

Endocrine regulation of lipid mobilization in human obesity

Citation for published version (APA):

Verboven, K. (2017). *Endocrine regulation of lipid mobilization in human obesity: unraveling the role of atrial natriuretic peptide*. Universiteit Hasselt en Maastricht University. <https://doi.org/10.26481/dis.20170703kv>

Document status and date:

Published: 01/01/2017

DOI:

[10.26481/dis.20170703kv](https://doi.org/10.26481/dis.20170703kv)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
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Maastricht University

Doctoral dissertation submitted to obtain the degrees of
- Doctor of Biomedical Sciences | UHasselt
- Doctor | Universiteit Maastricht

Kenneth Verboven

DOCTORAL DISSERTATION

Endocrine regulation of lipid mobilization in human obesity: unraveling the role of atrial natriuretic peptide

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D/2017/2451/21

The public defence will take place on Tuesday 4 July 2017 at 10.00 a.m.

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Endocrine regulation of lipid mobilization in human obesity: unraveling the role of atrial natriuretic peptide

Dissertation

to obtain the degree of Doctor at Maastricht University, on the authority of the Rector Magnificus Prof. dr. Rianne M. Letschert, and a degree of Doctor of Biomedical Science at the Transnational University Limburg, on the authority of the Rector Prof. dr. Luc De Schepper,

In accordance with the decision of the Board of Deans to be defended in public on Tuesday 4th of July 2017 at 10.00 hours in Maastricht

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Endocrine regulation of lipid mobilization in human obesity

unraveling the role of atrial
natriuretic peptide

The research presented in this thesis was performed within NUTRIM School of Nutrition and Translational Research in Metabolism, which participates in the Graduate School VLAG (Food, Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Arts and Sciences, and within the Faculty of Medicine and Life Sciences of Hasselt University. Part of the research described in this thesis was funded by a research grant of the Research Foundation Flanders (FWO).



Financial support by Hartcentrum Hasselt for the publication of this thesis is gratefully acknowledged.

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Chapter 1

GENERAL INTRODUCTION

Obesity

In the last decades, the worldwide prevalence of overweight and obesity has risen enormously, affecting all ages and socioeconomic groups ¹⁻³. This unfortunate trend is driven by an increased consumption of energy-dense foods and the declining physical activity level, of which the relative contribution in obesity has been the subject for debate recently ⁴⁻⁷. Traditionally, obesity is defined by the body mass index (BMI, defined as weight in kilograms divided by the square of height in meters (kg/m^2)), a simple surrogate measure used to determine excess body weight in adult populations and individuals. A BMI ≥ 25 kg/m^2 indicates overweight and a BMI ≥ 30 kg/m^2 is classified as obesity ¹. However, recent findings suggest the inadequacy of BMI as a measure for general obesity categorization ^{8,9} but there is still considerable controversy as to which measurement parameter most accurately defines unhealthy body fat distribution ¹⁰. BMI based categorization revealed that worldwide 39% of the adults (> 1.9 billion) are overweight, being even > 60% in Europe, and 13% of the adults (> 600 million) are obese. These numbers are also observed in Belgium, where 67% of the adults deal with overweight and even 22% are diagnosed with obesity ¹. This trend is even more alarming as overweight and obesity is rising in children (even under the age of 5), reaching already more than 41 million children worldwide. It is clear that the increasing obesity prevalence is one of the major health concerns of our modern society since overweight and obesity increase the risk for developing metabolic complications such as insulin resistance (IR), type 2 diabetes mellitus (T2DM) ¹¹, hyperlipidemia, hypertension, atherosclerosis and cardiovascular diseases ¹². Moreover, obesity is associated with mental disorders ¹³ and several types of cancer ^{14,15}, and stroke, congestive heart failure, pulmonary embolism, asthma, gallbladder disease and gout are more common in obese versus lean individuals ¹⁶. Increased body mass can also lead to biomechanical anomalies like obstructive sleep apnea ¹⁷, chronic pain and osteoarthritis ¹⁶, which together with the aforementioned comorbidities all result in a lower quality of life ¹⁸ and an increased mortality ¹⁹. Furthermore, obesity is likely to be associated with higher healthcare costs as it is related to more comorbidity, secondary care management and reduced ability for employment, indicating its high socio-

economic burden. Hence, effective strategies are necessary to promote a healthier weight status, which has also economic benefits ²⁰.

Inter-organ crosstalk in obesity-related insulin resistance

The dysbalance between energy intake (that is, nutrient excess) and energy expenditure in obesity results in a disproportionate fat accumulation in adipose tissue ²¹ and lipid overflow to non-adipose tissues like skeletal muscle ²² and the liver ²³, thereby causing IR and inflammation, two central factors contributing to the development of cardiometabolic disease ²⁴⁻²⁷ (Figure 1). With respect to IR, the ectopic lipid deposition disrupts insulin-mediated glucose uptake and glycogen storage, likely through direct interaction of lipid-intermediates with insulin signaling, thereby reducing insulin-stimulated translocation of cell membrane glucose transporters, or through activation of inflammatory pathways ²⁸⁻³². Consequently, obesity-related insulin resistant states that progress towards the prediabetic state can be classified as impaired fasting glucose (IFG, fasting glucose > 5.6 mmol/L) or impaired glucose tolerance (IGT, 2h oral glucose tolerance test (OGTT)-derived glucose 7.8 – 11.0 mmol/L), two intermediate states of impaired glucose metabolism preceding T2DM and associated with increased cardiovascular and chronic kidney disease risk ³³. Depending on the class (impaired fasting glucose, impaired glucose tolerance or a combination of both), these conditions may differ in the site of IR (that is, peripheral and/or hepatic IR) ³⁴⁻³⁶, representing different etiologies towards T2DM ³⁷.

Long-term regulation and control of body weight (body composition) relies upon a large regulatory network integrating different metabolic organs and pathways with the aim of controlling energy intake, energy expenditure and substrate utilization. This network comprises a close inter-organ crosstalk between the adipose tissue, skeletal muscle, pancreas and liver in order to adapt to varying conditions (Figure 1). Normally, in the postprandial state dietary fatty acids are (for a large part) taken up and stored by the adipose tissue (mainly as triglycerides). However, in obesity the chronic positive energy balance enlarges the adipocytes (which become hypertrophic to accommodate this extra energy) which may lead to an impaired lipid buffering of the adipose tissue and IR ^{26,38,39}. This is further characterised by a disturbed lipoprotein lipase (LPL)-

mediated lipid uptake ^{40,41}, an impaired hormone-mediated lipid breakdown through receptor and post-receptor defects ^{42,43} and a reduced adipose tissue blood flow (leading to less triglyceride delivery to the adipose tissue) ^{44,45}. Once adipose tissue storage capacity is exceeded, lipids spill over to non-adipose tissues and are deposited ectopically ⁴⁶ when lipid supply exceeds fat oxidative capacity. There are indications that in particular skeletal muscle is characterised by an inability to increase fat oxidation upon increased supply, also indicated as metabolic inflexibility ²⁶. Besides the adipose tissue expandability hypothesis, a new concept, 'the personal fat threshold', was recently introduced to indicate that the physiological mechanisms underlying an individual's susceptibility to develop adverse effects of excess fat, such as T2DM, at a particular weight may vary between individuals ⁴⁷. With respect to ectopic skeletal muscle lipid accumulation, the overload of lipids is stored into lipid droplets as triglycerides (TAG) or lipid intermediates such as diacylglycerol (DAG), fatty acyl-CoA and ceramides, which interfere with insulin signaling ^{22,48} and thus may promote IR in the long term ⁴⁹⁻⁵¹. In this regard, the location and type (saturation) of bioactive lipid fractions as well as their turnover rate at the level of the skeletal muscle might be of major importance as opposed to the total amount *per se* ^{48,52,53}.

In the obese state, adipocyte hypertrophy is further characterized by a pro-inflammatory state in which an increased or altered production and secretion of pro-inflammatory cytokines (e.g. tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), IL-1 β , chemokines (e.g. monocyte chemoattractant protein-1 (MCP-1)) and adipokines (e.g. leptin, adiponectin) is present ^{54,55}. This increased local inflammation further attracts immune cells, such as macrophages ⁵⁶, thereby substantially increasing immune cell proportions in the obese adipose tissue ⁵⁷. In turn, these immune cells can switch from an anti-inflammatory (M2) to a pro-inflammatory (M1) phenotype (at least in animal studies) ^{58,59}, a phenotypic distinction that may be more complex in the human situation ⁶⁰, involving several other pathways besides its classical activation ⁶¹. Hence, the present pro-inflammatory cells and/or processes leading to increase local and even systemic low-grade inflammation may lead to impairments in insulin signaling and contribute to the development of IR in peripheral insulin-sensitive metabolic tissues (e.g. muscle and liver) ^{55,62} thereby increasing cardiometabolic risk.

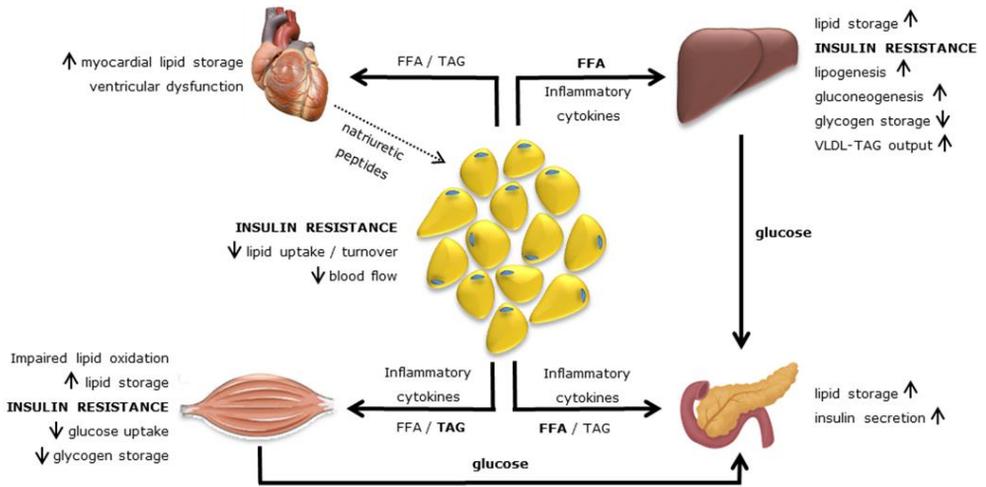


Figure 1 – Inter-organ crosstalk in the obese insulin resistant state. During long-term positive energy balance (when energy intake is much greater than energy expenditure), adipose tissue exceeds its lipid buffering capacity resulting in lipid overflow and lipid storage in non-adipose tissues (ectopic fat storage), which is associated with the systemic low-grade inflammation and the development of insulin resistance. Substrate fluxes between tissues depicted by arrows (bold font indicates increased plasma concentrations and substrate fluxes in human obesity-related insulin resistance). FFA, free fatty acids; TAG, triacylglycerol; VLDL, very low density lipoprotein.

As already indicated before, altered storage, localization and composition of lipids and lipid-metabolites might result in lipotoxicity thereby interfering with insulin signaling and promoting IR^{23,63,64} in skeletal muscle^{31,65} and liver^{23,26}. In addition to an increased supply of lipids to the skeletal muscle, disturbances in lipid handling may also contribute to the impaired insulin action at the level of the muscle. Skeletal muscle of obese insulin resistant individuals often displays a suboptimal fat oxidative capacity⁶⁶, presumably related (at least in part) to an impaired fatty acid uptake, altered intramuscular lipolysis and attenuation of the mitochondrial capacity^{42,49,67}. The resulting accumulation of lipid metabolites may lead to lipotoxicity and related IR in metabolically compromised conditions^{31,37,66,68}. Furthermore, the capacity of pancreatic β -cells to produce insulin upon glucose stimulation is also blunted upon long-term lipotoxic conditions and ectopic pancreatic lipid accumulation^{69,70}. Moreover, the liver is an important site for ectopic lipid accumulation, mainly in the postprandial phase as a consequence of the impaired insulin-mediated anti-lipolysis present in the obese

state ⁷¹. Indeed, several studies indicated the compelling association between hepatic lipid content and obesity ⁷²⁻⁷⁴. In many obese individuals, ectopic lipid accumulation is also observed in and around the heart ⁷⁵, which is believed to be harmful for cardiac function ⁷⁶. The presence of ectopic lipids in multiple non-adipose tissues, besides their excessive storage within distinct adipose tissue depots, suggest that targeting adipose tissue lipid metabolism may be a possible effective method to prevent and/or treat metabolic disorders.

Recently, research indicated that the gut, in particular the gut microbiota composition and its products, may have an impact on host metabolism, thereby being an important player in inter-organ crosstalk. Studies have shown that alterations in the gut microbiota composition and function (microbiome) affect host adipose tissue, skeletal muscle and liver lipid and glucose metabolism. The gut microbiota has been proposed to be involved in the development of IR and impaired glucose metabolism ⁷⁷ as it regulates host energy and substrate metabolism ⁷⁸⁻⁸⁰, possibly via alterations in the production of bile acids, incretins, short chain fatty acids and inflammatory factors ⁸¹⁻⁸⁴. Nevertheless, a recent study showed that interference with adult microbiota by a 7-day antibiotic treatment has no clinically relevant impact on metabolic health in obese humans ⁸⁵, indicating that the microbiota may not play a physiologically significant role in all metabolic conditions. Of interest, gut-derived short chain fatty acids, which are formed after fermentation of dietary fibres, have anti-lipolytic potency, thereby possibly affecting peripheral lipid metabolism ⁸⁶⁻⁸⁸. Furthermore, angiotensin-like protein 4 (ANGPTL4), a protein that is expressed and secreted by numerous cell types ⁸⁹ (including endocrine cells within the gut ⁹⁰, is able to modulate peripheral lipid metabolism under various conditions ⁹¹⁻⁹⁴.

As obesity increases the risk for both T2DM and cardiovascular disease, animal studies have postulated that alterations in either myocardial lipid uptake or fatty acid oxidative capacity may lead to the accumulation of various lipid intermediates that are linked to the progression of ventricular dysfunction, and thus cardiac lipotoxicity ⁹⁵. Indeed, a possible role of the heart with respect to inter-organ crosstalk becomes more plausible as the heart-adipose tissue connection has been of special interest in metabolic research during the last decade ⁹⁶. In particular, the heart is able to produce specific peptides (natriuretic

peptides), which have beside cardiovascular effects ⁹⁷, several metabolic effects at the level of different insulin sensitive tissues ⁹⁸⁻¹⁰⁰. The understanding of this inter-organ crosstalk (Figure 1) and the link between cardiovascular and metabolic effects is rapidly evolving as further examples of important physiological connections between tissues and systems are unveiled ^{96,99,100}. However, with respect to the bi- or multi-directional communication between the heart and different metabolic tissues such as the adipose tissue, skeletal muscle, liver and pancreas, much work remains to be done, to identify future targets for the prevention and treatment of cardiometabolic diseases.

Adipose tissue (dys)function in obesity-related insulin resistance

The adipose tissue organ is composed of different discrete anatomical depots ¹⁰¹, all with a different size and metabolic function ^{102,103}. Regarding upper-body adipose tissue depots, the visceral adipose tissue (VAT) confers increased risk for metabolic complications and cardiovascular risk factors compared to subcutaneous adipose tissue (SCAT) ¹⁰⁴⁻¹⁰⁷. Intriguingly, insulin resistant SCAT was suggested to contribute to ectopic VAT accumulation ¹⁰⁸, presumably through a compensatory uptake of dietary lipids in VAT in case of an impaired SCAT buffering of dietary lipids ^{109,110}. This increased VAT may subsequently cause hepatic lipid accumulation and IR via increased delivery of lipids through the portal vein (portal hypothesis ¹¹¹) ¹¹². In contrast, impaired SCAT lipid buffering contributes for the largest part to systemic lipid overflow leading to ectopic lipid accumulation in peripheral tissues like skeletal muscle and IR.

During the last decades adipose tissue function, besides protecting internal organs and storing excessive energy in the form of fat, became the subject of numerous investigations in human (patho)physiology ^{30,113}. Dysfunctional adipose tissue is a known concept, associated with an increased cardiometabolic risk and represented by an altered adipose tissue morphology, an impaired lipid buffering capacity (due to the presence of anomalies in lipid uptake, storage and release (lipid mobilization)) and modified secretion of endocrine, paracrine and autocrine signals that could contribute to local and systemic low-grade inflammation. All these characteristics of adipose tissue (dys)function will be discussed in detail below.

Adipocyte hypertrophy versus hyperplasia

In humans, the total number of adipocytes is determined during childhood and adolescence. The adipocyte number remains stable during adulthood although cells are renewed throughout life ¹¹⁴, implying proper neogenic and apoptotic capacity of the adipose tissue ¹¹⁵. Data from early onset obesity revealed a yearly turnover of ~8% of the adipocyte population ¹¹⁶. However, these data might not reflect adipocyte turnover in adults who have gained weight over several years in adulthood, thereby first increasing their adipocyte size rather than the adipocyte number ¹¹⁶. In line, during the average 10 year life span of human adipocytes, the triglyceride content is renewed 6 times, on average ¹¹⁷. In case of long-term energy overload, adipocytes store this energy by expanding and increasing in size, a process called hypertrophy ³⁸. Hyperplasia is a second adaptive response of the adipose tissue ¹¹⁸, an adipogenic process where pluripotent mesenchymal stem cells are committed to the preadipocyte lineage and/or preadipocytes undergo multiple mitosis cycles and differentiate into adipocytes ¹¹⁹. When the hyperplastic potential of the adipose tissue is limited because of impaired differentiation, the expandability capacity decreases ¹²⁰⁻¹²³. This in turn affects other tissues since adipocytes fully filled are extremely insulin resistant and therefore are restricted in their buffering capacity ^{124,125}. Of interest, in overweight and obese individuals a high amount of triglyceride is stored in the adipocytes which may result in a reduced triglyceride extraction, in particular in the postprandial state, as compared with lean individuals. This impaired lipid turnover reflects a reduced capacity to adjust to chronic lifestyle changes, thereby favoring fat mass accumulation ^{117,126}. In line, a low lipid turnover was associated with obesity ¹²⁶ and hypertrophic dysfunctional AT ¹²⁰. Indeed, this limited adipocyte and triglyceride turnover is an important characteristic of adipose tissue hypertrophy ^{120,126}. Furthermore, this lipid removal rate was positively associated with the lipolytic capacity of adipocytes and was inversely related to IR ¹¹⁷. Additionally, adipocyte hypertrophy has been shown to be a key phenotypic characteristic of non-obese T2DM patients ¹²⁷. Furthermore, many studies show adipocyte hypertrophy in obesity, IR and T2DM ^{116,128-130}, which was associated with IR independently of adiposity indices ^{39,131,132}. In obese T2DM patients, subcutaneous adipocyte number was lower compared to BMI-matched obese individuals without metabolic complications

^{39,133-137}. Yet, whether adipogenesis is an adaptive or maladaptive contributor to obesity-related IR or T2DM is still an open question ^{120,138}.

Adipose tissue lipid mobilization

In times of a negative energy balance, such as during fasting or physical exercise in order to fulfill other organs' needs, adipocytes are able to mobilize their stored (TAG) via adipocyte lipid mobilization (also called lipolysis). Hydrolysis of TAG into glycerol and free fatty acids (FFA) during lipolysis is achieved via the consecutive action of three lipases, which act sequentially: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) ¹³⁹⁻¹⁴¹. The main lipolytic regulators of human adipose tissue lipolysis are catecholamines (secreted by the adrenal medulla and sympathetic nervous system) and natriuretic peptides (secreted by the heart), while anti-lipolysis is mainly mediated by insulin (Figure 2).

Adrenergic signaling in the adipose tissue is mainly mediated by the catecholamines adrenalin and noradrenalin (which are synthesized in the chromaffin cells of the adrenal gland medulla as a response to direct stimulation from the sympathetic nervous system (mediated by adrenocorticotrophic hormone and/or cortisol) ¹⁴²) ^{42,143-145}. These hormones accomplish their lipolytic regulation by binding to different adrenergic receptors, for which their affinity (together with the relative abundance of each receptor on the adipocyte membrane) determines whether they stimulate or inhibit lipolysis. In this respect, β -adrenergic receptors on the adipocyte surface activate lipolysis, while anti-lipolytic signals are transferred by the α_2 -adrenergic receptors ¹⁴⁶. For β -adrenergic receptors, three subtypes exist (β_1 , β_2 and β_3) of which only the β_1 and β_2 are involved in human lipolysis regulation ¹⁴⁷⁻¹⁵⁰. The β_3 -adrenoceptor has been shown to be present in human ¹⁵¹ as well as in rodent adipocytes ¹⁵² and to participate in lipolysis ¹⁵¹, via an ATGL-mediated mechanism (at least in mice) ¹⁵³. A clear proof of a physiological relevance of the β_3 -adrenoceptor in human obesity is still lacking ¹⁵⁴⁻¹⁵⁶. However recent human data in young lean metabolically healthy individuals showed that the β_3 -receptor agonist mirabegron can stimulate human brown adipose tissue thermogenesis and may be a promising treatment for metabolic disease ¹⁵⁷. These β -adrenergic

receptors are coupled to a G-protein, containing a stimulating G_s subunit which interacts with adenylyl cyclase upon stimulation ¹⁵⁸, thereby converting ATP to cyclic adenosine monophosphate (cAMP) resulting in elevated intracellular cAMP levels. This second messenger activates protein kinase A (PKA) ¹⁵⁹, which phosphorylates the lipid droplet-associated protein PLIN1 ¹⁶⁰ and cytosolic HSL ¹⁶¹⁻¹⁶³ which becomes activated and translocated to the lipid droplet surface ¹⁶⁴ where it docks on the phosphorylated PLIN1 and gains access to its substrate (that is, diacylglycerols) ^{165,166}. Phosphorylation of PLIN1 stimulates the release of comparative gene identification-58 (CGI-58), a co-activator of ATGL ^{167,168}. ATGL hereby becomes fully activated, resulting in the initiation of the lipolytic cascade and the generation of diacylglycerols serving as a substrate for HSL. In contrast, the α_2 -adrenergic receptors are coupled to a G-protein containing the inhibitory G_i subunit, thereby inhibiting adenylyl cyclase and PKA activity ¹⁵⁸.

In the beginning of this millennium, natriuretic peptides (NPs), including atrial (ANP) and B-type (BNP) NPs, produced by the myocardium (by increasing NPs gene transcription and secretion of stored granules ¹⁶⁹) in response to myotube distension ¹⁷⁰ such as during exercise have been shown to be potent stimulators of human adipose tissue lipolysis ¹⁷¹. By stimulating the guanylyl cyclase-linked type-A NP receptor (NPRA) on the adipocyte surface, NPs cause the conversion of intracellular GTP into cGMP. This results in the activation of protein kinase G (PKG), which activates the lipolytic cascade by the phosphorylation of PLIN1 and HSL ^{172,173}. Limited data is available with respect to ANP-mediated activation of ATGL ¹⁷⁴ NP-induced lipolysis is completely independent from the catecholamine-induced (cAMP/PKA mediated) lipolysis, as they rely on different pathways ^{175,176} (Figure 2). However, an additive lipolytic effect occurs when human adipocytes are stimulated simultaneously with ANP and a beta-adrenergic agonist (e.g. isoproterenol) ¹⁷⁷. Unlike the cAMP-dependent pathway, NP-mediated lipolysis is unresponsive to the anti-lipolytic effects of insulin-mediated phosphodiesterase 3B (PDE3B) activation ^{171,176}. However, the NP pathway is counter-regulated via the hydrolysis of cGMP by other members of the PDE family, like PDE5 ^{178,179}. Indeed, PDE5 expression and activity have been found in isolated human adipocytes from both subcutaneous ¹⁷⁸ and visceral ¹⁸⁰ adipose tissue. However, this enzyme appears to be insufficient to control ANP-mediated lipolysis ¹⁷⁸.

Hence, at present, details of the regulatory pathway counteracting ANP-mediated lipolysis *in vivo* are incompletely understood¹⁷⁹. In addition, binding of NPs to the type C natriuretic peptide receptor (NPRC), also expressed on the adipocyte plasma membrane (amongst other tissues¹⁸¹⁻¹⁸³) but lacking guanylyl cyclase activity, mainly facilitates scavenging of its ligands, internalization of ligand-receptor complexes and recycling of NPRC, together with lysosomal degradation of its ligands^{182,184}. Expression of the functional NPRA and scavenging NPRC receptors appears to be regulated in an opposite way. In the fasted state, *NPRA* gene expression increases while *NPRC* gene expression decreases¹⁸⁵. In contrast, high fat feeding and exposure to insulin greatly increased NPRC expression while reducing NPRA expression^{183,186,187}. The local tissue specific and systemic effects of NP are thought to depend on the ratio between the signaling receptor NPRA to the clearance receptor NPRC^{187,188}. In addition to catecholamines and natriuretic peptides, a number of other hormones are known to participate in adipocyte lipolysis regulation, including growth hormone¹⁸⁹⁻¹⁹³, cortisol¹⁹⁴⁻¹⁹⁶, thyroid hormones¹⁹⁷, parathyroid hormone^{198,199}, androgens²⁰⁰⁻²⁰⁴ and glucagon^{205,206}. However, their impact on adipocyte lipolysis is much more reduced as opposed to the aforementioned major regulators and their pathways are briefly reviewed elsewhere²⁰⁷.

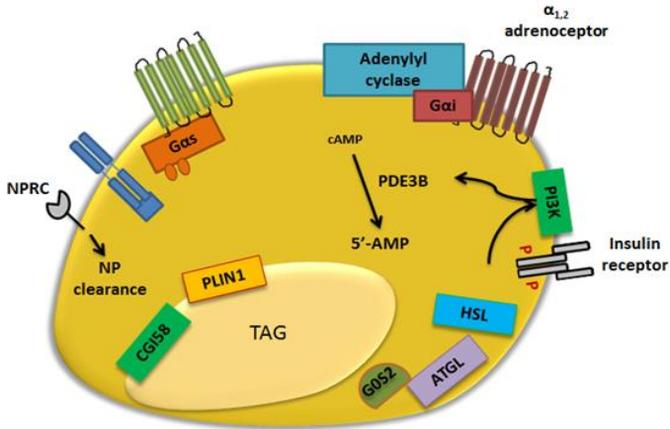
An alternative pathway that recently has been proposed to play a role in lipid breakdown is autophagy. Although autophagy (that is, a 'self-digestion' system that degrades unnecessary or dysfunctional cellular components to provide essential nutrients for cellular survival in times of energy deprivation) is generally seen as a non-selective process, studies clearly associate autophagy and the selective breakdown of lipids in murine adipocytes and hepatocytes, a process termed lipophagy²⁰⁸⁻²¹¹. The potential hypotheses described in literature, indicating emerging roles of autophagy in cellular metabolism and metabolic diseases are beyond the scope of this thesis and are therefore referred to elsewhere^{212,213}.

Insulin is considered being the main factor in the inhibition of adipocyte lipolysis. The binding of insulin to its receptor, which possesses intrinsic tyrosine kinase activity, leads to autophosphorylation and subsequent phosphorylation of the IRS1/2²¹⁴. Subsequently, this promotes phosphatidylinositol 3-kinase (PI3K)

which increases phosphatidylinositol-3,4,5-triphosphate (PIP₃)²¹⁵, thereby causing phosphorylation and activation of Akt (that is, PKB). Ultimately, Akt/PKB activates PDE3B and the degradation of cAMP²¹⁶. Via this signaling pathway, insulin prevents the phosphorylation of HSL and PLIN1, thereby suppressing catecholamine-stimulated (not NP-stimulated) adipocyte lipolysis (Figure 2).

Besides the catecholamine-mediated α_2 -adrenergic receptor mediated activation of G_i coupled proteins, several other factors arising from different cell types (such as stroma vascular fraction cells, sympathetic nerve terminals, the gut or adipocytes themselves) have been shown to inhibit lipolysis. In particular, GPR109A and GPR81, two G-protein coupled receptors with an inhibitory effect (G_i coupled receptors), are of special interest^{217,218}. GPR109A is highly expressed on adipocytes, binding ketone bodies of which plasma levels are increased during fasting²¹⁸. GPR81 has a binding affinity for lactate and counterbalances catecholamine-induced lipolysis^{217,219-222}. Furthermore, short chain fatty acids receptors (GPR41 and GPR43 receptors) are present in human adipose tissue²²³⁻²²⁵, via which their ligands have been shown to modulate adipocyte lipolysis by anti-lipolytic²²⁶ or lipolytic effects *in vitro*²²⁷. However, the impact of short chain fatty acids on lipolysis in human adipocytes needs to be investigated in more detail⁸⁸.

Anti-lipolytic signals



Lipolytic signals

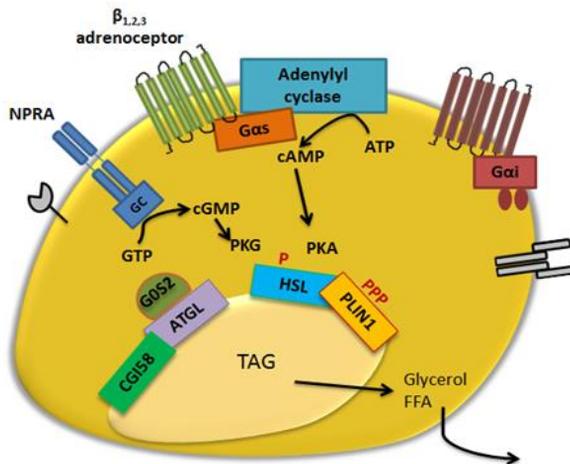


Figure 2 – Main regulators of adipose tissue lipolysis. *Upper panel*: anti-lipolytic signals. Insulin activation of PDE3B through a PI3K-dependent pathway and adenylyl cyclase inhibition through Gi protein-coupled receptor attenuate cAMP production and PKA activation. Binding of NPRC in anti-lipolytic conditions favors natriuretic peptide clearance. *Lower panel*: lipolytic signals. Catecholamines (adrenalin and noradrenalin) and natriuretic peptides (ANP, BNP) act through β -adrenoceptors and NPRA, respectively. Subsequently, PKA and PKG activation leads to HSL and PLIN1 phosphorylation. Phosphorylated HSL translocates to the lipid droplet surface to exert its function. Phosphorylated PLIN1 promotes lipid droplet fragmentation and ATGL co-factor CGI58 release, which interacts with and activates ATGL. ATGL, adipose triglyceride lipase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CGI58, comparative gene identification 58; FFA, free fatty acid; G0S2, G₀/G₁ switch gene 2; GC, guanylyl cyclase; HSL, hormone sensitive lipase; NPRA, natriuretic peptide receptor type A; NPRC, natriuretic peptide receptor type C; P, phosphate group; PDE3B, phosphodiesterase 3B; PI3K, phosphatidylinositol 3 kinase; PKA: protein kinase A; PKG, protein kinase G; PLIN1, perilipin 1; TAG, triacylglycerol.

Depot-specific regulation of lipid mobilization

Fat distribution, like android versus gynoid, rather than fat mass *per se* has profound implications with respect to metabolism and hence risk for cardiometabolic disease. With respect to adrenoceptors, intracellular lipases and lipid droplet-associated proteins, these are known to be differentially expressed in VAT and SCAT, contributing to depot-specific differences in lipolytic capacity²²⁸⁻²²⁹. From *in vitro* studies, VAT adipocytes were shown to display lower basal (that is, non-stimulated) lipolysis but higher adrenergic responsiveness²³⁰⁻²³³. This aspect might be explained by a lower expression level of anti-lipolytic α_2 -adrenergic receptors, while β -adrenoceptors are expressed to a higher extent in the VAT, thus contributing to a higher adrenergic responsiveness of this depot^{234,235}. With respect to anti-lipolysis, visceral adipocytes were reported to be more insulin resistant as compared to subcutaneous adipocytes²³⁶, implying that the relatively small contribution of VAT lipolysis to systemic FFA concentrations during the fasted state (as compared to SCAT) may be more pronounced during insulin-mediated suppression of lipolysis or in the postprandial state (reaching up to 40%)²³⁶⁻²³⁷. Physiologic factors addressing the cause(s) of these depot differences need to be explored further *in vivo* and might include changes in innervation, blood flow, or other factors affecting triglyceride synthesis and storage or inflammatory profile and adipokine secretion²³⁸. In addition, upper body subcutaneous depots (that are, femoral, gluteal and abdominal) have a higher responsiveness to β -adrenergic agonists, while lower body adipocytes are more responsive to α_2 -adrenergic agonists²³⁴⁻²³⁹. With respect to the anti-lipolytic effect of insulin in these different depots, data are inconclusive^{240,241}.

Furthermore, the lipolytic effects of natriuretic peptides are thought to depend on the ratio between the signaling receptor NPRA to the clearance receptor NPRC^{187,188}. However, data about depot-specific natriuretic peptide receptors are currently lacking in lean individuals. In obese adipose tissue, VAT NPRA and NPRC mRNA expression was shown to be increased compared to SCAT in the obese state¹⁸³. Moreover, NPRA expression is decreased in obese as compared to lean SCAT, both at mRNA and protein level^{242,243}.

The depot-specific differences in lipolytic activity may also be mediated by downstream differences in the lipolytic pathway, such as altered lipase or lipid-droplet-associated protein expression profiles. While ATGL mRNA expression is comparable between VAT and SCAT ²⁴⁴⁻²⁴⁶, HSL mRNA expression and activity is shown to be higher in SCAT compared to VAT, which explains the higher lipolytic activity of the SCAT as compared to VAT ²⁴⁷. However, other studies did not observe differences in HSL mRNA or protein expression between both depots ²⁴⁸⁻²⁵¹, indicating that the direction of the adipose tissue depot difference in lipase expression is unclear. Of interest, higher lipase protein expression (that is, HSL and ATGL) has been shown for large adipocytes as opposed to small adipocytes from the same subject ²⁵², indicating the important association between adipocyte size and lipase expression/activity.

The lipid droplet-associated protein PLIN1, the major important perilipin family member involved in the regulation of adipose tissue lipolysis ²⁵³, differs in depot expression as well. In the obese state, PLIN1 mRNA expression has been reported to be increased in VAT ^{254,255}, but unaffected in SCAT ²⁵⁴. In contrast, PLIN1 protein expression is reduced in both obese VAT and SCAT as compared to lean adipose tissue depots ²⁵⁴, and among obese individuals, the protein levels in SCAT are lower in insulin resistant compared to insulin sensitive individuals ²⁵⁶.

Lipolytic impairments in obesity-related insulin resistance

It has been suggested that plasma FFA concentrations increase in proportion with the degree of obesity ²⁵⁷. This increase was believed to result from an increased basal lipolysis in the obese state, since plasma FFA are predominantly derived from adipose tissue lipolysis ¹⁴⁰. However, the question whether an FFA overload exists in obesity became a matter of discussion ²⁵⁸. In light of basal (that is, spontaneous or non-stimulated) lipolysis, data are inconsistent showing basal lipolysis to be increased ²⁵⁹, unchanged ²⁶⁰ or even decreased ^{261,262} in obese SCAT, depending on the degree of IR or method of expression. It might be evident to state that in obese individuals basal lipolysis in fasting conditions (measured as plasma FFA concentrations) is increased due to the increased adipose tissue mass. However, plasma FFA concentrations have shown to be not increased in proportion to the amount of fat mass, indicating basal lipolysis to be

even lower in obese versus lean individuals ^{258,262-264}. Nevertheless, the importance of basal lipolytic rate in IR was corroborated by a bariatric surgery-induced weight loss study, in which obese individuals with the highest decrease in basal lipolysis (as determined by spontaneous glycerol release from isolated mature adipocytes *in vitro*) were those with the greatest improvement in insulin sensitivity ²⁶⁵. Adipose tissue IR (that is, the blunted suppression of lipolysis during hyperinsulinemia), has been associated with glucose intolerance and adiposity ^{43,266,267}. Also, in human cohorts with a wide spectrum of adiposity, basal lipolysis (spontaneous glycerol release from isolated mature adipocytes *in vitro*) has been proven to be a determinant of variations in insulin sensitivity ²⁶⁵. Accordingly, the SCAT of obese non-diabetic and type 2 diabetic individuals is resistant to the anti-lipolytic effect of insulin. Recently, a cohort with a wide ranging in glucose tolerance showed that fasting adipose tissue IR, quantified by means of the fasting FFA times fasting insulin ²⁶⁸⁻²⁷², progressively rises over the span of glucose tolerance (ranging from normal glucose tolerance to impaired glucose tolerance to T2DM) ²⁷³. Furthermore, the enlargement of adipocytes (that is, hypertrophy) was suggested to be a major contributor to IR ^{126,265}, a phenotype that has been linked to increased lipolytic rates as well, independent of BMI or age ^{252,274}.

In line with the above-mentioned findings, an *in vivo* blunted whole-body catecholamine-induced lipolysis has been observed in obese individuals ^{42,275-280}. *In vitro* the site of this reduced catecholamine-stimulated lipolytic response was shown to be the abdominal SCAT ^{147,259,281-285}. Only some ²⁷⁸, but not all ^{286,287}, *in situ* microdialysis studies support this observation. It is however unclear whether these defects in lipolysis are of primary origin or rather a secondary result in the etiology of obesity and related IR ^{42,140}. Of interest, lipolytic defects may be causally related to obesity development, a theory that has been observed in childhood onset obesity ^{288,289} and in first-degree relatives of obese individuals ²⁹⁰. On the other hand, reductions in lipolytic activity have been proposed to be secondary to obesity development by others ¹²⁶, maybe caused by hyperinsulinemia ²⁶⁶. Regarding ANP-mediated lipolysis, Rydén *et al.* has recently showed an impaired ANP-mediated lipolysis *in vitro* and *in situ* in human SCAT of obese women compared to non-obese counterparts ²⁴². Of interest, ANP-mediated lipolysis was comparable between subcutaneous

adipocytes compared to visceral adipocytes of obese individuals ²⁹¹. However, these studies investigating depot-specific ANP-mediated lipolysis did not include lean individuals, nor obese individuals with type 2 diabetes. The decreased NP-induced lipolysis may be the result of an impaired receptor signaling in subcutaneous adipocytes ²⁴², an anomaly that might be caused by the hyperinsulinemic state that is frequently observed with obesity ^{174,183,187,243,292}. The lipolytic impairments summarized here may be an early factor in the development of increased fat stores and obesity-related metabolic complications. Therefore, a better insight into the role of these anomalies in obesity pathophysiology as well as lipolytic endocrine regulation is warranted.

Adipose tissue inflammation in obesity

The presence of immune cells in adipose tissue has been known for decades ²⁹³. The first report providing the basis for the hypothesis that local inflammation was present in the obese adipose tissue dates from the early nineties, where Hotamisligil *et al.* ²⁹⁴ reported *TNF- α* gene and protein expression to be increased in obese compared to lean adipose tissue, suggesting their role in whole-body insulin sensitivity. The origin of this pro-inflammatory cytokine is believed to be adipose tissue macrophages ^{295,296} surrounding dead or apoptotic adipocytes ²⁹⁷. Moreover, several adipose tissue-derived cytokines, such as monocyte chemoattractant protein 1 (MCP-1) and IL-6, are known to be altered in the obese state ^{298,299}. These cytokines initiate the migration of monocytes from the circulation into the adipose tissue, where they further differentiate into macrophages ³⁰⁰, contribute to maintain adipose tissue inflammation and attract other immune cells ³⁰¹. Moreover, different immune cells are activated within hypertrophic adipose tissue (in case of resident immune cells) or attracted to it, especially in the VAT (mainly based on rodent studies) ^{302,303}. Upon migrating, macrophages populate the adipose tissue, reaching cell proportions up to 50% in obese individuals ²⁹⁵, in order to remove cell debris, remodel the adipose tissue and serve as a lipid buffer in order to reduce lipid overload ³⁰⁴. Indeed, FFA serve as ligands for the toll-like receptor 4 (TLR4) complex ³⁰⁵, activate classical inflammatory response, and drive adipose tissue macrophage accumulation ^{306,307}. Switching macrophage phenotype is also associated with adipose tissue inflammation ³⁰⁸⁻³¹⁰ because macrophages can exist along a spectrum of

phenotypes ³¹¹, the poles being M1 (or classically-activated) and M2 (or alternatively-activated) ³¹². M1 macrophages display a pro-inflammatory appearance (characterized by expression of cytokines like TNF- α , IL-6 and IL-1 β) while M2 macrophages are involved in tissue remodeling and wound healing, expressing anti-inflammatory cytokines like IL-10 and IL-1 receptor antagonist. During obesity progression, more inflammatory M1 macrophages infiltrate the adipose tissue, causing IR and maintaining inflammation ³¹³, while adipose M2 macrophage numbers remain stable (or even slightly increase), resulting in a vicious inflammatory cycle ³¹⁴. Following these processes, a shift towards an increased, pro-inflammatory M1/M2 ratio culminates ^{299,315,316}. The above mentioned division in inflammatory M1 macrophages and anti-inflammatory M2 macrophages is merely based on animal studies whilst in humans, the distinction between different macrophage phenotypes may be more complex, as other activation pathway(s) may define their properties ^{60,113,311}.

Still, the dynamic changes in which different immune cells initiate and maintain adipose tissue inflammation are incompletely understood. Other immune cells beside macrophages are likely to be involved in the initiation of local inflammation, all exerting negative effects on insulin sensitivity ³⁰³. Indeed, T lymphocytes ³¹⁷⁻³¹⁹, B lymphocytes ^{320,321}, NK cells ³²², neutrophils ³²³, dendritic cells ³²⁴ and mast cells ³²⁵ have been found to reside in the adipose tissue in the obese state, where they may contribute to adipose tissue inflammation and IR. Of interest, T and B lymphocytes gained considerable attention because of the observed early influx into the adipose tissue upon high-fat diet in mice ^{317,326}, thereby preceding macrophage infiltration into the adipose tissue ^{309,326}. The proinflammatory features of B cells in a high-fat diet setting was also reported in mice ³²¹, which can also contribute to a decreased glucose tolerance via orchestrating T cell populations and releasing pathogenic antibodies ³²⁰. Furthermore, reduced proportions of regulatory T cells in adipose tissue, and thus a downscaled dampening of proinflammatory signaling, have been found to precede macrophage accumulation in abdominal (epididymal) adipose tissue in mice and omental adipose tissue in obese individuals ³²⁷. This depletion of regulatory T cells has been implicated in IR ^{328,329}, presumably by exerting their anti-inflammatory properties thereby preventing accumulation of pro-

inflammatory cells in the adipose tissue, as was shown in murine visceral adipose tissue³²⁹. In addition, it was recently proposed that NK cells might be of importance in obesity-related IR and adipose tissue inflammation and it has been shown that manipulations of NK cells (either with neutralizing antibodies or genetic ablation) altered metabolic outcomes in obese animals³²². These data indicate a strong interplay between adaptive (*e.g.* B cells and T cells) and innate immunity (*e.g.* macrophages and NK cells), which determines the inflammatory characteristics of obese adipose tissue. Therefore, gaining insights in inflammation-related concepts in the pathogenesis of obesity-induced IR is important, especially because the early local inflammation is likely to escalate when not counteracted, thereby contributing to systemic (low-grade) inflammation, IR and lipotoxicity in tissues like skeletal muscle and liver^{30,113,330}. Of interest, VAT inflammation (as demonstrated by increased macrophage infiltration in VAT) was proposed to be the strongest correlate of IR in obese human with a wide range of insulin sensitivity^{331,332}. Notwithstanding the pathogenic properties of adipose tissue inflammation, one should keep in mind that these immune cells also interact with adipose tissue lipid metabolism, affecting the lipid storage or buffering capacity of the adipose tissue and lipolysis^{304,333}, and promote adipogenesis³³⁴. The latter data indicate that low-grade adipose tissue inflammation is essential for a healthy adipose tissue expansion^{297,335}. Whether inflammation is the trigger or result of obesity-associated defects remains an important area for future human studies.

Prevention and treatment strategies for obesity and related cardiometabolic diseases

In the treatment of obesity, and indirectly its related cardiometabolic risks, creating a negative energy balance is imperative. The most common strategies to achieve weight loss include decrease in energy intake, increase in energy expenditure or a combination between both³³⁶. Lifestyle interventions including combined diet and physical activity have been shown to be effective in the prevention of diabetes, reducing diabetes incidence by 47-57% over a 3-6 year timeframe^{337,338}.

Combined caloric restriction and physical activity interventions have been shown to be superior to diet-only intervention in terms of (in particular) weight management after weight loss and weight loss *per se*, as indicated in several meta-analyses³³⁹⁻³⁴¹, even in the initial-onset phase (during the first 6 months)³⁴². Physical activity interventions without caloric restriction, including several types of interventions, such as endurance exercise, walking interventions, resistance training or habitual activity (number of steps throughout the day), have been shown to induce weight reductions (depending on the exercise modalities used), although to a lower extent (2-3%) as compared to combined treatments³⁴³⁻³⁴⁷. However, endurance exercise is also associated with other health benefits like improvements in blood lipid profiles, insulin sensitivity and a reduction in inflammatory markers as recently reviewed by Ross *et al.*³⁴⁸. In this type of interventions, the intensity of the exercise seems to play an important role in the magnitude of weight loss and in improving metabolic profile³⁴⁹. Weight loss is assumed to include both loss of fat mass and fat-free mass. However, a restricted reduction in fat-free mass is associated with a lesser reduction in resting energy expenditure^{350,351}. Therefore, the use of resistance training in addition to combined intervention might be important to preserve or potentially increase fat-free mass³⁵², thereby also improving glycemic control and insulin sensitivity (independent of changes in BMI)^{353,354} and other risk markers for cardiometabolic health³⁵⁵. Recently, high-intensity interval training was shown to have positive effects on glucose control and cardiometabolic health in populations at risk of cardiac complications and metabolic disease³⁵⁶. In short, combined lifestyle interventions (including diet plus exercise) as well as endurance exercise interventions of moderate- to high-intensity (with or without resistance training) may help individuals with obesity to achieve a clinically relevant weight loss (3-5% of initial body weight³⁵⁷), improve weight maintenance and cardiometabolic risk profile.

Despite the effectiveness of dietary and lifestyle modifications in the treatment of obesity and its related complications, these lifestyle changes are difficult to maintain and weight regain is typically found. Therefore, several anti-obesity and antidiabetic treatments have been developed, which are approved by the US Food and Drug Administration (FDA), while many other potential therapies are currently being investigated³⁵⁸. Currently, five FDA-approved anti-obesity drugs

are on the market (ADA obesity treatment, 2016), which act centrally (Saxenda, Belviq, Qsymia and Mysimba)³⁵⁹⁻³⁶³ or peripherally (Orlistat)³⁶⁴.

Of special interest, to promote insulin sensitivity, inhibition of adipose tissue lipolysis might be an attractive therapeutic strategy in obese individuals. In this regard, preclinical studies in mouse models revealed that partial inhibition adipose tissue lipolysis protects against IR without affecting body weight via increased *de novo* lipogenesis^{265,365}. However, in clinical research, no pharmacological lipase (ATGL or HSL) inhibitors have been used so far. Nicotinic acid is a recognized lipid lowering agent able to bind GPR109A³⁶⁶⁻³⁶⁸, a G_i coupled receptor responsible for partial inhibition of adipose tissue lipolysis^{217,218}, which results in reduced circulating FFA concentrations and an increased plasma glucose clearance in humans³⁶⁹⁻³⁷¹. However, chronic treatment with nicotinic acid might even raise plasma FFA levels, thereby deteriorating glucose metabolism and insulin sensitivity^{371,372}. Moreover, an analog of nicotinic acid, acipimox, has a more favorable profile on insulin sensitivity via decreased circulating FFA and an increased glucose clearance as shown in obese diabetic and non-diabetic individuals^{373,374}. Despite some contrasting results showing decreased³⁷⁵⁻³⁷⁷, increased^{378,379} or even unaffected³⁸⁰ plasma FFA concentrations, chronic acipimox treatment has been reported to moderately improve glycemic control and reduce glycosylated hemoglobin levels in non-insulin dependent diabetic individuals³⁷⁸, increase insulin signaling in skeletal muscle of obese insulin resistant individuals³⁷⁷ and decrease hepatic glucose production in obese and T2DM individuals³⁷⁵⁻³⁷⁷.

Outline of this thesis

This thesis focuses on the endocrine regulation of adipose tissue lipid mobilization and the impact of exercise intervention in obese individuals, with a specific emphasis on the natriuretic peptide system and in particular atrial natriuretic peptide (ANP). The main goal of the current thesis was to gain more insight into the endocrine regulation of lipid mobilization in human obesity by combining cross-sectional and human clinical studies, together with *ex vivo* and *in situ* studies directed at understanding the underlying molecular mechanisms.

Aim 1: To determine the role of adipocyte ANP-mediated lipolysis in different adipose tissue depots under metabolically compromised conditions

First, **Chapter 2** provides a comprehensive review on the role of natriuretic peptides in the control of lipid metabolism and insulin sensitivity. This review provides data on the natriuretic peptide system, its signaling mechanisms, as well as its associations with cardiometabolic diseases. In particular, the metabolic roles of natriuretic peptides in adipose tissue, skeletal muscle and liver function are discussed in relation to the control of insulin sensitivity. Finally, human studies modulating the natriuretic peptide system by exercise or exercise intervention are implemented in this overview. In light of the growing body of evidence regarding the importance of ANP in stimulating adipose tissue lipid mobilization, it remains unknown whether this effect is comparable between adipose tissue depots, especially in distinct metabolic phenotypes. Accordingly, in **Chapter 3**, an unique design enabled us to investigate ANP-mediated lipid mobilization *ex vivo* in isolated subcutaneous and visceral mature adipocytes of lean, obese and obese type 2 diabetic individuals. Additionally, the molecular ANP signaling pathway was examined in more detail in these individuals.

Aim 2: To assess the relative contributions of adipose tissue dysfunction characteristics to whole-body insulin resistance

Different determinants of adipose tissue dysfunction contribute to the development of insulin resistance, but the relative contributions of an impaired adipose tissue lipid metabolism and inflammation remains to be elucidated. **Chapter 4** provides novel insights into the associations between subcutaneous and visceral adipose tissue morphology, lipid mobilization, inflammation and whole-body insulin resistance in a cohort of lean and obese individuals with or without type 2 diabetes. For this study, analyses on *ex vivo* lipolysis and adipose tissue morphology were combined with a detailed quantification of adipose tissue immune cell populations using state-of-the-art methodology (flow cytometry) in paired subcutaneous and visceral adipose tissue samples.

Aim 3: To gain insight in the physiological regulation of adipose tissue lipid mobilization and the effects of non-pharmacological and pharmacological intervention in obese insulin resistant men

Further extending on our outcome of the study outlined in **Chapter 3**, where we studied *ex vivo* ANP-mediated lipolysis in insulin resistance, the relevance of subcutaneous lipid mobilization under local adrenergic blockade during acute exercise was examined in **Chapter 5** in an *in situ* setting in lean and obese individuals, as well as the potential effects of exercise intervention on the subcutaneous adipose tissue lipid mobilization were studied. More specifically, we explored ANP-mediated subcutaneous lipid mobilization during an acute exercise bout in lean individuals and obese insulin sensitive or insulin resistant individuals, by using the golden standard microdialysis technique. In addition, systemic responses with respect to whole-body lipid mobilization were assessed. Furthermore, in a training intervention study, the effects of 12 week supervised exercise training on ANP-mediated lipid mobilization *in situ* and whole-body lipid mobilization were assessed in obese insulin resistant individuals.

Besides non-pharmacological treatment strategies, preclinical studies suggest that partial inhibition of adipose tissue lipolysis may be a promising strategy to alleviate insulin resistance in obese pre-diabetic people. In **Chapter 6**, the clinical impact of pharmacological inhibition of adipose tissue lipid mobilization, using acipimox, during moderate-intense endurance-type exercise on postprandial glycemic control was examined in males obese type 2 diabetic patients.

Finally, in **Chapter 7**, the main findings from these studies described in this thesis are discussed in a broader perspective and suggestions for future research are provided.

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Chapter 2

Natriuretic peptides in the control of lipid metabolism and insulin sensitivity

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revision submitted

Abstract

Natriuretic peptides (NP) have long been known for their cardiovascular function. However, a growing body of evidence emphasizes the role of NP in human substrate and energy metabolism, thereby connecting the heart with several insulin sensitive organs like adipose tissue, skeletal muscle and liver. Obesity may be associated with an impaired regulation of the natriuretic peptide system, also indicated as a natriuretic handicap. Evidence points towards a contribution of this natriuretic handicap to the development of obesity, type 2 diabetes mellitus and cardiometabolic complications, although the causal relationship is not fully understood. Nevertheless, targeting the natriuretic peptide pathway may offer novel future therapeutic potential for improving metabolic health in obesity and type 2 diabetes mellitus. This review will focus on current literature regarding the metabolic roles of NP with emphasis on lipid metabolism and insulin sensitivity. Furthermore, it will be discussed how exercise and lifestyle intervention may modulate the NP-related metabolic effects.

Introduction

Obesity is one of the major health problems of the twenty-first century as it is closely associated with the development of chronic metabolic diseases, including cardiovascular disease, insulin resistance (IR) and type 2 diabetes mellitus (T2DM) ¹⁻³. Different insulin sensitive organs tightly orchestrate energy and substrate metabolism in the human body. Therefore, alterations in these organs may contribute to the development of disturbances in fatty acid (FA) metabolism, ultimately leading to impaired glucose metabolism, IR and cardiometabolic disease. Next to the liver, skeletal muscle, the gastrointestinal tract and the pancreas, the adipose tissue (AT) is an important central organ in the inter-organ crosstalk in human energy and substrate metabolism. The AT is the primary site for long-term energy storage, mainly as triglycerides. However, a chronic excessive energy intake, like in obesity, results in enlargement (hypertrophy) of existing adipocytes. Since adipocytes have a limited expansion capacity, hypertrophy will lead to a reduced buffering capacity and dysfunctional AT may develop ^{4,5}. This AT dysfunction is further characterized by an altered lipid storage capacity and adipokine release, immune cell infiltration and low-grade inflammation, plays an important role in the development and/or progression of IR by promoting ectopic lipid storage and systemic low-grade inflammation ⁶⁻¹⁰. Physical activity intervention, whether or not combined with diet, may reduce the progression towards T2DM ^{11,12}, possibly due to modulation of AT, liver and/or skeletal muscle FA metabolism ¹⁰.

Only recently, research proposed natriuretic peptides (NPs) as important endocrine hormones implicated in the regulation of whole-body energy and substrate metabolism ¹³⁻¹⁶. Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), both secreted by the heart, and C-type natriuretic peptide (CNP), mainly expressed by vascular endothelial cells and to a lower extent in cardiomyocytes ¹⁷, are the three NP family members that may play a role in human substrate and energy metabolism. Besides their cardiovascular and renal effects (described extensively by Volpe *et al.* ¹⁸), NPs play a role in different metabolic processes including lipid mobilization in human white AT ^{13,15}, energy dissipation in brown AT, browning of white AT ¹⁹ and fat oxidation in human skeletal muscle ²⁰, possibly influencing whole-body FA metabolism, glucose

homeostasis and insulin sensitivity. In addition to their wide range of metabolic effects in human insulin sensitive tissues, plasma NP levels were also reported to be negatively associated with cardiometabolic diseases²¹⁻²⁶ and have been even suggested to have a predictive value in the development of new onset T2DM²⁵. However, a better understanding of the interaction between NPs and tissue energy, FA and glucose metabolism is necessary to obtain a better insight in the role of NPs in the development of IR, T2DM and cardiometabolic complications.

In this review, we will discuss the current knowledge regarding the role of NPs in the control of human lipid metabolism and insulin sensitivity. At first, NP secretion, clearance and signaling is discussed. Subsequently, the role of NPs in the etiology of obesity and T2DM is discussed, as well as their effects on AT, skeletal muscle and liver metabolism. Moreover, the benefits of lifestyle and/or exercise interventions with respect to NPs in the control of insulin sensitivity are considered.

Secretion and processing of NP

The discovery of the endocrine properties of the heart by deBold in 1981, as shown by a potent natriuretic and diuretic effect of atrial myocardial extracts in rats²⁷, led to the reexamination of the function of the earlier discovered atrial myocardium granules²⁸. The dual nature of atrial cardiomyocytes (i.e. secretory-contractile function) became obvious and research led to the identification of atrial natriuretic peptide (ANP)²⁹ and later the other NP-hormone family members B-type natriuretic peptide (BNP), which is found at highest levels in cardiac ventricles, C-type natriuretic peptide (CNP), which is mainly expressed in and produced by endothelial cells¹⁷. CNP, previously thought to act as a neuropeptide in the central nervous system^{30,31}, is mainly viewed as a peptide regulating vascular blood pressure³² and bone growth³³, although a minor role in metabolic regulation has been suggested^{34,35}. In mammals, dendroaspis natriuretic peptide (DNP) (of which the synthesis and secretion sites have not been identified) exerts renal actions via its specific receptor³⁶ but because it has not been well studied with respect to metabolic effects in humans, DNP will not be further discussed in the current review.

Currently, ANP and BNP are the main metabolically active NPs described in literature and will therefore be discussed in more detail in the present review. These peptides have a 17-amino acid ring structure in common, formed by an intramolecular disulfide linkage, of which the sequence is highly preserved within the biologically active form of these peptides³⁷. Structural differences between NP family members are due to specific amino- and carboxy-terminal extensions³⁸. At rest, ANP (normal concentration range 5-50 pg/mL) is mainly produced by the (right) atrial myocardium as a prohormone³⁹. The intracellular granules of the atrial myocardium contain proANP which is cleaved by corin (type II transmembrane serine protease expressed primarily in the heart) during secretion, resulting in a 28-amino acid, bioactive α ANP peptide⁴⁰, with a very short plasma half-life of about 2-4 min⁴¹, and inactive fragments (N-terminal ANP and mid-regional-proANP) which have a longer plasma half-life (about 40-50 min)^{41,42}. BNP is mainly produced and secreted by the ventricular myocardium as preproBNP⁴³. To become biologically active, preproBNP is cleaved to proBNP and subsequently, like for ANP, a cardiac protease, corin or furin, is responsible for the conversion to the 32-amino acid BNP (range plasma levels 0-65 pg/mL) which is secreted in the circulation having a plasma half-life of about 15-25 min⁴⁴, and the inactive N-terminal fragment proBNP⁴⁵. The latter inactive fragment has a plasma half-life of about 60-120 min and a plasma concentration in the range of 7-220 pmol/L in healthy individuals⁴¹. While the structure of BNP varies distinctly among species, ANP is strongly homologous between human and rodent⁴⁵. The post-translational processing, cleavage and degradation sites of NPs were recently reviewed by Volpe *et al.*¹⁸.

Determinants of NP secretion

Mechanical stretch of cardiomyocytes is the most important trigger for NP release in the circulation⁴⁵. Atrial wall stretching causes an increase in ANP gene transcription and increased release of stored granules⁴⁶. Ventricular wall stress, in case of volume or pressure overload, is mainly responsible for BNP transcription and secretion³⁸. In addition, hypoxic conditions or myocardial ischemia are potent inducers of ANP secretion due to the presence of hypoxia-responsive elements in the promotor region of the ANP gene⁴⁷.

Another potent stimulus for NP secretion is cold exposure. Animals and humans exposed to cold display a greater expression of ANP (mRNA) and BNP (mRNA and protein) in the heart, combined with higher plasma levels, which are suggested to be direct responses of elevated sympathetic nervous system activation in the heart ¹⁹.

Besides mechanical stretch and cold exposure, endocrine regulation of NP secretion is present as well. Sex steroids, thyroid hormones, glucocorticoids, endothelin-1, angiotensin II and inflammatory cytokines (tumor necrosis factor- α , interleukin-1 and -6) all are able to modulate NP secretion ^{38,45,48}, the latter stimulating BNP transcription and translation *in vitro* in murine cardiomyocyte cultures ⁴⁹ and *in vivo* secretion into the plasma in human transplant patients specifically ⁵⁰. In this regard, it was shown that the GLP1 receptor agonist liraglutide was able to induce a significant increase in ANP secretion *in vivo* in T2DM ⁵¹ due to the presence of GLP1 receptors on right atrial cardiomyocytes ⁵². ANP and BNP plasma levels also increase with age, possibly due to an age-related reduction in coronary blood flow reserve and thus increased myocardial ischemia ⁵³⁻⁵⁵. Modulation by sex steroids may result in gender dependent regulation of NP levels ⁵⁵⁻⁵⁷. The latter is already observed in pubertal versus post-pubertal adolescents, where NP concentrations are lower in post-pubertal boys compared with pubertal boys ⁵⁸. Estrogens might have a stimulatory effect on the production and secretion of ANP and BNP by the cardiomyocyte, whereas androgens may have an inhibitory effect ⁵⁶. In part, the increased NP levels in women might have clinical implications for gender-related difference in relative risk of developing metabolic and cardiovascular disease. Furthermore, during adolescence NPs levels seem to increase progressively in girls ^{53,54}, probably the result of an interaction between the increased estrogen concentration and ANP transcription and secretion or via the regulation of the NPs receptor expression ⁵⁹. Plasma CNP levels alternatively decrease during adolescence until the age of fifty, whereupon they tend to increase. CNP concentrations are higher in men than in women as testosterone and growth hormone are able to induce CNP ⁶⁰. Finally, metabolically compromised conditions like obesity, insulin resistance and T2MD may be characterized by altered systemic NP concentrations, which will be discussed more extensively later in this review.

NP receptors and signaling

To exert their main biological effects NPs bind to NP receptors, of which three subtypes have been described (reviewed recently by Kuhn) ⁶¹. ANP and BNP bind with a high affinity to a membrane-bound receptor, containing a transmembrane segment, with specific guanylyl cyclase (GC) activity called NP receptor A (NPRA). CNP is mainly bound to NP receptor B (NPRB) ⁶², similar in structure and function to NPRA and mainly expressed by chondrocytes, thereby playing a role in long bone growth ⁶³. Ligand binding to a NPRA homodimer results in the internalization of the bound ligand-receptor complex and the activation of cytosolic GC, the catalytic effector of the receptor, subsequently causing hydrolysis of GTP into cyclic GMP (cGMP). This second messenger is able to activate various biological responses via cGMP-dependent protein kinases, cGMP-gated ion channels or other effector proteins ⁶⁴. A large proportion of the ligand-bound receptor undergo lysosomal degradation, in which about 75% of the internalized ANP is processed in the lysosomes and 25% is released as intact molecules through a recycling pathway. A small amount of internalized receptors is recycled back to the plasma membrane or released into the cell exterior ⁶⁵. The NP receptor C (NPRC) is the third subtype, having an extracellular domain that is partly homologous to those of NPRA receptors and thus has the ability to bind NPs, with the highest affinity to ANP and lowest to BNP ⁶⁶. However, NPRC lacks GC activity and instead its main function is to facilitate scavenging of its ligands, internalization of ligand-receptor complexes and recycling of NPRC, together with lysosomal degradation of its ligands ^{67,68}. Besides via lysosomal clearance, NPs can be degraded intracellularly by endopeptidases including neutral endopeptidase (NEP) ⁶⁹, which is also produced in adipocytes ⁷⁰. In addition, insulin-degrading enzyme (IDE) enzymatically cleaves NP ^{45,71} and dipeptidyl peptidase-IV (DPPIV or CD26) cleaves the N-terminal peptide of NPs thereby lowering biological activity ⁷². Another route to clear circulating NP is via secretion into body fluids like urine (via glomerular filtration) and bile ⁴⁵.

The main effector receptor for ANP and BNP, NPRA, is highly expressed throughout the cardiovascular system (vascular smooth muscle and endothelial cells with only a limited expression in the heart), in kidney and adrenal gland, as well as in different metabolic organs like skeletal muscle, pancreas, liver, brain,

gut and AT ^{37,73,74}. Expression of the scavenging NPRC, the most widely expressed NPs receptor, is mainly present in the AT, kidneys, lungs, the cardiovascular system and blood monocytes ^{68,75,76}. Interestingly, NPRA and NPRC display diurnal regulations (in antiphase of one other) in the rodent white AT ⁷⁷, not in the heart muscle ⁷⁸, which together with the circadian regulated plasma NPs ^{79,80}, may be a characteristic for energy homeostasis during the day. Furthermore, the local tissue specific and systemic effects of NP are thought to depend on the ratio between the signaling receptor NPRA to the clearance receptor NPRC ^{81,82}. Collectively, NPs mediate their effects via NP receptors, of which three subtypes have been described. The diverse effects of NPs, systemically as well as the local tissue effects, are determined by NP receptor expression profiles and their ligand-affinity.

Systemic NP deficiency in obesity, insulin resistance and type 2 diabetes mellitus

Evidence from several epidemiological studies demonstrated an inverse association between systemic NPs levels (both ANP and BNP) and body weight (mostly expressed by body mass index (BMI)) ^{21,23,83-87}. The inverse relationship between NP concentrations and BMI was found also in the presence of left ventricular hypertrophy ⁸⁸. However, these studies lack detailed body composition analyses thereby not being able to differentiate between fat accumulation or fluid retention as a cause of the increased BMI. In contrast, other small cohorts showed that there is no or even a positive relationship between circulating NPs levels and BMI ^{89,90}. Furthermore, NPs may affect AT distribution ^{91,92}. Variations in regional and particularly visceral adiposity were strongly related to circulating N-terminal-pro-BNP. The relationship of NPs with subcutaneous adiposity was less strong ⁹². This relation could be partly moderated by the hyperinsulinemic state that is frequently observed in visceral adiposity, as high insulin levels have been shown to suppress NPs secretion and activity ^{93,94}. The Dallas Heart Study recently showed that both BNP and N-terminal-proBNP are inversely related to visceral and liver fat, while being positively associated with lower gluteofemoral body fat, independent of insulin sensitivity ⁹⁵.

Of interest, it is important to take into account obesity comorbidities including the presence of cardiac burden in considering these controversial results⁹⁶ as obesity is often linked to volume overload, leading to left ventricular hypertrophy and left ventricular dysfunction⁹⁷. The decrease in systemic NPs concentrations may be accompanied by higher blood aldosterone concentrations, as a consequence of an impaired NPs-mediated renin-angiotensin-aldosterone system inhibition^{98,99}, thereby leading to obesity-related hypertension²² or an increased incidence of all-cause mortality⁸⁶. The substantial role of NPs deficiency in the pathogenesis of obesity-related hypertension was recently corroborated in healthy obese men, showing a negative association between serum mid-regional-proANP and mean 24-hour systolic ambulatory blood pressure¹⁰⁰. Moreover, research indicated that NPs deficiency might enhance cardiovascular risk¹⁸. Therefore, achieving normal range NPs levels is preferential with respect to obesity-related hypertension¹⁰¹.

As obesity is often associated with IR or T2DM, a link between the NPs system, obesity and T2DM seems plausible. Recent research indicated that NPs deficiency might increase the risk of T2DM onset¹⁰². Indeed, two recent prospective cohort studies showed evidence supporting this hypothesis^{25,26}. Results of the Malmö Diet and Cancer Study showed mid-regional-proANP plasma levels to be inversely associated with new-onset diabetes development (*i.e.*, diabetes incidence) and an impaired glucose metabolism over the 16-year follow-up period of the study, which was also true for N-terminal-proBNP plasma levels, although not statistically significant²⁵. In this regard, mid-regional-proANP is believed to be a better predictor of T2DM incidence compared to N-terminal-proBNP²⁵, the latter being more sensitive to mild forms of left ventricular dysfunction¹⁰³ which is relatively frequent (even subclinically) in the obese state¹⁰⁴. Circulating ANP (measured as mid-regional-proANP) within the normal high range (~212-372 pg/mL) was associated with lower risk of IR during a 16-year follow-up period in a middle-aged cohort¹⁰⁵. This association was independent of diabetes risk factors (including waist circumference, plasma levels of triglycerides, HDL-cholesterol, systolic blood pressure, antihypertensive treatment, age and sex) or renal function (cystatin C)²⁵. In the Atherosclerosis Risk in Communities Study (a community-based population study), it was shown that having low N-terminal-proBNP levels (lowest quartile, <31pg/mL) was

associated with higher risk of incident diabetes over a 12 year follow-up period in subjects without T2DM at baseline. These results were consistent across race, gender and BMI categories ²⁶, and were independent of age ^{106,107}. Of interest, statistical adjustment for BMI did not abrogate the association between low NP levels and diabetes onset ¹⁰⁸. These results are in line with the Framingham Heart Study and the Malmö Diet and Cancer Study, which showed that lower N-terminal-proBNP levels were associated with higher incidence of IR in lean as well as in obese subjects in a cross-sectional study ²³. Additionally, prospective cohort data from the Women's Health Study showed that subjects with N-terminal-proBNP levels near the upper limit of the normal range (>117 pg/mL) have a significantly lower risk of developing diabetes ¹⁰⁹.

Thus, there is consistent evidence that increased NP concentrations are protective against IR ⁹⁷ and T2DM ¹⁰⁹⁻¹¹¹. In line, lower blood glucose concentrations were transiently observed upon systemic BNP infusion during intravenous glucose tolerance testing in young, healthy lean men with normal glucose tolerance ¹¹². Moreover, in a random subset of a general middle-aged population (age >45 years) a genetic variant of the *ANP* gene (single nucleotide polymorphism rs5068) was associated with higher N-terminal-proANP levels and a beneficial cardiometabolic profile (*i.e.* reduced systemic blood pressure, BMI, waist circumference and a lower risk of metabolic syndrome) compared to the A/A carriers ⁸⁴. This *ANP* gene-polymorphism was accompanied with a lower incidence of T2DM after a 14-year follow-up ¹¹³. Similarly, a genetic polymorphism in the promotor region of the *BNP* gene (T-381C polymorphism) is associated with higher plasma BNP levels and lower risk of T2DM in several population samples including individuals with normoglycemia, impaired glucose tolerance and T2DM ¹¹⁴. Together these findings suggest NPs to be protective against IR and development of T2DM.

Altogether, the presence of a NPs deficiency in metabolic disease is generally accepted but the cause remains incompletely understood. Nonetheless, it is imperative to understand the etiology of this anomaly, especially because of the pleiotropic protective cardiovascular and metabolic effects of NPs, as will be described later. Further studies are necessary to establish the clinical relevance of using mid-regional-ANP and/or N-terminal-proBNP as biomarkers for diabetes

prediction because multiple mechanisms are thought to contribute to the anti-diabetic role of NPs ¹⁵.

Underlying mechanism for systemic NP deficiency in obesity

Several potential explanations for the observed systemic NPs deficiency in human obesity, and more general human metabolic disease, have been proposed, apart from common variants of the human *ANP* and *BNP* genes that affect circulating NP concentrations ^{109,111,115}. One explanation could be that the NP deficiency may be due to an increased NP degradation, which is mainly fulfilled by NPRC-mediated lysosomal breakdown as mentioned before ^{69,116}. Indeed, mRNA expression of NPRC is increased in human AT of obese ^{73,76,81} and obese hypertensive patients ²². In addition, hyperinsulinemia increased NPRC expression *in vitro* in 3T3-L1 adipocytes ⁸¹, human adipocytes ^{81,117} and in human subcutaneous AT of healthy, moderately obese individuals with normal glucose tolerance during hyperinsulinemic-euglycemic and hyperinsulinemic-hyperglycemic clamps ⁷⁶, mainly through the phosphatidylinositol 3-kinase (PI3K) pathway ⁸¹. Together, these data suggest that the AT may be responsible for the increased NP clearance and lowering systemic NP availability in obese insulin resistant conditions ^{76,118}.

However, results from the Dallas Heart Study showed that the association between BMI and circulating NP levels is explained by the amount of lean mass, and not AT mass, indicating that lean tissue could also be important for plasma NP regulation ⁸³. Indeed, upregulation of NPRC in human skeletal muscle tissue, next to down-regulation of the NPRC expression in the AT and skeletal muscle of obese and/or obese diabetic humans and mice has been found ^{119,120}. Thus, besides AT, also skeletal muscle may contribute to the NP deficiency observed in T2DM in the long-term, certainly considering that muscle mass accounts for up to 40% of total body weight. Therefore, even a moderate increase in skeletal muscle NPRC expression could markedly reduce NPs plasma levels ¹¹⁹. Moreover, previous work of Sarzani *et al.* with a genetic *NPRC* variant shows that a reduced NP clearance (or resulting increased systemic levels) might be associated with a reduced content of upper body fat and a lower risk of developing abdominal obesity ⁹¹.

Additionally, it has been shown that NPRC mRNA expression is down-regulated *in vitro* following starvation in human differentiated adipocytes¹¹⁷ and *in vivo* in rat white and brown AT¹²¹ while the opposite was true under high fat feeding in wild-type mice skeletal muscle, white and brown AT¹²². Like NPRC expression, NEP expression is increased in the plasma (protein) and AT (mRNA) of obese subjects, although there is no direct evidence for an increased NEP activity in human obesity¹²³. Together, these findings propose an altered NPs receptor ratio in obese insulin resistant or T2DM individuals mainly due to an increased expression of adipose NPRC and NEP, possibly leading to elevated NP clearance thereby reducing circulating NPs.

On the other hand, a decreased cardiac ANP and BNP release in metabolic disease has also been proposed. Not only circulating NPs levels but also the side products of NPs release, the aminoterminal cleavage products N-terminal-proANP and N-terminal-proBNP are reduced in obesity. These proteins are structurally distinct and are biologically inactive compounds, which makes NPRC mediated clearance of these components rather unlikely^{83,124}. Of interest, NPs levels in the aortic root and the coronary sinus were observed to be negatively correlated with BMI¹²⁵. These findings suggest that besides the above indicated impaired clearance, a reduced cardiac NPs release might potentially contribute to the systemic NPs deficiency in metabolic diseases as well. This hypothesis was further acknowledged by the notion that cardiac ANP and BNP mRNA expressions were reduced in obese Zucker fatty rats and *db/db* mice^{126,127}. Putative impairments in cardiac NPs secretion in human metabolically compromised conditions needs to be further explored.

NP and its role in inter-organ crosstalk

NPs are long known for their cardiovascular and renal actions¹⁸ resulting in the use of NPs (or their fragments) as cardiovascular biomarkers in the clinic¹²⁸. Over the last two decades, pioneering studies by the group of Lafontan *et al.* suggested a protective role for the heart in metabolic diseases¹²⁹. The inter-organ crosstalk effectuated by ANP and BNP secretion by the heart integrates effects on AT function, skeletal muscle, liver, gut, central nervous system and pancreas, as indicated in Figure 1.

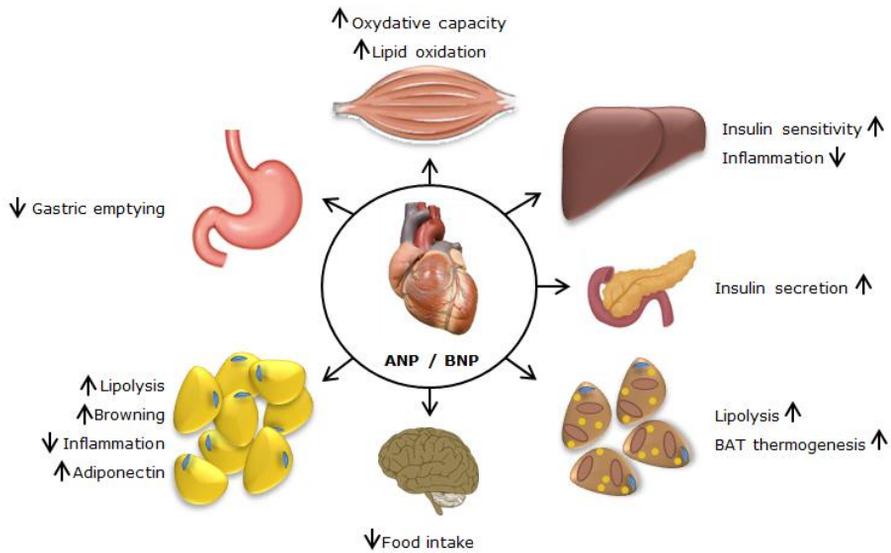


Figure 1 – Overview of the multiple metabolic actions of natriuretic peptides in the control of lipid metabolism and insulin sensitivity.

NP and adipose tissue function

As the human white AT is one of the main sites of NP receptor expression, investigating the effects of NPs on AT function seems obvious, in particular in the obese state where AT mass is expanded. Next, the effects on AT lipolysis, brown AT metabolism and the role of NP in AT lipid metabolism in obesity and T2DM will be discussed in more detail.

NP and adipose tissue lipolysis

The potency of inducing a lipolytic effect by NPs was first described in isolated human adipocytes, with ANP being the most potent activator of lipolysis as compared to BNP and CNP, the latter only showing a minor maximal lipolytic response (17% as compared to maximal stimulation with isoproterenol) ¹²⁹. In addition, these lipolytic properties seem to be primate-specific, which may be due to the differential NPRC clearance receptor expression profiles in other mammalian adipocytes, especially in rodents, making ANP-mediated lipolysis less likely due to increased NPs clearance and degradation ¹³⁰. The signaling

pathway relies on cGMP-dependent activation of protein kinase G (PKG), thereby promoting phosphorylation of perilipin-1 (PLIN-1) and hormone sensitive lipase (HSL) to trigger triglyceride hydrolysis^{129,131,132}, in which adipose triglyceride lipase (ATGL) might be involved as well¹¹⁷, the latter probably via a different signaling pathway (*i.e.* AMP-activated kinase)¹³³ as compared with HSL activation (*i.e.* PKA and PKG)¹³⁴. NPs-induced lipolysis is completely independent from the catecholamine-induced (cAMP/PKA mediated) lipolysis, as they rely on different pathways^{135,136}. However, an additive lipolytic effect occurs when human adipocytes are stimulated with ANP and a beta-adrenergic agonist (*e.g.* isoproterenol) simultaneously¹³⁷. Infusion studies of human ANP (hANP), either intravenous or *in situ* through microdialysis in the subcutaneous AT, showed promotion of lipid mobilization in healthy subjects, also in the presence of local beta-adrenergic blockade¹³⁵. Infusing intravenous hANP (doses from 6.25-25 ng*kg⁻¹min⁻¹), corresponding to the physiological range observed during moderate exercise, stimulates whole-body lipid mobilization and oxidation (in a dose dependent way), even in the postprandial state^{132,136,138}. Furthermore, exercise-induced increases in systemic ANP concentrations (which may vary depending on the exercise/subjects' characteristics) lead to an increase in lipid mobilization, at least in lean healthy subjects¹³⁹. In human obesity, lipolytic catecholamine-resistance is most commonly observed in the subcutaneous AT in the obese insulin resistant state¹⁴⁰⁻¹⁴². In line, regarding ANP-mediated lipolysis, an impaired ANP-mediated lipolysis *in vitro* and *in situ* in human subcutaneous AT of obese women and overweight men compared to non-obese counterparts has been reported¹²⁰. ANP-mediated lipid mobilization was reported to be comparable in subcutaneous adipocytes compared to visceral adipocytes of obese women¹⁴³. The blunted ANP-mediated lipolysis in the subcutaneous AT of obese insulin resistant individuals may be in part due to an up-regulation of NPRC and a down-regulation of NPRA mRNA and protein in human subcutaneous adipocytes¹²⁰.

An interaction between the NPs' system and the anti-lipolytic hormone insulin was first suggested by Endre *et al.*¹⁴⁴, who showed that hyperinsulinemic euglycemic clamping caused a decrease in serum ANP in normotensive and hypertensive men. Insulin inhibits the catecholamine-induced lipolysis via activation of phosphodiesterase 3B (PDE3B), but does not have a direct anti-

lipolytic effect on the cGMP-PKG dependent lipolytic pathway of ANP ¹⁴⁷. However, insulin might attenuate ANP-mediated lipolysis by inducing NPRC expression, as described earlier in this review. Of interest, the presence of low-glucose conditions together with insulin stimulation abolished NPRC expression to basal levels, indicating the existence of a “nutritional signaling” in NPRC regulation ¹¹⁷. The relative ratio of NPRA to NPRC mRNA levels in subcutaneous AT was decreased depending on glucometabolic status as patients with T2DM had the lowest ratio compared to subjects with normal glucose tolerance or impaired glucose metabolism ¹⁴⁶. In parallel with improvements in insulin sensitivity, 12 weeks of treatment with pioglitazone, a thiazolidinedione that affects insulin sensitivity ¹⁴⁷, increased the relative NPRA to NPRC mRNA ratio with the greatest effect on NPRC mRNA levels in subcutaneous AT in patients with T2DM ¹⁴⁶. Acute increases in systemic blood glucose decreased circulating N-terminal-proANP in lean, overweight and obese subjects, a mechanism mediated through glucose-induced miR-425 expression ¹⁴⁸, a negative regulator of NPRA ¹⁴⁹. Insulin thus seems to be a key hormone in the connection between glucose/lipid metabolism and NPs’ metabolic activities.

Together, one can suggest that improving NP-mediated lipid mobilization in the obese insulin resistant and/or T2DM state might be a potential novel strategy to improve lipid metabolism in metabolic disease.

NP and adipokines

Another way to link NPs to AT function is the ability of NPs to alter expression and secretion of adiponectin, an adipokine with insulin sensitizing properties, both *in vitro* ¹⁵⁰ and *in vivo* ¹⁵¹ in healthy subjects. Moreover, adiponectin is positively associated with NPs ^{95,106,152,153}. Other insulin desensitizing mediators frequently linked to NPs include TNF- α or interleukin-6. The secretion of these pro-inflammatory factors was reduced by treating human AT pieces with physiological ANP concentrations *in vitro*, possibly through a direct effect on both adipocytes and macrophages ¹⁵⁴. In this regard, reducing pro-inflammatory cytokines and increasing adiponectin secretion from AT could indirectly ameliorate insulin sensitivity by NPs.

NP and brown adipose tissue metabolism

Next to white AT, the presence and the importance of human brown AT (BAT) for human metabolic diseases receives increasing attention ¹⁵⁵⁻¹⁵⁸, although the quantitative importance of BAT in human energy and substrate metabolism remains uncertain. BAT is a thermogenic tissue having the ability to dissipate energy in the form of heat, thereby maintaining body temperature. Substrates including glucose and free fatty acids, delivered by white AT lipolysis, are necessary for heat dissipation, a process that is mediated by mitochondrial inner membrane uncoupling protein 1 (UCP-1) ¹⁵⁹. In addition, UCP-1 may have a regulatory function in whole-body energy homeostasis ¹⁶⁰. However, most of these data are derived from rodent studies and because adult human BAT may have a differential gene expression profile as either rodent BAT or beige fat ¹⁶¹, its physiological function in humans still needs to be determined in more detail. Support for a role of NPs in non-shivering thermogenesis was recently provided by showing that cold exposure acutely increases cardiac BNP secretion in mice, resulting in an increased NPRA/NPRC ratio in white AT ¹⁹. Mechanistic experiments indeed showed that ANP (and BNP) might activate mitochondrial biogenesis and uncoupling in human and mouse white adipocytes, in an AMPK dependent manner ¹⁹. Chronic BNP treatment of *db/+* and *db/db* mice further confirmed these findings, showing increased UCP-1 expression and browning of the white fat pads ¹²⁶. ANP treatment also enhanced mitochondrial function in human adipocytes ¹⁶². Taken together, studies have shown that the NP system is able to induce a thermogenic process in the AT and to induce brown AT activation. However, this knowledge is only limited to *in vitro* experiments. Since cold exposure is able to increase both NPs secretion and brown AT activation, the role of NPs in white AT “browning” is of particular interest in the human *in vivo* situation, making this connection a potential target for treating metabolic disease. Until today, the role of NPs in human brown AT remains elusive.

NP and skeletal muscle metabolism

The mobilization of free fatty acids from AT depots by NPs provides substrates for energy production by oxidative tissues ^{132,135}. However, not only AT lipid mobilization has been shown to be susceptible to NPs but also enhancement of AT and muscle lipid oxidation. Birkenfeld *et al.* observed an acute increase in

whole-body lipid oxidation (predominantly resulting from increased muscle lipid oxidation) following intravenous ANP infusion. Additionally ANP infusion leads to higher energy expenditure in the postprandial state ¹³⁸. The oxidative effect of ANP, as well as BNP, was later confirmed *in vitro* in human muscle cells and *in vivo* in mice (*in vitro* and *in vivo*) showing increased skeletal muscle mitochondrial biogenesis, respiration and lipid oxidation upon chronic overexpression of BNP or cGMP-dependent protein kinase in transgenic mice ¹²². This enhanced oxidative metabolic state was protective for high fat diet induced obesity and glucose intolerance in mice. A physiological role of NPs in the regulation of skeletal muscle oxidative capacity in human primary myotubes was established by showing that ANP, BNP and cGMP analogs induce peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) expression, mitochondrial oxidation and lipid (palmitate) oxidation *in vitro* ²⁰. In addition, PGC-1 α expression was associated with NPRA expression in skeletal muscle of healthy human subjects ²⁰. This proposes that NPs affect mitochondrial respiration and lipid oxidation in skeletal muscle through a cGMP dependent pathway, which was shown to be mediated by the induction of transcription and protein expression of PGC-1 α and several OXPHOS complexes (complex I and complex IV), accompanied by an unchanged peroxisome proliferator activated receptor (PPAR) δ expression and mitochondrial DNA content ²⁰.

Moreover, in skeletal muscle of obese and glucose intolerant humans and mice an altered NPRA/NPRC protein ratio was recently reported to be accompanied with a diminished phosphorylation and activation of p38 MAPK, a downstream effector of the NPs receptor signaling pathway. However, chronic treatment of mice with obesity-induced glucose intolerance and T2DM with BNP showed a reduced total diacylglycerol content in skeletal muscle, which was accompanied with higher oxidative capacity and *PGC-1 α* gene expression ¹¹⁹. This observation was further confirmed in human primary myotubes, showing that increased NP mediated lipid oxidation was accompanied by reduced *de novo* ceramide production ¹¹⁹. These studies indicate the importance of NPs signaling in skeletal muscle lipid oxidative capacity, which is imperative for long-term maintenance of insulin sensitivity in obesity and T2D.

Yet, recent data indicate a conceivable interaction between the NPs system and an exercise induced myokine called musclin¹⁶³. This skeletal muscle protein is homologous to members of the NPs family¹⁶³ and their interaction results in the amelioration of the NPs' effectiveness¹⁶⁴. Its physiologically relevant interaction was shown *in vitro* and *in vivo* and indicates the NPRA-mediated increase in skeletal muscle mitochondrial biogenesis to be potentiated by a musclin-NPRC interaction during exercise in mice¹⁶⁴. Musclin is significantly upregulated in skeletal muscle of obese IR mice¹⁶³ and its gene expression is known to be increased upon palmitate treatment in C2C12 myotubes¹⁶⁵ and high fat diet in rats¹⁶⁶. Therefore, dysregulation of musclin might play a role in IR of skeletal muscle and thus needs further investigation to elucidate the molecular mechanisms by which expression of muscle can be changed in human metabolic conditions. Furthermore, as musclin was proposed to exert its effects on glucose uptake in skeletal muscle via PPAR- γ ¹⁶⁷, this also suggests a possible role for musclin in AT metabolism which needs to be explored in humans in the future.

NP and liver function

Hepatic IR is an additional hallmark frequently observed in the metabolic syndrome and develops in case of an imbalance between fatty acid supply and utilization of fatty acids by hepatocytes and myocytes, leading to ectopic lipid accumulation and whole-body IR¹⁶⁸. In obesity the presence of non-alcoholic fatty liver (NAFL) is frequently observed¹⁶⁹, which may further lead to non-alcoholic steatohepatitis, liver cirrhosis or even liver carcinoma¹⁷⁰. Recent studies show inverse relationships between NPs, in particular N-terminal-proBNP, and liver fat content in individuals without diabetes or self-reported liver disease¹⁷¹, as well as between NPs and liver function as indicated by aminotransferases enzymes in individuals without cardiovascular disease¹⁷². Additionally, NPs could ameliorate hepatic function as the presence of NPs receptors was shown in the human liver¹⁷³. More precisely, these receptors were found on Kupffer-cells, resulting in a hepatoprotective effect of ANP by reducing Kupffer-cell-derived oxidant stress¹⁷⁴ and inhibiting LPS-induced release of pro-inflammatory TNF- α via a cGMP-mediated signaling¹⁷⁵. ANP or its analogs inhibited hepatic glycolysis and stimulated gluconeogenesis and cGMP production in perfused livers of fed rats¹⁷⁶. Besides, ANP also induces hepatic

lipid oxidation in healthy lean individuals, thereby reducing lipid spill-over and ectopic lipid deposition¹³⁸. Consequently, lower liver TAG content was observed in BNP- or cGKI-transgenic mice on a high fat diet¹²². These findings were later confirmed in a cGKI knock out model, indicated by the presence of IL-6 mediated liver inflammation, fasting hyperglycemia and reduced insulin signaling¹⁷⁷. These data together suggest a direct role of NPs in liver lipid catabolism next to indirect effects via AT mass reduction¹²².

NP and control of insulin sensitivity

A role of NPs in the regulation of insulin sensitivity is plausible since several studies indicated an inverse association between NP deficiency and IR in human cohorts^{21,24,87,108}. Moreover, the broad range of metabolic effects in insulin sensitive tissues makes these peptides putative targets for lifestyle and exercise interventions in metabolic diseases like obesity and T2DM. By enhancing adipocyte lipid mobilization, AT function and skeletal muscle oxidative capacity, NP might contribute to a reduction in ectopic lipid accumulation and thus an improved insulin sensitivity. However, unraveling the mechanistic link between NPs signaling and insulin sensitivity remains incompletely understood.

NP and insulin sensitivity

A role of NPs and their signaling pathway(s) in the control of insulin sensitivity was corroborated by BNP- and cGK-I-transgenic animals, which were protected against the development of diet-induced IR and glucose intolerance¹²². In line, insulin sensitivity and glucose tolerance improved with chronic BNP infusion in obese diabetic db/db mice^{119,126}, which was accompanied by a reduced lipid content in liver and skeletal muscle^{119,122,126}. On the contrary, genetic knock-down of the NPs signaling cascade impaired fasting glycemia in mice, possibly reflecting an attenuated insulin-mediated regulation of hepatic gluconeogenesis¹⁷⁷. Mechanisms responsible for the improvements in the NP-induced metabolic effects may relate to NP secretion as well as NPs receptor expression and post-receptor signaling. Indeed, in humans, whole-body insulin sensitivity was recently shown to strongly correlate with NPRA in subcutaneous AT¹⁴⁶ and skeletal muscle¹¹⁹. Next to the observed downregulated NPRA (both at mRNA and protein) in obese insulin resistant individuals^{120,146}, AT¹⁴⁶ and skeletal

muscle ¹¹⁹ NPRC expression was increased and was negatively associated with whole-body insulin sensitivity. Furthermore, caloric restriction-induced weight loss resulted in an increased NPRA/NPRC ratio in the skeletal muscle tissue of obese subjects and was related to improvements in insulin sensitivity ¹¹⁹. Additionally, NP degradation by NEP ⁶⁹ may contribute to the development of IR (as was shown in obese Zucker rats) ^{178,179}. Indeed NEP expression in plasma and adipocytes was positively associated with obesity and cardiometabolic risk in the presence of IR ¹²³.

NP and insulin secretion

In addition to changes in insulin sensitivity, NPs may also affect pancreatic insulin secretion. Increased insulin levels were observed during ANP infusion in healthy subjects ^{138,180}, while others showed no alterations during physiological infusion ¹⁸¹. This effect of NPs on insulin concentration could be (partly) mediated by an increased secretion since NPRA receptors were shown to be present on pancreatic α and β cells ¹⁸². Furthermore, β cell insulin content, fasting glycemia as well as islet size and β cell mass were shown to be attenuated in the NPRA knock out state ¹⁸², the latter being confirmed in isolated rat pancreatic islands ¹⁸³.

Impact of exercise/lifestyle intervention on NP-related metabolic effects

The use of exercise as a non-pharmacological strategy to combat NP deficiency is appropriate as several studies have shown that exercise increases ANP levels, at least in healthy subjects ^{139,184-186}. In particular, ANP secretion is enhanced by increasing venous return and cardiac filling pressure (*i.e.* cardiac output) ¹⁸⁷⁻¹⁸⁹, while only slightly increasing ¹⁸⁷ or even not altering circulatory BNP levels ¹⁸⁸ in healthy volunteers, possibly indicating opposing regulation in cardiac atria and ventricles. One study reported plasma BNP levels to be positively associated with physical activity levels, although this remains controversial ^{190,191}. Exercise in elderly, healthy subjects caused significantly increased proANP and N-terminal-proBNP levels ¹⁹². Of interest, data about exercise effects on NPs in metabolic conditions are scarce. Tanaka *et al.* showed that NP secretion (ANP and BNP) is more sensitive to sympathetic activity in normotensive subjects

compared to hypertensive patients, where increased NPs levels may represent cardiac stress ¹⁹³. In overweight and obese patients mid-regional-proANP concentrations increased upon incremental exhaustive exercise both before and after diet intervention, with no difference in exercise response between these conditions ¹⁹⁴. Moreover, NPs were also evaluated in relation to resistance training ^{195,196}. Resistance training induces a significant increase in N-terminal-proBNP, which might be partly due to myocardial damage ¹⁹⁵. However, N-terminal-proBNP concentrations did not change in elderly following resistance training ¹⁹⁶. The latter two studies indicate that the effects of strength training on NPs concentrations are still controversial and thus need more investigation, especially in metabolically compromised conditions.

With respect to CNP, exercise preconditioning (that is, an enhancement of the tolerance of an ischemic heart to subsequent ischemic insult by a single bout of interval exercise) promotes the secretion of CNP in rodents, thereby elevating CNP levels in the myocardium and protecting against high-intensity exercise-induced myocardial injury ¹⁹⁷. Indeed, exercise training (more specifically walking or walking plus resistance exercise training) increased plasma CNP levels, which may be one of the mechanisms through which exercise intervention may reverse endothelial-dependent dysfunction in middle-aged individuals with impaired glucose tolerance ¹⁹⁸. This supports the use of (combined) exercise training to prevent T2DM.

NP and its role in intervention-induced adipose tissue and muscle phenotype changes

However, mechanisms responsible for the exercise-/exercise training-induced enhancement of the NP deficiency in metabolic disease might include multiple paths. In this respect, AT expression levels of functional NPRA do increase with regular physical activity ¹⁹⁹, an effect that was absent after caloric restriction ¹⁹⁴. In line, long-term aerobic exercise training (partly) recovered the reduced ANP responsiveness in situ in white AT of overweight subjects ¹⁸⁶ and in obese women with polycystic ovary syndrome in vitro and in vivo, independently of AT mass loss ²⁰⁰. Additionally, ANP-induced increase in AT blood flow, a functional AT characteristic, which has been shown to be impaired in young obese men ¹³⁵,

also improved following long-term aerobic exercise training ¹⁸⁶. As an alternative, water-based exercise, which is known to be beneficial compared to land-based exercise regarding injury risk and joint stress in obesity, has been shown to be not advantageous over moderate intensity land-based exercise with respect to lipid mobilization or fat oxidation ²⁰¹. However, exercise in water did show higher circulatory ANP levels in rats ²⁰² and in humans despite no change in lipid oxidation ²⁰¹. Moreover, as exercise training is supposed to enhance mitochondrial function at the level of the skeletal muscle ²⁰³, this improvement could be partly due to an increased NPRA expression and signaling as indicated by increased expression of PGC1- α and several oxidative phosphorylation genes following an 8-week aerobic training program in obese subjects ²⁰. Of interest, the anti-diabetic drug pioglitazone improves insulin sensitivity, which was accompanied by an increased NPRA/NPRC ratio in subcutaneous AT ¹⁴⁶. Liraglutide-induced weight loss in obese subjects with T2DM was correlated with change in NPs levels, although the mechanism responsible remained elusive ⁵¹. Together, these data indicate that the NPs signaling pathway may be a suitable target to improve insulin sensitivity in exercise-based lifestyle interventions.

Diet-induced ¹³⁰ and gastric bypass-mediated weight loss ¹²⁰ confirmed the above indicated reversibility of the reduced maximal ANP responsiveness in the subcutaneous AT of obese women, postulating that the observed impairments in NPs-mediated metabolic effects are secondary to the obese state. With respect to caloric restriction, Sarzani et al. showed that fasting restored NPs signaling by reducing NPRC expression in the AT ¹²¹ which was later confirmed by others ¹²². Besides, weight loss studies, either involving caloric restriction or gastric bypass surgery, indicated increased systemic NPs levels (*i.e.* NT-proBNP) ²⁰⁴⁻²⁰⁹, which was not confirmed in all studies ^{194,210,211}. Comparing these interventions, it was recently shown that gastric bypass surgery resulted in a greater systemic increase in NT-proBNP as compared to lifestyle intervention in morbidly obese patients ²¹², indicating that the amount of weight loss is associated with the increase in systemic NPs concentrations. Non-pharmacological interventions attenuate NPs levels by altering their signaling pathways and consequently their metabolic effects, which are proposed to result in an improve insulin sensitivity. However, as many studies also observe changes in the inactive fragments (NT-proANP and NT-proBNP), which are not cleared by NPRC, these findings suggest

not only changes in signaling but also adjustments in cardiac production and release following weight loss. In this regard, improvements in other comorbidities which could affect the NPs system following this type of interventions should be taken into account as well.

Despite, the use of exercise training, weight loss interventions or a combination between both, and their interaction with NPs related improvements in insulin sensitivity need further investigation. However, the present clinical studies do not indicate a causal relationship but they do indicate the presence of a strong association between the NPs system and insulin sensitivity management, which needs further focus in upcoming human non-pharmacological intervention studies.

Conclusions

The potential role of NPs as an important metabolic target affecting insulin sensitivity in metabolically comprised conditions has been put forward over the last decade. Preclinical and clinical research indicated the presence of a NP deficiency in obesity and T2DM, which is a well-accepted anomaly that could result from inadequate cardiac NPs production and secretion, as well as an increased degree of peripheral degradation. Of interest, several impairments in NPs receptor and post-receptor signaling have been observed in peripheral tissues like the AT or the skeletal muscle tissue of patients with metabolic disease, including overweight, obesity, IR and type 2 diabetes. Despite several animal and human data suggest a causal link between these NPs (signaling) deficiencies and the development of T2DM, the exact molecular mechanism remains incompletely understood. Unraveling the molecular background of these anomalies could therefore further highlight the emerging role of NPs in metabolic diseases. From a clinical point of view, it remains important to investigate therapeutic options to restore this NP deficiency. Next to implementing pharmacological intervention strategies, lifestyle interventions (including physical activity and diet) are of interest in the treatment of metabolic disease. Indeed, previous studies indicated diet-induced weight loss and physical exercise training to sensitize NPs signaling in AT and skeletal muscle tissue, two main metabolic organs with a role in obesity-related IR and T2DM. However, selecting the appropriate intervention with respect to reducing cardiovascular and metabolic risk factors is of special interest to further improve cardiometabolic health and reduce the development and progression of obesity-related risk factors. Therefore, long-term human intervention studies are needed to clarify the role of NPs on control of body weight and insulin sensitivity, including investigation of the underlying molecular machinery.

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Chapter 3

Attenuated atrial natriuretic peptide-mediated lipolysis in subcutaneous adipocytes of obese type 2 diabetic men

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Published in *Clinical Science*, (2016) 130, 1105-1114

Abstract

Aims/hypothesis Catecholamines and atrial natriuretic peptide (ANP) are major regulators of adipocyte lipolysis. Although obesity is characterized by catecholamine resistance in subcutaneous adipose tissue (SCAT), data on ANP lipolytic response and sensitivity in different adipose tissue depots of metabolically distinct humans are scarce.

Methods Ex vivo catecholamine- and ANP-induced lipolysis was investigated in adipocytes derived from SCAT and visceral (VAT) depot of lean (n=13) and obese men, with (n=11) or without (n=18) type 2 diabetes (HbA1c < or ≥6.5%). Underlying molecular mechanisms were examined by looking at functional receptors in the NP signaling pathway on mRNA and protein level.

Results Maximal ANP- and catecholamine-induced lipolysis in SCAT was blunted in obese type 2 diabetic compared with age-matched lean men whilst non-diabetic obese subjects showed intermediate responses. This blunted ANP-mediated lipolytic response was accompanied by lower mRNA and protein expression of the type-A natriuretic peptide (NP) receptor and higher mRNA but reduced protein expression of the scavenging type-C receptor. Maximal ANP-induced lipolysis was lower in VAT compared to SCAT but not different between groups.

Conclusions/interpretation Collectively, our data show that both ANP- and catecholamine-mediated lipolysis is attenuated in SCAT of obese men with type 2 diabetes, and might be partially explained by NP receptor defects. Therefore, improving maximal ANP responsiveness in adipose tissue might be a potential novel strategy to improve obesity-associated metabolic complications.

Introduction

Adipose tissue (AT) dysfunction is a commonly observed feature of metabolic dysfunction in obesity ¹. Since the subcutaneous adipose tissue (SCAT) has only a limited expandability, ectopic lipid accumulation contributes to the onset or worsening of obesity-related insulin resistance ^{2,3}. As obesity and associated insulin resistance are important risk factors for the development of cardiometabolic diseases like type 2 diabetes mellitus ⁴, it is imperative to investigate AT function in obese insulin resistant subjects with and without type 2 diabetes. An important characteristic when examining AT function is the capacity to break down intracellular stored lipids, a process called lipolysis ⁵. This hydrolysis of triacylglycerol (TAG) stored in adipocytes involves different lipases (i.e. adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL)), and their activity is regulated by several hormones including catecholamines, insulin and natriuretic peptides (NP) ⁶. In the obese state, SCAT adipocytes show an increased ⁷, unchanged ⁸ or decreased ^{9,10} basal (non-stimulated) fasting lipolysis (depending on the degree of insulin resistance and hyperinsulinemia or method of expression), while catecholamine resistance of SCAT lipolysis is most commonly observed ^{7,10,11}. An increased catecholamine sensitivity has been reported for visceral AT (VAT) ^{12,13}. Differences at receptor and post-receptor signaling may contribute to lipolytic differences between depots ¹⁴⁻¹⁷.

Natriuretic peptides, including atrial, B- and C-type natriuretic peptide, display primate-specific effects in human white adipocytes. Of all NP family members, Sengen et al. showed ANP to be the most potent activator of lipolysis in human adipocytes ¹⁸. Moreover, limited data are available about the role of atrial natriuretic peptide (ANP) in human AT lipolysis, of which an overview is shown in Table 3. In 2000 the discovery of the in vitro lipolytic potency ¹⁸ and later the ability of increasing plasma non-esterified fatty acids (NEFA) upon intravenous infusion of ANP, suggested its contribution to an enhanced lipid catabolism in humans ¹⁹⁻²¹. Until today, only one in vivo study indicated an attenuated ANP-induced lipolysis in the SCAT of young overweight/obese men and women ²². However, data on depot-specific differences in ANP sensitivity in obesity are scarce as only two studies suggested ANP-mediated lipolysis to be

similar in SCAT and VAT of morbidly obese subjects ^{23,24}. However, these studies did not include lean subjects, nor obese subjects with type 2 diabetes. The lipolytic effect of ANP is determined by binding to the NP receptor type-A (NPRA) which stimulates cGMP-dependent activation (phosphorylation) of the lipid-droplets associated protein perilipin 1 (PLIN1) and HSL ^{18,20,25}. Next to NPRA, ANP-mediated lipolysis is dependent on NP clearance, predominantly mediated by the NP receptor type C (NPRC) ²⁶⁻²⁸ which is abundantly present in human white AT ^{29,30}. An increased expression of NPRC in the SCAT of obese hypertensive patients compared to non-obese and normotensive subjects has been observed ³¹, which is associated with a reduced NP availability in these patients and possibly a lower NP-mediated lipid mobilization. In addition, insulin has been shown to regulate NPRC expression in human adipocytes ^{24,30,32}. Yet, the possible relevance of insulin-induced upregulation of NPRC and the effect of obesity per se on ANP-mediated AT lipolysis remains unclear and needs further investigation. (Patho)physiological variations in different components of the ANP-mediated lipolytic pathway should be investigated thoroughly, especially under metabolically compromised conditions like obesity or type 2 diabetes ³³.

In the present study, the aim was to investigate whether ex vivo ANP-mediated lipolysis is altered in adipocytes derived from SCAT or VAT of obese subjects with or without type 2 diabetes, compared to age-matched lean men. Furthermore, the underlying molecular mechanism was examined by looking at functional receptors in the NP signaling pathway both at transcriptional (mRNA) and translational (protein) level.

Methods

Subjects – For this study, male subjects who were scheduled to undergo abdominal or bariatric surgery were recruited. The decision to undergo surgery was made independently of this research protocol, between patient and physician. The study sample size was based on previous studies assessing AT lipolysis in non-obese and obese subjects ^{11,12}. The control group consisted of 13 lean male subjects (age 51 ± 5 years; BMI 23.9 ± 1.1 kg/m²; HbA1c $5.3 \pm 0.2\%$ (35 ± 2 mmol/mol)). The obese group was composed of 18 male subjects without type 2 diabetes (age 47 ± 5 years; BMI 38.3 ± 3.9 kg/m²; HbA1c $5.5 \pm 0.3\%$ (37 ± 3 mmol/mol)) and 11 male subjects with type 2 diabetes (age 51 ± 7 years; BMI 38.1 ± 3.7 kg/m²; HbA1c $7.2 \pm 0.9\%$ (56 ± 10 mmol/mol)). Presence of type 2 diabetes was defined as glycated hemoglobin (HbA1c) $\geq 6.5\%$ (45 mmol/mol) or the use of glucose lowering medication based on a clinical diagnosis (on average 2.5 years of diagnosis, ranging from newly diagnosed to 6 years). Clinical characteristics and use of glucose/lipid lowering and anti-hypertensive medication of the subjects included in the study are summarized in Table 1. Major exclusion criteria were presence of a history of heart, lung or kidney disease and/or presence of endocrine anomalies. The study protocol was approved by the Medical Ethical Committee of Jessa Hospital in accordance with the Declaration of Helsinki, and all subjects gave their written informed consent before participating in the study (ClinicalTrials.gov accession number NCT02598544).

Anthropometric measurements and blood sampling - Body weight, height, waist/hip circumference and blood pressure were determined at the morning of surgery (between 7-10 am). Fat and lean body mass were estimated by bioelectrical impedance analyses (Bodystat 1500; Bodystat Ltd., Isle of Man, U.K.). Fasting venous blood samples were collected after an overnight fast (at least 10 hours) for the measurement of plasma ANP, glucose, triglycerides, NEFA, serum insulin and HbA1c. Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA-IR), calculated from fasting glucose and insulin, according to the formula: fasting insulin (μ U/l)*fasting glucose (mmol/l)/22.5 ³⁴. Plasma levels of ANP were measured using the RayBio ANP Enzyme Immunoassay Kit (Tebu-Bio, Boechout,

Belgium). Plasma glucose was measured by the glucose oxidase method using a AU2700 analyzer (Beckman Coulter, Brea, CA, USA). Serum insulin was assessed by immunoassay (ADVIA Centaur Insulin IRI; Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). Plasma NEFA (NEFA C kit; Wako Chemicals, Neuss, Germany) and TAG (Sigma, St. Louis, MO) were determined by an enzymatic colorimetric quantification on a COBAS FARA centrifugal spectrophotometer (Roche, Diagnostica, Basel, Switzerland). HbA1C was assessed by high performance liquid chromatography using a HA-8160 Hi-Auto A1C analyzer (Menarini, Zaventem, Belgium).

Adipose tissue biopsies - After an overnight fast (at least 10 hours) abdominal subcutaneous (SCAT) and omentus majus (VAT) AT biopsies were collected from all participants during abdominal surgery (under general anesthesia). For each AT depot, approximately 1–5 g AT was collected. Tissue was immediately brought to the laboratory for further processing or frozen in liquid nitrogen for gene and protein expression analyses.

Adipocyte isolation and lipolysis experiments - Mature adipocytes were obtained by collagenase digestion of AT fragments in Dulbecco's Modified Eagle's Medium(DMEM)-Ham's F12 under gentle shaking (60 cycles/min) at 37°C³⁵. The resulting suspension was filtered through a 200 µm filter and adipocytes were washed once with DMEM-Ham's F12 to eliminate collagenase. Isolated mature adipocytes were diluted in DMEM-Ham's F12 supplemented with 3% bovine serum albumin for lipolysis assays and incubated with increasing final concentrations of isoprenaline (ISO, a non-selective beta-adrenergic agonist; 10⁻¹⁰–10⁻³ mol/l) or human ANP (Bachem, Weil am Rhein, Germany) (10⁻¹⁰–10⁻⁴ mol/l) in duplicate at a final volume of 100µl for 3h at 37°C. Following incubation, 60µl cell free aliquots of the infranatant were collected for glycerol determination (lipolysis index) using the EnzyChrome™ Adipolysis assay kit (Gentaur, Eersel, The Netherlands). Glycerol release was expressed per 100 mg of lipids, or per cell number, as previously described^{33,36,37}. In addition, glycerol release at maximum effective concentration (compared to baseline) was determined. Lipid content was determined by a quantitative calorimetric method (ABX Pentra Triglycerides CP reagent, HORIBA Instruments LTD, The Netherlands). In brief, pooled cell suspension aliquots were mixed with 5%

Nonidet P40 substitute (NP-40, Sigma Aldrich, The Netherlands) and heated twice (95°C) for 2 minutes and subsequently centrifuged for 2 minutes at 14.000 rpm at 4°C. Supernatant was collected and used for enzymatic TAG determination after incubation (20 minutes at 37°C) using a Spectramax M2 microplate reader (Molecular devices, Berkshire, UK).

Gene expression experiments – Total AT RNA was isolated using the TRIzol® reagent (Invitrogen, Paisley, UK). Reverse transcription of 300 ng of total RNA was performed using iScript cDNA synthesis kit (Bio-Rad) and real-time PCR was carried out on the MyiQ single color RT-PCR detection system (Bio-Rad). Reactions were performed in a total volume of 25 µL containing 5.5 µL complementary DNA (cDNA), 12.5 µL SYBR green master mix (IQ SYBR Green Supermix) and gene specific primers for *NPRA* and *NPRC* (Biologio, Nijmegen, The Netherlands). Gene expression was normalized using *18S* ribosomal RNA. *NPRA* forward primer 5' GCAAAGGCCGAGTTATCTACAT and reverse primer 5'AAGAAAACGTAGTCCTCCCA. *NPRC* forward primer AGACTACGCCTTCTTCA ACATTG and reverse primer 5' GCTTCAAAGTCGTGTTTGTCTCC.

Protein isolation – AT biopsies (100-300 mg) were ground to a fine powder under liquid nitrogen and homogenized in 200-600 µL radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease/phosphatase inhibitor cocktail (Cell Signalling Technology Europe, Leiden, The Netherlands). Lysates were vortexed for 5 min at room temperature and centrifuged at 14.000 rpm for 30 min at 10°C. Infranatant was carefully transferred to new tubes. Protein concentrations were determined using the Pierce® BCA protein assay kit (Santa Cruz Biotechnology Inc., Heidelberg, Germany). Samples were stored at -80°C prior to analysis.

Western blotting – 25 µg protein was separated on Any kD SDS-PAGE gels (Bio-Rad Laboratories, Veenendaal, The Netherlands) and subsequently blotted semi-dry onto nitrocellulose membrane using Trans-Blot® Turbo Transfer System (Bio-Rad Laboratories, Veenendaal, The Netherlands). Following transfer, membranes were blocked for 1 h in blocking buffer (Tris-buffered saline with 0.1% Tween 20 (TBS-T), 5% nonfat dry milk). The membranes were incubated overnight at 4°C with primary antibodies diluted in blocking buffer.

Next, membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. The primary antibodies used were anti- α -NPRC antibody (Sigma SAB2501867, 1:1000 dilution), anti- α -NPRA antibody (Abcam ab154266, 1:1000 dilution) and anti- β -actin antibody (Santa Cruz, #sc-47778, 1:1000 dilution). Secondary antibodies were horse-radish peroxidase-conjugated-IgG anti- α -swine or anti- α -rabbit antibody (DAKO, Heverlee, Belgium). Antigen-antibody complexes were visualized by chemiluminescence using SuperSignal™ West Femto extended Duration Substrate (Life Technologies, Gent, Belgium). As positive control for NPRC, 1 μ g of a transient overexpression lysate of NPRC was loaded (Origene, #LY424462). Visualization and analysis was performed using a Chemidoc XRS system (Bio-Rad Laboratories, CA, USA) and Quantity One software.

Statistical analysis – All data are presented as mean \pm SEM. Shapiro-Wilk tests indicated that data were not normally distributed. Therefore, group differences were analyzed using nonparametric Kruskal Wallis and Mann-Witney U tests and depot differences within groups using Wilcoxon Signed rank tests. Statistical significance was set at $P < 0.05$ (2-tailed). Statistical analyses were performed using SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Subjects' characteristics

Subjects' characteristics and use of glucose/lipid lowering and anti-hypertensive medication are displayed in Table 1. All groups were matched for age, and obese subjects differed from lean controls in several anthropometric and metabolic parameters, having higher body weight, and body fat, while their lipid profile (circulating lipids (NEFA and TAG)) and insulin sensitivity (as indicated by the higher HOMA-IR) were more disturbed. Obese subjects with type 2 diabetes showed higher HbA1C and fasting glucose levels compared to lean subjects and obese subjects without type 2 diabetes. Fasting ANP levels were significantly higher in obese type 2 diabetic men (29.4 ± 1.7 pg/ml) compared to lean men (23.7 ± 1.4 pg/ml; $P < 0.01$), with intermediate values for obese men without type 2 diabetes. Both obese men with and without type 2 diabetes were on lipid (n=8) and/or blood pressure (n=17) lowering medication (including beta-blockers, diuretics, calcium channel blockers, angiotensin converting enzyme (ACE) inhibitors or a combination). Eight of the obese type 2 diabetic men were on blood glucose lowering medication.

Table 1 – Subjects’ clinical characteristics

	Lean (13)	Obese without type 2 diabetes (18)	Obese with type 2 diabetes (11)
Age, years	51 ± 1	48 ± 1	51 ± 2
BMI, kg/m ²	23.9 ± 0.3	37.9 ± 1.0 *	38.0 ± 1.1*
Waist circumference, cm	91.3 ± 1.6	128.3 ± 2.2 *	126.1 ± 1.9 *
Waist-to-hip ratio	0.96 ± 0.01	1.08 ± 0.01 *	1.08 ± 0.01 *
Fat mass, kg	18.6 ± 1.1	45.8 ± 2.7 *	42.9 ± 2.0 *
Body fat, %	23.8 ± 1.2	36.6 ± 1.1 *	36.7 ± 1.0 *
Fasting glucose, mmol/l	5.6 ± 0.1	5.7 ± 0.2	7.8 ± 0.7 *,†
Fasting insulin, mU/l	9.3 ± 1.1	21.9 ± 2.6 *	21.3 ± 3.2 *
Fasting NEFA, μmol/l	295 ± 56	759 ± 88*	872 ± 69*
Fasting triglycerides, mmol/l	1.1 ± 0.1	1.2 ± 0.1*	1.2 ± 0.2
Fasting ANP, pg/ml	23.7 ± 1.4	25.3 ± 1.6	29.4 ± 1.7*
HbA _{1C} , %	5.3 ± 0.1	5.5 ± 0.1	7.3 ± 0.3 *,†
HbA _{1C} , mmol/mol	35 ± 1	37 ± 1	56 ± 3 *,†
HOMA-IR	2.3 ± 0.3	5.7 ± 0.8 *	7.9 ± 1.7 *
Glucose lowering medication	-	-	8/11
Lipid lowering medication	-	4/18	4/11
Anti-hypertension medication	-	9/18	8/11

Values are means ± SEM. BMI, body mass index; NEFA, non-esterified fatty acids; ANP, atrial natriuretic peptide; HOMA-IR, homeostatic model assessment of insulin resistance. *Significantly different from lean group ($P < 0.01$); † significantly different from obese group without type 2 diabetes ($P < 0.01$).

Attenuated maximal ISO- and ANP-mediated lipolysis in mature adipocytes derived from the SCAT of obese men

Glycerol release, a marker of complete TAG hydrolysis, was used to compare basal, ISO- and ANP-mediated adipocyte lipolysis in freshly isolated mature adipocytes of the SCAT and the VAT (Table 2). Basal lipolysis per cell was significantly higher in obese men with and without type 2 diabetes compared with lean men in both the SCAT and VAT. No differences between groups were observed in basal (non-stimulated) glycerol release when adjusted for the amount of lipids.

Incubation of freshly isolated mature adipocytes with ISO or ANP caused a concentration-dependent increase in glycerol release in both AT depots (Supplemental Figure 1). When expressing ISO-induced glycerol release per cell

number, responses in both AT depots (Table 2) were significantly higher in both obese and obese diabetic men as compared to lean men. Maximal ISO-mediated lipolysis (expressed as fold-change over basal) was significantly reduced in the SCAT of obese (2.5–2.7-fold) compared to lean (3.8-fold) men ($P < 0.05$). This blunted maximal lipolytic response was most pronounced in adipocytes derived from obese subjects with type 2 diabetes and intermediate in obese subjects without type 2 diabetes. In VAT, no difference in maximal ISO-mediated lipolysis was observed between groups (Table 2). However, when comparing both depots, a higher maximal ISO-mediated lipolytic response was observed in adipocytes derived from VAT compared to SCAT of obese and obese type 2 diabetic subjects, which was not observed in lean controls (Table 2). In contrast to maximal response, β -adrenergic receptor sensitivity did not differ between groups or depots (data not shown).

Maximal ANP-mediated lipolysis expressed per cell number was not different between groups in the SCAT. However in the VAT, a higher maximal ANP response per cell was observed in adipocytes derived from obese compared to lean men, which was most obvious in obese subjects with type 2 diabetes as compared to lean men (4.3 vs. 1.4, $P < 0.05$). When corrected for basal lipolysis, maximal ANP-mediated lipid mobilization was significantly lower in the adipocytes derived from SCAT of obese type 2 diabetic subjects as compared to lean subjects (2.4 vs. 4.9, $P < 0.05$). In VAT, no differences in maximal ANP response were observed between groups when expressed as fold-change over basal lipolysis. When comparing both depots, lean subjects showed a significantly higher maximal ANP responsiveness in the adipocytes derived from the SCAT compared to the VAT (1.6-fold), while this depot difference was absent in both obese groups. In contrast to maximal lipolytic response, ANP-sensitivity did not differ between groups or depots (data not shown).

Table 2 – Basal and maximal ISO- and ANP-mediated lipolytic response in SCAT and VAT adipocytes of lean, obese and obese diabetic men

	SCAT			VAT		
	Lean (13)	Obese (18)	Obese diabetics (11)	Lean (13)	Obese (18)	Obese diabetics (11)
Basal						
Glycerol ($\mu\text{mol/l/3h}$ incubation)	12.3 \pm 2.7 [†]	8.6 \pm 1.2 [†]	10.5 \pm 2.0	20.0 \pm 5.3	13.4 \pm 2.2	13.6 \pm 1.7
per 100mg lipids	7.7 \pm 2.1	6.5 \pm 0.8	7.0 \pm 1.5	8.4 \pm 2.3	9.1 \pm 2.9	6.4 \pm 0.9
per 10 ⁶ cells	0.44 \pm 0.09	1.48 \pm 0.23**	1.32 \pm 0.15**	0.67 \pm 0.20	1.19 \pm 0.16**	1.81 \pm 0.52*
Isoprenalin(10⁻⁶ mol/l)						
per 100mg lipids	24.0 \pm 5.1	15.8 \pm 1.6	20.8 \pm 8.1	45.9 \pm 19.0	48.5 \pm 18.6	22.7 \pm 4.4
per 10 ⁶ cells	1.62 \pm 0.31	3.65 \pm 0.54**	3.94 \pm 1.51	2.42 \pm 0.73	4.99 \pm 0.78**	6.06 \pm 1.55
fold compared to basal	3.78 \pm 0.50	2.72 \pm 0.26 [†]	2.54 \pm 0.78*	4.69 \pm 0.79	4.44 \pm 0.41	3.72 \pm 0.72
ANP(10⁻⁴ mol/l)						
per 100mg lipids	26.0 \pm 5.1	17.2 \pm 2.2	18.5 \pm 7.3	23.1 \pm 7.2	21.7 \pm 7.1	15.4 \pm 2.8
per 10 ⁶ cells	1.86 \pm 0.31	4.08 \pm 0.64	3.70 \pm 1.54	1.45 \pm 0.33	2.52 \pm 0.49	4.30 \pm 1.16*
fold compared to basal	4.97 \pm 0.88 [†]	3.09 \pm 0.46	2.41 \pm 0.82*	2.98 \pm 0.50	2.23 \pm 0.31	2.60 \pm 0.51

Values are means \pm SEM. Basal and maximal hormone-induced lipolysis are expressed as amount of glycerol release (($\mu\text{mol/l/3h}$ incubation)) from mature adipocytes, corrected for amount of lipids, cell number or relative to basal glycerol release. * $P < 0.05$; ** $P < 0.01$ compared to lean control group; [†] $P < 0.05$ between depots of same subject group.

Reduced NPRA and increased NPRC expression in the adipose tissue of obese subjects

In line with the attenuated maximal ANP response in adipocytes derived from SCAT of obese men, NPRA mRNA expression was lower in adipose tissue biopsies derived from obese compared to lean SCAT (0.63 vs. 1.00, $P < 0.01$, Figure 1A). In VAT, NPRA mRNA expression was comparable between groups (Figure 1A). In contrast to NPRA, the NPRC transcript was more abundant in both SCAT and VAT biopsies of obese (1.83 and 1.17, respectively) and obese type 2 diabetics (1.78 and 1.34, respectively) compared to lean men (1.00 ($P < 0.05$) and 0.54 ($P < 0.01$), respectively), resulting in a decreased NPRA:NPRC ratio (i.e. NPRR) in obese subjects (Figure 1A). When comparing both depots, NPRC mRNA expression was lower in the VAT compared to the SCAT of lean and obese men ($P < 0.05$), while NPRA expression was higher in the VAT compared to the SCAT of obese diabetic subjects ($P < 0.05$, Figure 1A).

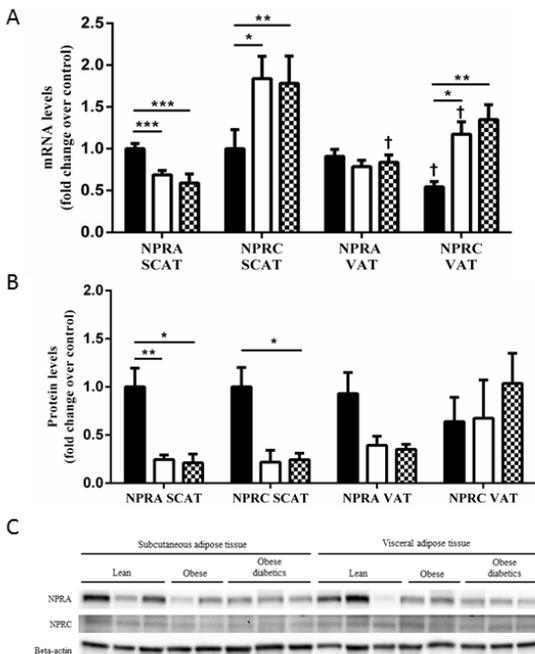


Figure 1 - mRNA and protein expression of NPRA and NPRC in SCAT and VAT of lean, obese and obese diabetic men. (A) mRNA expression of NPRA and NPRC were determined in paired AT samples from 30 obese and 14 lean subjects. (B) Protein expression data of NPRA was derived from paired AT samples of lean ($n=7$), obese ($n=7$) and obese diabetic men ($n=6$). Protein expression data of NPRC was derived from paired AT samples of lean ($n=6$), obese ($n=4$) and obese diabetic men ($n=6$). (C) Representative western blot for NPRA and NPRC in SCAT and VAT of lean ($n=3$) and obese men with ($n=3$) and without type 2 diabetes ($n=2$). Black bars indicate lean, white bars indicate obese and white squared bars indicate obese diabetic men. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; † $P < 0.05$ between depots of same subject group.

Western Blot analysis (Figure 1B and C) revealed that both receptors were downregulated at the protein level in SCAT biopsies of obese subjects. Both obese (4.1-fold for NPRA ($P < 0.01$) and 4.5-fold for NPRC ($P = 0.05$)) and obese type 2 diabetic men (4.7-fold for NPRA ($P < 0.05$) and 4.1-fold for NPRC ($P < 0.05$)) showed lower NP receptor levels compared with lean men. No difference in NP receptor protein content was found in the VAT between groups (Figure 1B). When comparing between depots, a trend towards a higher NPRC protein expression was found in the VAT of obese subjects with type 2 diabetes compared to the SCAT (4.2-fold, $P=0.07$). Additionally, NPRA protein expression tended to be lower in SCAT compared to VAT in non-diabetic obese subjects ($P = 0.07$) (Figure 1B).

Discussion

In the present study, we investigated for the first time both catecholamine- and ANP-mediated lipolysis in visceral and subcutaneous adipocytes derived from lean compared to obese men with and without type 2 diabetes. Our data clearly demonstrate an attenuated maximal responsiveness to the two major lipolytic hormones, ANP and catecholamines (i.e. ISO) in the SCAT of obese men (independent of diabetes status). The impaired ANP-mediated lipolysis was accompanied by a differential NP receptor expression pattern. Of interest, in lean men, ANP-mediated lipid mobilization was higher in SCAT than VAT but this depot difference disappeared in both obese groups.

In the current study basal and ANP-mediated glycerol release, expressed per cell, were increased in obese hypertrophic subcutaneous adipocytes, although this did not reach statistical significance. These observations are in agreement with recent *ex vivo* data from Rydén et al. indicating comparable ANP-mediated lipolysis in obese compared to non-obese women when expressed per fat cell number²². Maximal ISO-mediated glycerol release from subcutaneous adipocytes was higher in obese when expressed per cell and lower when expressed per cell mass (i.e. lipid content), a consequence of the increased average size and mass of adipocytes in obese men. In line, *ex vivo* basal and maximal stimulated (ANP- and ISO-mediated) lipolysis has been shown to be higher in large versus small adipocytes within the same depot of healthy lean and obese men and women^{38,39}. However, when maximal ANP- and ISO-induced glycerol release were expressed as a ratio of basal release, we observed an attenuated maximal lipolytic responsiveness to both ANP and ISO in subcutaneous adipocytes of obese men (independent of diabetes status). Previously, both a decreased maximal catecholamine-induced lipolytic response and attenuated beta-adrenergic receptor sensitivity have been observed in SCAT of obese subjects^{12,13}. With respect to ANP, previous data from Rydén et al. also reported only an attenuated maximal ANP response and no difference in ANP sensitivity between obese and non-obese women²². Table 3 gives a detailed overview of the *in vivo* and *in vitro* human studies on ANP-mediated lipolysis in adipose tissue performed so far. Moreover, we observed a more pronounced decrease in maximal ANP-mediated lipolysis in obese type 2 diabetic men

compared to obese insulin resistant men, illustrating that this impaired ANP response in the SCAT is related to severity of metabolic abnormalities. Together these data indicate the presence of a state of impaired lipid turnover, which together with the attenuated maximal catecholamine- and ANP-mediated lipolytic response in SCAT may contribute to an impaired adipose tissue lipid metabolism in obesity and the subsequent development of insulin resistance.

Of interest we observed a reciprocal regulation of ANP- and catecholamine-mediated lipolysis in subcutaneous and visceral adipocytes from lean men, which was not present in obese men. The higher ISO- and lower ANP-induced lipolysis in visceral compared to subcutaneous adipocytes of lean men, might be partly explained by differences in receptor density or function or post-receptor signaling ¹⁴. In line with our observations in obese men, Dicker et al. investigated potential depot-specific differences in ANP-mediated lipolysis in middle-aged, morbidly obese women and did not find any difference between visceral and subcutaneous differentiated preadipocytes (Table 3) ²³. Consistent with these data our data showing no depot differences in ANP-mediated lipolysis between depots in the obese groups as compared to a reduced ANP-mediated lipolysis in VAT as compared to SCAT in the lean group, support the presence of an ANP-resistant phenotype at the level of SCAT in obese subjects.

Since the net lipolytic effect of ANP is determined by both NPRA and scavenging NPRC ³⁶, we investigated the expression level of both receptors to get some more insight on the underlying mechanism responsible for the observed attenuated maximal ANP responsiveness in the SCAT of obese men. In line with our ex vivo data, we found a decreased expression of NPRA in SCAT, both at mRNA (as shown previously ²⁴) and protein level, while VAT showed comparable levels for NPRA (mRNA and protein level) between groups. Regarding ANP scavenging, NPRC mRNA levels were higher in obese compared to lean men in both AT depots. However, this did not translate into higher NPRC protein levels, which might be a compensatory mechanism for the observed higher circulating ANP levels in obese men. Due to the absence of a catalytic GC domain, the ANP scavenging NPRC receptor has a very minor role in ANP-mediated lipolysis. Its main function is to facilitate scavenging of its ligands, internalization of ligand-receptor complexes and recycling of NPRC, together with degradation of its

ligands ²⁶. However, the neutral endopeptidase neprilysin, an enzyme responsible for processing of NPs, was shown to have a minor function in human adipocytes and therefore, in contrast to NPRA, NPRC might not play a major role in the regulation of ANP-mediated lipolysis in human adipose tissue ³³. In addition, comparable NPRA and NPRC levels were observed between both adipose tissue depots in lean as well as in obese men. Furthermore, as maximal lipolytic response is determined by both receptor and post-receptor signaling, also more downstream impairments might have contributed to the observed blunted lipolytic response in the SCAT of obese men and the depot-specific regulation of ANP- and ISO-mediated lipolysis as observed in lean men. In line, others and we have shown previously that expression and activity of the lipid droplet associated lipases ATGL and HSL is decreased in SCAT of obese insulin-resistant men ^{11,40,41}, possibly contributing to the observed attenuated ANP- and ISO-mediated lipolysis in subcutaneous adipocytes of obese men. Another important downstream second messenger is cGMP, which is formed by the soluble guanylyl cyclase-pathway or via membrane-bound guanylyl cyclase upon binding of NP to its NPRA receptor ⁴². Evidence about the importance of the NPRA-linked cGMP/PKG pathway in browning of white AT and increasing energy expenditure is accumulating ⁴³. In combination with the results regarding an imperative role of NP in AT lipolysis, these data suggest that the NP system might be a promising target for anti-obesity drug development ²⁸. Unfortunately, clinical trials investigating the effects of (non)pharmacological stimulation of NP on obesity and related cardiometabolic disease are currently lacking ⁴⁴.

Together, our data suggest a decreased number of functional NPRA receptors and decreased ANP scavenging in the SCAT of obese insulin resistant men, which is in line with our observed attenuated maximal ANP responsiveness in the SCAT and increased circulating ANP levels in obese men. However, this contrasts previous data showing lower circulating ANP levels in obese men ⁴⁵⁻⁴⁷. This discrepancy results probably from a worse cardiovascular health in our obese group, indicated by the elevated (systolic) blood pressure and use of anti-hypertensive drugs by more than 70% of the obese men as compared to 0-38.5% in other studies ^{45,46}.

Table 3 – Summary of studies on ANP-mediated lipolysis

Methodology	Participants	Adipose tissue	Effect on lipolysis	Study
<i>In vitro</i> studies				
differentiated preadipocytes stimulated with ANP (10^{-11} – 10^{-6} mol/L)	10 obese women (BMI 43.8 ± 3.5 kg/m ²)	SCAT vs. VAT	ANP-mediated lipolysis did not differ between adipose tissue depots	Dicker <i>et al.</i> (2008) ²³
<i>Ex vivo</i> isolated mature adipocytes				
stimulated with hANP (10^{-10} – 10^{-6} mol/L)	7 healthy lean (BMI 22.2 ± 0.7 kg/m ²) and 7 healthy obese (BMI 31.5 ± 1.4 kg/m ²)	SCAT	Comparable hANP responsiveness and sensitivity between lean and obese	Galitzky <i>et al.</i> (2001) ¹⁹
stimulated with ANP (10^{-11} – 10^{-6} mol/L) with or without prior ANP (10^{-6} mol/L) treatment (1h)	6 healthy lean/moderately overweight (BMI 24.4 ± 1.3 kg/m ²) women	SCAT	<i>In vitro</i> desensitization of ANP-induced lipolysis following prior ANP treatment	Moro <i>et al.</i> (2005) ⁵²
stimulated with increasing ANP concentrations (10^{-17} – 10^{-7} mol/L)	35 non-obese (BMI 24 ± 2 kg/m ²) and 87 obese (BMI 42 ± 6 kg/m ²) women	SCAT	Lower ANP responsiveness in SCAT of obese women but no difference in ANP sensitivity	Rydén <i>et al.</i> (2015) ²²
<i>In vivo</i> studies				
in situ microdialysis infusion (60min) with hANP (10^{-6} mol/L)	7 healthy lean (BMI 23.0 ± 1.6 kg/m ²) men	SCAT	SCAT lipolysis increased significantly (~250%) compared to baseline	Sengenès <i>et al.</i> (2000) ¹⁸
	7 healthy lean (BMI 22.2 ± 0.7 kg/m ²) and 7 healthy obese (BMI 31.5 ± 1.4 kg/m ²) men	SCAT	SCAT lipolysis increased significantly (~200%) compared to baseline, comparable between groups	Galitzky <i>et al.</i> (2001) ¹⁹
	7 healthy lean (BMI 23.0 ± 1.6 kg/m ²) and 9 healthy overweight (BMI 28.0 ± 0.3 kg/m ²) men	SCAT	26% reduction in ANP-induced lipolysis in SCAT of overweight men as compared to the lean	Rydén <i>et al.</i> (2015) ²²
intravenous infusion of hANP ($2 \cdot 10^{-12}$, $4 \cdot 10^{-12}$ and $8 \cdot 10^{-12}$ mol/kg·min over 45min each)	14 healthy lean (BMI 24.2 ± 0.4 kg/m ²) men	SCAT	Significant increase in SCAT lipolysis with increasing serum ANP concentrations ($r^2=0.84$)	Birkenfeld <i>et al.</i> (2005) ²⁰

Abbreviations: ANP, atrial natriuretic peptide; BMI, body mass index; hANP, human atrial natriuretic peptide; SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue.

Since it is evident that medication used in the treatment of cardiovascular disorders can modulate ANP levels and tissue responses this should be taken into account when interpreting the present data on ANP and catecholamine responsiveness in human AT of metabolically compromised donors ⁴⁸. In line with previous data from Pivovarova et al., showing an up-regulation of NPRC mRNA expression by insulin in the obese insulin-resistant state in VAT and SCAT ²⁴, we observed a higher NPRC mRNA expression in the SCAT and VAT in obese and obese diabetic subjects and an increased NPRC protein expression in VAT of obese diabetic subjects. Together, these data show that both adiposity and insulin sensitivity may influence ANP responsiveness in SCAT, which needs to be investigated in more detail in future studies aimed to improve insulin sensitivity (e.g. physical training or weight loss). Of interest, exercise training, which improves insulin sensitivity, is accompanied by an increased ANP-induced lipolysis in the SCAT, at least in young overweight men ⁴⁹. In addition, it was shown recently that substantial weight loss following bariatric surgery normalized ANP-induced lipolysis in the SCAT of obese women ²². Together, these data suggest that ANP-induced lipolysis in the SCAT is reversible attenuated in human obesity and the use of exercise to optimize intervention effects related to fat mass loss and insulin sensitivity in the obese seems a promising strategy.

Conclusion

In conclusion, our data show that maximal ANP- and catecholamine-induced lipolysis^{13,14} in the SCAT are attenuated in obese diabetic men as compared to lean controls with intermediate values for obese insulin-resistant men. This blunted ANP-mediated lipolytic response was accompanied by a decreased NPRA receptor expression, which was partly correlated with measures of adiposity and insulin sensitivity. These data suggest that improving maximal ANP responsiveness in the SCAT might be a potential novel strategy to improve lipid mobilization in obesity, which may possibly result in a more beneficial cardiometabolic profile. However, it remains to be determined if these improvements could be equally obtainable in different cardiometabolic phenotypes within the obese population⁵⁰. Lifestyle as well as pharmacological interventions might be beneficial in this regard⁵¹.

Acknowledgements

We thank all abdominal surgeons from the Jessa Hospital (Hasselt, Belgium) and Hospital East-Limburg (Genk, Belgium) and Sandra Latet (Obesity Center Hasselt) for their contributions to subject recruitment. We thank University Biobank Limburg (UBiLim, Jessa Hospital, Belgium) for their technical support in sample handling and storage.

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Supplemental data

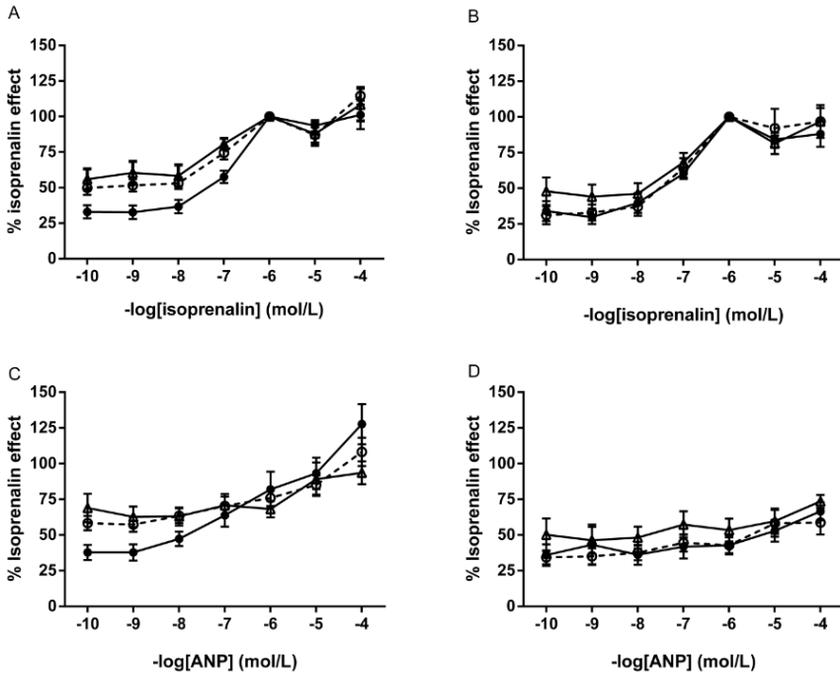


Figure S1 - Dose-response curves for ISO- or ANP-mediated lipolytic response in human mature adipocytes derived from the SCAT and VAT of lean, obese and obese diabetic male donors. Lipolysis induced by non-selective beta-adrenergic agonist isoprenaline (ISO) (**A,B**) or ANP (**C,D**) in subcutaneous (**A,C**) and visceral (**B,D**) isolated mature adipocytes of lean (n=13, black circles), obese (n=17, dotted line, white circles) and obese diabetic (n=11, white triangles). Lipolysis is expressed in percent maximum effect of isoprenaline, following incubation with increasing concentrations ISO (10^{-10} - 10^{-4} mol/l) or ANP (10^{-10} - 10^{-4} mol/l) for each adipose tissue depot respectively.

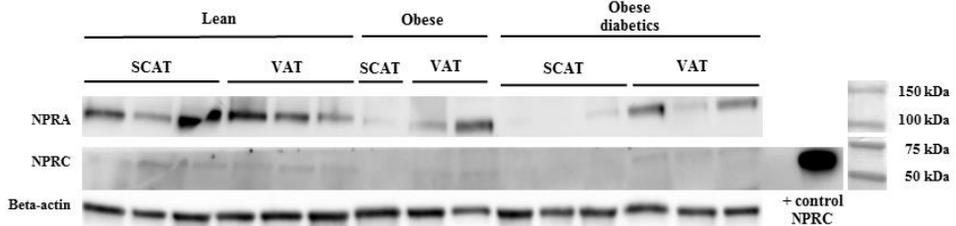


Figure S2 - Validation of NPRC protein expression in SCAT and VAT. In a subset of subjects (lean n=3, obese n=5) NPRC protein expression was validated using a positive control (transient overexpression NPRC lysate, 1 μ g) showing a band at predicted height \pm 60-70 kDa.

Chapter 4

Abdominal subcutaneous and visceral adipocyte morphology, lipolysis and inflammation in relation to insulin resistance in obese humans

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Submitted

Abstract

Aims/hypothesis - Obesity is associated with a disturbed adipose tissue (AT) function characterized by adipocyte hypertrophy, an impaired lipolysis and a pro-inflammatory phenotype, which contributes to insulin resistance (IR). Therefore, we investigated whether AT morphology, lipolysis and inflammation in different AT depots of obese individuals with and without type 2 diabetes mellitus (T2DM) are associated with whole-body IR.

Methods - Subcutaneous (SC) and visceral (V) AT biopsies from age-matched (39-65 year) lean (n=19, BMI 23.9±0.3 kg/m², HOMA-IR 2.1±0.2), obese (n=22, BMI 38.1±0.9 kg/m², HOMA-IR 5.6±0.7) and obese T2DM men (n=11, BMI 38.1±1.2 kg/m², HOMA-IR 7.9±1.7) were collected. AT phenotype was characterized by ex vivo measurement of basal, ANP- and isoprenalin-stimulated lipolysis (mature adipocytes), adipocyte size distribution (AT tissue sections) and AT immune cells (flow cytometry).

Results - Adipocyte hypertrophy was present in SCAT and VAT of obese compared to lean individuals. In VAT, CD45+ leukocytes and M1 macrophages were significantly increased in both obese groups compared to lean individuals. In SCAT no significant differences in immune cell populations between groups were found. In SCAT, multiple linear regression analysis, including age, fat mass and AT morphologic, lipolytic and immune cell markers showed that age ($\beta=0.341$) and total fat mass ($\beta=0.507$) independently contributed to HOMA-IR (adjusted R²=0.325). In VAT, adipocyte size ($\beta=0.619$), CD19+ B lymphocytes ($\beta=0.286$) and maximal isoprenalin responsiveness ($\beta=-0.313$) were significantly related to HOMA-IR, together accounting for 23% of the variance in HOMA-IR.

Conclusions/interpretation - Adipocyte hypertrophy in both SCAT and VAT is an important phenotype of IR. In hypertrophic visceral adipocytes, M1 macrophage- or B-cell-mediated inflammation may contribute to IR, while inflammation in SCAT does not seem to play a major role in IR.

Introduction

During the development of obesity, adipose tissue (AT) expansion frequently results in adipocyte hypertrophy (i.e. enlargement of the adipocyte), which is a known stressor for adipocytes ¹. Increases in AT mass and adipocyte volume result in a broad range of metabolic repercussions including, amongst others, a decreased insulin-mediated suppression ^{2,3} and attenuated catecholamine or atrial natriuretic peptide (ANP)-mediated stimulation of AT lipolysis ⁴⁻⁷. This altered AT function is characterized by an impaired lipid buffering capacity, especially in the post-prandial state ⁸. Subsequently, this may lead to systemic lipid overflow and ectopic lipid accumulation in several insulin sensitive peripheral tissues like skeletal muscle, liver, pancreas, the heart and kidneys, which relates to the development of peripheral and systemic insulin resistance (IR) ⁹.

Different immune cells are activated within hypertrophic AT (in case of resident immune cells) or attracted toward necrotic/apoptotic hypertrophic adipocytes, especially in the visceral AT (VAT), mainly based on rodent studies ^{10,11}. This VAT inflammation was proposed to be the strongest correlate of IR in human obesity ^{12,13}. In this regard, AT macrophages have gained much attention as important mediators of AT inflammation. In human obesity, AT macrophages display profound pro-inflammatory (M1) characteristics ^{14,15} and are thereby thought to be the major source of pro-inflammatory cytokines and chemokines. This in turn may affect local AT lipolysis and further impede adipocyte function ¹⁶ and reduce insulin sensitivity ¹⁷⁻¹⁹. However, recent evidence indicates the presence of other innate and adaptive immune cells in the AT (reviewed by Mraz et al.) ²⁰. The cross-talk and dynamics of immune cells initiating and orchestrating AT inflammation and an impaired lipid metabolism in different AT depots are incompletely understood in the obese non-diabetic or type 2 diabetic state.

It is still an ongoing debate whether an impaired AT lipolysis or inflammation, or an interaction between both, is a cause or rather a consequence of IR in the obese state. Animal models propose a causative role of AT inflammation in the pathogenesis of IR ²¹. In contrast, recent human studies indicated the development of hyperinsulinemia and dyslipidemia and (peripheral) IR upon overfeeding without affecting the inflammatory phenotype of the SCAT,

suggesting that AT inflammation is not necessary to evoke peripheral IR in humans ^{22,23}. Of interest, healthy AT expansion and remodeling implies a certain degree of AT inflammation contrasting the general belief that inflammation negatively affects metabolism ²⁴.

Therefore, gaining better insights into the association between AT lipolysis, inflammation and IR is imperative to improve future development of therapeutic strategies to ameliorate the adverse metabolic consequences of obesity. In the present study, we studied subcutaneous and visceral AT morphology (adipocyte size), ex vivo lipolysis and immune cell populations (flow cytometry) in relation to whole-body IR in obese non-diabetic and type 2 diabetic (T2DM) men, compared with age-matched lean men.

Research design and Methods

Subjects - For this study, lean and obese male age-matched individuals who were scheduled to undergo abdominal or bariatric surgery were recruited, as described previously in more detail ⁷. The lean control group consisted of 18 male individuals. The obese group was composed of 30 male individuals, including 19 individuals without type 2 diabetes and 11 individuals with type 2 diabetes. Presence of type 2 diabetes was defined as glycated haemoglobin (HbA1c) \geq 6.5% (45 mmol/mol) or the use of glucose lowering medication based on a clinical diagnosis (on average 2.5 years of diagnosis, ranging from newly diagnosed to 6 years). Major exclusion criteria were the use of exogenous insulin, presence or history of heart, lung or kidney disease and/or presence of endocrine anomalies. The study protocol was approved by the Medical Ethical Committee Jessa hospital, Hasselt, and Hasselt University, Belgium, in accordance with the Declaration of Helsinki (2008), and all individuals gave their written informed consent before participating in the study.

Anthropometric measurements and blood sampling - Body weight, height, waist/hip circumference and blood pressure were determined at the morning of surgery. Fat and lean body mass were estimated by bio-electrical impedance analyses (Bodystat 1500; Bodystat Ltd., Isle of Man, UK). Fasting venous blood samples were collected after an overnight fast for measurement of plasma glucose, serum insulin and HbA1C. Insulin sensitivity was assessed by the homeostatic model assessment index for insulin resistance (HOMA-IR), calculated from fasting glucose and insulin, according to the formula: fasting insulin (mU/l)*fasting glucose (mmol/l)/22.5 ²⁵. Plasma glucose concentration was measured by the glucose oxidase method using an AU2700 analyser (Beckman Coulter, Brea, CA, USA). Serum insulin concentration was assessed by immunoassay (ADVIA Centaur Insulin IRI; Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). HbA1C was assessed by high performance liquid chromatography using a HA-8160 Hi-Auto A1C analyser (Menarini, Zaventem, Belgium).

Abdominal subcutaneous and visceral adipose tissue biopsies - After an overnight fast, adipose tissue biopsies were taken from the abdominal subcutaneous adipose tissue depot (SCAT) and the distal portion of the omentum majus (VAT). The tissue samples were immediately placed in saline

and transported on ice to the laboratory for further processing. One portion of fresh AT was used for histological sections whereas another portion was used for isolation of the stromal vascular fraction (SVF) and mature adipocytes for lipolysis measurements.

Adipocyte size - A small part of the AT samples was fixed overnight in 4% paraformaldehyde and embedded in paraffin. Histological sections (8 μm) were cut, mounted on microscope glass slides and dried overnight in an incubator (37°C). Haematoxylin and eosin staining was used. Digital images were captured using a Leica DFC320 digital camera (Leica DM3000 microscope, Leica, Rijswijk, The Netherlands) at 20x magnification. Adipocyte size and distribution was performed in a blinded fashion (coefficient of variation <5%) using computerized morphometric analysis (Leica QWin V3, Cambridge, UK) of individual adipocytes (at least 400 adipocytes per sample), as described previously ²⁶.

Adipocyte isolation, stromal vascular preparation and lipolysis measurement - Mature adipocytes and stromal vascular cells were obtained, as described previously ⁷. Briefly, after collagenase digestion of AT fragments in Dulbecco's modified Eagle's medium (DMEM)-Ham's F12, the resulting suspension was filtered and mature adipocytes were diluted in DMEM-Ham's F12 supplemented with 3% BSA for lipolysis assays and incubated with isoprenaline (ISO, a non-selective β -adrenergic agonist; 10⁻⁶ M) or human ANP (Bachem) (10⁻⁴ M) for 3 h at 37°C. Following incubation, glycerol concentration in the medium (lipolysis index) was determined using the EnzyChrome™ Adipolysis assay kit (Gentaur) and expressed per cell number or as maximal responsiveness to adrenergic or ANP stimulation (compared with baseline).

Flow cytometry - Multicolour flow cytometry measurements were performed as described previously ²⁷. Briefly, SVF cells, which were obtained following collagenase digestion, were stained for flow cytometry using two antibody cocktails. Samples were measured with a FACS-Canto II (BD Biosciences). Results were analysed with FACSdiva (BD Biosciences) and FlowJo software. Since weight of the AT samples was unavailable, data are expressed as

percentage of cells relative to total number of cells (based on forward and side scatter plot).

Statistical analyses - All data are presented as mean \pm S.E.M. Shapiro-Wilk tests were used to examine normality and variables with a skewed distribution were ln-transformed prior to analysis. Group differences were analysed using one-way ANOVA. Post-hoc testing was performed using Bonferroni adjustment for multiple testing when one-way ANOVA showed a significant group difference. Correlation analysis was applied by Pearson's correlation. Multiple linear regression analyses including age, total fat mass and the AT phenotype markers that were significantly correlated with HOMA-IR (indicated in Table 3) were used to investigate whether AT morphology, lipolysis and inflammation were independently related to whole-body IR (dependent variable, expressed as HOMA-IR). These analyses were performed for both AT depots separately. Statistical significance was set at $p < 0.05$ (two-tailed). Analyses were performed using SPSS 22.0 for Windows (SPSS).

Results

Subjects' characteristics

There were no differences in age between lean individuals and both obese groups. As expected, lean individuals' body composition and metabolic parameters, including insulin sensitivity (expressed as HOMA-IR) and glycaemic control (indicated by fasting plasma glucose and HbA1c levels) were significantly different compared to the obese groups (Table 1). BMI, body fat percentage and body fat distribution was not significantly different between both obese groups. Fasting plasma glucose levels, whole-body IR (HOMA-IR) and glycated hemoglobin (HbA1c) were significantly elevated in the obese T2DM individuals as compared to obese non-diabetic individuals (Table 1).

Table 1 Characteristics of obese, obese diabetic individuals and healthy lean controls

Variable	Lean individuals	Obese insulin resistant individuals	Obese diabetic individuals	P _{group}
<i>n</i>	19	22	11	
Age, years	52 ± 1 (43-61)	48 ± 1 (39-63)	52 ± 2 (42-65)	0.224
BMI, kg/m ²	23.7 ± 0.3 (20.8-25.6)	38.1 ± 0.9 (30.4-49.2) ^c	38.1 ± 1.2 (33.8-47.6) ^c	<0.001
Body fat, %	23.2 ± 0.9 (14.6-30.0)	36.3 ± 0.9 (30.0-46.9) ^c	36.5 ± 1.1(29.1-43.1) ^c	<0.001
Fat mass, kg	17.9 ± 0.8 (11.1-24.9)	45.1 ± 2.6 (29.3-72.3) ^c	42.9 ± 2.2 (33.0-58.0) ^c	<0.001
Waist circumference, cm	90.8 ± 1.1 (78.5-99.0)	128.0 ± 2.3 (111.0-154.0) ^c	126.1 ± 2.1 (120.5-143.0) ^c	<0.001
Hip circumference, cm	93.3 ± 0.8 (88.5-99.5)	116.8 ± 2.1 (105.0-141.0) ^c	116.0 ± 2.1 (106.5-127.0) ^c	<0.001
Waist-to-hip ratio	0.97 ± 0.0 (0.89-1.02)	1.09 ± 0.0 (1.03-1.18) ^c	1.08 ± 0.0 (1.02-1.16) ^c	<0.001
Fasting plasma glucose, mmol/l	5.4 ± 0.1 (2.4-6.6)	5.9 ± 0.2 (4.7-10.1)	7.8 ± 0.7 (5.1-12.0) ^{c,d}	<0.001
Serum insulin, mU/l	8.7 ± 0.9 (2.1-17.0)	20.9 ± 2.2 (6.0-45.0) ^c	21.3 ± 3.2 (10.0-40.0) ^c	<0.001
HbA1c, %	5.2 ± 0.0 (5.0-5.8)	5.6 ± 0.1 (5.1-7.1)	7.2 ± 0.3 (6.0-9.0) ^{c,e}	<0.001
HbA1c, mmol/mol	34 ± 0 (31-40)	38 ± 1 (32-54) ^a	55 ± 3 (42-72) ^{c,e}	<0.001
HOMA-IR	2.1 ± 0.2 (0.3-4.5)	5.6 ± 0.7 (1.3-14.7) ^c	7.9 ± 1.7 (2.84-18.0) ^c	<0.001
Subcutaneous adipocyte diameter, μm (a)	64.5 ± 2.3 (51.7-85.7)	78.6 ± 2.0 (61.0-91.7) ^c	77.0 ± 1.9 (70.9-86.1) ^b	<0.001
Visceral adipocyte diameter, μm (a)	62.2 ± 3.8 (44.0-85.7)	80.6 ± 1.9 (62.0-91.6) ^c	81.6 ± 1.8 (72.4-88.1) ^c	<0.001

Data are mean±S.E.M (range). (a) Variable measured in 15 lean, 20 obese insulin resistant and 9 obese diabetic individuals. Significantly different from lean group (^a*p* < 0.05; ^b*p* < 0.01; ^c*p* < 0.001), significantly different from obese insulin resistant group (^d*p* < 0.01; ^e*p* < 0.001)

SCAT and VAT morphology

Mean adipocyte size was clearly elevated in both obese groups as compared to the lean group in both SCAT ($p < 0.001$ for obese individuals and $p < 0.01$ for obese T2DM) and VAT ($p < 0.001$ for both obese and obese T2DM individuals) (Table 1). However, there was no significant difference in mean adipocyte size between the different AT depots within obese groups (Table 1). The difference in mean adipocyte size between lean and obese individuals was attributed by a shift toward a significantly lower proportion of large (70 – 89 μm) and very large (> 90 μm) adipocytes, and a higher proportion of small (<50 μm) and medium size (50 – 69 μm) adipocytes in the lean individuals, in both the SCAT (Fig. 1A) and VAT (Fig. 1B). Detailed lipolysis data have been reported previously ⁷. Briefly, we observed an increased basal lipolysis in the SCAT and VAT of obese and obese diabetic individuals and an attenuated maximal ANP- and ISO-induced lipolysis in the SCAT of obese diabetic men as compared to lean individuals (with intermediate values for obese non-diabetic individuals).

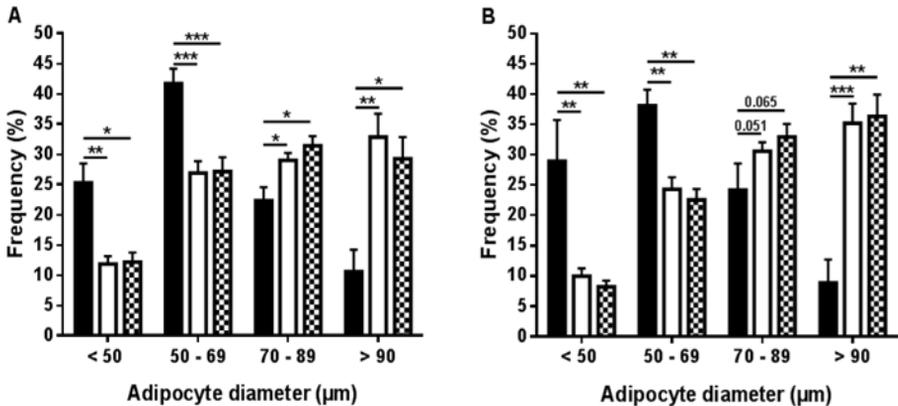


Figure 1 - Adipocyte size distribution. The frequency of small (<50 μm), medium (50-69 μm), large (70-89 μm) and very large (>90 μm) adipocytes was determined in SCAT (A) and VAT (B) of lean (black bars), obese insulin resistant (white bars) and obese diabetic (white squared bars) individuals. Obese groups clearly had higher proportions of large and very large adipocytes in combination with smaller proportions of small and medium size adipocytes, resulting in a higher mean adipocytes size compared to lean individuals both in SCAT ($p < 0.001$ for obese insulin resistant individuals and $p < 0.01$ for obese diabetic individuals) and VAT ($p < 0.001$ for both obese groups) Data are means \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Elevated inflammatory cell populations in the SVF of obese visceral AT

In SCAT, no significant differences were found in immune cell populations, expressed as percentage of total cells, between groups (Table 2). In contrast, the VAT of both obese groups showed a clear increase in proportion of leukocytes (CD45+) numbers, expressed as percentage of total cells, compared to lean controls ($p < 0.001$ for obese individuals and $p < 0.01$ for obese T2DM individuals). However, no significant differences were found between groups for the proportion of B-cells (CD19+), T-cell subsets (T-helper cells or cytotoxic T-cells) or NK cells (CD56+) (Table 2). For VAT macrophages, we observed an increased proportion of M1-macrophages (CD11+; expressed as percentage of total cells) in obese non-diabetic individuals ($p < 0.05$) compared to lean controls. A trend toward a higher proportion of CD11- M2 macrophages in obese VAT was observed ($p_{\text{group}}=0.066$) (Table 2). No group differences were observed when representing cell subsets as a fraction of total parent cells (either as % of CD45+ lymphocytes or as % of macrophages), thereby reflecting qualitative characteristics (Supplemental Table 1).

Table 2 Frequency of cell populations in subcutaneous and visceral SVF determined by flow cytometry

Variable	Lean individuals (n=18)	Obese insulin resistant individuals (n=18)	Obese diabetic individuals (n=8)	P _{group}
<i>Subcutaneous SVF</i>				
Total CD45+ leukocytes	41.3 ± 4.2 (4.3-70.4)	44.2 ± 4.0 (18.0-74.0)	38.7 ± 5.5 (23.5-74.0)	0.862
B cells	0.79 ± 0.16 (0.10-2.44)	1.26 ± 0.32 (0.14-5.20)	1.08 ± 0.36 (0.17-3.00)	0.300
T cells	21.9 ± 2.7 (1.8-48.9)	25.1 ± 1.8 (7.3-35.7)	24.9 ± 3.1 (16.3-42.3)	0.600
CD4+ T helper cells	12.0 ± 2.0 (0.8-33.3)	11.9 ± 1.0 (3.3-19.9)	14.0 ± 2.5 (7.1-28.1)	0.768
CD8+ cytotoxic T cells	6.5 ± 0.7 (0.6-10.8)	8.0 ± 0.7 (2.5-11.5)	7.7 ± 1.1 (3.8-13.1)	0.334
CD4+/CD8+ ratio	1.8 ± 0.2 (0.4-3.7)	1.5 ± 0.1 (0.8-2.9)	1.95 ± 0.2 (0.5-2.9)	0.764
NK cells	2.7 ± 0.5 (0.1-8.1)	3.7 ± 0.3 (1.8-6.1)	4.1 ± 1.0 (0.5-7.8)	0.211
M1 macrophages	6.6 ± 1.2 (0.2-16.1)	10.7 ± 1.2 (0.2-19.6)	5.6 ± 1.4 (0.3-12.0)	0.076
M2 macrophages	5.1 ± 1.0 (0.4-15.5)	5.9 ± 1.5 (0.6-22.8)	3.3 ± 0.9 (0.3-8.3)	0.706
M1/M2 ratio	2.1 ± 0.6 (0.4-11.5)	3.3 ± 0.6 (0.3-8.6)	2.4 ± 0.8 (0.5-8.2)	0.298
<i>Visceral SVF</i>				
Total CD45+ leukocytes	34.9 ± 3.5 (5.6-54.3)	57.3 ± 3.0 (33.0-93.8) ^c	53.5 ± 2.6 (41.6-65.7) ^b	<0.001
B cells	1.7 ± 0.5 (0.1-7.5)	3.9 ± 1.6 (0.0-30.4)	2.2 ± 0.9 (0.7-8.7)	0.377
T cells	28.7 ± 3.3 (3.3-59.3)	36.3 ± 3.2 (15.1-56.2)	33.9 ± 5.2 (12.7-51.4)	0.394
CD4+ T helper cells	15.3 ± 2.1 (1.7-31.8)	17.7 ± 1.7 (0.3-28.6)	17.2 ± 2.7 (6.6-28.9)	0.745
CD8+ cytotoxic T cells	10.1 ± 1.2 (0.9-18.3)	13.7 ± 1.8 (3.3-25.0)	13.5 ± 3.0 (0.9-26.4)	0.265
CD4+/CD8+ ratio	1.7 ± 0.2 (0.7-5.2)	1.7 ± 0.3 (0.0-5.8)	2.7 ± 1.3 (0.5-12.3)	0.695
NK cells	3.0 ± 0.4 (0.0-7.4)	3.5 ± 0.5 (0.2-8.9)	4.0 ± 1.0 (0.4-8.6)	0.725
M1 macrophages	2.9 ± 0.5 (0.1-9.0)	6.3 ± 0.9 (1.3-12.7) ^a	5.1 ± 0.9 (1.2-8.9)	0.010
M2 macrophages	3.5 ± 0.5 (0.1-8.5)	7.4 ± 1.4 (0.6-24.1)	7.2 ± 1.7 (0.9-17.0)	0.066
M1/M2 ratio	0.9 ± 0.1 (0.2-2.2)	1.4 ± 0.3 (0.1-5.3)	1.2 ± 0.4 (0.2-4.3)	0.725

Data are mean ± S.E.M (range). Cell frequencies are expressed as % of total cells; NK, natural killer; CD, cluster of differentiation; SVF, stromal vascular fraction. Significantly different from lean group (^ap < 0.05; ^bp < 0.01; ^cp < 0.001).

Table 3 Pearson correlation coefficients between AT lipolysis, AT size and AT immunophenotype of subcutaneous and visceral AT depots and whole-body IR

<i>Subcutaneous AT</i>	R	p value	<i>Visceral AT</i>	R	p value
Basal lipolysis, μmol glycerol (3h) per 10^6 cells	0.526	0.001	Basal lipolysis, μmol glycerol (3h) per 10^6 cells	0.126	0.444
Isoprenaline responsiveness, fold change to basal	-0.249	0.126	Isoprenaline responsiveness, fold change to basal	-0.364	0.023
ANP responsiveness, fold change to basal	-0.176	0.284	ANP responsiveness, fold change to basal	-0.187	0.261
Adipocyte diameter, μm	0.460	0.002	Adipocyte diameter, μm	0.603	< 0.001
Total CD45+ leukocytes, % of total cells	0.186	0.243	Total CD45+ leukocytes, % of total cells	0.515	< 0.001
CD3+ T cells, % of total cells	0.389	0.014	CD3+ T cells, % of total cells	0.143	0.366
CD3+CD4+ T-helper cells, % of live cells	0.218	0.182	CD3+CD4+ T-helper cells, % of total cells	0.008	0.960
CD3+CD8+ cytotoxic T cells, % of total cells	0.506	0.001	CD3+CD8+ cytotoxic T cells, % of total cells	0.254	0.110
CD4+/CD8+ T cell ratio	-0.067	0.685	CD4+/CD8+ T cell ratio	-0.083	0.605
CD56+ NK cells, % of total cells	0.452	0.004	CD56+ NK cells, % of live cells	0.157	0.320
CD11+ M1 macrophages, % of total cells	0.062	0.701	CD11+ M1 macrophages, % of total cells	0.261	0.095
CD11- M2 macrophages, % of total cells	-0.037	0.819	CD11- M2 macrophages, % of total cells	0.217	0.168
M1/M2 ratio	0.059	0.716	M1/M2 ratio	0.042	0.792
CD19+ B lymphocytes, % of total cells	0.174	0.289	CD19+ B lymphocytes, % of total cells	0.373	0.015

ANP, atrial natriuretic peptide; AT, adipose tissue; NK, natural killer; CD, cluster of differentiation

Determinants of whole-body insulin resistance

First, we investigated whether AT functional variables (adipocyte size, basal lipolysis and maximal lipolytic responsiveness to ISO or ANP) and AT immunophenotype were associated with whole-body IR, estimated using HOMA-IR (Table 3). In SCAT, basal lipolysis (expressed per cell number; $p=0.001$) and mean adipocyte size ($p=0.002$) were positively correlated with HOMA-IR. Furthermore, SCAT CD3+ T-cells ($p=0.014$), CD8+ cytotoxic T-cells ($p=0.001$) and CD56+ NK-cells ($p=0.004$) correlated positively with HOMA-IR (Table 3). In VAT, mean adipocyte size correlated positively ($p<0.001$), whereas maximal ISO responsiveness (fold change compared to basal) was negatively correlated ($p=0.023$) with HOMA-IR. Moreover, VAT CD45+ leukocytes ($p<0.001$) and CD19+ B-cells ($p=0.015$) were positively correlated with HOMA-IR, while VAT CD11+ M1-macrophages tended to show a positive association with HOMA-IR ($r=0.26$, $p=0.095$) (Table 3). Of interest, AT functional variables and AT immunophenotype showed some mutual correlations in the SCAT as well as in the VAT, respectively (Supplemental Table 2).

The variables significantly correlated with HOMA-IR (marked associations in Table 3) were subsequently analysed in a multiple linear regression analyses for both AT depots. In SCAT, only age ($\beta=0.341$, $p=0.037$) and total fat mass ($\beta=0.507$, $p=0.022$) were significantly related to HOMA-IR and together explained 32.5% of the variance in HOMA-IR. In VAT, adipocyte size ($\beta=0.619$, $p=0.001$), maximal ISO responsiveness ($\beta=-0.313$, $p=0.016$) and CD19+ B cells ($\beta=0.286$, $p=0.039$) accounted for 23% of the variance in HOMA-IR ($p_{\text{model}}<0.001$).

Discussion

To the best of our knowledge, this is the first study reporting detailed data on the relationship between depot-specific AT phenotype (i.e. morphology, lipolysis and inflammation) and whole-body IR (HOMA-IR) in lean, obese non-diabetic individuals and obese humans with type 2 diabetes. As reported previously ⁷, obese individuals have an attenuated basal lipolysis in both SCAT and VAT, and a decreased maximal ANP- and ISO-mediated lipolytic responsiveness in SCAT, compared to age-matched lean individuals. Here we demonstrate that this altered AT phenotype was further characterized by adipocyte hypertrophy and an increased immune cell infiltration, especially in the VAT. Of interest, in the VAT adipocyte size and CD19+ B cells were positively related to whole-body IR, while maximal ISO responsiveness was negatively related to whole-body IR. In contrast, none of the subcutaneous AT functional measures or immunophenotype were independently related to whole-body IR.

The present findings indicate that adipocyte hypertrophy in the obese SCAT and VAT is related to whole-body IR, which is in line with previous studies ²⁸⁻³⁰. In addition, VAT adipocyte hypertrophy was independently and positively associated ($\beta=0.619$, $p=0.001$) with whole-body IR, also after adjustment for age and total fat mass. Although adipocyte hypertrophy is the preferential expansion mechanism within the AT, in the chronic state of obesity SCAT adipogenesis is commonly impaired ³¹, possibly resulting in lipid spill-over to other tissues (the VAT as well) and thus the development of metabolic complications ³². The latter may explain our finding that adipocyte hypertrophy in VAT was an independent determinant of whole-body IR. Importantly, abdominal subcutaneous adipocyte hypertrophy may thus substantially contribute to expansion of VAT, which in turn results in worsening of whole-body IR.

The presence of a chronic pro-inflammatory AT microenvironment may limit adipocyte hypertrophy, by activating fibrotic signaling and impairing adipocyte functionality ³³, thereby contributing to metabolic alterations frequently observed in the obese state ^{34,35}. In addition to adipocyte hypertrophy, low-grade AT inflammation is essential for healthy AT expansion and remodeling, as

some cells are implicated in the clearance of cell debris released by necrotic adipocytes³⁶, act as a local buffer against increased lipid content³⁷, and promote angiogenesis³⁸. In this regard, our data show a significantly increased proportion of CD45+ leukocytes and CD11+ M1 macrophages in the VAT of obese and obese diabetic humans. In contrast, an inflammatory phenotype was less pronounced in the SCAT of obese individuals compared to lean or obese T2DM individuals, where we only observed a trend toward a higher proportion of CD11+ M1 (pro-inflammatory) macrophages in the obese SCAT (Table 2). Additionally, no immune-related markers in SCAT were significantly related to HOMA-IR, when adjusting for total fat mass and age. Indeed, the VAT is believed to have a more pronounced inflammatory phenotype than the SCAT³⁹, which may possibly be ascribed to an increased production of inflammatory mediators by adipocytes themselves and by cells in the SVF⁴⁰. Concerning the latter, one should be aware that SCAT is more important in quantitative terms, since SCAT accounts for approximately 80% of total AT mass. This may also explain why total fat mass rather than adipocyte morphology or the SCAT function was the strongest independent determinant of whole-body IR.

Therefore, in the VAT, the potential involvement of the elevated immune cells (i.e. CD45+ leukocytes and CD11+ M1 macrophages) in the development of AT inflammation and IR in obese humans is likely, indicated by a strong positive correlation between these parameters and HOMA-IR (Table 3). Notably, in the multiple linear regression analyses, VAT CD19+ B ($\beta=0.286$, $p=0.039$) cells were independently associated with HOMA-IR, also after adjustment for age and total fat mass. B lymphocytes are known to be elevated in obesity and IR, at least in diet-induced obese mice⁴¹. In epididymal AT, by orchestrating T cell populations and their activity (i.e. production of pro-inflammatory cytokines) and the release of pathogenic antibodies, B cells can decrease glucose tolerance⁴¹. Recently, the pro-inflammatory features of B cells in a high-fat diet setting were confirmed in obese mice⁴². These data, together with the present findings, suggest that B cells exert pro-inflammatory effects in obese AT and may contribute to IR. However, the precise mechanisms via which these cells impair insulin sensitivity require further investigation, especially in human obesity.

Inflammatory cells may have a modulatory function in chronically increased lipolysis. Indeed, beside the presence of inflammation additional mechanisms that may be (in)directly related to inflammation may be of importance in worsening of IR in obesity, as previously suggested ⁴³. Of interest, Kosteli et al. ³⁷ suggested that AT macrophages may buffer excess intracellular fatty acids upon increased lipolysis during fasting. The chronic elevation and uptake of lipids by macrophages may alter their phenotype ⁴⁴, promoting the progression to an inflammatory (CD11+) M1 polarization and AT inflammation ⁴⁵. However, in the present study no association between SCAT and VAT immunophenotype and measures of lipolysis were observed, suggesting that adipocyte lipolysis does not activate inflammatory pathways in human AT macrophages, as shown recently ⁴⁶. In line, only in the VAT a correlation between M1 and M2 macrophages and adipocyte size was found (Supplemental Table 2), respectively, suggesting that different immunological processes or alternative stimuli for AT macrophages (e.g. concentration of saturated fatty acids or ratio of monounsaturated to saturated fatty acids) could be present between abdominal SCAT and VAT depots in humans. Moreover, altered AT oxygen tension may have a putative role in AT dysfunction in obese insulin resistant conditions, as recently reviewed ⁴⁷. However, further studies are warranted to obtain more insight into the triggers of AT dysfunction within different AT depots.

Future studies should include human mechanistic experiments to strengthen these findings by focusing on intra-AT relationships and to address causality. In addition, it needs to be mentioned that a significant proportion of the obese patients in the present study received several forms of glucose lowering (72% of the obese diabetic men) or lipid lowering (22% of the obese insulin resistant men; 36% of the obese diabetic men) medication, which may impact the AT phenotype and have cofounded the present results ^{48,49}. Furthermore, the use of HOMA-IR as a surrogate markers to assess whole-body IR, may be a limitation of this study. It would be of interest to relate AT functional characteristics to tissue-specific (e.g. muscle, AT or liver) IR, and future studies should therefore use a two-step hyperinsulinemic-euglycemic clamp combined with a glucose tracer.

Conclusion

In conclusion, this study is the first to characterize the SCAT and VAT phenotype in detail in lean, obese and obese type 2 diabetic subjects. In VAT, adipocyte hypertrophy independently contributes to IR, possibly via pro-inflammatory M1 macrophage- and/or B cell-mediated inflammation. In SCAT, AT function or immunophenotype were only of minor importance with respect to whole-body IR. These alterations in AT mass and phenotypes likely impact cardiometabolic risk and may thus contribute to the development of type 2 diabetes.

Acknowledgements

We thank all abdominal surgeons from the Jessa Hospital (Hasselt, Belgium) and Hospital East-Limburg (Genk, Belgium) for their contributions to subject recruitment. We thank University Biobank Limburg (UBiLim, Jessa Hospital, Belgium) for their technical support in sample handling and storage. Yvonne Essers, Nicole Hoebbers, Maria Vroomen and José van de Gaar are greatly acknowledged for their technical support.

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Supplemental data

Supplemental Table 1 Frequency of cell populations in subcutaneous and visceral SVF, expressed as percentage relative to CD45⁺ leukocytes or to total macrophages

Variable	Lean individuals (n=18)	Obese insulin resistant individuals (n=18)	Obese diabetic individuals (n=8)	P _{group}
Subcutaneous SVF				
B cells (% of CD45 ⁺)	1.8 ± 0.2 (0.5-4.5)	2.0 ± 0.4 (0.4-7.2)	1.8 ± 0.5 (0.4-4.2)	0.901
T cells (% of CD45 ⁺)	50.1 ± 2.3 (32.4-69.4)	41.4 ± 3.3 (14.9-67.5)	46.2 ± 2.8 (33.3-55.5)	0.094
CD4+ T helper cells (% of CD45 ⁺)	25.9 ± 2.1 (10.1-42.6)	19.6 ± 1.7 (1.8-29.1)	25.5 ± 3.2 (14.1-39.2)	0.073
CD8+ cytotoxic T cells (% of CD45 ⁺)	16.4 ± 1.6 (8.1-39.8)	13.4 ± 1.5 (1.8-28.0)	14.4 ± 1.8 (8.7-26.0)	0.398
NK cells (% of CD45 ⁺)	6.3 ± 0.9 (0.7-17.8)	7.1 ± 0.9 (2.4-20.0)	7.1 ± 1.4 (1.1-11.6)	0.814
M1 macrophages (% of macrophages)	54.4 ± 5.1 (12.8-93.8)	63.3 ± 4.9 (5.2-83.9)	55.0 ± 8.3 (20.5-85.5)	0.431
M2 macrophages (% of macrophages)	45.5 ± 5.1 (6.1-87.1)	36.6 ± 4.9 (16.0-94.7)	44.9 ± 8.3 (14.4-79.4)	0.431
Visceral SVF				
B cells (% of CD45 ⁺)	3.6 ± 1.3 (0.3-23.0)	5.0 ± 1.8 (0.0-34.2)	3.6 ± 1.3 (1.0-13.0)	0.777
T cells (% of CD45 ⁺)	58.7 ± 2.7 (43.4-77.7)	52.5 ± 4.2 (17.0-83.0)	53.4 ± 6.8 (21.3-69.8)	0.453
CD4+ T helper cells (% of CD45 ⁺)	30.9 ± 2.0 (16.7-46.1)	25.8 ± 2.6 (0.6-46.4)	26.6 ± 3.3 (14.7-41.6)	0.288
CD8+ cytotoxic T cells (% of CD45 ⁺)	20.9 ± 1.6 (8.7-33.1)	19.1 ± 2.4 (0.0-34.7)	21.4 ± 4.3 (1.4-39.9)	0.787
NK cells (% of CD45 ⁺)	6.6 ± 1.0 (0.0-16.1)	5.2 ± 0.9 (0.3-15.5)	7.3 ± 2.2 (0.6-19.0)	0.503
M1 macrophages (% of macrophages)	45.3 ± 3.8 (19.4-79.4)	51.6 ± 5.0 (11.1-84.1)	46.4 ± 7.1 (23.5-81.2)	0.596
M2 macrophages (% of macrophages)	54.6 ± 3.8 (20.5-80.5)	48.3 ± 5.0 (15.8-88.8)	53.5 ± 7.1 (18.7-76.4)	0.596

Data are mean ± S.E.M (range). Cell frequencies are expressed as % of CD45⁺ leukocytes or % of total macrophages (as indicated); NK, natural killer; CD, cluster of differentiation; SVF, stromal vascular fraction.

Supplemental table 2 Pearson correlation coefficients between adipocyte size and lipolysis with immuno-phenotype in subcutaneous and visceral adipose tissue

<i>Subcutaneous adipocyte size (μm)</i>	R	p value	<i>Visceral adipocyte size (μm)</i>	R	p value
Total CD45+ leukocytes, % of total cells	0.172	0.308	Total CD45+ leukocytes, % of total cells	0.669	< 0.001
CD3+ T cells, % of total cells	0.269	0.118	CD3+ T cells, % of total cells	0.222	0.157
CD3+CD4+ T-helper cells, % of total cells	0.143	0.412	CD3+CD4+ T-helper cells, % of total cells	0.203	0.196
CD3+CD8+ cytotoxic T cells, % of total cells	0.381	0.024	CD3+CD8+ cytotoxic T cells, % of total cells	0.168	0.295
CD4+/CD8+ T cell ratio	-0.191	0.271	CD4+/CD8+ T cell ratio	0.140	0.384
CD56+ NK cells, % of total cells	0.161	0.355	CD56+ NK cells, % of total cells	0.072	0.649
CD11+ M1 macrophages, % of total cells	0.239	0.154	CD11+ M1 macrophages, % of total cells	0.534	< 0.001
CD11- M2 macrophages, % of total cells	0.177	0.295	CD11- M2 macrophages, % of total cells	0.425	0.005
M1/M2 ratio	0.013	0.940	M1/M2 ratio	0.098	0.536
CD19+ B lymphocytes, % of total cells	0.111	0.526	CD19+ B lymphocytes, % of total cells	0.357	0.020
<i>Subcutaneous basal lipolysis (per cell)</i>	R	p value	<i>Visceral basal lipolysis (per cell)</i>	R	p value
Total CD45+ leukocytes, % of total cells	-0.163	0.348	Total CD45+ leukocytes, % of total cells	0.338	0.044
CD3+ T cells, % of total cells	0.077	0.665	CD3+ T cells, % of total cells	0.091	0.597
CD3+CD4+ T-helper cells, % of total cells	0.043	0.809	CD3+CD4+ T-helper cells, % of total cells	0.181	0.292
CD3+CD8+ cytotoxic T cells, % of total cells	-0.072	0.687	CD3+CD8+ cytotoxic T cells, % of total cells	0.071	0.684
CD4+/CD8+ T cell ratio	0.245	0.163	CD4+/CD8+ T cell ratio	0.182	0.296
CD56+ NK cells, % of total cells	0.223	0.204	CD56+ NK cells, % of total cells	0.193	0.260
CD11+ M1 macrophages, % of total cells	-0.024	0.893	CD11+ M1 macrophages, % of total cells	0.261	0.125
CD11- M2 macrophages, % of total cells	-0.044	0.800	CD11- M2 macrophages, % of total cells	0.152	0.168
M1/M2 ratio	-0.014	0.935	M1/M2 ratio	-0.032	0.853
CD19+ B lymphocytes, % of total cells	0.196	0.267	CD19+ B lymphocytes, % of total cells	-0.179	0.296

Pearson correlations coefficients. NK, natural killer; CD, cluster of differentiation

Chapter 5

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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To be submitted

Abstract

Aims/hypothesis: 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 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. Obese individuals underwent 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 low-intense (40% VO_{2max}) 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 SCAT lipolysis (expressed as total area under the curve) was more pronounced in obese IS (81%) and IR (34%) as compared to lean individuals ($P_{group}=0.012$). 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 FFA, 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, the exercise training program improved metabolic profile and body composition in obese individuals, but did not affect abdominal SCAT lipolysis.

ClinicalTrial.gov accession number NCT02418728

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 μ l/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) ratio³⁸. 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 μ mol/l phentolamine ($\alpha_{1,2}$ -adrenergic receptor antagonist) (Regitin 10 mg/ml; Novartis Pharma BV, The Netherlands) and 100 μ mol/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 μ L/min after which the perfusion rate was increased to 2.0 μ L/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% $\text{VO}_{2\text{peak}}$ (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 analysed for glycerol, glucose and lactate concentrations by means of bioluminescence on an ISCUS clinical microdialysis analyser (M dialysis AB, Stockholm, Sweden). Ethanol concentrations in dialysate (out) and perfusate (in) were measured spectrophotometrically using a COBAS FARA semi-automatic analyser (Roche Diagnostics, Basel, 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™ Glycerol, Roche Biopharm, Switzerland), automated on a Cobas Fara spectrophotometric autoanalyser (Roche Diagnostics, Basel, Switzerland). Plasma FFA, glucose and lactate concentrations were measured with enzymatic assays on an automated spectrophotometer (ABX Pentra 400 autoanalyser, 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 \pm 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 analysed 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 analysed by means of paired *t*-test. Intervention effects in the obese groups were analysed 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 and healthy lean controls			
	Lean (<i>n</i> =10)	Obese insulin sensitive (<i>n</i> =10)	Obese insulin resistant (<i>n</i> =10)	<i>p</i> 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/m ²)	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				
VO_{2peak} (ml*min ⁻¹ *kg ⁻¹ (FFM))	62 ± 3	48 ± 2 **	48 ± 1 **	0.002
W_{peak} (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; W_{max} : 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 Supplemental Table S1.

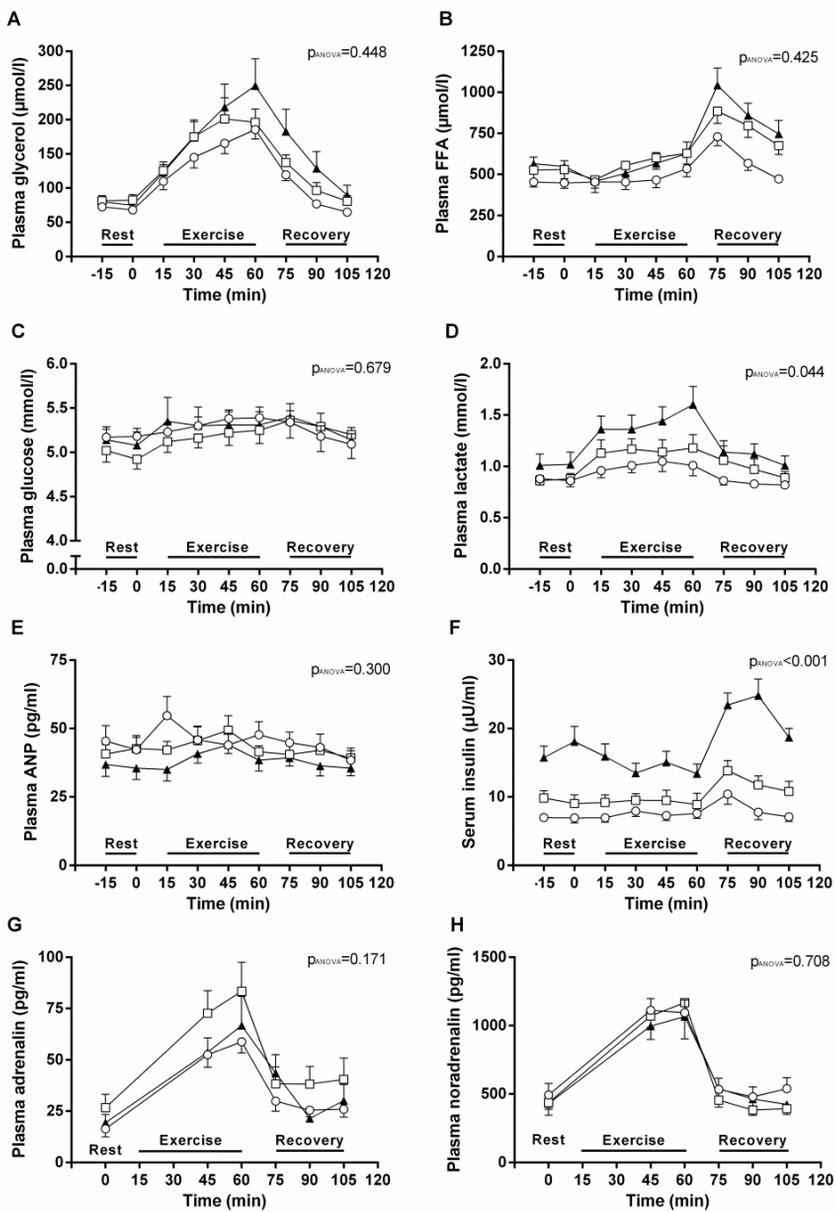


Figure 1 - Plasma glycerol, FFA, glucose, lactate, ANP, adrenalin, noradrenalin and serum insulin concentrations at rest, during exercise and recovery. Systemic glycerol (A), FFA (B), glucose (C), lactate (D), ANP (E), insulin (F), adrenalin (G) and noradrenalin (H) responses in lean (white circles), obese insulin sensitive (white squares) and obese insulin resistant (black triangles) individuals. Data are presented as mean \pm 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 (Supplemental 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 Supplemental Table S2.

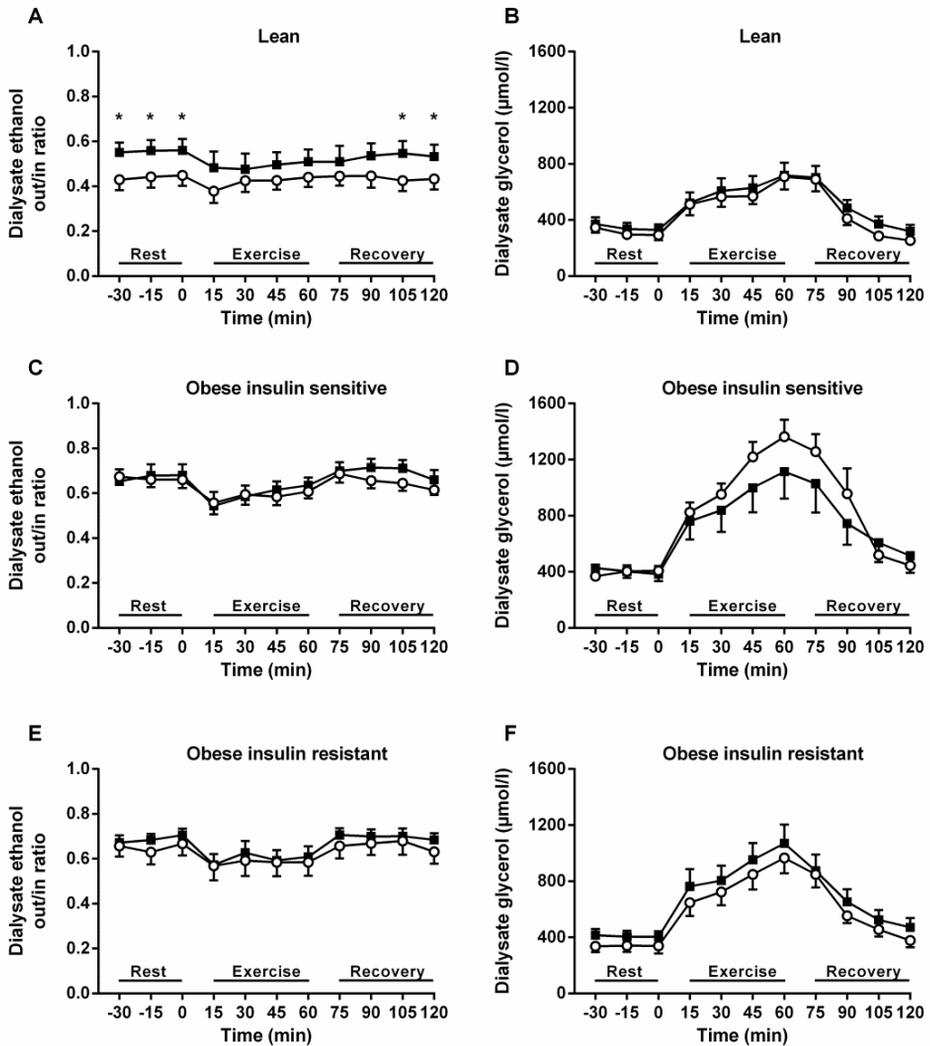


Figure 2 - Changes in subcutaneous adipose tissue extracellular glycerol concentration and adipose tissue blood flow indices. Subcutaneous adipose tissue ethanol ratio's in lean (A), obese insulin sensitive (B) and obese insulin resistant (C) individuals. Changes in extracellular glycerol concentration 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 \pm 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 Supplemental Table S2. Abdominal SCAT extracellular glucose and lactate concentrations and responses were comparable between groups at rest, during exercise and recovery (Supplemental Table S3).

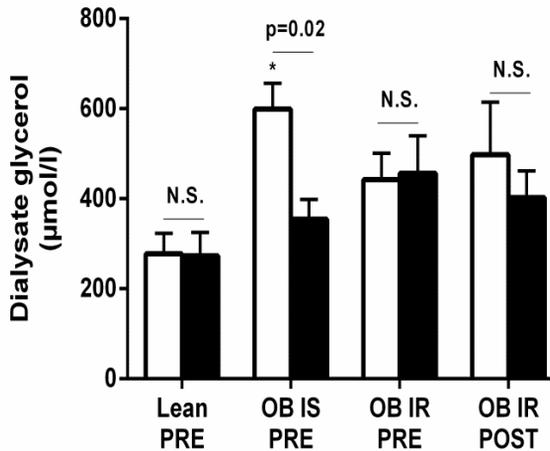


Figure 3 - Exercise-induced increase in subcutaneous adipose tissue extracellular glycerol concentration. Mean changes in subcutaneous adipose tissue extracellular glycerol concentration during 1 h of low-intense exercise (40% VO_{2peak}). Extracellular glycerol concentrations are determined in control 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 \pm SEM for pre-intervention (PRE) and post-intervention (POST) measurement. * 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).

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

Body composition	Obese insulin sensitive (<i>n</i> =8)		Obese insulin resistant (<i>n</i> =9)		P Time	P Group	P Time*Group
	Pre	Post	Pre	Post			
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/m ²)	32.7 ± 0.5	32.2 ± 0.3	34.1 ± 0.8	33.3 ± 0.8	0.002	0.225	0.550
Waist circumference (cm)	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							
VO _{2peak} (ml*min ⁻¹ *kg ⁻¹ (FFM))	49 ± 2	53 ± 1	48 ± 1	55 ± 2	0.012	0.800	0.521
W _{peak} (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; VO_{2 peak}: maximum oxygen uptake; Wmax: maximum power output

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 Supplemental Table S4.

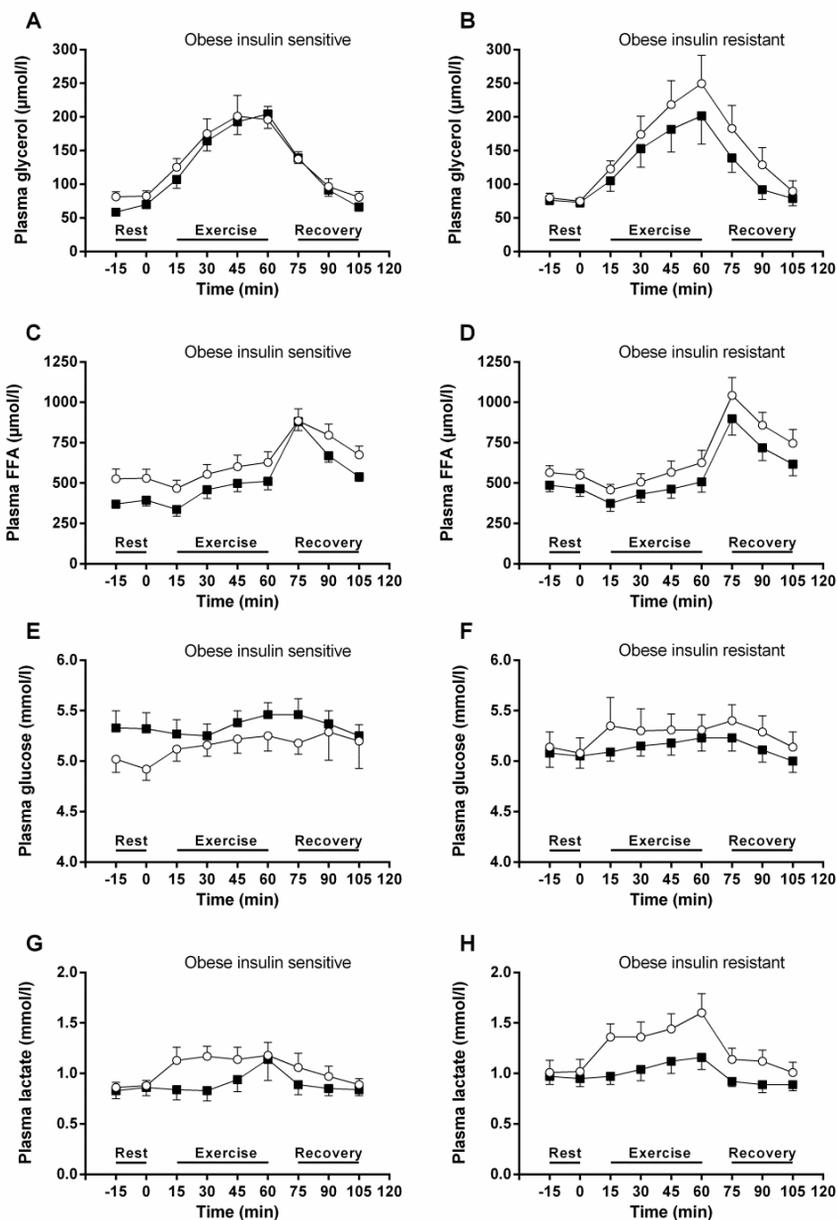


Figure 4 - Exercise training-induced changes in systemic plasma glycerol, FFA, glucose, lactate, ANP, adrenalin, noradrenalin and serum insulin in obese individuals. Systemic glycerol (A-B), FFA (C-D), glucose (E-F), lactate (G-H), insulin (I-J), ANP (K-L), adrenalin (M-N) and noradrenalin (O-P) of obese 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 \pm SEM.

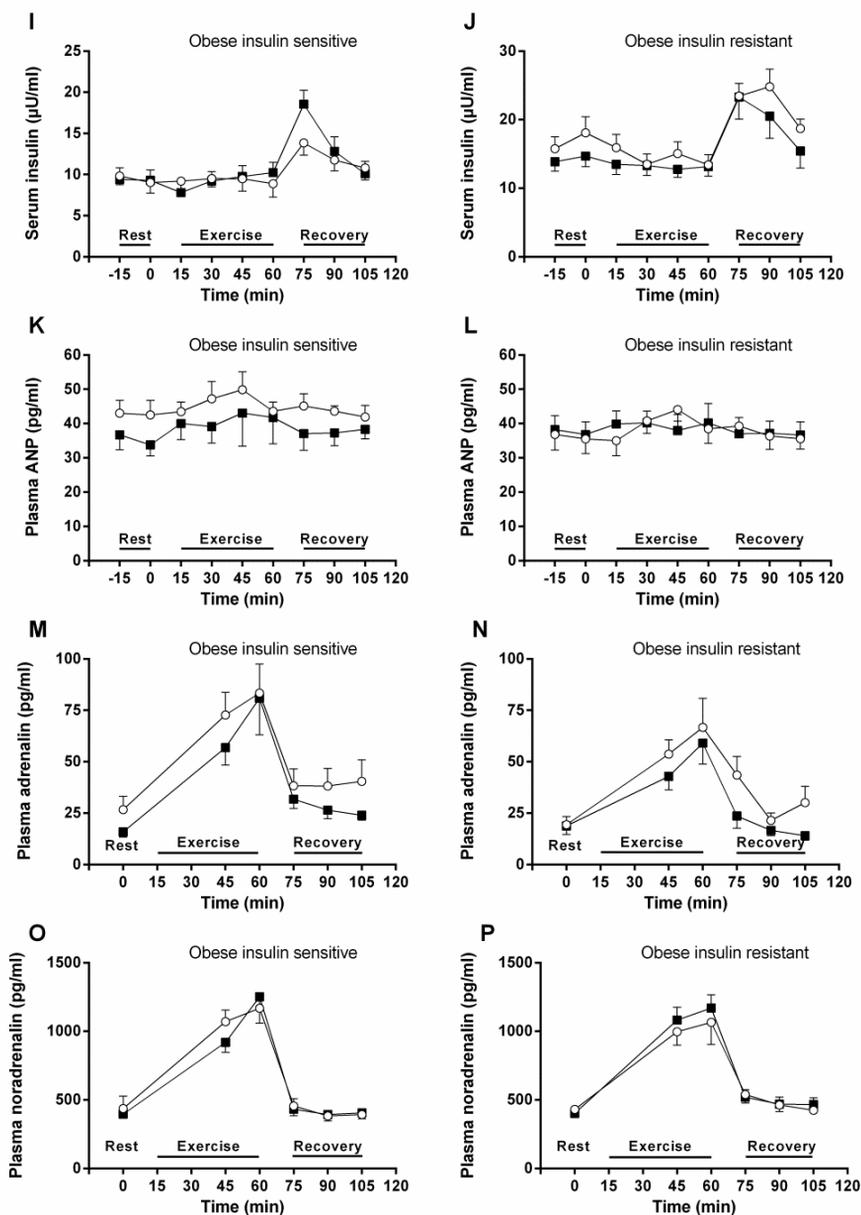


Figure 4 - continued. Exercise training-induced changes in systemic plasma glycerol, FFA, glucose, lactate, ANP, adrenalin, noradrenalin and serum insulin in obese individuals. Systemic glycerol (A-B), FFA (C-D), glucose (E-F), lactate (G-H), insulin (I-J), ANP (K-L), adrenalin (M-N) and noradrenalin (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 \pm 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).

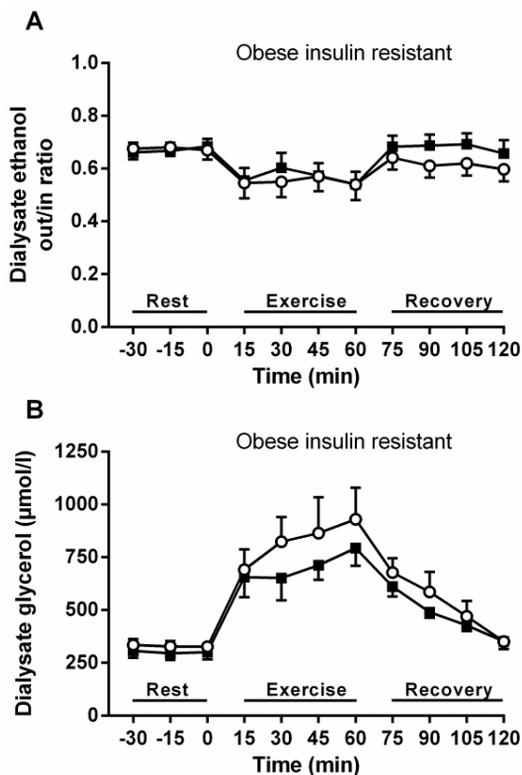


Figure 5 - Exercise training-induced changes in subcutaneous adipose tissue extracellular glycerol concentration and adipose tissue blood flow indices in obese insulin resistant individuals. Subcutaneous adipose tissue ethanol ratio (A) and extracellular glycerol concentration (B) in the obese insulin 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 \pm SEM.

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 ($\sim 40\%$) 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 obesity⁹, 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 α_2 -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*.

Conclusion

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.

Acknowledgments

The authors would like to thank all participants in this study and J. Stegen, W. Sluijsmans, H. Aydeniz, and Y. Essers for their excellent analytical and technical support (all from the Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center⁺, the Netherlands) and B. Froyen for his assistance in the experimental testing and training supervision as well as the department of ReGO (Jessa Hospital) for their accommodation.

Funding

Part of this work was financed by the Research Foundation - Flanders (FWO) (KAN 1507217N).

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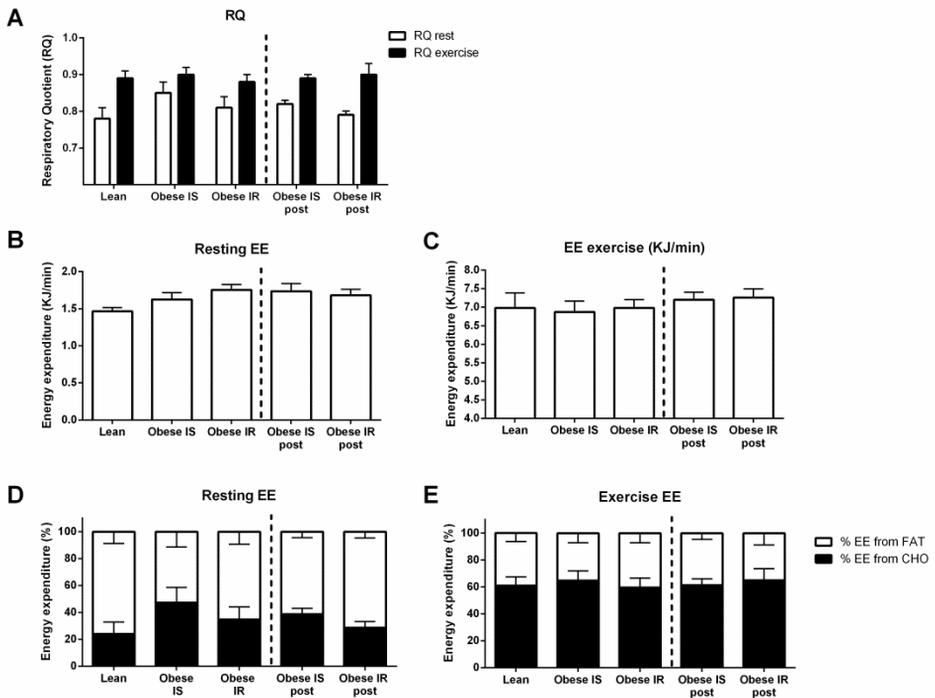
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Supplemental data



Supplemental Figure 1 - 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 \pm SEM.

Supplemental Table S1.

Average concentrations of circulating glycerol, insulin, FFA, glucose, lactate, ANP, adrenalin and noradrenalin at rest, during 1h of exercise and 1 h of 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)	ANOVA P-value
GLYCEROL							
Rest ($\mu\text{mol/l}$)	70 \pm 4	81 \pm 7	0.580	77 \pm 5	1.000	1.000	0.416
Exercise (AUC_{0-60}) ($\mu\text{mol/l} \cdot 60\text{min}$)	136 \pm 12	163 \pm 19	0.978	168 \pm 22	0.722	1.000	0.448
Recovery (AUC_{75-105}) ($\mu\text{mol/l} \cdot 45\text{min}$)	107 \pm 6	124 \pm 10	1.000	157 \pm 28	0.183	0.660	0.159
INSULIN							
Rest ($\mu\text{U/ml}$)	7.0 \pm 0.6	9.4 \pm 1.1	0.563	16.9 \pm 1.8	<0.001	0.001	<0.001
Exercise (AUC_{0-60}) ($\mu\text{U/ml} \cdot 60\text{min}$)	7.4 \pm 0.64	9.3 \pm 1.2	0.791	15.0 \pm 1.5	<0.001	0.005	<0.001
Recovery (AUC_{75-105}) ($\mu\text{U/ml} \cdot 45\text{min}$)	8.5 \pm 1.0	11.7 \pm 1.2	0.209	21.4 \pm 1.3	<0.001	<0.001	<0.001
FFA							
Rest ($\mu\text{mol/l}$)	451 \pm 31	528 \pm 58	0.658	557 \pm 36	0.292	1.000	0.224
Exercise (AUC_{0-60}) ($\mu\text{mol/l} \cdot 60\text{min}$)	466 \pm 42	555 \pm 56	0.628	531 \pm 47	1.000	1.000	0.425
Recovery (AUC_{75-105}) ($\mu\text{mol/l} \cdot 45\text{min}$)	601 \pm 37	777 \pm 64	0.189	864 \pm 82	0.019	1.000	0.020
GLUCOSE							
Rest (mmol/l)	5.2 \pm 0.1	5.0 \pm 0.1	0.722	5.1 \pm 0.1	1.000	1.000	0.488
Exercise (AUC_{0-60}) (mmol/l \cdot 60min)	5.3 \pm 0.1	5.1 \pm 0.1	1.000	5.3 \pm 0.2	1.000	1.000	0.679
Recovery (AUC_{75-105}) (mmol/l \cdot 45min)	5.3 \pm 0.1	5.3 \pm 0.2	1.000	5.3 \pm 0.1	1.000	1.000	0.975
LACTATE							
Rest (mmol/l)	0.9 \pm 0.1	0.9 \pm 0.05	1.000	1.0 \pm 0.1	0.628	0.600	0.339
Exercise (AUC_{0-60}) (mmol/l \cdot 60min)	1.0 \pm 0.7	1.1 \pm 0.1	0.100	1.4 \pm 0.1	0.044	0.313	0.044
Recovery (AUC_{75-105}) (mmol/l \cdot 45min)	0.9 \pm 0.1	1.0 \pm 0.1	0.730	1.2 \pm 0.1	0.057	0.679	0.061
ANP							
Rest (pg/ml)	43.8 \pm 5.2	41.7 \pm 3.2	1.000	36.2 \pm 4.0	0.548	0.793	0.356
Exercise (AUC_{0-60}) (pg/ml \cdot 60min)	47.2 \pm 4.8	45.9 \pm 3.5	1.000	39.2 \pm 2.2	0.452	0.680	0.300
Recovery (AUC_{75-105}) (pg/ml \cdot 45min)	42.7 \pm 4.4	44.0 \pm 2.3	1.000	37.5 \pm 3.2	0.864	0.616	0.390
ADRENALIN							
Rest (pg/ml)	16.4 \pm 3.8	26.7 \pm 6.5	0.561	19.4 \pm 4.0	1.000	1.000	0.376
Exercise (AUC_{0-60}) (pg/ml \cdot 60min)	54.0 \pm 5.7	76.2 \pm 11.2	0.233	58.0 \pm 8.1	1.000	0.429	0.171
Recovery (AUC_{75-105}) (pg/ml \cdot 45min)	35.2 \pm 3.2	48.2 \pm 9.0	0.537	39.4 \pm 5.9	1.000	1.000	0.380
NORADRENALIN							
Rest (pg/ml)	493 \pm 85	437 \pm 91	1.000	430 \pm 42	1.000	1.000	0.816
Exercise (AUC_{0-60}) (pg/ml \cdot 60min)	1107 \pm 81	1095 \pm 83	1.000	1007 \pm 110	1.000	1.000	0.708
Recovery (AUC_{75-105}) (pg/ml \cdot 45min)	610 \pm 71	540 \pm 48	1.000	580 \pm 56	1.000	1.000	0.725

Data are mean \pm 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.

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							
Resting ($\mu\text{mol/l}$)					0.279	0.266	0.674
	Control	311 \pm 34	403 \pm 41	339 \pm 46			
	α/β blocker	346 \pm 43	405 \pm 49	408 \pm 41			
Exercise (AUC_{0-60} , $\mu\text{mol/l} \cdot 60\text{min}$)					0.012	0.883	0.335
	Control	537 \pm 71	974 \pm 75	722 \pm 79			
	α/β blocker	570 \pm 110	851 \pm 116	840 \pm 123			
Recovery (AUC_{75-120} , $\mu\text{mol/l} \cdot 45\text{min}$)					0.014	0.894	0.348
	Control	467 \pm 69	909 \pm 77	624 \pm 82			
	α/β blocker	519 \pm 99	804 \pm 111	699 \pm 118			
ETHANOL RATIO							
Rest					0.003	0.037	0.190
	Control	0.44 \pm 0.05	0.66 \pm 0.03	0.64 \pm 0.05			
	α/β blocker	0.56 \pm 0.05	0.67 \pm 0.05	0.69 \pm 0.03			
Exercise					0.042	0.365	0.501
	Control	0.42 \pm 0.05	0.60 \pm 0.05	0.59 \pm 0.05			
	α/β blocker	0.50 \pm 0.05	0.59 \pm 0.05	0.60 \pm 0.05			
Recovery					0.003	0.025	0.620
	Control	0.44 \pm 0.04	0.65 \pm 0.05	0.66 \pm 0.05			
	α/β blocker	0.53 \pm 0.04	0.69 \pm 0.05	0.71 \pm 0.05			

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

Supplemental Table S3. Interstitial glucose and lactate concentrations 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)	P Group	ANOVA	
						P Treatment	P Treatment*Group
GLUCOSE							
Rest (mmol/l)					0.081	0.622	0.877
	Control	3.62 ± 0.31	2.88 ± 0.39	2.56 ± 0.48			
	α/β blocker	3.72 ± 0.45	2.86 ± 0.31	2.82 ± 0.23			
Exercise (AUC ₀₋₆₀)					0.811	0.194	0.655
	Control	4.30 ± 0.36	4.36 ± 0.73	4.60 ± 1.06			
	α/β blocker	4.24 ± 0.55	3.43 ± 0.38	3.93 ± 0.47			
Recovery (AUC ₇₅₋₁₂₀)					0.925	0.624	0.482
	Control	3.38 ± 0.30	3.45 ± 0.50	3.65 ± 0.92			
	α/β blocker	3.68 ± 0.41	3.32 ± 0.37	3.02 ± 0.16			
LACTATE							
Rest (mmol/l)					0.121	0.091	0.709
	Control	1.79 ± 0.35	0.98 ± 0.16	1.80 ± 0.60			
	α/β blocker	1.37 ± 0.37	0.78 ± 0.13	1.08 ± 0.14			
Exercise (AUC ₀₋₆₀)					0.354	0.078	0.449
	Control	2.63 ± 0.58	1.79 ± 0.41	3.16 ± 0.95			
	α/β blocker	2.36 ± 0.62	1.46 ± 0.34	1.94 ± 0.32			
Recovery (AUC ₇₅₋₁₂₀)					0.349	0.234	0.558
	Control	2.70 ± 0.64	1.62 ± 0.45	2.42 ± 0.90			
	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 + Propranolol).

P value were calculated via a 2-way repeated ANOVA.

Supplemental Table S4.

Average concentrations of circulating glycerol, insulin, FFA, glucose, lactate, ANP, adrenalin and noradrenalin at rest, during 1h of exercise and 1h of recovery, before and after 12 weeks of exercise training in obese insulin sensitive and obese insulin resistant individuals

	Obese insulin sensitive		Obese insulin resistant		ANOVA		
	Pre	Post	Pre	Post	P Time	P Group	P Time*Group
GLYCEROL							
Rest ($\mu\text{mol/l}$)	74.0 \pm 5.0	64.2 \pm 4.8	79.9 \pm 5.5	74.8 \pm 6.4	0.100	0.235	0.593
Exercise (AUC ₀₋₆₀) ($\mu\text{mol/l*60min}$)	157.3 \pm 22.7	150.3 \pm 12.7	175.7 \pm 24.1	144.0 \pm 23.6	0.026	0.730	0.500
Recovery (AUC ₇₅₋₁₀₅) ($\mu\text{mol/l*45min}$)	118.9 \pm 12.7	124.4 \pm 6.6	164.3 \pm 30.7	122.1 \pm 19.3	0.316	0.405	0.198
INSULIN							
Rest ($\mu\text{U/ml}$)	9.5 \pm 1.4	9.4 \pm 1.3	16.9 \pm 2.0	14.3 \pm 1.3	0.196	0.008	0.263
Exercise (AUC ₀₋₆₀) ($\mu\text{U/ml*60min}$)	9.2 \pm 1.5	9.1 \pm 1.2	14.8 \pm 1.7	13.4 \pm 1.3	0.313	0.021	0.393
Recovery (AUC ₇₅₋₁₀₅) ($\mu\text{U/ml*45min}$)	11.6 \pm 1.6	13.8 \pm 1.5	21.6 \pm 1.5	19.4 \pm 2.5	0.981	0.003	0.206
FFA							
Rest ($\mu\text{mol/l}$)	506 \pm 48	382 \pm 30	571 \pm 37	476 \pm 41	0.016	0.060	0.723
Exercise (AUC ₀₋₆₀) ($\mu\text{mol/l*60min}$)	533 \pm 55	436 \pm 42	540 \pm 51	439 \pm 47	0.040	0.930	0.954
Recovery (AUC ₇₅₋₁₀₅) ($\mu\text{mol/l*45min}$)	759 \pm 70	698 \pm 38	892 \pm 86	726 \pm 76	0.034	0.401	0.296
GLUCOSE							
Rest (mmol/l)	5.0 \pm 0.1	5.3 \pm 0.2	5.1 \pm 0.1	5.1 \pm 0.1	0.030	0.671	0.021
Exercise (AUC ₀₋₆₀) (mmol/l*60min)	5.2 \pm 0.1	5.3 \pm 0.1	5.3 \pm 0.2	5.1 \pm 0.1	0.973	0.790	0.202
Recovery (AUC ₇₅₋₁₀₅) (mmol/l*45min)	5.4 \pm 0.3	5.4 \pm 0.1	5.3 \pm 0.2	5.1 \pm 0.1	0.564	0.437	0.564
LACTATE							
Rest (mmol/l)	0.9 \pm 0.0	0.8 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.1	0.335	0.322	0.824
Exercise (AUC ₀₋₆₀) (mmol/l*60min)	1.1 \pm 0.1	0.9 \pm 0.1	1.4 \pm 0.1	1.0 \pm 0.1	0.016	0.127	0.609
Recovery (AUC ₇₅₋₁₀₅) (mmol/l*45min)	1.0 \pm 0.1	0.9 \pm 0.1	1.2 \pm 0.1	1.0 \pm 0.1	0.078	0.392	0.606
ANP							
Rest (pg/ml)	39.7 \pm 3.5	33.8 \pm 3.6	38.8 \pm 3.5	37.5 \pm 3.5	0.042	0.769	0.162
Exercise (AUC ₀₋₆₀) (pg/ml*60min)	44.6 \pm 4.7	38.1 \pm 5.2	41.1 \pm 2.5	39.1 \pm 3.5	0.043	0.819	0.256
Recovery (AUC ₇₅₋₁₀₅) (pg/ml*45min)	39.9 \pm 2.8	36.2 \pm 5.0	39.2 \pm 3.0	37.6 \pm 3.8	0.105	0.946	0.530
ADRENALIN							
Rest (pg/ml)	29.4 \pm 6.8	15.8 \pm 2.5	21.9 \pm 5.6	19.7 \pm 5.4	0.155	0.773	0.295
Exercise (AUC ₀₋₆₀) (pg/ml*60min)	85.6 \pm 7.1	62.9 \pm 10.4	58.0 \pm 9.2	48.1 \pm 7.2	0.029	0.057	0.354
Recovery (AUC ₇₅₋₁₀₅) (pg/ml*45min)	55.0 \pm 6.9	36.6 \pm 5.0	36.2 \pm 6.7	24.4 \pm 3.6	0.007	0.045	0.483
NORADRENALIN							
Rest (pg/ml)	452 \pm 118	395 \pm 28	442 \pm 45	400 \pm 41	0.468	0.973	0.912
Exercise (AUC ₀₋₆₀) (pg/ml*60min)	1078 \pm 92	1002 \pm 93	1043 \pm 118	1045 \pm 106	0.875	0.822	0.534
Recovery (AUC ₇₅₋₁₀₅) (pg/ml*45min)	519 \pm 50	552 \pm 68	584 \pm 63	608 \pm 56	0.613	0.377	0.930

Data are mean \pm 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 6

Adipose tissue lipolytic inhibition enhances the glucoregulatory properties of exercise in type 2 diabetes patients

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Submitted

Abstract

Aim - Exercise combined with adipose tissue lipolytic inhibition augments intramuscular lipid and glycogen use in type 2 diabetes mellitus (T2DM) patients. The present study investigates the impact of adipose tissue lipolytic inhibition during exercise on subsequent postprandial glycemic control in T2DM patients.

Methods - Fourteen male T2DM patients (age: 65 ± 2 years, HbA_{1c} $6.7 \pm 0.1\%$) participated in a double-blind placebo-controlled randomized cross-over study in which subjects performed 60 min of endurance-type exercise (at $45\% W_{peak}$) after being administered 250 mg of an nicotinic acid analog (acipimox; ACP) or a placebo (PLA). A control experiment was included in which no exercise was performed (CON). Plasma samples were obtained before, during and for up to 7.5 h after exercise.

Results - Sixty min of exercise at 73 ± 6 W did not significantly lower circulating plasma glucose and insulin excursions in PLA when compared with CON ($P=0.300$ and 0.778 for glucose and insulin, respectively). Acipimox administration strongly reduced circulating plasma FFA concentrations during exercise (175 ± 13 vs 535 ± 47 and 637 ± 58 mmol/L in the ACP vs CON and PLA, respectively; $P < 0.001$). Circulating plasma glucose (tAUC, 3500 ± 124 vs 3794 ± 172 and 3946 ± 183 mmol/L/450min) and insulin (tAUC, 76 ± 7 vs 106 ± 13 and 103 ± 13 nmol/L/450min) excursions were substantially lower during 7.5 h of recovery from exercise (*i.e.* postprandially) in ACP when compared with either CON or PLA ($P < 0.05$ and < 0.001 , respectively).

Conclusions - Exercise with adipose tissue lipolytic inhibition improves postprandial blood glucose regulation and thus clinical efficacy in male T2DM patients.

Introduction

In the treatment of type 2 diabetes mellitus (T2DM), tight glycemic control is important to reduce the likelihood of developing cardiovascular disease or other diabetes-related complications ^{1,2}. Despite the use of blood glucose-lowering medication, postprandial blood glucose excursions remain prevalent throughout the day in most T2DM patients ³. Postprandial hyperglycemia contributes substantially to the glycation of hemoglobin ⁴ and forms an early sign of metabolic abnormalities before developing T2DM ⁵. Adjuvant strategies are warranted to attenuate blood glucose excursions throughout the day and to improve glycemic control in T2DM patients ⁶. Along with proper dietary counseling and blood glucose-lowering medication, exercise forms another cornerstone in T2DM treatment ^{7,8}. It has been postulated that the glucoregulatory properties of each individual exercise session are of key importance to achieving long-term glycemic control targets ⁹. A single bout of exercise has been shown to enhance insulin sensitivity and improve glycemic control for up to 48 h ¹⁰⁻¹². Despite the use of different treatment strategies, many T2DM patients do not meet glycemic targets ⁸ and as these patients mostly suffer from poor exercise tolerance ⁷, we need to develop strategies that can augment the glucoregulatory properties of exercise and exercise training ¹³ in order to further improve the clinical efficacy of these treatment strategies.

Disturbances in fatty acid handling play an essential role in the development or progression of peripheral insulin resistance in T2DM ¹⁴. Impairments in the buffering capacity of the adipose tissue ¹⁴, as well as skeletal muscle lipid mobilization and oxidation ^{15,16} contribute to elevated plasma free fatty acids (FFA). Excess availability of plasma derived FFA results in ectopic lipid deposition in insulin sensitive tissues such as liver, pancreas, heart and skeletal muscle tissue ¹⁴. These metabolic disturbances cause a state of metabolic inflexibility ¹⁷, further promoting lipid deposition in skeletal muscle as opposed to their mobilization and oxidation, thereby increasing peripheral insulin resistance ¹⁸.

One of the mechanisms by which exercise is believed to improve insulin sensitivity is the mobilization and oxidation of the intramuscular lipid depots ¹⁹, which is largely determined by circulating plasma FFA concentrations ^{20,21}. The

elevated plasma FFA concentrations in obese or T2DM patients inhibit the hydrolysis of intramuscular lipid stores ²¹. Of interest, administration of the nicotinic acid analog acipimox has previously been shown to increase intramuscular triglyceride use during exercise in T2DM patients, reduce intramuscular lipid contents and lower circulating insulin concentrations in T2DM patients ²¹. We hypothesize that adipose tissue lipolytic inhibition during endurance-type exercise will help to further improve postprandial glycemic control in T2DM patients. To test our hypothesis, we determined the impact of a single bout of moderate-intense endurance-type exercise with or without adipose tissue lipolysis inhibition (acipimox administration) on subsequent postprandial blood glucose and insulin excursions throughout the day in male T2DM patients.

Methods

Subjects - A total of 18 male T2DM patients on blood glucose-lowering medication were selected to participate in this study. Patients were included based upon the following inclusion criteria: blood glycated hemoglobin (HbA1c) >6.5% (>48 mmol/mol), aged 45-75 y, body mass index (BMI) 27.5-35.0 kg/m², sedentary lifestyle (<2 h sports related activities per week) and Caucasian ethnicity. Exclusion criteria were: exogenous insulin therapy, self-reported coronary artery, pulmonary, renal or gastric disease, orthopedic symptoms that would interfere with exercise, or involvement in an exercise training or caloric restriction program within one year prior to the current study. Due to lack of motivation, four subjects withdrew from the study, leaving 14 subjects (Supplemental Fig. 1). This study was approved by the local medical ethical committee (Jessa Hospital and Hasselt University, Hasselt, Belgium), and the study was performed conform to the standards set by the latest revision (2013) of the Declaration of Helsinki. After careful explanation about the nature and risks of the experimental procedures, all subjects gave their written informed consent before participating in the study (study registration number NTR4710).

Screening and testing - Following a 2-day cessation of blood-glucose lowering medication intake, subjects arrived at the laboratory at 08.00 AM after an overnight fast. A fasting blood sample was obtained for blood HbA1c concentration (Hi-Auto A1c Analyzer, Menarini Diagnostics, Florence, Italy). Subjects underwent an evaluation of body composition by a dual x-ray absorptiometry scan (DEXA, Lunar DPXL, WI, USA), followed by a maximal cardiopulmonary exercise test on a cycle ergometer (eBike Basic, General Electric GmbH, Bitz, Germany) to assess peak oxygen uptake capacity (VO₂peak) and workload capacity (W_{peak}), using a 1-min work stage protocol (starting workload of 40 W, incremental workload of 20 W). VO₂ measurements were performed continuously (Jaeger Oxycon, Erich Jaeger GmbH, Germany), and VO₂peak was compared with normal age- and gender-related VO₂peak (expressed as %VO₂peak predicted)²². Heart rate (HR) was monitored continuously using a 12-leads electrocardiogram. All subjects cycled until exhaustion. The test was ended when subjects were no longer able to maintain a

cycling frequency of 55 rpm or higher. Peak exercise effort was confirmed when respiratory gas exchange ratio (RER) was ≥ 1.10 , in combination with dyspnea, leg and/or general fatigue.

Study design - Subjects participated in a randomized (double-blind, placebo-controlled) cross-over trial consisting of three conditions, interspersed by one week between treatments. Subjects were randomly assigned (Supplemental Fig. 1) to either a non-exercise condition (no exercise with placebo intake, CON), or to conditions in which a 60-min endurance-type exercise bout was performed exactly 60 min after per os administration of a nicotinic acid derivate (acipimox, ACP) or a placebo (PLA). Subjects were subsequently followed for 7.5 h in the laboratory. Twenty-two hours after cessation of exercise subjects returned to the laboratory for an oral glucose tolerance test (OGTT) (Fig. 1).

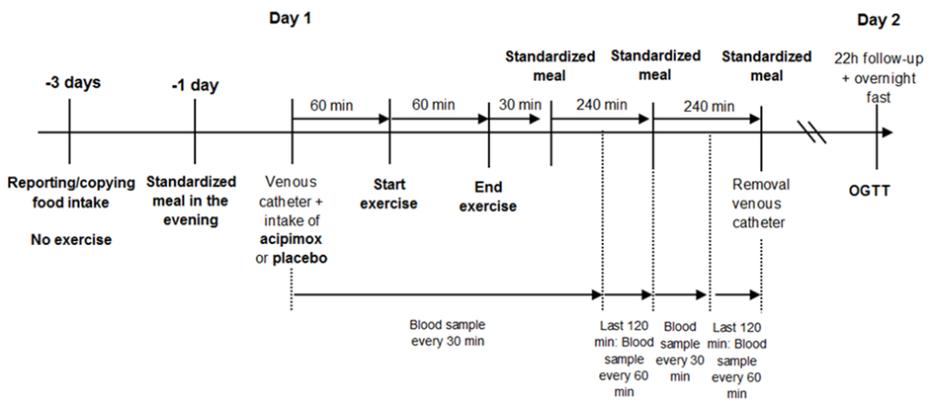


Figure 1 - Study design. Outline of the study protocol. On day 1, following administration of a placebo or the nicotinic acid analog acipimox, resting blood samples were collected for 60 min. In the three treatments (*i.e.* control condition, placebo intake with exercise and acipimox intake with exercise), fasting blood samples were collected at rest, during exercise and post-exercise at different time points for a total duration of 10.5 h. Twenty-two hours after cessation of exercise an oral glucose tolerance test was performed.

Study protocol - On day 1 of each intervention period, subjects arrived at the laboratory at 08.00AM following an overnight fast. An intravenous catheter was inserted in an antecubital vein for blood sampling purposes, one hour later followed by the oral administration of a placebo capsule (gelatin capsule containing starch) or a capsule of identical size and color containing 250 mg of the nicotinic acid analog acipimox (Nedios, Altana Pharma, Hoofddorp, The Netherlands). Except for flushing, no side-effects were experienced by the subjects after ACP administration. One hour after placebo or acipimox administration, subjects performed 60 min of endurance-type exercise on an electronically braked bike at 45% of their individual W_{peak} (PLA or ACP) or remained sedentary (CON). Heart rate (HR) was monitored continuously (Polar, Oy, Finland). Energy expenditure was calculated by use of ACSM equations²³.

Subjects next consumed three standardized meals during the day in the laboratory at 30 min (total energy: 2203 kJ (545 kcal); 18 g fat; 64 g carbohydrates; 25 g protein), 270 min (total energy: 2480 kJ (595 kcal); 36 g fat; 36 g carbohydrates; 27 g protein) and 510 min (total energy: 3292 kJ (788 kcal); 42 g fat; 71 g carbohydrates; 27 g protein) after cessation of exercise with ad libitum water consumption. Venous blood samples were collected every 30 or 60 min after consumption of the first and second standardized meal in EDTA-containing tubes and immediately centrifuged at 1,000 g for 5 min at 4°C. Plasma aliquots were frozen in liquid nitrogen and stored at -80°C until analysis. On day 2, subjects returned to the laboratory at 08.00 AM following an overnight fast (≥ 10 h) for an OGTT (starting at 22 h after completion of the exercise bout), in which blood glucose and insulin concentrations were assessed every 30 min.

Blood chemistry - Plasma glucose (A11A01667, Cobas Pentra semiautomatic analyzer, Roche, Basel, Switzerland), insulin (HI-14K, Human Insulin specific RIA Kit, Millipore, Billerica, MA, USA), FFA (NEFA HR (2) R1 set 434-91795 and Nefa HR (2) R2 set 436-91995 Wako, Neuss, Germany), triglyceride (A11A01640 (Roche), ABX Pentra Triglycerides CP, HORIBA ABX), and lactate (NAD 10127990001 and LDH 10127876001, Cobas Pentra semiautomatic analyzer, Roche, Basel, Switzerland) concentrations were assessed in duplo in each sample.

Medication, food intake and habitual physical activity - Except for screening, medication intake remained unchanged during the entire study period and was taken on the morning of each experimental visit (during the first standardized meal on day 1 and prior to the OGTT on day 2). Subjects maintained their normal habitual physical activity level and diet, but refrained from exhaustive physical activity three days prior to each visit. In addition, subjects recorded their dietary intake over three days prior to day 1 and copied their diet prior to each subsequent visit.

Statistical analysis - Data are presented as means \pm SEM. Shapiro-Wilk test indicated no normal distribution of data. To compare blood parameters (interval data) between different conditions, Friedman tests (for three related samples) were performed. In case of a significant main effect, post-hoc Wilcoxon signed ranks tests (for two related samples) were performed corrected for multiple testing (Bonferroni). Total areas under the curve (tAUC) of these blood parameters was calculated by the trapezoid method before and during exercise (08.00-10.30 AM; tAUC₀₋₁₅₀) and for a total of 7.5 h after exercise (11.00 AM - 06.30 PM; tAUC₁₈₀₋₆₃₀). In addition, tAUC during OGTT on day 2 was calculated. Statistical significance was set at $P < 0.05$ (two-tailed) for main effect and $P < 0.017$ (two-tailed) for two related samples. SPSS 22 for Windows was used to perform all calculations (IBM Corporation, Armonk, NY, USA).

Results

Subjects' characteristics

Fourteen male T2DM patients (age: 65 ± 2 y, HbA_{1c} $6.7 \pm 0.1\%$ (50 ± 2 mmol/mol), mean disease duration 10 ± 2 y) participated in this study. All subjects were either overweight or obese (BMI 30.2 ± 0.9 kg*(m²)⁻¹, whole-body fat $31 \pm 1\%$), showed a relative low exercise tolerance (VO_{2peak} 36 ± 2 mL*min⁻¹*kg FFM⁻¹ or $78 \pm 4\%$ of predicted norm) and maximal cycling power output (absolute W_{peak} 162 ± 12 W, relative W_{peak} 2.7 ± 0.1 W*kg FFM⁻¹). Except for one patient on monotherapy (DPP-4 inhibitor), all patients were treated with metformin ($n=11$) and/or other treatments including lipid lowering ($n=7$), antiplatelet ($n=6$), blood pressure lowering ($n=7$) or additional glucose lowering ($n=8$) therapy. Subjects' characteristics are summarized in Table 1.

Table 1 Subjects' characteristics

Variable		Medication use	
<i>n</i>	14		
Age, years	65 ± 2	Metformin (<i>n</i>)	11
BMI, kg*(m ²) ⁻¹	30.2 ± 0.9	Sulfonylurea (<i>n</i>)	2
Body fat, %	31 ± 1	DPP-4 inhibitor (<i>n</i>)	6
Fat free mass, kg	60.0 ± 2.1	GLP-1 receptor agonist (<i>n</i>)	1
		Statin (<i>n</i>)	7
HbA _{1c} , %	6.7 ± 0.1	Antiplatelet (<i>n</i>)	6
HbA _{1c} , mmol/mol	50 ± 2	Calcium antagonist (<i>n</i>)	3
Years since diagnosis	10 ± 2	ACE inhibitor (<i>n</i>)	3
		Angiotensin-2 receptor antagonist (<i>n</i>)	1
VO ₂ peak, ml*min ⁻¹ *kg ⁻¹ (FFM)	36.3 ± 2.0	Diuretics (<i>n</i>)	1
W _{max} , Watt*kg ⁻¹ (FFM)	2.7 ± 0.1	Beta blocker (<i>n</i>)	3
Maximal heart rate, beats*min ⁻¹	144 ± 4		
Maximal RER	1.17 ± 0.01		

Data are mean \pm SEM. HbA_{1c}, blood glycated hemoglobin; VO_{2peak}, whole-body peak oxygen uptake; BMI, body mass index; FFM, fat free mass; RER, respiratory exchange ratio.

Plasma FFA and triglycerides

All subjects performed two separate 60-min cycling exercise bouts at 45% of their W_{peak} (73 ± 6 W), eliciting a HR of 112 ± 4 and 116 ± 4 bts*min⁻¹ in PLA and ACP, respectively ($P > 0.05$). Energy expenditure during both exercise bouts was

estimated (heart rate based) to be 1.4 ± 0.1 MJ. In the fasted state, plasma FFA concentrations increased in both CON and PLA over time ($P=0.001$ and $P=0.001$, respectively), with a greater increase following the onset of exercise (PLA) when compared with CON ($P=0.022$) (Fig. 2A). Acipimox administration prevented the fasting- (CON) and exercise-induced (PLA) rise in circulating plasma FFA concentrations, resulting in lower plasma FFA concentrations when compared with both CON and PLA ($P<0.001$) (Fig. 2A). Overall $tAUC_{0-150}$ for the plasma FFA concentrations differed significantly between treatments ($P_{\text{treatment}}<0.001$) and averaged 79 ± 7 $\text{mmol} \cdot \text{L}^{-1} \cdot 150 \text{min}^{-1}$, 96 ± 8 $\text{mmol} \cdot \text{L}^{-1} \cdot 150 \text{min}^{-1}$ and 40 ± 2 $\text{mmol} \cdot \text{L}^{-1} \cdot 150 \text{min}^{-1}$ in CON, PLA and ACP, respectively (Table 2). After exercise, plasma FFA concentrations remained significantly lower in ACP when compared with both CON and PLA (Fig. 2A), whereby overall $tAUC_{180-630}$ for the postprandial plasma FFA concentrations differed significantly between treatments ($P_{\text{treatment}}=0.002$) and averaged 149 ± 15 $\text{mmol} \cdot \text{L}^{-1} \cdot 450 \text{min}^{-1}$, 170 ± 13 $\text{mmol} \cdot \text{L}^{-1} \cdot 450 \text{min}^{-1}$ and 115 ± 16 $\text{mmol} \cdot \text{L}^{-1} \cdot 450 \text{min}^{-1}$ in CON, PLA and ACP, respectively (Table 2). Plasma triglyceride concentrations slightly increased over time and did not show any differences between treatments in the fasted ($P_{\text{treatment}}=0.789$) or postprandial state ($P_{\text{treatment}}=0.458$) (Fig. 2B; Table 2).

Plasma glucose and insulin

ACP did not affect exercise-induced plasma lactate levels when compared with PLA ($P=0.683$), while plasma lactate concentrations were higher during exercise in PLA (3.14 ± 0.30 $\text{mmol} \cdot \text{L}^{-1}$, $P=0.001$) and ACP (3.02 ± 0.23 $\text{mmol} \cdot \text{L}^{-1}$, $P=0.001$) treatments when compared with CON (1.14 ± 0.10 $\text{mmol} \cdot \text{L}^{-1}$) (Fig. 3C). Postprandial plasma lactate concentrations did not differ between treatments ($P_{\text{treatment}}=0.395$) (Fig. 3C). Plasma glucose concentrations (Fig. 3A) and $tAUC_{0-150}$ (Table 2) were not significantly different between treatments during exercise ($P_{\text{treatment}}=0.607$ and $P_{\text{treatment}}=0.607$, respectively). Plasma insulin concentrations (Fig. 3B) and $tAUC_{0-150}$ (Table 2) were not significantly different between treatments during exercise ($P_{\text{treatment}}=0.751$ and $P_{\text{treatment}}=0.223$, respectively).

Table 2 Plasma glucose, insulin, free fatty acid, triglyceride and lactate concentrations during different treatments before and after exercise

	Treatment			P _{TREATMENT}
	Control (no exercise + placebo)	Exercise+ placebo	Exercise+ acipimox	
<u>Fasting and during exercise</u>				
tAUC glucose (mmol/L/150min)	1094 ± 56	1119 ± 65	1096 ± 61	0.607
tAUC insulin (nmol/L/150min)	11 ± 0.9	11 ± 1	10 ± 1	0.223
tAUC lactate (mmol/L/150min)	177 ± 16	314 ± 30 *	305 ± 20 *	<0.001
tAUC triglycerides (mmol/L/150min)	210 ± 36	207 ± 28	217 ± 38	0.789
tAUC free fatty acids (mmol/L/150min)	80 ± 7	97 ± 9 *	40 ± 11 *#	<0.001
<u>Postprandial during the day</u>				
tAUC glucose (mmol/L/450min)	3794 ± 172	3946 ± 183	3500 ± 124 #	0.011
peak glucose (mmol/L)	11.7 ± 0.6	12.2 ± 0.6	11.1 ± 0.6	0.526
tAUC insulin (nmol/L/450min)	106 ± 13	103 ± 13	76 ± 7 *#	<0.001
peak insulin (pmol/L)	414 ± 93	388 ± 88	263 ± 36 *#	0.013
tAUC lactate (mmol/L/450min)	827 ± 101	841 ± 77	790 ± 73	0.395
tAUC triglycerides (mmol/L/450min)	858 ± 134	817 ± 94	800 ± 126	0.458
tAUC free fatty acids (mmol/L/450min)	149 ± 16	170 ± 13	115 ± 16 *#	0.002
<u>During OGTT</u>				
tAUC glucose (mmol/L/120min)	1586 ± 83	1577 ± 93	1580 ± 86	0.607
peak glucose (mmol/L)	15.6 ± 0.8	15.5 ± 0.9	15.7 ± 0.8	0.807
tAUC insulin (nmol/L/120min)	31 ± 2	30 ± 3	31 ± 4	0.931
peak insulin (pmol/L)	361 ± 41	347 ± 41	368 ± 55	0.807

Data are expressed as means ± SEM. *Significantly different as opposed to control condition (p<0.017). #Significantly different as opposed to exercise with placebo intake (p<0.017). tAUC, total area under the curve; OGTT, oral glucose tolerance test.

Following exercise, postprandial plasma glucose concentrations were substantially lower in ACP when compared with PLA ($P_{\text{treatment}}=0.011$). This resulted in overall significantly different plasma glucose tAUC₁₈₀₋₆₃₀ between treatments, with lower plasma glucose concentrations in ACP when compared with PLA ($P=0.009$) and a strong trend for lower plasma glucose concentrations in ACP when compared to CON ($P=0.041$) (Table 2; Fig. 4). tAUC₁₈₀₋₆₃₀ averaged $3794 \pm 172 \text{ mmol} \cdot \text{L}^{-1} \cdot 450\text{min}^{-1}$, $3946 \pm 183 \text{ mmol} \cdot \text{L}^{-1} \cdot 450\text{min}^{-1}$ and $3500 \pm 124 \text{ mmol} \cdot \text{L}^{-1} \cdot 450\text{min}^{-1}$ in CON, PLA and ACP, respectively, while postprandial peak plasma glucose concentrations (measured during the entire postprandial timeframe) did not differ between treatments ($P_{\text{treatment}}=0.526$) (Table 2). Following exercise, ACP substantially reduced postprandial plasma insulin

excursions when compared with CON ($P=0.002$) or PLA ($P=0.001$) ($P_{\text{treatment}} < 0.001$). These reductions resulted in overall significantly lower insulin $\text{tAUC}_{180-630}$ between treatments (Table 2; Fig. 4). Hence, $\text{tAUC}_{180-630}$ averaged $106 \pm 13 \text{ nmol} \cdot \text{L}^{-1} \cdot 450 \text{ min}^{-1}$, $103 \pm 13 \text{ nmol} \cdot \text{L}^{-1} \cdot 450 \text{ min}^{-1}$ and $76 \pm 7 \text{ nmol} \cdot \text{L}^{-1} \cdot 450 \text{ min}^{-1}$ in CON, PLA and ACP, respectively (Table 2). In addition, postprandial peak plasma insulin concentrations (measured during the entire postprandial timeframe) were substantially reduced in ACP compared with CON and PLA ($P_{\text{treatment}} = 0.013$). Compared with CON (peak insulin: $414 \pm 93 \text{ pmol} \cdot \text{L}^{-1}$, $P=0.005$) and PLA (peak insulin: $388 \pm 88 \text{ pmol} \cdot \text{L}^{-1}$, $P=0.022$), ACP significantly lowered postprandial peak insulin concentrations (peak insulin: $263 \pm 36 \text{ pmol} \cdot \text{L}^{-1}$) (Fig. 2D; Table 2).

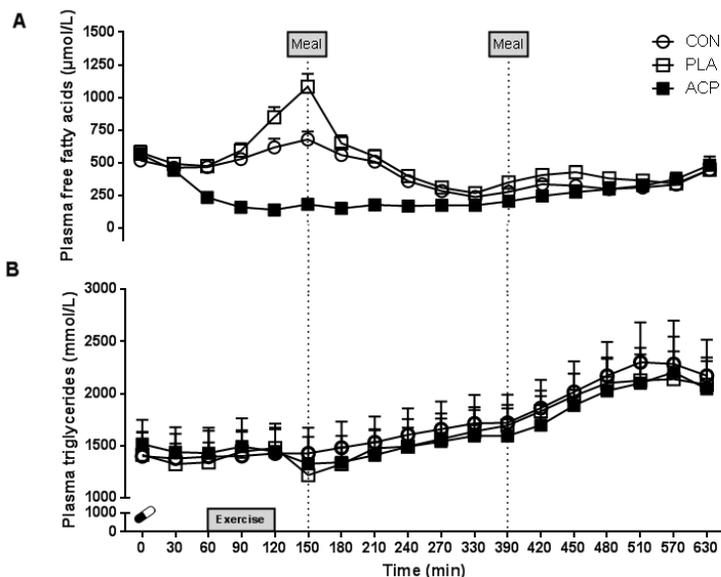


Figure 2 - Plasma metabolite concentrations. Data represent means + SEM; $n = 14$. Plasma FFA (A) and plasma triglycerides (B) concentrations at rest, during endurance-type exercise (or the control condition) and in the subsequent postprandial state.

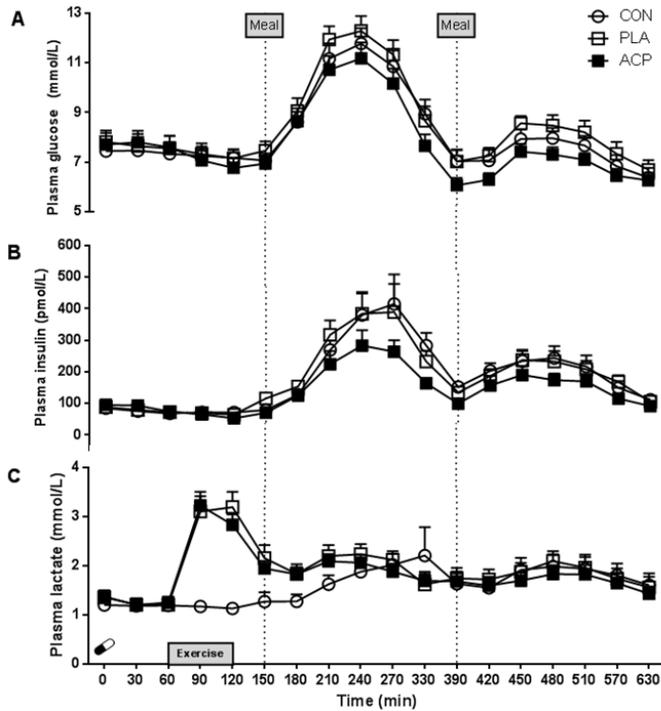


Figure 3 - Plasma metabolite concentrations. Data represent means + SEM; $n = 14$. Plasma glucose (A), plasma insulin (B) and plasma lactate (C) concentrations at rest, during endurance-type exercise (or the control condition) and in the subsequent postprandial state.

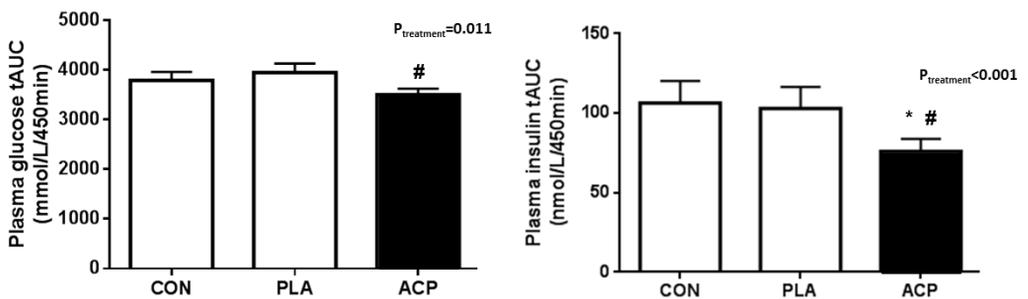


Figure 4 - Postprandial plasma glucose and plasma insulin responses expressed as total area under the curve. Data represent means + SEM; $n = 14$. Plasma glucose and plasma insulin concentrations were determined in the postprandial phase following exercise for up to 7.5 h (tAUC₁₈₀₋₆₃₀). P-values represent treatment (main) effect. * Significantly different from control condition (CON) ($P < 0.001$); # Significantly different from exercise with placebo intake (PLA) ($P < 0.017$).

Oral glucose tolerance test

The OGTT performed 22 h after exercise showed no differences between CON, ACP and PLA with respect to fasting plasma glucose (7.7 ± 0.4 , 7.5 ± 0.4 , and 7.9 ± 0.3 $\text{mmol}\cdot\text{L}^{-1}$, respectively; $P_{\text{treatment}}=0.526$) and fasting plasma insulin concentrations (89.5 ± 10.4 , 83.2 ± 10.3 , and 94.3 ± 12.4 $\text{pmol}\cdot\text{L}^{-1}$, respectively; $P_{\text{treatment}}=0.751$), peak plasma glucose (15.6 ± 0.8 , 15.7 ± 0.8 , and 15.5 ± 0.9 $\text{mmol}\cdot\text{L}^{-1}$, respectively; $P_{\text{treatment}}=0.807$) and insulin concentrations (361 ± 41 , 368 ± 55 , and 347 ± 41 $\text{pmol}\cdot\text{L}^{-1}$, respectively; $P_{\text{treatment}}=0.807$) or in their corresponding tAUC_{0-120} ($P_{\text{treatment}}=0.607$ and $P_{\text{treatment}}=0.931$, respectively) between treatments (Table 2).

Discussion

In the present study, we show that inhibiting adipose tissue lipolysis during a single bout of endurance-type exercise reduces plasma FFA availability in male T2DM patients. The lower plasma FFA concentrations during exercise were associated with a reduced postprandial rise in circulating blood glucose and insulin concentrations for at least 7.5 hours after cessation of exercise, when compared with exercise performed without adipose tissue lipolytic inhibition. These glucoregulatory benefits of combining exercise with adipose tissue lipolytic inhibition were no longer present 24 hours after cessation of exercise.

Patients with T2DM generally suffer from exercise intolerance and muscle weakness ⁷. In line, our patients were sedentary and showed a relative low physical fitness upon medical screening (Table 1) as compared with age-matched healthy individuals ²⁴. The applied 60 min bout of moderate-intensity exercise performed at 45% W_{max} (which was in accordance to the most recent clinical guidelines for T2DM treatment ⁸) represented an intense effort for these patients, but the absolute workload was merely 73 ± 6 W. Despite the effort, the exercise bout did not substantially improve glycemic control during exercise (PLA). Furthermore, postprandial glucose as well as insulin excursions throughout 7.5 h of recovery from exercise did not substantially differ from the non-exercise control trial (CON). Regardless of these somewhat unexpected findings, the current results clearly substantiate our opinion that new strategies that can help to augment the (clinical) benefits of exercise should be explored. Optimized therapies are warranted to further improve glycemic control in those patients that have been sedentary and are commencing a lifestyle intervention program.

Previous work has shown that the pool of intramuscular lipids are not mobilized during exercise when circulating plasma FFA concentrations are moderately elevated (exceeding $300\text{-}500 \mu\text{mol}\cdot\text{L}^{-1}$), especially in obese and type 2 diabetic individuals ²⁵. Using a nicotinic acid analog to specifically block adipose tissue lipolysis ²⁶, we previously showed that the exercise-induced increase in circulating plasma FFA concentrations can be prevented, thereby allowing the intramuscular lipid pool to be mobilized in both healthy athletes ²⁰ as well as

T2DM patients ²¹. In line, circulating plasma FFA concentrations during moderate-intense endurance-type exercise were successfully lowered by providing our patients with 250 mg acipimox 60 min prior to this exercise bout. Accordingly, we hypothesize that under these conditions exercise allows both intramuscular lipid as well as glycogen pools to be mobilized ^{20,25,27}, thereby increasing peripheral insulin sensitivity and, as such, improving glycemic control ²⁸.

Exercise in combination with adipose tissue lipolytic inhibition (ACP) substantially reduced subsequent postprandial circulatory glucose and insulin responses for up to 7.5 h after cessation of exercise. A substantial decline in postprandial circulatory glucose ($-11\pm 3\%$) and insulin ($-25\pm 4\%$) concentrations were observed following ACP when compared to PLA (Fig. 4). This indicates that adipose tissue lipolytic inhibition during low- to moderate-intense exercise can strongly increase insulin action. This results in lower postprandial blood glucose excursions with less circulating insulin, making this type of combinational exercise as effective as exercise bouts of either higher intensity ²⁹ or longer duration ³⁰. Still, it remains unclear whether the volume of endurance exercise affects glycemic control, or whether the different exercise characteristics modulate the impact of exercise on glycemic control ⁹. The benefits of exercise with adipose tissue lipolytic inhibition on glycemic control were evident for the remainder of the day after cessation of exercise, an important finding with respect to the importance of postprandial plasma glucose excursions in predicting secondary diabetes complications ³¹. However, at 24 h after exercise, we no longer observed greater whole-body glucose tolerance in ACP or PLA treatments, as no differences in circulatory plasma glucose or insulin responses were observed following the OGTT (Table 2). Therefore, the need for structured exercise or increased physical activity on a daily basis should be emphasized in this population.

In the present study, combining moderate-intense endurance-type exercise with adipose tissue lipolytic inhibition (ACP) was shown to improve postprandial glycemic control more effectively when compared with both the exercise only treatment (PLA) as well as the non-exercise control treatment (CON). Thus, combining exercise with adipose tissue lipolytic inhibition is postulated to be a

more effective interventional strategy to augment the glucoregulatory benefits of exercise intervention in T2DM treatment. Opposed to the combination therapy, one could suggest that adipose tissue lipolytic inhibition might be used without implementing exercise. However, the chronic use of nicotinic acid (or its analogs) is contraindicated. Previous work has shown that more prolonged use of acipimox can eventually lead to tolerance development as indicated by unaffected or even increased circulatory plasma FFA concentrations³²⁻³⁶. We hypothesize that intermittent use of acipimox, applied prior to exercise, might form an appropriate therapeutic strategy to allow intramuscular lipid and glycogen utilization, thereby further enhancing peripheral insulin sensitivity and, as such, improve postprandial glycemic control in patients with T2DM.

This combinational approach seems a promising alternative to enhance the clinical benefits of exercise therapy with equal effort of the patient on the long term, although one should be cautious about the potential side-effects of this combined therapy when used chronically. However, future work should be performed to explore the more prolonged benefits of combining low-intensity exercise training with adipose tissue lipolytic inhibition on glycemic control and clinical outcome in various diabetes subpopulations.

Conclusion

In conclusion, inhibition of adipose tissue lipolysis during exercise reduces circulatory plasma FFA concentration and lowers subsequent postprandial blood glucose and insulin excursions throughout the remainder of the day in male T2DM patients. These findings introduce the combined use of exercise with adipose tissue lipolytic inhibition as an effective interventional strategy to augment exercise-induced improvements in glycemic control in T2DM patients.

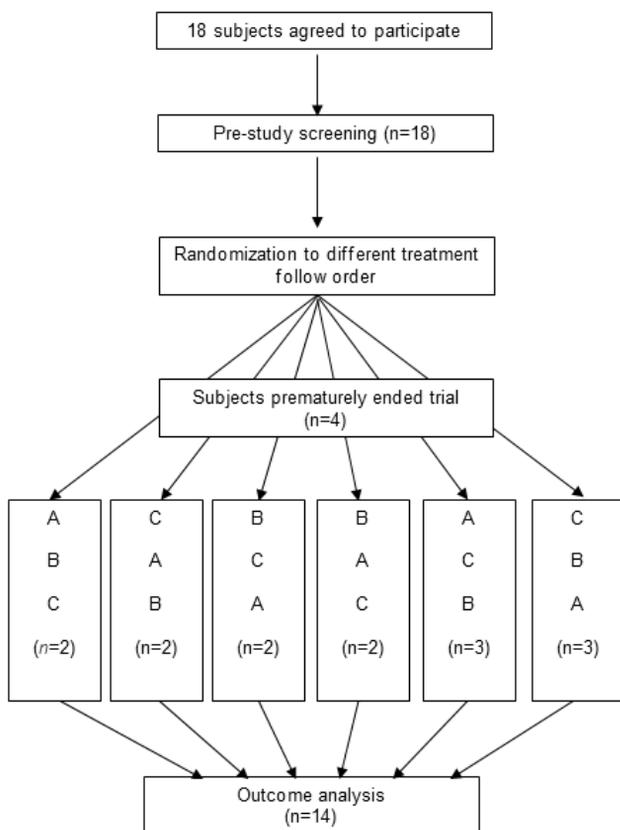
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Supplemental data



Supplemental Figure S1 - Flowchart and treatment randomization

Chapter 7

General Discussion

Obesity is a major ongoing health problem in our Western society ^{1,2}, a tendency that is mainly driven by changing lifestyle factors like increased energy intake ^{3,4} and reduced physical activity ⁵. Excess adiposity and an impaired adipose tissue (AT) function does not only imply an increased risk for developing metabolic complications such as insulin resistance (IR), type 2 diabetes mellitus (T2DM) and cardiovascular diseases ⁶, but also affects quality of life ⁷ and reduces cardiometabolic health as it is often accompanied by the presence of hypertension, hyperlipidemias or metabolic syndrome ^{1,8,9}.

Adipose tissue dysfunction has been recognized as an imperative contributor to the obesity-related complications ^{10,11}, in which an impaired lipid buffering capacity ¹²⁻¹⁴ and a reduced lipid turnover ¹⁵⁻¹⁸ play an important role. Whereas excess AT is associated with the development of local and systemic low-grade inflammation ¹⁹⁻²², altered storage, localization, handling and composition of lipids and lipid-intermediates might result in lipotoxicity, interfering with insulin signaling and promoting IR ²³⁻²⁶ in skeletal muscle ²⁷⁻³¹ and liver ^{24,32}, thereby worsening cardiometabolic health. In particular, the lipid mobilization related impairments observed in obesity-related IR (as described earlier in this thesis) may favor increased fat stores and thus may be of primary importance for obesity-related metabolic complications. Consequently, improving AT lipid mobilization and other AT functional characteristics is a major concern in the prevention and treatment of obesity and obesity-related metabolic diseases. However, the importance of sub-phenotyping was recently recommended by the CardioMetabolic Health Alliance to provide tailored care ³³. Therefore, more insight into the dysfunctional AT characteristics of human obesity is required to more effectively direct prevention and treatment in metabolic diseases.

Therefore, in this thesis, by combining state-of-the-art *in vivo* clinical research with *in vitro* (*ex vivo*) mechanistic work, we investigated the endocrine regulation of AT lipid mobilization (one of the hallmarks of AT function), the impact of exercise intervention in human obesity and its association to obesity-related IR in order to unravel its potential relevance in targeted obesity treatment.

Dysfunctional adipocyte lipolysis regulation in the obese state

Regulating lipid availability and thus the release of lipids from its storage depot(s) and the subsequent delivery to non-adipose tissues, is of the utmost importance in the obese state to preserve cardiometabolic health. The insulin resistant skeletal muscle tissue often displays an impaired ability to increase fat oxidation upon increased supply ²⁸, and it therefore is important to strictly regulate AT lipolysis in order to prevent ectopic lipid accumulation. It is known from *in vitro* ³⁴, *in situ* ³⁵ and *in vivo* ³⁶⁻⁴¹ studies that catecholamine-induced lipolysis is blunted in the AT of obese subjects ⁴², which was shown to be particularly present at the level of the abdominal subcutaneous adipose tissue (SCAT) ^{35,43-46}. Several physiological and molecular disturbances in the lipolytic pathway may underlie this catecholamine resistance. First, several receptor and post-receptor defects in the catecholamine signal transduction pathway are present in obese AT. A reduced number and function of β -adrenergic receptors ^{34,47} and an increased importance of the anti-lipolytic effects of α_2 -adrenergic receptors ^{48,49} have been reported in the insulin resistant SCAT. Next to catecholamines, of which adrenaline has been shown to be the major adrenergic regulator of adipocyte lipolysis ⁵⁰, other endocrine factors are involved in regulating lipid mobilization as will be discussed further in this chapter.

As described in **Chapter 2**, natriuretic peptides entered the lipolytic arena since the beginning of this millennium. Their role in metabolic control at the level of the AT, skeletal muscle and liver ⁵¹⁻⁵⁴, made this group of peptides attractive for human metabolic research. Moreover, their association with cardiovascular disease ⁵⁵⁻⁵⁸ together with the presence of a systemic natriuretic deficiency in metabolic diseases (of which the cause remains incompletely understood) ^{59,60} emphasized natriuretic peptides to be a potential target in the treatment of obesity and other (cardio)metabolic diseases ⁵⁴. Although, additional research such as human *in vivo* studies or fundamental studies with human tissue material is warranted to unravel the molecular mechanisms being involved, thereby getting more insight into the causality chain between the etiology of metabolic disease and the natriuretic deficiency.

With respect to the lipolytic properties of natriuretic peptides at the level of the AT^{61,62}, we investigated in **Chapter 3** whether adipocytes derived from abdominal subcutaneous and visceral (omental) AT of obese non-diabetic and obese diabetic individuals showed an impaired ANP-mediated lipolysis. We demonstrated an attenuated maximal responsiveness to ANP-mediated lipolytic stimulation in mature abdominal subcutaneous, but not visceral, adipocytes of obese men compared to lean individuals matched for age. This blunted maximal responsiveness was observed for both obese non-diabetic and obese diabetic men, indicating that this impairment can be attributed to the obese state *per se*. Additionally and in accordance with previous studies³⁴⁻⁴², the lipolytic response to isoprenaline, a non-selective β -adrenergic agonist mimicking catecholamine-induced lipolytic effects, was blunted as well in obese abdominal subcutaneous adipocytes.

Our data on an impaired ANP-mediated lipolysis are in accordance with findings in obese women⁶³, where ANP-mediated lipolysis showed a lower responsiveness in the abdominal SCAT of obese versus non-obese women. Of special interest and for the first time reported in scientific literature, in **Chapter 3** we showed that ANP-mediated lipolysis was reciprocally regulated in abdominal subcutaneous and visceral adipocytes in lean individuals, with abdominal subcutaneous adipocytes being more responsive to ANP stimulation as compared with visceral adipocytes. In contrast, this depot-specificity was absent in the obese non-diabetic and obese diabetic adipocytes. In line, previous data in middle-aged, morbidly obese women also showed no difference in ANP-mediated lipolysis between abdominal subcutaneous and omental (*i.e.* visceral) *in vitro* differentiated adipocytes⁶⁴. Thus, both ANP- and catecholamine-mediated lipolysis are impaired in abdominal SCAT and not in the visceral adipose tissue (VAT) depot in human obesity and/or diabetes. This impairment may promote and/or maintain excess body fat accumulation in the overweight or obese state.

Obesity or an impaired insulin sensitivity, a culprit of altered ANP signaling?

The presence of an impaired ANP-mediated lipolysis in abdominal SCAT in obese and obese diabetic subjects, led us to explore the underlying mechanism responsible for this anomaly. Natriuretic peptides exert their biological effects by binding the membrane-bound natriuretic peptide receptor A (NPRA), which stimulates the signaling cascade responsible for the functional/metabolic effects of ANP (and BNP) ⁶⁵. In contrast, besides intracellular degradation by neutral endopeptidase (NEP) ^{66,67}, the scavenging natriuretic peptide receptor type C (NPRC) is responsible for lysosomal degradation of its ligands such as ANP ^{68,69}.

Accordingly, in **Chapter 3**, the expression profiles of the functional NPRA and the scavenging NPRC were examined at the transcriptional (mRNA) as well as the translational (protein) level in order to (partly) unravel possible mechanisms for the impaired ANP-mediated lipolysis in human obesity. The scavenging NPRC is believed to affect ANP lipolytic effectiveness by binding ANP and subsequent intracellular degradation, precluding ANP from stimulating adipocytes ^{68,69}. The observed decrease in NPRA expression in SCAT of obese non-diabetic and obese diabetic individuals might be partly responsible for the observed attenuated ANP functionality. Besides, an increased NPRC mRNA expression was observed in both at the SCAT and VAT of obese non-diabetic and obese diabetic men. In addition, our NPRC mRNA levels were positively associated with whole-body IR (expressed as HOMA-IR) in both AT depots (with only VAT NPRC mRNA being associated independent of BMI), while the NPRA mRNA expression in SCAT was negatively associated with whole-body IR (independent of BMI) in our cohort. Our findings are in accordance with animal ⁷⁰ and human data ^{71,72} showing a decreased natriuretic peptide receptor ratio (i.e. NPRA/NPRC) to be associated with obesity, glucose intolerance and IR.

Altered natriuretic peptide receptor expression might contribute to triglyceride accumulation, enlarged adipocytes, favoring IR and lipid abnormalities. Of interest, a reduced *NPRC/NPRA* gene expression ratio in VAT was associated to clinical metabolic parameters such as total serum cholesterol, LDL-cholesterol, non-HDL cholesterol, next to fasting insulinemia in overweight, insulin sensitive (mean HOMA-IR 1.6) men and women ⁷³. Nevertheless, the differential NPRC

mRNA expression in the obese groups in the present study did not translate into a differential NPRC protein expression. This might possibly be explained by the rapid turnover of the NPRC receptor, whereby ligand-receptor complexes are rapidly internalized and degraded by lysosomes ⁶⁸.

However, according to classic receptor pharmacology principles ⁷⁴ differences in maximal responsiveness might reflect post-receptor signaling events. With respect to ANP-induced signal transduction, cyclic guanosine monophosphate (cGMP)-mediated signaling recently has been demonstrated to be repressed in visceral, but not subcutaneous, AT of obese humans, as shown by a decreased mRNA expression of protein kinase G (a downstream mediator of cGMP-mediated signaling in adipocytes) ⁷⁵. In line, lipid droplet associated lipases (ATLG and HSL) ⁷⁶⁻⁸⁰ and associated protein perilipin 1 ^{80,81} have been described to be decreased in SCAT of obese insulin resistant men, possibly contributing to the observed attenuated maximal ANP-mediated lipolysis in **Chapter 3**. Expression of phosphodiesterase 5 (PDE5), which hydrolyzes cGMP and thus impairs ANP-mediated signaling, was comparable between obese and non-obese individuals ⁶³. Moreover, the physiological relevance of PDE5 function in AT has been questioned since pharmacological inhibition in human subcutaneous ⁸² and visceral ⁸³ adipocytes did not affect lipolytic rate. Despite the very limited evidence for a physiologically relevant role of AT cGMP signaling, we cannot draw firm conclusions about the cause of the impaired ANP-mediated lipolysis in human obesity from the present study and available literature. Nevertheless, targeting this pathway could be metabolically beneficial ⁸⁴⁻⁸⁷.

Overall, in **Chapter 3** both ANP- and catecholamine-mediated lipolysis were attenuated in SCAT of obese men with type 2 diabetes, that might be partially explained by NP receptor defects. Additionally, downstream signaling defects might have contributed to the blunted maximal ANP responsiveness as well. Therefore, improving maximal ANP responsiveness in adipose tissue, or lipolytic regulation in general, might be a potential future strategy to improve obesity-associated metabolic complications

Adipose tissue dysfunction and obesity-related insulin resistance - different disparities matter

Dysfunctional adipose tissue is a known concept, associated with an increased cardiometabolic risk and characterised by an altered adipose tissue morphology, lipid metabolism and secretion of endocrine, paracrine and autocrine signals contributing to local and systemic low-grade inflammation and whole-body IR ^{10,88,89}. In **Chapter 4**, we studied the relevance of subcutaneous and visceral AT morphology, *ex vivo* lipolysis and immune cell populations in relation to whole-body IR in obese non-diabetic and type 2 diabetic (T2DM) men, compared with age-matched lean men.

Our data clearly indicated that visceral adipocyte hypertrophy is independently and positively associated with whole-body IR and thereby confirming previous studies ⁹⁰⁻⁹². This adipose tissue hypertrophy was accompanied by increased immune cell infiltration (CD45⁺ leukocytes and pro-inflammatory M1 macrophages in particular), predominantly in the VAT of obese and obese diabetic individuals, which confirms previous studies showing an increased inflammation of this depot as compared to its subcutaneous counterpart ^{93,94}. Furthermore, multiple regression analyses with whole-body IR as a dependent variable revealed that VAT B-lymphocytes were independently associated with HOMA-IR. In SCAT, body fat mass was the major determinant of whole-body IR, whilst none of the AT functional characteristics were independently associated with whole-body IR.

Our data might partly reflect the commonly observed impaired buffering capacity of SCAT in the obese insulin resistant state ⁹⁵, which contributes to visceral adipocyte hypertrophy as a result of ectopic lipid deposition in this AT depot ^{96,97}. Notably, several human studies indicated the development of hyperinsulinemia, dyslipidemia and (peripheral) IR upon overfeeding without affecting the inflammatory phenotype of the SCAT ^{98,99}. In contrast to the role of AT immune cells in healthy AT expansion ¹⁰⁰⁻¹⁰², chronic AT inflammation results from the presence and/or activation of different immune cells in the hypertrophic AT ¹⁰³⁻¹⁰⁶. The systemic inflammation in the obese insulin resistant state probably results from the pro-inflammatory phenotype of obese AT ^{107,108} and

systemic inflammation may lead to impairments in insulin signaling and contributes to the development or maintenance of IR in insulin-sensitive metabolic tissues^{21,109} and T2DM and its complications¹¹⁰. Our novel results in human AT depots of metabolically compromised patients substantiate the role of adaptive immune cells (B lymphocytes) in the process of local AT inflammation in VAT, thereby (partly) confirming previous work in animals¹¹¹⁻¹¹³.

Taken together, the data in **Chapter 4** showed that different cell populations in the immune landscape attribute to the known sub-clinical inflammatory state in obesity¹⁰² thereby broadening the classical view of AT macrophages being the major culprit of the inflammatory state in obesity^{20,114}. We here confirm that the role of lymphocytes and macrophages in the obesity-related inflammatory carousel is undoubtable¹¹⁵. The interplay of immune and other AT cells orchestrating AT inflammation and function, as well as the mechanisms via which these cells impair insulin sensitivity require further investigation in preferentially longitudinal studies in metabolic disease.

Modulation of adipose tissue lipid metabolism

Exercise

Based on our observed attenuated ANP-mediated lipolysis in obese and obese diabetic SCAT (shown in **Chapter 3**), we examined the physiological relevance of exercise-induced ANP-mediated lipolysis in the abdominal SCAT of obese individuals *in situ* in **Chapter 5**. By locally and entirely blocking adrenergic-mediated lipolytic stimulation during microdialysis at the level of the abdominal SCAT, using proper dosing of propranolol (a nonselective β -adrenergic receptor antagonist)¹¹⁶ and phentolamine (an $\alpha_{1,2}$ -adrenergic receptor antagonist)^{117,118}, we could investigate non-adrenergic-mediated lipolysis in the SCAT of lean insulin sensitive (IS), obese IS and obese insulin resistant (IR) individuals during rest, low-intensity endurance-type exercise and post-exercise recovery in the fasted state.

Our microdialysis data showed a reduced increase in exercise-mediated SCAT glycerol release in lean as compared to obese IS individuals. This discrepancy between phenotypes was, most likely, explained by a significant lower AT blood

flow in the obese abdominal SCAT, thereby decreasing the shunting of mobilized lipids ¹¹⁹⁻¹²¹ into the circulation. Although the difference in local AT blood flow (with or without pharmacological modification) hampers the lean vs obese comparison, this parameter certainly needs to be included in studies investigating local tissue effects.

Comparing obese IS and obese IR individuals (which displayed a comparable AT blood flow level), we observed a local reduction of SCAT lipolysis in the obese IS, not in obese IR, individuals following local α -/ β -adrenergic blockade. These findings propose an interaction of AT adrenergic responsiveness with whole-body IR, which is in line with previous work ^{34,41,47} and suggests a catecholamine-resistant phenotype of the SCAT during exercise especially in the obese IR state. In addition, the presence of higher anti-lipolytic insulin ^{29,122,123} and lactate ^{124,125} concentrations in the obese IR might have contributed to the attenuated adrenergic-mediated SCAT lipolysis as compared to the obese IS group.

Our data postulate for a major role of non-adrenergic regulators of abdominal SCAT lipolysis during low-intensity endurance-type exercise. Several endocrine regulators with pro- or anti-lipolytic actions have been shown to regulate lipolysis only to a minor extent during this type of exercise ¹²⁶, so the main regulator (besides catecholamines and insulin) of SCAT lipolysis is suggested to be ANP ^{61, 127-129}, especially in the overweight/obese state ^{130,131}. Our data showed that more than half of the lipolytic response remained present under local α -/ β -adrenergic blockade in the lean and obese (IS and IR) state. Therefore these results suggest a major role of non-adrenergic- (probably ANP-) mediated AT lipolysis during exercise in abdominal SCAT, which is even sustained in the obese IR state as the anti-lipolytic action of insulin does not affect ANP-mediated lipolytic signaling ¹³²⁻¹³⁴. Notably, the lack of an ANP receptor antagonist limits the strength of the proposed contribution of ANP in the physiological control of adipose tissue lipid mobilization. Still, the relevance of ANP is, in our opinion, established as it is currently the most important factor governing AT lipolysis during acute exercise bouts. Understanding its modulatory properties in the lipolytic arena therefore seems imperative in order to orchestrate the synergy between the different lipolytic regulatory pathways to further ameliorate AT metabolism in metabolic compromised conditions.

Exercise intervention has been known for its important role in causing fat mass loss and ameliorating metabolic profiles (including insulin sensitivity) in obese individuals ¹³⁵. In line, as described in **Chapter 5**, a 12-week exercise intervention (including endurance- and resistance-type exercise training) improved physical fitness and body composition in obese individuals. Of interest, exercise-induced systemic plasma responses (in particular plasma glycerol, FFA and lactate) improved in both obese groups and whole-body IR improved in the obese IR individuals. Together these data indicate the clinical efficacy of our exercise training program, which was applied to the current guidelines for obesity treatment ¹³⁶.

Previous studies in overweight and obese individuals have shown that exercise training has beneficial effects on adipocyte lipolysis and its regulation, including improvements in insulin ¹³⁷ and adrenergic sensitivity ¹³⁷⁻¹⁴³ at the level of the SCAT of obese individuals. Moreover, long-term endurance exercise training demonstrated a recovery of the reduced subcutaneous ANP responsiveness and ANP-mediated lipolysis in overweight individuals and obese women *in situ* ¹⁴⁴ and in obese women with polycystic ovary syndrome *in vitro* and *in situ* ¹⁴², indicating the susceptibility of ANP-mediated AT lipolysis. In **Chapter 5**, the effect(s) of a combined endurance and resistance exercise intervention on the endocrine regulation of abdominal SCAT lipolysis were determined in obese IR individuals. However, exercise-induced abdominal SCAT lipolysis was not changed as a result of exercise intervention in the obese IR individuals. Since we only observed a weak trend towards a small increment of adrenergic blockade efficacy in the SCAT of obese IR individuals following exercise intervention, these modest improvements may result from the low number of individuals included or the intensity of the applied exercise program, which can modulate the clinical efficacy in metabolic disease ^{145,146}. In addition, combining calorie restriction together with (high intensity) exercise training may be preferred ¹⁴⁷.

Overall, the results described in **Chapter 5** postulate for a major role ANP-mediated lipolysis during exercise in both lean and obese individuals and an apparent reduced adrenergically-mediated lipolysis in obese IR versus obese IS individuals. However, regardless of improvements in metabolic profile and body composition, adipocyte lipolytic impairments remain present in the obese state.

Therefore, optimized therapies are warranted to achieve enhancements in AT lipolysis regulation, especially in metabolically compromised individuals.

Pharmacological targeting of adipose tissue lipid metabolism during exercise

The role of lipotoxicity and, in particular, lipid spill-over has been known to impair insulin signaling and to promote IR at the level of different insulin-sensitive organs including skeletal muscle and liver, making AT lipid metabolism an important target to improve glucose homeostasis ³². In line, preclinical studies ¹⁴⁸⁻¹⁵⁴ have suggested that partial inhibition of lipolysis, using nicotinic acid or its analog acipimox, may be a promising therapeutic strategy in obese metabolically compromised people. Therefore, the clinical study described in **Chapter 6** substantiated the role of fatty acids in the regulation of glycemic control in obese men with T2DM.

In **Chapter 6**, our double-blind, placebo-controlled cross-over trial in obese T2DM individuals showed that inhibiting *in vivo* AT lipolysis by means of acipimox (a well-known analog of nicotinic acid specifically inhibiting adipose HSL ¹⁵⁵) during a single bout of endurance-type exercise in the fasted state resulted in alleviated consecutive postprandial glycemia and insulinemia, as opposed to exercise alone or a sedentary control experiment. However, the beneficial glucoregulatory effects of endurance-type exercise in the fasted state under acipimox intake in **Chapter 6** were only present in the subsequent two postprandial phases but were no longer present the morning after, during an oral glucose tolerance test.

In clinical treatment T2DM it is of major importance to enhance the efficacy of exercise bouts in T2DM patients as many T2DM patients suffer from an impaired exercise tolerance ¹⁵⁶. In addition, to achieve long-term glycemic control targets ¹⁵⁷, it is important to optimize/maximize the glucoregulatory properties of each individual acute exercise bout ^{158,159}, for which our applied combined approach (including acipimox intake during endurance-type exercise) described in **Chapter 6** could be a potential option. In this regard, sedentary T2DM patients might benefit the most from this combined therapy when starting the exercise intervention (since their exercise capacity is mostly limited at this point). Upon

enhanced exercise tolerance, exercise training can become the main focus, thereby gradually reducing acipimox dosage.

Of interest, one should be aware of the possible deteriorating effects of chronic treatment with nicotinic acid, which affect glucose metabolism and insulin sensitivity in the opposite way ^{149,150} due to a rebound effect on plasma FFA concentrations ^{151,160,161}. Therefore, the use of acipimox is preferred because of its more favorable effects on insulin sensitivity ^{152,153,162}, glycated hemoglobin ¹⁶³ and glucose metabolism ^{162,164-167}.

The clinical suitability of this therapy in combination with exercise has definitely some promising potential implications, in which the intermittent use of acipimox (only during exercise) may be a therapeutic option in metabolic disease to circumvent the potential rebound effect of chronic acipimox treatment and to alleviate the often observed insulin resistant state. To fully realize the potential of acipimox-mediated lowering of plasma FFA on improving glucose control, net FFA fluxes rather than solely circulating FFA should be achieved ^{148,154,168}. In this respect, intermittent exposure profiles in humans might improve the current clinical use and metabolic outcomes of acipimox treatment ^{154,161,169}.

Of interest, as the feeding status might influence the efficacy of metabolic outcome ¹⁶⁹, the practical use of this combined therapy might, in our opinion, be limited to the fasted (as opposed to the post-prandial) state. Consequently, these questions need to be addressed in future human intervention studies.

Conclusion and future perspectives

This thesis focuses on the endocrine regulation of AT lipid mobilization and the impact of exercise intervention in obese individuals, with a specific emphasis on the atrial natriuretic peptide (ANP), a heart-derived protein involved in human substrate and energy metabolism.

We found a pronounced attenuation of ANP-mediated lipolysis in the abdominal SCAT of obese individuals due to alterations at the receptor level, although this resistant-like phenotype was not observed in visceral adipocytes. In our exercise intervention study investigating the regulation of SCAT lipolysis in obese IS and IR individuals, the physiological relevance of this regulatory pathway was confirmed in the obese state. Of interest, controlling AT function in the obese state is crucial to maintain whole-body insulin sensitivity, the latter which was shown to be modulated by an exercise training program in obese individuals or its combination with partial pharmacological modulation of AT lipolysis in T2DM patients.

The main outcomes of this thesis provide evidence for recommending the importance of a proper regulation of AT lipid metabolism in order to maintain a 'healthy' AT function. We showed the presence of an impaired adipocyte lipolysis regulation in the SCAT of obese insulin resistant and obese T2DM patients, which could not be reversed by moderate-intense exercise intervention. Optimizing the clinical efficacy of exercise in the treatment of obesity and T2DM is of primary importance to improve obesity-related metabolic complications and therefore new strategies need to be explored in clinical practice. However, several questions and issues should be addressed in future research:

1. Our main finding in **Chapter 3** was the presence of an impaired ANP-responsiveness at the level of the SCAT in obesity-related IR and type 2 diabetes. Hence, the importance of ANP-mediated regulation during exercise of adipocyte lipolysis in the obese state was proposed in **Chapter 5**. However, despite several human studies suggested a metabolic role of natriuretic peptides including ANP, the lack of suitable ANP receptor antagonists limits the conclusive nature of these suggestions in humans. Therefore, the need for this type of molecules in human *in vivo* experiments is imperative to further unravel the

complexity of human adipocyte lipolysis regulation. In addition, tissues biopsies of adipose tissue and skeletal muscle should be taken to elaborate on underlying mechanisms.

2. In **Chapter 5**, we suggest the importance of ANP to sustain a proper lipolytic response during exercise in the obese state, whereby its role in the obese insulin resistant state might be of relatively increased importance due to the presence of catecholamine resistance. However, we did not observe any difference in systemic ANP concentrations between different obese phenotypes, nor between obese and lean individuals, which contrasts previous findings indicating the presence of a systemic natriuretic deficiency in human metabolic diseases, of which the cause remains to be determined. Therefore, more fundamental approaches are necessary in order to clarify the molecular mechanisms contributing to this natriuretic deficiency (e.g. attenuated cardiac secretion mechanisms or increased systemic/peripheral clearance of these peptides). Such experiments could include human intervention or prospective studies, mechanistic experiments with cultures of cardiomyocytes (principal producers of natriuretic peptides) or *in vitro* work on the tissue level to further exploring the difficulties with respect to tissue-specific receptors and signaling pathways.

3. In our studies, we included male obese and obese diabetic individuals. In literature, evidence also highlights gender differences in adipose tissue function, with a plausible role of sex hormones in differential regional white adipose tissue function. Still, the mechanisms controlling the regulation of gender-specific fat distribution remain poorly understood. Of interest, the repercussions of adipose tissue depot- and gender-specific effects on adipocyte function, as well as the relationship to cardiometabolic health and the efficacy of diet/exercise intervention are not fully elucidated and warrant further analysis.

4. Getting more insights into the molecular changes in the different lipolytic cascades would apply not only to obesity prevention but also to the elucidation of a methodology for advances in exercise efficacy. Especially with respect to ANP and its associated lipolytic signaling pathway, a lot of aspects are to be explored in future research. Together with mechanistic cell culture studies, these

experiments would gain more fundamental data for future optimization of exercise based therapies in the prevention of chronic metabolic disease.

5. As AT function, and in particular AT lipid metabolism, might be modified by both pharmacological and non-pharmacological intervention, the question remains which are the true molecular mechanisms going on during these intervention, especially with respect to metabolic organs like the AT or the skeletal muscle tissue. These mechanisms might (partly) clarify the observed integral lipolytic impairments present in the obese state, even following exercise intervention. Furthermore, the effects on AT function are mostly multifactorial (*e.g.* ameliorating AT lipid metabolism, AT inflammation, amongst others) leaving a lot of opportunities to investigate in metabolic research. Elucidating these multiple sub-characteristics of AT function and the effects of distinctive therapeutical approaches hereon may serve as base for improving obesity and metabolic disease treatment in clinical practice.

6. The important observation of our exercise intervention study, described in **Chapter 5**, that a 12-week training intervention did not translate into pronounced improvement of the lipolytic response effects at the SCAT in our obese populations with different insulin sensitivity despite improvement in body composition and metabolic profile, also raised further questions. First, is a three month period of this type of intensity, as prescribed by international guidelines¹³⁶, sufficient to induce changes in AT lipolysis regulation in these phenotypes? Second, are interventional approaches equally effective in improving metabolic health or AT function in different metabolic profiles (*e.g.* fat distribution, insulin sensitivity, cardiac burden, amongst others), or do we need tailored therapies for different metabolic profile (personalized medicine)? The use of combinational therapy (*e.g.* as applied in **Chapter 6**), including lifestyle changes, diet intervention and/or pharmacological methods could be of great interest to investigate in future metabolic research.

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ADDENDUM

Valorisation

The present thesis describes the potential of targeting the endocrine regulation of adipocyte lipolysis in human obesity treatment and prevention. An appropriate adipose tissue lipid metabolism is imperative to maintain normal adipose tissue function in human metabolically compromised conditions like obesity or type 2 diabetes mellitus. The valorization potential of the current results will be described in terms of social and economic relevance and implications for specific target groups and future innovative development.

Social and economic relevance of the obesity epidemic

Currently, our society is facing an alarming increase of overweight and obesity prevalence, which even extends to developing countries. According to latest statistics of the World Health Organisation (2014), Europe had the second highest proportion of individuals with overweight or obesity, preceded by the Americas. In Belgium and the Netherlands, 67% and 49% of the adult population deals with overweight and even 22% and 14% are diagnosed with obesity, respectively. The future perspectives on the development of obesity are disconcerting since it is predicted that the number of individuals with obesity will increase even more in the next two decades. This is a perplexing trend as individuals with obesity are more prone to develop metabolic diseases, cardiovascular diseases, mental disorders and certain types of cancer. Moreover, obesity (together with obesity-related complications) will further hamper efforts for healthcare cost management and thus not only affects public health but also has major socio-economic consequences. Therefore, new or optimized strategies for treatment and prevention are warranted to counteract this global obesity epidemic. Obtaining more insights into the adipose tissue physiology, and in particular adipose tissue lipid metabolism, is crucial with respect to treatment optimization and therefore was the main focus of the current thesis.

Development of obesity and obesity-related health complications

Obesity results from a chronic positive energy balance in which energy intake exceeds energy expenditure. Consequently, the energy surplus is stored within the adipose tissue, which gradually expands and may result in adipose tissue dysfunction. The main pillars of restoring the energy balance are reducing energy intake in relation to energy expenditure and increasing energy

expenditure. However, despite diet intervention being one of the most studied interventions in obesity treatment, many people often cannot maintain their initial weight loss due to a high relapse (weight regain) rate in this type of interventions. Contrarily, stimulating energy expenditure could be a suitable alternative to restore or even create a negative energy balance, in which increased physical activity or exercise is a common used strategy. Despite the efficacy of exercise, with respect to induce weight loss and promote healthier metabolic outcomes, many people often have difficulties maintaining regular physical activity regimes in their everyday life, ultimately limiting weight loss and in particular limiting weight maintenance after weight loss. Furthermore, the observed biomechanical anomalies often present in the obese state frequently hamper the ability to perform physical exercise. Developing alternative (combinational) strategies to improve clinical efficacy (i.e. maximizing weight loss and restoring metabolic complications) in the obese state are therefore imperative. With respect to weight loss, the stimulation of adipocyte lipolysis, a physiological process that is tightly regulated in an endocrine manner, is essential to reduce adipose tissue mass in the context of a negative energy balance.

Long-term regulation and control of body weight (body composition) relies upon a large regulatory network integrating different metabolic organs, in which the white adipose tissue is the major place for long-term energy storage in the form of triacylglycerol in adipocytes. In obesity, the chronic positive energy balance enlarges the adipocytes which may lead to an impaired lipid buffering capacity of the adipose tissue, which together with intrinsic lipolytic impairments may be identified as putative key factors in the pathophysiology of obesity and obesity-related metabolic and cardiometabolic complications. Gaining better insight into the role of these anomalies in obesity pathophysiology as well as lipolytic endocrine regulation is warranted in human metabolically compromised conditions. Therefore, in this thesis we explored whether natriuretic peptides, and in particular atrial natriuretic peptide (ANP), are substantially involved in the physiological regulation of adipocyte lipolysis in the obese insulin resistant state. Moreover, another goal was to explore the effect(s) of combined exercise intervention on this endocrine regulation system. As confirmed in the present thesis, there is now significant evidence that ANP is involved in the regulation of

adipocyte lipolysis, especially in the abdominal subcutaneous adipose tissue. However, in the obese insulin resistant or obese diabetic state, adipose tissue lipolysis displays a catecholamine- and ANP-resistant phenotype, which was not improved upon exercise intervention in the obese insulin resistant state. Therefore, finding appropriate strategies to alleviate the observed lipolytic impairments in the obese insulin resistant state are of major importance for body weight control and to improve adipose tissue function and metabolic profile in humans with obesity and thus more research in humans is required.

Target groups

Given the development of obesity associated metabolic disease affecting millions of people in our current societies, finding strategies to prevent and treat overweight, obesity and obesity-related health risks are crucial to improve global health and to counteract exploding healthcare costs. Indeed, in the obese state, even modest weight loss might lead to substantial beneficial health effects. Therefore, optimizing clinical efficacy of intervention programs, in terms of adipose tissue mass reduction and metabolic health, are of primary interest. In this respect, developing strategies, which are able to restore known lipolytic impairments present in the obese insulin resistant adipose tissue could be of major interest. To develop targeted exercise interventions or better pharmacological therapies should include fundamental as well as clinical work in human individuals with obesity and/or obesity-related metabolic complications.

In order to tackle obesity development, and in particular the development of its associated metabolic diseases like type 2 diabetes mellitus and cardiovascular disease, health care professionals, health institutions (including hospital settings, rehabilitation and sports centers), governments and general society need to be informed about these targeted exercise or combinational treatment (e.g. lifestyle intervention including exercise, diet and/or pharmaceutical treatments) strategies. In science, the aim is to make results available for the scientific community and health professionals through publication in international peer-reviewed journals and presentation on (inter)national scientific meetings. However, translation of scientific knowledge to society is essential. Beyond academia and scientific journals and meetings, our research

has been published in newspapers which enabled us to reach a broader audience to take notice of our findings. In this thesis, by performing in vitro work and clinical intervention studies with human subjects, we demonstrated a pronounced attenuation of ANP-mediated lipolysis in the abdominal subcutaneous adipocytes of obese individuals. The physiological relevance of this pathway in adipose tissue lipolysis was confirmed in the obese state as well, although exercise intervention could not restore the observed anomaly. Yet, exercise training in individuals with obesity or its combination with partial pharmacological inhibition of adipose tissue lipolysis in patients with type 2 diabetes was shown to modulate whole-body insulin sensitivity. Thus, the results presented in this thesis are of clinical relevance for a large number of people and therefore may be used by health care professionals to optimize lifestyle programs for individuals with overweight or obesity. In order to stimulate the translation of these findings to society and obtain awareness and support from the general public or policy makers, communicating these observations outside science is of considerable importance. Of interest, next to lifestyle and behavioral intervention to target obesity or its metabolic complications, pharmacological (co)interventions may be an alternative to alleviate adipose tissue function in the obese state. Targeting adipose tissue lipid metabolism or adipocyte function via pharmaceutical agents requires a good understanding and collaboration between universities and the pharmaceutical industry.

Activities and products

The research described in this thesis has been conducted at the department of Human Biology (Maastricht University), the Rehabilitation Research Center (Hasselt University) in collaboration with Heart Center Hasselt and Abdominal Surgery of Jessa Hospital and Hospital East-Limburg (Belgium). This unique collaboration between Universities and hospitals is a good example of how basic science can be performed in a hospital setting, combining knowledge of scientists and medical specialists across borders. The collaboration between basic scientists and professionals involved in obesity management is essential given that the current applied physical activity modalities in obesity rehabilitation, which are applied very frequent at both aforementioned hospital departments, might not be appropriate to restore adipose tissue function in

these patients. In order to increase the knowledge on white adipose tissue function and thereby improve prevention and/or treatment of human metabolically compromised conditions, strong national and international collaboration between different disciplines is of utmost importance.

The results described in this thesis are published or will be published in original research articles in scientific journals in the field of obesity and diabetes. Accordingly, these articles can be found online (accessible to those interested). The present results help to understand white adipose tissue physiology in humans and can be used for future studies on exploring optimized strategies to combat the obesity epidemic. The key role of adipose tissue function in metabolic health is currently subject of debate in the field of obesity research. The main outcomes of this thesis provide evidence for recommending the importance of a proper regulation of adipose tissue lipid metabolism in order to maintain a 'healthy' adipose tissue function. Moreover, this thesis showed that exercise training was not able to improve adipose tissue lipolysis despite improvements in metabolic disturbances, indicating the need for substantial weight loss to further restore adipose tissue function. However, the repercussions of adipose tissue depot- and sex-specific effects on adipocyte function, as well as the relationship to cardiometabolic health and the efficacy of diet/exercise intervention are important aspects, which require further research. This might also imply that stratification of individuals with obesity based on metabolic health characteristics is necessary to optimize and create tailored prevention and treatment strategies.

Innovation

By combining state-of-the-art *in vivo* clinical research and laboratory analyses, we were able to investigate for the first time the contribution of ANP in the regulation of adipocyte lipolysis in individuals with obesity. Moreover, the use of exercise intervention was shown to be insufficient to restore the observed impaired adipocyte lipolysis regulation in the obese insulin resistant state, leaving some opportunities for future research (as described in Chapter 6 and 7 of this thesis).

The current findings indicate a possible important role of non-adrenergically mediated lipolysis, most likely (partly) mediated by ANP, especially during physical activity or exercise. Together with the observations of the presence of a putative natriuretic deficiency in metabolic disease (as described in Chapter 2), this might indicate that the natriuretic peptide system or some of their components (*e.g.* their receptors or downstream mediators at the level of the adipose tissue) are candidate drug targets with therapeutic potential in obesity and type 2 diabetes, population in which poor adherence to lifestyle intervention is frequently observed. However, the therapeutic potential should be explored guardedly in future research, with special attention to cardiovascular complications. Of interest, efficient and selective (*i.e.* adipose tissue specific) ANP receptor agonists and antagonists are warranted to firmly delineate the physiological relative contribution of the different lipolytic agents in adipocyte lipolysis.

APPENDICES

Summary

Over the last decades, the prevalence of obesity has dramatically increased, reaching epidemic proportions and which is expected to rise even more the coming decennia. Obesity is an important risk factor for developing cardiometabolic complications, having a substantial impact on general health status and thus health care. Due to an imbalance between energy storage and energy expenditure in the obese state, energy is stored in the adipose tissue. Upon excessive weight gain, adipose tissue dysfunction develops, which is associated with ectopic lipid accumulation and the development of metabolic complications like insulin resistance or type 2 diabetes mellitus (**Chapter 1**). Therefore, an appropriate adipose tissue lipid metabolism is imperative to maintain healthy adipose tissue function in human obesity. Consequently, improving adipose tissue lipid mobilization (and other adipose tissue functional characteristics) is a major concern in the prevention and treatment of obesity and obesity-related metabolic diseases. As it is important to strictly regulate adipose tissue lipid mobilization (also called lipolysis) in order to prevent ectopic lipid accumulation, the studies described in this thesis investigated the endocrine regulation of adipose tissue lipid mobilization. Moreover, the impact of exercise intervention (and its combination with pharmaceutical inhibition of adipose tissue lipolysis) on adipose tissue lipolysis in human obesity and its association with obesity-related insulin resistance were investigated in order to unravel its potential in obesity treatment and prevention.

Accumulating evidence indicates that natriuretic peptides are involved in metabolic control at the level of insulin-sensitive tissue including adipose tissue, skeletal muscle and liver, making these peptides attractive for human metabolic research. Moreover, their role in the regulation of adipose tissue lipid mobilization (**Chapter 2**) led us to explore whether natriuretic peptides, and in particular atrial natriuretic peptide (ANP), are substantially involved in the physiological regulation of adipose tissue lipolysis in the obese insulin resistant state.

In **Chapter 3**, we demonstrated an attenuated maximal responsiveness to ANP-mediated lipolytic stimulation in isolated mature abdominal subcutaneous, but not visceral, adipocytes of obese men with and without type 2 diabetes compared to age-matched lean individuals. This blunted maximal

responsiveness might be explained by natriuretic peptide receptor defects (and post-receptor signaling defects) present in the obese insulin resistant state. Interestingly, the depot-dependent differential regulation of ANP-mediated lipolysis in abdominal subcutaneous and visceral adipocytes of lean individuals was absent in adipocytes of individuals with obesity, thereby promoting and/or maintaining excess body fat accumulation in the obese state.

Next to adipose tissue lipid metabolism, other dysfunctional adipose tissue phenotypic characteristics (e.g. adipocyte hypertrophy, adipose tissue inflammation) may contribute to an increased cardiometabolic risk in the obese state. In **Chapter 4**, we demonstrated the importance of visceral adipocyte hypertrophy and visceral B-lymphocytes as independent contributors to whole-body insulin resistance (as assessed by the surrogate marker HOMA-IR). With respect to the subcutaneous adipose tissue depot, fat mass rather than adipose tissue function variables was related to whole-body insulin resistance. The sub-clinical adipose tissue inflammatory state in the human obesity was corroborated by increased expression profiles of total lymphocytes and pro-inflammatory M1-macrophages in the visceral adipose tissue (as compared to lean individuals), thereby indicating the presence of an orchestrated interplay between different cell types within the obese adipose tissue.

As described in **Chapter 3**, mechanistic work using *in vitro* (*ex vivo*) techniques indicated an ANP-resistant like phenotype at the level of the subcutaneous adipose tissue of individuals with obesity. The physiological relevance of ANP-mediated lipolysis during exercise was examined by using state-of-the-art microdialysis experiments at the level of the subcutaneous adipose tissue of individuals with obesity. The results of **Chapter 5** indicate a major role of non-adrenergic regulators of adipocyte lipolysis during low-intensity endurance-type exercise in human obesity, presumably ANP-driven. However, regardless of improvements in metabolic profile and body composition, adipocyte lipolytic impairments remain present in the obese state following an exercise training program, suggesting the need for optimized therapeutic strategies to prevent or treatment obesity. Therefore, in **Chapter 6**, we demonstrated the clinical suitability and efficacy of a combined approach in which pharmacological lowering of plasma free fatty acids by means of the nicotinic acid analog

acipimox, used as a tool to inhibit adipose tissue lipolysis, together with low- to moderate-intensity endurance-type exercise in the fasted state was able to alleviate consecutive postprandial glycemia and insulinemia in obese individuals with type 2 diabetes.

In conclusion, and based on all data gathered in this thesis, a proper regulation of adipose tissue lipid metabolism is of utmost importance in order to maintain a 'healthy' adipose tissue function. The role of ANP in regulating adipocyte lipolysis was shown to be of physiological relevance, especially in metabolically compromised conditions like the obese insulin resistant or type 2 diabetic state, where we observed a blunted catecholamine- and ANP-mediated lipolytic response at the level of the subcutaneous adipose tissue. The observed lipolytic impairments could not be reversed by exercise intervention, postulating the need for novel add-on therapies to further optimize the efficacy of (combined) prevention and treatment strategies implemented in the clinical care of individuals with obesity.

APPENDICES

Samenvatting

De prevalentie van zwaarlijvigheid of obesitas is de afgelopen decennia enorm toegenomen, waarbij reeds epidemische proporties worden aangenomen. De huidige voorspellingen geven aan dat deze cijfers de komende decennia zelfs nog meer zullen stijgen. Obesitas is een sterke risicofactor voor de ontwikkeling van cardiovasculaire en metabole complicaties, wat een aanzienlijke impact heeft op de algemene gezondheid en leidt tot een stijgende druk op de gezondheidszorg. Bij obesitas wordt energie opgeslagen in het vetweefsel omwille van een onevenwicht tussen energie verbruik en energie opslag. Ten gevolge van aanhoudende gewichtstoename zal zich vetweefsel dysfunctie ontwikkelen, hetgeen wordt geassocieerd met ectopische vetstapeling en de ontwikkeling van metabole complicaties zoals insuline resistentie en diabetes mellitus type 2 (**Hoofdstuk 1**). Met oog op het behoud van een gezonde vetweefselfunctie bij personen met obesitas is een adequaat vetmetabolisme ter hoogte van het vetweefsel van noodzakelijk. Verbeteren van vetmobilisatie (en andere functionele vetweefsel kenmerken) is bijgevolg van primair belang in functie van preventie en behandeling van obesitas en de hiermee gepaarde metabole aandoeningen. Gezien het belang van een strikte regulatie van de vetmobilisatie (ook vetweefsel lipolyse genoemd), bestudeerden de studies beschreven in het huidige proefschrift de hormonale regulatie van de vetmobilisatie ter hoogte van het vetweefsel. Verder werd de invloed van een trainingsinterventie (en de combinatie met farmacologische inhibitie van vetweefsel lipolyse) op de vetweefsel lipolyse onderzocht bij personen met obesitas en werd het verband tussen vetweefsel lipolyse en obesitas-gerelateerde insuline resistentie bestudeerd met oog op de potentiële relevantie binnen de behandeling en preventie van obesitas.

In de vakliteratuur is er steeds meer bewijs voor het feit dat natriuretische peptiden een rol van betekenis hebben binnen de metabole controle door hun effecten ter hoogte van insuline gevoelige weefsels zoals het vetweefsel, het spierweefsel en de lever, wat deze peptiden aantrekkelijk maakt binnen het humaan metabool onderzoek. Gezien hun rol in het reguleren van vetweefsel lipolyse (**Hoofdstuk 2**), leidde dit ons ertoe om te onderzoeken of natriuretische peptiden, en specifiek het atriaal natriuretisch peptide (ANP), betrokken zijn in de fysiologische regulatie van vetweefsel lipolyse bij obese, insuline resistente personen.

In **Hoofdstuk 3** werd een verstoorde maximale responsiviteit voor ANP-gemedieerde vetcel lipolyse beschreven in geïsoleerde, mature subcutane, niet viscerale, vetcellen van obese mannen met of zonder type 2 diabetes, in vergelijking met slanke mannen met een vergelijkbare leeftijd. Deze verstoring kan (deels) verklaard worden door verschillende expressieprofielen van receptoren voor natriuretische peptiden (naast veranderingen post-receptor signaalmoleculen) bij obese insuline resistente individuen. Opmerkelijk genoeg was het depot-afhankelijk effect, aanwezig bij vetcellen van slanke mannen, waarbij subcutane vetcellen meer responsiviteit vertoonden voor ANP dan viscerale vetcellen, niet aanwezig bij obese mannen, hetgeen wellicht het behoud van overtollige vetmassa bij personen met obesitas in de hand werkt.

Naast een adequaat vetmetabolisme ter hoogte van het vetweefsel kunnen andere kenmerken van een dysfunctioneel vetweefsel (bv. vetcel hypertrofie, vetweefsel inflammatie) eveneens bijdragen aan een verhoogd cardio-metabool risico bij personen met obesitas. In **Hoofdstuk 4** werd het belang van viscerale vetcel hypertrofie en viscerale B-lymfocyten als onafhankelijke variabelen voor insuline resistentie (bepaald aan de hand van de surrogaat index HOMA-IR) aangetoond. Ter hoogte van het subcutaan vetweefseldepot droeg voornamelijk de vetmassa, meer dan andere merkers van vetweefselfunctie, bij aan de variatie in insuline resistentie. De subklinisch inflammatoire status van het vetweefsel, hetgeen prevalent aanwezig is bij obese individuen, werd verder bevestigd door een verhoogde aanwezigheid van totale lymfocyten alsook pro-inflammatoire M1 macrofaag populaties in het visceraal vetweefseldepot van personen met obesitas (in vergelijking met slanke personen). Deze bevindingen geven aan dat er in het obese vetweefsel inderdaad een georkestreerd samenspel tussen verschillende celtypes aanwezig is.

Zoals beschreven in **Hoofdstuk 3**, werd aan de hand van mechanistische *in vitro* (*ex vivo*) methoden een ANP-resistent fenotype van het subcutaan vetweefsel bij personen met obesitas aangetoond. De fysiologische relevantie van de ANP-gemedieerde lipolyse tijdens inspanning werd vervolgens onderzocht gebruik makend van "gouden standaard" microdialyse experimenten ter hoogte van het abdominaal subcutaan vetweefsel van personen met obesitas. De bevindingen uit **Hoofdstuk 5** wijzen op een belangrijke aandeel

van niet-adrenerge regulatoren in het lipolytisch proces ter hoogte van de subcutane vetcel tijdens laag-intense uithoudingsinspanning bij personen met obesitas, vermoedelijk ANP-gemedieerd. Echter, de verstoringen op gebied van vetcel lipolyse bleven aanwezig na een gecombineerd trainingsprogramma, ondanks positieve veranderingen in metabool profiel en lichaamssamenstelling ten gevolge van dit trainingsprogramma bij personen met obesitas, hetgeen erop duidt op de nood aan geoptimaliseerde therapeutische strategieën ter bevordering van de preventie en behandeling van obesitas. Bijgevolg werd in **Hoofdstuk 6** de toepasbaarheid en klinische effectiviteit van een gecombineerde aanpak aangetoond. Deze combinatietherapie omvatte het farmacologisch verlagen van de circulerende vrije vetzuren, gebruik makend van acipimox (een analoog van nicotinezuur) om de vetweefsel lipolyse te remmen, naast een laag- tot matig-intense uithoudingsinspanning in gevaste toestand bij obese personen met type 2 diabetes en resulteerde in een substantiële verbetering van de glycemie en insulinemie tijdens de daaropvolgende postprandiale fasen.

Uit de resultaten die in dit proefschrift werden behaald kan worden geconcludeerd dat een adequaat regulatie van het lipidenmetabolisme ter hoogte van het vetweefsel van cruciaal belang is om een 'gezonde' vetweefselfunctie na te streven. In dit proefschrift werd ook de fysiologische rol van ANP in de regulatie van vetcel lipolyse beschreven, voornamelijk in obesitas-gerelateerde metabole aandoeningen zoals insuline resistentie of type 2 diabetes mellitus, condities waarbij een verstoorde catecholamine- en ANP-gemedieerde regulatie van vetcel lipolyse werd waargenomen op het niveau van het abdominaal subcutaan vetweefsel. De geobserveerde lipolytische defecten konden niet worden gemedieerd door middel van trainingsinterventie, hetgeen suggereert dat er een essentiële nood is aan nieuwe therapieën met als doel de klinische effectiviteit van (al dan niet gecombineerde) preventie- en behandelingsstrategieën te optimaliseren in functie van een verbeterde zorg voor personen met obesitas.

Curriculum Vitae

Kenneth Verboven was born on 23rd of January 1989 in Sint-Truiden, Belgium. After completing secondary school at Royal Atheneum 1 Hasselt, Belgium, he started, in 2007, a Bachelor in Biomedical Sciences at the Transnational University Limburg (tUL), a unique partnership of two universities in two countries: Hasselt University and Maastricht University. In 2012, he obtained his Master of Science in Biomedical Sciences at the tUL, Belgium/The Netherlands. In 2012, he worked as a scientific researcher at the Jessa Hospital in Hasselt, Belgium. In January 2013, Kenneth Verboven started as a joint PhD candidate at the Department of Rehabilitation Sciences of the Biomedical Research Institute of Hasselt University, Belgium, and the Department of Human Biology of Maastricht University Medical Centre + (MUMC+), the Netherlands, under the supervision of Prof. dr. Ellen Blaak and Prof. dr. Dominique Hansen. His research focuses on the endocrine regulation of adipose tissue lipolysis in human *in vivo* studies including fundamental *in vitro* methodology. His work has been presented at several national and international conferences (European Obesity Summit 2016, 52nd Annual Meeting of the European Association for the Study of Diabetes (EASD) and 24th European Congress on Obesity 2017). In 2017, he received a Travel Scholarship for the annual ECO meeting 2017, Porto, Portugal.

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Dankwoord

Ondanks het feit dat ik een persoon ben van weinig woorden wil ik via dit dankwoord toch enkele woorden richten aan de prachtige mensen die ik tijdens mijn doctoraatsonderzoek (beter) heb leren kennen. Ik had het geluk aan twee onderzoeksinstellingen te mogen werken, en dus dubbel zoveel leuke collega's met stuk voor stuk een aandeel in dit geheel, waar ik iedereen héél dankbaar voor ben!

Ellen, dankjewel dat je me in 2013 wilde ontvangen om samen dit gezamenlijk PhD-project uit de grond te stampen. Vanaf de start kreeg ik een gevoel van vertrouwen, ook al was het weer een Belg die in je lab terecht was gekomen. Heel dankbaar ben ik voor de kans die ik in jouw groep kreeg, dit opende heel wat perspectieven waar we in Hasselt nooit aan hadden durven denken bij de start van het project gezien onze onervarenheid in het humaan metabool onderzoek. Ik heb nog steeds veel bewondering voor al je wetenschappelijke inzichten die onze studieprotocollen na elk overleg weer net dat tikkeltje verbeterden, ook al moest er soms worden terug gegaan naar het oorspronkelijk idee :) Bedankt Ellen voor de leerrijke, maar ook gezellige jaren, welke ik nog lang zal blijven koesteren! Ik kijk er alvast heel erg uit om onze samenwerking te blijven onderhouden.

Dominique, bedankt voor alles! Al in 2012 maakte ik, tijdens mijn seniorstage op REVAL, kennis met je onuitputtelijke gedrevenheid en ambities. Dit werkte zo aanstekelijk dat ik graag inging op je vraag om bij jou een PhD te beginnen, een beslissing waar ik geen moment over heb getwijfeld! Bedankt dat je me van het begin de vrijheid en het vertrouwen hebt gegeven om zelfstandig mijn ding te doen. Altijd stond je deur open, al kwam ik weer een keer vragen om een (dure) bestelling te plaatsen. Je enthousiasme telkens ik met nieuwe analyses aan kwam lopen werkte inspirerend, al moest ik je wilde ideeën voor nieuw onderzoek soms wat temperen :) Tot op vandaag bewonder ik nog steeds je passie waarmee je telkens nieuwe studies uitdenkt, uitvoert en opschrijft. Dominique, een dikke merci!

Johan, ik wil je graag heel erg bedanken voor al je inzichten en ondersteuning om mij vertrouwd te maken met het hele lipolyse-gebeuren! Je kritische blik op

telkens nieuwe versies van papers zijn van goudwaarde, alsook de motiverende woorden op het einde van al onze overlegmomenten. De aanpassing naar de Nederlandse manier van werken verliep 'smooth' mede dankzij jouw waardevolle tips die je me regelmatig hebt gegeven. Een welverdiende dank u wel voor je bijdrage in dit geheel!

Bert, ook voor jou alleen maar woorden van dank. Je kennis omtrent de inspanningsfysiologie heeft mijn biomedische kennis heel erg verreikt. Je introduceerde me destijds in het klinische werkveld, een gouden combinatie met het fundamentele onderzoek bleek achteraf! Je stond altijd klaar om een logistieke oplossing te bedenken wanneer ik al je apparatuur weer een keer nodig had om proefpersonen te testen, nooit was dit teveel gevraagd (wat ik heel erg heb weten appreciëren). Ik hoop dat we in de toekomst onze expertises verder kunnen delen! Bert, bedankt!

I would also like to thank the members of the Assessment Committee and the corona of my thesis defence, **Prof. Marleen van Baak, Prof. Mikael Rydén, Prof. Patrick Rensen, Prof. Marleen van Greevenbroek, Prof. Paul Dendale, Prof. Virginie Bito** for your time and effort that you put in reading and reviewing this thesis.

Ik bedank ook graag mijn **co-auteurs** voor hun input en kritische commentaren om de papers in deze thesis te vormen tot hoe ze nu zijn geworden. **Cédric**, un grand remerciement pour toutes les recommandations sur les expérimentations *in vitro*. **Kristiaan, Katrien, Coen** en **Casper**, bedankt om me wegwijs te maken in de wondere wereld van de inflammatie. **Gijs**, heel erg bedankt voor je nuttige suggesties om in de manuscripten de nuances net wat anders aan te brengen. Je kwam telkens weer met nieuwe interpretaties van onze resultaten, al was het op de gang of met een biertje op een of ander congres. Het was een plezier om met je samen te werken, bedankt Gijs! **Luc, Lex** en **Jan-Willem**, bedankt om telkens tijd te maken om ons gezamenlijk verhaal steeds weer sterker te maken. Dit heeft mijn visie op wat dingen zeker verbreed, wat in de toekomst nog zijn nut zal bewijzen.

Bedankt ook aan de mensen van de afdeling Cardiologie, het Hartcentrum Hasselt en het Revalidatie- en Gezondheidscentrum van het Jessa Ziekenhuis. In

het bijzonder wil ik **dr. Dendale** bedanken om telkens de mogelijkheid te bieden al onze metingen en experimenten in een veilige, perfect uitgeruste omgeving te laten plaats vinden. Een expert in eigen gebied met een passie voor zijn vak en wetenschappelijk onderzoek, buitengewoon bewonderenswaardig! Natuurlijk wil ik ook **Jan, Kim, Katrien, Ines, An, Frank en Toon** van harte bedanken voor de talrijke, gezellige babbels in het ReGo. Ook al was ik een 'buitenstaander', ik heb me steeds welkom gevoeld en was zeer blij om deel te mogen uitmaken van jullie ROPARUN-team (#blijdatjeinmijnteamzit)!

In de eerste fase van mijn promotietraject heb ik het voorrecht gehad om samen te werken met de afdelingen Abdominale Heelkunde van het Jessa Ziekenhuis en het Ziekenhuis Oost-Limburg. Daarom een welgemeende dankjewel aan **dr. Knol, dr. Bouckaert, dr. Dams, dr. Vangertruyden, dr. Houben en dr. Verhelst. Sandra, Peggy en Marleen**, bedankt voor het steeds tijdig aanleveren van jullie patiëntgegevens!

Anne, Frank en Ines, jullie hebben ook een groot aandeel in dit verhaal. Onze samenwerking zat steeds goed, ik kon telkens op jullie hulp en expertise rekenen wanneer er weer eens een bloedafname of een biopt moest gebeuren. Bedankt!

Mijn paranimfen. **Inez**, je was degene bij wie mijn onderzoeksloopbaan is begonnen. Als student BMW wist je me in 2012 te overtuigen om met dit doctoraatsproject te starten, bedankt daarvoor! We hebben een lange, maar gezellige, tijd samen de bureau gedeeld (samen met **An**), waar je ook altijd klaar stond om een handje te helpen wanneer het nodig was. Het was een leuke tijd en ik ben blij dat je, mede in functie als mijn paranimf (ook al is dit voor ons Belgen een nieuwigheid), samen dit hoofdstuk met mij mag afsluiten! Ik hoop dat we nog veel contact zullen houden! **Rudi**, we hebben mekaar vooral beter leren kennen in de tweede fase van onze PhD's. Gelukkig, want je was (en bent nog steeds) een topcollega! Tijdens onze gezamenlijke studie was het een plezier om steeds aan een testdag te starten, ook al was het weer vroeg in de ochtend. Ook onze belevenissen tijdens de ECO zullen altijd bij blijven (Gothenburg Memories) ;) Bedankt voor je bijdrage in dit geheel! **Inez en Rudi**, ik wens jullie alle geluk en veel succes toe in jullie verdere leven!

Mijn CRI collega's. De afgelopen 4 (en zelf 5, als ik terug denk aan mijn laatstejaarsstage als student BMW) jaren waren fantastisch! Niet alleen op professioneel vlak vormen we een hecht team waarin iedereen steeds voor mekaar klaar staat en waarbij geen inspanning teveel is, maar ook daarbuiten zijn jullie allemaal topmensen! De gezellige CRI-etentjes, de vrijdagavond borrels en verschillende andere randactiviteiten maakten deel uit van het geheel, een betere werkplek kan ik me niet wensen. Bedankt **Inez, An, Jana, Lisa, Charly, Chris, Pieter, Dominique, Martijn** en **Bert!**

Mijn collega's van HB. Mijn tijd bij HB was een geweldige ervaring. Als nieuweling werd ik meteen in jullie groep opgenomen. Gezien mijn beperkte expertise werd ik tijdens de afgelopen jaren omringd door experts in hun domein, waarbij jullie me met raad en daad bijstonden wanneer ik weer een (voor mij) nieuwe analyse moest uitvoeren. Vooral de inzet van onze gezellige en über sympathieke laboranten **Nicole** en **Yvonne** zijn van onschatbare waarde. Congressen werden ervaren als citytrips, waar we memorabele momenten beleefden (en hopelijk nog zullen beleven)! Bedankt **Rudi, Johan, Gijs, Nicole, Yvonne, Max, Birgitta, Dorien, Emmanuel, Ruth, Jasper, Mattea, Ellen, Manuel, Adriyan** en **Rens** voor de leuke tijd!

Graag wil ik ook de leden van de **vakgroep RWK**, met in het bijzonder **Peter** en **Monique**, bedanken voor hun vertrouwen en steun. Ook alle andere **REVAL-collega's** wil ik hierbij bedanken voor de geweldige tijd die we samen hebben beleefd. De sfeer onder de 'jonge garde' op de eerste verdieping van gebouw A was altijd top. Tussen de wetenschappelijke discussies door was er steeds ruimte voor een grap, een drink of een collectieve sportprestatie. Bedankt **Jonas, Anouk, Rob, Wout, Lousin, Steffi, Fanny, Deborah, Liesbet, Tom, Ilse L, Bart, Ilse B, Inez, Joke, Koen, Lotte** en natuurlijk mijn eigen **bureaumaatjes van A1.06!**

De mensen van **Universitaire Biobank Limburg** verdienen natuurlijk ook een woord van dank. **Dr Rummens, Loes, Tine**, bedankt om steeds klaar te staan en me verder te helpen bij de opslag van onze stalen of het uitvoeren van onze analyses. Ook een dank u wel aan alle **laboranten** van het **klinische labo** van

het Jessa Ziekenhuis om altijd met een glimlach onze stalen te verwerken en ons tijdig de nodige uitslagen te bezorgen.

Geen onderzoek zonder toplaboranten! Mijn dank gaat daarom ook uit naar **Jos, Wendy, Nicole, Yvonne, Antoine** en **Joan** om steeds hun expertise te delen met mij en de gevraagde analyses in een recordtijd uit te voeren! Ook bedankt aan **Paul**, je zorgde steeds voor de nodige voorraad stikstof zodat ik vroeg in de ochtend steeds kon starten met mijn experimenten! Ik heb veel van jullie geleerd, dit zal ik de rest van mijn loopbaan meedragen!

Deze thesis was niet tot stand gekomen zonder de vrijwillige deelname van mijn **proefpersonen**. Ik ben me er van bewust dat er veel van jullie werd gevraagd: bloed, zweet, vet, spier, scans enz. Wat voor mij vanzelfsprekend was, was voor jullie iets nieuws. Graag wil ik alle deelnemers bedanken voor hun inzet en vrijwillige deelname aan al onze studies!

Een warm en verwelkomend secretariaat is eveneens deel van de geoliede machine en is dus van groot belang geweest tijdens de totstandkoming van deze thesis. **Anne, Maya, Veronique, Rani, Desiree, Cleo en Yolanda**, jullie weten op ieders probleem een oplossing te vinden. Jullie zijn de *matresfamilias* van de afdelingen waar je altijd terecht kan, ook al is het maar voor een korte (maar gezellige) babbel! Bedankt!

Doorheen de afgelopen promotiejaren was er op tijd en stond nood aan afleiding, om alle zorgen van het onderzoek en de deadlines even te vergeten en de gedachten te verzetten. Hier hebben heel wat vrienden hun steentje toe bijgedragen, ook al moesten ze telkens opnieuw hetzelfde verhaal aanhoren. Bedankt aan alle **BMW-vrienden**, **'De mannen van Biomedische'** en alle **badminton-** en **niet-badmintonvrienden!**

Ten slotte wil ik mijn familie graag dubbel en dik bedanken voor de steun en raad doorheen de afgelopen jaren, maar ook al de jaren die hieraan vooraf zijn gegaan. Ik zou hier nooit gestaan hebben zonder jullie! Jullie, en ook mijn schoonfamilie, waren ook steeds bereid om de jaarlijkse bbq's en verjaardagsfeestjes op een moment in te plannen zodat ik er toch steeds kon bij

zijn, dit heb ik altijd weten te appreciëren! **Mama, Papa, Bomma, Oma, Bompa, Opa, Pa** en **Kimberley**, ook al hadden jullie vaak geen idee waarmee ik al die tijd precies mee bezig was, jullie waren altijd oprecht geïnteresseerd, trots en wisten me steeds te motiveren. Jullie deden me altijd weer opnieuw beseffen dat er sommige dingen en vooral sommige personen belangrijker zijn dan werk. BEDANKT!

Liefste **Anne**, voor u alleen maar de allergrootste woorden van dank. Bedankt voor je begrip als ik 's avonds of in het weekend moest/wilde werken of wanneer ik weer voor enkele dagen naar het buitenland moest. Nooit is iets je teveel, je staat altijd voor mij (en alle anderen) klaar. Het gezegde "achter elke man staat een sterke vrouw" is je op het lijf geschreven, getuige hiervan je onvermoeibare inzet om ons toekomstig huisje in orde te krijgen (ook al had ikzelf hier het afgelopen jaar jammer genoeg weinig tijd voor)! Bedankt voor je liefde en om er altijd te zijn! Binnenkort is het onze beurt om te gaan genieten in ons stulpje op de Bosstraat!