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The correlation of liver fat content and insulin resistance with skeletal muscle energy metabolism

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“Der Zweifel ist der Beginn der Wissenschaft.

Wer nichts anzweifelt, prüft nichts.

Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind“

Teilhard de Chardin

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SUMMARY

Skeletal muscle insulin resistance has been implicated in the development of fatty liver and dyslipidemia by changing the partitioning of postprandial substrate fluxes. On the other hand, insulin resistance may be due to reduced mitochondrial function. We examined the association between mitochondrial activity and insulin sensitivity of skeletal muscle and steatosis in a larger human cohort.

We analyzed muscle mitochondrial activity from ATP synthase flux (fATP) and ectopic lipids in skeletal muscle and liver by multinuclear magnetic resonance spectroscopy from 113 volunteers with and without diabetes. Insulin sensitivity was assessed from M-values using hyperinsulinemic-euglycemic clamps, and/or from the OGIS using oral glucose tolerance tests (OGTT).

Muscle fATP correlated negatively with hepatic lipid content and degree of glycemic as assed from hemoglobin A1c (HbA1c). After model adjustment for study effects and other confounders, fATP showed a strong negative correlation with hepatic lipid content and a positive correlation with insulin sensitivity and fasting C-peptide. The negative correlation of muscle fATP with age, HbA1c and plasma free fatty acids was weakened after adjustment. Of note, body mass, muscle lipid contents, plasma lipoproteins and triglycerides did not associate with fATP.

The association of impaired muscle mitochondrial activity with hepatic steatosis supports the concept of a close link between altered muscle and liver energy metabolism as early abnormalities promoting insulin resistance.

ZUSAMMENFASSUNG

Die Insulinresistenz der Skelettmuskulatur beeinflusst die Verteilung der postprandialen Substratzufuhr. Dadurch soll die Insulinresistenz an der Entstehung von Fettleber und Dyslipidämie beteiligt sein. Andererseits gibt es Hinweise, dass Insulinresistenz durch verminderte mitochondriale Aktivität induziert wird. Diese Studie untersucht das Verhältnis von mitochondrialer Aktivität zur Insulinresistenz sowie zur Fettleber in einer größeren Menschenkohorte.

Die muskuläre mitochondriale Aktivität, gemessen als ATP-Synthase Flussrate (fATP) und die im Skelettmuskel und Leber gespeicherten Triglyzeride wurden mit der Magnetresonanzspektroskopie (MRS) bei 113 Menschen mit oder ohne Diabetes bestimmt. Die Insulinsensitivität wurde durch den M-Wert, der aus dem hyperinsulinämisch-euglykämischen Clamp-Test erfasst wird, und den OGIS, der aus dem oralen Glukosetoleranz-Test resultiert, erhoben.

Muskuläre fATP korreliert negativ mit dem Fettgehalt der Leber und dem HbA1c-Wert. Nach Adjustierung für Studieneffekte und weitere Faktoren zeigte fATP einen engen Zusammenhang mit dem Fettgehalt der Leber. Die negative Korrelation zwischen fATP und Alter, HbA1c und freien Fettsäuren im Plasma wurde durch die Adjustierung abgeschwächt. Darüber hinaus zeigte sich weder ein Zusammenhang von fATP mit BMI, noch mit IMCL, Lipoproteinen im Plasma und Triglyzeriden.

Die enge Korrelation von verminderter mitochondrialer Aktivität und Fettgehalt der Leber unterstützt die These, dass verminderter Muskel- und Leberstoffwechsel als frühe Veränderungen die Entwicklung von Insulinresistenz fördern.

ABBREVIATIONS

AACE	American Association of Clinical Endocrinologists	**JNK1**	Junk-kinase1
ADA	American Diabetes Association	**Ln**	Natural logarithm
AHA	American Heart Association	**Log**	Logarithm
ALT	Alanine Aminotransferase	**M-value**	Index for whole body glucose metabolism
AST	Aspartate Aminotransferase	**mmHg**	Millimeters of mercury
ATP	Adenosine triphosphate	**Mr-value**	Calculated index for whole body glucose metabolism
ATPIII	Adult treatment Panel III	**mRNA**	Messenger Ribonucleic acid
BMI	Body mass index	**MRS**	Magnetic resonance spectroscopy
β	Beta-Coefficient	**NAFL**	Non-alcoholic fatty liver
°C	Degree Celsius	**NCEP**	National Cholesterol Education program
^{13}C	Carbon-13	**NHLBI**	National heart, lung and blood institute
ChREBP	Carbohydrate responsive element binding protein	**OGIS**	Oral Glucose Insulin Sensitivity
CoA	Coenzyme A	**OGTT**	Oral glucose tolerance test
CPT1	Carnitine palmitoyl transferase 1	**^{31}P**	^{31}Phosphate
CVD	Cardiovascular disease	**PAI I**	Plasminogen activator inhibitor
DAG	Diacyglycerol	**PCr**	Phosphocreatine
DNA	Deoxyribonucleic acid	**PGC1α**	Peroxisome proliferator-activated receptor-γ coactivator 1-α
fATP	unidirectional flux through ATP synthase, fATP synthase flux	**PKC**	Protein kinase C

FFA	Free fatty acids	**QUICKI**	Quantitative insulin sensitivity check index
G6P	Glucose-6-Phosphate	**r**	Correlation coefficient
GDM	Gestational diabetes mellitus	**ROC**	receiver operating characteristic
GLUT	Glucose transporter	**ROS**	reactive oxygen species
HbA1C	Hemoglobin A1c	**SD**	Standard deviation
HCL	Hepatocellular Lipid	**SOCS3**	Suppressor of cytokine signaling 3 protein
HDL	High density lipoprotein	**SREBP-1c**	Sterol regulating element binding protein 1c
HOMA	Homeostasis model assessment	**T1DM**	Type 1 diabetes mellitus
IFG	Impaired fasting glucose	**T2DM**	Type 2 diabetes mellitus
IGT	Impaired glucose tolerance	**TG**	Triglyceride
IKK	Inhibitor of Kappa B-Kinase	**VLDL**	Very low density lipoprotein
IMCL	intramyocellular lipid	**VO_2max**	Whole body maximal oxygen consumption
IR	Insulin receptor	**WHO**	World Health Organization
IRS	Insulin receptor substrate		

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1 INTRODUCTION

The prevalence of type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) is steadily increasing [2]. The metabolic syndrome is a major risk factor for CVD. The World Health Organization (WHO), American Association of Clinical Endocrinologist (AACE), National Cholesterol Education program (NCEP) European Group for Studying Insulin Resistance (EGIR) and International Diabetes Federation (IDF) include in their definition of the metabolic syndrome either glucose intolerance or insulin resistance. Insulin resistance seems to be involved also in the development of hepatic steatosis and dyslipidemia and is associated with alterations of muscle mitochondrial function.

1.1 The metabolic syndrome

The metabolic syndrome describes different combinations of risk factors for CVD. CVD includes a number of abnormalities: coronary heart disease, high blood pressure, and heart failure. According to the WHO, CVD is the leading cause of death in the whole world [3]. From 2006 to 2015, CVD is estimated to increase by 17 % [2]. This shows the importance of the metabolic syndrome and necessity to prevent CVD.

In 1988, Reaven *et al.* reviewed that patients who develop CVD have several risk factors that commonly cluster together and named the combination of risk factors Syndrome X. It includes high blood pressure, dyslipidemia, obesity, and hyperglycemia [4]. Syndrome X was also called insulin resistance syndrome [4]. Today, it is mostly called metabolic syndrome, but causal interaction of the factors that would justify it as a syndrome has been repeatedly questioned and discussed.

Nowadays, there are different definitions of the metabolic syndrome. The following definition are widely accepted:

- WHO,
- NCEP,
- AACE,
- EGIR,
- IDF.

First of all, the WHO defined risk factors in 1998 [5]. This definition focuses on insulin resistance coexisting with two other risk factors to make the diagnosis. It is the only definition including microalbuminuria. The components defined by the WHO are:

- high blood pressure (≥140/90 mmHg)
- low high density lipoprotein (HDL) cholesterol [<35 mg/dl (0.9 mmol/l) in men, <39 mg/dl (1.0 mmol/l) in women]
- increased plasma triglycerides [≥150 mg/dl (1.7 mmol/l)]
- increased body mass index (BMI) (≥30 kg/m²)
- microalbuminuria (≥20 μg/min)
- mandatory: insulin resistance, glucose intolerance or T2DM

Second, the AACE defined following risk factors in 2003:

- high blood pressure (≥135/85 mmHg)
- low HDL cholesterol [<40 mg/dl (1.03 mmol/l) in men, <50 mg/dl (1.3 mmol/l) in women]
- increased triglycerides [≥150 mg/dl (1.7 mmol/l)]
- increased body mass index (BMI) (≥25 kg/m²)
- increased fasting glucose (between 110 and 126 mg/dl), raised blood glucose 2 h after glucose load blood glucose (>140 mg/dl)
- other factors: family history (T2DM, hypertension, CVD), polycystic ovary syndrome, sedentary lifestyle, advancing age, ethnic group

The AACE listed these risk factors, but did not define a certain number of risk factors needed to diagnose the metabolic syndrome [6].

The third one is the NCEP's Adult Treatment Panel III (ATP III). The definition lists seven components of the metabolic syndrome in 2004 comprising the following:

- high waist circumference (≥102 cm in men, ≥88 cm in women)
- increased triglycerides [≥150 mg/dl (1.7 mmol/l)]
- low HDL[<40 mg/dl (1.03 mmol/l) in men, <50 mg/dl (1.3 mmol/l) in women]
- high blood pressure (≥130 mmHg systolic blood pressure or ≥85 mm Hg diastolic blood pressure)
- increased fasting glucose (≥110 mg/dl)

- proinflammatory state
- prothrombotic state

Three out of five components have to be present to diagnose the metabolic syndrome [7]. Furthermore, ATP III does not recommend an oral glucose tolerance test (OGTT) in patients without elevated fasting plasma glucose. The NCEP reports that the information gained by an OGTT is not cost-effective for diagnostic purposes [8]. This recommendation differs from the ones by AACE and WHO, where an OGTT is recommended [5, 6].

The EGIR released their definition of the metabolic syndrome in 1999.

- Insulin resistance

Insulin resistance is mandatory to diagnose the metabolic syndrome. Furthermore, two or more of the following need to be present [9]:

- Central obesity: waist circumference (male ≥94cm, female ≥80cm)
- high TG (≥2.0 mmol/l)
- low HDL-C (<1.0 mmol/l)
- Hypertension: blood pressure (≥140/90 mmHg)
- Fasting plasma glucose (≥6.1 mmol/l)

The definition of the IDF was updated in 2005 and now comprises five features. For its diagnosis the following component is required [10]:

- high waist circumference (male >94 cm, female >80 cm).

And two of the following conditions:

- high TG [≥150 mg/dl (≥1.7 mmol/l)]
- low HDL-cholesterol [male <40 mg/dl (1.03 mmol/l), female 50 mg/dl (1.29 mmol/l)]
- high blood pressure (>130/85 mmHg)
- high fasting glucose [>100 mg/dl (5.6 mmol/l)].

In 2004 the American Heart Association (AHA) and the National Heart, Lung, and Blood Institute (NHLBI) reported that patients with metabolic syndrome have a higher risk for

T2DM than healthy humans [8]. This differs to the definition of the ATPIII and AACE, that included glucose abnormalities. However, it is not known yet which risk factors of the metabolic syndrome directly cause the development of overt T2DM [11].

Abdominal obesity is strongly related to many metabolic risk factors. These include hypertension, high serum cholesterol, low HDL cholesterol, and hyperglycemia [8]. Adipose tissue releases free fatty acids (FFA) and adipocytokines such as plasminogen activator inhibitor 1 (PAI-1). These products aggravate the metabolic risk factors while the adipokine, such as adiponectin, seems to be protective.

For some investigators [12, 13], insulin resistance is the main cause of the metabolic syndrome because it influences or even induces the main metabolic risk factors. However, Grundy *et al.* predict that it is complicated to identify the unique role of insulin resistance since it is associated with obesity. Nevertheless, they do not deny a significant role of insulin resistance. For example, hyperinsulinemia raises circulating triglycerides by enhancing the output of very low density lipoproteins (VLDL) [8]. In addition, insulin resistance in skeletal muscle advances artherogenic dyslipidemia by increasing the *de novo* lipogenesis in liver [14]. Some even suggest that treating insulin resistance in skeletal muscle by reversing defects in insulin-stimulated glucose transport might be the best way to prevent the development of the metabolic syndrome [14].
But there is no clinical evidence yet that the treatment of insulin resistance improves the other components of the metabolic syndrome. Also, the mechanism behind the association of insulin resistance and most of the components of the metabolic syndrome are still obscure [15].
Furthermore, the prevalence of the metabolic syndrome rises with age. This shows that age also influences the development of the metabolic risk factors [16].

Moreover, independent factors seem to mediate specific risk factors of the metabolic syndrome [8]. The remaining factors are regulated by acquired and genetic factors, as well as inter-dependent mechanisms.
Dyslipidemia is related to obesity and insulin resistance and additionally contributes to CVD. Also, blood pressure is a strong predictor of CVD and is therefore often caused by obesity [8].

Other factors that have been shown to be risk factors for CVD, namely plasma free fatty acids and hepatic steatosis, are yet not part of the "metabolic syndrome concept". Of note,

the hepatic steatosis might contribute to both, the development of whole body insulin resistance and the increased output of artherogenic lipoproteins. Thus, the interaction of liver and muscle energy metabolism seems of pivotal interest to identify novel targets for the prediction and therapy of insulin resistance and CVD.

1.2 Insulin resistance

Insulin resistance is a condition in which cells do not respond efficiently to insulin stimulation. Insulin resistance mainly affects skeletal muscle, hepatic, adipose tissues and all tissues with insulin reception.

1.2.1 Prediabetes and T2DM

T2DM is the most frequent form of diabetes mellitus. About 90 % of all diabetes mellitus patients have T2DM. The incidence of T2DM is the highest between 40 and 60 years. Nevertheless, T2DM incidence at younger age is becoming more common [17]. Prediabetes is a precursor of T2DM and comprises impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) [6].

T2DM is often companioned by complications. Complications of T2DM are due to macrovascular and microvascular damage. Macrovascular complications are ischemic heart disease, stroke and peripheral vascular disease. Microvascular complications comprise retinopathy, nephropathy and neuropathy.

The American Diabetes Association (ADA) has defined T2DM, IFG and IGT in 2003 [6]. IFG is diagnosed when the plasma glucose is between the normal fasting rate [≥5.5 mmol/l (100 mg/dl)] and the diagnostic cut off for diabetes [<7 mmol/l (126 mg/dl)]. IGT is diagnosed when plasma glucose concentration is ≥7.8 mmol/l (140 mg/dl) 2 h after a 75-g oral glucose load, but lower than the diagnostic cut off for T2DM, <11.1 mmol/l (200 mg/dl). The definition also includes fasting plasma glucose which has to be lower than 7.0 mmol/l (126 mg/dl). T2DM is determined as fasting plasma glucose concentrations higher than or equal to 7.0 mmol/l (126 mg/dl) and/or higher than 11.1 mmol/l (200 mg/dl) 2 h after ingestion of 75 g glucose [6].

The ADA definition of IFG was based on a ROC curve analyses, which includes data of four studies (Pima Indian, Mauritius, San Antonio and Hoorn). It identified the baseline fasting plasma glucose level, which showed the highest sensitivity and specificity for

prediction of diabetes over a period of 5 years. The result was that the cut off points for fasting glucose were changed from 6.1 mmol/l (110 mg/dl) to 5.5 mmol/l (100 mg/dl) [18].

A common characteristic of prediabetes and T2DM is the insulin resistance of skeletal muscle, adipose tissue and liver. IFG is a risk factor for T2DM. It is mainly caused by impaired suppression of hepatic glucose output and impaired insulin secretion [5]. IGT is a risk factor for T2DM and is also associated with increased risk of CVD [19]. There is evidence that IGT is also related to skeletal muscle insulin resistance and impaired insulin secretion [20].

In a recent study, persons with IGT were observed over a time period of 11 years. About 30 % of them developed diabetes, 35 % persisted with IGT, 5 % changed to IFG and 30 % reverted to normal glucose tolerance [21]. Another study showed that humans with IGT have a 6-times higher relative risk for developing T2DM than persons with normal glucose tolerance. Persons with IFG have a relative risk of 4.7 times for developing T2DM. Humans who have IFG and IGT have an even greater risk of 12 times. Moreover, persons with IGT have an increased relative risk of all-cause mortality that is 1.48 times compared to the risk of healthy humans. A 1.66 times higher relative risk of serious cardiovascular outcome has also been reported. Humans with IFG have a lower risk, but still higher than glucose tolerant humans, with the relative risk for cardiovascular events and all-cause mortality ranging from 1.19-1.28 compared to healthy humans [22].

1.2.2 Pathogenesis of insulin resistance

In insulin sensitive humans, insulin stimulates glycogen storage in skeletal muscle and the liver, promotes skeletal muscle glucose uptake and inhibits endogenous glucose production in the liver. Furthermore, insulin inhibits lipolysis in adipose tissue.

Insulin interacts with the insulin receptor b subunit of muscle cells and adipose cells. The insulin receptor b subunit is a tyrosine kinase. It phosphorylates tyrosine of various proteins including insulin receptor substrate (IRS) itself [23]. The activated IRS stimulates downstream molecules, such as phosphatidylinositol-3-kinase (PI3K). PI3K is a key signal transducer for the insulin-mediated glucose transport through glucose transporter 4 (GLUT 4) into the muscle cell membrane [24] and into adipocyte. GLUT 2 shuttles glucose into hepatocytes. This transporter is independent of insulin.

During postprandial hyperinsulinemia, the insulin sensitive skeletal muscle takes up the majority of blood glucose. In insulin resistant individuals the influx of glucose into skeletal muscle cells is reduced [25]. Instead, the liver takes up more glucose according to the higher abundance, but increases the depletion of glycogen and increases gluconeogenesis, thereby promoting hyperglycemia. Insulin resistance also decreases muscle glycogen storage and induces *de novo* lipogenesis in the liver after carbohydrate ingestion. This could increase VLDL production and in turn raises plasma levels of triglycerides and low density lipoprotein (LDL) cholesterol [14]. Additionally, lipolysis is induced in adipose tissue leading to increased plasma levels of FFA [26]. All these metabolic changes increase blood glucose and promote dyslipidemia. Hypercaloric nutrition and a lack of exercise amplify these processes. During high FFA supply in plasma, lipid tissue, liver [hepatocellular lipid (HCL)] and muscle cells [intramyocellular lipid (IMCL)] are taking up lipids and store them as TG [27].

The underlying cellular mechanisms might be that a high glucose concentration in liver activates the carbohydrate responsive element-binding protein (ChREBP), which induces lipogenesis. Increased lipogenesis causes high generation of malonyl-coenzyme-A (malonyl-CoA) which inhibits the carnitine palmitoyl transferase I (CPT-I) [28]. CPT-I is an enzyme, which controls the access of FFA to the mitochondria. As a result, less FFA are transported into the mitochondria and more triglycerides are stored as HCL [29].

Research on the effects of high FFA plasma levels on β-oxidation in skeletal muscle showed controversial results. On the one hand, Holloway *et al.* suggest that increased plasma FFA impairs the lipid induced peroxisome proliferation-active receptor (PPAR) - peroxisome proliferation-activated receptor gamma coactivator 1 alpha (PGC1α) interaction. The PPA-PGC1α interaction takes part in the regulation of β-oxidation in insulin sensitive humans. They concluded that high FFA plasma levels reduce β-oxidation [30].

On the other hand, Koves *et al.* showed that FFA increase the rate of β-oxidation in mitochondria induced by elevated PPAR levels and increased substrate supply. When rates of β-oxidation are inadequately higher than rates of the tricarboxylic cycle activity due to lower glucose uptake and glycolysis rates, this higher rate can be accompanied by incomplete fat oxidation. This leads to intramuscular accumulation of acylcarnitines and increased export of acylcarnitines into the circulation. However, it is yet unclear whether these compounds are effective mediators of insulin resistance or solely inactive

biomarkers. As a result of inefficient β-oxidation, intracellular lipid metabolites such as acyl-CoA accumulate also [29]. A high concentration of acyl-CoA leads to synthesis of triglycerides, which are stored as IMCL. Furthermore, other lipid metabolites such as diacyglycerol (DAG) and ceramides accumulate [27]. It is known that DAG and ceramides via protein kinase C phosphorylate the serin/threonin of insulin receptor substrates, so that insulin signaling is impaired [31]. Furthermore, accumulated acyl-CoA are believed to stimulate reactive oxygen species (ROS) generation. ROS have a toxic effect and thereby damage cellular structures such as mitochondria [32]. The capacity of β-oxidation may decrease secondary to this lipid-induced damage of mitochondria. This may lead to a *circulus vitiosus:* more accumulation of lipids that raises ROS, which in turn damage mitochondria [31]. This is amplified by the fact that insulin activates sterol regulatory element-binding protein 1c (SREBP-1c), PPARγ and transcription factors for enzymes of the lipogenesis (acetyl-CoA carboxylase and free fatty acid synthase). As a result, *de novo* synthesis of FFA and triglyceride further increases [29, 33].

Previous studies showed the effect of increased lipid availability over different time periods. Triglyceride infusion caused insulin resistance in insulin sensitive humans [34]. In these studies, the flux through adenosine triphosphate synthase (fATP) and the concentration of glucose-6-phosphate (G6P) was assessed by magnetic resonance spectroscopy (MRS). Triglyceride infusions for 3 h had no effect on the mitochondrial activity in participants, but it decreased the glucose disposal [35]. Lipid infusions for 6 h lowered the mitochondrial membrane potential. The membrane potential is necessary to create the electromotive driving force for the adenosine triphosphate (ATP) synthesis, so it impairs the mitochondrial activity [36]. Lipid infusions for 48 h decreased the expression of nuclear encoded mitochondrial genes and PPARγ coactivator 1 (PGC1) [37].

1.2.3 Relationship between insulin sensitivity and muscle mitochondrial function

Using MRS, muscle mitochondrial activity can be assessed *in vivo* during fasting and during other metabolic conditions (see below). Thereby, it has been shown that muscle mitochondrial activity of healthy humans can adapt to different metabolic conditions. This is referred to as mitochondrial plasticity [38, 39]. Accordingly, mitochondrial activity increases up to 90 % after 1 h to 2 h, and oxidative capacity up to 42 % after 8 h during a hyperinsulinemic-euglycemic clamp [40-44].

Two mechanisms might promote mitochondrial plasticity:
The first mechanism is that expression and activation of key enzymes of the respiratory chain is raised. These enzymes increase mitochondrial activity. The second mechanism describes that the high energy demand [high adenosine diphosphate (ADP): ATP ratio] or energy supply due to insulin-stimulated glucose uptake raise mitochondrial activity [38].

However, in insulin resistant humans the mitochondrial activity or oxidative phosphorylation is not increased during a hyperinsulinemic-euglycemic clamp. This was observed in patients with overt T2DM and their first degree relatives as well as in patients with type 1 diabetes mellitus (T1DM) [40-44, 46] and healthy humans after a 6 h lipid infusion [43]. This might affect postprandial substrate oxidation in skeletal muscle and might promote storage of fuels in adipose tissue but also ectopically in skeletal muscle and the liver. Thus, cellular concentrations of lipid metabolites that induce insulin resistance increase. Likewise, insulin resistance correlates with reduced mitochondrial oxidative capacity in myocytes [27].

Moreover, it has been shown that insulin resistant humans have reduced mitochondrial content [47-50] and/or impaired oxidative capacity [51-53]. Participants with T2DM have a reduced *in vivo* sub maximal ADP-stimulated oxidative phosphorylation in skeletal muscle compared to insulin sensitive humans [49, 51]. Impaired function and/or reduced density of mitochondria might cause the decreased fATP of overt T2DM and their first-degree relatives [41, 51, 54]. Furthermore, fATP is an index for ATP production via nutrient oxidation according to prevalent metabolic conditions such as fasting or hyperinsulinemia [55]. The impaired adaptation of the ATP synthesis rate could be an early indicator of muscular insulin resistance and metabolic inflexibility [38].

One reason for the impaired adaptation function and/or reduced capacity of mitochondria could be damage of the mitochondrial deoxyribonucleic acid (DNA). Increased lipid availability in skeletal muscle induces oxidative stress via ROS and down regulation of antioxidative enzymes [56-58]. This causes damage to mitochondrial DNA.

Another reason for decreased mitochondrial action could be the reduction of substrate supply. Bonadonna *et al*. described a relationship between insulin resistance and decreased glucose transport, phosphorylation and reduced insulin-stimulated glycogen synthesis in skeletal muscle cells of humans with T2DM [59]. Glucose transport and/or

phosphorylation control the extent of myocellular insulin-stimulated glucose disposal and the glycogen synthesis in humans with T2DM [60]. Later, these results were extended to first degree relatives of patients with T2DM and obese patients [39]. The diminished glucose transport and phosphorylation lead to a slower and smaller increase of myocellular G6P compared to insulin sensitive humans [60]. The lower substrate supply was assumed to prevent the increase in ATP synthesis. An adequate substrate supply is necessary to suit the insulin stimulated energy demand [61]. Nevertheless, it was shown that even double raised substrate supply of G6P during hyperinsulinemic-hyperglycemia clamps could not raise mitochondrial activity in patients with T2DM [41]. According to this, the oxidative phosphorylation capacity *ex vivo* under conditions of excess of substrate was reduced. In conclusion, substrate availability is unlikely to cause the impaired adaptation of insulin stimulated mitochondrial activity [40].

However, it was demonstrated that FFA impair muscle glucose transport and phosphorylation [62, 63]. Plasma FFA are often increased in obese people and persons with T2DM compared to healthy humans [64]. This might explain insulin resistance in obese humans and insulin sensitive persons during high fat diet [62, 63]. This could be explained by the following hypothesis. Impaired lipid oxidation combined with increased lipid availability leads to higher accumulation of lipid intermediates and IMCL [54, 55, 65]. Lipid intermediates are DAG, acyl-CoAs or ceramides. The lipid intermediates might activate atypical protein kinase C (PKC) isoforms, which phosphorylate inhibitory serine binding sites of IRS1. This induces impaired insulin signal transduction, which leads to insulin resistance [63, 66-69].

An oversupply of lipids also induces stimulation of inflammatory pathways [63]. The inflammatory kinases [Inhibitor of kappa B-kinase-β (IKK-β), jun-kinase-1 (JNK-1) and suppressor-of-cytokine-signalling-3-protein (SOCS-3)] also phosphorylate inhibitory serine of IRS-1. Therefore, these directly inhibit the glucose transport into the muscle cells and glucose phosphorylation [11].

1.2.4 Relationship between hepatic mitochondrial function and hepatic steatosis

About 70 % of obese humans and patients with T2DM have hepatic steatosis [70]. Ten to 20 % of humans with hepatic steatosis develop steatohepatitis [71]. Hepatic steatosis, also called non-alcoholic fatty liver (NAFL), is defined as liver containing more than 5.5 % fat. This accounts for women who consume less than 20 g of alcohol and men who

consume less than 40 g of alcohol per day [17]. Hepatic steatosis is accompanied by insulin resistance, decreased activity of the respiratory chain enzymes and alterations of the morphology of mitochondria [72]. Muscle insulin resistance has been suggested to predispose for NAFL and artherogenic dyslipidemia [73]. However, the impact of the interaction between liver and muscle metabolism for the development of T2DM is yet unknown.

Some studies have shown that elevated plasma FFA, high glucose and insulin levels activate lipogenic transcription factors (SREBP-1c, ChREBP) and lipogenic enzymes (acetyl-coA-carboxylase, fatty acid synthase). These lipogenic transcription factors and lipogenic enzymes stimulate the *de novo* synthesis of FFA and triglycerides. FFA and triglycerides accumulate as HCL in the liver or are secreted as VLDL into the bloodstream [29, 33]. Short-chain FFA are supplied to ß-oxidation [74]. Lipid metabolites (DAG, ceramides and lipid peroxide) and proinflammatory cytokines inhibit the insulin cascade [75]. A reason for the alterations of the morphology of liver mitochondria might be an elevated concentration of ROS and lipid peroxides, which affect the mitochondria [27]. *In vivo* studies showed that participants with steatosis had a reduced ability to recover ATP reserves in the liver, which is a measurement for oxidative capacity [76]. It has been shown that the liver phosphorus metabolites as well as hepatic fATP relate to steatosis and insulin resistance at the level of the liver and skeletal muscle [77, 78]. These studies support the concept that the interaction between liver and muscle energy metabolism contributes to the development of insulin resistance.

1.3 Assessment of insulin sensitivity

To assess insulin sensitivity there are different clinical tests. The Oral glucose tolerance test (OGTT) is a test which is easily practiced in clinical settings. Therefore, the hyperinsulinemic-euglycemic-clamp test is the gold standard for measuring insulin resistance.

1.3.1 Oral glucose tolerance test (OGTT)

The OGTT is a non-steady state measurement to determine changes in plasma insulin and glucose concentration during glucose loading.

At the beginning the test, humans who are fasted for 8 to 12 h have to drink a solution with 75 g glucose (normally dextrose diluted in 300 ml water). Then, blood samples are

drawn at intervals of 15 or 30 min during the next 2 to 3h. Plasma glucose of an insulin sensitive participant would increase in the first hour, followed by a decrease returning to the fasting plasma glucose level in 2 to 3 h from the beginning.

To evaluate the results, there are three indices to describe insulin sensitivity: ISI-COMP, MCRest and oral glucose insulin sensitivity (OGIS). All three of them correlate well with the hyperinsulinemic-euglycemic clamp. We will concentrate on OGIS:

$$OGIS = f(FPG, PG_{90}, PGG_{120}, FPI_b, PI_{90}, D)$$

PG and PI are plasma glucose and plasma insulin concentration at the time determined by the subscript. D is the oral glucose dose (g/m^2 body surface area). The function f is complex and can be viewed at http://www.isib.cnr.it/bioing/ogis/home.html. ($r=0.73$, $p<0.0001$ with clamp-derived data) [79].

1.3.2 Hyperinsulinemic-euglycemic clamp test

The hyperinsulinemic-euglycemic clamp is the gold standard for measuring the whole body insulin sensitivity. This technique is a steady state measurement and is a more complex test [80].

At first, a priming dose of insulin is infused intravenously for 5 to 15 min. After the first insulin doses, the participants reach a hyperinsulinemic plateau. Thereafter, a continuous infusion of about 40 $mU.min^{-1}.m^{-2}$ body surface area [81] insulin is necessary to uphold the hyperinsulinemic plateau. There are also protocols using higher insulin doses (i.e. 80 $mU.min^{-1}.m^{-2}$ body surface area). The infused insulin causes a glucose uptake in different tissues. This promotes glycogen storage, while oxidation rates increase and plasma glucose decreases. The plasma glucose is maintained within the euglycemic range (90 to 100 mg/dl). To prevent the plasma glucose from dropping below 90 mg/dl the investigator infuses either 10 % or 20 % glucose solution with varying rates adjusted to changes of the blood glucose levels. During the test the plasma glucose is monitored at 5 to 10 min intervals [81, 82].

1.4 Markers of insulin sensitivity

To evaluate insulin sensitivity, different markers where developed. There is the fasting insulin blood concentration, homeostasis model assessment (HOMA), quantitative

insulin sensitivity check index (QUICK), TG/HDL, the OGIS and the Index for whole body glucose metabolism (M-value).

1.4.1 Fasting insulin

Fasting insulin concentration is a crude surrogate marker for insulin sensitivity, which can be easily used in clinical practice [83]. Although fasting insulin levels correlate quite well with insulin resistance, its value for assessing insulin sensitivity is limited. It is important that the blood sample is taken after sufficient overnight fasting so that the rapidly changing glucose levels over the course of the day cannot disarrange the measure of insulin [84]. One problem is imperfect standardization of insulin assays. ADA Task Force implements a certification by a central laboratory for standardization of the assay of fasting insulin [85]. A disadvantage of fasting insulin as surrogate marker for insulin resistance is that it does not bestride low insulin secretion during hyperglycemia in glucose-intolerant subjects [84]. Furthermore, there are limitations because of high rates of false-positive results. Taking into consideration all of these limitations, only high values of plasma fasting insulin suggest insulin resistance may predict the development of T2DM [86].

1.4.2 Homeostasis model assessment (HOMA)

The homeostasis model assessment (HOMA) is a current index to estimate insulin resistance in the fasting state. It is calculated from insulin or C-peptide concentrations and fasting plasma glucose [87]. The HOMA-R is given as the product of fasting insulin concentration times fasting glucose concentration divided by the constant k. HOMA-R is expressed as percentage of the normal level [88]. This model rests upon the extension of fasting glycemia, which is determined by combined ß-cell function and extent of insulin resistance [89]. The HOMA-R cannot be used in patients with lack of insulin secretion, uncontrolled diabetes and those who take drugs directly affecting insulin secretion.

The coefficients of variation of HOMA are ~30 % indicating rather poor reproducibility, but can be somewhat improved when specific insulin assays are used and when applied to larger cohorts [90, 91].

1.4.3 Quantitative insulin sensitivity check index (QUICKI)

The QUICKI is also an index to estimate insulin sensitivity [92]. A study with 22 participants resulted in the equation:

$$\text{QUICKI} = [1/(\log(\text{fasting plasma insulin}) + \log(\text{fasting plasma glucose})]$$

Another study approved the strong correlation with the clamp-derived measure of insulin sensitivity ($r=0.78$, $p<10^{-12}$) [92]. Furthermore, QUICKI is more precise than HOMA over a wide range of insulin sensitivities. The revised QUICK index also considers plasma FFA levels and shows an even stronger correlation with hyperinsulinemic-euglycemic clamp ($r=0.86$, $P<0.0001$) [93, 94].

1.4.4 Ratio of triglycerides to high density lipoprotein

The ratio of TG/HDL has been discussed to be a surrogate marker for insulin resistance. In one study it was investigated as a marker for insulin resistance as defined by fasting insulin concentration and as a predictor of insulin resistance as measured by the HOMA index. The study results showed that TG/HDL is an acceptable marker and predictor for insulin resistance in Caucasians but a poor marker and predictor for insulin resistance in African Americans [95].

1.4.5 Oral glucose insulin sensitivity (OGIS)

The OGIS is a marker for insulin resistance drawn from the OGTT. It includes the glucose and insulin concentrations and the time indicated by the subscript and the oral glucose dose which was applied during the OGTT. The OGTT has some limitations: variable rates of gastric emptying and variable release of gastrointestinal hormones, mainly the incretins [79].

1.4.6 M-Value

The M-value [M, $mg.kg^{-1}.min^{-1}$] is also a marker for insulin resistance. It is in an indicator for the whole body glucose disposal. It is measured during a hyperinsulinemic-euglycemic clamp, which is the gold standard measure of the whole body insulin sensitivity. The average glucose infusion rate over a time period of 100 to 120 min after start of hyperinsulinemia is defined as M-value [80]. Values below 4.7 $mg.kg^{-1}.min^{-1}$ indicate insulin resistance [81].

1.5 Assessment of mitochondrial function and ectopic lipid deposition

Noninvasive measurement of mitochondrial function is done by phosphorus MRS (^{31}P MRS), while proton (H^1) MRS can be used to quantify ectopic lipid disposition in skeletal muscle and liver.

1.5.1 Assessment of mitochondrial function

^{31}P MRS is a non-invasive *in vivo* technique to measure biochemical fluxes and concentrations of phosphorus metabolites [39]. The ^{31}P MRS also allows to determine phosphocreatine kinetics following exhaustive exercise and thereby to assess the maximal mitochondrial oxidative capacity *in vivo* [96]. It is also possible to measure the fATP directly by using the magnetization saturation transfer experiment. This is managed by the measurement of the exchange between inorganic phosphate (Pi) and γ-ATP [97, 98]. In this process, the steady state Pi magnetization is quantified with selective continuous wave irradiation of the γ-ATP resonance. This has to be compared with the magnetization of a field with the irradiation placed symmetrically downfield from the Pi frequency. The fractional reduction in Pi magnetization is a consequence of the exchange of saturated γ-ATP with non-saturated Pi nuclei. Adequate fractions of Pi magnetization are assessed by variable interpulse delay lengths. These fractions are needed to calculate the spin lattice relaxing time for Pi. The Forsen and Hoffman formula yields the unidirectional fATP (μmol.g muscle^{-1}.min^{-1}) [98]:

$$k1 = [(Mo - Mz)/Mo] \cdot (1/T1*),$$

While k1 is the pseudo-first-order rate constant M_o with the equilibrium P_i magnetization, M_z is the steady state P_i magnetization and T_1^* is the spin lattice relaxation time for P_i when ATP is saturated. The spectra measures the fractional reduction of P_i magnetization upon γATP saturation $(M_o\text{-}M_z)$ $/M_o$. This technique allows for the determination of suprabasal dynamic mitochondrial activity. Of note, oxidation and phosphorylation energy coupling can be measured by combined C^{13}/P^{31} MRS [54].

1.5.2 Assessment of ectopic lipid deposition

^{1}H-MRS was devolved to exactly quantify intracellular lipid disposition in liver and skeletal muscles *in vivo* [39]. This is more practical in studies than *in vitro* measurements because it is not invasive. During ^{1}H spectra analysis, ^{1}H peaks correspond to lipids.

These lipids are mainly methylene and methyl protons of TG acyl chains within muscle. The ^{1}H-MRS technique was validated against biochemical measurements [99, 100].

2 AIM OF THE STUDY

The aim of this study was to evaluate the correlation between muscle mitochondrial activity with insulin sensitivity, steatosis and parameters of the metabolic syndrome.

In this study, we formulated three hypotheses:

- Skeletal muscle fATP correlates positively with insulin sensitivity, postulating that intact muscle mitochondrial function allows for adequate lipid oxidation and thereby improves insulin sensitivity or *vice versa*.
- Lower skeletal muscle fATP relates to components of the metabolic syndrome, assuming that impaired muscle mitochondrial activity shifts lipids towards hepatocytes and adipocytes, where they lead to lipid synthesis and increase body mass.
- Low skeletal muscle fATP relates positively to liver fat content, assuming that also lipid storage within hepatocytes results from lower muscle oxidative capacity.

To explore the role of the mitochondrial function we used fATP as measure of mitochondrial activity. The individual data of participants were collected out of six clinical experimental studies performed by the research groups of Michael Roden. The study benefits from using identical methodology applied by the same team, which allows for combined analyses.

3 METHODS

We collected individual data from six clinical experimental studies and analyzed them by univariate and multivariate linear (log-linear) regression analyses.

3.1 Participants

The data of participants included in our study were gathered out of six clinical experimental studies published between 2007 and 2011. Our collection includes 113 individuals, enough to achieve a representative cohort. All underwent measurement of fATP, HCL and IMCL within the studies [35, 41, 46, 101-103]. The number of participants, the methods and the glucose tolerance status of the individuals of each study are summarized in Table 1.

The first study [101] includes 14 volunteers, seven controls with normal glucose tolerance and seven participants previously suffering from acromegaly. They underwent an oral glucose testing (OGTT) to evaluate the insulin sensitivity (OGIS), ^{31}P MRS to quantify fATP in calf muscle and ^{1}H MRS for assessment of HCL and IMCL of tibialis anterior and soleus muscle. In addition, whole body oxidative capacity was quantified by indirect calorimetry and spiroergometry. The participants with a history of acromegaly showed a reduced insulin secretion, a lower oxidative capacity, impaired muscular ATP synthesis and higher liver fat content than controls. The authors draw the conclusion that regardless of the successful treatment of acromegaly, the alterations in ß-cells of pancreas and the production of ATP in skeletal muscle are reduced.

The second study [35] includes seven male glucose-tolerant volunteers without family history of T2DM. Before the experiment, the participants were on an isocaloric diet (60 % carbohydrate/20 % protein/20 % fat) for three days and did not perform any kind of exercise. Thereafter, they fasted overnight for 12 h. All volunteers underwent two experiments in a random crossover design. One was during saline infusions, the other during intravenous lipid infusion (Intralipid 20 %; Pharmacia & Upjohn, Vienna, Austria). In both experiments, the participants underwent a hyperinsulinemic-euglycemic clamp for assessment of insulin sensitivity (M-value), ^{1}H MRS for assessment of IMCL of soleus muscle and ^{31}P MRS to quantify fATP in calf muscle. The result of the study is that a 3 h lipid infusion does not affect fATP, but still reduces the whole body glucose uptake. The authors suggest that lipid induced insulin resistance seems to be caused by

mechanisms which decrease glucose uptake rather than by interaction of fatty acid metabolites with mitochondrial function.

The third study [102] was performed with 31 participants: 23 glucose tolerant women who had a history of gestational diabetes mellitus (GDM) and eight normal glucose tolerant women who have not had GDM during their pregnancies. All deliveries were four to five years before the experiments. They underwent an OGTT to determine insulin sensitivity (OGIS). To scale HCL and IMCL of soleus muscle, they conducted ^{1}H MRS. They measured fATP in skeletal muscle via ^{31}P MRS. Further, they used bio impedance analysis to consider the body fat mass. In the end, they did an indirect calorimetry to assess resting energy expenditure. In this study, women who had GDM exhibited higher hepatocellular lipids, lower insulin sensitivity in skeletal muscle and decreased ATP synthesis. The authors suggest that increased hepatic lipid storage is an early and cardinal sign of women after GDM.

The fourth study [46] involves eight humans suffering from type 1 diabetes mellitus (T1DM) with excellent glucometabolic control, 10 humans suffering from T1DM with poor glucometabolic control and 16 glucose tolerant volunteers. In this study, they measured myocellular fATP by ^{31}P MRS and HCL and IMCL of soleus muscle by ^{1}H MRS. They underwent hyperinsulinemic-euglycemic clamps to estimate insulin sensitivity from M-values. The authors found that non-obese patients with T1DM with good glycemic control are still at risk to develop insulin resistance. This study suggests that glucose toxicity could be the reason for a lower fATP, which is associated with insulin resistance.

In the fifth study [41] 30 participants volunteered: 10 young, lean and 10 elderly humans with normal glucose tolerance and 10 well controlled overweight patients with T2DM. The volunteers underwent hyperinsulinemic-euglycemic clamps, ^{1}H MRS to quantify IMCL of soleus muscle and HCL and ^{31}P MRS to measure fATP during fasting and hyperinsulinemia. They found that participants with well-controlled T2DM had lower fATP compared to lean healthy humans. This is caused by insulin sensitivity and lipid availability. Moreover, they had a reduction of insulin-stimulated glucose disposal but no abnormal glucose transport or phosphorylation. Insulin stimulated fATP was impaired only in T2DM but normal in elderly and in young lean healthy humans, suggesting the adaptation of muscle mitochondria to metabolic conditions is impaired in patients with T2DM, implying impaired mitochondrial plasticity[38].

The last study [104] includes 24 non-obese first degree relatives of T2DM, and 12 volunteers with normal glucose tolerance. OGTT, ^{1}H MRS to assess HCL and IMCL of soleus muscle and ^{31}P MRS to quantify fATP were performed. The participants went through the test at rest and 48 hours after three bouts of exercise. Furthermore, they went through exercise testing and their genes were examined for polymorphisms in genes regulating mitochondrial function. The authors conclude that some individuals benefit from the short-term exercise improving their insulin sensitivity and fATP, while there are non-responders who do not improve neither insulin sensitivity nor mitochondrial activity. The susceptibility to lifestyle intervention was determined by a specific SNP encoding for complex I of the mitochondrial chain, suggesting that the metabolic response to exercise training depends on the genetic constitution.

Table 1: Description of the participants collectives and the applied methods

Study	[41]	[101]	[43]	[104]	[46]	[102]
n	31	7	7	36	16	31
Patient collective	T2DM, controls	controls	controls	Relatives of T2DM, controls	controls	GDM, controls
Hyper-insulinemic-euglycemic clamp	+	-	+	-	-	-
OGTT	-	+	-	+	+	+
^{1}H MRS	+	+	+	+	+	+
^{31}P MRS	+	+	+	+	+	+

All studies are done in the same laboratory by the same probers and some are done in the same time. T2DM: Type 2 diabetes mellitus, GDM: Gestation diabetes mellitus, controls: normal glucose tolerance, n: number of participants, MRS: *In vivo* magnetic resonance spectroscopy. Patients with T1DM and previous acromegaly were excluded.

Table 1 shows an overview of the methods used in the respective studies as well as the numbers of participants and the patients collective.

The institutional ethics board of the medical university of Vienna authorized the study protocols and the subjects gave written informed consent after the nature and possible consequences of the studies had been explained to all participants (EK 258/2002, EK 178/2003).

3.2 Measures of insulin sensitivity

Some of these studies performed an OGTT and assessed the dynamic insulin sensitivity with the OGIS, as a measure of glucose clearance. This method has been validated against whole body insulin sensitivity assessed from the hyperinsulinemic-euglycemic clamp [105]. The other studies performed hyperinsulinemic-euglycemic clamps only obtained M-values during the last 30 min of the clamps to assess whole-body insulin sensitivity [41].

Thus, the participants of these six studies were subjected either to OGTT or to hyperinsulinemic-normoglycemic clamp. Thirty individuals had data from both, OGTT and hyperinsulinemic-normoglycemic clamp. The strong correlation between OGIS and M-value allows a reconstruction of M-value (Mr-value) from the OGIS [Figure 1]. Based on 170 data of individuals who underwent OGIS and hyperinsulinemic-euglycemic clamp Pacini *et al.* derived a formula for the calculation of the Mr-value[mg/min/kg] out of OGIS [in ml/min/m^2] data [1]:

$$\mathrm{Mr-value} = 0.023 \cdot \mathrm{OGIS} - 4.061$$

Comparing the true M-value with calculated Mr-value in 170 subjects, the true M-value has a mean value of 4.29±0.24 and the calculated Mr-value has a mean value of 4.26±0.18. The paired T-test leads to p=0.8663. The Bland-Altmann Plot showed that only 13 out of 170 subjects are external to the mean different ±1.96 standard deviation (SD). M-value and Mr-value correlate tightly with each other so that this equation was applied to the present analysis.

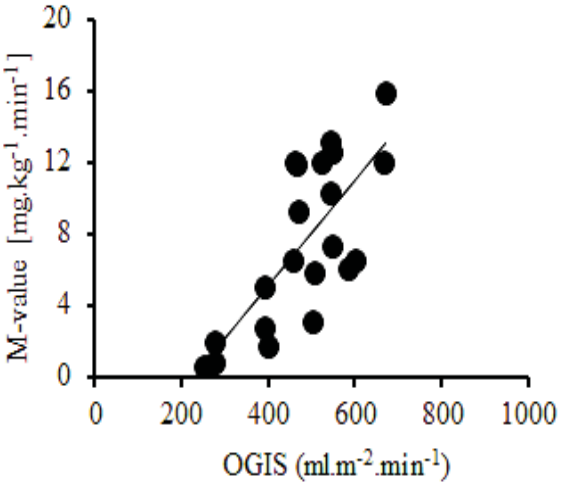

Figure 1: Relationship between oral glucose insulin sensitivity (OGIS) and index for whole body glucose metabolism (M-value)

3.3 *In vivo* magnetic resonance spectroscopy (MRS)

All studies used the MRS in which the individuals are lying supine inside a 3 Tela Medspec MR spectrometer (Bruker Biospin, Ettlingen, Germany). To assess fATP from exchange between Pi and ATP, they used ^{31}P-MRS with a surface coil positioned ~2 cm into the medial head of the right gastrocnemius muscle and the saturation transfer experiment [41, 43, 54]. Further, to measure HCL [106] and IMCL in soleus muscle [43] they employed ^{1}H-MRS.

3.4 Analytical procedures

Each study had the same analytical procedures. Plasma glucose was assessed by the glucose oxidase method (Glucose analyzer II, Beckman Coulter, http://www.beckmancoulter.com). Plasma free fatty acids (FFA) were assayed microfluorometrically in samples containing orlistat to prevent *in vitro* lipolysis (WAKO Chem USA Inc., http://www.wakousa.com) [43]. Plasma lactate was measured via enzymes (Roche, http://www.roche.com/home.html) [43]. Plasma insulin and C-peptide were assessed by commercial double antibody RIA [43].

3.5 Data collection

This study is a meta-analysis, and on the base of this study the formula for the Mr-value was developed. The data were collected from six clinical experimental studies, which were conducted in the research group of Michael Roden from 2007 to 2011. The major advantage of these clinical experimental studies resides in the use of identical technology and equipment in one single center.

3.6 Data processing

We defined inclusion and exclusion criteria for the data of the six clinical studies. Furthermore, we used the AACE definition of the metabolic syndrome, prediabetes and T2DM.

3.6.1 Inclusion criteria

General exclusion criteria for the six studies were:

- Age: 25-60 years
- BMI <30 kg/m^2 (requirement for MRS studies)
- Normal routine lab tests (blood cell count, kidney, liver, pancreas, thyroid and neuromuscular function)
- Availability within the area throughout the study
- Ability to understand and sign the consent forms
- No family history for controls

The analyses include participants who either had a normal glucose tolerance, prediabetes, or T2DM. All the participants had ^{1}H MRS and a ^{31}P MRS. Furthermore, they had either a hyperinsulinemic-euglycemic clamp or an OGTT. We only involved data that were acquired without or before intervention.

Further inclusion criteria were that all participants had to have following data completed: fATP, age, sex, BMI, and either OGIS or M-value.

3.6.2 Coding of variables

To define the parameters of the metabolic syndrome we used the definition of the AACE [6]. Our database contains all required parameters but blood pressure. Other definitions include parameters that our database does not contain.

- high blood pressure (>140 mmHg/90 mmHg)
- low HDL cholesterol (female <1.3 mmol/l, male <1.0 mmol/l)
- increased triglycerides (≥150 mg/dl)
- increased body mass index (BMI) (≥25 kg/m²)
- increased fasting glucose (IFG, IGT)

We did not include blood pressure to analyze the relationship between fATP and parameters of the metabolic syndrome. The reason is that blood pressure was just recorded in one study [103]. The number of participants with measured blood pressure is too small for the purpose of a statistical analysis.

The population was divided in three different glucose tolerance status. The first one is normal glucose tolerance, the second prediabetes and third T2DM. Normal glucose tolerance is defined as fasting glucose <100 mg/dl. Prediabetes can be divided into IFG and IGT. IFG is defined as fasting plasma glucose ≥100 mg/dl to 125 mg/dl [6]. IGT is determined as fasting plasma glucose <126 mg/dl and 2-h plasma glucose ≥140 mg/dl to199 mg/dl [6]. Participants with a fasting plasma glucose ≥126 mg/dl and/or a 2-h plasma glucose >200 mg/dl [6] or a known diagnosis of T2DM were designated as T2DM.

3.6.3 Exclusion criteria

General exclusion criteria for the six studies were:

- Overt diabetes mellitus
- Severe bone and joint diseases, myopathy, neuropathy, cardio-, cerebro- and peripheral vascular diseases, untreated atrial hypertension
- Regular heavy physical activity: history of exercise training during last 2 years
- Current smoking
- Current drug treatment
- Contraindications for MRS studies: claustrophobia and metallic implants
- Pregnancy

Further, for our analysis participants with T1DM are not included in the collective because of the different pathogenesis and different therapy. Also, previous acromegaly patients and patients after GDM are excluded because of their complex disease and its independent pathogenesis. Moreover, participants who participated in more than one study are just announced once.

Figure 2 shows the flow chart for developing the study population. The database of the six studies comprised of 183 humans. Patients with a diabetes type other than type 2 diabetes, such as acromegaly (n=6) or diabetes mellitus type 1 (n=19), were excluded from further analyses. From the remaining 158 persons, data on fATP were lacking in 15 persons. After exclusion of those and of persons in whom M or Mr were missing, 113 participants fulfilled the inclusion criteria and remained in the analyses. A real, i. e. clamp-derived, M-value was present in 43 persons and a calculated, i. e. OGTT-derived, Mr-value was available in 70 persons. Further conditions reduced the numbers of participants for specific analyses as indicated.

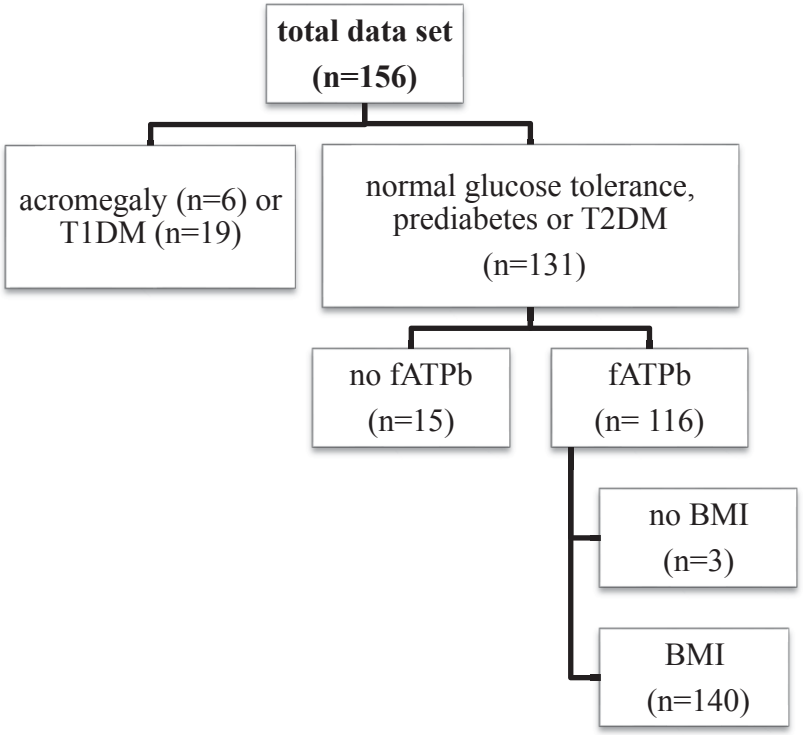

Figure 2: Flow chart of including criteria: presence of flux through ATP synthase (fATPb), body mass index (BMI). All participants had age and sex data. Only those data sets that satisfied the depicted criteria were included into analyses. T2DM: Type 2 diabetes mellitus.

3.7 Statistical analyses

We applied univariable and multivariable linear (log-linear) regression analyses to evaluate three main relationships. In order to find out how fATP is connected with insulin sensitivity we investigated the relationship between fATP and surrogates of insulin sensitivity. Additionally, we analyzed the relationship between fATP and parameters of

glucose metabolism. Furthermore, we assessed if fATP is associated with the metabolic syndrome. We analyzed the relationship of fATP and parameters of the metabolic syndrome such as BMI, fasting plasma triglycerides, HDL and fasting insulin. The final analyses of the relationships between fATP and parameters of lipid metabolism were performed in order to look at the interaction between muscle fATP with lipid parameters and to investigate the role of mitochondrial activity in hepatic steatosis. The data are presented as mean ± standard deviation (SD).

All analyses were done using the SAS for Windows Version 9.2 (SAS Institute, Cary, North Carolina, USA) software. For all analyses we took the logarithm naturalist (ln) of fATP, because fATP has an ln-normal distribution. Furthermore, M-value, HCL, fasting plasma C-peptide, insulin, FFA, TG and the TG/HDL were transformed to their natural logarithms.

We performed five sequential analyses employing different models (M1 to M5) for each independent variable. The first model (M1) was unadjusted. The second model (M2) was adjusted for basic study differences in order to control for effects of study groups, investigators and times of investigation. model 3 (M3) was adjusted for study effects as well as for age and sex. The fourth model (M4) was adjusted for study effects, age, sex and BMI. The last model (M5) is adjusted for study effects, age, sex, BMI and glucose tolerance status (normal glucose tolerance, prediabetes, T2DM). Exceptions are the analyses of the relationship between fATP (ln) and age and between fATP (ln) and BMI. For the analysis of fATP (ln) and age we calculated the models M2, M4 and M5 without adjusting for age. Similarly, for analysis of the relationship between fATP (ln) and BMI we calculated the models M2, M3 and M5 without adjusting for BMI.

We extended the five models for the analysis of fATP and M-value with model 6 (M6). In M6, we adjusted for study effects, age, sex, BMI, glucose tolerance status and additionally for HCL so that the relationship with fATP can be described independently of the influence of HCL.

Relationship between fATP and insulin sensitivity or glucose metabolism

To examine a possible correlation between fATP and glucose metabolism, we analyzed parameters which correlate with insulin sensitivity, insulin secretion and glycemic control.

Parameters of the insulin sensitivity were M-value, fasting insulin and TG/HDL [107]. Fasting insulin and C-peptide served as parameters of the insulin secretion. Glycemic control was assessed by hemoglobin A1c (HbA1c) and fasting plasma glucose.

We performed six linear or log linear regression analyses of:

- fATP (ln) and M-value (ln),
- fATP (ln) and fasting plasma glucose,
- fATP (ln) and HbA1c,
- fATP (ln) and C-peptide (ln),
- fATP (ln) and fasting insulin (ln),
- fATP (ln) and triglycerides/HDL (ln).

In addition, we examined how age interacts with fATP by linear regression analysis of:

- fATP (ln) and age.

The inclusion criteria for the analysis of the relationship between fATP and M-value was that fATP, age, sex, BMI and M-value (either measured or calculated) are existing. 113 participants fulfill the requirements. So the analysis of fATP (ln) and M-value (ln) was done with data of 113 persons. Values of fasting plasma glucose, fasting insulin and age were available from all 113 participants, so the analyses were done with the same number of data (n=113). For all other analyses we used a smaller collective which, in addition to age, sex, BMI and M-value, also had the value of HbA1c (n=86), C-peptide (n=84) or TG/HDL (ln) (n=91).

Relationship between fATP and parameters of the metabolic syndrome

To define the parameters of the metabolic syndrome, we used the definition of the AACE [6]. To analyze the role of fATP for the metabolic syndrome, we did a linear or a log-linear regressions analysis of:

- fATP (ln) and HDL cholesterol,
- fATP (ln) and triglycerides (ln),
- fATP (ln) and BMI,
- fATP (ln) and fasting plasma glucose (ln).

Also, for these analyses fATP, age, sex, BMI and M-value were required to be complete (n=113). So the analyses of fATP (ln) and BMI, fasting plasma glucose and age were done with data of 113 persons. But the analyses of the relationship between fATP and triglycerides (n=105) or HDL (n=98) were done with a smaller population because triglycerides and HDL were not available from all 113 participants.

Relationship between fATP and lipid parameter

To discover this relationship we made four log-linear regressions of fATP (ln) and different lipid parameters:

- fATP (ln) and HCL (ln)
- fATP (ln) and IMCLs (ln),
- fATP (ln) and fasting FFA (ln).

The analysis of fATP (ln) and TG/HDL (ln) is a component of the section "Relationship between fATP and insulin sensitivity or glucose metabolism". The analyses of fATP (ln) and fasting triglycerides (ln) or HDL (ln) are mentioned in the section "Relationship between fATP and parameters of the metabolic syndrome".

For this analysis the inclusion data (fATP, age, sex, BMI and M-value) were presumed as well. The analyses of fATP (ln) and HCL (ln) encompass data of 95 participants. The analysis of fATP (ln) and IMCLs (ln) have a number of 112 subjects. The analyses of fATP (ln) and fasting FFA (ln) include data of 104 humans.

To sum up, most analyses were done with the same collective (n=113). The analyses of fATP (ln) and HbA1c, C-peptide, TG/HDL, TG, HDL, IMCL or FFA were done with a smaller collective based on data availability. If these parameters also were inclusion criteria, the number of the population would have been reduced.

4 RESULTS

In the following the results of the analyses are listed up.

4.1 Characterization of the study population

The present study includes 113 participants out of the six clinical studies with 52 male and 61 female subjects. The average age of the study population is 40 years with a SD of ±13 years. 87 persons have a normal glucose tolerance, 16 have prediabetes (IFG/IGT) and 10 of them have T2DM [Table 2]. The participants were rather insulin sensitive (M-value and Mr-value: 7.5±4.6 and 7.0±2.7 $mg.min^{-1}.kg^{-1}$) or metabolically well controlled (HbA1c: 5.52±0.62 %, TG: 9549 mg/dl, FFA: 462±206 µmol/l). The mean BMI was 25.0±3.5 kg/m^2.

Table 2: Baseline characteristics of the study population.

Study	n	m/f	age [years]	Glucose tolerance
Szendroedi *et al*. 2008 [101]	7	4/3	45±11	3/4/0
Brehm *et al*. 2010 [35]	7	7/0	26±2	7/0/0
Szendroedi *et al.* 2007 [41]	31	19/12	47±16	19/2/10
Kacerovsky *et. al*. 2011 [46]	5	5/0	27±1	5/0/0
Kacerovsky-Bielesz *et al.* 2012 [104]	36	17/19	39±12	28/8/0
Prikoszovich *et al*. 2011 [102]	27	0/27	36±5	25/2/0
Total	**113**	**52/61**	**40±13**	**87/16/10**

Baseline characteristics of the study population (mean ± SD) of all participants showing the number of participants per subgroup, sex [male (m)/female (f)], mean age and glucose tolerance: normal glucose tolerance (NGT)/impaired fasting glucose (IFG) or impaired glucose tolerance (IGT)/type 2 diabetes (T2DM).

4.2 Correlation of fATP with parameters of lipid metabolism and fat distribution

To analyze the role of parameter of lipid metabolism and ectopic lipid deposition for mitochondrial activity, we did six linear regression analyses. In these analyses fATP (ln) was the dependent variable and the independent variables for each analysis was one of the following: TG (ln), HDL-C, plasma FFA, BMI (ln), HCL and IMCLs (ln).

Table 3: **Associations between fasting muscle mitochondrial activity and lipid metabolism and fat distribution.**

Independent Variable	N		Model M1	M2	M3	M4	M5
Plasma TG (ln)	105	β	-0.0350	-0.0425	-0.0192	-0.0047	-0.0067
		p-value	0.5323	0.4502	0.7427	0.9389	0.9135
Plasma HDL-C	98	β	0.0759	0.0911	0.1504	0.1430	0.1372
		p-value	0.3872	0.3008	0.1199	0.1641	0.1836
Plasma FFA (ln)	104	β	-0.0981	**-1.2135**	-0.0930	-0.0989	-0.0831
		p-value	0.0759	**0.0379**	0.1291	0.1090	0.1883
BMI	113	β	-0.0119	-0.0131	-0.0099	-	-0.0087
		p-value	0.1120	0.0968	0.2189	-	0.2769
HCL (ln)	95	β	**-0.0809**	**-0.0805**	**-0.0662**	**-0.0684**	**-0.0759**
		p-value	**0.0015**	**0.0025**	**0.0185**	**0.0264**	**0.0232**
IMCL (ln)	112	β	0.0270	0.0614	0.0691	0.0752	0.0728
		p-value	0.5591	0.2321	0.1731	0.1371	0.1542

Linear regression analyses with fATP(ln) as dependent variable and Plasma triglyceride (TG), plasma high density lipoprotein cholesterol (HDL), Plasma free fatty acid (FFA), body mass index (BMI), hepatocellular lipid (HCL) and intramyocellular lipid (IMCL) as independent variables model M1 unadjusted; model M2 adjusted for study effects; model M3 adjusted for study effects, age and sex; model M4 adjusted for study effects, age, sex and BMI; model M5 adjusted for study effects, age, sex, BMI and glucose tolerance status.

HCL correlates negatively to fATP (ln) unadjusted (M1: p=0.002, β=-0.081) and adjusted (M2: p=0.002, β=-0,080; M3: p=0.0186, β=-0.066; M4: p=0.026, β=-0.068; M5: p=0.023, β=-0.076) [Figure 3, Table 3].

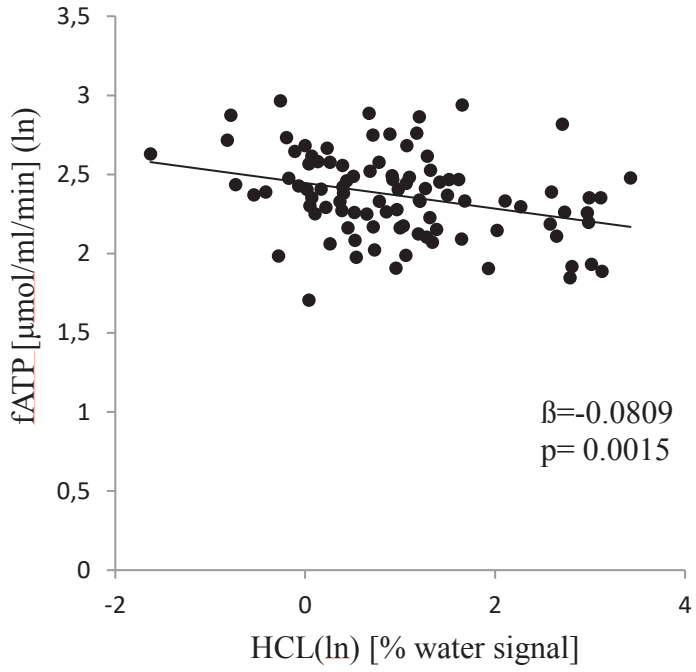

Figure 3: Association between flux through ATP synthase during fasting [fATP (ln)] with HCL, n=95.

Also fATP (ln) and fasting FFA (ln) are related, when adjusted for study effects (M2: p=0.038, β=-0.121) [Table 3]. But it is not associated in model M1 (p=0.076, β=-0.098), M3 (p=0.129, β=-0.093), M4 (p=0.109, β=-0.099) or M5 (p=0.188, β=-0.083).
Between fATP (ln) and IMCLs (ln) is no relationship (M1: p=0.559, β=0.027; M2: p=0.232, β=0.068; M3: p=0.173, β=0.069; M4: p=0.137, β=0.075; M5: p=0.154, β=0.073). The results of the analyses between fATP (ln) and BMI or TG (ln) are mentioned above.

4.3 Correlation of fATP with parameters of glucose metabolism and age

To analyze the relationship between fATP and parameters of glucose metabolism, we performed six linear regression analyses with fATP (ln) as a dependent variable and for each analysis one of the following independent variables: M-value, C-peptide (ln), fasting insulin (ln), fasting plasma glucose, HbA1c and TG/HDL (ln) [Table 4].

Table 4: Association between fasting muscle mitochondrial activity [fATP (ln)], glucose metabolism and age.

Independent Variable	n		Model M1	M2	M3	M4	M5
M-value (ln)	113	β	0.0672	**0.2264**	**0.1905**	**0.1814**	**0.1984**
		p-value	-0.2434	**0.0015**	**0.0141**	**0.0331**	**0.0430**
Fasting plasma	113	β	-0.002	-0.0016	0.0029	-0.0003	0.0038
glucose		p-value	0.0719	0.1821	0.2184	0.8121	0.1182
TG/HDL (ln)	91	β	-0.0604	-0.0674	-0.0587	-0.0628	-0.0629
		p-value	0.2375	0.1950	0.2904	0.3016	0.3060
HbA1c	86	β	**-0.1532**	**-0.1382**	**-0.1088**	-0.0986	-0.0791
		p-value	**0.0005**	**0.0037**	**0.0489**	0.0687	0.3196
Plasma	84	β	-0.0793	**-0.3937**	**-0.3629**	**-0.3271**	**-0.3085**
C-peptide (ln)		p-value	0.1375	**0.0001**	**0.0003**	**0.0051**	**0.0167**
Plasma	113	β	-0.0513	-0.00003	-0.0116	0.0190	0.0157
insulin (ln)		p-value	0.2579	0.9996	0.8439	0.7660	0.8060
Age	113	β	-0.0039	**-0.0052**	-0.0052	-0.0045	-0.0033
		p-value	0.0522	**0.0200**	0.0245	0.543	0.1869

Linear regression analyses with log-transformed flux through ATP synthase [fATP(ln)] as dependent variable and insulin sensitivity [M-value or OGIS-derived reconstructed Mr-value], fasting plasma glucose, triglyceride to high density lipoprotein cholesterol ratio [TG/HDL (ln)], hemoglobin A1c (HbA1c), C-peptide (ln) and fasting insulin (ln) as independent variables model M1 unadjusted; model M2 adjusted for study effects; model M3 adjusted for study effects, age and sex; model M4 adjusted for study effects, age, sex and BMI; model M5 adjusted for study effects, age, sex, BMI and glucose tolerance status.

The analysis of fATP (ln) and insulin sensitivity [assessed as M-value (ln) or Mr-value (ln)] showed that fATP (ln) correlates positively with insulin sensitivity. In the unadjusted model M1 ($p=0.243$, $\beta=0.067$) there is no correlation. However, it correlates when it is adjusted for the model M2 ($p=0.002$, $\beta=0.226$), M3 ($p=0.014$, $\beta=0.191$), M4 ($p=0.033$, $\beta=0.181$) and M5 ($p=0.043$, $\beta=0.198$) [Table 4]. To evaluate the influence of HCL for this relationship, we did another analysis, which is adjusted for study effects, age, sex, BMI, glucose tolerance status and HCL. The positive correlation between fATP (ln) and

insulin sensitivity still remains (M6: p=0.043, β=0.225, n=95). In conclusion, the relationship between insulin sensitivity and fATP (ln) is independent from HCL.

Fasting plasma glucose does not correlate with fATP (ln) (M1: p=0.072, β=-0.002; M2: p=0.218, β=0.182; M3: p=0.218, β=0.003; M4: p=0.812, β=-0.0003; M5: p=0.118, β=0.004) [Table 4]. Also, the direction of the relationship between fATP and fasting plasma glucose is not consistent.

HbA1c also correlates with fATP (ln) effects (M1: p=0.001, β=-0.153) [Figure 4] when adjusted for study effects (M2: p=0.004, β=-0.183) and adjusted for study effects, sex and age (M3: p=0.049, β=-0.109). There is no correlation when it is adjusted additional to BMI (M4: p=0.069, β=-0.099) and glucose tolerance status (M5: p=0.320, β=-0.079) [Table 4]. High values of HbA1c relate to low values of fATP (ln).

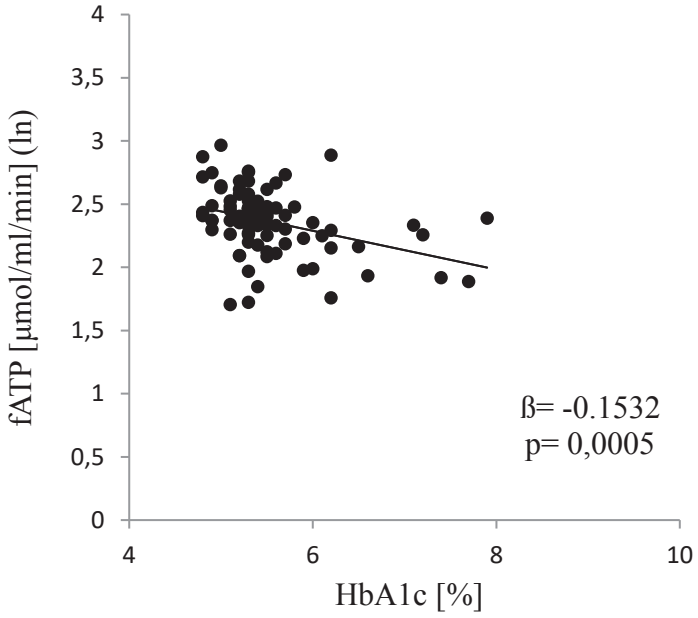

Figure 4*:* Association between flux through ATP synthase during fasting [fATP (ln)] with hemoglobin A1c (HbA1c, n=86),

There is a relationship between C-peptide (ln) and fATP (ln) when it is adjusted for model M2 (p=<0.001, β=-0.394, n=84), M3 (p=<0.001, β=-0.363, n=84), M4 (p=0.005, β=-0.327, n=84) or M5 (p=0.017, β=-0.309, n=84). However, it does not correlate when it is not adjusted (M1: p=0.138, β=-0.079).

Fasting insulin (ln) and fATP (ln) are not related (M1: p=0.745, β=-0.002, M2: p=0.572, β=0.004; M3: p=0.725, β=0.002; M4: p=0.392, β=0.006; M5: p=0.411, β=0.006). Further, there is no relationship between TG/HDL (ln) and fATP (ln) (M1: p=0.238, β=-0.060; M2: p=0.195, β=-0.067; M3: p=0.290, β=-0.059; M4: p=0.302, β=-0.063; M5: p=0.306, β=-0.063)**.**

Age and fATP are related to each other when adjusted for the study effect (M2: p=0.020, β=-0.005). The older a person, the lower is fATP. The unadjusted analysis shows a borderline significance (M1: p=0.052, β=-0.004) [Table 4, Figure 5]. When the analysis is additionally adjusted for sex and BMI in model M4 and further for glucose tolerance status in M5 there is no correlation (M4: p=0.054, β=-0.005; (M5: p=0.187, β=-0.003; M4 and M5 are not adjusted for age in this case) [Table 4].

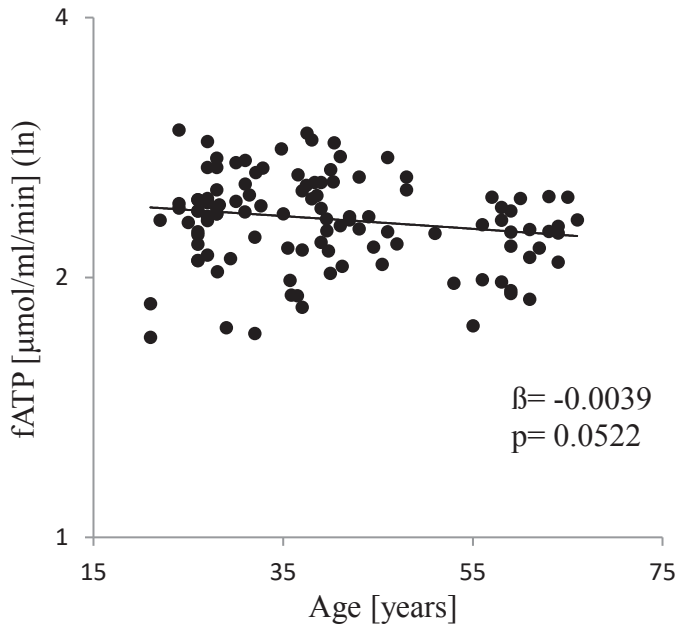

Figure 5: Correlation between flux through ATP synthase during fasting [fATP (ln)] and age [years], n=113.

4.4 Correlation of fATP with features of the metabolic syndrome

In order to analyze the relationship between fATP and parameters of the metabolic syndrome, we did five linear regression analyses with fATP (ln) as a dependent variable and for each analysis one of the following independent variables: BMI, fasting FFA,

fasting HDL and fasting plasma glucose. Blood pressure was not measured in the majority of studies, therefore there are not enough data to be included in the analyses. No parameters of the metabolic syndrome correlate with fATP (Table 5).

Table 5: Association between fasting muscle mitochondrial activity and parameters of metabolic syndrome

Independent Variable	n		Model M1	M2	M3	M4	M5
BMI	113	β	-0.0119	-0.0131	-0.0099	-	-0.0087
		p-value	0.1120	0.0968	0.2189	-	0.2769
Plasma TG (ln)	105	β	-0.0350	-0.0425	-0.0192	-0.0047	-0.0067
		p-value	0.5323	0.4502	0.7427	0.9389	0.9135
Plasma HDL-C	98	β	0.0759	0.0911	0.1504	0.1430	0.1372
		p-value	0.3872	0.3008	0.1199	0.1641	0.1836
Fasting plasma glucose	113	β	-0.002	-0.0016	0.0029	-0.0003	0.0038
		p-value	0.0719	0.1821	0.2184	0.8121	0.1182

Linear regression analyses with fATP (ln) as dependent variable and body mass index (BMI), plasma triglyceride (TG), plasma high density lipoprotein cholesterol (HDL-C) and fasting plasma glucose as independent variables model M1 unadjusted; model M2 adjusted for study effects; model M3 adjusted for study effects, age and sex; model M4 adjusted for study effects, age, sex and BMI; model M5 adjusted for study effects, age, sex, BMI and glucose tolerance status.

5 DISCUSSION

The main finding of this study is the strong and independent correlation between fasting muscle mitochondrial activity and ectopic lipid deposition in the liver. Further, this analysis supports previous reports on a positive relationship of fATP with age and insulin sensitivity in humans with different glucose tolerance status. Moreover, fATP relates negatively with fasting plasma FFA positively to glycemia as assessed from HbA1c. Lastly, in this study cohort, fATP did not relate to any other parameter of lipid metabolism or intramyocellular lipid storage.

5.1 Low Muscle fATP relate to an high amount of HCL

One of the main findings of this study is a correlation between basal flux through muscle ATP synthase and ectopic lipid deposition in liver. This relationship is strong and independent. Further, we could show a weak and negative association of mitochondrial activity and fasting plasma FFA. All other parameters of lipid metabolism did not show a relationship to fATP.

A decreased mitochondrial activity in muscle during fasting comes along with high HCL levels. In the cohort of the present study intramyocellular lipids and lipid metabolism did not relate to fATP. Our cohort included healthy glucose tolerance humans, patients with prediabetes and well-controlled T2DM without clinical evidence for cardiovascular disease. Furthermore, we detected a weak relationship between plasma FFA and fATP. However, one of the substudies found a strong association [41]. That study provided all those participants of this present meta-analysis who had overt T2DM and rather low fasting plasma FFA. IMCL is not associated with fATP in the present analysis. In accordance, some [108] but not all studies found higher IMCL in patients with T2DM and no correlation with insulin resistance [49] [51], which is in line with our finding.

The most relevant finding of the present study is the strong correlation between HCL and fATP in a large cohort with mostly glucose tolerant humans. There was no association between HCL and fATP in the substudies. An explanation could be that the substudies had smaller numbers than the present study. Still, other studies already assumed a relationship between HCL and fATP, because they found that humans with higher HCL had decreased fATP values [41, 101, 102].

About 70 % of obese and patients with T2DM have hepatic steatosis [70]. Hepatic steatosis is accompanied by insulin resistance, decreased activity of the respiratory chain enzymes and alterations of the morphology of mitochondria [72]. But it is not fully understood what part the hepatic mitochondrial dysfunction takes in the development of insulin resistance and for accumulation of HCL [17]. Also, the relationship between hepatic steatosis and mitochondrial function of the skeletal muscle is yet unknown. A recent study investigated the relationship between liver steatosis and muscle insulin resistance. They investigated liver and muscle biopsies from pathological obese patients during a bariatric surgery [109]. In these samples, the activity of complex I and complex III were higher in obese patients with hepatic steatosis than in controls. Furthermore, the activity of complex I and III were positively associated to alanine-aminotransferase (ALT) levels and the activity of complex III was negatively associated with aspartate aminotransferase (AST) levels [109]. They were able to show that the activity of complex I increases with progressive steatosis and fibrosis degree. Furthermore, they detected higher mitochondrial content in skeletal muscle via measurement of the citrate synthase activity, which leads to the suggestion that enzyme activity reflects the adjustment of skeletal muscle mitochondria to fat overload. It seems to be difficult to determine the specific effect of insulin resistance and alterations of skeletal muscle mitochondrial function on the progress of steatosis. High-caloric diet and low physical activity lead to substrate overflow and encourage the process of steatosis and reduction of muscle mitochondrial activity. In turn, steatosis and decreased muscle mitochondrial activity are tightly related to whole body insulin resistance. Some investigators consider insulin resistance as a main causal factor of hepatic steatosis and the metabolic syndrome. The reason is that insulin resistance influences and conduces to the other metabolic risk factors [12, 13].

A hypothesis of Peterson *et al.* was that reduction of the non-oxidative storage of carbohydrates in skeletal muscle (glycogen synthesis), which appears in insulin resistant humans, leads to a change of carbohydrate fluxes so that they are directed to hepatic *de novo* lipogenesis. Further, *de novo* lipogenesis causes dyslipidemia and hepatic steatosis [14]. In a study insulin resistant healthy humans and controls matched for age, BMI and physical activity were investigated after a mixed meal. They assessed HCL, IMCL, hepatic *de novo* synthesis and glycogen synthesis. Compared to controls, insulin resistant subjects had 60 % lower net muscle glycogen synthesis. Instead, net HCL synthesis and *de novo* lipogenesis were two times greater in insulin resistant humans. They concluded

that skeletal muscle insulin resistance could be responsible for the induction of postprandial hepatic triglyceride synthesis and later contribute to hepatic steatosis. Limitations of that study were that fasting or postprandial skeletal muscle mitochondria activity was not measured and further the *de novo* lipogenesis was also influenced higher postprandial insulin levels. Petersen *et al.* also exhibited that hyperinsulinemia due to raising *de novo* lipogenesis in liver promote artherogenic dyslipidemia [14]. Accordingly, hepatic steatosis is tightly associated with hepatic insulin resistance and is a risk factor for prediabetes and T2DM [110].

Moreover, a negative correlation between increased HCL and whole body insulin resistance as well as a negative correlation between HCL and hepatic insulin resistance were reported [111, 112]. Our findings fit the suggestion that development of insulin resistance starts in skeletal muscle and then leads to insulin resistance of the liver [113]. The background is that during insulin resistance the insulin-stimulated uptake of glucose is reduced in skeletal muscle [25], which in turn is increased by the liver. To supply the skeletal muscle with glucose the liver induces the depletion of glycogen, gluconeogenesis and lipolysis in adipose tissue [26]. All this causes a switch from muscle glycogen storage to *de novo* lipogenesis in the liver [14]. High amounts of lipids are stored in lipid tissue and also as HCL and IMCL [27]. In the present study, mitochondrial activity and HCL were assessed during fasting conditions. This way the suggestion can be tested that also during fasting state the interplay between skeletal muscle and liver substrate utilization contribute to the development of hepatic steatosis. Because insulin resistance and skeletal muscle mitochondrial function is closely related to obesity [8] it is difficult to detect the unique role of both. In the present study, we suggest an independent relationship between HCL and fATP because it did not depend on BMI and insulin sensitivity. To prevent the metabolic syndrome, some investigators suggested that a treatment of insulin resistance of skeletal muscle via reversing defects in insulin-stimulated glucose transport might be the best way [14, 114]. Still it is difficult to determine the specific effect of improving insulin sensitivity on HCL because most of the lifestyle interventions include weight loss, and it has been shown that weight loss improves hepatic steatosis [115]. Hepatic steatosis induced by overload of nutrient leads to high blood glucose level and high plasma FFA. As a result, hyperglycemia and high plasma FFA levels might lead to damage of mitochondria, decreased lipid oxidation and lipid induced insulin resistance in skeletal muscle. This pathophysiologic concept is substantiated strongly by the findings of the

present study, in which the independent relationships between HCL, fasting plasma FFA, HbA1c levels, insulin resistance and mitochondrial impairment were shown.
Fasting plasma FFA correlates with muscle mitochondrial activity in the present analysis. Fasting plasma FFA was the only lipid parameter that was related to fATP. This is in consensus with one of our substudies performed in overt T2DM [41].

5.2 Muscle fATP correlates positively with insulin sensitivity

In the present study we confirmed that fATP correlates positively with insulin sensitivity in a large study population (n=113). The M-value was used as an index of insulin sensitivity and showed a correlation with skeletal muscle fATP. This relationship remains when additionally adjusted for HCL. This indicates a strong and independent relationship. In addition, fATP relates positively to good glucometabolic control as assessed from HbA1c levels.

The substudies have already suggested that there is a positive correlation between insulin sensitivity and muscle fATP, which we could now confirm in a large study cohort. Muscle fATP was lower in participants with T2DM compared to young lean healthy humans. Some studies compared lean healthy humans to patients with insulin resistant states such as family history for T2DM, history of gestational diabetes [102] or for acromegaly [101]. In these studies, fATP was lower in humans with insulin resistant state [101, 102]. Other studies showed an impaired increment of fATP in humans with insulin resistance during hyperinsulinemia [41, 43], after exercise [103], during fasting [41, 42, 51, 54, 101, 102, 116] or in healthy humans during acute lipid-induced insulin resistance [43]. This impaired mitochondrial plasticity was suggested to be an early indicator of muscle insulin resistance and metabolic inflexibility [38]. However, in these small groups fATP did not correlate with insulin resistance.

We found a relationship between insulin sensitivity and fATP despite the fact that the individuals of our cohort were mainly glucose tolerant (87 of 113) and insulin sensitive (M-value and Mr-value were 7.52±4.63, and 6.95±2.69 $mg.min^{-1}.kg^{-1}$, means ±SD). The association between insulin sensitivity and fATP still remained upon adjustment for age, sex body mass, glucose tolerance or HCL. This strong association suggests that reduced mitochondrial activity might be an early step during the development of insulin resistance. There is no relationship between fATP and M-value when it is unadjusted but

as soon as adjusted for study effects the relationship is consisting. This shows that the study effects influence the relationship between fATP and M-value considerably.

Notwithstanding that all six clinical experimental studies were implemented in the same laboratory, by the same investigators and some of them even during the same time period, a limitation is that some studies only had participants with normal glucose tolerance, which fitted in this analysis [35, 46].

Additionally, other studies also had patients with prediabetes [101-103] and one study with prediabetes and T2DM [41]. The adjustment for study effect might flatten the difference of the study population. However, this is just a small limitation, because all in all the majority of the population has a normal glucose tolerance, and that applies to all included studies. As mentioned above, the adjustment to glucose tolerance status does not change the relationship. Nevertheless, there is another limitation to mention. If the glucose infusion is greater than 8, the calculated M-value tends to underrate the true M-value (R=0.61). That could influence the analyses so that the regression is in fact more significant but is underestimated by our analyses, which further strengthens our conclusions. It prevents false-positive findings, and the association existed even despite from this, which again shows the strength of the correlation. The effect is minimized through the adjustment to study effects. This could also be the reason for the big influence of the study effects on the relationship between fATP and M-value. Other studies have also shown a lower fATP in patients with insulin resistance [41, 51, 54], in healthy humans during lipid infusion induced insulin resistance [43] and during hyperinsulinemia [40-44, 117]. The damage of mitochondrial proteins could be the cause for the impairment of mitochondrial function. In turn, the damage of mitochondrial proteins could be promoted by oxidative stress and subsequently long-term high oxidation rates caused by increased substrate flux rates (hyperglycemia, hyperlipidemia). The impairment of mitochondrial function seems to be an early abnormality rather than a precondition of the developing of insulin resistance [38]. Hyperinsulinemia increases ATP synthesis and messenger ribonucleic acid (mRNA) transcripts of mitochondrial mitobiogenesis [40] whereas lipid exposition inhibits the insulin effect on mitochondria [118]. Furthermore, *in vitro* it was shown that in contrast to lean healthy humans, insulin does not stimulate the mitochondrial function in patients with T2DM and thereby does not increase the production of ATP [40]. Furthermore, it was demonstrated that insulin stimulated fATP is strictly decreased in patients with T2DM and their relatives [39]. Accordingly, insulin

resistance at the level of mitochondria has been suggested to explain lower mitochondrial capacity rather than insufficient substrate oxidation causing impaired glucose uptake [119]. Some life style intervention studies found correlations between improvement of insulin sensitivity and mitochondrial function. They submitted that raising glucose and lipid oxidation rates could underlie reduction of insulin resistance [120]. Correspondingly, a positive correlation between insulin stimulated glucose disposal and post-absorptive fatty acid oxidation was found after exercise intervention [121]. Moreover, citrate synthase activity and change in protein expression of complexes III and IV after physical training correlated with insulin sensitivity [122]. After exercise both insulin sensitivity and mitochondrial oxidative capacity improve [123]. But only few cross sectional studies found correlations between insulin sensitivity and markers of mitochondrial function [48, 124]. Most other studies found no correlations between insulin resistance and mitochondrial function although via *ex vivo* measurements they detected impaired mitochondrial function such as reduced mitochondrial content [51, 55, 125, 126], oxidative enzyme capacity [52, 108] and abnormal mitochondrial morphology [127] in humans with insulin resistance compared to controls. A limitation of these findings could be the small cohorts of the studies. However, some of these studies suggested dissociation between mitochondrial function and insulin sensitivity. Accordingly, the muscle mitochondrial response to four months of aerobic exercise training was similar in all age-groups, but elderly people did not show an improvement in insulin sensitivity [128]. Furthermore, mitochondrial capacity in skeletal muscle was not stimulated by weight loss although it increases in insulin action [129]. Impairment of mitochondrial function could rather result from oxidative stress occurring during long-term lipid and glucose mediated greater oxidative substrate flux rates. The greater oxidative flux rates cause oxidative stress and thereby damage mitochondrial proteins [38]. Altogether, analyzing the larger cohort revealed that reduced mitochondrial activity might be an early abnormality during development of insulin resistance.

In this study we also report a negative association between fATP and age, which was present particularly after adjustments. Age is accompanied with lower physical activity as a causal factor of mitochondrial biogenesis [131, 132]. The fact that insulin sensitivity decreases with age [133] can explain the higher incidence of T2DM in the elderly. However, there are contradictory results of studies, which leads to assume another instance which relates to aging. For example in some studies, groups of different age were matched for extent of physical activity and showed similar results for insulin sensitivity

[134-137]. But all in all they also found that in elderly the function of mitochondria decreases [134-137]. Our analysis also reflects that aging correlates with declined fATP. Another aspect is that elderly have more body fat content compared to young, which could influence the relationship. Furthermore, a study showed that in elderly humans, ectopic lipid disposition such as IMCL and HCL are elevated and fATP is reduced by about 40 % [133]. In our study, these effects were present. Upon adjusting for study effects and BMI the relationship was only borderline (M3: $p=0.054$, $\beta=-0.005$), suggesting that this relationship might be influenced by BMI.

Another surrogate for insulin resistance is fasting insulin [130]. In this analysis of data of 113 different humans, it did not relate to fATP. Fasting insulin is easy to implement in clinical practice and is described to correlate well with insulin resistance [83]. However, there are some problems about this surrogate marker. Firstly, there is no standardization of the insulin assay procedure [84]. Another disadvantage is that it does not bestride low insulin secretion during hyperglycemia in diabetes or glucose-intolerant subjects [84]. Further, there are limitations due to a high value of false-positive results [84]. All these disadvantages explicate that the non-significant relationship between fATP and fasting insulin do not contradict the finding of this study showing that mitochondrial activity correlates with insulin sensitivity.

Fasting plasma glucose also does not correlate with fATP. This raises the question why M-value related to fATP but fasting plasma glucose did not. Fasting plasma glucose is measured in blood samples after an 8-10 hours fasting period. The M-value results from the hyperinsulinemic-euglycemic clamp, which is explained in detail in the introduction. It represents the average glucose infusion rate over a time periods of 80 to 120 min [80], thus M-value shows a dynamic process and is classified as the gold standard for the *in vivo* assessment of whole body insulin sensitivity in humans [45]. Fasting plasma glucose on the other hand is not such a sensitive surrogate. In conclusion, M-value is a much better parameter to estimate insulin resistance.

C-peptide strongly correlates with fATP when adjusted. The fact that fasting insulin does not correlate with fATP could be an argument for the better validity of C-peptide than fasting insulin to indicate the insulin resistance.

5.3 Muscle fATP and parameters of the metabolic syndrome

The second aim was to evaluate our hypothesis that muscle fATP relates to components of the metabolic syndrome. In this study we could show that there is no correlation between skeletal muscle fATP and components of the metabolic syndrome.

In former times, some investigators [12, 13] already discussed insulin resistance as the main cause of the metabolic syndrome because it influences or even causes the main metabolic risk factors. Some even assume that treating insulin resistance in skeletal muscle by reversing defects in insulin-stimulated glucose transport might be a possible way to prevent the development of the metabolic syndrome [14]. Also, the mechanism behind the association of insulin resistance and most of the components of the metabolic syndrome are still obscure [15]. However, there is no clinical proof for this concept.

Peterson *et al.* suggested that insulin resistance in skeletal muscle reduces the non-oxidative storage of carbohydrates in skeletal muscle. This leads to diversion of ingested carbohydrate as substrate for *de novo* lipogenesis [14]. They found that after two high-carbohydrate mix meals insulin resistant individuals had 60 percent lower net muscle glycogen synthesis compared to matched insulin sensitive humans. Furthermore, the net hepatic triglyceride synthesis was 2.5 fold greater and the hepatic *de novo* lipogenesis was 2.2 fold greater in insulin resistant participants than in insulin sensitive participants. The increased hepatic *de novo* lipogenesis was accomplished by 80 percent higher plasma triglyceride concentration and 20 percent lower HDL concentration [14]. Both low HDL and high triglyceride concentration are associated with metabolic syndrome and hence with artherogenic processes.

Nevertheless, our analysis could not show a relationship between the triglycerides or HDL and fATP. The relationship between HDL and fATP still shows a positive direction of the relationship, which matches with the finding above [14]. The lower HDL, the lower is fATP. HDL is the only lipoprotein that has the ability to transport cholesterol out of tissues, therefore it has an anti-artherogenic function. BMI shows the relation of the weight and height of one person. The fact that there is no relationship between BMI and fATP shows that for the development of mitochondrial dysfunction the ectopic lipid disposition is more important than the whole lipid disposition. The waist circumference could be a better parameter to estimate the risk for T2DM and the metabolic syndrome. The National Cholesterol Education program (NCEP) Adult Treatment Panel III (ATP

III) used in their definition of the metabolic syndrome instead of BMI waist circumference as a parameter [7].

6 CONCLUSION

Muscle insulin resistance is involved in the development of fatty liver and dyslipidemia by changing the partitioning of postprandial substrate fluxes. Moreover, insulin resistance may be due to reduced mitochondrial function. We examined the association between mitochondrial activity and insulin sensitivity of skeletal muscle and steatosis in a larger human cohort. We verified two of our hypotheses.

Firstly, skeletal muscle fATP correlates positively with insulin sensitivity in a large study population. This implicates that individuals with insulin resistance may have reduced mitochondrial activity or density. Moreover, it suggests that mitochondrial abnormalities may contribute to the developing of insulin resistance.

Secondly, low skeletal muscle fATP are observed in humans with hepatic steatosis. The strong association between hepatic lipid content and lower muscle mitochondria activity supports the concept of a close connection between alterations of skeletal muscle and liver energy metabolism. This link can be interpreted as an early abnormality that drives insulin resistance.

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EIDESSTATTLICHE VERSICHERUNG

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

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