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Determinants of insulin sensitivity in obesity and recent-onset type 2 diabetes mellitus

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Summary

Insulin resistance, a key feature of type 2 diabetes (T2D), has been associated with inherited and acquired abnormalities of skeletal muscle mitochondrial function. Increased availability of free fatty acids (FFA) is involved in insulin resistance, impairment of mitochondrial function and non-alcoholic fatty liver disease (NAFLD). However, it remains unclear, which factors influence the associations of insulin sensitivity with mitochondrial function in patients with recent-onset, well-controlled T2D and whether specific lipid metabolites contribute to insulin resistance and abnormal mitochondrial function and thereby promote NAFLD.

These questions were addressed by one study in 136 patients with metabolically well-controlled, recent-onset T2D, enrolled in the German Diabetes Study (GDS), and another study in 21 participants (7 lean controls and 14 obese with or without NAFLD) of the Bariatrix study. All volunteers underwent thorough metabolic phenotyping using gold standard methodology, including hyperinsulinemic-euglycemic clamps for measuring insulin sensitivity (M-value). GDS participants also underwent indirect calorimetry and spiroergometry to assess metabolic flexibility (ΔRQ) and maximal oxygen uptake (VO_2max), respectively, as measures of whole body energy metabolism. In the Bariatrix study, direct measurement of hepatic oxidative capacity and inflammation was combined with targeted lipidomics to quantify sphingolipid concentrations in various tissues.

The first study revealed that in recent-onset T2D, ΔRQ and VO_2max independently associate with the M-value, even upon various adjustments and that only fasting FFA could abolish these relationships. ΔRQ associated positively with FFA, whereas VO_2max was lower in the carriers of a polymorphism in the fat and obesity-related (*FTO*) gene and related negatively with C-reactive protein. The second study found that NAFLD patients feature higher total hepatic ceramide levels. Specific serum ceramide species correlated with peripheral insulin resistance. Particularly, hepatic ceramide 16:0 and lactosylceramides also correlated with increased oxidative stress and inflammation in the liver.

In conclusion, circulating FFA play a central role even in the early course of T2D, affecting muscle mitochondrial function and peripheral insulin sensitivity. In particular, increased serum and hepatic sphingolipid species seem to be important for the development of insulin resistance and hepatic inflammation and the progression of NAFLD.

Zusammenfassung

Die Insulinresistenz, ein Hauptmerkmal des Typ-2-Diabetes (T2D) ist mit vererbten und erworbenen Störungen der mitochondrialen Funktion des Skelettmuskels assoziiert. Die erhöhte Verfügbarkeit von freien Fettsäuren (FFS) bewirkt Insulinresistenz, beeinträchtigt die mitochondriale Funktion und begünstigt die Entstehung einer nicht-alkoholischen Fettleber (NAFLD). Derzeit ist dennoch unklar, welche Faktoren die Assoziation von Insulinsensitivität und mitochondrialer Funktion bei Patienten mit neu diagnostiziertem, metabolisch gut eingestelltem T2D beeinflussen und ob spezifische Lipidmetabolite zu Insulinresistenz, abnormer mitochondrialer Funktion und so zu NAFLD beitragen.
Diese Fragen wurden in einer Studie mit 136 neu-diagnostizierten, gut eingestellten Patienten mit T2D, Teilnehmern der Deutschen Diabetes Studie (GDS) und in einer weiteren Studie mit 21 Teilnehmern der Bariatrix-Studie (7 normalgewichtige und 14 übergewichtige Patienten mit und ohne NAFLD) untersucht. Alle Teilnehmer wurden einer ausführlichen metabolischen Phänotypisierung mittels Goldstandardmethoden, wie hyperinsulinämisch-euglykämischen Clamps zur Bestimmung der Insulinsensitivität (M-Wert) unterzogen. Bei den Teilnehmern der GDS Studie wurden zusätzlich eine indirekte Kalorimetrie und eine Spiroergometrie zur Messung der metabolischen Flexibilität (ΔRQ) und der maximalen Sauerstoffaufnahme (VO_2max) als Parameter des Energiestoffwechsels durchgeführt. In der Bariatrix-Studie wurde die direkte Messung der hepatischen oxidativen Kapazität und Inflammation mit „Targeted Lipidomics“ zur Bestimmung von Sphingolipiden in verschiedenen Geweben kombiniert.
Die erste Studie zeigte, dass ΔRQ und VO_2max, trotz verschiedener Adjustierungen, unabhängig voneinander mit dem M-Wert in neu-diagnostizierten T2D-Patienten assoziiert sind. Diese Korrelationen wurden nur nach Adjustierung für FFS aufgehoben. ΔRQ war mit FFS positiv assoziiert und VO_2max war bei den Trägern eines Polymorphismus im *FTO*-Gen erniedrigt und korrelierte negativ mit dem C-reaktiven Protein (CRP). Die zweite Studie ergab, dass Patienten mit NAFLD eine höhere hepatische Konzentration der Gesamt-Ceramide aufweist. Spezifische Serum-Ceramide sind mit der peripheren Insulinresistenz assoziiert. Im Besonderen korrelierten das hepatische Ceramid 16:0 und Lactosylceramide mit erhöhtem oxidativen Stress und Inflammation in der Leber.
Zusammenfassend spielen zirkulierende FFS auch in den Frühstadien der Krankheitsmanifestation des T2D eine Hauptrolle, in dem sie die mitochondriale Funktion des Skelettmuskels und der peripheren Insulinsensitivität beeinträchtigen. Spezifische Sphingolipide im Serum und in der Leber scheinen für die Insulinresistenz, hepatische Inflammation und die Progression der NAFLD von Bedeutung zu sein.

List of abbreviations

ADA: American Diabetes Association

OGTT: oral glucose tolerance test

HbA1c: hemoglobin A1c

MODY: maturity-onset diabetes of the young

EGP: endogenous glucose production

FFA: free fatty acids

IDF: International Diabetes Federation

IFG: impaired fasting glucose

IGT: impaired glucose tolerance

PCOS: polycystic ovary syndrome

TGs: triglycerides

HDL: high-density lipoprotein

LDL: low-density lipoprotein

NAFLD: non-alcoholic fatty liver disease

IRS: insulin receptor substrates

GLUT 2, 4: glucose transporter 2, 4

IMCL: intramyocellular lipid

PKC: protein kinase C

DAG: diacylglycerols

ATP: adenosine triphosphate

PCr: phosphocreatine

RQ: respiratory quotient

VO_2max: maximal oxygen uptake

SNP: single nucleotide polymorphism

AMP: adenosine monophosphate

TLR-4: toll-like receptor 4

ROS: reactive oxygen species

H_2O_2: hydrogen peroxide

GDS: German Diabetes Study

ECG: electrocardiogram

BW: body weight

FFM: fat free mass

FM: fat mass

REE: resting energy expenditure

LOX: lipid oxidation

GOX: glucose oxidation

POX: protein oxidation

Hs CRP: high-sensitive C-reactive protein

GSC: glucose space correction

FLI: fatty liver index

HIS: hepatic insulin sensitivity

TCA: tricarboxylic acid

CSA: citrate synthase activity

TBARS: thiobarbituric acid reactive substances

ANOVA: analysis of variance

WHR: waist-to-hip ratio

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1. Introduction

1.1 Type 2 diabetes mellitus

In the year 2015, 415 million people worldwide were suffering from diabetes according to International Diabetes Federation (IDF), with 59.8 million of cases in Europe. Of note, an estimated number of about 193 million people worldwide remain undiagnosed. In Germany, nearly 6.5 million patients were diagnosed with diabetes in the year 2015. The world prevalence of diabetes is estimated to rise more in the age group of 20-79 years up to 7.7% and 439 million patients by the year 2030 and reach up to 642 million people in the year 2040 [3]. Factors that seem to contribute to this rise in diabetes prevalence include population growth, ageing of population, urbanization and most importantly the increase in obesity and physical inactivity, as risk factors for the development of T2D. This comprises not only a social, but also economic burden, since rising prevalance is tightly associated with increased direct and indirect healthcare costs in Germany, with cardiovascular complications, diabetes duration and insulin therapy correlating positively with more increased costs [4].

1.1.1 Definition and diagnosis

Diabetes is a metabolic disease defined by chronic hyperglycemia. According to American Diabetes Association (ADA) guidelines there are four ways to diagnose diabetes mellitus, i.e using as diagnostic criteria: fasting plasma glucose, 2-hour plasma glucose in 75 g oral glucose tolerance test (OGTT), hemoglobin A1c (HbA1c) and random plasma glucose (Table 1) [5].

Table 1: Criteria for diagnosis of prediabetes and diabetes

Diagnostic criteria	Prediabetes	Diabetes mellitus
HbA1c*	5.7-6.4 %	≥ 6.5 %
OR		
Fasting plasma glucose**	100-125 mg/dl (5.6-6.9 mmol/l)	≥126 mg/dl (7.0 mmol/l)
OR		
Random plasma glucose	-	≥ 200 mg/dl (11.1 mmol/l) and classical symptoms of hyperglycemia
OR		
2-h plasma glucose during an OGTT***	140-199 mg/dl (7.8 – 11.0 mmol/l)	≥ 200 mg/dl (11.1 mmol/l)

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

****No caloric intake for at least 8 hours.**

***** The test should be performed using a glucose load equivalent of 75 g anhydrous glucose dissolved in water.**

Individuals that do not meet the criteria of diabetes diagnosis but are above the normal range are described as having intermediate hyperglycemia or prediabetes, consisting of two entities known as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT) [5].

The disease is classified in 4 subtypes:

1) Type 1 diabetes (T1D): Immune-mediated beta cell destruction with subsequent insulin deficiency.

2) Type 2 diabetes (T2D): The hallmark of type 2 diabetes is insulin resistance combined with relative insulin deficiency.

3) Specific diabetes types including genetic defects of β-cells (e.g MODY: maturity-onset diabetes of the young), genetic mutations of insulin receptors, drug-induced diabetes (e.g glucocorticoids), diseases of exocrine pancreas (e.g pancreatitis, cystic

fibrosis), virus-infection-induced diabetes, diabetes associated with Down or Turner syndrome [5, 6].

4) Gestational diabetes that is diagnosed during pregnancy and resolves after delivery [5, 6].

T1D and T2D comprise the major diabetes types. T1D accounts for 5-10% of all diabetes types and is the result of an immune-mediated β-cell destruction and is usually diagnosed in children, adolescents and young adults. It displays a subacute to acute disease manifestation, often with presence of ketoacidosis, reduced to absent insulin secretion and the presence of diabetes-associated autoantibodies in 90-95% of the cases at diagnosis [6].

T2D is characterized by a wide range of insulin resistance and insulin deficiency and comprises 90-95% of all diabetes cases. T2D is usually accompanied by obesity and physical inactivity and is commonly diagnosed in older ages than T1D and at more advanced stages [6]. The initial defect is insulin resistance with subsequent compensatory increase in pancreatic insulin secretion, which at some point declines and hyperglycemia prevails.

1.1.2 Risk factors for the development of T2D

Multiple factors have been associated with increased risk of T2D development. These can be mainly categorized into non-modifiable and modifiable. The most important non-modifiable risk factor is age, with prevalence of T2D rising with age up to 8th decade among men and women [7]. Furthermore, T2D has been shown to have a strong genetic basis with the mode of inheritance being more influenced by the maternal environment [8]. T2D is highly concordant in monozygotic twins and as child of a parent with T2D one has a 40% risk of developing the disease [7]. Ethnicity seems to also play a role in increasing the T2D development risk, with some ethnic groups being more prone to the disease, likely due to a genetic basis. Hispanics have an increased prevalence of the disease by 1.9 times compared to Caucasians [9]. Other ethnic groups, that show susceptibility are Afro-Caribbeans and Asian Indians [10]. Another risk factor for the development of the disease is the presence of gestational diabetes, which is defined as any degree of impaired glucose tolerance with onset or first recognition during

pregnancy [11]. It affects about 3-5% of all pregnancies and women with gestational diabetes in their history have a 7.5-fold increased risk for the development of T2D [11]. Women with polycystic ovary syndrome (PCOS) comprise 10% of women in reproductive age and most of them are characterized by increased insulin resistance and impaired β-cell function compared to age- and BMI-matched controls [12]. Around 30% of women with PCOS have an IGT and show accelerated progression to T2D, whereas 10% are already diagnosed with a T2D [12].

As to modifiable risk factors, the most important is overweight and obesity (BMI≥25 kg/m^2). It is estimated that 90% of T2D patients are obese, however only 20-25% of obese individuals develop a T2D. More important is the localization of obesity, with central obesity being tightly associated with insulin resistance, T2D and cardiovascular risk [13]. Waist circumference provides a measure for central adiposity with cut-off points ranging according to ethnicity. In this context, we should refer to the metabolic syndrome, as an important risk factor for cardiovascular events, T2D and all-cause mortality [14]. According to the new IDF definition, an individual suffers from the metabolic syndrome, if it suffers from central obesity (defined by waist circumference) and has 2 more of the following factors:

- Triglycerides (TGs) ≥150 mg /dl (1.7 mmol/l) or treatment for this lipid abnormality
- High-density lipoprotein cholesterol (HDL) < 40 mg/dl (1.03 mmol/l) in males
  or <50 mg/dl (1.29 mmol/l) in females,
  or treatment for this lipid abnormality
- Systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg, or treatment for previously diagnosed hypertension
- Dysglycemia defined as fasting blood glucose ≥ 100 mg/dl (5.6 mmol/l) or T2D.

According to these criteria, metabolic syndrome is not only a risk factor for the development of T2D, but may also be part of the disease. Obesity is the result of increased food intake and decreased physical activity and genetic predisposition can

also play an important role. As far as diet is concerned, foods rich in saturated fats, red and processed meat, as well as sugar-sweetened beverages are associated with increased diabetes risk, whereas a high intake of vegetables, coffee and fiber-rich foods decreases the risk of diabetes development and is associated with improved glycemic control in patients with established T2D [15, 16]. Furthermore, diets characterized by high glycemic index or high glycemic loads are associated with increased risk for T2D [17]. Higher levels of physical activity are associated with lower risk of T2D development. A program consisting of increased physical activity and moderate weight loss could decrease the risk of T2D development by 58% [18]. The effect of exercise on lowering diabetes risk is explained by beneficial acute and chronic effects on insulin action and skeletal muscle insulin sensitivity and can be achieved by either aerobic or resistance training interventions [19]. Increased physical activity also provides additional effects on circulating lipids, blood pressure and mortality [18]. Non-alcoholic fatty liver disease (NAFLD) is also in close association with obesity, and a risk factor for the metabolic syndrome, cardiovascular disease, T2D and its complications [20].

IFG and IGT are early abnormalities, also often referred to as prediabetes and comprise a further risk factor for the development of T2D [7]. These entities are estimated to be associated with a yearly conversion rate of 5-10% into T2D [21]. Lifestyle interventions and treatment with metformin have been shown to reduce the development of T2D in individuals with either or both entities, with lifestyle intervention consisting of weight loss and 150 minutes of physical exercise weekly being more effective than metformin treatment (risk reduction of 58% vs. 31%, respectively) [22].

Moreover, premature infants and low body weight at birth has been associated with decreased insulin sensitivity and risk for T2D later on in life [23]. A further modifiable risk factor for T2D is therapy with drugs, such as glucocorticoids, antihypertensives (β-blockers, thiazide diuretics), immunosuppressives, atypical antipsychotic agents and drugs used for HIV-infection [24]. Finally, psychosocial stress and depression are also associated with an increased risk of T2D development by up to 37% [25].

1.2 Skeletal muscle insulin resistance

1.2.1 Insulin signaling

The initial step of this cascade is the binding of insulin to its receptor leading to activation (phosphorylation) of tyrosine kinase of the insulin receptor and activation of phosphatidylinositol-4,5-bisphosphate 3-kinase-protein kinase B (PI3K-Akt). The insulin receptor consists of 2 extracellular α subunits, that bind insulin and 2 β subunits with tyrosine kinase activity. After insulin binds to the α subunit, β subunit is transphosphorylated, which results in kinase activation and subsequent activation of a number of intracellular substrates, such as the insulin receptor family (IRS1-4). IRS proteins, in turn, interact with the p85 regulatory subunit of PI-3 kinase, leading to activation of enzyme PI3K-Akt, which leads to translocation of glucose transport 4 (GLUT4) in the cellular membrane and increased glucose uptake (75). Activation of PI3 kinase also promotes glycogen, lipid and protein synthesis as well as control of hepatic gluconeogenesis, being mainly responsible for the metabolic effects of insulin (76). The Ras pathway on the other hand is responsible for activation of transcription factors and stimulation of growth promoting actions of insulin, promoting insulin´s mitogenic effects (77).

1.2.2 Definition of skeletal muscle insulin resistance

Impairment of insulin action includes diminished insulin responsiveness and insulin sensitivity. In vitro, decreased insulin responsiveness is defined by a lower maximal effect of insulin, whereas lower insulin sensitivity is defined by a higher insulin concentration eliciting the maximal response to insulin, i.e. a right shift of the dose response curve [26]. In vivo, the gold-standard method to assess insulin sensitivity is hyperinsulinemic-euglycemic clamp technique. When combined with isotope dilution techniques clamp can additionally provide information on tissue-specific insulin sensitivity in vivo [26].

Several mechanisms can induce insulin resistance by interfering with insulin signaling cascade, such as elevated circulating lipids, elevated FFA, hyperglycemia, oxidative stress, inflammation as well as genetic variations (Figure 1).

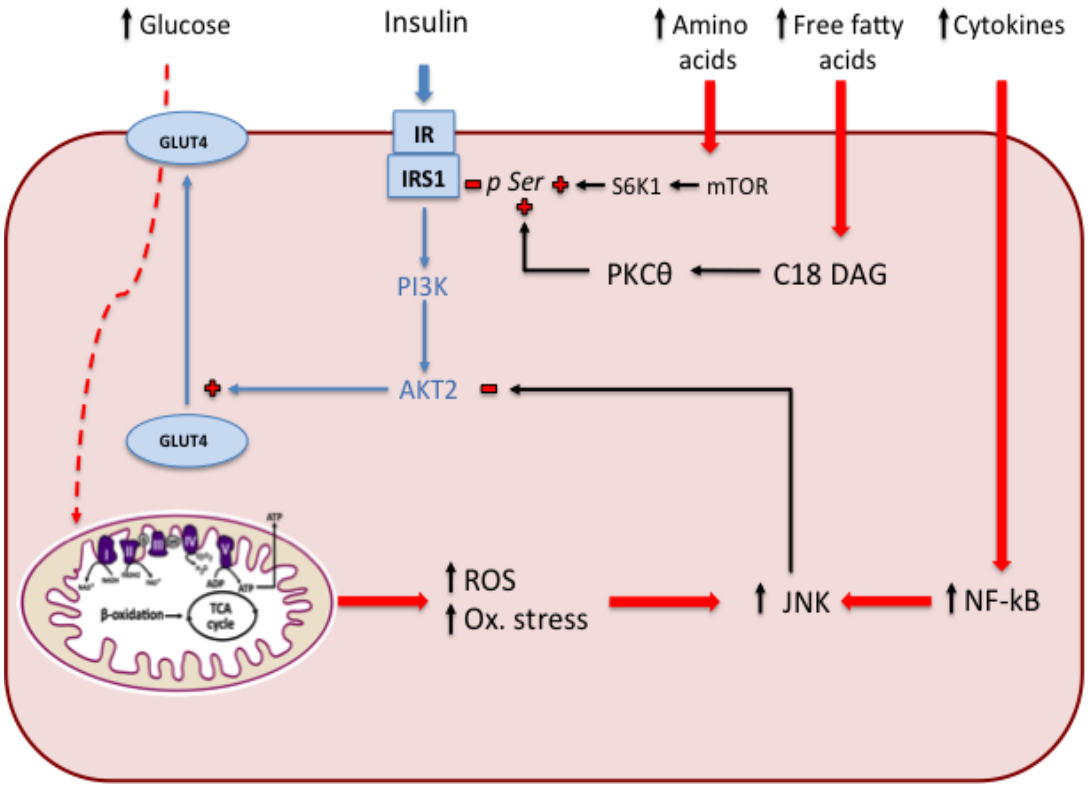

Figure 1. Mechanisms underlying insulin resistance in type 2 diabetes

A main proposed mechanism is the inhibition of insulin signaling, as a result of chronic hyperglycemia, increased plasma FFA and amino acids and inflammatory processes. JNK: c-Jun-N terminal kinase pathway, PKC: protein kinase C activity AGE: advanced glycation end products, FFA: free fatty acids, IRS-1: insulin receptor substrate 1 ROS: reactive oxygen species, DAG: diacylglycerol

Modified from Kaul, Apostolopoulou, Roden, 2015 [27].

1.2.3 Excess lipid availability

Increased energy intake, adipose tissue inflammation and impaired mitochondrial function have been linked to insulin resistance and ectopic fat accumulation.

Ectopic fat accumulation in skeletal muscle is increased among obese, sedentary patients with T2D and is already present at the insulin resistant state [28]. Moreover, intramyocellular lipid (IMCL) content correlates with the degree of insulin resistance in healthy non-obese humans [29]. Increasing plasma FFAs with lipid infusions in healthy volunteers was associated with increased IMCL content and associated with the observed insulin resistance, suggesting that fat accumulation in skeletal muscle is an early step in the development of insulin resistance [30].

According to the Randle hypothesis, increased fatty acid oxidation in cases of lipid oversupply lead through increases in acetylCoA/CoA and NADH/NAD+ ratios to inhibition of phosphofructokinase, increases in glucose-6-phosphate, inhibition of hexokinase and thus increased intracellular glucose and diminished glucose transport [31]. Non-invasive magnetic resonance spectroscopy studies have shown that muscle glycogen synthesis accounts for the majority of insulin-stimulated glucose uptake in insulin-sensitive persons. In insulin resistance and T2D, however, such studies showed lower increases in insulin-stimulated glucose-6-phosphate and diminished glycogen synthesis, indicating that abnormal glucose transport and phosphorylation activity are mainly responsible for insulin resistance in humans [32].

Studies applying lipid infusions in vivo showed that acute elevation of plasma FFA also results in decreased muscle glucose transport/phosphorylation and glycogen synthesis along with blunted IRS-1 tyrosine phosphorylation and increased PKC θ activity [33]. At six hours following an oral lipid challenge, skeletal muscle insulin resistance was found by reduced insulin-mediated non-oxidative glucose utilization along with increases in muscle C18:2 membrane DAG species and PKC θ activation [34]. A subsequent intravenous lipid challenge study also leading to skeletal muscle insulin resistance, reported sequential increases in diacylglycerol (DAG) species (especially C16:0, C18:0, C18:1, C18:2 and C20:4), followed by PKC θ activation and inhibitory serine phosphorylation of IRS-1 in healthy, lean individuals [35].

Of note, increased FFA have also been implicated in impairing mitochondrial function by promoting incomplete fatty acid oxidation and oxidative stress generation [36]. Insulin-stimulated ATP production was inhibited six [37], but not three hours [38] after an intravenous lipid challenge, although insulin resistance was already present after three hours, suggesting that mitochondrial function impairment appears as a result of insulin resistance. Furthermore, expression of mitochondrial genes appears decreased 48 hours after lipid infusions [39].

1.2.4 Skeletal muscle mitochondrial function

A major task of mitochondria is the production of ATP via oxidative phosphorylation within the electron transport chain of inner mitochondrial membrane, driven by a proton gradient. The role of mitochondria in insulin resistance and T2D has been addressed by several studies with various and often contradictory results. This could in part be caused by differences in terminology used for alterations in different mitochondrial features or content [40].

1.2.4.1 Definition of mitochondrial function

Mitochondrial activity can be defined as the resting oxidative phosphorylation flux in certain metabolic states, such as fasting, hyperinsulinemia or hyperlipidemia. It is defined as the flux through ATP synthase, given by the ADP:ATP ratio, and can be assessed in vivo, by measuring ATP saturation transfer using ^{31}P nuclear magnetic resonance spectroscopy [40].

Oxidative phosphorylation capacity reflects maximal ADP-stimulated oxidative phosphorylation under conditions of maximal energy demand with unlimited substrate and oxygen supply. It can be assessed ex vivo by measuring oxygen flux during high-resolution respirometry experiments in isolated mitochondria or in permeabilized tissue fibers [40].

Submaximal oxidative phosphorylation represents non-resting activity stimulated by energy demanding standardized procedures. This can be assessed in vivo either by

measuring local skeletal muscle phosphocreatine (PCr) recovery upon submaximal exercise using ^{31}P magnetic resonance spectroscopy or by measuring whole-body maximal oxygen uptake (VO_2max) using spiroergometry. Of note, oxidative capacity measured by these procedures is lower than that measured ex vivo in isolated mitochondria or permeabilised fibers, when ATP synthesis is measured with unlimited supply of substrates [40].

Mitochondrial plasticity is defined as the ability of the mitochondria to respond to altered metabolic conditions by changing their activity, number or oxidative phosphorylation capacity [40].

Mitochondrial coupling represents the molar ratio of the yield of ATP per oxygen consumption (P/O ratio) [40].

1.2.4.2 Mitochondrial function in insulin resistance and T2D

In several studies, mitochondrial function has been shown impaired in patients with insulin resistance and T2D. This could be due either to reduced mitochondrial content [41, 42] or to true intrinsic defects with impairment of oxidative capacity in skeletal muscle [43-45]. Studies applying in vivo measurements of mitochondrial function, such as PCr recovery, found lower submaximal ADP-stimulated oxidative phosphorylation in skeletal muscle [46, 47]. Basal mitochondrial function exhibited stronger associations with insulin sensitivity than IMCL in T2D patients than BMI-matched control persons [47]. Further studies examined skeletal muscle biopsies from T2D patients and controls and found reduced oxidative phosphorylation applying ex vivo respirometry measurements in the T2D group compared to healthy controls [44, 48]. Interestingly, this reduction remained after controlling for mitochondrial content and was further confirmed in isolated mitochondria in one study [45], thereby suggesting reduced capacity rather than mitochondrial content underlying the observed differences.

Some studies have confirmed the reduction in measures of mitochondrial content among patients with T2D, such as citrate synthase and complexes I and III of mitochondrial respiratory chain [41, 44, 49, 50]. On the other hand, when comparing with BMI-matched controls, some studies did not find such differences so that one could argue that the observed changes are a result of obesity, rather than insulin resistance [42, 43, 45].

Some of the observed differences could also result from the measures used as markers of mitochondrial content. Transmission electron microscopy is regarded as the gold-standard method for measuring mitochondrial fractional area [51]. A study that applied electron microscopy in obese, insulin resistant individuals and lean controls found a 40-% reduction in mitochondrial content in the obese group, and mitochondrial content associated with insulin sensitivity, lipid oxidation and metabolic flexibility [41]. Peroxisome proliferator-activated receptor-γ coactivator 1 (PGC-1) is a key component in the transcription of genes of oxidative phosphorylation and stimulates mitochondrial biogenesis. Its expression has been found to be reduced in patients with T2D and their relatives and was associated with insulin resistance [52, 53].

Oxidative phosphorylation was decreased by 40% among elderly individuals compared to young controls [54] and 30% lower in insulin-resistant offsprings of patients with T2D along with 60% lower glucose uptake than healthy insulin-sensitive controls [55], also matched for their physical activity, which provides evidence for a genetic component of the observed mitochondrial impairments.

Evidence of possible associations between mitochondrial function and insulin sensitivity was provided by lifestyle-intervention studies in patients with T2D. Interventions with exercise programs in T2D patients showed increase in mitochondrial content, as measured by citrate synthase and NADH oxidase activity [56-58], but also qualitative improvements, as measured by increased ADP-stimulated oxidative phosphorylation [46, 59].

A common approach in several clinical studies for measurement of mitochondrial plasticity and submaximal oxidative phosphorylation has been the measurement of metabolic flexibility under conditions of insulin stimulation and maximal oxygen uptake during exhaustive physical tests. Both methods can provide information on mitochondrial function in vivo in healthy individuals and patients with T2D.

1.2.4.3 Metabolic flexibility

Metabolic flexibility is defined as the ability of healthy individuals to switch from lipid to glucose oxidation in the postprandial, insulin-stimulated state [60].

A common method to assess metabolic flexibility is indirect calorimetry during the

fasted state and during the steady-state of hyperinsulinemic-euglycemic clamp. The measurement of respiratory quotients (RQ) results from assessment of inhaled O_2 and exhaled CO_2 rates. A low RQ (~0.7) is indicative of reliance on lipid oxidation, whereas a higher RQ (~1.0) shows reliance on carbohydrate oxidation [60]. Acute hyperinsulinemia causes short-term plasticity of skeletal muscle mitochondria [40], so that in lean healthy individuals skeletal muscle displays the ability to switch from reliance to lipid oxidation during fasting conditions to the suppression of lipolysis and augmented glucose uptake and subsequent oxidation during hyperinsulinemia.

Patients with insulin resistance and T2D have shown impaired capacity to oxidize fat during fasting, which is reflected by a higher RQ during the fasted state compared to lean, insulin sensitive persons. On the other hand, during insulin-stimulated conditions insulin resistant patients failed to increase their glucose oxidation and had increased rates of lipid oxidation resulting in a lower insulin-stimulated RQ compared to insulin sensitive individuals [60, 61].

At present it remains unclear whether this lower ΔRQ among patients with insulin resistance results from impaired mitochondrial function or rather from diminished glucose uptake due to insulin resistance. A study addressing this question showed that decreased ΔRQ among insulin resistant patients was no longer present after adjusting for glucose infusion rates [60]. On the other hand, lower ΔRQ could also represent insufficient insulin-mediated suppression of lipolysis in insulin resistant individuals with subsequent development of ectopic fat accumulation and insulin resistance [62]. Elevation of circulating FFA resulting from an imbalance between FFA uptake and oxidation or alternatively resulting from adipose tissue inflammation [62] gives rise to myocellular lipotoxins, which interfere with insulin signaling leading to impaired glucose uptake and phosphorylation. In line with this, Roden et al found impaired glucose uptake, glucose phosphorylation and muscle glycogen synthesis in the presence of increased circulating FFA [63].

Taken together, metabolic flexibility reflects the ability of the cell to synchronize fuel selection according to fuel availability, probably reflects mitochondrial function and could be impaired by elevation of circulating lipids. These associations remain unknown in patients with recent-onset T2D and could contribute to understanding the sequence of events leading to insulin resistance.

1.2.4.4 Maximal oxygen uptake

Muscle mitochondrial oxidative capacity reflects ADP-stimulated oxidative phosphorylation at submaximal energy demand during exercising and represents another important feature of mitochondrial function [40].

Measurement of VO_2max during exhaustive incremental exercise can provide an in vivo measure of cardiorespiratory fitness and reflect oxidative capacity. Patients with long-term diabetes have lower VO_2max than age- and BMI-matched healthy control individuals [64-66]. On the other hand, physical exercise can increase insulin sensitivity in patients with T2D [67]. Improvements in whole-body insulin sensitivity achieved by exercise training programs in long-term T2D also relate to restoration of in vivo mitochondrial function, increases in mitochondrial content and VO_2max (Table 2) [59, 68]. Differences in the mitochondrial response on exercise training have been attributed to different types of training protocol applied, gene polymorphisms but also use of medication, such as statins, metformin and β-blockers [69].

However, aerobic capacity, as expressed by VO_2max, is the result of functional capacities of all systems involved in supply, transportation and energetic transformation from the working muscle. A reduction in VO_2max during spiroergometry not only indicates impaired mitochondrial activity, but may also result from diminished substrate supply, calcium release and lactic acidosis during exercising [40]. Table 2 summarizes studies reporting VO_2max and ΔRQ differences among patients with T1D and T2D.

Table 2: Studies on maximal oxygen uptake (VO_2max) and metabolic flexibility (ΔRQ) in healthy individuals (CON), type 1 (T1D) and type 2 diabetes (T2D) patients.

Reference	Patient cohort	Other parameters measured	Findings on VO_2max/ ΔRQ
Haufe et al., Diabetes, 2010 [70]	138 overweight healthy individuals	Body composition, abdominal fat, IMCL, IHL (MRS), IS by OGTT	Correlation between VO_2max and IS explained by IHL
Vella et al., Metab Syndr Relat Disord, 2016 [71]	68 normal-weight women	Body composition IS by OGTT	VO_2max inverse correlations: HOMA-IR, fasting insulin, 2-h insulin
Röhling et al., Diab Care, 2017 [65]	163 T1D, 188 T2D, CON	HRV during clamp	↓VO_2max in T2D, HRV correlated with VO_2max in T1D
Gusso et al., Diabetologia, 2008 [66]	8 T2D, 12 T1D, 10 CON	Stroke volume, body composition	↓VO_2max in T2D and T1D
Demir, Jpn Heart J, 2001 [72]	275 T2D patients	Plasma lipids, HbA1c	HbA1c and hypercholesteremia affected VO_2max
Galgani et al., Diabetes, 2008 [60]	59 T2D 42 CON	Body composition IS by clamp	↓ΔRQ in T2D than CON / Difference abolished after adjustment for WGD
Van de Weijer, PLOS One, 2013 [64]	49 T2D, 54 CON	IMCL, PCr recovery	↓VO_2max and ΔRQ ↓ PCr recovery T2D WGD predicted ΔRQ
Meex et al., Diabetes, 2010 [59]	18 T2D, 20 CON 12-w exercise	PCr recovery IS by clamp IMCL	PCr recovery ↓ T2D ΔRQ, IS ↓ in T2D

IMCL: intramyocellular lipid content, IHL: intrahepatic lipids, MRS: magnetic resonance spectroscopy, IS: insulin sensitivity, OGTT: oral glucose tolerance test, HOMA-IR: homeostasis model assessment of insulin resistance, FFM: fat free mass, HRV: heart rate variability, HbA1c: glycosylated hemoglobin, WGD: whole-body glucose disposal, PCr: phosphocreatine, 12-w: 12-week

1.2.5 Single nucleotide polymorphisms affecting mitochondrial function and insulin sensitivity

Certain single nucleotide polymorphisms (SNPs) of genes have been variably associated with VO_2max, lipolysis and/or insulin sensitivity. These genes encode the peroxisome proliferator-activated receptor γ and δ (PPARγ, PPARδ) or the β-adrenergic receptor (ADRB2). PPAR γ-coactivator (PGC)-1α is a transcriptional coactivator of the *PPAR* gene family that regulates insulin sensitivity and enzymes involved in oxidative phosphorylation. PPARδ is a nuclear transcription factor that promotes expression of enzymes involved in fatty acid uptake and lipid oxidation as well as mitochondrial uncoupling [73]. Targeted expression of PPARδ in skeletal muscle can promote a skeletal muscle fiber switch towards type I oxidative fibers [73]. Increased expression of PPARδ is associated with increased fat oxidation, reduction of intramyocellular fat and attenuation of metabolic syndrome profiles [74]. Polymorphisms of *PPARδ* gene identified differential responses of insulin sensitivity and VO_2max after moderate intensity training [75].

PPARγ is mainly expressed in adipose tissue and regulates the development of fat cells and lipid storage [76]. A Pro12Ala polymorphism in the *PPARγ* gene has been associated with decreased risk of T2D and a more insulin-sensitive phenotype [77, 78]. This polymorphism was also associated with improvement in insulin sensitivity in response to exercise [79].

Futhermore, β-adrenergic receptors modulate blood flow, lipolysis in adipose tissue and insulin secretion, thus exerting local and systemic effects on obesity, insulin resistance and cardiorespiratory fitness [80]. A SNP in *ADRB2* gene was found to be associated with obesity and insulin resistance in obese postmenopausal females [80].

A SNP in *NADH dehydrogenase (ubiquinone) 1 β subcomplex 6* (*NDUFB6*) gene has also been differently associated with the response of insulin sensitivity and VO_2max to exercise training [81]. *NDUFB6* gene encodes a subunit of complex I, which is located in the mitochondrial inner membrane, required for electron transfer activity. Decreased expression of this gene is associated with insulin resistance and T2D [53, 82]. Carriers of the G/G allele of rs540467 SNP in *NDUFB6* gene increased their myocellular ATP

synthesis by 26% after a long-term training intervention [81], whereas G/A allele carriers resisted in increasing their flux through ATP synthase and insulin sensitivity after three bouts of exercise [83].

A further gene that gained attention during the last years is the *fat mass and obesity associated (FTO)* gene, which is associated with BMI and body fatness, however, its function remains unclear. On the one hand, its central distribution in the hypothalamus implies a role in the regulation of energy balance [84], other data however imply also a possible role in the regulation of lipolysis [85].

1.3 Hepatic insulin resistance and NAFLD

1.3.1 Definition of hepatic insulin resistance

Liver plays a central role in glucose homeostasis by storing excess glucose and producing it via hepatic gluconeogenesis and glycogenolysis in times of low glucose availability. In hepatocytes decreased insulin response leads to diminished hepatic glycogen synthesis and impaired suppression of gluconeogenesis, which results in increased endogenous glucose production (EGP). In terms of a dose-response curve of insulin action on EGP, a shift to the right defines hepatic insulin resistance [26].

Measurement of basal (fasting) and insulin-stimulated EGP during a hyperinsulinemic-euglycemic clamp using isotope dilution techniques with stable isotope-labelled glucose molecules are the gold-standard measures of hepatic insulin sensitivity. Hepatic insulin resistance indices that require basal EGP and fasting insulin concentrations are also used for assessment of hepatic insulin sensitivity [26, 86].

1.3.2 Insulin signaling and lipotoxins

It is known that triglyceride accumulation in skeletal muscle and in the liver is associated with insulin resistance. Lipidomic analyses have identified specific lipotoxins, especially DAGs and sphingolipids that may interfere with insulin signaling and cause hepatic and peripheral insulin resistance. DAGs function as second messenger signaling lipids. PKC θ and PKC ε activation by DAGs have been associated with insulin resistance in skeletal muscle and liver respectively. Sphingolipid metabolites on the other hand, such as ceramides serve functions such as cell recognition and signaling involved in cellular apoptosis, inflammation, proliferation and differentiation. Furthermore they are involved in PKC ζ activation, reduced Akt/PKB activation and insulin resistance.

1.3.2.1 Diacylglycerols

The role of DAGs in induction of skeletal muscle insulin resistance via PKC θ activation and serine phosphorylation of insulin receptor has been reported in numerous studies, as described in the respective chapter. Hepatic insulin resistance has been also associated with increased DAGs in livers of mice and rats on high-fat diet without changes in ceramide species [87]. In human liver some studies have shown that hepatic DAG accumulation is associated with hepatic insulin resistance, supporting the hypothesis that NAFLD-associated hepatic insulin resistance is the result of increased PKCε activation [88]. PKCε is highly abuntant in the liver and its activation is responsible for reductions in insulin-stimulated hepatic glycogen synthesis, increased hepatic glyconeogenesis through reduced activation of FOXO1 and as a result increased glucose release through glucose transporter-2 (GLUT2) and hyperglycemia [89]. The specific role of PKCε was examined in knock-out mice, which were protected by lipid-induced hepatic insulin resistance despite the presence of steatosis and was associated with direct interaction with the insulin receptor, reducing its kinase activity in vivo and

in vitro [90]. However, other studies failed to find associations between increased hepatic DAGs and hepatic insulin resistance [91, 92], postulating that the chain length of fatty acids, degree of saturation and compartmentalization of DAGs may be more important than total DAG per se.

1.3.2.2 Sphingolipids

In the last years, sphingolipids have been associated with peripheral and hepatic insulin resistance, increased oxidative stress and impaired mitochondrial function in rodent and human studies. In mammals ceramides are generated by at least 6 ceramide synthases (CerS) 1-6, that produce ceramide sets with dinstict chain lengths.

Figure 2 depicts pathways of ceramide formation in the cell:

1) de novo biosynthesis, a pathway localized in endoplasmic reticulum that starts with condensation of serine and palmitoyl-CoA and results in formation of 3-ketosphingonine, dihydrosphingosine and dihydroceramides and finally, after removal of two hydrogens to ceramide formation;

2) activation of inflammatory pathways triggered by recognition of saturated fatty acids, activation of toll-like- receptor 4 (TLR4) and an increase in ceramide biosynthetic enzymes;

3) breakdown of more complex sphingolipids by sphingomyelinases (also known as the salvage pathway);

4) disruption of the endoplasmic reticulum homeostasis [93].

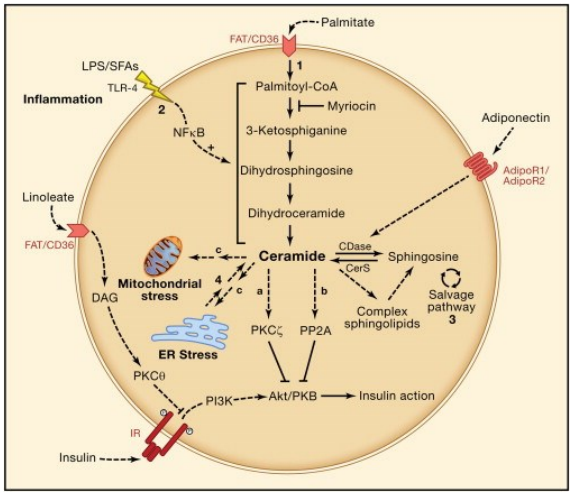

Figure 2: Pathways of cellular ceramide formation [93]

Ceramides are generated through: 1) de novo biosynthesis from palmitate, 2) activation of inflammation through recognition of saturated fatty acids, 3) breakdown of more complex sphingolipids (salvage pathway) and 4) endoplasmic reticulum (ER stress)

Induction of insulin resistance by ceramides has been associated with inhibitory effects in Akt/PKB translocation either through ceramide-mediated PKC ζ activation [94] or activation of a cytosolic phosphatase (PP2A) that dephosphorylates Akt/PKB [95]. Furthermore, ceramide is an obligate mediator of TLR4-induced insulin resistance in vivo and in vitro [96].

In rodent studies inhibition of ceramide synthesis prevents obesity-induced insulin resistance and the onset of diabetes [97]. In humans, studies on the relationship between peripheral insulin resistance and ceramide levels have shown contradictory results. In obese insulin resistant humans, increased ceramide levels in skeletal muscle have been reported, accompanied by reduced levels of activated Akt/PKB [98]. Lifestyle modification studies, such as exercise intervention, revealed a reduction in skeletal muscle ceramide content along with improved insulin sensitivity [99]. Other intervention studies have also found different effects between diet-induced weight loss and exercise intervention studies, with both interventions improving insulin sensitivity and reducing DAGs, but only exercise reducing ceramides [100], despite increased intramyocellular lipid content after exercise [100, 101]. On the other hand, some studies failed to find associations between skeletal muscle ceramides and insulin resistance

[102, 103]. In skeletal muscle, lipid-induced insulin resistance was associated with specific DAG-species but not with total ceramides [35].

Rodent studies using high-fat diet or in leptin-deficient ob/ob mice have shown controversial results as to the association between hepatic ceramide accumulation and hepatic insulin sensitivity [96, 104, 105]. Hepatic overexpression of ceramidases with subsequently increased ceramide degradation prevents from hepatic fat accumulation and leads to improvements in hepatic insulin action and adipose tissue upon high-fat diet [106]. Recent data support that insulin resistant humans display increased hepatic saturated TAGs, dihydroceramides and ceramides along with decreased serum adiponectin concentrations compared to NAFLD of different origins [107]. Both insulin resistance and NAFLD have been linked to abnormal mitochondrial function in people with obesity and T2D and ceramides seem to also have deleterious effects on mitochondrial function. Obese people with NAFLD feature increased hepatic mitochondrial function, which declines during progression to NASH, a situation, where reactive oxygen species (ROS) production and oxidative DNA damage prevail [108, 109]. Ceramides arising from lipid oversupply may also exert direct effects on mitochondrial transport chain with hydrogen peroxide (H_2O_2) emission [110], opening of mitochondrial permeability pore and cytochrome c release [111].

1.3.3 NAFLD

1.3.3.1 Definition and diagnosis of NAFLD

NAFLD is characterized by hepatic fat accumulation of more than 5.5% after exclusion of secondary causes in males consuming less than 30 g and females consuming less than 20 g alcohol per day and ranges from simple steatosis to non-alcoholic steatohepatitis, fibrosis, cirrhosis up to hepatocellular carcinoma [112, 113]. According to the EASL-EASD-EASO clinical practice guidelines for the management of NAFLD [114], abdominal ultrasound is the first-line cheap and non-invasive diagnostic tool in the clinical practice for detection of simple steatosis, although it only provides qualitative assessment, is subjective and has poor sensitivity for mild steatosis (<20% liver fat).

Quantitative assessment of liver steatosis can be achieved by ^{1}H-MRS, which is, however, generally applied only in clinical research studies because of it is costly and time consuming. Validated scores using also liver function tests include the fatty liver index (FLI), NAFLD liver fat score and SteatoTest, which can be used whenever imaging tools are not available. For NASH diagnosis, ultimately a liver biopsy is needed to confirm hepatocyte ballooning, lobular inflammation and fibrosis.

1.3.3.2 The relevance of NAFLD for T2D

NAFLD is closely associated with obesity and T2D, with its prevalence reaching up to 75% in obese populations [115, 116] and 50-75% in diabetes patients using ultrasound measurements to diagnose steatosis [114, 117].

NAFLD is also strongly associated with whole-body, hepatic and adipose tissue insulin resistance [118-120]. Increased fat accumulation in the liver can result from: (i) increased lipid dietary intake, (ii) increased FFA release due to suppressed lipolysis of adipose tissue and/or (iii) increased hepatic de novo lipogenesis. On a controlled diet with 30% fat intake approximately 60% of liver fat derived from circulating FFA from adipose tissue, underlining the importance of adipose tissue insulin resistance with subsequent impaired inhibition of lipolysis in the pathogenesis of NAFLD. Excess dietary fat intake not only has direct effects in the development of fatty liver but also results in obesity and whole-body insulin resistance, followed by diminished suppression of lipolysis, further increased FFA release in the circulation and induction of hepatic insulin resistance. Increased de novo lipogenesis can also be the result of insulin resistance, as hyperinsulinemia stimulates lipogenic enzymes through overexpression of sterol receptor binding protein 1-c (SREBP-1c) with subsequent development of hepatic steatosis [121].

Circulating hormones secreted by adipose tissue, such as adiponectin have been associated with decreased hepatic fat content and increased insulin sensitivity by stimulating fatty acid oxidation and glucose use in the liver through activation of AMP-activated protein kinase [122, 123]. This describes another mechanism, through which obesity can be linked to hepatic fat accumulation.

2. Aims and hypotheses

We tested the following hypotheses:

1. Impairment of whole-body energy metabolism is associated with whole-body insulin resistance in newly-diagnosed T2D and is influenced by lipidemia, glycemia and gene polymorphisms.
2. Specific sphingolipid metabolites are elevated in insulin resistant individuals and associate with NAFLD.

The first hypothesis was tested in a larger near-normoglycemic cohort (n=136) of T2D patients, participating in the German Diabetes Study (GDS) with known disease duration of less than 12 months, by examining associations of clinical parameters of mitochondrial function and insulin sensitivity with levels of lipidemia, inherited and acquired factors.
The second hypothesis was tested in a smaller obese, insulin resistant group (n=21) undergoing bariatric surgery, by assessing differences in specific sphingolipid metabolites that could interfere with peripheral and/or hepatic insulin sensitivity, ectopic fat accumulation and NAFLD.

3. Material and methods

3.1 German Diabetes Study (GDS)

3.1.1 Study design

GDS is an ongoing prospective observational study that examines patients within the first 12 months of diabetes diagnosis employing intensive metabolic phenotyping [124]. Patients aged 18-69 years are followed for at least 20 years at 5 year-intervals and including annual telephone interviews. Inclusion and exclusion criteria are summarized in Table 3. The main inclusion criterion is diagnosis of diabetes according to current ADA recommendations. Patients with type 3 (e. g. pancreoprive) or type 4 (gestational) diabetes are not included in the study. The aims of the study are: (i) to identify subphenotypes of diabetes mellitus, (ii) to find predictors of diabetes-associated comorbidities, (iii) development of individualized intervention strategies, (iv) to research the effect of known and novel risk factors on disease progression. The study is conducted according to the Declaration of Helsinki. Informed consent was obtained from all volunteers prior to inclusion into the trial (ClinicalTrial.gov registration no: NCT01055093), which was approved by the ethics board of Heinrich Heine University Düsseldorf (reference number 4508).

Table 3. Key inclusion and exclusion criteria of the GDS [124]

Inclusion criteria	Exclusion criteria
Diagnosis of T1D and T2D, maturity onset diabetes of the young (MODY) and latent autoimmune diabetes of the adult (LADA) Diabetes onset within the last 12 months T1D diagnosis based on ketoacidosis or immediate insulin requirement, presence of at least one islet cell directed autoantibody or C- peptide levels below detection limit Age of 18–69 years	Secondary diabetes (Type 3, e.g. pancreoprive) Gestational diabetes, pregnancy Poor glycemic control (HbA1c >9.0 %) Hyperlipidemia (triglycerides and low- density lipoproteins ≥double upper reference limit) Heart failure (New York Heart Association class ≥II) Renal disease (serum creatinine ≥1.6 mg/dL) Liver disease (aspartate aminotransferase and/or alanine aminotransferase and/or gamma glutamyltransferase ≥double upper reference limit) Peripheral artery occlusive disease IV Venous thromboembolic events Anemia or blood donation Participation in a clinical study within the past 3 months Acute infection, increased leukocytes, immunosuppressive therapy, autoimmune diseases, infection with human immunodeficiency virus, other severe diseases (e.g., active cancer disease) Psychiatric disorders

3.1.2 Subcohort of GDS

For the current study, a total of 136 patients with T2D (93 men and 43 women) within the first 12 months of diabetes diagnosis was included, for whom M-value, spiroergometry and indirect calorimetry data were available. For spiroergometry, specific exclusion criteria applied, such as electrocardiogram (ECG) abnormalities (ST segment alterations, arrhythmia), unstable angina pectoris and uncontrolled hypertension [124].

3.1.3 Methods employed in the GDS cohort

For the current analysis, all participants underwent spiroergometry, hyperinsulinemic-euglycemic clamps for measurement of peripheral insulin sensitivity combined with indirect calorimetry, bioelectrical impendance, laboratory measurements and genotyping.

3.1.3.1 Spiroergometry

On the first day of examination, each participant performed an incremental exhaustive exercise test on an electronically braked cycle ergometer (Ergoline ergometrix 900, Bitz, Germany) at 60 revolutions/min [1]. Respiratory gas exchange measurements were determined by open-air spirometry (Masterscreen CPX; Jäger/Viasys, Hoechberg, Germany). During exercising, work load was increased in 10 Watt/min increments, and the incremental portion of the test lasted 12-15 minutes. Arm blood pressure, heart rate and a 12-lead ECG were recorded every 2 minutes during the test to monitor physical well-being. Capillary blood was drawn from the ear every two minutes and then every minute after the anaerobic threshold for the measurement of lactate. At the end of the test, VO_2max, related to body weight (VO_2max/kg BW) or to fat free mass (VO_2max/kg FFM), were recorded. Exhaustion was achieved when one of the following criteria was

met: (i) respiratory ratio >1.1, (ii) peak heart rate within 10 beats of age-predicted maximum, (iii) maximal ventilation (in ml/l)/maximal oxygen uptake (in ml/min) >30. Age-predicted maximum heart rate was calculated according to the formula of Haskell and Fox: heart rate max=220-age (years) [125].

3.1.3.2 Indirect calorimetry

In order to measure resting energy expenditure (REE) and respiratory quotient (RQ), oxygen consumption and carbon dioxide production were measured with an automated respiratory gas analyzer using a ventilated hood system and the Vmax Encore 29n (SensorMedix; delivered by Cardinal Health Germany). The RQ was measured as the quotient VCO_2/VO_2 at rest and during the last 30 minutes of the clamp. Resting energy expenditure (REE) was calculated using the Weir equation: $REE=(3.94*VO_2 + 1.11*VCO_2)*1.44$ [126].

Lipid oxidation (LOX) and glucose oxidation (GOX) were calculated using the following formulas and assuming that protein oxidation (POX) covered 15% of total REE in healthy individuals under postabsorptive conditions: $LOX=((1.67*VO_2-1.67*VCO_2)*1.44)-0.307*POX$, $GOX=((4.55*VCO_2-3.21*VO_2)*1.44)-0.459*POX$ [126].

After the measurement, a recalibration was performed as previously described [127]. ΔRQ was assessed as the increase in RQ during a euglycemic-hyperinsulinemic clamp and reflects metabolic flexibility [128].

3.1.3.3 Laboratory measurements

All measurements were performed on the first study day in 10-to-12-hours fasted patients [129]. Blood samples were taken from an antecubital vein and collected in tubes, were immediately centrifuged and plasma was frozen and stored at -80°C until further analysis. Blood glucose was measured by the hexokinase method (Epos analyser 5060, Eppendorf, Hamburg, Germany), serum concentrations of insulin and C-peptide

chemoluminimetrically, FFA microfluorimetrically (n=78) and TG on a Cobas c311 (Roche Diagnostics, Mannheim, Germany). High-sensitive C-reactive protein (hsCRP), a measure of sublinical inflammation was measured on a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany).

3.1.3.4 Measurement of body composition and habitual physical activity

Fat mass (FM) and fat free mass (FFM), both in kg, were measured using bioelectrical impendance analysis during the first day of study [130]. This non-invasive method involves placement of two electrodes on the right hand and right foot and determines resistance to the current flow as it passes through the different tissues. Questionnaires for assessment of physical activity were filled out according to the original paper of Baecke and adapted in case of pensioners or unemployed participants [131, 132].

3.1.3.5 Hyperinsulinemic-euglycemic clamp tests

All of the participants underwent a modified Botnia clamp, as previously described and validated [124, 133, 134]. All participants were asked to refrain from vigorous physical activity 3 days prior to investigation. Patients had stopped their oral glucose lowering medication 3 days prior to the clamp and/or injected their last insulin dosis on the evening before examination day. On the morning of the examination, two venous catheters were inserted in the antecubital veins of both arms for blood sampling and infusion of glucose and insulin. A primed [0.36 mg (fasting blood glucose in mmol/l) × (5 mmol/l)$^{-1}$ × (body weight (BW) in kg)$^{-1}$ min^{-1} for 5 min], constant intravenous infusion [0.036 mg × (BW in kg)$^{-1}$ min^{-1}] of the [6,6-$_2H^2$]glucose for assessment of endogenous glucose production (EGP) was started at −120 min and this infusion rate remained unchanged until the end of the clamp. At 0 min an intravenous glucose tolerance test was started by a bolus of glucose (1 mg/kg BW in a 30% (w/v) solution containing 1.98% [6,6-$_2H^2$]glucose) within 30 s into one forearm vein. After 60 min a

primed [10 mU (BW in kg) $^{-1}$ min^{-1} for 10 min] constant infusion of short-acting human insulin [1.5 mU (BW in kg) $^{-1}$ min^{-1}] (Insuman Rapid; Sanofi, Frankfurt, Germany) was started and continued for 180 minutes until the end of the clamp. Blood glucose was measured every 5 minutes, insulin/C-peptide every 20 minutes. A 20-% dextrose infusion was periodically adjusted to maintain euglycemia at 5 mmol/l. Whole body insulin sensitivity was measured from whole-body mean glucose infusion rates during the last 30 minutes of the clamp (M-value: mg glucose x (BW in kg)$^{-1}$ x min^{-1} after substracting glucose space correction (GSC). GSC was calculated as (G180-G150)/30, where G180, G150 are the glucose values at the timepoints 180 and 150 of the clamp (in mg/dl) and 30 is the time interval between these two measurements (in minutes).

(A) (B)

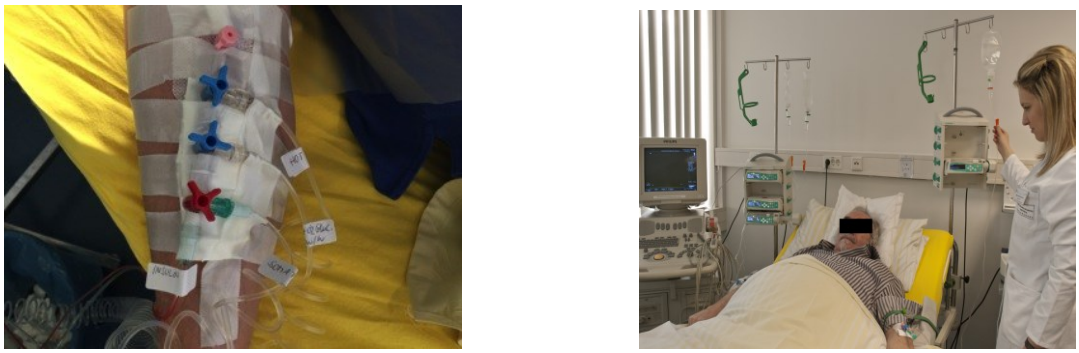

Figure 3: (A): Vein catheter during clamp test for administration of insulin, glucose and [6,6-$_2$H^2]glucose infusions, (B): Setting of a clamp test, infusions administered on the right patient side, blood glucose measurements on the left patient side.

2.1.3.6 Assessment of hepatic steatosis (FLI)

For assessment of liver steatosis, we calculated FLI, a measure of hepatic steatosis previously validated against the ultrasound [135], as follows:

FLI=logistic (0.953*ln (triglycerides) + 0.139*BMI +0.718*ln(γ-glutamyltranferase) +0.053*waist-15.745)*100, where logistic(x)=1/(1+e-x) denotes the logistic function and ln the natural logarithm.

3.1.3.6 Genotyping

Genetic analyses were performed in 134 of our 136 patients to examine possible associations of genetic variations associated with higher VO_2max or insulin sensitivity. Genomic DNA was extracted from PBMCs (Peripheral Blood Mononuclear Cell) using the Qiagen Blood and Tissue kit (Qiagen, Hilden, Germany). Genotyping was performed by allelic discrimination with TaqMan® pre-designed SNP genotyping assays and chemistry (ThermoFisher, Darmstadt Germany) on a StepOne Plus Real time PCR System (ThermoFisher). The following assays were used for: *PPARγ*: rs1801282 (C_1129864), *NDUFB6*: rs540467 (C_2334430), *PG-C1A*: rs8192678 (C_1643192), *PPARα*: rs2267668 (C_15872729), *ADRB2* rs1042713 (C_2084764), and *FTO* rs9939609 (C_30090620).

3.1.3.7 Statistical analyses

Data are presented as means and standard deviations (±SD) or medians (25th/75th percentiles), as appropriate [1]. Variables with skewed distribution (M-value, insulin, triglycerides) were log-transformed before analyses. The logit transformation (logit(x) = ln(x/(1-x)) was applied to the FLI index. Pearson correlation analyses, adjusted for sex were used to study associations between VO_2max, ΔRQ, M-value, C-peptide and anthropometric variables. Multiple regression analyses were carried out to investigate the relationships of VO_2max and ΔRQ with the M-value and to adjust for potential confounders. Such models were also used to assess differences of VO_2max, ΔRQ and insulin sensitivity between genotype groups. P-values from two-sided tests ≤5% were considered to indicate significant differences. Analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, USA).

3.2 Bariatrix study

3.2.1 Study design

Bariatrix is an ongoing prospective study that includes obese humans undergoing bariatric surgery and healthy humans undergoing elective abdominal surgery such as cholecystectomy or herniotomy for non-malignant diseases (registered clinical trial NCT01477957) [108]. Bariatric surgery procedures include (i) gastric bypass, (ii) sleeve gastrectomy and (iii) adjustable gastric band. One week prior to surgery, participants come to clinical research center and undergo metabolic phenotyping, including blood drawings, hyperinsulinemic-euglycemic clamp tests and tissue biopsies. All participants maintain stable body weight for at least two weeks before surgery and are advised not to follow a hypocaloric diet.

Inclusion criteria are age $\geq$ 30 and $\leq$70 years and BMI: 20-25 kg/m^2 for the lean control group, 35-39.9 kg/m^2 or $\geq$ 40.0 (adiposity grade 2 or 3).

Exclusion criteria included acute infections within two weeks prior to examination, immunosuppressive therapy, autoimmune diseases, anemia or blood donation, heart failure (New York Heart Association class $\geq$II), renal failure (serum creatinine $\geq$1.5 mg/dL), untreated thyreoid disease, pregnancy, HIV infection, infection with hepatitis B or C, liver disease other than NAFLD, active cancer disease, psychiatric disorders.

Aims of the study are to investigate: (i) the role of oxidative stress and inflammation on hepatic and peripheral insulin sensitivity, (ii) changes in insulin sensitivity and lipid metabolism after bariatric surgery and (iii) energy metabolism in skeletal muscle and liver and markers for oxidative stress and inflammation after bariatric surgery. Participants are followed-up 2 weeks, 3, 6 and 12 months after surgery.

They give written informed consent prior to inclusion in the study, which was approved by the institutional review board of Heinrich-Heine-University Düsseldorf (reference number 3516) and conducted according to the Declaration of Helsinki.

The current analysis included 14 obese patients, stratified by body mass index (BMI) >30 kg/m^2 with (NAFLD+) or without hepatic steatosis (NAFLD-) and 7 lean healthy individuals serving as control group (CON).

3.2.2 Classification of NAFLD

The classification of obese patients into NAFLD-, NAFLD+ and NASH was based on histology of liver tissue obtained during the surgery using hematoxylin-eosin and Masson´s trichrome by experienced histopathologists. NAFLD was defined by the presence of >5% steatotic hepatocytes in a liver tissue section [136]. The NAFLD activity score (NAS) was used to define NASH. This score is the sum of individual scores for hepatic steatosis, lobular inflammation and hepatic ballooning and can be classified from 0 to 8. A score of 1-2 rules out NASH, whereas NASH is defined by a NAS of 5-8. Values of 3 and 4 indicate borderline NASH [137].

3.2.3 Methods employed in Bariatrix

The methods used in the current analysis included an OGTT, measurement of hepatic and peripheral insulin sensitivity by hyperinsulinemic-euglycemic clamp tests, tissue biopsies and measurement of hepatic mitochondrial function, oxidative stress and inflammation in the circulation and liver tissue.

3.2.3.1 OGTT

On the screening day, all participants without known diabetes received 75 g of dextrose dissolved in 300 ml solution (ACCU-CHECK® Dextrose O.G-T, Roche Diagnostics, Mannheim, Germany), which they drank within 5 minutes [138]. Three days prior to investigation, patients were instructed to ingest a diet containing at least 150 g carbohydrates per day. An intravenous catheter was placed for blood sampling before and 10, 20, 30 minutes and in 30-minute intervals until 180 minutes. At these timepoints, blood glucose, insulin, c-peptide, inflammation markers and adipokines were measured.

3.2.3.2 Hyperinsulinemic-euglycemic clamp test

One week prior to surgery participants arrive at the clinical research center at 08:00, where they received two venous catheters in the antecubital veins of both arms for blood sampling and infusions of glucose and insulin [108]. All patients were asked to refrain from vigorous physical activity for 3 days prior to the clamp test. Patients with T2D were asked to refrain from oral-glucose lowering medication for 3 days before the test.

A primed-continuous infusion for 10 min ([3.6 mg fasting glucose (mg/dl)/90 (mg/dl)]/[min × kg body weight]), and for 360 min (0.036 mg/[min × kg body weight]) of 98% enriched [6,6-$_2H^2$]glucose was administered until the end of the clamp test to measure EGP.

The clamp protocol is different than that of GDS with a lower insulin infusion dose and without IVGTT prior to clamp initiation. More specifically, insulin (Actrapid; Novo Nordisk) was administered as a primed-continuous infusion (40 mU/[m^2 body surface area × min]) from 0 to 180 min. Blood glucose measurements were performed every 5 minutes and a 20% dextrose infusion labeled with [6,6-$_2H^2$]glucose (2% enriched) was adjusted to maintain normoglycemia (5 mmol/l).

Whole-body insulin sensitivity was measured from whole body mean glucose infusion rates (M-value) with glucose space correction, as in GDS.

Hepatic insulin sensitivity index (HIS) was calculated as the quotient 100/(fasting EGP × fasting insulin concentration). Hepatic insulin sensitivity was assessed as insulin-mediated EGP suppression.

3.2.3.3 Tissue biopsies during surgery

Samples from liver, skeletal muscle, subcutaneous and visceral adipose tissue were obtained on the day of the surgery [108]. More specifically, samples were obtained from the lower part of the right liver lobe, rectus abdominis muscle, the superficial layer of

subcutaneous abdominal adipose tissue lying directly beneath the skin and visceral abdominal adipose tissue surrounding intra-abdominal organs, respectively (amounts ranging between 200 and 1000 mg). Serum samples for quantification of sphingolipid concentrations were also obtained during surgery. All specimens were taken by the surgeon at 30 minutes after induction of anesthesia according to standardized protocols [108]. Aliquots of 100 mg fresh liver sample were fixed in 1% formaldehyde for histological examination, 50 mg were transferred into ice-cold preservation solution (BIOPS) for high resolution respirometry and 200 mg were transferred into isolation buffer (MIBO6) for isolation of mitochondria. Samples from liver and other tissues were all rapidly snap-frozen in liquid nitrogen and stored at -80^{o}C until further analysis.

3.2.3.4 High resolution respirometry of hepatic mitochondria

High resolution respirometry was applied in liver tissue and in isolated mitochondria upon exposure to various substrates for β-oxidation and tricarboxylic acid (TCA) cycle and ADP titration. More specifically, substrates associated with TCA cycle activity (glutamate, succinate) or with β-oxidation (octanoyl-carnitine) were added to hyperoxygenized 4 mg liver tissue or 0.1 mg of isolated mitochondria. Maximal uncoupled (state u) respiration was measured after exposure to the mitochondrial uncoupler (FCCP) and adjusted for individual citrate synthase activity (CSA), as a measure of for mitochondrial content. For CSA measurement 10 mg of liver tissue was homogenized in buffer.

Respiration measurements were performed in specifically designed respiratory chambers (Oroboros oxygraphs, Innsbruck) duplicate at 37^{o}C after incubation using medium MiRO5 for liver tissue, or a mitochondrial respiration buffer MiRO1 for isolated mitochondria.

The protocol used for liver tissue included sequential titration with following substrates: malate (5 µl), octanoyl-carnitine (20 µl), ADP (20 µl followed by 10 µl titration until stable response), glutamate (10 µl), succinate (20 µl), cytochrome c (3 µl), FCCP (0.5 µl titration) [108].

3.2.3.5 Hepatic lipid peroxidation, oxidative stress and systemic inflammation

As a marker of lipid peroxidation, thiobarbituric acid reactive substances (TBARs) were measured in serum and liver tissue fluorometrically (BioTek, Bad Friedrichshall, Germany) [139]. TBARs are formed as byproducts of lipid peroxidation and include hydroperoxides and aldehydes, which increase as a response to oxidative stress and can be detected using thiobarbituric acid as a reagent.

High resolution respirometry with Amplex Red was used to quantify hydrogen peroxide (H_2O_2) emission from liver tissue, which reflects ROS production from complexes I+III after adding succinate and antimycin A [140]. Amplex Red is a colorless substrate that reacts with H_2O_2 with a 1:1 stoichiometry to produce highly fluorescent resorufin.

Catalase activity and 8-oxo-guanosine in hepatic tissue lysates were measured with ELISA (ELISA, Trevigen) in hepatic tissue lysates [108].

Interleukin 6 (IL-6), tumor necrosis factor alpha (TNFα) and interleukin 1 receptor antagonist (IL1-RA) were quantified using Quantikine HS (IL-6, TNFα) or Quantikine (IL-1RA) ELISA kits (R&D Systems, Wiesbaden, Germany) [141].

C-Jun N-terminal kinase (JNK) belongs to the mitogen-activated protein kinases that are activated in response to various stimuli such as stress and inflammatory signals. Hepatic JNK phosphorylated at Thr183 and Tyr185 (p-JNK Thr183/Tyr185), as a measure of hepatic inflammation, was quantified using specific antibodies (Cell Signaling Technology) [108].

3.2.3.6 Laboratory measurements

Blood glucose was measured with Cobas c311 (Roche Diagnostics, Mannheim, Germany), serum concentrations of insulin and C-peptide were measured with RIA (Millipore, St Charles, Miss, USA), FFA microfluorimetrically [124]. Total adiponectin was measured in serum and plasma samples with Millipore's Human Adiponectin ELISA.

3.2.3.7 Sphingolipid measurements in different tissues and serum

For sphingolipid measurements samples from serum, liver, skeletal muscle, abdominal and subcutaneous adipose tissue were shipped to the University of Texas, Dallas, USA, where the group of Prof. Dr. Philipp Scherer (Touchstone Diabetes Center, Department of Internal Medicine) performed all analyses. Sphingolipids were quantified using liquid chromatography-mass spectrometry methodology (LC-MS/MS) [96]. Flash frozen tissue samples (40 mg) were homogenized in 2.0 ml of organic extraction solvent (isopropanol: water: ethyl acetate, 25:10:65; v:v:v). Immediately afterwards, 20 µl of internal standard solution was added (Avanti Polar Lipids, AL Ceramide/Sphingoid Internal Standard Mixture II diluted 1:10 in ethanol). The mixture was vortexed and sonicated in ultrasonic bath during 40 minat 40°C. Then the samples were allowed to reach room temperature and 1.5 ml HPLC grade was added. Two-phase liquid extraction was performed, the supernatant was transferred to a new tube and the pellet was re-extracted. Supernatants were combined and evaporated under nitrogen. The dried residue was reconstituted in 200 µl of HPLC solvent B (methanol/formic acid 99:1; v:v, containing 5 mmol/l ammonium formate) for LC-MS/MS analysis. Lipid separation was achieved on a 2.1 (i.d.) x 150 mm Kinetex C8, 2.6 micron core-shell particle (Phenomenex, Torrance, CA) column. Plasma sphingolipids were quantified using a similar methodology requiring 50 µl of serum. Sphingolipids were quantified using a Nexera X2 UHPLC system coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer operating the Dual Ion Source in Electrospray positive mode. Sphingolipids species were identified based on exact mass and fragmentation patterns, and verified by lipid standards. The concentration of each metabolite was determined according to calibration curves using peak-area ratio of analyte vs. the corresponding internal standard. Calibration curves were generated using serial dilutions of each target analyte. Sphingolipid true standards were purchased from Avanti Polar Lipids (Alabaster, Al).

3.2.3.8 Statistical analysis

For the Bariatrix study sample size calculation was performed as follows:

$N = \frac{(Z\alpha+Z\beta)^2 s^2}{d^2}$, where N is the required sample size, Zα and Zβ are the constants set according to α and β error (Zα =1.96, Zβ =0.84), s^2 represents the variance of each main parameter according to previous studies and d is the estimated effect size. With the main parameter being whole-body insulin sensitivity and a dropout percentage of 10%, we calculated 15 participants for each of the 3 groups of bariatric operation procedures.

For the current analysis, differences in metabolic characteristics among the three groups of patients were calculated with one-way ANOVA using Tukey statistical hypothesis testing. Comparisons between sphingolipid levels were calculated using two-tailed t-tests assuming unequal variances. One-way ANOVA t-test was performed in all correlations with $R^2 \geq 0.2$. P-values from two-sided tests less than or equal to 5% were considered to indicate significant differences.

4. Results

4.1 Patients´ characteristics of the GDS analysis

For the current analysis, 136 patients (93 males and 43 females) with newly diagnosed T2D were included [1]. Patients characteristics are summarized in Table 4. Both groups were of comparable age and overweight to slightly obese, with females having a higher BMI than males. Patients were moderately insulin resistant and had a near normo-glycemic control. Males had a higher waist-to-hip ratio (WHR) than females. Females showed lower FFM, elevated FFA and hsCRP levels compared to males. The two groups had comparable RQ during fasting and during stimulation by insulin, as well as ΔRQ, as a measure of metabolic flexibility. VO_2max was higher among males when expressed as ml per total body weight, however when divided by FFM, the group difference lost significance. Fasting blood glucose, low-density lipoprotein (LDL)-cholesterol and fasting serum TGs were comparable between the two patient groups.
LOX and GOX during fasting and insulin-stimulated conditions was comparable between males and females. Males showed a higher REE during both conditions compared to the female group. These data are summarized in Table 5.

Table 4. Characteristics of patient cohort of GDS analysis [1].

Parameter	Male T2D	Female T2D	P
N	93	43	
Age [years]	51.4 ± 10.5	51.9 ± 9.9	0.939
BMI [kg/m^2]	**30.9 ± 6.1**	**33.4 ± 7.5**	**0.046***
WHR	**0.99 ± 0.06**	**0.90 ± 0.05**	**<0.0001***
Fasting blood glucose [mg/dl]	130 ± 36	131 ± 24	0.217
Fasting serum insulin [µU/ml]	13.9 (8.7, 22.0)	16.4 (11.6, 23.4)	0.175
HbA1c [%]	6.5 ± 0.9	6.4 ± 1.0	0.861
HbA1c [mmol/mol]	47.0 ± 10.3	46.8 ± 11.2	0.861
M-value [mg.(kg BW)$^{-1}$.min^{-1}]	6.7 (4.9, 8.4)	6.1 (4.2, 7.5)	0.118
M-value [mg.(kg FFM)$^{-1}$.min^{-1}]	9.4 (7.6,11.9)	10.8 (7.4,13.0)	0.261
RQ during fasting	0.80 ± 0.06	0.81 ± 0.05	0.491
RQ during clamp	0.92 ± 0.06	0.92 ± 0.06	0.496
ΔRQ	0.11 ± 0.06	0.12 ± 0.06	0.959
VO_2max/kg BW [ml.min.(kg BW)$^{-1}$]	**21 ± 4**	**16 ± 3**	**<0.0001**
VO_2max/kg FFM [ml.min^{-1}.(kg FFM)$^{-1}$]	30 ± 5	28 ± 5	0.058
FFM [kg]	**68 ± 10**	**51 ± 6**	**<0.0001**
FM [kg]	**32 ± 12**	**39 ± 13**	**0.001**
Fasting free fatty acids [µmol/l]	**606 ± 183**	**807 ± 332**	**0.003**
Fasting low density lipoprotein[mg/dl]	136 ± 33	142 ± 40	0.301
Fasting triglycerides [mg/dl]	127 (102, 205)	133 (95,183)	0.263
Fasting lipid oxidation [mg.(kg BW) ^{1}.min^{-1}]	0.80 ± 0.28	0.71 ± 0.24	0.08
hsCRP [mg/dl]	**0.25 (0.12, 0.46)**	**0.34 (0.14, 0.75)**	**0.023**

Data are presented as mean ± SD. M-values, insulin levels and blood triglycerides, are presented as median (first quartile; third quartile). BMI: body mass index, WHR: Waist-to-hip ratio, RQ: respiratory quotient, ΔRQ: change of the respiratory quotient during the clamp compared to fasting, VO_2max: maximal oxygen uptake, BW: body weight, FFM: fat free mass

Table 5. Lipid, glucose and protein oxidation and resting energy expenditure in patients with type 2 diabetes (T2D).

Parameter	**Males T2D**	**Females T2D**	**P**
LOX during fasting [mg.kg (BW) $^{-1}$.min^{-1}]	0.80 ± 0.28	0.72 ± 0.23	0.11
LOX during clamp [mg.kg (BW) $^{-1}$.min^{-1}]	0.24 ± 0.28	0.19 ± 0.27	0.28
GOX during fasting [mg.kg (BW) $^{-1}$min^{-1}]	1.04 ± 0.73	1.03 ± 0.56	0.96
GOX during clamp [mg.kg (BW) $^{-1}$.min^{-1}]	2.64 ± 0.87	2.52 ± 0.83	0.33
POX during fasting [mg.kg (BW) $^{-1}$.min^{-1}]	0.52 ± 0.06	0.48 ± 0.06	0.0005
POX during clamp [mg.kg (BW) $^{-1}$·min^{-1}]	0.55 ± 0.07	0.51 ± 0.06	0.001
REE during fasting[kcal/d]	1972 ± 324	1645 ± 272	<0.0001
REE during clamp [kcal/d]	2068 ± 286	1724 ± 254	<0.0001

Data are presented as mean ± SD. P-values describe differences between the men and women with type 2 diabetes. LOX: lipid oxidation, GOX: glucose oxidation, POX: protein oxidation, REE: resting energy expenditure, BW: body weight

4.1.1 Associations of insulin sensitivity with VO_2max and ΔRQ

The main hypothesis of this study was to examine associations of clinical parameters of mitochondrial function (metabolic flexibility and maximal oxygen uptake) with insulin sensitivity (Figure 4). ΔRQ correlated with M-values across all patients, in males (r=0.34, p=0.0007) and only marginally in females (r=0.28, p=0.056).

VO_2max correlated with M-values in males (r=0.43, p<0.0001), but only marginally in females (r=0.29, p=0.055).

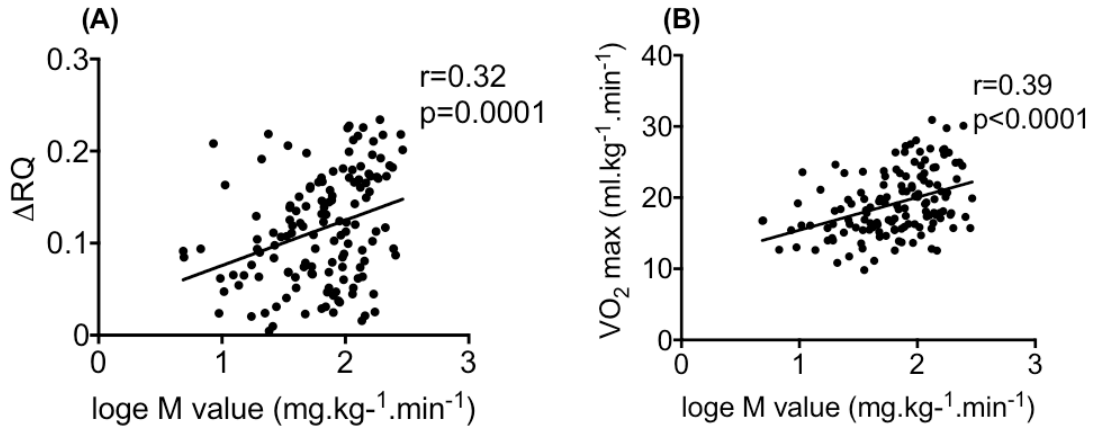

Figure 4: (A): Correlation of the change of respiratory quotient (ΔRQ) with the logarithm of M value (B): Correlation between maximal oxygen uptake related to whole body weight (VO_2max) with M value in the whole group of T2D patients [1]

VO_2max also correlated with fasting C-peptide (r=-0.35, p<0.001) and WHR (r=-0.28, p=0.0007), but not with fasting LOX (r=0.14, p=0.10).

Next we were interested in the association of metabolic flexibility with oxidative capacity. We did not find any correlation between ΔRQ and VO_2max without or with adjustment for M-values (r=-0.07, p=0.41) (Figure 5). M-values related to C-peptide (r=-0.52, p<0.0001), WHR (r=-0.42, p<0.0001) and FFM (r=-0.46, p<0.0001).

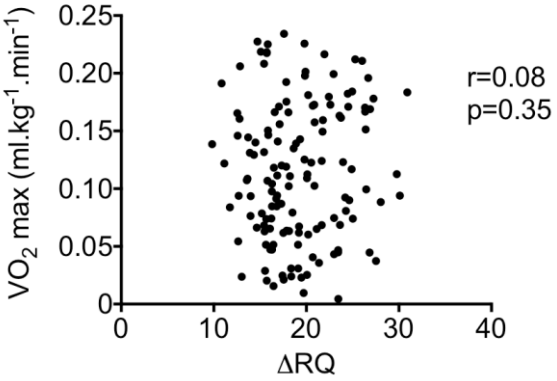

Figure 5: Association between maximal oxygen uptake related to whole-body weight (VO_2max) with the change of respiratory quotient (ΔRQ) in the whole group of T2D patients [1]

4.1.2 Associations of HbA1c, FFA and hsCRP with insulin sensitivity, VO_2max and ΔRQ

There was no association between glycometabolic control, as assessed by HbA1c with ΔRQ (r=-0.02, p=0.79), VO_2max (r=-0.06, p=0.42), or with M-values (r=-0.13, p=0.10) Circulating FFA correlated positively with metabolic flexibility (ΔRQ), but neither with maximal oxygen uptake (VO_2max) nor insulin sensitivity (M-value) (Figure 6).

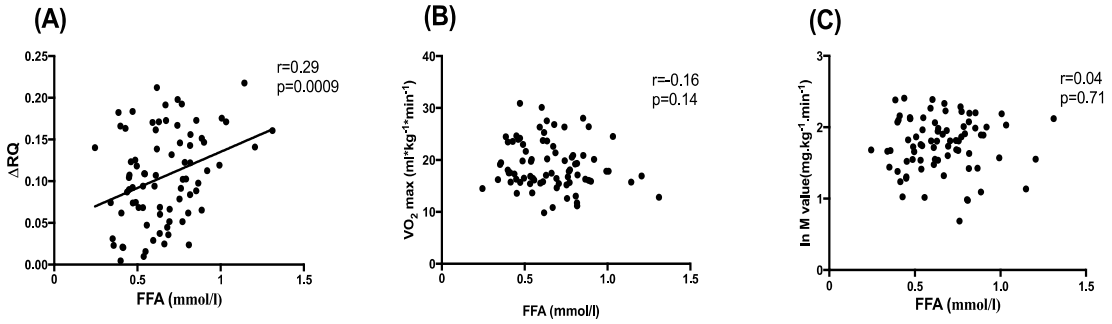

Figure 6 (A): Association between the change of respiratory quotient (ΔRQ) and free fatty acids (FFA), (B) Association between maximal oxygen uptake related to whole body weight (VO_2max) with free fatty acids (FFA) (C): Association between insulin sensitivity (M-value) and FFA in the whole group of type 2 diabetes (T2D) patients [1].

As far as the role of inflammation in the main parameters is concerned, hsCRP correlated negatively with VO_2max (r=-0.35, p=0.0004) and M-value (r=-0.19, p=0.03). RQ during fasting conditions (fasting RQ) correlated negatively with circulating FFA (r=-0.23, p=0.03), but not with M-value (r=0.01, p=0.86) or VO_2max (r=-0.002, p=0.97).

FLI, as a measure of hepatic steatosis correlated negatively with M-value (r=-0.59, p<0.0001), VO_2max (r=-0.42, p<0.0001) and ΔRQ (r=-0.21, p=0.01), but not with FFA (r=0.13, p=0.24).

4.1.3 Multiple regression analysis for determinants of insulin sensitivity

In order to investigate the relationships of metabolic flexibility and oxidative capacity with the M-value and to adjust for potential confounders we performed multiple regression analysis (Table 6). ΔRQ and VO_2max associated with insulin sensitivity across the whole group, when adjusted for sex (ΔRQ: β=1.92, p=0.0001, VO_2max: β=0.04, p<0.0001). Further adjustments for age, anthropometric parameters such as BMI, WHR and fat mass, inflammation markers (hsCRP), hepatic steatosis (FLI), medication that could interfere with mitochondrial function or insulin sensitivity such as metformin, statins and β-blocker or combinations of these variables, did not affect associations of ΔRQ and VO_2max with M-values.

Interestingly, we found that after further adjustment for levels of lipidemia (circulating FFA) in the subcohort of 78 patients for whom FFA concentrations were available the correlations of ΔRQ and VO_2max with M-values were abolished.

Table 6: Multiple regression analysis for the impact of VO_2max and ΔRQ on M-value (ln) [1].

Model	ΔRQ		VO_2max	
	β	p	β	p
Model 1: adjusted for sex	1.93	0.0001	0.04	<0.0001
Model 2: Model 1 + adjusted for age, BMI	1.52	0.0001	0.02	0.002
Model 3: Model 2 + adjusted for FLI	1.27	0.004	0.02	0.009
Model 4: Model 2 + adjusted for WHR and FM	1.37	0.002	0.02	0.032
Model 5: Model 2 + adjusted for hsCRP	1.36	0.004	0.03	0.001
Model 6: Model 2 + adjusted for statins, β-blocker, metformin	1.56	0.001	0.02	0.006
Model 7: Model 4 + adjusted for FLI, hsCRP, statins, β-blocker, metformin	1.20	0.012	0.02	0.049
Model 8: Model 2 + adjusted for FFA	1.51	0.044	0.02	0.045
Model 9: Model 7 + adjusted for FFA	1.20	0.139	0.02	0.078

Regression coefficients (β) and *P* values were calculated using linear regression analyses with M-value (ln) as dependent variable.
VO_2max: maximal oxygen uptake, ΔRQ: change of the respiratory quotient during the clamp compared to fasting, WHR: waist-to-hip ratio, FM: fat mass, FFA: free fatty acids, FLI: fatty liver index

4.1.4 The role of single nucleotide polymorphisms on insulin sensitivity, VO_2max and ΔRQ

All SNPs analyzed were in Hardy–Weinberg equilibrium (HWE, P>0.05) and therefore suitable for further analyses. Carriers of the A allele in the rs9939609 SNP of the *FTO* gene exhibited lower (p=0.04) VO_2max than homozygous carriers of the T allele, after adjustment for age, sex and BMI (Figure 7). Other examined single nucleotide polymorphisms did not associate with VO_2max, ΔRQ or M-values (Table 7). Of note, there were no significant interactions between sex and tested genotypes.

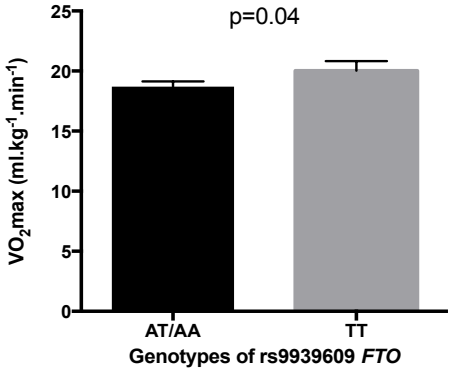

Figure 7: VO_2max among carriers of the A allele and homozygous carriers of the T allele

Table 7. Association of single nucleotide polymorphisms with VO_2max, ΔRQ and M-value in newly diagnosed patients with type 2 diabetes (T2D) [1].

	VO_2max Δ [95%CI] p		**ΔRQ** Δ [95%CI] p		**M-value** Δ_{rel} [95%CI]	p
PPARG (CG/GG vs. CC)	0.46 [-1.05, 1.96]	0.55	-0.010 [0.03, 0.15]	0.42	3.5% [-9.6%, 18.6%]	0.61
PPARD (AG/GG vs. AA)	0.74 [-0.59, 2.07]	0.27	0.007 [-0.01, 0.03]	0.51	1.1% [-12.3%, 11.5%]	0.85
PGC-1α (CT/TT vs. CC)	0.38 [-0.86, 1.63]	0.55	-0.006 [-0.03, 0.01]	0.55	2.6% [-8.3%, 14.8%]	0.66
ADRB2 (GA/AA vs. GG)	-0.23 [-1.60,1.13]	0.74	-0.003 [-0.03, 0.02]	0.79	-1.7% [-13.1%, 11.2]	0.79
NDUFB6 (GA/AA vs. GG)	0.69 [-0.58, 1.97]	0.28	-0.008 [-0.03, 0.01]	0.43	-0.6% [-11.5%, 11.5%]	0.91
FTO (TA/AA vs. TT)	-1.39[-2.73, -0.04]	0.04	-0.007 [-0.03, 0.01]	0.51	-0.13% [-11.6%, -12.8%]	0.98

Δ (Δ_{rel}) and corresponding confidence intervals and p-values describe differences (relative changes) between the carriers of at least one minor allele and the homozygotes of the major allele with respect to the examined parameters. Comparisons are adjusted for sex, age and BMI. VO_2max: maximal oxygen uptake, ΔRQ: change of the respiratory quotient during the clamp compared to fasting

4.2 Patients´characteristics of the Bariatrix analysis

For this analysis, we included a total of 21 participants, 7 lean controls, 7 obese patients without NAFLD and 7 obese patients with NAFLD. The groups had comparable age, sex and circulating FFA (Table 8). BMI was similar in NAFLD- and NAFLD+, but higher in both obese groups than in CON. The two obese groups were equally insulin resistant in the periphery. Whole-body insulin sensitivity of the obese groups was markedly lower than in CON. As far as hepatic insulin sensitivity is concerned, mean HIS was nominally higher in CON, but not significantly different from that of obese groups. Per definition of the groups, hepatocellular lipid content (HCL) was similar in CON and NAFLD-, but 14-fold higher in NAFLD+. Circulating TGs were comparable among the three groups. LDL-cholesterol was significantly higher in the two obese groups compared to controls. Liver enzymes serum glutamic-oxaloacetic transaminase (SGOT) and glutamate pyruvate transaminase (SGPT) were not different among the groups, however there was a trend towards increased SGPT in the NAFLD group compared to controls ($p=0.06$).

Table 8. Participant characteristics of the Bariatrix analysis.

	CON	NAFLD (-)	NAFLD (+)
Number (females)	7 (5)	7 (6)	7 (4)
Age (years)	40 ± 14	43 ± 7	49 ± 9
BMI ($kg.m^{-2}$)	25.2 ± 3.3$^{***\#\#\#}$	49.5 ± 8.3	55.6 ± 7.4
Waist circumference (cm)	82.8 ± 13.1$^{***\#\#\#}$	125.7 ± 19.8§	149.0 ± 12.1
Peripheral insulin sensitivity ($mg.kg^{-1}.min^{-1}$)	7.4 ± 2.2$^{**\#\#}$	3.1 ±1.7	1.6 ± 0.1
HIS ($(mg.kg^{-1}.min^{-1})/(\mu U/ml))^{-1}$	15.1 ± 13.9	4.8 ± 2.1	1.7 ± 0.1
FFA (µmol/l)	617 ± 218	790 ±142	535 ± 349
HbA1c (%)	5.2 ± 0.2	5.6 ± 0.5	6.0 ± 0.9
SGOT (U/l)	20.4 ± 2.6	20.0 ± 4.2	22.1 ± 4.0
SGPT (U/l)	20.2 ± 7.1	21.8 ± 6.4	30.6 ± 8.7
LDL-cholesterol (mg/dl)	83.8 ± 23.6$^{*\#\#}$	126.7 ± 24.3	120.8 ± 21.0
TG (mg/dl)	126.0 ± 49.9	124.4 ± 47.1	170.8 ± 98.8
HCL (%)	0 (0,5)$^{\#\#\#}$	2 (0,5)§§§	40 (10,50)

Data are presented as mean ± SD. HCL (%) is presented as median (q1,q3). T2D: type 2 diabetes, BMI: body mass index, HIS: hepatic insulin sensitivity index, FFA: free fatty acids, SGOT: serum glutamic-oxaloacetic transaminase, SGPT: glutamate pyruvate transaminase, LDL-cholesterol: low-density-lipoprotein cholesterol, HCL: hepatocellular lipids
* p≤0.05, ** p≤0.01, ***p<0.001 CON vs NAFLD (-)
#p≤0.05, ## p≤0.01, ###p<0.001 CON vs NAFLD (+)
§p≤0.05, §§ p≤0.01, §§§p<0.001 NAFLD (-) vs NAFLD (+)

4.2.1 Sphingolipid concentrations in humans with or without NAFLD

In serum, total ceramides did not show differences between the groups, whereas total dihydroceramides were higher in both obese groups than in CON (Figure 8).

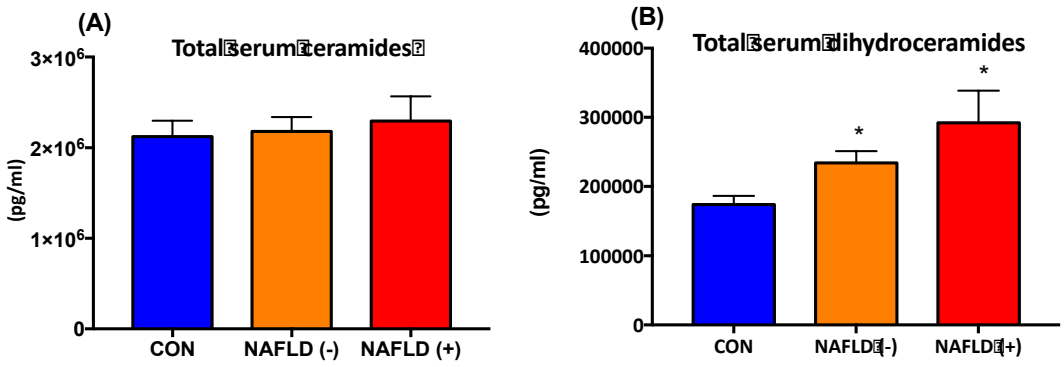

Figure 8: Total serum ceramide (A) and dihydroceramides (B) in healthy controls (CON), obese patients without (NAFLD -) and with NAFLD (NAFLD +). *p<0.05 NAFLD (+) and NAFLD (-) compared to CON

Detailed analysis of dihydroceramide species identified differences among the groups. More specifically, dihydroceramides 20:0 and 24:1 were increased in both NAFLD+ and NAFLD-, while dihydroceramide 16:0 and 22:0 were higher only in NAFLD+ and NAFLD-, respectively (p=0.03) (Figure 9). Hexosylceramide 24:0 was lower in NAFLD+ than in both NAFLD- and CON (p=0.03).

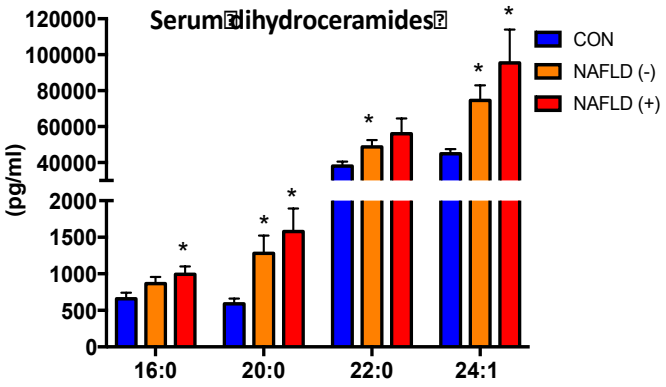

Figure 9: Specific serum dihydroceramide species in healthy controls (CON), obese patients without (NAFLD -) and with NAFLD (NAFLD +). *p<0.05 NAFLD (+) and NAFLD (-) compared to CON

Total liver tissue ceramides were increased by 8% and 19% in NAFLD+ compared to NAFLD- and CON (19406 vs. 17734 and 16427 pg/mg wet tissue), respectively (Figure 10A). As to specific ceramide species, ceramides 16:0 and 18:0 were higher in NAFLD+ and NAFLD-, whereas ceramides 20:0, 22:0 and 24:1 were higher only in NAFLD+ (Figure 10B).

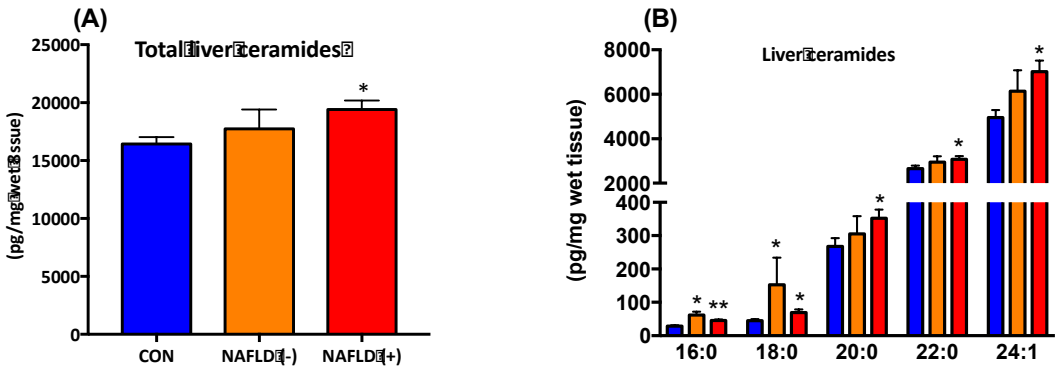

Figure 10: Total liver ceramide (A) and ceramide species (B) in healthy controls (CON), obese patients without (NAFLD -) and with NAFLD (NAFLD +). *p<0.05 NAFLD (-) and NAFLD (+) compared to CON, **p<0.01 NAFLD (+) compared to CON

Total dihydroceramides were also increased only in NAFLD+ by 12% compared to CON (9399 vs. 8421 pg/mg wet tissue). Dihydroceramide species 16:0 and 24:1 were increased in NAFLD+ group, whereas dihydroceramide 22:0 was increased in both obese groups (Figure 11).

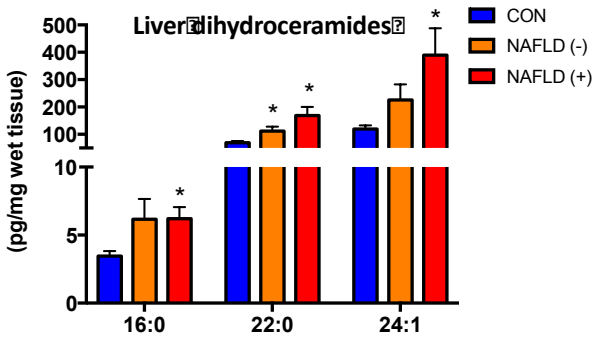

Figure 11: Specific liver dihydroceramide species in healthy controls (CON), obese patients without (NAFLD -) and with NAFLD (NAFLD +). *p<0.05 NAFLD (-) and NAFLD (+) compared to CON

NAFLD+ featured lower hexosylceramides 14:0 and 24:0 (p=0.03 and 0.02, respectively), but higher hexosylceramide 18:0 than both NAFLD- and CON (p=0.04). Liver sphingomyelins 22:0 and 24:0 were reduced in both obese groups (p=0.04). Sphingosine and sphinganine were increased by 25% and 40% respectively in NAFLD+ (p=0.04).

Skeletal muscle total ceramides, as well as total ceramides in subcutaneous adipose tissue did not differ among the three groups (Figure 12). We found however, that patients with NAFLD exhibited reduced total ceramide content in their visceral fat compared to controls.

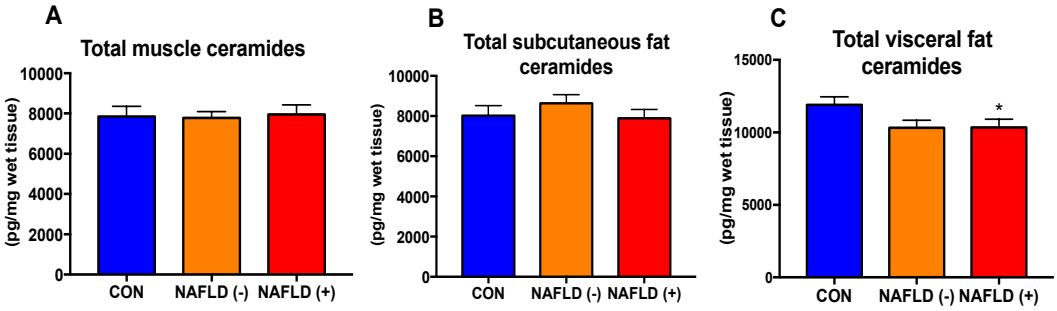

Figure 12: Total ceramides in skeletal muscle (A), subcutaneous (B) and visceral adipose tissue (C) in healthy controls (CON), obese patients without (NAFLD -) and with NAFLD (NAFLD +). *p<0.05 NAFLD (+) compared to CON

4.2.2 Associations of sphingolipids with whole-body and hepatic insulin sensitivity

Serum ceramide species, 14:0 (r=0.61, p=0.02), 16:0 (r=0.56, p=0.04) and 20:0 (r=0.57, p=0.03) correlated negatively with whole-body insulin sensitivity, as assessed by the M-value. Total serum dihydroceramides (r=-0.58, p=0.03),

dihydroceramide 16:0 (r=-0.56, p=0.04), 20:0 (r=-0.61, p=0.02) and 22:0 (r=-0.55, p=0.04) as well as sphingomyelin 18:1 (r=-0.58, p=0.03) also correlated negatively with insulin sensitivity. In liver, only ceramide 16:0 (r=0.57, p=0.03) and dihydroceramide 22:0 (r=-0.55, p=0.04) correlated negatively with insulin sensitivity (Figure 13).

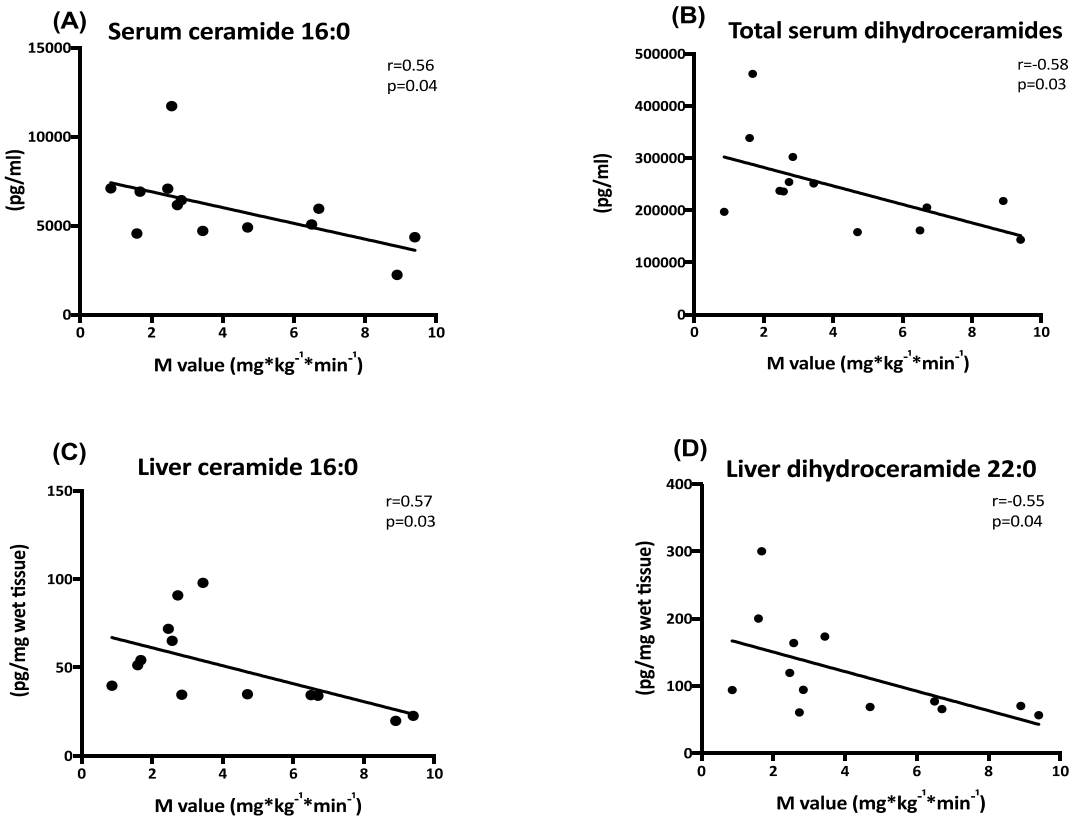

Figure 13: Correlations between serum ceramide 16:0 (A), total serum dihydroceramides (B), liver ceramide 16:0 (C) and liver dihydroceramide 22:0 with peripheral insulin sensitivity (M value)

Specific ceramide species in serum, subcutaneous and visceral fat as well as skeletal muscle tightly and positively correlated with basal (fasting) EGP while hepatic ceramide species did not relate to basal EGP. Of note, HIS, as a measure of hepatic insulin sensitivity, correlated negatively with serum hexosylceramide 18:0 (r=-0.75, p=0.007) and 20:0 (r=-0.72, p=0.01).

4.2.3 Sphingolipids and adiponectin concentrations

Serum adiponectin correlated positively with whole-body (M value: $r=0.73$, $p=0.02$) and hepatic insulin sensitivity (HIS: $r=0.65$, $p<0.03$). Selectively in visceral fat tissue, ceramide 24:1, total hexosyl- and subspecies 24:0 (all $r>0.44$, $p<0.04$) and even more total lactosyl- and subspecies 14:0, 16:0, 24:0 and 24:1 ($r>0.76$, $p<0.001$) related positively to circulating adiponectin concentrations.

4.2.4 Sphingolipids and hepatic oxidative capacity

Serum total dihydroceramides ($r=0.62$, $p=0.003$) and subspecies 24:0 ($r=0.58$, $p=0.007$) and 24:1 ($r=0.59$, $p=0.005$) were associated with higher rates of maximal hepatic uncoupled respiration from both TCA cycle and beta oxidation, prior to correction for CSA. Thereafter, only total serum lactosylceramides and subspecies 16:0 (Figure 14) as well as sphingomyelins 16:0 and 18:0 correlated positively with rates of state u respiration.

Hepatic sphinganine ($r=0.50$, $p=0.03$), deoxysphinganine ($r=0.58$, $p=0.01$) and deoxysphingosine ($r=0.58$, $p=0.01$) related positively to rates of state u respiration from TCA cycle substrates, before CSA correction. After CSA correction, hepatic lactosylceramide 16:0 (Figure 13) and sphingomyelin 18:1 correlated positively with state u respiration from TCA cycle and from β oxidation, respectively.

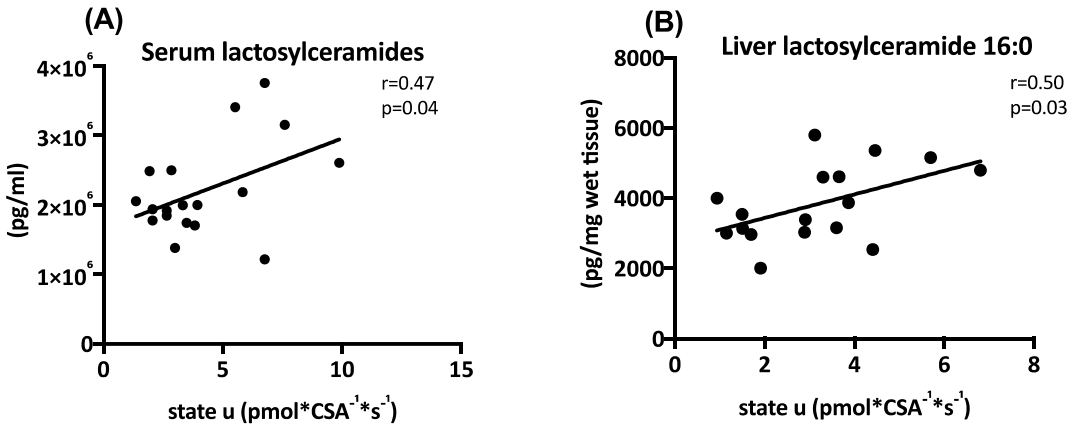

Figure 14: Correlations between serum lactosylceramides (A) and liver lactosylceramide 16:0 (B) with maximal uncoupled respiration in the liver (state u) corrected with citrate synthase activity (CSA).

4.2.5 Hepatic ceramide species, oxidative stress and inflammation

Hepatic H_2O_2 emission from liver was positively associated with hepatic ceramide 16:0, hexosyl- (16:0, 20:0, 22:0 and 24:0) and lactosyl-ceramide (18:0, 20:0 and 24:0) species (Figure 15 A, B). Liver TBARs further correlated positively with total hepatic ceramides, ceramide 24:1 and dihydroceramide 24:1 (Figure 15 C, D).

Liver p-JNK correlated positively with total hepatic dihydroceramides, ceramide 22:0, total lactosylceramides and the subspecies 16:0, 20:0 and 22:0 as well as with hexosylceramides 16:0 and 22:0 (Figure 16). While there were correlations of serum ceramide species 14:0 ($r=0.60$, $p=0.007$) and serum sphingosine ($r=0.57$, $p=0.01$) with circulating TNFα concentrations, neither serum nor hepatic sphingolipids correlated with circulating IL-6 levels.

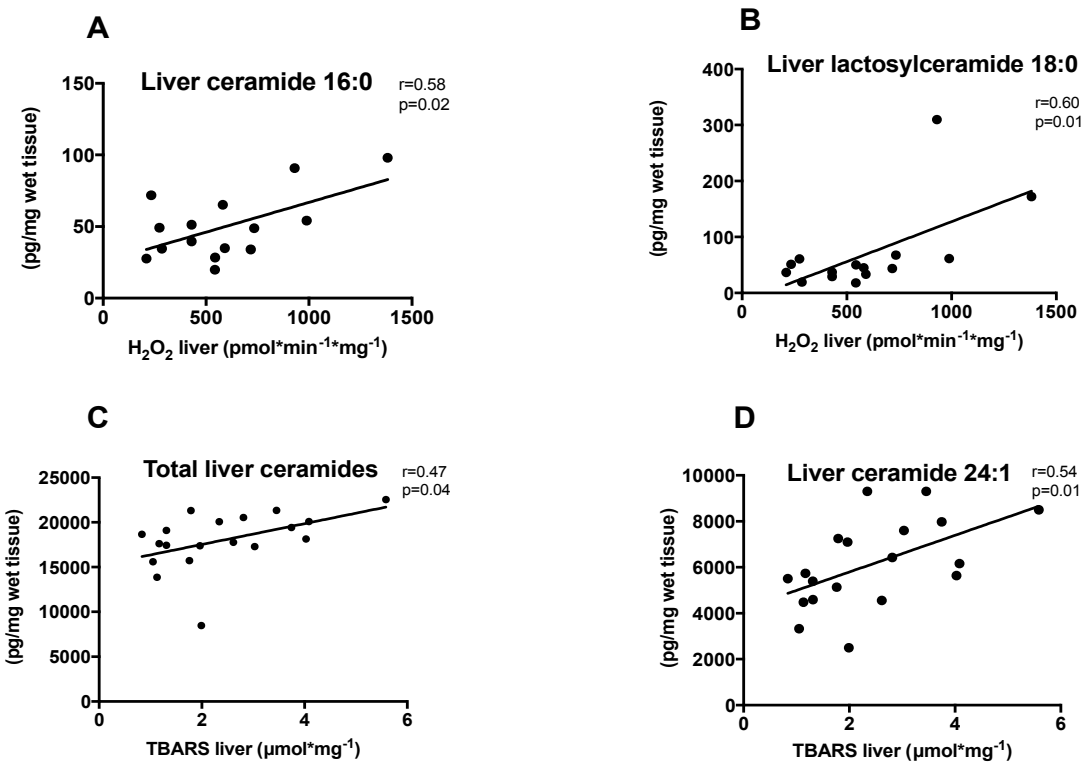

Figure 15: Correlations between liver ceramide 16:0 (A) and liver lactosylceramide 18:0 (B) with H_2O_2 emission in the liver and total liver ceramides (C) and liver ceramide 24:1 (D) with TBARs in the liver.

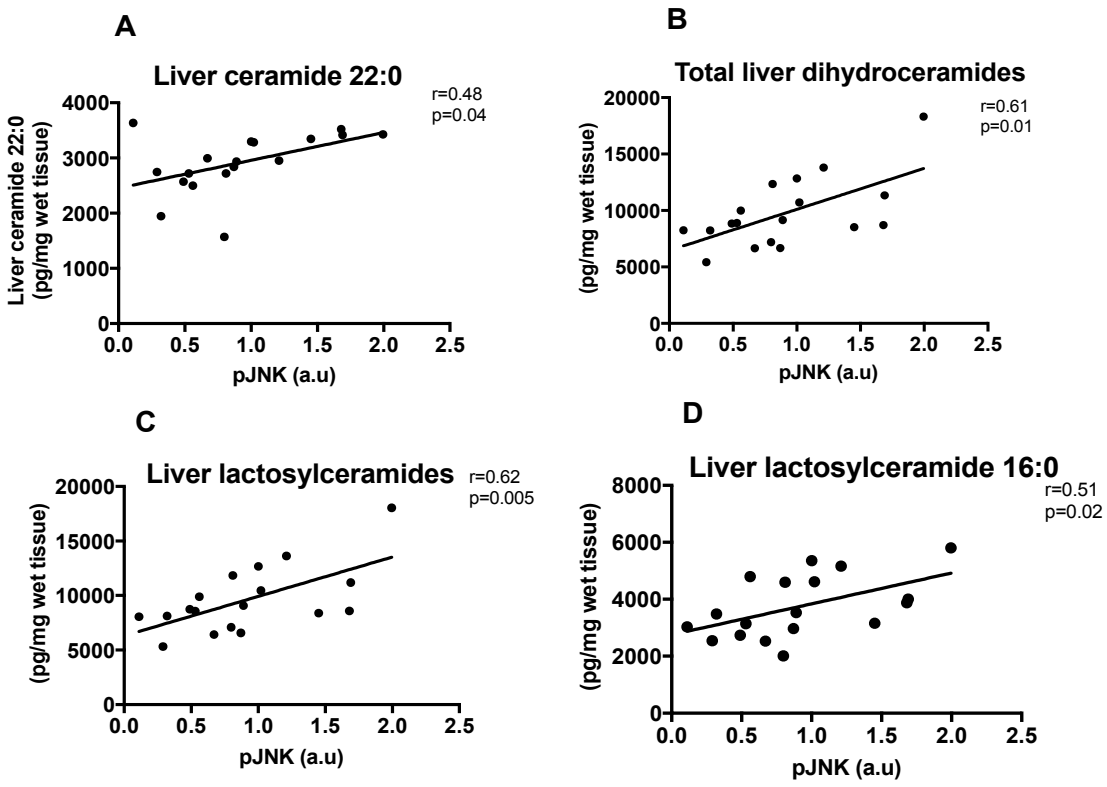

Figure 16: Correlations between liver ceramide 22:0 (A), total liver ceramides (B), liver lactosylceramides (C), and liver lactosylceramide 16:0 with pJNK in the liver.

5. Discussion

Collectively, these studies showed (i) that circulating FFA affect muscle mitochondrial function and peripheral insulin sensitivity even in the early course of T2D and (ii) that certain serum and hepatic sphingolipid species relate to peripheral insulin resistance and hepatic inflammation in the context of NAFLD.

5.1 Skeletal muscle mitochondrial function and insulin resistance

5.1.1 Metabolic flexibility and insulin resistance

Among newly-diagnosed, well-controlled patients with T2D, we found that metabolic flexibility (ΔRQ) and oxidative capacity (VO_2max) of skeletal muscle independently associate with whole-body insulin sensitivity [1]. However, these two parameters did not associate with each other indicating that they relate to dinstict features of muscle mitochondrial function. More specifically, ΔRQ associated with levels of lipidemia, whereas VO_2max was determined by a genetic polymorphism of the *FTO* gene and subclinical inflammation, as assessed by hsCRP levels.

Among patients with long-standing T2D, studies demonstrated a positive correlation between ΔRQ and insulin sensitivity [48, 73, 142]. The present study extends this finding to patients with recent-onset T2D. It is currently unclear whether the positive correlation of impaired ΔRQ with insulin resistance in T2D patients is a result of diminished glucose uptake, reflecting insulin resistance with subsequent decreased cellular glucose uptake and oxidation. Indeed, after correction for glucose disposal rates, ΔRQ was no longer decreased in a T2D group compared to healthy controls [60]. Other studies reported that under conditions of enhanced mitochondrial activity following exercise interventions in T2D (Table 2) [59] or prediabetes [143], changes in metabolic flexibility did not parallel improvements in insulin sensitivity suggesting that metabolic flexibility does not only reflect insulin sensitivity, but is also controlled by

mitochondrial oxidative capacity.

In the present study, ΔRQ did not associate with VO_2max even upon adjustment for M-values. Moreover, we found that ΔRQ correlated positively with circulating free fatty acids, and fasting RQ correlated negatively with free fatty acids, suggesting higher rates of fasting lipid oxidation [35]. Increased circulating lipids are known to interfere with insulin signaling and reduce ATP synthase flux (fATP), therefore lowering basal mitochondrial activity. Of course, fasting FFA vary substantially among obese individuals and T2D patients [144]. However, elevated FFA during lipid infusions or prolonged fasting can cause insulin resistance and diminished insulin-stimulated ATP synthesis in skeletal muscle [37].

Finally, metabolic flexibility could reflect insulin-mediated suppression of lipolysis and thereby insulin sensitivity in adipose tissue [62, 145]. It has been proposed that inflammation of adipose tissue could lead to increased lipolysis and subsequently ectopic fat accumulation and insulin resistance [62]. In accordance with this concept, we show that ΔRQ correlated negatively with fatty liver index (FLI), as a measure of hepatic fat accumulation and positively insulin sensitivity. We, however, did not find an association with hsCRP, as a marker of subclinical inflammation.

5.1.2 Maximal oxygen uptake and insulin resistance

This study also showed that VO_2max correlates positively with the M-value in patients with short known duration of T2D [1]. VO_2max during exhaustive physical activity is not only an indicator of cardiometabolic fitness, but also a surrogate marker of mitochondrial oxidative capacity and is often impaired in T2D (Table 2) [64-66]. In overweight and obese healthy persons, the association of VO_2max with the M-value was explained mainly by intrahepatic lipid content (Table 2) [70]. In our cohort, adjustment for hepatic steatosis in the multiple regression analysis models weakened, but did not abolish the correlation between VO_2max and M-value. However, patients with lower VO_2max had a higher degree of hepatic steatosis as assessed by FLI. Impaired muscle mitochondrial function could lead to decreased lipid oxidation and promote ectopic fat accumulation in liver and skeletal muscle, resulting in hepatic steatosis and insulin resistance. However, this study did not detect any association between fasting lipid

oxidation and VO_2max. On the other hand, we found that VO_2max correlated with hsCRP, which suggests a role of subclinical inflammation already in the early course of T2D.

5.1.3 The role of glycemia, lipidemia and anthropometric parameters

This study also investigated the effect of glycemic control, lipidemia, subclinical inflammation, anthropometric variables and inherited factors on the associations of mitochondrial function with insulin sensitivity. To this end, we used multiple regression analysis models and adjusted stepwise for potential confounders [1].

Hyperglycemia may contribute to the development and acceleration of insulin resistance and oxidative stress, interfering with mitochondrial function. In our study, we did not detect any association of muscle insulin sensitivity, metabolic flexibility and oxidative capacity with glycemic control, as assessed by HbA1c. This can be explained by the good glycemic control and short diabetes duration of our patient group.

Further adjustments performed for sex, age, fat mass, WHR, subclinical inflammation, hepatic steatosis and patient medication that could interfere with the tested variables did not abolish the significant relationships of metabolic flexibility and oxidative capacity with insulin sensitivity, suggesting that these parameters comprise early abnormalities in the course of T2D.

A major finding of this study was that adjusting for circulating FFA abolished the association of VO_2max and ΔRQ with the M-value. This underlines the superior importance of circulating lipids in the early course of T2D. Interference of DAG species with insulin signaling cascade through PKCθ activation is an initial step in the development of insulin resistance, which could in turn impair maximal oxidative capacity during diabetes progression [29, 35]. Other studies have underlined the role of ceramides, as lipid metabolites with deleterious effects on insulin signaling and induction of TLR4-associated insulin resistance that also associate with damages in mitochondrial electron transport chain [93, 146].

5.1.4 The role of single nucleotide polymorphisms

SNPs of certain genes have been associated with insulin resistance, oxidative metabolism, as well as response of maximal oxygen uptake and insulin sensitivity to exercise training interventions. In the current study, we analyzed genes with known relationships to insulin sensitivity and/or energy metabolism (*PPARγ, NDUFB6, PGC1α, PPARδ, ADRB2* and *FTO*) and examined associations with ΔRQ, VO_2max and M values in recent onset patients with T2D.

The main finding regarding in this regard was an association between the presence of the A allele in rs9939609 SNP in the *FTO* gene and lower VO_2max after adjustment for age, sex and BMI. This association, however, disappeared after Bonferroni correction for our SNP analyses. The *FTO* gene is associated with BMI and body fatness and with increased risk for T2D [147], its function remains largely unknown. The central distribution of the gene in the hypothalamus implies a role in the regulation of energy balance [148]. Overexpression of this gene in mice was related with increased food intake and adiposity without impact on energy expenditure. In humans, at risk SNPs were associated with increased food intake, but not with altered resting energy expenditure [84]. On the other hand, a study in 306 healthy women found elevated mRNA levels of the *FTO* gene in adipose tissue of obese participants, a finding that supports a role of the gene in the regulation of lipolysis [85].

Our data suggest that the presence of this allele has a direct effect on VO_2max, that is neither mediated by BMI nor relates to metabolic flexibility, thus indicating rather a central than a peripheral function of the *FTO* gene. In contrast to our findings, another study failed to detect an association of this SNP with VO_2max and basal metabolic rate, which might result from the higher fitness levels of this healthy overweight cohort (VO_2max >27 ml/kg/min) [148].

This study failed to support a relevant role of other gene polymorphisms in the *NDUFB6*, *PGC1α*, *PPARδ* and *ADRB2* genes on metabolic flexibility, maximal oxygen uptake or insulin sensitivity in this cohort of recent-onset, well-controlled T2D patients after adjustments for age, sex and BMI. Of note, the sample size might have been too small to draw valid conclusions on the interaction between energy metabolism and insulin sensitivity.

5.2 Specific lipid metabolites in insulin resistance

The findings of the first study, the GDS analysis, provided evidence for a major role of lipidemia in recent-onset T2D and the associations of insulin resistance with mitochondrial function. Previous studies have supported a role of toxic lipid intermediates in the initiation of insulin resistance, mainly DAGs and ceramide species [35, 93]. NAFLD is tightly associated with obesity, hepatic and peripheral insulin resistance and T2D [117, 119, 149] with recent data suggesting that hepatic ceramide levels may dissociate liver steatosis from insulin resistance in NAFLD of different origins [107]. Against this background, the second study analyzed ceramide species in serum and in highly metabolically-active tissues (liver, skeletal muscle, visceral and subcutaneous adipose tissue) of morbidly obese, insulin-resistant patients undergoing bariatric surgery and healthy controls undergoing elective abdominal surgery.

5.2.1 The role of sphingolipids in hepatic and peripheral insulin resistance

This study demonstrated that obese patients with NAFLD had increased total liver ceramides and dihydroceramides, sphingosine and sphinganine. Furthermore, specific ceramide species (20:0, 22:0, 24:0) were found to be characteristic for the presence of NAFLD, whereas others are increased in insulin resistance without NAFLD. A previous study that examined hepatic lipid content in insulin resistant patients with NAFLD (low HOMA-IR group) and NAFLD due to PNPLA3 genotype (high HOMA-IR group) reported increased total hepatic ceramides and dihydroceramides, as an indicator of de novo ceramide synthesis in the insulin resistant group [107]. In our study, total ceramides in other tissues were not different among the three groups, a finding that supports the concept of a direct effect of hepatic de novo ceramide synthesis with insulin resistance and the progression of NAFLD.

We further investigated associations between hepatic and peripheral insulin sensitivity with ceramide species in serum and liver and found that serum ceramide species 14:0,

16:0 and 20:0, total dihydroceramides and species correlated negatively with whole-body insulin sensitivity, as assessed by hyperinsulinemic-euglycemic clamps. As far as liver sphingolipid species are concerned, we found negative correlations of hepatic ceramide 16:0 and dihydroceramide 22:0 with the M-value. A previous study reported increased hepatic ceramide 16:0 in insulin resistant patients [107]. Total hepatic ceramides did not associate with the M-value, which extends previous findings using HOMA-IR for assessment of insulin resistance [88]. Total hepatic ceramides did not associate with basal EGP or hepatic insulin sensitivity index in our study, which is in line with previous findings in obese patients [150]. However, we demontrated inverse associations of serum hexosylceramides 18:0 and 20:0 with hepatic insulin sensitivity.

5.2.2 The role of sphingolipids in hepatic mitochondrial function

This study also provided evidence that serum total dihydroceramides and subspecies, hepatic sphinganine and deoxysphinganine correlated with increased maximal uncoupled respiration of hepatic mitochondria, as assessed with high resolution respirometry. Increased lipid oxidation and oxidative capacity may represent compensatory mechanisms in the initial stages of NAFLD and contributes to its progression among obese patients [108, 109]. Thus, the observed associations could result from increased lipid oxidation rates of hepatic mitochondria in obesity.

Oxidative stress and more specifically H_2O_2 emission have been associated with NAFLD progression [108], whereas ceramides, especially lactosylceramides, N-acetyl-sphingosine and GD3 ganglioside have been shown to induce ROS production and subsequently cause opening of mitochondrial permeability pore and cytochrome c release in isolated mitochondria of rat liver [110]. More recently, lactosylceramides have been identified as mediators of oxidative stress by inhibiting mitochondrial respiratory chain and increasing sensitivity of mitochondrial permeability transition pore to Ca^{2+} in heart mitochondria of diabetic mice [146]. In the present study, we found that hexosyl- and lactosylceramide species (16:0, 20:0, 22:0, 24:0 and 18:0, 20:0, 14:0 respectively) were positively correlated with H_2O_2 in the liver. We also provide further evidence of the role of ceramides in induction of oxidative stress, since total liver ceramides associated with increased lipid peroxidation, as assessed by TBARs.

5.2.3 The role of sphingolipids in hepatic and systemic inflammation

Elevated hepatic dihydroceramides, lactosylceramides and hexosylceramides were also associated with increased hepatic inflammation, as reflected by hepatic p-JNK in the present study. Saturated fats lead to activation of TLR4 receptors and subsequently transcription of proinflammatory cytokines, such as IL-6 and TNFa and increase cell ceramide levels [93]. This notion is further supported by experiments in TLR-knockout mice, proving a protected phenotype against lipid-induced insulin resistance [151].
We also found that circulating TNFa levels correlated with serum ceramide 14:0 and sphingosine, which underlines the role of ceramides not only in hepatic but also in systemic inflammation.

5.2.4 Hepatic ceramide 16:0 in insulin resistance, NAFLD and oxidative stress

Of special interest is our finding that hepatic ceramide 16:0 was not only elevated in NAFLD, but also linked to both insulin resistance and increased ROS production. Ceramide 16:0 has been involved in the pathogenesis of diet-induced insulin resistance, since high-fat diet has been shown to upregulate C16:0 ceramide synthesis and provide increased amount of palmitate finally inhibiting insulin signaling by antagonizing PI3K/Akt signaling and inhibiting mitochondrial electron transport chain, thereby suppressing β-oxidation [152]. Liver-specific knock-out of ceramide synthase enzyme (CerS) 6 reduced the concentrations of ceramide 16:0 and dihydroceramides and improved glucose tolerance with parallel elevation of palmitate oxidation [152].
The observed increase in hepatic ceramide 16:0 might be the result of upregulated hepatic CerS6 in obesity, which could lead to hepatic mitochondrial adaptation with greater hepatic oxidative capacity in NAFLD [108].

5.3 Strengths and limitations

Our analysis of the GDS focused on identifying determinants of insulin resistance in recent-onset T2D and to explore the associations of insulin resistance with clinical parameters of mitochondrial function (metabolic flexibility and oxidative capacity). The main strength of this study is the large cohort group of T2D patients that underwent thorough phenotyping as well as genotyping, using gold-standard methods. As far as the limitations are concerned, VO_2max does not purely reflect mitochondrial function, since oxygen supply capacity, determined by cardiorespiratory fitness also interferes with maximal oxygen uptake achieved during exhaustive tests in physiological conditions. Furthermore, ΔRQ is also influenced by fasting glycemia and circulating FFA. In order to overcome these limitations we used multiple regression analysis models to adjust for potential confounders.

Our analysis of the Bariatrix study included a smaller group of 21 patients that not only underwent intensive phenotyping using gold-standard methods, but also intensive lipidomic analysis in various tissues and in serum from obese patients with and without NAFLD, but also from a healthy control group. The main limitation of this study is the cross-sectional study design, which does not allow us to draw conclusions regarding causality of the observed associations.

5.4 Conclusions

First, these studies found that in patients with recent-onset T2D, impaired mitochondrial function as reflected by metabolic flexibility and oxidative capacity of skeletal muscle mitochondrial are early, but dinstict defects in the course of peripheral insulin resistance and diabetes. Our genetic analysis identified an association of a polymorphism in the *FTO* gene with lower VO_2max levels, underlining the additional role of inherited parameters in mitochondrial function. The observed associations of these mitochondrial parameters with insulin sensitivity were only abolished after adjustment for circulating FFA in multiple regression analysis models, thus underpinning the important contribution of lipidemia to mitochondrial impairment and inhibition of insulin

signaling with subsequent impairment of insulin sensitivity.

Second, the sphingolipid analysis confirmed elevation of several species in insulin resistance and NAFLD, but also identified specific hepatic sphingolipids, such as ceramide 16:0, dihydroceramides and lactosylceramides that specifically relate to hepatic oxidative stress and hepatic inflammation. From these data we conclude that these species contribute to deterioration of insulin sensitivity and progression from simple hepatic steatosis to inflammatory liver disease in humans.

Taken together, these studies underline the deleterious role of lipidemia on the progression of insulin resistance and impairment of mitochondrial function, not only in skeletal muscle, but also at the level of the liver, where inflammation and ROS production may lead to NAFLD progression. These studies have clinical implications suggesting that therapeutic and preventive interventions should aim at reduction of lipid availability and improvement of mitochondrial function.

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