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Effects of circulating lipids on mitochondrial structure and function

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Effects of circulating lipids on mitochondrial structure and function

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List of Abbreviations

^{1}H MRS	proton magnetic resonance spectroscopy
ADP	adenosine 5`diphosphate potassium salt
ANT	adenine nucleotide translocator
ATP	adenosine triphosphate
BIOPS	biopsy preservation solution
BMI	body mass index
BSA	body surface area
CoA	coenzyme A
DM	diabetes mellitus
DNA	desoxyribonucleic acid
DTT	dithiothreitol
ECM	extracellular matrix
EGTA	ethylene glycol tetraacetic acid
EM	electron microscope
et al.	et alia (and others)
ETC	electron transport chain
FAD	flavin adenine dinucleotide
fATP	ATP synthetic flux (unidirectional flux through ATP synthase)
FBG	fasting blood glucose
FCCP	carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone
FFA	free fatty acids
GIR	glucose infusion rate
glutamate	L-glutamic acid sodium salt
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IMCL	intramyocellular lipids
IMF	intermyofibrillar
KH_2PO_4	potassium dihydrogen phosphate
KOH	potassium hydroxide
l	liter

m	meter
malate	L-malic acid
MES	2-(N-morpholino)ethanesulfonic acid
mg	milligram
$MgCl_2*6H_2O$	magnesium chloride hexahydrate
miRO5	mitochondrial respiration medium
mmol	millimol
mU	milliunits
M-value	total glucose disposal rate
Na_2ATP	adenosine 5`-triphosphate disodium salt
NAD	nicotinamide adenine dinucleotide
nmol	nanomol
O_2	oxygen
pyruvate	pyruvic acid sodium salt
pH	decimal logarithm of the reciprocal of the hydrogen ion activity
pmol	picomol
RCR	respiratory control ratio
ROS	reactive oxygen species
s	second
SAP	saponine
SEM	standard error of the mean
SS	subsarcolemmal
succinate	succinate disodium salt hexahydrate
T2DM	type 2 diabetes mellitus
TAG	triacylglycerol
TLR	toll like receptors
UCR	uncoupling control ratio
µmol	micromol

A. Abstract

Insulin resistance is the hallmark of type 2 diabetes mellitus, a condition with life threatening complications affecting millions of people worldwide. Increases in circulating free fatty acids, inflammatory pathways and abnormal features of mitochondrial function may contribute to or even cause insulin resistance. This study aimed at elucidating the effect of a short-term increase in circulating free fatty acids on mitochondrial function and morphology. Healthy volunteers received lipid infusions and underwent muscle biopsies for analyzing mitochondrial function by high resolution respirometry and sub-cellular morphology by electron microscopy. Short-term lipid elevation resulted in an increase in muscular lipid accumulation after 2.5 h and 4 h in the form of lipid droplets, in particular in the regulatory subsarcolemmal area. The respiratory control ratio was lowered after 4 h of lipid infusion, indicating impaired mitochondrial respiration. In addition, mitochondrial size correlated negatively with respiratory capacity as represented by the respiratory control ratio, suggesting that smaller mitochondria were more efficient in substrate oxidation. No correlations were found between mitochondrial functional parameters and lipid content. Taken together, these findings showed a direct interplay between circulating free fatty acids and mitochondrial respiration. Short-term lipid infusion directly increased muscular lipid content, which emphasized the dynamic and fast reacting nature of this compartment.
Lipid droplet content per se did not affect mitochondrial function, which indicated that further metabolic pathways might underlie the functional impairment.

B. Zusammenfassung

Insulinresistenz ist die vordergründige Pathologie des Diabetes mellitus Typ 2, einer lebensbedrohlichen Krankheit, die Millionen Menschen weltweit betrifft. Eine Erhöhung der freien Fettsäuren im Blut, inflammatorische Stoffwechselprozesse sowie eine Veränderung der mitochondrialen Funktion tragen zu Insulinresistenz bei oder können diese sogar verursachen.

Ziel der vorliegenden Studie war, den Effekt einer kurzzeitigen Erhöhung der freien Fettsäuren auf die mitochondriale Funktion und Morphologie zu überprüfen. Vor, während und im Anschluss an eine Lipidinfusion wurde gesunden Studienteilnehmer/innen eine Muskelbiopsie entnommen, um die Mitochondrienfunktion mittels hochauflösender Respirometrie und die zelluläre Mitochondrienmorphologie mittels Elektronenmikroskopie zu untersuchen.

Eine kurzzeitige Lipiderhöhung resultierte in einer Erhöhung der muskulären Lipidansammlungen nach 2,5 und 4 h in Form von Fetttröpfchen. Dieser Effekt findet besondere Ausprägung in der regulatorischen Region, direkt unterhalb des Sarkolemms. Zudem wurde nach 4 h eine Abnahme der mitochondrialen Effektivität, repräsentiert durch die Respirations-Kontroll-Rate, beobachtet. Auch lag eine negative Korrelation zwischen der Größe der Mitochondrien und der respiratorischen Kapazität in Form der Respirations-Kontroll-Rate vor, was vermuten lässt, dass kleinere Mitochondrien effizienter in der Substratoxidation waren. Zwischen Mitochondrienfunktion und Fetttröpfchengehalt des Muskels zeigten sich keine signifikanten Korrelationen.

Zusammenfassend wiesen die Ergebnisse auf einen direkten Zusammenhang zwischen erhöhten freien Fettsäuren und mitochondrialer Respiration hin. Kurzzeitige Erhöhung der Lipide steigerte direkt den muskulären Fetttröpfchengehalt, was auf dynamische und schnell reagierende Charakteristika dieses Kompartiments hindeutete.

Der erhöhte Fetttröpfchengehalt des Muskels per se schien keinen direkten Einfluss auf die Mitochondrienfunktion zu haben, was darauf hinweist, dass der Beeinträchtigung der Funktion und Morphologie der Mitochondrien zusätzliche Signaltransduktionswege zugrunde liegen.

C. Introduction

C.1. Diabetes mellitus

Diabetes mellitus (DM) either results from an insufficiency of pancreatic beta cells to secrete insulin in response to increased glucose levels (Type 1) [1] or from a decrease of the ability of peripheral tissues to respond to insulin, termed as insulin resistance (Type 2, T2DM) [2]. Common to both types is persistent hyperglycemia, leading to severe and life threatening complications in untreated patients [3].

T2DM, accounting for 90% of diabetes cases, has reached epidemic proportions around the world. In 2010, a global prevalence of 6.6% (285 million affected people) for diabetes was reported, causing 4 million deaths in the 20 – 79 year age cohort. It is predicted that this prevalence will rise to 7.8% in 2030 [4]. Every 5 seconds a patient is diagnosed with diabetes, and every 10 seconds someone dies of it [5]. In the United States 25.8 million people are diagnosed with diabetes, resulting in total costs of 174 billion US$ [6].

It is generally believed that insulin resistance is the primary abnormality underlying T2DM [7]. Insulin resistance describes the insufficient ability of peripheral tissues to act properly in response to insulin stimulation [8]. T2DM patients have typically a compensatory hyperinsulinemic state to account for the increased glucose concentrations [9]. The resulting hyperglycemia is mainly caused by an inability of liver and muscle tissue to respond adequately to elevated insulin levels [10, 11], which will be described in detail in the following paragraphs.

C.2. Pathophysiology of T2DM

C.2.1. *Insulin resistance in T2DM*

The development of T2DM is likely driven by several external factors [12] as well as genetic predispositions [13, 14]. There is an intimate correlation between the rise in obesity, the consumption of diets rich in fat and carbohydrates, low in fiber and inadequate amounts of physical activity in developed countries [15, 16]. These risk factors, together with metabolic and inflammatory disturbance by environmental factors, ultimately result in peripheral insulin resistance, the major hallmark of T2DM

[17-19]. It has been shown, however, that the risk of developing insulin resistance and T2DM can be reduced effectively by inducing weight loss and by performing regular exercise [12]. The underlying mechanisms of insulin resistance involve ectopic lipid deposition leading to lipotoxicity, adipose tissue inflammation and mitochondrial dysfunction, and will be described in more detail in the following paragraphs.

Insulin regulates glucose homeostasis in response to changing nutritional glucose availability during periods of feeding. It is produced by the pancreatic beta cells and its secretion is stimulated by high blood glucose levels, free fatty acids (FFA) or branched chain amino acids [20]. Furthermore, insulin stimulates the storage of energy in muscle and adipose tissue, but inhibits degradation and release into the circulation by inhibition of glycogenolysis, lipolysis and protein catabolism [21, 22].

In the liver, insulin inhibits glucose production by stimulating glycogenesis, lipogenesis and protein synthesis. In insulin resistant states such as T2DM, hepatic glucose production is increased, leading to an increase in blood glucose concentrations [23]. FFA induce hepatic insulin resistance through a stimulatory allosteric effect on enzymes regulating gluconeogenesis [24, 25].

In patients with T2DM and in obese non-diabetic humans, fasting FFA in the plasma are elevated when compared to lean, non-diabetic individuals [26]. Insulin resistance, caused by artificially elevated triacylglycerols (TAG) and FFA via infusion of lipids, results in the accumulation of intracellular lipids, in particular in muscle and hepatic tissue [27, 28].

The resulting down regulation of oxidative enzymes and an increase in oxidative stress with generation of reactive oxygen species is hypothesized to damage cellular structures, such as the cellular genome and proteome [29, 30]. Furthermore, mitochondrial function, which is responsible for the energy production of skeletal muscle cells, is correlated with insulin sensitivity [31], although the existence of a direct causality, however, remains unrevealed. In states of insulin resistance, oxidative phosphorylation has also been shown to be impaired [32], which will be explained in more detail in the following paragraphs.

C.3. Mechanisms of insulin resistance in T2DM

Insulin resistance is defined as a state where the cellular response to the action of insulin is diminished. This condition mainly limits the uptake of circulating glucose from the bloodstream into tissues. Pancreatic beta cells secrete more insulin to compensate for the increased glucose concentrations [9]. Once the insulin production cannot compensate for the diminished peripheral insulin function, blood glucose levels rise and T2DM becomes overt [17-19]. The resulting hyperglycemia is mainly caused by an inability of liver and muscle tissue to respond adequately to elevated insulin levels [10, 11].

C.3.1. *Fat accumulation & lipotoxicity*

In patients with T2DM and in obese non-diabetic humans, fasting FFA in the plasma are elevated when compared to lean, non-diabetic individuals [26, 33]. The delivery of surplus FFA to non-adipose tissues such as skeletal muscle, liver and heart, causes cellular dysfunction and is consequently termed lipotoxicity [34-36]. Lipotoxicity causes insulin resistance in liver and skeletal muscle [37, 38]. Intramyocellular lipid (IMCL) accumulations are important energy sources within muscle tissue [39]. However, surplus IMCL depositions within muscle tissue have been reported to correlate with insulin resistance [40-44]. On the other hand, physical exercise increases IMCL content while also lowering muscular insulin resistance [45, 46]. For this, the term “athlete´s paradox” has been introduced [47]. It can be speculated that IMCL accumulation is not the cause but rather a consequence of excess dietary fat supply or disturbed utilization. An increase in FFA and metabolites would in turn promote progression of T2DM through interference with insulin signaling [11]. There is evidence that some lipid metabolites, mainly diacylglycerol, but maybe also long-chain fatty acyl-CoA and ceramides, rather than IMCL directly cause insulin resistance [25, 48-51]. These toxic lipid metabolites can impair insulin signaling and decrease enzyme activities underlying insulin resistance [52]. The resulting down regulation of oxidative enzymes and an increase in oxidative stress with generation of reactive oxygen species (ROS) is hypothesized to damage cellular structures, such as the cellular genome and proteome [25, 29, 30]. In addition, other pathologies can be attributed to lipotoxic

effects: cardiac dysfunction and apoptosis has been associated with lipid accumulation in the heart of T2DM patients [53]. Persistent exposure to surplus of FFA in beta-cells inhibits insulin secretion and lowers insulin gene expression [51].

It is furthermore known that the risk for the development of insulin resistance and T2DM can be reduced effectively by inducing weight loss and by performing regular exercise [12]. Skeletal muscle accounts for most of the insulin-stimulated non-oxidative glucose disposal through storage in the form of glycogen. Exercise therefore directly affects glucose disposal through increased activity of skeletal muscle [9].

C.3.2. *Inflammation*

Inflammation, as characterized by an increase in inflammatory cytokines produced by macrophages, negatively affects insulin sensitivity. Macrophages expand and infiltrate adipose tissue in obese individuals [54]. The increased production of TNF-α, IL-6, resistin as well as several other cytokines from adipose tissue as well as macrophages create a subacute inflammatory state in obese individuals that promotes insulin resistance [55]. In addition, there is an intimate link between lipotoxicity and inflammation. Toll like receptors (TLRs) are cellular pattern recognition modules involved in pathogen identification. The fact that some TLRs like TLR4 recognize lipid conjugates lends to the involvement of lipids in immune activation [56]. An additional aspect of immune activation through surplus lipids is their aforementioned promotion of cellular stress and ROS formation, which is a known mechanism of immune activation [57]. Inflammation is therefore believed to be a strong contributor to obesity-associated diseases, in particular insulin resistance and T2DM

C.3.3. *Mitochondria and their role in T2DM*

Mitochondrial function, which is responsible for the oxidative glucose utilization of skeletal muscle cells, is correlated with insulin sensitivity [31]; the existence of a direct causality, however, remains unrevealed. Mitochondrial function depends on extrinsic as well as intrinsic factors. Extrinsic factors include reduced mitochondrial density, lower blood perfusion of the skeletal muscle [58] as well as differences in insulin stimulation [59]. Intrinsic factors are defined as a decreased respiratory capacity per mitochondrion [60] and are apparent in patients with T2DM [61]. These factors can either be inherited

or acquired but ultimately result in reduced mitochondrial function and insulin resistance, the order of which still remains elusive [62].

In states of insulin resistance, oxidative phosphorylation has also been shown to be impaired [32], which will be explained in more detail in the following paragraphs.

The causality between mitochondrial function and insulin resistance remains to be elucidated and forms the basis of this thesis.

C.4. Structure and function of mitochondria

Mitochondria are essential membrane-encapsulated organelles that carry specific components of the cellular metabolic machinery, encoded by nuclear as well as mitochondrial nucleic acids [63]. They are composed of an outer and an inner membrane, which enclose a matrix region. Between the outer and inner membrane is another compartment, which is referred to as the intermembrane space [64]. Morphometric analysis of the skeletal muscle mitochondria based on electron microscopy (EM) images indicate smaller mitochondria in obese or T2DM patients compared to lean humans. In addition, mitochondrial size was found to correlate negatively with insulin sensitivity [65].

The inner membrane folds into cristae, which contain transmembrane proteins of the electron transport chain (ETC) as well as the mitochondrial ATP-synthase [66]. Inside mitochondria, many essential cellular processes take place, such as the conversion of acetyl-CoA derived from glycolysis and beta-oxidation in the tricarboxylic acid cycle, catabolism of amino acids, ketogenesis as well as the production of reactive oxygen species [67].

C.4.1. *Oxidative phosphorylation*

Oxidation of energy containing carbohydrates leads to a reduction of nicotinamide adenine dinucleotide (NAD+) and flavin adenine dinucleotide (FAD) to the electron donors $NADH+H^+$ and $FADH_2$. The ETC is composed of four large protein complexes and transports electrons from the aforementioned reduced equivalents (NAD+, FAD) to molecular oxygen (Figure 1). During electron transfer, protons (H+) are translocated across the mitochondrial membrane, thereby creating an H+ gradient.

This proton gradient drives the phosphorylation of adenosine diphosphate (ADP) into the energy-rich ATP, used, i.e., for muscle contraction [68, 69].

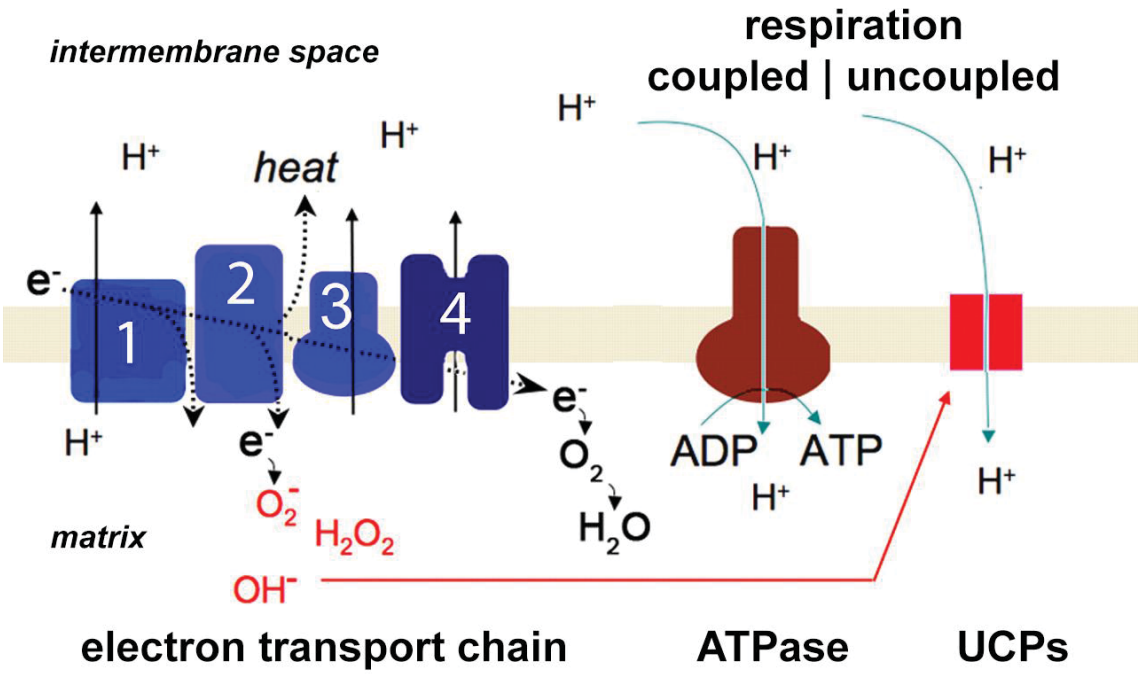

Figure 1. Oxidative phosphorylation in the inner mitochondrial membrane

1: complex I, NADH: ubiquinone-oxidoreductase; 2: complex II, succinate: ubichinon-oxidoreductase; 3: complex III, ubichinol: cytochrome c-oxidoreductase; 4: complex IV, cytochrome c oxidase; UCP = uncoupling proteins. Figure adapted from [70].

The efficiency of oxidative phosphorylation, electron transport, generation of a proton gradient and proton flux coupled to the ATP-synthase, can differ between tissue types, metabolic conditions and individuals [70]. The re-entry of protons within the matrix can also occur by bypassing the ATP-synthase, which is termed as mitochondrial uncoupling [71]. This non ATP-synthase-coupled proton leak results in energy inefficiency and might be a regulatory pathway of energy expenditure or a mechanism to protect mitochondria against lipid-induced oxidative damage [72]. Oxidative phosphorylation efficiency can also be impaired by an imbalance between electron flow and proton transfer or by electron-loss from the ETC before reaching complex IV [70]. Overall, mitochondrial oxidative phosphorylation is regulated by several factors, like mitochondrial enzyme activity, substrate availability, mitochondrial content as well as the concentration of key regulatory metabolites (ADP, NAD+) [73]. These processes

are in turn controlled by major rate-limiting steps of ATP synthesis [74]. For example, modifications of the adenine nucleotide translocator (ANT) properties result in reduced oxidative phosphorylation. In this case, the diminished exchange of extramitochondrial ADP and intramitochondrial ATP lead to impaired mitochondrial function [75]. Changes in functional mitochondrial parameters, including oxidative phosphorylation capacity as well as changes in mitochondrial size and density in response to altered metabolic conditions are commonly referred to as mitochondrial plasticity [9].

C.4.2. *Subcellular distribution of muscle mitochondria*

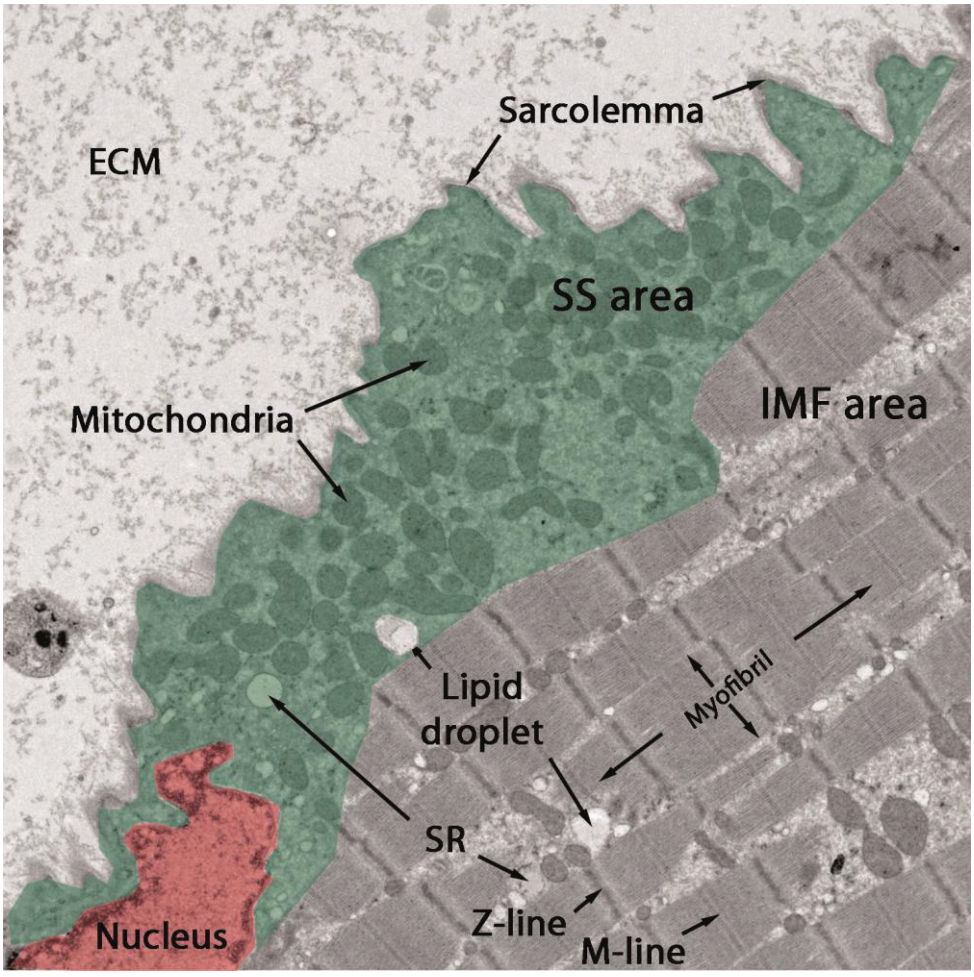

Figure 2. Microscopic structures within muscle cells

Representative EM micrograph of a negative stain tissue sample is shown. Mitochondria, lipid droplets, IMF and SS area (false-colored green) are labeled. The nucleus is false-colored in red. SR = sarcoplasmatic reticulum and T-tubules, ECM = extracellular matrix. Magnification = 6300.

Two biochemical populations of mitochondria, which are located in different regions of the muscle cell, can be distinguished [76]. The majority of mitochondria are located within the intermyofibrillar (IMF) area, between contractile filaments mostly adjacent to Z-lines, as can be seen in Figure 2. Mitochondria in the IMF area are ascribed to function mainly during muscle contraction [77].

The subsarcolemmal (SS) area contains about 10–15% of the total mitochondrial volume. Mitochondria in this region are located adjacent to the sarcolemma and the nuclei. SS mitochondria may provide energy for peripheral nuclei and membrane function, and play a role in substrate transport processes and insulin signaling [77], although their exact role is still unknown.

In a previous study, sophisticated isolation procedures have been used to separate SS and IMF mitochondria [78]. A disproportional reduction of ETC activity in the SS mitochondrial fraction was found in obese and T2DM patients compared with lean volunteers, which was not attributed to reduced mitochondrial content [78], indicating a true functional impairment. This is in line with previous results [61]. In general, the role of these mitochondrial subpopulations in the development in insulin resistance is unknown, as well as the impact of lipid surplus on mitochondrial morphology.

C.5. Hypothesis and Aims

Under insulin resistant conditions, reduced oxidative phosphorylation capacity, submaximal oxidative phosphorylation and/or content of mitochondria have been observed in insulin responsive tissues [9]. In addition, long-term accumulation of lipids can disturb many cellular signaling cascades, including the insulin-induced activated pathway for glucose disposal [79, 80]. Furthermore, it has been shown that temporary elevation of plasma FFA by intravenous infusions inhibits insulin stimulation of mitochondrial ATP synthesis, assessed as unidirectional ATP synthetic flux (fATP), and cellular glucose transport in young, healthy humans [79]. It has not yet been investigated whether elevated FFA impairs mitochondrial oxidative capacity acutely through direct disposition of lipids in muscle tissue. This led us to hypothesize that the temporary elevation of circulating FFA through short-term lipid infusion:

- causes a physiological response by lowering the mitochondrial respiration rate in permeabilized muscle fibers
- elicits an increase in the number of lipid droplets in muscle tissue
- lowers muscular mitochondrial size, content and density, in particular in the SS area

The aims of this study were to evaluate the effects of short-term lipid infusions in healthy male and female participants on the following:

- mitochondrial oxidative capacity *ex vivo*
- lipid droplet count as a measure of intramuscular lipid accumulation
- morphology of mitochondrial subpopulations

To address this, thirteen healthy volunteers underwent biopsies from the *M. vastus lateralis* before and after 2.5 h and 4 h intravenous lipid infusion. Mitochondrial oxidative capacity was measured *ex vivo* using high resolution respirometry. Size, content and density of lipid droplets and mitochondria were assessed by EM within the SS and IMF areas.

D. Materials and Methods

D.1. Study design and inclusion criteria

D.1.1. *Recruitment of volunteers*

Participants of this study represent a subgroup of [81]. Healthy, young, lean men and women (day 5 – 9 of their menstrual cycle) without any family history of T2DM were recruited via online advertisement. Participants were sedentary (< 2 h exercise session per week). Age and body mass index (BMI) needed to be in the range of 20-40 years and < 27 kg/m^2, respectively. Exclusion criteria were smoking and immune-modulatory or metabolic medication. All participants were instructed to avoid extra physical activity and eat carbohydrate-rich meals three days before the clamp test and present after an over-night fasting period. The study was approved by the institutional ethics board of the Heinrich-Heine University, Düsseldorf (# 3157, 11.03.2009).

D.1.2. *Hyperinsulinemic–euglycemic clamp*

To inhibit endogenous insulin secretion, a 0 h – 6.5 h pancreatic clamp was performed using an infusion of somatostatin (0.1µg/kg*min, UCB Pharma, Figure 3). As a tracer, D-[6,6-$^2H_2$]-glucose infusion was administered from -1 h to 6.5 h (3.6 mg/kg*fasting glucose [mg/dl] / 90 [mg/dl] for 5 min, then 0.036 mg/kg*min). For standardization of fasting insulin levels, 6 mU/m² insulin (Actarapid, Novo Nordisk) was administered for 0 h – 4 h. From 4 h – 6.5 h, a hyperinsulinemic-euglycemic clamp (40 mU/m²*min insulin) was performed [79, 82, 83], paralleled by a variable 20% glucose infusion to maintain glucose levels at 5 mmol/l. Every 5 to 10 minutes, plasma blood glucose values were monitored and the 20% glucose infusion rate adapted accordingly and reported as the glucose infusion rate (GIR). During the hyperinsulinemic-euglycemic clamp, the endogenous glucose output is assumed to be suppressed. Therefore, the infused amount of glucose required to maintain constant glucose levels directly reflects the whole body glucose metabolism [84, 85].

The M-value is calculated using the following equations, adapted from [86]:

$$M = GIR - SC$$

M = whole body glucose metabolism
GIR = glucose infusion rate
SC = space correction

Space correction is calculated using the following equation:

$$SC\ (\frac{mg}{kg * min}) = \frac{(\ G(end)\ -\ G(start))\ *10\ *\ (0.19\ *\ body\ weight)}{20\ *\ body\ weight}$$

G = plasma glucose concentration

To afford an increase in circulating FFA, a 20% triglyceride injectable emulsion infusion (Intralipid, Fresenius Kabi) was administered from 0 h - 6.5 h (10 ml/h during first 10 min, 90 ml/h thereafter), combined with an infusion of 0.2 mU/kg*min heparin (Leo Pharma, Denmark) to induce lipase mediated cleavage of triglycerides into FFA and glycerol.

D.1.3. *Muscle biopsy*

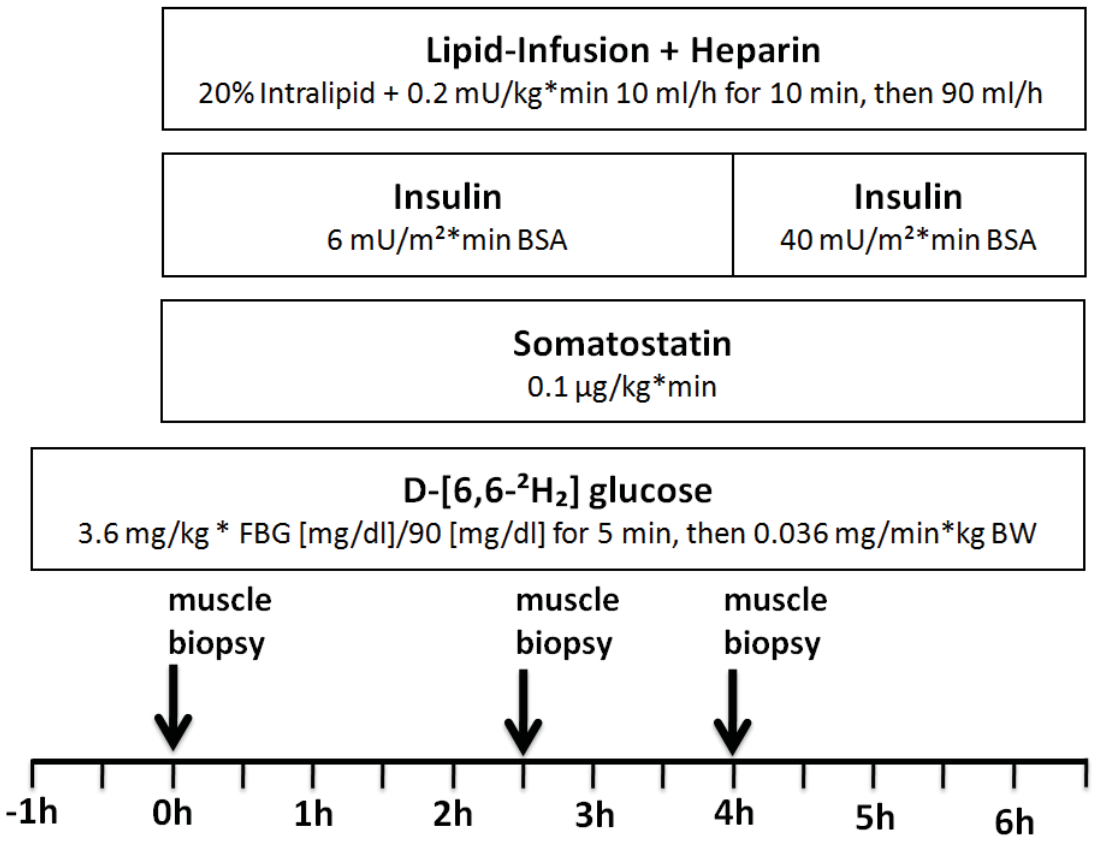

Figure 3. Study protocol

Study protocol for lipid infusion and hyperinsulinemic-euglycemic clamp. Arrows indicate muscle biopsies. BSA: body surface area, FBG: fasting blood glucose, BW: body weight. Adapted from [81].

Muscle biopsies were taken from the *M. vastus lateralis*, according to the technique of Bergström et al. [87] before and during the infusion of Intralipid (at 2.5 h and 4 h, Figure 3).

D.2. Mitochondrial respiration measurements

D.2.1. *Preparation of the muscle fibers*

Muscle tissue was kept on ice in preservation medium (BIOPS: 10 mmol Ca-EGTA, 0.1 mmol Ca, 5.77 mmol Na_2ATP, 6.56 mmol $MgCl_2*6H_2O$, 20 mmol taurine, 15 mmol Na_2 phosphocreatine, 20 mmol imidazole, 0.5 mmol DTT, 50 mmol MES, pH 7.1) [88,

89] until further processing for a maximum of 12 h. Then, the tissue was transferred to petri dishes for mechanic separation of muscle fibers, which was performed on ice using sharp forceps. The sarcolemma of the muscle fibers was permeabilized according to Veksler et al. [90], in which 25 µl SAP solution (5 mg/ml saponine in H_2O) was added to 2.5 ml BIOPS. After 30 min incubation of the muscle fibers on ice, the fibers were washed twice for 10 min in 3 ml mitochondrial respiration buffer (MiRO5: 0.4 mmol EGTA, 3 mmol $MgCl_2*6H_2O$, 125 mmol KCI, 0.3 mmol DTT, 5 mmol KH_2PO_4, 20 mmol HEPES, pH 7.1). For measuring the wet weight of the prepared muscle fibers, samples were dabbed and softly rolled on a filtration paper five times in a standardized manner. The fiber amount for subsequent respiratory measurements ranged from 1-3 mg.

D.2.2. *Instrumental respiratory measurements*

The O_2 consumption of permeabilized muscle fibers in MiRO5 buffer was quantified using a two chamber OROBOROS O2K Oxygraph System (Innsbruck, Austria). This method allows measuring the different states of mitochondrial respiration and yields a direct measure of oxidative capacity *ex vivo*. Each measurement was performed in duplicate at 37°C and under hyperoxygenated conditions (~400 nmol/ml) to avoid any limitation of oxygen supply. Mitochondrial oxidative capacity was expressed as state 3, state u and state 4 respiration as described in the following paragraph. The O_2 consumption (or flux) is given by the slope of the O_2 concentration (nmol/ml), expressed as pmol/s and corrected for the wet weight of the fibers (pmol/mg*s). All substrates were added in excess to avoid a substrate limitation. FCCP (cyanide p-(trifluoro-methoxy)phenyl-hydrazone) was titrated until the maximal response was reached. Two different substrate titration protocols, in which O_2 consumption equilibration was reached after the addition of every substrate, were applied.

The first protocol was initiated by the addition of 5 µl malate (0.8 mol), 10 µl glutamate (2.0 mol) and 10 µl ADP (0.5 mol) to reach state 3 respiration, on complex I of the ETC. State 3 is defined as the state of maximal mitochondrial respiration coupled to oxidative phosphorylation. Then, 20 µl succinate (1.0 mol) were added to reach state 3 respiration on complex I and II. State 4 is the constant state, which is reached after all added ADP is converted to ATP. Sequentially, 5 µl of cytochrome C (4.0 mmol) was

added as a validation control. The cytochrome oxidase, located in the mitochondrial intermembrane space, can reduce cytochrome C, which cannot cross membranes. A rise of the respiration rate under elevated external cytochrome C concentrations indicates damage to the outer mitochondrial membrane. To uncouple the mitochondrial oxidative phosphorylation from substrate oxidation, incremental 0.2 µl titration steps of FCCP (1.0 mmol) were performed until the maximal mitochondrial respiration, independent of the phosphorylation system, was reached (state u). The measurement was terminated when no further increase of the O_2 consumption could be detected upon additional FCCP titration.

In the second protocol, 5 µl of malate (0.8 mol) was added, followed by the addition of 10 µl of ADP (0.5 mol). Then, the instrument titration pump was used to titrate glutamate (2 mol) in 1 µl steps every 1.5 min in order to determine the enzyme kinetics of NADH dehydrogenase. After reaching the maximal respiration for complex I, 1 µl rotenone (0.1 mmol) was added for inhibition of complex I and a second titration with 2.0 mol succinate (2 µl steps every 2 min) was started to evaluate enzyme kinetics of succinate dehydrogenase. As for the first protocol, 5 µl of cytochrome C (4.0 mmol) were added. Finally, 2 µl oligomycin (4.0 mg/ml) were added as an inhibitor of ATP-synthase to evaluate the respiration due to 'leak' in the resting state, uncoupled from ATP production (state 4). Then the respiratory control ratio (RCR), i. e., the ratio of state 3 to state 4 respiration and the uncoupled control ratio (UCR), i. e., the ratio of state u to state 4 respiration were calculated.

D.3. Morphological analysis

D.3.1. *Electron microscopy*

EM images were made in the cellular morphology unit, at the German Diabetes Center by Kay Jeruschke and Esther Phielix. In brief, the biopsies were fixated and dehydrated directly after muscle biopsies were taken for later embedding in Epon to preserve morphology of mitochondria and lipid droplets. The membranes were negatively stained with osmium tetroxide using standard procedures. Unbiased microscopic imaging and analysis were ensured by randomization in a blinded fashion. On average, ~100 pictures were analyzed per participant.

D.3.2. *Picture analysis*

EM image analysis was performed with the Java-based, National Institutes of Health (NIH) program ImageJ. In the following paragraph, the quantification of mitochondrial size using Image J from EM micrographs will be described.

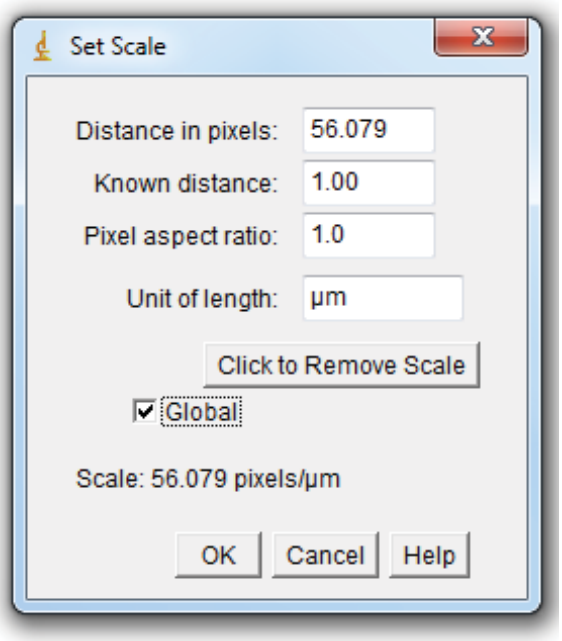

Figure 4. ImageJ settings for distance and area quantification

Distance in pixels and unit of length are supplied in order to adjust the image scale for subsequent analyses at a magnification of 2500 in this example.

The resolution of the charge-coupled device camera used for image acquisition was 2048 by 2048 pixels. The electro-optical magnification, which is reported for each picture is now used to calculate the actual number of pixels per µm of sample area. 2048 pixels were divided by the known length of the sample cut-out of this magnification in µm. This value was used to calibrate ImageJ as shown in Figure 4.

D.3.3. *Area quantification*

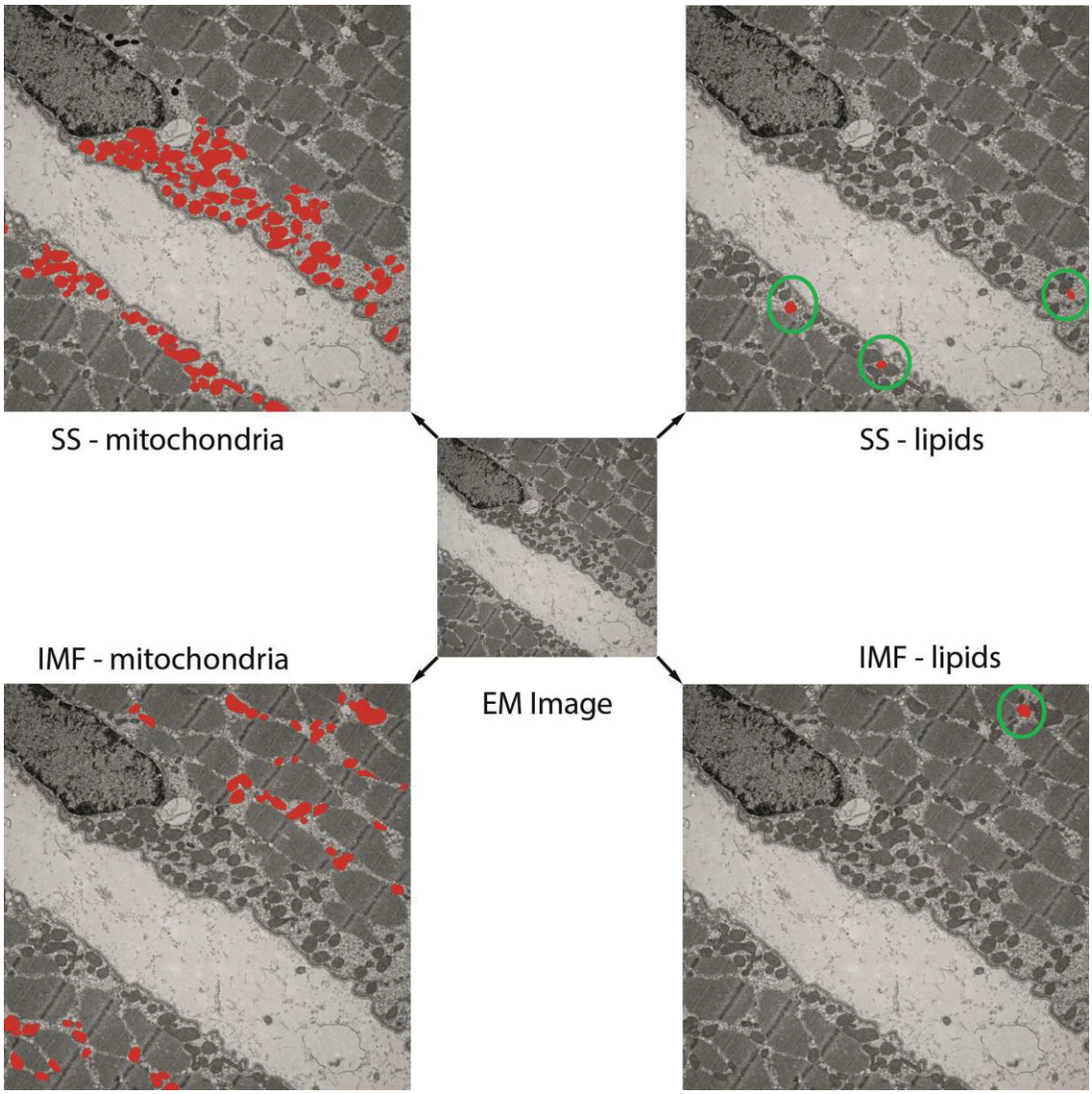

Figure 5. Histological distribution of mitochondria in muscle tissue

Respective mitochondria (left panels) or lipid droplets (right panels) are false-colored in red. Lipid droplets are marked by green circles. Magnification: 6300.

Using the ImageJ 'Freehand selections' tool, individual microscopic structures were encircled and the area calculated using the 'Measure and Fill' tool. Mitochondria and lipid droplets as shown in Figure 5 were analyzed both in the IMF and SS area. In addition, the total size of the IMF, SS and nuclear area was determined using the Polygon selection tool. Raw data in groups was transferred to Excel for further processing.

D.3.4. *Statistics*

Data are reported as means ± SEM. Differences between groups were analyzed by analysis of variance (ANOVA) using Tukey post hoc testing when appropriate. Correlations are calculated according to Spearman (non-parametric test) for calculating a linear relationship between parameters. Thirteen participants were analyzed and only pictures with magnifications greater than 5000 were included, amounting to 691 pictures in total (table 5).

Analyses of distinct mitochondrial and lipid populations were performed by grouping micrographs according to the presence of an SS area, and tested for changes upon lipid infusion.

Mitochondrial size was calculated as the surface area of all mitochondria divided by the number of mitochondria per picture. Content was calculated as IMF or SS area divided by the number of mitochondria per picture. Density was calculated as content multiplied by size. Outliers were defined as ± 1.5 times of the standard deviation and were excluded from the final analysis (2.1%). The one-sided paired T-test was performed when a deviation in one direction was assumed. A significance level of 0.05 was selected. All calculations were performed using Microsoft Office Excel 2007 and SPSS version 19.

E. Results

E.1. Participants characteristics

Thirteen healthy male and female humans fulfilled the inclusion criteria and underwent short-term intravenous lipid infusions for studying the above described parameters of mitochondrial morphology and function (Table 1). They were young, lean, normoglycemic and insulin sensitive at whole body level.

Table 1. *Participants characteristics*

Age (y)	30 ± 2
Body mass (kg)	73.3 ± 3.0
BMI (kg/m²)	23 ± 1
Fasting plasma glucose (mmol/l)	5.2 ± 0.7
Fasting plasma FFA (µmol/l)	426 ± 72
Fat free mass (kg)	56.8 ± 2.6
M-value (during lipid infusion) (µmol/kg*min) [mg/kg*min]	19.9 ± 4.2 [3.6 ± 0.8]
M-value per fat free mass (µmol/kg*min) [mg/kg*min]	25.7 ± 5.4 [4.6 ± 1]

Data are represented as mean ± SEM. BMI: Body Mass Index, FFA: free fatty acids, M-value: whole body glucose metabolism. Subgroup of probands as reported in study [81].

E.2. Mitochondrial respiration during lipid infusion

After 4 h lipid infusion, only RCR was decreased compared to baseline conditions, while all other parameters of mitochondrial respiration such as UCR, state 3 on glutamate, state 3 on succinate and state u were unchanged (Figure 6). A low RCR rate is indicative of reduced mitochondrial capacity and high proton leakage. As this value depends on both state 3 and state 4, it serves as a sensitive measure of ATP synthase coupled oxidative phosphorylation [91].

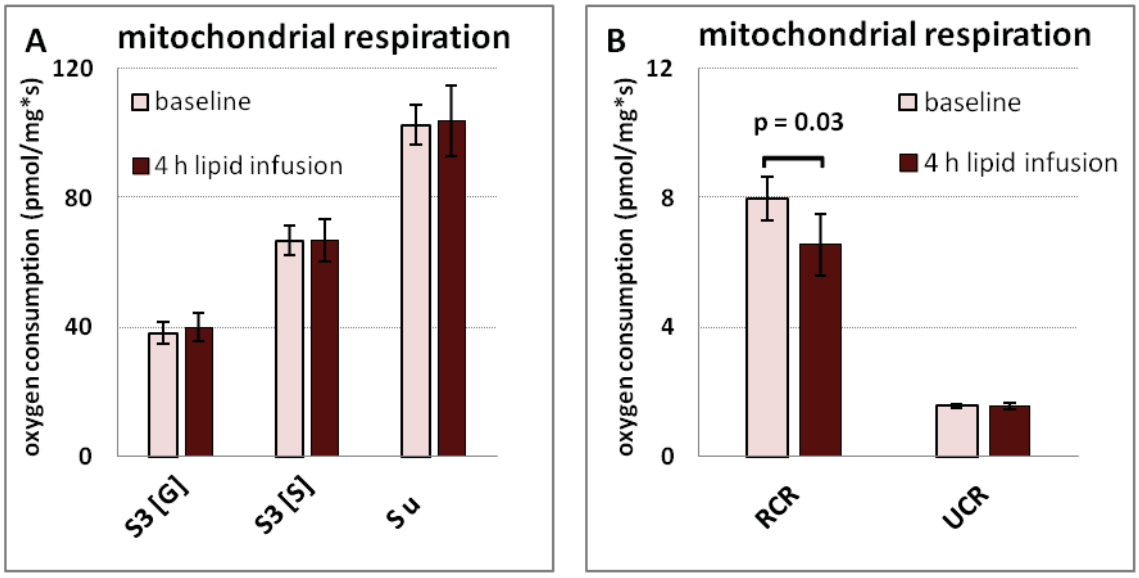

Figure 6. Mitochondrial respiration before and after a 4 h lipid infusion

Mitochondrial respiration measured before (baseline) and after 4 h lipid infusion. S3: state 3, [G]: glutamate, [S]: succinate, S u: uncoupled state. RCR: Respiratory Control Ratio, UCR: Uncoupled Control Ratio. Data are given as a mean ± SEM and only p values < 0.05 are indicated.

E.3. Subcellular changes during lipid infusion

Sections of skeletal muscle fibers were analyzed by EM and individual subcellular structures were evaluated by graphical analyses.

Table 2. *Mitochondrial morphology during lipid infusion*

			infusion duration [h]	baseline	lipid infusion	Δ	Δ %	p
IMF	mito	Size (µm²)	2.5	0.099 ± 0.005	0.113 ± 0.014	0.01	14%	0.41
		Content (#/µm²)	2.5	0.583 ± 0.037	0.603 ± 0.033	0.02	3%	0.68
		Density (%)	2.5	5.847 ± 0.589	6.895 ± 1.159	1.05	18%	0.40
	lipids	Size (µm²)	2.5	0.236 ± 0.020	0.242 ± 0.038	0.01	3%	0.87
		Content (#/µm²)	2.5	0.015 ± 0.002	0.022 ± 0.003	0.01	47%	**0.02**
		Density (%)	2.5	0.316 ± 0.053	0.491 ± 0.059	0.18	55%	0.05
SS	mito	Size (µm²)	2.5	0.133 ± 0.009	0.145 ± 0.015	0.01	9%	0.55
		Content (#/µm²)	2.5	3.205 ± 0.285	3.006 ± 0.285	-0.20	-6%	0.69
		Density (%)	2.5	41.295 ± 1.695	41.320 ± 1.929	0.02	0%	0.99
	lipids	Size (µm²)	2.5	0.215 ± 0.019	0.236 ± 0.036	0.02	10%	0.69
		Content (#/µm²)	2.5	0.080 ± 0.026	0.117 ± 0.028	0.04	46%	0.24
		Density (%)	2.5	1.828 ± 0.808	3.121 ± 1.253	1.29	71%	0.45
IMF	mito	Size (µm²)	4	0.098 ± 0.005	0.146 ± 0.040	0.05	49%	0.28
		Content (#/µm²)	4	0.585 ± 0.019	0.646 ± 0.076	0.06	10%	0.48
		Density (%)	4	5.773 ± 0.392	6.265 ± 0.457	0.49	9%	0.32
	lipids	Size (µm²)	4	0.234 ± 0.024	0.271 ± 0.029	0.04	16%	0.32
		Content (#/µm²)	4	0.009 ± 0.002	0.019 ± 0.004	0.01	111%	0.08
		Density (%)	4	0.418 ± 0.228	0.772 ± 0.260	0.35	85%	0.11
SS	mito	Size (µm²)	4	0.144 ± 0.011	0.258 ± 0.099	0.11	79%	0.28
		Content (#/µm²)	4	2.637 ± 0.307	2.591 ± 0.204	-0.05	-2%	0.91
		Density (%)	4	36.194 ± 2.439	39.151 ± 1.336	2.96	8%	0.17
	lipids	Size (µm²)	4	0.237 ± 0.032	0.268 ± 0.065	0.03	13%	0.52
		Content (#/µm²)	4	0.029 ± 0.008	0.066 ± 0.015	0.04	128%	**0.04**
		Density (%)	4	0.688 ± 0.221	1.903 ± 0.537	1.22	177%	**0.04**

Morphometric analysis of IMF (intermyofibrillar) and SS (subsarcolemmal) mitochondria (mito) and lipid droplets (lipids) before and after lipid infusion for the indicated periods. Data are represented as mean ± SEM.

After 2.5 h lipid infusion, IMF lipid droplet content was increased by 47% compared with baseline. In addition, SS lipid droplet content and total SS lipid density had increased by 128% and 177%, respectively, after 4 h of lipid infusion (Table 2). Overall, mitochondria in the SS area were about 47% larger than mitochondria in the IMF area (Table 2).

In addition, after both 2.5 h and 4 h lipid infusion, a trend towards larger mitochondrial size and density in the IMF and SS region was observed.

E.4. Distinct populations of IMF mitochondria

For this specific analysis, EM micrographs were categorized into two groups based on the presence of mitochondria of the IMF area. The first group included all micrographs where just an IMF area was included in the section. In the second group, only micrographs that included both SS and IMF regions were analyzed. IMF area morphological parameters were then compared between these subgroups.

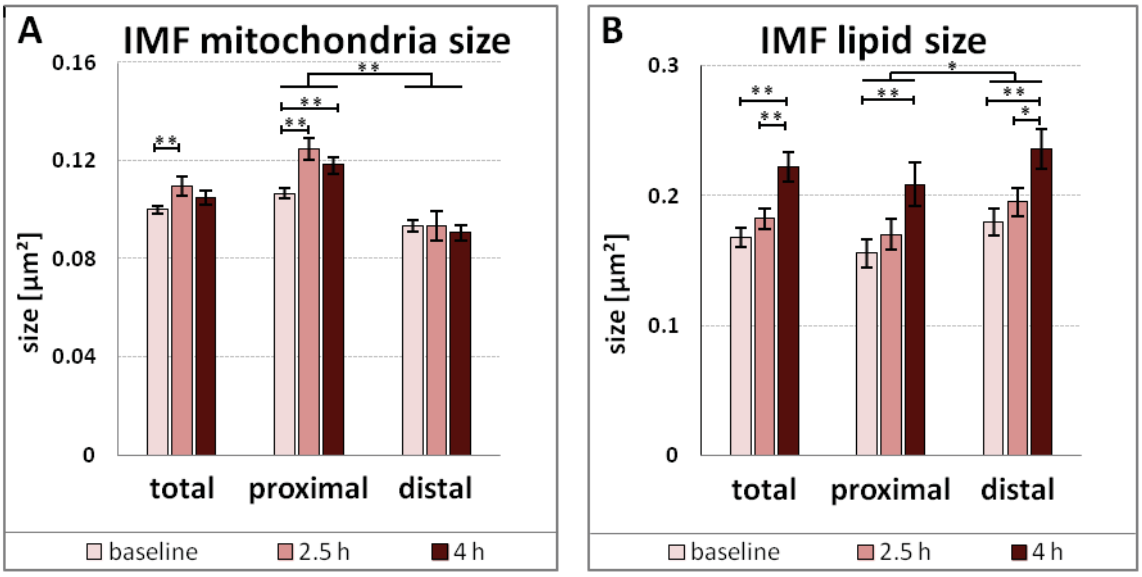

Figure 7. Morphology of subcellular structures during lipid infusion

Total: All micrographs ([baseline] n = 331, [2.5 h] n = 181, [4 h] n = 178). Proximal: Only micrographs where SS and IMF area were visible ([baseline] n = 165, [2.5 h] n = 94, [4 h] n = 91). Distal: Micrographs where only an IMF area was visible ([baseline] n = 166, [2.5 h] n = 87, [4 h] n = 87). Data are represented as mean ± SEM, * = $p < 0.05$, ** = $p < 0.01$.

The average IMF mitochondrial size in proximity to an SS area (proximal) was larger compared to IMF mitochondria more distant from the SS area. The mitochondrial size increase at 2.5 and 4 h vs. baseline as reported in the previous paragraph was significant when analyzing the subgroup of proximal mitochondria separately (Figure 7, Panel A). IMF lipid size was affected in both regions upon lipid treatment (Figure 7, Panel B).

E.5. Relationship between mitochondrial function and lipid content

To this end, correlations between mitochondrial respiration (expressed as RCR ratio), SS area lipid content and IMF area lipid content were calculated individually. At baseline as well as after 4 h lipid infusion, no significant correlations between RCR and SS lipid content (#/µm²) or IMF lipid content (#/µm²) were found (Figure 11, Appendix).

E.6. Correlation of mitochondrial morphology and function

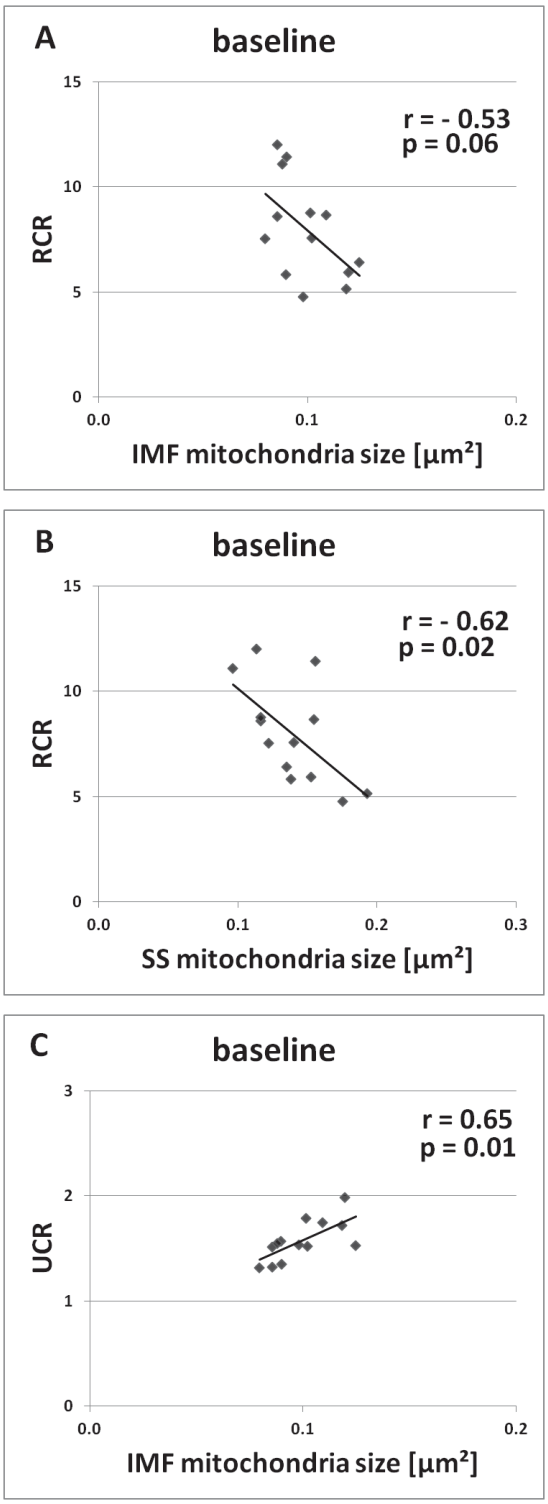

Figure 8. Morphological and functional correlations of mitochondrial parameters

Correlation of RCR (respiratory control ratio) and mitochondria size [µm²] at baseline (IMF area: A, SS area: B). Panel C shows the relationship between UCR (uncoupled control ratio) and mitochondrial size [µm²] in the IMF area. Coefficient of correlation represented as (r).

RCR correlated negatively and UCR positively with mitochondrial size at baseline in the IMF area (Figure 8, Panels A, C). The same effect was also seen in the SS area where a negative correlation was found between RCR and mitochondrial size (Figure 8, Panel C). After short-term lipid infusion, no correlations were observed.

E.7. Dependence of mitochondrial and lipid morphology

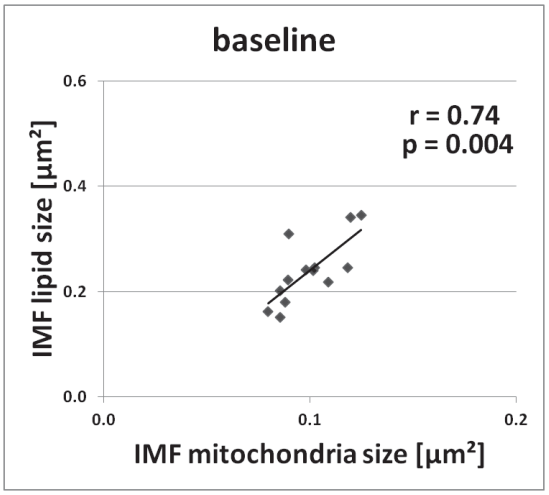

Figure 9. Morphological correlation of mitochondrial and lipid size

Correlation of mitochondrial and lipid droplet size (µm²) in the IMF area at baseline. Coefficient of correlation represented as (r).

At baseline, lipid droplet size correlated positively with mitochondrial size (Figure 9). Strikingly, at 2.5 h and 4 h, no correlations of these parameters were detected.

G. Discussion

This study tested the effects of short-term lipid elevation in young and lean participants on mitochondrial morphology and function.
Our morphological assessment of subcellular organelle distributions showed:

- an increase of lipid droplet content in the SS and IMF area
- a positive correlation between lipid droplet and mitochondrial size before lipid infusion
- the presence of distinct sub-populations of IMF area mitochondria and lipid droplets dependent on their distance to the SS area

On a functional level, we found:

- mitochondrial respiration (RCR) decreased upon short-term lipid infusion
- mitochondrial function correlated negatively with mitochondrial size, which indicated a higher efficiency of smaller mitochondria

Mitochondrial size, content and density did not decrease upon lipid infusion within the experimental observation time.

Elevation of circulating FFA leads to subcellular lipid accumulation

Skeletal muscle cells are believed to store increased circulating FFA in the form of lipid droplets [92]. This study used a novel preparation procedure for EM micrographs, which preserved lipid droplets. Using EM, the present study clearly demonstrated increased storage of lipid droplets in different sub-cellular compartments. To our knowledge, this is the first time such quantification was performed on a morphological level. In obese individuals [93], and upon 4 h lipid elevation [94], increased levels of IMCL have been demonstrated using ^{1}H nuclear magnetic resonance spectroscopy, and a positive correlation to insulin resistance was shown. The present study reports a rise in IMF lipid droplet content as early as 2.5 h after commencement of lipid elevation. This might imply a higher sensitivity of a morphological quantification method over ^{1}H MRI imaging. It is important to note that the onset of insulin resistance is only detectable after a delay of 3-4 h [94, 95] which indicates that the effect on insulin sensitivity is

indirect. It was speculated that the processing of FFA into the cellular storage form of TAG and their deposition in lipid droplets may explain this delay [89].

Functionally, the formation of lipid droplets involves the expression of perilipin (Plin) family proteins [96-99], which in turn form the outer layer of the lipid droplet. The formation of lipid droplets has been extensively studied in the context of liver disease, where inactivating mutations of adipocyte-Plin1 result in steatosis and lipodystrophy, along with insulin resistance [99, 100]. Lipid storage in the form of TAG requires cellular enzymes which condense the FFA to glycerol. In humans, this reaction is catalyzed by the DGAT1 enzyme. Inactivation of this enzyme promotes oleic acid-induced cell death in cultured fibroblasts, whereas over-expression augmented this effect [101], which points toward involvement of the formation of TAG in lipid detoxification. This notion is further corroborated by the observation that athletes have high amounts of skeletal muscle lipid droplets albeit no insulin resistance [102, 103], which might be explained by a higher expression of the DGAT1 enzyme [97, 104]. However, transport and signaling processes might be affected by the elevated lipid content observed in this study, resulting in a reduced RCR rate but not affecting other functional parameters. Taken together, this supports the hypothesis that deposition of surplus lipids into lipid droplets may serve as a detoxification mechanism.

Importantly, the increase in lipid droplet content and density was more pronounced in the SS area. Marked compositional differences between SS and IMF mitochondria were reported [76], indicating divergent involvements in cellular processes. Our results indicated that not only mitochondria, but also lipid storages in the IMF and SS area differ in their functional role. This is supported by our finding that the increase in lipid content was significantly higher in the SS area compared to the IMF area, which might imply that this region is responsible for immediate reactions to changes in the nutritional states.

Effect of elevated circulating FFA on mitochondrial morphology

Previous studies assessed the effect of lipid infusions on mitochondrial gene expression as a marker for mitochondrial density. It was reported that prolonged (48 h), but not short-term (6 h) exposure to circulating FFA lowers the expression of genes of oxidative phosphorylation [105, 106], as does a high fat diet [107]. Mitochondrial density as

assessed by quantification of mitochondrial proteins after a 6 h lipid infusion also reported no differences [106].

In the present study, short-term lipid infusion for 4 h did not induce immediate changes in mitochondrial size, content and density as quantified by EM, consistent with previous observations. Of note, in T2DM there is a plethora of contradictory results generated using different methodologies. By EM, skeletal muscle mitochondria in T2DM were reported to be reduced in size [65] and number [78]. In addition, a reduction in mitochondrial DNA copy number was reported [108]. Other studies report no alterations in mitochondrial DNA copy number [59, 109], mitochondrial content as represented by citrate synthase activity in isolated mitochondria [110, 111] or mitochondrial density as represented by mitochondrial protein levels [112] in T2DM. It can be speculated that those inconsistencies could be attributed to different methodologies used. As shown in this report, and found by another study [76], SS and IMF mitochondria react differently to lipotoxic stress, and the different methodologies used in the aforementioned studies might put different emphases on SS vs. IMF mitochondria, resulting in conflicting outcomes.

Effect of an increase in lipid droplets on mitochondrial respiration

Previously, it has been shown that intravenous lipid infusions do impair substrate phosphorylation in peripheral muscle tissue [27] although mitochondrial content remains unchanged [106]. In our experiments, the lipid infusion over a short period of time resulted in a decrease in the RCR, expressed as state 3/state 4 respiration. Although RCR became affected upon lipid exposure, neither absolute values for state 3 respiration on glutamate and succinate nor state u were affected. A previous short-term lipid infusion study using hyperinsulinemic-euglycemic clamps also found no changes in absolute respiratory values [91]. A recent report compared the effects of substrate overload through transient elevation of FFA by lipid infusion in lean athletes, lean sedentary and obese sedentary subjects. Here, the transient lipid elevation reduced state 3 and increased state 4 respiration as well as RCR, consistent with our finding of a reduced RCR [113].

Unidirectional ATP flux was found to be lower upon lipid infusion [79], which could have been reflected here by a lowered RCR, and therefore implied a negative effect on

mitochondrial respiratory capacity. After a 3 h lipid infusion, overall mitochondrial ATP synthesis was not affected, but whole-body insulin-stimulated glucose uptake was reduced [80]. Taken together, these data suggest that lipid elevations may mildly impair mitochondrial respiration with a delay of 3-4 h. It might be speculated that this happens through uncoupling of the mitochondrial membrane to increase substrate flux [113-115], or as a result of diminished substrate supply through limited glucose transport and utilization. This in turn limits ATP synthesis in states of insulin stimulated energy expense [62]. Consistent with this hypothesis, transient reduction of circulating FFA by use of acipimox did not result in increased insulin sensitivity [116].

At baseline, no correlation between lipid droplet content and RCR was observed, showing that lipid droplet content *per se* did not influence mitochondrial function in this group. It should however been noted, that only young, lean, healthy humans were included, a group with generally low amounts of IMCL when compared to obese insulin-resistant humans [117]. Therefore, it cannot be concluded whether a correlation between mitochondrial function and lipid droplet content exists when insulin resistant groups with T2DM would be included. Our study was not sufficiently powered to derive meaningful correlations, which could have indicated if lipotoxicity affects mitochondrial function directly. Also, no methodology was applied to assess previously discussed lipotoxic pathways, involving ROS production or lipid intermediates [48-51, 118]. Recently, Phielix et al. [102] found higher lipid content in muscle cells obtained from endurance-trained athletes paralleled by higher oxidative capacity, when compared to untrained participants [103]. In the present study, participants were sedentary, performing physical exercise less than two times per week. This might account for the fact that although IMCL content was increased upon lipid infusion, no profound effects on respiration were observed. It has therefore been speculated that lipid metabolites, rather than IMCL play a pivotal role in peripheral insulin resistance [119, 120]. Our results are in strong support of this hypothesis.

Subcellular differences in mitochondrial function

Interestingly, the present study shows that mitochondria in the SS area were larger than in the IMF area, which might have been a consequence of their different involvements in energy homeostasis and signaling processes. As discussed before, conflicting results

about effects on mitochondrial density in T2DM patients exist. On a functional level, SS mitochondria participate mostly in regulatory processes such as signal transduction and ion- as well as substrate homeostasis [121]. Therefore, lipotoxic effects may underlie some pathological aspects observed in T2DM patients.

Increased lipid exposure of skeletal muscle correlates negatively with insulin stimulated glucose disposal [122]. This might underlie the lower energy demand of muscle in T2DM. In our system, this was recapitulated by the lower RCR of the treated muscle fibers. In a study where SS and IMF mitochondria were separated by a differential centrifugation procedure, only the underrepresented SS mitochondria [78] were affected in T2DM patients [78]. Our results showed that RCR decreases upon 4 h of lipid infusion, which could point toward lower mitochondrial function, although absolute respiratory parameters (state 3 on glutamate and succinate; state u) remained unaffected. This raises the question which subtypes of mitochondria (SS or IMF area) contribute to what extent to the cumulative signal. Indeed, mitochondria in the SS area were bigger in size than those in the IMF area. Therefore, it can be speculated that mostly SS mitochondria were affected; however, the experimental design of the present study does not allow distinguishing between SS and IMF mitochondria since activity is measured in unfractionated muscle fibers.

Existence of distinct IMF mitochondrial populations?

Our EM micrographs contained either SS and IMF areas or an IMF area alone. Upon categorizing the morphological data, it became apparent that IMF mitochondria proximal to an SS area were larger than those located more distal. This might implicate different functional roles of IMF mitochondria based on their environment, as seen for SS and IMF mitochondria in general [76].

Lipid droplet distributions were also analyzed and no differences between regions were observed. As EM pictures are two dimensional sections, this categorization is not rigorous and for subsequent studies, standardized criteria for EM micrograph categorization should be set out.

Relationship between mitochondrial size and lipid droplets

A positive correlation between lipid droplet size and mitochondrial size in the IMF area at baseline before lipid infusion (Figure 10) was observed. This indicated a well-ordered link between intramuscular lipid storage in the form of lipid droplets and the size of surrounding mitochondria. After 4 h lipid infusion, this correlation disappeared, indicating that the interplay between lipid droplets and mitochondria has been substantially impaired by the net increase in lipid accumulates in the cells.

Correlation of mitochondrial size with function

Although no significant differences in mitochondrial size before and after lipid infusion were observed, the variance in mitochondrial size did correlate with functional parameters, especially RCR. It was shown that mitochondrial size correlates positively with insulin sensitivity [65]. Here, these findings were extended by showing that mitochondrial size correlated negatively with RCR and positively with UCR (uncoupled control ratio, the ratio of state u to state 3 respiration), indicating a prominent functional impairment as discussed later.

Previously, increased respiration rates were shown to correlate with mitochondrial matrix volume in rats [123, 124]. In the SS area, and to a lesser extent also in the IMF area, we cannot recapitulate these findings. A possible explanation might be that mitochondrial matrix volume is not linearly related to mitochondrial size.

Negative correlations at baseline between mitochondrial size and RCR were observed, which implicated a lower overall respiratory capacity of bigger mitochondria. A high UCR reflected a lower efficiency of the respiratory process and correlated positively with mitochondrial size at baseline. Together, this may imply that smaller mitochondria were more efficient in substrate oxidation (Figure 10).

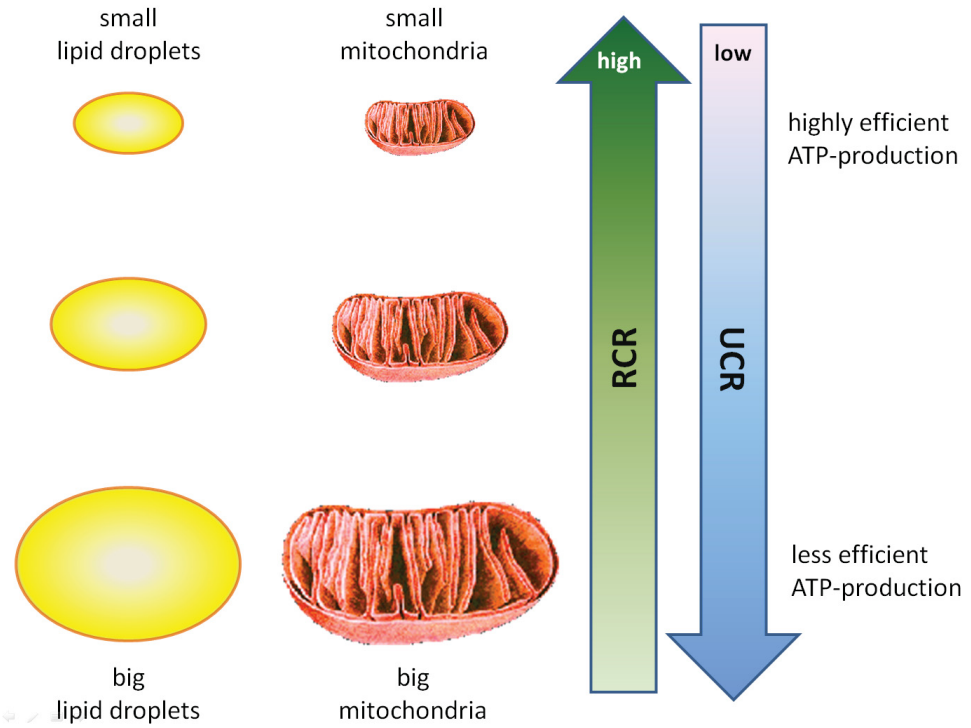

Figure 10. Mitochondrial size correlates with functional parameters at baseline

Further studies should be directed to elucidate mitochondrial plasticity more intensively under altered metabolic circumstances, like excess availability of different lipid species, exercise or cold exposure. To this end, it can be speculated that the reduced overall respiratory capacity is a physiological response to protect mitochondria [72], however a correlation with markers of lipid breakdown and insulin sensitivity would be required to corroborate this phenomenon. There is also a need for further studies on the relationship between markers of lipid break down products and insulin sensitivity [125].

H. Conclusion

The novelty of this study resides in examining the effect of acute lipotoxicity on muscle energy metabolism by the combined analysis of features of mitochondrial function using high resolution respirometry and morphological assessment of mitochondrial and lipid droplet size, content and density by EM.

- Short-term lipid infusion directly resulted in an increase in muscular lipid content, in particular in the regulatory SS area, which emphasized the dynamic nature of this compartment
- Lipid elevation indeed affected mitochondrial respiration, but effects were weak and the only affected parameter was the RCR rate
- Mitochondrial size was related to mitochondrial function and smaller mitochondria in healthy individuals were clearly more efficient

The lack of a correlation between lipid droplet count and mitochondrial RCR rate supports the concept that intracellular metabolic pathways and probably lipid breakdown products rather than lipids themselves negatively affect mitochondrial respiration.

J. Appendix

J.1. Chemicals, reagents and instruments

Table 3. *Chemicals*

Item	Vendor
Actarapid	Novo Nordisk, Denmark
ADP (adenosine 5`diphosphate potassium salt)	Sigma-Aldrich, St. Louis, USA
BSA (bovine serum albumin fraction V)	Sigma-Aldrich, St. Louis, USA
Cytochrome C from horse heart	Sigma-Aldrich, St. Louis, USA
DTT (dithiothreitol)	Sigma-Aldrich, St. Louis, USA
EGTA (ethylene glycol tetraacetic acid)	Sigma-Aldrich, St. Louis, USA
Ethanol, absolute, analysis-grade	Merck, Darmstadt, Germany
FCCP (carbonyl cyanide 4-(trifluoro-methoxy) phenylhydrazone	Sigma-Aldrich, St. Louis, USA
Heparin	Leo Pharma, Denmark
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich, St. Louis, USA
Imidazole	Fluka, St. Louis, USA
Intralipid	Fresenius Kabi, Germany
KH_2PO_4	Merck, Darmstadt, Germany
K-lactobionate	Fluka, St. Louis, USA
KOH	Sigma-Aldrich, St. Louis, USA
L-Glutamic acid monosodium salt hydrate	Sigma-Aldrich, St. Louis, USA
L-Malic acid	Sigma-Aldrich, St. Louis, USA
MES (2-(N-morpholino)ethanesulfonic acid)	Sigma-Aldrich, St. Louis, USA
$MgCl_2*6H_2O$	Schalau, Barcelona, Spain
Na_2ATP (adenosine 5`-triphosphate disodium salt)	Sigma-Aldrich, St. Louis, USA
Na_2 Phosphocreatine	Sigma-Aldrich, St. Louis, USA
NaCl	Invitrogen, Carlsbad, USA
Oligomycin	Sigma-Aldrich, St. Louis, USA
Rotenone	Sigma-Aldrich, St. Louis, USA
Saponine	Sigma-Aldrich, St. Louis, USA
Sodium pyruvat	Sigma-Aldrich, St. Louis, USA

Sodium succinate dibasic hexahydrate	Sigma-Aldrich, St. Louis, USA
Sucrose	Roth, Karlsruhe, Germany

Table 4. *Laboratory instrumentation and equipment*

Item	**Vendor**
Reaction tubes	Sarstedt, Nuembrecht, Germany
Forceps	A. Dumont & Fils, Montignez, Switzerland
Injection pump – TIP 2k	Oroboros Instruments, Austria
Oxygraph-2k	Oroboros Instruments, Austria
Pair of scissors	KLS Martin Gruppe, Tuttlingen, Germany
Parafilm	Pechiney Plastic Packaging, Menasha, USA
Petri dishes	Greiner, Austria
Pipette + tips	Eppendorf, Germany
Syringes	Hamilton, Switzerland
Weighing balance - XP205 Delta Range	Mettler Toledo, USA

Magnification	**n**	**%**
6.150	35	5,1
6.300	590	85,4
8.000	48	6,9
10.000	18	2,6
Total	***691***	***100***

Table 5. *EM micrograph distribution per magnification*

Participant ID	n	%
1	39	5,6
2	64	9,3
4	60	8,7
5	53	7,7
6	38	5,5
7	60	8,7
8	54	7,8
9	64	9,3
10	35	5,1
11	25	3,6
12	57	8,2
13	57	8,2
14	85	12,3
Total	***691***	***100,0***

Table 6. *EM micrograph distribution per participant*

Time of biopsies	n	%
Baseline, before lipid infusion	331	47,9
After 2.5 h lipid infusion	181	26,2
After 4 h lipid infusion	179	25,9
Total	***691***	***100,0***

Table 7. *EM micrograph distribution per time*

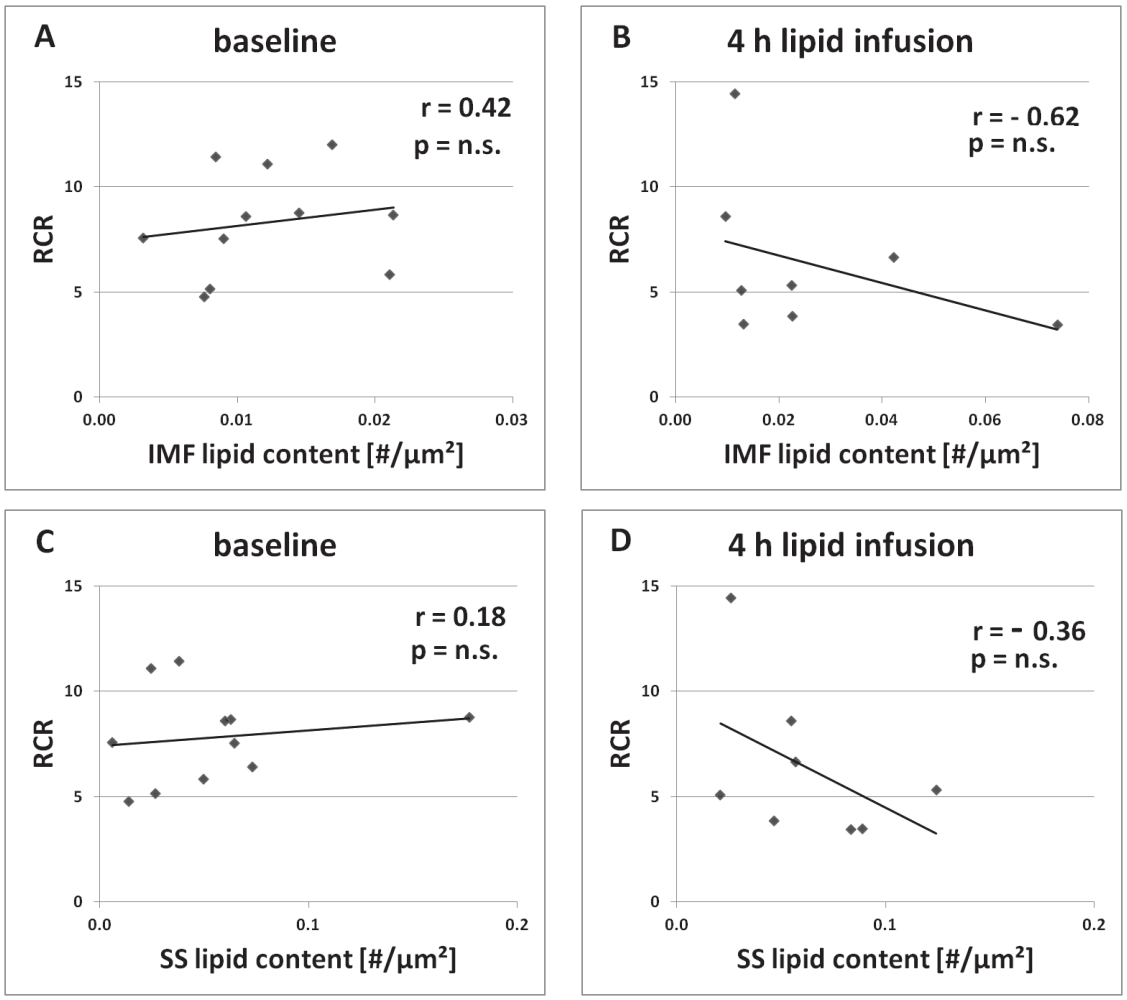

Figure 11. Correlations of morphological and functional parameters

Correlation of RCR (respiratory control ratio) and lipid content (#/µm²) at baseline (IMF area: A, SS area: C) and after 4 h lipid infusion (IMF area: B, SS area: D). Coefficient of correlation represented as (r), n.s. = non significant ($p > 0.05$).

K. Bibliography

1. Atkinson, M.A. and G.S. Eisenbarth, *Type 1 diabetes: new perspectives on disease pathogenesis and treatment.* Lancet, 2001. **358**(9277): p. 221-9.
2. Stumvoll, M., B.J. Goldstein, and T.W. van Haeften, *Type 2 diabetes: principles of pathogenesis and therapy.* Lancet, 2005. **365**(9467): p. 1333-46.
3. Jeong, I.K. and G.L. King, *New perspectives on diabetic vascular complications: the loss of endogenous protective factors induced by hyperglycemia.* Diabetes Metab J, 2011. **35**(1): p. 8-11.
4. Colagiuri, R., *Diabetes: a pandemic, a development issue or both?* Expert Rev Cardiovasc Ther, 2010. **8**(3): p. 305-9.
5. Federation, I.D., *World Diabetes Media Kit*. 2007, International Diabetes Federation, Brussels, Belgium
6. Prevention., C.f.D.C.a., *National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States.* Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, 2011.
7. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-9.
8. Reaven, G.M. and Y.D. Chen, *Role of insulin in regulation of lipoprotein metabolism in diabetes.* Diabetes Metab Rev, 1988. **4**(7): p. 639-52.
9. Szendroedi, J., E. Phielix, and M. Roden, *The role of mitochondria in insulin resistance and type 2 diabetes mellitus.* Nat Rev Endocrinol, 2012. **8**(2): p. 92-103.
10. Lewis, G.F., et al., *Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes.* Endocr Rev, 2002. **23**(2): p. 201-29.
11. Roden, M., *Muscle triglycerides and mitochondrial function: possible mechanisms for the development of type 2 diabetes.* Int J Obes (Lond), 2005. **29 Suppl 2**: p. S111-5.
12. Knowler, W.C., et al., *Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin.* N Engl J Med, 2002. **346**(6): p. 393-403.
13. Scott, L.J., et al., *A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants.* Science, 2007. **316**(5829): p. 1341-5.
14. Sladek, R., et al., *A genome-wide association study identifies novel risk loci for type 2 diabetes.* Nature, 2007. **445**(7130): p. 881-5.

15. Khaodhiar, L., S. Cummings, and C.M. Apovian, *Treating diabetes and prediabetes by focusing on obesity management.* Curr Diab Rep, 2009. **9**(5): p. 348-54.

16. Roglic, G., et al., *The burden of mortality attributable to diabetes: realistic estimates for the year 2000.* Diabetes Care, 2005. **28**(9): p. 2130-5.

17. Yach, D., D. Stuckler, and K.D. Brownell, *Epidemiologic and economic consequences of the global epidemics of obesity and diabetes.* Nat Med, 2006. **12**(1): p. 62-6.

18. Szendrodi, J. and M. Roden, *[The adipose tissue as an endocrine organ]*. Acta Med Austriaca, 2004. **31**(4): p. 98-111.

19. Lumeng, C.N. and A.R. Saltiel, *Inflammatory links between obesity and metabolic disease.* J Clin Invest, 2011. **121**(6): p. 2111-7.

20. Saltiel, A.R. and C.R. Kahn, *Insulin signalling and the regulation of glucose and lipid metabolism.* Nature, 2001. **414**(6865): p. 799-806.

21. Kahn, S.E., R.L. Hull, and K.M. Utzschneider, *Mechanisms linking obesity to insulin resistance and type 2 diabetes.* Nature, 2006. **444**(7121): p. 840-6.

22. Rains, J.L. and S.K. Jain, *Oxidative stress, insulin signaling, and diabetes.* Free Radic Biol Med, 2011. **50**(5): p. 567-75.

23. Fery, F., *Role of hepatic glucose production and glucose uptake in the pathogenesis of fasting hyperglycemia in type 2 diabetes: normalization of glucose kinetics by short-term fasting.* J Clin Endocrinol Metab, 1994. **78**(3): p. 536-42.

24. Lam, T.K., G. van de Werve, and A. Giacca, *Free fatty acids increase basal hepatic glucose production and induce hepatic insulin resistance at different sites.* Am J Physiol Endocrinol Metab, 2003. **284**(2): p. E281-90.

25. Roden, M., *The liver in focus.* Diabetologia, 2016. **59**(6): p. 1095-7.

26. Baldeweg, S.E., et al., *Insulin resistance, lipid and fatty acid concentrations in 867 healthy Europeans. European Group for the Study of Insulin Resistance (EGIR).* Eur J Clin Invest, 2000. **30**(1): p. 45-52.

27. Roden, M., et al., *Rapid impairment of skeletal muscle glucose transport/phosphorylation by free fatty acids in humans.* Diabetes, 1999. **48**(2): p. 358-64.

28. Krebs, M., et al., *Free fatty acids inhibit the glucose-stimulated increase of intramuscular glucose-6-phosphate concentration in humans.* J Clin Endocrinol Metab, 2001. **86**(5): p. 2153-60.

29. Turner, N., et al., *Excess lipid availability increases mitochondrial fatty acid oxidative capacity in muscle: evidence against a role for reduced fatty acid*

oxidation in lipid-induced insulin resistance in rodents. Diabetes, 2007. **56**(8): p. 2085-92.

30. Bonnard, C., et al., *Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice.* J Clin Invest, 2008. **118**(2): p. 789-800.

31. Simoneau, J.A. and D.E. Kelley, *Altered glycolytic and oxidative capacities of skeletal muscle contribute to insulin resistance in NIDDM.* J Appl Physiol, 1997. **83**(1): p. 166-71.

32. Petersen, K.F., et al., *Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes.* N Engl J Med, 2004. **350**(7): p. 664-71.

33. Knebel, B., et al., *Specific Metabolic Profiles and Their Relationship to Insulin Resistance in Recent-Onset Type 1 and Type 2 Diabetes.* J Clin Endocrinol Metab, 2016. **101**(5): p. 2130-40.

34. Lowell, B.B. and G.I. Shulman, *Mitochondrial dysfunction and type 2 diabetes.* Science, 2005. **307**(5708): p. 384-7.

35. Jucker, B.M., et al., *13C and 31P NMR studies on the effects of increased plasma free fatty acids on intramuscular glucose metabolism in the awake rat.* J Biol Chem, 1997. **272**(16): p. 10464-73.

36. Shulman, G.I., et al., *Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy.* N Engl J Med, 1990. **322**(4): p. 223-8.

37. Unger, R.H., *Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.* Diabetes, 1995. **44**(8): p. 863-70.

38. Unger, R.H., *Lipotoxic diseases.* Annu Rev Med, 2002. **53**: p. 319-36.

39. Dagenais, G.R., R.G. Tancredi, and K.L. Zierler, *Free fatty acid oxidation by forearm muscle at rest, and evidence for an intramuscular lipid pool in the human forearm.* J Clin Invest, 1976. **58**(2): p. 421-31.

40. Randle, P.J., et al., *The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus.* Lancet, 1963. **1**(7285): p. 785-9.

41. Cline, G.W., et al., *Impaired glucose transport as a cause of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes.* N Engl J Med, 1999. **341**(4): p. 240-6.

42. Dresner, A., et al., *Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity.* J Clin Invest, 1999. **103**(2): p. 253-9.

43. Griffin, M.E., et al., *Free fatty acid-induced insulin resistance is associated with*

activation of protein kinase C theta and alterations in the insulin signaling cascade. Diabetes, 1999. **48**(6): p. 1270-4.

44. Yu, C., et al., *Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle.* J Biol Chem, 2002. **277**(52): p. 50230-6.

45. Goodpaster, B.H., et al., *Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5755-61.

46. Pruchnic, R., et al., *Exercise training increases intramyocellular lipid and oxidative capacity in older adults.* Am J Physiol Endocrinol Metab, 2004. **287**(5): p. E857-62.

47. Dube, J.J., et al., *Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited.* Am J Physiol Endocrinol Metab, 2008. **294**(5): p. E882-8.

48. Schmitz-Peiffer, C., *Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply.* Cell Signal, 2000. **12**(9-10): p. 583-94.

49. Itani, S.I., et al., *Lipid-induced insulin resistance in human muscle is associated with changes in diacylglycerol, protein kinase C, and IkappaB-alpha.* Diabetes, 2002. **51**(7): p. 2005-11.

50. Cooney, G.J., et al., *Muscle long-chain acyl CoA esters and insulin resistance.* Ann N Y Acad Sci, 2002. **967**: p. 196-207.

51. Summers, S.A., *Ceramides in insulin resistance and lipotoxicity.* Prog Lipid Res, 2006. **45**(1): p. 42-72.

52. Obanda, D.N. and W.T. Cefalu, *Modulation of cellular insulin signaling and PTP1B effects by lipid metabolites in skeletal muscle cells.* J Nutr Biochem, 2013. **24**(8): p. 1529-37.

53. van de Weijer, T., V.B. Schrauwen-Hinderling, and P. Schrauwen, *Lipotoxicity in type 2 diabetic cardiomyopathy.* Cardiovasc Res, 2011. **92**(1): p. 10-8.

54. Kohlgruber, A. and L. Lynch, *Adipose Tissue Inflammation in the Pathogenesis of Type 2 Diabetes.* Curr Diab Rep, 2015. **15**(11): p. 92.

55. Shoelson, S.E., J. Lee, and A.B. Goldfine, *Inflammation and insulin resistance.* J Clin Invest, 2006. **116**(7): p. 1793-801.

56. Lee, J.Y., et al., *Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4.* J Biol Chem, 2001. **276**(20): p. 16683-9.

57. Kohchi, C., et al., *ROS and innate immunity.* Anticancer Res, 2009. **29**(3): p. 817-21.

58. Morino, K., et al., *Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents.* J Clin Invest, 2005. **115**(12): p. 3587-93.

59. Phielix, E., et al., *Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals.* Diabetologia, 2010. **53**(8): p. 1714-21.

60. Brands, M., A.J. Verhoeven, and M.J. Serlie, *Role of mitochondrial function in insulin resistance.* Adv Exp Med Biol, 2012. **942**: p. 215-34.

61. Phielix, E., et al., *Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients.* Diabetes, 2008. **57**(11): p. 2943-9.

62. Koliaki, C. and M. Roden, *Alterations of Mitochondrial Function and Insulin Sensitivity in Human Obesity and Diabetes Mellitus.* Annu Rev Nutr, 2016.

63. Ballard, J.W. and M.C. Whitlock, *The incomplete natural history of mitochondria.* Mol Ecol, 2004. **13**(4): p. 729-44.

64. Benard, G. and R. Rossignol, *Ultrastructure of the mitochondrion and its bearing on function and bioenergetics.* Antioxid Redox Signal, 2008. **10**(8): p. 1313-42.

65. Kelley, D.E., et al., *Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes.* Diabetes, 2002. **51**(10): p. 2944-50.

66. Vonck, J. and E. Schafer, *Supramolecular organization of protein complexes in the mitochondrial inner membrane.* Biochim Biophys Acta, 2009. **1793**(1): p. 117-24.

67. Starkov, A.A., *The role of mitochondria in reactive oxygen species metabolism and signaling.* Ann N Y Acad Sci, 2008. **1147**: p. 37-52.

68. Chance, B. and G.R. Williams, *The respiratory chain and oxidative phosphorylation.* Adv Enzymol Relat Subj Biochem, 1956. **17**: p. 65-134.

69. Saraste, M., *Oxidative phosphorylation at the fin de siecle.* Science, 1999. **283**(5407): p. 1488-93.

70. Patti, M.E. and S. Corvera, *The role of mitochondria in the pathogenesis of type 2 diabetes.* Endocr Rev, 2010. **31**(3): p. 364-95.

71. Harper, J.A., K. Dickinson, and M.D. Brand, *Mitochondrial uncoupling as a target for drug development for the treatment of obesity.* Obes Rev, 2001. **2**(4): p. 255-65.

72. Schrauwen, P., J. Hoeks, and M.K. Hesselink, *Putative function and physiological relevance of the mitochondrial uncoupling protein-3: involvement in fatty acid metabolism?* Prog Lipid Res, 2006. **45**(1): p. 17-41.

73. McBride, H.M., M. Neuspiel, and S. Wasiak, *Mitochondria: more than just a powerhouse.* Curr Biol, 2006. **16**(14): p. R551-60.

74. Cortassa, S., et al., *Control and regulation of integrated mitochondrial function in metabolic and transport networks.* Int J Mol Sci, 2009. **10**(4): p. 1500-13.

75. Gouspillou, G., et al., *Alteration of mitochondrial oxidative phosphorylation in aged skeletal muscle involves modification of adenine nucleotide translocator.* Biochim Biophys Acta, 2010. **1797**(2): p. 143-51.

76. Cogswell, A.M., R.J. Stevens, and D.A. Hood, *Properties of skeletal muscle mitochondria isolated from subsarcolemmal and intermyofibrillar regions.* Am J Physiol, 1993. **264**(2 Pt 1): p. C383-9.

77. Hood, D.A., *Invited Review: contractile activity-induced mitochondrial biogenesis in skeletal muscle.* J Appl Physiol, 2001. **90**(3): p. 1137-57.

78. Ritov, V.B., et al., *Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes.* Diabetes, 2005. **54**(1): p. 8-14.

79. Brehm, A., et al., *Increased lipid availability impairs insulin-stimulated ATP synthesis in human skeletal muscle.* Diabetes, 2006. **55**(1): p. 136-40.

80. Brehm, A., et al., *Acute elevation of plasma lipids does not affect ATP synthesis in human skeletal muscle.* Am J Physiol Endocrinol Metab, 2010. **299**(1): p. E33-8.

81. Szendroedi, J., et al., *Role of diacylglycerol activation of PKCtheta in lipid-induced muscle insulin resistance in humans.* Proc Natl Acad Sci U S A, 2014. **111**(26): p. 9597-602.

82. Phielix, E., et al., *Effects of pioglitazone versus glimepiride exposure on hepatocellular fat content in type 2 diabetes.* Diabetes Obes Metab, 2013. **15**(10): p. 915-22.

83. Nowotny, B., et al., *Mechanisms underlying the onset of oral lipid-induced skeletal muscle insulin resistance in humans.* Diabetes, 2013. **62**(7): p. 2240-8.

84. DeFronzo, R.A., J.D. Tobin, and R. Andres, *Glucose clamp technique: a method for quantifying insulin secretion and resistance.* Am J Physiol, 1979. **237**(3): p. E214-23.

85. Ferrannini, E. and A. Mari, *How to measure insulin sensitivity.* J Hypertens, 1998. **16**(7): p. 895-906.

86. Roden, M.D., *Clinical diabetes research : methods and techniques*. 2007, Chichester: John Wiley.

87. Bergstrom, J., et al., *Diet, muscle glycogen and physical performance.* Acta Physiol Scand, 1967. **71**(2): p. 140-50.

88. Gnaiger, E., et al., *Mitochondrial respiration in the low oxygen environment of the cell. Effect of ADP on oxygen kinetics.* Biochim Biophys Acta, 1998. **1365**(1-2): p. 249-54.

89. Saks, V.A., et al., *Permeabilized cell and skinned fiber techniques in studies of mitochondrial function in vivo.* Mol Cell Biochem, 1998. **184**(1-2): p. 81-100.

90. Veksler, V.I., et al., *Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers.* Biochim Biophys Acta, 1987. **892**(2): p. 191-6.

91. Brands, M., et al., *Short-term increase of plasma free fatty acids does not interfere with intrinsic mitochondrial function in healthy young men.* Metabolism, 2011. **60**(10): p. 1398-405.

92. Walther, T.C. and R.V. Farese, Jr., *Lipid droplets and cellular lipid metabolism.* Annu Rev Biochem, 2012. **81**: p. 687-714.

93. Sinha, R., et al., *Assessment of skeletal muscle triglyceride content by (1)H nuclear magnetic resonance spectroscopy in lean and obese adolescents: relationships to insulin sensitivity, total body fat, and central adiposity.* Diabetes, 2002. **51**(4): p. 1022-7.

94. Boden, G., et al., *Effects of acute changes of plasma free fatty acids on intramyocellular fat content and insulin resistance in healthy subjects.* Diabetes, 2001. **50**(7): p. 1612-7.

95. Roden, M., et al., *Mechanism of free fatty acid-induced insulin resistance in humans.* J Clin Invest, 1996. **97**(12): p. 2859-65.

96. Aon, M.A., N. Bhatt, and S.C. Cortassa, *Mitochondrial and cellular mechanisms for managing lipid excess.* Front Physiol, 2014. **5**: p. 282.

97. Greenberg, A.S., et al., *The role of lipid droplets in metabolic disease in rodents and humans.* J Clin Invest, 2011. **121**(6): p. 2102-10.

98. Kimmel, A.R. and C. Sztalryd, *Perilipin 5, a lipid droplet protein adapted to mitochondrial energy utilization.* Curr Opin Lipidol, 2014. **25**(2): p. 110-7.

99. Szendroedi, J. and M. Roden, *Perilipin 5: From fatty liver to hepatic lipodystrophy?* Hepatology, 2014.

100. Gandotra, S., et al., *Perilipin deficiency and autosomal dominant partial lipodystrophy.* N Engl J Med, 2011. **364**(8): p. 740-8.

101. Listenberger, L.L., et al., *Triglyceride accumulation protects against fatty acid-induced lipotoxicity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3077-82.

102. Phielix, E., et al., *High oxidative capacity due to chronic exercise training attenuates lipid-induced insulin resistance.* Diabetes, 2012. **61**(10): p. 2472-8.

103. Sparks, L.M., et al., *ANT1-mediated fatty acid-induced uncoupling as a target for improving myocellular insulin sensitivity.* Diabetologia, 2016. **59**(5): p. 1030-9.

104. Ikeda, S., et al., *Up-regulation of SREBP-1c and lipogenic genes in skeletal muscles after exercise training.* Biochem Biophys Res Commun, 2002. **296**(2): p. 395-400.

105. Richardson, D.K., et al., *Lipid infusion decreases the expression of nuclear encoded mitochondrial genes and increases the expression of extracellular matrix genes in human skeletal muscle.* J Biol Chem, 2005. **280**(11): p. 10290-7.

106. Chavez, A.O., et al., *Effect of short-term free Fatty acids elevation on mitochondrial function in skeletal muscle of healthy individuals.* J Clin Endocrinol Metab, 2010. **95**(1): p. 422-9.

107. Sparks, L.M., et al., *A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle.* Diabetes, 2005. **54**(7): p. 1926-33.

108. Boushel, R., et al., *Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle.* Diabetologia, 2007. **50**(4): p. 790-6.

109. Asmann, Y.W., et al., *Skeletal muscle mitochondrial functions, mitochondrial DNA copy numbers, and gene transcript profiles in type 2 diabetic and nondiabetic subjects at equal levels of low or high insulin and euglycemia.* Diabetes, 2006. **55**(12): p. 3309-19.

110. Hey-Mogensen, M., et al., *Effect of physical training on mitochondrial respiration and reactive oxygen species release in skeletal muscle in patients with obesity and type 2 diabetes.* Diabetologia, 2010. **53**(9): p. 1976-85.

111. Mogensen, M., et al., *Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes.* Diabetes, 2007. **56**(6): p. 1592-9.

112. Meex, R.C., et al., *Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity.* Diabetes, 2010. **59**(3): p. 572-9.

113. Dube, J.J., et al., *Effects of acute lipid overload on skeletal muscle insulin resistance, metabolic flexibility, and mitochondrial performance.* Am J Physiol Endocrinol Metab, 2014. **307**(12): p. E1117-24.

114. Karakelides, H., et al., *Age, obesity, and sex effects on insulin sensitivity and skeletal muscle mitochondrial function.* Diabetes, 2010. **59**(1): p. 89-97.

115. Stump, C.S., et al., *Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthesis, and mRNA transcripts.* Proc Natl Acad Sci U

S A, 2003. **100**(13): p. 7996-8001.

116. Phielix, E., et al., *Reduction of non-esterified fatty acids improves insulin sensitivity and lowers oxidative stress, but fails to restore oxidative capacity in type 2 diabetes: a randomised clinical trial.* Diabetologia, 2014. **57**(3): p. 572-81.

117. Petersen, K.F., et al., *Reversal of muscle insulin resistance by weight reduction in young, lean, insulin-resistant offspring of parents with type 2 diabetes.* Proc Natl Acad Sci U S A, 2012. **109**(21): p. 8236-40.

118. Towler, D.A., *Mitochondrial ROS deficiency and diabetic complications: AMP[K]-lifying the adaptation to hyperglycemia.* J Clin Invest, 2013. **123**(11): p. 4573-6.

119. Chow, L., A. From, and E. Seaquist, *Skeletal muscle insulin resistance: the interplay of local lipid excess and mitochondrial dysfunction.* Metabolism, 2010. **59**(1): p. 70-85.

120. Samuel, V.T. and G.I. Shulman, *Mechanisms for insulin resistance: common threads and missing links.* Cell, 2012. **148**(5): p. 852-71.

121. Krssak, M., et al., *Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study.* Diabetologia, 1999. **42**(1): p. 113-6.

122. Sun, G., et al., *Skeletal muscle characteristics predict body fat gain in response to overfeeding in never-obese young men.* Metabolism, 2002. **51**(4): p. 451-6.

123. Halestrap, A.P., *The regulation of the matrix volume of mammalian mitochondria in vivo and in vitro and its role in the control of mitochondrial metabolism.* Biochim Biophys Acta, 1989. **973**(3): p. 355-82.

124. Lim, K.H., et al., *The effects of ischaemic preconditioning, diazoxide and 5-hydroxydecanoate on rat heart mitochondrial volume and respiration.* J Physiol, 2002. **545**(Pt 3): p. 961-74.

125. Martin, S.D. and S.L. McGee, *The role of mitochondria in the aetiology of insulin resistance and type 2 diabetes.* Biochim Biophys Acta, 2013.

L. Declaration

Herewith I declare that I prepared the Doctoral Thesis: **"Effects of circulating lipids on mitochondrial structure and function"** on my own and with no other sources and aids than quoted.

21.02.2017, Ulrike Schomburg

Ich versichere an Eides statt, dass die Dissertation **"Effects of circulating lipids on mitochondrial structure and function"** selbständig und ohne unzulässige fremde Hilfe erstellt und die hier vorgelegte Dissertation nicht von einer anderen medizinischen Fakultät abgelehnt worden ist.

21.02.2017, Ulrike Schomburg