid oxidation in skeletal muscle cells [199]. In cultured myocytes, CTRP9 activates phosphorylation of AMPK and protein kinase B (PKB) and promotes insulin stimulated glucose uptake [194]. It also reduced diet-induced weight gain, decreased insulin resistance and hepatic steatosis, with enhanced AMPK activation and fat oxidation in skeletal muscle in CTRP9 transgenic mice [200]. CTRP12, also known as Adipolin (adipose-derived insulin-sensitizing factor) [201], has been reported to activate insulin signaling in the liver and adipose tissue of obese mice [202]. Furthermore, omentin-1, also known as intelectin-1, is abundantly expressed in visceral fat tissue [203], is decreased in obese individuals [204] and increased insulin stimulated glucose uptake in cultured adipocytes *in vitro* [203].

In addition, dipeptidyl peptidase-4 (DPP4) is released by mature adipocytes and inhibits skeletal muscle insulin signaling [205, 206]. Beside lipolysis and insulin signaling, pro-inflammatory cytokines may also regulate adipocyte mitochondrial function [207]. Of note, not only adipose tissue but also other organs such as the liver, skeletal muscle, heart and pancreas may contribute to lipid-induced systemic inflammation in obesity via secretion of cytokines [208, 209].

Taken together, lipids are important triggers for adipose tissue inflammation and consequently insulin resistance in obesity. Therefore, interventions aimed at improving lipid metabolism may improve glucose metabolism via reduction of adipose tissue and systemic inflammation.

Putative nutritional targets to improve adipose tissue function

The balance between lipolysis, adipocyte differentiation and mitochondrial function within adipose tissue is important to maintain adequate lipid storage capacity of adipose tissue, thereby preventing lipid overflow in the circulation and ectopic fat deposition. Therefore, improving the lipid buffering capacity of adipose tissue has high potential to increase glucose tolerance and insulin sensitivity.

Presently, there is a clear need for additional studies to determine the regulators of LPL activity, including the involvement of proteins affecting post-translational mechanisms, like ANGPTL4, which may be regulated by diet [63].

Secondly, as discussed above partial HSL or ATGL inhibition may reduce lipid overflow and possibly improve metabolic profile. Interestingly, intravenous acetate infusion [210] or colonic acetate administration may reduce systemic FFA concentrations, through an effect on HSL phosphorylation [211]. Dietary manipulation of colonic acetate and short chain fatty acid (SCFA) concentration through pre- and probiotics may be a promising target in this respect.

Thirdly, modulation of the adipose tissue lipophagic pathway might be a potential target pathway as well. Recently, we have shown that dietary polyphenols including resveratrol and epigallocatechin-gallate (EGCG), found naturally in red wine and green tea have caloric restriction-like effects in overweight humans [212]. Interestingly, our microarray data showed that an improved adipose tissue lipolysis and function, is associated with selectively targeting of the master regulator of lipophagy, TFEB, in human adipose tissue following resveratrol supplementation [213]. However, it remains to be determined whether lipophagy-mediated lipid catabolism in adipose tissue is directly involved in the potential beneficial effects of polyphenols.

As indicated above, nutritional strategies to improve mitochondrial function, like specific polyphenols or a combination of polyphenols, may be effective in balancing lipid supply to utilization [212, 213], improving thereby adipose tissue function. Moreover, recent data from our group also suggest that a dysbalance between oxygen supply and oxygen utilization leading to an increased adipose tissue oxygen tension may induced adipose tissue dysfunction [214, 215], again illustrating the importance of a normal mitochondrial mass and function.

Factors affecting the activity and recruitment of BAT, may have positive effect with respect to adipose tissue function, body weight control and insulin sensitivity. Currently, most promising may be dietary components affecting our microbial bile acid metabolism, leading to increased circulating FGF21, a mediator of lipid and carbohydrate metabolism, also inducing recruitment of brown adipocytes [216].

The effect of dietary quality on adipose tissue function and ectopic fat accumulation remains an area of particular interest in composing optimal diets that minimize ectopic and abdominal fat accumulation. There is strong evidence that avoidance of high saturated fatty acids (SFA) diet contributes to lower health risks among obese, metabolic syndrome and diabetic patients [217]. There are indications that the monounsaturated fatty acids (MUFA) and/or PUFA have more beneficial effects compared to SFA on the action of insulin [218-221]. Research showed that n-3 PUFA (eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)) reduced LD formation in 3T3-L1 cells compared to SFA [222]. Further, long chain n-3 PUFA may increase fatty acid oxidation and mitochondrial biogenesis in adipose tissue [223, 224], may inhibit fat cell proliferation [225] and may limit fat cell hypertrophy and hyperplasia [226]. Additionally, positive effect have been ascribed to specifically n-6 fatty acids in reducing abdominal fat area, improving insulin sensitivity and in reducing visceral fat/subcutaneous fat ratio compared with a SFA diet [227, 228].

Dietary fat quality not only modulates lipid metabolism, it may also affect low-grade inflammation, as for instance seen by exposure of myotubes or adipocytes to SFA

which increased IL-6 mRNA and protein expression, possibly via activation of nuclear factor-κB (NF-κB) [229, 230]. A human intervention study with a SFA rich diet for 5 weeks showed higher concentrations of C-reactive protein (CRP), fibrinogen and IL-6 compared to a diet enriched in MUFA [231]. Substantial increase in the PUFA intake can also decrease low-grade inflammation as indicated by circulating CRP, IL-6 or soluble adhesion molecules [232, 233].

In summary, polyphenols, specific dietary fatty acids and pre- and probiotics may be promising nutritional components in improving the balance between lipolysis, autophagy and mitochondrial function and in stimulation of adipose tissue browning, thereby improving adipose tissue function and whole-body glucose homeostasis. For an overview of putative nutritional targets see Table 1.

ALTERED LIVER FUNCTION AND IMPAIRED GLUCOSE METABOLISM

Obesity is recognized as a major cause of the promotion of metabolic diseases including non-alcoholic fatty liver disease (NAFLD), which is not only linked to an impaired glucose metabolism and diabetes, but also evokes more severe liver diseases like non-alcoholic steatohepatitis (NASH), hepatic cirrhosis and eventually liver cancer.

Indeed, hepatic insulin resistance, which is defined as an impaired suppression of hepatic glucose production [234], may originate from the accumulation of lipid metabolites that interfere with insulin signaling [235]. This accumulation of lipids and lipid metabolites may be caused by multiple factors [236]. Besides that, liver lipid accumulation is also associated with the progression of ER stress, mitochondrial stress and an impaired autophagy, resulting in lipotoxicity [237]. An increased FFA release from visceral adipose tissue (the 'portal hypothesis') or increased intake of dietary fat, followed by a reduction in postprandial lipid clearance and an increased spillover of FFA in the circulation, due to a reduced lipid storage capacity, can explain this phenomenon [238-241]. Furthermore, mitochondrial dysfunction, associated with insulin resistance may precede liver fat accumulation by impairing fatty acid β-oxidation [242]. Additionally, an increased de novo lipogenesis highly contributes to liver lipid accumulation, ultimately leading to steatosis [243] (Figure 3).

When FA supply to the liver is increased, VLDL-TAG secretion by the liver is also increased, but it appears that this secretion of VLDL-TAG is not sufficient to compensate for the increased uptake, resulting in a net lipid accumulation [244], even though conditions of insulin resistance are marked by an increased production of VLDL-TAG [245]. Below, the mechanisms contributing to liver fat accumulation are briefly delineated.

Hepatic lipid uptake

In obesity, insulin resistance and T2D, circulating levels of FFA and lipoprotein remnants are increased [246-248] and this causes an increased storage of TAG and lipid metabolites in hepatocytes and may lead to hepatic insulin resistance. This may further increase circulating VLDL-TAG concentrations due to a decreased insulin-mediated suppression of VLDL production by the liver [249-252].

In the liver, beside LPL, hepatic lipase (HL) plays an important role in fatty acid metabolism, as it is both a phospholipase and a TAG lipase [253]. An increased HL activity promotes FFA uptake into hepatocytes and has been shown to be increased in obese humans [254-256], insulin resistant rodents [257], type 1 diabetic patients [258] and in NAFLD patients [259].

The rate of plasma FFA uptake by the liver is determined by both the plasma FA concentration and the hepatocellular capacity for FA uptake [260], which depends on the number and activity of transporter proteins on the plasma membrane of the hepatocyte. The main proteins in the hepatic transmembrane FA transport are FATP-2 [261], FATP-5 [262], caveolins, and to a lower extent fatty acid translocase CD36 [263, 264]. FATP-5 knockout mice show resistance to diet-induced obesity and hepatic lipid accumulation, but there is no evidence for involvement of this isoform in human obesity [265]. Also, silencing of hepatic FATP-5 in hyperglycemic mice showed a rapid (2-3 weeks) reduction in liver FFA uptake and a reduction in serum glucose levels, reaching normal glycaemia after 5 weeks [262]. Additionally, expression of FABP-4 and -5 correlated with hepatic fatty acid infiltration in NAFLD patients [266]. Interestingly, it was shown that specific bile acids (BAs) (ursodeoxycholic acid (UDCA) and deoxycholic acid (DCA)) inhibit liver-specific FATP-5 in mice [267], suggesting that specific bile acids can impact hepatic lipid metabolism. Caveolin proteins consisting out of caveolin-1, -2 and -3, play an important role in protein trafficking and the formation of lipid droplets. Caveolin-1 knockout mice have lower TAG accumulation in the liver and are resistant to the development of diet-induced obesity, suggesting that this protein may be of importance in liver fat accumulation and TAG synthesis [268]. Furthermore, rodent *in vivo* and *in vitro* data show that overexpression of caveolin-3 in liver resulted in an improved insulin receptor signaling, insulin sensitivity and glucose metabolism [269]. Normally, CD36 is not highly expressed in liver, but its expression was positively correlated with hepatic TAG content in NAFLD patients, underscoring the importance of this protein in lipid accumulation [270]. Moreover, CD36 deficiency increased insulin sensitivity in muscle, but resulted in an insulin resistant state in the liver of mice [271]. It is evident that further research is required to elucidate the role of these FA transporters in normal physiology as well as pathological conditions.

Hepatic lipolysis

Rodent *in vivo* and *in vitro* data showed that hepatic ATGL (also known as PNPLA2 and desnutrin) knockdown enhances glucose tolerance by increasing hepatic glucose utilization and improved insulin action from hepatic TAG accumulation [272]. Furthermore, HSL-knockout mice also showed reduced hepatic TAG stores and increased hepatic insulin sensitivity [273], but results are not consistent [274]. In adipose tissue, ATGL and HSL are highly expressed and active [275], but in the liver, the contribution of other lipases may also play a role in the catabolism of stored lipids. Several members of the carboxylesterase/lipase family and the patatin-like phospholipase domain-containing protein (PNPLA) family have been suggested as potential TAG hydrolases [276]. One of them, carboxyl esterase-3/triglyceride hydrolase-1 (Ces-3/Tgh-1, ortholog of human Ces-1), has gained

major interest because the recent characterization of Ces-3/Tgh-1-deficient mice provided compelling evidence that the enzyme participates in the assembly and secretion of hepatic VLDL [277]. PNPLA-4 and -5 exhibit TAG-hydrolase, DAG-transacylase and retinylester hydrolase activity *in vitro* [278], but whether these activities are also relevant *in vivo* remains to be determined [90]. The adiponutrin/PNPLA-3 I148M polymorphism was reported to be associated with insulin resistance and NAFLD, suggesting a role of importance in hepatic TAG accumulation [279-281]. Furthermore, LD-covering proteins also play a role in the pathophysiology in fatty liver disease, which is characterized by hepatocytes containing LD with excessive neutral lipids [282]. Studies showed that LD proteins such as PLIN-1 and PLIN-2 are highly expressed in liver steatosis [283, 284]. A high fat diet increases expression of PLIN-2 in a PPAR-γ dependent manner, resulting in the development of fatty liver [285-287]. Data from a rodent study showed that PLIN-2 knockout enhanced insulin action in the liver, whereas muscle and adipose tissue were not affected [288]. Furthermore, other LD-associated proteins such as FSP27 and CIDEC have also been characterized. FSP27 was expressed in the steatoic liver of a T2D mouse model and the expression was markedly decreased in livers lacking PPAR-γ. Forced expression of FSP27 in hepatocytes *in vitro* or *in vivo* led to an increase of LD through increased TAG levels, as reviewed elsewhere [289].

In addition to the classical lipolysis, recent evidence suggests a role for autophagy and lysosomal lipid degradation in liver LD (also termed lipophagy) [109, 290], via the action of lysosomal lipase (LAL) [109]. Even though this lipophagy accounts for a high percentage of the lipolysis that takes place in response to lipid challenges and during prolonged starvation in liver [109], also a certain percentage of degradation of lipids in lysosomes may occur continuously in many cell types (e.g. adipose tissue and skeletal muscle) [109]. Further research is warranted to determine the reasons behind the coexistence and possible co-regulation of the two different lipolytic pathways, the one mediated by the cytosolic lipases (i.e. ATGL and HSL) and the other mediated by the autophagic-lysosomal degradation system (i.e. LAL). It is possible that activation of one lipolytic pathway or the other may be related to total capacity (i.e. lipophagy may be able to degrade larger amounts of triglycerides in shorter time). However, it is also possible that the quality and type of the resulting lipolytic products differs between cytosolic and lysosomal lipases, but data on this are largely lacking. Lastly, in light of the growing evidence in support of the heterogeneity of the cellular LD, it is also plausible that the two lipolytic systems target different subpopulations of LD's [291].

In the presence of insulin resistance and hyperinsulinemia, hepatic autophagy has been reported to be reduced [292] and it has been shown that defective autophagy is causal to impaired hepatic insulin sensitivity and glucose homeostasis in obese mice [293]. Since most evidence of autophagy comes from rodent studies, more research is warranted to elucidate the exact mechanism of action of hepatic autophagy and possible lysosomal dysfunction in human insulin resistant subjects.
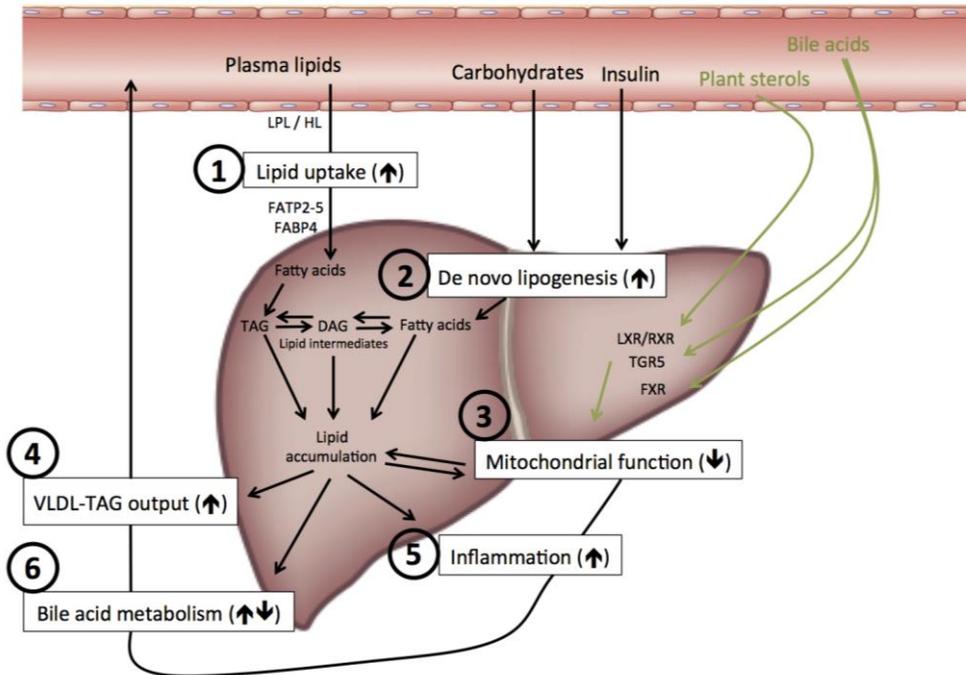
Figure 3. Disturbances in liver fatty acid metabolism

When circulating levels of FFA and lipoprotein remnants are increased, uptake and storage **(1)** of TAG and lipid metabolites in hepatocytes occur via increased LPL, HL activity and number and activity of transporter proteins (such as FATP2-5, FABP4) leading to hepatic insulin resistance. Increased insulin-stimulated de novo lipogenesis **(2)** contributes to hepatic lipid accumulation. In addition, under insulin resistant conditions hepatic mitochondrial function is decreased **(3)** and may inhibit FA oxidation, contributing to hepatic lipid accumulation, increased VLDL production **(4)** and an inflammatory state **(5)**. During conditions of hepatic lipid accumulation, an overproduction of VLDL occurs and together with an increased de novo lipogenesis results in hypertriglyceridemia, a condition often seen in insulin resistance and T2D. Finally, circulating bile acid composition **(6)** is altered in insulin resistant and T2D patients and might affect liver lipid metabolism via membrane (e.g. TGR5) and nuclear (e.g. RXR) receptor signaling. Interestingly, plant sterols have been shown to be effective nuclear receptor activators. Abbreviations: FFA: Free fatty acids; TAG: Triacylglycerol; DAG: Diacylglycerol; LPL: Lipoprotein lipase; HL: Hepatic lipase; FATP2-5: Fatty acid transport proteins 2-5; FABP4: Fatty acid binding protein 4; VLDL: Very-low-density lipoprotein; TGR5: G-protein coupled receptor TGR5; FXR: Farnesoid X receptor; LXR: Liver X receptor; RXR: Retinoid X receptor.
Solid lines indicate stimulation. Green lines indicate beneficial effects. Symbols: (↑↓): Altered; (↑): Increased; (↓): Decreased

De novo lipogenesis

On the other hand, increased insulin concentrations stimulate de novo lipogenesis (DNL), a process that is characterized by converting carbohydrates towards FAs and generating "new lipids" [294, 295]. The overproduction of VLDL together with an increased DNL, leads to hypertriglyceridemia, a condition often seen in insulin resistance and T2D [245]. During DNL, the conversion of glucose to fatty acids includes a coordinated series of enzymatic reactions of which fatty acid synthase (FAS) is the key rate-limiting enzyme that regulates the conversion of malonyl-CoA into palmitate, which is, thereafter, converted into complex fatty acids [296]. Knockout of FAS has been shown in mice to provoke a fatty liver phenotype upon high carbohydrate feeding, perhaps due to an increase in hepatic malonyl-CoA [297]. Another key enzyme is stearoyl-CoA desaturase 1 (SCD1), which plays a role in the desaturation of fatty acids and is predominantly expressed in liver. Depletion of SCD1 in mice showed a decreased lipogenesis and an increased β-oxidation [298]. During DNL, the final step of TAG synthesis is catalyzed by diacylglyceroltransferase (DGAT), which consists of DGAT1 and DGAT2. DGAT1 deficient mice are resistant to diet-induced obesity [299], have decreased levels of tissue TAG and are more insulin and leptin sensitive [300]. Furthermore, glucose and insulin regulate the expression of lipogenic enzymes via carbohydrate responsive element binding protein (ChREBP) [301] and sterol regulatory element binding protein-1 (SREBP1c) [302, 303], respectively. Both SREBP1c and ChREBP are involved in the transcriptional regulation of lipogenic genes and have been associated with increased DNL in NAFLD [304]. DNL is a highly regulated process and can lead to adverse metabolic consequences when dysregulated. Therapeutic targeting of this pathway may open a new window of opportunity for combating various lipogenesis-driven pathological conditions like obesity and insulin resistance.

Hepatic mitochondrial function and fatty acid oxidation

The majority of FAs are oxidized within mitochondria and lower amounts of oxidation takes place in peroxisomes [305], which are dynamic, multifunctional organelles that contribute to several anabolic and catabolic processes and are essential for human health and development (for review, see: [306, 307]). While medium- and short-chain FAs are thought to enter the mitochondrial matrix directly [308], long-chain FAs require carnitine palmitoyltransferase I (CPT-1) to enter the mitochondrial matrix. Insulin regulates CPT-1 by inhibiting transcription of the CPT-1 gene [309] and this could lead to increased concentrations of TAG and lipid metabolites inside hepatocytes. In contrast, the peroxisomal β-oxidation system is CPT-1 independent, but complete FA oxidation does not take place [310] and the chain-shortened acyl-CoAs are consequently exported to the mitochondria where they undergo complete β-oxidation [311-313].
Research showed that during insulin resistant conditions, hepatic mitochondrial function is altered [314-316]. During insulin resistant conditions, an increased circulating glucose concentration may downregulate PPAR-α [317], which inhibits FA-oxidation in men [318] and contributes to hepatic lipid accumulation and increased VLDL production [319]. Furthermore, as reviewed by Koliaki *et al.* [320],

an up-regulation of hepatic mitochondrial β-oxidation may occur as a result of lipid oversupply. This would stimulate hepatic ATP and reactive oxygen species production, possibly leading to local lipotoxicity, inflammation and oxidative stress and resulting in impairments in mitochondrial function and morphology. Since non-diabetic obese humans show normal or even greater hepatic mitochondrial function compared to lean humans, the authors suggest that hepatic energy metabolism transitionally adapts to chronic lipid overload by up-regulation of oxidative capacity in states of obesity and steatosis, which could be followed by a progressive decline in liver mitochondrial function during prolonged chronic insulin resistance, associated with T2D and NASH [320].

Bile acid metabolism

Once synthesized in the liver, the free bile acids (BAs) are conjugated with the amino acids taurine or glycine, secreted and stored in the gallbladder. After being released into the intestinal tract to facilitate the absorption of dietary fats and fat-soluble vitamins, about 95% of BAs are reabsorbed in the lateral ileum and transported back to the liver via the entero-hepatic circulation with only 5% of BAs excreted into the feces. The reabsorption of bile decreases the need for de novo bile acid synthesis [321, 322] and the loss of BAs in the feces represents the principal means of eliminating cholesterol from the body [321, 323, 324]. The synthesis of BAs by oxidation of cholesterol occurs exclusively in the liver and involves two different pathways. Firstly, the classical pathway converts cholesterol to 7α-hydroxyl-cholesterol to ultimately form 2 major primary BA products: chenodeoxycholic acid (CDCA) and cholic acid (CA) [325]. Secondly, the alternative pathway (the "acidic pathway") converts cholesterol to 27-hydroxy-cholesterol and produces mainly CDCA [326]. The intestinal microbiota metabolize primary BAs by dehydroxylation and deconjugation to form the secondary BAs lithocholic acid (LCA) and deoxycholic acid (DCA) [321, 323, 324]. The gut microbiota may not only regulate the conversion of BA, also the BA may regulate the microbiota composition [327]. Complementary to the aid of absorbing lipids and fat-soluble vitamins in the intestine, accumulating data also show that BAs have a signaling function in regulating biological processes by binding to the nuclear receptor farnesoid X receptor (FXR) and to the G-protein coupled receptor TGR5 (also known as GPR19) in gut and other tissues affecting glucose, lipid and energy metabolism [321, 323, 324], as discussed more extensively below.

FXR-receptor

The FXR-receptor is highly expressed in the liver, intestine, kidney and adrenal gland [321, 323, 324, 328] and is also shown to play a role in liver regeneration, inflammation and tumorgenesis [321, 324]. Both conjugated and unconjugated BAs can activate FXR and the order of potency is CDCA > LCA = DCA > CA. CA feeding increased hepatic expression of ApoC-2, a LPL activator, specifically through FXR as this effect was not observed in FXR$^{-/-}$ mice [329]. In addition, FXR activation increased the expression of VLDL receptor [330] and syndecan-1 [331], which are responsible for increased clearance of TAG-rich lipoprotein and remnant particles, respectively. Furthermore, BA sequestrants, such as cholestyramine,

were found to increase plasma TAG, mediated at least in part, through FXR [332], which increased plasma lipoprotein clearance. However, other mechanisms may also be involved in FXR-mediated lipid lowering effects. For example, PPAR-α was induced by CDCA and GW4064 (a synthetic FXR agonist) treatment in HepG2 cells and primary hepatocytes [333]. GW4064 treatment also increased the mRNA expression of PDK4, a PPAR-α target gene involved in the regulation of substrate metabolism, in both rat hepatoma cells, human primary hepatocytes and also resulted in a reduced plasma TAG concentration *in vivo* in mice [334]. BA or GW4064 induced activation of FXR also increased the expression and secretion of FGF21 [335], a cytokine modulating systematic carbohydrate and lipid metabolism and reducing hepatic TAG levels [336-338]. FGF21 has been reported to inhibit lipogenesis through suppressing the transcriptional activity of SREBP-1c [339] and therefore, the FGF21 pathway may play an important role in FXR-mediated decrease in hepatic TAG levels, which may be of importance in decreasing hepatic insulin resistance [340, 341]. Besides the nuclear receptor FXR, the liver X receptor (LXR-α and LXR-β) and retinoid x receptor (RXR) play an important role in regulating carbohydrate and lipid metabolism in humans [342, 343]. Interestingly, sitosterol, campesterol and certain oxidized derivatives of phytosterols (oxyphytosterols) are effective LXR activators [344]. A rodent study showed that LXR-α and LXR-α/β knockout mice remained glucose tolerant and insulin sensitive, while LXR-β knockout mice became highly insulin resistant after a high-fat diet [345]. Treatment of lean and ob/ob mice with the pharmacological LXR activator (GW3695) resulted in lower blood glucose levels and significantly improved whole body insulin sensitivity in the ob/ob mice, but no changes were found in the lean mice [346]. Furthermore, the synthetic LXR-α/β activator (T0901317) augmented diet-induced hyperlipidemia, normalized glucose tolerance and improved insulin-stimulated glucose uptake in isolated soleus muscle and completely restored glucose transporter 4 (GLUT4) expression and insulin-stimulated AS160 phosphorylation in rat muscle [347]. Although the vast majority of studies have been performed in non-adipose cells/tissues, results in recent years suggest that LXRs may have important modulatory roles on adipose lipid and glucose metabolism [348].

In addition, PPAR-γ and RXR agonists have complementary effects on glucose and lipid metabolism in human skeletal muscle [349], as well as in diabetic and obese rodent models [350]. It has also been shown that a RXR ligand (LG100754) improved insulin resistance *in vivo* in db/db mice [351] and a recent study showed that a novel RXR partial agonist (CBt-PMN (11b)) has a glucose-lowering effect and improved insulin secretion and glucose tolerance in the liver of KK-A^y mice [352].

TGR5-receptor

TGR5 is a family member of the G-protein coupled receptors (also known as GPR19) and is highly expressed in gallbladder, ileum and colon and in lower concentrations in BAT, liver, muscle and the central nervous system [336]. The activation of TGR5 is highest with the bile acid LCA, followed by DCA, CDCA and CA [323, 336] and results in the activation of PKA [336]. Furthermore, TGR5 activation regulates the expression of genes involved in inflammation [353],

increases energy expenditure in skeletal muscle and BAT [354] through stimulation of mitochondrial function [355-357] and modulates plasma glucose and lipid concentrations [336, 355, 356, 358]. BA mediated activation of TGR5 has beneficial metabolic effects and the BA composition is altered in patients with insulin resistance [359] and T2D [360]. Since gram-positive bacteria have a more pronounced effect on the transformation of primary to secondary BAs [361, 362], compared to most of the gram-negative bacteria [363], an alteration in the gut microbiota might have a distinct effect on bile acid metabolism and might be an effective strategy to improve insulin sensitivity. Indeed, in the study of Vrieze *et al.*, a vancomycin-induced decrease in gram-positive bacteria was associated with a reduced conversion of primary to secondary BAs and a tendency towards a reduced peripheral insulin sensitivity [364]. Also, transgenic liver overexpression of cholesterol 7α-hydroxylase, protected mice against high-fat diet induced obesity, fatty liver and insulin resistance [365]. Furthermore, glucose metabolism can also be improved in diabetic patients by administration of BA sequestrants, such as colesevelam [366, 367]. Thus, a promising nutritional strategy may be to alter the gut microbiota by modifying the diet by either prebiotics or probiotics and modulating thereby BA metabolism [368, 369].

Hepatic inflammation

Since insulin resistance and obesity are characterized by a low-grade inflammatory status [370], inflammation induced by the increased lipid accumulation, could also be an underlying cause for the development of hepatic insulin resistance. Of interest, ER stress has been shown to be involved in the development of hepatic inflammation and insulin resistance. First, ER stress can directly modify key hepatic enzymes involved in gluconeogenesis and lipogenesis, and stimulate stress kinases that interfere with insulin signaling. Secondly, ER stress may indirectly induce inflammation and lipotoxicity by promoting fat accumulation in hepatocytes [179, 371, 372].
Furthermore, hepatic peroxisomal FA oxidation causes an increased concentration of reactive oxygen species [373], which in excess cause a decrease in the natural antioxidant concentrations, leading to oxidative stress in hepatocytes [374, 375]. In turn, this oxidative stress causes hepatocyte degeneration and death [374] evoking an inflammatory response [376]. Specialized liver macrophages, called Kupffer cells, play a central role in this inflammatory process since *in vitro* stimulation of these cells by endotoxin (e.g. lipopolysaccharides (LPS)) or specific FAs (e.g. SFA) and their metabolites, lead to toll-like receptor signal transduction and the production of inflammatory cytokines, including TNF-α and IL-6 [377].
Furthermore, in analogy to the adipokines, the liver-derived proteins (known as hepatokines) [378] such as leukocyte cell-derived chemotaxin 2 (LECT2) regulate cross talk with other tissues and link obesity with skeletal muscle insulin resistance [379].
Taken together, the oversupply of lipids to the liver, together with impaired clearance of circulating remnants, increased de novo lipogenesis and decreased FA oxidation contribute to lipid accumulation in the liver, referred to as hepatic steatosis, which can progress to NAFLD, NASH and liver cancer [380].

Putative nutritional targets to reduce liver fat accumulation

All factors that improve adipose tissue function may reduce lipid overflow and spillover to the liver and will, as such, likely reduce hepatic steatosis.

Furthermore, it has been shown that BAs influence energy expenditure and glucose homeostasis via their effects on gluconeogenesis, insulin secretion and insulin sensitivity in both mouse and human studies [355, 358, 381]. BA-mediated activation of TGR5 has beneficial metabolic effects and the BA composition is altered in patients with insulin resistance. Secondary BAs can also affect host metabolism via binding to several nuclear receptors (FXR, LXR, RXR). Therefore, manipulating BA metabolism to increase concentrations of secondary BAs could be an attractive target to tackle obesity and insulin resistance [358, 381]. An alteration of the gut microbiota might have distinct effects on BA metabolism and might therefore be an effective strategy to improve insulin sensitivity. Beside that, modulating gut microbiota may affect fermentation products from dietary fibers like SCFA and monosaccharides affecting thereby liver gluconeogenesis and lipogenesis, which might directly or indirectly affect glucose homeostasis [382].

The nuclear receptor FXR and LXR are important in regulating liver, muscle and adipose tissue lipid and glucose metabolism in humans and it has been shown that sitosterol, campesterol and certain oxidized derivatives of phytosterols (oxyphytosterols) are effective LXR activators [344].

In summary, in particular nutritional factors targeting BA metabolism, gut microbiota like pre and probiotics and factors affecting the nuclear receptors FXR and LXR like plant sterols seem promising targets in improving liver fat metabolism thereby improving glucose homeostasis (Table 1).

IMPAIRMENTS IN SKELETAL MUSCLE METABOLISM

Accumulation of intramuscular TAG (IMTAG) has been associated with skeletal muscle insulin resistance in humans and is already present in young lean offspring of type 2 diabetic parents [12]. Both high fat diets and acute intravenous intralipid infusions result in increased IMTAG stores and a concomitant development of insulin resistance [383-386], highlighting an important role of lipid supply in fatty-acid induced insulin resistance. Nevertheless, also several studies report similar IMTAG concentrations in obese insulin sensitive and obese T2D subjects [387, 388] indicating that intramuscular lipids are not directly linked to insulin resistance. Further evidence against a direct role of IMTAG in insulin resistance originates from the observation that endurance trained athletes also have high IMTAG levels and are highly insulin sensitive [389-391]. This so called 'athletes paradox' may be explained by a higher muscle oxidative capacity [389], a higher antioxidant capacity in the athletic group [392] or a higher concentration of intramuscular lipid droplets around the mitochondria after endurance exercise [393]. On the contrary, in obese, insulin resistant and T2D patients, an increased IMTAG concentration has been linked with a reduced oxidative capacity [394-398], which could lead to FA storage, rather than oxidation and thereby provide a mechanism for lipid accumulation within skeletal muscle (Figure 4). By now, it is obvious that the relationship between IMTAG accumulation and insulin sensitivity is not as straightforward as originally thought, but rather the accumulation of bioactive lipid metabolites like diacylglycerol (DAG), long chain fatty acyl-CoA and ceramides are involved in FA-induced insulin resistance [399-403]. Furthermore, others and we recently highlighted that, besides the amount and type of lipid metabolite, also the cellular localization is of major importance for the development of insulin resistance [404-406]. Although results are not consistent [407-411], it was recently described that skeletal muscle inflammation and macrophage markers are increased and associated with insulin resistance in rodents as well as humans and that treatment of high-fat-diet fed mice with the PPAR-γ agonist rosiglitazone decreased muscle inflammation and improved local insulin signaling [412]. Also, several studies showed that muscle insulin resistance is associated with ER stress activation, as reviewed elsewhere [179, 413].
Taken together, it is obvious that the relationship between TAG or lipid metabolites is more complex as originally proposed and that understanding lipid turnover within skeletal muscle in addition to the role of increased lipid supply and uptake is crucial for elucidating the relationship between ectopic fat accumulation and insulin resistance. Therefore, the major components of skeletal muscle lipid turnover and intrinsic disturbances are discussed below.

Skeletal muscle lipid uptake

As mentioned before, there is mixed evidence for the notion that obesity is associated with increased fasting, postprandial, diurnal or nocturnal FFA concentrations. Instead, elevated circulating TAG concentrations in both the fasting and postprandial state may be far more striking associated with insulin resistance as compared to increased FFA concentrations. TAG concentrations have been reported to be increased in the obese insulin resistant state, which may be

ascribed to an increased liver VLDL production [92] or to impairments in adipose tissue TAG clearance from the circulation [68-73].

In human studies, postprandial systemic TAG concentrations and TAG extraction across forearm muscle were significantly elevated in subjects with impaired glucose metabolism (either subjects with impaired fasting glucose (IFG) or impaired glucose tolerance (IGT)) versus normal glucose tolerant controls [414]. Another study comparing insulin resistant versus control subjects with the same stable isotope methodology could not confirm an increased postprandial muscle TAG extraction, despite elevated TAG concentrations [92]. Although the mixed results remain to be elucidated, this study also confirms the apparent importance of TAG metabolism in insulin resistance, showing that the insulin resistant state was closely related to increased TAG rather than increased FFA concentrations, most likely due to a partitioning towards TAG synthesis in the liver.

The expression and activation of muscle LPL plays a major role in skeletal muscle TAG extraction. It was shown that in mice LPL deletion reduces lipid storage and increases insulin signaling in skeletal muscle [415] and that muscle specific overexpression of LPL causes muscle-specific insulin resistance by causing defects in muscle signaling and action [416], whilst skeletal muscle LPL knockout mice show opposite effects. Whether these effects are translational to humans is currently unknown. Most studies indicate that fasting raises total LPL activity in human skeletal muscle [417, 418], but results are not consistent [44]. Furthermore, a single bout of physical exercise also leads to a marked increase in LPL activity, protein, and mRNA in the exercising muscle [417, 419-422]. In contrast to adipose tissue and liver, insulin infusion decreased skeletal muscle LPL activity in humans [423]. Additionally, muscle LPL activity is inhibited at posttranslational level by ANGPTL4 [424] but little is known about the physiological and molecular mechanisms involved in the regulation of muscle LPL expression. It has been shown that T2D patients have significantly lower plasma ANGPTL4 levels as compared to healthy subjects, thereby suggesting a role for ANGPTL4 in diabetes [63]. Furthermore, skeletal muscle ANGPTL4 expression is increased in human muscle following fasting via elevation of plasma FFA. However, the functional implications of fasting and lipid-induced ANGPTL4 expression in muscle as well as possible impairments in insulin resistant conditions remain to be elucidated. In rodents, ANGPTL4 overexpression and treatment markedly decreased blood glucose concentration, improved glucose tolerance and hyperinsulinemia, but induced hyperlipidemia, fatty liver and hepatomegaly in mice [425]. The FA that are liberated after LPL-mediated lipolysis and those from the plasma FFA pool can be taken up in skeletal muscle via passive diffusion, depending on the concentration gradient over the muscle membrane and via membrane associated carrier proteins [426] like CD36, FABPpm and a family of fatty acid transport proteins (FATP 1-6) [427-430]. Of these FA transporters CD36 has been best characterized [46]. CD36 deficiency has been associated with a functionally important impairment in FA transport in muscle and adipose tissue [427, 431]. In humans, it has been shown that muscle CD36 protein expression may be acutely upregulated by insulin [80, 432] and that this upregulation may be more pronounced in insulin resistant conditions [80, 433]. Furthermore, it has been shown that CD36 translocation to the plasma membrane may be increased in muscle strips of obese subjects with T2D and that FA uptake, as measured with the giant vesicle model, was increased 4-

fold as compared to overweight and lean controls [434]. Nevertheless, several human *in vivo* studies showed that the FFA concentrations were comparable between groups and no difference was observed in skeletal muscle FA uptake during fasting or insulin-stimulated conditions in obese versus lean subjects [434], in patients with impaired glucose metabolism versus normal glucose tolerant subjects [414, 435] or in insulin resistant men versus controls [436]. However, results are contradicting, since 2 other studies showed a small increase in muscle FFA uptake during fasting in obese versus lean [437] or insulin resistant versus control subjects [92], despite comparable FFA concentrations. In summary, there may be more human evidence for an elevation of plasma TAG concentrations as compared to FFA in the obese insulin resistant or pre-diabetic state. Although mixed evidence is available for increased muscle TAG extraction, not much evidence is available from *in vivo* human studies for the notion that skeletal muscle FFA uptake may be largely increased in the insulin resistant state.

Skeletal muscle fatty acid storage

The FAs that enter the myocyte, bind to cytoplasmic FABPc for transport through the cell [438] and can either be directed towards storage in lipid droplets or towards the mitochondria for oxidation. The synthesis of TAG in the myocyte involves the activity of several glycerol-3-phosphates (GPAT1-4), lipin 1 and DGAT1-2 [439] whilst the activity of SCD1 or Δ-9-desaturase is particularly important for the further metabolism of SFA. Increased IMTAG synthesis via upregulation of lipogenic enzymes (DGAT1, GPAT an SCD1) has been linked to protection against FA-induced insulin resistance in rodents and humans [416]. Recently, Timmers *et al.* [440] showed that unilateral overexpression of DGAT-1 (involved in the conversion of TAG to DAG) in rat skeletal muscle, could rescue insulin sensitivity despite increased DAG and TAG concentrations, possibly by increasing DAG and TAG turnover [440]. Nevertheless, data on DGAT1 expression in human skeletal muscle are mixed with several studies showing no differences in DGAT1 expression between obese insulin resistant subjects and normal weight sedentary volunteers, endurance trained athletes [441] or in obese volunteers after weight loss [442]. One of the putative mechanisms regulating turnover of in particular SFAs may be related to the activity of SCD1 or Δ-9-desaturase, converting SFAs to MUFAs. Overexpression of SCD1 has been reported to protect L6 myotubes from FA-induced insulin resistance and increased TAG reesterification [443]. Nevertheless, evidence is mixed with another study showing increased SCD1 expression in skeletal muscle of extremely obese humans with severe muscle insulin resistance [444]. Less information is available on the synthesis rate of muscle TAG *in vivo* in humans. Bergman and coworkers [445] showed that IMTAG concentration and its fractional synthetic rate were not related to insulin action in smokers compared to non-smokers [445]. Additionally, the same group showed that obese prediabetic men had higher muscle TAG concentrations, a lower TAG fractional synthetic rate and a lower oxidative capacity in parallel to a reduced insulin action as compared to obese normal glucose tolerant men [446]. These disturbances in IMTAG metabolism were not found in women. In line, we recently showed that a more pronounced degree of insulin resistance in subjects with IGT (either isolated or in

combination with IFG) as compared to isolated IFG was accompanied by a reduced fractional synthesis of TAG from dietary palmitate, an increased saturation of the intramuscular FFA pool, a reduced saturation of the DAG and TAG pool and a reduced expression of genes involved in oxidative metabolism, confirming that a reduced muscle lipid synthesis and turnover may be an important characteristic of the insulin resistant muscle [447]. Finally, a reduced incorporation of FFA into TAG in primary myotubes from obese individuals with T2D has been recently observed. The data indicate that the ability to incorporate FAs into TAG is an intrinsic feature of human muscle cells that is reduced in individuals with T2D [448]. In summary, there is evidence that partitioning of FFA towards TAG synthesis may be beneficial for insulin sensitivity and that insulin resistance may be associated with a reduced fractional TAG synthesis in skeletal muscle.
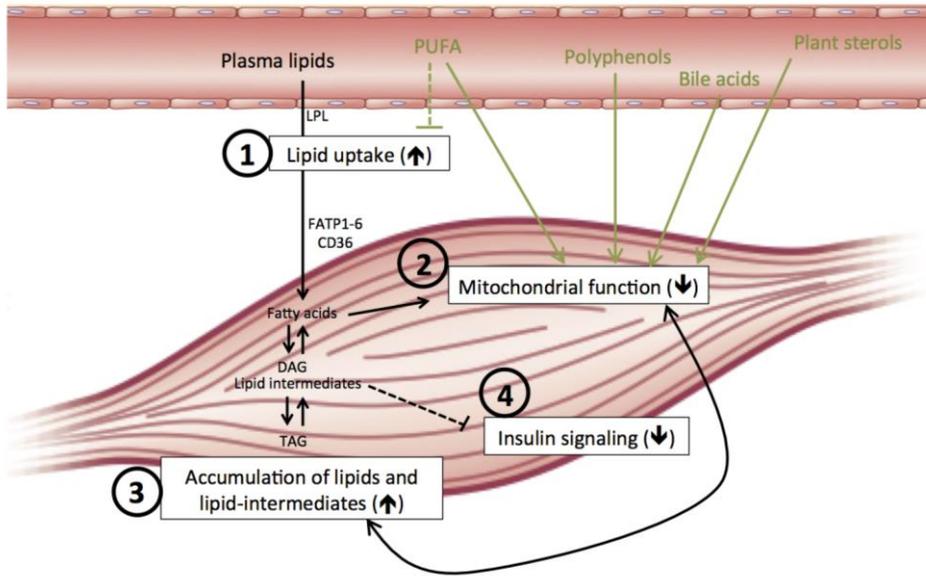
Figure 4. Disturbances in skeletal muscle fatty acid metabolism

Skeletal muscle fatty acid uptake, which is regulated via LPL and fatty acid transport proteins (FATP1-6, CD36), might be higher in subjects with impaired glucose metabolism compared to control subjects **(1)**. In addition, a reduced mitochondrial mass and/or mitochondrial function **(2)** have been proposed as underlying mechanisms for reduced muscle fat oxidation, contributing to the accumulation of TAG and lipid-intermediates (LCFA-CoA, DAG, ceramides, acylcarnitines) in myocytes **(3)**, interfering with insulin signaling **(4)** in the obese insulin resistant state. Finally, the capacity to increase intramyocellular fat oxidation during conditions of high FA supply has shown to be impaired in obese, T2D subjects. PUFA's, polyphenols, plant sterols and bile acids may have beneficial effects on skeletal muscle lipid metabolism.
Abbreviations: LPL: Lipoprotein lipase; FATP1-6: Fatty acid transport protein 1-6; CD36: Fatty acid translocase CD36; TAG: Triacylglycerol; DAG: Diacylglycerol; LCFA-CoA: long chain fatty acyl co-enzyme A; PUFA: Poly unsaturated fatty acids. Dashed lines indicate inhibition. Solid lines indicate stimulation. Green lines indicate beneficial effects. Symbols: (↑): Increased; (↓): Decreased

Skeletal muscle lipolysis

Besides FA uptake and incorporation into DAG and TAG, also intrinsic disturbances in skeletal muscle lipolysis may contribute to the accumulation of lipids and lipid metabolites in skeletal muscle of obese insulin resistant subjects. As in adipose tissue, the intramuscular lipid stores are hydrolyzed by phosphorylation and translocation of ATGL and HSL, which are under hormonal control of catecholamines, NPs and insulin. HSL deficiency in mice has been shown to increase DAG storage and signs of impaired skeletal muscle insulin sensitivity [274], while ATGL deficient mice have increased TAG accumulation in skeletal muscle and show improved glucose tolerance and insulin sensitivity [449]. In line, *in vitro* data of Badin *et al.* [450] showed that overexpression of ATGL in human myotubes promotes DAG and ceramide accumulation and disrupts insulin signaling and action. These data suggest that a dysbalance in lipase expression and activity contributes to an increased accumulation of lipotoxic metabolites, which might interfere with insulin signaling. As far as we know, there is no human *in vivo* evidence for a significant improvement in insulin sensitivity following inhibition of muscle ATGL activity. However, it has been shown that ATGL deficiency in humans with neutral lipid storage disease with myopathy (NLSDM), when compared to healthy controls, show impaired insulin response to glucose, preserved whole-body insulin sensitivity and a shift toward glucose metabolism in the heart [451].

Furthermore, we showed that skeletal muscle of obese subjects exhibited a blunted β-adrenergic mediated lipolysis as compared to lean controls *in vivo* [452]. Additionally, under fasting conditions total glycerol release was reduced across forearm muscle of obese compared to lean men, which was accompanied by a reduced HSL protein content and an increased ATGL protein content without changes in CGI-58, a co-activator of ATGL [453]. This difference in lipase content was accompanied by a 60% lower ratio of DAG to TAG hydrolase activity implying incomplete muscle lipolysis. Nevertheless, the incomplete lipolysis was not accompanied by DAG accumulation, but total DAG content was rather decreased in obese subjects, again, as already indicated above not supporting an important role of total DAG content in lipid-induced insulin resistance. Furthermore, we recently showed that insulin-mediated suppression of skeletal muscle lipolysis is blunted in T2D compared to normal glucose tolerant subjects and that this is associated with increased accumulation of membrane saturated DAG and protein kinase C (PKC) activation [404]. These data are supported by several other observations that both cellular localization and composition of DAG influence the relationship to insulin sensitivity [405, 406]. It remains to be elucidated whether intracellular lipases directly contribute to membrane DAG accumulation in human skeletal muscle. However, there are indications that *in vitro* ATGL mainly generates specific DAG isoforms (1,3-DAGs and 2,3-DAGs) but not 1,2-DAGs, of which the latter is found in the plasma membrane and able to activate PKC, suggesting that muscle lipases are not directly involved in membrane DAG accumulation [90]. Nevertheless, targeting skeletal muscle lipases may constitute an interesting strategy to improve insulin sensitivity in obesity and T2D.

Finally, accumulating evidence suggests that LD covering proteins play an important role in skeletal muscle lipid turnover and the development of insulin

resistance. The best-characterized group of LD coat proteins is the PLIN family. As mentioned above, PLIN-1 is adipose tissue specific, although PLIN1 mRNA expression is observed in human skeletal muscle [454], while PLIN-2 and PLIN-5 mRNA and protein are abundantly expressed in skeletal muscle [455, 456]. PLIN-3 and PLIN-4 are also present in skeletal muscle, but little is known to date about their function in muscle lipid metabolism. It was recently reported that PLIN-5 protein is highly expressed in skeletal muscle of endurance athletes, corresponding to their higher LD volume and their higher insulin sensitivity [456]. Nevertheless, another study in humans showed no correlation between PLIN-5 protein content in skeletal muscle and insulin sensitivity in obese T2D subjects and BMI-matched control subjects [457]. PLIN-5 and PLIN-2 are co-localized with the LD [458] and recent research using *in vitro* and *in vivo* methods showed that PLIN-2 is an important facilitator of IMCL storage and that by improving IMCL storage, PLIN-2 protects against lipotoxicity improving thereby insulin sensitivity [455]. The role of LD dynamics in myocellular insulin resistance is beyond the scope of this review and has been reviewed in more detail elsewhere [399]. Taken together, although the field is still in its infancy, mounting evidence suggests a role for LD covering proteins in the protection against muscle lipotoxicity and insulin resistance. One could hypothesize that storage of lipids in LD is not as harmful as opposed to accumulating lipids elsewhere in the cell (e.g. the plasma membrane).

Skeletal muscle fat oxidation

The dynamics of fat oxidation and fine tuning with FA uptake and intramyocellular TAG turnover may be very important to prevent accumulation of bioactive lipid metabolites. To meet the complexity of changes in fuel oxidation, the concept of metabolic flexibility has been introduced, defined as the capacity to increase fat oxidation upon increased FA availability and to switch between fat and glucose as the primary fuel source after a meal [459]. The insulin resistant muscle may be characterized by a metabolic inflexibility to regulate substrate oxidation. Indeed, the capacity to increase intramyocellular fat oxidation during conditions of increased FA supply such as fasting has shown to be impaired in obese, T2D subjects [396, 460-462] and also during exercise and β-adrenergic stimulation in obese and T2D subjects [463, 464]. Additionally, the postprandial suppression of muscle fat oxidation has shown to be impaired in T2D and obesity [396, 435]. Together these data suggest that an impaired metabolic flexibility may contribute to the accumulation of lipid metabolites and may be driven by lipid supply from either extracellular or intracellular sources (lipid turnover) as well as intrinsic impairments in mitochondrial function.

Interestingly, a study in human myotubes indicated that elevated extracellular FAs increase their own oxidation, which may in turn inhibit the oxidation of intramyocellular lipids [465]. Also, inhibition of intracellular adipose tissue lipolysis with acipimox decreased adipose tissue derived FA availability and increased the oxidation of IMTAG derived FA during rest and during exercise [466]. Nevertheless, since circulating FFA concentrations may only be slightly elevated or not increased at all in insulin resistant conditions (see above), an increased lipid overflow from FFA is probably not a major contributor to an impaired utilization of IMTAG in the

obese insulin resistance state. Since there is increasing support for the notion that lipid overflow in insulin resistance under physiological conditions is rather characterized by increased circulating TAG concentrations than by increased FFA concentrations, it is a plausible option that increased TAG-derived FA oxidation may impair IMTAG utilization in the obese insulin resistant state. However, this option remains to be investigated.

Furthermore, differences in IMTAG oxidation may be caused by differences in IMTAG lipolysis. Interestingly, lipolysis from IMTAG might be necessary for the maintenance of oxidative gene expression and FA oxidation. Recent findings indicate that in particular ATGL-mediated lipolysis generates lipid ligands for PPAR activation and the subsequent transcription of oxidative genes [90]. It was shown that a cycle of FA esterification and rehydrolysis is required for activation of PPAR-α and normal mitochondrial and oxidative phosphorylation, at leasat in cardiomyocytes [467]. *In vivo* tracer data show that in patients with NLSDM (caused by mutations in the ATGL gene) fat oxidative capacity is blunted and can be partly reversed by PPAR-α treatment [468, 469]. These data highlight the importance of ATGL activity in regulating muscle fat oxidation. Furthermore, it was shown recently that activation of NP signaling in human skeletal muscle enhances mitochondrial oxidative metabolism and fat oxidation [470]. However, further studies are required linking lipolysis and NP signaling to muscle oxidative metabolism and insulin resistance in human.

Finally, a reduced FA transport across the mitochondrial membrane and a reduced mitochondrial mass and/or mitochondrial function have been proposed as underlying causes of a reduced muscle fat oxidation in insulin resistance. In rodent models of obesity and insulin resistance an increased content of malonyl-CoA, an allosteric inhibitor of mitochondrial FA transport, has been shown in skeletal muscle in combination with hyperglycaemia and hyperinsulinaemia and a reduced lipid oxidation [471]. Also, in human skeletal muscle it has been shown that a combination of hyperglycaemia and hyperinsulinaemia increases malonyl-CoA, inhibits functional carnitine palmitoyl transferase 1 (CPT1b, muscle isoform) activity and shunts long chain FA away from oxidation and towards storage in human muscle [472]. Furthermore, two studies have shown that an improvement in fat oxidation by exercise or by lifestyle intervention was accompanied by decreased acetyl-CoA carboxylase (ACC) mRNA expression, involved in the formation of malonyl-CoA, suggesting that a reduced inhibition of mitochondrial FA transport through malonyl-CoA may have contributed to the improved fat oxidative capacity [473, 474]. Furthermore, a lowered mitochondrial transport through CPT1 may contribute to the reduced fat oxidation. In line, heterozygous CPT1b deficiency results in mitochondrial abnormalities and lipid accumulation with elevated TAG and ceramide content at least in mice cardiomyocytes [475]. Moreover, it has been shown that an increased CPT1b activity is sufficient to improve high-fat diet induced insulin resistance [476]. Peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α) is involved in mitochondrial biogenesis by supporting the transcriptional activity of nuclear respiration factor 1 (NRF1), thereby regulating the transcription of genes involved in oxidative metabolism. The expression of genes responsible for oxidative phosphorylation is coordinately downregulated in muscle of T2D individuals [477, 478] and muscle specific overexpression of PGC-1α in rodents has been shown to improve lipid utilization, insulin signaling and

glucose transport [479]. *In vivo* mitochondrial capacity and function have been reported to be reduced in T2D, independent of mitochondrial content [388, 480, 481], although data are not consistent [482, 483]. Nevertheless, a reduced mitochondrial capacity and a reduced activity of TCA cycle [484] and citrate synthase (-14%) [485] have also been reported in primary myotubes from T2D subjects, suggesting that skeletal muscle mitochondrial function is intrinsically impaired in insulin resistance. Moreover, a marked reduction in mitochondrial oxidative and phosphorylation activities, together with intramyocellular lipid accumulation, were found in skeletal muscle in insulin resistant offspring of T2D subjects [486, 487]. Furthermore, the definition of mitochondrial dysfunction may also include a dysbalance balance between β-oxidation and TCA cycle activity resulting in an accumulation of incompletely oxidized, fatty acid products (i.e. acylcarnitines), which can impair skeletal muscle insulin sensitivity, as extensively reviewed elsewhere [488].

In summary, skeletal muscle fat oxidation is reduced in the obese insulin resistant state. This impaired fat oxidation may contribute to the accumulation of lipid metabolites and may be driven by lipid supply from either extracellular or intracellular sources as well as intrinsic impairments in mitochondrial function. However, whether reduced mitochondrial function is causally related to insulin resistance or rather a consequence of the sedentary lifestyle needs to be investigated in more detail in future research.

Putative nutrional targets to improve skeletal muscle lipid metabolism

An improvement in adipose tissue and liver function may reduce lipid overflow and low-grade systemic inflammation, thereby reducing the supply of lipids and inflammatory mediators to skeletal muscle, which likely improves skeletal muscle function.

As indicated above, targeting skeletal muscle lipases, LD covering proteins and skeletal muscle fat oxidation/mitochondrial function to improve metabolic flexibility may constitute an interesting strategy to improve insulin sensitivity in obesity and T2D by limiting the accumulation of bioactive lipid metabolites interfering with insulin sensitivity.

As discussed earlier in this review, specific polyphenols or a combination of polyphenols may affect human lipid metabolism. The polyphenolic compound resveratrol, which is abundantly present in grape skin, red wine and peanuts, has been shown to improve *in vitro* FA oxidation and insulin sensitivity in murine adipocytes [489], as well as in C2C12 myotubes [490] and improved skeletal muscle mitochondrial function in rodents by activating Sirtuin-1 (SIRT-1) and PGC-1α [491]. In humans, on the other hand, resveratrol supplementation has recently been shown to improve metabolic profile and flexibility, skeletal muscle mitochondrial function, energy expenditure, glucose control and insulin sensitivity especially in patients with T2D [492, 493]. We have recently also shown that short-term supplementation of the combination of EGCG and resveratrol may increase resting energy expenditure and improve metabolic flexibility [212]. Nevertheless, results of clinical trials are contradictory, as recently discussed [494].

Additionally, dietary fat quality may differentially affect (skeletal muscle) lipid

metabolism and insulin sensitivity [218, 495]. Indeed, we recently showed that a PUFA rich meal acutely improved insulin sensitivity in obese insulin resistant subjects as compared to a high-SFA meal, which was accompanied by a reduced TAG-derived skeletal muscle FA uptake, an altered intramyocellular lipid partitioning and a more oxidative transcriptional phenotype [496]. Additionally, studies in human myotubes showed that palmitate induced a lower lipolysis rate [497] and follows a different metabolic pathway as compared to oleic acid [497,498]. Also, a lower palmitate oxidation has been shown in myotubes of T2D subjects as compared to control, whilst oleate oxidation was not significantly different [499]. These studies indicate that dietary fat quality may modulate the nature of lipid-induced insulin resistance through affecting pathways of fatty acid partitioning. Further studies are required to investigate the impact of gender and dietary fat quality on FA dynamics and to investigate the causative role of an impaired TAG synthesis capacity in the etiology of insulin resistance. In summary, polyphenols and modulation of dietary fat quality may affect storage of bioactive lipid metabolites in skeletal muscle, thereby improving insulin sensitivity and glucose homeostasis. Beside that, manipulation the nuclear FXR and LXR receptors by modulating bile acid metabolism or by ingestion of plant sterols may also have positive effects on muscle lipid metabolism and insulin sensitivity, but apparently more human evidence is required. For a summary see Table 1.

Tabel 1: Summary of pathways, possible targets and (dietary) components in adipose tissue, liver and gut and skeletal muscle

Tissue	Pathway	Target	Dietary intervention or component	Evidence for improved glucose metabolism	Strength of evidence for improved glucose metabolism
Adipose tissue	Fatty acid trapping	Stimulation of LPL	Dietary modulation of ANGPTL4 [63]	[425]	Rodent - in vivo/ex vivo
	Lipolysis	Selective HSL and/or ATGL inhibition	Pre- and probiotics affecting SCFA metabolism [210], [211]	[100, 101]	Rodent - in vitro/in vivo Human - in vitro/in vivo
	Lipolysis	Modulation of alternative pathway of lipid breakdown (lipophagy)	Polyphenols [212, 213]	Currently unknown	Currently unknown
	Mitochondrial function and browning	Increase mitochondrial function	Dietary fat quality, PUFA quantity [223, 224]	[146]	Rodent - in vivo/in vitro
			Polyphenols [212] [492]	[492]	Human – in vivo
		Increase BAT activity	Capsinoids [163]	[157]	Human – in vivo
			Increase FGF21 via BA metabolism / gut microbiota [165] [335]	[619]	Rodent - in vivo
	Inflammation	Decrease low-grade inflammation	Dietary fat quality, PUFA quantity [229-233]	[173]	Rodent/human - in vivo
Liver & Gut	Bile acid manipulation	FXR and TGR5 receptors, Increase FGF21	Modulation bile acid metabolism by pre- and probiotics, FXR and TGR5 agonists [333-335, 337-341] [356, 357, 359, 360]	[356]	Rodent - in vivo
	Bile acid manipulation	Activate LXR	Plant sterols [344]	[346, 347]	Rodent - in vivo Rodent - in vivo/ex vivo
	Gut microbiota manipulation	Alter gut microbiota (to alter secondary BA and SCFA)	Pre-, probiotics [366] [369]	[366, 367]	Human - in vivo
Skeletal muscle	Lipolysis	Selective HSL, ATGL inhibition	To be identified	[450, 451]	Human - ex vivo/in vitro Human - in vivo
	TAG extraction	Reduce TAG-derived- FA uptake	PUFA [496]	[496]	Human - in vivo
	Mitochondrial function	Increase SIRT-1, PGC-1α, fat oxidation	Polyphenols, PUFA [212] [491, 492] [496]	[479] [490]	Rodent - in vitro Rodent - in vitro/in vivo
	Gut microbiota manipulation	Activate LXR, TGR5	Plant sterols, Pre-, probiotics [344] [369]	[346, 347] [356]	Rodent - in vivo Rodent - in vivo/ex vivo Rodent - in vivo

PANCREATIC BETA-CELL FUNCTION

T2D is a multifactorial disease that develops when insulin resistance is accompanied by pancreatic β-cell failure [2, 3], which is defined as a deterioration of β-cell function as well as loss of functional β-cell mass. In obese conditions, lipid accumulation of TAG in pancreatic islets occurs [500], which is aggravated by the simultaneous presence of hyperglycemia [501-503]. Elevated lipid accumulation can damage the β-cell functionality (Figure 5) and evidence for this mainly comes from studies with isolated islets exposed to high concentrations of FFA for periods of 24-48 h [504, 505], while evidence from *in vivo* studies mainly comes from studies with ZDF rats, since suitable pancreas specimens from individuals with increasing degrees of β-cell dysfunction are scarce [235]. This decreased β-cell functionality may lead to a decreased insulin secretion even when demands for secretion are increased. However, in order to maintain normal glucose homeostasis, a compensatory increased insulin secretion occurs. Prediabetic patients however, in the long run, fail to compensate adequately for the greater insulin requirements [2, 506-508] and will ultimately develop T2D. In this type 2 diabetic state, a decreased β-cell mass [509] and an increased level of β-cell apoptosis [510-514] occurs. However, the rate of β-cell replication and neogenesis was not different between a diabetic and a control group [510].

Putative targets to improve insulin secretion

The increased rate of β-cell apoptosis in T2D can originate from several mechanisms [515], including oxidative stress [13, 509, 516, 517], inflammation [518, 519], ER-stress [520-523], mitochondrial overload [524, 525] and lipotoxicity [526]. As extensively reviewed elsewhere, human and animal data suggest that there might be direct effects of bioactive compounds (including polyphenols, vitamins and carotenoids) on enhancing insulin secretion and preventing β-cell apoptosis, and some compounds might modulate β-cell proliferation [527]. Targeting these processes within the pancreatic islets, as already discussed in the above paragraphs, may result in decreased β-cell apoptosis and maintain the insulin secretory capacity, and as such could be a potential therapy to reduce the incidence of T2D.
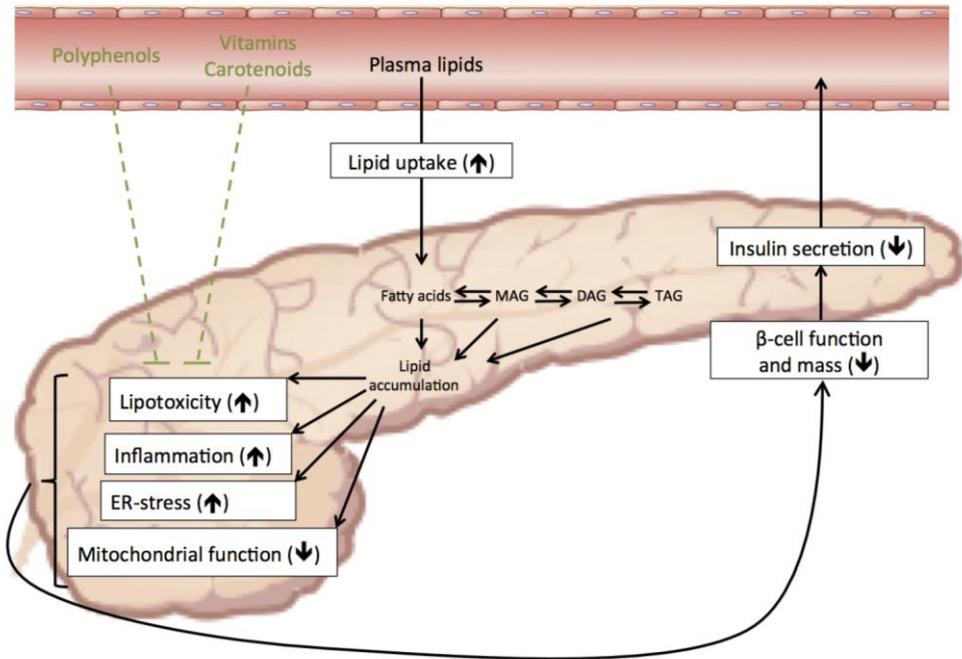
Figure 5. Disturbances in pancreatic fatty acid metabolism

Lipid accumulation in pancreatic islets can result in inflammation, ER-stress, mitochondrial overload and lipotoxicity. These mechanisms damage the β-cell function and lower β-cell mass, resulting in a decreased insulin secretion and ultimately leading to the development of T2D. Polyphenols, vitamins and carotenoids may counteract these negative effects.
Abbreviations: TAG: Triacylglycerol; DAG: Diacylglycerol; MAG: monoacylglycerol; FFA: Free fatty acids; ER-stress: Endoplasmic reticulum stress. Dashed lines indicate inhibition. Solid lines indicate stimulation. Green lines indicate beneficial effects. Symbols: (↑): Increased; (↓): Decreased

THE INTESTINE AND GUT MICROBIOTA IN INSULIN RESISTANCE

The intestinal tract has traditionally been seen as a passive organ, but currently it is seen as an active organ that may affect substrate metabolism via the secretion of several factors such as free fatty acids, incretins and inflammatory factors [528] and may contribute to the development of obesity and insulin resistance [427, 529, 530]. The intestine plays an important role in lipid homeostasis and contributes significantly to the systemic plasma cholesterol and lipid concentrations [531]. Fat absorption by the small intestine is a very efficient process, which requires several transport mechanisms, as reviewed elsewhere [532]. Fatty acids taken up in the enterocyte can either be oxidized by the enterocyte, [533-535], stored as intracellular LD's [536] or excreted as chylomicrons, which is related to hypertriglyceridemia [537, 538] contributing to T2D [539], metabolic syndrome [540] and coronary artery disease [541, 542]. Targeting these processes can significantly affect postprandial lipid concentrations and insulin sensitivity. Improving intestinal fatty acid oxidation, for example by PPAR-α activation [543] might contribute to reduced lipid accumulation in other organs, such as liver and skeletal muscle. Furthermore, also PUFAs [544, 545] and DAG [546] appear to be effective inducers of enterocyte fatty acid oxidation and may be beneficial in the anti-diabetic and anti-obesity effects in various tissues. Besides fatty acid oxidation, also the absorption and excretion of fatty acids might be altered. As reviewed elsewhere, there are several components that might influence intestinal lipid metabolism [547]. For example, JTT-130, a novel intestine-specific inhibitor of microsomal triglyceride transfer protein (MTP), which is involved in the mobilization and secretion of TAG rich lipoproteins from enterocytes and hepatocytes [548], has been shown to suppress the absorption of dietary fat and cholesterol in the intestine, decreased plasma TAG and total cholesterol levels [549] and improved glucose and lipid metabolism in rodents [550-552].

Furthermore, recent evidence showed that a high-fat diet induced intestinal inflammation [553, 554], which is related to obesity and insulin resistance [555-557]. Interestingly, it has recently been shown in beta7 integrin-deficient mice (Beta7(null)) that suppression of the gut immune system decreases HFD-induced insulin resistance [558]. Furthermore, intestinal ER stress has been found in obesity [559] and might contribute to intestinal and systemic inflammation [560], leading to insulin resistance [561]. Taken together, this makes the intestine an attractive therapeutic target.

Importantly, not only the intestine itself, but also its microbiota play an important role in the intestinal lipid metabolism, obesity and insulin resistance. The human adult intestines contain more than 10^{14} bacteria from over 1000 species and the genetic material of the intestinal microbes is collectively called the microbiome [562]. The vast majority of all gut microbes include the phyla Gram-negative *Bacteroidetes, Proteobacteria* and *Verrucomicrobia* as well as the Gram-positive *Firmicutes* and *Actinobacteria* [562-566]. Growing evidence indicates that also our gut and its microbiota play a crucial role in substrate metabolism and the development of obesity, cardiometabolic diseases and T2D, as reviewed extensively elsewhere [567, 568] (Figure 6).

Obese and T2D subjects seem to be characterized by an altered composition of gut microbiota compared to lean and normal glucose tolerant individuals,

respectively [569-572], although conflicting results have also been reported [570, 573-575]. Interestingly, germ-free mice infected with the gut microbiota of conventionally raised mice demonstrated increased body weight, insulin resistance and glucose intolerance together with reduced food intake and increased oxygen consumption [576]. The body fat gain was related to suppressed gut expression of the LPL inhibitor ANGPTL4, leading to increased fat accumulation in adipocytes and increased energy harvest [570, 577-579].

In obese men, seven days treatment with the antibiotic vancomycin modulated their gut microbiota and decreased peripheral insulin sensitivity [364]. Finally, the transfer of intestinal microbiota from human lean donors to individuals with the metabolic syndrome increased insulin sensitivity, along with increase of intestinal butyrate-producing microbiota [580].

Several mechanisms link our gut microbiota to obesity and diabetes (diabesity), as extensively reviewed elsewhere [581]. Firstly, alterations in gut microbiota composition can increase the amount of LPS through increased production, reduced breakdown or increased translocation across the gut wall, thereby inducing a strong immune response to protect the organism from bacterial infection [582]. This LPS-induced inflammatory state is referred to as 'metabolic endotoxemia' and is accompanied by body weight gain and insulin resistance in animal models [583]. In addition, major microbial products from the fermentation of indigestible carbohydrates, like the SCFAs acetate, butyrate and propionate may affect energy and fat metabolism through various mechanisms [584-587]. The type of substrate is the primary determinant of the SCFA production rate and SCFA ratio, compared to the composition of the intestinal microbiota [584] and the level of substrate fermentability varies with fiber solubility. Soluble fibers are fermented more completely than insoluble fibers [588] and highly fermentable substrates produce higher amounts of SCFA [589], which have physiological effects in different tissues [590]. At first, it has been proposed that they may play a role in harvesting extra energy from the diet, which would be a promoting factor for body weight gain. However, evidence that this largely contributes to human energy balance is not yet convincing [591]. On the other hand, increased SCFA concentrations have rather been associated with distinct generally positive, metabolic effects, affecting hormonal release of glucagon-like peptide 1 (GLP1) and peptide YY (PYY), cell proliferation and differentiation [584, 592-595], regulate adipocyte lipolysis [210, 211], modulate inflammation, affecting adipose tissue fat storage and ectopic fat storage, as has been extensively reviewed elsewhere [596]. Effects of SCFAs may be related to the activation of their related G-protein coupled receptors GPR41 (also known as free fatty acid receptor 3 (FFAR3)) and GPR43 (FFAR2) [596], which are present in the gut epithelium but also in peripheral tissues like adipose tissue and skeletal muscle [595, 597]. Thus, manipulation of SCFA production by prebiotics and probiotics (or a combination of both) may, among other processes, differentially affect human fat and glucose metabolism.

Furthermore, besides converting non-digestible fibers, the microbiota are also closely linked to BA metabolism. On the one hand, microbiota can transform primary to the so-called secondary BAs [598], while on the other hand BAs by themselves exert an anti-microbial effect on the microbiota [599, 600]. As discussed above, the secondary BAs can affect host metabolism via binding to

several nuclear receptors and GPRs and altering the intestinal microbiota could be a potential therapeutic target to reduce obesity and insulin resistance.

Putative nutritional targets to modulate gut microbiota composition and function

Altering the diet, either via specific dietary FA or combinations of pre- and probiotics, can rapidly change the human gut microbiota [601-603] and can improve metabolic status [604, 605]. By fermenting dietary fibers that humans cannot (fully) digest themselves, the human microflora produces SCFA [565, 570, 588, 606], which may play an important role in glucose homeostasis via modulation of human substrate metabolism, as briefly discussed above. Besides endogenous SCFA production, exogenous administration of SCFA has been studied in the context of interest. Thus, an alteration of the SCFA production by microbiota, i.e. via intake of pre- and probiotics, may be a useful tool to prevent obesity and obesity-related insulin resistance. Additionally, prebiotics may also directly affect postprandial glycemic and insulinemic responses by slower glucose absorption [607].

Research has shown that FAs exert their effect on bacterial growth and survival, in part, via altering the microbial cell membrane fluidity and disrupting cell membranes of certain bacteria [608, 609]. As reviewed by Alcock *et al.* [610], unsaturated fatty acids had a higher anti-microbial effect compared to SFA and within the group of SFA, short chain saturated FA (4 to 12 carbons in length) had a higher bactericidal effect, compared to long chain saturated FA (more than 12 carbons). Furthermore, while MUFA had a lower anti-microbial effect then PUFA of the same chain length, the n-3 PUFA had a greater effect compared to n-6 PUFA, but results are not consistent [610]. Although human evidence is scarce, research showed that the type of dietary fat has an effect on the faecal microbiota. Fava *et al.* [611] showed that a diet high in SFA increased *Faecalibacterium prausnitzii* and that MUFA-rich diets reduced the total bacterial numbers, but not the specific bacterial groups. In contrast, Simões *et al.* [612] showed that high habitual intake of MUFA was associated with lower numbers of *bifidobacteria* and slightly higher numbers of *Bacteroides* spp., that habitual n-3 PUFA intake had a significant positive association with *Lactobacillus* group abundance and that a higher habitual n-6 PUFA intake was associated with a decreased number of *bifidobacteria*. Santacruz *et al.* [613] showed that the numbers of *Lactobacilli* remained the same, eventhough the total PUFA consumption was greatly reduced.

Furthermore, it was shown that a high-fat diet significantly reduced the *bifidobacteria* [614]. In addition, high-fat diets seem to increase metabolic endotoxemia, which is related to an inflammatory state [583, 615, 616].

The altered gut microbiota composition also affects BA metabolism in rodents [617, 618] as well as in humans [364]. As such, it might be a possible target to combat the development towards obesity and insulin resistance (Table 1), as discussed above. However, further human studies are necessary to understand the exact mechanisms of action of the human microbiome.
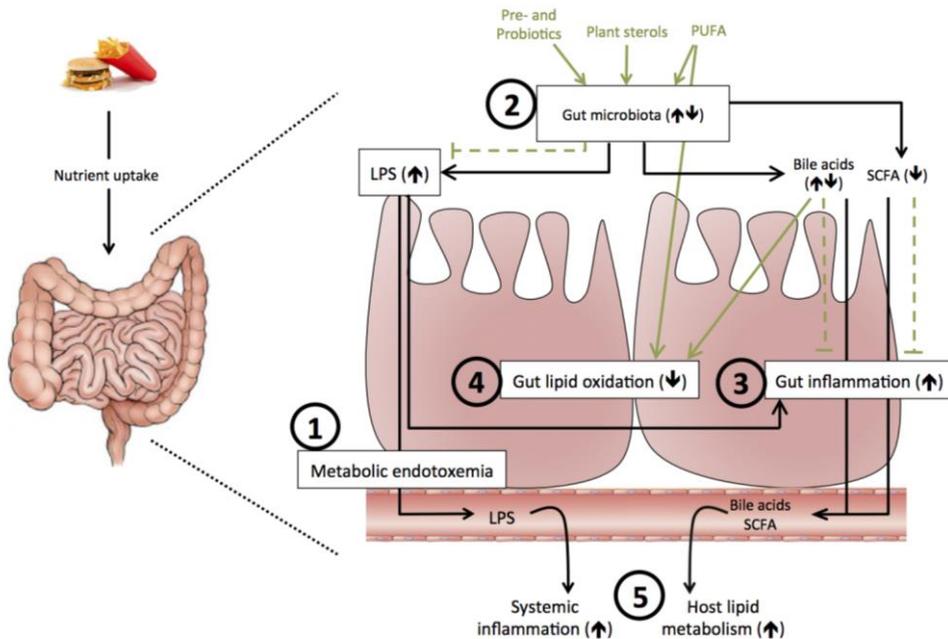
Figure 6. Disturbances in gut microbiota composition and lipid metabolism in the gut affect host metabolism

An altered gut microbiota composition, as seen in obesity and T2D, may increase local and systemic lipopolysaccharide (LPS) concentrations, referred to as metabolic endotoxemia **(1)** that can lead to systemic inflammation. Besides LPS, also bile acid composition and SCFA concentrations are altered. Pre- and probiotics, plant sterols and PUFAs exert a beneficial effect on gut microbiota composition **(2)**, leading to an altered SCFA production, bile acid composition and a decreased LPS concentration, thereby affecting gut inflammation **(3)** and gut lipid metabolism **(4)**. In addition, changes in systemic SCFA, bile acids and LPS affect host lipid metabolism and inflammation in adipose tissue, liver and skeletal muscle **(5)**.
Abbreviations: PUFA: Poly unsaturated fatty acids; SCFA: short chain fatty acid; LPS: Lipopolysaccharide. Dashed lines indicate inhibition. Solid lines indicate stimulation. Green lines indicate beneficial effects. Symbols: (↑↓): Altered; (↑): Increased; (↓): Decreased

SUMMARY

This review focuses on the disturbances in fatty acid metabolism at both the tissue and whole-body level that play a role in the etiology of insulin resistance, β-cell dysfunction and an impaired glucose metabolism. The most important pathways related to lipid metabolism in adipose tissue, liver, skeletal muscle, pancreas and gut have been discussed, and targets to improve lipid metabolism and glycaemia, which may be modulated by diet, have been described. A detailed overview of pathways and potential targets to improve lipid metabolism and glycaemia in adipose tissue, liver, skeletal muscle, pancreas and gut, which can be modulated by dietary interventions and food components, is provided in Table 1, and will be explained in more detail below.

Within adipose tissue, the balance between fatty acid extraction, lipolysis, adipocyte differentiation and mitochondrial function is important to maintain adequate lipid storage capacity. Improving the lipid storage capacity of adipose tissue prevents lipid overflow in the circulation and subsequent ectopic fat deposition, and therefore has high potential to improve glucose tolerance and insulin sensitivity. Increasing adipose tissue fatty acid uptake, possibly via stimulation of LPL, might reduce lipid overflow. On the other hand, a reduced lipid overflow might also be achieved by partial inhibition of lipolysis (partial lipase inhibition) and/or by modulation of the alternative pathway of lipid breakdown (lipophagy). There is some evidence that partial lipolysis inhibition may be achieved by modulation of SCFA metabolism through pro- and prebiotics and that the lipophagic pathway may be modulated by specific polyphenols, like resveratrol. Furthermore, adipose tissue mitochondrial function seems important in balancing lipid supply and utilization, and thereby affects adipose tissue function. Improved adipose tissue mitochondrial function may be achieved by nutritional strategies such as supplementation with specific polyphenols (or a combination of polyphenols) and altering dietary fatty acid composition. Dietary fat quality not only modulates lipid metabolism but may also affect low-grade inflammation, which in turn may lower the risk of developing insulin resistance and T2D.

An attractive target to tackle obesity and insulin resistance could be manipulation of the bile acid metabolism and/or gut microbiota composition. On the one hand, bile acids influence energy expenditure and glucose homeostasis via their effects on gluconeogenesis, insulin secretion and insulin sensitivity, and an altered concentrations of secondary bile acids can affect host metabolism via binding to several nuclear receptors (e.g. FXR, LXR, RXR), some of which can be activated by sitosterol, campesterol and certain oxidized derivatives of phytosterols (oxyphytosterols). On the other hand, an alteration of the gut microbiota by modulation of the dietary fat quality or pre- and probiotics might have a distinct effect on bile acid composition and metabolism and may also affect fermentation products from dietary fibers (e.g. SCFA and monosaccharides), affecting thereby liver gluconeogenesis and lipogenesis, pathways directly or indirectly affecting glucose homeostasis.

Targeting skeletal muscle lipid turnover and the balance between lipolysis, FA uptake and mitochondrial function/fat oxidation may constitute an interesting strategy to improve insulin sensitivity in obesity and T2D by limiting the accumulation of bioactive lipid-intermediates interfering with insulin sensitivity and

glucose homeostasis. The most promising dietary intervention to reduce FA uptake and thereby muscle fat accumulation might be an improvement of the adipose tissue lipid buffering capacity through the manipulation of dietary fat quality. On the other hand, an increased activation of SIRT-1 and PGC-1α may result in an enhanced skeletal muscle mitochondrial biogenesis/function, fat oxidation and metabolic flexibility. Putative dietary strategies for the latter pathways may be modulation of the dietary polyphenol content or the modulation of dietary fat quality. In addition, stimulation of the nuclear FXR and LXR receptors by modulation of bile acid metabolism or by ingestion of plant sterols may have positive effects on skeletal muscle lipid metabolism and insulin sensitivity.

Finally, preventing β-cell apoptosis, and modulation of β-cell proliferation by bioactive compounds (including polyphenols, vitamins and carotenoids) may maintain the insulin secretory capacity, and as such could be a potential target to reduce the incidence of T2D.

To conclude, the main most promising dietary interventions or components to target fatty acid metabolism in order to improve glucose metabolism would be components to alter the gut microbiota composition, like pre- and probiotics, thereby affecting SCFA- and bile acid metabolism. Furthermore, the use of (specific) polyphenol(s) to increase adipose tissue function possibly by affecting the lipolytic pathway or the alternative pathway for lipid breakdown (lipophagy) and/or adipose tissue and skeletal muscle mitochondrial function may be promising strategies to improve glucose metabolism. Lastly, manipulation of the dietary fat quantity and quality is of particular interest to improve adipose tissue function and skeletal muscle lipid turnover and mitochondrial function. Further human intervention studies are required targeting specific fatty acid related pathways to translate the above indications into relevance for the control of glucose homeostasis and insulin sensitivity.

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CHAPTER 3

Improved insulin sensitivity with angiotensin receptor neprilysin inhibition in individuals with obesity and hypertension

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ABSTRACT

Natriuretic peptide (NP) deficiency and sustained renin-angiotensin system activation are associated with impaired oxidative metabolism and predispose to type-2 diabetes. We hypothesized that sacubitril/valsartan (LCZ696), which augments NP through neprilysin inhibition while blocking angiotensin II type-1 (AT_1)-receptors, improves insulin sensitivity, lipid mobilization, and oxidation. After 8 weeks of treatment of obese patients with hypertension, sacubitril/valsartan 400 mg q.d., but not amlodipine 10 mg q.d., was associated with a significant increase from baseline in the insulin sensitivity index (hyperinsulinemic-euglycemic clamp), and tended to be higher in patients treated with sacubitril/valsartan compared to amlodipine. Abdominal adipose tissue interstitial glycerol concentrations increased with sacubitril/valsartan, but decreased with amlodipine. Whole-body lipolysis and substrate oxidation did not change with either treatment. Results confirm that sacubitril/valsartan treatment leads to a metabolic benefit in the study population and supports the relevance of neprilysin inhibition along with AT_1-receptor blockade in the regulation of human glucose and lipid metabolism.

INTRODUCTION

Obesity, type-2 diabetes mellitus (T2DM), and cardiovascular diseases are interrelated. Patients with obesity and T2DM are at increased risk for arterial hypertension and heart failure [1, 2], whereas patients with arterial hypertension or heart ,failure frequently exhibit impaired muscular oxidative capacity predisposing them to insulin resistance through accumulation of toxic lipid intermediates [3, 4].

The endopeptidase neprilysin is ubiquitously expressed, including in human adipocytes [5]. Its plasma activity positively correlates with measures of obesity and insulin resistance in humans, implicating neprilysin in the regulation of cardiometabolic crosstalk presumably through cleavage of neprilysin substrates with cardiometabolic effects, such as natriuretic peptides (NPs) and angiotensin II [6]. Indeed, patients with obesity [7] or heart failure exhibit absolute or relative natriuretic peptide deficiency, respectively. Circulating atrial natriuretic peptide levels are inversely related to plasma glucose and insulin concentrations, and low atrial natriuretic peptide levels predict the development of both arterial hypertension and diabetes mellitus in humans [8]. Furthermore, polymorphisms in the genes encoding atrial natriuretic peptide and B-type natriuretic peptide contribute to the variability in the risk for type 2 diabetes after adjustment for age, gender, and body mass index [9, 10]. NP promotes lipid mobilization from adipose tissue [11], increases postprandial lipid oxidation [12], elicits adiponectin release, and enhances muscular oxidative capacity [13, 14]. In contrast to atrial natriuretic peptide, angiotensin II promotes insulin resistance, and angiotensin II type-1 (AT_1)-receptor blockade modestly improved insulin sensitivity and pancreatic beta-cell function in humans [15].

Sacubitril/valsartan (LCZ696), a novel angiotensin receptor neprilysin inhibitor, was recently approved in the United States, Europe, and many other countries to reduce the risk of cardiovascular death and hospitalizations in patients with chronic heart failure (New York Heart Association classes II–IV) and reduced ejection fraction [16]. Upon oral administration, sacubitril/valsartan provides exposure to sacubitril (AHU377), a prodrug that is rapidly metabolized to the biologically active neprilysin inhibitor, sacubitrilat (LBQ657), and to the AT_1-receptor blocker valsartan. This active drug augments neprilysin substrates, such as natriuretic peptides, while inhibiting AT_1-receptor mediated responses [17].

We hypothesized that sacubitril/valsartan, through its combined mechanism of action, improves peripheral insulin sensitivity and increases lipid mobilization from subcutaneous abdominal adipose tissue in patients with obesity and arterial hypertension.

We conducted a mechanistic metabolic profiling study to investigate the effects of 8-week treatment with sacubitril/valsartan and amlodipine on peripheral insulin sensitivity, abdominal subcutaneous adipose tissue lipolysis, whole-body lipolysis, and energy expenditure and substrate oxidation in patients with obesity and elevated blood pressure. In this study, the patient population served as an exemplary human model for relative natriuretic peptide deficiency and impaired insulin sensitivity, and was specifically selected to support the comparison of sacubitril/valsartan with a metabolically neutral comparator, amlodipine.

RESULTS

Subjects

Of the 98 patients enrolled, (sacubitril/valsartan=50; amlodipine=48), 92 patients (93.9%) completed the study. All patients were included in the safety analysis set (Figure 1). Patients with missing baseline or post-treatment assessments were excluded from the pharmacodynamic analysis set, resulting in a maximum of 96 patients (sacubitril/valsartan=50; amlodipine=46) included in the pharmacodynamic analysis set.

Patient demographics and baseline characteristics are summarized in Table 1 and were comparable between the groups.
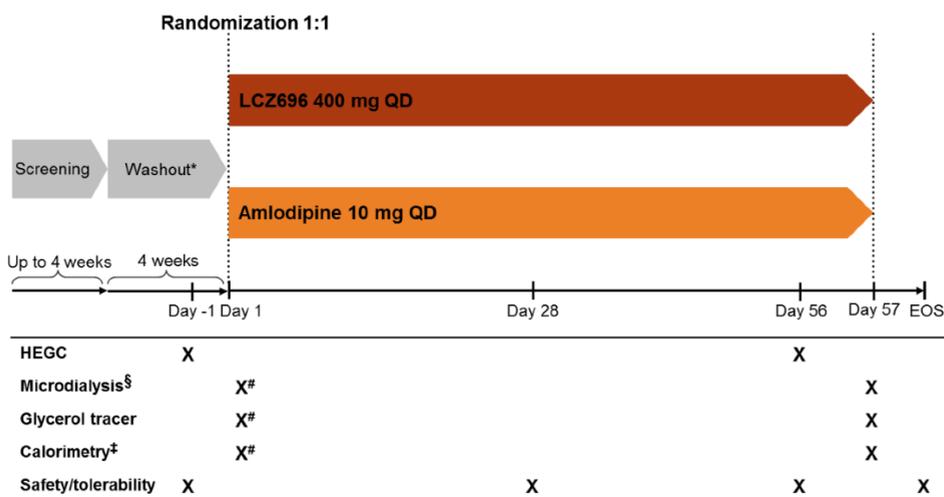

Figure 1. Study design

*Indicates those who had to discontinue antihypertensive medication. # Indicates those who had to discontinue before the first dose. § Indicates subcutaneous adipose tissue microdialysis.
‡ Indicates indirect calorimetry by Spirometry. EOS, end of study; HEGC, hyperinsulinemic-euglycemic glucose clamp.

Table 1. Patient demographics and baseline characteristics

Parameter	LCZ696 (400 mg) N = 50	Amlodipine (10 mg) N = 48
Age, y	51.9 (9.6)	50.5 (9.4)
Weight, kg	101.2 (17.3)	104.3 (14.1)
Waist circumference, cm	112.1 (10.5)	113.9 (10.0)
BMI, kg/m^2	32.6 (4.6)	33.3 (4.4)
Gender, male, no. (%)	41 (82)	35 (72.9)
Race, white, no. (%)	50 (100)	48 (100)
msSBP, mmHg	141.7 (11.1)	139.7 (12.6)
msDBP, mmHg	90.2 (6.1)	91.9 (6.0)
Mean sitting pulse rate, bpm	70.6 (9.7)	70.3 (9.5)
Insulin SI (µg/kg*min/(mmol/L*pmol/L))	1.6 (1.0)	1.6 (0.9)*
GIR (mg/min)	364.7 (192.5)[†]	401.8 (189.6)[‡]
Metabolic clearance rate (mg/(min*kg))	64.4 (33.3)[†]	71.1 (31.2)[‡]

Data are mean (SD) unless specified otherwise. BMI, body mass index; bpm, beats per minute; GIR, glucose infusion rate; msSBP, mean seated systolic blood pressure; msDBP, mean seated diastolic blood pressure; SI, sensitivity index.   * N = 45; † N = 48; ‡ N = 42.

Peripheral insulin sensitivity

Sacubitril/valsartan, but not amlodipine, was associated with a significant increase from baseline in mean insulin sensitivity index (SI), as evidenced by the 95% confidence interval (CI; mean change from baseline (95% CI); sacubitril/valsartan 0.192 (0.025–0.359) µg/kg*min/(mmol/L*pmol/L); and amlodipine 0.065 (–0.116 to 0.246) µg/kg*min/(mmol/L*pmol/L); Figure 2a). At week 8, the SI trended to be higher after treatment with sacubitril/valsartan vs. amlodipine (sacubitril/valsartan 1.87 µg/kg*min/(mmol/L*pmol/L); amlodipine 1.76 µg/kg*min/(mmol/L*pmol/L)); however, the treatment difference did not reach statistical significance as evidenced by the 97.5% CI (estimated treatment difference (97.5% CI), 0.128 (–0.155 to 0.410) µg/kg*min/(mmol/L*pmol/L)). Importantly, 8 weeks of treatment with sacubitril/valsartan, but not amlodipine, showed a significant increase from baseline and a significant treatment difference in favor of sacubitril/valsartan in glucose infusion rate (GIR; adjusted mean (95% CI) at week 8: LCZ696 445.3 (412.3–478.3) mg/min; amlodipine 390.5 (355.2–425.8) mg/min; estimated change from baseline: sacubitril/valsartan 58.4 (25.4–91.4) mg/min; and amlodipine 3.6 (–31.7 to 38.9) mg/min; adjusted treatment difference 54.8 (6.4–103.3) mg/min, p=0.027; Figure 2b), the GIR normalized by body weight (adjusted mean (95% CI) at week 8: sacubitril/valsartan 4.49 (4.15–4.83) mg/(min*kg); amlodipine 3.88 (3.51–4.24) mg/(min*kg); estimated change from baseline: sacubitril/valsartan 0.64 (0.31–0.98) mg/(min*kg); amlodipine 0.03 (–0.33 to 0.39) mg/(min*kg); adjusted treatment difference 0.61 (0.12–1.11) mg/(min*kg), p=0.016; Figure 2c), and metabolic clearance rate of glucose (adjusted mean (95% CI) at week 8: sacubitril/valsartan 79.1 (73.1–85.0) (L*mg)/(min*mmol); amlodipine 69.4 (63.0–75.8) (L*mg)/(min*mmol); estimated change from baseline: sacubitril/valsartan 10.7 (4.8–16.7) (L*mg)/(min*mmol); amlodipine 1.1 (–5.3 to 7.5) (L*mg)/(min*mmol); and adjusted treatment difference 9.7 (0.9–18.4) (L*mg)/(min*mmol), p=0.031; Figure 2d).

To assess any significant effect of body weight and waist circumference, the treatment difference for GIR was obtained from a similar analysis of covariance model with additional covariates: change in body weight at week 8 and waist circumference at screening. There was no significant covariate effect in either case (body weight, estimated treatment difference (95% CI): 57.57 (8.98–106.15), p=0.0208; waist circumference 50.86 (2.28–99.44), p=0.0404) compared to the actual treatment difference for GIR (54.85 (6.39–103.30), p=0.0270). Scatter plot of GIR vs. body weight and change in GIR vs. change in body weight did not display any apparent trend of change at day 56 from baseline (Supplementary Figure S1a and S1b, respectively).

Plasma insulin had reached steady-state values over the last 30 minutes of the clamp procedure (Figure 3).

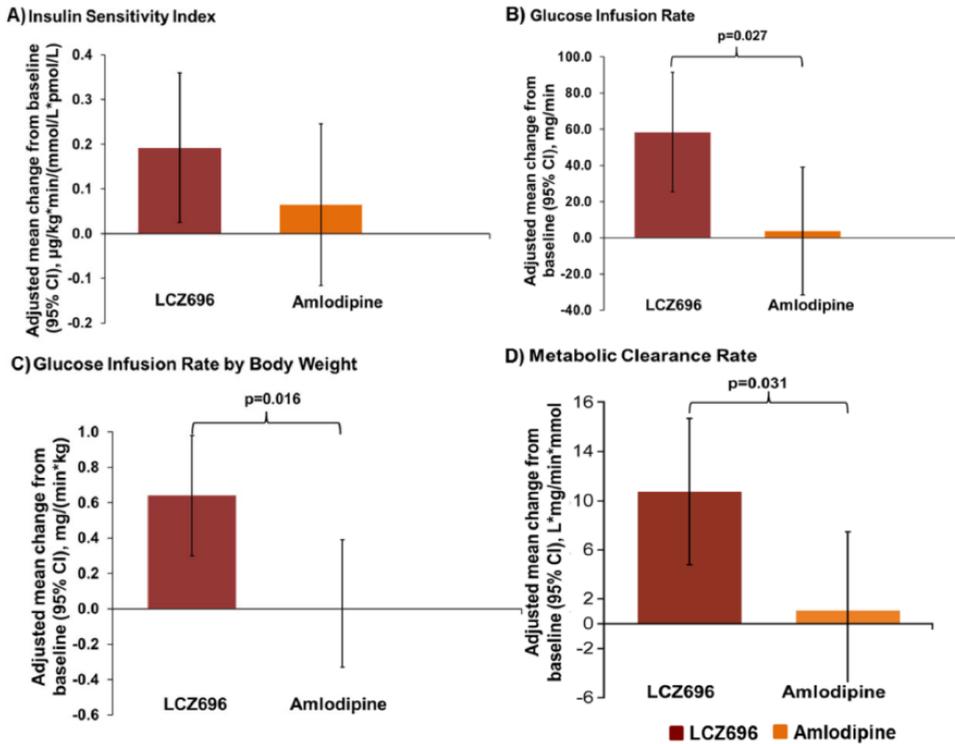**Figure 2.** Mean changes from baseline in insulin sensitivity variables after 8-week treatment with sacubitril/valsartan vs. amlodipine.

Insulin sensitivity index (**A**); glucose infusion rate (GIR) (**B**); GIR by body weight (**C**); and metabolic clearance rate (**D**). CI, confidence interval.
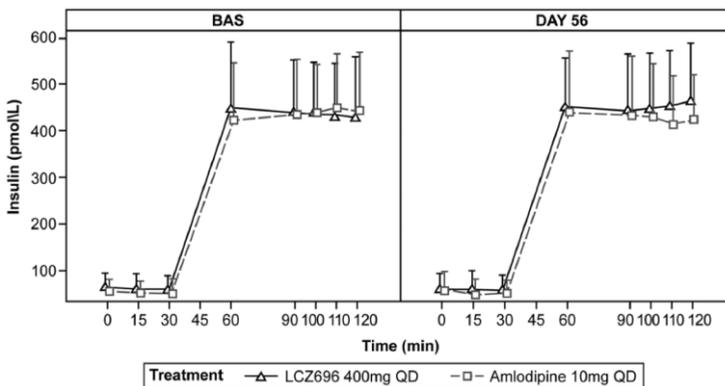

Figure 3. Insulin profiles during the hyperinsulinemic-euglycemic glucose clamp.

BAS, baseline.

Abdominal subcutaneous adipose tissue lipolysis

The change in ethanol ratio from baseline was significant in the sacubitril/valsartan group at the 45-minute (p=0.027) and 30 + 45-minute (p=0.042) time points. However, there was no significant difference between treatment groups at any of the time points (Figure 4a). An increase in interstitial glycerol from baseline to week 8 was observed at all time points in the sacubitril/valsartan group (adjusted geometric mean ratio to baseline (95% CI) at 30 + 45-minute time points 1.05 (0.93–1.18)), but the change did not reach statistical significance. Compared with the amlodipine group, the change from baseline in glycerol levels was significantly higher with sacubitril/valsartan (adjusted geometric mean ratio between treatments (95% CI) at 30 + 45-minute time points 1.22 (1.03–1.45), p=0.026; Figure 4b). Glucose and lactate levels did not differ significantly from baseline in any of the treatment groups and there was no difference between the treatment groups after 8 weeks of treatment (Figure 4c and 4d).
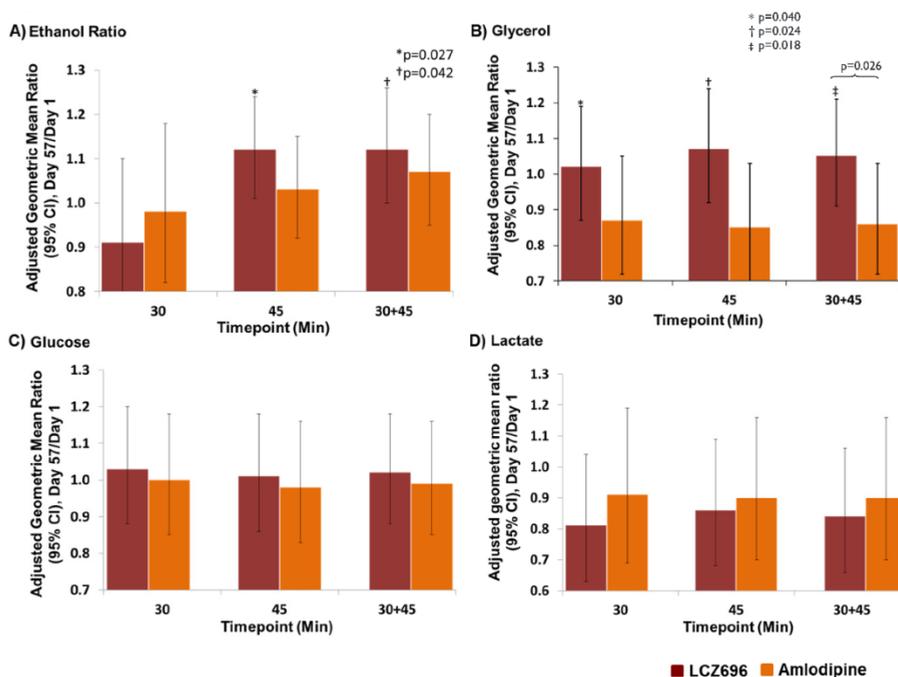
Figure 4. Adjusted geometric mean ratios of local adipose tissue lipolysis variables after 8-week treatment with sacubitril/valsartan and amlodipine.

Ethanol ratio (**A**); glycerol (**B**); glucose (**C**); and lactate (**D**). Data were analyzed for repeated measurements on log transformed values with treatment, visit, time and treatment*visit*time interaction as fixed effect assuming an unstructured covariance. Subjects with missing data were excluded from the analysis. The number of missing values ranged from 3 to 6 and 3 to 7 across all variables in the sacubitril/valsartan and amlodipine groups, respectively. CI, confidence interval.

Whole-body lipolysis

The rate of appearance (95% CI) of endogenous plasma glycerol at baseline on day 1 was 172.8 (158.0–189.0) and 200.6 (182.4–220.6) µmol/kg.min^{-1} in the sacubitril/valsartan and amlodipine groups, respectively. Whole-body glycerol rate of appearance was not significantly affected by amlodipine treatment, but was significantly lower at week 8 compared to baseline in the sacubitril/valsartan group at the 30 + 45-minute time point (geometric mean ratio (95% CI), 0.93 (0.87–1.00), p=0.045; Figure 5). There was no change from baseline in the sacubitril/valsartan group at the 45-minute time point. There were no differences between treatment groups in whole-body lipolysis after 8 weeks of treatment.
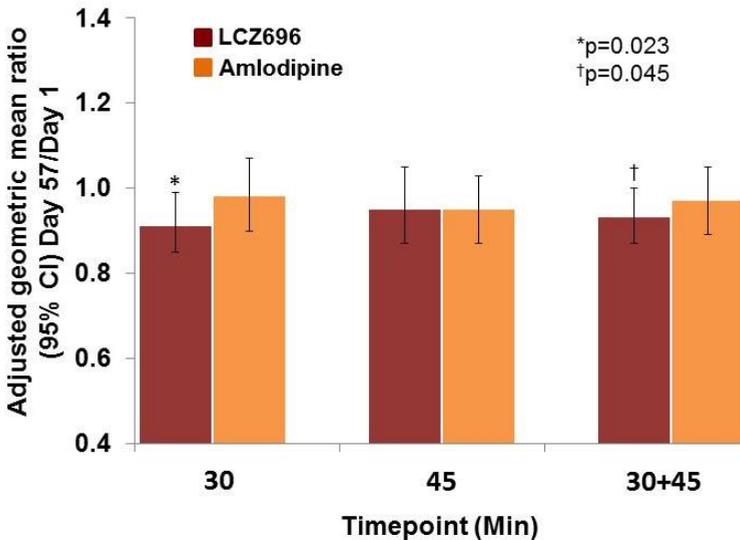
Figure 5. Whole-body lipolysis (rate of glycerol appearance).

The p value compared with baseline. CI, confidence interval.

Energy expenditure and substrate oxidation

At baseline, the mean respiratory quotient and resting energy expenditure were comparable between groups (mean (SD); respiratory quotient: LCZ696 0.773 (0.063), amlodipine 0.768 (0.051); resting energy expenditure: sacubitril/valsartan 2,134.81 (397.98) kcal; and amlodipine 2,180.30 (548.78) kcal). At week 8, respiratory quotient and resting energy expenditure were not significantly different from baseline in either of the treatment groups or between the treatment groups (mean treatment difference at week 8 (95% CI); respiratory quotient, 0.012 (–0.028 to 0.051), p=0.557; resting energy expenditure, 231.26 (–149.75 to 87.24) kcal, p=0.601).

Circulating metabolites, insulin and catecholamines in the fasted state

At week 8, there were no significant differences from baseline in either treatment group or between treatment groups in fasting plasma glucose, insulin, glycerol, or serum free fatty acids (Supplementary Table S1). However, sacubitril/valsartan, but not amlodipine, was associated with a significant decrease from baseline in plasma epinephrine (ratio to baseline (95% CI) for sacubitril/valsartan, 0.81 (0.72–0.91), p<0.001), resulting in a significant treatment difference (sacubitril/valsartan vs. amlodipine treatment ratio (95% CI), 0.77 (0.66–0.91), p=0.003). Conversely, amlodipine was associated with a significant increase from baseline in norepinephrine (ratio to baseline (95% CI) for amlodipine, 1.20 (1.07–1.33), p=0.001); however, the difference between treatments at week 8 was not statistically significant.

Blood pressure

Baseline data for mean seated blood pressure is presented in Table 1. At week 8, mean seated blood pressure had decreased more in the sacubitril/valsartan group compared with the amlodipine group (systolic blood pressure: -21.0 ± 16.1 mmHg vs. -12.4 ± 14.7 mmHg; diastolic blood pressure: -12.4 ± 9.1 mmHg vs. -10.0 ± 7.8 mmHg; Figure 6).

Safety Assessments

Of the 98 patients, 67 patients (68.4%) experienced at least one adverse effect (AE). Most AEs were mild or moderate in intensity. The overall AE incidence was lower in the sacubitril/valsartan compared with the amlodipine group (60.0% vs. 77.1%; Supplementary Table S2). Five patients discontinued due to AEs. Two patients discontinued due to a serious adverse event, which was unrelated to the study drug (ruptured cerebral aneurysm in one patient in the sacubitril/valsartan group and nephrolithiasis in one patient in the amlodipine group). Three patients discontinued due to AEs (pruritus in one patient in the sacubitril/valsartan group, and a single case of hypertension and peripheral edema in the amlodipine group). The most commonly occurring AEs were nasopharyngitis, peripheral edema, and headache, each of which had an overall incidence of >10% (Supplementary Table S2). AEs suspected to be related to the study drug by the investigators occurred more frequently in the amlodipine group than in patients receiving sacubitril/valsartan (46% vs. 24%). Peripheral edema was more common in the amlodipine group, whereas pruritus occurred only in the sacubitril/valsartan group (Supplementary Table S2). Two patients in the sacubitril/valsartan group reported mild orthostatic hypertension, both of which resolved by the end of the study. Both of these events were suspected to be related to the study drug. No deaths were reported in the study.
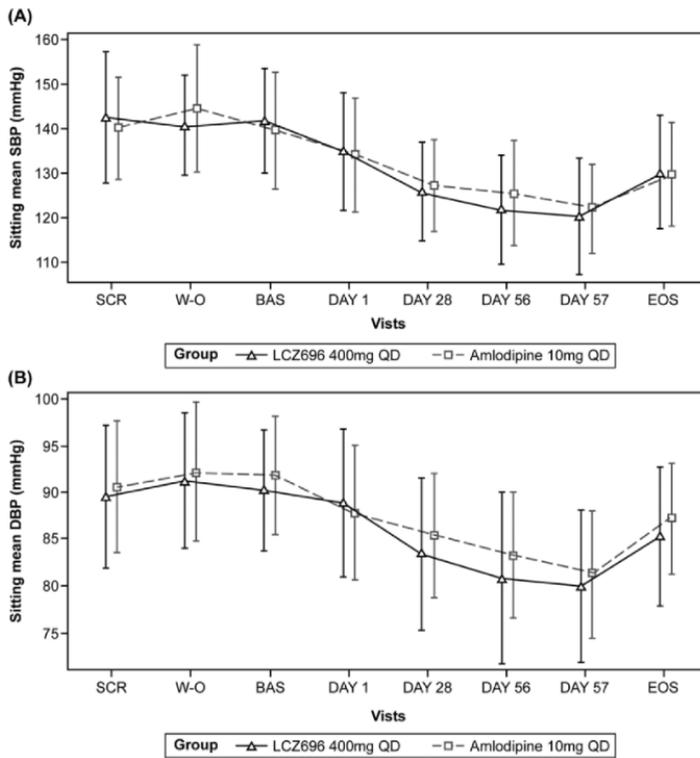
Figure 6. Arithmetic mean (SD) of mean seated systolic blood pressure (SBP) (**A**); and mean seated diastolic blood pressure (DBP) (**B**) after 8-week treatment with sacubitril/valsartan and amlodipine.

BAS, baseline; EOS, end of study; SCR, screening; W-O, washout.

DISCUSSION

Treatment with sacubitril/valsartan compared with amlodipine for 8 weeks improved peripheral insulin sensitivity in obese patients with elevated blood pressure. This improvement was not explained by changes in body weight or waist circumference. Furthermore, sacubitril/valsartan significantly increased abdominal subcutaneous adipose tissue lipolysis without changing whole-body lipolysis and plasma free fatty acid concentrations. Therefore, this study is the first to show that simultaneous neprilysin inhibition and AT_1-receptor blockade regulates glucose and lipid metabolism in humans, supporting the concept that neprilysin substrates and AT_1-receptor blockade have an important role in the crosstalk between the cardiovascular system and metabolism in humans. These findings imply that sacubitril/valsartan may improve cardiovascular and metabolic health in patients with cardiovascular disease.

The metabolic effects of sacubitril/valsartan observed here could be mediated through increased availability of neprilysin substrates, AT_1-receptor inhibition, or both mechanisms combined. Indeed, individuals with obesity exhibit increased renin angiotensin system (RAS) activity [18, 19] both systemically and at the tissue level. Although genetically modified animals overexpressing RAS components are characterized by insulin resistance that improved with pharmacological RAS inhibition [20], the improvement in insulin resistance after RAS inhibition in humans has been less consistent [16]. Among patients with impaired glucose tolerance and cardiovascular disease or risk factors, the use of valsartan for 5 years, along with lifestyle modification, led to a 14% relative reduction in T2DM incidence [21]. However, ramipril compared with placebo did not reduce the risk of new onset T2DM in patients with impaired fasting glucose levels [22]. Overall, the improvement of glucose metabolism after RAS inhibition seems to be modest and may not solely explain the improvement in insulin sensitivity observed with sacubitril/valsartan treatment. Moreover, AT_1-receptor blockade does not increase abdominal subcutaneous adipose tissue lipolysis, which was observed with sacubitril/valsartan treatment [23, 24].

The present study suggests that neprilysin inhibition contributed to the metabolic effect of sacubitril/valsartan treatment. Neprilysin degrades multiple peptides that have the potential to modulate lipid and glucose metabolism, such as natriuretic peptides, bradykinin, endothelin-1, and glucagon-like peptide 1. Therefore, we cannot distinguish the contribution of individual neprilysin substrates to the observed metabolic response. Eventually, the pharmacological effect of neprilysin inhibition will depend on the net effect on all biologically relevant neprilysin substrates. For example, bradykinin has been suggested to improve insulin sensitivity and attenuate lipolysis, whereas endothelin-1 promotes insulin resistance and increases lipolysis [25, 26]. Given the minor contribution of neprilysin relative to angiotensin converting enzyme and aminopeptidase [27] and the proven clinical safety of sacubitril/valsartan [16], it seems unlikely that an increase in bradykinin explains the observed metabolic response. Likewise, sacubitril/valsartan was shown to decrease, not increase, endothelin-1 plasma concentrations in patients with heart failure and reduced ejection fraction. Indeed, neprilysin contributes to both endothelin-1 formation from its precursors and endothelin-1 degradation [28]. Hence, an involvement of endothelin-1 in mediation

of metabolic effects of sacubitril/valsartan is also unlikely. Although the effect of sacubitril/valsartan on multiple other metabolically relevant neprilysin substrates has not been evaluated, its effect on the NP system, including increased NP and cyclic guanosine monophosphate levels, has been well established in nonclinical and clinical studies [17, 28, 29]. Given the strong evidence for an effect of NPs on lipolysis and glucose metabolism in conjunction with the demonstrated increase in NP and cyclic guanosine monophosphate availability with sacubitril/valsartan [17], we propose that increased NP availability may have contributed, at least in part, to metabolic improvements observed in our study.

NPs are more potent in stimulating human adipose tissue lipolysis than the prototypical β-adrenoreceptor agonist isoproterenol [9]. Due to high NP clearance receptor expression in adipose tissue of various animal species, NP-induced lipolysis is only observed in primates [30], thus limiting the utility of standard preclinical animal models. The present study, for the first time, examined the long-term effects of simultaneous neprilysin inhibition and AT_1-receptor blockade on abdominal subcutaneous and whole-body lipolysis in humans. The sustained increase in subcutaneous adipose tissue lipolysis is consistent with the observation that ex vivo adipose tissue lipolysis is not desensitized in patients with heart failure despite increased NP levels [30]. The observation that whole-body lipolysis does not increase with sacubitril/valsartan treatment suggests that increased adipose tissue lipolysis may be balanced by reduced lipolysis elsewhere in the body, however, further studies are required to elucidate the mechanism.

In the present study, patients treated with sacubitril/valsartan tended to exhibit lower norepinephrine and epinephrine plasma concentrations compared with patients treated with amlodipine. Given the central role of catecholamines in regulation of lipid mobilization, the expected response is reduced whole-body and adipose tissue lipolysis. Instead, sacubitril/valsartan-treated patients showed increased adipose tissue lipolysis with sustained whole-body lipolysis, presumably through increased availability of neprilysin substrates.

Unopposed increases in adipose tissue lipolysis could promote ectopic fat storage and insulin resistance through accumulation of toxic lipid intermediates, including diacylglycerol [3]. However, NPs acutely increase postprandial fatty acid oxidation in humans, and circulating levels of the anti-inflammatory and insulin sensitizing adipokine adiponectin [13]. Thus far, data on metabolic effects of sustained NP activation were limited to nonclinical experiments. Nonclinical and *in vitro* studies have demonstrated that NP activation and transgenic overexpression enhanced mitochondrial oxidative capacity and lipid oxidation in human skeletal myotubes and in adipocytes [14], and protected mice from high-fat diet induced obesity and insulin resistance [31], respectively. Additionally, chronic NP infusion upregulated muscular oxidative capacity in obese and diabetic mice, thereby ameliorating lipotoxicity and insulin resistance [32]. The present clinical study did not indicate alterations in whole-body fat oxidation by sacubitril/valsartan treatment. We speculate that, after treatment with sacubitril/valsartan, NP-mediated changes in oxidative metabolism at the organ level that cannot be captured by indirect calorimetry may have attenuated accumulation of toxic lipid intermediates and subsequent impairment of insulin signaling [3]. It is also possible that augmented NP availability increased peripheral glucose and insulin supply through vascular

actions. Augmenting cyclic guanosine monophosphate signaling through phosphodiesterase 5 inhibition with sildenafil also improved insulin sensitivity in overweight individuals with prediabetes [33].

Combination of state-of-the-art methodologies in a comparatively large sample size is a particular strength of our study. In fact, hyperinsulinemic euglycemic clamp testing, $[1,1,2,3,3-^2H]$-glycerol tracer kinetics, and microdialysis technics are considered gold-standards in assessing insulin sensitivity, whole-body lipolysis, and subcutaneous adipose tissue lipolysis, respectively. The total daily dose of sacubitril/valsartan used in this study (400 mg) is known to provide superior blood pressure control in patients with arterial hypertension when administered once daily [34] and to reduce cardiovascular mortality and hospitalizations due to heart failure in patients with systolic dysfunction when administered as sacubitril/valsartan 200 mg twice daily compared with standard-of-care RAS inhibition. Therefore, the findings of this study are relevant for the future clinical use of sacubitril/valsartan.

We studied patients representing a human model for impaired insulin sensitivity. Given the close association between adiposity and risk for T2DM, and the epidemic increase in the incidence of T2DM in recent years, cardiovascular medications with added benefits on insulin sensitivity are highly desirable. Neprilysin activity seems to be upregulated in obese and insulin-resistant individuals [6], such that sacubitril/valsartan directly targets a mechanism mediating relative natriuretic peptide deficiency. Furthermore, heart failure, for which sacubitril/valsartan is approved for, is also associated with insulin resistance and muscular metabolic abnormalities. Patients with heart failure exhibit a reduction in oxidative slow twitch (type I) relative to glycolytic fast twitch (type II) muscle fibers along with reduced mitochondrial density [35]. This intrinsic impairment in skeletal muscle metabolism likely limits exercise capacity and may contribute to associated insulin resistance in patients with heart failure [36]. Decreased skeletal muscle oxidative capacity in heart failure is prevalent and clinically relevant because poor exercise tolerance and impaired insulin sensitivity predict mortality risk in these patients [37]. Therefore, metabolic improvements with sacubitril/valsartan may also be beneficial in patients with heart failure, thereby differentiating sacubitril/valsartan from currently available cardiovascular drugs.

The main limitation of our study was the fact that the change in the primary endpoint SI between interventions was not statistically significant. To account for an interim analysis conducted for SI, the p value for equality of treatments was reported at 2.5% level of significance for SI, whereas it was reported at 5% level of significance for all other glucose-clamp derived measurements. Nonetheless, there was a clear trend for improvement of SI with sacubitril/valsartan compared to amlodipine. Furthermore, GIR with or without adjustment for body weight, an established marker for insulin sensitivity, was significantly improved with sacubitril/valsartan, as were all other glucose-clamp derived measurements of insulin sensitivity, supporting the overall conclusion. Moreover, the fact that we used amlodipine as the metabolically neutral comparator does not allow distinguishing individual contributions of neprilysin inhibition and angiotensin receptor blockade to the effects of sacubitril/valsartan observed in this study. Furthermore, we cannot completely rule out a carryover effect of metabolic testing, particularly insulin infusion, on lipolysis measurements obtained on the following study day. However, all patients underwent all study assessments in the same

sequence suggesting that an impact on the comparison of investigational treatments is unlikely. Finally, our study was conducted in a relatively homogenous white population. Although there is no ethnic sensitivity with regard to exposure to LCZ696 analytes (sacubitril, sacubitrilat, and valsartan), extrapolation to other ethnic groups requires careful consideration.

CONCLUSION

In conclusion, our study demonstrated that sacubitril/valsartan treatment compared with amlodipine resulted in improved peripheral insulin sensitivity and increased abdominal subcutaneous adipose tissue lipid mobilization, but did not show significant effects on whole-body lipolysis, energy expenditure, and substrate oxidation. Moreover, our study highlights the utility of mechanistic profiling studies in discerning metabolic drug actions that could go undetected in clinical development.

METHODS

Study design and participants

This was a multicenter, randomized, double-blind, double-dummy, active-controlled, and parallel-group study. Eligible subjects were ≥18 years of age with abdominal obesity (waist circumference ≥102 cm for men and ≥88 cm for women) and elevated blood pressure either untreated (mean seated systolic blood pressure ≥130 mmHg and <180 mmHg at screening) or treated with up to two classes of antihypertensive therapy (mean seated systolic blood pressure ≤160 mmHg at screening and <180 mmHg at the end of the 4-week washout period). Women had to be of nonchild-bearing potential. Key exclusion criteria were severe hypertension (mean seated diastolic blood pressure ≥100 mmHg and/or mean seated systolic blood pressure ≥180 mmHg at screening or at the end of the washout period), type 1 or type 2 diabetes mellitus, fasting plasma glucose ≥126 mg/dL or HbA1c ≥6.5%), dyslipidemia requiring pharmacological therapy with fibrates or nicotinic acid, concomitant use of antihypertensive medications, antidiabetic medications or drugs that may affect glucose or lipid metabolism, previous or current diagnosis of cardiac structural and functional abnormalities, history or current diagnosis of heart failure (New York Heart Association classes II-IV), history of myocardial infarction, coronary bypass surgery or percutaneous coronary intervention during the 6 months before screening, history of angioedema, or known hypersensitivity to the study drugs.

The study included a screening period of up to 4 weeks followed by a 4-week washout period and an 8-week randomized, double-blind, and double-dummy treatment phase (Figure 1). Patients receiving antihypertensive medications at the time of screening discontinued the therapy during the washout period.

In the double-blind treatment period, patients were randomized to receive sacubitril/valsartan 400 mg q.d. or amlodipine 10 mg q.d. along with matching placebos for 8 weeks. Patients were stratified into four groups based on the baseline Homeostasis Model Assessment of Insulin Resistance (<2.5 and ≥2.5) and use of statin therapy within 8 weeks before randomization. Stratification was intended to ensure that within each stratum the number of subjects receiving either LCZ696 or amlodipine is comparable, thereby avoiding any impact of a potential imbalance in baseline characteristics on study outcome.

All participants provided written informed consent before screening. The study protocol was reviewed by the independent ethics committee or institutional review board for each center, conducted in accordance with the Declaration of Helsinki, and registered at clinicaltrials.gov under the identifier NCT01631864.

Peripheral insulin sensitivity

Changes in insulin sensitivity were measured from baseline to week 8 for sacubitril/valsartan 400 mg q.d. vs. amlodipine 10 mg q.d. by a hyperinsulinemic-euglycemic glucose clamp. Assessments were performed on day -1 (baseline) and on day 56 under fasting conditions. A dorsal hand vein was catheterized and kept warm for arterialized blood sampling and the contralateral arm was catheterized for glucose and insulin infusions. The procedure consisted of a 2-hour primed infusion

of insulin (a priming dose over the first 10 minutes (103 mU/m^2/min at 0–5 minutes and 57 mU/m^2/min at 5–10 minutes) followed by a continuous infusion at 40 mU/m^2/min thereafter until 2 hours) and a variable glucose infusion to achieve steady-state plasma insulin levels while maintaining blood glucose levels at 90.1 mg/dL (5.0 mmol/L). Blood samples were collected continuously (automated clamp) or at approximately 5 minute intervals (manual clamp) to determine glucose levels during hyperinsulinemic- euglycemic clamp testing. The last 30 minutes (minutes 90 - 120) of the clamp were considered as the steady-state period and the mean GIR was calculated for this period.

Insulin SI was calculated from steady-state GIRs and plasma insulin and glucose concentrations (SI: glucose infusion rate/(plasma glucose x plasma insulin), µg/kg*min/(mmol/L*pmol/L)). Whole-body glucose disposal rate (M, mg/min) was calculated from mean GIRs at steady state to assess peripheral insulin sensitivity. Finally, the metabolic clearance rate was calculated from the M-value and the mean blood glucose concentration at steady state (metabolic clearance rate: M/blood glucose, min*mmol). The glucose disposal rate was expressed per unit of insulin at steady state, calculated from M-value and plasma insulin concentrations (M/I, U/min).

Lipolysis

Lipolysis was assessed on day 1 before the first dosage and on day 57. One microdialysis probe was placed in the subcutaneous adipose tissue 6–8 cm lateral from the umbilicus with the patient under local anesthesia and sterile conditions, as described previously [38]. The process comprised a recovery phase of 60 minutes during which the probes were infused with perfusion solution (50 mM ethanol + T1 perfusion solution; CMA Microdialysis AB, Stockholm, Sweden) at a flow rate of 0.3 µL/min followed by a flow calibration phase of 60 minutes. During the flow calibration phase, the perfusion rate was maintained at 0.3 µL/min for the first 30 minutes and increased thereafter to 2.0 µL/min for the remaining 30 minutes. Microdialysate samples were collected at 30 minutes and 45 minutes in the flow calibration phase.

Glycerol (an indicator of local lipolysis), glucose, and lactate concentrations were measured in microdialysates. The ethanol ratio (ratio of ethanol concentration in dialysate to ethanol concentration in perfusate) was measured as an indicator of adipose tissue blood flow.

Whole-body lipolysis was assessed using [1,1,2,3,3-^{2}H]-glycerol tracer kinetics. A glycerol tracer bolus (3.0 µg/kg) was injected at the start of the microdialysis flow calibration phase, followed by a continuous infusion (0.1 µg/kg/min) until the end of the sampling period. Blood samples were collected at 0, 30, 40, 50, and 60 minutes during the flow calibration phase and at 15, 30, and 45 minutes thereafter. The rate of appearance of endogenous glycerol was calculated as the ratio of the glycerol tracer infusion rate to the plasma glycerol tracer enrichment. At steady state, glycerol rate of appearance was calculated from glycerol tracer enrichment using Steele's equation.

Statistical analysis

Based on 80% power to detect a difference of 0.1 in SI at week 8 between the two treatment groups at 2.5% significance level with two-sided alternative, 90 subjects (45 in each group) were needed to complete the study. SI at 8 weeks was analyzed using the analysis of covariance model with treatment as a fixed effect and baseline insulin sensitivity as a covariate for assessing the difference in the mean effect of sacubitril/valsartan vs. amlodipine. For the purpose of a two-sided hypothesis of equality of treatment effects at 2.5% level of significance after the completion of the study, the point estimate and 97.5% CI for the difference along with the p-value for equality of the two treatments were reported. The smaller level of significance (2.5%) was chosen to account for the conduct of an interim analysis. Differences between treatments for change in SI from baseline were analyzed as described above. Similar analyses at 5% level of significance were performed for all other clamp-derived measures of insulin sensitivity.

For abdominal subcutaneous adipose tissue microdialysate data (ethanol ratio, dialysate lactate, dialysate glucose, and plasma glycerol, glycerol, NEFA, glucose, insulin, adrenalin, and noradrenalin) at rest, data were analyzed using repeated measures analysis on log transformed values with treatment, visit, time and treatment*visit*time interaction as fixed effects. Ratio to day 1 (day 57 vs. day 1) at each treatment and ratio of sacubitril/valsartan vs. amlodipine for ratio to day 1 along with the corresponding 95% CIs and p-values are presented for 30 and 45-minute microdialysis time points combined. Comparison of 30 and 45-minute microdialysis time points demonstrated steady state of the microdialysis experiment.

Oxidative metabolism was analyzed using the analysis of covariance with treatment as fixed effect and baseline as covariate and the treatment mean difference with 95% CIs and p-values are reported.

For biomarkers, data were analyzed using repeated measures of analysis on log transformed values with treatment, visit, time and treatment*visit*time interaction as fixed effects. Ratio to day 1 (day 57 vs. day 1) at each treatment and ratio of sacubitril/valsartan vs. amlodipine for ratio to day 1 along with the corresponding 95% CIs and p-values are presented.

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SUPPLEMENTARY MATERIAL

Energy Expenditure and Substrate Oxidation

Energy expenditure and substrate oxidation were assessed in parallel by indirect calorimetry using a ventilated hood system. The ventilated hood measurements were recorded for 30 minutes in the resting state (during the flow calibration phase) and averaged for further analysis. Resting energy expenditure (REE) was calculated using the abbreviated Weir equation (REE = [3.9 (VO2) + 1.1 (VCO2)] 1.44) (with VO2 and VCO2 expressed as mL/min).

Circulating metabolites, insulin, and catecholamines

Fasting plasma concentrations of free fatty acids, glycerol, glucose, insulin, epinephrine, and norepinephrine were evaluated at baseline (Day 1) and on Day 57 at rest and concurrently with the microdialysis experiment. Blood samples were taken at 30 minutes and 45 minutes following the calibration flow phase.

Blood Pressure

Office BP was measured at screening, during washout and throughout the study at baseline, week 4, week 8 and end of study using the same arm and the same automated equipment with an appropriate cuff size. Measurements were performed in triplicates at 2-minute intervals after patients have been sitting for 15 minutes with the back supported and both feet on the floor. During the home stay period, patients were given a home measurement device and instructed to monitor BP twice weekly at approximately the same time each morning (7–9 AM).

Safety Assessments

Safety assessments included adverse events (AEs) and serious adverse events (SAEs) and were regularly monitored throughout the study based on hematology, blood chemistry and urine analysis as well as assessments of vital signs (BP and pulse measurements), ECG, physical condition and body weight.
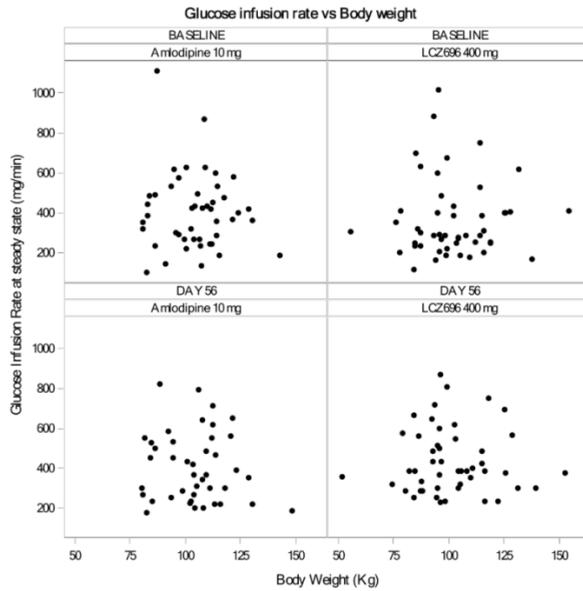
Supplementary Figure 1A. Comparison of glucose infusion rate with body weight at baseline and Day 56.
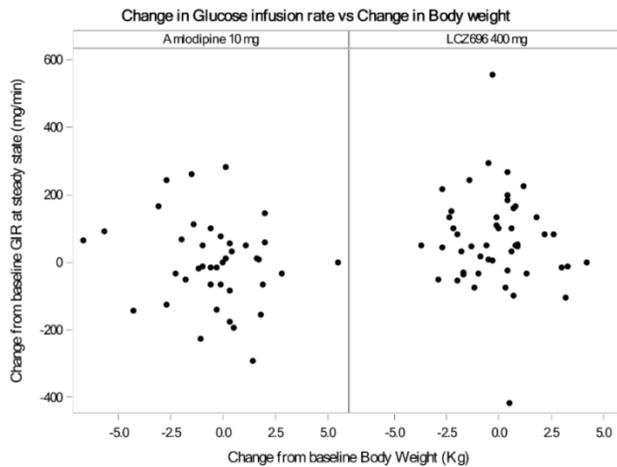
Supplementary Figure 1B. Change from baseline in glucose infusion rate vs. body weight following 8-week treatment with LCZ696 vs. amlodipine

Supplementary Table 1. Mean Change in Biomarkers from Baseline to Day 57

Biomarker Adjusted means (95% CIs)	LCZ696 (400 mg q.d.) (n=50)	Amlodipine (10 mg q.d.) (n=46)	Treatment difference (LCZ696 vs. Amlodipine)
Glucose (mmol/L)			
Baseline	5.64 (5.51, 5.78)	5.52 (5.39, 5.65)	-
Day 57	5.66 (5.55, 5.78)	5.50 (5.38, 5.62)	-
Ratio to baseline	1.00 (0.99, 1.02)	1.00 (0.98, 1.01)	1.01 (0.98, 1.03)
Insulin (pmol/L)			
Baseline	41.09 (34.68, 48.67)	37.54 (31.45, 44.79)	
Day 57	41.07 (34.57; 48.79)	37.91 (31.61, 45.47)	
Ratio to baseline	1.00 (0.88, 1.14)	1.01 (0.88; 1.16)	0.99 (0.82, 1.20)
Glycerol (µmol/L)			
Baseline	85.39 (77.97; 93.52)	91.61 (83.32, 100.73)	
Day 57	84.48 (76.85, 92.87)	87.90 (79.51, 97.18)	
Ratio to baseline	0.99 (0.91, 1.08)	0.96 (0.87; 1.05)	1.03 (0.91, 1.17)
Non-esterified fatty acids (mmol/L)			
Baseline, adjusted mean	0.63 (0.56, 0.70)	1.03 (0.91, 1.17)	
Day 57	0.60 (0.53, 0.68)	1.03 (0.91, 1.17)	
Ratio to baseline	0.96 (0.86, 1.06)	0.92 (0.82, 1.03)	1.04 (0.89, 1.21)
Epinephrine (pg/mL)			
Baseline	25.23 (21.20, 30.03)	20.98 (17.49, 25.16)	
Day 57	20.42 (17.19, 24.25)	21.91 (18.28, 26.26)	
Ratio to baseline	0.81 (0.72, 0.91)[a]	1.04 (0.93, 1.18)	0.77 (0.65, 0.91)[b]
Norepinephrine (pg/mL)			
Baseline,	217.3 (188.3, 250.7)	230.8 (198.8; 267.9)	
Day 57	236.8 (204.2, 274.5)	276.2 (236.4; 322.9)	
Ratio to baseline	1.09 (0.98, 1.21)	1.20 (1.07, 1.33)[c]	0.91 (0.78, 1.06)

[a] p<0.001; [b] p=0.002; [c] p=0.001
Data was analyzed using repeated measurements on log transformed values with treatment, visit, time and treatment*visit*time interaction as fixed effect assuming an Unstructured covariance. Values are then log back transformed.

Supplementary Table 2. Incidence of AE occurring in more than one patient - safety analysis set

	LCZ696 400 mg N = 50, n (%)	Amlodipine 10 mg N = 38, n (%)
Subjects with AEs, n (%)	30 (60.0)	37 (77.1)
Nasopharyngitis	9 (18.0)	8 (16.7)
Peripheral edema	1 (2.0)	16 (33.3)
Headache	4 (8.0)	6 (12.5)
Pruritus	5 (10.0)	-
Diarrhea	3 (6.0)	1 (2.1)
Dizziness	3 (6.0)	1 (2.1)
Pollakisuria	2 (4.0)	1 (2.1)
Circulatory collapse*	-	2 (4.2)
Fatigue	1 (2.0)	1 (2.1)
Gastroenteritis	2 (4.0)	-
Orthostatic hypotension	2 (4.0)	-
Pruritus generalized	2 (4.0)	-
Toothache	1 (2.0)	1 (2.1)

AE, adverse event
* Vasovagal reaction coded as circulatory collapse according to MedDRA

CHAPTER 4

Effect of sacubitril/valsartan on exercise induced lipid metabolism in individuals with obesity and hypertension

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ABSTRACT

Background: sacubitril/valsartan (LCZ696), a novel angiotensin receptor-neprilysin inhibitor, was recently approved for the treatment of heart failure with reduced ejection fraction. Neprilysin degrades several peptides that modulate lipid metabolism, including natriuretic peptides. In this study, we investigated the effects of 8 weeks' treatment with sacubitril/valsartan on whole body and adipose tissue lipolysis and lipid oxidation during defined physical exercise compared with the metabolically neutral comparator amlodipine.

Methods: This was a multicenter, randomized, double-blind, active-controlled, parallel-group study enrolling subjects with abdominal obesity and moderate hypertension (mean sitting systolic blood pressure [msSBP] ≥130-180 mmHg). Lipolysis during rest and exercise was assessed by microdialysis and [1,1,2,3,3-^{2}H]-glycerol tracer kinetics. Energy expenditure and substrate oxidation were measured simultaneously using indirect calorimetry. Plasma non-esterified fatty acids, glycerol, insulin, glucose, adrenaline and noradrenaline concentrations, blood pressure and heart rate were also determined.

Results: Exercise elevated plasma glycerol, free fatty acids and interstitial glycerol concentrations and increased the rate of glycerol appearance. However, exercise-induced stimulation of lipolysis was not augmented upon sacubitril/valsartan treatment compared with amlodipine treatment. Furthermore, sacubitril/valsartan did not alter energy expenditure and substrate oxidation during exercise compared with amlodipine treatment.

Conclusion: Sacubitril/valsartan treatment for 8 weeks did not elicit clinically relevant changes in exercise-induced lipolysis or substrate oxidation in obese patients with hypertension, implying that its beneficial cardiovascular effects cannot be explained by changes in lipid metabolism during exercise.

INTRODUCTION

Fatty acids are stored in the form of triglycerides in the adipose tissue and are released during lipolysis to fuel lipid oxidation in energy consuming tissues. Lipolysis and skeletal muscle lipid oxidation decrease following carbohydrate ingestion and increase in the fasting state or during physical exercise [1]. An imbalance between fatty acid mobilization and utilization may adversely affect cardiovascular and metabolic health. Acute experimental increases in circulating fatty acids in humans worsened hepatic [2] and skeletal muscle [3] insulin sensitivity and endothelium-mediated vasodilation [4]. Chronic increase in fatty acid availability promotes hepatic, skeletal muscle and myocardial lipotoxicity, dyslipidemia, insulin resistance, and type 2 diabetes mellitus [5, 6]. Conversely, interventions that reduce fatty acid levels, improve metabolic health [5]. These observations are highly relevant for cardiovascular medications with the potential to affect lipid turnover. Sacubitril/valsartan, comprising a novel neprilysin inhibitor pro-drug sacubitril and angiotensin receptor blocker (valsartan) has been approved for the treatment of chronic heart failure (NYHA Class II-IV) with reduced ejection fraction [7]. The endopeptidase neprilysin is ubiquitously expressed, including in human adipocytes, and degrades multiple peptides such as natriuretic peptides (NPs), angiotensin II, bradykinin and endothelin that may modulate lipid metabolism [8, 9]. Notably, NPs potently augment human adipose tissue lipolysis, postprandial lipid oxidation, and skeletal muscle oxidative capacity [9], whereas, angiotensin II elicits more subtle changes in fatty acid turnover [10].

Given the role and association of aberrant NP- and renin-angiotensin-aldosterone signaling in cardiovascular diseases and metabolic dysfunction, we hypothesized that simultaneous blockade of angiotensin receptor and neprilysin with sacubitril/valsartan can potentially ameliorate metabolic dysfunction, especially lipid-turnover, compared with amlodipine. In the present study, we investigated the effects of 8-weeks treatment with sacubitril/valsartan compared with the metabolically neutral comparator amlodipine on whole-body and adipose tissue lipolysis, energy expenditure and substrate oxidation during defined physical exercise, which is known to stimulate NP release and induces lipolysis and lipid oxidation.

METHODS

Study design

The study design, key inclusion and exclusion criteria of the patients and the primary results of this study have been described earlier [11]. Briefly, this was a multicenter, randomized, double-blind, double-dummy, active-controlled, parallel-group study enrolling adult subjects with abdominal obesity (waist circumference ≥102 cm for men and ≥88 cm for women) and moderate hypertension (mean sitting systolic blood pressure [msSBP] ≥130 and<180 mmHg). Key exclusion criteria were severe hypertension (msSBP>180 mmHg), type 1 or 2 diabetes (fasting plasma glucose ≥126 mg/dL or HbA1c ≥6.5%), dyslipidemia requiring therapy with fibrates or nicotinic acid, concomitant use of antihypertensive, anti-diabetic or other

medications that affect glucose and/or lipid metabolism, and a history or current diagnosis of heart failure (NYHA class II-IV).

The study included a screening period of up to 4-weeks followed by a 4-week washout period and an 8-week, randomized, double-blind, double-dummy treatment phase. Patients receiving antihypertensive medications at the time of screening discontinued the therapy during the washout period. During the treatment period, patients were randomized to receive either sacubitril/valsartan 400 mg every day (QD) or amlodipine 10 mg QD along with a matching placebo for 8 weeks. Patients were stratified into 4 groups based on baseline homeostatic model assessment of insulin resistance (HOMA-IR) and statin use.

All patients provided written informed consent before screening. The clinical study protocol was reviewed and approved by the Independent Ethics Committee or Institutional Review Board at each center and, conducted in accordance with declaration of Helsinki and registered at clinicaltrials.gov under the identifier NCT01631864.

Exercise test

An incremental exercise test on a bicycle ergometer was conducted before the start of the intervention (Day -14) to determine the maximal aerobic capacity (VO$_2$peak) at volitional exhaustion by measuring the individual maximum workload prior to stopping for exhaustion or until pre-defined heart rate or blood pressure (BP) criteria were met. At baseline (Day 1) and after 8 weeks (Day 57), subjects exercised at 50% of VO$_2$peak (as determined on Day -14) for a period of 60 min.

Measurement of lipolysis

Local adipose tissue and whole body lipolysis were assessed at baseline and after 8 weeks of treatment, as described previously [11]. Local adipose tissue lipolysis was measured by microdialysis and assessed during a 45 min interval at rest, followed by a 60 min interval during which the patients exercised at 50% of their individual VO$_2$peak. Dialysates were collected from abdominal subcutaneous adipose tissue at the lower right abdominal quadrant at rest and at 15-min intervals during exercise. Concentrations of glycerol (as an indicator of lipolysis), glucose and lactic acid in dialysates were measured. The ethanol outflow/inflow ratio (ratio of ethanol concentration in the dialysate and the perfusate) was measured as an indicator of adipose tissue blood flow.

Whole body lipolysis was estimated using [1,1,2,3,3-^{2}H]-glycerol tracer kinetics following an intravenous glycerol bolus (2 μmol·kg^{-1}) after insertion of the microdialysis catheter, approximately 60-min before the baseline measurements started, and subsequent infusion at an infusion rate of 0.1 μmol·kg^{-1}min^{-1} at rest and 0.2 μmol·kg^{-1}min^{-1} during exercise. Blood samples were collected at 15-min intervals at rest and during exercise. The rate of appearance (Ra) of endogenous glycerol was calculated as the ratio of glycerol tracer infusion rate to plasma glycerol tracer enrichment. At steady state, glycerol Ra was calculated from glycerol enrichment using Steele's equation.

Energy expenditure and substrate oxidation

Energy expenditure and substrate oxidation during rest and exercise were assessed by indirect calorimetry using a ventilated hood system. The ventilated hood measurements were recorded for 30 minutes in the resting phase with the patient in supine position and during the last 10 minutes of the 60-min exercise period.

Circulating metabolites and hormones

Samples for fasting plasma biomarkers (non-esterified fatty acid [NEFA], glycerol, glucose, insulin, adrenaline and noradrenaline) were collected at baseline (Day 1) and on Day 57 at rest and during exercise concurrently with microdialysis measurements.

Blood pressure

Office BP was measured at screening, during washout and throughout the study at baseline, Week 4, Week 8 and at the end of study using the same automated equipment with an appropriate cuff size. Measurements were performed in triplicate at 2-min intervals after patients had been sitting for 15 minutes with the back supported and both feet on the floor. BP was also measured during the exercise phase. During the home stay period, patients were given a home measurement device and instructed to monitor BP twice weekly at approximately the same time each morning.

Statistical analysis

Following 8 weeks of treatment with sacubitril/valsartan or amlodipine, assessments of local adipose tissue lipolysis, whole body lipolysis, oxidative metabolism, BP and biomarkers during exercise were performed as pre-specified study objectives.

For abdominal subcutaneous adipose tissue microdialysate data (ethanol ratio, dialysate lactate, dialysate glucose, dialysate glycerol), plasma biomarkers (glycerol, NEFA, glucose, insulin, adrenaline and noradrenaline) and whole-body lipolysis (rate of glycerol appearance) data for 45 minutes at rest and 4 time-points during exercise (15, 30, 45 and 60 minutes) were analyzed using repeated-measures analysis on log-transformed values with treatment, visit, time and treatment*visit*time interaction as fixed effects. Geometric mean ratios of each exercise time-point to 45 minutes resting for each day and treatment, ratios of Day 57 to Day 1 for each treatment and each exercise time-point, and the ratio between sacubitril/valsartan and amlodipine for Day 57 to Day 1 were calculated.

Oxidative metabolism was analyzed using analysis of covariance (ANCOVA) with treatment as the fixed effect and baseline as the covariate. Oxidative metabolism during exercise was analyzed using analysis of variance (ANOVA) for repeated measurements with treatment, visit and treatment*visit interaction as fixed effects. Mean difference to Day1 (Day 57 vs. Day 1) for each treatment along with the corresponding 95% CIs and p-values are presented. Data for exercise and resting phase were analyzed for each day and treatment with a mixed-effects linear model with phase (exercise or resting) as the fixed effect and subject as the random effect

to obtain the mean difference estimate and 95% CI for exercise vs. rest comparison. Respiratory quotient (CO_2/O_2 ratio) was calculated at each of the Days 1 and 57 at rest and during exercise. A statistical comparison of the quotients was made between rest and exercise within each day.

RESULTS

Exercise testing

On Day 1, 39 patients in the sacubitril/valsartan group and 24 patients randomized to the amlodipine group completed the constant workload exercise for 60 minutes. On Day 57, 36 patients treated with sacubitril/valsartan and 23 treated with amlodipine completed the exercise for 60 minutes. Similar observations were made in patients completing only 45 and 60 minutes of exercise, suggesting that 8 weeks of treatment of patients with obesity and hypertension with sacubitril/valsartan or amlodipine did not have any clinically relevant impact on the exercise duration. Oxygen consumption and workload were comparable between Day 1 and Day 57 in both treatment groups (Supplementary Table 1).

Plasma glucose and insulin concentrations

With exercise, plasma glucose concentrations increased in the amlodipine group for all time-points and for 30 (p=0.017), 45 (p=0.002) and 60 (p<0.001) minutes in the sacubitril/valsartan group on Day 1. On Day 57, the increase was significant during 60 min of exercise in the sacubitril/valsartan group (p=0.031) but the difference was not significant at any time point in the amlodipine group. A decrease in glucose concentration was noticed on Day 57 in both the treatment groups as compared to baseline (Day 1), with the difference being significant only in the amlodipine group at 30 (p=0.017) and 45 (p<0.001) minutes of exercise. However, no statistically significant differences in glucose concentrations were observed between the treatment groups at any time-point.

A decrease in insulin concentrations with increasing exercise duration was observed in both treatment groups. When compared with resting insulin concentrations, a significant decrease was observed at 45 min (p=0.015) and 60 min (p<0.001) on Day 1 and at 45 min (p=0.044) on Day 57 in the sacubitril/valsartan group. However, exercise-induced decreases in insulin concentrations were not statistically significant in the amlodipine group, either on Day 1 or Day 57. After 8 weeks of treatment, compared with baseline, insulin concentrations were significantly lower in the amlodipine group at all time-points except 60 minutes, while the change was not significant at any time-point in the sacubitril/valsartan group. Significant differences in insulin concentrations were observed at 30 min (p=0.017) and 45 min (p=0.027) between treatment groups on Day 57 compared to baseline.

Subcutaneous adipose tissue lipolysis during exercise

Compared with resting measurements, microdialysate glycerol concentrations increased during exercise, indicating increased subcutaneous adipose tissue lipolysis in both the amlodipine and sacubitril/valsartan groups on Day 1 as well as on Day 57. Compared with baseline, microdialysate glycerol concentrations during exercise were numerically lower in the amlodipine group on Day 57. In the sacubitril/valsartan group, microdialysate glycerol concentrations increased similarly at the beginning and at the end of treatment, but this increase was not statistically significant (Figure 1). Microdialysate glucose concentrations were comparable between sacubitril/valsartan and amlodipine at baseline [sacubitril/valsartan vs. amlodipine: 15 min (1.07 vs. 0.94 mmol/L); 30 min (1.06 vs. 1.02 mmol/L); 45min (1.05 vs 0.99 mmol/L); 60 min (1.03 vs. 0.91 mmol/L)] and on Day 57 [15 min (1.12 vs. 0.95 mmol/L); 30 min (1.08 vs. 0.94 mmol/L); 45min (1.07 vs 1.02 mmol/L); 60 min (1.06 vs. 1.01 mmol/L)]. No statistically significant differences in glucose levels from baseline to Week 8 were observed between the two treatment groups. A similar trend was observed with lactate levels.

Ethanol ratios were comparable between sacubitril/valsartan and amlodipine on Day 1 [sacubitril/valsartan vs. amlodipine: 15 min (0.42 vs. 0.43); 30 min (0.42 vs. 0.44); 45 min (0.43 vs 0.45); 60 min (0.47 vs. 0.46)]. Ethanol ratios increased on Day 57 in both the sacubitril/valsartan and amlodipine groups, but remained comparable between treatment groups [15 min (0.49 vs. 0.49); 30 min (0.51 vs. 0.49); 45 min (0.53 vs. 0.51); 60 min (0.55 vs. 0.53)]. These data suggest that there was no relevant change in blood flow that needs to be accounted for when interpreting glycerol measurements.
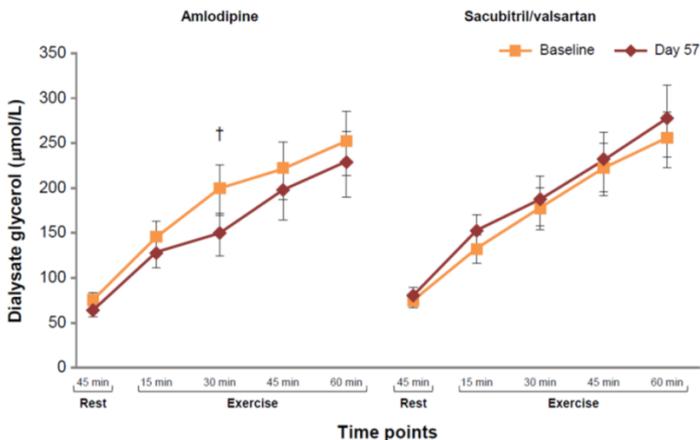
† p=0.003 vs. baseline

Figure 1. Comparison of local adipose tissue lipolysis (dialysate glycerol) variable during exercise following 8-weeks of treatment with sacubitril/valsartan and amlodipine

Error bars indicate 95% CI

Whole-body lipolysis

Plasma glycerol concentrations increased with exercise in both treatment groups, both on Day 1 and Day 57 [amlodipine group, Day 1 vs. Day 57: resting (89.77 vs. 88.04 µmol/L); 15 min (141.12 vs. 119.56 µmol/L); 30 min (184.78 vs. 156.03 µmol/L); 45 min (216.04 vs. 179.27 µmol/L); 60 min (224.85 vs. 191.95 µmol/L) and sacubitril/valsartan, Day 1 vs. Day 57: resting (85.64 vs. 83.93 µmol/L); 15 min (139.3 vs. 126.92 µmol/L); 30 min (177.65 vs. 157.29 µmol/L); 45 min (205.68 vs. 189.84 µmol/L); 60 min (225.62 vs. 205.26 µmol/L)]. Compared to baseline, plasma glycerol levels were lower in both the treatment groups on Day 57. While the change from baseline to Day 57 was significant at all time-points in the amlodipine group ($p<0.05$), it was significant at 30 minutes in the sacubitril/valsartan group ($p=0.012$). The differences in plasma glycerol levels between treatment groups were not significant.

As compared with glycerol Ra following 45 minutes rest, a significant increase was observed during exercise at all time-points in both treatment groups on Day 1 and Day 57 ($p<0.001$). The change from baseline to Day 57 was statistically significant in the sacubitril/valsartan group at 15 min ($p=0.026$), 30 min ($p=0.012$) and 45 min ($p=0.035$), but was not significant at any time-point in the amlodipine group (Figure 2A). However, there was no significant difference between treatment groups at any time-point.

Plasma NEFA concentrations decreased on Day 57 at 15 minutes in the sacubitril/valsartan group ($p=0.018$) and at 15 min and 30 min ($p<0.05$) in the amlodipine group. No significant differences were observed between treatment groups. When compared with NEFA levels at rest (45 minutes), the levels were lower during the initial phases of exercise, but increased gradually with increasing exercise duration in both treatment groups (Figure 2B).

A
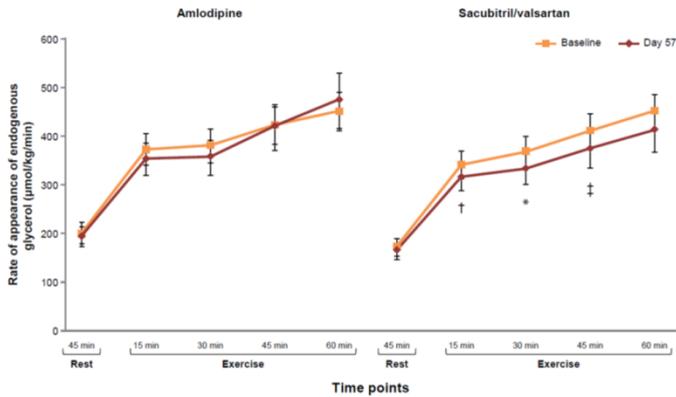
† p=0.026,* p=0.012, ‡ p=0.035 vs. baseline

B
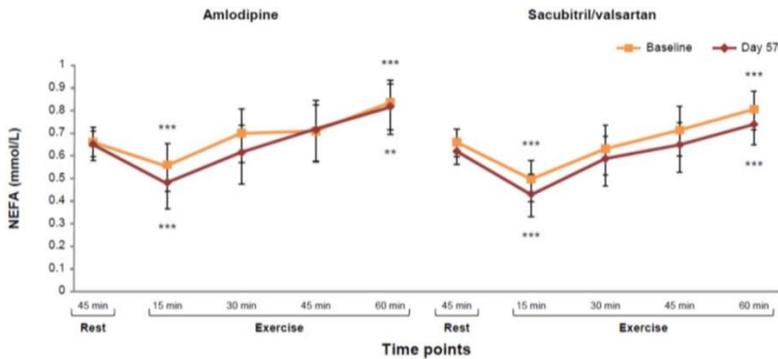
*** p<0.001, ** p<0.002 vs. 45 min rest

Figure 2. Whole body lipolysis at exercise: comparison of rate of glycerol appearance between treatments (**A**) and plasma concentration of NEFA (**B**).

Error bars indicate 95% CI

Oxidative metabolism during exercise

Oxygen consumption was comparable between the sacubitril/valsartan and amlodipine groups at baseline (O_2 consumption: amlodipine, 1.31 ± 0.45 L/min; sacubitril/valsartan, 1.40 ± 0.41 L/min) and on Day 57 (amlodipine, 1.27 ± 0.39 L/min; sacubitril/valsartan, 1.37 ± 0.44 L/min) and no differences were found between treatment groups.

The respiratory quotient significantly increased during exercise in both the treatment groups, on Day 1 and Day 57 (Figure 3). The respiratory quotient was comparable between treatments at baseline and on Day 57.
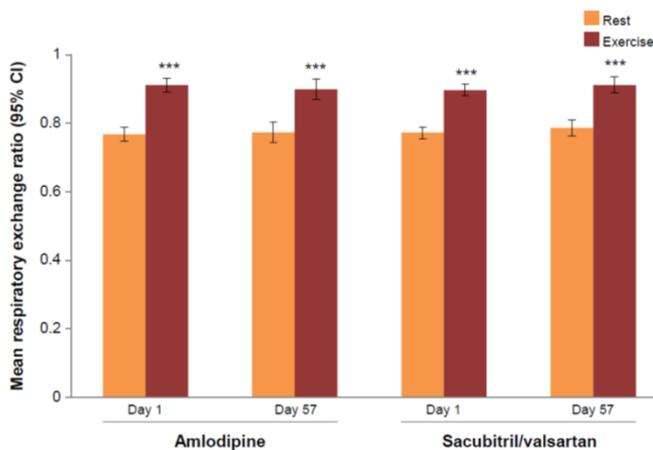
*** p<0.001, exercise vs. rest

Figure 3. Oxidative metabolism-comparison of respiratory quotient between resting and exercise status- carbon dioxide to oxygen ratio.

Error bars indicate 95% CI

Plasma catecholamine concentrations

When compared with resting levels, adrenaline concentrations increased significantly during exercise at all time-points in both treatment groups on Day 1 and 57 (Figure 4A). Compared with baseline, a significant reduction in adrenaline concentrations were observed on Day 57 in the amlodipine groups at all time-points while the decrease was not statistically significant in the sacubitril/valsartan group. However, no significant differences were observed in the adrenaline concentrations between treatment groups at any time-point, except at 30 minutes (p=0.012).

Plasma noradrenaline levels were significantly increased during exercise in both the treatment groups on Day 1 and Day 57 (p<0.001) when compared with resting

levels (Figure 4B). Noradrenaline levels increased incrementally during exercise on Day 1 and Day 57 in both the treatment groups, with no significant differences between treatments.

A
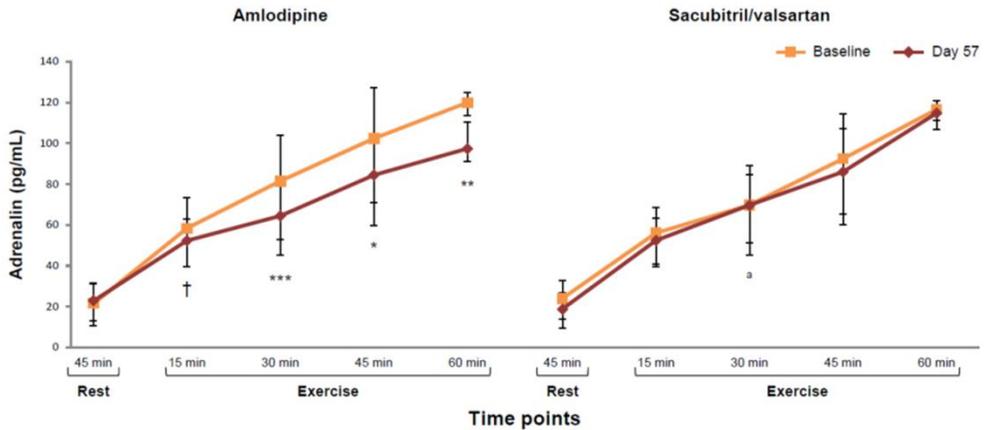
† p=0.044, *p=0.022, **p=0.019, ***p<0.001 vs. baseline. a p=0.012 vs. amlodipine

B
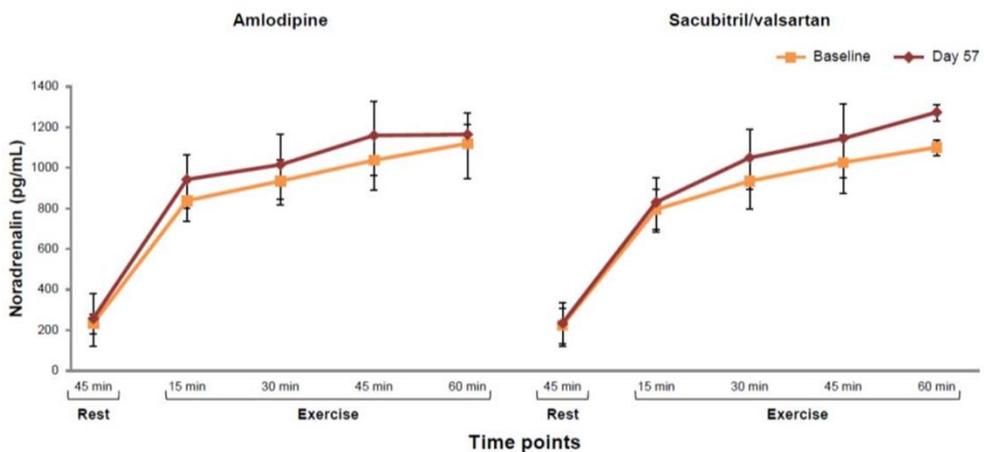
p<0.001 vs. rest

Figure 4. Analysis of plasma biomarkers during exercise: adrenaline (**A**) and noradrenaline (**B**)

Error bars indicate 95% CI.

Blood pressure

After 8-weeks of treatment, systolic BP, diastolic BP and pulse pressure decreased from baseline in both treatment groups at rest. Systolic and diastolic BP and pulse rate values increased during exercise in both treatment groups on both Day 1 and Day 57 without clinically relevant differences between treatment groups (Table 1).

Table 1. Comparison of BP and pulse rate between treatments during exercise and rest

Parameter	Day	Rest/Exercise	LCZ696 (400 mg QD) N=50	Amlodipine (10 mg QD) N=48
SBP (mmHg)	Day 1	Rest	137.9 ± 14.7	135.8 ± 12.8
		Exercise	157.5 ± 23.8	146.5 ± 23.3
	Day 57	Rest	119.9 ± 14.3	125.9 ±10.2
		Exercise	146.8 ±21.9	137.6 ± 20.5
DBP (mmHg)	Day 1	Rest	88.0 ± 10.6	86.8 ± 8.9
		Exercise	82.8 ±12.6	84.3 ± 12.4
	Day 57	Rest	77.2 ± 8.5	81.2 ± 7.2
		Exercise	78.4 ± 12.3	78.8 ± 11.9
Pulse rate (BPM)	Day1	Rest	67.4 ± 8.6	66.7 ± 9.3
		Exercise	121.2 ± 15.4	111.4 ± 25.6
	Day 57	Rest	65.2 ± 8.7	69.2 ± 10.5
		Exercise	123.5 ± 18.6	115.6 ± 16.5

Data are expressed as mean ± SD.
SBP, systolic blood pressure; DBP, diastolic blood pressure, BPM, beats per minute

DISCUSSION

The present study demonstrated that treatment with sacubitril/valsartan compared with amlodipine for 8 weeks did not elicit relevant changes in exercise-induced lipolysis and substrate oxidation in obese patients with hypertension. The exercise-induced increase in abdominal subcutaneous adipose tissue and whole-body lipolysis was not augmented following sacubitril/valsartan treatment compared with amlodipine treatment. Moreover, the shift in substrate oxidation towards carbohydrate catabolism during exercise was comparable in both treatment groups, implying that sacubitril/valsartan did not significantly affect lipid utilization during acute exercise. We have previously observed significantly improved whole-body insulin sensitivity and a modest increase in resting abdominal subcutaneous adipose tissue lipolysis with no marked changes in whole-body lipolysis with sacubitril/valsartan compared with amlodipine treatment [11]. Overall, these findings imply that the beneficial cardiometabolic effects of sacubitril/valsartan may not be explained by changes in lipid mobilization or oxidation.

In this study, we used state-of-the-art methodology including $[1,1,2,3,3-^2H]$-glycerol tracer kinetics and abdominal subcutaneous adipose tissue microdialysis to assess whole-body and local lipolysis, respectively, in a large patient sample. Furthermore, we treated patients with a total daily dose of sacubitril/valsartan which provided superior BP control in patients with arterial hypertension (400 mg QD) [12] and reduced cardiovascular mortality and heart failure hospitalizations in patients with heart failure and reduced ejection fraction (HFrEF) (200 mg twice daily) compared with standard-of-care renin-angiotensin system (RAS) inhibition [7]. This study, therefore, was appropriately designed to study the effect of sacubitril/valsartan on lipid turnover.

Our study extends previous investigations on the role of neprilysin substrates and angiotensin II type-1 (AT_1)-receptors in the regulation of lipid turnover. All components of the RAS are expressed in adipose tissue, and AT_1-receptors have been implicated in the regulation of adipose tissue differentiation, inflammation, and metabolism [10]. Conflicting findings have been reported with respect to the effects of angiotensin II on adipose tissue lipolysis. More specifically, both increased [13, 14] and decreased [15] subcutaneous adipose tissue lipolysis have been demonstrated [14]. Moreover, intravenous angiotensin II infusions and angiotensin converting enzyme inhibition did not elicit major changes in whole-body lipolysis as determined by glycerol tracer kinetics [16]. AT_1-receptor blockade in human subjects did not increase lipolytic gene expression or lipolysis in abdominal subcutaneous adipose tissue [17, 18]. However, long-term AT_1-receptor blockade altered intramuscular lipid partitioning, manifested by decreased saturation of skeletal muscle triacylglycerol and diacylglycerol stores, reduced postprandial fatty acid spillover and lipolysis [19]. Overall, angiotensin II actions on AT_1-receptors appear to have modest effects on lipid turnover. Although postprandial fatty acid handling has not been examined in this study, the present findings suggest that AT_1-receptor blockade in the context of neprilysin inhibition by sacubitril/valsartan does not have clinically relevant effects on lipid mobilization or utilization.

Neprilysin degrades multiple peptides potentially modulating lipid metabolism such as natriuretic peptides (NPs), bradykinin, endothelin-1, and glucagon-like peptide 1 (GLP-1) [20]. We cannot discern contributions of individual neprilysin substrates to

the observed metabolic response. While bradykinin has been suggested to attenuate lipolysis, endothelin-1 may increase lipolysis. However, endothelin-1 was significantly decreased following treatment of patients with HFrEF with sacubitril/valsartan for 21 days, and no changes in lipolysis for GLP-1 at high concentrations have been reported [21-25]. Among neprilysin substrates, lipolytic effects of NPs are particularly striking. NPs are more potent in stimulating human adipose tissue lipolysis than the prototypical β-adrenoreceptor agonist isoproterenol [26]. Since NP-induced lipolysis is observed only in primates, the utility of many preclinical animal models is limited [27]. NPs are released during physical exercise and have been suggested to provide lipid fuel to the working skeletal muscle. Excess NP-mediated lipid mobilization has been suggested as a potential limitation of therapeutic neprilysin inhibition. In fact, *ex vivo* lipolysis of subcutaneous adipose tissue was not desensitized in patients with heart failure despite increased circulating NP levels [28]. However, the lack of changes in exercise-induced lipolysis by sacubitril/valsartan observed in the present study is clinically reassuring.

Given the potent acute effect of NPs on human lipolysis, our findings are somewhat unexpected. Plasma noradrenaline and adrenaline increased to a similar extent at baseline and following treatment with amlodipine and sacubitril/valsartan, suggesting that opposing changes in sympatho-adrenal activity did not mask a direct treatment effect on lipolysis. The reduction in catecholamine concentrations observed with sacubitril/valsartan in this study is consistent with our previous observation [11]. Conflicting observations have been reported with respect to the effect of amlodipine therapy on noradrenaline concentrations [29-31] while valsartan treatment has been demonstrated to attenuate increases in plasma noradrenalin concentrations with larger reductions from baseline associated with lower risk of mortality and morbidity [32, 33]. While sacubitril/valsartan improved insulin-mediated glucose disposal compared with amlodipine [11], potential anti-lipolytic effects of insulin in adipose tissue following sacubitril/valsartan have not been investigated before. We cannot completely rule out that improved insulin action in adipose tissue confounded our analysis. However, an alternative and more likely explanation is that NP actions in adipose tissue are not or to a lesser degree dependent on neprilysin activity. Indeed, a study in isolated human adipocytes suggests that clearance via the natriuretic peptide type-C receptor, the so-called scavenger receptor, may be more important than neprilysin activity to control local NP availability [34]. Indeed, completely abolishing neprilysin activity using thiorphan did not modify atrial natriuretic peptide (ANP)-mediated lipolysis [34].

Noteworthy, we conducted our study in obese patients with hypertension. Given the differences in neurohormonal activity between patients with hypertension and heart failure, the extent to which our findings can be extrapolated to patients with HFrEF remains to be elucidated. However, a recent post-hoc analysis of the PARADIGM-HF trial showed that in patients with heart failure and type 2 diabetes mellitus, treatment with sacubitril/valsartan resulted in greater reductions in HbA1$_c$ concentrations compared to those treated with enalapril. Moreover, sacubitril/valsartan treated patients with type 2 diabetes were less likely to require initiation of insulin treatment during the trial suggesting potential metabolic benefits of sacubitril/valsartan therapy in HF patients [35]. Finally, the selection of

amlodipine as a metabolically neutral comparator does not allow distinguishing the contributions of neprilysin inhibition from those of AT_1-receptor blockade to the effects of sacubitril/valsartan observed in this study.

In conclusion, our study demonstrated that sacubitril/valsartan treatment did not result in clinically relevant changes in exercise-induced abdominal subcutaneous adipose tissue and whole-body lipolysis, energy expenditure and substrate oxidation compared with amlodipine. This finding is relevant because neprilysin substrates, particularly NPs, have been implicated in lipolysis and the pathogenesis of cardiac cachexia. Our findings further support the idea that neprilysin is of lesser importance in regulating NP availability in the vicinity of adipocytes.

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CHAPTER 5

The effects of angiotensin receptor neprilysin inhibition by sacubitril/valsartan on adipose tissue transcriptome and protein expression in obese hypertensive patients

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ABSTRACT

Increased activation of the renin-angiotensin system is involved in the onset and progression of cardiometabolic diseases, while natriuretic peptides (NP) may exert protective effects. We have recently demonstrated that sacubitril/valsartan (LCZ696), a first-in-class angiotensin receptor neprilysin inhibitor, which blocks the angiotensin II type-1 receptor and augments natriuretic peptide levels, improved peripheral insulin sensitivity in obese hypertensive patients. Here, we investigated the effects of sacubitril/valsartan (400mg QD) treatment for 8 weeks on the abdominal subcutaneous adipose tissue (AT) phenotype compared to the metabolically neutral comparator amlodipine (10mg QD) in 70 obese hypertensive patients. Abdominal subcutaneous AT biopsies were collected before and after intervention to determine the AT transcriptome and expression of proteins involved in lipolysis, NP signaling and mitochondrial oxidative metabolism. Both sacubitril/valsartan and amlodipine treatment did not significantly induce AT transcriptional changes in pathways related to lipolysis, NP signaling and oxidative metabolism. Furthermore, protein expression of adipose triglyceride lipase (ATGL) ($P_{time*group}$=0.195), hormone-sensitive lipase (HSL) ($P_{time*group}$=0.458), HSL-ser660 phosphorylation ($P_{time*group}$=0.340), NP receptor-A (NPRA) ($P_{time*group}$=0.829) and OXPHOS complexes ($P_{time*group}$=0.964) remained unchanged. In conclusion, sacubitril/valsartan treatment for 8 weeks did not alter the abdominal subcutaneous AT transcriptome and expression of proteins involved in lipolysis, NP signaling and oxidative metabolism in obese hypertensive patients.

INTRODUCTION

Obesity is strongly associated with cardiometabolic risk factors [1], which is reflected by an increased risk for arterial hypertension, heart failure and type 2 diabetes mellitus (T2DM) [2]. An impaired adipose tissue function and excessive fat mass in obesity represent key factors in the development of insulin resistance and related chronic diseases, including cardiovascular disease and T2DM [3]. Evidence suggests that impaired insulin sensitivity in obesity might be related to an altered renin-angiotensin system (RAS) and natriuretic peptide (NP) signaling in adipose tissue. Blockade of the RAS using angiotensin-converting enzyme (ACE) inhibitors or angiotensin type-1 receptor blockers (ARB) has been shown to improve insulin sensitivity and beta-cell function [4] and reduces the incidence of T2DM [5] as reviewed elsewhere [6]. However, results are not consistent [7]. In addition, NPs are positively associated with insulin sensitivity and low atrial natriuretic peptide (ANP) concentrations are associated with an increased risk of developing arterial hypertension and T2DM [8]. In accordance, neprilysin (NEP), which is involved in the degradation and inactivation of NP, is linked to insulin resistance, increased blood pressure and impaired lipid metabolism [9]. Therefore, combined RAS blockade and NEP inhibition might have synergistic beneficial effects on blood pressure and peripheral insulin sensitivity. We recently demonstrated that combined ARB and NEP inhibition, using sacubitril/valsartan (LCZ696), improved peripheral insulin sensitivity following 8 weeks of treatment compared to amlodipine (AMLO) in obese hypertensive patients [10]. However, the mechanisms underlying these beneficial effects remain to be established.

Evidence suggests that both the RAS and ANP affect adipose tissue metabolism, thereby determining insulin sensitivity [6, 11]. It has been shown that valsartan (ARB) reduced adipocyte size, increased adipose tissue blood flow and decreased gene expression of angiogenesis, adipogenesis and macrophage infiltration markers [12], which may have contributed to the valsartan-induced increased insulin sensitivity [4]. Furthermore, angiotensin II inhibited lipolysis *in vitro* in mature human adipocytes [13], although conflicting findings on adipose tissue lipolysis *in vivo* in humans have been reported [14, 15]. ANP has been shown to increase adipose tissue lipid mobilization and oxidation [11] and we and others have recently demonstrated that ANP-mediated lipolysis is impaired in subcutaneous mature adipocytes from obese men with and without T2DM [16, 17] .

Therefore, it is hypothesized that ARB and NEP inhibition with sacubitril/valsartan may affect adipose tissue function, thereby contributing to the observed improved peripheral insulin sensitivity in obese individuals [10]. The present study investigated the effects of sacubitril/valsartan compared to amlodipine treatment for 8 weeks on the abdominal subcutaneous adipose tissue transcriptome and protein expression profiles in obese hypertensive individuals.

METHODS

Study design

Ninety-eight obese hypertensive patients participated in a multicenter, randomized, double-blind, parallel-group study to investigate the effects of sacubitril/valsartan (400mg QD) compared with amlodipine (10mg QD) treatment for 8 weeks. A detailed description of the inclusion and exclusion criteria is described elsewhere (clinicaltrials.gov - NCT01631864). Before and after treatment, abdominal subcutaneous adipose tissue biopsies were collected by needle aspiration under local anesthesia after an overnight fast. We determined adipose tissue gene expression profiles using microarray analysis in a subgroup of 70 patients who had a RNA Integrity Number (RIN) ≥5.0, as described in detail below. Next, in a subgroup (*N*=12-13), we determined the expression of proteins involved in the lipolytic pathway, the natriuretic peptide signaling pathway and mitochondrial oxidative phosphorylation. Subjects showing a treatment-induced increase in adipose tissue lipolysis *in vivo* following sacubitril/valsartan and unchanged lipolysis after amlodipine treatment were included in the latter analyses.

All patients gave written informed consent before participation. The Independent Ethical Committee or Institutional Review Board of each center reviewed the study protocol. The study was performed in accordance with the Declaration of Helsinki.

Adipose tissue transcriptomics

First, the integrity of abdominal subcutaneous adipose tissue total RNA was determined using the sample RNA Integrity Number (RIN), generated using a Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Foster City, CA, USA). RIN values <5.0 indicated high level of sample total RNA degradation and were excluded from analyses [18]. Biopsies of 70 patients (*N* sacubitril/valsartan=36, *N* amlodipine=34) were eligible for transcriptional analyses (RIN ≥5.0).

Whole-transcriptome analysis was carried-out using Affymetrix HG-U133plus2.0 oligonucleotide microarrays (Affymetrix Inc., Santa Clara, CA, USA) and the microarray files were pre-processed using the Robust Multi-array Average algorithm (RMA). The Chip Definition File (CDF) used for the RMA procedure corresponded to the public domain Michigan University Entrez CDF version 17.0. Transcripts showing median expression higher than 6 (log2-value) were combined and considered for statistical analyses. Evaluations comprised unsupervised (hypothesis-free) and supervised (targeted) assessments of which the latter explored the longitudinal treatment-induced effects on specific transcripts linked to lipolysis, fatty acid oxidation, mitochondrial biogenesis and adipokines.

Adipose tissue protein expression

A detailed description of the analysis can be found in the Supplementary Material. Briefly, abdominal subcutaneous adipose tissue was ground to a fine powder under liquid nitrogen and homogenized in RIPA buffer. The homogenate was lysed, vortexed and centrifuged and the supernatant was collected and stored at -80°C. The protein concentration was determined by the Bradford-based protein assay. Next, solubilized proteins (15 μg) were separated on a precast gel and transferred

onto a nitrocellulose membrane and quantitative Western Blot analysis was performed to determine the levels of proteins involved in the lipolytic pathway (adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and HSL serine 660 phosphorylation), the natriuretic peptide signaling pathway (natriuretic peptide receptor A (NPRA)) and mitochondrial oxidative phosphorylation (OXPHOS).

Statistics

Statistical analyses of gene expression data was performed with R v.3.2.2 (R Development Core Team, Vienna, http://www.R-project.org) and the Bioconductor limma R package v.3.26.8. Statistical significance was set at nominal P≤0.05 and P-values were corrected for multiple testing using the Bonferroni method. A total of 8319 transcripts (out of assessed 18898 transcripts/microarray) fulfilled the expression level filtering criteria and the Bonferroni adjusted threshold was therefore defined as $P<6.01\cdot10^{-6}$. Longitudinal treatment-induced transcriptional changes were expressed as Ratio Change from baseline (RC; (post-treatment level)·(pre-treatment level)$^{-1}$). Thresholds for minimum relevant treatment-induced effects were defined as 0.66≥RC≥1.50 (i.e. RC range equivalent to absolute fold changes≥1.5).

Protein expression data are expressed as mean±SEM. All variables were checked for normal distribution by Shapiro-Wilk test and variables were Ln-transformed to satisfy conditions of normality (HSL, HSL S660 phosphorylation, NPRA and OXPHOS). Data was analyzed using two-way repeated measures ANOVA, with time (pre, post) and treatment (sacubitril/valsartan, amlodipine) as factors. Bonferroni post-hoc correction was applied when a significant time*treatment interaction was found. Calculations were performed using SPSS v.21 for Mac OSX (IBM, Chicago, IL, USA) and P≤0.05 was considered statistically significant.

RESULTS

Subject characteristics

Baseline characteristics of patients contributing to the present study are shown in Table 1. Importantly, the two groups were well matched and there were no major differences in clinical characteristics between groups.

Transcriptomics

At baseline, no significant differences in the abdominal subcutaneous adipose tissue transcriptome were found between treatment arms. Unsupervised assessments revealed 1443 longitudinally modulated transcripts (nominal P<0.05), but none of these fulfilled both thresholds for Bonferroni adjusted P-value and minimum treatment-induced RC effect. A set of selected transcripts is shown in Supplementary Table S1.

Supervised analyses identified no significant treatment-induced changes (defined based on the two thresholds stated above) in gene expression levels of transcripts involved in lipolysis, NP signaling, oxidative metabolism and adipokines (Figure 1).

More specifically, gene expression of ATGL (*PNPLA2*), MGL (*MGLL*), lipoprotein lipase (*LPL*), perilipin-1 (*PLIN1*) and fatty acid binding protein 4 (*FABP4*) was not significantly altered by sacubitril/valsartan treatment (Figure 1). In addition, gene expression of natriuretic peptide receptors (*NPR1*, *NPR3*), adipokines (adiponectin (*ADIPOQ*), leptin (*LEP*)) and oxidative metabolism markers (peroxisome proliferator-activated receptor gamma co-activator-related 1 (*PPRC1*), peroxisome proliferator-activated receptor gamma co-activator 1 alpha (*PPARGC1A*), nuclear respiratory factor 1 (*NRF1*), acyl-coenzyme A oxidase 1 (*ACOX1*) and uncoupling protein 2 (*UCP2*)) were not significantly affected (Figure 1).
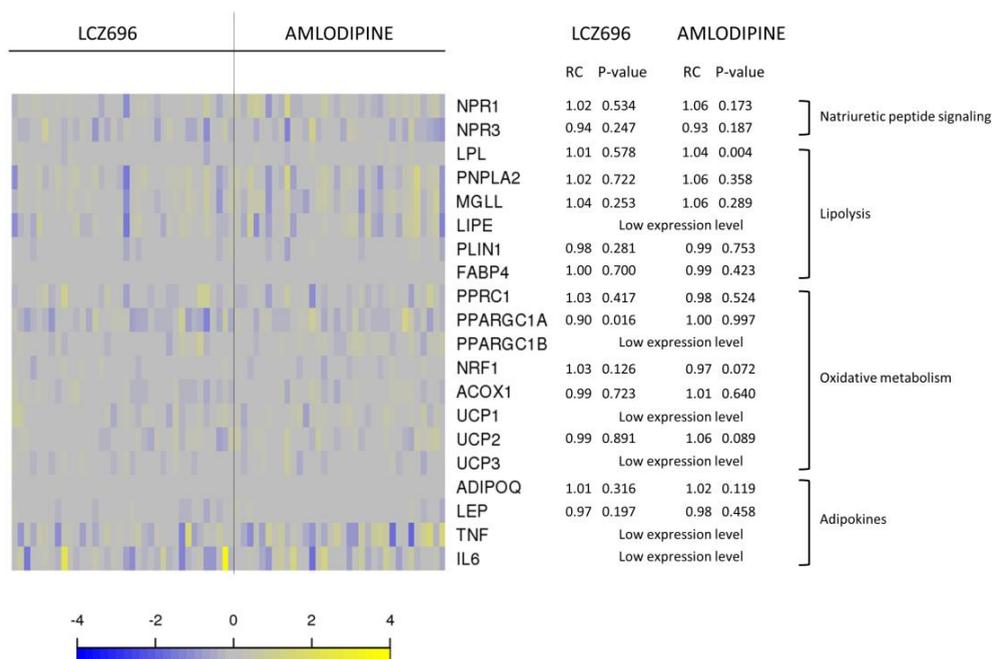
Figure 1. Targeted assessment of selected treatment-induced transcriptional changes in abdominal subcutaneous adipose tissue.

The heatmap plot shows per patient longitudinal treatment-induced gene expression changes [defined as log2(post treatment expression) - log2(pre-treatment expression)] for selected transcripts related to natriuretic peptide signaling, lipolytic pathway, oxidative pathway and adipokines in abdominal subcutaneous adipose tissue (n=70 patients; grouped per treatment arm). Color in the heatmap reflects transcript change from baseline per subject. Blue: downregulated transcripts; yellow: upregulated transcripts. The adjacent table depicts the corresponding transcript ratio change from baseline (RC) and P-value per treatment arm.

Table 1. Baseline clinical characteristics of patients involved in transcriptomic and protein expression analyses

Transcriptomic analyses

Parameter		Sacubitril/valsartan ($N = 36$)	Amlodipine ($N = 34$)	Total ($N = 70$)
Age (yrs.)		52.4 (8.56)	51.6 (9.21)	52.0 (8.83)
Gender (N)	Male	28	25	53
	Female	8	9	17
Weight (kg)		100 (18.2)	104 (15.8)	102 (17.1)
Waist circumference (cm)		111.0 (10.53)	113.4 (10.81)	112.2 (10.66)
BMI (kg·(m^2)$^{-1}$)		32.4 (4.77)	33.6 (4.59)	33.0 (4.69)
Mean sitting SBP (mm Hg)		143.2 (13.26)	138.8 (11.61)	141.0 (12.60)
Mean sitting DBP (mm Hg)		89.6 (7.79)	90.4 (5.87)	90.0 (6.89)
Glucose infusion rate (μmol·kg bw^{-1}·min^{-1})		344.5 (161.96)	409.1 (192.69)	375.9 (179.22)

Protein expression analyses

Parameter		Sacubitril/valsartan ($N = 15$)	Amlodipine ($N = 12$)	Total ($N = 27$)
Age (yrs.)		53.6 (6.92)	54.9 (7.04)	54.2 (6.87)
Gender (N)	Male	13	8	21
	Female	2	4	6
Weight (kg)		108 (19.2)	102 (14.4)	105 (17.1)
Waist circumference (cm)		114.6 (12.98)	111.2 (12.89)	113.1 (12.80)
BMI (kg·(m^2)$^{-1}$)		34.3 (5.89)	33.1 (3.93)	33.8 (5.06)
Mean sitting SBP (mm Hg)		147.2 (10.62)	137.8 (12.59)	143.0 (12.27)
Mean sitting DBP (mm Hg)		92.0 (6.10)	87.5 (5.63)	90.0 (6.22)
Glucose infusion rate (μmol·kg bw^{-1}·min^{-1})		362.6 (141.71)	401.1 (104.46)	379.7 (125.74)

Data are expressed as mean (SD).

Protein expression

Sacubitril/valsartan treatment did not significantly change protein expression of ATGL (LCZ696: 1.00±0.21 vs. 0.85±0.19 AU; AMLO: 1.00±0.26 vs. 1.36±0.30 AU; P_{time}=0.583, $P_{time*treatment}$=0.195; Figure 2A), HSL (LCZ696: 1.00±0.23 vs. 1.38±0.24 AU; AMLO: 1.00±0.26 vs. 1.00±0.18 AU; P_{time}=0.141, $P_{time*treatment}$=0.458; Figure 2B), HSL serine 660 phosphorylation (LCZ696: 1.00±0.16 vs. 1.04±0.14 AU; AMLO: 1.00±0.25 vs. 0.71±0.19 AU; P_{time}=0.551, $P_{time*treatment}$=0.340; Figure 2C) or NPRA (LCZ696: 1.00±0.24 vs. 0.99±0.29 AU; AMLO: 1.00±0.36 vs. 0.96±0.30 AU; P_{time}=0.775, $P_{time*treatment}$=0.829; Figure 2D). Furthermore, total OXPHOS protein expression (LCZ696: 1.00±0.22 vs. 1.76±0.48 AU; AMLO: 1.00±0.15 vs. 1.74±0.50 AU; P_{time}=0.125, $P_{time*treatment}$=0.964; Figure 2E) remained unchanged following treatment. More specifically, OXPHOS complexes I-V were not affected (data not shown). Representative Western Blots are presented in Supplementary Figure S1.
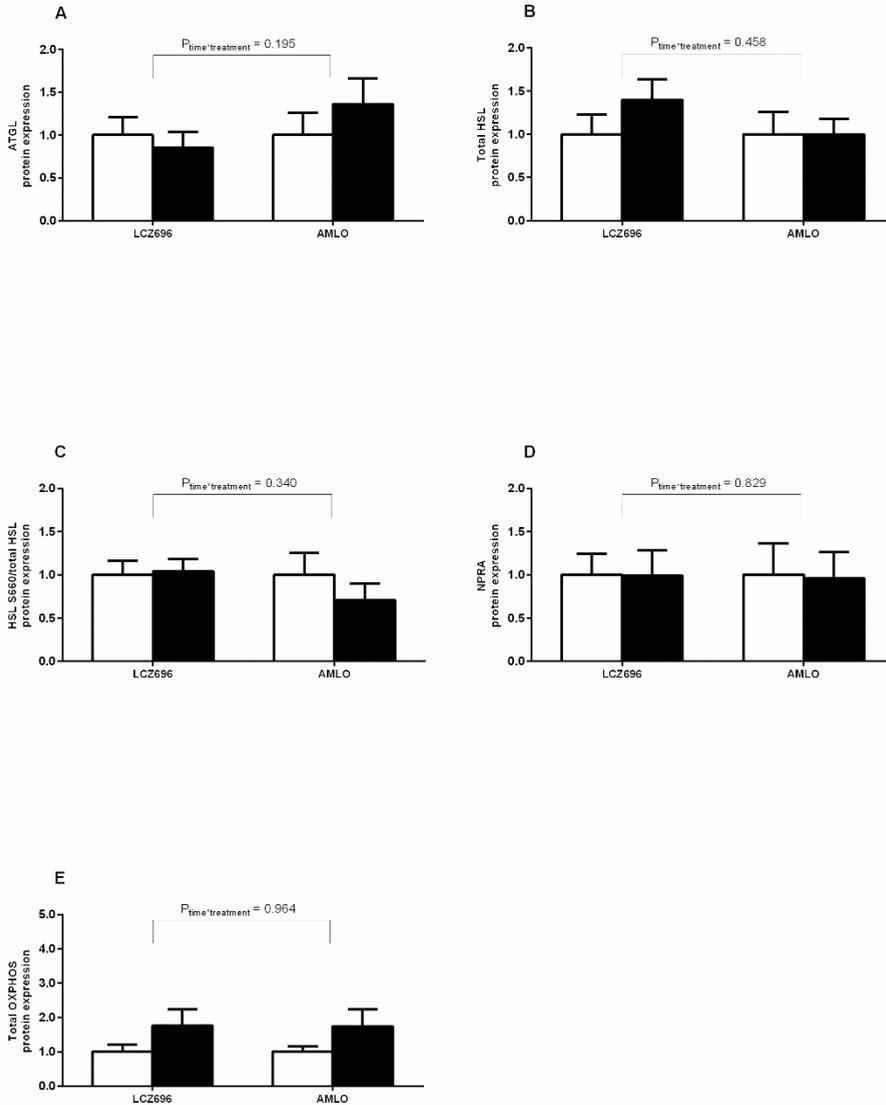
Figure 2. Protein expression in abdominal subcutaneous adipose tissue.

Expression of proteins involved in the lipolytic pathway (ATGL, N=12, **A**; HSL, N=13, **B**; HSL serine 660 phosphorylation, N=13, **C**), the natriuretic peptide signaling pathway (NPR-A, N=12, **D**) and mitochondrial oxidative metabolism (total OXPHOS, N=13, **E**) expressed as fold change relative to baseline for each treatment arm (Sacubitril/valsartan: LCZ696 or Amlodipine: AMLO). Data are expressed as mean ± SEM. White bars: baseline values; black bars: post-treatment values. Representative Western Blots are presented in Supplementary Figure S2.

DISCUSSION

Here, we investigated the effects of sacubitril/valsartan versus amlodipine treatment for 8 weeks on the abdominal subcutaneous adipose tissue transcriptome and protein expression in obese hypertensive patients. We demonstrated that sacubitril/valsartan treatment did not significantly alter adipose tissue gene and protein expression of factors related to lipolysis, natriuretic peptide signaling and oxidative metabolism.

We recently demonstrated that in obese hypertensive patients sacubitril/valsartan treatment, which provides simultaneous ARB blockade and NEP inhibition, significantly increased peripheral insulin sensitivity [10]. Furthermore, sacubitril/valsartan slightly but significantly increased abdominal subcutaneous adipose tissue lipolysis, although no changes in whole-body lipolysis were observed [10]. Therefore, we hypothesized that RAS blockade and NEP inhibition has synergistic beneficial effects on abdominal subcutaneous adipose tissue metabolism and might underlie the observed improvement in insulin sensitivity. We showed that the abdominal adipose tissue phenotype was not significantly affected by 8 weeks of sacubitril/valsartan treatment in obese hypertensive patients. First, sacubitril/valsartan treatment did not reveal any transcriptional changes in abdominal subcutaneous adipose tissue, as determined by an unsupervised analysis. More specific, no treatment-induced changes in expression of genes involved in lipolysis, the NP signaling pathway and mitochondrial oxidative pathway were found. Secondly, the expression of proteins involved in these pathways, as well as post-translational modification of HSL, remained unchanged after the intervention.

Evidence suggests that both the RAS and NP system may affect adipose tissue metabolism, thereby contributing to improved insulin sensitivity [6, 11]. It has previously been shown that angiotensin II decreased adipose tissue lipolysis *in vivo* in humans [14, 19, 20] and in human isolated adipocytes [13], which seems to be mediated via the angiotensin II type-1 receptor. However, increased adipose tissue lipolysis has also been reported [15]. In accordance with our results, long-term ARB treatment with valsartan improved insulin sensitivity in subjects with impaired glucose metabolism [4], but adipose tissue gene and protein expression of several lipolytic enzymes remained unchanged [12].

Several studies have shown that ANP promotes adipose tissue lipid mobilization and oxidation in healthy individuals [11] via cGMP-mediated phosphorylation of HSL [21, 22]. Furthermore, ANP induced mitochondrial biogenesis and uncoupling in human adipocytes from healthy, non-diabetic women [23]. Here, we did not find significant changes in gene and protein expression of markers involved in lipolysis, phosphorylation of HSL and mitochondrial oxidative metabolism, which may be explained by reduced ANP-mediated signaling in the study population. Indeed, an impaired ANP-mediated lipolysis has recently been described *in situ* in subcutaneous adipose tissue and *in vitro* in subcutaneous adipocytes from obese individuals [16, 17]. Moreover, it has been shown that NPRC (NP clearance receptor) is increased in adipose tissue of obese hypertensive patients compared to lean and normotensive individuals [24], together with increased NEP expression in obesity [9]. These data suggest reduced NP signaling and increased NP clearance in adipose tissue in obesity. This may explain the unaltered

subcutaneous adipose tissue metabolic phenotype following sacubitril/valsartan treatment in the present study.

Due to conservative multiple testing correction, we may have missed relevant treatment-induced changes in gene expression. However, the observed relative changes in gene expression were very modest, revealing only minor, potentially not physiologically-relevant, changes in gene expression.

CONCLUSION

The present study demonstrated that simultaneous RAS blockade and NEP inhibition following sacubitril/valsartan treatment for 8 weeks did not significantly alter the adipose tissue metabolic phenotype in obese hypertensive patients. More specifically, abdominal subcutaneous adipose tissue gene and protein expression of factors involved in lipolysis, natriuretic peptide signaling and mitochondrial oxidative metabolism remained unchanged. Therefore, alterations in the adipose tissue metabolic phenotype do not seem to contribute to the improved peripheral insulin sensitivity following 8 weeks of sacubitril/valsartan treatment [10].

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SUPPLEMENTARY MATERIAL

Protein expression analysis

Adipose tissue (~500 mg) was ground to a fine powder under liquid nitrogen and homogenized in radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris (Calbiochem)-HCl (Merck, Darmstadt, Germany) buffered saline (Merck) with 0,1% SDS (Bio-Rad Laboratories Inc, Hercules, CA, USA), 1% Na-Deoxycholate (Sigma-Aldrich, St. Louis, MO, USA), 1% NP-40 (Fluka) and a protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Beverly, MA, USA). The homogenate was lysed on iced and vortexed for 5 min and centrifuged at 20,000 g for 30 min at 10°C. The supernatant was carefully collected and aliquots were stored at -80°C. The protein concentration was determined by the Bradford-based protein assay (Santa Cruz Biotechnology, Dallas, TX, USA).

Next, solubilized proteins were separated on a precast gel (Criterion™ TGX any kD, Bio-Rad Laboratories Inc, Hercules, CA, USA) and transferred onto a nitrocellulose membrane (Trans Blot® Turbo™ transfer system; Bio-Rad). Differences in loading were adjusted to total adipose tissue protein content (via Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining), and appropriate positive controls (lysates of abdominal subcutaneous adipose tissue) were included.

Thereafter, quantitative Western Blot analysis was performed to determine the levels of ATGL (Cell Signaling Technology, Beverly, MA, USA), HSL (kind gift from Prof. Cecilia Holm, [Department of Cell and Molecular Biology, Lund University, Sweden]), HSL serine 660 phosphorylation (Cell Signaling Technology, Beverly, MA, USA) and NPRA (Abcam, Cambridge, MA, USA). The secondary antibody was a horseradish peroxidase (HRP) swine-anti-rabbit antibody (DakoCytomation, Glostrup, Denmark). Furthermore, OXPHOS blots were probed with Total OXPHOS Antibody Cocktail (Abcam, Cambridge, MA, USA) and a secondary HRP-conjugated Rabbit-anti-Mouse antibody (DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized using chemiluminescence by a ChemiDoc™ XRS apparatus (Bio-Rad) and analyzed with Quantity One® software (Bio-Rad), which calculated the optical density units that are expressed as average intensity [average intensity = total intensity of the rows of pixels inside the band boundary divided by the number of rows, minus the background intensity].

SUPPLEMENTARY TABLE S1. Set of selected genes for the untargeted assessment of transcriptional changes in abdominal subcutaneous adipose tissue.

A total of 1443 transcripts were modulated by either sacubitril/valsartan (n=916) or amlodipine (n=668) treatment (nominal P≤0.05, no multiple correction applied). A set of selected genes with corresponding ratio changes from baseline (RC) and P-value per treatment arm is shown.

BIOLOGICAL PATHWAY	GENES ID	LCZ696		AMLO	
		RC	P-value	RC	P-value
INFLAMMATION	ALOX5AP	0.76	0.001	0.93	0.483
	AOX1	0.93	0.083	0.89	0.025
	BLNK	0.91	0.040	0.96	0.430
	BTK	0.88	0.011	0.94	0.337
	C3AR1	0.90	0.072	0.88	0.024
	CCL16	1.14	0.009	1.01	0.838
	CCL18	1.24	0.040	0.97	0.781
	CD14	0.89	0.035	0.95	0.351
	CD163	0.94	0.222	0.91	0.027
	CD44	0.95	0.044	0.89	0.000
	CD74	0.98	0.530	0.94	0.028
	CLU	0.96	0.036	0.98	0.372
	CTSS	0.85	0.009	0.88	0.118
	CXCL9	1.20	0.042	1.15	0.134
	CYBB	0.86	0.003	0.93	0.215
	E2F3	0.95	0.020	1.00	0.887
	FCER1G	0.87	0.023	0.87	0.042
	FYB	0.85	0.050	1.01	0.944
	FYN	0.98	0.075	0.96	0.036
	GAB2	0.96	0.131	0.95	0.027
	GBP2	0.91	0.005	0.96	0.236
	HCLS1	0.90	0.022	0.98	0.687
	HLA-DMA	0.99	0.660	0.93	0.033
	IFI30	0.89	0.153	0.77	0.000
	IFIT2	0.91	0.009	1.03	0.593
	IFNGR1	0.99	0.649	0.95	0.021
	IRF1	0.90	0.040	1.09	0.134
	ITGB2	0.85	0.031	0.82	0.020
	LCP1	0.85	0.016	0.82	0.019
	LYN	0.87	0.009	0.98	0.823
	MAP2K1	1.03	0.048	1.04	0.119
	MAP2K4	0.94	0.004	0.98	0.414
	MAP3K7	0.97	0.044	0.93	0.004
	MAX	0.96	0.041	0.98	0.514
	PAK2	0.97	0.023	0.99	0.447
	PIK3CB	0.96	0.027	0.97	0.290
	PLA2G7	1.01	0.917	0.71	0.001
	PLCG1	1.07	0.010	0.99	0.748
	PPP3CC	1.00	0.927	0.96	0.047
	PTPN6	0.88	0.042	0.98	0.779
	PTPRC	0.83	0.017	1.00	0.989
	RAC2	0.85	0.034	0.96	0.652
	RIPK1	0.98	0.249	0.95	0.021
	STAT1	0.93	0.006	1.03	0.316
	SYK	0.85	0.001	0.89	0.078

BIOLOGICAL PATHWAY	GENES ID	LCZ696		AMLO	
		RC	P-value	RC	P-value
OXIDATIVE METABOLISM	COX11	1.05	0.001	1.03	0.128
	COX4I2	1.09	0.016	1.11	0.024
	COX7A2	1.01	0.312	1.03	0.049
	COX7A2L	0.97	0.028	0.98	0.288
	IDH2	1.02	0.530	1.10	0.011
	NDUFA5	1.01	0.831	1.06	0.049
	NDUFB4	1.03	0.179	0.94	0.047
	NDUFS1	1.00	0.939	1.08	0.010
	PDP1	0.94	0.002	0.99	0.497
	PPARGC1A	0.89	0.016	1.00	0.997
	SLC25A11	1.01	0.804	1.10	0.009
	SLC25A16	0.99	0.820	1.10	0.019
	SLC25A18	1.08	0.041	1.07	0.117
	SLC25A27	0.99	0.862	0.92	0.046
	SLC25A33	1.04	0.337	1.10	0.038
	SUCLG1	0.96	0.017	1.02	0.417
NATRIURETIC PEPTIDE SIGNALING	ADM	0.95	0.027	0.95	0.071
	EDN1	1.00	0.958	0.82	0.006
	FAP	1.27	0.000	1.05	0.361
	GUCY1A2	1.12	0.000	1.11	0.001
	GUCY1A3	1.14	0.000	1.07	0.047
	GUCY1B3	1.12	0.000	1.04	0.150
	MME	1.05	0.177	1.11	0.001
	PDE3A	1.06	0.048	1.04	0.295
	PDE9A	1.09	0.016	0.99	0.797
	RAPGEF5	1.09	0.031	1.04	0.362
LIPID METABOLISM	ACACA	1.06	0.153	1.14	0.008
	ADIPOR1	0.91	0.006	0.99	0.802
	ANG	0.94	0.076	0.91	0.037
	CIDEA	0.90	0.096	0.85	0.024
	ELOVL5	0.99	0.522	1.03	0.048
	ELOVL6	1.09	0.431	1.42	0.003
	ELOVL7	0.93	0.095	0.90	0.047
	GPAM	1.01	0.498	1.08	0.002
	LPL	1.01	0.578	1.04	0.004
	PPARGC1A	1.08	0.016	0.93	0.997
	PLA2G4C	0.89	0.043	1.00	0.096
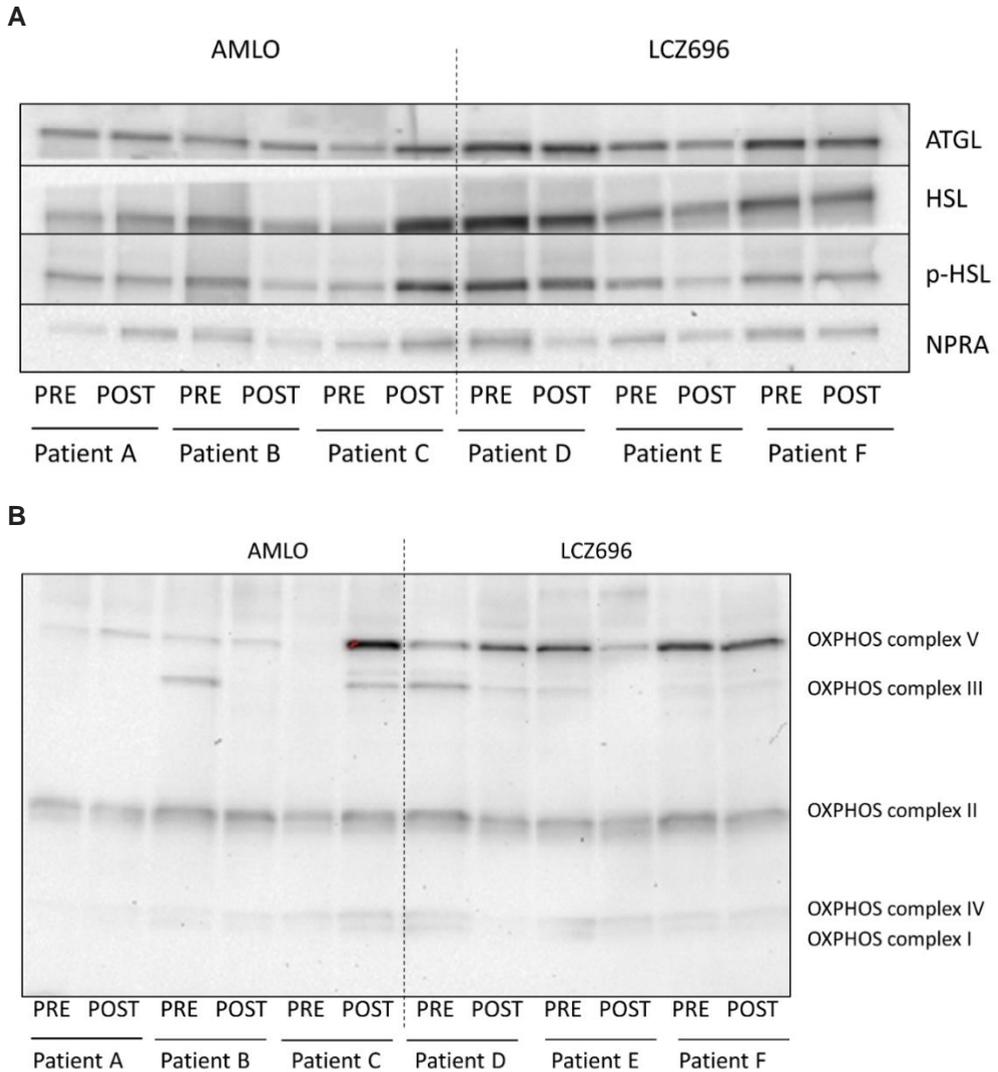
SUPPLEMENTARY FIGURE S2. Representative Western Blots for proteins involved in the lipolytic pathway, the natriuretic peptide signaling pathway and mitochondrial oxidative metabolism.

Membranes were probed with antibodies directed against total ATGL, total HSL, phosphorylated HSL (p-HSL) on Ser660, NPRA (**A**) and OXPHOS protein expression (**B**).

CHAPTER 6

Exercise training-induced effects on the abdominal subcutaneous adipose tissue phenotype in obese humans

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ABSTRACT

Aims/hypothesis: Rodent studies have indicated that physical exercise may improve adipose tissue (AT) function. We investigated the effects of a 12-weeks supervised, progressive exercise training program on adipocyte morphology and abdominal subcutaneous AT function in well-phenotyped, obese subjects.

Methods: 21 obese men (14 metabolically compromised - non-alcoholic fatty liver and/or type 2 diabetes - and 7 metabolically healthy controls) participated in a 12-weeks supervised, progressive, combined exercise training program. At baseline and after intervention, abdominal subcutaneous AT biopsies were collected to determine 1) adipocyte morphology, 2) gene expression of markers for lipolysis, inflammation, browning, adipokines and mitochondrial biogenesis/function, 3) protein expression of mitochondrial oxidative phosphorylation (OXPHOS) complexes and 4) ex vivo basal and β_2-adrenergic stimulated lipolysis.

Results: At baseline, AT gene expression of HSL (P=0.005), CGI-58 (P<0.001) and PGC-1α (P=0.037) were significantly lower in the metabolically compromised as compared to metabolically healthy obese subjects. Mean adipocyte diameter and total OXPHOS protein content in AT were comparable between groups. The exercise training program, which increased maximal aerobic capacity (P_{time}<0.001) and muscle strength (P_{time}<0.001), slightly reduced AT mass (~ 0.7 kg, P_{time}=0.037) but did not affect abdominal subcutaneous adipocyte size (P_{time}=0.860), AT gene expression of markers for mitochondrial biogenesis and function, browning, lipolysis, inflammation and adipokines, total OXPHOS protein content (P_{time}=0.826) and β_2-adrenergic sensitivity of lipolysis (P_{time}=0.555), irrespective of baseline metabolic status.

Conclusions/interpretation: A 12-weeks supervised, progressive exercise training program did neither alter abdominal subcutaneous adipocyte morphology and AT gene and protein expression of markers related to adipose tissue function, nor β_2-adrenergic sensitivity of lipolysis in obese subjects, irrespective of baseline metabolic status.

INTRODUCTION

The obesity epidemic is paralleled by a tremendous increase in the prevalence of obesity-related diseases, including type 2 diabetes (T2DM), non-alcoholic fatty liver (NAFL), cardiovascular disease and certain types of cancer [1]. A sedentary lifestyle is a major contributor to obesity and related complications. In line, increased habitual physical activity and exercise training may have beneficial effects on insulin sensitivity and glucose homeostasis in obese, insulin resistant and T2DM patients [2-4]. Therefore, increasing physical activity is a recommended lifestyle modification in the prevention and treatment of obesity-related disorders, including T2DM [5].

Since skeletal muscle is responsible for the majority of glucose disposal, adaptations in skeletal muscle metabolism are thought to play a central role in the exercise training-induced improvement of insulin sensitivity. Adipose tissue dysfunction in obesity, however, represents a key step in the development of obesity-related insulin resistance and chronic diseases [6, 7]. The reason for this is that adipocyte hypertrophy in obesity promotes low-grade inflammation and decreases the adipose tissue lipid buffering capacity. Consequently, lipids accumulate in non-adipose tissues (e.g. skeletal muscle and liver) when lipid supply exceeds fat oxidation, thereby accelerating the development and progression of insulin resistance and chronic metabolic diseases [6-8].

Interestingly, there is evidence that exercise training may improve white adipose tissue function [9]. Several rodent studies demonstrated that exercise training increased adipose tissue mitochondrial biogenesis [10, 11] and function [12-14], induced browning of white adipose tissue [10, 11, 13, 15, 16] and altered adipokine expression [17, 18]. Furthermore, transplantation of white adipose tissue from trained animals to untrained recipients markedly improved skeletal muscle glucose uptake [13], suggesting that improvement of adipose tissue function may contribute to the increased peripheral insulin sensitivity after exercise training. However, human studies that investigated the effects of exercise training on the adipose tissue phenotype are scarce. Exercise training has been shown to increase gene expression of peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) [19] and oxidative metabolism markers [20], yet conflicting data regarding the expression of adipokines and markers of lipolysis in human adipose tissue have been reported [21-29]. Furthermore, the evidence that exercise training enhances adipose tissue lipolysis, assessed either *ex vivo* in isolated adipocytes or *in vivo* at rest and in response to a lipolytic stimulus, is inconsistent and complicated by confounding factors such as recent energy balance, as reviewed [30]. Importantly, most human studies that have investigated the exercise training-induced effects on adipose tissue metabolism did not perform detailed metabolic phenotyping. Therefore, it remains to be established whether the metabolic phenotype at baseline determines study outcomes.

The aim of the present study was to investigate the effects of a 12-weeks supervised, progressive, combined exercise training program on abdominal subcutaneous adipocyte morphology, adipose tissue gene expression of markers related to mitochondrial biogenesis/function, browning, lipolysis, inflammation and adipokines and protein expression of mitochondrial oxidative phosphorylation (OXPHOS) in obese, metabolically healthy and metabolically compromised

individuals, matched for age and BMI. Furthermore, using isolated adipocytes from these subjects, we determined the exercise training-induced effects on *ex vivo* basal and β_2-adrenergic stimulation of lipolysis.

METHODS

Study design

Twenty-one sedentary, middle-aged (40–70 yrs), overweight/obese men (14 NAFL/T2DM and 7 age and BMI-matched metabolically healthy control subjects) participated in the present study, which was conducted within the framework of a larger clinical trial designed to primarily investigate the effects of exercise training on liver fat content, hepatic, adipose tissue and peripheral insulin sensitivity [31]. Control subjects (n=7) had low liver fat content (all ≤4%), as measured with proton magnetic resonance spectroscopy (^{1}H-MRS), in the absence of liver dysfunction (defined as alanine aminotransferase (ALAT) >2.5 times normal values) and had to be normoglycemic according to the WHO criteria. Subjects were defined as NAFL patients (n=7) when having a liver fat content ≥5% as measured with ^{1}H-MRS, in addition to a fasting plasma glucose concentration <7.0 mmol/l. Furthermore, at screening, T2DM patients (n=7) were allowed to be on sulphonyl urea, metformin, dipeptidyl peptidase-4 inhibitors therapy (or a combination) for at least 6 months with stable dosage for at least 2 months or on a dietary treatment for 6 months, with fasting plasma glucose concentrations ≥7.0 and <10.0 mmol/l. Liver fat content was not a selection criteria for T2DM patients. All subjects gave written informed consent before participation in the study. The Medical Ethical Committee of Maastricht University Medical Center[+] approved the study protocol, which was performed according the principles expressed in the Declaration of Helsinki.

All participants were asked not to change their habitual dietary intake during the study period. General exclusion criteria were unstable body weight, cardiovascular disease, impaired renal function, hemoglobin <7.5 mmol/l, blood pressure >160/100 mmHg, participation in a weight-loss or exercise program, history of substantial alcohol use (>3 units/day), history of drug abuse, use of beta-blockers, anti-thrombotic medication, insulin therapy and use of medication known to interfere with glucose homeostasis (except for T2DM patients).

At screening, routine laboratory analyses and physical examinations were performed, medical history was checked and a resting electrocardiogram (ECG) was taken. Maximal power output (W_{max}) and maximal aerobic capacity (VO_{2max}) were assessed during a graded cycling test with concurrent ECG until exhaustion. Body composition was determined using DEXA (Hologic Discovery A, Waltham, MA, USA). Furthermore, a two-step hyperinsulinemic-euglycemic clamp with primed D-[6,6-[2]H$_2$]-glucose was performed to assess peripheral, hepatic and adipose tissue insulin sensitivity, as described elsewhere [31].

Exercise training protocol

Subjects participated in a 12-weeks supervised, progressive exercise training program. Aerobic exercise training was performed on a cycle ergometer twice a week for 30 min at 70% W_{max}, which was determined just before the start of the intervention. Resistance exercise training, which focused on large muscle groups (chest press, lat pull down, leg extension, shoulder press, horizontal row, leg press, triceps extensions and biceps curls), was performed once a week and comprised three series of ten repetitions at 60% of subjects' previously determined one repeated maximum (1RM). The 1RM test was preceded by a familiarization trial. Warming-up and cooling-down sessions of 5 min were performed on a stationary bike at 45% W_{max}. Every 4 weeks, 1RM and VO_{2max} were reassessed and training loads were adjusted accordingly to assure that the training stimulus was maintained. At baseline and after the 12-weeks training program, several measurements were performed, as described below.

Adipose tissue biopsies

After an overnight fast, an abdominal subcutaneous adipose tissue biopsy (~1g) was collected 6–8 cm lateral from the umbilicus, under local anesthesia (2% lidocaine) by needle biopsy. The biopsy was washed with sterile saline and visible blood vessels were removed. One part of the biopsy was snap frozen in liquid nitrogen and stored at -80°C for gene and protein expression analyses, whereas two parts were processed for determination of adipocyte morphology and measurement of *ex vivo* lipolysis, as described below.

Adipocyte morphology

A part of the adipose tissue biopsy was fixed overnight in 4% paraformaldehyde and embedded in paraffin for histological sections (8 μm). Sections were cut from paraffin-embedded tissue, mounted on microscope glass slides and dried overnight in an incubator at 37°C. The sections were stained with hematoxylin (VWR, Radnor, PA, USA) and eosin (Klinipath BV, Duiven, The Netherlands). Digital images were captured with a Leica DFC320 digital camera (Leica, Rijswijk, The Netherlands) at x20 magnification (Leica DM3000 microscope, Leica, Rijswijk, The Netherlands) and computerized morphometric analysis (Leica QWin V3, Cambridge, UK) of individual adipocytes was performed in a blinded manner. Approximately 400 adipocytes per sample were measured.

Gene expression

Total RNA was extracted from frozen adipose tissue biopsies (~500 mg) using Trizol chloroform extraction (Invitrogen, Cergy Pontoise, France) and 300ng RNA was reversed transcribed using iScript cDNA synthesis kit (BIO-RAD). Gene expression for markers of lipolysis (*ATGL* [*PNPLA2*], *HSL* [*LIPE*], *CGI-58* and *PLIN1* [perilipin 1]), inflammation (*TNFα*, *IL-6*, *MCP-1* [*CCL2*], *CD68*), browning (*CIDEA*, *PRDM16*), mitochondrial biogenesis (*PGC-1α* [*PPARGC1A*]) and adipokine expression (*ADIPOQ* and *LEP*) (supplementary table 1 for primer sequences) was determined in a total volume of 25 μL containing 12.5 ng cDNA using SYBR-Green based qPCR (iCycler/MyIQ, BIO-RAD). Results were

calculated via the $2^{-\Delta CT}$ method and normalized for *18S* (housekeeping gene) ribosomal RNA.

Protein expression

A detailed description can be found in the supplementary material. Briefly, subcutaneous adipose tissue (~500mg) was ground to a fine powder under liquid nitrogen and homogenized in radioimmunoprecipitation assay buffer. The homogenate was lysed, vortexed and the supernatant was collected and stored at -80°C. The protein concentration was determined by the Bradford-based protein assay (Santa Cruz Biotechnology, Dallas, TX, USA).

Next, solubilized proteins (15 μg) were separated on a precast gel (Criterion™ TGX any kD, Bio-Rad Laboratories Inc, Hercules, CA, USA) and transferred onto a nitrocellulose membrane (Trans Blot® Turbo™ transfer system; Bio-Rad).

Thereafter, quantitative Western Blot analysis was performed to determine the levels of OXPHOS proteins. OXPHOS blots were probed with Total OXPHOS Antibody Cocktail (Mitoscience/Abcam, Cambridge, MA, USA) and a secondary horseradish peroxidase (HRP)-conjugated Rabbit-anti-Mouse antibody (DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized using chemiluminescence by a ChemiDoc™ XRS apparatus (Bio-Rad) and analyzed with Quantity One® software (Bio-Rad), which calculated the optical density units that are expressed as average intensity.

Ex vivo adipocyte lipolysis

Ex vivo adipocyte lipolysis was determined in 15 individuals and mature adipocytes were isolated from the subcutaneous adipose tissue following collagenase digestion. First, digestion was performed for 60 min at 37°C in a Krebs-Ringer phosphate buffer, containing 100 mg glucose/100 ml and 4% bovine serum albumin with 2 mg/ml collagenase (Sigma-Aldrich, Zwijndrecht, The Netherlands). Secondly, adipocytes (~5,000-10,000 cells/incubation) were incubated with or without increasing concentration of salbutamol (specific β_2-adrenergic receptor agonist) (10^{-9}–10^{-4} M; GlaxoSmithKline, Zeist, The Netherlands) for 2 h at 37°C in Krebs–Ringer phosphate buffer. Thereafter, incubation medium was collected and stored at -80°C until analysis. Glycerol concentration in the medium, which is an indicator of complete TAG hydrolysis (lipolysis), was determined using the EAPL-200 EnzyChrom Adipolysis Assay Kit (Glentaur Europe BVBA, Kampenhout, Belgium).

Biochemistry

Arterialized blood samples were collected and immediately centrifuged at 4°C for 10 min at 1000 *g* and plasma was snap frozen in liquid nitrogen and stored at -80°C until further analysis. Plasma non-esterified fatty acid (NEFA; Wako NEFA C test kit; Wako Chemicals, Neuss, Germany) and glucose (hexokinase method; LaRoche, Basel, Switzerland) concentrations were measured with enzymatic assays, whereas triacylglycerol (TAG) concentrations were measured colorimetrically (Roche, Vienna, Austria), automated on a Cobas Fara/Mira. Plasma insulin and serum liver function parameters (aspartate aminotransferase

(ASAT), alanine aminotransferase (ALAT), γ-glutamyl transpeptidase (GGT)) were routinely measured and analyzed at the clinical chemistry department in the hospital.

Statistics

Student's unpaired *t*-test was used for baseline comparisons between groups. The effects of exercise training in metabolically healthy and metabolically compromised obese subjects were compared by two-way repeated measures ANOVA, using time (baseline and post-intervention) as within-subject factor and group as between-subject factor. When a significant time*treatment interaction was observed, post-hoc analysis with Bonferroni correction was applied to identify significant within-group effects. All variables were checked for normal distribution and were Ln-transformed to satisfy conditions of normality. All data are presented as means ± SEM. Calculations were done using SPSS 21 for Mac OS X (IBM, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

RESULTS

Anthropometric and clinical characteristics

Subject characteristics before and after the 12-weeks supervised, progressive exercise training program are summarized in Table 1. At baseline, fasting plasma glucose ($P=0.009$) and HOMA-IR ($P=0.016$) were significantly higher, whereas peripheral ($P<0.001$), adipose tissue ($P=0.048$) and hepatic insulin sensitivity ($P<0.001$) were significantly lower in the obese metabolically compromised as compared to age and BMI-matched obese metabolically healthy controls (Table 1). As expected, VO_{2max} ($P_{time}<0.001$), W_{max} ($P_{time}<0.001$) and 1RM ($P_{time}<0.001$) were significantly increased following the exercise training intervention in both groups. The training program significantly improved peripheral insulin sensitivity to a similar extent in all subjects ($P_{time}=0.015$; $P_{time*group}=0.106$), but did not significantly affect hepatic ($P_{time}=0.213$) and adipose tissue insulin sensitivity ($P_{time}=0.943$) (Table 1). Furthermore, total fat mass ($P_{time}=0.037$) and body fat percentage ($P_{time}=0.008$) were slightly but significantly decreased, whereas body weight ($P_{time}=0.866$), BMI ($P_{time}=0.890$), fat free mass ($P_{time}=0.309$) and plasma glucose, NEFA and TAG concentrations remained unaltered after the training intervention (Table 1).

Adipocyte morphology

At baseline, no differences in mean adipocyte size and adipocyte size distribution were observed between groups (Figure 1A). The training intervention did not affect mean adipocyte size, neither in the total group (62.9 ± 1.4 vs. 63.3 ± 1.4 µm, P=0.860, Figure 1A) nor in both groups separately (Figure 1A). In line, adipocyte size distribution was not affected by the exercise training in the total group (Figure 1B) and subgroups (Figure 1C).
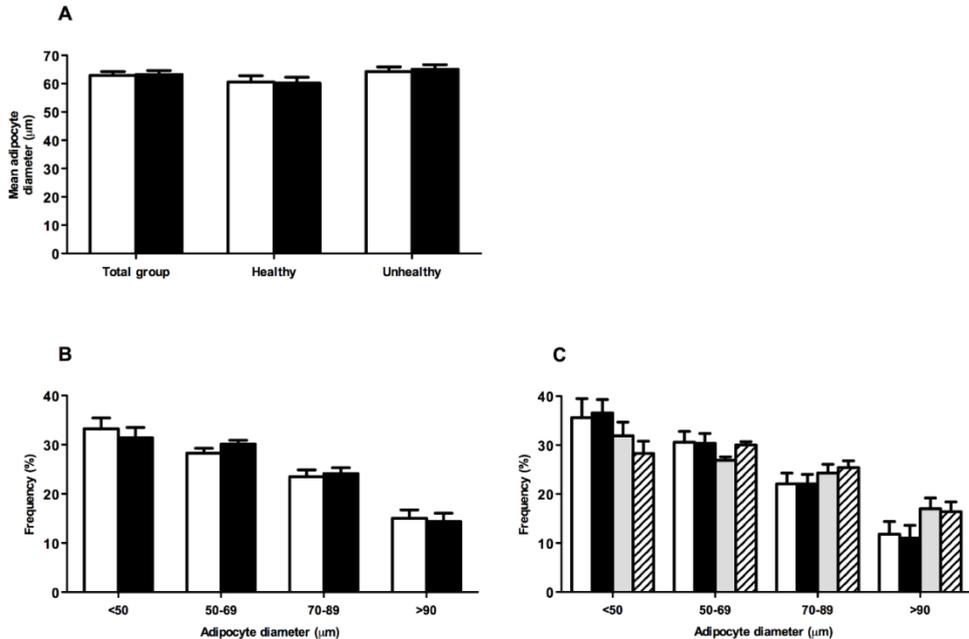
Figure 1. Exercise training-induced effects on adipose tissue morphology.

Mean adipocyte size (**A**) and adipocyte size distribution in the total group (**B**); adipocyte size distribution in the metabolically healthy and metabolically compromised subjects (**C**).
Panel A and B: white bars, baseline values; black bars, post-intervention values.
Panel C: white and grey bars, baseline values of the metabolically healthy and compromised subjects, respectively; black and striped bars, post-intervention values of the metabolically healthy and compromised subjects, respectively.

Table 1. Anthropometric and clinical subject characteristics before and after the 12-weeks supervised, progressive exercise training program.

|	Total group		Obese metabolically healthy		Obese metabolically compromised		P_{time}	P_{group}	$P_{time*group}$
	Baseline	Post intervention	Baseline	Post intervention	Baseline	Post intervention			
Age (years)	58.1 ± 1.6	-	60.1 ± 2.3	-	57.1 ± 2.2	-	-	-	-
Body weight (kg)	95.4 ± 2.6	95.4 ± 2.6	93.6 ± 5.5	93.9 ± 5.6	96.3 ± 3.6	96.2 ± 3.8	0.866	0.676	0.707
BMI (kg·m⁻²)	30.0 ± 0.6	30.0 ± 0.6	29.7 ± 1.2	29.8 ± 1.2	30.1 ± 0.8	30.0 ± 0.8	0.890	0.849	0.684
Fat mass (kg)	27.9 ± 1.2	27.2 ± 1.3	27.4 ± 3.0	26.8 ± 3.0	28.3 ± 1.4	27.4 ± 1.6	0.037	0.806	0.838
Fat free mass (kg)	65.7 ± 1.5	66.0 ± 1.6	64.4 ± 3.0	64.8 ± 3.2	66.4 ± 2.2	66.6 ± 2.4	0.309	0.602	0.772
Body fat percentage (%)	28.8 ± 0.7	28.2 ± 0.7	28.6 ± 1.5	28.0 ± 1.6	28.9 ± 0.8	28.3 ± 0.9	0.008	0.888	0.907
VO$_{2max}$ (ml·min⁻¹·kg⁻¹)	26.9 ± 0.9	29.5 ± 1.0	26.9 ± 1.8	29.3 ± 2.0	27.0 ± 1.0	29.6 ± 1.1	<0.001	0.929	0.910
W$_{max}$ (W·kg⁻¹)	2.0 ± 0.8	2.4 ± 1.0	2.1 ± 0.2	2.4 ± 0.2	2.0 ± 0.1	2.3 ± 0.1	<0.001	0.805	0.927
Strength (kg)	83.4 ± 3.8	97.1 ± 4.7	78.2 ± 6.8	91.2 ± 9.6	88.5 ± 5.2	101.8 ± 5.8	<0.001	0.355	0.822
Fasting plasma glucose (mmol·l⁻¹)	6.6 ± 0.5	6.6 ± 0.5	5.2 ± 0.2	5.2 ± 0.1	7.2 ± 0.8	7.4 ± 0.9	0.959	0.040	0.982
Fasting insulin (mU·l⁻¹)	13.1 ± 1.5	12.4 ± 1.4	9.8 ± 1.3	9.8 ± 1.1	13.7 ± 2.1	13.7 ± 2.0	0.353	0.188	0.748
HOMA-IR	3.7 ± 0.5	3.4 ± 0.4	2.3 ± 0.3	2.3 ± 0.2	4.1 ± 0.6	4.0 ± 0.6	0.399	0.048	0.751
Fasting plasma NEFA (µmol·l⁻¹)	702 ± 31.7	648 ± 38.8	669 ± 46.6	633 ± 90.4	735 ± 44.3	670 ± 36.2	0.184	0.566	0.691
Fasting plasma TAG (mmol·l⁻¹)	1.6 ± 0.1	1.6 ± 0.2	1.4 ± 0.2	1.4 ± 0.3	1.6 ± 0.2	1.7 ± 0.2	0.712	0.302	0.709
Rd (µmol·kg⁻¹·min⁻¹)	11.5 ± 2.2	14.2 ± 2.8	22.1 ± 2.9	27.8 ± 2.5	6.7 ± 1.1	8.0 ± 1.9	0.015	<0.001	0.106
EGP suppression (%)	-48.6 ± 4.5	-53.4 ± 5.6	-71.0 ± 3.6	-78.3 ± 10.6	-38.4 ± 2.8	-42.0 ± 2.5	0.213	<0.001	0.663
NEFA suppression (%)	-61.6 ± 3.2	-62.1 ± 3.2	-69.8 ± 2.4	-68.9 ± 3.0	-56.8 ± 4.4	-58.1 ± 4.4	0.943	0.036	0.736

Data are expressed as mean ± SEM (n=21). VO$_{2max}$: maximal aerobic capacity; W$_{max}$: maximal power output; TAG: triacylglycerol; R$_d$: glucose rate of disappearance (reflects peripheral insulin sensitivity); EGP: endogenous glucose production (EGP suppression reflects hepatic insulin sensitivity); NEFA: non-esterified fatty acid (NEFA suppression reflects adipose tissue insulin sensitivity).

Gene expression

At baseline, gene expression of *HSL* (0.35 ± 0.08 vs. 0.45 ± 0.11, P=0.005), *CGI-58* (0.39 ± 0.04 vs. 0.75 ± 0.19, P<0.001) and *PGC-1α* (-0.35 ± 0.22 vs. 0.00 ± 0.13, P=0.037) was significantly lower in the obese metabolically compromised as compared to obese metabolically healthy individuals. In the total group, the exercise training did not alter adipose tissue gene expression of *ATGL* (P_{time}=0.332, Figure 2A), *HSL* (P_{time}=0.862, Figure 2B), *PLIN1* (P_{time}=0.614, Figure 2C) and *CGI-58* (P_{time}=0.546, Figure 2D). Furthermore, the inflammatory markers *TNFα* (P_{time}=0.604, Figure 2E), *IL-6* (P_{time}=0.507, Figure 2F), *MCP-1* (P_{time}=0.222, Figure 2G) and *CD68* (P_{time}=0.688, Figure 2H) were unchanged after the intervention. Next, gene expression of the browning markers *CIDEA* (P_{time}=0.943, Figure 2I) and *PRDM16* (P_{time}=0.839, Figure 2J) and *PGC-1α* (P_{time}=0.835, Figure 2K), a major regulator of mitochondrial biogenesis and function, remained unchanged following the exercise training. Finally, gene expression of *LEP* (P_{time}=0.840, Figure 2L) and *ADIPOQ* (P_{time}=0.413, Figure 2M) was also not significantly altered after the 12-weeks training program. In line, no significant differences in exercise-induced alterations in these parameters were observed between groups, except for a slight but significant change in *CGI-58* ($P_{time*group}$=0.037).
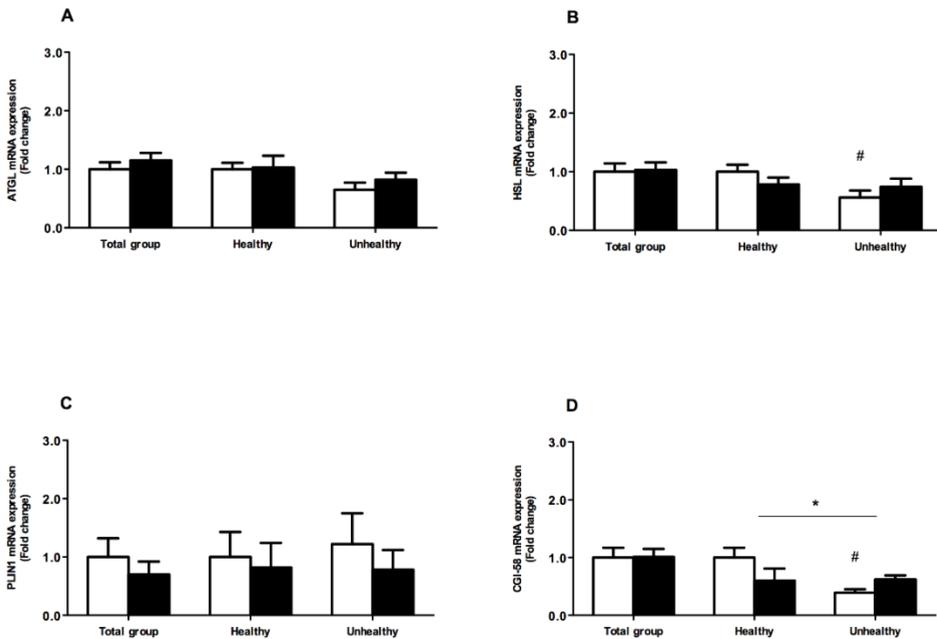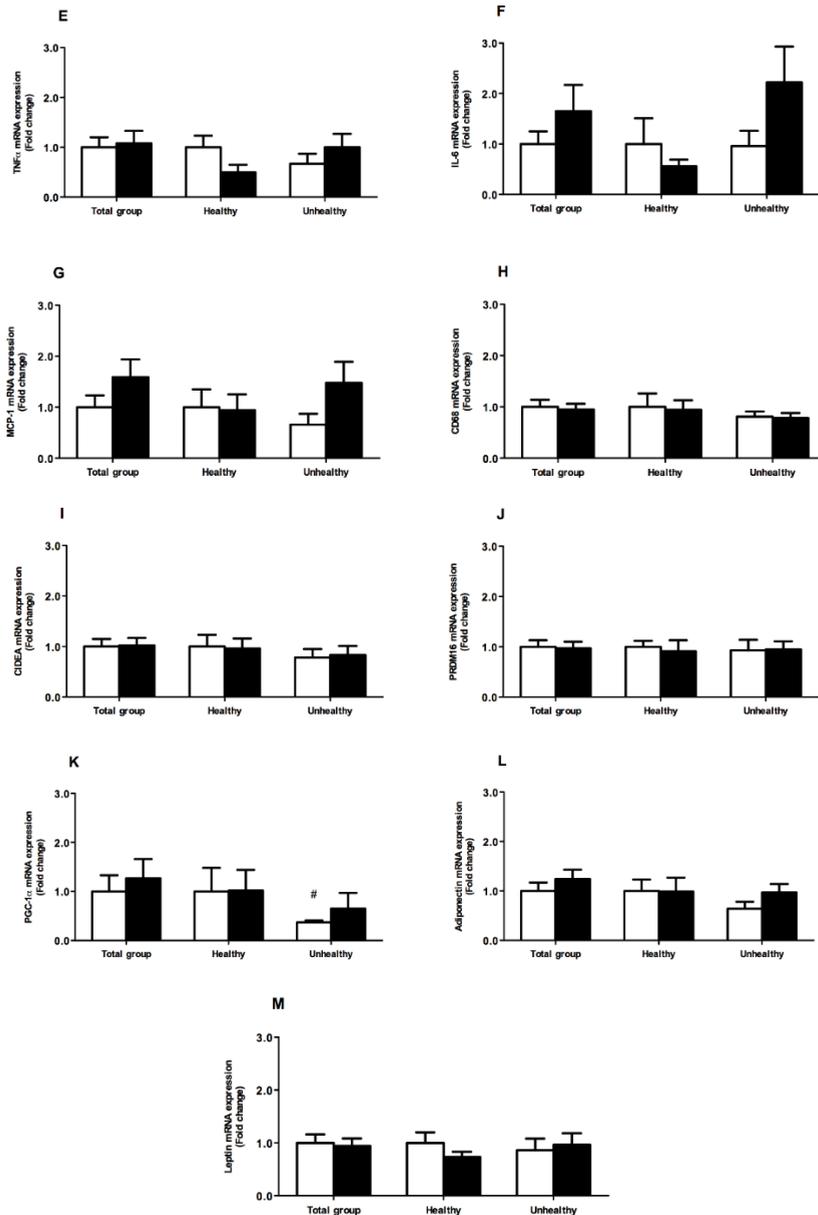
Figure 2. Exercise training-induced effects on adipose tissue gene expression.

Genes involved in lipolysis (**A-D**), inflammation (**E-H**), browning (**I-J**), mitochondrial biogenesis and function (**K**) and adipokine expression (**L-M**) are expressed as fold change relative to the baseline values of the total group and of the obese metabolically healthy control group.
* $P_{time*group} < 0.05$; #$P < 0.05$ compared to baseline value of obese metabolically healthy control group. White bars, baseline values; black bars, post-intervention values.

Protein expression

At baseline, total OXPHOS protein content was not significantly different between groups (P=0.176). In the total group, total OXPHOS protein expression remained unchanged following the training program (27.0 ± 9.7 vs. 24.9 ± 6.9 AU, P_{time}=0.826, Figure 3A). More specific, OXPHOS complex I (2.9 ± 1.2 vs. 2.9 ± 1.0 AU, P_{time}=0.857, Figure 3B), complex II (9.2 ± 2.4 vs. 8.9 ± 1.7 AU, P_{time}=0.804, Figure 3C), complex III (4.7 ± 2.7 vs. 2.1 ± 1.0 AU, P_{time}=0.549, Figure 3D), complex IV (0.8 ± 0.2 vs. 0.9 ± 0.3 AU, P_{time}=0.870, Figure 3E) and complex V (14.5 ± 6.8 vs. 13.3 ± 5.3 AU, P_{time}=0.666, Figure 3F) were not affected. In line, no significant differences in exercise-induced alterations in OXPHOS protein complexes were observed between groups (Figure 3A-F).
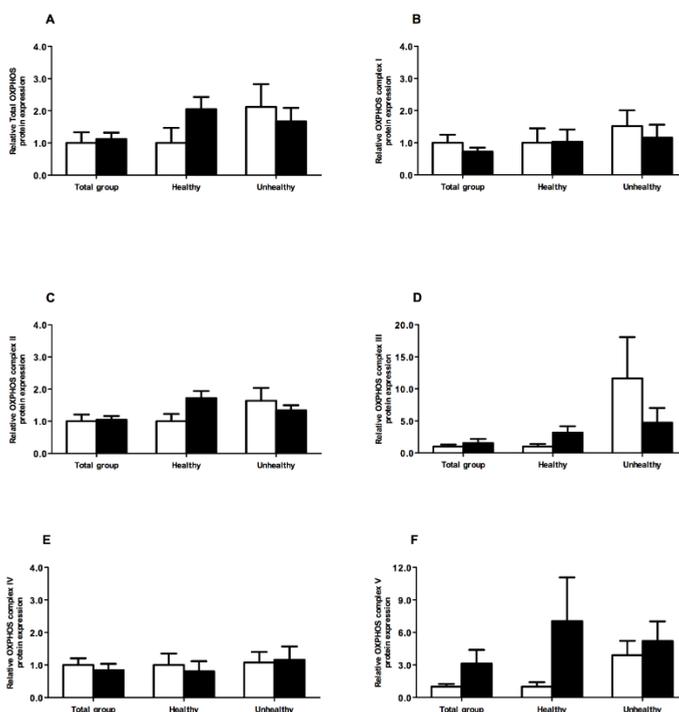
Figure 3. Exercise training-induced effects on adipose tissue mitochondrial oxidative phosphorylation (OXPHOS) protein expression.

Protein content of total OXPHOS (A), OXPHOS complex I (B), OXPHOS complex II (C), OXPHOS complex III (D), OXPHOS complex IV (E) and OXPHOS complex V (F), expressed as fold change relative to baseline values of the total group (time effect) and of the obese metabolically healthy group (time*treatment interaction).
White bars, baseline values; black bars, post-intervention values.

Ex vivo adipocyte lipolysis

The potency of salbutamol to stimulate lipolysis was determined by its EC_{50}, which represents the concentration of agonist inducing 50% of its maximal lipolytic response. The dose-response curve for salbutamol on ex vivo adipocyte lipolysis is presented in supplementary Figure S1. In the total group, the training intervention did not induce a significant change in basal lipolysis (7.0 ± 0.9 vs. 5.9 ± 0.6 µmol glycerol·10^7 cells^{-1}·2h incubation^{-1}, P=0.108), maximal lipolysis (18.1 ± 2.1 vs. 14.5 ± 1.9 µmol glycerol·10^7 cells^{-1}·2h incubation^{-1}, P=0.111) or the potency of salbutamol (-logEC_{50}) (6.0 ± 0.2 vs. 5.8 ± 0.3, P=0.555).

DISCUSSION

The aim of the present study was to investigate the effects of a 12-weeks supervised, progressive exercise training program on the abdominal subcutaneous adipose tissue phenotype in metabolically healthy and metabolically compromised, well-phenotyped obese individuals. Here, we demonstrate that exercise training did neither alter abdominal subcutaneous adipocyte morphology and adipose tissue gene expression of markers for mitochondrial biogenesis/function, browning, lipolysis, inflammation and adipokines, adipose tissue OXPHOS protein content, nor β_2-adrenergic stimulation of adipocyte lipolysis in obese subjects, irrespective of baseline metabolic status. These data suggest that alterations in the phenotype of abdominal subcutaneous adipose tissue do not significantly contribute to the exercise-induced improvement in peripheral insulin sensitivity in obese men when adipose tissue mass is only slightly reduced (~0.7 kg).

The training program induced a significant increase in aerobic capacity, maximal power output and maximal muscle strength, indicating that the supervised, progressive nature of the program was successful regarding enhancement of physical fitness. This was accompanied by a slight but significant decrease in fat mass and body fat percentage. In agreement with the findings in a larger study population [31], we observed that peripheral insulin sensitivity was significantly increased, whereas hepatic and adipose tissue insulin sensitivity remained unchanged after the training program.

Exercise training interventions may affect adipocyte morphology in humans [30]. We demonstrated that a 12-weeks exercise training intervention did not significantly alter mean adipocyte size or adipocyte size distribution, despite a 0.7 kg decrease in total fat mass. In contrast, Despres *et al.* [32] demonstrated that 20 weeks of endurance training decreased mean adipocyte size in young men, but not in women. Importantly, they observed a more pronounced reduction in body weight (~3.0 kg). Thus, a more prolonged intervention period, leading to a more pronounced decrease in adipose tissue mass, seems necessary to induce beneficial changes in adipocyte morphology.

Since an altered rate of lipolysis is one of the characteristics of adipose tissue dysfunction and relates to peripheral insulin resistance [8], we determined adipose tissue gene expression of lipolytic enzymes and genes encoding lipid droplet-associated proteins. At baseline, gene expression of *HSL* and *CGI-58* was lower in obese metabolically compromised as compared to obese metabolically healthy subjects. These findings are in line with previous studies from our group and

others, showing a reduced expression of lipolytic genes in obese insulin resistant [33] and patients with T2DM [34] as compared to obese insulin sensitive individuals. Conflicting results on the impact of exercise training on basal and stimulated adipose tissue lipolysis in humans has been reported, as extensively reviewed [30]. Here, we show that exercise training did not alter the expression of genes related to lipolysis under fasting conditions in obese individuals, irrespective of baseline metabolic status, except for a slight change in *CGI-58*. It has previously been demonstrated that β_2-adrenergic stimulation of lipolysis is impaired in obese as compared to lean subjects, whereas β_1-adrenergic receptor sensitivity was comparable between groups [35]. We therefore determined if exercise training altered β_2-adrenergic sensitivity of lipolysis. In agreement with unchanged adipose tissue gene expression of lipolytic markers, the potency of the β_2-adrenergic receptor agonist salbutamol to stimulate abdominal subcutaneous adipocyte lipolysis was comparable before and after the exercise training program. Furthermore, basal and maximal β_2-adrenergic receptor-mediated lipolysis remained unchanged after exercise training. The present findings are in agreement with a previous study showing no improvement of β_2-adrenergic stimulation of lipolysis after 12 weeks of training in obese non-diabetic men [36]. However, in contrast to the present findings, a decreased basal lipolysis was found after exercise training in the latter study [36]. This may be explained by the modest loss of fat mass in the present study, which did not result in a reduction in adipocyte size. Indeed, it has previously been demonstrated that substantial weight loss, which decreased adipocyte size, increased and normalized the sensitivity to catecholamine-stimulated lipolysis in obese subjects [37].

In addition to impairments in lipolysis, a pro-inflammatory phenotype of adipose tissue is associated with insulin resistance in obese subjects and T2DM patients [6-8]. In the present study, no changes in macrophage infiltration and inflammatory markers in adipose tissue were found following exercise training. The absence of alterations in the inflammatory profile after exercise training is in agreement with most previous studies in obese subjects [22, 23, 25, 26]. Furthermore, we found no effects of exercise training on adipose tissue gene expression of leptin and adiponectin, which is in accordance with observations in both lean and overweight/obese humans [25, 26, 38]. Nevertheless, some studies have shown a reduction in adipose tissue gene expression of *MCP-1* [22] and an increase in adiponectin expression [23] following exercise training in obese subjects. The present findings further support the notion that a reduction in adipocyte size is required to achieve beneficial changes in the adipose tissue phenotype.

Rodent studies demonstrated that increasing brown adipose tissue mass/activity or inducing browning of white adipose tissue via cold exposure or other stimuli might be a promising strategy for the treatment of obesity and obesity-related impairments in glucose homeostasis [39]. Interestingly, physical exercise has been shown to induce browning of white adipose tissue in rodents, possibly via the secretion of the myokine irisin [15], although findings are controversial [40], or via increased natriuretic peptide concentrations [41]. However, the expression of browning markers in white adipose tissue of endurance-trained athletes was not different from lean sedentary controls [42]. In the present study, the expression of *PRDM16* and *CIDEA*, markers of beiging/browning of white adipose tissue, did not change following the intervention. In agreement with our findings, no exercise

training-induced changes in browning markers in white adipose tissue were found in lean [43, 44] and overweight [20, 44] subjects.

The oxidative phenotype of white adipose tissue is impaired in obesity and seems related to an altered glucose homeostasis in rodents and humans [45-48]. Here, we found that baseline gene expression of *PGC-1α* was significantly lower in obese metabolically compromised as compared to metabolically healthy obese subjects, although this did not translate into significant differences in OXPHOS protein content between these BMI-matched groups. Furthermore, the exercise training did not induce alterations in adipose tissue gene expression of *PGC-1α*, which is in accordance with most [20, 38, 43], but not all previous studies [19, 49]. Finally, we observed no significant exercise-induced alterations in adipose tissue OXPHOS protein content after the training intervention, which is in line with high-intensity interval training in healthy lean [43] and overweight [50] subjects. In contrast to human data, previous rodent studies showed that endurance training had beneficial effects on intra-abdominal and epididymal white adipose tissue mitochondrial activity in rats [12], indicating species differences in mitochondrial protein expression and/or function in response to exercise.

In conclusion, the present study demonstrated that a 12-weeks supervised, progressive exercise training intervention, which improved physical fitness and peripheral insulin sensitivity, had no significant effects on abdominal subcutaneous adipocyte morphology, adipose tissue gene and protein expression of markers related to adipose tissue function, nor β_2-adrenergic sensitivity in obese subjects, irrespective of their baseline metabolic status. Noteworthy, we cannot exclude that exercise training may induce beneficial alterations in the adipose tissue phenotype after a more prolonged intervention period, leading to a more pronounced loss of fat mass, or in other fat depots.

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SUPPLEMENTARY MATERIAL

Protein expression analysis

Firstly, subcutaneous adipose tissue (500mg) was ground to a fine powder under liquid nitrogen and homogenized in radioimmunoprecipitation assay buffer (10mM Tris (Calbiochem)-HCl (Merck, Darmstadt, Germany) buffered saline (Merck) with 0,1% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories Inc, Hercules, CA, USA), 1% sodiumdeoxycholate (Sigma-Aldrich, St. Louis, MO, USA), 1% NP-40 (Fluka) and a protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Beverly, MA, USA). The homogenate was lysed on iced and vortexed for 5 min and centrifuged at 20,000 g for 30 min at 10°C. The supernatant was carefully collected and aliquots were stored at -80°C. The protein concentration was determined by the Bradford-based protein assay (Santa Cruz Biotechnology, Dallas, TX, USA).

Next, solubilized proteins were separated on a precast gel (Criterion™ TGX any kD, Bio-Rad Laboratories Inc, Hercules, CA, USA) and transferred onto a nitrocellulose membrane (Trans Blot® Turbo™ transfer system; Bio-Rad). Differences in loading were adjusted to total protein content (via Ponceau S (Sigma-Aldrich, St. Louis, MO, USA) staining) and appropriate positive controls were included.

Thereafter, quantitative western blot analysis was performed to determine the levels of the OXPHOS proteins. OXPHOS blots were probed with Total OXPHOS Antibody Cocktail (Mitoscience/Abcam, Cambridge, MA, USA) and a secondary horseradish peroxidase (HRP)-conjugated Rabbit-anti-Mouse antibody (DakoCytomation, Glostrup, Denmark). Antigen-antibody complexes were visualized using chemiluminescence (ECL) by a ChemiDoc™ XRS apparatus (Bio-Rad) and analyzed with Quantity One® software (Bio-Rad), which calculated the optical density units that are expressed as average intensity ([average intensity = total intensity of the rows of pixels inside the band boundary divided by the number of rows, minus the background intensity]).
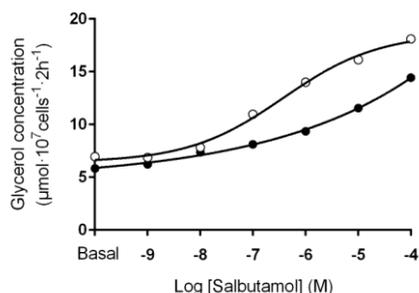
Supplementary Figure S1. The effects of salbutamol on ex vivo adipocyte lipolysis before and after exercise training in the total group (n=15).

Glycerol release into the medium (μmol glycerol$\cdot 10^7$ cells$^{-1}\cdot$2h incubation^{-1}) was used as an indicator of lipolysis.
Open circles represent baseline values; closed circles represent post-intervention values.

Supplementary Table S1: RT-qPCR Primer Sequences

The following RT-qPCR primer sequences were used for gene expression analysis.
Upper sequences represent forward primers (5' - 3'), while lower sequences represent reverse primer (5' - 3').

Genes	Sequences
ATGL	GTGTCAGACGGCGAGAATG TGGAGGGAGGGAGGGATG
HSL	GCGGATCACACAGAACCTGGAC AGCAGGCGGCTTACCCTCAC
CGI-58	CAGCATCCAGTCCTTACGACCA GTTCAGTCCACAGTGTCGCAGA
PLIN1	CTCTCGATACACCGTGCAGA TGGTCCTCATGATCCTCCTC
TNFα	CCGAGTGACAAGCCTGTAGC GAGGACCTGGGAGTAGATGAG
IL-6	AAATTCGGTACATCCTCGACGG GGAAGGTTCAGGTTGTTTTCTGC
MCP-1	CCCCAGTCACCTGCTGTTAT TCCTGAACCCACTTCTGCTT
CD68	CCCTATGGACACCTCAGCTTT GAAGGACACATTGTACTCCACC
CIDEA	TCAGACCTTGGGAGACAACACG CGAAGGTGACTCTCGCTATTCC
PRDM16	CAGCCAATCTCACCAGACACCT GTGGCACTTGAAAGGCTTCTCC
PGC-1α	TCTGAGTCTGTATGGAGTGACAT CCAAGTCGTTCACATCTAGTTCA
ADIPOQ	TGGTGAGAAGGGTGAGAA AGATCTTGGTAAAGCGAATG
LEP	GAACCCTGTGCGGATTCTTGT TCCATCTTGGATAAGGTCAGGAT

CHAPTER 7

Coordinated regulation of adipose tissue adrenergic- and non-adrenergic-mediated lipolysis during exercise in lean and obese individuals:
the effect of exercise training

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ABSTRACT

Background: Adipose tissue dysfunction, which includes impairments in (adipose tissue) lipolysis, contributes to insulin resistance. Subcutaneous adipose tissue (SCAT) lipolysis in obesity is characterized by catecholamine resistance and an impaired ANP responsiveness. It remains to be established whether exercise training improves non-adrenergically-mediated lipolysis, next to the adrenergic pathway, in metabolically compromised conditions. The aim of the present study was to investigate the effect of local combined α- and β-adrenergic receptor blockade on SCAT lipolysis in obese insulin sensitive (IS), obese insulin resistant (IR) and age-matched lean IS men. Moreover, obese individuals underwent endurance and resistance exercise training to improve metabolic profile and (non-)adrenergically-mediated SCAT lipolysis.

Methods: Abdominal SCAT lipolysis was investigated in 10 obese IS, 10 obese IR and 10 age-matched lean IS men using microdialysis in the presence or absence of local combined α- and β-adrenergic receptor blockade at rest, during 60 min of low-intense (40% VO2max) endurance-type exercise and recovery. Systemic responses were investigated using venous blood sampling. Obese individuals participated in a supervised, endurance and resistance exercise training intervention for 12 weeks (3 sessions/week) after which the microdialysis measurements were repeated in obese IR men.

Results: Exercise-induced increase in abdominal SCAT lipolysis (expressed as total area under the curve) was more pronounced in obese IS (81%) and IR (34%) as compared to lean individuals (P_{group}=0.012). Abdominal SCAT lipolysis was significantly reduced (~40%) following local combined α-/β-adrenoceptor blockade in obese IS individuals only. Despite improvements in body composition, physical fitness and exercise-induced changes in circulating free fatty acids, lactate and adrenalin, exercise intervention did not significantly affect (non-)adrenergically-mediated lipolysis in abdominal SCAT of obese IR individuals.

Conclusion: Our findings indicate a major contribution of non-adrenergically mediated lipolysis during exercise in abdominal SCAT of lean and obese individuals. Furthermore, a 12-week exercise training program improved metabolic profile and body composition in obese individuals, but did not affect abdominal SCAT lipolysis.

INTRODUCTION

Adipose tissue (AT) dysfunction is commonly observed in human obesity and contributes to insulin resistance (IR) and chronic metabolic diseases, including cardiovascular disease, type 2 diabetes mellitus (T2D) and certain types of cancer [1, 2]. Disturbances in AT lipid metabolism, including a decreased lipid uptake and impairments in lipid mobilization are closely linked to ectopic fat deposition and obesity-related IR [3]. An important function of the AT is to release fatty acids through lipolysis [4, 5], especially during fasting and increased energy demanding conditions such as exercise. Multiple endocrine factors affect the activity of lipid droplet-associated proteins and lipases, thereby regulating the release of free fatty acids (FFA) and glycerol [6]. However, impairments in the regulation of lipolysis have been identified in subcutaneous AT (SCAT) of obese humans [7], including a blunted catecholamine-mediated lipolysis [8, 9]. More specific, β-adrenergically-mediated lipolysis is reduced [9] and inhibitory α_2-adrenoceptors become predominant on adipocytes in the obese insulin resistant state [10] [11], leading to a blunted adrenergically-mediated lipolysis [9, 12]. Of interest, local β-adrenergic blockade (alone or in combination with α_2-adrenergic blockade) in SCAT, inhibits exercise-induced lipolysis only to a minor extent at low-to-moderate intensities in healthy lean [13-15] and overweight individuals [16]. In this respect, Moro *et al.* [16] demonstrated that non-adrenergically-mediated lipolysis in SCAT substantially contributes to lipid mobilization during exercise in healthy young lean men [15] and healthy young overweight men [16]. Other key regulators of lipolysis are insulin [17] and lactate [18], which both exert an inhibitory role in the physiological control of AT during exercise [17-19].

More recently, evidence has emerged that natriuretic peptides (NP) not only affect the cardiovascular system, but also have pronounced effects in several key metabolic organs such as AT and skeletal muscle [20]. Interestingly, several studies have indicated that the circulating NP concentrations are reduced in human obesity and T2D [21-23]. The latter findings, together with evidence that reduced systemic NP concentrations increase the risk of developing T2D [24, 25], highlight the importance of NP in metabolic disease. Of the NP family, atrial natriuretic peptide (ANP) has been shown to be the most potent stimulator of human AT lipolysis [26], via guanylyl cyclase-coupled natriuretic peptide receptor type A (NPRA)-mediated activation of hormone-sensitive lipase (HSL) [27, 28]. Interestingly, we have recently found that maximal ANP responsiveness is impaired in isolated abdominal subcutaneous adipocytes of obese non-diabetic and T2D men [29]. In line, Rydén and colleagues [30] have recently shown a blunted lipolytic effect of ANP in isolated abdominal subcutaneous adipocytes of obese women and *in situ* (microdialysis) in abdominal SCAT of overweight men under resting conditions. Importantly, however, the physiological role of exercise-induced ANP-mediated lipolysis in human obesity remains to be established.

It has been shown that endurance exercise training can partly improve β-adrenoceptor activity, reduce anti-lipolytic α_2-adrenoceptor sensitivity in human SCAT [31-33], and alleviate ANP-mediated lipolysis in subcutaneous adipocytes in young, metabolically healthy overweight individuals [28, 34]. However, to date, it remains elusive if endurance and resistance exercise intervention improves ANP-induced activation of lipolysis in metabolically compromised conditions.

The aim of the present study was to compare the effect of local combined α- and β-adrenoceptor blockade on local SCAT lipolysis at rest, during low-intensity endurance-type exercise and during recovery from exercise in middle-aged obese insulin sensitive (IS), obese insulin resistant (IR) and age-matched lean IS men. In addition, we investigated whether a 12-week endurance and resistance exercise training improved the metabolic profile in obese men and (non-)adrenergically-mediated abdominal SCAT lipolysis in obese IR men.

METHODS

Subjects

Ten middle-aged healthy lean insulin sensitive (IS), 10 obese IS and 10 obese insulin resistant (IR) men, matched for age and BMI (obese groups) participated in the present study. Subjects were included when they had a stable body weight for at least 3 months prior to the start of the intervention and had no contraindications for participation in an exercise training intervention based on their medical history. Major exclusion criteria were a history, or clinical symptoms, of heart, lung or kidney disease, presence of endocrine anomalies and/or the use of beta-blockers, glucose or lipid-lowering medication. Insulin sensitivity was assessed via homeostasis assessment of insulin resistance (HOMA-IR) [35]. Subjects were classified as insulin sensitive or insulin resistant when HOMA-IR was ≤ 2.3 [36] or ≥ 3.8 [37], respectively. Height, weight, waist and hip circumference and blood pressure were measured during screening. Body composition was measured using a Dual Energy X-ray Absorptiometry scan (Hologic Series Delphi-A Fan Beam X-ray Bone Densitometer). One week before the investigational protocol, peak oxygen uptake (VO_{2peak}) was determined during a maximal cardiopulmonary exercise test performed on an electrical braked cycle ergometer (Gymna Ergofit Cycle 400, Bilzen, Belgium) by using an incremental procedure (work rate increased by 15W/min until volitional exhaustion). Heart rate (electrocardiography) was monitored continuously and VO_{2peak} was measured using a Metalyzer II (Cortex Medical, Leipzig, Germany). The study was approved by the Medical Ethical Committee of the Jessa Hospital and Hasselt University, Hasselt, Belgium, and performed in accordance with the declaration of Helsinki (2008). All individuals gave written informed consent prior to the start of the study.

Experimental protocol

Subjects arrived at the hospital at 07:30 AM after an overnight fast. They were instructed to consume a standardized meal and snack the evening before the test day (total energy: 2628 kJ (626 kcal); 23.4g fat (10.4g saturated fat); 73.8g carbohydrates (of which 6.8g sugar); 28.8g protein; 2.9g salt; 2.3g fibres) and to abstain from exhausting activities 48 hours prior to the experimental protocol. On arrival, a catheter was inserted into the antecubital vein for blood sampling. Two microdialysis catheters (CMA 63, CMA Microdialysis AB, Stockholm, Sweden) were inserted percutaneously into SCAT after epidermal anesthesia (EMLA® crème: lidocaine 2.5% and prilocaine 2.5%, AstraZeneca AB) at a distance of 6-8

cm from the umbilicus (one probe on the left side and one probe on the right side of the umbilicus). The probes were connected to a microinfusion pump (Harvard apparatus, Plato BV, Diemen, The Netherlands) and perfused with Ringer solution (in mmol/l: 147 sodium, 4 potassium, 2.25 calcium and 156 chloride; Fresenius Kabi BV, 's Hertogenbosch, The Netherlands) at a perfusion rate of 2.0 µl/min. Ethanol (50 mmol/l) was added to the perfusate to semi-qualitatively estimate changes in local adipose tissue blood flow (ATBF), using the ethanol outflow/inflow (out/in) ratio [38]. A higher ethanol out/in ratio, corresponding to a lower ethanol wash-out, reflects a lower regional ATBF.

One microdialysis catheter was perfused with Ringer solution (control), while the contralateral catheter was perfused with Ringer, supplemented with 100 µmol/l phentolamine ($\alpha_{1,2}$-adrenergic receptor antagonist) (Regitin 10 mg/ml; Novartis Pharma BV, The Netherlands) and 100 µmol/l propranolol (nonselective β-adrenergic receptor antagonist) (propranolol hydrochloride, Dociton 1 mg/ml, Mibe GmbH, Germany), concentrations that completely suppress lipolysis [28, 39, 40]. After a 60-min equilibration period (recovery from insertion), two 30-min fraction of dialysate were collected at a flow rate of 0.3 µL/min after which the perfusion rate was increased to 2.0 µL/min for the remaining of the experiment. During the resting phase, three 15-min fractions of the outgoing dialysate were collected from both sites to determine the extracellular glycerol concentration (reflecting basal lipolysis). Next, subjects performed a single bout of endurance exercise for 60 min at 40% of their VO_{2max} on a cycle ergometer while heart rate was monitored continuously (Polar, Kempele, Finland). Exercise was followed by a 60-min recovery period in supine position. During exercise and recovery, dialysate samples were collected at 15 min intervals without disconnecting the microdialysis probes from the microinfusion pumps.

Ethanol concentrations were determined both in the ingoing (perfusate) and outgoing (dialysate) fluid to assess the ethanol out/in ratio as an indicator of local nutritive blood flow. Ethanol concentrations were determined at the same day, whereas dialysate samples for measurement of extracellular glycerol, glucose and lactate concentrations were immediately frozen and stored at -80 °C until analysis. Venous blood samples were taken at rest, during exercise and recovery in prechilled 20 mL tubes at 15 min intervals throughout the study protocol.

Indirect calorimetry

Substrate utilization and energy expenditure were determined at rest and during submaximal exercise via indirect calorimetry using a Metalyzer II (Cortex Medical, Leipzig, Germany). Substrate oxidation rates (g/min) and energy expenditure were calculated from VO_2 and VCO_2 [41, 42]. Water intake was allowed *ad libitum* during the exercise and recovery period.

Exercise training protocol

Obese IS and IR subjects participated in a supervised, exercise training program for 12 weeks (3 sessions per week) [43]. Subjects were asked not to change their habitual diet during the intervention period. Each training session started with cycling (Excite Bike, Technogym, Zaventem, Belgium) for 45min at 65% VO_{2peak} (heart rate based) from which mean heart rate, mean workload and total energy

expenditure (calories) were collected. Next, resistance exercises of 5 large muscle groups were performed at 65-70% of 1 RM (leg press, leg curl, leg extension, vertical traction, arm curl and chest press; Technogym). Training volume and load were gradually increased during the intervention whereby resistance training was increased every 3 weeks. Training sessions were supervised to assure compliance and safety of the participants. After the 12 weeks of exercise training the experimental protocol was repeated and venous blood samples were taken at rest, during exercise and recovery at 15 min intervals. In addition, SCAT microdialysis was performed in obese IR subjects, as described above.

Biochemical analysis

Microdialysate samples were analyzed for glycerol, glucose and lactate concentrations by means of bioluminescence on an ISCUS clinical microdialysis analyzer (M dialysis AB, Stockholm, Sweden). Ethanol concentrations in dialysate (out) and perfusate (in) were measured spectrophotometrically using a COBAS FARA semi-automatic analyzer (Roche Diagnostics, Basal, Switzerland) and using a standard ethanol assay kit (Boehringer Mannheim, Germany).
Blood samples were centrifuged at 4°C for 10 min at 1200 g and plasma and serum was stored at -80°C until further analysis. Plasma free glycerol was measured after precipitation with an enzymatic assay (Enzytec^{TM} Glycerol, Roche Biopharm, Switzerland), automated on a Cobas Fara spectrophotometric autoanalyzer (Roche Diagnostics, Basel, Switzerland). Plasma FFA, glucose and lactate concentrations were measured with enzymatic assays on an automated spectrophotometer (ABX Pentra 400 autoanalyzer, Horiba ABX, Montpellier, France). Plasma ANP concentrations were measured using an enzyme immunoassay (RayBiotech, Norcross GA, USA). Catecholamine concentrations (adrenalin and noradrenalin) were determined using high performance liquid chromatography with electrochemical detection (ClinRep® Complete Kit for Catecholamines in Plasma, RECIPE chemicals & Instruments GmbH, Munich, Germany). Serum insulin concentrations were determined with radioimmunoassay kits (Human Insulin specific RIA Kit, Millipore Corporation, MA, USA).

Statistical analysis

All data are expressed as means ± SEM. Normal distribution was tested by the Kolmogorov-Smirnov test. Subjects were excluded from analyses when dialysate samples of 2 subsequent time points were missing, in order to maintain paired samples. Dialysate and systemic exercise responses were expressed as the area under the curve (AUC) and the incremental area under the curve (iAUC), calculated by the trapezoid method. Cross-sectional analyses (differences between groups and conditions) for the microdialysis lipolysis data were analyzed with a two-way repeated-measures ANOVA. In case of significance, post-hoc analyses with Bonferroni correction were applied to identify significant within-group effects. Differences in plasma concentrations and substrate metabolism between groups were tested with a one-way ANOVA and differences within groups were analyzed by means of paired *t*-test. Intervention effects in the obese groups were analyzed with a two-way repeated-measures ANOVA (with pre- and post-intervention as conditions), with Bonferroni post-hoc correction to detect within-group effects.

Three subjects dropped out of the exercise intervention, due to medical (n=1) or motivational reasons (n=2) and were therefore excluded from the intervention (pre vs. post) analyses. SPSS 21 for Macintosh OS X was used to perform all calculations (IBM Corporation, Armonk, NY, USA). The level of statistical significance was set at $p<0.05$ (2-tailed), while $p<0.10$ was considered a tendency.

RESULTS

BASELINE

Anthropometric and clinical characteristics

Subjects' characteristics are presented in Table 1. By design, there was a significant difference between the lean and the obese IS and/or obese IR group with respect to body weight, BMI, WH-ratio, whole-body fat percentage, android (*i.e.* trunk region) and gynoid (including hip and leg regions) fat mass (all $p<0.05$). Furthermore, HOMA-IR and fasting serum insulin concentrations were significantly higher in obese IR compared to the lean and obese IS individuals ($p<0.001$ for both parameters in both groups). Obese IS and obese IR individuals only differed in android fat mass, which was higher in the obese IR group ($p=0.017$) (Table 1). With respect to physical fitness, $VO_{2peak/FFM}$ and $W_{peak/FFM}$ were significantly lower in the obese IS and obese IR group as compared to the lean group ($p<0.01$ and $p<0.001$, respectively) (Table 1), while maximal heart rate and maximal respiratory quotient (RQ) were comparable between groups.

Table 1. Characteristics of obese insulin sensitive, obese insulin resistant individuals and healthy lean controls

	Lean (n=10)	Obese insulin sensitive (n=10)	Obese insulin resistant (n=10)	P ANOVA
Age, years	45 ± 2	47 ± 2	43 ± 1	0.527
Cardiometabolic risk				
Fasting plasma glucose (mmol/L)	5.5 ± 0.0	5.2 ± 0.1	5.8 ± 0.2 †	0.050
Fasting serum insulin (mU/L)	7.3 ± 0.6	9.2 ± 0.6	19.6 ± 1.6 *** †	<0.001
HOMA-IR	1.8 ± 0.1	2.1 ± 0.1	5.0 ± 0.4 *** †	<0.001
Systolic BP (mmHg)	122 ± 2	135 ± 6	143 ± 6	0.066
Diastolic BP (mmHg)	72 ± 1	81 ± 4	86 ± 5	0.108
Body composition				
Body weight (kg)	79.9 ± 2.9	101.5 ± 3.2 **	109.6 ± 4.7 ***	<0.001
Body mass index (kg/m²)	23.7 ± 0.4	32.6 ± 0.4 ***	33.9 ± 0.7 ***	<0.001
Waist-to-hip ratio	1.00 ± 0.00	1.04 ± 0.01 *	1.05 ± 0.01 *	0.010
Fat mass (kg)	16.4 ± 1.1	30.0 ± 1.7 ***	34.2 ± 1.8 ***	<0.001
Fat percentage (%)	22.0 ± 0.8	31.4 ± 0.9 ***	33.2 ± 1.1 ***	<0.001
Fat free mass (kg)	57.4 ± 1.8	65.0 ± 1.5	68.4 ± 2.8 **	0.004
Exercise capacity				
VO2 peak (ml*min^{-1}*kg^{-1} (FFM))	62 ± 3	48 ± 2 **	48 ± 1 **	0.002
Wmax (Watt*kg^{-1} (FFM))	4.9 ± 0.1	3.7 ± 0.2 ***	3.4 ± 0.1 ***	<0.001

Data are mean ± SE. * Significantly different from lean group $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
† Significantly different from obese insulin sensitive group ($p < 0.05$). FFM: fat free mass; HR: heart rate; RER: respiratory exchange ratio; VO$_2$ peak: maximum oxygen uptake; Wmax: maximum power output

Systemic responses during rest, exercise and recovery phase

Under resting conditions, plasma glycerol, FFA, glucose, lactate, ANP, adrenalin and noradrenalin concentrations were comparable between groups, while fasting serum insulin concentration was higher in obese IR compared to lean and obese IS individuals (Figure 1 A-H). During exercise, plasma concentrations of glycerol, FFA, glucose, ANP, adrenalin and noradrenalin increased to the same extent in all groups (Figure 1). Exercise increased plasma lactate concentrations in all groups, which was most pronounced in obese IR as compared to lean individuals (p_{ANOVA}=0.044) (Figure 1 D). The exercise-induced increase in plasma ANP was similar in all groups (p_{ANOVA}=0.300), but peak plasma ANP concentrations were reached earlier during exercise in the lean compared to the obese groups (p=0.034) (Figure 1 E). Serum insulin levels were significantly higher during exercise in obese IR as compared to obese IS and lean individuals, with no differences between the latter two groups (Figure 1 F). During exercise, the increase in plasma adrenalin and noradrenalin concentrations was comparable between groups (Figure 1 G-H). During recovery, plasma glycerol, glucose, adrenalin, noradrenalin and ANP concentrations decreased back to baseline concentrations (Figure 1), while plasma lactate concentrations tended to remain elevated in the obese IR group (p_{ANOVA}=0.061) (Figure 1 D). Furthermore, plasma FFA concentrations peaked in the first 15 minutes of the recovery period and

remained significantly elevated in obese IR as compared to lean individuals (p_{ANOVA}=0.020) (Figure 1 B). Serum insulin concentrations remained significantly elevated during recovery in the obese IR group compared to the obese IS and lean group (both p_{ANOVA}<0.001), with no differences between the latter two groups (Figure 1 F). Detailed systemic plasma responses during baseline, exercise and recovery are shown in Supplementary Table S1.
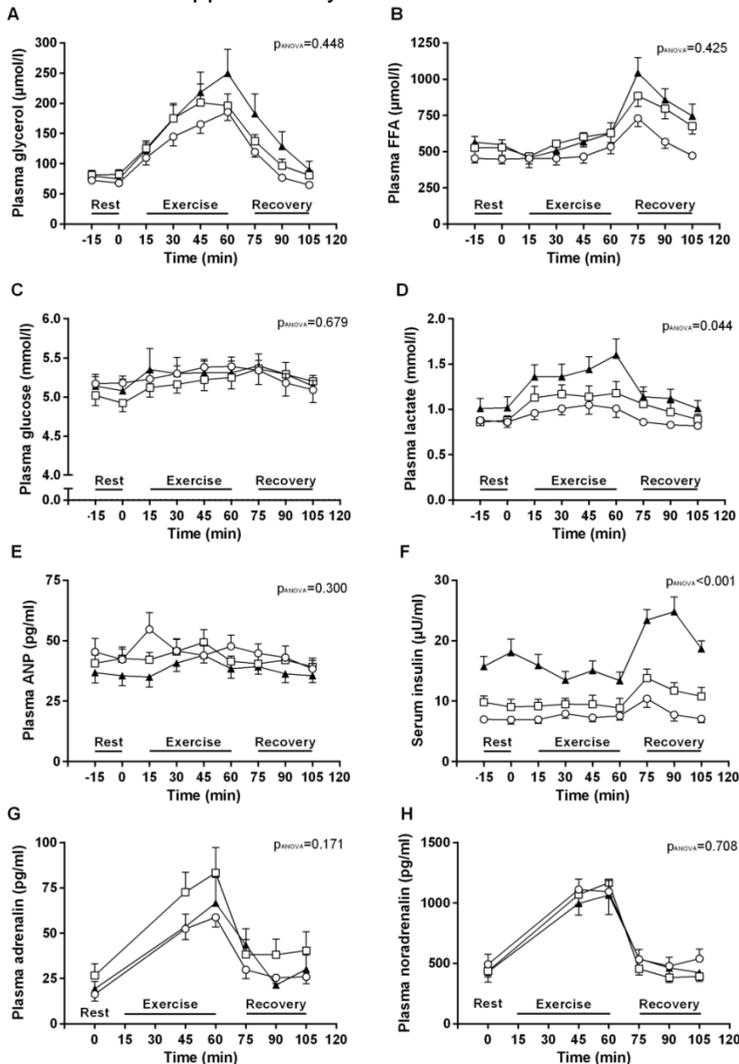
Figure 1. Plasma glycerol, FFA, glucose, lactate, ANP, adrenalin, noradrenalin and serum insulin concentrations at rest, during exercise and recovery.

Systemic glycerol (**A**), FFA (**B**), glucose (**C**), lactate (**D**), ANP (**E**), insulin (**F**), adrenalin (**G**) and noradrenalin (**H**) responses in lean (white circles), obese insulin sensitive (white squares) and obese insulin resistant (black triangles) individuals. Data are presented as mean ± SEM. P_{ANOVA} values represent differences in exercise-induced systemic responses between groups.

Substrate oxidation and energy expenditure

Substrate oxidation and energy expenditure were determined at rest and during exercise. Whole-body energy expenditure (kJ/min), RQ, carbohydrate and fat oxidation (as percentage of energy expenditure) were not different between groups at rest, nor during exercise (Supplementary Figure 1 A-E).

Microdialysis

Abdominal subcutaneous adipose tissue blood flow

At rest, lean individuals had a significantly lower ethanol out/in ratio, reflecting a higher ATBF, compared to both obese groups (p<0.01), whilst no significant difference in ATBF was observed between both obese groups (Figure 2 A, C and E). Local α-/β-adrenergic blockade induced a significant increase in ethanol out/in ratio in the lean group (Figure 2 A), reflecting a reduced ATBF, while this effect disappeared during exercise. Moreover, this adrenergic sensitivity of ATBF was not observed in the obese IS or IR group (Figure 2 C and E). Exercise induced a decrease in ethanol out/in ratio, reflecting an increase in ATBF, in all groups. This exercise-induced increase in ATBF tended to be higher in lean as compared to obese IS and obese IR individuals (p=0.093, p=0.087, respectively) (Figure 2 A, C and E). During recovery, ATBF returned to resting levels, with a significantly higher ATBF (*i.e.* a lower ethanol out/in ratio) in the lean group compared to both obese groups (Figure 2 A, C and E). Details with respect to ethanol out/in ratio during baseline, exercise and recovery are shown in Supplementary Table S2.
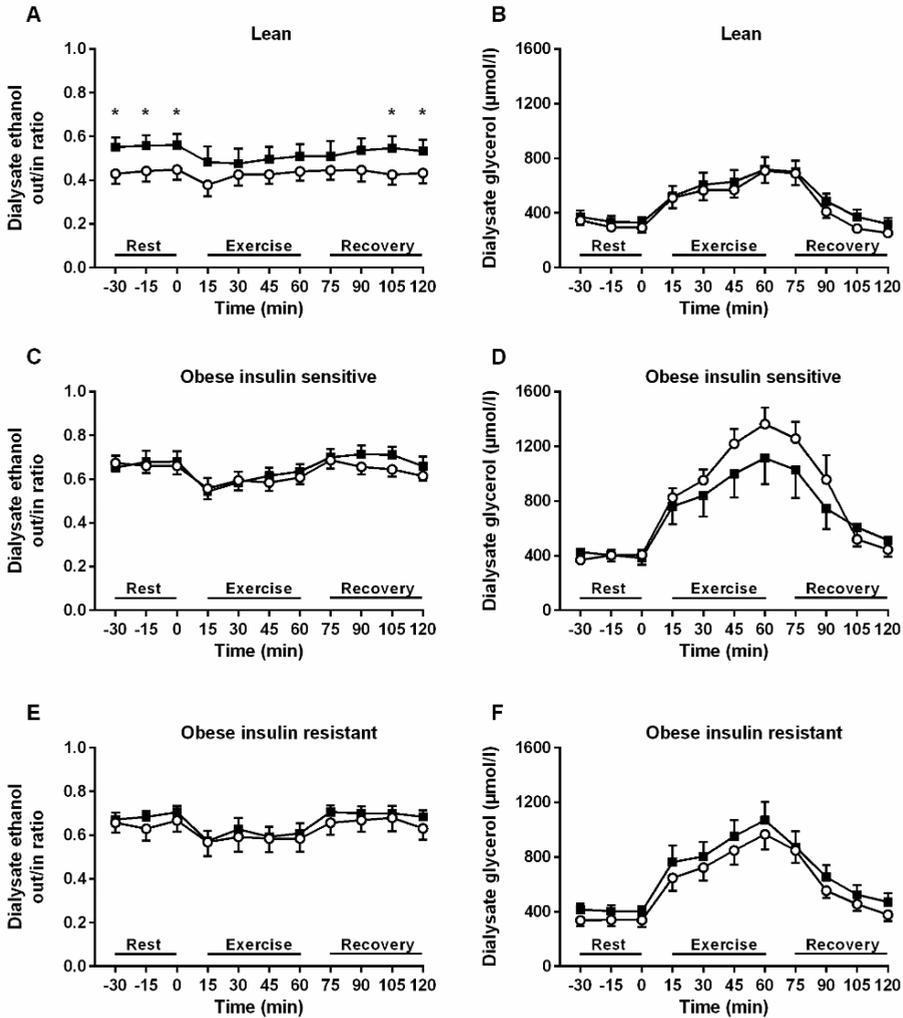
Figure 2. Changes in subcutaneous adipose tissue extracellular glycerol concentration and adipose tissue blood flow indices.

Subcutaneous adipose tissue ethanol ratio's in lean (**A**), obese insulin sensitive (**B**) and obese insulin resistant (**C**) individuals. Changes in extracellular glycerol concentration in lean (**D**), obese insulin sensitive (**E**) and obese insulin resistant (**F**) individuals at rest, during exercise and recovery in control probe (white circles) and the probe perfused with phentolamine and propranolol (black squares). Data are presented as mean ± SEM. * Significantly different from the control probe (p<0.05).

Abdominal subcutaneous adipose tissue lipolysis

In SCAT, resting extracellular glycerol concentrations were comparable between groups (Figure 2 B, D and F). Local α-/β-adrenergic blockade had no significant effects on resting extracellular SCAT glycerol concentration in either of the groups (Figure 2 B, D and F). During exercise, extracellular glycerol concentration significantly increased in all groups. In obese IS individuals, exercise-induced increase in glycerol concentration (AUC_{0-60}) was higher compared to lean (p=0.011), but not obese IR individuals (p=0.816) ((Figure 2 B, D and F). Local α-/β-adrenergic blockade induced a significant reduction in the exercise-induced increase in extracellular glycerol in the obese IS group (p=0.020), but not in the lean IS or obese IR group (Figure 3). During recovery, extracellular glycerol concentrations decreased in all groups, but remained significantly elevated in the obese IS as compared to the lean group, with no differences between both obese groups (Figure 2 B, D and F). Additionally, there were no significant effects of α-/β-adrenergic blockade on the extracellular glycerol concentration during recovery in any group (Figure 2 B, D and F). Details with respect to dialysate glycerol concentrations at rest, during exercise and recovery are shown in Supplementary Table S2.

Abdominal SCAT extracellular glucose and lactate concentrations and responses were comparable between groups at rest, during exercise and recovery (Supplementary Table S3).
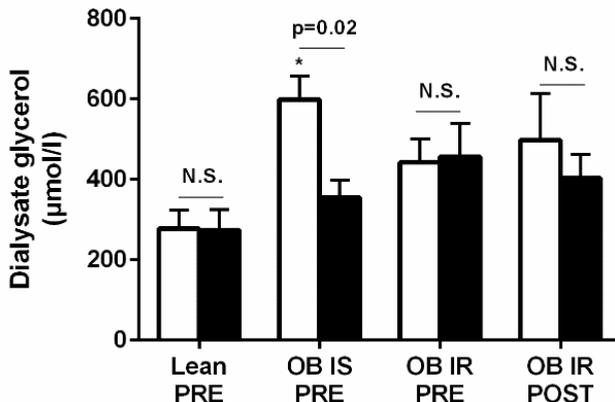
Figure 3. Exercise-induced increase in subcutaneous adipose tissue extracellular glycerol concentration.

Mean changes in subcutaneous adipose tissue extracellular glycerol concentration during 1 h of low-intense exercise (40% VO2peak). Extracellular glycerol concentrations are determined in control probe (white bars) and the probe perfused with phentolamine and propranolol (black bars). Changes were calculated by the difference between the mean glycerol concentrations during exercise and the baseline concentration (p_{group}=0.009, $p_{treatment}$=0.069, $p_{treatment*group}$=0.035). Data are presented as mean ± SEM.
* Significantly (p<0.05) different compared to the control probe from the lean group; N.S.: not significant.

EXERCISE TRAINING INTERVENTION

Anthropometry, exercise capacity and systemic responses

In both obese groups, exercise training led to a significant reduction in body weight, BMI, whole-body fat percentage as well as android and gynoid fat mass (Table 2). Whole-body insulin sensitivity (HOMA-IR) was significantly improved in the obese IR group (p_{time}=0.005), but not in the obese IS group. Furthermore, physical fitness ($VO_{2peak/FFM}$ as well as $W_{max/FFM}$) improved significantly following the 12-week exercise training (Table 2).

The training intervention induced a significant reduction in resting plasma FFA (Figure 4 C and D) and tended to reduce fasting ANP concentrations in both obese groups (Figure 4 K and L). Resting blood glucose concentration increased in the IS group but not in the IR group (Figure 4 E and F). The training intervention did not induce significant changes in plasma glycerol, insulin, lactate, adrenalin or noradrenalin concentrations. However, resting insulin concentrations, as well as insulin concentration during exercise and recovery remained elevated in the obese IR compared to obese IS group (Figure 4 I-J).

The exercise-induced increase in plasma FFA (Figure 4 C and D), lactate (Figure 4 G and H) and adrenalin (Figure 4 M and N) concentrations were significantly blunted after intervention. Peak blood ANP concentrations (Figure 4 K and L) tended to be reduced in both obese groups. The increase in plasma glycerol, insulin and glucose concentrations during exercise remained unchanged.

In the recovery period, beside reduced plasma FFA (Figure 4 C and D) and adrenalin concentrations (Figure 4 M and N), no significant training-induced changes were observed in plasma glycerol, glucose, lactate, ANP, noradrenalin or serum insulin concentrations (Figure 4). In addition, the training intervention did not induce changes in whole-body energy expenditure and substrate oxidation (Supplemental Figure 1 A-E). Detailed post-intervention systemic plasma responses at rest, during exercise and recovery are shown in Supplementary Table S4.
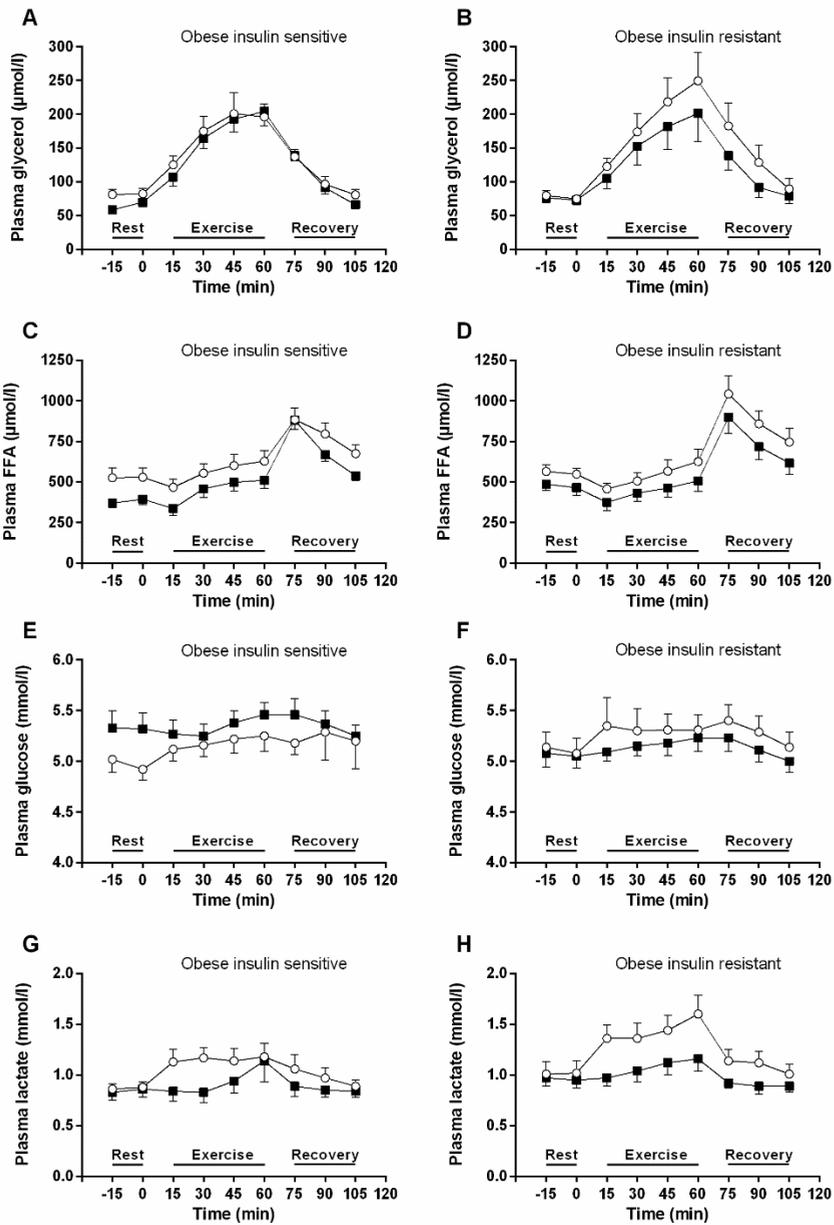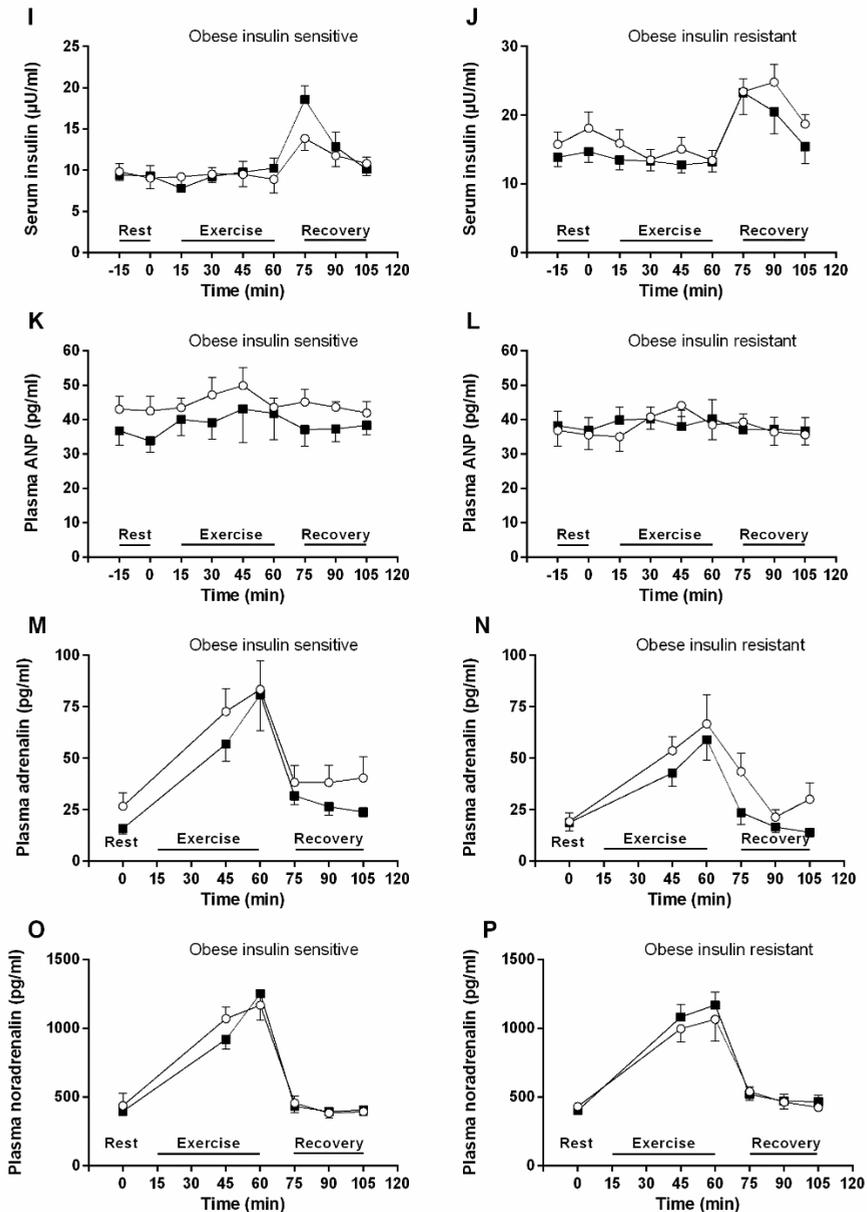
Figure 4. Exercise training-induced changes in systemic plasma glycerol, FFA, glucose, lactate, ANP, adrenalin, noradrenalin and serum insulin in obese individuals.

Systemic glycerol (**A-B**), FFA (**C-D**), glucose (**E-F**), lactate (**G-H**), insulin (**I-J**), ANP (**K-L**), adrenalin (**M-N**) and noradrenalin (**O-P**) of obese insulin sensitive and obese insulin resistant individuals at baseline (white circles) and after 12 weeks of exercise training intervention (black squares). Data are presented as mean ± SEM.

Abdominal subcutaneous adipose tissue blood flow and lipolysis

Following exercise intervention, SCAT lipolysis was investigated in the obese IR group only. Ethanol out/in ratio (Figure 5 A) as well as resting, exercise-induced and recovery-related extracellular glycerol concentration (Figure 5 B) were not altered after exercise intervention. Additionally, α-/β-adrenergic blockade had no significant effect on resting, exercise-induced or recovery-related extracellular glycerol concentration in SCAT following the exercise training program (Figure 5 B; Figure 3).
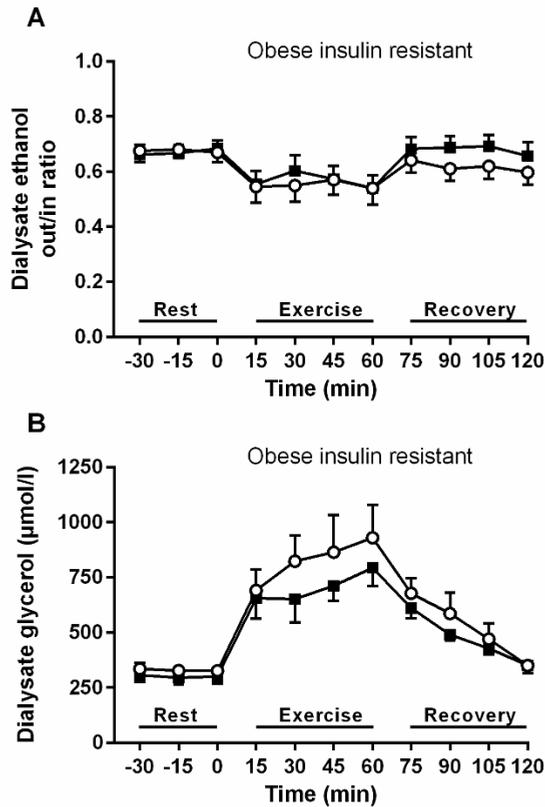
Figure 5. Exercise training-induced changes in subcutaneous adipose tissue extracellular glycerol concentration and adipose tissue blood flow indices in obese insulin resistant individuals.

Subcutaneous adipose tissue ethanol ratio (**A**) and extracellular glycerol concentration (**B**) in the obese insulin resistant individuals at rest, during exercise and recovery after 12 weeks of exercise training in control probe (white circles) and the probe perfused with phentolamine and propranolol (black squares). Data are presented as mean ± SEM.

Table 2. Body composition and exercise capacity before and after 12 weeks of supervised endurance and resistance training

	Obese insulin sensitive N = 8		Obese insulin resistant N = 9		P_{Time}	P_{Group}	$P_{Time*Group}$
	PRE	POST	PRE	POST			
Body composition							
Body weight (kg)	104.5 ± 3.2	102.8 ± 2.6	110.6 ± 5.1	108.3 ± 5.0	0.002	0.360	0.585
Body mass index (kg/m²)	32.7 ± 0.5	32.2 ± 0.3	34.1 ± 0.8	33.3 ± 0.8	0.002	0.225	0.550
Waist-to-hip ratio	108.8 ± 1.6	108.8 ± 1.5	117.6 ± 3.3	113.5 ± 1.3	0.039	0.004	0.076
Fat mass (kg)	31.22 ± 1.91	29.11 ± 1.69	35.00 ± 1.92	33.07 ± 2.02	<0.001	0.168	0.833
Fat percentage (%)	31.6 ± 1.1	30.0 ± 1.0	33.7 ± 1.1	32.8 ± 1.2	<0.001	0.150	0.267
Fat free mass (kg)	66.8 ± 1.4	67.2 ± 1.3	68.5 ± 3.1	67.5 ± 3.3	0.377	0.779	0.037
Exercise capacity							
VO2 peak (ml*min^{-1}*kg^{-1} (FFM))	49 ± 2	53 ± 1	48 ± 1	55 ± 2	0.012	0.800	0.521
Wmax (Watt*kg^{-1} (FFM))	3.8 ± 0.2	4.1 ± 0.1	3.4 ± 0.1	4.0 ± 0.1	<0.001	0.382	0.069

Data are mean ± SE. FFM: fat free mass; VO2 peak: maximum oxygen uptake; Wmax: maximum power output.

DISCUSSION

The present study is the first to investigate (non-)adrenergically-mediated lipolysis during low-intensity endurance-type exercise in abdominal subcutaneous adipose tissue (SCAT) in middle-aged obese IS and obese IR men as compared to age-matched lean IS men. Furthermore, we examined whether a 12-week exercise intervention altered resting and exercise-induced (non-)adrenergically-mediated SCAT lipolysis in obese IR individuals. Here, we demonstrated that the exercise-induced increase in abdominal SCAT extracellular glycerol concentration (reflecting local lipolysis) was more pronounced in obese as compared to lean individuals, which may at least partly be explained by the higher adipose tissue blood flow (ATBF) in lean individuals. Exercise-induced SCAT lipolysis was substantially reduced (~40%) following local combined α-/β-adrenergic blockade in obese IS individuals, but not in obese IR or lean IS individuals. Finally, the exercise training intervention improved body composition, physical fitness and exercise-induced systemic responses in both obese groups, and insulin sensitivity in the obese IR group. However, this was not accompanied by changes in adrenergically- and non-adrenergically-mediated lipolysis in the SCAT of obese IR individuals. Collectively, our findings indicate that exercise-induced lipolysis is predominantly mediated by non-adrenergic factors, most likely mediated by natriuretic peptides (NP) in middle-aged lean IS, obese IS and obese IR individuals.

The present study showed a more pronounced increase in exercise-mediated SCAT extracellular glycerol concentration in obese IS as compared to lean individuals. Although the lipolytic response in abdominal SCAT is often blunted in human obesity [9], the higher exercise-induced extracellular glycerol levels in both obese groups is likely explained by the substantially lower ATBF in the obese state, which contributes to higher extracellular glycerol concentrations due to a lower removal of glycerol from the AT [16, 44]. Local α-/β-adrenergic blockade substantially reduced basal ATBF in lean but not in the obese individuals. This might suggest that adrenergic sensitivity of ATBF is reduced in the obese state, which is in line with previous findings [39, 45]. Thus, these differences in ATBF between lean and obese individuals should be taken into account when interpreting local SCAT lipolysis.

Interestingly, combined α-/β-adrenergic receptor blockade reduced exercise-induced SCAT lipolysis in obese IS men but not in lean and obese IR individuals. In obese IR individuals, the lipolytic activity of the β-adrenergic receptors is attenuated [9, 11, 12], while an increase in anti-lipolytic α₂-adrenergic receptors in SCAT reduce exercise-mediated lipolysis [39]. In addition, obese IR individuals often display lower plasma catecholamine (adrenaline, noradrenaline) responses to physical exercise [46], although the latter was not observed in the current study. Therefore, the reduced exercise-mediated lipolytic response upon combined α-/β-adrenergic receptor blockade in the obese IS individuals, as opposed to the obese IR group, might suggest differences in adrenergic receptor expression and sensitivity. The blunted lipolytic response in obese IR individuals might also be explained by the significantly higher fasting and exercise induced serum insulin levels. Since the anti-lipolytic effects of insulin might be normal or only slightly impaired in obese AT [47-49], the observed hyperinsulinemia in the obese IR group might have contributed to the attenuated adrenergically-mediated SCAT lipolysis

as compared to the obese IS group, as previously shown [50]. In addition, increased plasma lactate concentrations in obese IR individuals, may have contributed to the reduced lipolytic response, since lactate has been shown to inhibit lipolysis in mice [51] and human primary adipocytes *in vitro* [18].

The present study implies that non-adrenergic regulators of lipolysis play a major role during low-intensity endurance-type exercise. It has previously been demonstrated that propranolol [28] and phentolamine [39, 40] fully inhibit adrenergically-mediated SCAT lipolysis at the concentrations used in the present study. The exercise-induced lipolytic response was only suppressed to a minor extent in all groups, clearly indicating a major contribution of non-adrenergic components to SCAT lipolysis during exercise. Importantly, other (anti-)lipolytic factors, such as the parathyroid hormone, cortisol and growth hormone are less important during the type and duration of exercise as applied in the current study [7, 16]. Noteworthy, atrial natriuretic peptide (ANP) may be responsible for the exercise-induced increase in SCAT lipolysis in the present study, especially since ANP is one of the major lipolytic hormones produced upon exercise [26, 52], next to sympathetic nervous system activation. In line with our findings, it has previously been shown that non-adrenergically mechanisms are involved in SCAT lipolysis, accounting for ~65% of the exercise-mediated lipolysis in young healthy lean and overweight men [15, 16]. Previously, a lower *in vivo* ANP responsiveness in SCAT of overweight (31) and obese (30) individuals was observed. In line, the non-adrenergically regulation of SCAT lipolysis has been reported to be more pronounced in young healthy overweight as compared to lean men [16], suggesting that ANP-mediated SCAT lipolysis is particularly important in the overweight and obese state. Interestingly, in contrast to obese IS individuals, we found that SCAT lipolysis in obese IR men was not affected by local α-/β-adrenergic blockade, which may propose an interaction between SCAT adrenergically-mediated lipolysis and whole-body insulin resistance. These findings support a catecholamine-resistance phenotype of the SCAT during exercise especially in the obese IR state. Therefore, a major role for ANP in SCAT lipolysis during exercise can be suggested, which is sustained in the obese IR state.

To ensure adequate fatty acid delivery to the working skeletal muscles during exercise, the sustained ANP-mediated lipolytic response, the adrenergically-mediated lipolysis and a blunted insulin- and lactate-mediated inhibition of lipolysis, might have contributed to the more pronounced SCAT lipolysis as observed in the obese IS men. Because most of the anti-lipolytic action of insulin is mediated through stimulation and activation of cellular phosphodiesterase-3B [53, 54], which degrades cAMP, attenuation of the ANP-related SCAT lipolysis by insulin is unlikely, since the latter is cGMP-mediated [27]. Likewise, it has been shown that NP receptor expression (both functional NPRA and scavenging NPRC) in SCAT associates with whole-body insulin sensitivity [55].

The 12-week exercise intervention increased insulin sensitivity in the obese IR group. While plasma glycerol, FFA, lactate, ANP and adrenalin concentrations were significantly reduced after the intervention, only minor reductions were observed for circulating glucose, insulin and noradrenalin concentrations. However, local exercise-induced abdominal SCAT lipolysis was not improved following the exercise intervention. Moreover, the efficiency of local α-/β-adrenergic blockade was not affected by the exercise intervention. Together, these data suggest that

even after a substantial improvement in metabolic profile (as indicated by our systemic responses) and body composition after a 12-week exercise intervention, lipolytic disturbances remain unaffected in SCAT of obese IR individuals. Optimized therapies are warranted to achieve enhancements in the regulation of SCAT lipolysis, especially in metabolically compromised individuals.

Although evidence suggests that exercise training induces beneficial changes in SCAT insulin sensitivity [56], SCAT adrenergic sensitivity [56-58] as well as ANP-mediated lipolysis [59], future mechanistic studies are needed to obtain a better understanding of the hormonal (exercise-induced) lipolytic regulation in lean and obese individuals with a different degree of insulin sensitivity. Unfortunately, a NPRA receptor agonist/antagonist for use in humans is currently unavailable, which hampers strong conclusions about the physiological role of ANP in human AT lipolysis *in vivo*.

In conclusion, this study is the first to report the integrated physiological role of adrenergically- and non-adrenergically-mediated SCAT lipolysis during low-intensity endurance-type exercise in middle-aged lean IS, obese IS and obese IR men, and the effects of a 12-week supervised exercise intervention on these processes in obese individuals. The present data demonstrate a major role for non-adrenergically regulated lipolysis in SCAT during low-intensity exercise, likely involving ANP-mediated lipolysis. Furthermore, the exercise training intervention was not accompanied by changes in SCAT lipolysis in obese IR individuals, regardless of improvements in metabolic profile and body composition.

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SUPPLEMENTARY MATERIAL
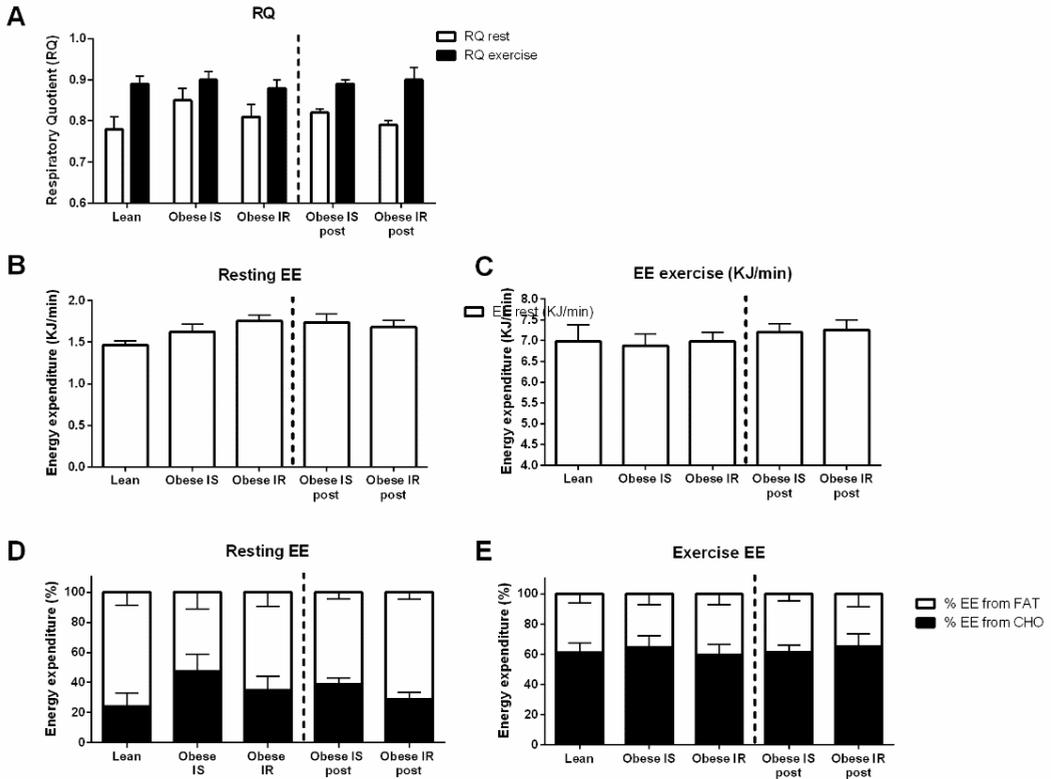
SUPPLEMENTARY FIGURE S1. Fasting and exercise-induced whole-body energy expenditure and substrate oxidation before and after exercise intervention.

Indirect calorimetry was performed during resting conditions and during an acute exercise bout of moderate intensity. Mean O_2-consumption and CO_2-production over 10 min were used for calculations of respiratory quotient (A), energy expenditure and substrate oxidation during fasting (B + D) and during exercise (C + E). Post intervention data are added for both obese groups (A-E). CHO, carbohydrates; EE, energy expenditure; IR, insulin resistant; IS, insulin sensitive. Data are presented as mean± SEM.

Supplementary Table S1. Mean plasma and serum concentrations of systemic glycerol, insulin, FFA, glucose, lactate, ANP, adrenalin and noradrenalin at rest, during low-intense exercise and recovery in obese insulin sensitive, obese insulin resistant and lean individuals

	Lean (n=10)	Obese insulin sensitive (n=10)	P (vs lean)	Obese insulin resistant (n=10)	P (vs lean)	P (vs OB IS)	P (ANOVA)
GLYCEROL							
Baseline (μmol/l)	70.5 ± 4.8	81.8 ± 7.5	0.580	77.5 ± 5.5	1.000	1.000	0.416
Exercise (AUC$_{0-60}$) (μmol/l*60min)	136.73 ± 12.5	163.2 ± 19.4	0.978	168.48 ± 22.8	0.722	1.000	0.448
Recovery (AUC$_{75-105}$) (μmol/l*45min)	107.2 ± 6.5	124.2 ± 10.4	1.000	157.0 ± 28.4	0.183	0.660	0.159
INSULIN							
Baseline (μU/ml)	7.0 ± 0.6	9.4 ± 1.1	0.563	16.9 ± 1.8	<0.001	0.001	<0.001
Exercise (AUC$_{0-60}$) (μU/ml*60min)	7.4±0.64	9.3±1.2	0.791	15.0±1.5	<0.001	0.005	<0.001
Recovery (AUC$_{75-105}$) (μU/ml*45min)	8.5 ± 1.0	11.7 ± 1.2	0.209	21.43 ± 1.3	<0.001	<0.001	<0.001
FFA							
Baseline (μmol/l)	451.1 ± 30.9	528.4 ± 58.3	0.658	556.7 ± 36.3	0.292	1.000	0.224
Exercise (AUC$_{0-60}$) (μmol/l*60min)	466.4 ± 42.6	555.2 ± 56.0	0.628	530.9 ± 46.9	1.000	1.000	0.425
Recovery (AUC$_{75-105}$) (μmol/l*45min)	600.7±37.2	777.6 ± 63.8	0.189	864.5±81.9	0.019	1.000	0.020
GLUCOSE							
Baseline (mmol/l)	5.2 ± 0.1	5.0 ± 0.1	0.722	5.1 ± 0.1	1.000	1.000	0.488
Exercise (AUC$_{0-60}$) (mmol/l*60min)	5.3 ± 0.1	5.1 ± 0.1	1.000	5.3 ± 0.2	1.000	1.000	0.679
Recovery (AUC$_{75-105}$) (mmol/l*45min)	5.3 ± 0.1	5.3 ± 0.2	1.000	5.3 ± 0.1	1.000	1.000	0.975
LACTATE							
Baseline (mmol/l)	0.9 ± 0.1	0.9 ± 0.0	1.000	1.0 ± 0.1	0.628	0.600	0.339
Exercise (AUC$_{0-60}$) (mmol/l*60min)	1.0 ± 0.7	1.1 ± 0.1	0.100	1.4 ± 0.1	0.044	0.313	0.044
Recovery (AUC$_{75-105}$) (mmol/l*45min)	0.9 ± 0.1	1.0 ± 0.1	0.730	1.2 ± 0.1	0.057	0.679	0.061
ANP							
Baseline (pg/ml)	43.8 ± 5.2	41.7 ± 3.2	1.000	36.2 ± 4.0	0.548	0.793	0.356
Exercise (AUC$_{0-60}$) (pg/ml*60min)	47.2 ± 4.8	45.9 ± 3.5	1.000	39.2 ± 2.2	0.452	0.680	0.300
Recovery (AUC$_{75-105}$) (pg/ml*45min)	42.7 ± 4.4	44.0 ± 2.3	1.000	37.5 ± 3.2	0.864	0.616	0.390
ADRENALIN							
Baseline (pg/ml)	16.4 ± 3.8	26.7 ± 6.5	0.561	19.4 ± 4.0	1.000	1.000	0.376
Exercise (AUC$_{0-60}$) (pg/ml*60min)	54.0 ± 5.7	76.2 ± 11.2	0.233	58.0 ± 8.1	1.000	0.429	0.171
Recovery (AUC$_{75-105}$) (pg/ml*45min)	35.2 ± 3.2	48.2 ± 9.0	0.537	39.4 ± 5.9	1.000	1.000	0.380
NORADRENALIN							
Baseline (pg/ml)	492.6 ± 84.7	437.1 ± 91.4	1.000	430.3 ± 42.2	1.000	1.000	0.816
Exercise (AUC0-60) (pg/ml*60min)	1107.1 ± 80.8	1095.6 ± 83.1	1.000	1007.3 ± 110.6	1.000	1.000	0.708
Recovery (AUC75-105) (pg/ml*45min)	610.1 ± 70.9	539.8 ± 48.3	1.000	580.2 ± 56.1	1.000	1.000	0.725

Data are mean ± SEM. AUC$_{0-60}$ (area under the curve during exercise (from timepoint 0 till 60min)), AUC$_{75-105}$ (area under the curve during the recovery period (from timepoint 75 until 105min)). Group effects were tested with a one-way ANOVA with Bonferroni post-hoc.

Supplementary Table S3. Interstitial glucose and lactate concentrations in the control probe and the probe containing phentolamine and propranolol in the obese insulin sensitive, obese insulin resistant and lean individuals.

	Lean (n=10)	Obese insulin sensitive (n= 10)	Obese insulin resistant (n= 10)	P_{Group}	ANOVA $P_{Treatment}$	$P_{Treatment*Group}$
GLUCOSE						
Baseline (mmol/l)						
Control	3.62 ± 0.31	2.88 ± 0.39	2.56 ± 0.48	0.081	0.622	0.877
α/β blocker	3.72 ± 0.45	2.86 ± 0.31	2.82 ± 0.23			
Exercise (AUC$_{0-60}$)						
Control	4.30 ± 0.36	4.36 ± 0.73	4.60 ± 1.06	0.811	0.194	0.655
α/β blocker	4.24 ± 0.55	3.43 ± 0.38	3.93 ± 0.47			
Recovery (AUC$_{75-120}$)						
Control	3.38 ± 0.30	3.45 ± 0.50	3.65 ± 0.92	0.925	0.624	0.482
α/β blocker	3.68 ± 0.41	3.32 ± 0.37	3.02 ± 0.16			
LACTATE						
Baseline (mmol/l)						
Control	1.79 ± 0.35	0.98 ± 0.16	1.80 ± 0.60	0.121	0.091	0.709
α/β blocker	1.37 ± 0.37	0.78 ± 0.13	1.08 ± 0.14			
Exercise (AUC$_{0-60}$)						
Control	2.63 ± 0.58	1.79 ± 0.41	3.16 ± 0.95	0.354	0.078	0.449
α/β blocker	2.36 ± 0.62	1.46 ± 0.34	1.94 ± 0.32			
Recovery (AUC$_{75-120}$)						
Control	2.70 ± 0.64	1.62 ± 0.45	2.42 ± 0.90	0.349	0.234	0.558
α/β blocker	2.65 ± 0.84	1.38 ± 0.36	1.52 ± 0.16			

Data are mean ± SEM. Control (probe containing Ringer), α/β blocker (probe containing Phentolamine

Supplemental Table S2. Interstitial glycerol concentrations and ethanol ratio in the control probe and the probe containing phentolamine and propranolol in obese insulin sensitive, obese insulin resistant and lean individuals

	Lean (n=10)	Obese insulin sensitive (n=10)	Obese insulin resistant (n=10)	ANOVA P Group	P Treatment	P Treatment*Group
GLYCEROL						
Baseline (µmol/l)				0.279	0.266	0.674
Control	311.4 ± 33.7	403.6 ± 41.1	338.9 ± 46.0			
α/β blocker	345.8 ± 42.7	404.8 ± 49.2	407.8 ± 41.5			
Exercise (AUC$_{0-60}$)				0.012	0.883	0.335
Control	537.2 ± 71.0	973.7 ± 74.9	721.6 ± 79.4			
α/β blocker	570.5 ± 110.5	851.1 ± 116.4	839.7 ± 123.5			
Recovery (AUC$_{75-120}$)				0.014	0.894	0.348
Control	466.8 ± 69.2	909.6 ± 77.4	624.3 ± 82.7			
α/β blocker	519.5 ± 99.3	803.8 ± 111.1	699.0 ± 118.7			
ETHANOL RATIO						
Baseline				0.003	0.037	0.190
Control	0.44 ± 0.05	0.66 ± 0.03	0.64 ± 0.05			
α/β blocker	0.56 ± 0.05	0.67 ± 0.05	0.69 ± 0.03			
Exercise				0.042	0.365	0.501
Control	0.42 ± 0.05	0.60 ± 0.05	0.59 ± 0.05			
α/β blocker	0.50 ± 0.05	0.59 ± 0.05	0.60 ± 0.05			
Recovery				0.003	0.025	0.620
Control	0.44 ± 0.04	0.65 ± 0.05	0.66 ± 0.05			
α/β blocker	0.53 ± 0.04	0.69 ± 0.05	0.71 ± 0.05			

Data are expressed as mean ± SEM. Control (probe containing Ringer), α/β blocker (probe containing Phentolamine + Propranolol). P-values were calculated via a 2-way repeated ANOVA.

Supplementary Table S4. Mean plasma and serum concentrations of circulating glycerol, insulin, FFA, glucose, lactate, ANP, adrenalin and noradrenalin at rest, during exercise and recovery, before and after 12 weeks of exercise training in the obese insulin sensitive and obese insulin resistant subjectss

	Obese insulin sensitive		Obese insulin resistant		ANOVA		
	Pre	Post	Pre	Post	P_{Time}	P_{Group}	$P_{Time*Group}$
GLYCEROL							
Baseline (μmol/l)	74.0 ± 5.0	64.2 ± 4.8	79.9 ± 5.5	74.8 ± 6.4	0.100	0.235	0.593
Exercise (AUC_{0-60})	157.3 ± 22.7	150.3 ± 12.7	175.7 ± 24.1	144.0 ± 23.6	0.026	0.730	0.500
Recovery (AUC_{75-105})	118.9 ± 12.7	124.4 ± 6.6	164.3 ± 30.7	122.1 ± 19.3	0.316	0.405	0.198
INSULIN							
Baseline (μU/ml)	9.5 ± 1.4	9.4 ± 1.3	16.9 ± 2.0	14.3 ± 1.3	0.196	0.008	0.263
Exercise (AUC_{0-60})	9.2 ± 1.5	9.1 ± 1.2	14.8 ± 1.7	13.4 ± 1.3	0.313	0.021	0.393
Recovery (AUC_{75-105})	11.6 ± 1.6	13.8 ± 1.5	21.6 ± 1.5	19.4 ± 2.5	0.981	0.003	0.206
FFA							
Baseline (μmol/l)	506.2 ± 48.5	382.2 ± 30.2	571.3 ± 37.1	476.5 ± 41.1	0.016	0.060	0.723
Exercise (AUC_{0-60})	533.0 ± 55.4	436.7 ± 42.6	540.5 ± 51.3	439.0 ± 47.7	0.040	0.930	0.954
Recovery (AUC_{75-105})	759.0 ± 70.1	698.0 ± 38.5	892.5 ± 86.1	726.4 ± 76.1	0.034	0.401	0.296
GLUCOSE							
Baseline (mmol/l)	5.0 ± 0.1	5.3 ± 0.2	5.1 ± 0.1	5.1 ± 0.1	0.030	0.671	0.021
Exercise (AUC_{0-60})	5.2 ± 0.1	5.3 ± 0.1	5.3 ± 0.2	5.1 ± 0.1	0.973	0.790	0.202
Recovery (AUC_{75-105})	5.4 ± 0.3	5.4 ± 0.1	5.3 ± 0.2	5.1 ± 0.1	0.564	0.437	0.564
LACTATE							
Baseline (mmol/l)	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.335	0.322	0.824
Exercise (AUC_{0-60})	1.1 ± 0.1	0.9 ± 0.1	1.4 ± 0.1	1.0 ± 0.1	0.016	0.127	0.609
Recovery (AUC_{75-105})	1.0 ± 0.1	0.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.078	0.392	0.606
ANP							
Baseline (pg/ml)	39.7 ± 3.5	33.8 ± 3.6	38.8 ± 3.5	37.5 ± 3.5	0.042	0.769	0.162
Exercise (AUC_{0-60})	44.6 ± 4.7	38.1 ± 5.2	41.1 ± 2.5	39.1 ± 3.5	0.043	0.819	0.256
Recovery (AUC_{75-105})	39.9 ± 2.8	36.2 ± 5.0	39.2 ± 3.0	37.6 ± 3.8	0.105	0.946	0.530
ADRENALIN							
Baseline (pg/ml)	29.4 ± 6.8	15.8 ± 2.5	21.9 ± 5.6	19.7 ± 5.4	0.155	0.773	0.295
Exercise (AUC_{0-60})	85.6 ± 7.1	62.9 ± 10.4	58.0 ± 9.2	48.1 ± 7.2	0.029	0.057	0.354
Recovery (AUC_{75-105})	55.0 ± 6.9	36.6 ± 5.0	36.2 ± 6.7	24.4 ± 3.6	0.007	0.045	0.483
NORADRENALIN							
Baseline (pg/ml)	452.1 ± 118.1	395.6 ± 28.2	442.5 ± 45.8	400.8 ± 41.7	0.468	0.973	0.912
Exercise (AUC_{0-60})	1078.5 ± 92.2	1002.6 ± 93.3	1043.1 ± 118.7	1045.6 ± 106.2	0.875	0.822	0.534
Recovery (AUC_{75-105}) (pg/ml*45min)	519.6 ± 50.7	552.8 ± 68.1	584.7 ± 63.4	608.1 ± 56.8	0.613	0.377	0.930

Data are mean ± SEM. AUC0-60 (area under the curve during exercise (from timepoint 0 till 60min)), AUC75-105 (area under the curve during the recovery period (from timepoint 75 until 105min)). Group effects were tested with a one-way ANOVA with Bonferroni post-hoc.

CHAPTER 8

GENERAL DISCUSSION

The prevalence of obesity has increased enormously over the last decades and is associated with an increased risk for metabolic impairments and chronic diseases such as insulin resistance [1], type 2 diabetes [2] and cardiovascular diseases [3]. Obesity results from a chronic positive energy balance that leads to an increased amount of adipose tissue mass. However, adipose tissue mass *per se* does not seem to be the most important contributor to the development of obesity-related disorders. Body fat distribution and adipose tissue dysfunction play a more prominent role in the determination of cardiometabolic health [4, 5]. Therapies to reduce obesity and related comorbidities can include dietary manipulation (as extensively discussed in **Chapter 2**), physical activity strategies and a pharmacological approach.

There is evidence to suggest that both the renin-angiotensin system (RAS) and the natriuretic peptide (NP) system can influence cardiometabolic risk. Indeed, several RAS components, which are also present in different key metabolic organs such as the adipose tissue, skeletal muscle and the liver, are increased in obesity and insulin resistance [6-9]. Also, reduced circulating NP concentrations have been observed in obesity and type 2 diabetes [10]. An increased RAS activity [6] and reduced NP concentrations [10] have detrimental metabolic effects and may increase disease progression [11-15]. Recently, a novel dual acting drug, sacubitril/valsartan, has been developed that facilitates the beneficial effects of the NP system, while inhibiting the detrimental effects of the RAS [16]. This combination therapy may have beneficial synergistic effects [17, 18] with respect to disease progression. Indeed, sacubitril/valsartan has been shown to be superior in reducing the risks of cardiovascular death or hospitalization for heart failure as compared to monotherapy with enalapril [19]. Due to the common pathophysiological impairments in individuals with cardiovascular and several metabolic diseases (e.g. reduced oxidative capacity, altered lipolysis, increased inflammation, insulin resistance), treatment with sacubitril/valsartan could induce clinical benefits in both patients with cardiovascular and/or metabolic diseases. However, it remains to be established whether sacubitril/valsartan may also have (superior) beneficial metabolic effects as compared to other blood pressure lowering agents.

Beside a pharmacological approach, changes in lifestyle are effective in preventing the development of type 2 diabetes and related cardiometabolic complications [20, 21]. Increasing physical activity levels lead to increased energy expenditure, improved physical fitness and contribute to an improved metabolic health [22, 23], which could be attributable to changes in adipose tissue metabolism. Although several rodent studies suggest that exercise training may improve adipose tissue metabolism and function [24], human data are limited and need further investigation [24-26].

Therefore, in the present thesis, the metabolic effects of pharmacological treatment with sacubitril/valsartan, as well as exercise-training induced effects on adipose tissue metabolism and the metabolic profile were investigated. In this chapter, our findings will be discussed and put into a broader perspective.

PHARMACOLOGICAL TREATMENT WITH SACUBITRIL/VALSARTAN

Combination therapies that simultaneously target more than one biological pathway or mechanism may be more effective in reducing disease progression because of additional and/or synergistic effects as compared to monotherapies [17, 18]. Therefore, due to common involved pathways, it is tempting to postulate that combination therapy using sacubitril/valsartan through its distinct mechanisms of action, may target risk factors for both cardiovascular (e.g. hypertension) and metabolic diseases such as an impaired lipid mobilization, lipid oxidation and substrate utilization, as will be discussed below.

Effects on insulin sensitivity

In **Chapter 3**, we investigated the effects of 8 weeks treatment with sacubitril/valsartan in obese hypertensive patients on peripheral insulin sensitivity. Compared to the metabolically neutral comparator, the calcium antagonist amlodipine, peripheral insulin sensitivity was significantly improved after sacubitril/valsartan, independent of changes in body weight or waist circumference. The increased peripheral insulin sensitivity may be due to valsartan-induced AT_1-receptor inhibition, increased availability of neprilysin (NEP) substrates or both mechanisms combined. Unfortunately, the present study design does not allow any conclusions regarding the separate effects of valsartan and sacubitril on peripheral insulin sensitivity.
The improvement in peripheral insulin sensitivity following sacubitril/valsartan is in line with most [27-31] but not all [32] previously performed human studies using valsartan treatment. Importantly, however, the latter study [32] investigated the effects of a lower dosage of valsartan (40 mg/day vs. 400 mg/day in our study) after 4 weeks of treatment and used surrogate markers of insulin sensitivity rather than the hyperinsulinemic-euglycemic clamp, which is the golden-standard. Taken together, our data and previous studies collectively show that valsartan improves peripheral insulin sensitivity in humans.
Although it has been shown that interference with the RAS improves glucose metabolism and reduces the incidence of type 2 diabetes [6], the valsartan-induced effects may not solely explain the observed improvement in peripheral insulin sensitivity with sacubitril/valsartan treatment in the present study and most likely also NEP inhibition is involved. The effects of NEP inhibition *per se* on insulin sensitivity have not been investigated previously, although it is known that a reduced NP system activity is associated with insulin resistance and type 2 diabetes and that modulation of the NP system can be protective against the development of insulin resistance [10].
An increased adipose tissue lipolysis, accompanied by an increased fatty acid oxidation has beneficial effects on insulin sensitivity [33, 34]. Therefore, we hypothesized that enhanced lipolysis and whole-body fat oxidation contributed to the improved peripheral insulin sensitivity following sacubitril/valsartan treatment, as described in **Chapter 3** and **Chapter 4**, since it has been shown that activation of NP signaling induces lipolysis [35-39], promotes fatty acid oxidation [36, 37, 40] and enhances mitochondrial oxidative metabolism [41-43].
While monotherapy with valsartan increased peripheral insulin sensitivity by approximately 10% in subjects with impaired glucose metabolism [27, 44],

treatment with sacubitril/valsartan showed an increase of ~20% in insulin sensitivity, with no marked effects of amlodipine treatment. Although the previous study [27] used a lower dosage of valsartan as compared to our study (320 mg/day vs. 400 mg per day, respectively), this difference is not likely to explain the additional 10% increase in insulin sensitivity after treatment with sacubitril/valsartan. Therefore, these data suggest that the NEP inhibitor sacubitril has significantly contributed to the effects on peripheral insulin sensitivity that were found in the present study.

Effects on subcutaneous adipose tissue lipolysis and substrate oxidation

Since adipose tissue metabolism and function and substrate oxidation are important factors that contribute to peripheral insulin sensitivity, we investigated sacubitril/valsartan-mediated effects on abdominal subcutaneous adipose tissue lipolysis, whole-body lipolysis and substrate oxidation, both at rest and during an acute bout of exercise.

After 8 weeks of treatment, there was a slight, but significant increase in abdominal subcutaneous adipose tissue lipolysis at rest in the sacubitril/valsartan as compared to the amlodipine group (**Chapter 3**). Surprisingly, the increased subcutaneous adipose tissue lipolysis at rest did not translate into significant changes in whole-body lipolysis, as measured with the stable isotope $[1,1,2,3,3-{}^2H]$-glycerol (**Chapter 3**). Together, these findings either suggest that the increased subcutaneous adipose tissue lipolysis might be paralleled by a reduced lipolysis in other adipose tissue depots or, more likely, that the effects on lipolysis may not be physiologically relevant. An increased adipose tissue lipolysis has beneficial effects on insulin sensitivity, only when the mobilized fatty acids are oxidized at an increased rate [33, 34].

In **Chapter 3**, we found no significant changes in total energy expenditure and substrate oxidation in resting conditions. In **Chapter 4**, we examined the effects of sacubitril/valsartan on abdominal subcutaneous and whole-body lipolysis during an acute bout of aerobic exercise, which is known to stimulate lipolysis [45, 46], and observed no significant differences in abdominal subcutaneous adipose tissue lipolysis, whole-body lipolysis, energy expenditure and substrate oxidation between groups. Thus, there were no differences between resting (**Chapter 3**) and exercise (**Chapter 4**) conditions, except for a slightly higher abdominal subcutaneous adipose tissue lipolysis under resting conditions (**Chapter 3**). Therefore, an increased fat oxidation does not seem to contribute to the improved peripheral insulin sensitivity following sacubitril/valsartan treatment.

Next, we examined the effects of sacubitril/valsartan on adipose tissue gene and protein expression, as described in **Chapter 5**, and we found no significant changes in the expression of genes and proteins of factors involved in lipolysis, natriuretic peptide signaling and mitochondrial oxidative metabolism.

These results are in line with a previous study that did not show an increased adipose tissue lipolytic gene and protein expression after 26 weeks of valsartan treatment [44]. Furthermore, AT_1-receptor blockade did not increase abdominal subcutaneous adipose tissue lipolysis [47] and, therefore, it seems unlikely that AT_1-receptor blockade with sacubitril/valsartan treatment contributed to the mild alteration in lipid mobilization. Rather, a NEP inhibition-mediated increased NP

availability could have contributed to the observed effects on subcutaneous adipose tissue lipolysis, since NP have been shown to affect adipose tissue lipid mobilization and oxidation in lean and overweight men and women [35-40].

Taken together, alterations in adipose tissue lipolysis or whole-body substrate oxidation at rest and during exercise do not seem to contribute to the sacubitril/valsartan-induced improvement in peripheral insulin sensitivity. The underlying mechanisms remain to be elucidated, but seem to be explained by metabolic effects in other tissues (e.g. visceral adipose tissue, the liver or skeletal muscle).

EXERCISE TRAINING INTERVENTIONS TO IMPROVE METABOLIC HEALTH

Regular physical exercise has beneficial effects on cardiometabolic health and improves glucose tolerance, insulin sensitivity and circulating lipid concentrations [48, 49]. These exercise-induced improvements in cardiometabolic risk profile have largely been attributed to changes in skeletal muscle metabolism and function, but physical exercise may also induce alterations in other metabolically active tissues, including the adipose tissue. Indeed, several rodent studies suggest that exercise training may improve adipose tissue metabolism and function [24], although the evidence for this in humans is limited [24-26].

Effects on insulin sensitivity

In **Chapter 6** and **Chapter 7** of this thesis, we investigated the metabolic effects of a 12-week supervised, combined endurance and resistance exercise training program in sedentary, middle-aged overweight/obese men with or without metabolic impairments (**Chapter 6**) and sedentary, middle-aged obese insulin sensitive, obese insulin resistant and age-matched lean insulin sensitive men (**Chapter 7**).

Both exercise training programs improved aerobic capacity, maximal power output and maximal muscle strength, indicating that the supervised, progressive nature of both programs was successful with respect to enhancement of physical fitness. This enhanced physical fitness was accompanied by improved peripheral insulin sensitivity, as determined using either a hyperinsulinemic-euglycemic clamp (**Chapter 6**) or HOMA-IR (**Chapter 7**). These findings are in line with previous studies showing an improved insulin sensitivity after 12 weeks of exercise in obese individuals [50, 51] and could, among other mechanisms, be explained by an altered body composition [52]. In **Chapter 6**, we observed a modest but significant reduction in body fat mass (~0.7 kg) and body fat percentage (~0.6 %) in both obese groups, without significant changes in fat-free mass. In **Chapter 7**, we observed a significantly reduced fat mass (~2.1 kg in the obese insulin sensitive group and ~1.9 kg in the obese insulin resistant group), body fat percentage (~1.6 % in the obese insulin sensitive and ~0.9 % in the obese insulin resistant group), BMI and body weight, while fat-free mass remained unchanged and was not different between groups. Previous data showed that increased fat-free mass (e.g. skeletal muscle cross-sectional area) and improved skeletal muscle function (e.g. skeletal muscle capillarization, oxidative capacity) are associated with improved insulin sensitivity [53-56], while reduced fat-free mass induced insulin resistance

[57, 58]. However, our data suggest that peripheral insulin sensitivity can improve without significant changes in fat-free mass.

Although exercise training significantly increased peripheral insulin sensitivity it did not induce changes in adipose tissue and hepatic insulin sensitivity, as described in **Chapter 6**. These findings are in contrast with previous rodent [59, 60] and human [61] studies. The unaltered adipose tissue and hepatic insulin sensitivity may be explained by the rather minor changes in fat mass loss. Nevertheless, our data showed that exercise training beneficially improved physical fitness, body composition and peripheral insulin sensitivity, irrespective of the baseline metabolic status.

Effects on subcutaneous adipose tissue metabolism and adipokine expression

Subcutaneous adipocyte size is closely associated with adipose tissue function and insulin resistance [4] and adipocyte hypertrophy is correlated with impairments in adipose tissue metabolism [62]. Since marked weight loss is associated with decreased adipocyte size in subcutaneous adipose tissue, which is accompanied by improved insulin sensitivity [63-66], we investigated if exercise training could alter adipocyte morphology, thereby contributing to an improved subcutaneous adipose tissue metabolism and whole-body insulin sensitivity.

In **Chapter 6**, we found that physical exercise training did not change abdominal subcutaneous adipocyte morphology, neither mean adipocyte size nor the proportion of small and large adipocytes, in metabolically healthy obese and metabolically compromised obese subjects. In contrast, a previous study demonstrated a decreased adipocyte size in young men, but not in women, after 20 weeks of endurance training [67]. Importantly, in the latter study, a more pronounced reduction in body weight (~3.0 kg) was achieved. Since we observed a weight reduction of only ~0.7kg in **Chapter 6**, it is likely that a more pronounced decrease in adipose tissue mass is needed to induce significant changes in adipocyte morphology and, consequently, adipose tissue metabolism. While exercise training duration was comparable between the exercise training studies in chapter 6 and 7, we observed a greater reduction in fat mass in **Chapter 7**, which is likely due to the higher training volume. In **Chapter 6**, subjects performed three training sessions per week, of which two sessions consisted of 30min cycling at 70% VO_{2max} and one training session included resistance training at 60% of 1RM (3 sets of 10 reps). In **Chapter 7**, the participants performed three training sessions per week of which all consisted of 45min cycling at 65% VO_{2max} combined with resistance training at 65% of 1RM (4 sets of 10 reps). Since a greater reduction in body weight (fat mass) induces more favorable metabolic effects [68, 69], the greater reduction in fat mass, as observed in **Chapter 7**, could have altered adipocyte morphology and improved adipose tissue metabolism, although this was not investigated.

However, it remains to be established how much weight loss is needed to induce a significant reduction in adipocyte size and which exercise training modality is most optimal [63, 68, 70].

Since adipocyte hypertrophy is associated with impairments in adipose tissue metabolism [4, 62], the minor reduction in fat mass and the lack of changes in adipocyte morphology seems to explain the unaltered expression of genes and proteins related to adipose tissue metabolism, as reported in **Chapter 6**. More specific, there were no exercise training-induced changes in expression of genes involved in inflammation (e.g. TNF-a, IL-6, MCP-1 and CD68), which have been implicated to induce insulin resistance. Additionally, gene expression of the adipokines leptin and adiponectin, which are related to adipocyte size [71, 72], also remained unchanged after exercise training. These observations are in line with most other exercise training studies in obese subjects [73-78]. Furthermore, an adequate mitochondrial function is essential to maintain adipose tissue function, glucose homeostasis [79] and protects against insulin resistance and type 2 diabetes [80]. Browning of white adipose tissue may increase cellular energy expenditure [81, 82], thereby improving whole-body glucose homeostasis and insulin sensitivity in humans [83]. We did not found changes in browning markers after training, which is in line with studies in lean [84, 85] and overweight [85, 86] subjects, but in contrast to rodent data [41, 87]. Furthermore, the exercise training intervention did not induce changes in mitochondrial gene expression and did not alter mitochondrial oxidative phosphorylation protein expression, which was in accordance with most [78] [84, 86, 88], but not all previous studies [89, 90]. When investigating adipose tissue lipolysis, which is one of the characteristics of adipose tissue dysfunction and relates to peripheral insulin resistance [91], we observed no major significant exercise training-induced effects on expression of genes related to lipolysis. Furthermore, *ex vivo* basal and maximal lipolysis, as well as β_2-adrenergic sensitivity of lipolysis in mature human adipocytes, were not affected by the exercise training program. The latter observation is in agreement with a previous study in obese non-diabetic men [92] and may be explained by the minor change in fat mass loss, since it has previously been demonstrated that substantial weight loss (BMI: -6.1 kg/m^2) increased and normalized the sensitivity to catecholamine-stimulated lipolysis in obese subjects [93].

Overall, **Chapter 6** demonstrated that a 12-weeks supervised, progressive exercise training intervention did not significantly affect abdominal subcutaneous adipocyte morphology, adipose tissue gene and protein expression of markers related to adipose tissue function, nor β_2-adrenergic sensitivity in obese subjects, irrespective of their baseline metabolic status.

Disturbances in subcutaneous adipose tissue (SCAT) lipolysis have been reported in obese humans [46], including a reduced adrenergically-mediated lipolysis [94-96]. On the other hand, non-adrenergically-mediated lipolysis in SCAT substantially contributes to lipolysis during exercise in healthy young lean [38] and healthy young overweight men [39]. Atrial natriuretic peptide (ANP) is the most potent stimulator of human adipose tissue lipolysis [35] and one of the major lipolytic hormones produced upon exercise [35, 97]. However, ANP responsiveness has been shown to be impaired in middle-aged obese men with or without type 2 diabetes [98] and in middle-aged obese women and overweight men [99].

It has been shown that endurance exercise training can partly improve β-adrenoceptor sensitivity and reduce anti-lipolytic α_2-adrenoceptor sensitivity in human SCAT [76, 100, 101], and can alleviate ANP-mediated lipolysis in

subcutaneous adipocytes in young, metabolically healthy overweight individuals [102, 103]. However, it remained to be investigated if combined endurance and resistance exercise training could improve ANP-mediated lipolysis in metabolically compromised conditions.

In **Chapter 7**, we aimed to investigate the effects of local combined α- and β-adrenergic blockade on SCAT lipolysis in obese insulin sensitive, obese insulin resistant and age-matched lean insulin sensitive men. Therefore, we determined *in situ* SCAT lipolysis at rest, during a single bout of low-intensity endurance exercise and during recovery in age-matched obese insulin sensitive, obese insulin resistant and lean insulin sensitive men in the presence or absence of combined α- and β-adrenergic receptor blockade in abdominal SCAT. Next, the obese individuals participated in a 12-week combined endurance and resistance exercise training intervention to investigate whether exercise training was able to improve (non-) adrenergic-mediated abdominal SCAT lipolysis in obese insulin resistant subjects.

Our findings indicated a major contribution of non-adrenergically mediated lipolysis during exercise in abdominal SCAT of lean and obese individuals. Additionally, we found a greater increase in extracellular glycerol concentration in SCAT during exercise in obese insulin sensitive as compared to lean insulin sensitive individuals. Although extracellular glycerol concentration is a reflection of lipolysis, the higher exercise-induced lipolysis in the obese insulin sensitive group is likely explained by the lower adipose tissue blood flow (ATBF) in the obese state, which contributes to higher extracellular glycerol concentrations due to a lower removal of glycerol from the adipose tissue [39, 104]. Local α-/β-adrenergic blockade substantially reduced basal ATBF in lean but not in the obese individuals, which suggests that adrenergic sensitivity of ATBF is reduced in the obese state, which has been shown before [105, 106].

The exercise-induced SCAT lipolysis following combined α-/β-adrenergic blockade was substantially reduced in obese insulin sensitive but not in lean insulin sensitive or obese insulin resistant men. This might suggest differences in adrenergic receptor expression and/or sensitivity. Previous data showed that the lipolytic activity of the β-adrenergic receptors is attenuated in obese insulin resistant individuals [95, 96, 107], while inhibitory α_2-adrenoceptors become predominant on adipocytes in the obese insulin resistant state [107, 108] and reduce exercise-mediated SCAT lipolysis [105]. Additionally, obese insulin resistant individuals often show lower plasma catecholamine (adrenaline, noradrenaline) responses to physical exercise [109], although we did not find this in **Chapter 7**. Furthermore, the blunted SCAT lipolysis in the obese insulin resistant individuals, as compared to the obese insulin sensitive group, might also partly be explained by the significantly higher insulin concentrations, as shown previously [110]. In addition, increased plasma lactate concentrations, as observed in the obese insulin resistant individuals, may have contributed to the reduced lipolytic response, since lactate has been shown to inhibit lipolysis in mice [111] and human primary adipocytes *in vitro* [112].

In all groups, we found a major contribution of non-adrenergic components in SCAT lipolysis during low-intensity exercise, which is likely involving ANP-mediated lipolysis. This observation is in line with previous studies, which showed that non-adrenergically-mediated mechanisms are involved in SCAT lipolysis, accounting for

~65% of the exercise-mediated lipolysis in young healthy lean and overweight men [38, 39]. We found that SCAT lipolysis in obese insulin resistant men, compared to the obese insulin sensitive group, was not affected by local α-/β-adrenergic blockade, which may point toward an interaction between SCAT adrenergically-mediated lipolysis and whole-body insulin resistance. These findings support a catecholamine-resistant phenotype of the SCAT during exercise, especially in the obese insulin resistant state.

The 12-week combined endurance and resistance exercise training intervention did not improve exercise-induced abdominal SCAT lipolysis. Furthermore, the efficiency of local α-/β-adrenergic blockade was not affected and these data combined suggest that the lipolytic disturbances in SCAT of obese insulin resistant individuals remain unaffected after 12 weeks of exercise training. This observation is in contrast with previous studies that showed beneficial changes in SCAT insulin sensitivity [61], SCAT adrenergic sensitivity [61, 92, 113] as well as ANP-mediated lipolysis [114] after exercise training in obese subjects.

Taken together, the results described in **Chapter 7** show a major role for non-adrenergically-mediated lipolysis in SCAT during low-intensity exercise, likely involving ANP-mediated lipolysis. Furthermore, our data suggest that even after a substantial improvement in body composition, physical fitness and insulin sensitivity, lipolytic disturbances remain unaffected in SCAT of obese insulin resistant individuals after a 12-week exercise training intervention.

CONCLUSION AND FUTURE PERSPECTIVES

This thesis describes the effects of a pharmacological intervention as well as physical exercise interventions to improve metabolic health in obese individuals, with a focus on adipose tissue metabolism.

In **Chapter 3**, we found improved peripheral insulin sensitivity after 8 weeks of combination therapy with sacubitril/valsartan in obese hypertensive subjects, which was accompanied by an increased abdominal subcutaneous adipose tissue lipolysis at rest. However, this finding did not translate into alterations in whole-body lipolysis, energy expenditure and substrate oxidation. In **Chapter 4**, no changes were found for these parameters during a single bout of moderate-intensity endurance exercise. Therefore, it seems that sacubitril/valsartan had no clinically relevant effects on adipose tissue and whole-body lipolysis and substrate utilization. In line, in **Chapter 5**, no significant effects on gene and protein expression of markers related to lipolysis, natriuretic peptide signaling and mitochondrial oxidative metabolism were found. Therefore, we conclude that treatment with sacubitril/valsartan improved peripheral insulin sensitivity, without altering the abdominal subcutaneous adipose tissue metabolic phenotype and substrate oxidation.

In **Chapter 6**, we showed that a 12-weeks supervised, progressive, combined endurance and resistance exercise training program improved body composition, physical fitness and peripheral insulin sensitivity in sedentary, middle-aged overweight/obese men with or without metabolic impairments. However, no significant effects on hepatic and adipose tissue insulin sensitivity, abdominal

subcutaneous adipocyte morphology, adipose tissue gene and protein expression of markers related to adipose tissue function, nor β_2-adrenergic sensitivity of abdominal SCAT lipolysis were observed in obese subjects, irrespective of their baseline metabolic status.

In **Chapter 7**, we showed a major contribution of non-adrenergically-mediated lipolysis during exercise in SCAT of sedentary, middle-aged obese insulin sensitive, obese insulin resistant and age-matched lean insulin sensitive men. Furthermore, 12 weeks of supervised, combined endurance and resistance exercise training did not significantly improve exercise-induced abdominal SCAT lipolysis.

The outcomes of this thesis provide evidence for an improved metabolic health after sacubitril/valsartan treatment and exercise training interventions, but several questions and issues should be addressed in future research:

1.   Since we used a combination drug, we cannot distinguish between the individual effects of sacubitril (NEP inhibition) and valsartan (RAS blockade) on the improved peripheral insulin sensitivity. It would be of interest to investigate the effects of NEP inhibition compared to placebo, valsartan (at a similar dosage as applied in this thesis), or a metabolically neutral blood pressure lowering agent and/or sacubitril/valsartan, to elucidate whether the observed effects of sacubitril/valsartan on peripheral insulin sensitivity are attributable to the slightly higher valsartan concentration as compared to a previous study [27], or whether the effects are attributable to the additional effects of increased neprilysin substrates, such as NP concentrations.

2.   We treated obese hypertensive patients with sacubitril/valsartan for 8 weeks, since literature reports show that 8 weeks of treatment is sufficiently long to demonstrate metabolic effects of several drug classes used for the treatment of hypertension and heart failure, including ACE inhibitors and ARBs [27, 115, 116]. However, it remains to be investigated whether metabolic effects would be more pronounced after a more prolonged treatment period. Furthermore, the persistence and durability of the metabolic effects after cessation of treatment also remains to be established.

3.   It remains to be established whether sacubitril/valsartan has effects on other key metabolic organs (i.e. visceral adipose tissue, the liver and skeletal muscle), which may underlie the observed improvement in peripheral insulin sensitivity.

4.   Future studies should investigate the exercise-mediated metabolic responses in females, since there is a sex difference in metabolic regulation [117-119], related to circulating sex hormones [120, 121]. Interestingly, there is a sex difference with respect to circulating NP concentrations [122], and estrogen administration in postmenopausal women increased circulating ANP concentrations [123]. The sex dependent NP regulation might contribute to the well-known differences in cardiovascular risk between men and women. Clearly,

results obtained in a male population cannot simply be extrapolated to the female population.

5.   There is a large inter-individual variation in several metabolic health outcomes following different interventions, including exercise training [124]. Metabolic phenotyping at baseline makes it possible to stratify subjects into different subgroups and may improve the effectiveness of a particular intervention in a specific subgroup of the population [125]. However, we do not find clear evidence that obese individuals with a different metabolic phenotype at baseline, differentially affected exercise training-induced study outcomes. Thus, based on the findings described in this thesis, we cannot conclude that a subgroup-based approach is more beneficial than a population-based approach. Therefore, studies including more detailed metabolic phenotyping such as tissue-specific profiling are needed not only to identify individuals or subgroups at increased risk of developing metabolic diseases but also to design optimized prevention and treatment strategies for specific subgroups of the population, and ultimately leading to personalized exercise strategies.

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CHAPTER 9

ADDENDA

SUMMARY

Obesity is associated with an increased risk for metabolic impairments and chronic diseases, including insulin resistance, type 2 diabetes and cardiovascular diseases. Strategies to reduce body weight and obesity-related comorbidities include dietary (as discussed in **Chapter 2**), pharmacological and physical activity interventions. This thesis describes the effects of a pharmacological intervention as well as physical exercise interventions to improve metabolic health in obese

individuals, with a focus on adipose tissue metabolism.
An increased renin-angiotensin system activity and a lower activity of the natriuretic peptide system have been linked to the development of type 2 diabetes and cardiovascular disease. Combination therapy with sacubitril/valsartan, a combined angiotensin receptor blocker (ARB) and neprilysin (NEP) inhibitor, facilitates the beneficial effects of the natriuretic peptide system, while inhibiting the detrimental effects of the renin-angiotensin system.

In **Chapter 3**, we performed a multi-centre, randomized, double-blind, double-dummy, parallel-group study to assess the effects of 8 weeks treatment with sacubitril/valsartan as compared to amlodipine on whole-body insulin sensitivity, determined by a hyperinsulinemic-euglycemic clamp in 98 obese hypertensive patients. We found that sacubitril/valsartan significantly improved peripheral insulin sensitivity without affecting body weight or waist circumference. Furthermore, abdominal subcutaneous adipose tissue lipolysis at rest was slightly but significantly increased in the sacubitril/valsartan as compared to the amlodipine group. Surprisingly, the increased subcutaneous adipose tissue lipolysis at rest did not translate into significant changes in whole-body lipolysis, as measured using the stable isotope $[1,1,2,3,3-^2H]$-glycerol. Moreover, we also found no significant changes in total energy expenditure and substrate oxidation in resting conditions.

In **Chapter 4**, we extended the outcome of this multi-centre trial by investigating the effects of sacubitril/valsartan on abdominal subcutaneous adipose tissue and whole-body lipolysis as well as energy metabolism and substrate oxidation during a single bout of moderate-intensity aerobic exercise, which is known to stimulate lipolysis. We observed no significant effects of sacubitril/valsartan as compared to amlodipine on abdominal subcutaneous adipose tissue lipolysis, whole-body lipolysis, energy expenditure and substrate oxidation. Therefore, it seems that sacubitril/valsartan had no physiological relevant effects on adipose tissue lipolysis, whole-body lipolysis and substrate utilization.

To obtain more detailed insight into possible mechanisms underlying the findings described in Chapters 3 and 4, we assessed the effects of sacubitril/valsartan on abdominal subcutaneous adipose tissue gene expression patterns using microarray analysis and determined adipose tissue protein expression profiles in **Chapter 5**. We showed no significant changes in expression of genes and proteins of factors involved in lipolysis, natriuretic peptide signalling and mitochondrial oxidative metabolism.
Collectively, these data indicate that alterations in abdominal subcutaneous adipose tissue lipolysis, whole-body lipolysis or whole-body substrate oxidation at rest and during exercise, do not seem to contribute to the sacubitril/valsartan-

induced improvement in peripheral insulin sensitivity. It remains to be established whether sacubitril/valsartan has effects on other key metabolic organs, which may underlie the observed improvement in peripheral insulin sensitivity.

Beside pharmacological therapy, changes in lifestyle are effective in preventing the development of type 2 diabetes and related cardiometabolic complications. There is some evidence, mainly from rodent studies, that exercise training may improve adipose tissue function, thereby reducing obesity-related insulin resistance and other comorbidities. However, human studies that investigated the effects of exercise training on adipose tissue function are limited. Therefore, a second objective of this thesis was to determine the effects of physical exercise training on abdominal subcutaneous adipocyte morphology, adipose tissue gene and protein expression of markers related to adipose tissue function and *ex vivo* adipocyte lipolysis in metabolically healthy and metabolically compromised individuals.

In **Chapter 6**, we investigated the effects of a 12-weeks supervised, progressive, combined endurance and resistance exercise training program on insulin sensitivity and adipose tissue function in metabolically healthy and metabolically compromised sedentary, middle-aged men. We found that 12 weeks of exercise training improved body composition, physical fitness and peripheral insulin sensitivity, as assessed by a two-step hyperinsulinemic-euglycemic clamp. However, no significant effects on hepatic and adipose tissue insulin sensitivity, abdominal subcutaneous adipocyte morphology, adipose tissue gene and protein expression of markers related to adipose tissue function, nor $β_2$-adrenergic sensitivity of abdominal subcutaneous adipose tissue lipolysis were observed in obese subjects, irrespective of their baseline metabolic status. Importantly, since we observed only a slight but significant decrease in fat mass, it is likely that a more pronounced decrease in adipose tissue mass is needed to induce significant changes in adipocyte morphology and, consequently, adipose tissue metabolism.

Also, since atrial natriuretic peptide (ANP) increases during exercise and plays an important role in adipose tissue lipolysis, we investigated abdominal subcutaneous adipose tissue (non-)adrenergically-mediated lipolysis before, during and after a single bout of endurance exercise and after 12-weeks of exercise training in metabolically healthy and metabolically compromised individuals in **Chapter 7**. Therefore, we investigated the effect of local combined α- and β-adrenoceptor blockade on local subcutaneous adipose tissue lipolysis at rest, during low-intensity endurance-type exercise and during recovery from exercise in sedentary, middle-aged obese insulin sensitive, obese insulin resistant and age-matched lean insulin sensitive men. In addition, we investigated whether a 12-week supervised, progressive, combined endurance and resistance exercise training improved the metabolic profile in obese men and (non-)adrenergically-mediated abdominal subcutaneous adipose tissue lipolysis in obese insulin resistant individuals. We demonstrated a major contribution of non-adrenergically-mediated lipolysis during exercise in all groups. Furthermore, we showed that the exercise training intervention improved body composition, physical fitness and exercise-induced changes in circulating free fatty acids, lactate and adrenalin concentrations in both obese groups and insulin sensitivity in the obese insulin resistant group. However,

this was not accompanied by changes in adrenergically- and non-adrenergically-mediated lipolysis in the subcutaneous adipose tissue of obese insulin resistant individuals. Together, these data suggest that even after a substantial improvement in metabolic profile and body composition after a 12-week exercise intervention, lipolytic disturbances remain unaffected in subcutaneous adipose tissue of obese insulin resistant individuals. Optimized therapies are warranted to achieve enhancements in the regulation of subcutaneous adipose tissue lipolysis, especially in metabolically compromised individuals.

Combined, these data indicate that even after exercise-induced improvements in body composition, physical fitness and peripheral insulin sensitivity, changes in abdominal subcutaneous adipose tissue metabolism and function are lacking and will most likely occur only after a more pronounced decrease in adipose tissue mass. Currently, it remains to be established which exercise training duration and modality is most optimal to induce beneficial effects in abdominal subcutaneous adipose tissue.

VALORIZATION

SOCIAL RELEVANCE

The worldwide prevalence of obesity has increased enormously over the last decades and numbers are still increasing every year. According to the World Health Organization, 13% of the world's adult population (11% of men and 15% of women) was obese in 2014, while in the same year, obesity affected around 18,5% of men and 19% of woman in The Netherlands [1]. If post-2000 trends continue, this global prevalence of obesity is suggested to reach 18% in men and 21% in women by 2025. Obesity is associated with an increased risk of developing chronic diseases, including insulin resistance [2], type 2 diabetes [3], cardiovascular diseases [4] and certain types of cancer [5, 6]. To reduce these obesity related comorbidities, nowadays, millions of people are in need of medication, such as glucose-, cholesterol- and/or blood pressure lowering medication, and surgical treatments such as gastric bypass or cardiovascular surgery. Since obesity is a major public health issue and one of the most important risk factors for the development of metabolic diseases, it is clear that the increasing obesity prevalence has major socioeconomic consequences [7]. National and international guidelines recommend changes in modifiable lifestyle characteristics, such as diet and physical activity for both prevention and management of metabolic diseases [8]. While weight loss has been shown to be effective in reducing disease risk, implementation of the recommended lifestyle in the long-term is often hard to maintain by the majority of people. Therefore, to reduce the incidence of obesity and thereby partly improving global health, it is important to obtain better insights in the development and treatment of obesity and related metabolic diseases and to implement new treatment strategies. The results described in this thesis contribute to a better understanding of the role of adipose tissue metabolism in cardiometabolic health and obesity, and provide leads for possible treatment strategies to reduce or prevent obesity and cardiometabolic complications.

TARGET GROUPS

Scientific community

The results described in this thesis have and will become available to the scientific community via publication of scientific articles in international peer-reviewed journals. Additionally, results have been presented at (inter)national conferences to scientists as well as physicians, healthcare professionals and dieticians, working in the fields of obesity, diabetes and metabolism.

Industry

A part of this thesis was accomplished by the close collaboration between academia and industry and research outcomes are of valuable information to the academic community and both the nutritional and the pharmaceutical industry. The industrial partners can translate the research outcomes to develop improved or novel treatment strategies or products that help to prevent or reduce the prevalence of obesity and obesity-related complications. More specific, the nutritional industry can translate the results from chapter 2 in defining new

nutritional targets. The pharmacological industry can use the results from this thesis to develop and/or implement new or improved pharmacological therapies or to expand the rationale for prescribing certain cardiovascular drugs in metabolically compromised conditions, since in our studies combination therapy with sacubitril/valsartan was shown to improve both cardiovascular and metabolic risk factors.

Health care professionals

Healthcare professionals (e.g. dieticians, physiotherapists and physicians) play an important role in stimulating a healthy lifestyle among patients and people at increased risk of developing obesity and related complications. On the other hand, physicians prescribe drugs to reverse for example cardiovascular risk (e.g. hypertension) or to improve glucose metabolism and insulin sensitivity. Although the results from this thesis do no provide direct guidelines for healthcare, they provide better insight in metabolic parameters that can be targeted through certain interventions, applied by physicians and other healthcare professionals. Furthermore, this thesis might provide an indication for more targeted prevention programs in high risk groups of the population (i.e. people with both cardiovascular and metabolic risk factors) and it gives more insight in the mechanism of action of a cardiovascular drug (i.e. sacubitril/valsartan) that also has effects on insulin sensitivity and which therefore may alter the rationale for prescription.

ACTIVITIES AND PRODUCTS

In this thesis, we have put forward fatty acid metabolism-related pathways in several metabolically active organs that can be targeted by dietary interventions, thereby improving whole-body glucose metabolism and insulin sensitivity. The results from the nutritional review can potentially lead to novel functional foods or food supplements (e.g. pre- and probiotics, polyphenols, plant sterols, improved dietary fat quality). However, before these novel products will become available on the market, more scientific research is necessary to confirm mechanisms, safety and health benefits in individuals at increased risk of developing cardiometabolic diseases.

The results from the pharmacological intervention with sacubitril/valsartan, which has recently been approved by the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of heart failure [9], showed that the combination therapy has a positive effect on cardiovascular and metabolic risk factors. We showed that treatment with sacubitril/valsartan, as compared to the metabolically neutral calcium antagonist amlodipine, improved peripheral insulin sensitivity in obese hypertensive patients. This knowledge will be immediately used by the pharmaceutical industry. Moreover, sacubitril/valsartan may be a promising alternative for the treatment of hypertension in patients who are at increased risk for developing chronic metabolic diseases (e.g. hypertensive individuals with impaired glucose metabolism).

The exercise training intervention studies showed that exercise training beneficially affects body composition and physical fitness and is effective to improve obesity-related disturbances like whole-body insulin resistance. Although 12 weeks of exercise training induced a slight but significant reduction in fat mass, no significant changes in abdominal subcutaneous adipocyte morphology, adipose tissue function, and abdominal subcutaneous adipose tissue lipolysis were observed in obese subjects, irrespective of their baseline metabolic status. Furthermore, we showed that 12 weeks of exercise training did not improve disturbances in subcutaneous adipose tissue lipolysis in obese insulin resistant individuals. It seems that a more pronounced decrease in adipose tissue mass is needed to induce significant changes in adipose tissue metabolism. Currently, it remains to be established which exercise training duration and modality is most optimal to induce beneficial effects in abdominal subcutaneous adipose tissue.

INNOVATION & IMPLEMENTATION

All results described in this thesis are novel findings and have, partly, been performed at the Department of Human Biology and Movement Sciences of Maastricht University Medical Center+ and in close collaboration with other universities and industrial partners within The Netherlands and Europe, using state-of-the-art methodologies for both *in vivo* and *ex vivo* analyses.

An attractive approach, as described in this thesis, is the application of combination therapy, which simultaneously targets more than one biological pathway or mechanism and therefore may be more effective in reducing disease progression because of additional and/or synergistic effects as compared to monotherapies [10] [11]. With respect to the pharmacological treatment with the combination drug, sacubitril/valsartan, this thesis is the first to describe the beneficial metabolic effects (i.e. improved insulin sensitivity) in obese hypertensive patients. However, to implement these results in treatment strategies for the hypertensive population, more research is necessary to unravel the underlying mechanisms in different metabolic tissues (e.g. skeletal muscle) and long-term health outcomes of therapy with the combinational drug sacubitril/valsartan.

The innovative aspect of the exercise training interventions, was the investigation of exercise training-induced effects on abdominal subcutaneous adipose tissue, since these studies have mainly been performed in rodents whereas human studies are scarce. Our results contribute to the knowledge and provide better insight in exercise training-mediated metabolic changes in abdominal subcutaneous adipose tissue.
Furthermore, metabolic phenotyping at baseline makes it possible to stratify subjects into different subgroups and may improve the effectiveness of a particular intervention in a specific subgroup of the population [12]. However, in this thesis we did not find clear evidence that metabolic phenotype at baseline affected exercise training-induced study outcomes. Therefore, before extrapolating our findings to a larger population, more research is necessary in larger study populations following different intervention strategies (e.g. nutritional, pharmacological as well as

prolonged exercise training interventions). Studies including more detailed metabolic phenotyping such as tissue-specific profiling are needed not only to identify individuals or subgroups at increased risk of developing metabolic diseases, but also to design optimized prevention and treatment strategies for specific subgroups of the population. These promising strategies will be further investigated by the current project team by performing human intervention trials including *in vivo* and laboratory analyses, performed via state-of-the-art research methodologies.

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CURRICULUM VITAE

Rudi Stinkens was born on June 2nd 1983 in Sittard, The Netherlands and raised in Belgium. He studied Social and Technical Sciences and completed secondary school at the Sint-Augustinus Insituut in Bree, Belgium in 2001. Consecutively, he started a Bachelor of Science in Nutrition and Dietetics at the Katholiek Hogeschool Kempen in Geel, Belgium, where he graduated in 2005. During the following years, he specialised in sports nutrition at both the HAN University of Applied Sciences in Nijmegen, The Netherlands and the Artesis & Plantijn Hogeschool in Antwerpen, Belgium, while working in the food industry.

In 2010 he decided to make a switch in his career and started the Master of Science program in Physical Activity and Health at Maastricht University, The Netherlands, where he graduated in 2011. During this Master program, he investigated astaxanthin supplementation in endurance trained athletes during a 6-months internship at the department of Human Movement Sciences at Maastricht University. Following his internship, he performed several months pro deo research towards beet root juice supplementation, under the supervision of Dr. Cermak N. and Prof. van Loon LJ.

In November 2012, he started as a Ph.D. candidate at the department of Human Biology at Maastricht University (NUTRIM School of Nutrition and Translational Research in Metabolism), under supervision of Prof. Dr. Ellen Blaak and Dr. Gijs Goossens. His research is described in this thesis and is entitled "Adipose tissue metabolism and cardiometabolic health in obesity - Effects of pharmacological and lifestyle interventions". During his Ph.D., Rudi was selected by the European Association for the Study of Obesity (EASO) to attend the 1st Young Investigators United summer school in Lisbon, Portugal (2015). He was also selected as one of the 10 best candidates from the Netherlands to present his research findings at the North European Young Diabetologists (NEYD) meeting in cooperation with the Danish Diabetes Academy in Snekkersten, Denmark (2016). He presented his research findings at several national and international conferences. Furthermore, he received a travel grant from The Netherlands Association for the Study of Obesity (NASO) to present his research at the 1st European Obesity Summit in Göteborg, Sweden (2016) as well as a travel grant from the European Association for the Study of Diabetes (EASD) to present his research at the 53rd Annual Meeting of the European Association for the Study of Diabetes (EASD) in Lisbon, Portugal (2017).

LIST OF PUBLICATIONS

FULL PAPERS

Improved insulin sensitivity with angiotensin receptor neprilysin inhibition in individuals with obesity and hypertension
Jordan J, **Stinkens R**, Jax T, Engeli S, Blaak EE, May M, Havekes B, Schindler C, Albrecht D, Pal P, Heise T, Goossens GH, Langenickel TH.
Clin Pharmacol Ther. 2017 Feb;101(2):254 - 263

Targeting fatty acid metabolism to improve glucose metabolism
Stinkens R, Goossens GH, Jocken JW, Blaak EE.
Obes Rev. 2015 Sep;16(9):715-757

Astaxanthin supplementation does not augment fat use or improve endurance performance
Res P, Cermak NM, **Stinkens R**, Tollakson TJ, Haenen GR, Bast A, van Loon LJC.
Med Sci Sports Exerc. 2013 Jun;45(6):1158-65

No improvement in endurance performance after a single dose of beetroot juice
Cermak NM, Res P, **Stinkens R**, Lundberg JO, Gibala MJ, van Loon LJC.
Int J Sport Nutr Exerc Metab. 2012 Dec;22(6):470-8

Effect of sacubitril/valsartan on exercise induced lipid metabolism in individuals with obesity and hypertension
Engeli S, **Stinkens R**, Heise T, May M, Goossens GH, Blaak EE, Jax T, Albrecht D, Pal P, Tegtber U, Haufe S, Langenickel TH, Jordan J.
Submitted

The effects of angiotensin receptor neprilysin inhibition by sacubitril/valsartan on adipose tissue transcriptome and protein expression in obese hypertensive patients
Stinkens R, van der Kolk BW, Jordan J, Jax T, Engeli S, Heise T, Jocken JW, May M, Schindler C, Havekes B, Schaper N, Albrecht D, Kaiser S, Hartmann N, Letzkus M, Langenickel TH, Goossens GH, Blaak EE.
Submitted

Exercise training-induced effects on abdominal subcutaneous adipose tissue phenotype in obese humans
Stinkens R*, Brouwers B*, Jocken JW, Blaak EE, Theunissen-Beekman KF, Hesselink MK, van Baak M, Schrauwen P, Goossens GH.
To be submitted   * Shared first authorship

Coordinated regulation of adipose tissue adrenergic- and non-adrenergic-mediated lipolysis during exercise in lean and obese individuals: the effect of exercise training
Stinkens R*, Verboven K*, Hansen D, Wens I, Frederix I, Eijnde BO, Jocken JW, Goossens GH[#], Blaak EE.[#]
Submitted   * Shared first authorship, [#] Shared last authorship

ABSTRACTS

Coordinated regulation of adipose tissue adrenergic- and non-adrenergic-mediated lipolysis during exercise in lean and obese individuals: the effect of exercise training
Stinkens R, Verboven K, Hansen D, Wens I, Frederix I, Eijnde BO, Jocken JW, Goossens GH, Blaak EE.
Diabetologia, 2017; 60(Suppl.1):1

Exercise training-induced effects on abdominal subcutaneous adipose tissue gene and protein expression and adipose tissue morphology in obese humans
Stinkens R, Brouwers B, Jocken JW, Blaak EE, Hesselink M, Schrauwen P, Goossens GH.
Obesity Facts, 2016; 6(9) (Suppl 1):38

LCZ696 improves lipid mobilization from adipose tissue: A randomized, double-blind, active-controlled, parallel-group study in obese hypertensive patients
Jordan J, **Stinkens R**, Jax T, Engeli S, Haufe S, Blaak EE, May M, Havekes B, Schindler C, Albrecht D, Pal P, Schaper N, van der Kolk B, Tegtbur U, Heise T, Goossens GH, Langenickel TH.
Obesity Facts, 2016; 6(9) (Suppl 1):152

LCZ696 improves lipid mobilization from adipose tissue: a randomized, double-blind, active-controlled, parallel-group study in obese hypertensive patients
Jordan J, **Stinkens R**, Jax T, Engeli S, Haufe S, Blaak EE, May M, Havekes B, Schindler C, Albrecht D, Pal P, Schaper N, van der Kolk B, Tegtbur U, Heise T, Goossens GH, Langenickel TH.
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Metabolic benefits of LCZ696: a randomized, double-blind, active-controlled, parallel-group study in obese hypertensive patients
Jordan J, **Stinkens R**, Jax T, Engeli S, Blaak EE, May M, Havekes B, Schindler C, Albrecht D, Pal P, Heise T, Goossens GH, Langenickel TH.
Diabetes, 2015; 64 (Suppl 1):A496-A574