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Impact of lipid metabolism on skeletal muscle mitochondrial function in type 2 diabetes mellitus patients

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Dissertation

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Summary

Elevated plasma free fatty acids (FFA) may lead to ectopic lipid storage in skeletal muscle and liver, which is characteristic of type 2 diabetes mellitus (T2DM). Intramyocellular lipid content (IMCL) has been associated with insulin resistance, metabolic inflexibility and impaired mitochondrial oxidative capacity. Reduction of circulating FFAs by antilipolytic agents may therefore improve insulin sensitivity. However, the effect of alterations of circulating lipids on skeletal muscle mitochondrial function remains unclear.

The impact of FFAs on energy metabolism was examined in (i) a cross-sectional study, employing high-caloric meal ingestion in healthy, obese and T2DM patients, and in (ii) an interventional study, employing the antilipolytic agent Acipimox in T2DM patients. The cross-sectional study tested, whether ingestion of a high-caloric lipid-enriched meal would impair mitochondrial function, metabolic flexibility and insulin sensitivity, but increase IMCL. The interventional study tested, whether Acipimox would improve muscle mitochondrial oxidative capacity, metabolic flexibility, insulin sensitivity and IMCL in T2DM patients.

To allow for comparison, both studies applied identical methods. High-resolution respirometry allowed determining mitochondrial oxidative capacity *ex vivo*, while phosphorus magnetic resonance spectroscopy (^{31}P-MRS) made it possible to assess mitochondrial function *in vivo.* Insulin sensitivity and metabolic flexibility were analyzed by hyperinsulinemic-euglycemic clamps along with indirect calorimetry measurements. Thiobarbituric acid reactive substances (TBARS) served as a measure for oxidative stress. IMCL and liver fat content were measured by proton MRS (^{1}H-MRS), while IMCL was quantified histochemically in the interventional study.

The cross-sectional study revealed that glucose concentrations were higher in T2DM patients in comparison to obese participants and healthy controls before and after meal ingestion and circulating FFA were increased both in T2DM and in obese participants than in healthy lean humans. T2DM and obese humans had also lower whole body insulin sensitivity. *Ex vivo* mitochondrial oxidative capacity (state 3 respiration) was reduced in T2DM patients than in healthy participants. However, *in vivo* mitochondrial function, metabolic flexibility, TBARS and IMCL did not differ between groups. The interventional study showed that Acipimox decreased insulin sensitivity compared to control condition and placebo administration. Acipimox did neither affect mitochondrial density, mitochondrial oxidative capacity, IMCL content nor metabolic flexibility. Of note, plasma FFAs were increased upon Acipimox administration compared to both control and placebo conditions. Liver fat storage tended to be lower upon Acipimox compared to the control period, but not compared to the placebo period.

These studies showed that insulin resistance does not necessarily require impaired mitochondrial capacity and that circulating FFAs rather associate with insulin resistance than with abnormal mitochondrial function. In contrast to the initial hypothesis, even short-term Acipimox treatment increased circulating FFAs, likely due to the known 'rebound'-effect, which masked any possible beneficial effect of preceding FFA lowering on muscle mitochondrial function, metabolic flexibility, insulin sensitivity and IMCL content. In conclusion, circulating FFAs have no major direct impact on muscle mitochondrial function and FFA-lowering by Acipimox fails to improve metabolic disturbances and cannot be recommended for treatment of T2DM patients.

Zusammenfassung

Erhöhte Fettsäurespiegel im Plasma können zu Lipideinlagerungen im Skelettmuskel und der Leber führen, was ein Merkmal des Typ 2 Diabetes mellitus (T2DM) ist. Intramyocelluläre Lipide (IMCL) sind assoziiert mit Insulinresistenz, metabolischer Inflexibilität und verminderter mitochondrialer Kapazität. Antilipolytische Wirkstoffe können die Konzentration freier Fettsäuren (FFA) im Plasma reduzieren und dadurch die Insulinsensitivität steigern. Die Wirkung des veränderten FFA Spiegels auf die mitochondriale Funktion im Skelettmuskel ist unklar.
Die Auswirkungen des FFA Spiegels auf den Energiemetabolismus wurde in (i) einer Querschnittstudie untersucht, in welcher eine hoch-kalorische Mahlzeit bei gesunden, übergewichtigen und T2DM Patienten verabreicht wurde, sowie in (ii) einer Interventionsstudie, in welcher die Wirkung des antilipolytischen Wirkstoffes Acipimox bei T2DM Patienten ermittelt wurde. In der Querschnittstudie wurden die mitochondriale Funktion, die metabolische Flexibilität, die Insulinsensitivität sowie die muskuläre Lipideinlagerung prä- und postprandial untersucht, während die gleichen Parameter in der Interventionsstudie nach Acipimox Einnahme erhoben wurden.
Um eine Vergleichbarkeit der Studien zu gewährleisten, wurden identische Messmethoden angewendet. Die Funktion der Mitochondrien *ex vivo* wurde anhand eines Respirationstests erhoben, während die Funktion *in vivo* mittels einer Phosphor-MRS Untersuchung (^{31}P-MRS) ermittelt wurde. Zur Bestimmung der Insulinsensitivität und metabolischen Flexibilität wurden euglykämische-hyperinsulinämische Clamps durchgeführt, kombiniert mit indirekten Kalorimetrie Messungen. Thiobarbitursäure-reaktive Substanzen (TBARS) dienten als Messgröße für den oxidativen Stress. Die Fetteinlagerung in der Leber und der Muskulatur wurden mittels Protonen-MRS (^{1}H-MRS) erhoben, während die muskuläre Lipideinlagerung in der Interventionsstudie histo-chemisch bestimmt wurde.
In der Querschnittstudie waren die Plasma Glukose Konzentrationen bei T2DM Patienten prä- und postprandial erhöht im Vergleich zu übergewichtigen und gesunden Patienten, während die FFA Spiegel sowohl bei T2DM als auch bei übergewichtigen Patienten höher waren als in der Kontrollgruppe. T2DM und übergewichtige Patienten wiesen eine verringerte Insulinsensitivität auf. Die mitochondriale Funktion *ex vivo* (Respriationsstadium 3) war bei T2DM Patienten reduziert im Vergleich zu der gesunden Patientengruppe. Die mitochondriale Funktion *in vivo*, die metabolische Flexibilität, TBARS und der IMCL Gehalt unterschieden sich nicht zwischen den Gruppen. Acipimox setzte die Insulinsensitivität herab im Vergleich zur Placebo Einnahme und Kontrollbedingungen. Acipimox hatte keine Auswirkungen auf die mitochondriale Dichte, die mitochondriale Kapazität, den IMCL Gehalt sowie auf die metabolische Flexibilität. Der FFA Spiegel im Plasma war jedoch deutlich erhöht nach Acipimox Einnahme im Vergleich zu Kontrollbedingungen und Placebo Einnahme. Die Lipideinlagerungen in der Leber waren während Acipimox Einnahme geringer im Vergleich zur Kontrollgruppe, jedoch nicht im Vergleich zur Placebo Einnahme.
Die Studien zeigten, dass Insulinresistenz nicht zwangsläufig mit verminderter mitochondrialer Kapazität einhergeht und dass FFAs eher mit Insulinresistenz als mit veränderter mitochondrialer Funktion assoziiert sind. Die Acipimox Behandlungen führte zu einer Erhöhung des FFA Spiegels, was auf einen *Rebound*-Effekt zurückzuführen ist, wodurch die metabolischen Parameter negativ beeinflusst wurden. Schlussendlich zeigten die vorliegenden Studien, dass FFAs keine direkte Auswirkung auf die mitochondriale Funktion in der Muskulatur haben und dass Acipimox zu keiner Verbesserung der metabolischen Störungen von T2DM Patienten führt und daher als Medikament in der Langzeit-Therapie des T2DM ungeeignet ist.

List of abbreviations

^{31}P-MRS	Phosphorus magnetic resonance spectroscopy
^{1}H-MRS	Proton magnetic resonance spectroscopy
ADA	American Diabetes Association
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BP	Blood pressure
bpm	Beats per minute
BMI	Body mass index
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CoQ	Coenzyme Q
DAG	Diacylglycerol
DEXA	Dual-energy x-ray absorptiometry
EGP	Endogenous glucose production
ETC	Electron transport chain
FCCP	Fluoro-carbonyl cyanide phenylhydrazone
FFA	Free fatty acid
FPG	Fasting plasma glucose
GH	Growth hormone
GIR	Glucose infusion rate
GLUT4	Glucose transporter 4
HCL	Hepatocellular lipids
HDL	High-density lipoprotein
HRmax	Maximal heart rate
IDF	International Diabetes Federation
IRS	Insulin receptor substrate
IMCL	Intramyocellular lipids
LCFA-CoA	Long-chain fatty acyl coenzyme A
MetS	Metabolic syndrome
MMT	Mixed meal test
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
NA	Nicotinic acid
NAFLD	Non-alcoholic fatty liver disease
PCr	Phosphocreatine
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
PKC	Protein kinase C
Rd	Rate of glucose disposal
rmANOVA	Analysis of variance for repeated measurements
ROS	Reactive oxygen species
RQ	Respiratory quotient
T2DM	Type 2 diabetes mellitus
TBARS	Thiobarbituric acid reactive substances
TG	Triglyceride
UCP	Uncoupling protein
VLDL	Very-low-density lipoprotein
VO_2max	Maximal oxygen consumption

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1. Introduction

1.1 Type 2 diabetes mellitus

1.1.1 Definition and description

Diabetes comprises of metabolic diseases, which have gained importance worldwide. Impairments of insulin secretion and/or diminished peripheral insulin action are the hallmark of diabetes, which is defined by chronic hyperglycemia. According to the American Diabetes Association (ADA), there are four ways to diagnose diabetes (1), which are listed below:

1. $HbA_{1c} \geq 6.5$ %*

   OR

2. Fasting plasma glucose (FPG) $\geq$ 126 mg/dl (7.0 mmol/l)**

   OR

3. Symptoms of hyperglycemia and plasma glucose $\geq$ 200 mg/dl (11.1 mmol/l)***

   OR

4. 2-h plasma glucose $\geq$ 200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test ****

* The test should be performed in a laboratory using a method that is certified by the NGSP (National Glycohemoglobin Standardization Program) and standardized by the assay according to DCCT (Diabetes Control and Complications Trial).

** Fasting is defined as no caloric intake for at last 8 hours.

*** The classic symptoms of hyperglycemia are defined as polyuria, polydipsia and unexplained weight loss.

**** The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

The disease is classified in two major types, type 1 and type 2 diabetes, however, other specific types of diabetes exist as well, with the classification being based on the causes inducing diabetes mellitus. Inherited genetic defects of ß-cells, as well as genetic mutations of peripheral insulin receptors and diseases of the exocrine pancreas belong to the category of specific diabetes types, as well as drug-induced diabetes, endocrinopathies, virus-infection induced diabetes and diabetes associated with genetic

syndromes like Down´s syndrome or Turner´s syndrome (1). Gestational diabetes is another subgroup of diabetes disease (1). A more common form of the disease is the immune-mediated diabetes, also known as type 1 diabetes mellitus, as the incidence accounts for 5-10% of diabetes (1). This form of diabetes is characterized by an absolute deficiency of insulin secretion reduced to an autoimmune reaction towards ß-cells of the pancreas. Type 1 diabetes is primarily diagnosed during childhood, but it can also occur at any age, depending on a rapid or slow autoimmune destruction of ß-cells and also environmental factors like virus infections or respiratory diseases during infancy are known to influence the progression of the disease (1). Type 2 diabetes mellitus (T2DM) is the most common form of diabetes, as 90% of the people affected are diagnosed with this form (2). T2DM mainly appears at older age, however, in recent years the number of teenagers affected has raised tremendously due to the upward tendency of physical inactivity and obesity, which are two important risk factors in the progression of the disease. In contrast to type 1 diabetes, T2DM is often diagnosed upon advanced progression of the disease, attributable to ambiguous symptoms at the onset of the disease. T2DM is characterized by hyperglycemia, induced by diminished peripheral insulin action, also termed insulin resistance. The interaction between liver and muscle metabolism plays a key role in the development of insulin resistance (3). Both liver and muscle cells are important in keeping glucose levels at a stable equilibrium by storage of glucose in times of glucose surplus or on the other hand by hepatic gluconeogenesis in times of glucose deficiency. Disturbances of these processes in patients with T2DM, according to a resistance towards insulin, result in diminished insulin-mediated glucose uptake, e.g. in muscle cells, and elevated endogenous glucose production (EGP) even in times of increased plasma glucose concentrations, which both intensifies the hyperglycemic state. In the early stages of the disease, an initial increase of pancreatic insulin secretion can be observed, which is considered to be a compensatory mechanism in order to decrease hyperglycemic conditions, which failed within the progression of the disease due to exhaustion of pancreatic ß-cells (4). Furthermore, insulin resistance of adipocytes causes enhanced lipolysis, which results in an elevated release of free fatty acids (FFA) into the circulation (5). FFAs are known to increase hepatic gluconeogenesis through stimulation of key gluconeogenic enzymes (6). Indeed, it has been shown that both hepatic glucose production and plasma glucose levels rise correspondingly to the concentration of plasma FFAs in diabetic patients (6). Moreover, chronic oversupply of circulating lipids intensifies ectopic fat accumulation

in skeletal muscle cells, which both interferes with the cellular insulin signaling pathway and impairs mitochondrial oxidative capacity (7, 8, 9). Accordingly, it has been suggested, that a reduction of circulating FFAs through decreased lipolysis in adipose tissue increases whole body and hepatic insulin sensitivity, followed by enhanced peripheral glucose uptake as well as diminished EGP production, resulting in decreased plasma glucose levels (9). Furthermore, it has been speculated, that a reduction of serum FFA levels beneficially affects ectopic fat storage and muscle mitochondrial function, which forms the scope of this thesis.

1.1.2 Epidemiology

In Germany, the prevalence for T2DM was nearly 0.5% in 1950, whereas nowadays nearly 7.0% of the German population suffers from this disease (10). In the year 2000, there were already approximately 170-190 million people worldwide with the diagnosis of diabetes and it is predicted that it will rise up to 300-366 million in 2030 (11, 12). The International Diabetes Federation (IDF) even suspects a rise of the affected people up to 380 million in 2025 worldwide (13). The estimated number of diabetes patients may however be higher due to the difficulty of calculating the percentage of unidentified people suffering from the disease. Some studies speculate on the interrelationship between the progression of T2DM and population growth, longevity, urbanization and increased prevalence of obesity and physical inactivity (11, 14). Accordingly, it is suspected that India and China will have the highest numbers of people diagnosed with T2DM in 2030 because of population aging and westernization (11, 14).

1.1.3 Risk factors

Multiple factors, such as genetic predisposition, environmental- and lifestyle factors, contribute to the onset of T2DM. The ADA describes the patients´ age as the most influential risk factor in the pathogenesis of T2DM (15), as insulin resistance is known to be a potential concomitant phenomenon during aging (16). Furthermore, genetic predisposition for diabetes is known to enhance the probability to come down with diabetes disease, as 40% of children of T2DM patients are susceptible towards diabetes (15). Genetic predisposition varies between ethnic groups, whereas Hispanics, Caribbeans and Asian Indians are said to have an increased risk in comparison to Caucasians, for example (15). Moreover, women who suffer from the polycystic ovary

syndrome are at higher risk for the onset of T2DM and also women who were affected from gestational diabetes during pregnancy have a 7.5 -fold increased risk for diabetes (15). Furthermore, infants who are suffering from a low birth weight are expected to have diminished ß-cell function in adulthood and rank therefore among the group of risk patients for diabetes disease (15).

Moreover, the interplay between different features, which induce metabolic disorders are defined by the IDF as the metabolic syndrome (MetS) (17). Most of the people who suffer from the MetS exhibit insulin resistance, which is considered to be the most important risk factor in the pathogenesis of T2DM (17, 18). Organizations like the World Health Organization or the European Group for the Study of Insulin Resistance mentioned obesity, insulin resistance, dyslipidemia as well as hypertension as keywords in the progression of the MetS (17). The IDF published the latest definition of the MetS and they concluded that a person suffers from the MetS, if central obesity, defined by waist circumference, plus any two of the factors mentioned below are diagnosed:

- increased triglycerides (TG): ≥ 1.7 mmol/l (150 mg/dl)
- reduced high-density lipoprotein (HDL)-cholesterol: < 1.03 mmol/l (40 mg/dl) in males and < 1.29 mmol/l (50 mg/dl) in females (or specific treatment for these lipid abnormalities)
- increased blood pressure (BP) (systolic BP ≥ 130 or diastolic BP ≥ 85 mmHg) (or treatment of previously diagnosed hypertension)
- increased FPG [FPG ≥ 5.6 mmol/l (100 mg/dl)] (or previously diagnosed T2DM) (17).

Additionally, a link was found between psychosocial diseases, particularly depressive disorders, and both the MetS and T2DM (15).

Obesity correlates closely with insulin resistance and is therefore a risk factor of T2DM (17, 19). Abdominal fat storages entail the main risk (17), which can be estimated by waist circumference, and values of ≥ 94 cm for European males and ≥ 80 cm for European females are considered to be alarming (17). It is appreciated that about 90% of T2DM patients are obese, however only 20-25% of the obese population suffers from overt diabetes (20). Obesity results from a hyper-caloric diet, accompanied by physical inactivity. Accordingly, lifestyle intervention, intending a balanced nutrition including an increased intake of fibre and monounsaturated fatty acids, as well as regular physical

activity, are shown to beneficially influence the body fat ratio and therefore diabetes disease (10, 15). In obese patients, increased FFA flux into the circulation was observed, inducing both lipotoxicity and ectopic lipid storage in peripheral tissues, which in turn provoke insulin resistance.

1.2 Pathogenic features of type 2 diabetes mellitus

1.2.1. Insulin resistance

The endocrine effect of insulin is most important for the whole body glucose metabolism, as insulin mediates peripheral glucose uptake and cellular storage in form of glycogen, reduces hepatic glucose output and inhibits adipose tissue lipolysis, which prevents the release of FFAs into the circulation. Insulin resistance is considered to be the core factor in the pathogenesis of T2DM, as a resistance to insulin induces metabolic disturbances like compromised peripheral glucose disposal, hyperglycemia and elevated plasma FFA levels (16, 21, 22). Moreover, insulin resistance is known as a syndrome with accompanying symptoms like obesity, T2DM, hypertension, as well as atherosclerotic cardiovascular disease, dyslipidemia and hyperinsulinemia (16). Impaired insulin action can be either inherited or acquired during life, especially due to obesity and aging (16). The interplay between environmental and genetic influences defines the onset of diabetes (23, 24).

Most importantly, insulin resistance occurs several years before the manifestation of T2DM, indicating that the degree of insulin resistance is an expressive predictor for the progression of the disease (25). In the early stages of insulin resistance, peripheral glucose uptake is impaired, which is initially counter-regulated by an increased insulin secretion of pancreatic β-cells, in order to maintain euglycemia (16). Subsequently, if counter-regulation via hyperinsulinemia failed, due to β-cell exhaustion, fluctuating and increasing plasma glucose levels, paralleled by restricted insulin secretion, proceed to impair glucose tolerance and contribute to the development of diabetes (16) (Fig. 1).

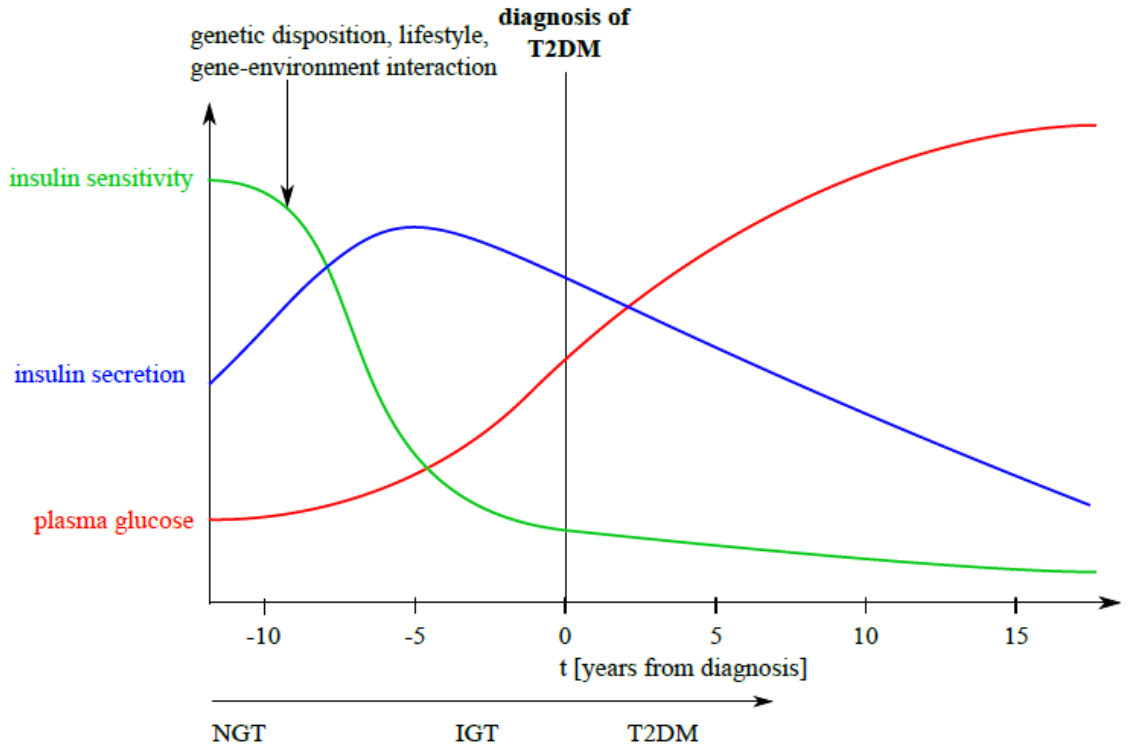

Fig. 1: Pathogenesis of T2DM. The first symptoms of impaired glucose metabolism develop several years before manifestation of diabetes, caused by genetic predisposition and/or environmental influences. The pre-diabetic state is characterized by diminished insulin sensitivity (green line), initially counter-regulated by enhanced pancreatic insulin secretion (blue line), in order to keep glucose levels in a physiological range (red line). After reaching a maximum, the insulin secretion rate constantly decreases, due to severe β-cell defects, paralleled by a reduction of insulin sensitivity. Plasma glucose levels increase and impaired glucose tolerance occurs. The decade after the onset of T2DM is characterized both by hyperglycemia, which approaches its maximum and by reduced sensitivity for insulin. NGT, normal glucose tolerance; IGT, impaired glucose tolerance; T2DM, type 2 diabetes mellitus [adapted from Ramlo-Halsted and Edelman (26)]

To gain more insight into the metabolic defects underlying impaired insulin action, it is of highest interest to research the cellular mechanisms in more detail. Skeletal muscle accounts for more than 80% of insulin-stimulated whole body glucose disposal (12, 16). Accordingly, especially skeletal muscle cells of insulin resistant patients are known to face with multiple metabolic defects like complications of the insulin signaling pathway, reduced glycogen synthesis and glycolysis, as well as increased intramyocellular lipid (IMCL) accumulation (2, 9, 27, 28). Impaired insulin signaling in T2DM patients is not only proven in skeletal muscle cells but also in liver and adipocytes. Insulin binding to its receptor, receptor phosphorylation and subsequently phosphorylation of the insulin receptor substrate (IRS) is impaired (21). Both receptor- and postreceptor defects may be responsible for diminished insulin action (29), whereas the restricted insulin-mediated cellular glucose transport is said to be the decisive factor in the pathomechanism of insulin resistance (25). Accordingly, diminished expression

of glucose transporter 4 (GLUT4) was observed in adipose tissue of diabetes patients, as well as an impaired insulin-mediated fusion of GLUT4 with the plasma membrane of skeletal muscle cells (21, 27). Subsequently, diminished peripheral glucose uptake restricts both the oxidative and non-oxidative glucose pathway, especially in skeletal muscle cells (16). Besides the restricted cellular glucose uptake in T2DM patients, another decisive factor responsible for the diminished pathway of glycogen synthesis is suspected to be the decreased enzyme activity of hexokinase II and glycogen synthase, which is even shown in offspring of T2DM patients, indicating that insulin resistance is genetically determined (16, 25).

Furthermore, a positive correlation between the plasma content of FFAs and both hepatic and muscular insulin resistance was verified (21), meaning the higher the plasma FFAs, the more insulin resistant people are. Elevated release of FFAs from adipose tissue is the key mediator in the development of insulin resistance (27), caused by impaired insulin-mediated suppression of lipolysis (30). Impaired insulin-mediated suppression of EGP was found in times of increased plasma FFA levels, reflecting impaired hepatic insulin sensitivity (31). Accordingly, adipocytes are known to be the most highly insulin-responsive cell types, which underlines the close correlation between obesity and insulin resistance (21) and furthermore, IMCL accumulation, initiated by increased circulating FFAs, contribute to insulin resistance of skeletal muscle (30, 32). Intramyocellular accumulation of harmful lipid metabolites, like diacylglycerol (DAG) and ceramides interfere with insulin signaling, whereas the inhibition of IRS via DAG is mediated by activation of protein kinase C (PKC) (25, 33).

Furthermore, insulin resistance is associated with diminished mitochondrial oxidative capacity, which contributes to a reduced adaption of fuel availability and fuel oxidation, termed metabolic inflexibility (22). Moreover, the cellular energy demand cannot be met, as the mitochondrial adenosine triphosphate (ATP) generation is known to be impaired in insulin resistant patients, due to reduced activity of the electron transport chain (ETC) (22). Also the mitochondrial content is reduced in insulin resistant individuals (22).

Taken together, insulin resistance is the key factor in the pathogenesis of T2DM. Impaired insulin-mediated suppression of lipolysis in adipocytes was found in insulin resistant patients, which contributes to elevated plasma FFA levels and subsequently to

enhanced EGP as well as increased IMCL accumulation, which intensify a preexisting resistance to insulin. Also mitochondrial activity is reduced in insulin-resistant individuals, which is coupled with metabolic inflexibility and results in impaired cellular energy supply, as ATP generation via the ETC is diminished.

1.2.2 Metabolic flexibility

Metabolic flexibility is defined as the ability of healthy lean individuals to switch from lipid to glucose oxidation in the postprandial, insulin-stimulated state and from carbohydrate to lipid oxidation during fasting (12). Patients with T2DM are characterized as metabolic inflexible, as they are not able to adapt fuel oxidation to fuel availability compared to healthy controls (34). The respiratory quotient (RQ) reflects the switch between carbohydrate and lipid oxidation, which allows drawing conclusions about metabolic flexibility (34). A low RQ value (~0.7) is indicative for a higher reliance on fat oxidation, whereas an increased RQ (~1.0) refers to glucose oxidation (34). Accordingly, in healthy individuals the RQ is diminished during fasting conditions, revealing the preferred oxidation of lipids, whereas increased values are observed in the postprandial state, indicating enhanced glucose oxidation (35). Transferred to the metabolic pathophysiology of T2DM patients, metabolic inflexibility is characterized by an impaired capacity to oxidize fat during fasting conditions, reflected by an increased RQ, and the inability to intensify glucose oxidation in the postprandial state, reflected by a decreased RQ in comparison to the values of healthy controls (30). Consequently, the total range of RQ changes is diminished in patients with T2DM compared with healthy controls, referred to as metabolic inflexibility (Fig. 2) (34).

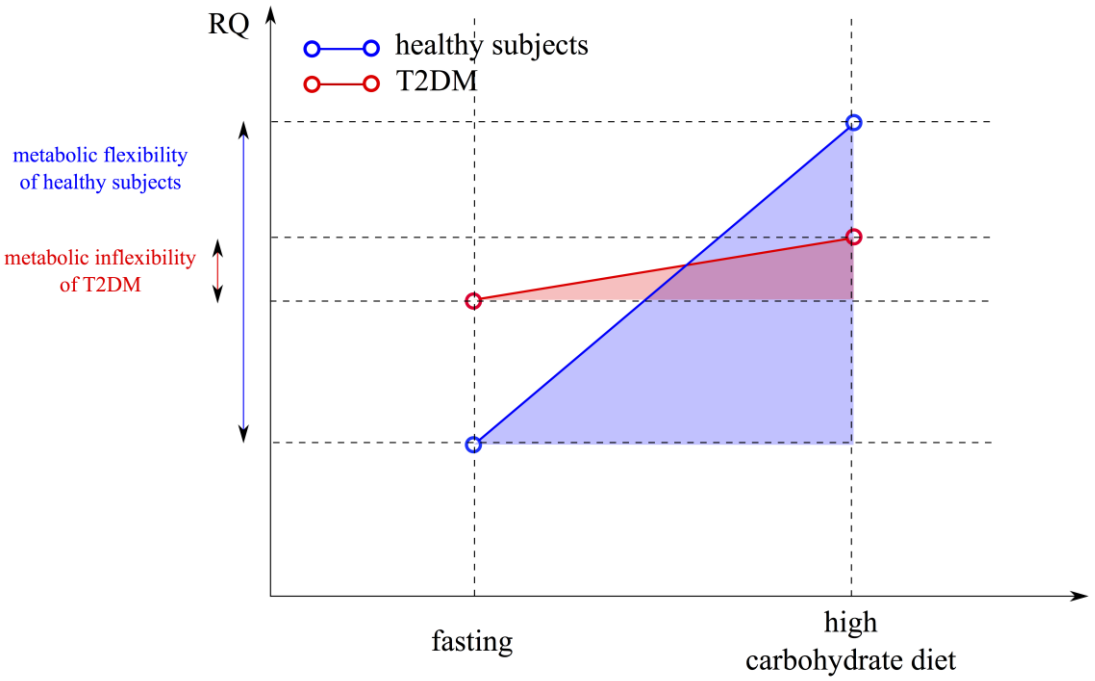

Fig. 2: Illustration of the metabolic inflexibility of T2DM patients in comparison to healthy controls. RQ values are compared between T2DM patients (red line) and healthy controls (blue line) during fasting condition and upon a carbohydrate-rich diet (insulin-stimulated state). The diminished RQ value of healthy controls in the fasting state indicates a preferred lipid oxidation rate, which rapidly switches to elevated RQ values upon a carbohydrate-rich diet, revealing the predominance of glucose oxidation. In contrast, the fasting RQ value of T2DM patients is elevated, displaying a diminished rate of lipid oxidation compared to healthy controls and the value does hardly increase to glucose oxidation in the postprandial state. Based on the total range of the RQ changes, illustrated by the arrows on the y-axis, the metabolic flexibility can be assessed, revealing that healthy individuals are metabolic flexible as they are able to adapt fuel oxidation to fuel availability, whereas T2DM patients are considered to be metabolic inflexible. RQ, respiratory quotient; T2DM, type 2 diabetes mellitus patients [adapted from Kelley and Mandarino (36)]

A close correlation was found between metabolic flexibility and insulin sensitivity (34), although, in some studies it was shown that improved metabolic flexibility may not necessarily be paralleled by enhanced insulin sensitivity (7, 37). Therefore, it can be concluded that metabolic inflexibility is not a simple reflection of insulin resistance, but controlled differently, i.e. by mitochondrial oxidative capacity (12, 34). The latter is already known to be of major importance in the development of metabolic inflexibility, as a correlation was found between mitochondrial disturbances, metabolic inflexibility and insulin resistance (12, 34). Diminished mitochondrial oxidative capacity of skeletal muscle cells, which is known as a particular feature of T2DM patients, leads to an imbalance between FFA uptake and FFA oxidation, which initiates IMCL accumulation

and lipotoxicity (12, 30, 34). Besides impaired mitochondrial function, elevated plasma FFAs also promote intracellular lipid accumulation. Ectopic storage of lipid intermediates are known to interfere with the insulin signaling pathway, resulting in diminished cellular glucose uptake even in times of glucose surplus, which in turn reflects metabolic inflexibility (34). In line, impaired glucose phosphorylation and decreased glucose oxidation was found in times of elevated plasma FFA levels, reflected by diminished intramyocellular concentrations of glucose-6-phosphate (32, 38). Also Roden et al. found an inhibitory effect of FFAs on whole body glucose disposal due to diminished glucose transport and phosphorylation (27, 39). Furthermore, they observed reduced glycogen synthesis caused by decreased glycogen synthase activity due to elevated FFAs (39). Taken together, not only mitochondrial oxidative capacity but also plasma FFA concentrations are known to be core components in regulating the metabolic balance (35).

In conclusion, lowering of plasma FFA levels, either by changes in dietary and physical activity (7, 30) or by lipid lowering agents, is suggested to improve metabolic flexibility.

1.2.3 Mitochondrial function

The key task of mitochondria is the generation of ATP via oxidative phosphorylation within the respiratory chain of the inner mitochondrial membrane, mainly driven by the proton gradient generated by the ETC. Mitochondrial oxidative capacity is shown to be impaired in T2DM patients and in first-degree relatives at a risk to develop diabetes (40, 41, 42). It has been speculated that decreased mitochondrial oxidative capacity in T2DM patients is attributable to reduced true intrinsic mitochondrial impairments, as well as diminished mitochondrial density with age and obesity (12, 19, 28). Diminished mitochondrial ATP production in T2DM patients is assumed to be rather related to a reduction in mitochondrial content than to impairments of mitochondrial quality (19). In line, a reduction of approximately 38% of mitochondrial density was shown in insulin resistant relatives of T2DM patients (43). However, other studies argue the converse by suggesting that rather intrinsic mitochondrial defects than diminished mitochondrial content are responsible for mitochondrial impairments (12, 40). Accordingly, mitochondrial oxidative capacity was still impaired if normalized for mitochondrial DNA (mtDNA) content and furthermore, studying the intrinsic mitochondrial capacity

by *ex vivo* measurements, true intrinsic defects were found (12, 40). Skeletal muscle mitochondria of diabetic and obese humans were shown to be smaller and also mitochondrial abnormalities like large vacuoles as well as visional structural derangements were found compared to healthy controls (7, 12, 44). mtDNA is known to be susceptible towards damages, possibly via mitochondrial production of reactive oxygen species (ROS), and the repair mechanism of the mtDNA is less effective, contributing to mutations of the mtDNA (23, 44, 45). Interestingly, it was found that both reduced mitochondrial size and mitochondrial damages correlate with the degree of insulin resistance (2, 12, 40, 44). Additionally, mitochondrial biogenesis is already impaired, if insulin resistance is proven, prior to the diagnosis of T2DM (32).

An expressive parameter for the determination of mitochondrial oxidative capacity *in vivo* is the calculation of phosphocreatine (PCr) resynthesis rate using phosphorus magnetic resonance spectroscopy (^{31}P-MRS) measurements. In T2DM patients, a prolonged PCr resynthesis rate was found in previous studies, indicating impaired mitochondrial function *in vivo* (12, 40). Moreover, a correlation was also found between PCr half-time and plasma glucose concentration, as well as between PCr resynthesis rate and maximal oxygen consumption (VO_2max) (12). Accordingly, both diminished values of VO_2max and rising plasma glucose concentrations are related to the extent of impaired mitochondrial function.

Compromised activity of maker enzymes, necessary for oxidative phosphorylation processes, are held responsible for restrictions of the ETC activity in T2DM patients (10, 12, 28, 40, 44), which results in an imbalance of substrate metabolism in skeletal muscle (12, 28). Abnormal mitochondrial function results in IMCL accumulation, as well as ROS production, which both impairs insulin signaling (2, 12, 40, 44, 45). In addition, the ectopic lipid accumulation in skeletal muscle seems even to influence mitochondrial biogenesis, as it was found that the mtDNA content is lower in obese people or in T2DM affected. A link between increased body mass and mitochondrial impairments was assumed (28). It has been speculated that reduced levels of peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) causes mitochondrial abnormalities, as this cofactor is considered to be both a core component in the transcription of genes involved in oxidative phosphorylation processes and a stimulator of mitochondrial biogenesis (7, 12). An interrelation between plasma lipid levels and mitochondrial function was presumed, as an inverse correlation between

plasma FFA levels and PGC-1α messenger RNA (mRNA) was found, which leads to the conclusion that lipid oversupply hinders mitochondrial gene expression and therefore induces abnormal mitochondrial function (46).

Moreover, the intracellular glucose storage via glucose phosphorylation is mediated by an ATP dependent enzyme, hexokinase II (44). With regard to this interdependency, if the ATP level is lowered due to mitochondrial alterations, the storage of glucose is limited, which contributes to diminished glucose uptake via negative feedback coupling, followed by hyperglycemia.

Diminished ATP production was not only found in T2DM patients but also in first-degree relatives of T2DM patients, which provide evidence for the genetic component of mitochondrial impairments. Resting muscular ATP synthesis rate was decreased by 40% in insulin resistant patients in comparison to healthy humans and furthermore, the children of T2DM patients were shown to have a 60% lower rate of cellular glucose uptake as well as a reduction of resting ATP synthesis rate by 30% (40). Nonetheless, mitochondrial function of diabetic patients was completely restored due to exercise training via enhanced mitochondrial biogenesis, indicating that lifestyle changes overcome genetic disturbances of mtDNA (7, 28).

As an inverse correlation was found between mitochondrial impairments and cellular insulin sensitivity, the questions still arises, if diminished oxidative capacity is a feature of insulin resistance or on the other hand, if it causes insulin resistance (2). Previous studies provide evidence for the latter suggestion, as compromised mitochondrial function was even found in glucose tolerant offspring of T2DM patients and in first-degree relatives accompanied with physiological values of insulin sensitivity (7, 12, 40).

1.2.4 Oxidative stress

A further accompanying phenomenon of impaired mitochondrial function is the rising content of ROS. ROS are byproducts of the ETC with the particular feature of causing oxidative stress, which in consequence initiates cellular damages or even apoptosis. Mitochondrial ROS production is a feature of T2DM and it is linked with impairments of insulin signaling (2). In times of enhanced ß-oxidation rate, electrons contained in the oxidative products of NADH and $FADH_2$ are donated to the ETC in order to intensify the electrochemical proton gradient, which drives the ATP generation at complex V of

the ETC. A possible side effect of an oversupply of equivalents donated to the ETC is an overproduction of ROS molecules, if both the proton electrochemical gradient is large and if adenosine diphosphate (ADP) regeneration exceeds a maximum (2, 23, 47). In times of electron surplus, electrons were presented at coenzyme Q (CoQ) within the ETC, whereas electrons are transferred to ROS molecules like O_2^-, H_2O_2 or OH^- (2, 23). These molecular species are characterized by a tremendously substrate reactivity and the capability of damaging cellular structures. Thiobarbituric acid reactive substances (TBARS) are a measure for oxidative stress and are classified as products of cellular damage initiated by oxidative stress.

Furthermore, a dependency between the enzyme activity of ATP synthase and the proton electrochemical gradient was observed, since the lower the ATP synthase activity the higher the electrochemical gradient, which in turn stimulates ROS generation (47). This assumption allows the following conclusion: the lower the ATP synthase activity the higher the amount of cell-damaging ROS, which is coupled with mitochondrial impairments, inducing insulin resistance.

Uncoupling proteins (UCP) of the inner mitochondrial membrane, like UCP2 and UCP3, are able to protect against ROS-related cellular damage. These proteins appear to be involved in the reduction of the electrochemical gradient through elimination of protons (23), aiming to limit the ROS production. Complementary, it was also proven that UCP2 as well as mRNA of UCP3 in skeletal muscle cells are upregulated due to chronically elevated plasma FFA levels (47, 48). This interrelation reveals the strong association between the expression of UCP and lipid metabolism and furthermore, it indicates that an oversupply of circulating lipids initiates cellular stress, causing mitochondrial alterations or even apoptosis.

1.2.5 Intramyocellular fat storage

A negative correlation was shown between IMCL content and whole body insulin sensitivity (49). Increased ectopic fat accumulation, especially in skeletal muscle cells, was found in patients with T2DM compared to healthy controls, which coexists with elevated levels of plasma FFAs, as well as obesity, physical inactivity and mitochondrial disturbances (50, 51). These pathophysiological parameters are known as predictors for the onset of diabetes disease, as a correlation was observed between

IMCL content and the degree of insulin resistance (12, 21, 46, 49, 50, 51), reflected by decreased insulin sensitivity in times of elevated IMCL content (51).

Nearly 40% increase in insulin resistance is said to be attributable to an intensified IMCL accumulation (52). Furthermore, a strong dependency between plasma FFA concentrations and IMCL content was assumed, as fluctuations of IMCL storages were initiated by changes of plasma FFA concentrations (52). Despite, not FFAs itself but rather some lipid metabolites are stored in skeletal muscle cells in form of TGs, DAG, long-chain fatty acyl CoA (LCFA-CoA) and ceramides (10, 46, 47). The latter three are known to interfere with the insulin signaling cascade, partially through activation of the PKC (12, 46, 50, 52) (Fig. 3).

Moreover, a negative correlation was found between IMCL content and insulin-stimulated whole body glucose uptake, as it was suggested that lipid metabolites are able to compromise the membrane fusion of GLUT4 within the sarcolemma, inducing impaired insulin-mediated glucose uptake (21, 50), followed by both impaired cellular glucose oxidation and diminished storage of glucose (9, 49) (Fig. 3). Accordingly, insulin resistant relatives of T2DM patients are known to have a reduced glucose disposal rate by 60% accompanied by an elevation of IMCL about 60% in comparison to healthy controls (43).

Intramyocellular accumulation of lipid metabolites like LCFA-CoA, DAG and ceramides cause insulin resistance and subsequently insulin-dependent mitochondrial processes are impaired, which in turn leads to an imbalance between cellular FFA uptake and mitochondrial substrate metabolism (12, 38, 50). In line, a preferred ectopic accumulation of lipids was found near the sarcolemma of skeletal muscle cells, providing evidence for the interdependency of IMCL accumulation and mitochondrial alterations, as mitochondria are located in next proximity of the subsarcolemm region (50, 52). Supporting this suggestion, it was found that lipid oversupply impairs mRNA expression of PGC-1α, resulting in diminished expression of genes involved in mitochondrial oxidative phosphorylation processes, revealing that an enhancement of IMCL storages intensifies preexisting mitochondrial impairments (46) (Fig. 3).

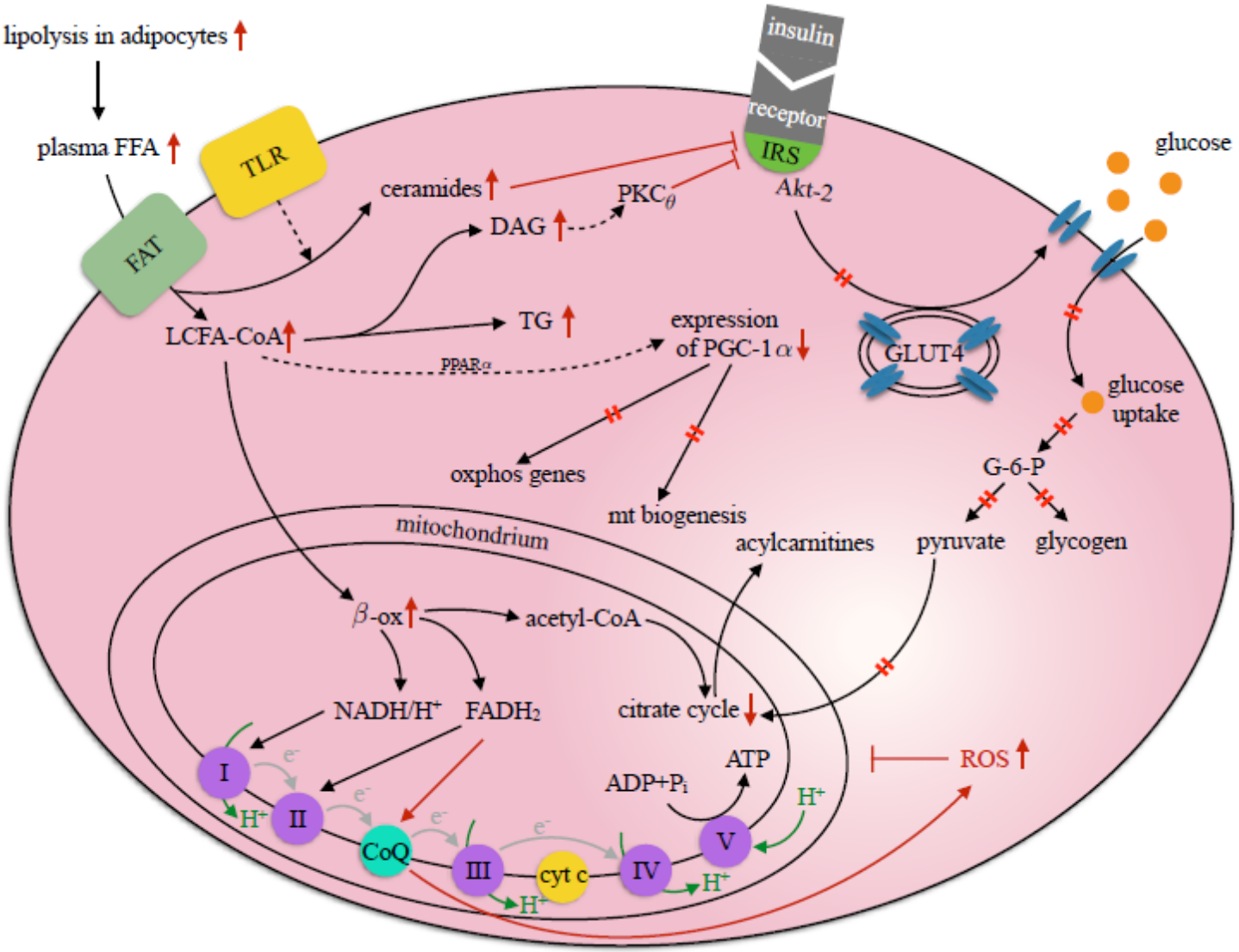

Fig. 3: Impacts of FFA oversupply on skeletal muscle cells. Metabolic pathways (black arrows), are partially impaired by an intracellular surplus of lipid metabolites (red double bars). The dashed lines indicate stimulating pathways. FFAs are taken up via FAT and lipid intermediates are stored intracellularly as TGs. Lipid metabolites like ceramides and DAG impair the insulin signaling cascade, the latter one via activation of PKCθ. Insulin-stimulated postreceptor effects are interfered, e.g. the membrane fusion of GLUT4, which impairs the cellular glucose uptake. Subsequently, both glycogen synthesis and oxidative glucose metabolism are restricted, which leads to diminished substrate supply for the mitochondrial citrate cycle. Furthermore, the oversupply of LCFA-CoA induces an increased mitochondrial β-oxidation, which enhances the proton gradient of the electron transport chain. However, if the proton gradient is large, or if ADP regeneration has reached a maximum, the equivalent of $FADH_2$ is donated to CoQ, which generates ROS, leading to cellular damages. FFA, free fatty acid; FAT, fatty acid translocator; TLR, toll like receptor; LCFA-CoA, long-chain fatty acyl Co-A, DAG, diacylglycerol; TG, triglyceride; PKCθ, activated protein kinase C; IRS, insulin receptor substrate; GLUT4, glucose transporter 4; G-6-P, glucose-6-phosphate; PPARα, peroxisome proliferator-activated receptor α; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; oxphos genes, genes involved in oxidative phosphorylation; β-ox, β-oxidation; I-V, complex I-V of the respiratory chain; CoQ, coenzyme Q, cyt c, cytochrome c; ROS, reactive oxygen species; ADP, adenosine diphosphate; P_i, inorganic phosphate; ATP, adenosine triphosphate [adapted from König et al. (10), Maassen et al. (23), Szendroedi et al. (22), Corpeleijn et al. (30), Boss et al. (47)]

1.2.6 Hepatocellular fat storage

Non-alcoholic fatty liver disease (NAFLD) is a concomitant phenomenon in patients who suffer from overt T2DM and it can be defined as a metabolic disorder, which is associated with ectopic fat accumulation in the liver (53). 50-75% of T2DM patients are suffering from NAFLD, examined by ultrasound measurements (53). Undoubtedly, hepatic insulin resistance correlates closely with NAFLD, whereas fatty liver seems to intensify a preexisting insulin resistance (54, 55). The pathological status of NAFLD allows drawing conclusions about the progression of diabetic disease (53). Importantly, fasting serum insulin is the most expressive parameter to assess the hepatic lipid content, as the insulin clearance correlates negatively with hepatic fat storage (56). The higher the fasting serum insulin, the lower the hepatic insulin clearance, which reflects diminished hepatic insulin sensitivity as a result of ectopic lipid accumulation (56).

Interestingly, besides T2DM, also obesity, dyslipidemia and the MetS are linked closely to NAFLD (53, 54, 57), and accordingly fatty liver is verified in 48% of patients with MetS (53). In line, 4-fold higher values of hepatic fat storages were observed in patients with the MetS compared to patients without the syndrome (56). Additionally, it was proven that all features of the MetS, defined by the IDF, correlate with the content of hepatocellular lipids (HCL), whereas waist circumference turned out as the feature most closely correlated (53, 54, 56).

HCL accumulation leads to impaired insulin-mediated suppression of EGP as well as an overproduction of very-low-density lipoproteins (VLDL) accompanied by diminished values of HDL-cholesterol as well as hypertriglyceridemia (43, 55, 56, 57). Taken together, fatty liver induces dyslipidemia, whereas the VLDL quantity is proportional to liver fat content and serum TG levels admit conclusions about the progression of hepatic insulin resistance (54, 56, 57). Interestingly, the content of HCL is suggested to be a more expressive parameter for the evaluation of whole body insulin resistance in comparison to IMCL levels (43). Moreover, ectopic lipid accumulation in liver correlates negatively with fasting muscle mitochondrial function, indicating a close interrelation between muscle and liver metabolism (3).

Impaired insulin-mediated suppression of lipolysis in adipose tissue causes an excess portal FFA flux to the liver, contributing to hepatic fat storages, besides dietary fat intake and hepatic *de novo* lipogenesis (53, 54). It was suggested that 59% of the

triacylgylcerol that accumulates in the liver is attributable to circulating FFAs (53). This statement implies the explanation of NAFLD especially observed in T2DM patients, since plasma FFA oversupply is known to be a feature of T2DM (53). Even if a comparison was done among T2DM patients, it was found that diabetes patients, who are additionally suffering from NAFLD disease, exhibit higher plasma levels of FFAs than T2DM patients without NAFLD, followed by an increased insulin resistance (54).

Furthermore, the impairment of skeletal muscle glucose metabolism is considered to be a driving force of hepatic de novo lipogenesis (43). Also the hormone level of adiponectin, which is secreted from adipose tissue in order to stimulate the hepatic fatty acid oxidation, is inversely correlated with both hepatic fat content and hepatic insulin resistance (53, 54). Adiponectin levels were diminished in patients with NAFLD, which in turn leads to impaired hepatic fatty acid oxidation and therefore stimulates ectopic accumulation of lipids (53, 57).

On the basis of NAFLD, a certain danger of developing a subsequent steatohepatitis cannot be neglected, since the calculated risk is given as 2.6-fold increased (53). Rising serum concentrations of liver enzymes like alanine aminotransferase and aspartate aminotransferase are an expressive indicator for hepatic steatosis (56).

2. Study design

2.1 Cross-sectional study

In the cross-sectional study, ten young, lean, insulin sensitive patients were included as well as ten glucose tolerant but insulin resistant obese patients and ten T2DM patients. During the first visit, the volunteers had to run through specific examinations to determine, if they are suitable for participating. This screening included the anamnesis of medical history, an electrocardiogram, body weight and height measurement, determination of waist-hip ratio and a blood survey. If the volunteers were included, the patients avoided any physical activity and had withdrawn their glucose- and lipid-lowering medication three days prior to the measurements. Moreover, the participants were asked to have a balanced iso-caloric diet and to refrain from alcohol ingestion three days prior to the tests. A hyperinsulinemic-euglycemic clamp was performed in order to determine whole body insulin sensitivity, paralleled by indirect calorimetry measurements to analyze metabolic flexibility. *Ex vivo* mitochondrial oxidative capacity was measured by high-resolution respirometry, whereas the *in vivo* mitochondrial function was determined by ^{31}P-MRS measurements. Ectopic fat storage in skeletal muscle was analyzed by proton MRS (^{1}H-MRS) (Fig. 4).

Mixed meal test (MMT)

In the present cross-sectional study, the influence of high-caloric meal ingestion on plasma FFA concentrations, glucose metabolism and TBARS was examined in lean, insulin sensitive patients compared with obese, insulin resistant but glucose tolerant patients as well as elderly T2DM patients. After an overnight fast, the participants arrived at the Clinical Research Center and received a high-caloric mixed meal (652 kcal, 55% carbohydrates, 15% protein, 35% fat). An intravenous catheter was placed for obtaining blood samples before and 15, 30, 60, 90, 120, 180 and 260 min after meal ingestion (Fig. 4).

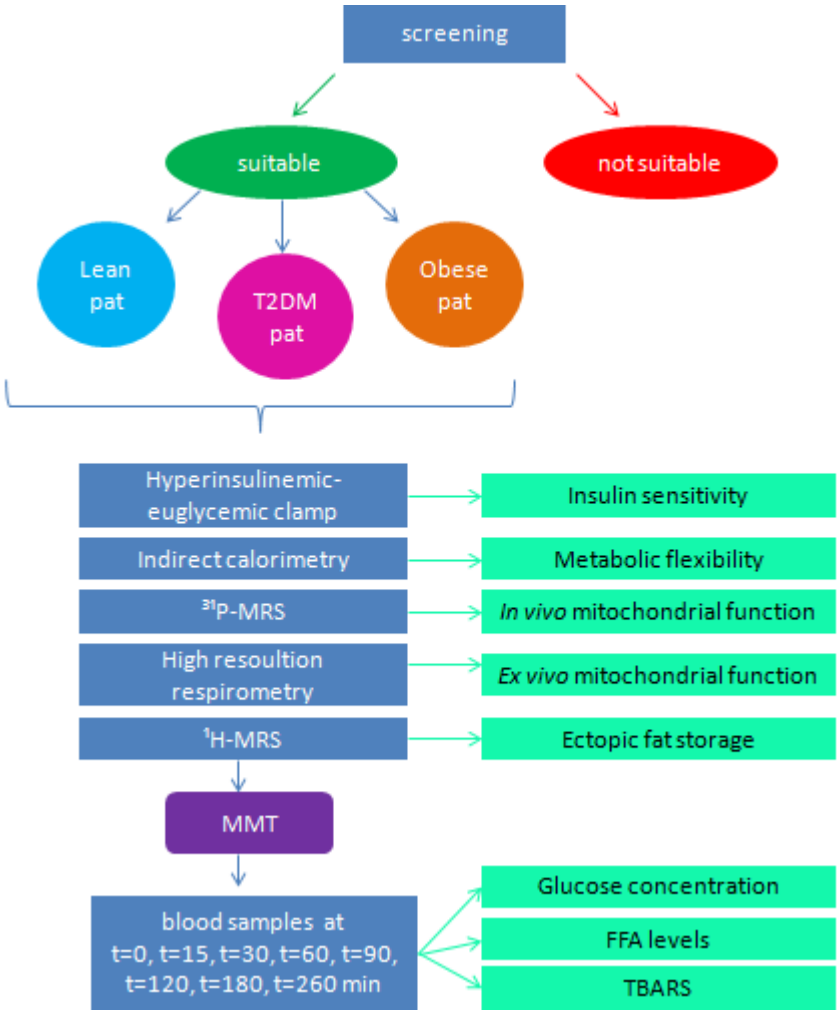

Fig. 4: Study design of the cross-sectional study. First, a screening was performed in order to decide, if the volunteers were suitable for participating in the study. Lean, insulin sensitive individuals, T2DM patients and insulin resistant but glucose tolerant obese patients were included. After inclusion, patients had to run to further examinations like a hyperinsulinemic-euglycemic clamp, in order to examine insulin sensitivity, as well as indirect calorimetry measurement to evaluate metabolic flexibility. Moreover, a ^{31}P-MRS of the skeletal muscle was performed to assess *in vivo* mitochondrial function and a high-resolution respirometry was done to determine *ex vivo* mitochondrial function. A ^{1}H-MRS measurement was applied to analyze ectopic fat storage in skeletal muscle. Next, a MMT was performed and blood samples were taken before, 15, 30, 60, 90, 120, 180 and 260 minutes after ingestion of a high-caloric meal, in order to measure glucose and FFA levels, as well as TBARS in the pre- and postprandial state. Pat, patients; T2DM, type 2 diabetes mellitus; ^{31}P-MRS, phosphorus magnetic resonance spectroscopy; ^{1}H-MRS, proton magnetic resonance spectroscopy; MMT, mixed meal test; FFA, free fatty acids; TBARS, thiobarbituric acid reactive substances

2.2 Interventional study

The interventional study was a randomized, double-blind, placebo-controlled intervention study, in which eleven patients with T2DM were included. The total duration of the study was nine weeks, including eight visits per patient. Comparable to the cross-sectional study, a first screening was applied to evaluate if the patients were suitable for participating. If the volunteers were included, the study started with an aerobic cycling test and a dual-energy x-ray absorptiometry (DEXA) measurement. Afterwards, a MRS examination of the skeletal muscle was done, as well as a ^{1}H-MRS of the liver, followed by a hyperinsulinemic-euglycemic clamp including a muscle biopsy, blood sampling and indirect calorimetry measurements. Subsequently, all participants were randomized, receiving the daily oral medication of 750 mg Acipimox or placebo (Cellulosum microcryst PH 102), splitted in doses of 250 mg thrice daily. After this interval of Acipimox or placebo administration, measurements mentioned above were performed once again. Afterwards, a four week washout period followed and the oral anti-diabetic medication was reestablished. After this wash-out period the patients got placebo or Acipimox medication depending on what they got previously and measurements were repeated (Fig. 5).

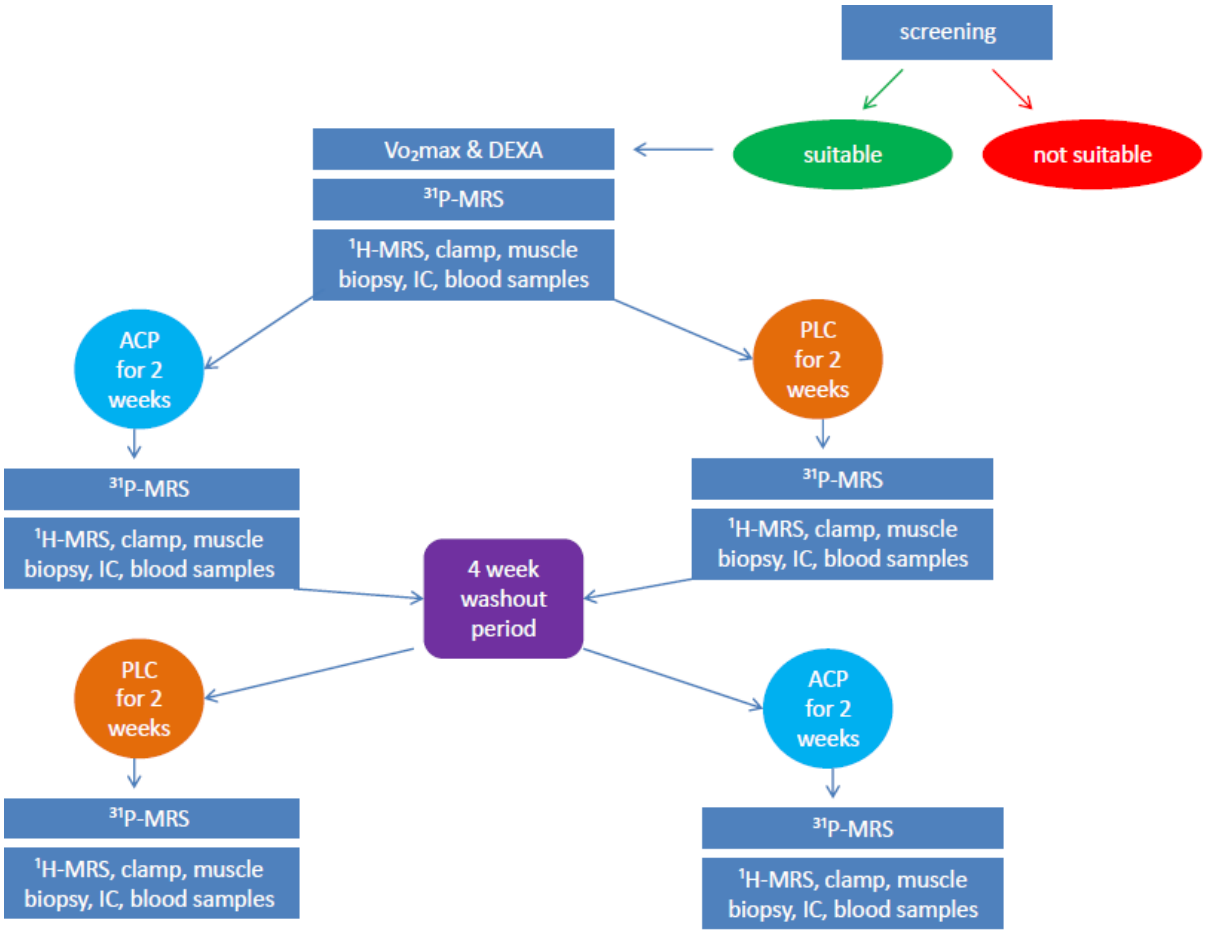

Fig. 5: Study design of the interventional study. First, a screening was performed to decide, if the volunteers were suitable for participating in the study. If patients were included, they had to run to further examinations, including a VO_2max measurement and a DEXA scan. Afterwards, a ^{31}P-MRS of the skeletal muscle was performed. Subsequently, a ^{1}H-MRS was done, followed by a 2-step clamp including a muscle biopsy, indirect calorimetry measurements and blood sampling. Hereinafter, the patients got placebo or Acipimox in random order for a period of two weeks. Measurements of ^{31}P-MRS and ^{1}H-MRS as well as a 2-step clamp containing a muscle biopsy, indirect calorimetry measurements and blood sampling were done once again. Afterwards, a four week washout period followed, whereas the oral anti-diabetes medication was reestablished. Subsequently, patients were administered placebo or Acipimox for two weeks, depending on what they got previously and measurements were repeated. VO_2max, maximal oxygen consumption; DEXA, dual-energy x-ray absorptiometry; ^{31}P-MRS, phosphorus magnetic resonance spectroscopy; ^{1}H-MRS, proton magnetic resonance spectroscopy; IC, indirect calorimetry; ACP, Acipimox; PLC, placebo

Intervention concept - Acipimox

We used a nicotinic acid (NA) derivate, which contains the active ingredient Acipimox, aiming to lower the concentration of plasma FFAs (46). In comparison to NAs, Acipimox is known to have a prolonged duration of action (5, 58), whereas the efficacy is appreciated to last for 8 hours (59). Because of the prolonged half-time, Acipimox is considered to be less susceptible to a rebound effect of lipolysis (59, 60). Side effects of

Acipimox include flushing, palpitation and gastrointestinal disturbances. Acipimox is suggested to affect G_i-protein coupled receptors, exclusively found in adipose tissue (4), which beneficially influences the peripheral glucose metabolism (54). It is speculated that Acipimox is a potent inhibitor of lipolysis in adipose tissue (6, 46), exerting its effect through inhibition of adenylate cyclase, which leads to diminished intracellular concentrations of cyclic adenosine monophosphate (cAMP) (4, 59, 61). Subsequently, protein kinase A is less active which results in a lack of activation of hormone-sensitive lipase (4, 5, 59, 61, 62, 63) followed by a reduced conversion of TG into fatty acids, which finally is accompanied by a diminished release of fatty acids into the circulation (46) (Fig. 6).

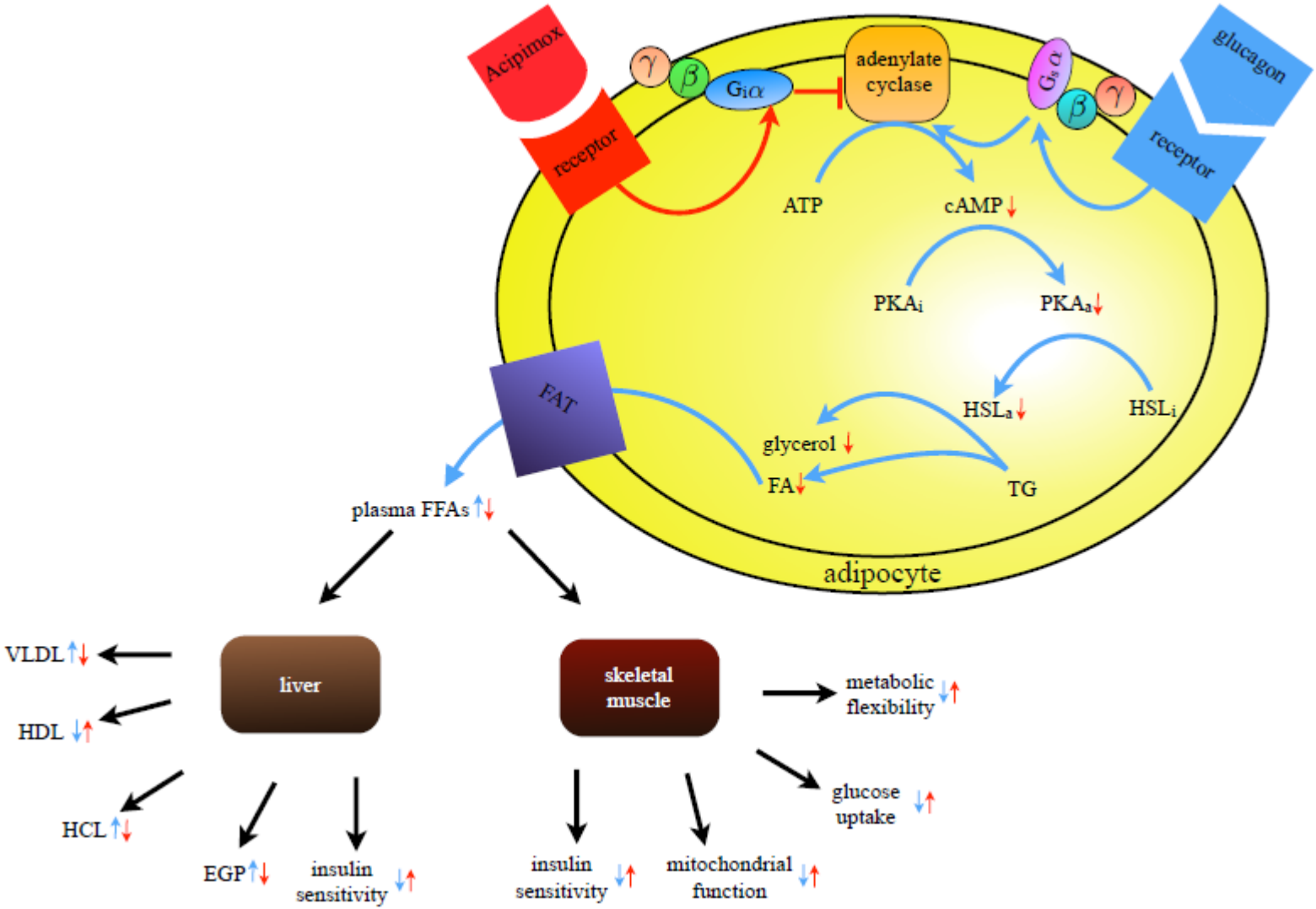

Fig. 6: Intracellular effects of Acipimox on adipocytes. Lipolysis of adipocytes is stimulated by glucagon (blue arrows), which docs on its G_s-protein coupled receptor, localized in the plasma membrane of adipocytes. Through activation of the $G_s\alpha$-subunit, adenylate cyclase is stimulated, which metabolizes ATP into cAMP. cAMP activates PKA and in turn, PKA phosphorylates HSL, leading to the transformation of TG into glycerol and FA. FA metabolites are released into the circulation via FAT. The impact of plasma FFAs on skeletal muscle and liver is shown by the blue arrows next to the metabolic parameters. The suggested metabolic inhibition of lipolysis upon Acipimox is shown by the red arrows. Acipimox exerts its effect via docking on a G_i-protein coupled receptor, localized in the plasma membrane of adipocytes, and subsequently, the metabolic pathway of lipolysis is hindered, leading to diminished FA release. Decreased plasma FFA levels beneficially affect the metabolic pathways of skeletal muscle and liver (displayed by the red arrows next to the metabolic parameters). γ, γ-subunit of the G-protein; β, β-subunit of the G-protein; $G_s\alpha$, α-subunit of the stimulatory G-protein; $G_i\alpha$, α-subunit of the inhibitory G-protein; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA_i, inactive protein kinase A; PKA_a, active protein kinase A; HSL_i, inactive hormone sensitive lipase; HSL_a, active hormone sensitive lipase; TG, triglyceride; FA, fatty acid; FAT, fatty acid translocator; FFA, free fatty acid; EGP, endogenous glucose production; HCL, hepatocellular lipids; HDL, high-density lipoprotein; VLDL, very-low-density lipoprotein

Accordingly, it was shown that Acipimox treatment for seven days caused a FFA decline of approximately 60% (46). Beneficial effects on glucose disposal rate and plasma glucose levels, as well as increased metabolic flexibility were also confirmed upon Acipimox administration (46, 58, 59, 62). In addition, lowering of FFA availability reduces ectopic lipid storages, i.e. in skeletal muscle or liver, which was proven upon Acipimox therapy (64). However, it was also observed, that FFA concentrations were increased upon Acipimox treatment for a period of three month, which was explained by a possible 'rebound' effect of lipolysis (65). These disturbances due to Acipimox administration were followed by interferences of metabolic pathways. Moreover, it is speculated that this rebound effect appears mainly during long-term treatment with Acipimox (59, 65), since contrary results were obtained during short-term administration of Acipimox (46). Previous Acipimox studies and their findings on circulating FFAs, whole body insulin sensitivity, mitochondrial function, ectopic fat storage, EGP as well as on metabolic flexibility are summarized in table 1.

Ref	Patients	Dosage (daily)	Duration	FFA	Ins sens	Mt fct	Ectopic fat storage	EGP	Met flex
4	FH T2DM	6x250mg	48 hours	↓	↑	n.a.	n.a.	n.a.	n.a.
46	FH T2DM	4x250mg	7 days	↓	↑	PGC-1α↓	IMCL ↓	↔	↑
60	T2DM	3x250mg	1 day	↓	↑	n.a.	n.a.	n.a.	↑
59	T2DM	2x250mg	1 day	↓	↑	n.a.	n.a.	↔	↑
65	T2DM	3x250mg	3 month	↑	↔	n.a.	n.a.	n.a.	n.a.
66	Healthy men	3x250mg	1 day	↓	n.a.	n.a.	n.a.	n.a.	n.a.
67	T2DM	3x250mg	Overnight	↓	n.a.	n.a.	n.a.	↔	↑
58	T2DM	3x250mg	Overnight	↓	↑	n.a.	n.a.	↓	↑
68	T2DM	3x250mg	3 month	↓	↑	n.a.	n.a.	n.a.	n.a.
69	T2DM	1x500mg	4 weeks	↓*	↑	n.a.	n.a.	n.a.	n.a.
		4x250mg	4 days	↓**	n.a.	n.a.	n.a.	n.a.	n.a.
		12x125mg	4 days	↓***	n.a.	n.a.	n.a.	n.a.	n.a.
64	T2DM	4x250mg	7 days	↓	↑	n.a.	IMCL ↓	↔	↑
63	Lean, young patients	4x250mg	7 days	↓	↑	n.a.	IMCL ↔	n.a.	n.a.
61	T2DM	12x125mg	3 days	↓	↑	n.a.	n.a.	n.a.	n.a.
70	T2DM	3x250mg	12 weeks	↔	↑	n.a.	n.a.	n.a.	n.a.
5	Healthy patients	1x250mg	1 day	↓	↑	n.a.	n.a.	↓	↑

Table 1: Overview of previous Acipimox studies. Ref, references; FFA, free fatty acids; Ins sens, insulin sensitivity; Mt fct, mitochondrial function; EGP, endogenous glucose production; Met flex, metabolic flexibility; FH T2DM, family history of type 2 diabetes mellitus; T2DM, type 2 diabetes mellitus; IMCL, intramyocellular lipids; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1-α; n.a., not available; *, nocturnal FFA levels were lowered upon bed-time administration of Acipimox, but a daytime rebound of FFAs was observed; **, FFA rebound was observed, if plasma Acipimox levels were decreased; ***, rebound rise of FFAs from the 3rd day of medication

2.3 Aims and hypotheses

The following hypotheses were investigated:

Primary hypothesis:

Lipid lowering improves mitochondrial function of skeletal muscle in T2DM patients.

Secondary hypotheses:

1. High-caloric meal ingestion affects mitochondrial function in insulin resistant patients.
2. Lipid lowering improves whole body- and hepatic insulin sensitivity in T2DM patients.
3. Lipid lowering lowers ectopic lipid accumulation in T2DM patients.
4. Lipid lowering improves metabolic flexibility in T2DM patients.

According to these hypotheses, we compared the effect of high-caloric meal ingestion on skeletal muscle energy metabolism in patients who were differently sensitive to insulin. Next, the effects of Acipimox were examined in patients with T2DM.

2.4 Inclusion and exclusion criteria

In the cross-sectional study, ten young (age of 27.5 ± 0.9 years), lean (body mass index (BMI) of 23.0 ± 0.5 kg/m²) insulin sensitive patients, as well as ten glucose tolerant but insulin resistant obese patients (BMI of 34.5 ± 0.5 kg/m²) with comparable age (31.3 ± 2.6 years) and ten elderly (63.3 ± 1.1 years) T2DM patients (BMI of 32.5 ± 0.7 kg/m²) were included. The insulin sensitive participants and the obese volunteers were matched for sex and age, whereas the T2DM patients were matched for sex and body fat mass with the obese participants. In every group, the male-to-female ratio was found to be 1:1. Before inclusion, every patient had to run through physical and laboratory examinations and the medical history was checked. T2DM patients were only included, if they never used insulin or thiazolidinediones. In addition, volunteers who cannot be examined by MRS, because of electronic implants or claustrophobia, were not suitable for the participation.

The Acipimox study was performed with nine men and two postmenopausal women with T2DM, if the manifestation of diabetic disease persisted for minimal 1.5 year. All

participants were obese ones with a BMI between 30 – 35 kg/m^2 and advanced age (58.7 ± 1.6 years). Humans with abnormal or impaired liver, renal and/or gastric function were excluded. Moreover, insulin-dependent diabetic patients and patients who suffer from diabetes related co-morbidities, like retinopathy and/or diabetic foot, could not take part in this study. Qvigstad et al. reported a negative correlation between HbA_{1c} and the effect of Acipimox during lipid infusion (71). This finding pointed towards a beneficial therapeutic effect of Acipimox in well-controlled diabetes patients. Accordingly, only well controlled T2DM patients were included in the present study, having an HbA_{1c} below 8%. Dietary habits and body weight needed to be stable during the last six months, with having no weight loss or gain more than 3 kg. All patients were taking glucose-lowering medication, meaning sulphonylurea-derivates with or without using metformin, with a stable dose for at least six months.

2.5 Methods

2.5.1 Approval by the ethics board

The studies were approved by the medical ethics committee of the Heinrich-Heine University of Düsseldorf. The cross-sectional study was registered with the number 3638 (registered clinical trial: NCT0122905) and the interventional study was registered with the number MO-LKP-450 (registered clinical trial: NCT00943059).

2.5.2 Maximal aerobic cycling test

Physical activity is known to influence mitochondrial function, indicating the importance of similar levels of daily physical activity among the participants. Aerobic exercises were performed on a cycling ergometer to measure VO_2max (72). After a warming-up period of five minutes (50 Watt), the workload increased every 2.5 minute with 50 W until 80% of the patients´ maximal heart rate (HRmax) was reached. The theoretical HRmax was calculated according to the formula of Haskell and Fox, considering the heart beats per minute (bpm): HRmax [bpm] = 220 – age [years]. In case 80% of the theoretical heart rate was reached, the workload was increased with 25 W instead of 50 W until maximal exhaustion was reached. Throughout the cycling test, oxygen consumption was measured in order to determine VO_2max [ml/kg/min]. During cycling, blood pressure values were monitored and an electrocardiogram was continuously done to monitor physical well-being.

2.5.3 DEXA method

DEXA was used to quantify the body composition. Total body fat [expressed as % and kg], as well as fat free mass [kg], and total body water [l] were obtained. A low dose of radiation was used in order to get a two dimensional picture of body composition. DEXA measurements are based on the assumption, that the body consists of three components, which include fat mass, fat free mass as well as bone tissue. Based on different x-ray photoenergy-absorption rates of these tissues, a detailed analysis of body composition can be done. Detailed body composition gives more valuable information in addition to the standard measures of body weight and BMI.

2.5.4 Blood sample analyses

Blood samples were taken from an antecubital vein and collected in tubes containing ethylenediaminetetraacetic acid. The samples were immediately centrifuged and plasma was frozen in liquid nitrogen and stored at -80°C until assayed. In the cross-sectional study, glucose concentrations were examined by the glucose oxidase method (EKF biosen C-Line glucose analyzer; EKF Diagnostic GmbH, Barleben, Germany) and plasma FFA levels were analyzed by microfluorometric measurements (Wako Chem USA Inc., Richmond, VA) before and 30, 60, 90, 120, 180 and 260 minutes after meal ingestion. TBARS were also examined fluorometrically (BioTek, Bad Friedrichshall, Germany) before and 120, 180 and 260 minutes after meal ingestion. In the interventional study, glucose and FFA levels were analyzed by enzymatic assays (glucose: hexokinase method, Roche, Basel, Switzerland; FFA: Wako Nefa C test kit; Wako Chemicals, Neuss, Germany) and blood samples were taken throughout the 2-step clamp (40, 72).

2.5.5 Hyperinsulinemic-euglycemic clamp

The 2-step clamp technique was used to measure hepatic and peripheral insulin sensitivity through determination of EGP, as well as quantification of whole body glucose disposal rate (Rd) (40, 51). Alternatively, the M-value was determined in the cross-sectional study as a measure for whole body insulin sensitivity. The 2-step clamp includes the baseline period, the low insulin infusion clamp (10 mU/m^2/min) and the high insulin infusion clamp (40 mU/m^2/min). The patients were asked to avoid any extra physical activity as well as to consume carbohydrate-rich meals during three days prior to the clamp. After an overnight fast at t=0 min, a primed continuous infusion (0.04 mg/kg/min) of the glucose tracer ([6,6- $^2H_2$] glucose) (Cambridge Isotope

Laboratories, Andover, MA, USA) was started to determine baseline rates of non-insulin-stimulated whole body and hepatic insulin sensitivity at the end of the baseline period, if steady clamp conditions were reached (7, 37, 72). During both the low- and high insulin clamp, glucose infusion rates were adjusted by a variable 20% glucose infusion in order to maintain euglycemic plasma glucose levels of 5 mmol/l (90 mg/dl). The glucose infusion rate (GIR) provides information about the sensitivity for insulin, since the higher the levels of glucose, which were needed to produce an euglycemic state, the more insulin sensitive the patient is. The GIR during the low insulin phase merely reflects the insulin sensitivity of the liver, by percentage of insulin-suppressed EGP, whereas the GIR of the high insulin phase merely reflects the glucose uptake by skeletal muscle. Also rates of whole body and hepatic insulin sensitivity were determined, if the steady state condition of the low- and high insulin infusion phases were reached. Additionally, a continuous somatostatin infusion (0.1 µg/kg/min) was started for the duration of the clamp in order to inhibit the pancreatic hormone secretion. Accordingly, blood was sampled and indirect calorimetry measurements were performed during the clamp in order to determine whole body carbohydrate- and lipid oxidation rates. Prior to the start of the low insulin clamp, a muscle biopsy of the *m. vastus lateralis* was taken (Fig. 7).

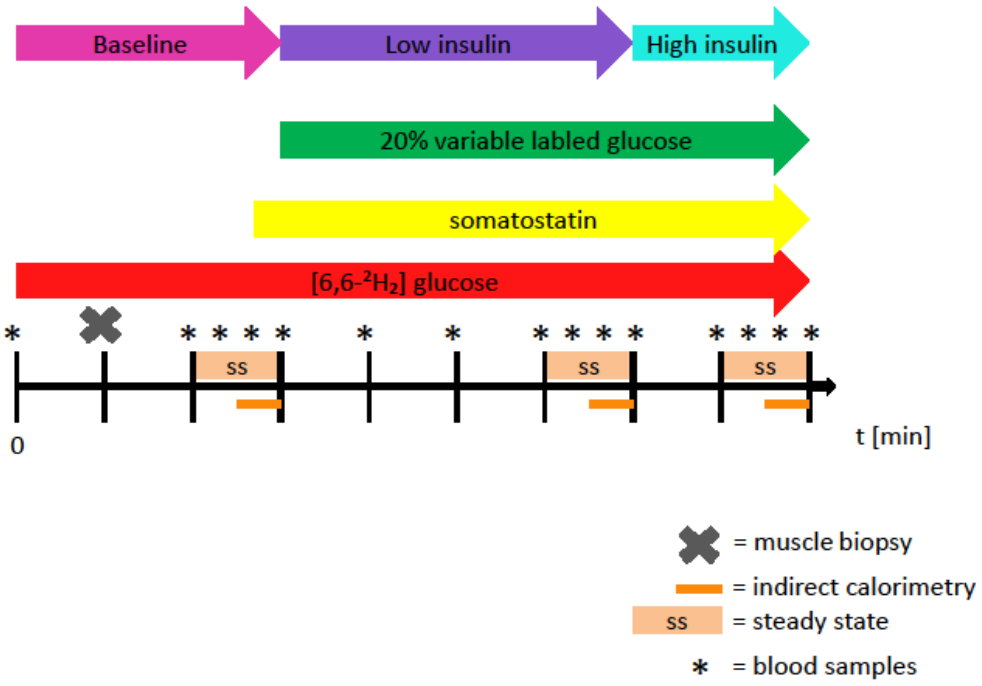

Fig. 7: Timeline of the 2-step clamp. Prior to the baseline period, a fasting blood sample was taken. The baseline period was started with an infusion of a glucose tracer ([6,6-$^2H_2$] glucose) and blood glucose levels were clamped at 5 mmol/l. During the baseline period a muscle biopsy of the *m. vastus lateralis* was taken. Prior to the low insulin period, a somatostatin infusion was started and continued for the duration of the clamp. Hereinafter, the low insulin period was started, paralleled by a variable co-infusion of 20% glucose to maintain euglycemic conditions. The concentration of insulin was upregulated upon the high insulin period. During the steady state conditions of each period, blood samples were taken and indirect calorimetry measurements were performed.

2.5.6 Indirect calorimetry

Indirect calorimetry (Vmax Encore 90n, Care Fusion, Hochberg, Germany) was used to measure substrate oxidation rates and metabolic flexibility (7). Oxygen consumption and carbon dioxide production were measured during the steady state periods of the hyperinsulinemic-euglycemic clamp in order to calculate the RQ. The RQ indicates the relative substrate oxidation of carbohydrates (RQ=~1) or lipids (RQ=~0.7) (73), whereas protein oxidation is neglected (7). Calculations of lipid- and carbohydrate oxidation were performed by stoichiometric equations according to Frayn, considering the body weight (BW), as well as oxygen consumption and carbon dioxide production (40, 73):

Glucose oxidation [nmol/kg/min] = $[(4{,}585*VCO_2-3{,}226*VO_2)/BW/180*1000]*10^3$

Lipid oxidation [nmol/kg/min] = $[(1{,}695*VO_2-1{,}701*VCO_2)/BW/860*1000]*10^3$

2.5.7 Magnetic resonance spectroscopy (MRS)

^{31}P-MRS measurements of the *m. vastus lateralis* were performed on a 3.0 T whole body scanner (Intera, Philips Health Care, Best, The Netherlands) both in the cross-sectional study and in the interventional study, in order to determine mitochondrial function of skeletal muscle *in vivo* through quantification of phosphorus energy metabolites (12). Phosphorus spectra were obtained every 4 seconds during rest, during knee-extension exercise and during the recovery phase (post-exercise). All participants performed a weight lifting knee-extension exercise for 5 minutes whilst lying in a spin position in the scanner. Exercise was performed at 50-60% of their maximal weight lifting capacity, which was determined previously at a different day. Low- to medium-intensity exercises were done to prevent the formation of muscular lactate, which is known to affect PCr recovery time (12, 40, 74). During exercise, the PCr content temporarily diminishes, but restores rapidly during recovery from exercise. The PCr resynthesis rate during recovery time depends on mitochondrial function, as it is driven almost purely oxidatively (12). A prolonged PCr resynthesis rate, which is a feature of diabetes disease, is an indicator for impaired mitochondrial function (7, 75). As PCr recovery follows mono-exponential kinetics, data was fitted against the time using Matlab software (Mathworks Inc, Natick, MA, USA) for the determination of the PCr recovery half-time.

In the same whole body scanner, a ^{1}H-MRS was performed in *m. vastus lateralis* in order to measure the content of IMCL in the cross-sectional study and to determine the HCL content of the liver in the interventional study (40). Magnetic resonance images were done to select the volume of interest in order to acquire ^{1}H-MRS spectra especially from this region. During measurements, the strong water signal, which dominates the proton spectra, was suppressed. The measurements were repeated without suppression of the water signal in order to determine the intensity of the water signal. The lipid/water ratio indicates the intensity of the lipid signal. During measurements, the patient was requested to breath in a regular 4-s rhythm to minimize motion artifacts.

2.5.8 Muscle biopsy

Muscle biopsies from the *m. vastus lateralis* were taken before starting the low-insulin infusion phase during the clamp under local anaesthesia (2% lidocaine).

2.5.8.1 Mitochondrial density

Mitochondrial density was determined in the interventional study by a quantitative real-time polymerase-chain-reaction, based on the TaqMan probe method (40). The ratio between mtDNA, reflected by the expression of NADH dehydrogenase subunit 1 (ND1), and the relative amounts of nuclear DNA, reflected by the expression of lipoprotein lipase, were analyzed. The proportion between mtDNA to nuclear DNA gives evidence of the cellular tissue concentration of mtDNA. The mtDNA copy number is considered to be a marker of mitochondrial density, expressed as arbitrary units [AU].

2.5.8.2 Muscle fiber type quantification

To quantify muscle fiber type, the muscle fibers were differentiated in the interventional study histochemically through utilization of an IgM antibody against myosin heavy chain type 1. Consequently, only type 1 muscle fibers were stained green, which enables the visualized classification between type 1 and type 2 fibers. For the identification of the cellular membranes, an IgG antibody against the membrane protein laminin was used. Additionally, the diazo dye oil red O was utilized to stain lipid droplets, which accumulated in the myocytes, revealing the IMCL content per cell.

2.5.8.3 Muscle fiber permeabilization

Both in the cross-sectional study and in the interventional study, ~20 mg muscle tissue was immediately transferred into a conservation medium termed as ‘BIOPS’ (OROBOROS Instruments, Innsbruck, Austria). The muscle fibers were dissected on ice using sharp forceps and visible fat cells were removed. Muscle fibers were incubated for 30 minutes in BIOPS containing 5 mg/ml saponine to permeabilize the muscle cell membrane. After this permeabilization step, the muscle fibers were washed twice in the mitochondrial respiration buffer ‘MiRO5‘ (OROBOROS Instruments), in which each washing step took 10 min. After the last washing step, the muscle fibers were transferred into the oxygraph. About 2-4 mg wet weight muscle fibers were used for the measurements (40). The rest of the muscle biopsy was stored at -80°C and used for later analysis.

2.5.9 High-resolution respirometry

A two-chamber oxygraph (OROBOROS Instruments) was used both in the cross-sectional study and in the interventional study to quantify *ex vivo* mitochondrial respiration through polarographic measurements of oxygen consumption (40, 72).

In the cross-sectional study, oxygen flux was analyzed upon addition of malate (2.0 mmol/l) to analyze state 2 respiration, followed by glutamate (10.0 mmol/l) as a substrate for complex I and ADP (1.0 mmol/l) to evaluate state 3 respiration of complex I. Next, succinate (10.0 mmol/l) was added, with or without pyruvate (5.0 mmol/l), to obtain state 3 respiration via activating both complex I and II. Also, cytochrome c (10 µmol/l) was added to test the mitochondrial membrane integrity. Afterwards, the uncoupler fluoro-carbonyl cyanide phenylhydrazone (FCCP) (0.1 mmol/l) in titration steps of 1.0 µl was added until maximal uncoupled respiration (state u) was reached. The addition of an uncoupling agent initiates the loss of the electrochemical proton gradient, followed by enhanced oxygen consumption, which enables the measurement of maximal enzyme capacity of the ETC (12).

To determine oxidative phosphorylation, two different protocols of substrate addition were used in the interventional study. In protocol 1, malate (4.0 mmol/l) was added to analyze state 2 respiration, followed by glutamate (8.0 mmol/l) as a substrate for complex I of the ETC and ADP (1.6 mmol/l) to evaluate state 3 respiration of complex I. Thereafter, succinate (8.0 mmol/l) was added to obtain state 3 respiration via activating both complex I and II of the ETC. Additionally, cytochrome c (10 µmol/l) was added to test the mitochondrial membrane integrity. At least titrations (in steps of 0.5 µl of 1.0 mmol/l) of FCCP were added until maximal uncoupled respiration was reached. In protocol 2, malate (4.0 mmol/l), octanoyl-carnitine (40 µmol/l) and ADP (1.6 mmol/l) were added to reach state 3 respiration on octanoyl-carnitine. Subsequently, glutamate (8.0 mmol/l), succinate (8.0 mmol/l) and cytochrome c (10 µmol/l) were added. FCCP (in steps of 0.5 µl of 1.0 mmol/l) was titrated to determine maximal mitochondrial oxidative capacity.

Oxygen consumption was corrected for muscle wet mass (2-4 mg) and was given as oxygen flux, expressed as pmol/(s*mg). Values were normalized for mtDNA content given as (pmol/(s*mg))/mtDNA copy number.

2.6 Calculations

Baseline and insulin-stimulated Rd of the interventional study were calculated by Steel`s single-pool non-steady-state equations. Volume of distribution was assumed to be 0.160 l/kg for glucose. Insulin stimulated glucose disposal (ΔRd) was computed as the difference between Rd during the 40 mU clamp minus Rd during baseline condition (7). The M-value was calculated in the cross-sectional study considering the GIR and the body weight. EGP was calculated in the interventional study as glucose appearance rate minus GIR (7). Insulin-stimulated EGP (Δ EGP) was computed upon high insulin infusion, expressed as EGP during the 40 mU clamp minus baseline values, and upon low insulin infusion, expressed as EGP during the 10 mU clamp minus baseline values. Carbohydrate and fat oxidation rates were calculated by stoichiometric equations according to Frayn, with protein oxidation considered negligible. The change in insulin-stimulated glucose and lipid oxidation rates (Δ glucose oxidation and Δ lipid oxidation) were calculated as oxidation rates during the 40 mU insulin clamp minus oxidation values at baseline conditions. The RQ was calculated in both of the studies by the ratio between carbon dioxide production to oxygen consumption (VCO_2/ VO_2). Metabolic flexibility was expressed as the change in RQ upon insulin stimulation compared to baseline values.

2.7 Statistical evaluation

Comparisons between groups of the cross-sectional study were done by performance of one-way analysis of variance (ANOVA) and the level of significance was set at α=0.05. A Tukey post-hoc test was applied for multiple testing.

Values of the interventional study were tested for significant differences between the three treatment periods (control condition, placebo administration, Acipimox administration). Significance was tested using one-way analysis of variance for repeated measurements (rmANOVA) and the level of significance was set at α=0.05. A Tukey post-hoc test was applied to correct for multiple testing.

Data were reported as mean ± SEM.

3. Results

3.1 Patients` characteristics

Ten lean, healthy patients, as well as ten T2DM patients and ten obese patients were included in the cross-sectional study. Patients` characteristics are shown in table 2.

Patients` characteristics of the cross-sectional study	Healthy patients	T2DM patients	Obese patients
Age [years]	27.5 ± 0.9	63.3 ± 1.1	31.3 ± 2.6
Body weight [kg]	69.3 ± 4.1	91.6 ± 4.3	102.8 ± 4.3
BMI [kg/m²]	23.0 ± 0.5	32.5 ± 0.7	34.5 ± 0.5
Waist/hip ratio	0.8 ± 0.0	1.0 ± 0.0	0.97 ± 0.0
Lean mass [kg]	51.0 ± 2.9	55.1 ± 3.8	61.7 ± 4.3
Fat [%]	21.9 ± 2.3	40.3 ± 1.2	39.2 ± 2.2
HbA_{1c} [%]	5.1 ± 0.1	7.5 ± 0.4	5.4 ± 0.2
Triglyceride [mg/dl]	86 ± 14	155 ± 27	188 ± 54
High-density lipoprotein [mg/dl]	64.8 ± 7.3	49.0 ± 3.3	54.2 ± 7.9
Low-density lipoprotein [mg/dl]	87.1 ± 9.6	143.1 ± 12.6	122.0 ± 12.8
Alanine aminotransferase [U/l]	14.4 ± 1.6	22.9 ± 3.9	33.7 ± 8.3
Gamma-glutamyltransferase [U/l]	14.3 ± 1.5	36.4 ± 9.5	35.0 ± 11.2
Aspartate aminotransferase [U/l]	20.1 ± 1.5	25.7 ± 3.0	24.4 ± 3.3

Table 2: Patients` characteristics of the cross-sectional study. Data are mean ± SEM. BMI, body mass index; T2DM, type 2 diabetes mellitus

Eleven patients with T2DM were included in the interventional study. The characteristics are shown in table 3.

Patients` characteristics of the interventional study	
Age [years]	58.7 ± 1.6
Body weight [kg]	104.6 ± 3.8
BMI [kg/m²]	35.7 ± 1.2
Waist/hip ratio	1.1 ± 0.0
Fat mass [kg]	37.8 ± 3.4
Free fat mass [kg]	64.3 ± 2.1
Fat [%]	37.2 ± 1.6
VO_2max [ml/kg/min]	19.6 ± 2.0
HbA_{1c} [%]	7.2 ± 0.3
Fasting blood glucose [mg/dl]	162 ± 20
Triglyceride [mg/dl]	209 ± 43
High-density lipoprotein [mg/dl]	47.5 ± 2.9
Low-density lipoprotein [mg/dl]	129.0 ± 8.3
Alanine aminotransferase [U/l]	35.6 ± 4.0
Gamma-glutamyltransferase [U/l]	49.3 ± 5.8
Aspartate aminotransferase [U/l]	25.1 ± 1.8

Table 3: Patients` characteristics of the interventional study. Data are mean ± SEM. BMI, body mass index; VO_2max, maximal oxygen consumption

3.2 Metabolic parameters

In the cross-sectional study, the glucose concentration in T2DM patients was approximately twice as high as values of healthy and obese participants both before and 15, 30, 60, 90, 120, 180 and 260 minutes after meal ingestion (figure 8, appendix table 4).

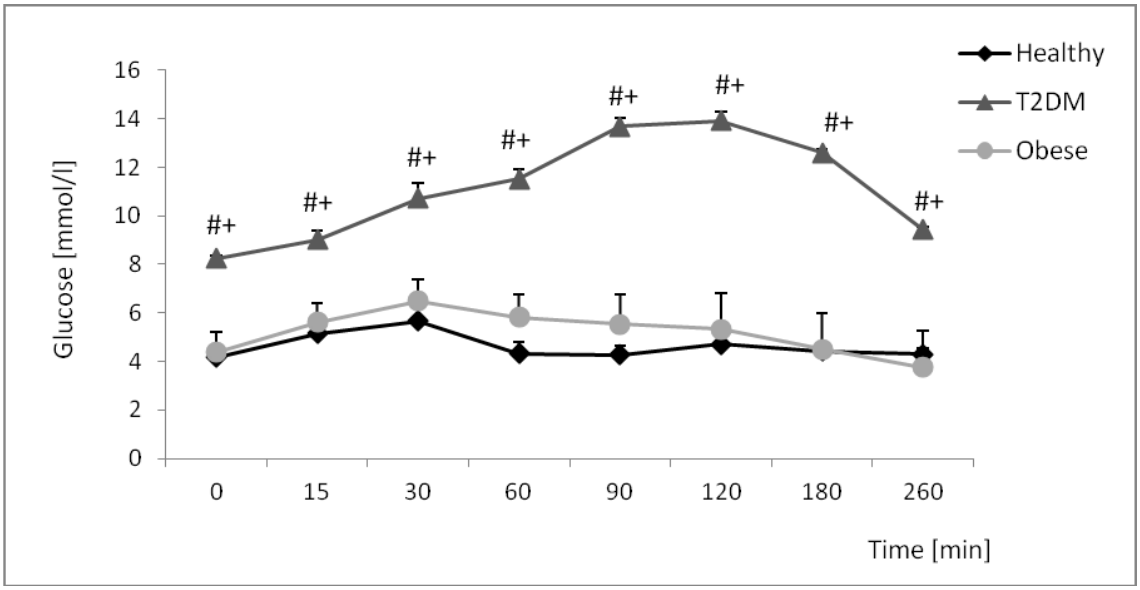

Fig. 8: Glucose concentrations [mmol/l] of the cross-sectional study, measured in healthy individuals (black diamonds), T2DM patients (dark-grey triangles) and obese humans (light-grey points), before and 15, 30, 60, 90, 120, 180 and 260 minutes after meal ingestion, data are mean ± SEM, #p≤0.05 T2DM: healthy, +p≤0.05 T2DM: obese; T2DM, type 2 diabetes mellitus

Plasma glucose values were measured in the interventional study upon baseline, low insulin and high insulin infusion of the 2-step clamp. Values differed in the baseline period, as the glucose concentrations upon placebo and Acipimox administration were nearly 30% higher compared to the control condition (9.95 ± 1.25 vs. 9.63 ± 1.12 vs. 7.62 ± 0.53 mmol/l, figure 9, appendix table 5). Glucose concentrations of the low insulin period were diminished upon control condition compared to Acipimox and placebo treatment, whereas the within group comparison showed only a tendency of increased glucose values upon Acipimox therapy in comparison to control condition (6.31 ± 0.25 vs. 5.43 ± 0.18 mmol/l, p=0.06). No differences were found between groups in the high insulin period (figure 9, appendix table 5).

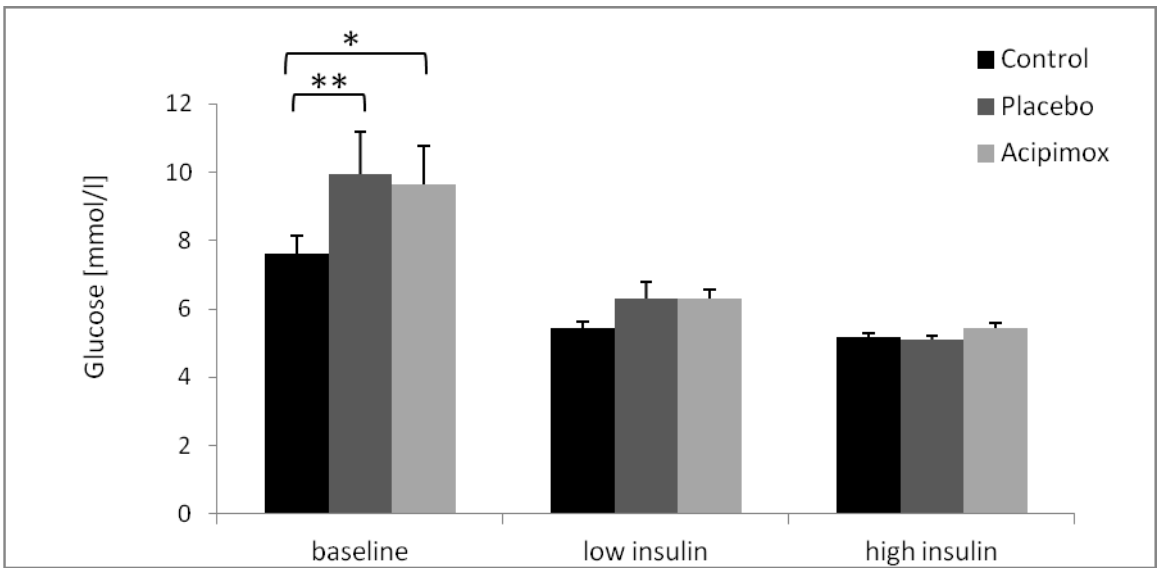

Fig. 9: Glucose concentrations [mmol/l] of the interventional study, upon control condition (black bars), placebo administration (dark-grey bars) and Acipimox administration (light-grey bars), in the baseline, low insulin and high insulin clamp period, data are mean ± SEM, *p≤0.05, **p≤0.01

In the cross-sectional study, the FFA concentration was approximately 1.6 fold higher both in T2DM patients and in obese patients compared to healthy patients before meal ingestion (516 ± 62 vs. 517 ± 41 vs. 318 ± 48 µmol/l, p≤0.05, figure 10). After meal ingestion, values of FFAs were elevated in T2DM patients compared to healthy participants (figure 10, appendix table 4). At t=260 minutes, FFA were lower in T2DM patients compared to obese patients (figure 10, p≤0.05, appendix table 4).

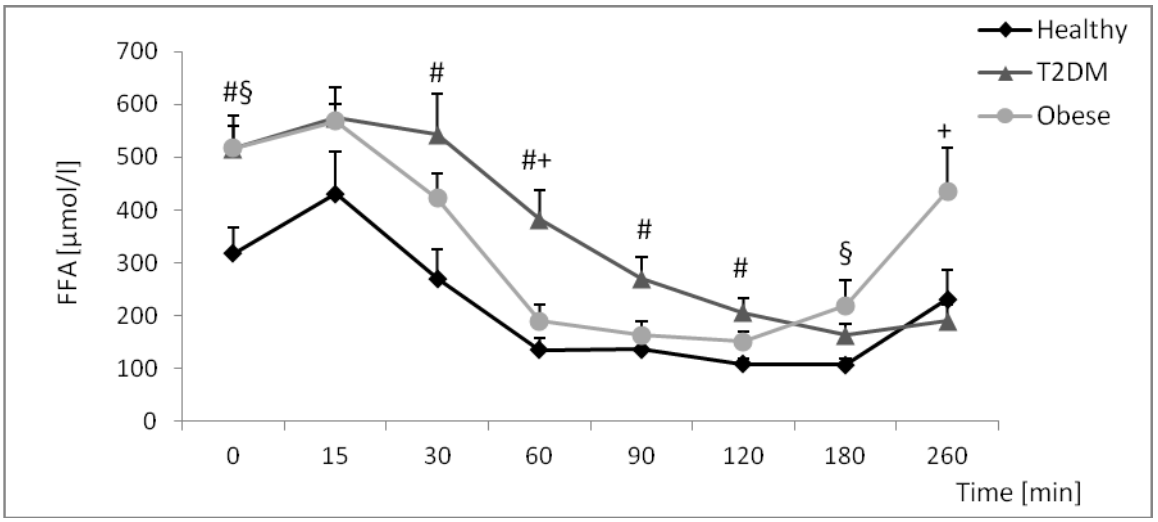

Fig. 10: FFA concentrations [µmol/l] of the cross-sectional study, measured in healthy individuals (black diamonds), T2DM patients (dark-grey triangles) and obese humans (light-grey points), before and 15, 30, 60, 90, 120, 180 and 260 minutes after meal ingestion, data are mean ± SEM, #p≤0.05 T2DM: healthy, +p≤0.05 T2DM: obese; §p≤0.05 obese: healthy; T2DM, type 2 diabetes mellitus; FFA, free fatty acids

FFA concentrations were also measured in the interventional study and were shown to be different between groups (appendix table 5). The FFA concentration during baseline period of the 2-step clamp was approximately twice as high upon Acipimox administration compared to placebo medication or control period (1175 ± 78 vs. 663 ± 51 vs. 623 ± 53 µmol/l, figure 11, p≤0.01). Values of FFAs reported for the low insulin period were 2.8-fold higher, if Acipimox was compared to placebo and 2.9-fold higher, if Acipimox was compared to control condition (appendix table 5, figure 11, p≤0.01). Also during high insulin infusion, the plasma concentration of FFA was increased upon Acipimox treatment compared to placebo and control group (336 ± 61 vs. 159 ± 19 vs. 135 ± 24 µmol/l, figure 11, p≤0.01).

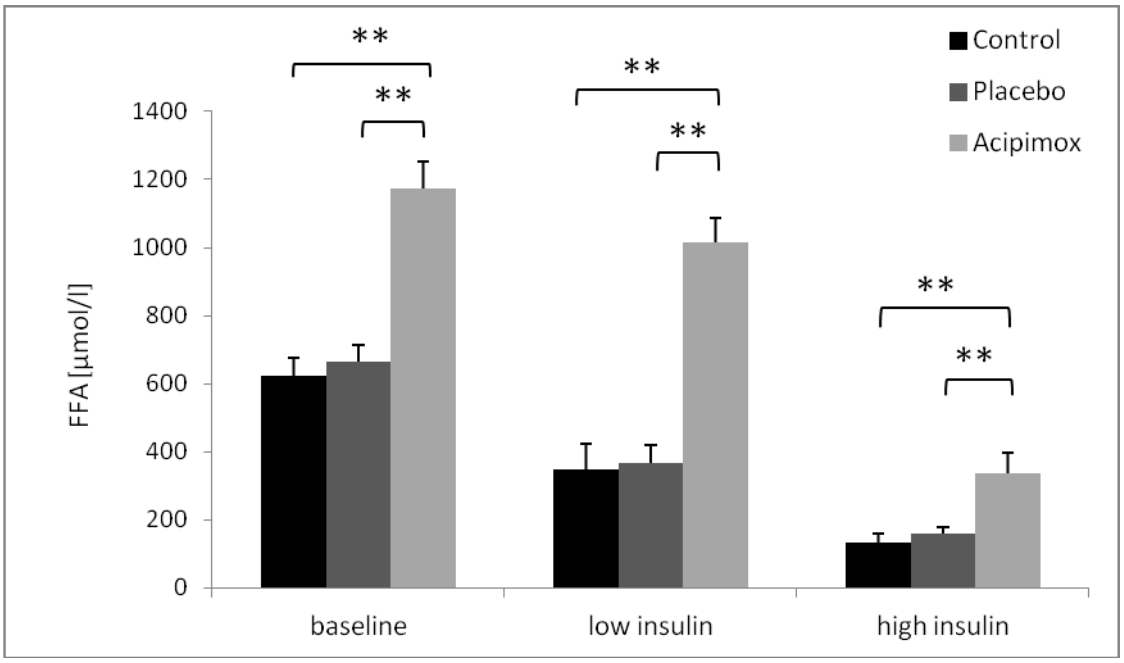

Fig. 11: FFA concentrations [µmol/l] of the interventional study during baseline measurements, the low insulin- and high insulin period upon control condition (black bars), placebo medication (dark-grey bars) and Acipimox administration (light-grey bars), data are mean ± SEM, **p≤0.01; FFA, free fatty acids

3.3 Oxidative stress

In the cross-sectional study, TBARS were not different between healthy, obese and T2DM patients, neither before nor after meal ingestion (appendix table 4, p>0.05). However, comparing the value of the fasted state with the value 180 minutes after meal ingestion in T2DM patients, values increased by 24% (2.6 ± 0.2 vs. 3.2 ± 0.2 µmol/l, figure 12).

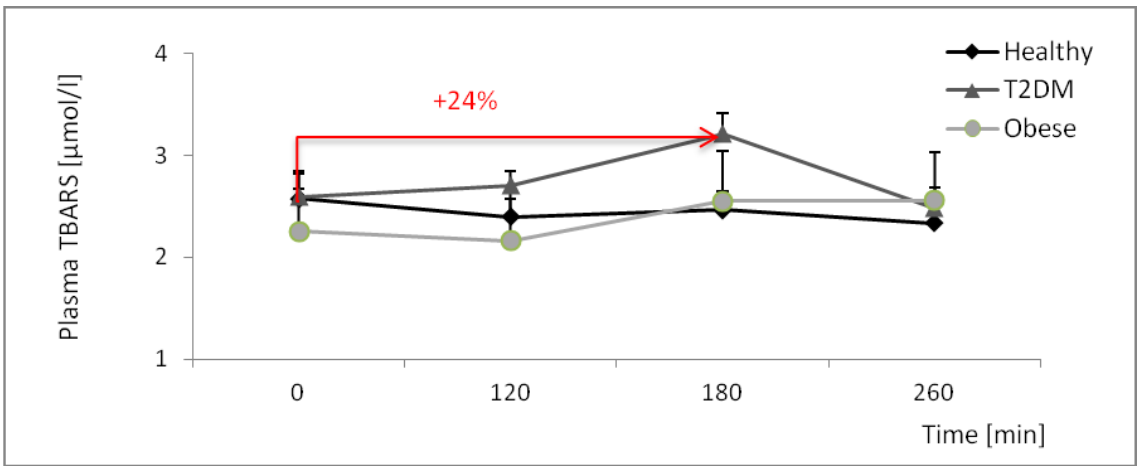

Fig. 12: TBARS [µmol/l] of the cross-sectional study, measured in healthy individuals (black diamonds), T2DM patients (dark-grey triangles) and obese humans (light-grey points), before and 120, 180 and 260 minutes after meal ingestion; the red arrow shows the increase of TBARS by 24%, if the preprandial state is compared to the value of 180 minutes after meal ingestion in T2DM patients, data are mean ± SEM, T2DM, type 2 diabetes mellitus; TBARS, thiobarbituric acid reactive substances

3.4 Whole body insulin sensitivity

In the cross-sectional study, healthy participants were about 75% more sensitive to insulin than T2DM patients (9.4 ± 1.3 vs. 2.4 ± 0.9 mg*kg^{-1}*min^{-1}) and nearly 65% more insulin sensitive than obese ones (9.4 ± 1.3 vs. 3.3 ± 0.5 mg*kg^{-1}*min^{-1}, figure 13, appendix table 4).

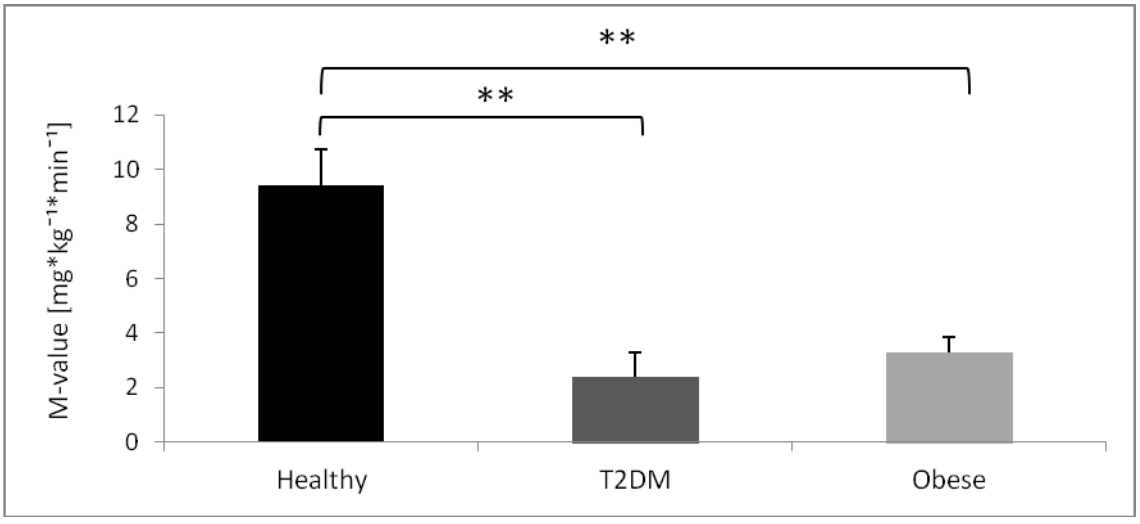

Fig. 13: Whole body insulin sensitivity [mg*kg^{-1}*min^{-1}] of the cross-sectional study, in healthy individuals (black bar), T2DM patients (dark-grey bar) and obese participants (light-grey bar), data are mean ± SEM, **p≤0.01; T2DM, type 2 diabetes mellitus

In the interventional study, insulin sensitivity did not differ between groups during baseline period and low insulin infusion (appendix table 5). Upon high insulin infusion, Rd was different between therapy conditions ($p \leq 0.01$). The within group comparison showed 2.4-fold higher Rd during control condition and placebo therapy both compared to Acipimox therapy (20.69 ± 3.96 vs. 20.93 ± 4.53 vs. 8.77 ± 1.09 µmol/kg/min, $p \leq 0.01$, figure 14). Values of calculated ΔRd, expressed as Rd during high insulin infusion minus Rd during baseline period, were decreased upon Acipimox medication compared to placebo medication (appendix table 5, $p \leq 0.05$).

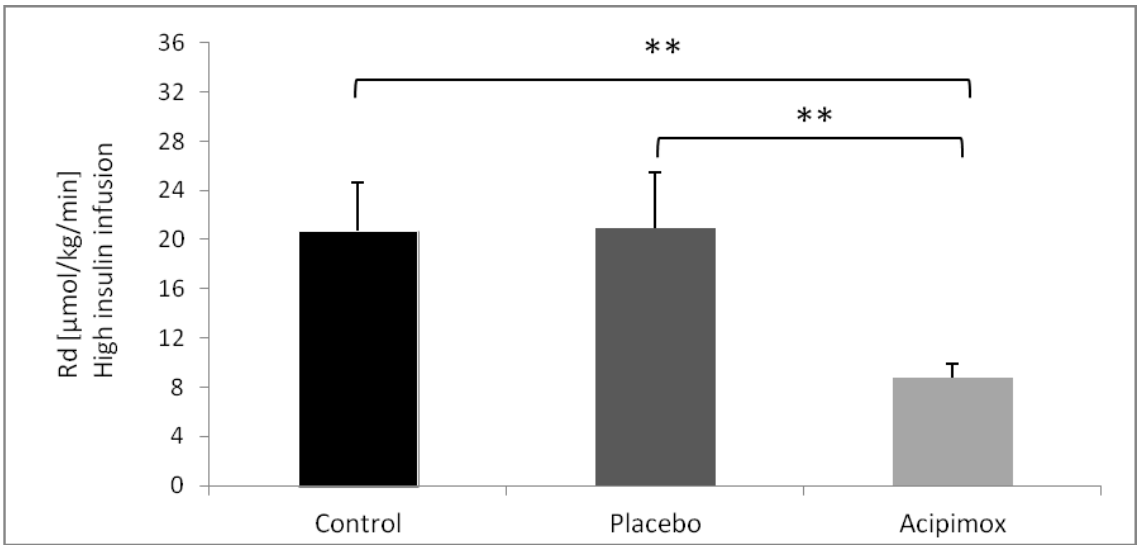

Fig. 14: Glucose disposal rate [µmol/kg/min] of the interventional study during the high insulin clamp period upon control condition (black bar), placebo administration (dark-grey bar) and Acipimox medication (light-grey bar), mean ± SEM, **p≤0.01; Rd, glucose disposal rate

3.5 Endogenous glucose production (EGP)

The EGP was similar between all groups of the interventional study during baseline condition and low insulin infusion (appendix table 5, $p > 0.05$). The insulin-suppressed EGP upon high insulin infusion tended to be lower during control condition and placebo treatment both compared to Acipimox (-0.93 ± 1.32 vs. 0.29 ± 1.31 vs. 2.87 ± 0.74 µmol/kg/min, $p = 0.09$). Calculations of ΔEGP, expressed as high insulin infusion minus baseline period, showed a reduced hepatic glucose production upon insulin stimulation during control period and placebo administration compared to the Acipimox treatment period (-12.87 ± 2.52 vs. -10.64 ± 1.27 vs. -5.77 ± 1.49 µmol/kg/min, $p = 0.04$), indicating diminished hepatic insulin sensitivity upon Acipimox. Performing the post-

hoc correction for multiple testing, a difference of ΔEGP (high insulin infusion minus baseline) was shown between Acipimox therapy and the control condition (figure 15, p=0.03). ΔEGP, expressed as low insulin infusion minus baseline condition, was 2.2-fold lower during control condition compared to Acipimox medication (appendix table 5, figure 15).

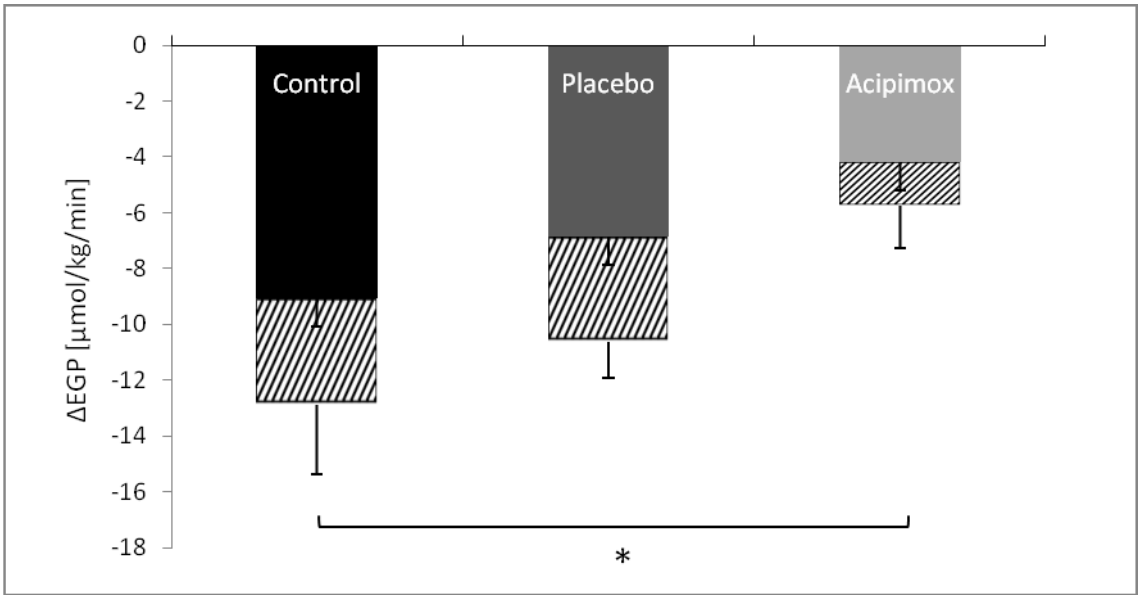

Fig. 15: Comparison of ΔEGP values [µmol/kg/min] of the interventional study. The hatched bars represent the ΔEGP of high insulin infusion minus baseline values. ΔEGP expressed as low insulin infusion period minus baseline is illustrated by the black bar (control condition), the dark-grey bar (placebo medication) and the light-grey bar (Acipimox administration). Values are mean ± SEM, *p≤0.05; EGP, endogenous glucose production

3.6 Metabolic flexibility

In the cross-sectional study, RQ was measured upon basal condition and during the clamp. RQ values were only different in the basal state, whereas values differed between healthy and obese patients (0.78 ± 0.01 vs. 0.87 ± 0.04, p≤0.05, figure 16, appendix table 4). ΔRQ values, expressed as RQ upon clamping minus basal RQ values, did not differ between groups (appendix table 4).

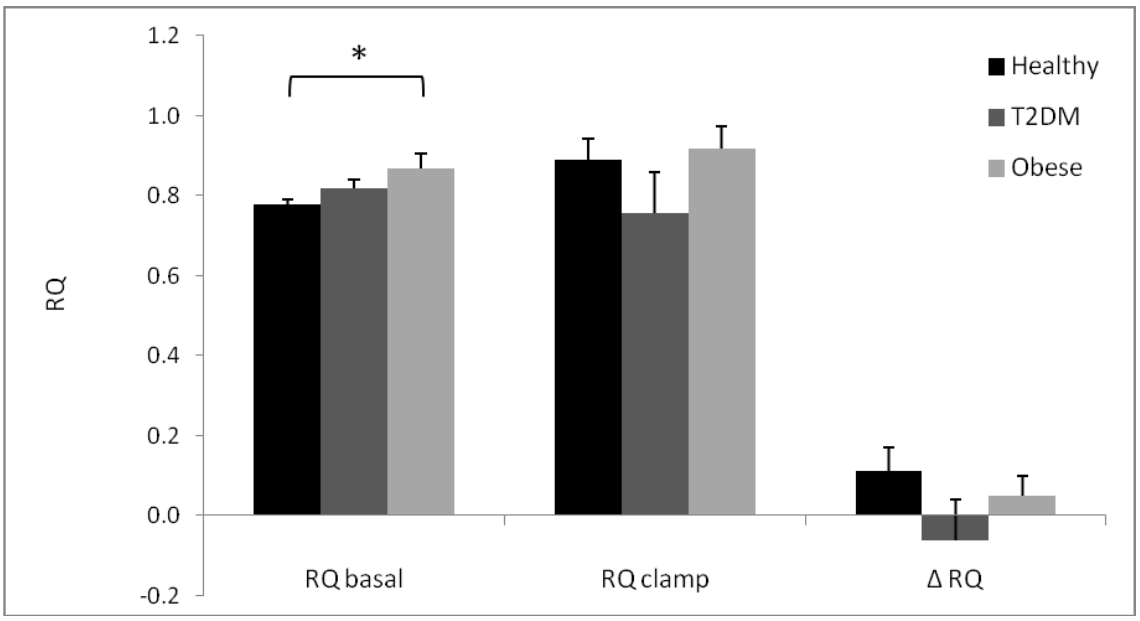

Fig. 16: RQ values of the cross-sectional study in healthy patients (black bars), T2DM patients (dark-grey bars) and obese patients (light-grey bars) upon basal condition and upon clamping. ΔRQ is expressed as clamp values minus basal values; values are mean ± SEM, *p≤0.05; RQ, respiratory quotient; T2DM, type 2 diabetes mellitus

RQ values of the interventional study were similar during baseline period in all treatment groups (appendix table 5, p>0.05). During the low insulin period, RQ tended to be lower during Acipimox medication compared to control condition and placebo administration (0.79 ± 0.02 vs. 0.86 ± 0.02 vs. 0.84 ± 0.03, p=0.07). The values of the high insulin period and the results of ΔRQ, expressed as high insulin minus baseline, showed no differences among conditions (appendix table 5, p>0.05). Lipid oxidation was similar between all groups during baseline condition (appendix table 5, p>0.05). During low insulin infusion, lipid oxidation tended to be higher upon Acipimox treatment compared to control and placebo values (1.1 ± 0.2 vs. 0.7 ± 0.1 vs. 0.8 ±0.1 nmol/kg/min, p=0.07, appendix table 5). The within group comparison showed, that values upon Acipimox treatment were approximately 1.6-fold higher compared to control condition (figure 17, p=0.05). Data of high insulin infusion, as well as Δlipid oxidation, expressed as high insulin infusion minus baseline, were not different between conditions (appendix table 5, p>0.05). Values reported for glucose oxidation did not differ between therapy groups during baseline, low insulin and high insulin periods (appendix table 5, p>0.05).

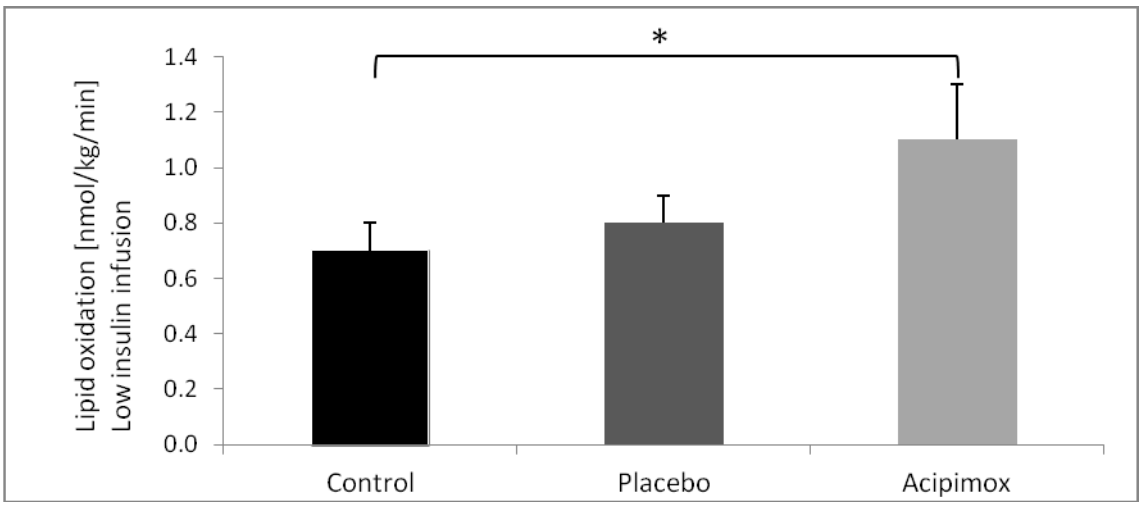

Fig. 17: Lipid oxidation [nmol/kg/min] of the interventional study during the low insulin clamping period upon control condition (black bar), placebo medication (dark-grey bar) and Acipimox administration (light-grey bar), data are mean ± SEM, *p≤0.05

3.7 Ectopic fat storage

In the cross-sectional study, IMCL values did not differ between groups (p>0.05, appendix table 4). No differences were found in the interventional study, if the IMCL content was compared between placebo and Acipimox therapy (1.7 ± 0.58 vs. 1.8 ± 0.39 %, p=0.78, appendix table 5). The HCL content was different between groups in the interventional study (appendix table 5, p≤0.05). The within group comparison showed a trend towards lower HCL content, if Acipimox was compared to control values (11.4 ± 1.9 vs. 16.0 ± 3.9 %, p=0.09), as well as a tendency of increased HCL content during control condition compared to placebo administration (16.0 ± 3.9 vs. 11.0 ± 2.2 %, p=0.06). HCL values upon placebo and Acipimox administration were comparable.

3.8 Muscle mitochondrial density

Mitochondrial density, measured as mtDNA copy number, was not different in the interventional study comparing control condition, placebo and Acipimox medication (2169 ± 464 vs. 1720 ± 244 vs. 2072 ± 273 AU, p=0.53, appendix table 5).

3.9 *In vivo* muscle mitochondrial function

PCr recovery half-time showed no differences in the cross-sectional study, if values of lean, insulin sensitive patients were compared with those of T2DM patients and obese humans (appendix table 4, p>0.05).

No differences of PCr recovery half-time was found between groups in the interventional study (appendix table 5, p>0.05).

3.10 *Ex vivo* muscle mitochondrial function

Evaluating the *ex vivo* measurements of the cross-sectional study, a difference was found, if state 3 respiration, complex I was compared (p=0.02, appendix table 4). Mitochondrial oxidative capacity of state 3 respiration at complex I was more than twice as high in lean, healthy individuals compared to T2DM patients (48.9 ± 3.9 vs. 24.0 ± 3.6 pmol/(s*mg), p=0.02, figure 18). No differences were found upon comparison of state 3 respiration at complex I+II and if values of state u were analyzed (appendix table 4).

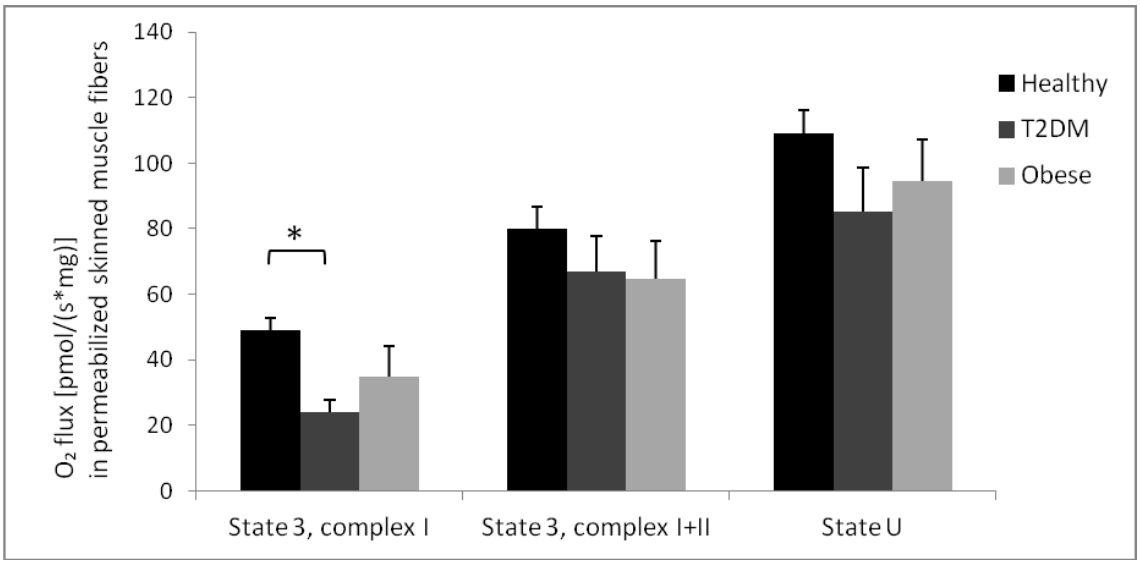

Fig. 18: Mitochondrial oxygen flux [pmol/(s*mg)] of the cross-sectional study measured in permeabilized skinned muscle fibers in healthy, insulin sensitive patients (black bars), in T2DM patients (dark-grey bars) and in obese patients (light-grey bars), data are mean ± SEM, *p≤0.05; T2DM, type 2 diabetes mellitus

In trace 1 of the interventional study, the addition of malate showed a difference between control condition and placebo administration (appendix table 6, p=0.02), as the mitochondrial oxidative capacity was approximately 47% lower during placebo medication compared to the control group (4.4 ± 0.8 vs. 8.3 ± 1.0 pmol/(s*mg), p=0.02, figure 19). If glutamate was added aside from malate, no differences were found (appendix table 6, p>0.05). Data between therapy groups were comparable, if ADP and succinate were added (appendix table 6). Values upon cytochrome c addition were similar between groups and also data of FCCP-driven state u (maximal respiratory capacity) showed no differences (appendix table 6, p>0.05). If the O_2 fluxes were

normalized for mtDNA content, no differences were found between groups (appendix table 6, p>0.05).

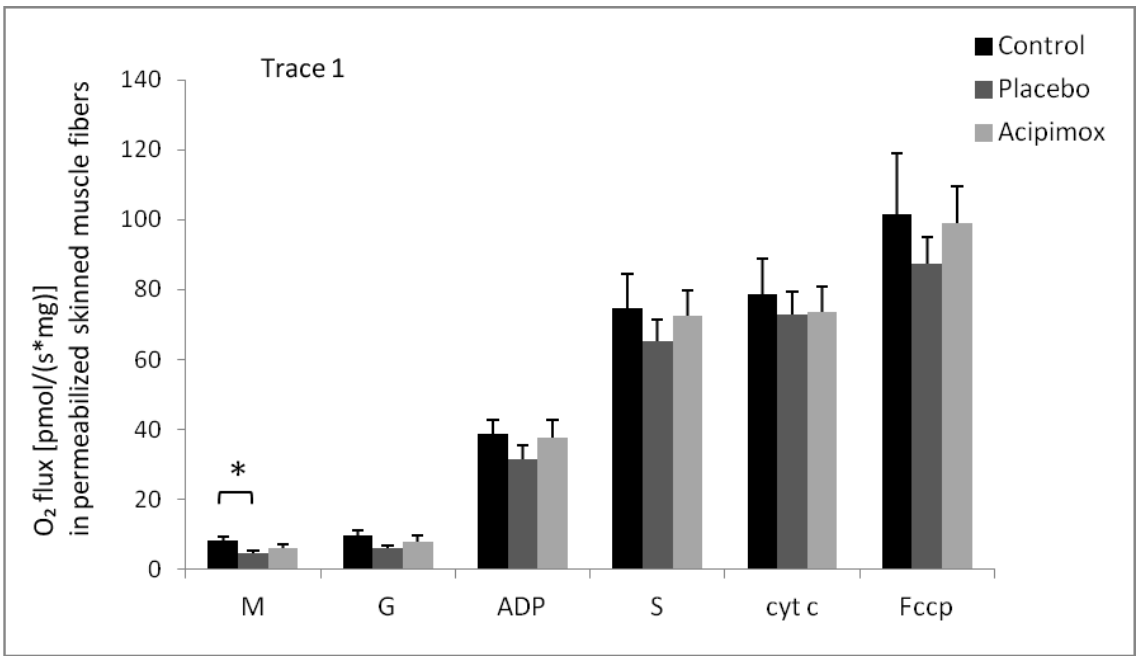

Fig. 19: Mitochondrial oxygen flux [pmol/(s*mg)] of the interventional study measured in permeabilized skinned muscle fibers upon control condition (black bars), placebo medication (dark-grey bars) and Acipimox administration (light-grey bars), data are mean ± SEM, *p≤0.05; M, malate; G, glutamate; ADP, adenosine diphosphate; S, succinate; cyt c, cytochrome c; FCCP, fluoro-carbonyl cyanide phenylhydrazone

In trace 2 of the interventional study, no differences were found upon the addition of malate, octanoyl-carnitine, glutamate, succinate, ADP, cytochrome c and FCCP (appendix table 6, p>0.05). However, values upon addition of succinate in permeabilized skinned muscle fibers showed a tendency to be lowered during placebo treatment compared to control condition (62.5 ± 7.8 vs. 78.3 ± 7.0 pmol/(s*mg), p=0.06). No differences were found, if values of trace 2 were normalized for mtDNA content (appendix table 6, p>0.05).

4. Discussion

In recent years, growing interest on the pathogenic influence of mitochondrial impairments on the development of T2DM rose. Energy metabolism of skeletal muscle was analyzed in the present studies, as both skeletal muscle accounts for the most insulin-sensitive tissue in humans and muscular function depends largely on mitochondrial capacity (2). A negative correlation between muscle mitochondrial function and elevated FFA levels was assumed (44, 76) and also insulin sensitivity, ectopic lipid accumulation and metabolic flexibility were suggested to be impaired by increased lipolysis in T2DM patients (30). Therefore, the present studies were performed to analyze the effect of lipotoxicity on skeletal muscle energy metabolism with a specific focus on muscle mitochondrial function. At first, we compared substrate kinetics between healthy, obese and T2DM patients during fasting and in the postprandial state, in order to evaluate the extent of altered skeletal muscle energy metabolism in diabetic patients. Subsequently, the interventional study was performed to examine the influence of lipid lowering on skeletal muscle metabolism and mitochondrial function in T2DM participants, based on the assumption that FFA levels were lowered upon Acipimox administration. Accordingly, we hypothesized, that both skeletal muscle energy metabolism and mitochondrial function would be impaired in T2DM patients compared to healthy controls before and upon meal ingestion, mainly attributable to lipotoxicity. Furthermore, we suggested that lipid lowering by Acipimox would improve mitochondrial function in skeletal muscle.

4.1 T2DM and metabolic parameters

In patients with T2DM, lipolysis is increased in adipose tissue, resulting in an enhanced release of FFAs into the circulation. In line, FFA levels were elevated in T2DM patients compared to healthy controls of the present cross-sectional study before and after meal ingestion. Also fasting and postprandial glucose concentrations of T2DM patients were approximately twice as high as in obese and controls. Hereinafter, Acipimox was utilized as a complementary substrate to insulin, aiming to inhibit the mobilization of FFAs in order to investigate the beneficial effects of decreased lipolysis on metabolic parameters of diabetic patients (77). Interestingly, we showed that a two week period of Acipimox treatment in T2DM patients resulted in elevated circulating FFAs instead of the hypothesized decrease, paralleled by increased plasma glucose levels. This so-called ‘rebound’ effect has been observed previously (65, 69, 78).

Diminished insulin sensitivity of adipose tissue is known to be the decisive factor provoking enhanced lipolysis, which in turn promotes insulin resistance (4). A correlation was found between overweight and insulin resistance (16), which confirms the results of the present cross-sectional study, as we found elevated plasma FFA levels not only in T2DM patients but also in obese ones in the fasted state, accompanied by diminished insulin sensitivity. Alterations in lipid metabolism are known to play a key role in the pathogenesis of T2DM through interference with glucose homeostasis (39). FFAs are suggested to provoke peripheral insulin resistance through inhibition of cellular glucose uptake, mediated by GLUT 4 (6, 39). Possibly the intrinsic activity of GLUT4 is reduced in times of elevated FFAs (39). It was also hypothesized, that FFAs are able to activate PKC-θ, leading to phosphorylation of IRS, which also reduces the peripheral glucose uptake (33). Contrary, a lack of effect of FFAs on insulin-stimulated glucose uptake was observed in other studies, whereas glucose metabolism of healthy, obese and T2DM patients was analyzed upon lipid infusion (39). However, these discrepant results were speculated to be falsified, maybe due to an insufficient duration of lipid infusion (39). Moreover, it was shown previously that a pharmacological elevation of FFA levels in insulin resistant individuals provoked impairments of pancreatic ß-cells, which modifies insulin secretion and in turn possibly intensifies a preexisting resistance towards insulin (4). Furthermore, FFAs are known to impair insulin-mediated suppression of hepatic glucose output, inducing increased plasma glucose levels (21). In conclusion, the results of the cross-sectional study point towards diminished insulin sensitivity of both adipose tissue and skeletal muscle of T2DM patients in comparison to healthy controls, as cellular glucose uptake was diminished, paralleled by rising FFA concentrations before and upon meal ingestion.

Moreover, in line with results of the interventional study, twofold elevated FFA plasma concentrations were found previously upon Acipimox administration for three month (65). In addition, a rebound phenomenon was observed at daytime, if Acipimox was only given once a day at bedtime during the total study duration of four weeks (69). Nevertheless, in previous short-term Acipimox studies, a reduction of FFA levels upon Acipimox therapy, accompanied by beneficial effects on insulin sensitivity and glucose metabolism, was observed (4, 5, 58, 59, 60, 68, 78), which provides evidence for the occurrence of the rebound effect mainly during long-term Acipimox treatment. The mechanisms inducing the rebound effect still need to be elucidated. It has been speculated that the rising plasma FFA levels were compensating the initial Acipimox-

induced FFA decrease in order to maintain the cellular energetic demand, since FFAs are major energy sources of the body (69, 79). Accordingly, this metabolic adjustment could be a counterbalance phenomenon to the acute impact of Acipimox. Furthermore, it was noticed that the serum concentrations of Acipimox seemed to be inversely correlated with plasma FFA concentrations, indicating that the period of FFA depression upon Acipimox administration is dose dependent until the rebound effect occurs (69). Surprisingly, if doses of Acipimox were given more frequently, in order to continuously suppress lipolysis by maintenance of stable serum Acipimox concentrations, the rebound effect also occurred (69). Another possible approach to explain the metabolic disturbances is the influence of the endocrine system, which also participates in mobilizing FFAs. Interestingly, the dimension of the FFA rebounding was diminished, when a hypophysectomy was performed in rats (79). It was also verified that growth hormone (GH) stimulates the FFA release from adipose tissue (62) via lipolytic enzymes like glucagon and cortisol in times of falling FFA levels (69) and interestingly, elevated GH levels during Acipimox treatment were found (69). Moreover, the rebound effect could possibly be mediated by GH, as the initial, Acipimox induced, decrease of plasma FFA levels is paralleled by an increase of GH secretion and in turn, GH is known to stimulate lipolysis (67).

As FFAs were elevated in the present cross-sectional study and Acipimox failed to lower plasma lipid levels, another study approach could be the administration of omega-3 fatty acids, contained in fish-oil, as these are known to positively affect lipid metabolism in patients with impaired glucose tolerance (80).

4.2 T2DM and whole body insulin sensitivity

In the cross-sectional study, we confirmed that both obese individuals and T2DM patients were less insulin sensitive compared to healthy controls, which was accompanied by elevated FFA levels and hyperglycemia. Upon Acipimox administration, some studies found that insulin-mediated glucose uptake was improved by lowering of FFAs (46, 59, 64, 60), but others did not (58, 67, 68). In the present interventional study, elevated FFAs upon Acipimox treatment showed no effect on whole body insulin sensitivity upon baseline and low insulin period during the 2-step clamp, but reduced peripheral insulin sensitivity in the high insulin period. Accordingly, these study results point towards impaired insulin sensitivity of skeletal muscle in obese and T2DM patients, as well as upon Acipimox therapy.

Both T2DM and obesity are known to be susceptible to elevated plasma FFA levels and diminished insulin sensitivity, however, central obesity was found to be more related to insulin resistance than peripheral lipid accumulation (21). In line, an inverse correlation was found between peripheral insulin sensitivity and FFA levels in the present cross-sectional study, which was also confirmed previously (64, 81). Additionally, it was suggested that an acute change of FFAs does not immediately induce insulin resistance, as the latency time amounts to two hours, as FFAs first need to enter the cell before provoking insulin resistance (52). An inhibitory effect of FFAs on glucose uptake was suspected to develop after more than 3 hours of fat infusion (39). The mechanism of FFAs to cause insulin resistance was not elucidated, however, the activation of the inflammatory NFκB/IκB pathway can be regarded as the main cause (81). Also fatty acid metabolites are able to inhibit the insulin transduction pathway (64), possibly by inhibiting the IRS (5). The latter suggestion is in line with the assumption that LCFA-CoA inhibits insulin action via activation of PKC, which subsequently hinders the insulin signaling cascade (52). In line, an increased activation of PKC in obese humans and T2DM patients was observed compared to healthy controls (82). Moreover, if a lipid infusion was performed in healthy individuals, muscular DAG molecules were shown to be elevated, paralleled by enhanced activation of PKC and increased phosphorylation of IRS (82). Furthermore, the contribution of FFA-mediated activation of the hexosamine pathway was suggested to provoke insulin resistance (55). Therefore, these results indicate, that elevated plasma FFA levels are able to hinder the insulin signaling pathway in skeletal muscle, which was confirmed by the present study results.

In the present interventional study, skeletal muscle insulin sensitivity was diminished accompanied by elevated plasma FFA levels. In contrast, previous studies found an improved insulin-stimulated glucose uptake in diabetes patients during short-term Acipimox medication (59). If Acipimox was given overnight, the cellular glucose uptake was twofold higher upon Acipimox treatment in comparison to placebo administration during insulin stimulation upon performance of a hyperinsulinemic-euglycemic clamp (60). Furthermore, diminished plasma concentrations of both insulin and glucose, accompanied by reduced plasma FFA levels, were observed, indicating that the improvement of peripheral insulin sensitivity, mediated by Acipimox, overcomes the decrease in plasma insulin levels (60). However, in line with our results, a stimulatory effect on insulin-mediated Rd upon 3-month of Acipimox treatment could not be confirmed in a previous study (65). The latter observation was assumed to be due

to the rebound effect of lipolysis, as insulin sensitivity was suggested to be negatively correlated with plasma FFA levels. Despite, in another study, an enhanced sensitivity for insulin was proven upon Acipimox administration independent of changes in plasma FFA levels, indicating a direct drug effect of Acipimox on insulin pathways, suggesting a negligible impact of FFA concentrations on insulin sensitivity (64). Taken together, the effect of Acipimox on insulin sensitivity was not elucidated so far, as ambiguous results were achieved in previous studies. However, the present study demonstrated a negative influence of Acipimox on skeletal muscle insulin sensitivity.

4.3 T2DM and hepatic insulin sensitivity

In the present interventional study, diminished hepatic insulin sensitivity upon Acipimox administration was found.

No direct drug effect of Acipimox on hepatic glucose production was proven previously (60), but it was speculated that the plasma level of FFAs correlates with the rate of EGP, as FFAs are known to stimulate key gluconeogenic enzymes in the liver (58). NADH and ATP, known as products of FFA oxidation, are provided for gluconeogenic processes, revealing the interrelation between EGP and lipolysis (67). Consequently, hepatic sensitivity to insulin inversely correlates with plasma FFAs (60), which is in line with the results of the present study. In contrast, a previous overnight Acipimox study showed increased insulin-mediated suppression of EGP in T2DM patients, indicating an improved hepatic sensitivity to insulin (58, 78). However, no beneficial effect upon a seven-day period of Acipimox medication on EGP was found, although fasting plasma FFAs were reduced (64) and similar results were proven upon acute administration of NA (83).

Furthermore, a dependency between the FFA mediated regulation of EGP and portal vein insulin concentrations was observed (31). It was suggested that lowering of FFAs only leads to decreased EGP, if glycogen storages are depleted or if glycogenolysis is additionally suppressed by insulin (69). Moreover, it was assumed that a short-term inhibition of lipolysis is initially compensated by the depletion of hepatic glycogen via hepatic auto-regulation, so that the total hepatic glucose output remains unaffected (60, 69). Subsequently, it was appreciated that the exhaustion of hepatic glycogen storages takes approximately 10 hours and therefore, hepatic glucose output merely reflects the rate of EGP upon prolonged suppression of lipolysis, if the liver is glycogen depleted

(58). Correspondingly, the discrepancies of previous study results could be explained by the latter suggestion, revealing that hepatic glycogen storages were not depleted upon short-term Acipimox administration, leading to a lack of effect on hepatic glucose production (67). Not only hepatic auto-regulation but also FFA-mediated insulin secretion is able to compensate the stimulatory effect of FFA on EGP, leading to the distinctions of previous study results (31).

Also the influence of the endocrine system is assumed to affect EGP, as the level of GH, glucagon and cortisol was found to be elevated upon Acipimox therapy and it was proven that these hormones are able to regulate gluconeogenesis and glycogenolysis (69).

4.4 T2DM and metabolic flexibility

The RQ level, a measure for metabolic flexibility, allows drawing conclusions about skeletal muscle glucose and lipid metabolism. Metabolic flexibility was suggested to correlate negatively with lipotoxicity. Comparisons of RQ measurements between the basal state and upon hyperinsulinemic-euglycemic clamp reflect the metabolic flexibility between the fasted state and during insulin stimulation. As described in previous papers (34, 35), we hypothesized that T2DM patients are metabolic inflexible in comparison to healthy controls and that Acipimox affects the metabolic pathways.

In the cross-sectional study no differences of metabolic flexibility between healthy and T2DM patients were found. In the interventional study, a trend of decreased RQ and increased lipid oxidation upon Acipimox was shown in the low insulin period, indicating a higher reliance on fat oxidation than on glucose oxidation. Glucose oxidation values were similar between all therapy groups of the interventional study. The results of the present studies pointed towards comparable metabolic flexibility of T2DM patients and healthy controls as well as a lack of effect of Acipimox treatment to improve metabolic flexibility in skeletal muscle.

A positive correlation between FFA levels and lipid oxidation and an inverse correlation between FFAs and glucose oxidation in skeletal muscle was verified in numerous studies (30, 34, 60, 69). Consequently, it was suggested that metabolic inflexibility is not an intrinsic defect of muscle cells but rather regulated differently, for example by extracellular fatty acid concentrations, whereas the mechanism of regulation still needs to be elucidated (35). Fatty acids have been suggested to impair glucose uptake und

utilization via substrate competition, as increased levels of both acetyl-CoA and citrate are known to impair pyruvate dehydrogenase, as well as phosphofructokinase activity and thereby inhibiting glucose oxidation and utilization (5, 58, 63). However, the interrelation between FFAs and metabolic inflexibility was not confirmed by the present study results, as a lack of impact of rising FFA levels on glucose oxidation was shown. In line, the inverse dependency between RQ and FFA levels was not always confirmed, as diminished RQ levels were observed in obese patients compared to lean controls upon fasting in a previous study (34). Furthermore, macronutrient dietary composition is considered to be a core component of fuel oxidation and it was found, that RQ levels, measured for 48 hours in a respiratory chamber, were similar between obese and lean controls, if macronutrient intake was carefully matched (34).

Previous short-term Acipimox studies showed that Acipimox is a potent inhibitor of lipolysis, resulting in a reduction of plasma FFAs, accompanied with a stimulatory effect on peripheral glucose oxidation both during the basal state and during insulin infusion, reflecting metabolic flexibility (4, 58, 59, 60). The enhanced glucose oxidation rate was explained by an improved, Acipimox induced, stimulation of glucose transport over the cell membrane (59). However, in another previous short-term Acipimox study, glucose oxidation of skeletal muscle was not affected by Acipimox, although GIR and insulin sensitivity was increased, accompanied by diminished plasma FFA levels and accordingly it was suggested that glucose was primarily metabolized via the non-oxidative pathway (5, 64). Another explanatory approach was the assumption that Acipimox mainly affects hepatic glucose metabolism rather than glucose utilization of skeletal muscle (58).

As discrepancies between previous study results complicate the comparability of the results achieved in both of the present studies, possible sources of error upon indirect calorimetry measurements were researched (59). It was criticized that only net rates of substrate utilization were measured upon indirect calorimetry, so that lipid and glucose oxidation rates were assessed regardless of a possible *de novo* lipogenesis or gluconeogenesis in adipocytes or liver. Correspondingly, it was speculate that both results of lipid- and glucose oxidation could be falsified and misinterpreted (59). Another possible approach to explain the discrepant results could be differences in diet composition of the participants within a few days before performing the measurements, as RQ is known to be highly sensitive towards differences of meal ingestion (30, 34).

However, in both of the present studies, the patients were asked to have a balanced iso-caloric diet three days prior to the tests, but nevertheless the comparability of nutrient supply among the participants remains questionable. Despite, RQ levels were measured upon a 2-step clamp in both of the studies, so that plasma glucose and insulin concentrations were carefully matched during measurements (34). Moreover, metabolic flexibility is affected by physical activity, aging, hormonal status as well as fiber type composition of skeletal muscle (30, 35). Furthermore, genetic factors, which are controlling metabolic pathways, have a major impact on metabolic flexibility (34). In the present studies, these variables partially differ between the participants. Inclusion of male and female volunteers in both of the studies leads to a lack of comparability because of differences concerning the hormonal status. Furthermore, the age was only matched between healthy and obese ones of the cross-sectional study, whereas T2DM patients were considerably older. However, in both of the studies all patients were matched for physical activity. Possibly, *in vitro* measurements with cultures of human myotubes are more suitable for the assessment of metabolic flexibility, as environmental factors and the influence of the physiological milieu were excluded (34, 35).

4.5 T2DM and ectopic fat storage

In the present interventional study, we found decreased levels of HCL upon Acipimox treatment and also upon placebo therapy compared to the control condition. This result was surprising, as elevated FFA concentrations were found upon Acipimox treatment, which were expected to be metabolized by the liver, leading to hepatic fat deposition (22, 53, 84).

FFA flux within the portal circulation was the most likely source of hepatic fat accumulation, besides hepatic *de novo* lipogenesis and dietary fat intake (53, 54). FFA release from visceral adipose tissue is the key factor leading to fatty liver in T2DM patients (54), which induces hepatic insulin resistance, possibly through suppression of IRS activity (53, 56). It was assumed that fifty-nine percent of the TG accumulation in the liver is attributable to circulating FFAs (53). Contrary, it was speculated previously, that fasting serum concentrations of FFAs are not linked with hepatic fat accumulation (57). Therefore, correlation factors influencing the liver fat deposition were analyzed (56). It was found that all parameters of the MetS, defined by the IDF, correlated with liver fat content, whereas fasting serum insulin seems to be the factor most closely

related (56). Taken together, hepatic lipid accumulation is not a simple reflection of FFA supply but controlled differently by various factors.

Regarding the ectopic lipid accumulation in skeletal muscle, no impacts were shown neither in T2DM patients of the cross-sectional study nor upon Acipimox administration in the interventional study, although increased IMCL contents were expected due to the elevated plasma FFA levels.

It was found in numerous papers, that the IMCL content depends largely on the plasma FFA concentration (46, 51, 52, 58, 64) and in line, a reduction of IMCL content was directly related to diminished plasma FFA concentrations in previous studies (46, 64). A dysbalance between uptake and oxidation of FFAs due to an oversupply of plasma lipids is suggested to induce IMCL accumulation, which subsequently contributes to lipotoxicity (30, 34). Despite, no changes of IMCL content in skeletal muscle were observed previously upon a prolonged suppression of FFA supply, indicating a lack of interdependency between these parameters (63). The latter observation could be explained by a cellular auto-regulation in times of diminished FFA levels in order to preserve cellular energy sources in form of intracellular TG storages and furthermore, an extended period of suppression of lipolysis could be necessary to induce intramyocellular depletion of lipid content (63). Additionally, upon elevation of plasma FFAs, no effect was found on IMCL accumulation in skeletal muscle in a previous study (32). In line, a lack of impact on FFA levels was found upon a 12-week period of Acipimox administration in T2DM patients, despite Acipimox was shown to beneficially influence plasma lipid levels, as serum cholesterol and serum triglyceride levels were lowered (70), which are both associated with intramyocellular lipid storages. So, it remains unclear if IMCL accumulation simply reflects FFA overload, as the present study results questions this assumption.

Moreover, previous studies indicated an association between IMCL content and impaired mitochondrial function, as intracellular lipid storages are shown to be located in close proximity to mitochondria, suggesting that not only FFA oversupply induces IMCL accumulation but also a dysbalance between cellular uptake of FFAs and impaired intracellular oxidation due to mitochondrial disturbances (50, 52).

Also an interrelation between IMCL content and insulin resistance was shown (51), whereas the TG content correlates closely with insulin resistance (21). However,

enhanced lipid storage was not only found in diabetic patients but also in healthy, young and highly trained athletes, which was paralleled with high insulin sensitivity in the latter mentioned subgroup, revealing that the IMCL content is not a simple reflection of insulin resistance (43, 50). On the one hand, IMCL can be regarded as energy sources in trained athletes and on the other hand it reflects the imbalance between energy supply and energy demand in insulin resistant patients (43). However, in the present studies, the interrelation between FFAs, IMCL accumulation and insulin resistance in T2DM patients was not elucidated.

4.6 T2DM and mitochondrial function

PCr recovery half-time, a measure for *in vivo* mitochondrial oxidative capacity, was found to be comparable between healthy controls, obese humans and T2DM patients in the present cross-sectional study. However, evaluating the *ex vivo* results, a diminished mitochondrial oxidative capacity was proven, if values of state 3 respiration of complex I were compared between T2DM patients and healthy controls. Diminished oxidative capacity at complex I in T2DM patients was also demonstrated previously (2, 4), whereas complex I capacity was reduced by 40% in skeletal muscle of T2DM and by 20% in obese patients compared to lean individuals (2). In comparison to the present cross-sectional study, we found reduced complex I activity by 51% in skeletal muscle of T2DM and by 28% in obese patients compared to healthy controls. Interestingly, TBARS, as a measure for oxidative stress, were not different in the cross-sectional study.

Correspondingly, results of the present interventional study showed that PCr recovery half-time upon Acipimox administration did not differ compared to placebo and control condition. Examinations of *ex vivo* mitochondrial function also resulted in similar results between the three therapy groups. Even if intrinsic mitochondrial function was adjusted for mtDNA copy number, values remained comparable, indicating a lack of effect of Acipimox on both mitochondrial oxidative capacity and mtDNA content. However, a beneficial impact of Acipimox on mitochondrial function was not expected in the present interventional study due to the rebound effect of lipolysis. Instead we have shown that elevated plasma FFA levels upon a two week Acipimox administration period did not influence the mitochondrial oxidative capacity.

Increased FFA levels were found to induce intensified intramuscular lipid deposits, which necessarily impairs mitochondrial function (85). In line, an association between increased intramuscular fat content and a reduction in mitochondrial oxidative phosphorylation activity was observed, as it was found that the mitochondrial activity was decreased by nearly 30% in insulin resistant patients paralleled by an increase of approximately 80% of IMCL content in comparison to insulin sensitive controls (42). In contrast, a dependency between IMCL content and mitochondrial oxidative capacity was disproved both *in vivo* and *ex vivo* in a previous animal study including Zucker diabetic fatty rats (86). Interestingly, increased IMCL levels were found, accompanied by unaltered mitochondrial function (86). It was suggested, that intracellular metabolites of FFAs induce mitochondrial impairments, rather than IMCL accumulation (76). So, the question arises, whether or not long-term elevation of FFAs would lead to a diminished function of mitochondria. Accordingly, a correlation was observed between increased FFA concentrations and diminished mitochondrial ATP synthesis and therefore it was concluded that FFAs cause an acquired mitochondrial defect (76, 87). These findings could possibly be explained by an inhibitory effect of FFAs on the ETC activity through decreasing the inner mitochondrial membrane potential or by downregulation of genes involved in oxidative phosphorylation processes (76, 87). Interestingly, in another study, no changes of ATPase activity were found in times of elevated plasma FFA levels (32). Furthermore, mutations of the mtDNA upon aging are known to be associated with mitochondrial impairments (23, 45). Therefore, comparisons of mitochondrial function between participants at different ages, as performed in the present cross-sectional study, remain questionable and complicate the comparability of previous study results. Furthermore, the impact of raising FFA levels on genetic parameters of mitochondria was proven, as decreased mRNA levels of mitochondrial enzymes, involved in mitochondrial oxidative processes, were found, indicating an inverse correlation between FFAs and mitochondrial gene expression (85). Researches for the effect of Acipimox on mitochondrial gene expression had proven diminished PGC-1α mRNA expression upon Acipimox administration accompanied by improved insulin sensitivity and diminished FFA levels (46). The decrease of mitochondrial gene expression in times of lowered FFAs levels could be explained by a compensatory homeostatic mechanism (46). Contrarily, other study results pointed towards an increase in mitochondrial enzymes paralleled by increased mitochondrial biogenesis upon elevated FFAs levels (88, 89).

Furthermore, it was suggested, that impaired mitochondrial function is linked with reduced mitochondrial density (4). Additionally, mitochondrial function was only different between healthy individuals and T2DM patients in a previous study, if *ex vivo* results of mitochondrial function were not normalized for mtDNA content (2, 90). The O_2-flux showed no difference between healthy participants and T2DM patients, if values were normalized for mtDNA content, revealing the major impact of mitochondrial content on mitochondrial function (90). Regarding the present interventional study, no difference of mitochondrial density was found between the groups, which possibly explained the lack of effect on both PCr recovery half-time and *ex vivo* measurements of mitochondrial O_2-flux. However, a reduced content of mitochondria in T2DM patients was proven in previous studies, inducing oxidative stress and therefore impaired mitochondrial biogenesis, which necessarily induces lowered mitochondrial function (90).

Furthermore, thyroid hormones are known to affect mitochondrial function by stimulating mtDNA transcription, up-regulation of PGC-1α and increasing mitochondrial biogenesis (45). Therefore, hypothyroidism is accompanied with reduced rates of oxidative phosphorylation, whereas hyperthyroidism is associated with elevated ROS levels (45). Also an interaction between mitochondria and steroid hormones was proven, as mitochondria are essential for steroidogenesis. Diminished testosterone levels were suggested to be paralleled with elevated FFA levels, insulin resistance and mitochondrial impairments (45). Therefore, comparisons of mitochondrial function among patients with different gender, like performed in both of the present studies, remain questionable.

Taken together, the effects of FFAs on mitochondrial function were paradoxical and further examinations are necessary.

4.7 Conclusion

In conclusion, we examined the acute impacts of meal ingestion on skeletal muscle energy metabolism in patients who are differently sensitive to insulin. We found in the cross-sectional study decreased insulin sensitivity, as well as increased glucose and FFA levels in T2DM patients compared to healthy controls both before and after meal ingestion, however, we did not verify a correlation between insulin resistance and mitochondrial impairments. Moreover, Acipimox enhanced plasma FFA levels instead of the hypothesized decrease, which influences the metabolic parameters analyzed in the present interventional study. Surprisingly, the expected increase of ectopic fat accumulation in liver and skeletal muscle, due to elevated FFA levels, was not shown but whole body- and the hepatic sensitivity to insulin were diminished. No benefits of Acipimox were shown on metabolic flexibility as well as on mitochondrial function.

A limiting factor of the present studies might be the small sample-size, which questions the expressiveness of the results received. On the other hand, volunteers included in the studies were well matched based on strict inclusion criteria in order to ensure comparable starting conditions amongst the participants and to minimize incalculable metabolic disturbances. Moreover, a large quantity of data was already received in both of the studies, as data of thirty patients were evaluated before and upon meal ingestion in the cross-sectional study and eleven patients had to run through three treatment arms in the interventional study. Nevertheless, a possible source of error leading to ambiguous results could be differences of age, merely between participants of the cross-sectional study, which may limit the comparability between the groups. Furthermore, the gender of the participants is known to influence the results achieved, as women are characterized by a different lipid metabolism in comparison to males (63). Supplementary, the determination of metabolic flexibility by indirect calorimetry measurements could be misinterpreted, as both lipogenesis and gluconeogenesis were not taken into account (59). Additionally, an extended duration of the interventional study, meaning Acipimox administration for more than two weeks, could be advisable for getting more expressive long-term results. Furthermore, a plausible cause initiating the rebound effect of lipolysis in the present study might be an inadequate dosage of Acipimox administration (58). In defense against this speculation, the rebound effect also occurred, if dosages of Acipimox were elevated up to 125 mg at 2-h intervals (69). Besides, if Acipimox was given in 2-h intervals for a treatment period of 4 days, the

Acipimox concentration was twice as high during the night compared with daytime concentrations, indicating that ingestion of food possibly interfere the absorption of Acipimox (69). Comparisons among previous Acipimox studies (table 1) are questionable, not only because of different dosages of Acipimox administration but also due to various study designs, including the application of different methods and dissimilar criteria for selecting the participants.

Taken together, a correlation between insulin resistance and mitochondrial impairments was not verified in both of the studies. Furthermore, the clinical implication of Acipimox therapy in T2DM patients would be that Acipimox is an inappropriate therapeutic agent for the treatment of T2DM.

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6. Appendix

	Unit		Healthy	T2DM	Obese	P-value
Glucose	mmol/l	t=0 min	4.17 ± 0.11	8.25 ± 0.87	4.37 ± 0.21	0.00**
		t=15 min	5.15 ± 0.34	9.03 ± 0.80	5.62 ± 0.31	0.00**
		t=30 min	5.66 ± 0.63	10.72 ± 0.90	6.49 ± 0.56	0.00**
		t= 60 min	4.32 ± 0.38	11.52 ± 0.95	5.82 ± 0.50	0.00**
		t=90 min	4.28 ± 0.36	13.69 ± 1.21	5.54 ± 0.37	0.00**
		t=120 min	4.70 ± 0.41	13.91 ± 1.50	5.34 ± 0.42	0.00**
		t=180 min	4.43 ± 0.13	12.60 ± 1.49	4.51 ± 0.24	0.00**
		t=260 min	4.29 ± 0.07	9.42 ± 1.55	3.76 ± 0.13	0.00**
FFA	µmol/l	t=0 min	318 ± 48	516 ± 62	517 ± 41	0.02*
		t=15 min	430 ± 79	575 ± 59	569 ± 32	0.19
		t=30 min	270 ± 55	542 ± 78	423 ± 46	0.02*
		t= 60 min	135 ± 22	382 ± 57	190 ± 30	0.00**
		t=90 min	136 ± 15	270 ± 42	163 ± 27	0.01*
		t=120 min	108 ± 10	205 ± 28	150 ± 19	0.01**
		t=180 min	106 ± 11	162 ± 23	219 ± 49	0.05
		t=260 min	232 ± 56	189 ± 31	436 ± 81	0.02*
TBARS	µmol/l	t=0min	2.6 ± 0.3	2.6 ± 0.2	2.3 ± 0.4	0.68
		t=120min	2.4 ± 0.2	2.7 ± 0.1	2.2 ± 0.4	0.39
		t=180 min	2.5 ± 0.2	3.2 ± 0.2	2.6 ± 0.5	0.18
		t=260min	2.3 ± 0.2	2.5 ± 0.2	2.6 ± 0.5	0.87
M-value	$mg*kg^{-1}*min^{-1}$		9.4 ± 1.3	2.4 ± 0.9	3.3 ± 0.5	0.00**
RQ		basal	0.78 ± 0.01	0.82 ± 0.02	0.87 ± 0.04	0.04*
		clamp	0.89 ± 0.05	0.76 ± 0.10	0.92 ± 0.06	0.29
		ΔRQ(clamp-basal)	0.11 ± 0.06	-0.06 ± 0.10	0.05 ± 0.05	0.26
IMCL	%		0.54 ± 0.15	0.83 ± 0.14	1.07 ± 0.49	0.33
PCr ½	sec		24.3 ± 4.8	33.5 ± 5.2	24.9 ± 1.9	0.32
Ex vivo State 3 complex I	pmol/(s*mg)		48.9 ± 3.9	24.0 ± 3.6	34.8 ± 9.5	0.02*
Ex vivo State 3 complex I+II	pmol/(s*mg)		80.1 ± 6.6	66.9 ± 10.7	64.9 ± 11.3	0.42
Ex vivo State u	pmol/(s*mg)		109.2 ± 6.9	85.2 ± 13.5	94.7 ± 12.6	0.26

Table 4: Substrate kinetics and mitochondrial function of the cross-sectional study. Data are mean ± SEM, *p≤0.05, **p≤0.01; T2DM, type 2 diabetes mellitus; FFA, free fatty acids; TBARS, thiobarbituric acid reactive substances; RQ, respiratory quotient; IMCL, intramyocellular lipids; PCr½, PCr-recovery half-time

	Unit	2-step clamp	Control	Placebo	Acipimox	P-value
Gluc	mmol/l	Baseline	7.62 ± 0.53	9.95 ± 1.25	9.63 ± 1.12	0.01**
		Low ins	5.43 ± 0.18	6.29 ± 0.48	6.31 ± 0.25	0.04*
		High ins	5.19 ± 0.10	5.10 ± 0.10	5.43 ± 0.17	0.13
		ΔGluc(high ins-baseline)	-2.43 ± 0.59	-4.85 ± 1.24	-4.20 ± 1.17	0.01**
FFA	µmol/l	Baseline	623 ± 53	663 ± 51	1175 ± 78	0.00**
		Low ins	349 ± 73	367 ± 52	1014 ± 74	0.00**
		High ins	135 ± 24	159 ± 19	336 ± 61	0.00**
		ΔFFA(high ins-baseline)	-488 ± 54	-504 ± 49	-839 ± 85	0.00**
Rd	µmol/kg/min	Baseline	12.39 ± 1.71	11.37 ± 1.31	9.07 ± 1.38	0.19
		Low ins	7.05 ± 0.52	7.55 ± 1.32	5.56 ± 0.62	0.15
		High ins	20.69 ± 3.96	20.93 ± 4.53	8.77 ± 1.09	0.00**
		ΔRd(high ins-baseline)	8.31 ± 4.27	9.57 ± 4.74	-0.30 ± 1.88	0.02*
EGP	µmol/kg/min	Baseline	11.95 ± 1.71	10.93 ± 1.31	8.64 ± 1.38	0.19
		Low ins	2.88 ± 0.77	4.05 ± 1.29	4.45 ± 0.61	0.41
		High ins	-0.93 ± 1.32	0.29 ± 1.31	2.87 ± 0.74	0.09
		ΔEGP(high ins-baseline)	-12.87 ± 2.52	-10.64 ± 1.27	-5.77 ± 1.49	0.04*
		ΔEGP(low ins-baseline)	-9.07 ± 1.40	-6.87 ± 1.42	-4.19 ± 1.41	0.05*
RQ		Baseline	0.78 ± 0.02	0.79 ± 0.02	0.77 ± 0.01	0.67
		Low ins	0.86 ± 0.02	0.84 ± 0.03	0.79 ± 0.02	0.07
		High ins	0.88 ± 0.02	0.86 ± 0.03	0.86 ± 0.03	0.75
		ΔRQ(high ins-baseline)	0.10 ± 0.03	0.05 ± 0.03	0.06 ± 0.03	0.51
Lip ox	nmol/kg/min	Baseline	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	0.95
		Low ins	0.7 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.07
		High ins	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2	0.77
		ΔLip ox(high ins-baseline)	-0.6 ± 0.2	-0.5 ± 0.2	-0.5 ± 0.2	0.85
Gl ox	nmol/kg/min	Baseline	5.1 ± 1.0	5.1 ± 0.6	6.4 ± 1.6	0.71
		Low ins	10.4 ± 1.6	7.8 ± 1.1	7.7 ± 2.2	0.43
		High ins	11.5 ± 1.2	10.9 ± 2.6	11.7 ± 2.6	0.94
		ΔGl ox(high ins-baseline)	6.5 ± 1.7	5.5 ± 2.5	5.3 ± 3.2	0.93
HCL	%		16.0 ± 3.9	11.0 ± 2.2	11.4 ± 1.9	0.04*
IMCL	%			1.7 ± 0.58	1.8 ± 0.39	0.78
Mt density	AU		2169 ± 464	1720 ± 244	2072 ± 273	0.53
PCr ½	sec		33.8 ± 4.1	32.4 ± 4.9	35.0 ± 4.7	0.45

Table 5: Whole body substrate kinetics of the interventional study. Data are mean ± SEM, *p≤0.05, **p≤0.01; Gluc, plasma glucose levels; FFA, free fatty acids; Rd, glucose disposal rate; EGP, endogenous glucose production; RQ, respiratory quotient; Lip ox, lipid oxidation; Gl ox, glucose oxidation; HCL, hepatocellular lipids; IMCL, intramyocellular lipids; Mt density, mitochondrial density; PCr½, PCr-recovery half-time; ins, insulin; AU, arbitrary units

	Unit	Substrates	Control	Placebo	Acipimox	P-value
Trace 1 Permeabilized skinned muscle fibers	pmol/(s*mg)	Malate	8.3±1.0	4.4 ± 0.8	6.0 ± 1.0	0.02*
		Glutamate	9.8 ± 1.2	6.0 ± 0.7	7.9 ± 1.8	0.12
		ADP	38.7 ± 4.0	31.4 ± 3.9	37.7 ± 5.0	0.32
		Succinate	74.7 ± 9.8	65.3 ± 6.0	72.4 ± 7.5	0.47
		Cyt c	78.8 ± 10.2	72.8 ± 6.7	73.5 ± 7.4	0.72
		FCCP	101.5 ± 17.4	87.2 ± 7.8	98.9 ± 10.5	0.55
Trace 1 Normalized for mtDNA content	(pmol/(s*mg))/ mtDNA copy number	Malate	6.1 ± 3.0	2.1 ± 0.3	3.1 ± 0.7	0.21
		Glutamate	8.2 ± 4.6	3.0 ± 0.3	3.8 ± 0.8	0.32
		ADP	19.9 ± 4.3	16.7 ± 2.5	18.8 ± 2.6	0.79
		Succinate	33.9 ± 4.0	34.7 ± 4.1	35.4 ± 3.6	0.96
		Cyt c	35.9 ± 4.4	38.4 ± 5.4	36.0 ± 3.8	0.90
		FCCP	46.7 ± 6.8	47.4± 7.4	46.8 ± 5.1	1.00
Trace 2 Permeabilized skinned muscle fibers	pmol/(s*mg)	Malate	9.2 ± 2.2	6.6 ± 1.4	7.3 ± 1.9	0.43
		Oct-car	12.1 ± 2.3	9.0 ± 1.8	10.1 ± 2.2	0.23
		ADP	30.5 ± 2.9	23.0 ± 3.5	27.3 ± 2.7	0.19
		Glutamate	43.1 ± 2.9	37.2 ± 4.2	39.5 ± 3.5	0.31
		Succinate	78.3 ± 7.0	62.5 ± 7.8	66.6 ± 7.1	0.06
		Cyt c	84.0 ± 8.5	73.7 ± 6.1	69.7 ± 8.1	0.13
		FCCP	108.9 ± 14.8	91.0 ± 5.1	82.6 ± 7.1	0.30
Trace 2 Normalized for mtDNA content	(pmol/(s*mg))/ mtDNA copy number	Malate	3.9 ± 1.3	3.7 ± 1.0	2.9 ± 0.3	0.65
		Oct-car	5.3 ± 1.4	5.0 ± 1.3	4.4 ± 0.8	0.63
		ADP	11.9 ± 1.7	12.2 ± 1.7	12.9 ± 1.8	0.83
		Glutamate	17.2 ± 2.8	19.9 ± 2.1	18.2 ± 1.5	0.63
		Succinate	30.3 ± 4.3	32.0 ± 2.7	30.5 ± 2.7	0.92
		Cyt c	32.3 ± 4.6	38.5 ± 3.7	31.7 ± 2.8	0.38
		FCCP	41.5 ± 5.4	46.1 ± 4.6	43.5 ± 5.3	0.80

Table 6: *Ex vivo* mitochondrial function of the interventional study. O_2-flux given in pmol/(s*mg) in permeabilized skinned muscle fibers upon substrate addition according to trace 1 and trace 2 during control condition, placebo medication and Acipimox administration. Values received were also normalized for mitochondrial content. Data are mean ± SEM, *p≤0.05; mtDNA, mitochondrial DNA; ADP, adenosine diphosphate; Cyt c, cytochrome c; FCCP, fluoro-carbonyl cyanide phenylhydrazone; Oct-car, octanoylcarnitine

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8. Eidesstattliche Versicherung

Ich versichere an Eides statt, dass die Dissertation selbstständig und ohne unzulässige fremde Hilfe erstellt und die hier vorgelegte Dissertation nicht von einer anderen Medizinischen Fakultät abgelehnt worden ist.

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