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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Does the fat cell listen to what the heart tells in human obesity? Role of atrial natriuretic peptide in fat mobilization and metabolic health in human obesity

Promotor :
Prof. dr. Dominique HANSEN

Berten Froyen

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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FOREWORD

Personally, I want several persons for giving me to opportunity to be part in this study. It has been an amazing experience. I am grateful that I finally could experience the clinical aspect of biomedical research. Therefore, my gratitude goes to promotor Prof. Dr. Dominique Hansen to entrust me with this project. Furthermore, I especially want to thank Drs. Kenneth Verboven for the daily supervision and valuable feedback and comments during the preparation of this thesis. Moreover, I also thank my colleague student for the nice collaboration and advice he has given me regarding several aspects of this thesis. Lastly, I wish to thank all participants of this study.

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LIST OF ABBREVIATIONS

ABBREVIATION	DESCRIPTION
ANP	Atrial natriuretic peptide
AT	Adipose tissue
ATBF	Adipose tissue blood flow
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BF	Breathing frequency
BMI	Body mass index
BNP	B-type natriuretic peptide
cAMP	Cyclic adenosine monophosphate
CNP	C-type natriuretic peptide
CO_2	Carbon dioxide
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DMEM	Dulbecco's Modified Eagle's Medium
ECG	Electrocardiogram
FFA	Free fatty acids
h-ANP	Human ANP
HbA_{1c}	Glycated haemoglobin
HOMA-IR	Homeostasis model assessment index for insulin resistance
HR	Heart rate
HSL	Hormone-sensitive lipase
IR	Insulin-resistant
IRS	Insulin receptor
IS	Insulin-sensitive
LDL	Low-density lipoprotein
MGL	Monoacylglycerol lipase
mRNA	Messenger ribonucleic acid
NPR-A	Guanylyl cyclase linked ANP receptor
NPR-C	Scavenging ANP receptor
NS	Not significant
O_2	Oxygen
OGTT	Oral glucose tolerance test
PDE3B	phosphodiesterase-3B
PKA	Protein kinase A
PKB	Protein kinase B
RAAS	Renin-angiotensin-aldosterone system
RER	Respiratory exchange ratio
RNA	Ribonucleic acid
RPE	Ratings of perceived exertion
rpm	Revolutions per minute
RT-PCR	Real-time polymerase chain reaction
SBP	Systolic blood pressure
SCAT	Subcutaneous adipose tissue
SEM	Standard error mean
T2D	Type II diabetes
TAG	Triacylglycerol
VAT	Visceral adipose tissue
V_E	Expiratory volume
VO_{2max}	Maximal oxygen uptake
VO_{2peak}	Peak oxygen uptake
WHO	World Health Organization
WHR	Waist-hip ratio
wks	Weeks
W_{max}	Maximal cycling resistance

SAMENVATTING

Introductie: Atriaal natriuretic peptide is onlangs geïdentificeerd als een belangrijke stimulator van de lipolyse. Echter, in de obese populatie, lipolyse ten gevolge van ANP is verstoord. Dit fenomeen limiteert dat trainingsprogramma's een gewichtsverlies te veroorzaken om zo ontwikkeling van secundaire ziektes te blokkeren. Echter, recent is het aangetoond dat een 4-maanden durend trainingsprogramma de ANP-geïnduceerde lipolyse herstelde in het subcutaan vetweefsel van mensen met overgewicht. Om deze reden veronderstellen we dat een gelijkaardig trainingsprogramma ook de ANP-geïndiceerde lipolyse kan herstellen in mensen met obesitas.

Material & methoden: Dertig obese mannen van een middelbare leeftijd werden gerekruteerd. Voor en na het doorlopen van het trainingsprogramma, werden ze gescreend om cardiometabole gezondheid te bepalen (OGTT, uithoudingstest, DXA scan). Verder werd ook de ANP- eïnduceerde lipolyse opgemeten tijdens rust en inspanning via microdialyse.

Resultaten: Initieel hadden obese mannen een hoger vetmassa dan slanke personen. Vetvrije massa was gelijkaardig. Na het voltooien van het trainingsprogramma was de hoeveelheid vetmassa significant gedaald in obese mannen. Verder zagen we ook een verbetering van de fysieke conditie (hoger vermogen, O_2 opname alsook een hoger CO_2 eliminatie).

Discussie & Conclusie: Samenvattend, training interventie verbeterde aanzienlijk de cardiometabole gezondheid van mannen met obesitas, waardoor de kans op secundaire complicaties verlaagd werd. Desondanks blijft het onduidelijk of het trainingsprogramma ook de ANP-geïnduceerde lipolyse verhoogt, aangezien deze stalen nog niet geanalyseerd waren. Desalniettemin sluiten onze bevindingen dicht aanleunen bij een gelijkaardige studie in mensen met overgewicht, waarin een verhoogde ANP-geïnduceerde lipolyse werd waargenomen na training. Dit impliceert dat onze training misschien ook de ANP-geïnduceerde lipolyse verhoogd. Dit zal nog bepaald worden in komende weken.

ABSTRACT

Introduction: In the past decade, atrial natriuretic peptide (ANP) has been identified as an stimulator of lipolysis. However, in obese population, ANP-mediated lipolysis is blunted, thereby limiting the clinical effectiveness of training programs that aim to stimulate weight loss to prevent the development of secondary comorbidities (diabetes, cardiovascular disease and cancer). However, recently, it has been shown that a 4-month endurance training program restored ANP-mediated lipolysis in the subcutaneous adipose tissue (SCAT) of overweight subjects. We hypothesized that such a training program also enhances ANP-induced lipolysis in the SCAT of obese subjects.

Material & methods: Thirty middle-aged men (10 lean, obese insulin-sensitive and obese insulin-resistant) were recruited. Prior and after 4-months combined training (3 days/wk, endurance and strength at 65% $VO2_{max}$), subjects were screened (glucose tolerance test, DEXA scan and maximal endurance test) to assess their cardiometabolic health and physical fitness. ANP-mediated lipid mobilization in the SCAT was determined at rest, during exercise and recovery by *in situ* microdialysis.

Results: Prior exercise intervention, adipose tissue mass was significantly higher in obese subjects compared to lean controls, whereas lean tissue mass was equal. No baseline differences regarding physical fitness were present. At this moment, 11 obese subjects (4 insulin-sensitive and 7 insulin-resistant) have completed the training protocol. Overall, combined training significantly lowered adipose tissue mass in obese subjects, whereas lean tissue mass remained unchanged. Additionally, workload and CO_2 elimination significantly increased, indicating an improved aerobic capacity.

Discussion & Conclusion: Combined training improved physical fitness and cardiometabolic health of obese subjects, thereby decreases the chance of secondary comorbidities. However, it remains uncertain if these improvements are related to an enhanced ANP-responsiveness because microdialysis samples are not yet analyzed. But so far, our findings are in line with the study done in overweighed persons, which enlighten that ANP could be responsible. However, this will be checked in the near future.

1. INTRODUCTION

Obesity is a metabolic disease in which body fat accumulates in adipose and non-adipose tissues to the extend it may have a detrimental impact on health (1, 2). Generally, the degree of adiposity is expressed as the body-mass index (BMI), defined as weight in kilograms divided by height in square metre (kg/m²) (3). According to World Health organization (WHO) classification, a BMI from 25.0 to 29.9 kg/m² corresponds with overweight, while persons with BMI ≥ 30 kg/m² are classified as obese (Fig. 1) (4). However, the validity of the BMI scale is questioned, because it does not correct for inter-individual muscle mass differences. Waist circumference and waist-to-hip ratio (WHR) have proven to be better markers to determine an individual's risk of obesity-related comorbidities (3).

WHO Classification	BMI (kg/m²)
Underweight	< 18.50
Normal range	18.50–24.99
Overweight	25.00–29.99
Obesity class I	30.00–34.99
Obesity class II	35.00–39.99
Obesity class III	≥ 40.00

FIGURE 1. World Health Organization (WHO) classification for obesity based on the body mass index (BMI = weight in kilograms divided by height in square metre) (4).

1.1. Obesity: the major health problem of the 21th century

Over the last decades, worldwide prevalence of obesity has increased dramatically in both developed and developing countries. In 2015, the WHO estimated that 13% of the adult world population was obese (3, 4). A complex interplay between genetic and lifestyle/environmental factors is thought to be responsible for this negative evolution (3). Evolutionary, the biological processes controlling our eating behaviour have adapted to effectively store any food surpluses in times of food shortage to obtain a survival advantage. This concept is called the "thrifty genotype" (3, 5). However, in the modern timeframe, this genotype is deleterious as environmental and societal changes (urbanization, agriculture, marketing, etc.) have led to a permanent food abundance and increased consumption of energy-dense food sources, rich in fat and sugars, leading to an excessive energy uptake (3, 6). Additionally, the average amount of daily physical activity has been decreased over the past decades in the population, resulting in a decreased basal energy expenditure (3, 6). Together, the increased food intake and lowered physical activity evoke an imbalance between the energy uptake and expenditure, creating an energy overload inside the body (Fig. 2) (3, 6, 7).

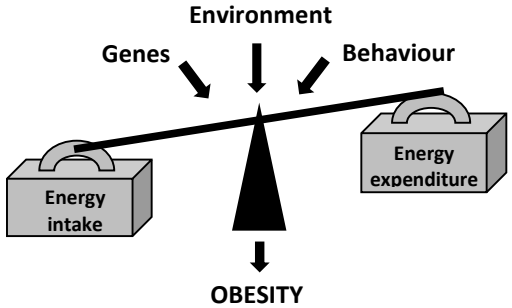

FIGURE 2. Factors contributing to obesity aetiology. Adapted from Markus A, 2005 (3)

Excessive amounts of energy are mainly stored in the white adipocytes of the subcutaneous adipose tissue (SCAT) and visceral adipose tissue (VAT) by a process called lipogenesis, which causes them to increase in size (7). Enlarged or hypertrophic adipocytes secrete an excessive amount of free fatty acids (FFAs) which are deposited in ectopic, non-adipose tissues (7, 8).

Ectopic FFA accumulations are the major inducers of the development of secondary obesity-related comorbidities. Ectopic FFA accumulations interfere with peripheral insulin sensitivity in liver, skeletal muscle and pancreas, causing an insulin-resistant state in obesity. Furthermore, FFA accumulations also induce hyperglycaemia by inhibiting insulin-mediated suppression of hepatic gluconeogenesis as well as insulin-mediated glucose uptake at the level of skeletal muscles cells (9). To neutralize the hyperglycaemic state, pancreatic β-islets insulin production escalates (i.e. hyperinsulinemia). However, over time, pancreatic FFA accumulations will induce β-cell dysfunction, which could result in the development of type 2 diabetes mellitus (T2D). Moreover, ectopic FFA accumulations have also been linked to other comorbidities including hypertension, stroke, cardiovascular disease (CVD) and several types of cancer (2, 3, 7).

1.2. Fat mass reduction needs efficient lipid mobilization

In the prevention and care of people with obesity, exercise interventions to reduce fat mass and restore insulin sensitivity are required, in addition to diet interventions, in order to prevent the development of obesity-related diseases (7, 10, 11). Fat mass reduction can only be achieved when sufficient lipid mobilization from adipose depots occurs, a process stimulated upon exercise (11). During physical activity, stored triacylglycerol (TAG) molecules inside the adipose tissue depots become hydrolysed into one molecule of glycerol and three FFA molecules by enzymes called lipases (i.e. monoacylglycerol lipase [MGL], hormone-sensitive lipase [HSL] and adipose triglyceride lipase [ATGL]) (Fig. 3) (8, 12). FFAs are then distributed via the systemic circulation to peripheral muscles where they become oxidized to produce adenosine triphosphate (ATP), the body's' major energy source. This process is tightly regulated by lipolytic (stimulatory) and lipogenic (inhibitory) hormones (8, 12, 13). The lipolytic rate is de facto dependent on (changes in) the ratio between lipolytic and lipogenic hormones (13).

Insulin, catecholamines and natriuretic peptides are considered to be the key regulators of adipose tissue lipolysis (8, 14). During fasting or exercise, among the factors that stimulate lipolysis, adrenal catecholamines (i.e. epinephrine and norepinephrine) are the most powerful (14). The lipolytic actions of catecholamines are mediated by selective binding to three different stimulatory G-coupled β-adrenoceptors which are located on the adipocyte cell membrane: β1, β2 and β3 adrenergic receptor (8, 12). Whereas the β3 receptor is barely expressed in human adipocytes, the β1 and β2 receptors are widely expressed in human tissues, including the adipose tissue depots (12, 13). Selective binding of catecholamines to adipose adrenergic receptors activates adenylate cyclase which increases intracellular levels of cyclic adenosine monophosphate (cAMP) (8, 12-14). Intracellular cAMP activates protein kinase A (PKA) which subsequently phosphorylates HSL and perilipin in order to trigger the breakdown of TAGs into glycerol and FFAs (Fig. 3) (8, 12-14).

Besides its lipolytic function, catecholamines also exert anti-lipolytic effects (12-14). Human adipocytes also express inhibitory G-coupled α2 receptors on their cell membrane, which degrades cAMP upon catecholamine binding (12, 13). Therefore, the magnitude of the lipolytic response is dependent of the β/α2 receptor expression ratio (12, 13). In this regard, the rate of lipolysis in the different adipose tissue depots differs due to an alternative adrenergic receptor distribution. For example, in lean subjects, the lipolytic rate is bigger in the VAT compared to the SCAT due to a predominating expression of β-adrenergic receptors in the VAT, while α2-adrenergic receptors are rather abundant in the SCAT (11). However, it must be kept in mind that the greatest absolute change in fat mass is still obtained in the SCAT since this is the greatest adipose tissue depot inside the human body (11).

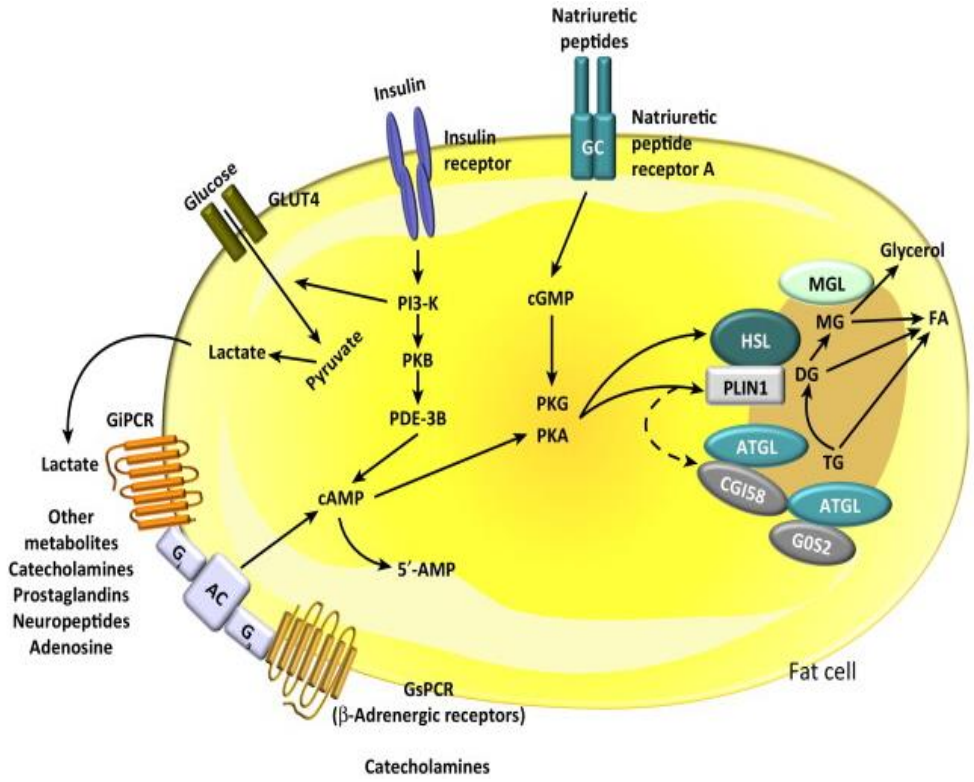

FIGURE 3. Overview of the hormonal regulation of the lipolysis in adipocytes. (15)

Besides catecholamines' inhibitory lipolytic functions, insulin is the most potent anti-lipolytic hormone (8, 12, 14). Insulin initiates a PI3K/PKB pathway in adipocytes after selective binding to membrane insulin receptors (IRS). Activated protein kinase B (PKB) acts by phosphorylating phosphodiesterase-3B (PDE3B), which subsequently suppresses adipose tissue lipolysis by catalysing the intracellular cAMP levels and dephosphorylating lipases such as HSL (Fig. 3) (8, 12, 14).

Besides catecholamines and insulin, several other lipolytic (growth hormone, cortisol, tumor necrosis factor alpha) and anti-lipolytic (adenosine, neuropeptide Y) hormones and factors are involved in the regulation of adipose tissue lipolysis (8, 12). However, their role and mechanism of action are characterized to a lesser extent and are beyond the scope of this thesis.

1.3. Atrial natriuretic peptide, a new key player in lipid mobilization

For many years, it was accepted that catecholamines were the only activator of lipolysis. However, this concept has been questioned by studies reporting a significantly remaining lipolytic response in the SCAT during a local blockade of the β-adrenergic lipolysis pathway (16). Natriuretic peptides, including atrial, B-type and C-type natriuretic peptides (ANP, BNP and CNP) has been identified as strong primate-specific stimulators of lipolysis. ANP is the most potent (ANP > BNP >> CNP) (17).

ANP is a cardiac hormone synthesized, stored and secreted by atrial cardiomyocytes (17, 18). Under normal (non-pathological) conditions, secretion of ANP from the intracellular granules of the atrial cardiomyocytes is limited. However, when atrial stretching occurs, ANP secretion increases rapidly (17, 18). Potential stimulators of atrial distention are volume and pressure overload resulting from physical exercise or pathological conditions (heart failure, infarction, stenosis) (17, 18). In addition, ANP also rises in case of myocardial (exercise-induced) hypoxia and ischemia due to the presence of hypoxia-responsive elements in the promotor region of the ANP gene (17). Other triggers of ANP release include inflammatory cytokines, cold exposure and humoral hormones (catecholamines, oestrogens, thyroid, angiotensin II, endothelin-1) (17). A growing body of evidence have shown that ANP is an important regulator of the food intake, insulin secretion, skeletal muscle energy consumption (17, 19, 20).

Originally, ANP has long been exclusively regarded as a cardio-renal hormone since it plays a key role in maintaining cardiovascular homeostasis. ANP protects the heart from CVD evoked by volume and pressure overload such as heart failure, hypertension and cardiac remodelling (17-19). Moreover, ANP stimulates renal diuresis and natriuresis and inhibits the renin-angiotensin-aldosterone system (RAAS) by suppressing the renin and aldosterone secretion. Secondly, ANP also reduces the vascular tone through abridgement of sympathetic signalling and vasodilation of blood vessels, thereby normalizing the blood pressure. Lastly, ANP also protects the heart against cardiac hypertrophy and fibrosis through activation of antifibrotic and antihypertrophic pathways (17).

In 2000, the unexpected discovery of ANP as a regulator of the adipose tissue lipolysis has led to the hypothesis that the heart is a member of a complex network of endocrine organs which controls energy metabolism (17, 20). Previously, *in vitro* studies in rodents already discovered the presence of guanylyl cyclase linked (NPR-A) and scavenging natriuretic peptide receptors (NPR-C) on the adipocyte surface (21). In the same period, Sarzani et al. found that ANP receptors are expressed on human adipocytes as well (22). Despite the fact that ANP generated cyclic guanylyl monophosphate as second messenger in rodent adipocytes, no FFA mobilization was reported (22).

The lipolytic effect of ANP has been demonstrated for the first time by Sengenès et al. In an *in vitro* study on isolated human subcutaneous adipocytes of lean and obese subjects, ANP stimulated lipolysis (indicated by a significant increase in glycerol levels) to the same extent as isoprenaline (a non-selective β-adrenoceptor agonist) (23). These observations were confirmed *in vivo* by the means of *in situ* microdialysis experiments. ANP infusion in human SCAT induced a marked increase

in local dialysate and plasma glycerol levels as well as an increased local blood flow, resulting in an enhanced lipid mobilization and gluconeogenesis. Plasma glucose and serum insulin remained unchanged during ANP infusion. Additionally, ANP-induced lipolytic response has been shown to be unrelated to sympathetic nervous activation and PDE-3B inhibition (19). Fundamental studies regarding the underlying pathway elucidated that ANP activates HSL via an cGMP-dependent pathway, comparable to the cAMP pathway for catecholamines (24). Activation of the NPR-A receptor induces an intracellular increase of cGMP, which activates protein kinase G (PKG). PKG subsequently phosphorylates HSL and perilipin, triggering adipocyte lipid mobilization (Fig. 3) (17, 24). On the other hand, the NPR-C receptor induces internalization and degradation of ANP, leading to a reduced availability of ANP ligand which thereby limits the rate of lipolysis (17, 24). Overall, total lipid mobilization from adipocytes is determined by the relative expression of these receptors in adipose tissue depots (11).

1.4. ANP-induced lipid mobilization is blunted in obese subjects

The discovery of ANP as an important regulator of the lipid mobilization yielded some possibilities to develop new strategies for anti-obesity therapies. Currently, therapeutic options like exercise training and diet intervention (or the combination of both) are used to induce weight loss and to prevent the development or worsening of secondary cardiometabolic diseases (7, 10). However in clinical practice, exercise interventions are not always adequate and show a large inter-individual variability in its clinical effectiveness (25, 26). In this regard, it has already been known that obese patients display lowered systemic epinephrine and growth hormone release at rest and during exercise. In addition, circulatory insulin levels are elevated, a phenotype that will very likely contribute to a suppressed lipolytic response, especially at the level of the SCAT (13).

During the past years, it has been demonstrated that obese subjects have an ANP and BNP deficiency in rest and during exercise, resulting in an impaired natriuretic response or "natriuretic handicap" (17, 27). This ANP deficiency has a high clinical relevance in obesity as it may enhance cardiovascular risk as well as the development of insulin resistance and T2D (17). Additionally, it also may lead to a suboptimal lipid mobilization response. Therefore, it can be assumed that the lack of a significant fat mass reduction following anti-obesity treatment can be due anomalies in ANP secretion or metabolic function (i.e. ANP-mediated lipolysis), additional to the known endocrine disturbances systemically at the level of the SCAT (13, 27).

Although it is known that ANP induces SCAT lipolysis in lean/overweight subjects, it remains unknown to what extent ANP plays a role in lipid mobilization in human obesity (28). The aetiology of the disturbed ANP physiology in obesity is currently an enigma. Wang et al. formulated two possible explanations for the observed natriuretic handicap in obesity. In their first hypothesis, they stated that reduced circulatory ANP levels in obese subjects could be attributed to an impaired synthesis and/or secretion of natriuretic peptides from atrial cardiomyocytes (17, 27). Although data regarding this assumption is limited in humans, several animal studies in rodents support their assumption by showing cardiac mRNA expression of natriuretic peptides to be lowered in Zucker fatty rats (29) and *db/db* mice (30).

The second hypothesis states that the reduced ANP plasma levels are the consequence of an increased clearance of ANP by NPR-C receptors, which are overexpressed in the adipose tissue of obese subjects (17, 27, 28, 31). Otherwise, the enhanced protein NPR-C expression in obese subjects could be due to the increased adipose tissue mass (11, 17, 27). In general, the increased NPR-C expression limits the biological effects of ANP at the site of the adipose tissue depots (17, 27). Although the exact mechanism remains elusive, it has been suggested that food intake and insulin resistance are important regulators of the NPR-C expression. Consumption of a high-fat diet augmented NPR-C expression in rodent adipose tissue (17). Recently, it was shown that serum insulin levels positively correlated with the adipose tissue NPR-C mRNA expression, suggesting an important link between insulin sensitivity and the blunted ANP-induced lipid mobilization (28, 31, 32). In this regard, it has been demonstrated that differential improvements regarding cardiometabolic health are achieved in insulin-sensitive (IS) obese and insulin-resistant (IR) patients following clinical training intervention (25). It has been proposed that the varying outcomes after training intervention are largely dependent of the hormonal status of each obese patient (13). Therefore, it has been suggested that the selection of an effective training program can be improved by differentiating between IS and IR obese subjects, with a specific focus on the hormonal status of each patient. However, data regarding the regulation of the natriuretic peptide system in these obese subpopulations are currently limited. Therefore, our first objective will be to determine whether the ANP-induced lipid mobilization is altered in adipocytes derived from the SCAT and VAT of obese subjects with a different insulin sensitivity (IS, IR and diabetic subjects), compared to age-matched lean men.

1.5. Moderate exercise training to restore the metabolic functions of ANP?

In clinical practice, training programs are the golden standard to lose weight and to improve their cardiometabolic risk profile in obese subjects (17, 27). Training programs aim to increase total energy expenditure in such a manner that it overrides the energy uptake, and thereby stimulate fat mass loss (3, 11). Generally, the reduction in body weight after physical exercise results from a reduction in adipocyte size (11, 33). However, there still remains controversy which regional adipose tissue depot is preferentially targeted during physical activity. As mentioned earlier, the VAT has a higher sensitivity to adrenergic stimuli and therefore the fat mass reduction is relatively the biggest in the VAT (11). However, the greatest absolute fat mass reduction is achieved in the SCAT since this is the largest fat depot. Additionally, it appears that the abdominal SCAT is more responsive than other subcutaneous depots in men, making SCAT is the most important supplier of lipids as energy source during physical activity (34).

Besides an enhanced energy expenditure, physical activity also induce beneficial acute and long-term changes in the structure and function of the adipose tissue (Fig. 4) (11). Over the past decade, many studies highlighted that adipose tissue lipolysis increases during acute exercise in lean subjects, even at a low training intensity (35, 36). The increased lipolytic rate during physical activity is attributed to an exercise-induced elevation of the circulatory plasma catecholamine and ANP levels as well as a decreased serum insulin concentration (11, 13, 28, 31, 37).

However, as mentioned earlier, the plasma catecholamine concentration in obese subjects is blunted, resulting in a lowered basal lipolytic response (13, 27). Since acute endurance exercise enhance the adrenergic responsiveness in lean subjects during and post-exercise, it has been assumed that acute endurance training induces the same outcome in obese subjects. *In situ* microdialysis demonstrated that acute endurance exercise indeed enhances β-adrenergic stimulated lipolysis in the SCAT obese subjects, but the exercise-induced lipolytic response is significantly lower compared to their lean counterparts (13, 14, 38). It has been hypothesized that the difference in the exercise-induced lipolysis can be attributed to either an increased expression by α2-adrenoceptors and/or a reduced expression or function of β2-adrenoceptors and HSL (13, 39). Besides an increased lipolytic response, acute endurance exercise also stimulates the adipose tissue blood flow (ATBF) (11, 37), muscular fat oxidation (11, 36, 37) and whole-body insulin sensitivity (37) in both lean and obese subjects during and post-exercise. Additionally, acute physical exercise has also a positive impact on several blood parameters including the diastolic blood pressure, total cholesterol, LDL cholesterol and glucose concentration in both groups (11, 14, 37, 39).

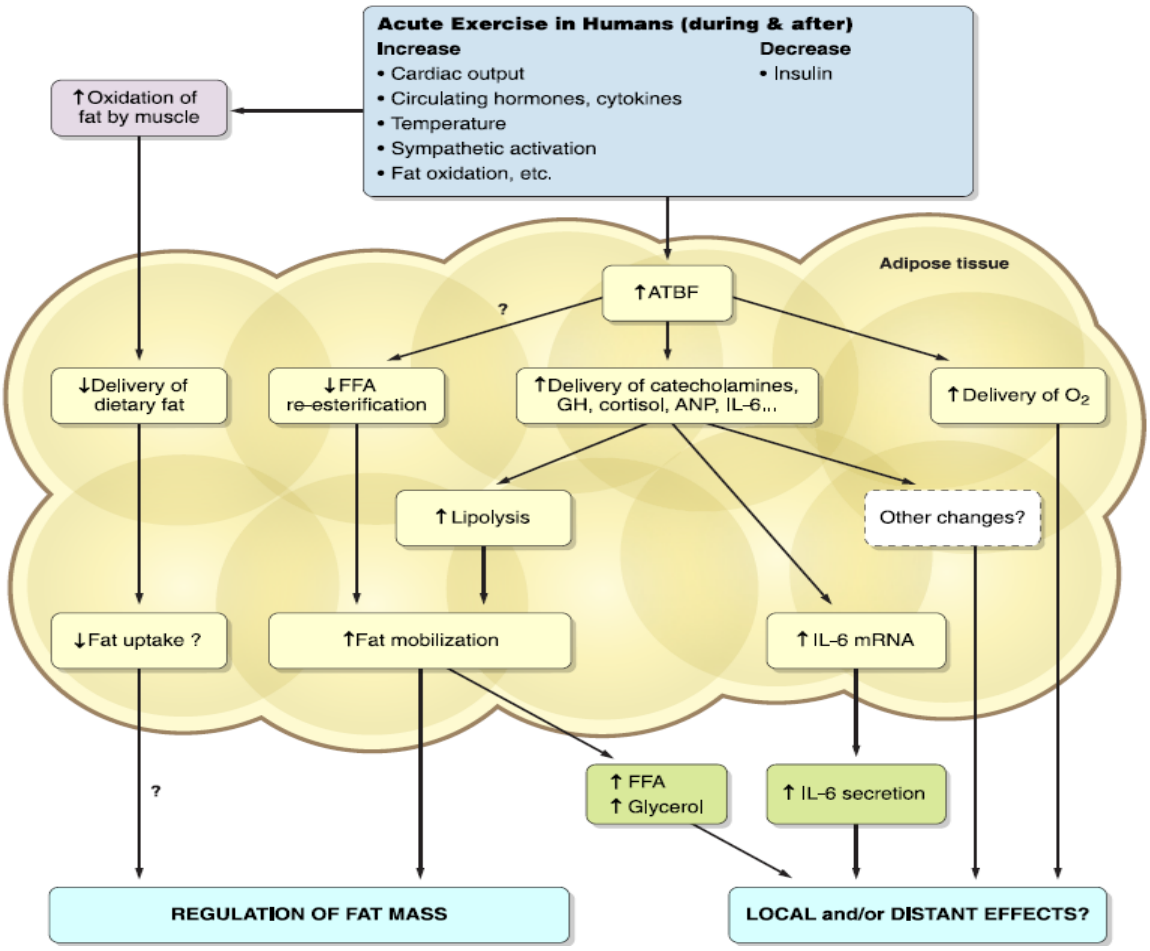

FIGURE 4. Schematic of the functional adaptations of acute physical exercise in the AT (11).

However, the adaptations in the structure and function of adipocytes in response to acute training are only transient and fades relatively fast in the post-exercise phase. In order to have a prolonged sustainment of acute adaptations, regular/chronic exercise (training) is required (11). It has been demonstrated that training induces a long-lasting increased fat oxidation and total energy expenditure in the post-exercise phase which is needed to maintain a negative energy balance (11, 33, 37, 40-42). Subsequently, this energy deficit increases the sensitivity of the adipose tissue to catecholamines and insulin, leading to an improved lipolytic response during and post-exercise (11, 33, 37, 40-42). Similar as acute exercise, training results in a long-lasting improvement of the ATBF (11, 37, 40) and the whole-body insulin sensitivity (41). Furthermore, it also shrinks the adipocytes to a smaller size which are more metabolically flexible (11). Lastly, regular physical activity also ameliorates blood plasma concentration of FFAs, glucose and cholesterol (37, 40).

Although the effects of training on the catecholamine function has been profoundly studied, less research has been examining the influence of training interventions on the metabolic effects of ANP in obese subjects. In 2005, Moro et al. demonstrated that a 4-month endurance training program enhanced the ANP-induced lipolytic response and ATBF in the SCAT of young overweight men (37). Additionally, aerobic training lowered resting respiratory exchange ratio (RER), plasma FFA, plasma glucose and low-density lipoprotein (LDL) levels and improved whole-body insulin sensitivity (37). However, the effect of such a training program has been only been limited examined in obese subjects. Therefore, the second objective of our project will be to investigate whether a 12-week combined training program (moderate aerobic endurance and strength training) enhances the ANP-induced lipid mobilization in the SCAT of IS and IR obese subjects.

It is imperative to understand whether the variability in adipose tissue mass loss originates from disturbances in ANP secretion/function in obese individuals. Such knowledge will very likely contribute to novel or improved therapies in the control/reduction of adipose tissue mass and hence improvement in cardiovascular and cardiometabolic disease risk factors in patients with obesity. In this present study, we hypothesize that IS and IR obese subjects have a differently blunted ANP-induced SCAT lipid mobilization which can be improved by a 12-week combined training program. Firstly, ANP-induced lipolysis and NPR expression was examined *in vitro* in the SCAT and VAT of middle-aged obese male subjects. In the second part, we assessed by *in situ* microdialysis if a 12-week moderate combined training enhances the ANP-mediated lipolytic response in SCAT of IS and IR male obese subjects.

2. METHODS

2.1. In vitro experiments

Subjects

A total of 52 Caucasian men between the ages of 40-65 years undergoing abdominal or bariatric surgery were recruited for the *in vitro* study. The decision to undergo surgery was made independently of this research protocol, between patient and physician. Selection of the participants and allocation to an experimental group (lean, obese IR or obese diabetic subgroup) was based on a screening evaluation of detailed medical history, BMI and glycated haemoglobin (HbA1c) levels. The obese IR group [49 ± 1 yr.; HbA_{1c} 5.65 ± 0.10% (38 ± 1 mmol/mol); n = 22] and obese diabetic group [52 ± 2 yr.; HbA_{1c} 7.29 ± 0.29% (56 ± 3 mmol/mol); n = 11] were selected for a BMI ≥ 30 kg/m² (37.8 ± 0.8 and 38.1 ± 1.1 kg/m² respectively). The control group consisted out of nineteen normal weight male volunteers (53 ± 1 yr.) with a BMI ranging from 20-25 kg/m² (23.8 ± 0.3 kg/m²) and HbA_{1c} index of 5.29 ± 0.05% (34 ± 1 mmol/mol). Presence of T2D was defined as HbA_{1c} ≥ 6.5% (45 mmol/mol) or the usage of glucose-lowering medication based on a clinical diagnosis. All selected volunteers had a stable body weight during the previous three months. 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 Hasselt University and Jessa Hospital. Prior to participation, all participants gave their informed consent (ClinicalTrial.gov accession number NCT02598544).

SCREENING TESTS

Anthropometric measurements

After an overnight fast (at least 10h fasting) and upon arrival at the hospital, several anthropometric and blood pressure measurements were performed. Body mass was measured to the nearest 0.1 kg, with the subject wearing no shoes, using a calibrated analogue weight scale. Height was determined with a stadiometer to the nearest 0.5 cm, while the subject standing in a complete upright position. The BMI was calculated as mass (kg) per square height (m²). The waist and hip circumference were measured using a measurement tape from which the WHR was calculated. Subjects' body composition (lean and adipose tissue mass) was determined using bioelectrical impedance analysis (Bodystat 1500, Bodystat Ltd., Isle of Man, UK).

Blood analyses

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 HbA_{1c}. Insulin sensitivity was assessed by the homeostasis model assessment index for insulin resistance (HOMA-IR), calculated from fasting glucose and insulin levels, according to the formula: fasting insulin (μU/l) x fasting glucose (mmol/l)/22.5 (43). 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 an 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).

Lipolysis measurements

Abdominal subcutaneous (SCAT) and omentus majus (VAT) adipose tissue biopsies were collected from all participants during abdominal surgery (under general anaesthesia). For each adipose tissue depot, approximately 1–5 g adipose tissue was collected. In order to obtain isolated mature adipocytes, a collagenase digestion was performed as described previously by Rodbell (44). Adipose tissue fragments were dissolved in Dulbecco's Modified Eagle's Medium (DMEM)-Ham's F12 under gentle shaking (60 cycles/min) at 37°C 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^{-4} M) or human ANP (h-ANP, 10^{-10} – 10^{-4} M) (Bachem, Weil am Rhein, Germany) in duplicate at a finale volume of 100 μl for 3h at 37°C. Following incubation, 60 μl cell free aliquots of the infranatant were collected for glycerol determination (lipolysis index) using the EnzyChromeTM Adipolysis assay kit (Gentaur, Eersel, The Netherlands). Glycerol release was expressed per 100 mg of lipids.

Quantification of NPR gene expression

Total adipose tissue RNA from isolated adipocytes 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 polymerase chain reaction (RT-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 1 μl forward and reversed gene primer (10 μM) for NPR-A and NPR-C (Biolegio, Nijmegen, The Netherlands). Gene expression was normalized using 18S ribosomal RNA. NPRA forward primer 5'-GCAAAGGCCGAGTTATCTACAT and reverse primer 5'-AAGAAAACGTAGTCCTCCCCA. NPRC forward primer 5'-AGACTACGCCTTCTTCAACATTG and reverse primer 5'-GCTTCAAAGTCGTGTTTGTCTCC.

Statistical analyses

All data are presented as mean ± SEM. Normality of each parameter was checked using Shapiro-Wilks, followed by an equal variance check using Brown-Forsythe tests. These tests indicated that the assumptions for parametric testing (normal distribution, equal variances) were unfulfilled for our data. Therefore, group differences were analysed using nonparametric Kruskall-Wallis tests followed by post-hoc Mann-Withney *U* tests. Depot differences within groups were analysed using Wilcoxon Signed rank tests. Statistical analyses were performed using JMP pro 12 for Windows 10. The level of statistical significance was set at $P \leq 0.05$.

2.2. In vivo experiments

Subjects

For the *in vivo* part, a total of 25 Caucasian aged 35 to 60 years were enrolled via advertisements in local newspapers and flyers in local hospitals and general practitioners. Thirty volunteers were selected and allocated to an experimental group (lean controls, IS obese or IR obese group) based on a detailed screening for their medical history, medication use, anaesthetics intolerance by the mean of questionnaires. Additionally, several anthropometric measurements (height, weight and waist/hip circumference), blood pressure measurement and an oral glucose tolerance test (OGTT) were performed to calculate their BMI and insulin sensitivity (HOMA-IR) respectively. The obese IS group (47 ± 2 yr.; HOMA-IR 2.15 ± 0.16; n = 10) and obese IR group (44 ± 2 yr.; HOMA-IR 4.78 ± 0.48; n = 10) were selected for a BMI ≥ 30 kg/m² (32.6 ± 0.4 and 34.0 ± 0.8 kg/m², respectively), while the BMI of lean subjects ranged between 20-25 kg/m² (24.0 ± 0.7 kg/m², 45 ± 5 yr.; HOMA-IR 1.81 ± 0.22; n = 5). All selected participant had a stable body weight during the previous three months. Major exclusion criteria were presence of a history of heart, lung or kidney disease and/or presence of endocrine anomalies. Additionally, subjects were excluded from the study if any orthopaedic, neurological problems or a history of coronary events and/or revascularization that could possibly interfere with exercise training. A summary of the group characteristics and inclusion/exclusion criteria is shown in the supplementary figure S1. The study was approved by the medical ethical committee of Hasselt University and Jessa Hospital. Prior to participation, all participants gave their informed consent after detailed explanation of the nature and the risks of the experimental procedures. The procedures were carried out in the Jessa Hospital and the BIOMED-REVAL institute at University Hasselt.

Experimental design

The trial was registered at www.clinicaltrials.gov (*identifier:* NCT02418728). Before and after the 3-month training protocol, all measurements and tests were carried out in the laboratory. Anthropometric parameters (adipose mass and lean mass) were measured using a total-body dual-energy x-ray absorptiometry (Hologic Series Delphi-A Fan Beam X-ray Bone Densitometer, Vilvoorde, Belgium). Respiratory gas exchange was measured by indirect calorimetry. (Sub)maximal oxidative exercise capacity test were performed and lipolytic response in SCAT and local blood flow responsiveness were assessed in situ by the microdialysis method. Participants were allocated to a specific experimental group based on their BMI and insulin sensitivity (determined by HOMA-IR). Therefore, neither participant nor the principle investigators could be blinded. Obese subjects were enrolled in a 12-week combined exercise program whereas lean controls did not participate in any training program. All tests were repeated for the obese subjects after completion of the training program. The primary outcome measure in this study was the SCAT lipolytic response during rest, exercise and recovery in basal conditions as well as under α and β-blockage. Body composition, maximal oxygen consumption, cardiovascular function, anthropometric characteristics and blood parameters (lipids, glucose, and insulin) were assessed and reported as secondary measures. A flow-chart of the study set-up can be found in supplementary figure S2.

Training protocol

For a period of 12 weeks, obese subjects were asked to visit the rehabilitation centre with a frequency of three training sessions per week. All sessions were supervised by the principle investigator or a research assistant. Exercise adherence (98%) and physical performance were monitored on each training session. Each session consisted of a 45-minute endurance bout (cycling) of which the intensity is compiled during the maximal cardiopulmonary exercise test (~65% of VO_{2peak}). Exercise intensity will be monitored continuously by means of heart rate registration (T31 coded transmitter, Polar, Kempele, Finland). The second part of a training session consisted of resistance training exercises (leg press, leg curl, leg extension, chest press, vertical traction and arm curl). Leg resistance training was performed unilaterally to avoid compensatory actions resulting from bilateral strength differences. To improve muscle fitness, sets of repetitions gradually increased during training intervention from 1 x 10 repetitions to 4 x 10 repetitions at a maximal attainable load with short resting periods of rest (± 1 min). The number of repetitions was determined beforehand, whereas the intensity was individually determined at the beginning of the training (~65% of the 1-repitition maximum). If possible, subjects were encouraged to increase the weight. At the end of the session, body weight and the ratings of perceived exertion (RPE) on a 20-point Borg scale were recorded. Food and calorie intake were not modified during the training protocol.

SCREENING TESTS

Index of insulin resistance or sensitivity

Prior training intervention, an OGTT to subjects' glucose tolerance (HOMA-IR). Subjects reported to our research facility following a 10-12 h overnight fast. Blood was collected from the antecubital vein. After a baseline blood sample (5mL fasting glucose and insulin), a 75 g glucose bolus was ingested. After 2h, a second blood sample was taken to determine subjects' 2h-glucose. The HOMA-IR was calculated as fasting insulin (μU/l) x fasting glucose (mmol/l)/22.5 (43).

Anthropometric measurements and body composition

After the OGTT, anthropometry was performed. Body mass was measured to the nearest 0.1 kg, with the subject wearing no shoes, using a calibrated analogue weight scale. Height was determined with a stadiometer to the nearest 0.1cm, while the subject standing in a complete upright position. BMI was calculated as body mass (kg) per square height (m^2). Waist and hip circumference were measured using a measurement tape from which the WHR was calculated. Body composition was determined using a duel energy x-ray absorptiometry scan (Hologic Series Delphi-A Fan Beam X-ray Bone Densitometer, Vilvoorde, Belgium). Adipose tissue mass and lean tissue mass were obtained for the whole body, legs, arms, trunk, gynoid and android region. Waist/hip fat mass ration (android fat [g]/ gynoid fat [g]), fat mass of the trunk/fat mass of the limbs and fat mass/height squared were automatically calculated.

Maximal oxygen consumption

Maximal oxygen uptake (VO_{2max}) was determined during a graded cycling test (cycling frequency: 70 rpm) on an electronically braked cycle ergometer (eBike Basic, General Electric GmbH, Bitz, Germany) with pulmonary gas exchange analysis (Jaeger Oxycon, Erich Haeger GmbH, Germany). Jaeger calibration (ambient conditions, volume calibration and O_2/CO_2 calibration) was performed at

the start of the endurance test. The cycling test started at 30W and was progressively increased by 15W every minute until exhaustion. Heart rate was continuously monitored using a 12-lead electrocardiogram (ECG) device. Maximal oxygen uptake as well as carbon dioxide removal (VO_{2max} and VCO_{2max}, ml/min), expiratory volume (V_E), breathing frequency (BF) and RER were collected breath-by-breath and averaged every 10 seconds. In addition, maximal cycling resistance (W_{max}), maximal heart rate (HR_{max}) were reported.

MICRODIALYSIS TRAIL PROTOCOL

Subjects were investigated at 7:30 AM after an overnight fast. The evening before the test day, all participants receive the same standardized meal (45 ± 1 kJ/kg, consisting of 33% of energy from carbohydrate, 47% from fat and 20% from protein). Upon arrival, two microdialysis probes (AURORA BOREALIS, Schoonebeek, The Netherlands) of 20 × 0.5 mm and 20-kDa molecular weight cutoff were inserted in the SCAT after local epidermal anesthesia (EMLA, AstraZeneca, Brussel, Belgium) at a distance of 10 cm to the right and left of the umbilicus. The probes were connected to a microperfusion pump (Harvard Apparatus; S.A.R.L., Les Ulis, France) and perfused at a rate of 2.0 μL/min with Ringer solution (147.2 mM sodium, 4.02 mM potassium, 2.24 mM calcium and 155.70 mM chloride). One probe (control) was perfused with Ringer solution, supplemented with ethanol (1.7 g/L) to estimate changes occurring in adipose tissue blood flow. The second probe was perfused with Ringer solution supplemented with respectively 100 μM phentolamine (a nonselective α-adrenergic antagonist; Novartis Pharma) and 100 μM propranolol (a nonselective β-adrenergic antagonist; Mibe GmhB) to block catecholamine-mediated lipolysis. After a 60-min calibration period, perfusion was set to a flow rate of 2.0 μl/min and 15-min fractions of the perfusate were collected during a 45-min resting period, a 1-hour exercise period (~40% VO_{2peak}) and a 1-hour recuperation period. Glycerol (used as lipolytic index) in dialysate (10 μl) was analyzed with ultrasensitive radiometry according to Bradley and Kaslow (45). Ethanol in dialysate and perfusate was determined with an enzymatic method. Additionally, SCAT biopsy were obtained at rest and after the exercise phase.

Blood analyses

Venous blood samples (18 mL) were collected from an indwelling polyethylene catheter inserted into the antecubital vein each 15 minutes during rest, exercise and recovery for the determination of metabolic and hormonal concentrations. Blood samples were immediately centrifuged at 3000 rpm at 4°C for 10 min. Plasma samples were collected, frozen in liquid nitrogen and stored at -80°C. Plasma samples were analysed using standardized accredited techniques. Plasma glucose (Uni Kit III, Roche, Basel, Switzerland), lactate, FFA (NEFA-C, Wako Chemicals, Neuss, Germany), TAG and free glycerol (148270, Roche Diagnostics, Indianapolis, IN, USA) concentrations were analyzed with a COBAS FARA semi-automatic analyzer (Roche). Insulin was analyzed by radioimmunoassay (Human Insulin RIA Kit, LINCO Research Inc, St. Charles, MO). Glycerol, glucose and lactate concentrations from the microdialysates were measured by bioluminescence after enzymatic oxidation of L-Lactate53.

Statistical analyses

All data are presented as mean ± SEM. Normality of each parameter was checked using Shapiro-Wilks, followed by an equal variance check using Brown-Forsythe tests. These tests indicated that the assumptions for parametric testing (normal distribution, equal variances) were unfulfilled for our data. Therefore, group differences were analysed using nonparametric Kruskall-Wallis tests followed by post-hoc Mann-Withney *U* tests. Additionally, the total area under the curve (AUC) was determined for each group using the trapezoid method. Depot differences within groups were analysed using Wilcoxon Signed rank tests. Pearson correlations were assessed to find relationship between plasma markers and the amount of lipid mobilization. Statistical analyses were performed using JMP pro 12 for Windows 10. The level of statistical significance was set at $P \leq 0.05$.

3. RESULTS

3.1. In vitro data

3.1.1. *Screening results*

Subjects' characteristics

A total of 52 male participants (mean age, 51 ± 1 yr.) were enrolled for the *in vitro* study of which 33 were obese (mean BMI, 37.9 ± 0.7 kg/m²). Lean subjects (n = 19) had a significantly lower body weight and BMI (77.4 ± 1.6 kg and 23.8 ± 0.3 kg/m²) compared to obese IR (123.0 ± 3.4 kg and 37.8 ± 0.8 kg/m²; $P < 0.001$) and diabetic subjects (117.1 ± 2.7 kg and 38.1 ± 1.1 kg/m²; $P < 0.001$). Moreover, lean subjects had a significantly lower WHR (0.97 ± 0.01; $P < 0.001$) compared to obese subjects (1.09 ± 0.01 and 1.09 ± 0.02 for obese IR and obese diabetics, respectively). Additionally, resting systolic blood pressures were significantly increased in the obese IR and obese diabetic subgroup (143 ± 3 and 147 ± 5 mmHg; $P < 0.01$) when compared to those of lean counterparts (130 ± 4 mmHg). No significant differences were found for weight, BMI and the resting diastolic blood pressure between both obese groups. Subjects' characteristics are depicted in Table 1.

Body composition

Adipose tissue mass, lean tissue mass as well as the fat percentage were significantly lower ($P < 0.001$) in lean subjects compared to obese counterparts. Body composition was comparable between the obese subpopulations (Table 1).

Glycemic control

Plasma glucose levels were significantly elevated in the obese IR (5.92 ± 0.27 mM; $P < 0.001$) compared to the obese diabetic subgroup (7.93 ± 0.68 mM; $P < 0.001$) and lean control group (5.47 ± 0.19 mM). Serum insulin concentrations were remarkably higher in obese IR and obese diabetic subpopulation (20.95 ± 2.25 mM and 30.27 ± 9.43 mM; $P < 0.001$) compared to lean subjects (8.73 ± 0.98 mM). Consequently, the HOMA-IR of obese subgroups (5.65 ± 0.72 and 11.55 ± 3.90 for obese IR and diabetics, respectively) was significantly higher compared to the lean group (2.19 ± 0.27 mM; $P < 0.001$). HbA1c levels of obese diabetics (56.18 ± 3.27 mM, 7.29 ± 0.29%) and obese IR subjects (38.35 ± 1.09 mM, 5.65 ± 0.10%) were significantly elevated ($P < 0.001$) compared to those of lean subjects (34.21 ± 0.61 mM, 5.29 ± 0.05%) (Table 1). According to the criteria of the American Diabetes Association, 34% of all participating subjects had an increased risk for the development of diabetes (HbA1c ≥ 5.7%) (46). In particular, 21% of all subjects (obese diabetics) possessed an impaired glucose tolerance (IGT), whereas 13% had an at-risk profile (one lean and six obese IR subjects).

Blood lipid profile

Blood analyses to determine subjects' lipid profile revealed significantly higher plasma FFA concentrations in the obese IR and diabetic population (759.15 ± 88.55 and 872.11 ± 100.43 mM; $P < 0.001$) compared to lean counterparts (295,89 ± 56.43 mM), while no significant differences in

plasma TAG was noticed between the lean, obese IR and obese diabetic group (1136.41 ± 88.55, 1273.73 ± 105.17 and 1270,74 ± 234.36 mM; $P > 0.05$). Plasma ANP levels were similar in lean and obese IR subjects (23.77 ± 1.41 and 25.41 ± 1.69 pg/mL; $P > 0.05$), but was significantly increased in obese diabetics (29.32 ± 1.76 pg/mL; $P < 0.05$) when compared to lean controls (Table 1).

TABLE 1. Subjects' characteristics

	Lean (*n* = 19)	Obese IR (*n* = 22)	Obese diabetics (*n* = 11)	*P*
General features				
Age (yr.)	53 ± 1	49 ± 1	52 ± 2	NS
Height (m)	1.80 ± 0.02	1.80 ± 0.02	1.76 ± 0.01	NS
Weight (kg)	77.4 ± 1.6	123.0 ± 3.4	117.1 ± 2.7	a,b
BMI (kg/m²)	23.8 ± 0.3	37.8 ± 0.8	38.1 ± 1.1	a,b
Waist circumference (cm)	90.9 ± 1.2	127.6 ± 1.9	126.2 ± 2.0	a,b
Hip circumference (cm)	93.3 ± 0.9	116.9 ± 1.8	116.0 ± 2.1	a,b
WHR	0.97 ± 0.01	1.09 ± 0.01	1.09 ± 0.02	a,b
SBP (mmHg)	130 ± 4	143 ± 3	147 ± 5	a,b
DBP (mmHg)	83 ± 2	83 ± 2	85 ± 3	NS
Body composition				
Adipose tissue mass (kg)	17.9 ± 0.9	45.0 ± 2.3	42.4 ± 2.0	a,b
Lean tissue mass (kg)	59.1 ± 1.4	77.4 ± 1.5	73.6 ± 1.3	a,b
Fat percentage (%)	23.2 ± 1.0	36.4 ± 0.9	36.7 ± 1.0	a,b
Glycemic control				
Glucose (mM)	5.47 ± 0.19	5.92 ± 0.27	7.93 ± 0.68	b,c
HbA_{1c} (mM)	34.21 ± 0.61	38.35 ± 1.09	56.18 ± 3.27	a,b,c
HbA_{1c} (%)	5.29 ± 0.05	5.65 ± 0.10	7.29 ± 0.29	a,b,c
Insulin (mM)	8.73 ± 0.98	20.95 ± 2.25	30.27 ± 9.43	a,b
HOMA-IR index	2.19 ± 0.27	5.65 ± 0.72	11.55 ± 3.90	a,b
Lipid profile				
FFA (mM)	295.89 ± 56.43	759.15 ± 88.55	872.11 ± 100.43	a,b
TAG (mM)	1136.41 ± 162.78	1273.73 ± 105.17	1270.74 ± 234.36	NS
ANP (pg/mL)	23.77 ± 1.41	25.41 ± 1.69	29.32 ± 1.76	b

Data are expressed as means ± SEM. P values were calculated using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests ($P < 0.05$).

a) Obese IR significantly different from lean subjects
b) Obese diabetic significantly different from lean subjects
c) Obese IR significantly different from obese diabetic subjects

Abbreviations: *IR, insulin-resistant; BMI, body mass index; WHR, Waist/hip circumference ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1c}, glycated hemoglobin; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; FFA, free fatty acids; TAG, Triacylglycerol; ANP, atrial natriuretic peptide; not significant NS; SEM, standard error mean*

Maximal isoprenaline-mediated lipolysis is equal in mature adipocytes derived from the SCAT and VAT of obese men compared to lean subjects

Basal glycerol release (basal lipolysis) at level of the SCAT (25.5 ± 8.5 vs. 15.5 ± 3.3 vs. 18.8 ± 4.7 μmol glycerol/100 mg TAG) and VAT was similar in all groups (54.9 ± 15.8 vs. 29.4 ± 6.2 vs. 33.5 ± 5.7 μmol glycerol/100 mg TAG), for lean, obese IR and obese diabetic, respectively.

Isoprenaline-induced stimulation of mature SCAT and VAT adipocytes enhanced the glycerol release in a dose-dependent manner in all three experimental groups (Fig. 5A and 5B) with maximal lipolytic responses obtained at an isoprenaline concentration of 10^{-6} M in both adipose depots. Maximal extracellular glycerol release from human SCAT adipocytes was comparable between groups (88.4 ± 24.9, 39.5 ± 9.2 and 71.6 ± 38.8 μM glycerol/100 mg TAG for lean, obese IR and obese diabetic subjects, respectively (Fig. 5A). Compared to basal values, extracellular glycerol release upon maximal stimulation from human SCAT adipocytes increased a 3.8 ± 0.3, 2.7 ± 0.2 and 2.5 ± 0.5 fold (obese diabetic significantly different from lean control; $p < 0.05$) (Fig. 5E).

Similarly as SCAT adipocytes, maximal lipolytic responses from mature VAT adipocytes were achieved at a isoprenaline concentration of 10^{-6} M (319.7 ± 120.2, 146.2 ± 52.6 and 159.5 ± 50.7 µM glycerol/100 mg TAG for lean, obese IR and obese diabetic subjects, respectively) (Fig. 5D). Extracellular glycerol release increased a 4.7 ± 0.4, 4.3 ± 0.4 and 3.7 ± 0.3 fold (lean, obese IR and obese diabetics, respectively) compared to basal values (not significant) (Fig. 5E). When comparing the isoprenaline-induced lipolytic fold increase between the adipose tissue depots, the maximal relative fold increase of extracellular glycerol in the SCAT was significantly ($P < 0.05$; data not shown) higher compared to the VAT in the obese IR group. No significant differences in lipolytic responses were noticed at any isoprenaline concentration between groups in the VAT and SCAT (Fig. 5A and 5B).

Maximal lipolytic response induced by h-ANP is attenuated in mature adipocytes derived from the SCAT of obese men

Incubation of mature adipocytes in h-ANP induced a significant dose- dependent enhancement of lipolysis in all three groups (Fig. 5C and 5D). Maximal lipolytic responses in the SCAT were obtained at an h-ANP concentration of 10^{-4} M (94.5 ± 20.6, 40.2 ± 9.1 and 71.6 ± 40.1 µM glycerol/100 mg TAG for lean, obese IR and obese diabetic, respectively) (Fig. 5C). Significant difference was noticed when comparing glycerol secretions of lean and obese IR subjects ($P = 0.043$). Compared to basal values, glycerol release increased a 4.9 ± 0.2, 3.0 ± 0.2 and 2.4 ± 0.6 fold for lean, obese IR and obese diabetic, respectively (Fig. 5E). Significant difference was noticed when comparing glycerol secretions of obese diabetics subjects ($P < 0.05$) with those of the lean controls.

Similarly as SCAT adipocytes, maximal lipolytic responses in VAT adipocytes were achieved at a h-ANP concentration of 10^{-4} M (194.7 ± 87.3, 59.5 ± 19.0 and 108.2 ± 34.9 µM glycerol/100 mg TAG for lean, obese IR and obese diabetic subjects, respectively) (Fig. 5D). Extracellular glycerol release increased a 3.0 ± 0.4, 2.2 ± 0.3 and 2.6 ± 0.3 fold (lean, obese IR and obese diabetics, respectively) compared to basal values (Fig. 5E). When comparing the h-ANP-induced lipolytic fold

increase between the adipose tissue depots, the maximal relative fold increase of extracellular glycerol in the SCAT was significantly ($P < 0.05$; data not shown) higher compared to the VAT in the control group. Additionally, significant differences ($P < 0.05$) in lipolytic responses were noticed at h-ANP concentrations between groups in SCAT adipocytes, starting from 10^{-6} M (Fig. 5A and 5B).

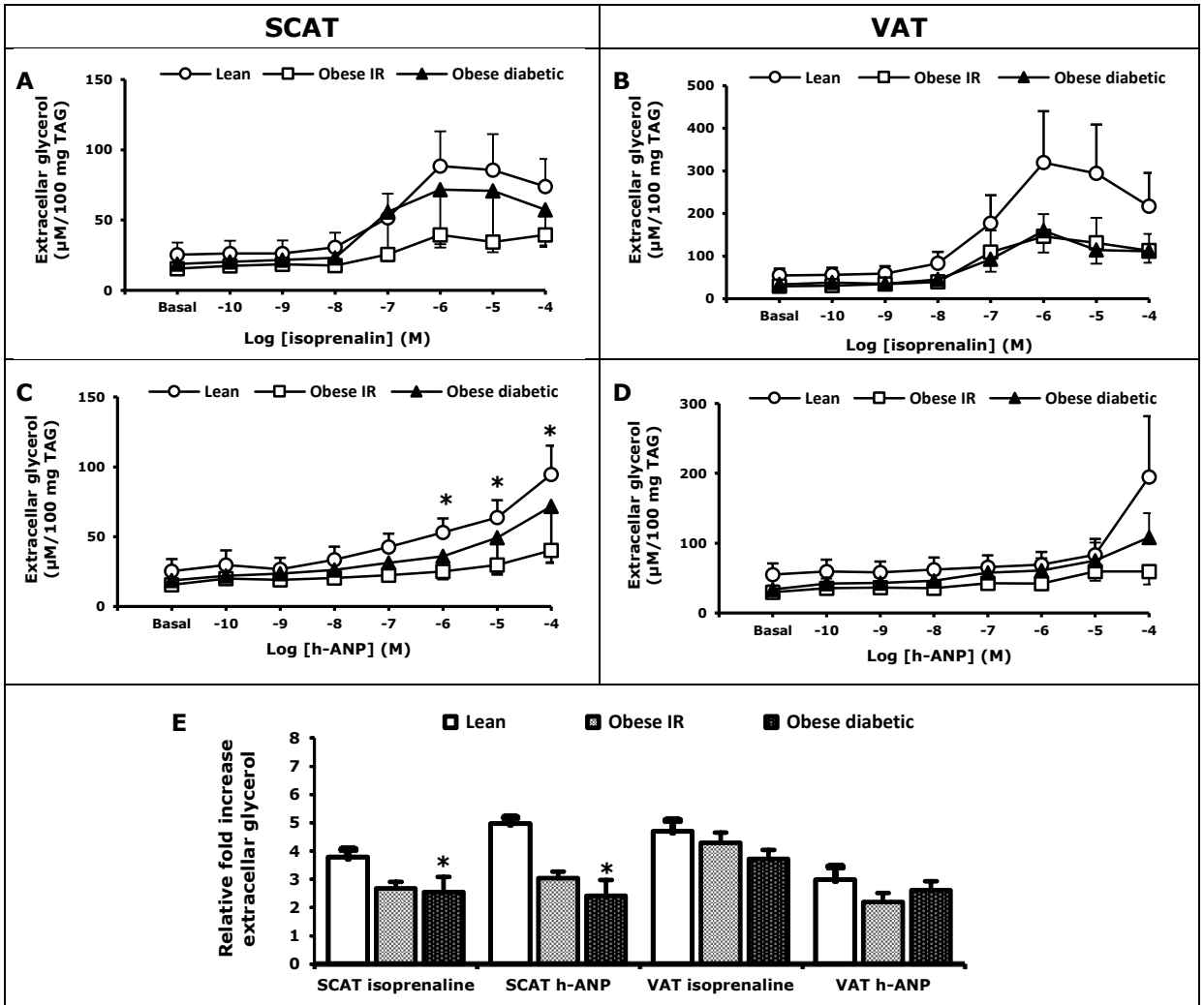

FIGURE 5. Effects of isoprenaline and h-ANP on the lipolysis *in vitro* at level of the SCAT and VAT

A + ***B.*** Extracellular glycerol release induced by administration of different concentrations of isoprenaline to isolated human SCAT (A) and VAT adipocytes (B) extracted from lean (○, n = 13), obese IR (□, n = 19) and obese diabetic (▲, n = 11) subjects. ***C*** + ***D.*** Extracellular glycerol release induced by administration of different concentrations of h-ANP to isolated human SCAT (C) and VAT adipocytes (D) extracted from lean (○, n = 13), obese IR (□, n = 19) and obese diabetic (▲, n = 11) subjects. Data are expressed as means ± SEM. The effects of isoprenaline and h-ANP at a given concentration were analyzed in adipose tissue depot using Kruskal-Wallis analysis followed by post-hoc Man-Whitney U-tests. *$P < 0.05$ compared to lean controls. ***E.*** Plots of the maximal relative fold glycerol increases. Values are expressed as means ± SEM. *$P < 0.05$ compared to lean controls.

Abbreviations: *SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; TAG, triacylglycerol; h-ANP, human ANP; IR, insulin-resistant*

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

Based on the lipolysis experiments, a disturbed ANP-induced lipolytic response in the SCAT of obese IR subjects was noticed. Based on previous literature, it was assumed that changes in the expression of functional ANP (NPR-A) and ANP scavenging receptors could be responsible for the attenuated ANP-mediated lipolysis in obese subjects (17). Therefore, the mRNA expression pattern of the NPR-A and NPR-C receptor in the SCAT of lean, obese IR and obese diabetic subjects was determined.

The relative mRNA expression of the NPR-A and NPR-C receptor in the SCAT and VAT are depicted in Fig. 6. At the level of the SCAT, the relative NPR-A mRNA expression was significantly decreased in the obese IR group (0.69 ± 0.05; $P < 0.05$) as well as in the obese diabetic subgroup (0.59 ± 0.11; $P < 0.05$). Additionally, the relative NPR-C mRNA expression was significantly increased in both obese groups (2.02 ± 0.31 vs. 1.78 ± 0.33; $P < 0.05$ for obese IR and obese diabetic, respectively) compared to lean controls. At the level of the VAT, no differences were found for the relative NPR-A mRNA expression between the different experimental groups (0.86 ± 0.08 vs. 0.92 ± 0.1 for obese IR and obese diabetic, respectively). Moreover, the relative NPR-C mRNA levels were significantly increased in the VAT of obese diabetics (2.12 ± 0.28; $P < 0.05$) while there was only an intermediate increased present in the VAT of obese IR subjects (1.85 ± 0.23).

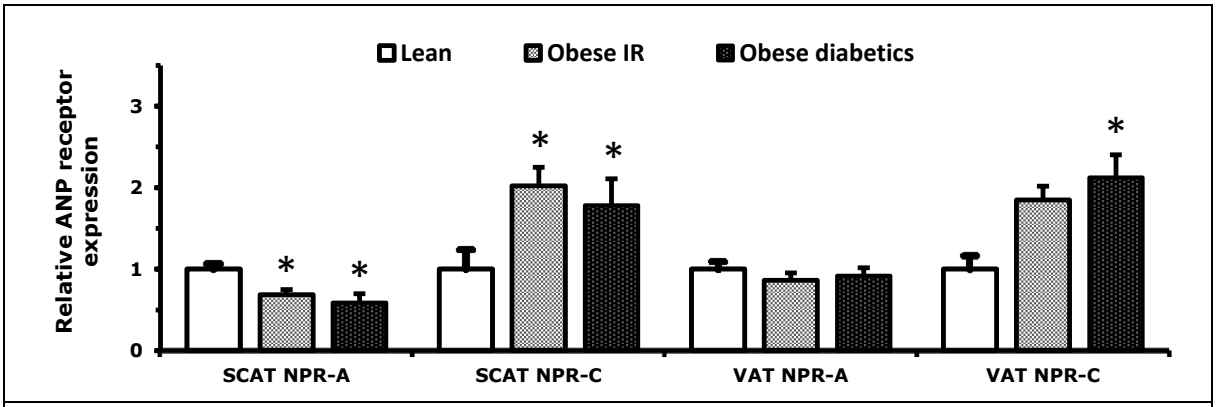

FIGURE 6. Relative NPR-A and NPR-C mRNA receptor expression in the major adipose tissue depots

Plots of the relative expression of the NPR-A and NPR-C receptor on isolated human SCAT and VAT adipocytes extracted from lean (n = 14), obese IR (n = 16) and obese diabetic (n = 11) subjects. Data are expressed as means ± SEM. The relative NPR-A and NPR-C receptor expression level in adipocytes of obese IR and diabetic subjects were compared to those of lean subjects using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests. *$P < 0.05$ compared to lean controls

Abbreviations: *SCAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; NPR-A, Guanylyl cyclase linked ANP receptor; NPR-C, Scavenging ANP receptor; IR, insulin resistant*

3.2. In vivo data

3.2.1. *Pre-intervention measurements*

Subjects' characteristics

A summary of the participants' characteristics are depicted in Table 2. A total of twenty-five male participants were recruited, matched for age. Prior training intervention, the BMI of obese IS (32.6 ± 0.4 kg/m²) and IR subjects (34.0 ± 0.8 kg/m²) was higher compared to lean counterparts ($P < 0.01$). Although waist and hip circumference ($P < 0.01$ and $P < 0.05$ respectively) were significantly higher in obese subjects, the waist/hip ratio remained similar between groups. Additionally, resting blood pressures were similar in all three groups.

Body composition

At baseline, total adipose tissue mass (30.0 ± 1.7 and 34.2 ± 1.9 kg for obese IS and obese IR, respectively) as well as the total body fat percentage (31.4 ± 2.9 and 33.3 ± 1.1% for obese IS and IR, respectively) were significantly higher ($P < 0.01$) in both obese subgroups compared to lean controls (18.2 ± 1.9 kg; 23.4 ± 1.3%). Additionally, the amount of lean tissue mass of obese IR subjects (65.9 ± 2.7 kg) was also higher compared to lean controls ($P < 0.01$). However, no differences were noticed in the total lean and fat mass between the obese subpopulations (Table 2).

Glycemic control

Fasting plasma glucose levels and 2-hour glucose were similar in all experimental groups before training intervention (Table 2). On the other hand, serum insulin levels were remarkably higher (19.6 ± 1.6 mU/L; $P < 0.01$) in the circulation of obese IR subjects compared to lean (7.6 ± 0.8 mU/L) and obese IS subjects (9.2 ± 0.7 mU/L), resulting in also a higher HOMA-IR index (1.81 ± 0.22, 2.15 ± 0.16 and 4.78 ± 0.48 for lean, obese IS and obese IR, respectively; $P < 0.01$). According to the criteria of the American Diabetes Association, 40% of all participant had an impaired fasting glucose level (≥100 mg/dL; two lean, two obese IS and 6 obese IR subjects) (46). Moreover, two of these obese IR subjects had even an impaired glucose tolerance (2h glucose ≥ 140 mg/dL; 8% of all subjects) (46).

Endurance capacity

At maximal exercise, no baseline differences were discovered in the gas exchange ($VO2_{max}$, HR_{peak}, RER, workload, V_E, $VCO2_{max}$ and BF) between groups (Table 2). Although, there was an inverse correlation between BMI and $VO2_{max}$/kg ($R^2 = 0.28$, $P = 0.0061$). Indirect calometry indicated a significantly reduced VO_{2max}/kg value (30.6 ± 1.4; $P < 0.05$) in the obese IR group compared to lean controls (38.6 ± 3.6). No significant difference were noticed regarding the VO_{2max}/lean mass.

TABLE 2 Baseline characteristics

	Lean (*n* = 5)	Obese IS (*n* = 10)	Obese IR (*n* = 10)	*P*
General features				
Age (yr.)	45 ± 5	47 ± 2	44 ± 2	NS
Height (m)	1.86 ± 0.04	1.76 ± 0.02	1.79 ± 0.03	NS
Weight (kg)	82.9 ± 5.4	101.5 ± 3.3	109.7 ± 4.7	a,b
BMI (kg/m²)	24.0 ± 0.7	32.6 ± 0.4	34.0 ± 0.8	a,b
Waist circumference (cm)	93.6 ± 2.0	108.1 ± 1.4	117.7 ± 3.3	a,b,c
Hip circumference (cm)	93.1 ± 2.6	103.1 ± 1.9	111.4 ± 3.0	a,b
WHR ratio	1.00 ± 0.01	1.05 ± 0.01	1.06 ± 0.02	NS
SBP (mmHg)	127 ± 3	136 ± 7	144 ± 7	NS
DBP (mmHg)	75 ± 2	81 ± 4	86 ± 5	NS
Body composition				
Lean tissue mass (kg)	56.3 ± 3.3	62.4 ± 1.5	65.9 ± 2.7	b
AT mass (kg)	18.2 ± 1.9	30.0 ± 1.7	34.2 ± 1.9	a,b
Fat percentage (%)	23.4 ± 1.3	31.4 ± 2.9	33.3 ± 1.1	a,b
Fat mass / Height² (kg/m²)	5.5 ± 0.4	10.0 ± 0.4	11.0 ± 0.5	a,b
Android/gynoid ratio	1.45 ± 0.95	1.29 ± 0.06	1.42 ± 0.04	c
% fat trunk / % fat legs	1.31 ± 0.11	1.19 ± 0.07	1.32 ± 0.06	NS
Trunk/limb fat mass ratio	1.39 ± 0.15	1.34 ± 0.09	1.64 ± 0.07	c
Glycemic control				
Fasting glucose (mg/dL)	96.6 ± 1.6	94.5 ± 3.2	105.4 ± 3.7	NS
Insulin (mU/L)	7.6 ± 0.8	9.2 ± 0.7	19.6 ± 1.6	b,c
Glucose 120' (mg/dL)	82.4 ± 4.0	103.4 ± 6.6	112.7 ± 12.7	NS
HOMA-IR index	1.81 ± 0.22	2.15 ± 0.16	4.78 ± 0.48	b,c
Endurance capacity				
Workload (W)	261 ± 23	240 ± 18	236 ± 12	NS
$VO2_{max}$ (L/min)	3.22 ± 0.44	3.10 ± 0.20	3.30 ± 0.16	NS
$VO2_{max}$ (mL/kg x min)	38.6 ± 3.6	30.5 ± 2.1	30.6 ± 1.4	b
$VO2_{max}$/lean mass (mL/kg x min)	56.6 ± 4.8	49.7 ± 2.9	50.2 ± 1.6	NS
$VCO2_{max}$ (mL/min)	3.97 ± 0.53	3.71 ± 0.22	3.85 ± 0.17	NS
RER	1.24 ± 0.01	1.21 ± 0.03	1.18 ± 0.02	NS
V_E (l/min)	116.7 ± 17.7	103.1 ± 5.1	110.0 ± 5.3	NS
BF (breaths/min)	37.1 ± 3.9	35.9 ± 1.8	36.8 ± 1.8	NS
HR (beats/min)	172 ± 3	168 ± 3	168 ± 3	NS

Data are expressed as means ± SEM. *P* values were calculated using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests ($P < 0.05$).

a) Obese IS significantly different from lean subjects
b) Obese IR significantly different from lean subjects
c) Obese IS significantly different from obese IR subjects

Abbreviations: *IR, insulin-resistant; BMI, body mass index; WHR, Waist/hip circumference ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; AT, adipose tissue; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; $VO2_{max}$, maximal oxygen consumption; $VCO2_{max}$, maximal carbon dioxide removal; RER, respiratory exchange ratio; V_E, expiratory volume; BF, breathing frequency; HR, heart rate; not significant NS; SEM, standard error mean*

Catecholamine-mediated lipolysis is blunted in the SCAT of obese subjects

Baseline extracellular glycerol concentration (EGC) was similar between groups in control probe (92.7 ± 7.4, 88.7 ± 5.3 and 85.1 ± 8.0 mM for lean, obese IS and obese IR, respectively) as well as in the probe containing Ringer solution supplemented with phentolamine and propanol (83.6 ± 9.7, 103.9 ± 11.6 and 87.9 ± 8.0 mM for lean, obese IS and obese IR, respectively) (Fig. 7A and 7B). Additionally, no difference in EGC at rest was found between the control probe and the probe containing phentolamine plus propanol in none of the groups (Supplementary Fig S3).

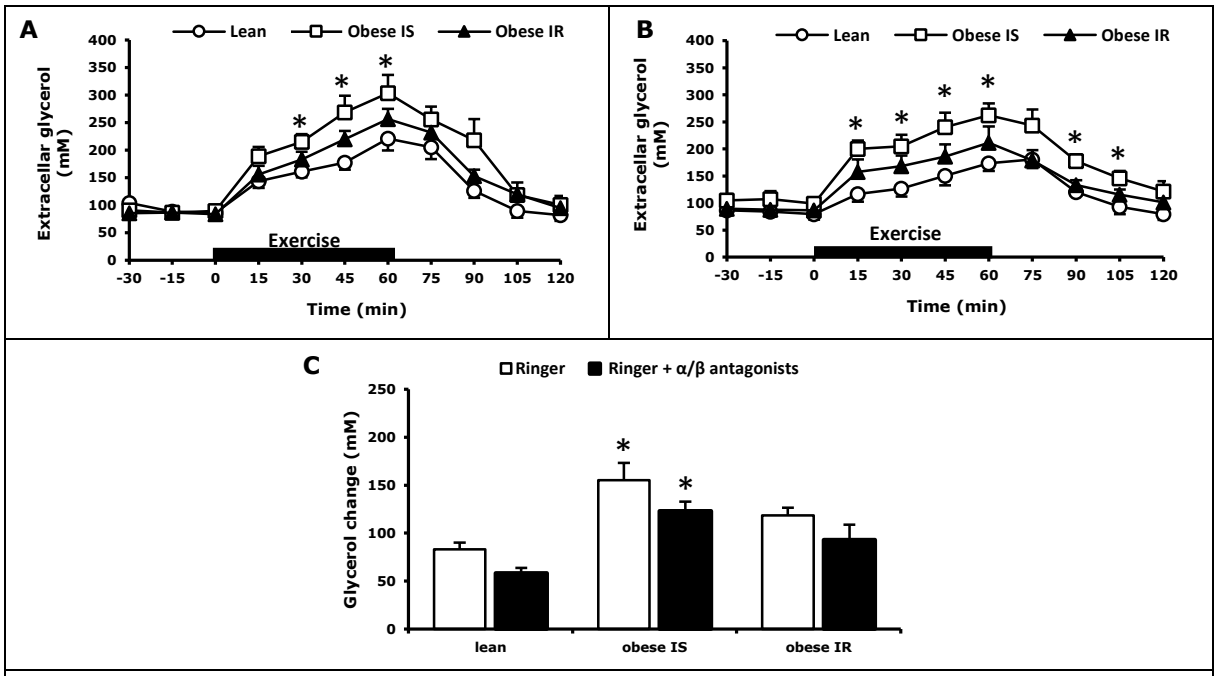

FIGURE 7. Extracellular glycerol release in SCAT tissue

A + ***B.*** Extracellular glycerol concentration at rest, during exercise and recovery in the dialysate of the control probe (A) and probe containing Ringer solution supplemented with phentolamine and propanol (B) in lean (○, *n* = 4), obese IS (□, *n* = 6) and obese IR (▲, *n* = 10) subjects. Data are expressed as means ± SEM. The effects of each probe at each time point were analyzed between groups using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests. **P* < 0.05 compared to lean controls. ***C.*** Plots of the maximal relative fold ECG increase in each probe of each group. Values are expressed as means ± SEM. **P* < 0.05 compared to lean controls.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; SCAT, subcutaneous adipose tissue; ECG, extracellular glycerol; SEM, standard error mean*

During a 1-hour endurance exercise (40% $VO2_{max}$), ECG gradually increased during exercise in both probes for all groups, reaching significant higher levels compared to basal ECG levels after 15' minutes of exercise in the control probe and the probe perfused with propanol plus phentolamine (Fig. 7A and 7B). In the control probe, EGCs were significantly higher ($P < 0.05$) in the dialysate of obese IR compared to lean subjects, starting after 30 min cycling and until the end of the exercise (Fig. 7A). The calculated AUCs of the control probe during exercise (duration 0-60 min) were 635.9 ± 35.9, 869.3 ± 72.6 and 726.8 ± 55.5 mM for lean, obese IS and obese IR subjects, respectively. The AUC of obese IS significantly higher compared to the AUC of lean controls ($P < 0.05$).

The EGC in the dialysate of the probe with pentolamine plus propanol were already significantly elevated ($P < 0.05$) after 15 min of exercise (Fig. 7B). The corresponding AUCs were 520.4 ± 57.4, 826.3 ± 72.6 and 660.9 ± 82.6 mM for lean, obese IS and obese IR subjects, respectively. The AUC of obese IS significantly higher compared to the AUC of lean controls ($P < 0.05$). Within each experimental group, no significant changes in the ECG during exercise were noticed when comparing the AUC of the control probe and probe perfused with phentolamine plus propanol (Supplementary Fig 3A-C). Additionally, the EGC increase during exercise did not differ between both probes within the same experimental group (Fig 7C).

During the recovery period, the EGCs of each experimental group returned back to basal levels. In the control probe, ECGs of each experimental group were similar during recovery. However, in the control probe with phentolamine plus propanol, the EGCs values ($P < 0.05$) remained significantly increased in the dialysate of obese IS subjects during recovery when compared to the ECGs lean controls (Fig. 8A and 8B). Furthermore, within each experimental group, no differences in EGC was found between the control probe and phentolamine plus propanol probe (Supplementary Fig 3A-C).

Plasma glycerol and FFA

Basal plasma glycerol levels (64.0 ± 7.9, 92.0 ± 9.6 and 76.6 ± 6.9 µM for lean, obese IS and obese IR subjects, respectively) were similar in all groups, whereas the baseline plasma FFA levels of obese IR subjects (391.6 ± 42.9, 573 ± 81.1 and 555.7 ± 44.7 µM for lean, obese IS and obese IR subjects, respectively) were significantly elevated ($P < 0.05$) compared to lean controls (Fig. 8). During exercise (40% VO_{2max}), plasma glycerol and FFA levels progressively increased similarly in all groups. When assessing the AUC method, the overall exercise-induced responses of glycerol (515.2 ± 70.1, 728.2 ± 95.3 and 685.4 ± 116.7 μM; $P < 0.05$) and FFA (1601.4 ± 258.7, 2342.6 ± 278.0 and 2058.8 ± 198.4 μM) were similar in the lean, obese IS and obese IR group, respectively. However, during the recovery period, plasma glycerol and FFA levels dropped at a slower rate in obese subjects than in lean controls, resulting to significantly increased plasma glycerol levels (obese IR) and increased FFA levels (obese IS and IR) compared to lean controls ($P < 0.05$).

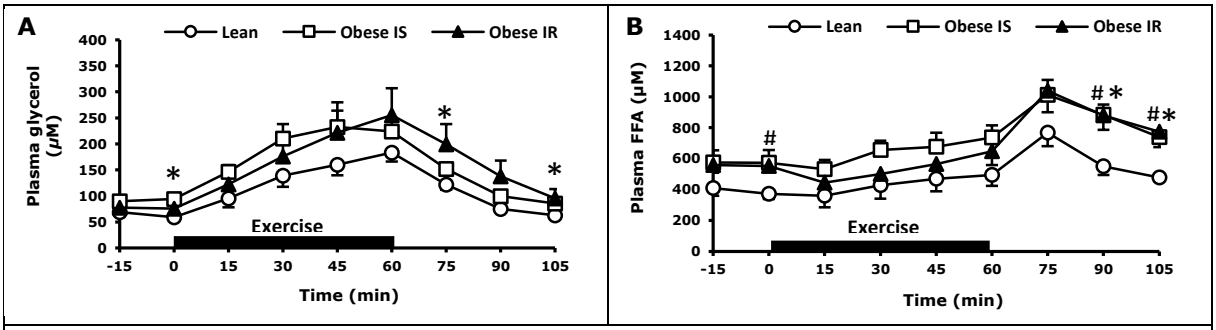

FIGURE 8. Plasma glycerol and FFA concentrations

Plasma glycerol (A) and plasma FFA concentration (B) at rest, during exercise and recovery in lean (○, n = 4), obese IS (□, n = 6) and obese IR (▲, n = 10) subjects. Data are expressed as means ± SEM. The plasma concentration of glycerol and FFA between groups at each time point were analyzed between groups using Kruskal-Wallis analysis followed by post-hoc Man-Whitney U-tests. *$P < 0.05$ compared to lean controls.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; FFA; free fatty acids; SEM, standard error mean*

Plasma glucose, insulin and lactate concentrations

Basal plasma glucose (5.15 ± 0.17, 4.78 ± 0.06 and 5.12 ± 0.17 mM) and lactate levels (0.82 ± 0.07, 0.90 ± 0.08 and 1.08 ± 0.13 mM) were between groups (lean, obese IS and obese IR subjects, respectively) (Fig. 9A and 9C). Plasma lactate levels remained significantly elevated ($P < 0.05$) in the obese IR group throughout the whole exercise (duration 0-60) and recovery phase compared to lean controls ($P < 0.05$). Plasma glucose levels did not changed in any group.

Basal plasma insulin levels were significantly elevated ($P < 0.05$) in the obese IR group compared to lean controls and obese IS subjects. During the exercise period, insulin levels of the obese IR group remained significantly higher. The exercise-induced AUCs values were 27.24 ± 4.03, 14.41 ± 4.96 and 60.78 ± 7.91 mM for lean, obese IS and obese IR subjects, respectively (data not shown). The post-exercise increase in insulin levels was higher in the obese IR group compared to obese IS and lean ($P < 0.05$), during the first part of the recovery phase (30 min) after which it returned to basal levels (Fig. 9B).

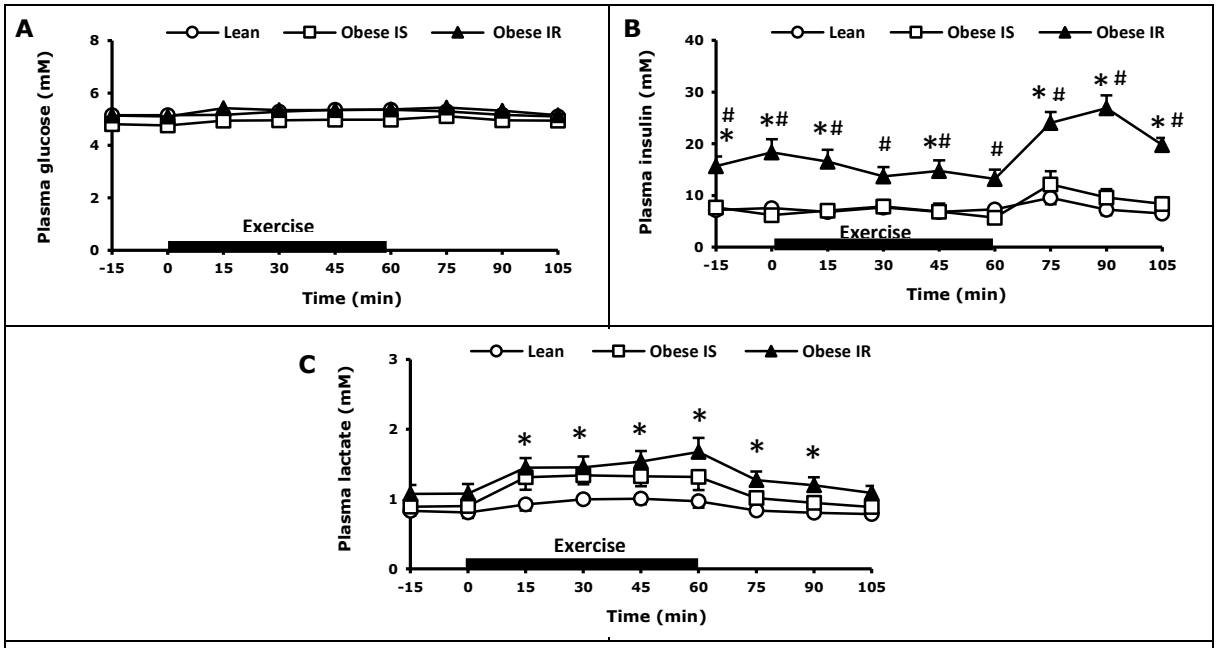

FIGURE 9. Plasma glucose, insulin and lactate concentrations

Plasma glucose (A), insulin (B) and lactate (C) concentrations at rest, during exercise and recovery in lean (○, $n = 4$), obese IS (□, $n = 6$) and obese IR (▲, $n = 10$) subjects. Data are expressed as means ± SEM. The plasma concentration of glycerol and FFA between groups at each time point were analyzed between groups using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests. *$P < 0.05$ obese IS compared to lean controls. #$P < 0.05$ obese IR compared to lean controls.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; SEM, standard error mean*

3.2.2. *Post-intervention measurements*

Intervention adherence

Adherence to the training intervention was good. A total of 98% (ranging from 94 % to 100%) of the supervised training sessions were attended with an average RPE Borg score of 13/20 (for both obese groups). Overall, three persons did not fully completed the study protocol owning to personal motivation (two obese IS) and due severe uprising orthopaedic problems (one obese IR).

Training outcomes

In order to evaluate the efficiency of the training program, the total calorie consumption and average workload were recorded each time after completion of the 45 min of endurance training (Fig. 10). Mixed model analysis demonstrated a progressively increased calorie consumption and workload increased progressively over time ($P < 0.05$) independent of group composition.

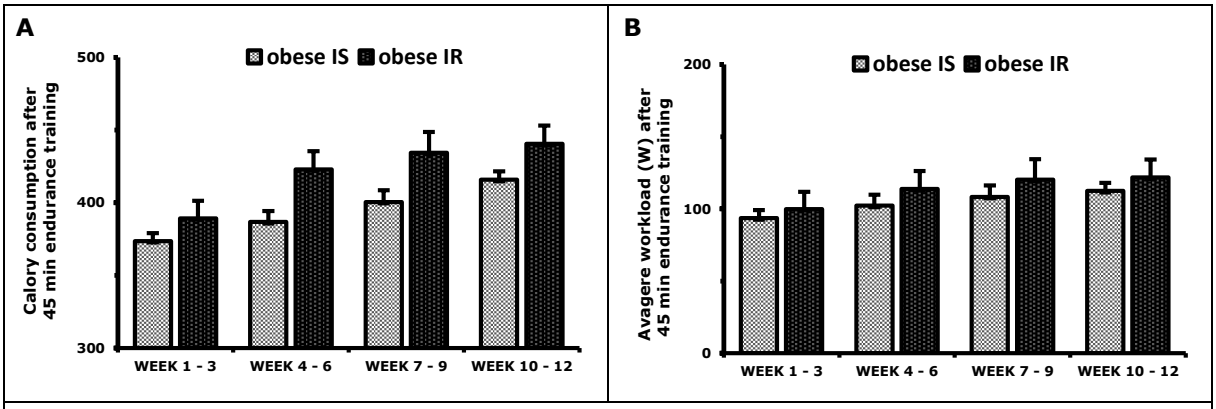

FIGURE 10. Effect of combined training on the calorie consumption and workload over time

Plots of the average calorie consumption (A) and workload (B) after 45 min endurance training in the obese IS (n = 4) and obese IR (n = 7) subgroup. Data are expressed as means ± SEM. Mixed model analysis ($P < 0.05$) was performed to evaluate insulin sensitivity- and time-dependent training effects on the average calorie consumption and workload.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; SEM, standard error mean*

Body composition

Within the obese IR group, body composition was improved after 12 wks combined training compared to baseline values. In particular, the average body weight decreased with 3% (0 wks, 112.9 ± 6.5; 12 wks, 110.0 ± 6.5; $P < 0.05$). Subsequently, the resulting BMI was also reduced about 3% (0 wks, 33.9 ± 1.1; 12 wks, 33.0 ± 1.1; $P < 0.05$). The weight and BMI decreases were attributed to a significant (6%) loss of adipose tissue (0 wks, 36.5 ± 2.2; 12 wks, 34.2 ± 2.5; $P < 0.05$), whereas no significant changes were observed in lean tissue mass.

Despite training intervention induced positive effects on body composition in obese IR subjects, no significant changes were observed in the obese IS group. However, similar trends were visible. Most likely, our current pool of obese IS subjects who completed the is probably too small to reach significance, since our power calculation stated that 6 persons/group were needed.

TABLE 3 Changes in body composition induced by 12-week combined training in obese IS and IR subjects

	0 wk		12 wk	
	Obese IS (*n* = 4)	**Obese IR (*n* = 7)**	**Obese IS (*n* = 4)**	**Obese IR (*n* = 7)**
Body composition				
Weight (kg)	107.9 ± 5.2	112.9 ± 6.5	105.0 ± 4.3	110.0 ± 6.5 [a]
BMI (kg/m²)	33.3 ± 1.0	33.9 ± 1.1	32.6 ± 0.7	33.0 ± 1.1 [a]
Waist circumference (cm)	111.5 ± 2.5	118.1 ± 2.2	110.5 ± 1.0	112.8 ± 2.1
Hip circumference (cm)	110.0 ± 2.5	112.5 ± 3.4	109.0 ± 1.5	109.5 ± 3.1
WHR ratio	1.01 ± 0.00	1.06 ± 0.04	1.01 ± 0.00	1.03 ± 0.02
Lean tissue mass (kg)	64.4 ± 2.3	66.7 ± 3.9	65.0 ± 2.4	65.5 ± 4.2
Adipose tissue mass (kg)	34.3 ± 2.6	36.5 ± 2.2	31.8 ± 2.5	34.2 ± 2.5 [a]
Fat percentage (%)	33.8 ± 1.4	34.5 ± 1.3	31.9 ± 1.4	33.4 ± 1.6
Fat mass / Height² (kg/m²)	11.0 ± 0.7	11.4 ± 0.6	10.2 ± 0.6	10.7 ± 0.7 [a]
Android/gynoid ratio	1.19 ± 0.03	1.36 ± 0.03	1.20 ± 0.02	1.34 ± 0.03
% fat trunk / % fat legs	1.00 ± 0.04	1.24 ± 0.04	1.04 ± 0.03	1.34 ± 0.02 [a]
Trunk/limb fat mass ratio	1.12 ± 0.05	1.55 ± 0.06	1.14 ± 0.08	1.62 ± 0.06

Data are expressed as means ± SEM. Within-group effects were calculated using Wilcoxon signed ranked rank tests. Trainings outcomes induced by 12-wks combined training between the obese subpopulation by using Man-Whitney U-tests.

[a] $P < 0.05$, within-group effect over 12 weeks combined training

Abbreviations: IS, insulin-sensitive; IR, insulin-resistant; BMI, body mass index; WHR ratio, Waist/hip circumference ratio; not significant NS; SEM, standard error mean

Endurance capacity

After 12 wks combined training, the physical fitness was reevaluated. An overview of the measurements is depicted in Table 4. Within the obese IR group, the average workload was increased about 15% (0 wks, 236 ± 16; 12 wks, 272 ± 16; $P < 0.05$). Furthermore, VCO_{2peak} values increased with 11% (0 wks, 33.9 ± 1.1; 12 wks, 33.0 ± 1.1; $P < 0.05$). Although the VO_{2max} increased 20% in the obese IS group and 15% in obese IR subjects, no statistical significance was reached, probably due uncomplete data collection. No other significant changes in gas exchange parameters were present, although the VO_{2max} increased . On contrary to obese IR subjects, no significant effects of combined training were noticed in the obese IS group. However, the limited number of post-training data is too small to probably induce statistical significance (estimated six subjects per group are needed).

TABLE 4 Changes in physical fitness induced by 12-week combined training in obese IS and IR subjects

	0 wk		12 wk	
	Obese IS (*n* = 4)	**Obese IR (*n* = 7)**	**Obese IS (*n* = 4)**	**Obese IR (*n* = 7)**
Physical fitness				
Workload (W)	210 ± 16	236 ± 16	251 ± 9	272 ± 16 [a]
$VO2_{max}$ (L/min)	2.71 ± 0.14	3.33 ± 0.23	3.33 ± 0.05	3.68 ± 0.21
$VO2_{max}$ (ml/kg x min)	24.8 ± 1.0	29.8 ± 1.9	31.5 ± 1.2	33.7 ± 1.7
$VCO2_{max}$ (L/min)	3.35 ± 0.16	3.85 ± 0.23	3.89 ± 0.04	4.29 ± 0.14 [a]
RER	1.25 ± 0.04	1.17 ± 0.04	1.18 ± 0.01	1.19 ± 0.03
V_E (L/min)	104.0 ± 6.6	108.8 ± 7.5	117.9 ± 6.2	118.4 ± 4.5
BF (breaths/min)	37.5 ± 4.3	35.5 ± 2.3	39.4 ± 3.8	36.9 ± 1.7
HR (beats/min)	163 ± 5	168 ± 4	162 ± 5	171 ± 4

Data are expressed as means ± SEM. Within-group effects were calculated using Wilcoxon signed ranked rank tests. Trainings outcomes induced by 12-wks combined training between the obese subpopulation by using Man-Whitney U-tests.

[a] $P < 0.05$, within-group effect over 12 weeks combined training

Abbreviations: IS, insulin-sensitive; IR, insulin-resistant; *$VO2_{max}$, maximal oxygen consumption; $VCO2_{max}$, maximal carbon dioxide removal; RER, respiratory exchange ratio; V_E, expiratory volume; BF, breathing frequency; HR, heart rate*; SEM, standard error mean

Plasma glycerol and FFA levels decreased after 12-wk combined training

After 3 months of training, basal plasma glycerol and FFA were lowered in both the obese IS group (92.0 ± 9.6 vs. 70.0 ± 3.4 μM and 573 ± 81.1 vs. 372.1 ± 35.5 μM) and the obese IR group (76.6 ± 6.9 vs. 73.3 ± 8.3 μM and 555.7 ± 44.7 ± 35.5 μM), before and after training respectively (Fig. 11 A-D). However, no statistical training differences were found ($P > 0.05$) for any parameter in both groups. During exercise, plasma glycerol levels significantly increased ($P < 0.05$) compared to basal levels (data not shown). Using the AUC method, no significant training-induced improvements were noticed regarding the overall exercise-induced responses of glycerol and FFAs in obese IS (728.2 ± 95.3 vs. 611.9 ± 85.4 μM and 2488.0 ± 277.6 vs. 1818.4 ± 217.0 μM) and obese IR subjects (685.4 ± 116.7 vs. 588.5 ± 122.9 and 2108.7 ± 258.7 vs. 1800.9 ± 230.6 μM) before and after 12 wks combined training, respectively. In the recovery period, plasma glycerol and FFA levels dropped back to basal values.

Plasma glucose, insulin and lactate levels decreased after 12-wk combined training

After 3 months of training, basal plasma glucose, insulin and lactate levels were not changed before and after intervention (Fig. 12 A-B). During exercise, plasma lactate levels were decreased, but no statistical differences were found ($P < 0.05$) compared to pre-intervention levels. Additionally, no significant training-induced improvements were noticed regarding the overall exercise-induced responses of glucose and insulin. In the recovery period, plasma glucose, insulin and lactate levels dropped back to basal values.

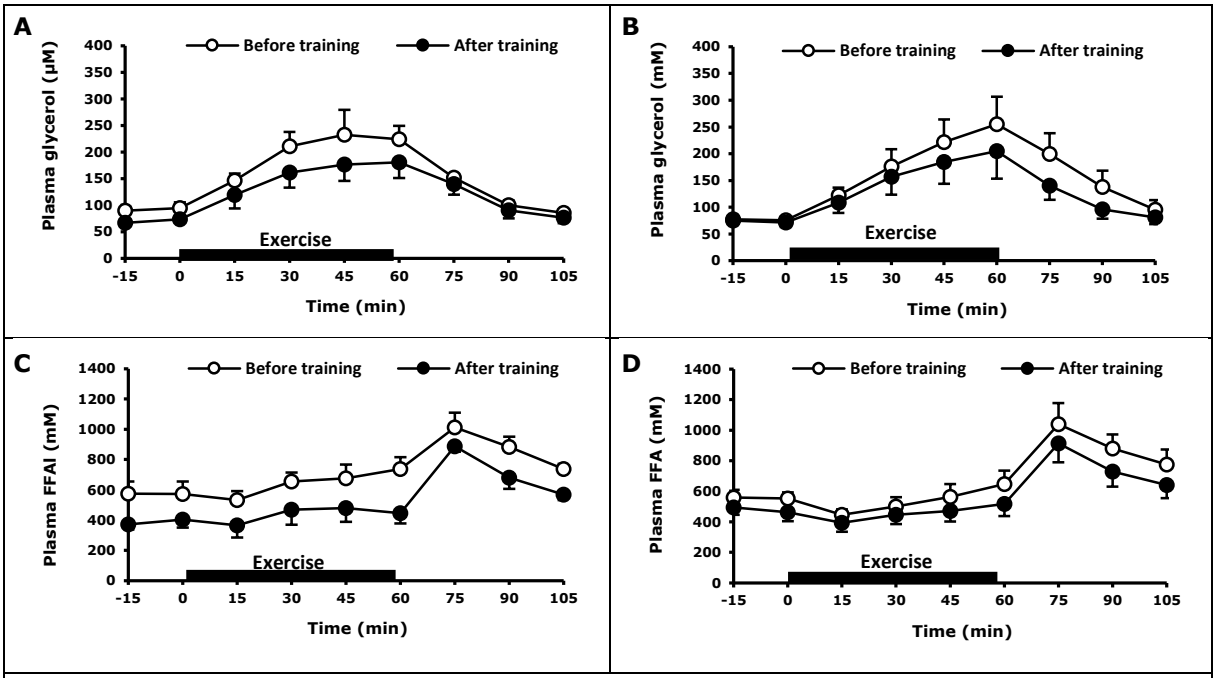

FIGURE 11. Effect of 12-wk combined training on plasma glycerol and FFA levels

A + ***B.*** Changes in plasma glycerol concentrations at rest, during exercise and recovery before (○) and after (●) training intervention in obese IS (A; *n* = 4) and obese IR subjects (B; *n* = 7). ***C*** + ***D.*** Changes in plasma FFA concentrations at rest, during exercise and recovery before (○) and after (●) training intervention in obese IS (C; *n* = 4) and obese IR subjects (D; *n* = 7). Data are expressed as means ± SEM. The plasma concentration of glycerol and FFAs before and after training at each time point were analyzed between groups using Wilcoxon Signed rank tests. **P* < 0.05 compared to pre-training values.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; FFA; free fatty acids; SEM, standard error mean*

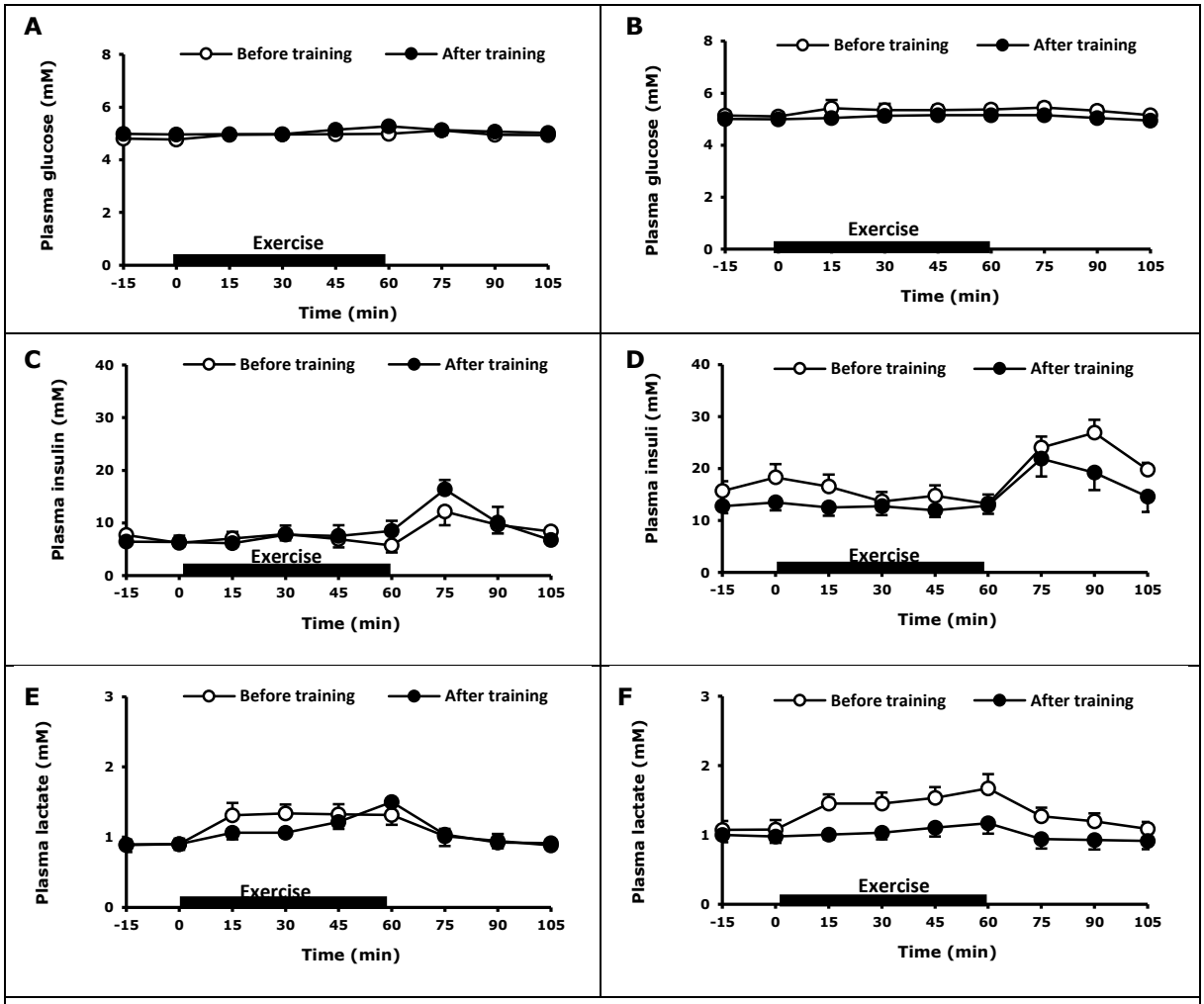

FIGURE 12. Effect of 12-wk combined training on plasma glycerol and FFA levels

A + ***B.*** Changes in plasma glucose concentrations at rest, during exercise and recovery before (○) and after (●) training intervention in obese IS (A; *n* = 4) and obese IR subjects (B; *n* = 7). ***C*** + ***D.*** Changes in serum insulin concentrations at rest, during exercise and recovery before (○) and after (●) training intervention in obese IS (C; *n* = 4) and obese IR subjects (D; *n* = 7). ***E*** + ***F.*** Changes in plasma lactate concentrations at rest, during exercise and recovery before (○) and after (●) training intervention in obese IS (C; *n* = 4) and obese IR subjects (D; *n* = 7). Data are expressed as means ± SEM. The plasma concentration of glucose, insulin and lactate before and after training at each time point were analyzed between groups using Wilcoxon Signed rank tests. **P* < 0.05 obese IS compared to pre-training values.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; SEM, standard error mean*

4. DISCUSSION

In the present study, we investigated the impact of obesity and insulin resistance on the ANP-mediated lipolysis *in vitro*. Furthermore, We also investigated if 12-week combined training counteract the natriuretic handicap which is typical in obesity and so improve subjects cardiometabolic health (*in* vivo part).

4.1. *In vitro* study

In the *in vitro* study, we investigated for the first time the lipolytic effects of catecholamine- and ANP on the lipolysis in mature adipocytes derived from the SCAT and VAT of obese IR and obese diabetic subjects and compared those with the lipolytic responses in lean subjects.

Based on the glycerol measurements, we clearly observed an attenuated maximal lipolytic responsiveness to both ANP and catecholamines (isoprenaline) in the SCAT of obese man. Additionally, the attenuated lipolytic responses were independent of subjects' insulin resistace, which is contradictory with the hypothesis that high insulin levels are responsible for the blunted ANP-mediated lipolysis (31, 32). However, we demonstrated that the blunted ANP-mediated lipolysis was accompanied by a differential expression pattern of the NPR-A and NPR-C receptor at the level of the SCAT, which is again agreement with the upper hypothesis.

4.1.1. *ANP-mediated and catecholamine-mediated lipolysis is blunted in obese subjects*

In the present study, the maximal isoprenaline-induced lipid mobilization was lowered in the SCAT of obese subjects when expressed per lipid content, although this does not reached statistical difference. This observation is in agreement with recent *ex vivo* data from Ryden et al. where they compared the catecholamine) and ANP-mediated glycerol release of obese and lean women (28). However, Ryden et al observed an elevated glycerol release from the SCAT of obese women (expressed per cell), which is not included in this thesis. They concluded that the different observations (expressed per cell or lipid content) probably would be a consequence of the hypertrophy of adipose cells in obese subjects (28). Indeed, large adipocytes had a higher lipid mobilization than small SCAT adipocytes, as confirmed in the SCAT of lean and obese men and women (47, 48). However, the maximal isoprenaline-induced glycerol release was significantly lower when expressed as the ratio of basal values in the SCAT of obese men (independent of the insulin status). However, this could be explained by the typical downregulation of β-adrenoceptors on the surface of SCAT adipocytes of obese subjects (17, 49).

Secondly, we noticed that the ANP-mediated glycerol release, when expressed per 100 mg lipids, was significantly downregulated in the SCAT of obese IR subjects. This observation is also in agreement with recent *ex vivo* data from Ryden et al. (28). However, when they expressed the ANP-mediated glycerol release in function of the number of adipocytes, then this significant difference disappeared. Therefore, Ryden et al. concluded that the ANP-mediated lipid mobilization is even more sensitive to the effects of obesity than the catecholamine-induced lipid mobilization (28).

Overall, the upper observations indicate the existence of an blunted catecholamine- and ANP-mediated lipid mobilization at the level of the SCAT in obese subjects, which causes an impaired lipid metabolism. Subsequently, FFA accumulations in the adipose tissue will lead to the development of insulin resistance.

When we compared the degree of catecholamine- and ANP-mediated lipolysis in SCAT adipocytes with VAT adipocytes, we discovered an antithetical regulation of the lipolysis in lean men, which was not present obese subjects. At level of the VAT, we reported an elevated catecholamine-induced and a decreased ANP-mediated lipolysis. This is probably the result of an alternative distribution of catecholamine and ANP receptors and the functioning of lipases, as mentioned earlier (11). In line with our results, Dicker et al. also did not find significant depot changes regarding the ANP-mediated lipolysis in VAT and SCAT adipocytes of obese women (50), which greatly support our theory that obese subjects possess an impaired ANP-resistant phenotype at the level of the SCAT.

4.1.2. *An increased NPR-A and decreased NPR-C expression pattern is present in the SCAT of obese subjects.*

Since the net lipolytic effect of ANP is determined by both NPRA and scavenging NPRC (17), the expression level of both receptors was investigated to unravel the potential underlying mechanism responsible for the observed attenuated maximal ANP responsiveness in the SCAT of obese men. By the means of RT-PCR, we observed a downregulated mRNA expression at the level of the SCAT in obese subjects, whereas NPR-A expression was similar in the VAT compared to lean subjects. On the other hand, the NPR-C expression was significantly elevated in the SCAT of both obese groups, whereas only a significant upregulation of NPR-C was present in the VAT of obese diabetic subjects. Together, our data revealed a decreased expression of NPR-A receptors and a concomitant increased NPR-C expression in the SCAT of obese IR, an expression pattern which predispose for lower circulating levels of ANP as seen in earlier research (27, 51). In this study, we also reported lower circulatory levels of ANP. Subsequently, the disturbed expression ANP receptor pattern explains the observed blunted ANP-mediated lipolytic response in the SCAT of obese subjects.

Of interest, Pivovarova et al. also reported high insulin levels upregulated NPR-C mRNA and protein expression in obese IR subjects and diabetic subjects, which supports our data (32). Therefore, it can be concluded that both adiposity and insulin sensitivity may determine the ANP responsiveness in the SCAT od obese subjects. However, this needs to be further investigated in detail by studies that aim to restore the insulin sensitivity in obese subjects (i.e. physical exercise or diets). Remarkably, exercise intervention has proven to ameliorate insulin sensitivity as well as increase the ANP-mediated lipolysis in the SCAT of overweight men (37). Therefore, we started an *in vivo* study in which we will evaluate if training also improves the insulin sensitivity and ANP-mediated lipolysis in the SCAT of obese IS and obese IR.

4.2. Training intervention study *(in vivo)*

In the *training* study, we investigated for the first time the effects of combined training on the ANP-mediated lipolysis in the SCAT of obese with a different whole-body insulin sensitivity (obese IS and IR subjects). Secondly, we also investigated the impact of combined training on the cardiometabolic health of these subjects (body composition, physical fitness and whole-body insulin sensitivity).

4.2.1. *Combined training improved body composition, endurance capacity, whole-body insulin sensitivity and potentially the ANP-mediated lipid mobilization in the SCAT of obese subjects*

Body composition

In the present study, we reported a higher BMI and WHR in obese subject compared to lean controls before training intervention. Similar observations were also observed in several other training studies in overweight and obese subjects (37, 40, 52). Anthropometry indicated that the elevated basal BMI and WHR result from an increased subtotal fat mass. After 12 wks combined training, BMI, WHR and subtotal adipose tissue mass were decreased in both obese groups, suggesting a positive training effect on body composition. Lean tissue remained similar before and after training. Similar anthropometric improvements were observed after aerobic training in overweight and obese men (37, 40). However, in contrast to our data, lean mass was also increased after completion of the training program.

Endurance capacity

Before training intervention, parameters reflecting subjects' physical fitness (VO_{2max}, RER and workload) were similar in all groups. Therefore, we concluded that obese subject are just as fit as lean subjects, regarding their insulin status. However, these observation seems contradictory, since many other studies observed lowered VO_{2max}, RER and workload values in obese subjects (37). After 12 wks combined training, VO_{2max}, maximal workload as well as VCO_{2max} were increased in obese men, which indicates an improved endurance capacity. Similar outcomes were reported in overweight and obese subjects after training intervention (37, 40, 41).

Insulin sensitivity

Basal plasma levels of glucose and insulin were elevated in obese subjects compared to lean counterparts, which suggested the presence of disrupted regulation of the glucose metabolism as seem as in other studies (32, 37, 41). In fact, 40% of all participants had impaired fasting glucose levels, whereas 8% even possessed an impaired glucose tolerance. Subsequently, HOMA-IR values were higher in obese subjects. A seen in overweight men and obese men (37, 40, 52), combined training reduced circulating levels glucose and insulin in both obese groups. Subsequently, HOMA-IR values were also improved in both obese groups, which indicates an improved whole-body glucose tolerance (37, 40, 52).

ANP-mediated lipolysis in the SCAT

Before training intervention, we did not observed basal differences in the plasma and dialysate concentrations of glycerol and FFA between groups, which indicates that amount of lipid mobilization of obese subjects at rest is similar compared to lean controls.

During physical exercise, we reported an increase of the glycerol dialysate concentrations in the both the control probe and the probe supplemented with propanol and phentolamine, indicating an upregulation of the lipid mobilization at the level of the SCAT. We noticed that obese IS subjects had the strongest increase dialysate glycerol concentrations. Their dialysate glycerol concentrations were significantly higher than the increase in obese IR and control subjects. This is presumably the result of a combination of increased adipose tissues mass, as well as an preserved insulin sensitivity (11). Within each experimental group, we did not reported any significant changes in the ECG during exercise when comparing the dialysate of the control probe and probe perfused with phentolamine plus propanol. Since propanol and phentolamine block catecholamine-induced lipolysis. Therefore, it could be we concluded that the increased lipolytic response is independent of catecholamine action, but rather induced by ANP and some other liopolytic factors (8, 12). These findings are in line with the study from Moro et al. (37). They observed a similar rise in SCAT lipid mobilization in overweight men during exercise (37).

At this moment, we did not yet analysed the glycerol dialysate after training intervention. Therefore, we do not yet know if training intervention ameliorate the ANP-induced SCAT. However, we noticed that after training, the plasma levels of plasma glycerol, FFA and lactate were reduced in obese men. These findings were also observed in the trainings study performed in overweight subjects (37). Moreover, Moro et al. observed an improved ANP-mediated lipolysis after training. This may implicate that ANP-mediated lipolysis is maybe also elevated in our training. However, this remains to be investigated in the near future.

Moro et al. assumed that the increased ANP-mediated lipolysis in overweight after training resulted from a changed NPR-A and NPR-C expression pattern. In line with this hypothesis, it has recently been suggested that weight loss improved whole-body insulin sensitivity and ANP-mediated lipolysis by decreasing the expression of scavenging NPR-C receptors as well as increased the number of functional NPR-A receptors on adipocytes of obese subjects (52, 53). Therefore, it can be hypothesized that combined training.

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. However, this needs to be further elucidated.

5. LIMITATIONS

The present study had some limitations. First of all, at the time that this thesis was written, the study was still in progress. Power analysis stated that at least six subjects had to complete the complete program in order to obtain legit results. However, only four obese IS finished their program by now. Our data indicate that body composition, endurance capacity and other factor are decreased/increased. However, no statistical was reached due to the limited data. Secondly, plasma ANP were not analysed at this moment. Therefore, we could only make assumption regarding ANP-mediated lipolysis before and after training.

6. CONCLUSION

In conclusion, the present study revealed that obese subjects have an impaired catecholamine-mediated and ANP-mediated lipolysis at the level of the SCAT. Moreover, the blunted ANP-mediated lipolysis results form an decreased expression of NPR-A receptors as well as an increased expression of NPR-C receptors. However, an increasing amount of evidence states that the blunted ANP-mediated lipolysis in obese subjects can be compensated by aerobic training. In our study, combined training improved the body composition and endurance capacity of obese subjects, leading to an improved cardiometabolic risk profile. Additionally, combined training lowered plasma glycerol, FFA and lactate levels, which suggested an improved ANP responsiveness at the level of the SCAT. However, future research is necessary to determine whether combined training improves ANP-mediated lipolysis in obese subjects

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8. ANNEX

	Lean control subjects (n = 10)	Insulin-sensitive obese subjects (n = 10)	Insulin-resistant obese subjects (n = 10)
Inclusion criteria:			
Age:	30 - 60 years	30 - 60 years	30 - 60 years
BMI:	20-25 kg/m²	≥ 30 kg/m²	≥ 30 kg/m²
Insulin sensitivity:	Insulin-sensitive (HOMA-IR < 2.3)	Insulin-sensitive (HOMA-IR < 2.3)	Insulin-resistant (HOMA-IR > 3.8)
Physical activity:	< 3h/week	< 3h/week	< 3h/week
Exclusion criteria:	• Heart, lung, kidney or hormonal diseases, cancer or type 2 diabetes • Use of beta blockers or glucose lowering medications • Orthopaedic or neurological problems that interferes with exercise training • Hypertension stage II (>160/100 mmHg) • History of coronary events/revascularization (PCI, CABG, and/or AMI), presence of chronic pulmonary or renal disease		
Additional remarks:	Power analysis (α = 0.05, power = 0.80, effect size = 11,8, two-tailed, calculated with GPower) stated that six subjects/group are required. We will include ten subjects/group in order to compensate for possible drop-outs. All groups will be matched for age. Additionally, obese groups are matched for BMI and medication use.		

FIGURE S1. Inclusion and exclusion criteria. (BMI = body mass index, HOMA-IR = Homeostatic Model Assessment of Insulin Resistance, PCI = Percutaneous coronary intervention, CABG = Coronary artery bypass grafting, AMI = acute myocardial infarction).

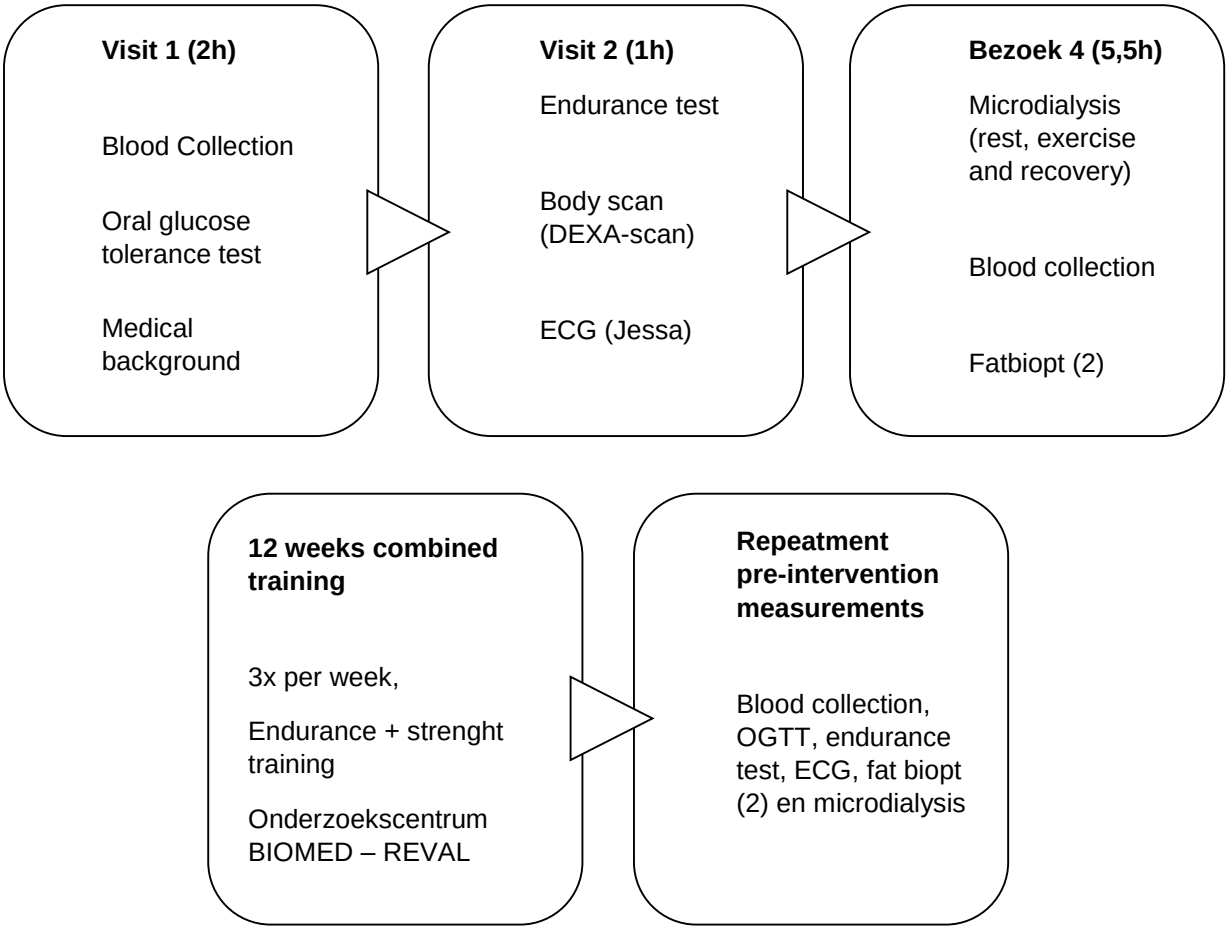

FIGURE S2. Set-up of the intervention study.

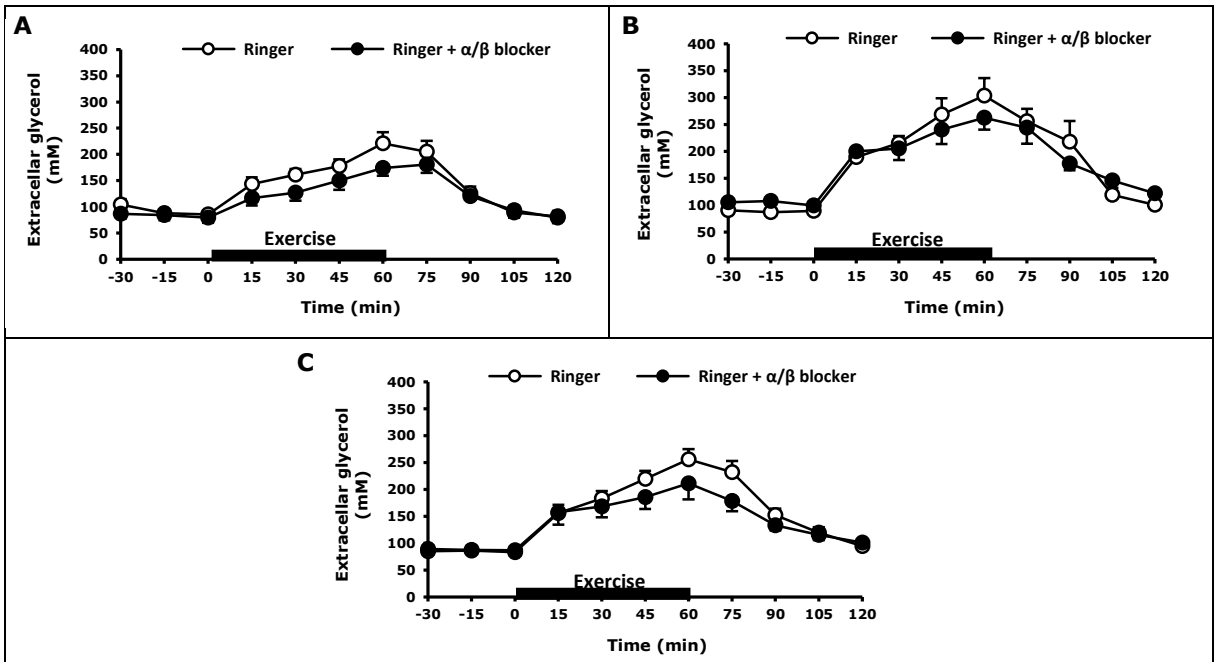

FIGURE S3. Extracellular glycerol release in SCAT tissue

Extracellular glycerol concentration at rest, during exercise and recovery in the dialysate of the control probe (○) and probe containing Ringer solution supplemented with phentolamine and propanol (●) in lean (A, *n* = 4), obese IS (B, *n* = 6) and obese IR (C, *n* = 10) subjects. Data are expressed as means ± SEM. The effects of each probe at each time point were analyzed between groups using Kruskal-Wallis analysis followed by post-hoc Man-Whitney *U*-tests. **P* < 0.05 compared to lean controls.

Abbreviations: *IS, insulin-sensitive; IR, insulin-resistant; SCAT, subcutaneous adipose tissue; ECG, extracellular glycerol; SEM, standard error mean*

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Does the fat cell listen to what the heart tells in human obesity? Role of atrial natriuretic peptide in fat mobilization and metabolic health in human obesity

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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