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Pathogenic mechanisms and innovative treatment concepts for diabetes-related steatotic liver diseases

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Original publications underlying this thesis

1. **Kahl S**, Nowotny B, Piepel S, Nowotny PJ, Strassburger K, Herder C, Pacini G, Roden M. Estimates of insulin sensitivity from the intravenous-glucose-modified-clamp test depend on suppression of lipolysis in type 2 diabetes: a randomised controlled trial. Diabetologia 2014; 57(10):2094-102.
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Frequently used abbreviations

^{1}H MRS	Proton magnetic resonance spectroscopy
^{31}P MRS	Phosphorus magnetic resonance spectroscopy
Adipo-IR	Fasting adipose tissue insulin resistance index
AI	Adaptation index
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BMI	Body mass index
BW	Body weight
CI	Confidence interval
CVOT	Cardiovascular outcome trial
DAG	Diacylglycerol
DI	Disposition index
DNL	de-novo lipogenesis
EGP	Endogenous glucose production
EMA	European Medicines Agency
EMPA	Empagliflozin
FDA	Food and Drug Administration
FIB-4	Fibrosis-4 Index
FLI	Fatty Liver Index
γ-GT	Gamma glutamyltransferase
GIR	Glucose infusion rate
GLP1-RA	Glucagon-like peptide 1 receptor agonist
GNG	Gluconeogenesis

GLYnet	Net glycogenolysis
GP	Glycogen phosphorylase
HbA1c	Hemoglobin A1c
HCC	Hepatocellular carcinoma
hsCRP	high-sensitivity C-reactive protein
HSI	Hepatic steatosis index
IGI	Insulinogenic index
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin 6
IL-18	Interleukin 18
ISIcomp	Composite insulin sensitivity index
IQR	Interquartile range
IVGTT	Intravenous glucose tolerance test
MRS	Magnetic resonance spectroscopy
MASL	Metabolic dysfunction-associated steatotic liver
MASLD	Metabolic dysfunction-associated steatotic liver disease
MASH	Metabolic dysfunction-associated steatohepatitis
MMTT	Mixed meal tolerance test
MUFA	Monounsaturated fatty acids
NAFLD-LFS	NAFLD liver fat score
NEFA	Non-esterified fatty acids
NFS	NAFLD fibrosis score
NIT	Non-invasive test
OGIS	Oral glucose insulin sensitivity (index)
OGTT	Oral glucose tolerance test
OXPHOS	Oxidative phosphorylation

P_i	Inorganic phosphate
PREDIM	PREDIcted-M index
QUICKI	Quantitative insulin sensitivity check index
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SAFA	Saturated fatty acids
SGLT2I	Sodium glucose co-transporter 2 inhibitor
T2D ´	Type 2 diabetes
TNFα	Tumor necrosis factor α
WHO	World Health Organization

Summary

Background: Insulin resistance, obesity and type 2 diabetes are closely associated with metabolic dysfunction-associated steatotic liver disease (MASLD). The presence of MASLD in turn increases the risk of liver, cardiovascular and malignant diseases. At the cellular level, these metabolic diseases are related to changes in hepatic energy, glucose and lipid metabolism and the mechanisms underlying the development and progression of MASLD are still incompletely understood. The basis of MASLD treatment is weight reduction through lifestyle changes, but particularly weight loss through bariatric surgery or medication, has shown the greatest efficacy in improving MASLD in people with and without type 2 diabetes in recent years.

The present studies therefore tested the following hypotheses:

(i) There is a need for detailed validation of clinical-metabolic measures in cohorts with different metabolic characteristics.

(ii) Prediabetes impairs liver mitochondrial plasticity in individuals with obesity.

(iii) The increased availability of lipids is the trigger of insulin resistance and altered liver energy, glucose, and lipid metabolism in metabolically healthy individuals.

(iv) MASLD and MASLD-related complications are improved by inhibition of sodium/glucose cotransporter-2 (SGLT2) in individuals with type 2 diabetes.

Methods: The hypotheses were tested in clinical-experimental studies in humans. Insulin sensitivity of muscle and liver was determined using hyperinsulinemic-euglycemic clamps combined with stably-labeled glucose or from the oral glucose tolerance test. Using magnetic resonance-based imaging and spectroscopy, the liver lipid and liver glycogen content as well as the absolute concentrations of energy metabolites (γ-ATP and P_i) in the liver were non-invasively quantified *in vivo*. The histological assessment of MASLD as well as the measurement of hepatic mitochondrial respiration and protein expression were carried out from liver biopsies.

Results: In people with type 2 diabetes, the comparison of the Botnia clamp test and the standard clamp test obtained similar values for whole-body insulin sensitivity, but the measured insulin sensitivity was influenced by the plasma concentrations of non-esterified fatty acids immediately before the start of the clamp test. Validation of various blood-based indices for assessing the risk of hepatic steatosis revealed moderate sensitivity and specificity in predominantly non-obese individuals without diabetes mellitus.

The relationship between insulin resistance and liver mitochondrial oxidative phosphorylation capacity was dependent on fasting plasma glucose levels and liver lipid content in individuals with grade 3 obesity. A direct positive association of both parameters was only found in a subgroup with normal glucose tolerance and without MASLD. There was evidence of impairment of the hepatic insulin signaling pathway and mitophagy, particularly in people with

obesity and prediabetes. In metabolically healthy individuals, concentrations of hepatic energy metabolites were positively associated with circulating levels of palmitoleic acid, but also certain amino acids, especially the essential amino acid leucine. Moreover, a single high-fat meal rich in saturated but not monounsaturated fatty acids caused changes in hepatic energy metabolism and liver lipid content, whereas both lipid challenges led to the acute development of insulin resistance in lean, healthy humans.

Antihyperglycemic therapy with the SGLT2 inhibitor (SGLT2I) empagliflozin reduced liver lipid content in individuals with type 2 diabetes. This was primarily mediated by the weight loss caused, but other mechanisms, such as an anti-inflammatory effect of reduced uric acid and increased adiponectin levels, may have contributed. In addition, independent of the risk of steatosis and fibrosis as assessed by non-invasive testing, empagliflozin treatment reduced the risk of cardiovascular and renal events in people with type 2 diabetes and overt cardiovascular disease.

Summary: Detailed method validation in cohorts with different metabolic risk profiles helps to avoid possible misinterpretations of data and understand the underlying mechanisms.

In individuals with grade 3 obesity, strategies to prevent dysglycemia and MASLD should be applied as early as possible. With regard to liver metabolism, a diet rich in unsaturated fatty acids should be preferred over a diet rich in saturated fatty acids. The SGLT2I empagliflozin effectively reduces the risk of the onset and/or progression of type 2 diabetes-related cardiometabolic complications.

Deutsche Zusammenfassung

Hintergrund: Insulinresistenz, Adipositas und Typ 2 Diabetes sind eng mit der metabolischen Dysfunktion-assoziierten steatotischen Lebererkrankung (MASLD) verbunden. Das Vorhandensein einer MASLD wiederum erhöht das Risiko für Leber-bezogene, kardiovaskuläre sowie maligne Erkrankungen. Auf zellulärer Ebene gehen diese metabolischen Erkrankungen mit Veränderungen des hepatischen Energie-, Glukose- und Lipidstoffwechsels einher und die Faktoren, welche zur Entstehung und Progression der MASLD beitragen, sind bislang nur unvollständig verstanden. Die Basis der Therapie der MASLD ist die durch Änderung des Lebensstils vermittelte Gewichtsreduktion, aber insbesondere ein durch bariatrische Chirurgie oder auch medikamentös bedingter Gewichtsverlust zeigte in den letzten Jahren die größten Erfolge zur Verbesserung der MASLD bei Personen mit und ohne Typ 2 Diabetes.
Die vorliegenden Untersuchungen prüften daher folgende Hypothesen:
(i) Es besteht die Notwendigkeit der detaillierten Validierung von klinisch-metabolischen Messgrößen in Kohorten mit unterschiedlichen metabolischen Eigenschaften.
(ii) Prädiabetes beeinträchtigt die mitochondriale Plastizität der Leber bei Personen mit Adipositas.
(iii) Die erhöhte Verfügbarkeit von Lipiden ist der Auslöser von Insulinresistenz und einem verändertem Energie-, Glukose- und Lipidstoffwechsel der Leber bei metabolisch gesunden Personen.
(iv) Die MASLD und MASLD-bedingte Komplikationen werden durch Hemmung des Natrium/Glukose-Cotransporter-2 (SGLT2) bei Personen mit Typ 2 Diabetes verbessert.
Methoden: Die Hypothesen wurden in klinisch-experimentellen Studien am Menschen geprüft. Die Insulinsensitivität von Muskel und Leber wurde mittels hyperinsulinämisch-euglykämischer Clamps und stabil-markierter Glukose oder aus dem oralen Glukosetoleranztest bestimmt. Mittels Magnetresonanz-basierter Bildgebung und -spektroskopie wurden der Leberlipid- und Leberglykogen-Gehalt sowie die absoluten Konzentrationen von Energieträgern (γ-ATP und P_i) in der Leber nicht-invasiv *in vivo* quantifiziert. Die histologische Beurteilung der MASLD sowie die Messung der hepatischen mitochondrialen Respiration und Proteinexpression erfolgte aus Leberbiopsien.
Ergebnisse: Der Vergleich von Botnia Clamp-Test und Standard-Clamp-Test bei Personen mit Typ 2 Diabetes ergab ähnliche Werte für die Gesamtkörperinsulinsensitivität, jedoch war die gemessene Insulinsensitivität beeinflusst durch die Plasmakonzentrationen nicht-veresterter Fettsäuren unmittelbar vor Beginn der Clamp-Untersuchung. Die Validierung verschiedener Blut-basierter Indizes zur Einschätzung des Risikos für eine hepatische Steatose ergab eine moderate Sensitivität und Spezifität bei vorwiegend nicht-adipösen Personen ohne Diabetes mellitus.

Die Beziehung von Insulinresistenz und mitochondrialer Kapazität der Leber war bei Personen mit Adipositas Grad 3 abhängig von der Nüchtern-Plasmaglukose sowie vom Leberlipidgehalt. Eine direkte positive Assoziation beider Parameter fand sich nur in der Subgruppe mit normaler Glukosetoleranz und ohne MASLD. Insbesondere bei Personen mit Adipositas und Prädiabetes ergaben sich Hinweise auf eine Beeinträchtigung des hepatischen Insulinsignalweges sowie der Mitophagie. Bei metabolisch gesunden Personen fand sich eine positive Assoziation zwischen den Konzentrationen hepatischer Energieträger und den zirkulieren Spiegeln von Palmitoleinsäure, aber auch bestimmten Aminosäuren, insbesondere der essentiellen Aminosäure Leucin. Des Weiteren konnte nachgewiesen werden, dass eine Fettmahlzeit reich an gesättigten, nicht jedoch reich an einfach ungesättigten Fettsäuren, bei schlanken, gesunden Personen eine Veränderung des hepatischen Energiestoffwechsels bedingt, obwohl beide Fettmahlzeiten zur akuten Entwicklung einer Insulinresistenz führen.
Die antihyperglykämische Therapie mit dem SGLT2-Inhibitor (SGLT2I) Empagliflozin verminderte den Leberlipidgehalt bei Personen mit Typ 2 Diabetes. Dies war vorwiegend über den verursachten Gewichtsverlust vermittelt, jedoch spielten möglicherweise auch andere Mechanismen, wie eine antiinflammatorische Wirkung gesenkter Harnsäure- und gesteigerter Adiponektinspiegel eine Rolle. Zudem reduzierte die Therapie mit Empagliflozin, unabhängig vom durch nicht-invasive Tests ermittelten Steatose- und Fibroserisiko, das Risiko für kardiovaskuläre und renale Ereignisse bei Personen mit Typ 2 Diabetes und manifester kardiovaskulärer Erkrankung.
Zusammenfassung: Eine detaillierte Methodenvalidierung in Kohorten mit unterschiedlichem metabolischen Risikoprofil trägt dazu bei, mögliche Fehlinterpretationen von Daten zu vermeiden und die dahinterstehenden Mechanismen zu verstehen.
Bei Personen mit Adipositas Grad 3 sollten Strategien zur Prävention von Dysglykämie und MASLD so früh wie möglich angewendet werden. Im Hinblick auf den Leberstoffwechsel ist eine Ernährung reich an ungesättigten Fettsäuren einer Ernährung reich an gesättigten Fettsäuren vorzuziehen. Der SGLT2I Empagliflozin reduziert effektiv das Risiko für das Auftreten und/oder die Progression von Typ 2 Diabetes-bezogenen kardiometabolischen Komplikationen.

Diese kumulative Habilitationsschrift besteht aus 8 begutachteten Originalarbeiten, die als Erstautorin oder Ko-Erstautorin publiziert wurden (Anlagen 1-8).

1. Introduction

1.1 The metabolic pandemic: obesity, type 2 diabetes and MASLD

Overweight and obesity closely relate to insulin resistance, type 2 diabetes and metabolic dysfunction-associated steatotic liver disease (MASLD) [1]. As per the definition of the World Health Organization (WHO), overweight is defined by a body mass index (BMI) of ≥25 kg/m^2 and obesity by a BMI ≥30 kg/m^2 [2]. Studies predict that more than half of the global population will be overweight or obese within the next 10 years, and in parallel, the economic impact of overweight and obesity is expected to rise to 4 trillion US dollars (USD) including both healthcare costs and effects on economic productivity [3]. In Germany, according to a self-report, already 53.5% of adults are overweight, and particularly obesity prevalence continues to increase [3,4]. Obesity is a risk factor for many secondary diseases, such as insulin resistance, type 2 diabetes, cardiovascular diseases, cancer and musculoskeletal disorders, and is accompanied by an increased risk of premature death [4].

Diabetes mellitus is diagnosed either by HbA1c levels (≥6.5%) or prevailing plasma glucose levels (fasting glucose ≥126 mg/dl; postprandial glucose ≥200 mg/dl) [5], and about 537 million people worldwide are affected [5]. In most cases, prediabetes, which is impaired fasting glucose (fasting glucose values ≥100 mg/dl) and/or impaired glucose tolerance (≥140 mg/dl for the 2-hour value of the oral glucose tolerance test), precedes manifest type 2 diabetes but already bears an increased risk of diabetes-related diseases [6]. Type 2 diabetes is the most common form of diabetes mellitus, representing ~90% of all diabetes cases. In Germany, about 6.2 million people are affected by diabetes [5]. The disease is related to its “classical“ complications, such as cardiovascular disease, diabetic retinopathy, neuropathy and nephropathy, but also to emerging complications concerning the liver, malignancies and psychiatric/psychological disorders [7]. Thus, diabetes is not only a large burden for the affected individuals, but also for the health care systems; in Germany in 2021, 6661 USD (~6120 Euro) were spent per adult person with diabetes [5].

Metabolic dysfunction-associated steatotic liver disease (MASLD) [8], formerly known as non-alcoholic fatty liver disease (NAFLD), encompasses a broad disease spectrum ranging from simple steatosis over inflammatory, pro-fibrotic steatohepatitis (MASH) to cirrhosis and hepatocellular carcinoma (HCC). Presence of steatosis is defined by a liver lipid content of ≥ 5.56% (assessed by magnetic resonance-based methods) or by ≥ 5% of hepatocytes having lipid droplets in liver histology [9,10], whereas the diagnosis of MASLD requires the presence of steatosis and at least one cardiometabolic risk factor (overweight, dysglycemia, hypertension, hypertriglyceridemia, or low high-density lipoprotein (HDL) cholesterol) in the

absence of relevant alcohol consumption [8]. The discrepancy between the new and old classifications “MASLD“ and “NAFLD“ is considered minimal, and findings from older NAFLD studies may therefore be reported under the new MASLD definition [11]. It is estimated that about 25% of the world’s adult population is affected by MASLD with 10-20% progressing to MASH [12]. MASLD is not only related to an increased risk of liver-related complications such as cirrhosis and HCC, but also of extrahepatic complications such as cardiovascular and renal disease as well as malignancies. Currently, fibrosis grade is thought to be the best predictor of morbidity and mortality risk in people with MASLD [13]. During the last decade, MASLD/MASH, together with alcohol-associated liver disease, became the most common indication for liver transplantation and was most frequent in people with HCC [14,15].

1.2 Pathogenesis

1.2.1 Pathogenesis of obesity and insulin resistance

In most cases, obesity develops due to overfeeding and excessive nutrient supply. Nutrients are stored in adipose tissue either by adipocyte hyperplasia or hypertrophy. Whereas adipocyte hyperplasia has been associated with a benign, anti-inflammatory phenotype, adipocyte hypertrophy is paralleled by cellular mechanical stress, endoplasmic stress and hypoxia, as well as the promotion of the immigration of pro-inflammatory macrophages, resulting in adipose tissue insulin resistance and increased rates of lipolysis [16]. There seems to be an individual threshold at which the adipose tissue switches from benign to pathologic lipid handling [17]. Two compartments have been established: subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), and especially VAT has been associated with deleterious metabolic effects [1,18]. Insulin-resistant adipose tissue is a major source of circulating non-esterified fatty acids (NEFA) as well as pro-inflammatory cytokines. Increased circulating NEFA levels promote lipotoxic effects in the liver and skeletal muscle, including the accumulation of toxic lipid intermediates, inflammation, altered mitochondrial function, increased oxidative stress and impaired insulin action [1].

On the cellular level, under physiological conditions, the binding of insulin to the insulin receptor activates its tyrosine kinase activity causing an autophosphorylation of several tyrosine residues in the β-subunit. This autophosphorylation triggers the activation of insulin receptor substrate (IRS) and phosphatidylinositol-3-Kinase (PI-3K), which in turn activate protein kinase B (also known as AKT), resulting in the regulation of glucose, lipid and protein metabolism [1]. In insulin resistance, these signaling pathways are disturbed.

1.2.2 Pathogenesis of type 2 diabetes

Type 2 diabetes results from a relative insulin deficiency in the presence of insulin resistance. As soon as the endogenous insulin secretion cannot cope with the prevailing insulin resistance, blood glucose rises continuously until it fulfills the criteria of prediabetes and, later on, manifest diabetes mellitus [19]. Over time, the enhanced insulin secretion rates decline again due to increasing β-cell failure (also referred to as Starling's curve [20]), which may ultimately result in the progression to absolute insulin deficiency in long-standing type 2 diabetes. There is consensus in the literature that the onset and pace of β-cell failure determine the rate of progression of hyperglycemia [21]. Several factors contribute to β-cell failure in type 2 diabetes, including age, genes, severity of insulin resistance, gluco- and lipotoxicity as well as defective incretin signaling [21].

In type 2 diabetes, basal rates of hepatic endogenous glucose production (EGP), and especially gluconeogenesis (GNG), are increased and contribute to both fasting and postprandial hyperglycemia [1]. In parallel, insulin resistance in skeletal muscle triggers impaired (post-)prandial muscle glucose disposal due to reduced myocellular glucose transport and phosphorylation, glycogen synthesis and glucose oxidation. Moreover, the chronically elevated circulating glucose and NEFA levels further aggravate insulin resistance in liver, muscle and adipose tissue, as well as pro-inflammatory processes (gluco- and lipotoxicity) (Figure 1).

The relative contributions of insulin resistance and β-cell failure to the development of type 2 diabetes differ in different populations. Moreover, individuals with first-degree relatives with type 2 diabetes bear a substantially increased risk of developing type 2 diabetes themselves [22]. Recently, genome-wide association studies (GWAS) essentially contributed to the understanding of the aetiological heterogeneity associated with the development and progression of type 2 diabetes [23].

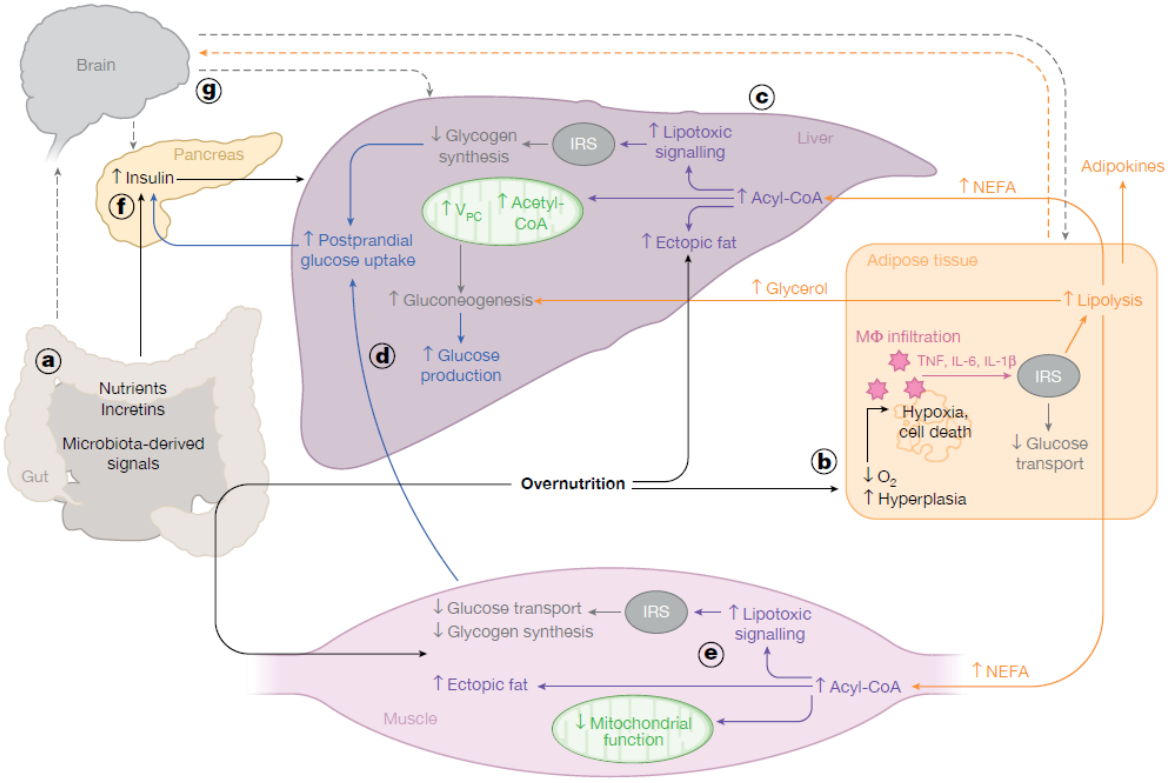

Figure 1: Pathogenic mechanisms of insulin resistance and type 2 diabetes. Illustration from Roden & Shulman [1] with the permission of Springer Nature. NEFA, non-esterified fatty acids; ROS, reactive oxygen species, IRS, insulin receptor substrate. a, overnutrition; b, adipose tissue insulin resistance; c, d hepatocellular insulin resistance and lipid accumulation; e, skeletal muscle insulin resistance; f, impaired insulin secretion; g, contribution of the brain via afferent and efferent signaling.

1.2.3 Pathogenesis of MASLD

Genetic and environmental factors contribute to the pathogenesis of MASLD. Family members of people with MASLD are at increased risk of developing MASLD themselves. One possible genetic factor is a single nucleotide polymorphism (SNP) in the patatin-like phospholipase domain-containing protein 3 (PNPLA3) gene, which has been associated with steatosis and progression to MASH-related fibrosis, cirrhosis and HCC [24], but, amongst others, also gene variants of transmembrane 6 superfamily 2 (TM6SF2), membrane-bound O-acyltransferase domain containing 7 (MBOAT7) and glucokinase regulatory protein (GCRK) may play a role [25]. As in type 2 diabetes, gluco- and lipotoxicity play a pivotal role in MASLD pathogenesis and progression [25]. Increased NEFA supply from insulin-resistant adipose tissue and enhanced hepatic de-novo-lipogenesis (DNL), paralleled by altered adipokine secretion patterns (i.e., reduced adiponectin, increased leptin levels), seem to be major contributors to increased liver lipid content (Figure 1) [26]. The liver encounters the increased lipid availability by upregulating its oxidative phosphorylation (OXPHOS) capacity (mitochondrial plasticity; [27]); however, this happens at the expense of increased production of

reactive oxygen species (ROS). ROS, in turn, trigger hepatic inflammation and cellular/mitochondrial damage. Thus, in the long term, hepatic mitochondrial adaption to increased substrate loads is lost in progressed states of MASLD [27]. Moreover, with increasing storage of hepatic triglycerides, toxic lipid intermediates (diacylglycerols (DAGs), ceramides) are produced, which interfere with hepatic insulin signaling, e.g., by the diacylglycerol-novel protein kinase C pathway. Certain lipid intermediates also trigger hepatic inflammation, which promotes pro-fibrotic processes by activating non-parenchymal Kupffer and Stellate cells [27]. Hyperglycemia further enhances DNL as well as the formation of advanced glycation end products (AGEs), contributing to impaired insulin signaling, oxidative stress and thus inflammatory and pro-fibrogenic activity. In addition, low adiponectin levels promote hepatic insulin resistance, inflammation and fibrosis [28].

Of note, not only mere overnutrition but also dietary patterns contribute to the development of metabolic diseases. A common feature of these pathologies is the presence of insulin resistance, with lipotoxic mechanisms playing a central role in their initiation and progression [18]. The Western diet, a modern dietary eating pattern, is characterized by increased intake of processed foods, red meat and saturated fatty acids (SAFA) [29]. It has been proposed that elevated SAFA consumption may relate to postprandially activated immune responses and pro-inflammatory conditions [30]. Moreover, SAFA have been associated with obesity, type 2 diabetes and MASLD [31].

1.3 Assessment of insulin sensitivity, β-cell function and MASLD

The gold-standard test for the assessment of insulin sensitivity is the hyperinsulinemic-euglycemic clamp test (HEC), which may be combined with other methods such as isotope dilution techniques for the assessment of hepatic insulin sensitivity and/or the intravenous glucose tolerance test (IVGTT) to be able to determine insulin secretion and insulin sensitivity in one experiment (the so-called Botnia clamp) [32]. Hyperglycemic clamps are another appropriate experimental set-up to measure β-cell function [32].
Whole-body but also tissue-specific insulin sensitivity may be calculated for fasting and/or insulin-stimulated (postprandial) conditions. At maximum insulin stimulation, the skeletal muscle accounts for about 85% of whole-body glucose uptake [32]. Thus, whole-body insulin sensitivity derived from the HEC mainly represents insulin-stimulated skeletal muscle glucose disposal and is calculated from the mean glucose infusion rate (GIR) during the last 30 minutes of the clamp test (steady state). Whole-body insulin sensitivity is expressed as the M-value, representing the average glucose disposal per minute and kg body weight (BW) during steady state conditions at a constant high insulin level. Rates of fasting and insulin-suppressed endogenous (predominantly hepatic) glucose production (EGP) are calculated from the tracer infusion rate of [6,6-$^{2}H_{2}$]glucose and its enrichment to the hydrogens bound to

carbon 6 divided by the mean percent enrichment of plasma [6,6-$^{2}H_2$]glucose using either steady-state or non-steady state equations [32] and are given as mg/min/kg BW. Hepatic insulin sensitivity is expressed as the ratio of insulin-suppressed EGP rates during the clamp test and fasting EGP rates ((1- $EGP_{clamp}/EGP_{fasting}$)*100). Adipose tissue insulin sensitivity may be addressed by different methods, using, e.g., the Adipo-IR index (fasting NEFA times fasting insulin levels [33]) for fasting conditions and the insulin-mediated NEFA suppression ((1-$NEFA_{clamp}/NEFA_{fasting}$)*100) during a clamp test [34].
However, as conducting HECs is time-consuming and costly, several indices for assessment of insulin secretion and whole-body insulin sensitivity were developed derived from the fasting state (i.e. for insulin secretion: ratio of fasting C-peptide and fasting insulin; for insulin sensitivity: QUICKI [35]) or dynamic conditions during the easier-to-perform oral glucose tolerance test (OGTT) and mixed-meal tolerance test (MMTT) (i.e., for insulin secretion: insulinogenic index [36], disposition index [37], adaptation index [38]; for insulin sensitivity: Matsudas index (ISIcomp) [39], oral glucose insulin sensitivity index (OGIS) [40], predicted-M index (PREDIM) [41]). All indices of insulin sensitivity have been validated against the gold standard for assessment of whole-body insulin sensitivity, the M-value [32].

The gold standard for MASLD assessment still remains liver biopsy, although this method is time-consuming, costly and bears rare but severe side effects such as bleedings [9, 10]. For estimation of steatosis and fibrosis grade, non-invasive magnetic resonance (MR)-based methods or transient elastography (TE) may be applied [10]. ^{1}H-MR spectroscopy (MRS) and MR imaging proton density fat fraction (MRI-PDFF) provide the most exact estimation of steatosis. MR elastography is applied for the most accurate, non-invasive estimation of fibrosis grade [42]. However, these methods are often not available in daily routine or large clinical studies. Therefore, also blood-based non-invasive tests (NITs) for estimation of steatosis and fibrosis risk based on simple demographic, anthropometric and laboratory parameters have been developed, providing better prediction than liver enzyme values alone [10]. The following NITs are examples of frequently used blood-based steatosis indices [43]: Fatty liver index (FLI) [44], hepatic steatosis index (HSI) [45], NAFLD liver fat score (NAFLD-LFS) [46], and Dallas steatosis index (DSI) [47]. The FLI uses triglycerides, BMI, waist circumference and gamma glutamyl transferase (γ-GT); the HSI is based on aspartate amino transferase (AST), alanine amino transferase (ALT), BMI and γ-GT, whereas the NAFLD-LFS includes the presence/absence of the metabolic syndrome and diabetes mellitus, as well as fasting insulin, AST and ALT. The DSI consists of a formula including ALT, BMI, age, sex, triglycerides, fasting glucose, diabetes mellitus, hypertension and ethnicity [43]. Although the FLI and HSI were initially developed in comparison to conventional ultrasound, both indices also performed with similar specificity and sensitivity when validated against liver histology and MRS [43]. In contrast, the NAFLD-LFS and DSI were developed based on direct

comparison with MRS. Also, several scores for assessment of fibrosis risk have been derived, including the fibrosis-4 index (FIB-4), which is calculated by a formula considering age, AST, platelet count and ALT [48], and the NAFLD fibrosis score (NFS), which includes age, BMI, glucose tolerance status, AST, ALT, platelet count and albumin [49]. Both indices were developed in comparison with liver histology for the identification of individuals at high risk of advanced fibrosis, which is commonly defined as fibrosis grade F3 or F4 [42].

1.4 Treatment

1.4.1 Current therapeutic concepts of type 2 diabetes

The treatment of newly diagnosed type 2 diabetes is based on nutritional counseling for a healthy diet (e.g., a Mediterranean diet) and encouragement to increase physical activity [50]. If glycemia does not improve with these measures after a few months, or in cases of high plasma glucose values at diagnosis, drug treatment is initiated. For many years, metformin was the first-line treatment, as it not only improves hyperglycemia but also associates with reductions in body weight and improvements in insulin sensitivity [18]. However, it also has relevant side effects, such as gastrointestinal intolerance and an increased risk of lactate acidosis. During the last few years, glucagon-like peptide 1 receptor agonists (GLP-1RA) and sodium glucose co-transporter 2 inhibitors (SGLT2I) have been increasingly used in type 2 diabetes treatment and included as first-line treatment options in international guidelines for several reasons [50]. On the one hand, both substance classes mediate effective improvement of glycemia with low risk for hypoglycemia and accompanied by body weight loss; on the other hand, they provide a risk reduction for cardiovascular and renal events in people with type 2 diabetes [18]. The unprecedented weight loss effects of GLP-1RA even lead to their approval in the indication of weight loss without a pre-existing condition of type 2 diabetes [51].

Sodium glucose co-transporter 2 (SGLT2) is expressed in the proximal tubules of the nephron in the kidneys, mediating glucose and sodium reabsorption from the primary urine back into the circulation. Inhibition of SGLT2 induces glucosuria with an average HbA1c reduction of ~0.5-1%. Of note, this glucose loss causes an energy deficit of about 200 kcal/day, resulting in an average body weight loss of about 2-4% [52,53]. The SGLT2I-induced natriuresis associates with a negative salt and water balance and in turn, a slight drop of blood pressure (3-6 mmHg systolic, 1-1.5 mmHg diastolic) [54], but also with the normalization of the tubulo-glomerular feedback, reduction in intraglomerular pressure and improvement of hyperfiltration, which is often found in early stages of diabetic nephropathy [55]. SGLT2I (canagliflozin, dapagliflozin and empagliflozin have marketing approval in the EU) proved consistent positive effects on cardiovascular and renal outcomes in people with and without type 2 diabetes in large cardiovascular outcome trials (CVOTs) [55]. A recent metaanalysis

specifically showed a reduction of heart failure events and cardiovascular death in different collectives at risk such as people with manifest heart failure, type 2 diabetes, chronic kidney disease and atherosclerotic cardiovascular disease [56]. The benefits of SGLT2I on cardiovascular outcomes are thought to occur through different mechanisms beyond the lowering of plasma glucose. SGLT2I have been reported to improve endothelial dysfunction and to decrease arterial stiffness and cardiac remodeling [55,57]. The underlying mechanisms may include the SGLT2I-mediated inhibition of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome and reduction of oxidative stress, most likely by modulating metabolic pathways and mitochondrial function [56].

GLP-1RA can be divided into short-acting (e.g., exenatide) and long-acting (e.g., liraglutide, dulaglutide, semaglutide) substances. Whereas the short-acting drugs primarily lead to the improvement of postprandial glycemia via slowing of gastric emptying, the long-acting molecules predominantly stimulate glucose-dependent insulin secretion and suppress glucagon secretion [58,59]. Moreover, all GLP-1RA can induce weight loss through central GLP-1 receptor activation in specific regions in the hypothalamus and the brainstem promoting satiety and decrease appetite [60,61]. The average HbA1c reduction with GLP-1RA from real-word data was reported to be ~1% in people with type 2 diabetes [62,63]. In CVOTs, GLP-1RA treatment has been shown to be beneficial in regard to cardiovascular and renal outcomes in people with and without type 2 diabetes [18].

1.4.2 Current therapeutic concepts of MASLD

As for type 2 diabetes, the basis of MASLD treatment is lifestyle intervention aiming at weight loss, and treatment success depends on the amount of weight loss achieved. Indeed, with ≥10% body weight reduction by lifestyle intervention, fibrosis regression was observed in 65% of the investigated individuals. However, in clinical practice, relevant weight loss by lifestyle intervention is achieved in only about 10% of the affected people and rarely maintained on the long-term [64].

Also, bariatric surgery has been proven highly effective for MASLD treatment [65]; however, the indication for surgery may not yet be based on the presence or severity of MASLD, but only on grade 2 obesity combined with comorbidities such as treatment-resistant type 2 diabetes or grade 3 obesity alone [9,10].

Recently, the first drug in the indication of MASLD, resmetirom, has received conditional approval by the American Food and Drug Administration (FDA). Resmetirom is a selective thyroid receptor β agonist, stimulating lipid oxidation and inhibiting lipogenesis in the liver. In phase 3 studies, resmetirom showed effective MASH resolution and fibrosis regression in people with and without type 2 diabetes [66]. The drug may be administered to people with

MASH and F2 to F3 fibrosis. Moreover, antihyperglycemic medications, especially GLP-1RA and SGLT2I have been tested in randomized clinical trials (RCTs) for their effects on MASLD as both substance classes mediate moderate (SGLT2I) to high (GLP-1RA) body weight reduction in people with type 2 diabetes [18]. Two RCTs with liraglutide and semaglutide in mixed collectives of people with and without type 2 diabetes provided evidence for higher rates of histological MASH resolution without worsening of fibrosis compared to placebo [67,68]. However, there was no improvement in fibrosis grade with either substance [18]. Nevertheless, for semaglutide, another respective RCT with a large collective and long study duration is ongoing (ESSENCE study; clinicaltrials.gov identifier NCT04822181). Of note, the recently approved dual GLP-1/gastric inhibitory peptide (GIP) receptor agonist tirzepatide, providing even greater effects on reduction of body weight than the most potent GLP-1RA semaglutide, also proved effective for MASH resolution without worsening of fibrosis in a recent phase 2 clinical trial in people with MASH and F2/F3 fibrosis [69]. Apart from their weight loss-mediating effects, GLP-1RA may increase adipose tissue and hepatic insulin sensitivity, paralleled by decreased DNL, even though there is consensus that hepatocytes, stellate and Kupffer cells do not express GLP-1 receptors [70] (Figure 2).

The effects of SGLT2I on MASLD components are less clear [10]. There is broad evidence for the beneficial actions of SGLT2I on hepatic steatosis based on the assessment of liver lipid content by imaging methods [18]. In contrast, only a few results from small pilot trials and one controlled trial investigating the SGLT2I-mediated effects on histological outcomes (steatosis, lobular inflammation, ballooning, and fibrosis) have been published [18]. Also, the mechanisms underlying SGLT2I-mediated MASLD alleviation are only incompletely understood; body weight reduction and/or anti-inflammatory actions on adipose tissue may contribute to the reduction of hepatic steatosis [18]. Pre-clinical studies reported increased fat utilization and browning in white adipose tissue, as well as attenuation of inflammation and insulin resistance with SGLT2I treatment [18,71] (Figure 2).

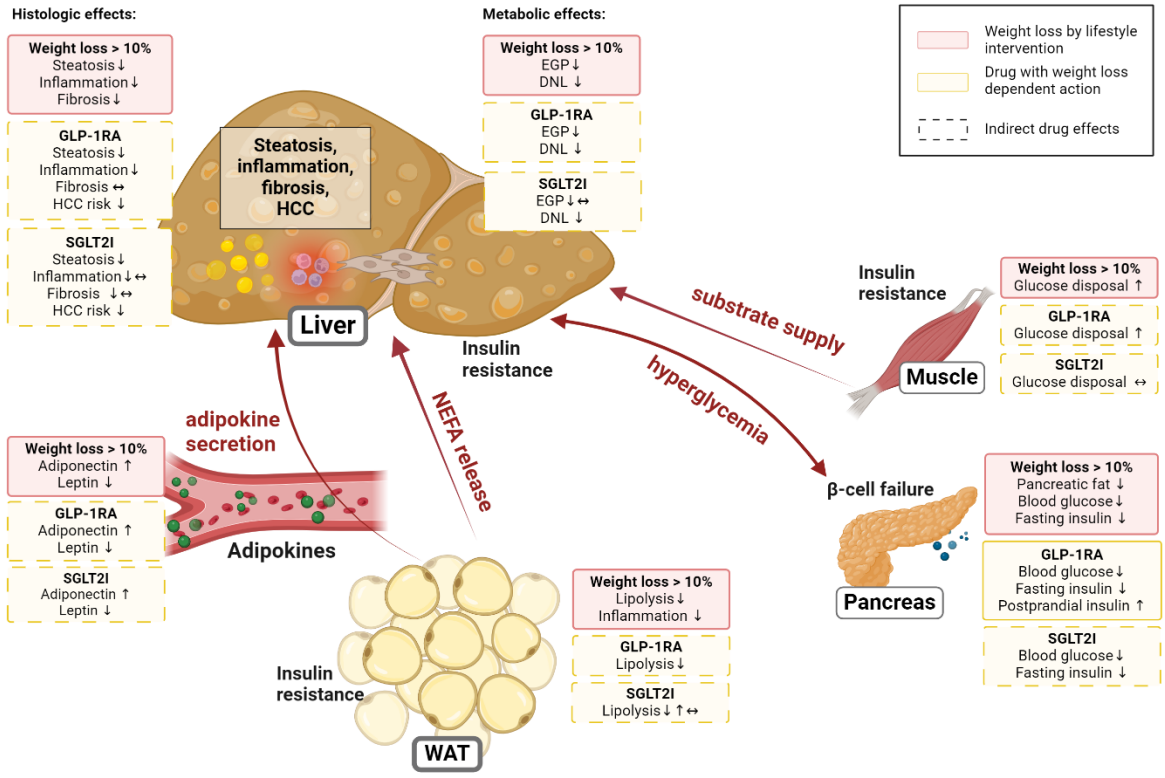

Figure 2. MASLD-related mechanisms of action of GLP-1RA and SGLT2I. Illustration adapted from Huttasch et al. [18], with the permission of Elsevier. DNL, de novo lipogenesis; EGP, endogenous glucose production; GLP-1RA, glucagon-like peptide 1 receptor agonist; HCC, hepatocellular carcinoma; SGLT2I, sodium glucose cotransporter 2 inhibitor; WAT, white adipose tissue.

2. Hypotheses

Insulin resistance and β-cell failure, present to different degrees in obesity, type 2 diabetes and MASLD, interfere with many cellular processes and may therefore also impact on frequently used measures in clinical-metabolic phenotyping [72]. Thus, there is a great need for validation of these measures in collectives with and without metabolic diseases.

Both gluco- and lipotoxicity exert detrimental effects on the liver. However, metabolically healthy people may compensate for transient, excessive substrate supply through adaptive mechanisms [73]. The metabolic conditions, that define the break-even point, at which compensatory mechanisms fail, are still not understood. Also, the impact of dietary lipid quality on the extent and nature of hepatic metabolic alterations has not yet been clearly defined.

In recent years, SGLT2Is have been increasingly prescribed for the treatment of type 2 diabetes, but also for the treatment of heart failure and chronic kidney disease [55]. Nevertheless, it is not clear if SGLT2I exert their beneficial effects in people with type 2 diabetes independent of metabolic control and concomitant diabetes-related diseases.

These gaps in knowledge led to the following set of hypotheses:

2.1 Validation of methods for assessment of insulin sensitivity and MASLD

2.1.1 The IVGTT of the BOTNIA clamp does not impact on the assessment of whole-body insulin sensitivity when compared to standard HEC in people with type 2 diabetes.

2.1.2 Blood-based NITs for estimation of steatosis risk provide only moderate performance in predominantly non-obese, non-diabetic collectives.

2.2. Pathogenesis of insulin resistance and MASLD

2.2.1 Glucose tolerance and the presence of MASLD determine the relationship of insulin resistance and hepatic mitochondrial OXPHOS capacity in people with obesity grade 3.

2.2.2 Circulating fatty acid and amino acid concentrations associate with hepatic phosphorus metabolites (γ-ATP and P_i levels) in metabolically healthy people.

2.2.3 Saturated and unsaturated fatty acids differentially affect insulin sensitivity as well as hepatic glucose, lipid and energy metabolism in young, lean individuals.

2.3. Pharmacological interventions for MASLD treatment

2.3.1 Empagliflozin reduces liver lipid content in people with well-controlled type 2 diabetes, with and without MASLD.

2.3.2 The empagliflozin-mediated reductions in cardiorenal events are independent of steatosis and fibrosis risk in people with type 2 diabetes and cardiovascular disease.

3. Results

The above-mentioned hypotheses were addressed in clinical-experimental studies in humans. The main findings are summarized in the following sections, and the full texts are attached to this document.

3.1 Validation of methods for the assessment of insulin sensitivity and hepatic steatosis

3.1.1 Values of whole-body insulin sensitivity obtained from the BOTNIA clamp are comparable to those from the standard hyperinsulinemic-euglycemic clamp (study 1) [74]

3.1.2 Blood-based indices of hepatic steatosis provide moderate performance in a collective of people from the general population (study 2) [75]

3.2. Role of dietary lipids and circulating metabolites in the pathogenesis of insulin resistance and MASLD

3.2.1 Dysglycemia and liver lipid content determine the relationship of insulin resistance and hepatic OXPHOS capacity in obesity (study 3) [76]

3.2.2 Circulating fatty acid and amino acid levels, but not insulin sensitivity, associate with hepatic energy metabolism in metabolically healthy humans (study 4) [77]

3.2.3 Saturated fat initiates insulin resistance and alterations in hepatic energy metabolism and liver lipid content in young, lean humans (study 5) [78]

3.2.4 Monounsaturated fat induces gluconeogenesis and insulin resistance but does not affect hepatic energy metabolism in young, lean humans (study 6) [79]

3.3 Effects of empagliflozin treatment on MASLD and cardiovascular risk in people with type 2 diabetes

3.3.1 Liver lipid content is reduced by empagliflozin treatment in people with well-controlled type 2 diabetes, with and without MASLD (study 7) [80]

3.3.2 Empagliflozin reduces cardiovascular events in people with type 2 diabetes irrespective of steatosis or fibrosis risk category (study 8) [81]

3.1.1 Values of whole-body insulin sensitivity obtained from the BOTNIA clamp are comparable to those from the standard hyperinsulinemic-euglycemic clamp

Background: The Botnia clamp includes an intravenous glucose tolerance test (IVGTT) immediately followed by a hyperinsulinemic-euglycemic clamp (HEC) to allow for the sequential assessment of insulin secretion and insulin sensitivity during one experimental day [32]. However, there is no data on the comparability of measures of insulin sensitivity (M-value) from HEC with and without a preceding IVGTT in people with type 2 diabetes. Thus, it cannot be excluded that the IVGTT-induced hormonal and metabolic responses acutely impact the prevailing insulin sensitivity.

Methods: In this cross-over study, 19 people with diet- or metformin-treated type 2 diabetes and good glycemic control were assigned to a Botnia clamp or standard HEC, each combined with isotope dilution technique, in random order spaced by a two-week interval. During clamp experiments, whole-body (M-value normalized for fat-free mass (FFM; M_{FFM}); M_{FFM} adjusted to the prevailing insulin levels (M_{FFM}/I)) and hepatic insulin sensitivity (insulin-stimulated EGP suppression) were assessed. Insulin-mediated NEFA inhibition was calculated by a previously described modeling method [82]. Further, dynamic indices of insulin secretion (acute insulin response (AIR) [32], disposition index (DI) [37]) were derived from insulin and C-peptide during the IVGTT.

Results: At HEC start (1 hour after start of the IVGTT or a saline bolus, respectively), circulating glucose, insulin and C-peptide were higher and NEFA levels were lower with the Botnia clamp compared to the standard protocol. During HEC, NEFA and insulin levels were comparable, whereas C-peptide remained higher during the Botnia HEC compared to the standard HEC ($p<0.01$). Plasma levels of inflammatory markers TNFα, monocyte chemoattractant protein-1 (MCP-1) and IL-1ra did not differ between both examinations. Measures of whole-body insulin sensitivity from the two experimental days were closely correlated (M_{FFM}: 8.7±0.8 and 8.1±0.7 mg*kg*FFM^{-1}*min^{-1}; CV 11.0%, $r=0.87$; $p<0.001$; M_{FFM}/I: 1.09±0.14 and 0.98±0.13 mg*kg FFM^{-1}*min^{-1}/pmol*l^{-1}; CV 12.8%, $r=0.93$, $p<0.001$), but nevertheless showed intra-individual variations. Endogenous glucose production and hepatic insulin sensitivity were comparable during fasting and HEC steady-state, but EGP was lower and hepatic insulin sensitivity was higher pre-clamp after the IVGTT compared to saline (both $p<0.01$). The M_{FFM} values from the Botnia clamp and standard HEC correlated with the corresponding fasting NEFA levels ($r=-0.58$, $p<0.01$ and $r=-0.59$, $p<0.01$) and even more strongly with the NEFA levels at the end of the IVGTT or saline period ($r=-0.86$, $p<0.001$ and $r=-0.69$, $p<0.01$). Intra-individual differences in whole-body insulin sensitivity obtained from the Botnia clamp and standard HEC correlated with differences in circulating NEFA concentrations 1 hour after start of the IVGTT/saline bolus between both tests (r=-

0.60, p<0.01), but neither with differences in the area under the curve (AUC) of C-peptide nor hepatic insulin sensitivity at the end of the IVGTT period.

Conclusions: The Botnia clamp and standard HEC provide similar estimates of insulin sensitivity in people with type 2 diabetes. However, insulin-mediated suppression of lipolysis with decreasing NEFA levels during the IVGTT may impact the measurement of whole-body insulin sensitivity to a variable degree. Thus, the discrimination between insulin-sensitive and insulin-resistant individuals may be affected when comparing M-values obtained from both the Botnia clamp and standard HEC in people with different adipose tissue insulin sensitivity and β-cell function.

Limitations: Differences in insulin sensitivity also occur because of intra-individual day-to-day changes, and the mean CV between both tests was 11%, as expected from the literature [32,72]. Thus, study 1 cannot rule out other factors impacting on prevailing insulin sensitivity, such as physical activity, diet or stress, although all participants were advised to follow a carbohydrate-rich diet for 3 days before the examinations and refrain from physical exercise.

Advantages: Study 1 was the first to show that the Botnia clamp and standard HEC yield similar estimates of whole-body insulin sensitivity (M_{FFM}) in people with type 2 diabetes. Moreover, study 1 was able to identify potential confounders for the assessment of whole-body insulin sensitivity when comparing the results from these settings.

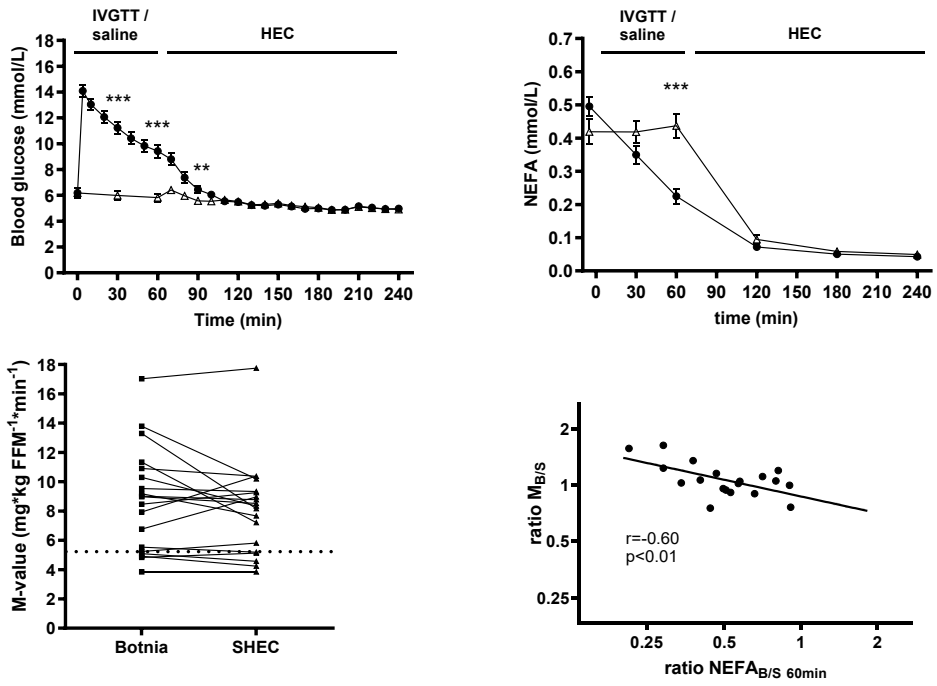

Figure 3: Comparison of metabolic conditions during the Botnia clamp and the standard hyperinsulinemic euglycemic clamp (SHEC). Adapted from Kahl et al. [74] with the permission of Springer Nature. IVGTT, intravenous glucose tolerance test. Dotted line, cut-off of 5.3 mg*kg FFM^{-1}*min^{-1} for discrimination of insulin-sensitive and insulin-resistant individuals [83]. Two out of 19 participants were grouped differently due to the M-value measured from Botnia clamp or standard HEC.

3.1.2 Blood-based indices of hepatic steatosis provide moderate performance in a collective of people from the general population

Background: The presence of MASLD increases the risk for complications in metabolic diseases, as shown for cardiovascular, renal, malign and liver-related events [18]. Thus, effective screening for MASLD is mandatory. The gold standard for the assessment of steatosis are imaging-based methods (MRI-PDFF, ^{1}H-MRS or transient elastography); however, these are often not available in clinical routine. Thus, in the last years, several blood-based non-invasive tests (NITs) for the estimation of steatosis risk have been developed. However, there is still limited data on their performance in different collectives with and without metabolic diseases.

Methods: This analysis included 92 non-obese humans without diabetes who underwent clinical examination, blood work, ^{1}H-MRS and an oral glucose tolerance test (OGTT) in the pre-tests for the German National Cohort („NAKO Gesundheitsstudie“ [84]). For the estimation of steatosis risk, the NAFLD liver fat score (NAFLD-LFS) [46], Hepatic Steatosis Index (HSI) [45] and Fatty Liver Index (FLI) [44] were calculated. Fasting and dynamic measures of insulin sensitivity (Quantitative insulin sensitivity check index (QUICKI) [35], oral glucose insulin sensitivity (OGIS) [40], composite insulin sensitivity (ISIcomp) [39]) and insulin secretion (fasting β-cell function, insulinogenic index (IGI), disposition index (DI) and adaptation index (AI) [36,37,85]) were derived from the OGTT. The performances of these indices were assessed from the respective area under the receiver operating characteristic curve (AROC).

Results: In the whole cohort, 17 out of 92 people (18%) had steatosis. Liver lipid content measured by ^{1}H-MRS ranged from 0.03 to 39.01% (median 2.49%; interquartile range (IQR) [0.62; 4.23]). All steatosis indices correlated with liver lipid content measured by ^{1}H-MRS (all $p<0.001$). Calculated values for the different indices were higher in people with steatosis compared to those without steatosis (NAFLD-LFS $p<0.05$; HSI $p<0.001$; FLI $p<0.01$). Across the whole group, NAFLD-LFS, HSI and FLI yielded AROCs of 0.70, 0.79, and 0.72, respectively and AROCs did not significantly differ from each other. Applying the cut-offs from the original publications yielded low sensitivity (Se) for NAFLD-LFS (0.35 [0.14; 0.62]), but high specificity (Sp) (0.91 [0.82; 0.96]), whereas HSI had maximal Se (1.00 [0.81; 1.00]) at the lower and acceptable Sp (0.75 [0.63; 0.84]) at the upper cut-off. FLI had a Se of 0.76 [0.50; 0.93] and a Sp of 0.83 [0.72; 0.90]. Raising the threshold for diagnosing steatosis to a liver lipid content greater than 5.56% improved AROCs for all indices, although the AROC of FLI did not further improve at a threshold of 7%. Liver lipid content measured by ^{1}H-MRS as well as all investigated indices were strongly and inversely correlated with the measures of insulin sensitivity and, to a lesser degree, with the indices of insulin secretion.

Conclusions: In a predominantly non-obese collective from the general population without diabetes, using NAFLD-LFS, HSI and FLI for assessment of steatosis risk yielded modest

diagnostic efficacy of 70–80% with lower sensitivities and/or specificities compared to the original publications. Of note, all indices were closely related to indices of insulin sensitivity and may therefore not only predict steatosis but also insulin resistance.

Limitations: Study 2 investigated a collective at low risk for hepatic steatosis as people did not have diabetes and had a low frequency of obesity. Thus, the collective of study 2 may not fully represent the group of people in which MASLD screening by blood-based NITs is performed in clinical routine. Further, the rather small sample size and predominantly low liver lipid contents may have contributed to the wide confidence intervals for sensitivity.

Advantages: A major strength of study 2 is the direct comparison of frequently used indices for the assessment of steatosis risk with the measurement of liver lipid content by ^{1}H-MRS in a cohort of predominantly non-obese people without diabetes and Caucasian ethnicity. Also, the results of study 2 support previous suggestions for the use of these steatosis indices as composite surrogate parameters for both liver lipid content and insulin sensitivity [86].

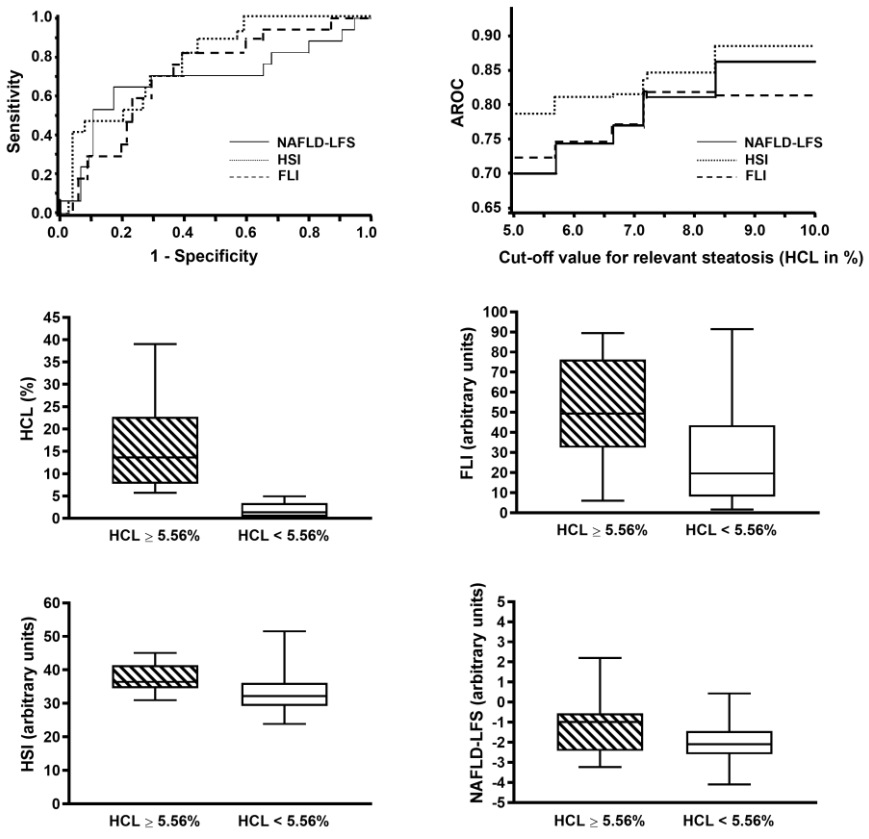

Figure 4. Performance of blood-based non-invasive tests for the estimation of steatosis risk in a predominantly non-obese, non-diabetic cohort from the general population. Adapted from Kahl et al. [75] under a Creative Commons Attribution (CC BY) license or other comparable license that allows free and unrestricted use. AROC, area under the receiver operating characteristics curve; HCL, hepatocellular (liver) lipid content; FLI, Fatty Liver Index; HSI, Hepatic Steatosis Index; NAFLD-LFS, NAFLD Liver Fat Score.

3.2.1 Dysglycemia and liver lipid content determine the relationship of insulin resistance with hepatic OXPHOS capacity in obesity

Background: Obesity, type 2 diabetes and MASLD share insulin resistance as a common central feature in their pathogenesis. Peripheral insulin resistance is causal to substrate oversupply (glucose, lipids) to the liver. The liver can adapt to excessive substrate availability by upregulating its energy metabolism. Hepatic mitochondrial OXPHOS capacity is increased in obesity and early MASLD, but decreased in type 2 diabetes and progressing MASLD. Generally, obesity-related insulin resistance precedes not only (pre)diabetes, but also MASLD. Only scarce information exists about the relationship between insulin resistance and hepatic mitochondrial OXPHOS capacity during the early course of the diseases.
Methods: Study 3 included 65 humans with obesity grade 3 and without diabetes (BMI 50±7 kg/m^2, HbA1c 5.5±0.4%) undergoing bariatric surgery. MASLD stages were assessed by histology, whole-body insulin sensitivity (PREDIcted-M index, PREDIM) [41] by oral glucose tolerance tests, and maximal ADP-stimulated mitochondrial OXPHOS capacity by high-resolution respirometry of liver samples. Multiple linear regression analyses with interaction terms were used to investigate the interplay of hepatic OXPHOS capacity and metabolic parameters.
Results: Prediabetes was present in 30 participants, and MASLD in 46 participants. In the whole collective, simple regression adjusted for age, sex and BMI did not detect an association of insulin sensitivity with hepatic OXPHOS capacity (β=-0.055, p=0.6067), but only in the subgroup of individuals without MASLD and with normal glucose tolerance (n=14; β=-0.54, p=0.0096). Interaction analyses revealed that the regression coefficient of OXPHOS capacity depended on fasting plasma glucose (FPG) and liver lipid content (β= 1.9268, p=0.0224 and β=0.1579, p=0.030, respectively). The respective slopes were negative for FPG ≤100 mg/dl but positive for FPG >100 mg/dl. Liver lipid content displayed a similar behavior, with a threshold value of 24%. Neither BMI, circulating triglycerides, high density lipoprotein (HDL) cholesterol, NEFA, Adipo-IR nor hsCRP interfered with the association of insulin sensitivity and OXPHOS capacity. OGTT 2-hour glucose values affected the association between insulin sensitivity and OXPHOS capacity normalized for citrate synthase activity (β=0.6222, p=0.0057). Prediabetes, particularly increased FPG levels, affected hepatic insulin signaling (lower protein kinase B (AKT) phosphorylation (phospho-AKT(Ser732)/AKT2)), mitochondrial dynamics (lower PTEN-induced kinase 1 (PINK1) phosphorylation (phospho-PINK1(Thr257)/PINK1)) and related positively to fibrosis prevalence. The presence of MASLD related to higher histological scores of hepatic inflammation and cellular damage (inflammation, ballooning) as well as higher lipid peroxidation (thiobarbituric acid reactive substances (TBARS)) in people with normal glucose tolerance.

Conclusions: Rising liver lipid contents and fasting plasma glucose concentrations are associated with a progressive decline of hepatic mitochondrial adaptation to increased energy supply in people with obesity grade 3.

Limitations: As study 3 was powered to detect an association of PREDIM with hepatic OXPHOS capacity, the analyses of interacting factors and of different subgroups have to be regarded as exploratory and do not allow final conclusions as to causality. Also, the lower number of males and the absence of lean people do not allow extrapolation to the general population.

Advantages: Study 3 applies state-of-the-art methods in humans for the assessment of hepatic OXPHOS capacity and physiologic insulin sensitivity. Moreover, it includes a comprehensive analysis of the hepatic expression of proteins involved in insulin signaling and mitochondrial dynamics in subgroups of different glucose tolerance and MASLD status, which allows for gaining mechanistic insights into the early metabolic and mitochondrial alterations occurring in insulin resistance.

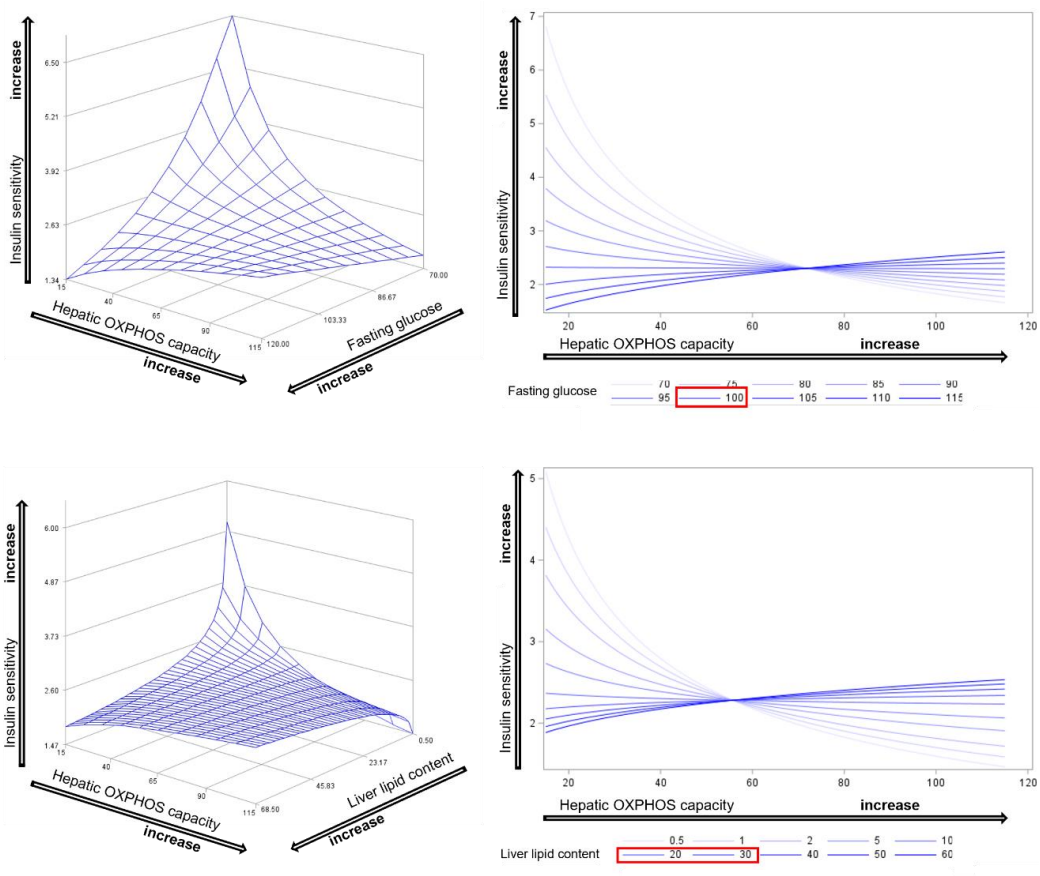

Figure 5: Predicted regression surfaces (left) for the association of insulin sensitivity with hepatic OXPHOS capacity and fasting glucose or liver lipid content, respectively. Regression lines (right) for given fasting glucose or liver lipid content and association of insulin sensitivity with liver OXPHOS capacity. For the graphical presentation, age and BMI were set to their mean values and sex was set to female. Adapted from Kahl et al. [76] with the permission of Elsevier.

3.2.2 Circulating fatty acid and amino acid levels associate with measures of hepatic energy metabolism in metabolically healthy humans

Background: Previous publications reported reduced hepatic energy metabolism in people with type 2 diabetes, which closely related to prevailing insulin sensitivity [87,88]. Of note, multiple factors as BMI, glycemia and lipidemia may contribute to the disturbed hepatic energy metabolism in metabolic diseases. However, neither the association of hepatic energy metabolism with insulin sensitivity, nor the factors which impact on hepatic energy metabolism in metabolically healthy people, have been investigated so far.
Methods: In total, 76 individuals participating in the pre-tests for the German National Cohort („NAKO Gesundheitsstudie“ [84]) were included in the analyses. People underwent a 75 g-OGTT, as well as MR spectroscopy ($^{31}P/^{1}H$-MRS) and blood work. In 62 out of 76 participants, targeted plasma metabolomics were performed. Indices of insulin sensitivity (OGIS [40], QUICKI [35], Hepatic-IR index [89], Liver-IR index [90], Adipo-IR [33]) were calculated from glucose and insulin values before and during the OGTT. The δ-9-desaturase (D9D) index was calculated to estimate DNL [91]. Pearson correlation analyses were performed for the dependent variables γ-ATP and P_i.
Results: Hepatic γ-ATP and P_i levels were positively related to 2-hour OGTT (both $p<0.01$), but not fasting glucose levels. Further, P_i was positively associated with fasting NEFA ($p<0.05$). Neither γ-ATP nor P_i correlated with the different indices of insulin sensitivity. Also, γ-ATP did not relate to any analyzed fatty acid species. In contrast, P_i was positively associated with myristoleic acid, cC16:1w5, palmitoleic acid and the D9D index with and without adjustment for age, sex, and BMI (all $p<0.05$). Hepatic levels of γ-ATP were positively associated with circulating leucine and ornithine concentrations in the unadjusted analyses and also adjusted for age, sex and BMI (all $p<0.05$). The correlation of γ-ATP and leucine levels also remained significant after correction for multiple testing. In multivariate regression models, age, sex, and BMI explained 9.8% of γ-ATP and 7.8% of P_i variability, respectively. Additional adjustment for leucine, as a significant independent predictor, explained 26% of γ-ATP variances, and the adjustment for age, sex, BMI and palmitoleic acid accounted for 15% of P_i variability.
Conclusions: Liver γ-ATP and P_i levels do not directly relate to insulin sensitivity in metabolically healthy non-obese humans. However, circulating concentrations of specific amino and fatty acids modulate hepatic energy metabolism in these people.
Limitations: The measurement of absolute concentrations of hepatic γ-ATP and P_i does not cover all aspects of mitochondrial function, but specifically addresses the flux through ATP synthase. Other relevant parameters of mitochondrial function as oxidative coupled/non-coupled capacity and TCA cycle activity need to be addressed by different techniques as

multitracer approaches and high resolution respirometry from liver tissue, which was not performed in study 4.

Advantages: This work can help to understand differences in hepatic energy metabolism in conditions of metabolic health and disease. A major strength of study 4 is the assessment of *in vivo* hepatic energy metabolism by a state-of-the art technique, ^{31}P-MRS, which is only available at a few centers worldwide. Further, ^{31}P-MRS has previously been performed mainly in conditions of metabolic disease. Thus, this study adds valuable data from metabolically healthy humans and allows for indirect comparison of factors regulating hepatic γ-ATP and P_i during different metabolic conditions.

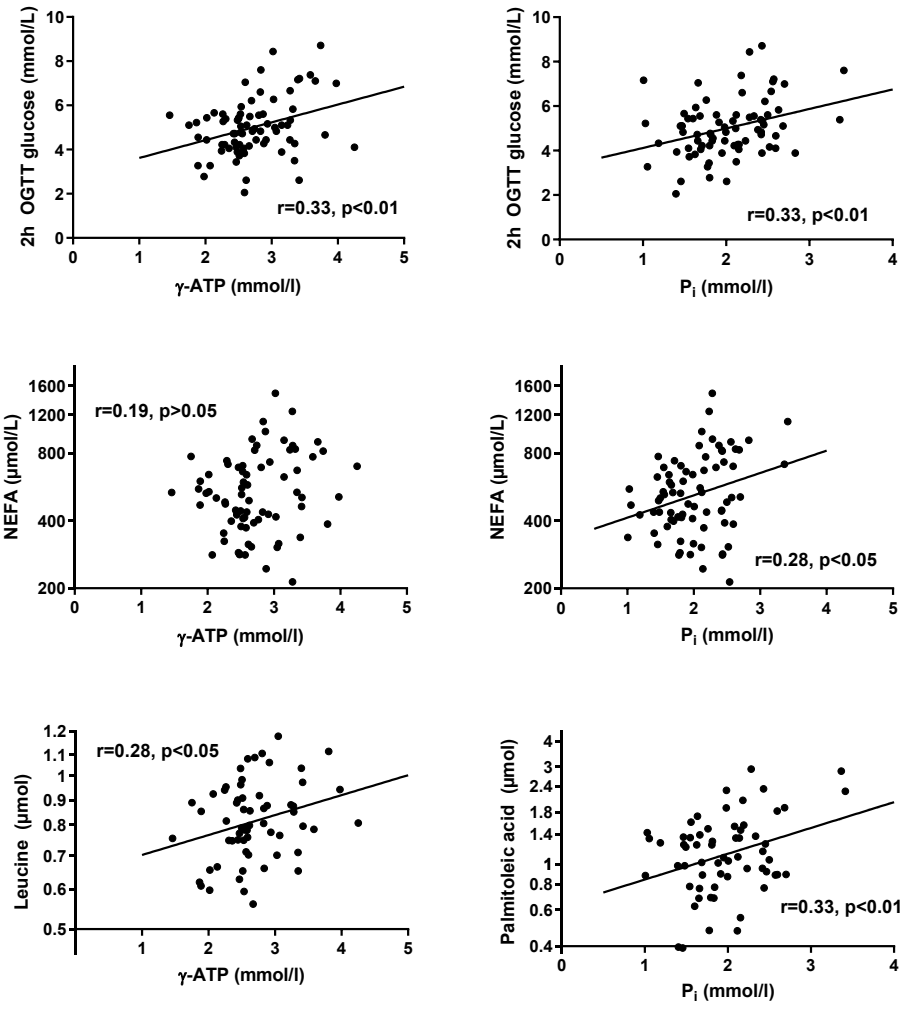

Figure 6: Correlations of hepatic phosphorus metabolite levels with circulating concentrations of fatty acids and amino acids. Adapted from Kahl et al. [77] with the permission of Oxford University Press. OGTT, oral glucose tolerance test; NEFA, non-esterified fatty acids. P_i, inorganic phosphate.

3.2.3 Saturated fat initiates insulin resistance and alterations in hepatic energy metabolism and liver lipid content in young, lean humans

Background: Liver lipid accumulation is promoted by NEFA derived from insulin-resistant adipose tissue, but also dietary fatty acids may directly contribute [1,92]. Especially long-chain saturated fatty acids (SAFA) have been associated with the pathogenesis of insulin resistance and MASLD [31,92]. However, which metabolic changes are acutely initiated by high SAFA intake is still largely unknown in humans. Thus, study 5 investigated the effects of a single oral lipid load rich in SAFA on whole-body, hepatic and adipose tissue insulin sensitivity as well as on hepatic energy metabolism and glucose fluxes in healthy lean humans complemented by liver transcriptomics in mice.

Methods: In this cross-over study, 14 lean, healthy individuals were included, which received palm oil (PO) and vehicle (VCL) in random order spaced by an 8 weeks interval. Insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamps (HEC) with isotope dilution technique. Liver lipid content was assessed by ^{1}H-MRS, hepatic energy metabolism (γ-ATP, P_i) by ^{31}P-MRS and hepatic glucose turnover by oral $^{2}H_2O$/acetaminophen, *ex vivo* ^{2}H MRS and *in vivo* ^{13}C-MRS. In mice receiving PO or VCL, identical clamp procedures and, in addition, hepatic transcriptome analyses were performed. Results were compared using a 2-tailed Student t test or ANOVA adjusted for repeated measures with Bonferroni's testing.

Results: Eight hours after oral administration of 1.18 g/kg body weight (BW) PO, whole-body, hepatic, and adipose tissue insulin sensitivity were decreased by 25%, 15%, and 34% compared to VCL, respectively (all $p<0.05$). Circulating levels of TNFα and interleukin (IL)-6 did not differ between PO and VCL, whereas plasma glucagon levels only rose by 41% after PO ingestion ($p<0.0001$ vs VCL). Further, liver lipid content increased from 0 to 6 hours by 35% ($p<0.01$; $p=0.085$ vs VCL) and γ-ATP by 16% ($p<0.05$; $p=0.57$ vs VCL). At 6 hours after PO ingestion, hepatic gluconeogenesis (GNG) increased by ~70%, and net glycogenolysis (GLYnet) declined by 20% (both $p<0.05$ vs VCL). The contribution of glycogen cycling to total glycogen phosphorylase (GP) flux was negligible with both PO and VCL. In mice, whole-body insulin sensitivity trended towards a reduction ($p=0.07$ PO vs VCL) and hepatic insulin sensitivity was lower with PO compared to VCL ($p<0.05$). Performing transcriptomics in mouse liver showed that PO differentially regulated predicted upstream regulators and pathways, including lipopolysaccharide (LPS), members of the toll-like receptor (TLR) and peroxisome proliferator-activated receptor (PPAR) families, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), and TNF-related weak inducer of apoptosis (TWEAK).

Conclusions: Already a single ingestion of a lipid load rich in SAFA initiated enhanced hepatic energy metabolism and lipid storage paralleled by reductions in whole-body and tissue-specific insulin sensitivity and increased hepatic GNG flux. On a cellular level, PO

ingestion mediated alterations in hepatic expression of genes modulating inflammatory pathways, which promote or prevent MASLD development.

Limitations: Study 5 included a relatively small number of humans and mice, so that more discrete effects of the oral lipid challenge may not have reached statistical significance. Further, the hepatic transcriptomics analyses from mice livers may not be necessarily fully transferable to humans. Study 5 did not perform liver proteomics analyses, which would have provided information if the observed transcriptional changes translated to changes on the protein level. Also, rates of de-novo lipogenesis (DNL), which may have also contributed to the increase in liver lipid content after PO intake, were not assessed.

Advantages: Study 5 was based on a translational approach in humans and mice, which allowed for performing mechanistic analyses that would not have been possible in humans due to ethical restrictions, as well as a direct comparison of the SAFA effects in both species. Moreover, the study profits from the use of near-physiologic administration of saturated fat resembling the lipid dose of a high-fat meal (e.g., salami pizza or cheeseburger and fries) and the comprehensive phenotyping of *in vivo* hepatic glucose and energy metabolism by state-of-the-art methodologies.

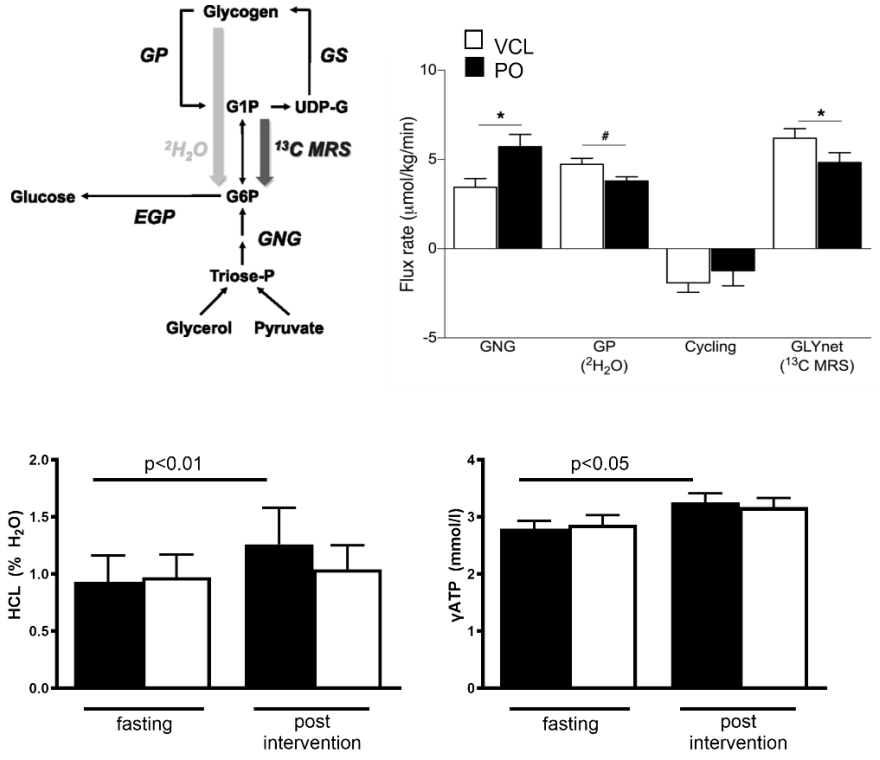

Figure 7. Schematic representation of hepatic glucose fluxes and effects of a single palm oil (PO) challenge (rich in saturated fatty acids (SAFA)) on hepatic glucose fluxes, liver lipid content and energy metabolism (γ-ATP). From Kacerovsky et al. [93] (with the permission of the American Diabetes Association) and adapted from Hernandez/Kahl et al. [78] with the permission of The American Society for Clinical Investigation (ASCI). GNG, gluconeogenesis; GP, glycogen phosphorylase; GLYnet, net glycogenolysis. Glycogen cycling = total glycogenolysis (measured by acetaminophen/^2H_2O) - GLYnet (measured by magnetic resonance spectroscopy (MRS)). *, p<0.05 and #, p=0.085 PO vs. VCL by 2-tailed t-tests.

3.2.4 Monounsaturated fat induces hepatic gluconeogenesis and whole-body insulin resistance, but does not affect hepatic energy metabolism in young, lean humans

Background: SAFA have previously been attributed with harmful effects as the promotion of insulin resistance and MASLD [31,92]. In contrast, monounsaturated fatty acids (MUFA) enriched e.g. in Mediterranean diets have been shown to be beneficial in regard to type 2 diabetes and MASLD [94] but also cardiovascular diseases [95]. Study 6 investigated the effects of a single lipid load rich in MUFA on insulin sensitivity and hepatic energy, glucose and lipid metabolism.

Methods: This cross-over study included 16 young, metabolically healthy individuals. Volunteers received canola oil (OIL; rich in MUFA) or water (vehicle, VCL) in random order on two different examination days. Insulin sensitivity was measured by hyperinsulinemic-euglycemic clamp tests (HEC) combined with isotope dilution. For assessment of hepatic glucose and energy metabolism, oral $^{2}H_2O$/acetaminophen, *in vivo* $^{13}C/^{31}P/^{1}H$-MRS as well as *ex vivo* ^{2}H-MRS were applied. Differences between treatment effects were tested using the classical crossover test; for analysis of time courses of distinct parameters, a mixed-model repeated-measures ANOVA was applied with adjustment for basal values and Bonferroni's correction.

Results: At 6 hours after intervention (pre-clamp), triglycerides and NEFA were higher with OIL compared to VCL and concentrations of plasma oleic acid increased by 66% with OIL ($p=0.017$ fasting vs. pre-clamp). During the pre-clamp period, the release of interleukin 18 (IL-18) was 54% higher after OIL intake than after VCL ($p< 0.05$), whereas interleukin-1 receptor antagonist (IL-1ra) levels were not different between the interventions. Pre-clamp hepatic insulin resistance (Hep-IR index) was 28% higher ($p=0.0037$), and whole-body glucose disposal (Rd), related to the prevailing insulin concentrations, was 27% lower after OIL compared to VCL ($p=0.0043$). During insulin-stimulated conditions (clamp), insulin-mediated EGP suppression was reduced by 21% ($p=0.0011$), and Rd was 18% lower ($p=0.011$) with OIL compared to VCL. GNG was 60% higher at six hours with OIL than with VCL ($p=0.022$) whereas GLYnet was reduced by 47% ($p=0.020$). Hepatic glycogen cycling was negligible with both interventions. No differences in hepatic γ-ATP or P_i levels, nor liver lipid content were observed between OIL and VCL.

Conclusions: A single oral lipid challenge rich in MUFA initiates insulin resistance in liver and skeletal muscle few hours after ingestion. Moreover, OIL increased GNG contribution to EGP, whereas – in contrast to PO – it did not alter hepatic energy metabolism and liver lipid content. Thus, the findings of study 6 support the proposed hepatoprotective effects of a Mediterranean diet rich in MUFA compared to a diet rich in SAFA, which are abundantly found in Western diets and ultraprocessed foods.

Limitations: Applying a pure lipid load may not fully resemble the effects of a whole meal, additionally containing carbohydrates and proteins as well as micronutrients and fiber. Also, the applied canola oil was rich in MUFA but contained also 25% of polyunsaturated fatty acids and 5% of SAFA. Thus, study 6 distinguished the effects of different oils on insulin sensitivity, rather than the effects of different fatty acids. Further, as liver biopsies were not possible due to ethical reasons, this study could not elucidate the cellular mechanisms underlying the alterations in hepatic insulin sensitivity and glucose fluxes.

Advantages: With the here applied study design, study 6 was able to monitor non-invasively *in vivo* hepatic metabolic fluxes by state-of-the-art techniques. Moreover, study 6 complements the previous findings on the acute effects of a lipid load rich in SAFA (study 5) and allows for indirect comparison of the effects of MUFA versus SAFA.

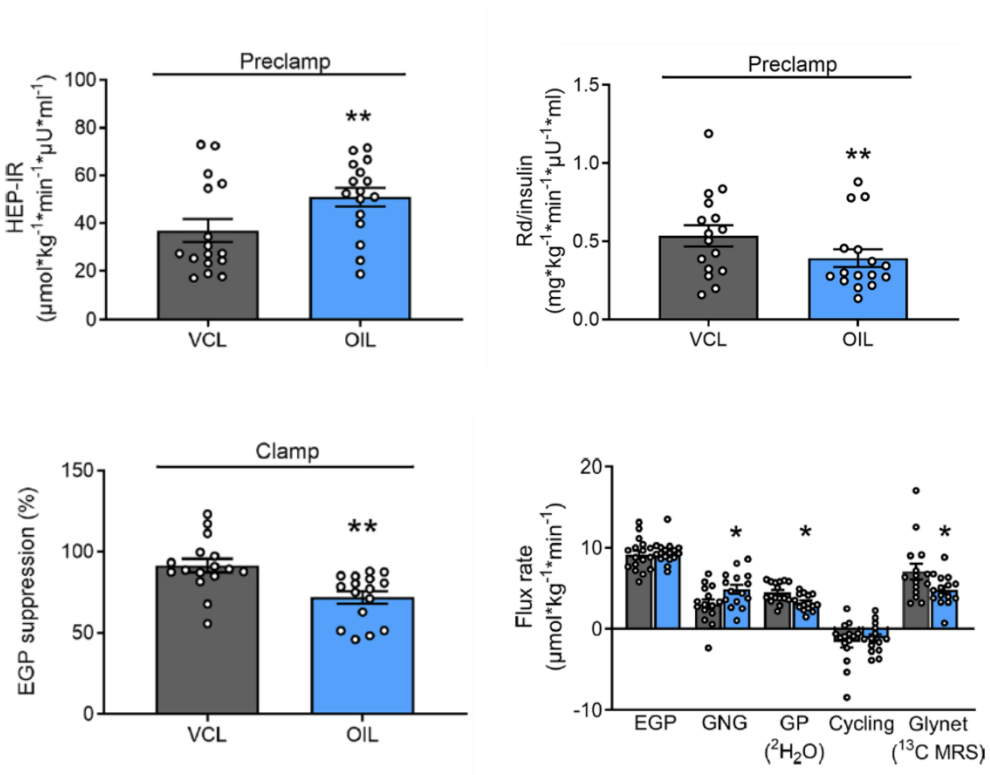

Figure 8. Changes in insulin sensitivity and hepatic glucose fluxes after a single canola oil (OIL) challenge rich in monounsaturated fatty acids (MUFA). Adapted from Sarabhai/Kahl et al. [79] with the permission of the American Society for Clinical Investigation (ASCI). VCL, vehicle; Hep-IR, hepatic insulin resistance; Rd, rate of disappearance of glucose from the circulation; EGP, endogenous glucose production.*, p<0.05; **, p<0.01 OIL vs VCL.

3.3.1 Liver lipid content is reduced by empagliflozin treatment in people with well-controlled type 2 diabetes with and without MASLD

Background: Weight loss is effective for the treatment of MASLD but is often difficult to achieve and even more to sustain. SGLT2Is not only improve glycemia but also lead to moderate reductions in body weight [18]. Further, SGLT2I may alleviate systemic and tissue-specific oxidative stress and inflammatory processes [96,97], mechanisms involved in the pathogenesis and progression of insulin resistance and MASLD [1]. Previous studies hinted to improvement in liver lipid content but also MASH-related histological parameters with SGLT2I treatment [98-103]. This multicenter, randomized, placebo-controlled clinical trial, examined the effectiveness of empagliflozin for the reduction of liver lipid content and the improvement of insulin sensitivity in people with type 2 diabetes and good metabolic control.
Methods: In total, 84 people with type 2 diabetes and short disease duration (HbA1c 6.6±0.5%; 39±27 months) were enrolled and randomized to either treatment with empagliflozin 25 mg/d or placebo for 24 weeks. The primary endpoint was defined as the change in liver lipid content from baseline to the end of the intervention period. Liver lipid content was assessed by MR-based methods (^{1}H-MRS or MRI-PDFF); whole-body and tissue-specific insulin sensitivity were measured by two-step clamps (low and high insulin conditions) combined with isotope dilution. Moreover, enzyme-linked immunosorbent assays (ELISAs) were performed for the measurement of high molecular weight (HMW) adiponectin and cytokeratin (CK)-18. Statistical comparisons were done by analysis of covariance (ANCOVA) with adjustment for respective baseline values, age, sex, and BMI.
Results: The treatment with empagliflozin resulted in a placebo-corrected absolute (1.8% [95% confidence intervall (CI) -3.4, -0.2]; p=0.02) and relative (-22% [-36, -7]; p=0.009) reduction in liver lipid content from baseline to 24 weeks of treatment, accompanied by a placebo-corrected body weight loss of -2.5 kg [-3.7, -1.4] (p<0.001). Additional adjustment for changes in body weight attenuated the difference in liver lipid content between empagliflozin- and placebo-treated individuals (placebo-corrected relative change: -6% [-23, 14]; p=0.50). In contrast, changes in HbA1c levels as well as changes in whole-body, hepatic and adipose tissue insulin sensitivity were comparable between the empagliflozin- and placebo-treated groups. Also, no differences in placebo-corrected changes in circulating cytokeratin (CK)-18 levels were observed. Of note, empagliflozin treatment induced a marked placebo-corrected increase in circulating HMW adiponectin of 36% [16, 60], while uric acid levels decreased (-74 mol/L [-108, -42]; both p<0.001).
Conclusions: Treatment with empagliflozin effectively reduced liver lipid content and body weight in metabolically well-controlled people with type 2 diabetes and a short disease duration. In addition, unchanged insulin sensitivity but decreased circulating uric acid, paralleled by increased HMW adiponectin levels, were observed with empagliflozin

treatment. Reduced uric acid levels and increased HMW adiponectin may contribute to reduced adipose tissue inflammation and, therefore – independently of weight loss – to the improvement of liver lipid content in metabolic disease.

Limitations: The results from study 7 may not apply to the full population of people with type 2 diabetes, as the investigated cohort only comprised people with good to excellent glycemic control, a short known disease duration, and rather mild liver disease. Thus, for people with uncontrolled type 2 diabetes and advanced liver disease, the results may differ. Moreover, the results (efficacy and safety) from study 7 cannot be extrapolated to non-diabetic collectives with MASLD, which may also benefit from empagliflozin treatment.

Advantages: Study 7 provides evidence that empagliflozin is effective for reducing liver lipid content without the necessity of major changes in glycemia or insulin sensitivity. Moreover, detailed metabolic phenotyping was applied to uncover mechanisms induced by empagliflozin that might contribute – apart from weight loss – to the previously observed improvements in liver lipid content but also other histological components of MASLD with SGLT2I treatment.

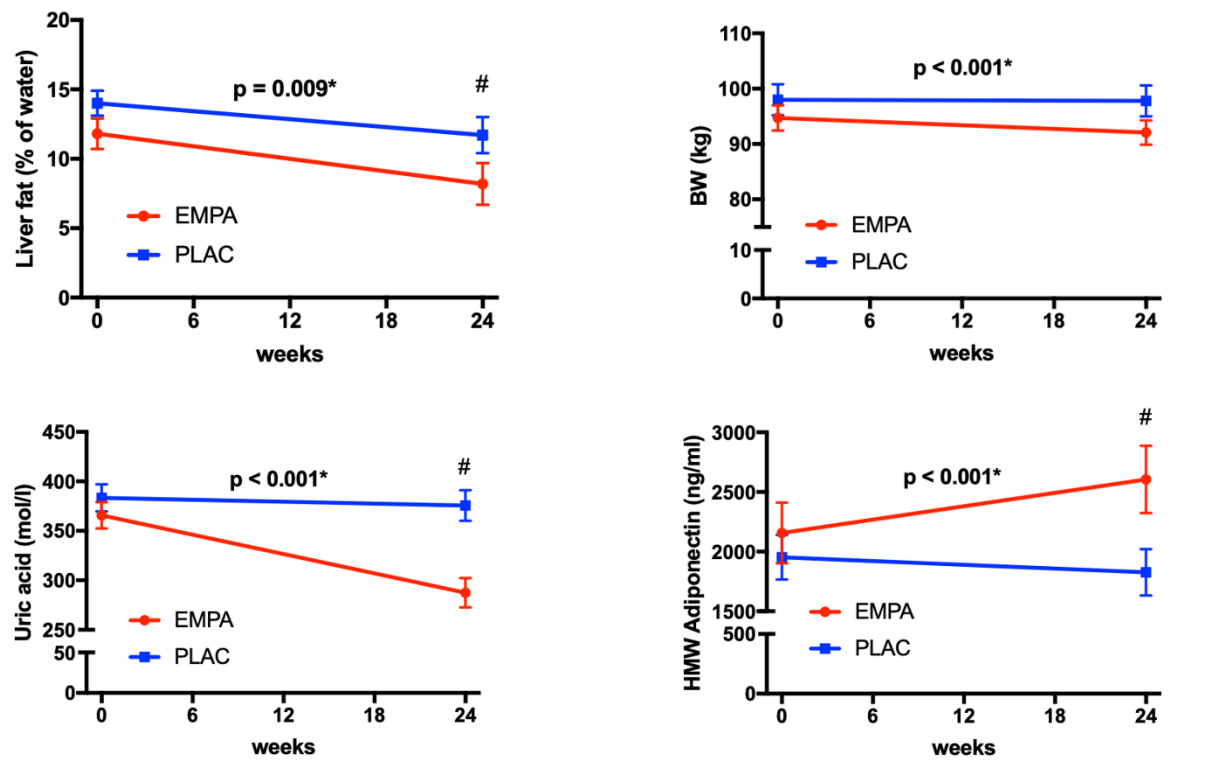

Figure 9. Changes in liver lipid content (liver fat), body weight (BW), uric acid and HMW adiponectin after empagliflozin treatment (EMPA, red) or placebo (PLAC, blue). Adapted from Kahl et al. [80] with the permission of the American Diabetes Association. *, significance levels of Δ parameter between treatment arms; #, p<0.05 EMPA vs PLAC at 24 weeks; p-values based on ANCOVA with adjustment for age, sex, BMI and respective baseline value. BW, body weight; HMW adiponectin, high molecular weight adiponectin.

3.3.2 Empagliflozin reduces cardiovascular events in people with type 2 diabetes irrespective of steatosis or fibrosis risk category

Background: People with type 2 diabetes are prone to developing MASLD with a prevalence among these of about 65% [104]. MASLD closely associates with an increased risk of cardiovascular disease [105]. SGLT2Is not only exert antihyperglycemic actions but also improve cardiovascular and renal outcomes [55]. It is unknown, whether the effectiveness of empagliflozin on cardiovascular and renal endpoints depends on the presence and/or severity of MASLD-related steatosis and fibrosis. Thus, the relationship between steatosis and fibrosis risk and cardiorenal outcomes in people with empagliflozin treatment was analysed in the randomized, placebo-controlled EMPA-REG OUTCOME trial.
Methods: In EMPA-REG OUTCOME, 7020 people with type 2 diabetes and cardiovascular disease were enrolled and randomized to either 10/25 mg/day empagliflozin or placebo [106]. Steatosis and fibrosis risk were calculated by the Dallas steatosis index (DSI) [47], the hepatic steatosis index (HSI) [45], the NAFLD fibrosis score (NFS) [49], and the Fibrosis-4 score (FIB-4) [48]. Mixed model repeated measures and Cox regression analyses were applied to examine changes from baseline in scores and their associations with cardiorenal outcomes and mortality, respectively.
Results: Applying DSI and HSI, 73% and 84% of the included participants had a score indicating high risk for steatosis, whereas calculating NFS and FIB-4 revealed 23% and 4% of individuals at high risk for advanced fibrosis. With empagliflozin treatment but not with placebo, the percentages of people at high steatosis risk, but not those at high risk for advanced fibrosis, slightly decreased over time (at 52, 108 and 164 weeks of follow-up compared to baseline). Importantly, a higher risk for cardiovascular events during follow-up was predicted by a high risk score for advanced fibrosis at baseline. In regard to cardiorenal and all-cause mortality outcomes, the beneficial effects of empagliflozin were sustained when performing separate analyses in the low, intermediate and high steatosis or fibrosis risk groups separately. Empagliflozin treatment slightly lowered platelet counts and increased serum albumin levels compared to placebo, with both biochemical parameters being included in the tested fibrosis scores. Across all derived steatosis and fibrosis risk groups, empagliflozin reduced body weight and HbA1c as compared to placebo at most timepoints.
Conclusions: Based on blood-based NITs, empagliflozin may alleviate steatosis but not fibrosis risk in people with type 2 diabetes and pre-existing cardiovascular disease. The empagliflozin-mediated beneficial effects on cardiorenal events and all-cause mortality associated with empagliflozin therapy were independent of the individual steatosis and fibrosis risks. However, baseline fibrosis risk was predictive of the future risk for cardiovascular events.

Limitations: Liver histology or imaging methods for the assessment of liver lipid content and fibrosis were not performed in EMPA-REG OUTCOME. Thus, the applied scores imply, but do not necessarily reflect, the individual steatosis or fibrosis grade. Also, laboratory parameters such as γ-GT or insulin, necessary for the calculation of other established steatosis indices (e.g., Fatty liver index (FLI) [44], NAFLD-LFS [46]), were not measured. Further, neither hepatitis serology nor assessment of alcohol intake were available to exclude other causes for hepatic steatosis than MASLD. Of note, in general, the use of steatosis and fibrosis biomarkers has not been extensively investigated for the assessment of longitudinal changes in response to pharmacological interventions [43].

Advantages: This post-hoc analysis from EMPA-REG OUTCOME included a very large number of enrolled participants with a follow-up interval of more than 3 years. Due to the placebo-controlled design of EMPA-REG OUTCOME, study 8 was able to evaluate empagliflozin actions (metabolic and cardiorenal outcomes) in people with type 2 diabetes, differing in their risk for steatosis and advanced fibrosis.

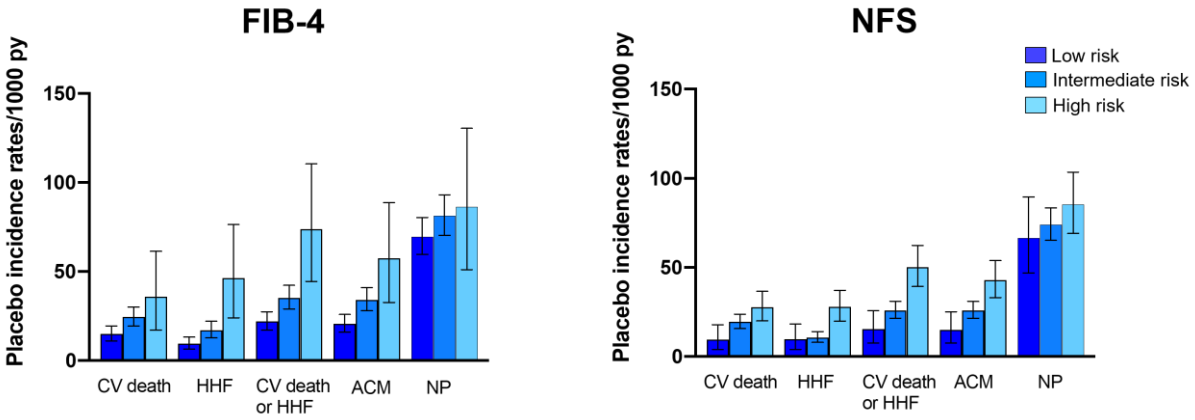

Figure 10. Placebo incidence rates/1000 patient years across subgroups by FIB-4 and NFS categories at baseline, respectively. Data are shown as placebo incidence rates/1000 py with 95% confidence intervals (CI). Py, patient years; CV, cardiovascular; HHF, hospitalization due to heart failure; ACM, all-cause mortality; NP, nephropathy. Figure created by S. Kahl based on data reported in [81] under the terms of a Creative Commons Attribution-NonCommercial License (CC BY-NC 4.0).

4. Conclusions

4.1 Validation of methods for the assessment of insulin sensitivity and hepatic steatosis in different metabolic conditions

4.1.1 Botnia clamp versus standard HEC

Type 2 diabetes is characterized by impaired insulin sensitivity and insulin secretion [1]. Whereas insulin sensitivity is frequently measured by HEC, insulin secretion can be addressed by the IVGTT, and the Botnia clamp combines both IVGTT and HEC [32]. However, endogenous hormonal and metabolic responses to the intravenous glucose load may interfere with the subsequent testing of insulin sensitivity, and already small differences in M-values may interfere with the discrimination between insulin-sensitive and insulin-resistant individuals, especially with M-values near the respective cut-off value [83]. Study 1 validated the Botnia Clamp procedure against the standard HEC in people with type 2 diabetes, which contributed essential knowledge to the correct interpretation of the M-values derived from the Botnia clamp, when compared to those from standard HEC. Circulating NEFA levels immediately before the start of the clamp procedure were identified as a relevant metabolic factor that may introduce bias in the assessment of insulin sensitivity. Previously, three studies compared the Botnia clamp versus standard HEC in people with different glucose tolerance [72,107,108] but did not focus on a collective of people with type 2 diabetes. Differences in both prevailing insulin secretion and insulin sensitivity may profoundly affect glucose handling during the IVGTT, including metabolic [109-111], hormonal [112,113], as well as inflammatory [114,115] responses. Most importantly, study 1 provided evidence that the Botnia clamp yields similar estimates of insulin sensitivity as the standard HEC in people with type 2 diabetes, which is in accordance with the other studies [72,107,108]. However, there was a trend toward higher M_{FFM} with the Botnia clamp compared to standard HEC, and specifically, the intraindividual differences in NEFA concentrations before the start of both HEC procedures were inversely correlated with differences in M_{FFM} between Botnia clamp and standard HEC. In this context, previous studies found a negative correlation of fasting NEFA levels and the M-value in metabolically healthy adults [116] and in first degree relatives of people with type 2 diabetes [108]. Study 1 extends the existing information on the relation of whole-body insulin sensitivity and fasting NEFA to HEC conditions, showing that the greater the lowering of NEFA levels before HEC start, the higher the insulin sensitivity during the subsequent clamp – although, statistically, M_{FFM} values obtained from the Botnia clamp were not different from those obtained from the standard HEC. Of note, during HEC, NEFA levels of Botnia clamp and standard HEC were comparable, further supporting the concept, that lipid preload is a major factor impacting insulin sensitivity [1]. In line with this, short-term lowering of plasma

NEFA by acipimox rapidly increased whole-body insulin sensitivity in obese people with and without type 2 diabetes [117].

During conditions of high circulating NEFA levels, NEFAs are taken up in the liver and skeletal muscle (see also 4.2). On a cellular level, they mediate lipotoxic effects through lipid intermediates (DAGs, ceramides) that activate specific isoforms of protein kinase C, which, in turn, interfere with insulin signaling by inhibitory serine phosphorylation of the insulin receptor substrate (IRS) or reduced proteinkinase B (AKT) activation [1,118]. Study 1 did not investigate the tissue-specific cellular insulin signaling, but it may be speculated that an IVGTT-mediated, reduced intracellular lipid load may have beneficially affected skeletal muscle glucose uptake and/or metabolic flexibility [118,119]. Of note, EGP was lower and insulin-dependent EGP suppression was higher at the end of the Botnia IVGTT compared to the saline control before standard HEC, but this could not explain the differences in M_{FFM} obtained from both experimental settings. Thus, NEFA effects on skeletal muscle rather than on the liver underlie the differences in M_{FFM} [32]. In addition, there were no differences in the measured circulating pro-inflammatory cytokines TNFα, IL-1RA or MCP-1 during the IVGTT and clamp period of the Botnia clamp compared to the standard HEC. In contrast, another study reported increases in plasma levels of IL-6, IL-18 and TNFα in response to acute hyperglycemia in healthy individuals and people with impaired glucose tolerance [19], which may be explained by differences in the examined cohorts, chosen inflammatory markers, and/or experimental set-up.

4.1.2 Non-invasive blood-based tests for assessment of steatosis

The accuracy of blood-based NITs for prediction of hepatic steatosis is still debated, and their performance may depend on the individual characteristics of the investigated collectives [120,121]. Hepatic steatosis relates to insulin resistance and hyperinsulinemia even in lean glucose-tolerant individuals [122]. In this context, Study 2 contributes knowledge to the optimized use of frequently applied steatosis indices not only in regard to their accuracy for the assessment of steatosis, but also to their use for estimation of insulin sensitivity and insulin secretion. Study 2 revealed that FLI, HSI and NAFLD-LFS offer modest sensitivity and specificity to detect steatosis and cannot substitute for exact liver lipid quantification by ^{1}H-MRS in a collective at low MASLD risk. In accordance, also the latest guidelines recommend the use of imaging-based but not blood-based NITs for MASLD screening in clinical routine [10,42,43]. Imaging-based NITs for detection of steatosis comprise conventional ultrasound (B-mode), transient elastography controlled attenuation parameter (TE-CAP), ^{1}H-MRS, and MRI-PDFF [42]. Of note, the sensitivity of conventional ultrasound to detect steatosis is about 91% in people with a liver lipid content of ≥30%, but only 64% for liver lipid contents <30% [123], indicating that ultrasound likely misses many cases of mild steatosis. Thus, especially in

steatosis grade 1 (defined as the presence of lipid droplet in 5 to 33% of the hepatocytes), optimized but simple screening procedures still remain to be identified as TE-CAP and MR-based methods are frequently not readily available. The clinical relevance of the assessment of mild cases of steatosis was demonstrated by a recent clinical study showing that liver lipid contents of ≥1.85% were already associated with insulin resistance and cardiovascular risk factors [124]. Study 2 found an acceptable correlation between steatosis indices and exact quantification of liver lipid content by ^{1}H-MRS in people with predominantly low liver lipid contents, which justifies their use as surrogate parameters of liver lipid content at least in larger epidemiological or clinical studies. Further, the results from study 2 revealed that all three indices were strongly and inversely correlated with measures of insulin sensitivity. Thus, they might serve as rough clinical estimates of insulin resistance in collectives of people with metabolic disease [125] but also as predictors of cardiometabolic risk (likelihood of developing diabetes or cardiovascular diseases), as insulin resistance and cardiometabolic risk are closely associated [126]. Recently, a study proposed using the FLI as a tool in clinical routine for selecting those people at higher risk for cardiovascular events who would profit from lifestyle changes and/or referral to a specialist for further examinations [127]. Thus, current non-invasive testing and risk stratification strategies represent relevant improvements in addressing the challenges posed by MASLD if correctly applied [128], underscoring the importance of performing validation studies such as in study 2.

Key messages

- **The Botnia clamp and standard HEC provide comparable estimates of whole-body insulin sensitivity in people with type 2 diabetes; however, discrimination between insulin-resistant and insulin-sensitive individuals may be affected by the individual degree of insulin-mediated inhibition of lipolysis.**
- **The assessment of steatosis risk by blood-based NITs has only moderate accuracy in non-obese, non-diabetic collectives.**
- **NITs may be valuable tools for identification of individuals at high cardiometabolic risk in primary care as well as in large epidemiological studies.**

4.2 Impact of circulating metabolites on insulin sensitivity and MASLD

4.2.1 Fasting and postprandial glucose levels

Hepatic energy metabolism is altered in metabolic disease [27], and previous studies revealed altered mitochondrial function in manifest type 1 [129] and type 2 diabetes [87], as well as in different MASLD states [73]. However, the analyses of the relationship between mitochondrial

respiratory capacity and insulin resistance – the central hallmark of metabolic diseases – revealed conflicting results [73,130]. Study 3 [76] provided a comprehensive analysis of this relationship, showing that fasting glycemia and liver lipid content interact with hepatic OXPHOS capacity in a collective of people with obesity grade 3 without manifest diabetes. Most interestingly, study 3 found a linear positive correlation of insulin resistance with hepatic OXPHOS capacity – in accordance with the concept of mitochondrial plasticity [27] – only in those individuals with normal glucose tolerance and without MASLD. This indicates that both gluco- and lipotoxic mechanisms contribute to insulin resistance as well as to impaired mitochondrial plasticity in people with grade 3 obesity. While hyperglycemia generally results from hepatic insulin resistance through impaired fasting and (post)prandial EGP suppression [131], prolonged mild dysglycemia can itself induce hepatic insulin resistance in glucose tolerant humans [132]. In line, in study 3, individuals with prediabetes presented with impaired AKT signaling along with a higher prevalence of fibrosis, extending the concept of glucotoxicity, reported for type 2 diabetes with progressing MASLD [133,134], to individuals with prediabetes. Several cellular mechanisms may be causal, such as the hexosamine pathway, reactive dicarbonyls and/or advanced glycation endproducts [132,135]. Study 3 also found lower PINK1 phosphorylation in individuals with prediabetes, likely reflecting decreased mitophagy, which has been previously suggested in states of overt hyperglycemia [136]. Particularly, fasting glucose might relate to liver injury in obesity grade 3 by showing a trend towards an association with lower PINK1 phosphorylation and more pronounced hepatic inflammation in study 3.

4.2.2 Circulating amino acids

Many cellular processes involved in insulin signaling and energy metabolism depend on the supply of specific metabolites, such as glucose and lipids, but also amino acids from the circulation [137,138]. However, the factors regulating hepatic energy metabolism in metabolically healthy humans are largely unknown. Study 4 [77] identified metabolic determinants of hepatic γ-ATP and P_i under physiological conditions in a cohort of middle-aged metabolically healthy individuals from the general population and contributed important information to the understanding of differences in the regulation of hepatic energy metabolism in people with and without metabolic disease. Study 4 did not observe any association of hepatic γ-ATP and P_i with different measures of insulin sensitivity, despite previous reports on negative associations of γ-ATP with liver lipid content and BMI [87,139]. Thus, in a metabolically healthy collective, such as in study 4, other than major metabolic factors impact hepatic energy metabolism. Study 4 found a positive relationship between hepatic P_i and fasting NEFA levels, which is in contrast to a previous study in a mixed collective of metabolically healthy people as well as people with obesity and type 2 diabetes [140]. This observation may be

explained by the findings of study 4, that in metabolic health, hepatic energy metabolism seems to be regulated independently of the individual degree of insulin sensitivity. Thus, in individuals with intact hepatic metabolic flexibility, higher substrate availability may merely promote charging of hepatic phosphorus pools and/or stimulation of energy-generating processes [141]. In the collective of study 4, γ-ATP levels were strongest and positively related to circulating leucine levels, also after adjustment for age, sex and BMI. Increased leucine levels are frequently found in insulin-resistant states [137]. Leucine belongs to the group of branched-chain amino acids, and alterations in their circulating concentrations have been linked to protective but also deleterious effects on mitochondrial function in metabolic diseases [137,142]. On a mechanistic level, data from preclinical studies indicated that leucin stimulated mitochondrial biogenesis in brown adipose tissue of obese mice [143]. Also, leucine increased lipid oxidation and oxygen consumption in skeletal muscle cells [144]. Moreover, leucine provides carbon skeletons to the tricarboxylic acid (TCA) cycle at the level of acetyl-coenzyme A, which may enhance TCA cycle flux [145]. Thus, the results from study 4 underpin the assumption that higher leucine levels are more associated with promoted mitochondrial performance than with pathologic metabolic conditions in metabolically healthy individuals.

4.2.3 Intrahepatic and circulating lipids

In addition to dysglycemia, study 3 [76] identified liver lipid content as a modulator of the association between whole-body insulin sensitivity and hepatic mitochondrial OXPHOS capacity. Liver lipid content per se unlikely plays an active role, but rather reflects other lipotoxic processes triggered by lipid intermediates such as DAGs and ceramides [146] (see also 4.1.1). A previous study reported that certain sphingolipids were not only increased in MASH but were also associated with hepatic OXPHOS capacity and oxidative stress [147]. Likewise, in study 3, individuals with normal glucose tolerance and MASLD featured higher hepatic TBARS levels along with greater steatosis, inflammation and ballooning when compared to those with normal glucose tolerance without MASLD. Of interest, study 3 found greater Adipo-IR and fibrosis grade in people with prediabetes compared to those with normal glucose tolerance, whereas neither Adipo-IR nor fibrosis grade differed between those with normal glucose tolerance with and without MASLD. Thus, adipose tissue insulin resistance with increased lipolysis is of relevance for MASLD pathogenesis as a major source of NEFA [26], but the missing association of Adipo-IR with hepatic OXPHOS capacity in study 3 may simply suggest that this parameter does not reflect the liver's ability to adapt to the excessive NEFA supply.

Study 4 [77] identified correlations of P_i with unsaturated fatty acid species, such as myristoleic acid, C16:1w5, palmitoleic acid, and adrenic acid. Myristoleic acid is abundant in milk fat and may also raise palmitoleic acid levels [148]. Thus, both palmitoleic and myristoleic acid levels

may, at least in part, depend on individual dietary habits. Palmitoleic acid may also derive from DNL as the main products of DNL include palmitic acid, palmitoleic acid, vaccenic acid, stearic acid, and oleic acid [149]. However, except for palmitoleic acid, none of those DNL-derived fatty acids was associated with hepatic P_i levels. Thus, study 4 suggests that in metabolically healthy individuals, palmitoleic acid levels seem to be regulated by dietary patterns rather than by DNL. Interestingly, palmitoleic acid was previously identified as an anti-inflammatory adipokine [150]. Further, stimulating PPARα-dependent effects of palmitoleic acid on mitochondrial metabolism have also been previously reported in murine liver [138].

The investigation of the acute development of hepatic and whole-body insulin resistance after a single intake of an oral lipid load rich in SAFA or MUFA (studies 5 [78] and 6 [79]) complements our understanding of the role of dietary lipids in the pathogenesis of metabolic disease. In study 5 [78], we found that in healthy, lean humans, a single palm oil intake, rich in SAFA, initiated insulin resistance, paralleled by changes in hepatic GNG, increased hepatic energy substrates, and liver lipid content. Study 6 reported similar findings with canola oil, rich in MUFA, in regard to insulin resistance and hepatic fluxes but could not identify changes in hepatic energy metabolism or liver lipid content [79]. Interestingly, in both studies, despite post-load increases in circulating triglycerides and NEFA, circulating levels of pro-inflammatory cytokines TNFα and IL-6 were unchanged compared to baseline conditions. Previous studies suggested the contribution of toll-like receptor 4 (TLR4) activation as a mechanism linking high fat intake to metabolic disease [151]. TLR4 is stimulated by lipopolysaccharide (LPS), an endotoxin derived from intestinal bacteria that induces a robust inflammatory response, but fatty acids are also able to induce TLR4 activation [152,153]. Further, high lipid consumption may increase circulating LPS levels through disruption of the intestinal barrier, particularly the tight junctions between the endothelial cells [154]. In a previous study, lean insulin-sensitive volunteers were administered either intravenous lipid (based on soybean oil rich in polyunsaturated fatty acids (PUFA)), oral lipid (soybean oil), or intravenous LPS [155]. Post-intervention, circulating inflammatory markers TNFα and IL-6 only rose with LPS, along with their mRNA expression in subcutaneous adipose tissue. Study 5 provided evidence that - considering TNFa and Il-6 as readouts of inflammation, also a lipid load rich in SAFA did not trigger an acute immune stimulation, despite their reported deleterious metabolic effects. In study 6, a moderate elevation of IL-18 levels was observed after canola oil intake, possibly reflecting a metabolic adaptation rather than a pro-inflammatory response, as IL-18 has been shown to increase skeletal muscle lipid oxidation [156]. Thus, both studies 5 and 6 strongly support the assumptions that (i) the acute effects of an oral lipid load are mediated by metabolic rather than endocrine changes, and (ii) acute lipid-induced insulin resistance happens regardless of the degree of lipid composition or saturation.

Notably, after a single oral lipid load, markedly altered hepatic glucose fluxes with increased rates of GNG, reduced GLYnet and a corresponding reduced GP flux were observed in both studies 5 and 6. Moreover, by applying combined tracer and imaging methods, rates of glycogen cycling (simultaneous synthesis and breakdown of glycogen) could be assessed, which have been reported to be elevated in states of impaired glucose handling, as in diabetes mellitus [93]. These comprehensive analyses expanded our knowledge on the acute effects of high lipid availability on *in vivo* glucose and glycogen fluxes in metabolically healthy humans [93,157,158]. The results may indicate that metabolically healthy humans are able to rapidly downregulate glycogenolysis under conditions of elevated gluconeogenesis to avoid futile cycling. Of interest, the oral lipid load also induced increases in circulating glucagon levels, which may have contributed to the modified hepatic fluxes, increased GNG and reduced glycogenolysis in the examined individuals [159].

Interestingly, the ingestion of a lipid load rich in SAFA but not in MUFA increased liver lipid content and hepatic γ-ATP in studies 5 and 6. This observation may be suggestive of fatty acid saturation-dependent effects on hepatic energy metabolism. The results from studies 5 and 6 are supported by other clinical studies on the long-term effects of a hypercaloric diet rich in either SAFA or unsaturated fatty acids in normal-weight individuals [160] or people with obesity [161], where a diet rich in SAFA induced a greater increase in liver lipid content than a diet rich in unsaturated fatty acids. Regarding the mechanisms underlying this observation, the direct quantification of sources of fatty acids accumulating in the liver showed that fatty acids are mainly derived from adipose tissue lipolysis (59%), but also DNL (26%), and diet (15%) [26]. Study 5 found reduced insulin-stimulated NEFA suppression during clamp conditions after SAFA compared to control, which is suggestive of increased adipose tissue lipolysis and thus adipose tissue insulin resistance. In accordance, a chronic hypercaloric diet rich in SAFA increased, whereas a diet rich in unsaturated fat decreased adipose tissue lipolysis [161]. The measurement of liver γ-ATP by *in vivo* ^{31}P-MRS is only performed at a few centers worldwide, and there is little available literature on γ-ATP levels in response to overfeeding. A previous study reported increased hepatic γ-ATP concentrations after a mixed-meal tolerance test (MMTT) in insulin-resistant obese humans but not in metabolically healthy people [140]. In the people with obesity, again, adipose tissue insulin resistance may have been causal to the observed changes in γ-ATP after meal intake because of increased postprandial insulin and NEFA availability at the liver promoting hepatic γ-ATP synthesis. Thus, studies 5 and 6 demonstrate the deleterious effects of hepatic lipid overload on metabolic homeostasis already in the acute setting and provide insights into the first steps in the transition from metabolic health to metabolic disease caused by unhealthy eating patterns.

The mechanisms underlying insulin resistance due to a high-fat diet seem to be similar in mice and humans, enabling translational studies [162,163]. Study 5 was designed as a translational study, expanding the human results to mice, complemented by liver transcriptomics [78]. Transcriptomics analyses revealed that palm oil intake acutely impacts on several predicted upstream regulators and pathways, including LPS, members of the TLR and peroxisome proliferator-activated receptor agonists (PPAR) families, nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) and TNF-related weak inducer of apoptosis (TWEAK). As discussed above, LPS and TLR-signaling may initiate the pro-inflammatory effects of high lipid loads [151-153]. Downregulation of TWEAK has been associated with obesity and MASLD [164], whereas NF-κB is involved in pro-inflammatory responses but also in hepatoprotective pathways [165]. Thus, study 5 was able to identify distinct signaling pathways that may be relevant for the early development of hepatic insulin resistance in conditions of lipid overload, contributing knowledge to the identification of new treatment targets for metabolic disease-related liver injury.

Key messages

- **Glycemia and liver lipid content impact mitochondrial plasticity in obesity and insulin resistance**
- **Specific circulating amino acids and fatty acids associate with hepatic energy metabolism in metabolic health**
- **A single lipid load acutely induces whole-body and tissue-specific insulin resistance independent of fatty acid composition**
- **Saturated, but not monounsaturated fatty acids acutely interfere with liver lipid content and hepatic energy metabolism**

4.3 Effects of empagliflozin on type 2 diabetes-related concomitant diseases

The increased risk of premature death in type 2 diabetes is mainly driven by detrimental effects on vascular integrity, i.e., cardiovascular disease [166]. However, at least in part, due to improved cardiovascular disease management, other diabetes-related diseases emerged, one of those being MASLD and especially advanced MASLD states [7]. In turn, MASLD drives an increased risk for cardiovascular but also liver-related events in people with and without type 2 diabetes, thus increasing morbidity and mortality rates [167]. Still, there is no approved pharmacological treatment for MASLD in Europe, as the only drug in the indication of MASH and F2/F3 fibrosis, resmetirom, has recently been approved by the American Food and Drug Administration (FDA), but not yet by the European Medicines Agency (EMA).

In this context, studies 7 [80] and 8 [81] contribute to the understanding of benefits and drawbacks for the currently available MASLD treatment strategies with antihyperglycemic drugs in people with type 2 diabetes and thus to evidence-based treatment decisions. Studies 7 and 8 provide evidence that the beneficial actions of SGLT2Is on metabolism also extend to the condition of MASLD. The results from study 7 showed that empagliflozin led to a placebo-corrected 2.3-fold greater reduction in liver lipid content after 24 weeks of treatment in people with type 2 diabetes [80]. There is now expert consensus that SGLT2I mediate a moderate reduction in liver lipid content in people with type 2 diabetes and the results of study 7 [80] contributed to achieving the respective guideline statements [9,10].

4.3.1 The EmLiFa001 trial

The magnitude of weight loss seems to be the major determinant for the amount of liver lipid reduction with both SGLT2I empagliflozin [80] and canagliflozin [100]. In study 7, the additional adjustment for changes in body weight attenuated the difference in changes in liver lipid content between empagliflozin- and placebo-treated individuals. Previous studies suggested that a clinically relevant reduction in liver lipid content starts with a loss of ≥5% of body weight, but even minor weight loss may be associated with marked reductions in liver lipid content [64]. This assumption is supported by study 7: a weight loss of ≥5% occurred in only 27% of empagliflozin-treated participants – despite a relative change in liver lipid content of -34% in the empagliflozin-treated group. Of note, there are also studies reporting an SGLT2I-mediated reduction in liver lipid content without weight loss [99,168], pointing to the weight loss-independent effects of empagliflozin [18]. In this regard, study 7 identified a placebo-corrected 23% decrease in uric acid and a 36% increase in plasma adiponectin levels with empagliflozin treatment [80]. There is evidence from the literature that SGLT2I mediate increased uric acid transport activity, resulting in increased urinary uric acid excretion [169]. Previously, high uric acid levels have been related to adipose tissue inflammation, insulin resistance and hypoadiponectinemia [170] and both elevated uric acid levels and decreased adiponectin levels have been linked to obesity and features of the metabolic syndrome, including type 2 diabetes and MASLD [171]. Thus, empagliflozin-induced increases in circulating adiponectin levels may activate hepatic β-oxidation of NEFA and decrease lipogenesis [172], contributing to liver lipid reduction independent of weight loss. Further, study 7 investigated changes in whole-body and tissue-specific insulin sensitivity of adipose tissue and liver before and after 24 weeks of treatment by gold-standard methods [32] but found no differences between both treatment arms. In accordance, also other studies testing the effects of canagliflozin [100] and dapagliflozin [98] on whole-body insulin sensitivity, reported no placebo-corrected treatment effects. However, improved hepatic insulin sensitivity was reported in response to treatment with canagliflozin [100], but not dapagliflozin [98]. The

observation that the improvements in hepatic insulin sensitivity associated with reductions in body weight [100] again support the concept of SGLT2I mediating their beneficial metabolic actions – at least in part – via body weight reduction. However, in study 7, the reductions in body weight with empagliflozin were not accompanied by improvements in hepatic insulin sensitivity. As the study with canagliflozin, but not study 7, found reductions in HbA1c with the respective SGLT2I treatment, glucotoxicity may be one mechanism causal to the observed differences in SGLT2I actions on hepatic insulin sensitivity [100].

4.3.2 The EMPA-REG OUTCOME trial

The EMPA-REG OUTCOME trial demonstrated a 35% decline in the rates of hospitalization for heart failure with empagliflozin treatment compared to placebo in people with type 2 diabetes [106]. In study 8, a post-hoc analysis from EMPA-REG OUTCOME was performed, suggesting that empagliflozin may reduce steatosis risk, but not the risk of advanced fibrosis in people with type 2 diabetes and manifest cardiovascular disease [81]. Of utmost importance for clinical practice, the results from study 8 point to effective cardiorenal risk reduction in all subgroups with low or high steatosis and fibrosis risk and thus contribute important information to decision-making in terms of personalized medicine. In accordance, former analyses from EMPA-REG OUTCOME reported that cardiorenal treatment benefits with empagliflozin were consistent across groups differing in age as well as in the underlying cardiovascular and heart failure risk [173,174]. Another major result from study 8 was that fibrosis, but not steatosis high risk categories assessed by FIB-4 and NFS related to increased placebo incidence rates of cardiovascular outcomes and all-cause mortality. Thus, FIB-4 and/or NFS might be useful predictors of cardiovascular morbidity and mortality risk in people with type 2 diabetes and MASLD and contribute to individual risk stratification [175-178]. The analyses performed in study 8 are of great scientific and clinical value, as large-scale randomized clinical trials (RCTs) that include imaging and/or liver histology for the assessment of SGLT2I-induced changes in MASLD parameters, and especially in MASLD-related fibrosis, are currently missing. In the large collective of EMPA-REG OUTCOME, study 8 confirmed the results from previous small scale studies with MR-based determination of changes in liver lipid content [80,99], showing that the percentages of participants at high steatosis risk, calculated by the DSI [47] and HSI [45], decreased over time with empagliflozin treatment compared to placebo. However, in contrast to previous studies, no empagliflozin-mediated reductions in the percentage of people grouped at high risk of advanced fibrosis using the FIB-4 [48] or NFS [49] were identified. A post-hoc analysis from the DURATION-8 trial, investigating the effects of dapagliflozin on fibrosis risk by NITs in people with type 2 diabetes, found a decrease in the percentages of individuals at high fibrosis risk from 0 to 52 weeks of treatment with NFS but not with FIB-4 [179]. Reduced fibrosis risk, as calculated by

FIB-4, was observed in a real-world analysis of people with type 2 diabetes who switched to SGLT2I treatment [180]. Also, a very recent study in 237 people with type 2 diabetes and MASLD reported a reduced risk for worsening of liver stiffness over a 52-month follow-up period with SGLT2I treatment [181]. In regard to the histological assessment of SGLT2I effects on MASLD-related fibrosis, the only available controlled trial found higher rates of MASH resolution and fibrosis regression compared to control after 72 weeks of ipragliflozin treatment [103]. In addition, another small-scale pilot study reported significantly reduced fibrosis after 24 weeks of empagliflozin treatment in people with type 2 diabetes and MASH when compared to pre-treatment and a historical control group [102]. In a similar collective, canagliflozin improved the composite histological endpoint at one year of treatment compared to pre-treatment [168]. However, looking at fibrosis separately, fibrosis grade only improved in 2 out of 7 individuals by 1 stage. The findings of study 8 do not support the previously reported beneficial effects of empagliflozin on hepatic fibrosis risk in a collective of people with type 2 diabetes and manifest cardiovascular disease. Considering all the above, several reasons may underlie these findings. On the one hand, individuals with type 2 diabetes and manifest cardiovascular disease often have progressive low-grade inflammation [182,183] that may increase platelet count [184]. Platelet count is included in the denominator in FIB-4 and subtracted in NFS. In study 8, platelet count was slightly lower with empagliflozin treatment compared to placebo throughout the study, maybe due to the previously reported anti-inflammatory effects of empagliflozin [97]. On the other hand, fibrosis scores may not detect smaller changes in fibrosis grade, especially in states of mild to moderate fibrosis, and the use of FIB-4 and NFS for monitoring MASLD progression has not been extensively tested in response to pharmacological treatment [24].

Key messages

- **Empagliflozin effectively lowers liver lipid content in individuals with type 2 diabetes**
- **Long-term empagliflozin treatment improves steatosis but not fibrosis risk assessed by blood-based NITs in people with type 2 diabetes and manifest cardiovascular disease**
- **Individuals with type 2 diabetes and cardiovascular disease benefit from empagliflozin-mediated cardiorenal risk reduction independent of steatosis or fibrosis risk**

5. Outlook

Obesity, MASLD and type 2 diabetes are major public health problems, both nationally and internationally, and a great challenge for both the affected individuals and the health care systems. A detailed understanding of the pathophysiological processes is needed to be able to develop, but also to individually select optimized treatments.

MASLD is a frequent comorbidity of obesity and type 2 diabetes. Still, there is debate about optimal MASLD screening strategies [9,10]. Although current imaging-based NITs for assessment of steatosis and fibrosis seem to have higher accuracy, blood-based NIT screening strategies are intensively investigated due to their simple mode of assessment, which may be performed in any clinical setting. In contrast to the established simple indices based on laboratory and anthropometric data, recent commercial but also academic approaches focused on the assessment of expression levels of proteins involved in fibrogenesis (e.g., N-terminal pro-peptide of type III collagen (PRO-C3), Enhanced Liver Fibrosis (ELF) test) or (multi)omics strategies for better accuracy of prediction of fibrosis [43]. Especially the assessment of fibrosis requires the development of dynamic disease-specific models, which may be supported by artificial intelligence (AI)-based approaches that are associated with relevant clinical outcomes. Steatosis indices need to be improved in regard to their ability to differentiate between MASLD and MASH.

Resmetirom was recently approved as the first drug for the treatment of MASH with F2/F3 fibrosis; however, it does not act on body weight and/or glycemia, factors renowned to promote the pathogenesis and progression of MASLD [10,18]. Moreover, approved pharmacological treatment options for early MASLD (≤F1 fibrosis) are still missing. GLP-1RAs, effectively lowering plasma glucose and body weight, mediate MASH resolution without worsening of fibrosis in people with and without type 2 diabetes; only fibrosis regression was not yet achieved [67,68]. Also, SGLT2I may serve as part of combinational treatment strategies to combat MASLD as they effectively lower liver lipid content [18,80]. Thus, metabolic drugs like SGLT2Is, GLP-1RAs or upcoming double [69] and triple agonists [185] may be especially effective in early MASLD stages, whereas pharmacological treatment of progressed MASLD may combine metabolic and liver-targeted drugs. Of importance, the ongoing COMBAT_T2_NASH trial (Combined active treatment in type 2 diabetes with NASH; clinicaltrials.gov identifier NCT04639414; sponsor: German Diabetes Center (DDZ)) will be the first clinical study reporting results of SGLT2I actions on histological endpoints, fulfilling the criteria of evidence level 2 (applying the Oxford Centre for Evidence-based Medicine system) that may result in strong recommendations in clinical guidelines [10]. In this multicenter, randomized, placebo-controlled double-blind clinical trial, people with type 2 diabetes and histologically proven MASH are randomized to either empagliflozin

monotherapy (10 mg/d), combined treatment with semaglutide (1 mg/week) and empagliflozin (10 mg/d), or a respective placebo for a treatment period of 48 weeks. The primary outcome is the percentage of people with histological MASH resolution without worsening of fibrosis at 48 weeks of treatment. The COMBAT_T2_NASH study will provide high quality data both on SGLT2I-mediated histological effects and on the add-on benefit of combined SGLT2I/GLP-1RA treatment in people with type 2 diabetes. First patient in (FPI) was in March 2021. The recruitment phase ended in March 2024, and the last patient out (LPO) is expected in January 2025.
Moreover, new substance classes such as mitochondrial uncouplers [186] (clinicaltrials.gov identifier NCT05822544) or targeting the NLRP3 (nucleotide-binding and oligomerization domain-like receptor [NOD]-like receptor pyrin domain-containing 3) inflammasome [187](clinicaltrials.gov identifier NCT06047262; with the German Diabetes Center (DDZ) heading a substudy on hepatic effects) are currently tested in clinical trials.

In conclusion, this work contributes valuable knowledge to the understanding of pathophysiology and optimized management of MASLD in people with and without type 2 diabetes by: i) detailed validation of methods for assessment of insulin resistance and hepatic steatosis, ii) elucidation of initial and pro-progressive metabolic alterations in lipid-induced insulin resistance and iii) characterization of the effects of SGLT2I on MASLD and its comorbidities in people with type 2 diabetes.

6. References

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8. Attached articles (study 1-8)

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ARTICLE

Estimates of insulin sensitivity from the intravenous-glucose-modified-clamp test depend on suppression of lipolysis in type 2 diabetes: a randomised controlled trial

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Abstract
Aims/hypothesis The combined IVGTT–hyperinsulinaemic–euglycaemic clamp (Botnia clamp) allows the assessment of insulin secretion and sensitivity in one experiment. It remains unclear whether this clamp yields results comparable with those of the standard hyperinsulinaemic–euglycaemic clamp (SHEC) in diabetes patients. We hypothesised that the IVGTT induces responses affecting insulin sensitivity assessment.
Methods Of 22 randomised diet- or metformin-treated patients with well-controlled type 2 diabetes, 19 randomly underwent a Botnia clamp and an SHEC, spaced by 2 weeks, in one clinical research centre in a crossover study. The main outcomes were whole-body and hepatic insulin sensitivity as measured by the clamp and [6,6-$^2H_2$]glucose. Substrate utilisation was assessed from indirect calorimetry and beta cell function from insulin dynamics during IVGTT.
Results The values of whole-body insulin sensitivity obtained from Botnia clamp and SHEC were correlated (r=0.87, p<0.001), but also revealed intra-individual variations. Hepatic insulin sensitivity did not differ between experiments during the clamp, but differed after IVGTT. The contribution of glucose oxidation to glucose disposal increased by 2.2±0.3 and 1.2±0.4 mg kg fat-free mass (FFM)$^{-1}$ min^{-1} (Botnia and SHEC, p<0.05), whereas lipid oxidation decreased by 0.8±0.1 and 0.4±0.1 mg kg FFM^{-1} min^{-1} (p<0.05) from baseline. Differences in NEFA (r=−0.60, p<0.01), but not C-peptide (r=−0.16, p=0.52) or hepatic insulin sensitivity between IVGTT and placebo before the clamps correlated with individual variations of insulin sensitivity.
Conclusions/interpretation The Botnia clamp provides similar estimates of insulin sensitivity as SHEC in patients with type 2 diabetes, but changes in NEFA during IVGTT may affect insulin sensitivity and thereby the discrimination between insulin-sensitive and insulin-resistant individuals.
Trial registration: ClinicalTrials.gov NCT01397279
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Keywords Glucose transport · Insulin resistance · Insulin sensitivity · Lipid metabolism · Metabolic physiology

Abbreviations
ACPR Acute C-peptide response
AIR Acute insulin response

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BSA	Body surface area
CSI	Calculated sensitivity index
DI	Disposition index
EGP	Endogenous glucose production
FFM	Fat-free mass
GIR	Glucose infusion rate
GOX	Glucose oxidation
I	Mean insulin concentration
IGT	Impaired glucose tolerance
IL-1RA	IL-1 receptor antagonist
LOX	Lipid oxidation
M	Whole-body insulin sensitivity
MCP-1	Monocyte chemoattractant protein 1
POX	Protein oxidation
R_{dFFM}	Rate of whole-body glucose disappearance
REE	Resting energy expenditure
SHEC	Standard hyperinsulinaemic–euglycaemic clamp

Introduction

Type 2 diabetes is characterised by abnormalities of both insulin sensitivity and beta cell function and metabolic phenotyping requires addressing both alterations [1]. The gold-standard technique for assessment of insulin sensitivity is the standard hyperinsulinaemic–euglycaemic clamp (SHEC)[2], while insulin secretion is measured with various techniques including the IVGTT, which primarily assesses first-phase insulin response [3].

The Botnia clamp combines both tests consecutively, providing a measure of both insulin sensitivity and beta cell function [4] and it could therefore serve as an ideal test for comprehensive metabolic phenotyping of larger cohorts comprising individuals in non-diabetic states with or without increased risk for diabetes [5]. However, the IVGTT period may influence the subsequent assessment of insulin sensitivity because of the variability of glucose loading and/or consecutive insulin responses. Surprisingly, the Botnia clamp has not been compared with SHEC in detail and in different metabolic states despite its broad use in clinical studies.

Three previous studies tested both Botnia clamp and SHEC in persons with different glucose tolerance states [4, 6, 7], but without focus on overt type 2 diabetes. However, the variation in residual beta cell function and insulin sensitivity between and within states of impaired glucose tolerance and diabetes [8, 9] will strongly affect the results of the Botnia clamp. The causal factors could be, in particular, the increased availability of NEFA [10–12], but also release of cytokines and hormones such as glucagon and cortisol [13, 14]. Elevated circulating NEFA promote insulin resistance by inhibiting insulin-mediated muscle glucose transport and suppression of endogenous glucose production (EGP) in liver [10, 15]. Rapid changes in glucose levels activates insulin release, which acts not only on muscle and liver but also suppresses adipose tissue lipolysis, thereby lowering plasma NEFA [16]. In addition, glucose challenge differently modulates secretion of glucagon and cortisol [17, 18] and increases several cytokines affecting insulin sensitivity, particularly in persons with impaired glucose tolerance (IGT) [19–21]. TNF-α impairs insulin action, whereas the IL-1 receptor antagonist (IL-1RA) can protect against beta cell dysfunction [22, 23]. Monocyte chemoattractant protein 1 (MCP-1) is secreted in response to insulin in insulin-resistant states and promotes macrophage infiltration of adipose tissue [24, 25].

We hypothesised that the preceding IVGTT induces endocrine and metabolic responses that affect insulin sensitivity assessed with the Botnia clamp. Thus, we compared the Botnia clamp with the SHEC by measuring tissue-specific insulin sensitivity, energy expenditure and substrate utilisation, circulating metabolites, hormones and cytokines in patients with type 2 diabetes.

Methods

Volunteers All participants gave their written informed consent before inclusion in the study (ClinicalTrial.gov registration no. NCT01397279), which was performed according to the principles of the Declaration of Helsinki (revised 2000) and approved by the local institutional ethics board. During the screening visit, all patients underwent detailed clinical examinations and routine blood testing. Inclusion criteria were age 30–69 years, confirmed diagnosis of type 2 diabetes [26] and negative islet autoantibody testing. Exclusion criteria were severe chronic diseases and anaemia.

Experimental design Patients were advised to stop metformin treatment at least 3 days before the experiments [27], alcohol consumption 24 h before and smoking 8 h before. All participants followed a carbohydrate-rich diet (60% carbohydrates) for 3 days and remained fasted for 10-12 h before the start of experiments. On the morning of an experiment, body height and weight were recorded and bioimpedance analysis performed to obtain fat-free mass (FFM) [28]. Intravenous catheters were inserted into the forearm veins of both arms for blood sampling and infusion of glucose and insulin. After baseline blood sampling, a primed [0.36 mg (fasting blood glucose in mmol/l) × (5 mmol/l)$^{-1}$ × (body weight in kg)$^{-1}$ min^{-1} for 5 min], constant intravenous infusion [0.036 mg × (body weight in kg)$^{-1}$ min^{-1}] of the 6,6-[$^{2}H_{2}$]glucose for assessment of EGP was started at −120 min and maintained at this rate throughout the experiment, which has been shown to suffice for metabolically well-controlled patients with type 2 diabetes [29].

At 0 min, a bolus of glucose during Botnia clamp (1 mg/kg body weight in a 30% (wt/vol.) solution

containing 1.98% [6,6-$^2H_2$]glucose) or a NaCl (154 mmol/l) bolus of identical volume during SHEC was injected within 30 s into the antecubital vein. Blood samples were obtained at −5, 0, 2, 4, 6, 8, 10, 20, 30, 40, 50 and 60 min during the IVGTT period and at −5, 30 and 60 min during the NaCl control period of SHEC. Each individual underwent both IVGTT and NaCl control experiments in random order spaced by 2 weeks interval.

For the hyperinsulinaemic–euglycaemic clamp from 60 to 240 min a primed [10 mU (body weight in kg)$^{-1}$ min^{-1} for 10 min] constant infusion of short-acting human insulin [1.5 mU (body weight in kg)$^{-1}$ min^{-1}] (Insuman Rapid; Sanofi-Aventis, Frankfurt am Main, Germany) was given.

Plasma glucose was measured at 5 min intervals throughout the clamp. A variable infusion of 20% glucose (wt/vol.) enriched with 2% [6,6-$^2H_2$]glucose (wt/vol.) was started to maintain blood glucose concentration at 5 mmol/l according to previous protocol [30].

Indirect calorimetry Indirect calorimetry was performed during fasting and clamp steady-state conditions (210–240 min) in the canopy mode for 20 min after 10 min adaptation time (Vmax Encore 29n, CareFusion, Höchberg, Germany) [31]. Rates of oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) were measured and RQ and resting energy expenditure (REE) were calculated according to Frayn [32], with fixed estimated protein oxidation rates of 15% of REE (POX) (g/day)=(0.15×REE in kJ/day)×16.74, glucose oxidation rate (GOX) (mg [kg FFM]$^{-1}$ min^{-1})=[(4.55 $\dot{V}CO_2$ − 3.21 $\dot{V}O_2$)×1.44] − (0.459 POX)×1,000 (1,440 kg FFM)$^{-1}$, and lipid oxidation rate (LOX) (mg [kg FFM]$^{-1}$ min^{-1}) = [(1.67 $\dot{V}O_2$ − 1.67 $\dot{V}CO_2$)×1.44) − 0.307 POX]×1,000 (1,440 kg FFM)$^{-1}$.

Assays Blood samples were immediately chilled, centrifuged and supernatant fractions stored at −20°C until analysis. Venous whole blood glucose was measured in duplicate by the glucose oxidase method using an EKF Biosen C-Line glucose analyser (EKF Diagnostic, Barleben, Germany) [33]. Insulin was measured by microparticle enzyme immunoassay (MEIA, AXSYM analyser, Abbott, Abbott Park, IL, USA), C-peptide by chemiluminescence immunoassay (Immulite1000, Siemens, Erlangen, Germany) and plasma glucagon by RIA (Millipore, St Charles, MO, USA). Plasma NEFA concentration was assayed with the microfluorimetric method (Wako, Neuss, Germany) and cortisol using fluorescence polarisation immunoassay (Axsym, Abbott). Plasma inflammatory markers MCP-1 and IL-1RA were analysed by Quantikine ELISA and TNF-α by Quantikine HS ELISA (R&D Systems, Wiesbaden, Germany) [23, 34].

Gas chromatography–mass spectrometry Determination of atom per cent enrichment (APE) of ^2H was done after deproteinisation and derivatisation to the aldonitrile-pentaacetate as described previously [35]. The analyses were performed on a gas chromatograph interfaced to a mass selective detector (Hewlett Packard 6890 and Hewlett Packard 5975).

Calculation of beta cell function AUCs of insulin and C-peptide were computed from the respective plasma concentrations during the first hour of IVGTT. The acute insulin response (AIR, pmol/l), as a measure of first-phase insulin secretion, was calculated from the average incremental insulin concentration during min 2–10 of IVGTT. The acute C-peptide response (ACPR, nmol/l) was calculated the same way as AIR but with C-peptide concentrations. The disposition index (DI), an index of beta cell function corrected for insulin sensitivity, was calculated as the product of AIR and calculated sensitivity index (CSI, a measure of insulin sensitivity during IVGTT) [3, 36].

Calculations of glucose and NEFA metabolism Whole-body insulin sensitivity (M value [mg kg FFM^{-1} min^{-1}]) was calculated from the mean glucose infusion rate (GIR) during the last 30 min of the clamp test normalised to FFM [3]. Rates of whole-body glucose disappearance (R_{dFFM}) and of EGP were calculated from the tracer infusion rate of [6,6-$^2H_2$]glucose and its enrichment to the hydrogens bound to carbon 6 divided by the mean per cent enrichment of plasma [6,6-$^2H_2$]glucose using non-steady state equations as described previously [37, 38]. M_{FFM}/ mean insulin concentration (I) or R_{dFFM}/I was calculated by dividing the M value or R_{dFFM} by I in pmol/l during steady state multiplied by 100 (M_{FFM}/I=[M_{FFM}/I] 100; R_{dFFM}/I=[R_{dFFM}/I] 100). Non-oxidative glucose disposal is given as the difference between the rate of disappearance (R_{dFFM}) and carbohydrate oxidation. NEFA inhibition was calculated as previously reported [39].

Power analysis The clamp test has a coefficient of repeatability of ~0.85–1.0 in non-diabetic individuals, corresponding to an intra-individual CV of ~10% [3]. Employing the Botnia clamp, the mean intra-individual CV for repeat M values was 9.0% in a collective of non-diabetic and diabetic individuals [6]. To our knowledge, no data exist about the intra-individual CV of M values regarding comparison of the Botnia clamp with the SHEC. Assuming that the CV of ratios of repeated measurements of insulin sensitivity from Botnia clamp and SHEC is comparable (~10%) and considering that the geometric mean of M value ratios is ≥1.1, a sample size of 16 paired experiments would be needed to reach a power of 0.95 based on a two-sided paired t test comparing the log-transformed M values of Botnia clamp and SHEC. With the actual number (n=19) of paired studies, we achieve a power of 0.98.

Statistical analysis Participant characteristics are expressed as mean ± SD for normally distributed variables, metabolic data

as mean ± SEM. Statistical analyses included Student's *t* test for paired analyses and Pearson correlation analysis for normally distributed and Wilcoxon's rank test and Spearman correlation for non-normally distributed samples. For comparing two time curves of blood glucose levels and GIRs, we tested specific time points (−5, 30, 60, 90, 120, 150, 180, 210 and 240 min and 60, 90, 120, 150, 180, 210 and 235 min, respectively) with a Bonferroni-adjusted multiple *t* test controlling the family-wise error rate at level 5%. Statistical significance of differences was defined at $p<0.05$. Calculations were performed using GraphPad Prism version 4.03 (GraphPad Software, La Jolla, USA) and SAS for Windows version 9.2 (SAS Institute, Cary, NC, USA). Data for insulin, C-peptide, NEFA, cortisol, GIRs, *M* values, MCP-1, TNF-α, IL-1RA, NEFA suppression and DI were $\log_e$-transformed to approximate normal distribution.

Results

Study population Nineteen (11 male, eight female) patients with type 2 diabetes (aged 53±10 years, BMI 32.4±6.1 kg/m^2, diabetes duration 21.5±9.7 months) and good metabolic control (HbA_{1c} 6.1±1.0% [43.0±10.4 mmol/mol]) with metformin and/or lifestyle intervention participated in the study (electronic supplementary material [ESM] Table 1; flow chart in ESM Fig. 1).

Metabolites, hormones, and cytokines During the Botnia clamp, the glucose bolus (IVGTT) induced a transient rise of blood glucose at 2–8 min to 14.9±0.5 mmol/l with a subsequent decrease to 9.4±0.5 mmol/l at 60 min, yielding an AUC_{Gluc} of 678±27 mmol/l. As expected, blood glucose did not increase and was lower before the start of the clamp (5.8±0.3 mmol/l, $p<0.001$) in the control period of SHEC (NaCl solution) (Fig. 1a). During the last 120 min of both clamp periods the mean blood glucose levels were comparable (Fig. 1a).

Serum insulin and C-peptide concentrations increased during IVGTT and remained higher before start of the Botnia clamp compared with SHEC (insulin 207±27 and 77±10 pmol/l, $p<0.001$; C-peptide 2.1±0.1 and 1.0±0.1 nmol/l, $p<0.001$) (Fig. 1b, c). Insulin levels did not differ between both clamp periods (Fig. 1b), while C-peptide remained higher during the Botnia clamp (Fig. 1c).

Plasma glucagon decreased after the start and was slightly lower at one time point of the IVGTT period of the Botnia clamp compared with the NaCl control period of SHEC (60 min 102±8 and 113±9 ng/l, $p=0.04$) but was comparable during the last 120 min of both clamp periods (ESM Fig. 2a). Plasma cortisol levels did not differ during any period of either experiment (ESM Fig. 2b).

Plasma NEFA were similar in the fasted state, but declined rapidly on glucose loading (IVGTT) without sex-specific differences (Fig. 1d, ESM Fig. 2c) and were markedly lower than in the NaCl control period of SHEC at 60 min (0.22±0.02 and 0.44±0.04 mmol/l, $p<0.001$). Plasma NEFA levels were comparable during both clamp periods (Fig. 1d). NEFA inhibition ranged from 2.32 to 7.55 μmol/l/min, with a mean of 3.85±0.28 μmol l^{-1} min^{-1}. Plasma TNF-α,

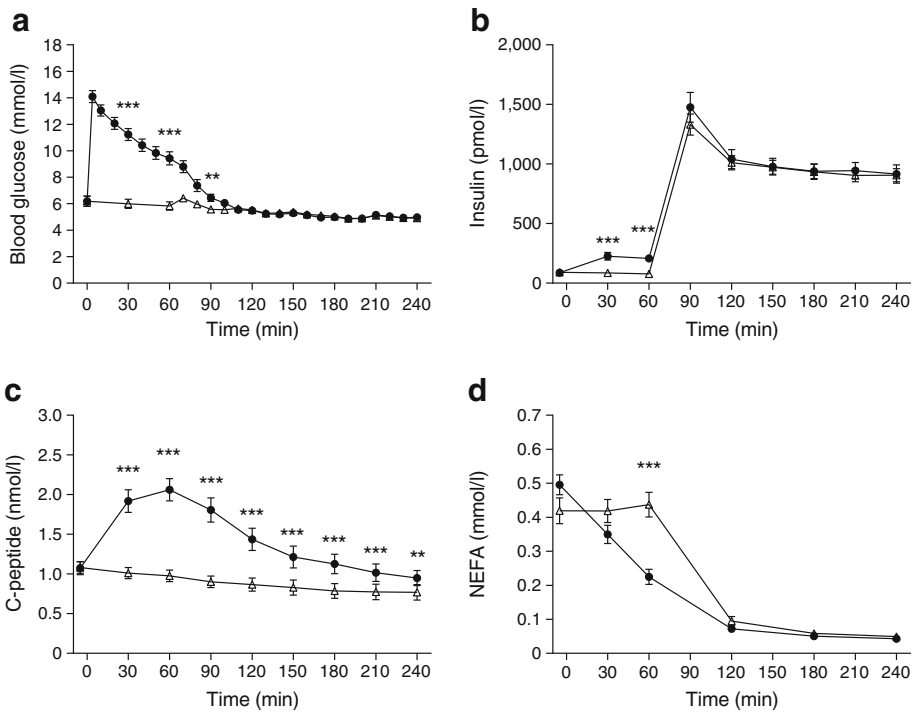

Fig. 1 Time course of venous blood glucose (**a**), insulin (**b**), C-peptide (**c**) and NEFA (**d**) during the Botnia clamp (black circles) and SHEC (white triangles), respectively. Data are shown as means ± SEM. Bonferroni-adjusted values: $^{**}p<0.01$; and $^{***}p<0.001$

MCP-1 and IL-1RA levels did not differ between experiments (ESM Table 2).

Insulin sensitivity and substrate utilisation GIRs tended to be higher with the Botnia clamp, but were not different from SHEC during the last 120 min of the clamp periods (Fig. 2a). Accordingly, M values (8.7±0.8 and 8.1±0.7 mg kg FFM^{-1} min^{-1}; CV 11.0%, r=0.87; p<0.001; ESM Fig. 3a) and M_{FFM}/I (1.09±0.14 and 0.98±0.13 mg kg FFM^{-1} min^{-1} (pmol l^{-1}) $^{-1}$ 100; CV 12.8%, r=0.93, p<0.001) were comparable and tightly correlated but showed intra-individual variation between both experiments (Figs. 2b, 3a, b and ESM Fig. 3b). There were no sex-specific differences in M values (ESM Fig. 3c). Of note, classification into insulin-sensitive and insulin-resistant persons at a defined cut-off of 5.3 mg kg FFM^{-1} min^{-1} [40] was different in two out of 19 participants based on their individual M values obtained from Botnia and SHEC (ESM Fig. 4).

EGP from Botnia and SHEC was similar at baseline (11.6±1.0 and 13.6±0.8 μmol kg^{-1} min^{-1}) and under insulin-stimulated conditions (6.4±1.0 and 5.9±0.7 μmol kg^{-1} min^{-1}), but differed at the end of IVGTT (4.8±1.2 and 10.1±1.0 μmol kg^{-1} min^{-1}, p<0.001). Thus, hepatic insulin sensitivity, as assessed by suppression of EGP, was different between Botnia clamp and SHEC after IVGTT (58±12% and 22±10%, p<0.01), but comparable at the end of clamp (51±6% and 66±4%) (Fig. 2c).

Also, the RQ was similar before and during both experimental conditions (Table 1). REE was slightly lower during fasting in the Botnia clamp than in SHEC and increased only during the clamp period of the Botnia clamp. Fasting substrate oxidation was comparable (Table 1). During the clamp periods, LOX declined and GOX increased in a more pronounced manner in the Botnia clamp compared with SHEC (ΔLOX between Botnia and SHEC, p<0.05; ΔGOX between Botnia and SHEC, p<0.05; Table 1). Finally, the rates of glucose disappearance (R_{dFFM} 9.1±0.9 and 8.3±0.7 mg kg FFM^{-1} min^{-1} for Botnia and SHEC), non-oxidative (4.6±0.7 and 4.6±0.7 mg kg FFM^{-1} min^{-1}) and oxidative glucose utilisation (4.5±0.3 and 3.7±0.3 mg kg FFM^{-1} min^{-1}) were similar during both clamp periods (Fig. 2d). Likewise, R_{dFFM}/I was similar between Botnia clamp and SHEC (1.12±0.14 and 1.01±0.13 mg kg FFM^{-1} min^{-1} [pmol l^{-1}]$^{-1}$ 100; CV 14.6%, r=0.89, p<0.001; ESM Fig. 3d).

Calculated indices of beta cell function and insulin sensitivity Beta cell function varied widely across the participants, the first phase of insulin secretion was mostly absent, whereas the second phase was detectable in most patients

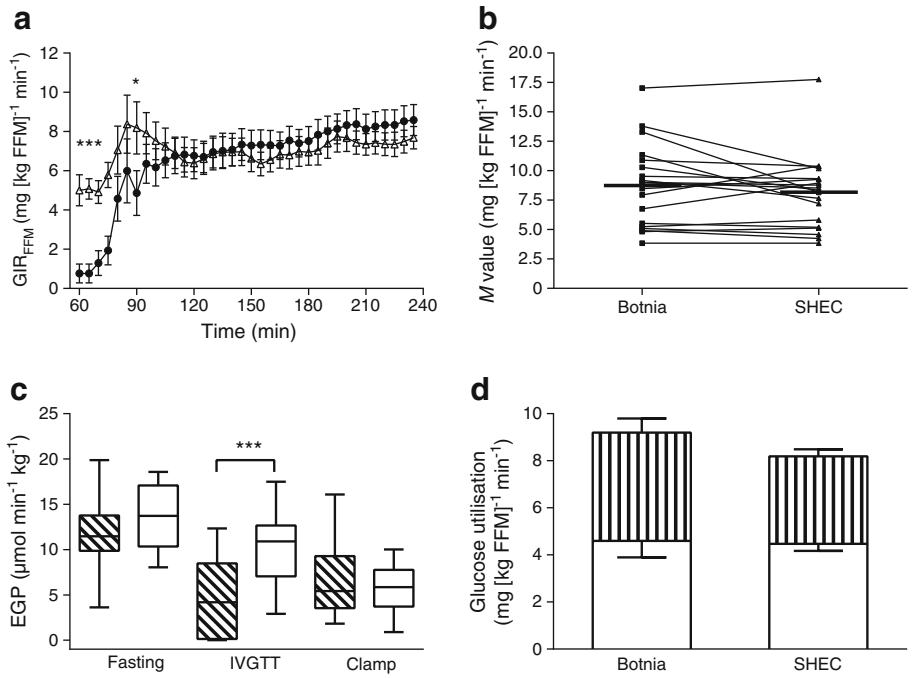

Fig. 2 (**a**) GIRs (mg [kg $FFM]^{-1}$ min^{-1}) during the Botnia clamp (black circles) and SHEC (white triangles). Data are shown as means ± SEM; *p<0.05; ***p<0.001 Botnia vs SHEC. (**b**) M values (mg [kg $FFM]^{-1}$ min^{-1}) obtained with the Botnia clamp (squares) and standard (triangles) for the examined individuals. Thin lines indicate corresponding values of a single participant; thick lines indicate mean of Botnia clamp and SHEC, respectively. (**c**) EGP (μmol [kg $FFM]^{-1}$ min^{-1}) during baseline, at the end of IVGTT and under clamp conditions from the Botnia clamp (hatched boxes) and SHEC (white boxes). ***p<0.001. (**d**) Non-oxidative (white bars) and oxidative (hatched bars) glucose utilisation (mg kg FFM^{-1} min^{-1}) during Botnia clamp and SHEC. Data are shown as means ± SEM

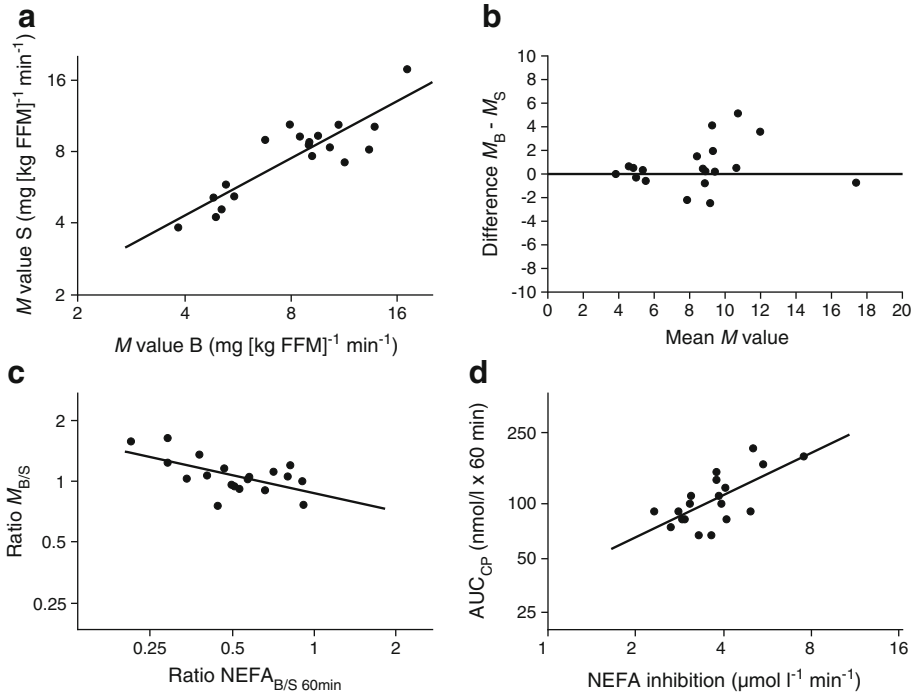

Fig. 3 (**a**) Correlation of *M* values for Botnia clamp and SHEC: $r=0.87$, $p<0.001$; *x* and *y* axes have a log base 2 scale. (**b**) Bland–Altman plot demonstrating the distribution of variability (difference between *M* value with Botnia clamp and *M* value with SHEC [M_B–M_S] [mg (kg FFM)$^{-1}$ min^{-1}] vs mean *M* value of the two tests [mg (kg FFM)$^{-1}$ min^{-1}]) of *M* values across a range of values. (**c**) Correlation of ratio $M_{B/S}$ (ratio *M* values for Botnia clamp/SHEC) with $NEFA_{B/S\ 60min}$ (ratio NEFA at min 60 for Botnia clamp and SHEC): $r=-0.60$, $p<0.01$; *x* and *y* axes have a log base 2 scale. (**d**) Correlation of NEFA inhibition with $AUC_{C\text{-peptide}}$, $r=0.68$, $p<0.01$; *x* axis shown with log base 2 scale and *y* axis with log base 10 scale. B, Botnia; CP, C-peptide; S, SHEC

(ESM Fig. 2d). AIR was 158±32 pmol/l with a maximal insulin response ranging from 36.6 pmol/l to 822 pmol/l. AUC_{Ins} was 12.1±1.7 nmol/l and $AUC_{C\text{-peptide}}$ 108.8±8.3 nmol/l, while CSI was 3.1 ± 0.410^4 min^{-1} (μU ml^{-1})$^{-1}$ and DI 59.5±6.7 common units.

Correlation of insulin sensitivity with beta cell function and NEFA The *M* values for Botnia and SHEC correlated with the corresponding fasting NEFA levels ($r=-0.58$, $p<0.01$ and $r=-0.59$, $p<0.01$) and even more strongly with $NEFA_{60min}$ ($r=-0.86$, $p<0.001$ and $r=-0.69$, $p<0.01$). The *M* value for Botnia did not correlate with NEFA inhibition, AIR, ACPR, AUC_{Ins} or $AUC_{C\text{-peptide}}$.

The differences in *M* values between Botnia clamp and SHEC (ratio $M_{B/S}$) correlated with differences in plasma NEFA levels at 60 min of IVGTT between both experiments (ratio $NEFA_{B/S\ 60min}$) (Fig. 3c) but not with NEFA inhibition. There was no correlation of the ratio $M_{B/S}$ with differences in ΔGOX or ΔLOX, AIR, ACPR, AUC_{Ins}, $AUC_{C\text{-peptide}}$ or DI as measures of insulin secretion and beta cell function or with EGP suppression at the end of IVGTT. Further, NEFA inhibition correlated strongly with AUC C-peptide (Fig. 3d),

Table 1 Energy expenditure and substrate utilisation

Variable	Botnia		SHEC		ΔClamp – fasting	
	Fasting	Clamp	Fasting	Clamp	Botnia	SHEC
RQ	0.84±0.01	0.95±0.02***	0.85±0.02	0.91±0.01**	0.11±0.02†	0.06±0.02
REE (kJ/day)	7,719±304‡	7,987±322*	7,900±365	7,973±359	267±129	74±102
LOX (mg kg FFM^{-1} min^{-1})	0.95±0.08	0.13±0.12***	0.86±0.13	0.42±0.12**	−0.82±0.12†	−0.44±0.14
GOX (mg kg FFM^{-1} min^{-1})	2.2±0.2	4.5±0.3***	2.5±0.3	3.7±0.3*	2.2±0.3†	1.2±0.4

Data given as means ± SEM

* $p<0.05$; ** $p<0.01$; and *** $p<0.001$ fasting compared with clamp conditions

† $p<0.05$ ΔBotnia vs ΔSHEC

‡ $p<0.05$ Botnia vs SHEC

AUC_{Ins} (r=0.51, p<0.05) and AIR (r=0.79, p<0.001), but not with EGP suppression after IVGTT. Additionally, EGP suppression after IVGTT did not correlate with any measure of insulin secretion.

Discussion

This study showed that the Botnia clamp provides estimates of insulin sensitivity similar to those with a 3 h SHEC using mU kg body weight min insulin in patients with overt type 2 diabetes. Nevertheless, individual changes in NEFA levels—reflecting variations of insulin secretion—but not differences in insulin-induced EGP suppression affected the assessment of insulin sensitivity and thereby the discrimination between insulin-sensitive and insulin-resistant individuals.

Previously, three studies found strong correlations (r=0.94 [4] and r=0.95 [6, 7]) between M values obtained from Botnia clamps and standard clamps in persons with normal and IGT [4, 6, 7]. This study is the first to address: (1) the performance in patients with overt type 2 diabetes; (2) the impact on hepatic insulin sensitivity; and (3) the influence of other metabolic and endocrine variables. Of note, we used slightly higher insulin doses in the Botnia clamp compared with most studies [4, 6, 7] and in the SHEC experiments, anticipating a pronounced insulin resistance in most patients with type 2 diabetes.

In general, our results confirmed the correlation of M values obtained from Botnia and a placebo-controlled SHEC protocol in a well-characterised cohort of type 2 diabetic patients despite intra-individual differences in M values between Botnia clamp and SHEC, even when using M/I or R_d/I. Of note, correlations tended to be stronger when adjusting for prevailing mean insulin concentrations during the steady state.

In this study on patients with type 2 diabetes, M values were within a rather small range so that even minor differences in the estimated insulin sensitivity led to changes in distinguishing between insulin-sensitive and insulin-resistant individuals. A recent clamp study employing an insulin infusion at 80 mU m^2 body surface area (BSA) min defined 5.3 mg kg FFM^{-1} min as a cut-off value for insulin resistance [40]. We used a constant insulin infusion of 1.5 mU kg body weight min, corresponding to a mean insulin dose of 68.6 mU m^2 BSA min. Using the above definition, two out of 19 investigated individuals (10%) would be grouped differently using the SHEC or Botnia clamp.

Nevertheless, differences in insulin sensitivity also occur because of intra-individual day-to-day changes, as the CV of repeat Botnia clamps and SHEC has been reported to be 9% and 10%, respectively [3, 6]. In the present study, the mean CV between both tests was 11% as expected; however, CV was greater than 20% in three out of 19 individuals. In this respect, circulating NEFA need to be taken into account: fasting NEFA level and M value correlated inversely in first-degree relatives of type 2 diabetic patients, but not healthy controls [7]. We extend this observation such that glucose loading induces changes in NEFA with an impact on the subsequent assessment of insulin sensitivity. The ratio $NEFA_{B/S\ 60min}$, reflecting intra-individual differences in NEFA levels at the start of clamp experiments, was strongly and negatively correlated with intra-individual differences in the M values of the Botnia clamp and SHEC (ratio $_{B/S}$). This suggests that the M value obtained by Botnia clamp increased with lower individual NEFA levels during the IVGTT period. However, the ratio $M_{B/S}$ was not correlated with NEFA inhibition, which is dependent on insulin action, although reduced C-peptide/insulin release yields a diminished NEFA inhibition (Fig. 3d). Thus, other factors apart from the IVGTT-mediated insulin release might act on NEFA levels as two participants with low FFA ratio showed rather small differences between the two M values. Other tested variables known to affect insulin sensitivity, such as sex and obesity, cannot explain this observation. Stress-enhanced lipolysis cannot be ruled out completely, but comparable mean cortisol levels during first phase of both experiments render this rather unlikely. Triacylglycerol levels or mitochondrial function might play a role [41, 42] but have not been assessed.

As NEFA levels during both clamp periods were comparable, NEFA ‘preload’ might be more important than the prevailing NEFA concentrations during the clamps. Increased endogenous lipolysis with elevated NEFA induced by lipopolysaccharide resulted in lower insulin sensitivity in the subsequent clamp phase despite similar NEFA levels during the steady state of the clamp [37]. Moreover, NEFA-induced insulin resistance of skeletal muscle occurs after several hours of lipid infusion, further emphasising the role of NEFA ‘preload’ [10, 15]. These effects of circulating NEFA are mediated by the cellular accumulation of lipid metabolites, which activate novel protein kinase C isoforms and cause inhibitory serine phosphorylation of insulin receptor substrates [43, 44] or directly reduce Akt activation [45]. Consequently, short-term lowering of plasma NEFA by acipimox rapidly increased whole-body insulin sensitivity [46]. Thus, it is conceivable that the lower NEFA levels during 60 min of IVGTT may be sufficient to raise whole-body insulin sensitivity in the Botnia clamp because of increased muscle glucose uptake or resulting from increased metabolic flexibility. Metabolic flexibility, defined as the whole-body capacity to adapt fuel oxidation to fuel availability [47] and assessed by the switch from fat to carbohydrate oxidation during a hyperinsulinaemic clamp test can be impaired by high NEFA levels [47]. REE was slightly increased only under Botnia clamp conditions; glucose utilisation increased more, as reflected by the higher ΔRQ, and was associated with stronger reduction of LOX and increase of GOX in the Botnia clamp compared with SHEC, but absolute GOX and LOX rates did not differ.

Plasma levels of metabolites and hormones were not different between clamp conditions, except for C-peptide remaining elevated in the Botnia clamp, indicating residual endogenous insulin secretion. Nevertheless, we found no correlation of C-peptide with measures of whole-body insulin sensitivity, rendering major effects of C-peptide on insulin sensitivity rather unlikely. Plasma insulin was identical, suggesting comparable insulin exposure at the level of the skeletal muscle without a detectable effect of IVGTT-mediated endogenous insulin secretion. Nevertheless, variable residual insulin secretion may have resulted in different portal insulin levels, reflected by increased peripheral C-peptide levels. EGP is sensitive to small changes in portal insulinaemia [48], which could explain the greater variability of EGP during the Botnia clamp and the differences in EGP after IVGTT by priming of the liver, thereby contributing to possible peripheral effects [49].

Also, the IVGTT period could be operative through affecting blood glucose levels and glucose effectiveness. Glucose effectiveness refers to the ability of glucose per se to suppress EGP, as seen in our study, and stimulate glucose uptake [50]. However, insulin-mediated EGP suppression and plasma glucose levels during the steady-state clamp periods were similar in both experiments and no association between EGP after IVGTT and peripheral insulin sensitivity was observed. This renders the effects of preceding IVGTT-induced hyperglycaemia on glucose-mediated glucose uptake unlikely. By design, we cannot prove a causal relationship between NEFA suppression and variations in insulin sensitivity and we cannot exclude a modulation by different EGP suppression.

Interestingly, there were no differences in circulating TNF-α, IL-1RA or MCP-1 during the Botnia clamp compared with SHEC. In general, data on cytokine release in response to a glucose challenge are scarce. One study reported increases in plasma levels of IL-6, IL-18 and TNF-α in response to acute hyperglycaemia in healthy individuals and IGT [19]. Further, IL-6, TNF-α and IL-18 were also increased after an oral glucose challenge in patients with IGT or type 2 diabetes, but not in individuals with NGT [20, 21]. The difference between the present and previous studies regarding TNF-α could result from the differences in mode, timing and amount of glucose loading.

In conclusion, the Botnia clamp provides estimates of insulin sensitivity comparable with those of the gold-standard clamp in patients with type 2 diabetes. Nevertheless, the calculated *M* value depends partly on changes in NEFA level, but not on any other tested variable known to affect insulin sensitivity or secretion on intravenous glucose loading. Thus, NEFA inhibition during IVGTT may contribute to changes in individual insulin sensitivity, affecting the discrimination between insulin-sensitive and insulin-resistant individuals.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement MR designed the study and headed the clinical experiments. SK, SP and BN researched the data. SK wrote the first draft of the manuscript and coordinated the inclusion of specific sections as outlined. PJN and CH conducted and wrote aspects of the laboratory analyses. KS supervised and interpreted the statistical analyses of the data. GP calculated indices of beta cell function and wrote the respective sections. All the authors contributed substantially to aspects of study design or the acquisition of data, contributed to drafting of the article or revised it critically for important intellectual content and gave final approval to the version to be published. MR is responsible for the integrity of the work as a whole.

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Comparison of Liver Fat Indices for the Diagnosis of Hepatic Steatosis and Insulin Resistance

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Abstract

Context: Hepatic steatosis, defined as increased hepatocellular lipid content (HCL), associates with visceral obesity and glucose intolerance. As exact HCL quantification by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) is not generally available, various clinical indices are increasingly used to predict steatosis.

Objective: The purpose of this study was to test the accuracy of NAFLD liver fat score (NAFLD-LFS), hepatic steatosis index (HSI) and fatty liver index (FLI) against ^{1}H-MRS and their relationships with insulin sensitivity and secretion.

Design, Setting and Participants: Ninety-two non-diabetic, predominantly non-obese humans underwent clinical examination, ^{1}H-MRS and an oral glucose tolerance test (OGTT) to calculate insulin sensitivity and β-cell function. Accuracy of indices was assessed from the area under the receiver operating characteristic curve (AROC).

Results: Median HCL was 2.49% (0.62;4.23) and correlated with parameters of glycemia across all subjects. NAFLD-LFS, FLI and HSI yielded AROCs of 0.70, 0.72, and 0.79, respectively, and related positively to HCL, insulin resistance, fasting and post-load β-cell function normalized for insulin resistance. Upon adjustment for age, sex and HCL, regression analysis revealed that NAFLD-LFS, FLI and HSI still independently associated with both insulin sensitivity and β-cell function.

Conclusion: The tested indices offer modest efficacy to detect steatosis and cannot substitute for fat quantification by ^{1}H-MRS. However, all indices might serve as surrogate parameters for liver fat content and also as rough clinical estimates of abnormal insulin sensitivity and secretion. Further validation in larger collectives such as epidemiological studies is needed.

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Introduction

Hepatic steatosis is the most frequent liver disease in Western countries, closely associates with insulin resistance, visceral obesity, dyslipidemia and type 2 diabetes (T2DM) and is now classified among non-alcoholic fatty liver diseases (NAFLD) in the absence of excessive alcohol intake [1]. The gold standard for diagnosis of NAFLD is the liver biopsy, which is only justified in severe liver disease [2]. ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS) allows for non-invasive quantification of hepatocellular lipid (HCL) content and for exact diagnosis of steatosis [2], while ultrasound and computed tomography provide rather semi-quantitative estimates [3].

As these techniques are time-consuming, expensive and often unavailable in daily routine, more simple tests have been developed based on routine laboratory and anthropometric parameters. The fatty liver index (FLI) [4], the hepatic steatosis index (HSI) [5] and the NAFLD liver fat score (NAFLD-LFS) [6] yielded satisfying results in their respective collectives, when validated against ultrasound (FLI, HSI) or ^{1}H-MRS (NAFLD-LFS). However,

despite the association of steatosis with impaired glucose tolerance [7], FLI and HSI seem to perform less well in insulin resistant states such as T2DM [8].

We aimed to test (i) the diagnostic accuracy of these three indices by comparison with exact quantification of HCL by ^{1}H-MRS and (ii) the relationships with insulin sensitivity and secretion in a non-diabetic, predominantly non-obese collective of white origin in which median liver fat content is supposed to be low and therefore diagnosis of steatosis appears more challenging. Of note, the FLI has been originally developed to detect steatosis, whereas HSI and NAFLD-LFS have been developed to detect NAFLD. To account for these differences, we also analyzed a subgroup of our collective with low-risk alcohol consumption [9].

Study Population and Methods

Study design

This study was performed in the context of the German National Cohort feasibility studies. The protocol is in line with the 1975 Declaration of Helsinki and was approved by the Bavarian Medical Association and the ethical board of Heinrich-Heine University Düsseldorf. All subjects gave their written informed consent to participate.

Overall, from July to October 2011, 148 residents of the Düsseldorf area, aged 22 to 70 years, were recruited from a random sample of the general population. 100 persons agreed to participate in additional clinical examination, blood sampling after 10 hours of fasting, a 2-hours oral glucose tolerance test (OGTT), liver ^{1}H-MRS and whole-body MR imaging (MRI). Persons with non-white origin, T2DM and/or with hepatitis B and C were excluded from analysis, because these conditions are known to specifically affect HCL [10] so that 92 subjects remained for further analyses.

Clinical examination

All participants underwent a structured interview including assessment of mean daily alcohol intake during 7 days using estimated ethanol contents of beverages (beer 5%, wine 12%, shots 40%). The World Health Organization definition was applied for low-risk alcohol (LRA) consumption [9].

Body weight was measured to the nearest 0.1 kg using a calibrated weighting scale (SECA 285; SECA, Hamburg, Germany). Body height and waist circumference (waist) were measured according to standard procedures. Values of systolic (SBP) and diastolic blood pressure (DBP) were measured thrice after 5 min rest in sitting position using a validated automatic device (OMRON HEM 705 IT, OMRON, Mannheim, Germany) and means of the last two measurements were used for analysis.

Oral glucose tolerance test (OGTT)

A 75 g-OGTT (Accu-Chek Dextro O.G-T., Roche, Basel, Switzerland) was performed after at least 10 hrs overnight fasting. Blood samples were drawn at −5, 30, 60 and 120 min of OGTT and dysglycemia was categorized according to international criteria [11].

Laboratory measurements

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transpeptidase (γ-GT) and HDL-cholesterol (HDL-C) were measured on a Cobas MODULAR analyzer (Roche, Basel, Switzerland). Triglycerides (TG) were measured on a Hitachi 912 analyzer (Roche, Basel, Switzerland). Blood glucose was measured from venous whole blood samples using an EPOS Analyzer 5060 (Eppendorf, Hamburg, Germany). Insulin was determined by microparticle enzyme immunoassay (MEIA) on an AXSYM analyzer (Abbot, Abbot Park, USA), C-peptide (CP) was measured chemiluminimetrically (Immulite1000, Siemens, Erlangen, Germany).

^{1}H-MRS and MRI

All measurements were performed using a 3-T MR scanner (Philips achieva, X-series, Eindhoven, Netherlands). For ^{1}H-MRS, a stimulated echo acquisition mode (STEAM) sequence (repetition time of 4 s, echo time of 10 ms) was performed on a volume of $3\times3\times2$ cm^3 in the liver. Spectra was collected without water suppression from 32 acquisitions and analyzed using the NUTS software package (Acorn NMR Inc, Livermore, CA, USA). HCL was quantified and corrected for T2 relaxation times with specific weighting for lipids as previously reported [12,13]. Steatosis was defined as HCL values ≥5.56% [14]. Whole body MRI was performed to quantify liver volume (HVOL), total (AT_{tot}), subcutaneous (SAT) and visceral (VAT) abdominal adipose tissue using transverse multi-slice turbo-spin echo sequences [15].

Indices of hepatic steatosis

NAFLD liver fat score (NAFLD-LFS) [6].

$$NAFLD-LFS = -2.89 + 1.18 * MS(yes=1/no=0) + 0.45 * T2DM(yes=2/no=0) + 0.15 * I_0 + 0.04 * AST - 0.94 * AST/ALT;$$

with I_0 (μU/ml) representing fasting insulin and AST, fasting AST levels (U/l). Values ≤−0.640 rule out, while values >−0.640 rule in NAFLD. Metabolic syndrome (MS) was defined according to the criteria of the International Diabetes Federation [16].

Hepatic steatosis index (HSI) [5].

$$HSI = 8 * ALT/AST + BMI + 2, \textit{ if } DM; +2, \textit{ if female};$$

with values <30 ruling out and values >36 ruling in steatosis.

Fatty liver index (FLI) [4].

$$FLI = logistic(0.953 * ln(TG) + 0.139 * BMI + 0.718 * ln(\gamma GT) + 0.053 * waist - 15.745) * 100;$$

where logistic(x) = $1/(1+e^{-x})$ denotes the logistic function and ln the natural logarithm. Values <30 rule out and values ≥60 rules in steatosis.

Index of percentage HCL

NAFLD-LFS_cont [6].

$$NAFLD-LFS\_cont(liver\ fat\%) = 10^{(-0.805 + 0.282 * MS(yes=1/no=0) + 0.078 * T2DM(yes=2/no=0) + 0.525 * log(I0 + 0.521 log(AST0) - 0.454 * log(AST0/ALT))}$$

log denotes the decadic logarithm.

Measures of insulin sensitivity and secretion

QUICKI. For fasting conditions, we applied the quantitative insulin sensitivity check index (QUICKI) calculated as $1/[\log(G_0) + \log(I_0)]$, where G_0 and I_0 are fasting glucose and insulin [17].

OGIS. Dynamic insulin sensitivity was assessed with the oral glucose insulin sensitivity index (OGIS), derived from a complex

Table 1. Participants' characteristics.

	No steatosis	Steatosis	No steatosis +LRA	Steatosis +LRA
N (m/f)	75 (29/46)	17 (7/10)	54 (25/29)	11 (6/5)
Age (years)	57.1±12.2	59.9±8.5	56.8±13.1	59.7±9.0
Alcohol (g/d)	18.1±16.1	26.1±18.8	11.1±9.7	18.1±12.9
BMI (kg/m^2)	25.3±4.1	28.2±2.8**	25.2±4.1	27.8±2.3§
Waist (cm)	87.1±12.4	94.6±8.5*	87.0±12.3	94.7±6.6§§
SBP (mmHg)	121.6±15.6	128.1±10.3	122.5±14.3	129.5±6.7§
DBP (mmHg)	72.54±8.7	79.3±8.0**	71.9±7.6	80.1±8.9§§
TG (mg/dl)	78 [60;117]	109[84;153]*	79[56;110]	125[87;153]§
HDL-C (mg/dl)	68.3±17.8	58.1±12.7*	67.3±18.2	55.7±12.7§
AST (U/L)	24[21;28]	25[22;32]	24[21;28]	23[21;31]
ALT (U/L)	18[14;25]	26[17;46]**	18[13;24]	26[17;29]§
ãGT (U/L)	20[14;30]	30[20;35]	21[14;30]	30[20;35]
MS (n)	6	4	3	2
G_0 (mg/dl)	75.7±8.3	77.6±10.7	75.5±7.9	77.2±9.5
G_{120} (mg/dl)	89.7±21.5	96.7±26.0	89.7±21.3	92.6±27.7
I_0 (μU/ml)	6[5;9]	8[6;13]*	6[5;9]	8[6;12]§
I_{120} (μU/ml)	37[24;57]	64[38;119]***	33 [23;57]	67[38;102]§
Hep_Extr (%)	69[62;74]	60[57;66]**	69[62;73]	59[56;66]§§
HCL (%)	1.3[0.4;3.4]	13.6[8.3;22.3]	1.1[0.4;2.9]	11.8[8.3;20.1]
HVOL (L)	1.6[1.4;1.8]	1.8[1.8;1.9]**	1.6[1.4;1.8]	1.8[1.7;1.9]§
AT_{tot} (L)	22[18;29]	29[27;32]***	22[18;27]	28[24;32]§
VAT (L)	2.9[1.7;4.4]	4.3[3.3;6.5]**	3.1[1.6;4.4]	4.6[3.3;6.5]§§§
SAT (L)	5.5[4.4;8.1]	7.4[6.6;9.4]	5.4[4.4;7.4]	7.4[5.6;9.4]

Normally distributed data given as mean±standard deviation; Log-normally distributed data as median [25%quartile;75%quartile];
*p<0.05;
**p<0.01 for steatosis vs no steatosis;
§p<0.05;
§§p<0.01,
§§§p<0.001 for steatosis vs no steatosis in LRA.
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mathematical model, which represents total glucose disposal or whole body insulin sensitivity [18].

ISIcomp. The Matsuda's index (ISIcomp) was used as another measure of dynamic insulin sensitivity and calculated as $= 10000/\sqrt{(G_0 \times I_0 \times G_m \times I_m)}$, where G_m and I_m are mean glucose and insulin concentrations during OGTT [19].

Fasting β-cell function. During fasting, β-cell function was calculated as CP_0/G_0.

Insulinogenic indices (IGI). During glucose loading, the insulinogenic index was calculated as $IGI\_Ins = (I_{30} - I_0)/(G_{30} - G_0)$, where I_{30} and G_{30} are insulin and glucose concentrations at 30 min of OGTT [20,21].

IGI_Ins reflects the appearance of insulin in the peripheral circulation.

For more precise assessing of β-cell (pancreatic, pre-hepatic) function, C-peptide levels were used to calculate the IGI_CP as $(CP_{30} - CP_0)/(G_{30} - G_0)$, where CP_0 and CP_{30} are C-peptide concentrations at fasting and 30 min of OGTT [21].

Disposition Index (DI). The DI is given as product of insulin sensitivity (OGIS) with post-hepatic insulin release function (IGI_Ins_{tot}) [22,23].

Adaptation Index (AI). The AI is the product of insulin sensitivity (OGIS) with β-cell function (IGI_CP_{tot}) [24,25].

Hepatic insulin extraction. Hepatic insulin extraction was approximated by a function of $1-(AUC_{Ins}/AUC_{CP})$ [26].

Statistical Analyses

The diagnostic performance of the indices was tested by the area under the receiver operating characteristic curve (AROC) [27]. Confidence bounds for comparison between AROC's were done as described [28]. The Clopper-Pearson method [29] was used to calculate exact confidence bounds for sensitivity (Se) and specificity (Sp) at different cut-off limits. The Youden index was calculated as sum of Se and Sp-1 [30].

Variables with skewed distribution were ln-transformed before correlation and regression analyses. Moreover, the logit transformation ($logit(x) = \ln(x/(1-x))$ was applied to the FLI index, divided by 100, to obtain a corresponding linear (approximately normally distributed) index given by

$$FLI_l = 0.953 * ln(TG) + 0.139 * BMI + 0.718 * ln(\gamma GT) + 0.053 * waist - 15.745.$$

This linear index has identical characteristics (ROC, Se, Sp) as the original index and was only used for regression analysis, for all other analyses we applied the original index.

P-values from two-sided tests less than 5% were considered to indicate statistically significant differences. For comparing two

Table 2. Correlation (R) of HCL and indices with insulin sensitivity, β-cell function, liver volume and visceral adipose tissue.

Variable		HCL_ln	NAFLD-LFS	HSI	FLI
Liver fat, volume and fat distribution					
HCL_ln	All	1	0.42***	0.46***	0.50***
	LRA	1	0.26*	0.37**	0.43***
HVOL_ln	All	0.36***	0.38***	0.45***	0.52***
	LRA	0.30*	0.32*	0.39**	0.48***
VAT_ln	All	0.52***	0.52***	0.58***	0.78***
	LRA	0.47***	0.39**	0.54***	0.76***
Insulin sensitivity					
ISIcomp_ln	All	−0.46***	−0.71***	−0.53***	−0.62***
	LRA	−0.34**	−0.56***	−0.48***	−0.59***
OGIS	All	−0.46***	−0.51***	−0.50***	−0.62***
	LRA	−0.39**	−0.27*	−0.43***	−0.55***
QUICKI	All	−0.38***	−0.68***	−0.42***	−0.55***
	LRA	−0.24*	−0.62***	−0.35**	−0.46***
β-cell function					
DI_ln	All	0.36***	0.57***	0.48***	0.47***
	LRA	0.24	0.46***	0.45***	0.47***
B-cell func_ln	All	0.28**	0.57***	0.47***	0.57***
	LRA	0.10	0.46***	0.45***	0.54***
AI	All	0.22*	0.35***	0.33**	0.34***
	LRA	0.14	0.25*	0.29*	0.29*
IGI_CP_ln	All	0.11	0.05	0.02	−0.02
	LRA	0.08	0.06	0.00	−0.03
IGI_Ins_ln	All	0.22*	0.26*	0.19	0.16
	LRA	0.15	0.25*	0.14	0.11
Hep _Extr_ln	All	−0.34***	−0.55***	−0.42***	−0.39***
	LRA	−0.24	−0.46***	−0.42***	−0.39***

*, $p<0.05$;
**, $p<0.01$;
***, $p<0.001$;
B-cell func, B-cell function.
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concentration-time curves, we tested specific time points with a Bonferroni-adjusted multiple t-test controlling the family-wise error rate at level 5%. All analyses were performed with SAS for Windows Version 9.2 (SAS Institute, Cary, North Carolina, USA).

Results

Clinical characteristics

Persons with steatosis had higher BMI, waist, DBP, TG, ALT, fasting and 2-hour insulin but lower HDL-C *(Table 1)*. Those with steatosis and low risk alcohol consumption (LRA) also had higher SBP. There were no differences between the respective subgroups with or without LRA.

HCL ranged from 0.03 to 39.01% (median 2.49%; interquartile range (0.62;4.23)) across the whole group (Figure S1 A) and from 0.05 to 30.34% (1.47% (0.60;4.02)) in the LRA subgroup. In the whole group, NAFLD-LFS, HSI and FLI ranged from −4.10 to 2.20, 23.87 to 51.52 and 1.61 to 91.44 with means of -1.81 ± 1.09, 33.71 ± 5.15 and 33.46 ± 26.68, respectively (Figure S1 B,C,D). NAFLD-LFS, HSI and FLI had comparable values in LRA subjects. All indices differed between persons with and without steatosis of the whole group (NAFLD-LFS: $p<0.05$; HSI: $p<0.001$; FLI: $p<0.01$) and LRA subgroups ($p<0.01$; $p<0.01$; $p<0.05$). All indices correlated with HCL and HVOL in the whole and LRA group. HCL and indices also related to AT_{tot}, SAT and VAT in the whole and LRA group, except for NAFLD-LFS, which did not correlate with AT_{tot} and SAT (Table 2, for AT_{tot} and SAT data not shown).

Diagnostic performance of indices

Across all persons, AROC's were 0.70(95% confidence interval [0.53;0.87]) for NAFLD-LFS, 0.79[0.68;0.90] for HSI, and 0.72[0.59;0.85] for FLI (Figure 1A). In the LRA subgroup, AROC's were 0.75[0.57;0.92], 0.80[0.68;0.92], and 0.75[0.63;0.88], respectively. AROC's did not differ from each other in the whole and LRA group.

Raising the threshold for diagnosing steatosis by HCL above 5.56% improved AROC's for all indices in the whole group (Figure 1B) and in LRA subjects (data not shown). However, AROC of FLI did not further improve at a threshold of 7%.

Applying the originally published cut-off values for each index, which rule in or out steatosis, yielded different diagnostic performance. NAFLD-LFS provided low Se (0.35[0.14;0.62]), but high Sp (0.91[0.82;0.96]). In contrast, HSI had maximal Se (1.00[0.81;1.00]) at the lower cut-off and acceptable Sp (0.75[0.63;0.84]) at the upper cut-off value. FLI had comparable Se (0.76[0.50;0.93]) and Sp (0.83[0.72;0.90]). Analysis of the LRA subgroup revealed similar results (data not shown). We also calculated positive (PPV) and negative predictive values (NPV) of the three indices, with NAFLD-LFS, HSI and FLI having a PPV of 0.46[0.19; 0.75], 0.25[0.16; 0.37] and 0.31[0.18; 0.47]. NPV for NAFLD-LFS, HSI and FLI were 0.86[0.76; 0.93], 0.88[0.77; 0.94] and 0.84[0.73; 0.91], respectively.

To determine optimal cut-off values for each index in our sample, we identified those values that maximize Youden's index. In the whole sample, the optimal cut-off values were −1.02 for NAFLD-LFS yielding a Se of 0.59[0.33; 0.82] and Sp of 0.89[0.80; 0.95], 35.0 for HSI (Se 0.76[0.50; 0.93]; Sp 0.70[0.59; 0.81]), and 29.2 for FLI (Se 0.82[0.56; 0.96]; Sp 0.61[0.49; 0.72]). In the LRA subgroup, the values were −1.12 for NAFLD-LFS (Se 0.64[0.31; 0.89]; Sp 0.87[0.75; 0.95]), 34.0 for HSI (Se 0.91[0.59; 1.0]; Sp 0.67[0.53; 0.79]) and 29.2 for FLI (0.91[0.59; 1.0];0.67[0.53; 0.79]).

After optimization of cut-off values, PPV were 0.56[0.31;0.79] for NAFLD-LFS, 0.37[0.21;0.55] for HSI and 0.33[0.19;0.49] for FLI. NPV were 0.91[0.81;0.96] for NAFLD-LFS, 0.93[0.83;0.98] for HSI and 0.94[0.83;0.99] for FLI. For the LRA subgroup, values were 0.77[0.48;0.95] (PPV) and 0.92[0.81;0.98] (NPV) for NAFLD-LFS, 0.55[0.35;0.74] (PPV) and 0.97[0.86;1.0] (NPV) for HSI, and 0.55[0.35;0.74] (PPV) and 0.97[0.86;1.0] (NPV) for FLI.

Finally, we examined whether specific indices can predict percentage of HCL by applying the previously proposed NAFLD-LFS_cont index using the identical parameters as NAFLD-LFS [6]. NAFLD-LFS_cont correlated with HCL across all ($r=0.42$, $p<0.001$) and LRA persons ($r=0.27$, $p<0.05$) (Figure 1C). However, the differences between observed and predicted ln-transformed HCL values (residuals) ranged from −3.9 to 2.5 (Figure 1D). Translated to the original scale, this means that the ratio of observed and predicted liver fat ranges from 0.02 to 12.2.

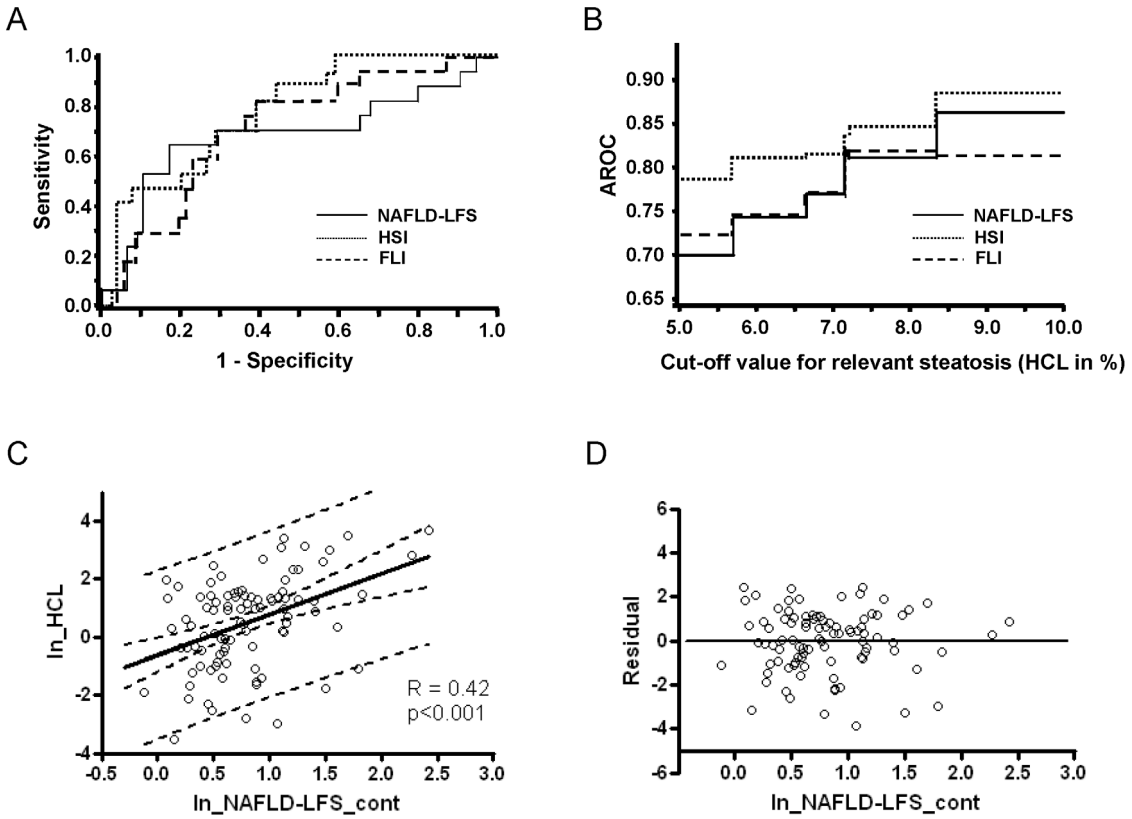

Figure 1. Performance of indices (all subjects). (A) ROC curves of NAFLD-LFS (black line), HSI (dotted line) and FLI (dashed line) (B) AROC's of NAFLD-LFS (black line), HSI (dotted line) and FLI (dashed line) for different HCL cut-offs defining steatosis (C) Correlation of HCL with NAFLD-LFS_cont. Black line, linear regression curve; inner broken lines, 95% confidence limits; outer broken lines, 95% prediction limits (D) Evaluation of goodness of fit by plotting residuals against HCL calculated by NAFLD-LFS_cont.
doi:10.1371/journal.pone.0094059.g001

Correlation of HCL and indices with glycemia, insulin sensitivity and β-cell function

Subjects with steatosis had similar blood glucose, but higher insulin and C-peptide during OGTT than those without steatosis (Figure 2 A,B). In LRA subjects, presumably due to low sample size, differences in insulin and C-peptide levels were less prominent (Figure 2 C,D).

HCL correlated inversely with fasting (QUICKI) and dynamic insulin sensitivity (OGIS, ISIcomp) and positively with fasting β-cell function and post-load insulin release (DI, AI, IGI_Ins) in all, but not in LRA subjects (table 2).

Also, the indices inversely and strongly correlated with QUICKI, OGIS and ISIcomp (table 2). Even after adjustment for age, sex, and HCL (model-1, table 3) and for LRA (model-2, table 3), FLI, NAFLD-LFS and HSI still related to all parameters of insulin sensitivity.

In all and LRA subjects, indices correlated with fasting β-cell function, DI and AI. Only NAFLD-LFS related to IGI_INS ($p<0.05$) (table 2). Applying model-1 on all subjects, correlations between indices and fasting β-cell function, DI and AI were still present. Also, applying model-2, correlations remained (table 3). LRA subjects showed comparable results with model-1, only HSI did not correlate with AI and NAFLD-LFS was not associated with OGIS (data not shown).

Hepatic insulin extraction differed between subjects with and without FL (table 1) and related to HCL and indices in all ($p<0.001$), but not in LRA subjects ($p = 0.06$). However, all indices correlated with hepatic insulin extraction in all and LRA subjects (table 2).

Discussion

In this non-diabetic, predominantly non-obese collective from the general population NAFLD-LFS, HSI and FLI offer a diagnostic efficacy of 70–80% with lower sensitivities and specificities compared with their original description. Interestingly, this study shows additional features of these indices as predictors of insulin resistance and - to less extent - insulin secretion.

Several factors might contribute to the lower than expected diagnostic efficacy of the indices, including selection and characteristics of the study populations (inclusion criteria, risk factor prevalence) as well as measurement technique [27]. In contrast to the populations from which the indices were derived, our study consists of a sample of non-diabetic, predominantly non-obese white persons from the general population. For NAFLD-LFS, the Finnish collective comprises persons without and with T2DM recruited on a 3-to-1 basis for metabolic studies [6]. HSI was derived from data of a Korean cross-sectional case-control study [5]. Finally, FLI however, was developed from data of the Dionysos Nutrition & Liver study, which included residents of Campogalliano in Italy [4,31], providing a real sampling of general population without particular bias in selection, but development of FLI was based on equally matched persons with and without suspected liver disease (SLD). Comparing these collectives shows marked differences in prevalence of risk factors.

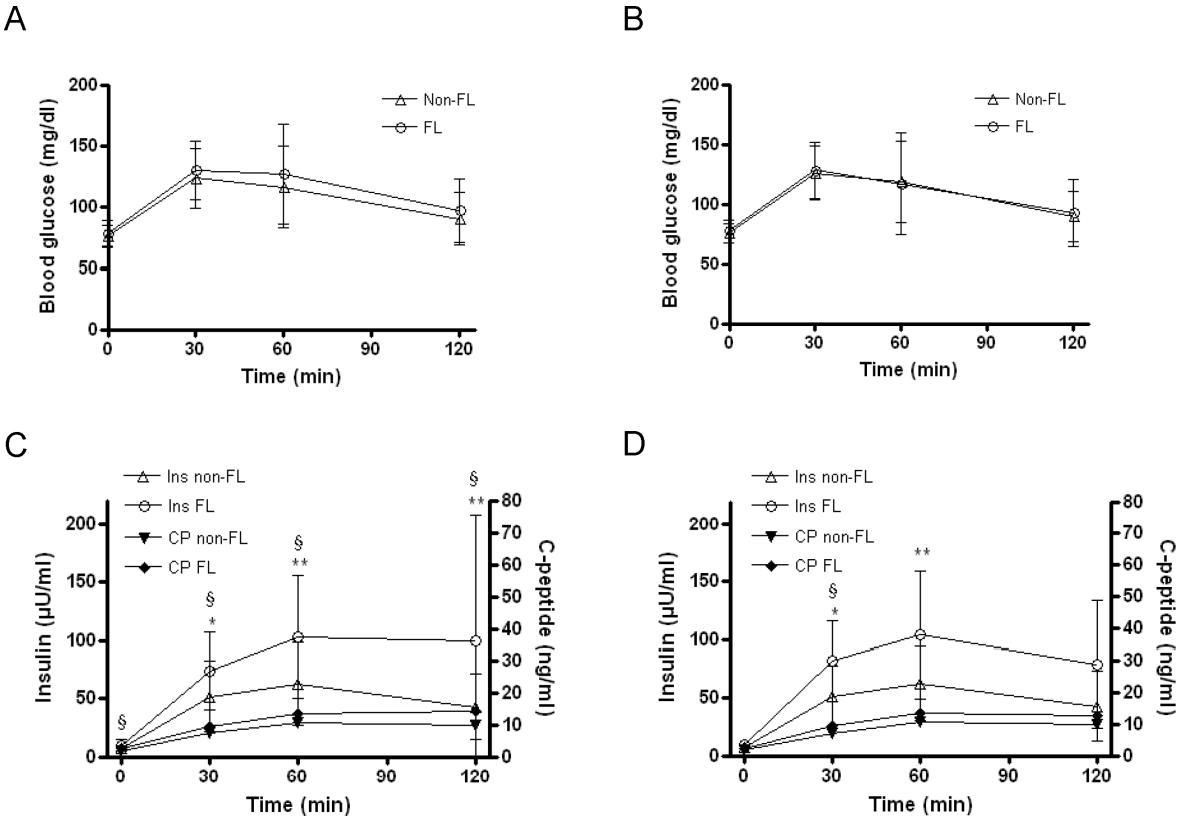

Figure 2. OGTT in subjects with and without steatosis. Plasma glucose (all: A, LRA: B), insulin and C-peptide (all: C, LRA: D) during OGTT in subjects without (non-FL) (insulin: open triangles, C-peptide: black triangles) and with steatosis (FL) (insulin: open circles, C-peptide: black squares). *, $p<0.05$; **, $p<0.01$ for insulin; §, $p<0.05$ for C-peptide.
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The NAFLD-LFS collective presents with already increased risk for metabolic diseases such as T2DM due to greater BMI, BP, TG and transaminases [6]. The HSI collective comprised exclusively Asians, who develop NAFLD at lower BMI with 3.5fold greater prevalence in males, both of which differing from whites [32]. Remarkably, both BMI and sex are variables of the HSI. The FLI collective comprised a white sample of the general population, but cases of SLD were matched with cases without SLD and therefore prevalence of the metabolic syndrome and T2DM might not be the same as in the general population [31].

The sensitivity of ultrasound to detect steatosis is about 91% in patients with HCL ≥30% [33], but only 64% for HCL <30%, indicating that ultrasound misses cases of mild steatosis. Thus, HSI and FLI, which have been validated against ultrasound, may have been rather designed to reliably identify patients with medium to severe fatty liver disease than those with mild steatosis. Testing the accuracy of FLI in a smaller group of women with previous gestational diabetes revealed a strong correlation with HCL measured by ^{1}H-MRS [34], whereas FLI and HSI performed less well in patients with T2DM [8]. We found reasonable AROCs for FLI and HSI, but lower diagnostic performance for NAFLD-LFS in our collective. The latter might be due to the lower mean HCL compared to the validation study of NAFLD-LFS [6]. NAFLD-LFS_cont was derived from the NAFLD-LFS collective and developed exclusively to predict percent HCL [6]. The present study showed that the residuals, i.e. the differences between observed and predicted HCL using this specific index, were in most cases as high as the value of HCL contents. This indicates that NAFLD-LFS_cont is not suitable for prediction of HCL at least in non-diabetic collectives with lower prevalence of steatosis. Thus, these scores offer overall modest performance in the clinical setting– even after optimizing cut-off values for our collective. In detail, sensitivity and specificity differ among the three indices between 0.59 and 0.82 for Se and 0.61 and 0.89 for Sp, respectively. This means that up to 41% of the investigated individuals may be classified as patients without FL, although having FL (false-negative rate) and up to 39% of the individuals may be grouped as FL positive, although having no FL (false-positive rate). These data do not support their use as screening tools, at least for populations with similar characteristics as in the present study with such non-obese persons. Additionally, the positive predictive values indicate that in case of a positive test result, the probability that the patient really has FL is only between 33 and 56%. It might be also critical to adjust cut-offs for FL indices for the tested cohort. Nevertheless, the acceptable correlation between fatty liver indices and exact quantification of HCL suggests that these indices might be appropriate surrogate parameters of liver fat content in large epidemiological studies. It is well accepted that hepatic steatosis associates with insulin resistance and hyperinsulinemia even in lean glucose-tolerant subjects [7]. Likewise, FLI correlates with insulin resistance and T2DM incidence [7,34,35]. Although NAFLD-LFS also predicted T2DM in a French cohort [36], its relationship with insulin resistance has not been assessed. To our knowledge, HSI has also not been analyzed with regard to insulin sensitivity and secretion. Here we clearly show that all three indices, strongly and inversely correlate with measures of insulin sensitivity.

Table 3. Association of indices with insulin sensitivity and β-cell function after adjustment for age, sex, HCL and LRA.

			Model-1 Age,Sex,HCL		Model-2 Age,Sex,HCL,LRA	
		Dependent	**Estimate (β)**	**Partial correlation**	**Estimate (β)**	**Partial correlation**
NAFLD-LFS	All	OGIS	−22.3***	−0.38	−22.9***	−0.39
HSI			−4.1**	−0.35	−4.3***	−0.35
FLI_l			−20.4***	−0.46	−21.1***	−0.46
NAFLD-LFS		QUICKI	−0.03***	−0.62	−0.03***	−0.64
HSI			−0.003**	−0.32	−0.003**	−0.33
FLI_l			−0.02***	−0.45	−0.02***	−0.46
NAFLD-LFS		ISIcomp_ln	−0.31***	−0.64	−0.33***	−0.66
HSI			−0.04***	−0.41	−0.04***	−0.42
FLI_l			−0.19***	−0.51	−0.20***	−0.52
NAFLD-LFS		Disposition Index_ln	0.22***	0.52	0.23***	0.55
HSI			0.03***	0.38	0.04***	0.40
FLI_l			0.13***	0.41	0.14***	0.43
NAFLD-LFS		Adaptation Index	0.04***	0.34	0.04***	0.36
HSI			0.006*	0.24	0.006*	0.24
FLI_l			0.03**	0.32	0.03**	0.33
NAFLD-LFS		B-cell func_ln	0.21***	0.53	0.19***	0.53
HSI			0.03***	0.40	0.03***	0.40
FLI_l			0.16***	0.56	0.16***	0.56

*, $p<0.05$;
**, $p<0.01$;
***, $p<0.001$;
B-cell func, B-cell function.
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Of note, less is known on an association between HCL and β-cell function. While HCL and fasting insulin may correlate [37], data on its relationship with dynamic/OGTT postload β-cell function in collectives with normal and impaired glucose tolerance was contradictory [38,39]. Here, we confirm that HCL relates to various parameters of β-cell function except IGI in all, but not in LRA subjects, and extend this finding to the three indices. The indices only failed to associate with IGI_Ins and IGI_CP, which might result from the pre-described rather low performance of IGI in small- to medium-sized collectives [21].

The novelty of the present study resides in the direct comparison of different indices with HCL measurement by ^{1}H-MRS in a single study population of non-diabetic, predominantly non-obese whites and the finding that - while not specific for prediction of hepatic steatosis - they at least partly reflect glucose homeostasis. On the other hand, this study has also certain limitations. First, this study has a rather small sample size and a collective with low mean HCL contents and prevalence of steatosis. This should not influence Se and Sp of the indices [27] and increases the relevance of these results for general screening of steatosis. However, the predominance of persons with low HCL contents might contribute to the wide confidence intervals for sensitivity and the low positive predictive values thereby underestimating the value of the indices. The small sample size may also add to the wide confidence intervals for AROCs of the tested indices. Moreover, when comparing AROCs of the different indices, we did not find significant differences in performances, but we cannot fully exclude that there might be differences in performance we cannot detect with our collective. Thus, further validation of these indices should be performed in larger cohorts.

Second, participants with significant consumption of alcohol were not omitted from the analysis of the whole collective, as the relative contribution of ethanol intake to the pathogenesis of NAFLD is still uncertain [4]. In their regression models, Bedogni et al. even report no association between ethanol intake and steatosis [4]. Recent data suggest that - despite the potential interactions between alcohol drinking and liver injury - moderate alcohol intake may have paradoxical, favorable and gender-dependent effects also in the liver [40,41]. However, as heavy drinking is known for its deleterious effects on the liver, we set maximum acceptable alcohol intake to 40 g/d for men and 20 g/d for women, which is below the levels set for heavy drinking (>60 g/d for men and >40 g/d for women), for analyses of the LRA subgroup. Of note, all analyses were also performed in this LRA subgroup, which gave similar results as reported for the whole group.

In conclusion, the tested fatty liver indices offer modest efficacy to detect steatosis and cannot substitute for exact fat quantification by ^{1}H-MRS. However, they might serve as surrogate parameters for liver fat content and also as rough clinical estimates of abnormal insulin sensitivity and secretion. Further validation in larger collectives such as epidemiological studies is needed.

Supporting Information

Figure S1 Comparison of HCL and indices in subjects with and without steatosis. Box plots of HCL (A), NAFLD-LFS (B), HSI (C) and FLI (D) scores.
(TIFF)

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Author Contributions

Conceived and designed the experiments: MR SK BN KS BK RL JHH. Performed the experiments: SK BN RL KK BK. Analyzed the data: SK KS RL JHH GG BH GP AG MR. Contributed reagents/materials/analysis tools: KS JHH GP. Wrote the paper: SK KS RL JHH GG GP MR. Revision of article: SK KS BN RL BK KK JHH GG BH GP AG MR. Final approval: SK KS BN RL BK KK JHH GG BH GP AG MR.

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Research Article
MASLD and Alcohol-Related Liver Diseases

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OF HEPATOLOGY

Dysglycemia and liver lipid content determine the relationship of insulin resistance with hepatic OXPHOS capacity in obesity

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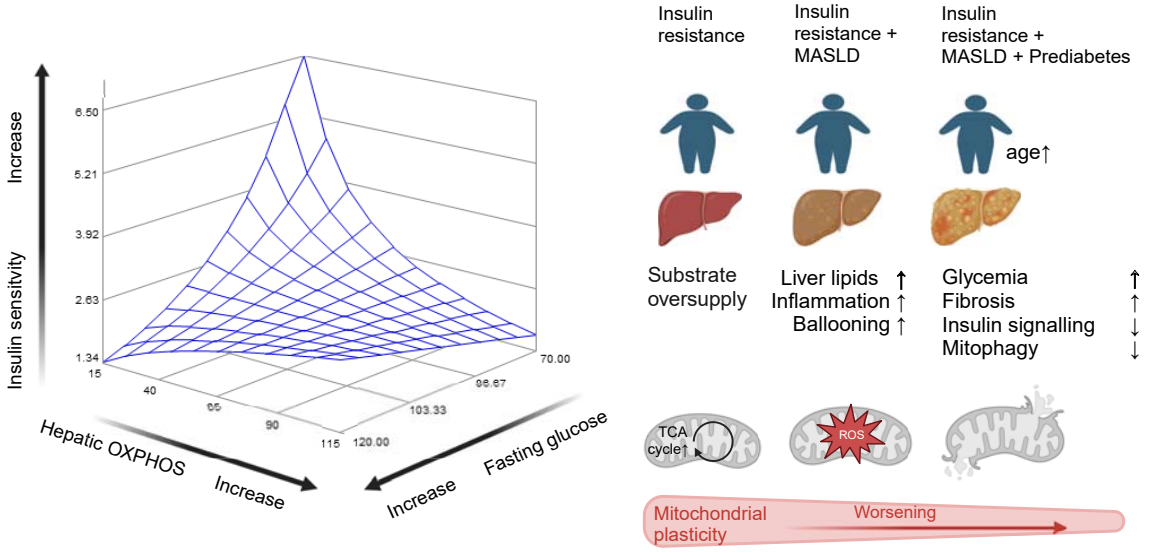
Graphical abstract



Highlights

- Glycemia and liver lipid content jointly affect the adaptation of hepatic oxidative capacity to insulin resistance.
- Prediabetes affects hepatic insulin signaling, mitochondrial dynamics and relates to fibrosis prevalence.
- Fasting plasma glucose predicts the decline of hepatic mitochondrial plasticity more robustly than 2-hour OGTT glucose.

Impact and implications

Mechanisms underlying the progression of metabolic dysfunction-associated steatotic liver disease (MASLD) are still unclear, but a better understanding of the pathogenesis of MASLD is essential for the development of targeted treatments. Adaptation of liver oxidative capacity was found to be impaired in people with diabetes and MASLD or liver fibrosis. Glycemia and liver lipid content affect the adaptation of hepatic oxidative capacity to insulin resistance in obesity. These results highlight the relevance of metabolically active drugs in individuals with grade 3 obesity and early MASLD.

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Research Article
MASLD and Alcohol-Related Liver Diseases

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Dysglycemia and liver lipid content determine the relationship of insulin resistance with hepatic OXPHOS capacity in obesity

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Background & Aims: Hepatic mitochondrial respiration is higher in steatosis, but lower in overt type 2 diabetes. We hypothesized that hepatic oxidative phosphorylation (OXPHOS) capacity increases with a greater degree of insulin resistance in obesity, independent of other metabolic diseases.

Methods: We analyzed 65 humans without diabetes (BMI 50 ± 7 kg/m^2, hemoglobin A1c 5.5 ± 0.4%) undergoing bariatric surgery. Metabolic dysfunction-associated steatotic liver disease (MASLD) stages were assessed by histology, whole-body insulin sensitivity (PREDIcted-M index) by oral glucose tolerance tests, and maximal ADP-stimulated mitochondrial OXPHOS capacity by high-resolution respirometry of liver samples.

Results: Prediabetes was present in 30 participants and MASLD in 46 participants, of whom 25 had metabolic dysfunction-associated steatohepatitis, and seven had F2–F3 fibrosis. While simple regression did not detect an association of insulin sensitivity with hepatic OXPHOS capacity, interaction analyses revealed that the regression coefficient of OXPHOS capacity depended on fasting plasma glucose (FPG) and liver lipid content. Interestingly, the respective slopes were negative for FPG ≤100 mg/dl, but positive for FPG >100 mg/dl. Liver lipid content displayed similar behavior, with a threshold value of 24%. Post-challenge glycemia affected the association between insulin sensitivity and OXPHOS capacity normalized for citrate synthase activity. Presence of prediabetes affected hepatic insulin signaling, mitochondrial dynamics and fibrosis prevalence, while the presence of MASLD was associated with increases in biomarkers of hepatic inflammation, cell damage and lipid peroxidation in people with normal glucose tolerance.

Conclusions: Increasing liver lipid contents and plasma glucose concentrations, even in the non-diabetic range, are associated with a progressive decline of hepatic mitochondrial adaptation in people with obesity and insulin resistance.

ClinTrials.gov identifier: NCT01477957.

Introduction

The continuously increasing prevalence of obesity causes a huge individual and societal burden, to which metabolic dysfunction-associated steatotic liver disease (MASLD; formerly nonalcoholic fatty liver disease (NAFLD)) is increasingly contributing.[1] Specifically, about 65% of people with type 2 diabetes not only have MASLD but are also at higher risk of accelerated progression to metabolic dysfunction-associated steatohepatitis (MASH) and fibrosis.[2,3] Over the last years, several drugs failed to hit their endpoints in phase III trials on MASH, partly due to the incomplete understanding of MASLD progression.[4] The first drug to receive accelerated approval by the FDA for the indication of MASH with fibrosis is the thyroid-hormone receptor agonist resmetirom,[5] which raised further interest in the role of hepatic energy metabolism in MASLD.

Insulin resistance represents the hallmark of both type 2 diabetes[6] and MASLD,[7] which, aside from genetic predisposition, results from energy overload worsening glycemia, lipidemia and intrahepatocellular lipid accumulation.[7,8] Up to a certain degree, the liver is able to compensate for this chronic energy overload by increasing its oxidative phosphorylation (OXPHOS) capacity (termed mitochondrial plasticity) at the expense of enhanced production of reactive oxygen species (ROS) and oxidative stress, promoting mitochondrial and

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cellular damage when antioxidant defense fails.[9] However, it is not fully understood which conditions trigger the break-even point, initiating the loss of the oversupply-driven hepatic mitochondrial plasticity.

Obesity-related steatohepatitis is associated with a decline of mitochondrial plasticity, which is further exacerbated by type 2 diabetes.[9] This could result from the OXPHOS-induced hepatocellular oxidative stress and altered mitochondrial turnover (mitophagy, mitochondrial fusion and fission),[10] but also from aggravating lipo- and glucotoxicity due to chronic nutrient overload,[6] adipose tissue dysfunction with lipid redistribution to the liver[7,8] and/or impaired insulin-dependent regulation of hepatic glucose and lipid metabolism involving protein kinase B (AKT) signaling, *i.e.* insulin resistance.[11]

Although insulin resistance generally not only precedes diabetes,[12] but also MASLD,[13] only scarce information exists about the relationship of insulin resistance with hepatic mitochondrial OXPHOS capacity during the early disease course. Hepatic OXPHOS capacity was negatively associated with steady-state whole-body insulin sensitivity (M-value) as assessed by the hyperinsulinemic-euglycemic clamp test,[14,15] but not when assessed by the HOMA-IR (homeostasis model assessment of insulin resistance), which reflects fasting whole-body (well correlated with hepatic) insulin sensitivity[16] in mixed populations of people with and without obesity or type 2 diabetes.[17] The PREDIcted-M index (PREDIM) is a validated, easier-to-perform measure of dynamic whole-body insulin sensitivity, which is derived from the oral glucose tolerance test (OGTT) and thereby better reflects physiologic conditions.[18] Thus, we investigated whether (i) whole-body insulin sensitivity (PREDIM) associates with hepatic mitochondrial OXPHOS capacity, (ii) glycemia and MASLD interact with this association and whether (iii) other metabolic factors such as circulating lipids, β-cell function or systemic low-grade inflammation impact this relationship in people with grade 3 obesity and MASLD but without overt diabetes.

Patients and methods

Study population

This study included 65 obese Caucasian individuals undergoing bariatric surgery from the BARIA_DDZ cohort (ClinTrials.gov identifier: NCT01477957), who were recruited between 2018 and 2023 and had a frequent-sampling OGTT as well as hepatic high-resolution respirometry (HRR) available (Fig. S1). Clinical characteristics and liver HRR measurements of some of the participants have been included in previous reports.[15,19] The BARIA_DDZ cohort study is being performed according to the Declaration of Helsinki (updated 2013 version). All participants were informed about the procedures and risks and provided their written consent to the protocol, approved by the ethics boards of Heinrich-Heine-University Düsseldorf and of the Medical Association North Rhine, both Germany (no. 2022-2021_1-andere Forschung erstvotierend/no. 2017222).

Metabolic characterization

Overnight-fasted participants arrived in the morning at the German Diabetes Center (DDZ) for a frequent-sampling OGTT, which comprised ingestion of 75-g dextrose solution (ACCU-CHECK® Dextro O.G.T.; Roche, Basel, Switzerland) with blood sampling at 30, 60, 90 and 120 min for measurements of glucose, insulin, C-peptide, non-esterified fatty acids (NEFAs) and triglycerides. Impaired fasting glucose (IFG; fasting plasma glucose (FPG) 100 to 125 mg/dl) and impaired glucose tolerance (IGT; 2-hour plasma glucose 140 to 199 mg/dl) were classified according to American Diabetes Association criteria.[20] Prediabetes was defined as either having isolated IFG (iIFG), isolated IGT (iIGT) or combined IFG/IGT. Within 6 weeks after the OGTT, participants underwent bariatric surgery, including an intra-surgical liver biopsy (median lag time between OGTT and surgery: 10 [6;14] days).

Liver biopsies

An experienced surgeon obtained the liver biopsies 30 min after the induction of anesthesia during bariatric surgery according to a standardized procedure.[14] About 20 mg of liver tissue was immediately transferred into ice-cold relaxing medium (BIOPS) for HRR, and remaining tissue was separately and immediately snap-frozen in liquid nitrogen and stored at -80 °C until further analyses.

Liver histology

Approximately 100 mg of fresh liver samples were fixed in 1% formaldehyde for histological examination. Histological analysis of liver tissue was performed by a blinded pathologist according to the NASH Clinical Research Network scoring system;[21] MASH was diagnosed when the steatosis grade was ≥1 and combined ballooning/lobular inflammation was present. Liver lipid content was assessed from histology by assessing the percentage of hepatocytes with lipid droplets.

Measurement of hepatic mitochondrial OXPHOS capacity

Ex vivo analysis of mitochondrial respiration was performed in permeabilized liver tissue using HRR (Oxygraph-2k, Oroboros, Innsbruck, Austria) with two different substrate-uncoupler-inhibitor (SUIT) protocols (fatty acid- (F-) NADH- (N-) and succinate- (S-) linked substrates (FNS-pathway)) as described.[14,15] Data are presented as mass-specific mitochondrial respiration (pmol O_2 s^{-1} mg^{-1} wet weight) and as mitochondrial-specific respiration ([pmol O_2 s^{-1} mg^{-1} wet weight/citrate synthase (CS) activity]; [pmol O_2 s^{-1} mg^{-1} wet weight/mitochondrial (mt) DNA]). For analyses, the results from the FNS-linked SUIT protocol ($[ETF+CI+II]_P$) were used to assess alterations in fatty acid oxidation-dependent respiration in metabolic disease, as reported previously.[22] Results from the additional NS-(glycolysis-)linked SUIT protocol in subgroups are indicated as "$[CI+II]_P$". For normalization to mitochondrial content,[9] we used CS activity and mtDNA as described before.[15]

Blood analyses

Venous whole-blood glucose concentrations were measured immediately using the glucose oxidase method (EKF Biosen C-Line glucose analyzer; EKF Diagnostics, Cardiff, Wales, UK).[23] If appropriate, glucose values were multiplied by factor 1.11 to obtain the plasma glucose equivalent. Serum metabolites, liver enzymes and high-sensitivity C-reactive protein (hsCRP) were measured on a Cobas C311 analyzer (Roche Diagnostics, Basel, Switzerland).[19] Plasma glycosylated hemoglobin A1c

(HbA1c) and serum insulin concentrations were measured as described before.[19,23]

Western blot analyses

Expression levels of proteins of interest were assessed as described.[19]

Statistical analysis

The power calculation was based on the primary aim of assessing the relationship between PREDIM and $[ETF+CI+II]_P$ in the whole cohort. A sample size of 65, $\alpha = 5\%$ and adjusting for three potential confounders (age, sex, and BMI), would allow for detection of a correlation (r) of ≥0.35 with a power of 80%. Further, with the sample size of this study and the observed effects, the *post hoc* determined power for detecting a significant interaction in the multivariable linear model including fixed effects (*e.g.*, $[ETF+CI+II]_P$, FPG) and age, sex and BMI as potential confounders was 0.64.

For participants' characteristics, normally and log-normally distributed data are presented as mean ± SD and median [IQR], as appropriate. Non-normally distributed parameters were ln-transformed before analyses.

Regression analyses adjusted for age, sex and BMI were used to identify associations between selected parameters in all participants as well as in subgroups with normal glucose tolerance (NGT) and prediabetes. Multiple linear regression with interaction terms was used to investigate the interplay of $[ETF+CI+II]_P$ and metabolic parameters (plasma glucose, HbA1c, triglycerides, NEFAs, hsCRP, liver lipid content, Adipo-IR, NAFLD activity score [NAS],[21] fibrosis, presence of MASH and IGI) with PREDIM. Cut-offs for inversion of the association between hepatic OXPHOS capacity and insulin sensitivity were calculated by an interaction model Y=a+b*X+c*Z+d*X*Z (equals Y = (a+b*X)+(c+d*X)*Z), reflecting a linear function in Z, with intercept and slope depending on X. The value of X where the slope prefix changes was identified by solving c+d*X = 0 (equals X=-c/d).

p values ≤0.05 were considered to indicate significant differences. As the power calculation based on the primary aim did not include an interaction term, all further analyses involving interaction terms were exploratory, and *p* values were not adjusted for multiple testing. Differences in parameters between subgroups were assessed by Wilcoxon's test or Fisher's exact test, as appropriate. All calculations were performed using SAS (Version 9.4; SAS Institute, Cary, NC, USA) and graphs were also generated using GraphPad Prism, Version 10.2.2 (GraphPad Software Inc., La Jolla, CA).

For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Results

Participants' characteristics

Out of 65 people without overt diabetes, 30 participants (46%) had prediabetes, comprising iIFG, iIGT and combined IFG/IGT (Table 1). Their concomitant medication is listed in Table S1. MASLD was diagnosed in 46 (71%) participants, of whom 25 had MASH. Clinically significant fibrosis (≥F2) was found in only seven participants (Table 2). Of the 46 participants with steatosis ≥1, four had two, 19 had three, 17 had four and six had all five cardiometabolic criteria of MASLD.

Regression analyses across the whole population

Neither simple unadjusted ($\beta = 0.0132$, $p = 0.9143$) nor age-, sex- and BMI-adjusted ($\beta = -0.055$, $p = 0.6067$, Table S2) regression analyses revealed an association between whole-body insulin sensitivity (PREDIM) and hepatic OXPHOS capacity ($[ETF+CI+II]_P$, FNS-pathway). Similar results were obtained after normalizing OXPHOS capacity to mitochondrial content using either CS activity or mtDNA (Tables S3 and S4).

Interaction analyses across the whole population

As an association of insulin sensitivity (PREDIM) and hepatic OXPHOS capacity may only hold true for specific metabolic conditions, we subsequently tested for interactions of glycemia, lipidemia, β-cell function, systemic low-grade inflammation and liver histological parameters with the above postulated association. These analyses revealed a complex association of insulin sensitivity with OXPHOS capacity when considering an interaction of OXPHOS capacity with FPG and liver lipid content (Tables 4 and 5 and Tables S5-9). While an inverse association was observed for FPG values ≤100.3 mg/dl, the relationship turned positive for values >100.3 mg/dl (Fig. 1A,B). Normalization for mitochondrial content had only a minor impact on the threshold value at which the prefix of slopes for given FPG levels changed (93.5 mg/dl using CS activity; 100.3 mg/dl using mtDNA) (Figs S2A,B and S3A,B). For liver lipid content, the slope was negative for values ≤24% and positive for values >24% (Fig. 1C,D). Normalization for mitochondrial content yielded thresholds at 11% and 20% using CS activity and mtDNA, respectively (Figs S2C,D and S3C,D). Neither BMI, circulating triglycerides, HDL-cholesterol, NEFAs nor hsCRP interfered with the association between insulin sensitivity and OXPHOS capacity (Table 3). Further testing the association of insulin sensitivity with OXPHOS capacity for an interaction with post-glucose load parameters revealed a significant interaction of 2-hour glucose with OXPHOS capacity upon normalization for CS activity (Table 3 and Table S10), with slopes being negative for ≤120 mg/dl and positive for >120 mg/dl (Fig. S2E,F). Of note, when combining FPG and liver lipid content in one model, we did not detect an independent interaction.

In the multivariate model testing both the impact of liver lipid content and 2-hour glucose on OXPHOS capacity, 2-hour glucose was independently associated with OXPHOS capacity normalized for CS activity only (Tables S11-14).

Clinical characteristics and hepatic OXPHOS capacity in subgroups differing in glycemia with or without MASLD

In the subgroups with NGT, those with MASLD (MASLD+) showed higher liver transaminases and insulin secretion but lower HDL-cholesterol than those without MASLD (MASLD-) (Tables S15-17). Compared to NGT, people with prediabetes were older and had a worse metabolic profile (higher insulin, triglycerides and hsCRP, but lower HDL-cholesterol). As expected, individuals with prediabetes had lower PREDIM, higher Adipo-IR and liver lipid content (Tables 1 and 3). Among those with prediabetes, individuals with iIFG were older and had

Table 1. Participants' characteristics.

	All	n	NGT	n	Prediabetes	n
N total (n males)	65 (8)		35 (7)		30 (1)	
iIFG/iIGT/IFG and IGT (n)	10/9/11		0/0/0		10/9/11	
Age (years)	40.2 ± 9.7	65	37.9 ± 8.6	35	**42.7 ± 10.4, *p* = 0.0489**	30
BMI (kg/m^2)	49.5 ± 7.2	65	48.8 ± 6.7	35	50.4 ± 7.7, *p* = 0.3909	30
HbA1c (%)	5.5 ± 0.4	64	5.3 ± 0.3	34	**5.6 ± 0.4, *p* = 0.0007**	30
Glucose (mg/dl)[§]	95.9 ± 10.9	65	89.0 ± 6.1	35	**104.0 ± 9.6, *p* <0.0001**	30
Insulin (μU/ml)[§]	16.9 [11.3–24.9]	60	13.4 [10.7–18.2]	33	**22.7 [14.8–29.1], *p* = 0.0026**	27
$iAUC_{Insulin\_0\text{-}120}$ (mU/ml)	8.3 [6.4–10.8]	65	7.1 [5.1–9.6]	35	**9.4 [7.8–13.9], *p* = 0.0492**	30
C-peptide (ng/ml)[§]	3.4 [2.6–4.4]	65	3.1 [2.4–3.8]	35	**3.7 [3.0–5.0], *p* = 0.0017**	30
$iAUC_{C\text{-}peptide\_0\text{-}120}$ (ng/ml)	846 [669–1,126]	65	771 [630–1,126]	35	954 [760–1,183], *p* = 0.0907	30
IGI_{INS} (A.U.)	1.4 [0.9–2.3]	64	1.8 [1.2–2.4]	34	1.2 [0.7–2.0], *p* = 0.1629	30
Disposition Index (A.U.)	27.2 [19.6–33.3]	65	28.7 [21.5–34.9]	35	22.4 [18.2–32.3], *p* = 0.2039	30
Adaptation Index (A.U.)	3.3 [2.6–3.9]	65	3.6 [3.2–4.3]	35	**2.8 [2.0–3.7], *p* = 0.0001**	30
Fasting hepatic insulin extraction (A.U.)	0.09 [0.07–0.11]	65	0.08 [0.07–0.11]	35	**0.10 [0.08–0.12], *p* = 0.0406**	30
Dynamic hepatic insulin extraction (%)	63.3 [55.0–68.7]	65	65.2 [55.0–69.4]	35	61.5 [54.3–66.7], *p* = 0.3054	30
Triglycerides (mg/dl)[§]	104 [80–157]	65	93 [69–133]	35	**138 [95–199], *p* = 0.0005**	30
HDL-cholesterol (mg/dl)	46.3 ± 11.9	65	45.1 ± 10.6	35	47.7 ± 13.4, *p* = 0.3958	30
NEFA (μmol/l)[§]	711 [547–844]	59	648 [483–868]	34	725 [631–794], *p* = 0.1065	25
PREDIM (mg/kg/min)	2.5 [1.9–3.0]	65	2.9 [2.4–3.4]	35	**1.9 [1.6–2.4], *p* <0.0001**	30
HOMA-IR (A.U.)	3.8 [2.6–6.2]	60	2.9 [2.3–4.3]	33	**5.7 [3.5–7.4], *p* = 0.0001**	27
Adipo-IR (A.U.)	10.7 [7.6–18.0]	54	9.3 [6.4–11.5]	32	**16.8 [10.8–25.8], *p* = 0.0008**	22
hsCRP (mg/dl)	0.8 [0.5–1.3]	65	0.6 [0.2–0.9]	35	**0.9 [0.6–1.6], *p* = 0.0020**	30

Adipo-IR, adipose tissue insulin resistance; A.U., arbitrary units; hsCRP, high-sensitivity C-reactive protein; iAUC, incremental area under the curve; IGI, insulinogenic index; iIFG, isolated impaired fasting glucose; iIGT, isolated impaired glucose tolerance; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; NEFA, non-esterified fatty acids; NGT, normal glucose tolerance; PREDIM, predicted-M (insulin sensitivity from the oral glucose tolerance test).
Data are shown as mean ± SD or median [IQR].
p values: NGT *vs.* prediabetes applying Wilxcoxon's test.
Values in bold denote statistical significance.
[§]Fasting values.

markedly lower plasma triglyceride and HDL-cholesterol than those with iIGT.

Hepatic OXPHOS capacity followed a specific pattern, with people with NGT/MASLD+ and iIGT presenting with the highest numerical OXPHOS capacity, NGT/MASLD– with intermediate, and iIFG as well as combined IFG/IGT with the lowest OXPHOS capacity, but without significant differences between the groups (NGT *vs.* iIGT: $p = 0.0915$, iIGT *vs.* combined IFG/IGT: $p = 0.0894$; Table S17).

Regression analyses in subgroups differing in glycemia with or without MASLD

Regression analyses revealed a direct association of insulin sensitivity with hepatic OXPHOS capacity only in people with both normal glucose and liver lipid metabolism, *i.e.* NGT/MASLD- (n = 14; $\beta = -0.54$, $p = 0.0096$), which remained robust upon adjusting for age, sex and BMI ($\beta = -0.49$, $p = 0.0182$). No such associations were found across NGT/MASLD- and NGT/MASLD+ combined ($p = 0.6657$) and those with prediabetes ($p = 0.3930$) (Fig. 2). Thus, these analyses indicate a major impact of combined glycemia and liver lipid content on the association of insulin sensitivity with hepatic OXPHOS capacity.

Impact of glycemia and liver lipid content on hepatic histology and mitochondrial dynamics

To further investigate how glycemia and liver lipid content may interfere with OXPHOS capacity, we performed regression

Table 2. MASLD staging of participants.

	All	NGT	Prediabetes	*p* values
MASLD [n/%]	46/71	21/60	25/83	*p* = 0.0903
MASH [n]	25	13	12	
Steatosis (0/1/2/3) [n]	19/25/15/6	14/14/6/1	5/11/9/5	*p* = 0.0606
Inflammation (0/1/2/3) [n]	15/34/14/2	7/21/6/1	8/13/8/1	*p* = 0.6208
Ballooning (0/1/2) [n]	38/13/14	21/7/7	17/6/7	*p* = 0.9425
Fibrosis (0/1/2/3/4) [n]	38/20/5/2/0	26/7/2/0/0	**12/13/3/2/0**	***p* = 0.0205**
NAS (0/1/2/3/4/5/6/7/8) [n]	8/12/10/10/13/6/5/1/0	6/7/5/5/8/3/1/0/0	2/5/5/5/5/3/4/1/0	*p* = 0.6561
BMI ≥ 25 kg/m^2 [n (%)]	65 (100)	35 (100)	30 (100)	
Fasting glucose ≥100 mg/dl or 2-hour OGTT levels ≥140 mg/dl or HbA1c ≥5.7% [n (%)]	16 (39)	0 (0)	14 (100)	
RR ≥130/85 or treatment [n (%)]	55 (84)*	28 (85)*	25 (83)	
Triglycerides ≥150 mg/dl or treatment [n (%)]	18 (28)	5 (14)	13 (43)	
HDL-cholesterol ≤40 mg/dl (m) or ≤50 mg/dl (f) [n (%)]	44 (65)	24 (69)	18 (60)	

HDL, high-density lipoprotein; MASLD, metabolic dysfunction-associated steatotic liver disease; MASH, metabolic dysfunction-associated steatohepatitis; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; RR, blood pressure (Riva-Rocci method).
p values NGT *vs.* prediabetes applying Fisher's exact test (only performed for histological parameters).
Data are shown as absolute (n) and relative numbers (%).
*n = 2 missing.

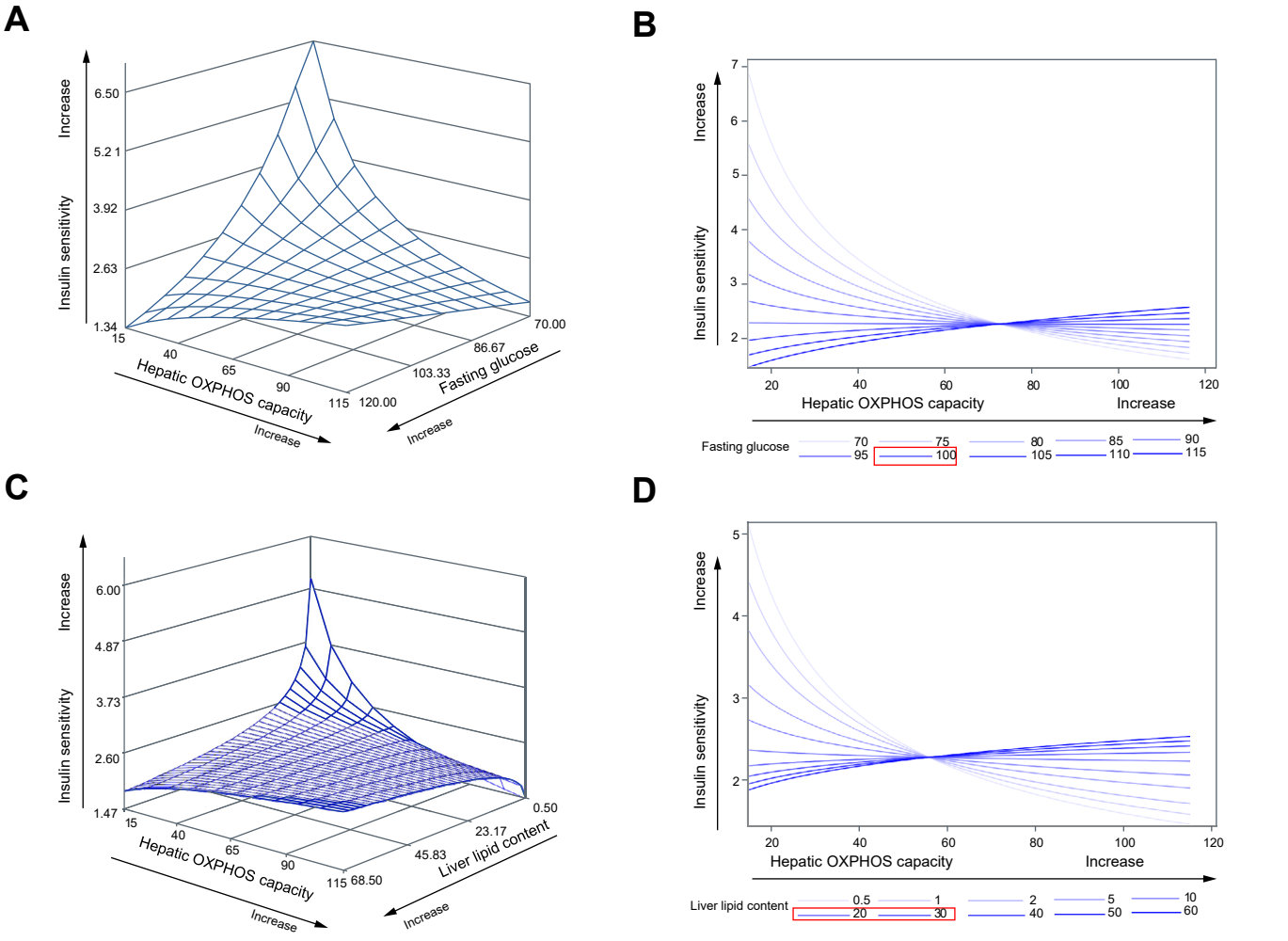

Fig. 1. Predicted regression surface and regression lines for the association of insulin sensitivity with hepatic OXPHOS capacity and fasting glucose or liver lipid content. Predicted regression surface for the association of insulin sensitivity with hepatic OXPHOS capacity ($[ETF+CI+II]_P$; given as O_2 flux in pmol*s^{-1}*mg wet weight^{-1}) and fasting glucose (mg/dl) (A) or liver lipid content (%) (C). Regression lines for given fasting glucose ((mg/dl); B) or liver lipid content (%); D) and association of insulin sensitivity with liver OXPHOS capacity. Figures are derived from multiple linear regression with interaction terms. For the graphical presentation, age and BMI were set to their mean values, and sex was set to female. OXPHOS, oxidative phosphorylation.

analyses to identify associations of fasting and 2-hour glucose levels as well as liver lipid content with hepatic histology and key proteins involved in mitochondrial dynamics (Table S19). Both fasting glucose and liver lipid content were positively related to fibrosis grade ($\beta = 0.0542$; $p = 0.0059$ and $\beta = 0.9013$, $p = 0.0026$) and liver lipid content was also positively related to hepatic inflammation and ballooning ($\beta = 0.6513$, $p = 0.0311$ and $\beta = 1.7279$, $p < 0.0001$). Further, fasting glucose showed a trend to a negative association with phospho-PINK1/PINK1 ($\beta = -0.0336$, $p = 0.0704$).

Table 3. Liver-related parameters in people with normal glucose tolerance and prediabetes.

Parameter	All (N = 65)	n	NGT (n = 35)	n	Prediabetes (n = 30)	n
Liver lipid content (%)	20 [2.5–40]	65	**10 [1–25]**	35	**30 [10–50], $p = 0.0048$**	30
Alanine aminotransferase (IU/L)	31 [21–39]	65	31 [18–39]	35	30 [21,46], $p = 0.5788$	30
Aspartate aminotransferase (IU/L)	23 [20–28]	65	23 [19–28]	35	23 [21–31], $p = 0.2867$	30
γ-glutamyl transferase (IU/L)	25 [17–35]	65	24 [16–33]	35	28 [17–41], $p = 0.2275$	30
$[ETF+CI+II]_P$ (pmol/s/mg wet weight)	38.4 [32.2–42.7]	65	39.4 [32.1–45.9]	35	37.2 [32.6–42.7], $p = 0.9740$	30
$[CI+II]_P$ (pmol/s/mg wet weight)	37.6 [26.3–44.6]	63	36.2 [25.5–46.1]	34	38.0 [29.1–42.0], $p = 0.6064$	29
iRCR (A.U.)	0.29 [0.21–0.35]	65	0.30 [0.21–0.33]	35	0.29 [0.24–0.38], $p = 0.1890$	30
PCR (A.U.)	0.62 [0.55–0.70]	58	0.62 [0.55–0.68]	30	0.62 [0.56–0.70], $p = 0.3248$	28
TBARS (pmol/mg protein)	398.9 [303–461]	56	415 [337–495]	34	368 [281–445], $p = 0.2838$	22

iRCR, inverse respiratory control ratio; NGT, normal glucose tolerance; PCR, phosphorylation control ratio; PRE, prediabetes. TBARS, thiobarbituric acid reactive substances. Data are shown as median [IQR].
p values: NGT *vs.* PRE applying Wilxcoxon's test.

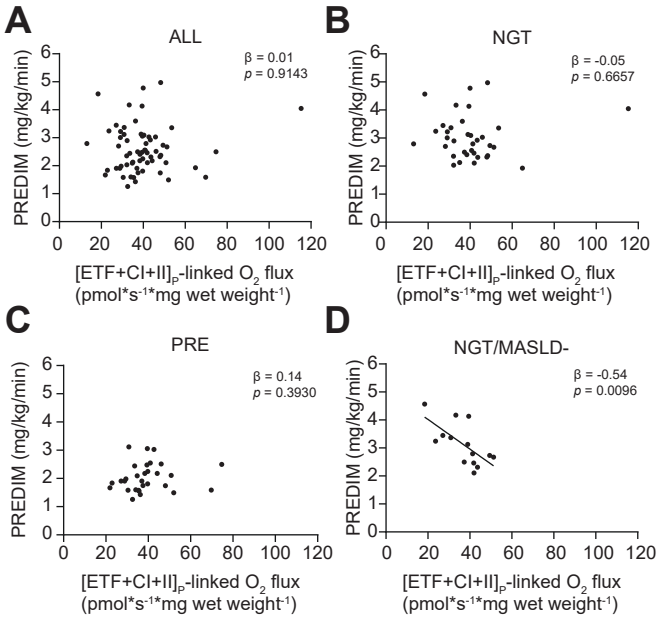

Fig. 2. Scatter plots depicting the relationship of whole-body insulin sensitivity (PREDIM) with hepatic OXPHOS capacity in different subgroups of glucose tolerance and MASLD status. (A) Whole cohort; (B) individuals with NGT; (C) individuals with PRE; (D) individuals with normal glucose tolerance without MASLD. $[ETF+CI+II]_P$, fatty acid- (F-) NADH- (N-) and succinate- (S-) linked maximal mitochondrial respiration in liver tissue. Beta coefficients and *p* values were derived from regression analysis. MASLD, metabolic dysfunction-associated steatotic liver disease; NGT, normal glucose tolerance; PRE, prediabetes; PREDIM, PREDIcted-M index.

Impact of presence of MASLD on hepatic histology and mitochondrial dynamics

To better understand the role of MASLD, independent of glycemia, on hepatic OXPHOS capacity, we compared the NGT/MASLD+ and NGT/MASLD-subgroups (Table S16). The NGT/MASLD + subgroup not only presented with hepatic steatosis, but also with greater inflammation and ballooning (Tables S16 and S17). This subgroup also showed higher liver TBARS (thiobarbituric acid reactive substances) and lower mtDNA levels, but no differences in hepatic FNS- and NS-dependent maximum OXPHOS capacity, respiratory control and coupling efficiency, nor levels of proteins involved in hepatic insulin signaling and mitochondrial dynamics (Table S17).

Impact of prediabetes on hepatic histology and mitochondrial dynamics

Finally, we compared liver histology and expression levels of the aforementioned proteins in the group with NGT to the group with prediabetes (Tables 1,2 and 5). In NGT and prediabetes groups, 21 (60%) and 25 out of 30 (83%) had MASLD, of whom 13 (62%) and 12 (48%) had MASH, respectively. The frequency of any fibrosis (≥F1), but not inflammation or ballooning, was higher in participants with prediabetes (Table 2). Prediabetes related to higher liver lipid content, whereas hepatic FNS- and NS-OXPHOS capacity and biomarkers of mitochondrial content did not differ between the groups (Table 5). In line with hepatic insulin resistance, prediabetes was associated with lower hepatic AKT phosphorylation (phospho-AKT(Ser732)/AKT2) and PINK1 phosphorylation (phospho-PINK1(Thr257)/PINK1) than NGT (Fig. 3; Table S18). Testing for a differential impact of iIGT and iIFG revealed a higher frequency of any fibrosis ≥F1, but no differences in expression levels of proteins involved in hepatic mitochondrial dynamics (Tables S16 and S17).

Discussion

This study found that the association of whole-body insulin sensitivity with hepatic mitochondrial OXPHOS capacity depends mostly on an interaction of OXPHOS capacity with fasting glycemia and liver lipid content in people with grade 3 obesity, even in the absence of diabetes mellitus. Only metabolically healthy people (no glucose intolerance, no MASLD) with grade 3 obesity exhibited a negative linear relationship between whole-body insulin sensitivity and hepatic mitochondrial OXPHOS capacity. This indicates that any decrease in

Table 4. Interaction tests of hepatic OXPHOS capacity ($[ETF+CI+II]_P$) with metabolic parameters on modeling insulin sensitivity (PREDIM) adjusted for age, sex and BMI.

	$[ETF+CI+II]_P$	$[ETF+CI+II]_P$/CS activity	$[ETF+CI+CII]_P$/mtDNA
Parameter	**β coefficient and *p* value**		
BMI	-0.0124, *p* = 0.4348	-0.0121, *p* = 0.2012	0.00154, *p* = 0.2012
Plasma glucose§	**1.9268, *p* = 0.0224**	**1.3410, *p* = 0.0280**	**2.0576, *p* = 0.0127**
2-hour plasma glucose	0.2153, *p* = 0.4711	**0.6222, *p* = 0.0057**	0.2036, *p* = 0.4813
HbA1c	0.2184, *p* = 0.5013	0.1595, *p* = 0.3507	0.1868, *p* = 0.4890
Liver lipid content	**0.1579, *p* = 0.030**	**0.0983, *p* = 0.0201**	**0.1485, *p* = 0.0461**
Fibrosis grade	0.0900, *p* = 0.6447	0.1651, *p* = 0.1852	0.2623, *p* = 0.1658
MASH	-0.2371, *p* = 0.2968	-0.2543, *p* = 0.0958	-0.2738, *p* = 0.2045
NAS	0.0794, *p* = 0.2047	0.0627, *p* = 0.0896	0.1074, *p* = 0.0905
Adipo-IR	0.0055, *p* = 0.9564	0.1155, *p* = 0.1914	-0.0092, *p* = 0.9278
NEFA§	0.0692, *p* = 0.8786	0.0606, *p* = 0.8529	-0.1062, *p* = 0.5769
Triglycerides§	0.2741, *p* = 0.1884	0.1956, *p* = 0.1363	0.1441, *p* = 0.5058
HDL-cholesterol§	-0.0005, *p* = 0.9597	0.0068, *p* = 0.3361	-0.0009, *p* = 0.8705
$IGI_{Insulin\_0\text{-}30}$	-0.0828, *p* = 0.5283	-0.1061, *p* = 0.3023	-0.0657, *p* = 0.4810
hsCRP	-0.0132, *p* = 0.9125	-0.0315, *p* = 0.7103	-0.0661, *p* = 0.2077

Adipo-IR, adipose tissue insulin resistance; CS, citrate synthase; $[ETF+CI+II]_P$, maximal oxidative phosphorylation (OXPHOS) capacity with convergent electron input through ETF, CI, and CII combined; HDL, high-density lipoprotein; hsCRP, high sensitive C-reactive protein; MASH, metabolic dysfunction-associated steatohepatitis; mtDNA, mitochondrial DNA; NAS, NAFLD activity score; NEFA, non-esterifed fatty acids.
Multiple linear regression with interaction terms.
§Fasting values.

Table 5. Interaction model of insulin sensitivity (log_e(PREDIM)) with hepatic OXPHOS capacity ($[ETF+CI+II]_P$) and fasting glucose adjusted for age, sex and BMI.

Effect	Estimate	Standard error	*p* value
Intercept	**39.8832**	**13.6077**	**0.0048**
$[ETF+CI+II]_P$ (log_e)	-8.8787	3.7329	0.0207
FPG (log_e)	**-8.2347**	**2.9849**	**0.0077**
$[ETF+CI+II]_P$ (log_e) * FPG (log_e)	**1.9268**	**0.8211**	**0.0224**
Age	0.0003	0.0031	0.9164
Sex=female	-0.1008	0.0951	0.2934
BMI	**-0.0205**	**0.0097**	**<0.0001**

$[ETF+CI+II]_P$, maximal oxidative phosphorylation (OXPHOS) with convergent electron input through ETF, CI, and CII combined; FPG, fasting plasma glucose.
Multiple linear regression with interaction terms.
Values in bold denote statistical significance.

insulin sensitivity results in the upregulation of hepatic oxidative capacity supporting the concept of mitochondrial plasticity in these metabolically healthy individuals. Interestingly, the presence of prediabetes, but not of MASLD, was associated with impaired hepatic insulin signaling (lower AKT2 phosphorylation) and mitophagy (lower PINK1 phosphorylation) and a higher frequency of hepatic fibrosis. In the context of the differential relationship of the individual diagnostic criteria of MASLD with insulin resistance,[24] the present study further underlines a specific role of dysglycemia in dissipation of the physiologic relationship between insulin sensitivity and hepatic energy metabolism in obesity, occurring early during MASLD.

Although most previous studies provided evidence for altered hepatic mitochondrial function using different methods in insulin-resistant states such as obesity and diabetes mellitus,[14,17,25–28] analyses of a possible relationship between mitochondrial respiration and insulin sensitivity revealed conflicting results.[14,16,17] The observation of lower hepatic mitochondrial respiration in both type 1 and type 2 diabetes[15,26,27,29] already suggested a role for hyperglycemia in MASLD pathogenesis. In the present study, fasting glycemia and liver lipid content jointly, but not independently, interacted with OXPHOS capacity in the multivariate model, indicating that both mechanisms of gluco- and lipotoxicity contribute to insulin resistance as well as to impaired mitochondrial plasticity in people with grade 3 obesity. Nevertheless, these results imply that early dysglycemia – in the absence of overt diabetes – already affects mitochondrial plasticity.

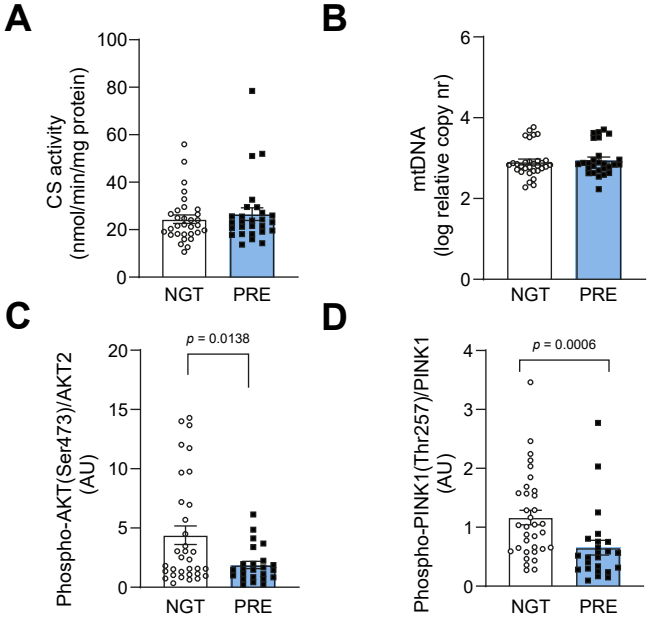

Fig. 3. Comparison of mitochondrial contents and phosphorylation of AKT2 and PINK1 in individuals with NGT and PRE. (A-D) CS activity (A), mtDNA copy number (B), ratios of phosphorylated to unphosphorylated protein levels for AKT2 (C) and PINK1 (D) in individuals with NGT and PRE. *p* values: NGT *vs.* PRE applying Wilxcoxon's test. AKT, protein kinase B; CS, citrate synthase; mtDNA, mitochondrial DNA; NGT, normal glucose tolerance; PINK1, PTEN-induced kinase 1; PRE, prediabetes.

While hyperglycemia generally results from hepatic insulin resistance through impaired suppression of hepatic glucose production,[30] prolonged mild dysglycemia can itself induce hepatic insulin resistance in glucose-tolerant humans.[31] In line, our prediabetes group presented with impaired hepatic insulin signaling via lower AKT2 phosphorylation along with a higher prevalence of fibrosis. Of note, all three AKT isoforms can also regulate mitochondrial oxidation, as recently reported in human hepatocellular carcinoma cells.[32] These findings extend the concept of glucotoxicity operating in type 2 diabetes with progressing MASLD[33,34] to individuals with prediabetes. Several cellular mechanisms have been implicated[35] such as the hexosamine pathway, reactive dicarbonyls and/or advanced glycation endproducts.[31,36] In a similar cohort, we found that hepatic methylglyoxal-derived hydroimidazolone isomer 1 correlated positively with fasting glycemia, while hepatic carboxymethyl-lysine correlated negatively with hepatic OXPHOS capacity.[15]

The present study also detected lower PINK1 phosphorylation in the prediabetes subgroup, likely reflecting decreased mitophagy, which has previously been suggested for overt hyperglycemic states.[37] In line, fasting glucose showed a trend towards an association with lower PINK1 phosphorylation and more pronounced hepatic inflammation in the regression analyses, further underscoring the contention that fasting glucose might relate to liver injury in obesity grade 3.

Finally, 2-hour glucose emerged as an interacting factor on hepatic OXPHOS capacity upon normalization for CS activity. While increased 2-hour glucose, *i.e.* impaired glucose tolerance, results more from extrahepatic insulin resistance,[38] fasting hyperglycemia is more closely related to hepatic insulin resistance.[39] Nevertheless, similar to previous studies, fasting and 2-hour glucose were well associated in our study, indicating that 2-hour glucose may represent another, albeit less sensitive, marker of the loss of hepatic mitochondrial plasticity. Of note, as HbA1c reflects overall glycemia[40] it

cannot differentiate between fasting and maximal postprandial glycemia, which might explain why it was not an interacting factor with hepatic OXPHOS capacity.

In addition to dysglycemia, the present study identified liver lipid content as a modulator of the association between whole-body insulin sensitivity and hepatic mitochondrial OXPHOS capacity. Liver lipid content unlikely plays an active role *per se*, but rather reflects other lipotoxic processes.[41] Although the contribution of *de novo* lipogenesis to hepatic triglyceride synthesis increases in obesity, the majority of the liver triglyceride pool arises from the redistribution of NEFAs and glycerol from insulin-resistant adipose tissue.[42] Within the liver, lipid intermediates such as diacyglycerols and ceramides promote hepatic insulin resistance, pro-inflammatory processes, oxidative stress and thereby liver disease.[6,43] In this context, we have previously detected that certain sphingolipids are not only increased in MASH, but also associated with hepatic oxidative capacity and stress.[44] Likewise, the NGT/MASLD+ group of the present study featured higher hepatic TBARS levels, reflecting greater lipid peroxidation, along with greater steatosis, inflammation and ballooning when compared to the NGT/MASLD- subgroup. The sustained importance of adipose tissue dysfunction is illustrated by the continuous worsening of Adipo-IR with increasing liver lipid contents and worsening of hepatic fibrosis in people with type 2 diabetes and MASLD.[41,45] The present study also found greater Adipo-IR and fibrosis grade in prediabetes than NGT, whereas neither Adipo-IR nor fibrosis grade differed between NGT/MASLD- and NGT/MASLD+. This does not downplay the relevance of Adipo-IR for MASLD, as the lack of an interaction of Adipo-IR with hepatic OXPHOS capacity may simply suggest that this parameter does not reflect the liver's ability to adapt to the excessive NEFA supply. Of note, Adipo-IR does not exploit the full insulin concentration-response curve and might be further affected by a lower hepatic insulin clearance with consecutively greater peripheral hyperinsulinemia as reported for recent-onset diabetes.[46]

This study benefits from the state-of-the-art methods used for direct assessment of hepatic OXPHOS capacity and physiologic insulin sensitivity in humans, as well as from the analysis of hepatic expression of proteins involved in insulin signaling and mitochondrial dynamics to gain mechanistic insights. Of note, the lower number of males and the absence of lean people do not allow for extrapolation to the general population. While BMI can negatively affect hepatic ATP recovery,[47] regression analyses were adjusted for all major confounders (age, sex and BMI), and BMI did not seem to be an independent interacting factor with hepatic OXPHOS capacity on the association with PREDIM in our cohort. Further, people with long-standing type 2 diabetes and/or fibrosis grade ≥F2 were not included to enabled investigations of the early changes of hepatic mitochondrial plasticity independently of drug treatment or progressive metabolic deterioration. As this cross-sectional study was powered to detect an association of PREDIM with hepatic OXPHOS capacity, analyses of interacting factors and of different subgroups must be regarded as exploratory and do not demonstrate causality. Further, the effects of the 2-week low-calorie diet before and the anesthesia during bariatric surgery on hepatic OXPHOS capacity cannot be excluded but should have identically affected all participants undergoing these standardized procedures. Finally, in the absence of a uniform gold standard for normalizing OXPHOS capacity to mitochondrial content, we applied two independent recommended surrogates, enabling comparison with previous studies.[9]

In obesity grade 3, whole-body insulin sensitivity is associated with hepatic maximum coupled mitochondrial OXPHOS capacity, only when fasting glycemia and liver lipid content are considered. Surprisingly, already small increases in plasma glucose correlate with early deterioration of hepatic mitochondrial plasticity under conditions of obesity, insulin resistance and MASLD, which may be even more relevant than the prevailing hyperlipidemia. Our data might help to explain why type 2 diabetes can contribute to MASLD progression and why metabolic drug concepts may be specifically efficient for the early treatment of MASLD, independently of the presence of overt diabetes.

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Abbreviations

CS, citrate synthase; FPG, fasting plasma glucose; HbA1c, hemoglobin A1c; HRR, high-resolution respirometry; (i)IFG, (isolated) impaired fasting glucose; iIGT, (isolated) impaired glucose tolerance; MASH, metabolic dysfunction-associated steatohepatitis; MASLD, metabolic dysfunction-associated steatotic liver disease; NEFAs, non-esterified fatty acids; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; OXPHOS, oxidative phosphorylation; PREDIM, PREDIcted-M index; ROS, reactive oxygen species; SUIT, substrate-uncoupler-inhibitor.

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Conflicts of interests

M.R. received lecture fees or served on advisory boards for AstraZeneca, Echosens, Eli Lilly, Madrigal, Merck-MSD, Novo Nordisk and Target RWE and performed investigator-initiated research with support from Boehringer Ingelheim, Novo Nordisk and Nutricia/Danone to the German Diabetes Center (DDZ). No conflicts of interest, financial or otherwise, are declared by the other authors.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M.R. and S.K. designed the study. S.K. and G.P. analyzed the results. S.K. drafted the manuscript. S.K., N.S., K.P., L.M., B.D., T.S., S.T., I.E., M.S., and F.A.G. contributed to the acquisition and analysis of data. K.S. provided statistical support. S.K., K.S., G.P., N.S., K.P., L.M., B.D., T.S., S.T., I.E., M.S, F.A.G., and M.R. revised and approved the final version of the manuscript. M.R. is the guarantor of this work, with full access to all data and analyses related to the content of this article.

Data availability statement

All data reported in this manuscript will be shared by the lead contact upon reasonable request. This manuscript does not report the original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

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Supplementary data

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Author names in bold designate shared co-first authorship

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Amino Acid and Fatty Acid Levels Affect Hepatic Phosphorus Metabolite Content in Metabolically Healthy Humans

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Objective: Hepatic energy metabolism negatively relates to insulin resistance and liver fat content in patients with type 2 diabetes, but its role in metabolically healthy humans is unclear. We hypothesized that intrahepatocellular γ-adenosine triphosphate (γATP) and inorganic phosphate (P_i) concentrations exhibit similar associations with insulin sensitivity in nondiabetic, nonobese volunteers.

Design: A total of 76 participants underwent a four-point sampling, 75-g oral glucose tolerance test (OGTT), as well as *in vivo* $^{31}P/^{1}H$ magnetic resonance spectroscopy. In 62 of them, targeted plasma metabolomic profiling was performed. Pearson correlation analyses were performed for the dependent variables γATP and P_i.

Results: Adjusted for age, sex, and body mass index (BMI), hepatic γATP and P_i related to 2-hour OGTT glucose ($r = 0.25$ and $r = 0.27$, both $P < 0.05$), and P_i further associated with nonesterified fatty acids (NEFAs; $r = 0.28$, $P < 0.05$). However, neither γATP nor P_i correlated with several measures of insulin sensitivity. Hepatic γATP correlated with circulating leucine ($r = 0.42$, $P < 0.001$) and P_i with C16:1 fatty acids palmitoleic acid and C16:1w5 ($r = 0.28$ and 0.30, respectively, $P < 0.01$), as well as with δ-9-desaturase index ($r = 0.33$, $P < 0.05$). Only the association of γATP with leucine remained important after correction for multiple testing. Leucine and palmitoleic acid, together with age, sex, and BMI, accounted for 26% and for 15% of the variabilities in γATP and P_i, respectively.

Conclusions: Specific circulating amino acids and NEFAs, but not measures of insulin sensitivity, partly affect hepatic phosphorus metabolites, suggesting mutual interaction between hepatic energy metabolism and circulating metabolites in nondiabetic humans. ***(J Clin Endocrinol Metab* 103: 460–468, 2018)**

Hepatic energy metabolism is altered in insulin-resistant and/or type 2 diabetes (T2D) patients with nonalcoholic fatty liver disease (NAFLD) (1, 2). Patients with T2D present with lower hepatic adenosine triphosphate (ATP), inorganic phosphate (P_i) concentrations, and ATP synthase flux (2, 3). On the other hand,

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Abbreviations: Adipo-IR, adipose tissue insulin resistance; ATP, adenosine triphosphate; au, arbitrary unit(s); AUC, area under the curve; BCAA, branched-chain amino acid; BMI, body mass index; D9D, δ-9-desaturase; DNL, *de novo* lipogenesis; HCL, hepatocellular lipid content; MRS, magnet resonance spectroscopy; NAFLD, nonalcoholic fatty liver disease; NEFA, nonesterified fatty acid; OGIS, oral glucose insulin-sensitivity index; OGTT, oral glucose tolerance test; P_i, inorganic phosphate; QUICKI, quantitative insulin-sensitivity check index; T2D, type 2 diabetes; TCA, tricarboxylic acid; γATP, γ-adenosine triphosphate.

obese individuals without nonalcoholic steatohepatitis exhibit increased hepatic oxidative capacity (1). These conflicting results may result from adaptive upregulation of oxidative phosphorylation in obesity (1), along with enhanced production of reactive oxygen species. Increased reactive oxygen species production, which is also linked to hyperglycemia and hyperlipidemia, can induce mitochondrial damage and thereby, contribute to lower mitochondrial function in T2D and nonalcoholic steatohepatitis (1, 3, 4).

In exercising skeletal muscle, ATP production is mainly regulated by training intensity, whereas in the liver, ATP-consuming metabolic processes challenge hepatic mitochondria (5, 6). Several factors, such as body mass index (BMI), glycemia, and hepatic insulin sensitivity (1, 2, 7), affect skeletal muscle and hepatic phosphorus metabolites in metabolic diseases, but there are no data on the factors regulating energy metabolism in nonobese nondiabetic persons. Cellular metabolic processes require substrate supply and signaling, particularly by branched-chain amino acids (BCAAs) (8, 9) and nonesterified fatty acids (NEFAs) (10). Although BCAAs have been suggested to improve energy metabolism (8), BCAAs are also increased in insulin-resistant states, such as obesity and T2D, and can induce insulin resistance (8, 9). In metabolic diseases, impaired BCAA metabolism could lead to accumulation of toxic BCAA metabolites, which would, in turn, impair mitochondrial function and give rise to stress signaling associated with insulin resistance (8). Likewise, chronic increases in NEFA can also induce insulin resistance (4) and have been linked to abnormal mitochondrial function (4). Although BCAA and NEFA likely reflect the risk for metabolic diseases (4, 8), their physiological roles for hepatic phosphorus metabolism remain unclear.

We have previously developed noninvasive ^{31}P magnet resonance spectroscopy (MRS) techniques, allowing for exact quantification of hepatic ^{31}P metabolite concentrations in a clinical setting (11, 12). With the use of these methods, we now aimed at identifying metabolic determinants of hepatic γ-ATP (γATP) and P_i under physiological conditions in a cohort of middle-aged metabolically healthy volunteers.

Participants and Methods

Study population

The study was performed as a feasibility study in the context of the national cohort; its protocol is in line with the Declaration of Helsinki (version 2008) and approved by the Ethics Board of the Bavarian Medical Association and of the Heinrich-Heine University Düsseldorf. All participants gave their written, informed consent before inclusion into the study. From the general population with residency in the Düsseldorf region, 496 persons, aged 20 to 70 years, were recruited from July 2011 to December 2012 by advertisement or via local residents' registration office (Supplemental Fig. S1). Of those, 270 persons without known diabetes mellitus underwent an extended examination, including standardized interview, anthropometry, and blood sampling, after 10 hours fast, as well as a 75-g oral glucose tolerance test (OGTT). Magnetic resonance imaging and MRS were performed in 120 of these individuals. Parts of these data have already been published (11, 13).

Clinical examination

Anthropometric data were measured, as reported previously (14).

OGTT

A standardized 75-g OGTT (ACCU-CHECK® Dextro O.G.T.; Roche, Basel, Switzerland) was performed after 10 hours overnight fasting with blood sampling, before and 30, 60, and 120 minutes after start. Dysglycemia was classified according to criteria of the American Diabetes Association.

Laboratory measurements

Measurements of glucose, alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, high-density lipoprotein-cholesterol, low-density lipoprotein-cholesterol, triglyceride, insulin, and C-peptide were performed, as reported previously (14). Hemoglobin A1c was determined on a VARIANT-II™ Analyzer (Bio-Rad Laboratories, Munich, Germany). Plasma NEFAs were determined using the NEFA-HR kit (Wako Chemicals, Neuss, Germany).

Metabolome analyses

Sodium-heparinate plasma samples were rapidly frozen and stored at −80°C. Targeted metabolic profiling of 294 blood metabolites was performed with the X MetaDis/DQTM kit at Biocrates Life Sciences (Innsbruck, Austria) (13). Fatty acid C18:0 was excluded from further analyses because of analytical errors. BCAAs were defined by the sum of isoleucine, leucine, and valine and δ-9-desaturase (D9D) index by the ratio of palmitoleic and palmitic acid (13).

Magnetic resonance imaging and MRS

All examinations were performed on a clinical 3-Tesla whole-body magnet (X-series Achieva; Philips, Best, The Netherlands), equipped with a 16-channel Torso XL phased-array receiver coil for ^{1}H MRS and a 14-cm circular ^{31}P surface coil (transmit-receive coil) using standardized procedures, as described (11). Liver triglyceride concentration [hepatocellular lipid content (HCL)] was assessed by ^{1}H MRS using the single voxel-stimulated echo acquisition mode (14); fat fraction was calculated as percentage of the ratio of lipids/(water + lipids), as described (11). For liver ^{31}P MRS, scout images were taken with the ^{1}H body coil in three orientations, followed by transverse T2-weighted images with multislice two-dimensional spin echo images and respiratory triggering to identify liver position clearly and place the volume of interest ($6 \times 6 \times 6$ cm) within the liver, avoiding skeletal muscle. Localized liver spectra were obtained using image-selected spectroscopy, with specifications, and analyzed as described before (11).

Measures of insulin sensitivity

From glucose, insulin, and C-peptide values, obtained during the OGTT, the following indices have been calculated for insulin sensitivity: oral glucose insulin-sensitivity index (OGIS), representing total glucose clearance or whole-body dynamic insulin sensitivity (15), and quantitative insulin-sensitivity check index (QUICKI) to assess fasting (hepatic) insulin sensitivity, calculated as 1/[log(G0) + log(I0)], where G0 and I0 are fasting glucose and insulin, respectively (16). The hepatic insulin resistance index (17) was calculated as the following: glucose area under the curve $(AUC)_{Gluc0\text{-}30}$ (g/dL) × $AUC_{I0\text{-}30}$ (U/dL) × min^2. Liver insulin-resistance index (relationship between insulin sensitivity and cardiovascular disease) (18) was calculated as the following: −0.091 + [log $AUC_{I0\text{-}120}$ (pmol/L) × 0.400] + [log fat mass (%) × 0.346] – [log high-density lipoprotein-cholesterol (mg/dL) × 0.408] + [log BMI (kg/m^2) × 0.435]. The adipose tissue insulin resistance (Adipo-IR) index was calculated as the following: fasting NEFA (mmol/L) × I0 (pmol/L) (19). *De novo* lipogenesis (DNL) was calculated as reported previously (13).

Statistical analyses

Normally distributed parameters are presented as means ± standard deviation and skewed distributed parameters as median (interquartile range). Pearson correlation analyses were performed for the dependent variables γATP and P_i. To account for potential confounders, such as age, sex, and BMI, partial (adjusted) Pearson correlations were calculated also. To improve normality, skewed distributed data were natural log transformed before analyses, as indicated (see Tables 1, 2, and 3). $P < 0.05$ was considered to indicate statistically significant differences. The main analyses (associations between hepatic γATP or P_i with markers of insulin resistance/amino acids/fatty acids) were adjusted for multiple testing using the Bonferroni method, as indicated in the respective table footnotes. Missing data were excluded from the analyses. No imputation methods were performed. Analyses were done using SAS Version 9.4 (SAS Institute, Cary, NC).

Results

Study population

Of 120 volunteers undergoing OGTT and MRS examinations, 76 entered the final analysis after exclusion of participants with missing measures and/or metabolic disease (Supplemental Fig. S1). Participants (26 men, 50 women) were middle aged (54 ± 13 years), nonobese (BMI 24.8 ± 3.2 kg/m^2), insulin sensitive (OGIS 461.2 ± 61.2 arbitrary units (au), QUICKI 0.46 [0.43;0.50]), and had low HCL (1.00 [0.41;2.76]%) and transaminases (alanine aminotransferase 18 [15;24] U/L, aspartate aminotransferase 23 [20;27] U/L). Fasting and 2-hour OGTT glucose were 4.2 ± 0.5 and 5.0 ± 1.3 mmol/L, respectively. Fasting NEFA and insulin were 516 [404; 714] and 42.6 [30.3;62.0] pmol/L, respectively. Hepatic γATP, P_i concentrations, and the Pi/γATP ratio were 2.73 ± 0.54, 2.02 ± 0.49, and 0.75 ± 0.18 mmol/L, respectively. Absolute concentrations of the measured amino acids and lipid metabolites are shown in Supplemental Tables S1 and S2.

Table 1. Correlation of Hepatic Phosphorus Metabolites With Clinical Parameters and Insulin Sensitivity Indices

	Unadjusted		Adjusted for Age, Sex, and BMI		Adjusted for Age, Sex, BMI, and P_i or γATP	
	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L
	Pearson Correlation Coefficient					
BMI, kg/m^2	0.11	0.07				
HCL, % (ln)	0.10	0.04	0.08	0.03	0.07	−0.007
NEFA, μmol/L (ln)	0.19	0.28[a]	0.18	0.28[a]	0.08	0.22
TG, mg/dL (ln)	0.10	0.03	0.07	0.009	0.07	−0.02
Fasting glucose, mg/dL	0.07	0.05	0.08	0.05	0.06	0.03
2-h Glucose, mg/dL	0.33[b]	0.33[b]	0.25[a]	0.27[a]	0.15	0.19
Fasting insulin, mU/L (ln)	−0.13	−0.12	−0.17	−0.14	−0.12	−0.08
HbA1c, %	0.19	0.06	0.12	−0.007	0.13	−0.06
OGIS, au	−0.10	−0.08	−0.002	−0.02	0.007	−0.02
QUICKI (ln)	0.11	0.09	0.15	0.12	0.11	0.06
Hepatic-IR index	−0.04	−0.12	−0.08	−0.14	−0.02	−0.12
RISC	0.11	−0.03	0.03	−0.06	0.05	−0.07
Adipo-IR index	0.03	0.08	0.003	0.06	−0.02	0.06
DNL index	−0.13	−0.008	−0.09	0.06	−0.13	0.11

Pearson and partial Pearson correlations of γATP and P_i with the respective variable are given. The Bonferroni-adjusted significance level is 0.0018 [0.05/(14 metabolic parameters × 2 hepatic phosphorus metabolites)]. After Bonferroni adjustment for multiple comparisons, none of the observed correlations remained statistically significant.

Abbreviations: HbA1c, hemoglobin A1c; ln, natural log transformed; RISC, relationship between insulin sensitivity and cardiovascular disease; TG, triglyceride.

[a] $P < 0.05$.

[b] $P < 0.01$.

Correlation analyses for hepatic phosphorus metabolites with glycemia and insulin sensitivity

Pearson correlation analyses revealed positive relationships of hepatic γATP concentrations with 2-hour OGTT glucose and of P_i with 2-hour OGTT glucose and NEFA, respectively (Fig. 1A–1D; Table 1). After adjustment for age, sex, and BMI, 2-hour OGTT glucose still related to both γATP and P_i content and NEFA levels to P_i concentrations. The adjustment of the analyses for P_i or γATP abolished these associations, pointing to the impact of the concentration of the respective other phosphorus metabolite on these relationships (Table 1). No associations with γATP or P_i content were found for any calculated measure of insulin sensitivity in unadjusted and adjusted analyses (Table 1). No association remained statistically significant after correction for multiple testing.

Correlation analyses for hepatic phosphorus metabolites with amino acids

Hepatic γATP was positively associated with plasma leucine (Fig. 1E) and ornithine. Hepatic P_i related negatively to threonine (Table 2). After adjustment for age, sex, and BMI, γATP still positively correlated with leucine and ornithine. Moreover, γATP associated with isoleucine, tryptophan, and BCAAs. The association of P_i with threonine disappeared after adjustment for age, sex, and BMI (Table 2). After additional adjustment for P_i, the correlation of γATP with isoleucine, leucine, ornithine, tryptophane, and BCAA remained, whereas additional correlations of γATP with tyrosine and phenylalanine were found (Table 2). After correction for multiple testing, only the association between γATP and leucine remained statistically significant (Table 2).

Correlation analyses for hepatic phosphorus metabolites with fatty acids species

Neither γATP nor P_i related to any analyzed saturated fatty acid species. Furthermore, γATP did not associate with any unsaturated fatty acid species (Table 3). P_i related positively to myristoleic acid (cC14:1w5), cC16:1w5, palmitoleic acid (cC16:1w7; Fig. 1F), adrenic acid (cC22:4w6), and the D9D index.

After adjustment for age, sex, and BMI, only the relationships of P_i with myristoleic acid, cC16:1w5, palmitoleic acid, and D9D index remained (Table 3). Further adjustment for P_i revealed negative relationships of γATP with cC16:1w5 and adrenic acid. After adjustment for

Table 2. Correlation of Hepatic Phosphorus Metabolites With Specific Amino Acids

	Unadjusted		Adjusted for Age, Sex, and BMI		Adjusted for Age, Sex, BMI, and P_i or γATP	
	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L
	Pearson Correlation Coefficient					
Alanine (Ala; ln)	0.16	0.04	0.08	−0.002	0.08	−0.03
Arginine (Arg; ln)	0.03	0.19	0.01	0.19	−0.06	0.20
Citrulline (Cit; ln)	0.16	−0.007	0.12	−0.08	0.16	−0.14
Glycine (Gly; ln)	0.14	−0.03	0.08	−0.10	0.13	−0.14
Histidine (His; ln)	−0.002	−0.06	0.05	−0.02	0.07	−0.05
Isoleucine (Ile; ln)	0.19	−0.09	0.32[a]	−0.005	0.35[b]	−0.14
Leucine (Leu; ln)	0.28[a]	0.06	0.42[c]	0.16	0.40[b]	−0.004
Lysine (Lys; ln)	0.22	0.13	0.15	0.07	0.13	0.01
Methionine (Met; ln)	0.05	−0.09	0.18	0.006	0.19	−0.07
Ornithine (Orn; ln)	0.35[b]	0.14	0.28[a]	0.05	0.28[a]	−0.06
Phenylalanine (Phe; ln)	0.23	−0.02	0.24	−0.02	0.26[a]	−0.12
Proline (Pro; ln)	−0.01	−0.08	0.06	−0.02	0.07	−0.04
Serine (Ser; ln)	0.15	−0.04	0.12	−0.05	0.15	−0.10
Threonine (Thr; ln)	−0.22	−0.28[a]	−0.17	−0.23	−0.10	−0.18
Tryptophane (Trp; ln)	0.23	−0.004	0.28[a]	0.05	0.29[a]	−0.07
Tyrosine (Tyr; ln)	0.25	−0.07	0.21	−0.10	0.26[a]	−0.20
Valine (Val; ln)	0.15	0.005	0.25	0.06	0.24	−0.03
BCAA (Ile/Leu/Val; ln)	0.22	−0.007	0.37[b]	0.09	0.37[b]	−0.06

Pearson and partial Pearson correlations of γATP and P_i with specific amino acids are given, respectively. The Bonferroni-adjusted significance level is 0.0014 [0.05/(18 amino acid parameters × 2 hepatic phosphorus metabolites)]. After Bonferroni adjustment for multiple comparisons, only the association of γATP and leucine remained statistically significant.

Abbreviation: ln, natural log transformed.

[a] $P < 0.05$.

[b] $P < 0.01$.

[c] $P < 0.001$.

Table 3. Correlation of Hepatic Phosphorus Metabolites With Free Fatty Acid Species

	Unadjusted		Adjusted for Age, Sex, and BMI		Adjusted for Age, Sex, BMI, and Pi or γATP	
	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L	γATP, mmol/L	P_i, mmol/L
	Pearson Correlation Coefficient					
SFA						
C14:0 (myristic; ln)	0.02	0.13	−0.05	0.09	−0.09	0.11
C15:0 (pentadecanoic; ln)	−0.13	0.07	−0.13	0.08	−0.17	0.14
C16:0 (palmitic; ln)	−0.06	0.09	−0.19	0.02	−0.21	0.10
C17:0 (heptadecanoic; ln)	0.0007	0.04	−0.08	−0.03	−0.08	0.004
C18:0 (stearic; ln)						
C19:0 (nonadecanoic; ln)	0.02	0.02	−0.16	−0.14	−0.12	−0.09
C20:0 (eicosanoic; ln)	−0.24	−0.18	−0.22	−0.16	−0.17	−0.09
MUFA						
cC14:1w5 (myristoleic; ln)	0.13	0.28[a]	0.08	0.28[a]	−0.02	0.27[a]
cC15:1w5, c10 (pentadecenoic; ln)	−0.14	0.10	−0.19	0.07	−0.23	0.15
cC16:1w5 (ln)	−0.04	0.32[a]	−0.14	0.30[a]	−0.28[a]	0.38[b]
cC16:1w7 (palmitoleic; ln)	0.09	0.33[b]	−0.03	0.28[a]	−0.15	0.31[a]
cC16:1w10 (sapienic; ln)	0.01	0.16	−0.13	0.10	−0.18	0.16
cC17:1w7, c10 (heptadecenoic; ln)	−0.16	−0.004	−0.23	−0.07	−0.22	0.02
cC17:1w8 (ln)	0.10	0.21	−0.03	0.11	−0.08	0.14
cC18:1w7 (vaccenic; ln)	−0.01	0.20	−0.17	0.10	−0.22	0.18
cC18:1w9 (oleic; ln)	0.01	0.12	−0.13	0.02	−0.14	0.07
cC20:1w9, c11 (eicosenoic; ln)	−0.02	0.13	−0.12	0.05	−0.15	0.10
PUFA						
cC18:2w6 (linoleic; ln)	0.05	0.08	−0.07	−0.04	−0.06	−0.01
cC18:3w3 (linolenic; ln)	0.03	0.13	−0.12	0.02	−0.13	0.06
cC18:3w6 (gamma linolenic; ln)	0.09	0.18	−0.005	0.13	−0.06	0.15
cC18:4w3 (stearidonic; ln)	0.03	0.20	−0.05	0.14	−0.11	0.17
cC20:2w6, c11, 14 (eicosadienoic; ln)	−0.02	0.16	−0.12	0.08	−0.17	0.14
cC20:3w6, c8, 11, 14 (eicosatrienoic; ln)	−0.05	−0.01	−0.05	0.02	−0.06	0.04
cC20:3w9 (mead; ln)	0.06	0.04	0.03	0.003	0.03	−0.009
cC20:4w6 (arachidonic; ln)	−0.17	0.04	−0.18	0.05	−0.22	0.13
cC20:5w3 (EPA; ln)	0.004	0.07	−0.13	−0.05	−0.12	0.003
cC22:4w6 (adrenic; ln)	−0.15	0.26[a]	−0.25	0.22	−0.37[b]	0.35[b]
cC22:5w3 (DPA; ln)	0.04	0.24	−0.12	0.13	−0.19	0.19
cC22:6w3 (DHA; ln)	0.03	0.14	−0.12	0.05	−0.14	0.10
Q_cC16_1__cC16_0	0.15	0.37[b]	0.08	0.33[a]	−0.05	0.33[a]

Pearson and partial Pearson correlations of γATP and P_i with specific amino acids are given, respectively. The Bonferroni-adjusted significance level is 0.00083 [0.05/(30 fatty acid parameters × 2 hepatic phosphorus metabolites)]. After Bonferroni adjustment for multiple comparisons, only the association of P_i and palmitoleic acid remained statistically significant.

Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; ln, natural log transformed; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

[a] $P < 0.05$.

[b] $P < 0.01$.

γATP, P_i related positively to myristoleic acid, cC16:1w5, palmitoleic acid, adrenic acid, and D9D index (Table 3). When corrected for multiple testing, none of the associations remained statistically significant (Table 3).

In multivariate regression models, age, sex, and BMI explained 9.8% of γATP and 7.8% of P_i variability, respectively. Additional adjustment for leucine, as a significant independent predictor, explained 26% of γATP variances, and the adjustment for age, sex, BMI, and palmitoleic acid accounted for 15% of P_i variability. A multivariate regression analysis containing variables age, sex, BMI, palmitoleic acid, and cC16:1w5 did not improve R^2 (0.16).

Predictors of hepatic phosphorus metabolite concentrations

These analyses identified leucine and C16:1 fatty acid palmitoleic acid as predictors of γATP and P_i content, respectively. We further confirmed that additional adjustment for selected parameters, indicative of insulin resistance and glucose intolerance, did not affect these relationships (Supplemental Table S3). The close association of γATP and leucine was not affected by adjustment for any of the chosen parameters. However, the relation of P_i and palmitoleic acid disappeared by adjustment for 2-hour OGTT glucose and NEFA levels, respectively.

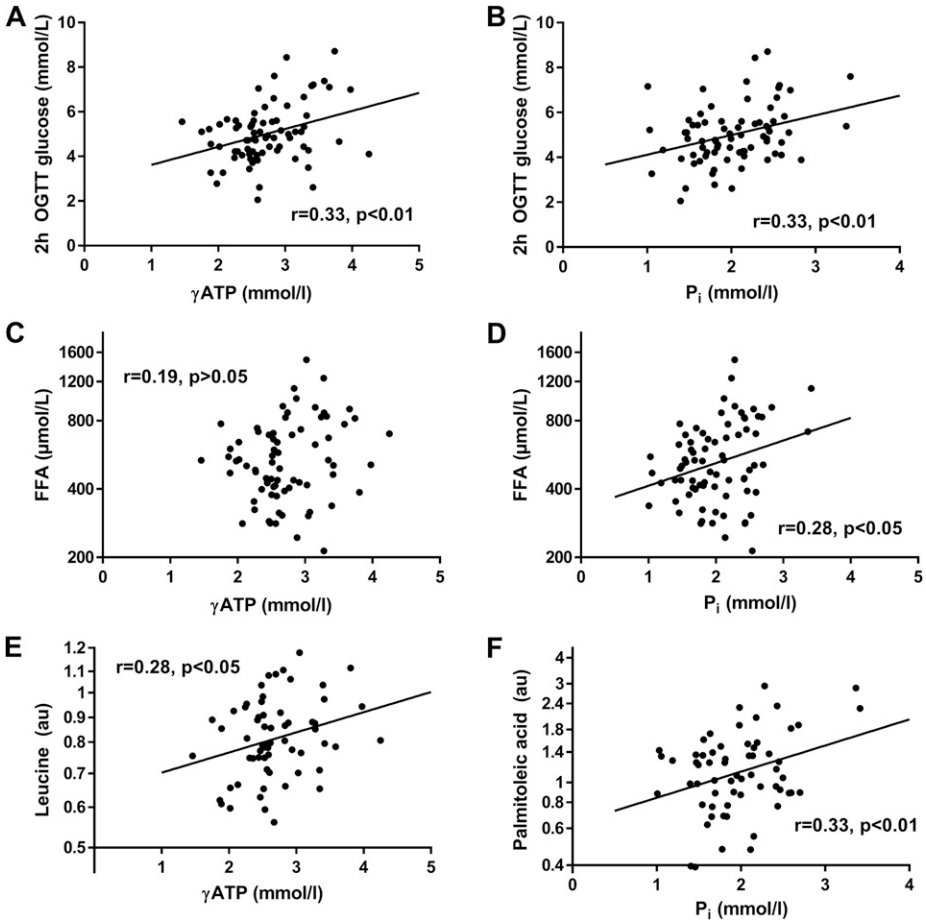

Figure 1. Correlations of γATP and P_i with plasma metabolites. (A, C, and E) Correlations of γATP with 2-hour OGTT glucose, NEFA, and leucine, respectively. (B, D, and F) Correlations of P_i with 2-hour OGTT glucose, NEFA, and palmitoleic acid, respectively. Before correlation analyses, NEFA and metabolites were log transformed to achieve normal distribution. Metabolites were adjusted further for plate of measurement (shown as au).

Discussion

This study found that specific essential amino acids and fatty acid species are independent predictors of hepatic γATP and P_i content in metabolically healthy humans, whereas measures of insulin sensitivity neither related to hepatic γATP nor to P_i content.

This cohort had low HCL, normal glucose tolerance, and insulin sensitivity, based on the OGTT analysis (15). Moreover, absolute concentrations of hepatic γATP and P_i were similar to nondiabetic cohorts with comparable baseline characteristics (2, 12). Thus, this cohort represents an appropriate group for investigation of hepatic energy status under physiological conditions.

Absolute hepatic concentrations of P_i and ATP have been reported to be positively associated with hepatic but not with peripheral insulin sensitivity and negatively with HCL in a mixed collective of patients with T2D and nondiabetic humans (2). Another study reported a negative correlation of ATP levels with BMI (20). Of note, our results do not confirm these associations in a collective of nondiabetic humans, as we did not observe any association of γATP and P_i with indices of fasting (QUICKI), dynamic peripheral (OGIS), or hepatic (Hepatic-IR index, relationship between insulin sensitivity and cardiovascular disease) and adipose tissue (Adipo-IR index) insulin sensitivity, HCL, or BMI, respectively. This might result from the fact that our collective excluded participants with hyperglycemia or hepatic steatosis. Furthermore, we used fasting and OGTT-derived measures of insulin sensitivity, but OGIS and M-values calculated from OGTT and hyperinsulinemic-euglycemic clamps, respectively, are closely correlated (7, 15).

Unexpectedly, we observed a positive relationship of both γATP and P_i with 2-hour OGTT glucose levels, which may serve as a rough predictor of insulin resistance (21). However, factors other than insulin resistance, such as duration of fasting and the amount of carbohydrate intake on the previous days, may influence OGTT glucose levels (22, 23). In T2D, the negative relationship between hepatic γATP and ATP synthase flux and insulin resistance was determined, at least in part, by fasting

plasma glucose and hemoglobin A1c, respectively (2, 3). Thus, the observed positive relationships of hepatic γATP and P_i with 2-hour OGTT glucose observed in this nonobese nondiabetic cohort are unlikely a result of insulin resistance. Of note, nondiabetic but obese persons even show greater hepatic oxidative capacity, despite peripheral insulin resistance (1).

We found a positive relation of P_i with circulating NEFA. In patients with T2D, no correlation was found for ATP flux and NEFA (3). Previous studies reported no association of γATP and plasma NEFA levels in metabolically healthy and diseased humans (12, 24), whereas P_i associated negatively with NEFA levels in a combined collective of healthy, obese, and T2D volunteers (12). Moreover, P_i did not correlate with the Adipo-IR index, suggesting that Adipo-IR cannot exclusively explain the relationship of P_i and NEFA. However, a discrete effect cannot be resolved by our study as a result of the limited sample size. Thus, in healthy humans with intact metabolic flexibility, higher substrate availability of either glucose or NEFA may charge hepatic phosphorus pools and/or stimulate energy-generating processes (25). Of note, further adjustment for γATP or P_i, respectively, abolished the previous associations, indicating that the relationship of 2-hour OGTT glucose with γATP and P_i, as well as of NEFA concentrations with P_i, also depends on the prevailing P_i and γATP levels, respectively. Absolute fasting γATP and P_i levels, as well as P_i/ATP ratios of our study, differed, in part, from those of the respective studies, which might also explain the observed differences in the correlation analyses (2, 3, 12). However, none of the associations of γATP or P_i with 2-hour OGTT glucose and of NEFA concentrations with P_i was statistically significant when corrected for multiple testing.

Interestingly, this study found a positive association of γATP with some essential amino acids and of P_i with some fatty acid species, respectively. People with insulin resistance or T2D typically present with higher levels of combined BCAA (leucine, isoleucine, valine) and/or other amino acids (phenylalanine, tryptophane, tyrosine, alanine, citrulline) and lower levels of threonine, glycine, and glutamine (9, 13). In our metabolically healthy cohort, γATP related strongest to leucine, independent of changes in P_i content. Leucine and isoleucine were reported to be elevated in obese compared with lean humans (9). In our cohort, γATP still related closely to leucine even after adjustment for age, sex, and BMI. Further investigation of parameters affecting the relation of γATP and leucine showed no effect on this interaction.

In obese mice, leucine increases ATP concentrations in brown adipose tissue as a result of stimulation of mitochondrial biogenesis (26), and in skeletal muscle cells, leucine stimulates fatty acid oxidation, as well as oxygen consumption (27). Moreover, leucine provides carbon skeletons to the tricarboxylic acid (TCA) cycle at the level of acetyl-coenzyme A, which may enhance TCA cycle flux (28). Thus, higher plasma leucine levels likely contribute to improved mitochondrial performance rather than reflect insulin resistance or impaired mitochondrial function in our collective of healthy humans.

This study observed relevant correlations of P_i with unsaturated fatty acid species, such as myristoleic acid, C16:1w5, palmitoleic acid, and adrenic acid. The absolute concentrations of these fatty acids were rather low, as previously reported (29). Whereas our assay does not distinguish between *cis* and *trans* fatty acids, the low contribution of *trans* fatty acids to the total circulating fatty acid pool, amounting to $<2\%$ (30), suggests that the observed correlations are mainly driven by the respective *cis* isomers. Myristoleic acid usually accounts for only small amounts of total fatty acids in animal tissues but is abundant in milk fat and may also raise palmitoleic acid levels (31). It is therefore conceivable that the circulating palmitoleic and myristoleic acid levels, at least partly, result from dietary intake.

We did not detect a correlation of the DNL index and γATP or P_i, respectively. The main fatty acid products of DNL include palmitic acid, palmitoleic acid, vaccenic acid, stearic acid, and oleic acid (32). Except for palmitoleic acid, we did not find a correlation of those fatty acids with P_i, further suggesting that the relationship of P_i and palmitoleic acid does not result from DNL. Nevertheless, palmitoleic acid *per se* and the D9D index have been proposed as markers of DNL (33).

The strong interaction between P_i and palmitoleic acid disappeared upon adjustments for fasting NEFA and 2-hour OGTT glucose levels. Fasting NEFA levels mainly reflect adipose tissue lipolysis, which also is tightly regulated by the glucose-regulating hormones insulin and catecholamines (34). This suggests that both NEFA supply to the liver and regulatory hormones contribute to this interaction. Palmitoleic acid has originally gained attention as an anti-inflammatory adipokine (35). In a murine model, chronic palmitoleic acid feeding enhances hepatic glucose uptake and fatty acid oxidation for energy production instead of storage through activation of adenosine 5′-monophosphate-activated protein kinase and fibroblast growth factor 21 -21, dependent on peroxisome proliferator-activated receptor α (10). Of note, *cis* monounsaturated fatty acids also strongly inhibit Ca^{2+} uptake, thereby inducing net Ca^{2+} efflux and higher extramitochondrial Ca^{2+} concentrations (36). Effects of palmitoleic acid and its ester on mitochondrial metabolism have also been reported in other tissues (37). Taken together, these relationships of palmitoleic and myristoleic acids with hepatic P_i might result directly or

indirectly from their interaction with mitochondrial Ca^{2+} handling and reduction in the mitochondrial membrane potential. As to possible hepatic effects of the other unsaturated fatty acids, adrenic acid may act as an inflammation enhancer in NAFLD (38), whereas there is currently no information on the physiological relevance of C16:1w5.

The strength of this study is the investigation of hepatic energy metabolism in a larger cohort of well-phenotyped, glucose-tolerant humans. Up until now, hepatic ATP and P_i content have mainly been studied in the context of metabolic diseases, such as T2D or NAFLD (2, 3, 6). This approach might help to differentiate among factors regulating ATP and P_i in nondiabetic, nonobese, non-NAFLD persons and in those with metabolic disease. This study also has some limitations. The main weakness resides in the inclusion of only healthy, nonobese individuals. Nevertheless, relationships of amino and fatty acid species with hepatic phosphorus metabolites have, to our knowledge, not been reported before, and this study provides that information.

Although measurement of absolute hepatic concentrations of ATP and P_i is reliable and highly reproducible, it covers only one aspect of hepatic energy metabolism—the flux through hepatic ATP synthase (3)—but does not address other features of mitochondrial function, such as TCA cycle activity and oxidative capacity, which may be differently affected in health and disease (1, 39, 40). Furthermore, the observed effect of leucine and palmitoleic acid on ATP and P_i levels is only modest, implying that other factors also regulate hepatic energy metabolism in healthy humans. Such factors might be less relevant in the presence of hepatic steatosis, insulin resistance, or hepatocellular injury (2, 6). Our data, from the examined cohort, suggest that hepatic concentrations of ATP and P_i relate to specific circulating amino acids and fatty acid species, but analyses could be considered exploratory as a result of the size of our cohort and the lack of statistical significance after Bonferroni correction for many associations. However, the strongest correlation found for hepatic γATP levels with leucine was still present after correction for multiple testing.

In conclusion, hepatic γATP and P_i do not relate to measures of insulin sensitivity in metabolically healthy nonobese humans. However, specific circulating amino acids and NEFAs partly affect the availability of hepatic phosphorus metabolites. These results point to a mutual interaction between hepatic energy metabolism and circulating metabolites and may therefore contribute to the better understanding of the differences in hepatic energy metabolism between glucose-tolerant and insulin-resistant humans.

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Acute dietary fat intake initiates alterations in energy metabolism and insulin resistance

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BACKGROUND. Dietary intake of saturated fat is a likely contributor to nonalcoholic fatty liver disease (NAFLD) and insulin resistance, but the mechanisms that initiate these abnormalities in humans remain unclear. We examined the effects of a single oral saturated fat load on insulin sensitivity, hepatic glucose metabolism, and lipid metabolism in humans. Similarly, initiating mechanisms were examined after an equivalent challenge in mice.

METHODS. Fourteen lean, healthy individuals randomly received either palm oil (PO) or vehicle (VCL). Hepatic metabolism was analyzed using in vivo $^{13}C/^{31}P/^{1}H$ and ex vivo ^{2}H magnetic resonance spectroscopy before and during hyperinsulinemic-euglycemic clamps with isotope dilution. Mice underwent identical clamp procedures and hepatic transcriptome analyses.

RESULTS. PO administration decreased whole-body, hepatic, and adipose tissue insulin sensitivity by 25%, 15%, and 34%, respectively. Hepatic triglyceride and ATP content rose by 35% and 16%, respectively. Hepatic gluconeogenesis increased by 70%, and net glycogenolysis declined by 20%. Mouse transcriptomics revealed that PO differentially regulates predicted upstream regulators and pathways, including LPS, members of the TLR and PPAR families, NF-κB, and TNF-related weak inducer of apoptosis (TWEAK).

CONCLUSION. Saturated fat ingestion rapidly increases hepatic lipid storage, energy metabolism, and insulin […]

Acute dietary fat intake initiates alterations in energy metabolism and insulin resistance

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CONCLUSION. Saturated fat ingestion rapidly increases hepatic lipid storage, energy metabolism, and insulin resistance. This is accompanied by regulation of hepatic gene expression and signaling that may contribute to development of NAFLD.

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Introduction

The pandemic of obesity, type 2 diabetes mellitus (T2DM) and nonalcoholic fatty liver disease (NAFLD) has frequently been associated with dietary intake of saturated fats (1) and specifically with dietary palm oil (PO) (2). According to current paradigms, chronic insulin resistance is the common feature of these diseases (3, 4) and relates to intracellular concentrations of triglycerides (TG) and lipotoxins (5). There is evidence that a chronic high-fat diet in mice and humans leads to insulin resistance via similar mechanisms (6, 7). Chronic insulin resistance comprises not only impaired muscle insulin action but also increased rates of endogenous glucose production (EGP) and gluconeogenesis (GNG) in obese and T2DM patients (8–10).

Still, only a few studies have addressed the initial effects of high-fat loading using either intravenous or enteral administration of lipids. These studies mainly focused on the role of skeletal muscle by assessing intramyocellular TG content (11), substrate oxidation, glycogen synthesis (12), or glucose disposal (13–17). Studies using parenteral administration of unsaturated lipids (18) or high-calorie mixed meals yielded conflicting results with regard to hepatic energy metabolism. One mixed-meal study found greater de novo lipogenesis without affecting hepatic glycogen metabolism (12), while an intravenous lipid infusion study failed to detect any effect on hepatic insulin sensitivity (19). Another study com-

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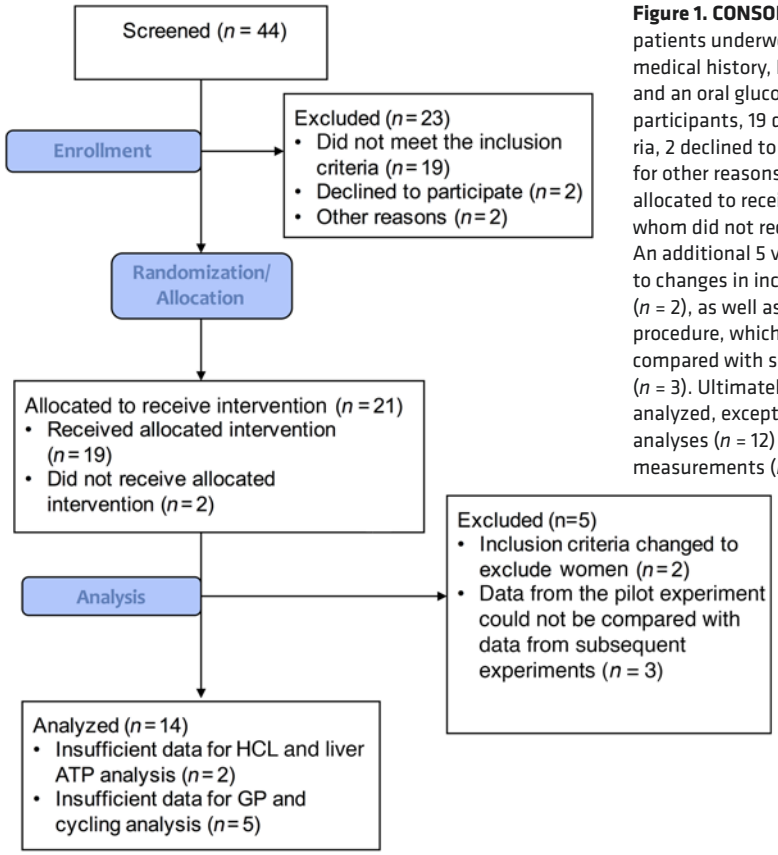

Figure 1. CONSORT flow diagram. Forty-four patients underwent screening, which included a medical history, BMI analyses, and bioimpedance and an oral glucose tolerance tests. Of the 44 participants, 19 did not meet the inclusion criteria, 2 declined to participate, and 2 were excluded for other reasons. Twenty-one participants were allocated to receive the intervention, two of whom did not receive the allocated intervention. An additional 5 volunteers were excluded due to changes in inclusion and exclusion criteria (n = 2), as well as changes in the experimental procedure, which yielded data that could not be compared with subsequently acquired data (n = 3). Ultimately, data from 14 individuals were analyzed, except for hepatic lipid and energy analyses (n = 12) and some hepatic glucose flux measurements (n = 9).

paring subacute oral ingestion of fatty acids with different compositions found an increase in the glucose infusion rate only after polyunsaturated fatty acid ingestion (20).

To overcome possible limitations of the previous studies, such as the use of nonphysiological routes of lipid administration or mixed meals, which introduce protein and carbohydrates as confounders, we designed a translational study concept comprising a clinical trial involving healthy humans and a corresponding study involving nonobese nondiabetic mice. The randomized crossover clinical trial examined the effects of a single oral challenge with PO, which is mainly composed of saturated fatty acids (2), versus vehicle (VCL) ingestion on whole-body insulin sensitivity (WBIS) and hepatic insulin sensitivity. Moreover, the contributions of hepatic glucose fluxes, i.e., GNG, net glycogenolysis (GLYnet), and the futile exchange between glycogenogenic and glycogenolytic pathways (glycogen cycling) to EGP as well as the effects of these fluxes on hepatocellular lipids (HCL) and phosphorous-containing metabolites were analyzed using combined in vivo multinuclear $^{13}C/^{31}P/^{1}H$ and stable isotope tracers to assess plasma glucose appearance rates and sources of EGP. In the mouse study, we examined the effects of a similar oral saturated fat challenge on insulin sensitivity and hepatic transcriptome changes.

Results

Studies in humans. A total of 14 young, lean, insulin-sensitive male volunteers (Figure 1 and Table 1) received either an oral dose of PO (~1.18 g/kg BW) or an identical volume of VCL on 2 occasions in random order, spaced by an 8-week interval.

PO results in increased circulating TG, glucagon, and incretins. After PO administration, TG in plasma rose by 59% (area under the time curve [AUC], $P < 0.001$) and by 156% in chylomicrons (AUC, $P = 0.009$) (Figure 2A). The AUC for plasma free fatty acids (FFA) (Figure 2B) and insulin concentrations (Figure 2C) was unchanged, while the AUC for plasma C-peptide was 28% higher after PO ingestion versus VCL ($P < 0.005$, Figure 2D). Of note, FFA were increased at 300, 420, and 480 minutes. Blood glucose levels were not different between PO- and VCL-treated groups (Figure 2E). Plasma glucagon rose by 41% (AUC, $P < 0.0001$) only after PO ingestion (Figure 2F). Also, glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) levels were markedly increased and remained elevated after PO ingestion (both $P < 0.005$) (Supplemental Figure 2; supplemental material available online with this article; https://doi.org/10.1172/JCI89444DS1). Circulating levels of TNF-α, IL-6, fetuin-A, chemerin, omentin, and cortisol were not different between PO and VCL groups ($P > 0.5$ for all) (Supplemental Table 1).

PO induces insulin resistance at whole-body, liver, and adipose tissue levels. Insulin sensitivity was measured using hyperinsulinemic-euglycemic clamp tests in healthy humans. Steady state was reached (Supplemental Figure 1), and pertinent parameters were analyzed during this time. PO ingestion reduced WBIS by 25% compared with VCL treatment ($P = 0.0005$, Figure 3A). Furthermore, after PO, volunteers also showed a decrease of 22% ($P = 0.002$) in the rate of glucose disappearance (Rd), mostly due to a 33% ($P = 0.01$) reduction in glucose oxidation (GOX), while the rate of nonoxidative glucose disposal remained unchanged

(Figure 3B). EGP increased by 10% ($P = 0.03$) at 240 minutes and by 82% ($P = 0.008$) at 480 minutes after PO compared with VCL treatment (Figure 3C). After PO, insulin-stimulated suppression of EGP was 15% lower than that detected after VCL ($P < 0.01$, Figure 3D). Finally, insulin-stimulated suppression of FFA (Figure 3E) and TG (Figure 3F) was 30% ($P = 0.0001$) and 80% ($P = 0.026$) lower after PO than after VCL treatment.

PO augments the contribution of GNG to EGP. In order to further analyze the PO-induced increase in EGP, we measured the contributions of gluconeogenic and glycogenolytic fluxes. We found that GNG increased by approximately 70% ($P = 0.01$), while GLYnet decreased by approximately 20% ($P < 0.05$) after PO. Glycogen phosphorylase (GP) flux tended to be lower in the PO arm ($P = 0.085$). The contribution of glycogen cycling to total GP flux was negligible in both study arms (Figure 4).

PO increases lipid oxidation rates. The respiratory quotient (RQ), defined as the rate of CO_2 production/O_2 consumption, was comparable in the period after PO or VCL ingestion but was reduced during the clamp only after PO (Figure 5A). Resting energy expenditure (REE) and lipid oxidation (LOX) rates increased markedly at 300 minutes after PO ingestion and remained elevated into the clamp (at 420 min) after PO (Figure 5, B and C). GOX decreased during the clamp following PO ingestion compared with GOX after VCL ingestion (Figure 5D).

PO raises hepatic ATP and lipid content. HCL and hepatic γATP increased by 33% ($P < 0.01$) and 16% ($P = 0.009$) from –120 minutes to 240 minutes after PO, respectively. At 240 minutes, γATP tended to be higher in the VCL arm ($P = 0.06$), while HCL levels did not differ between PO and VCL arms. Hepatic inorganic phosphate (P_i) and γATP/P_i did not change after PO or VCL (Table 2). When comparing the mean difference between –120 and 240 minutes after PO or VCL between interventions, only the change in HCL levels tended to be increased (Δ % HCL PO vs. VCL, $P = 0.085$; Δ γATP, $P = 0.57$; Δ P_i, $P = 0.14$; Δ γATP/P_i, $P = 0.22$).

Studies in mice. Two mouse cohorts received PO or VCL via gavage. One cohort then underwent hyperinsulinemic-euglycemic clamp tests under unrestrained conditions, whereas tissue and blood sample analysis was done for the other cohort.

PO increases circulating lipid levels. Plasma FFA levels increased by 45% (AUC, $P = 0.004$), while TG levels were not significantly different (AUC, $P = 0.08$) after PO (Figure 6, A and B). The post-intervention AUC for blood glucose and insulin was comparable between groups (Figure 6, C and D).

PO preferably induces hepatic insulin resistance. Insulin sensitivity was measured using hyperinsulinemic-euglycemic clamp tests in unrestrained mice. WBIS in mice trended toward a reduction ($P = 0.07$) (Figure 7A). Whole-body glucose uptake, given by Rd, was not different between interventions (Figure 7B). Residual EGP during the clamp tended to be higher ($P = 0.06$) (Figure 7C), and insulin-mediated suppression of EGP was 25% lower ($P = 0.04$) after PO (Figure 7D), reflecting marked hepatic insulin resistance. PO ingestion did not affect insulin-induced FFA suppression (Figure 7E) but reduced insulin-induced TG suppression by 83% ($P = 0.0039$) (Figure 7F). Of note, glucose uptake in the gastrocnemius muscle (Supplemental Figure 3A) and white adipose tissue (Supplemental Figure 3B) as well as rates of glycolysis did not differ between the PO- and VCL-treated groups (Supplemental Figure 3C). Interestingly, PO administration did not change hepatic TG content in mice (Supplemental Figure 3D).

PO causes changes in the expression of hepatic transcription factors. Livers from both mouse cohorts were used for analysis on Affymetrix Gene 2.1 ST microarrays. A total of 273 and 327 differentially regulated probe sets were obtained from each cohort. Array data were deposited in the NCBI's Gene Expression Omnibus (GEO) database (GEO GSE80259). Canonical pathways predicted to be differentially regulated by Ingenuity software after PO included TNF-like weak inducer of apoptosis (TWEAK) and aryl hydrocarbon receptor (AHR) under insulin-stimulated and noninsulin-stimulated conditions; phospholipase and D-myoinositol-5 phosphate signaling under insulin-stimulated conditions; and p38 MAPK, NF-κB, PPARα, and OX40 under noninsulin-stimulated conditions (Figure 8, A and B, all $P < 0.05$). Several upstream regulators involved in hepatic fatty acid metabolism and inflammatory processes were predicted by Ingenuity software to be regulated by PO ingestion. These upstream regulators included LPS, which was activated with the most certainty, with a Z score of 1.9 and 2.3 under insulin- and noninsulin-stimulated conditions, respectively, TLR family members (TLR9 and TLR3 under insulin-stimulated conditions as well as TLR4 under noninsulin-stimulated conditions), PPARα, and FOXO1 (all $P < 0.01$) (Figure 8, C and D).

Table 1. Anthropometric and blood parameters of study participants

Parameter	Mean ± SEM
n (men/women)	14 (14/0)
Age (yr)	25.8 ± 1.4
BMI (kg/m^2)	22.5 ± 0.3
Waist circumference (cm)	79.5 ± 1.4
Lean BW (kg)	59.2 ± 2.1
TG (mg/dl)	78.4 ± 9.8
FFA (μmol/l)	386.9 ± 32.9
ALT (U/l)	24.4 ± 3.2
AST (U/l)	26.0 ± 2.5
Fasting blood glucose (mg/dl)	76.1 ± 2.0
2-hour postprandial blood glucose (mg/dl)	76.4 ± 4.7
HCL (% H_2O)	0.60 ± 0.09

ALT, alanine aminotransferase; AST, aspartate.

Table 2. Hepatocellular lipids and hepatic phosphorus-containing metabolites

Time (min)	–120		240	
Group	VCL	PO	VCL	PO
HCL (% H_2O)	0.97 ± 0.20	0.93 ± 0.23	1.04 ± 0.21	1.26 ± 0.32^{A,D}
γATP (mmol/l)	2.86 ± 0.17	2.79 ± 0.14	3.17 ± 0.16^{C}	3.25 ± 0.16^{B}
P_i (mmol/l)	2.35 ± 0.04	2.61 ± 0.07	2.61 ± 0.15	2.65 ± 0.20
γATP/P_i	1.21 ± 0.07	1.07 ± 0.04	1.26 ± 0.10	1.35 ± 0.17

Data represent the mean ± SEM. $n = 12$. $^{A}P < 0.01$, $^{B}P < 0.05$, and $^{C}P = 0.066$, for –120 minutes compared with 240 minutes; $^{D}P = 0.085$, for the mean difference from –120 to 240 minutes between groups.

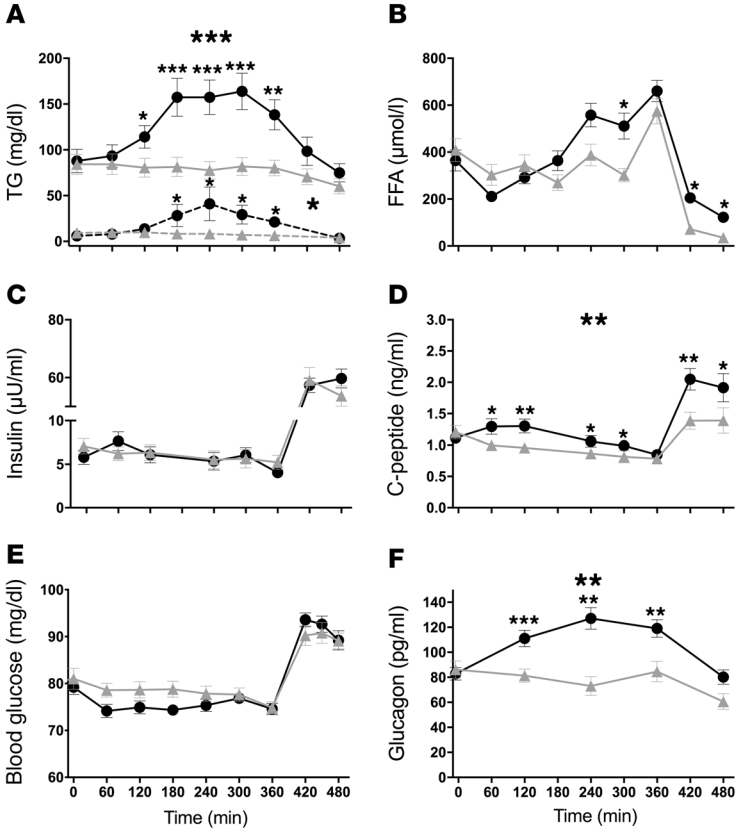

Figure 2. Time courses of circulating metabolites and hormones in humans. VCL (gray triangles) or PO (black circles) was administered at 0 minutes to lean, healthy men, and the hyperinsulinemic-euglycemic clamp was started at 360 minutes. TG circulating in plasma (solid line) and in chylomicrons (dashed line). The AUC was 59% and 156% higher after PO ingestion, respectively (**A**). Circulating FFA (**B**). Time courses for insulin (**C**), C-peptide (AUC 28% higher after PO) (**D**), blood glucose (**E**), and glucagon (AUC 41% higher after PO) (**F**). Values represent the mean ± SEM. $n = 14$; chylomicron TG $n = 6$. $^{***}P < 0.001$, $^{**}P < 0.005$, and $^{*}P < 0.05$, by paired, 2-tailed t test. Large asterisks refer to AUC differences; small asterisks refer to differences per time point.

Except for LPS, the analysis did not reveal whether the pathways and upstream regulators were activated or inhibited.

Under insulin- and noninsulin-stimulated conditions, PO resulted in upregulation of the following transcripts: miR1970, LPS-regulated genes (*Ifit3*, *Clec4a3*, *Slc22a3*, and *C3ar1*) (21–24), *G0s2*, and *Ar16*, while *Tweak* gene expression was downregulated. Opposing regulatory transcripts, i.e., those that were upregulated under insulin-stimulated conditions and downregulated under noninsulin-stimulated conditions, were also found and included, for example, transcripts involved in cell growth (*Map3k13* and *Slc30af*) (25, 26) and FFA metabolism and development of NAFLD (fatty acid–binding protein 5 or *Fabp5*) (27), as well as the predicted pseudogenes *Gm3601* and *H2-K2* (Figure 8E).

Discussion

This study demonstrates that a single oral dose of saturated fat increases hepatic TG accumulation, insulin resistance, GNG, and ATP concentrations in the human liver. Ingestion of saturated fat also induces peripheral insulin resistance in skeletal muscle and adipose tissue. In mice, a single saturated fat load preferentially induces hepatic insulin resistance and also affects hepatic gene expression and signaling, which could contribute to the promotion of NAFLD.

PO induced a marked increase in plasma FFA concentrations in both humans and mice, but no alterations in circulating inflammatory markers or adipokines, such as TNF-α, IL-6, fetuin A, chemerin, and omentin. This finding indicates that the acute effects of PO are mediated by metabolic rather than endocrine changes and is partly in line with findings obtained after intravenous infusion of soybean oil, which showed no changes in circulating cytokines (19), or after ingestion of cream, which resulted in increased expression of TNF-α but not IL-6 (28). Interspecies differences could also be due to the administration of emulsified versus pure PO.

Notably, a single dose of PO markedly altered hepatic glucose fluxes and resulted in increased rates of GNG, reduced GLYnet, and a corresponding trend toward reduced GP flux. This finding extends our previous results on the effects of parenteral administration of polyunsaturated lipids (29). The present study also provides a comprehensive analysis of in vivo hepatic glucose and glycogen fluxes in humans that includes an assessment of glycogen cycling, which ensured correct estimation of GLY and GNG contributions to EGP (30). In chronic insulin-resistant states, such as occurs in T2DM or type 1 diabetes mellitus (T1DM), elevation of GNG and EGP coexists with enhanced glycogen cycling (30). Surprisingly, oral lipid loading stimulated GNG and hepatic insulin resistance without affecting glycogen cycling, which remained negligible, as was reported for healthy humans in the fasted state (30, 31). This indicates that healthy humans can rapidly downregulate GLY under conditions of elevated GNG to avoid futile cycling. The absence of any effect on glycogen cycling might be due to the prevailing euglycemia and basal peripheral insulinemia compared with the hyperglycemic hypoinsulinemic conditions in the aforementioned study. Consequently, the augmented glycogen cycling observed in insulin-resistant states reflects chronically abnormal hepatic energy metabolism rather than an immediate physiological response to changes in dietary lipid supply. Of note, these changes occur in the presence of higher glucagon concentrations, which are likely due to FFA-induced glucagon stimulation (32). Specifically, the increase in GNG and the decrease in GLYnet upon PO administration could, at least in part, result from the increase in circulating glucagon. Even small changes in plasma glucagon can modify GLYnet independently of insulin in healthy humans (33).

Interestingly, ingestion of PO increased hepatic γATP and HCL concentrations, along with the induction of LOX and insulin resistance. Recently, we reported that a test involving ingestion of a mixed-meal with 35% fat content increases hepatic γATP exclusively in insulin-resistant obese humans but not in lean, nondiabetic or T2DM individuals (34). This suggests that the stimulatory effect of saturated fat on hepatic energy metabolism is dose dependent and may be linked to the onset of insulin resistance, but not the insulin sensitivity state per se. In line with this, recent studies report the

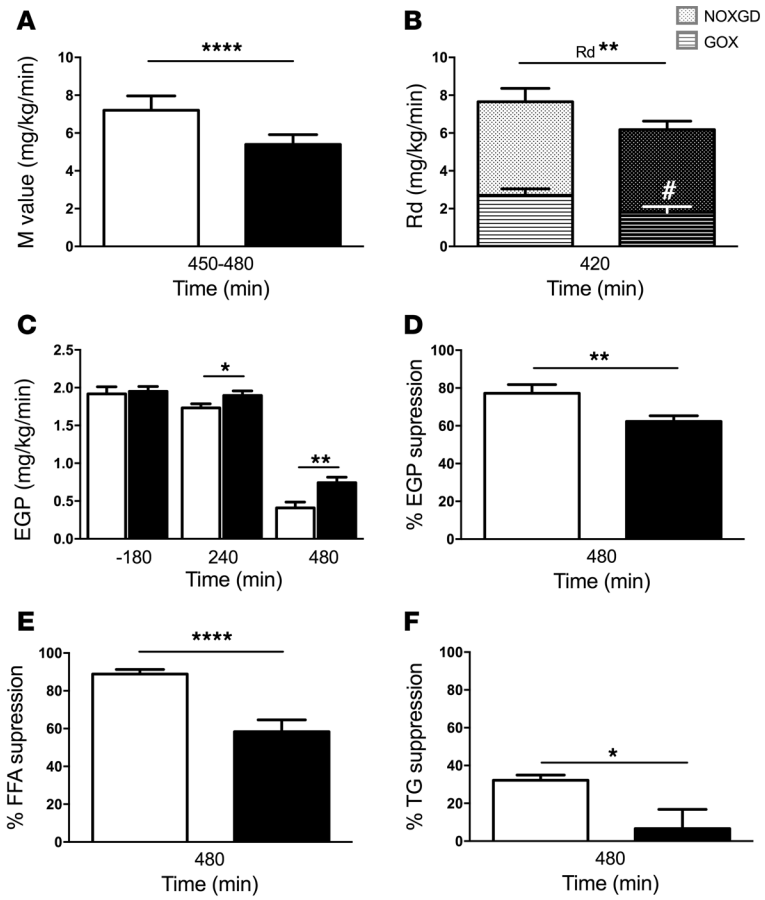

Figure 3. Parameters of insulin resistance in human volunteers after VCL or PO during clamp experiments. VCL, white bars; PO, black bars. (**A**) WBIS, reflected by the M value. (**B**) Rd and its components GOX and nonoxidative glucose disposal (NOXGD). (**C**) EGP denoting hepatic insulin sensitivity at baseline (–180 min), after PO or VCL ingestion (240 min), and under insulin-stimulated conditions during the clamp (480 min). (**D**) Insulin-induced EGP suppression as an indicator of hepatic insulin sensitivity. (**E**) Insulin-induced FFA suppression reflecting adipose tissue insulin sensitivity and (**F**) the percentage of insulin-induced TG suppression. Data shown represent the mean ± SEM. $n = 14$. $^{****}P = 0.0005$, $^{**}P < 0.01$, and $^{*}P < 0.05$; $^{\#}P < 0.05$, for GOX PO versus VCL. *P* values were determined by 2-tailed *t* test and ANOVA.

upregulation of hepatic mitochondrial capacity in obese, insulin-resistant, but not nondiabetic, humans in the absence of progressive NAFLD (35) and that T2DM patients have decreased ATP turnover associated with increased HCL (36). Collectively, these data suggest that an acute rise in lipid availability and oxidation can upregulate γATP production and HCL deposition in young, healthy individuals with conserved mitochondrial plasticity, even after the development of acute insulin resistance. The minor increase in HCL has to be considered in the context of lean, insulin-sensitive individuals. In this case, it is reasonable to assume that an increase in HCL after just 1 dose of PO probably contributes to altered hepatic metabolism. Furthermore, PO, like meal ingestion, probably induced individual time courses of increases, so that maximum γATP increases may have been missed in the absence of continuous magnetic resonance spectroscopy (MRS) monitoring, which would have been impossible, given the current experimental design.

Upon PO ingestion, human volunteers developed generalized insulin resistance, while mice responded primarily with hepatic insulin resistance. This may have resulted from species differences in insulin-sensitive tissues and lipid flux–buffering capacities of adipose tissue. Under physiological conditions, adipose tissue can buffer fatty acid flux into the bloodstream, thereby avoiding excessive exposure to lipotoxic stimuli. This is accomplished by suppression of FFA and TG release as well as increased clearance of circulating TG (37). Under the present experimental conditions, the high-dose PO likely impaired all 3 mechanisms in humans and to some extent in mice. The higher plasma TG concentrations observed in our study could have been due to higher rates of intestinal lipid absorption, altered hepatic lipid handling, or lower insulin sensitivity of the adipose tissue, as reflected by reduced insulin-induced suppression of FFA. Humans and mice both featured impaired insulin-induced TG suppression after PO ingestion. This is partly in line with findings in humans after a fat-free test meal, in which T2DM patients were also unable to suppress plasma TG in contrast to lean, healthy men (38). The inability of our cohort to suppress TG secretion may have therefore at least partly resulted from an altered apolipoprotein metabolism associated with insulin-resistant states and with decreased insulin-mediated fatty acid trapping by adipose tissue (37). In order to maintain low levels of HCL, the liver can only make use of either LOX or of lipid export of apolipoproteins (39). Here, we show that a single dose of PO induces both mechanisms, but nonetheless leads to increased HCL content. Of note, these changes occurred in the face of increased C-peptide, but unchanged insulin concentrations. The mismatch between insulin and C-peptide concentrations is likely explained by increased insulin clearance and turnover. While lower concentrations of palmitate decrease receptor-mediated insulin degradation in rat hepatocytes, higher concentrations of palmitate concentrations enhance this degradation (40). This can also explain the lack of increased insulin concentrations in the face of increased GLP-1 and GIP. In humans, most studies on insulin extraction used infusions of mainly polyunsaturated lipids, which revealed either unchanged or lower splanchnic insulin extraction (17, 41). Likewise, oral intake of soybean oil containing 61% polyunsaturated fatty acids resulted in increased concentrations of both insulin and C-peptide (19).

The marked alterations in hepatic glucose fluxes in humans and the predominant hepatic insulin resistance in mice raise interest in the effects of saturated fatty acids on hepatic gene expression, which has been previously examined mostly upon exposure to polyunsaturated fatty acids (42). The present study showed that a single dose of PO differentially regulated the canonical pathways TWEAK and AHR. The AHR pathway promotes NAFLD via upregulation of fatty acid transport (42), which is in line with the observed upregulation of *Fabp5* and increase in HCL content in humans. The TNF-related TWEAK is known to promote cell turnover homeostasis through the NF-κB and p38 MAPK pathways and could serve as a biomarker of obesity and T2DM (43). TWEAK was also found to be associated with reduced TG accumulation in pal-

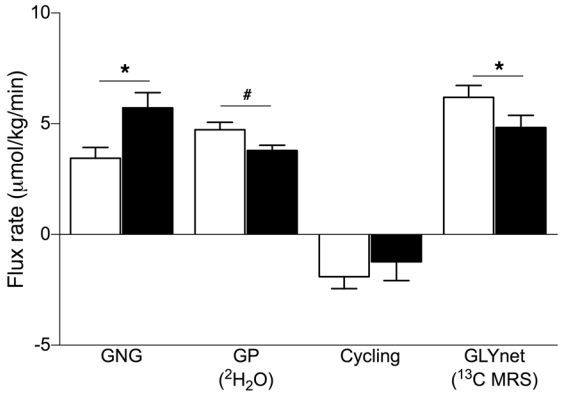

Figure 4. Hepatic glucose fluxes in humans. The rates of GNG, GP flux, glycogen cycling (cycling), and GLYnet were analyzed using in vivo $^{13}C/^{31}P/^{1}H$ and ex vivo ^{2}H MRS combined with $^{2}H_2O$ ingestion, after either VCL (white bars) or PO (black bars) treatment, in lean, insulin-sensitive, male volunteers. Data represent the mean ± SEM. $n = 14$; GP and cycling $n = 9$. $^{*}P < 0.05$ and $^{\#}P = 0.085$, by 2-tailed t test.

mitic acid–treated hepatocytes and may be involved in hepatic tissue repair (44). The present study cannot prove the activation status of TWEAK, thus making it difficult to interpret the observed downregulation of the *Tweak* gene and its receptor (Tnfrsf12a). However, recent findings of decreased circulating TWEAK concentrations correlating with obesity and concomitant NAFLD (45) could suggest that inhibition of the TWEAK pathway might increase susceptibility to hepatic injury.

The expression analyses further predicted PO-induced activation of LPS with or without insulin stimulation. This study also found upregulation of *Ifit3*, *Clec4a3*, *Slc22a3*, and *C3ar1*, genes regulated by LPS-stimulated macrophages. These data are in line with the reported increase in LPS concentrations upon high-fat ingestion (28, 46) resulting from lipid-induced disruption of the intestinal barrier (28). Interestingly, LPS is also known to decrease TWEAK signaling (47).

Downstream from LPS, NF-κB was found to be differentially regulated by PO. Generally known for its proinflammatory properties, NF-κB is also an important antiapoptotic factor (48, 49). LPS activation and TNF collectively lead to increased TLR4 expression, proinflammatory cytokine production, and inflammation, on the one hand, and NF-κB activation and cytoprotection on the other (48). NF-κB activation leads to modest and short-lived JNK activation, in turn inducing antiapoptotic genes, such as c-FLIP (a caspase 8 inhibitor) and X-linked inhibitor of apoptosis (48). As a result, the active NF-κB pathway is critical for LPS-induced resistance to hepatotoxicity (50). Additionally, a high-fat diet and obesity are associated with prolonged JNK activation and TNF-induced cell death (51, 52). This alludes to an adaptation, which is lost upon repeated and/or sustained exposure to hepatotoxic stimuli leading to NAFLD and steatohepatitis. Furthermore, the constitutive activation of NF-κB has been associated with severe hepatic and moderate peripheral insulin resistance (53). The present data suggest that a single PO challenge promotes pathways of LPS- and TLR4-mediated inflammation and cytotoxicity, which are buffered by the activation of NF-κB, which in turn contributes to insulin resistance. This study also found altered regulation of other putative cytoprotective mechanisms including the phospholipase C4 pathway, which is important for hepatic regeneration (54), and PPARα, which serves as both a canonical pathway and an upstream regulator protecting against NAFLD progression (29).

Our analyses of the differential regulation of single genes by saturated fat revealed several genes of interest. The observed upregulation of *G0s2* may contribute to decreased TG clearance, thereby promoting NAFLD (55). The observed greater expression of *Arl6* in the present study may also serve to protect against NAFLD,

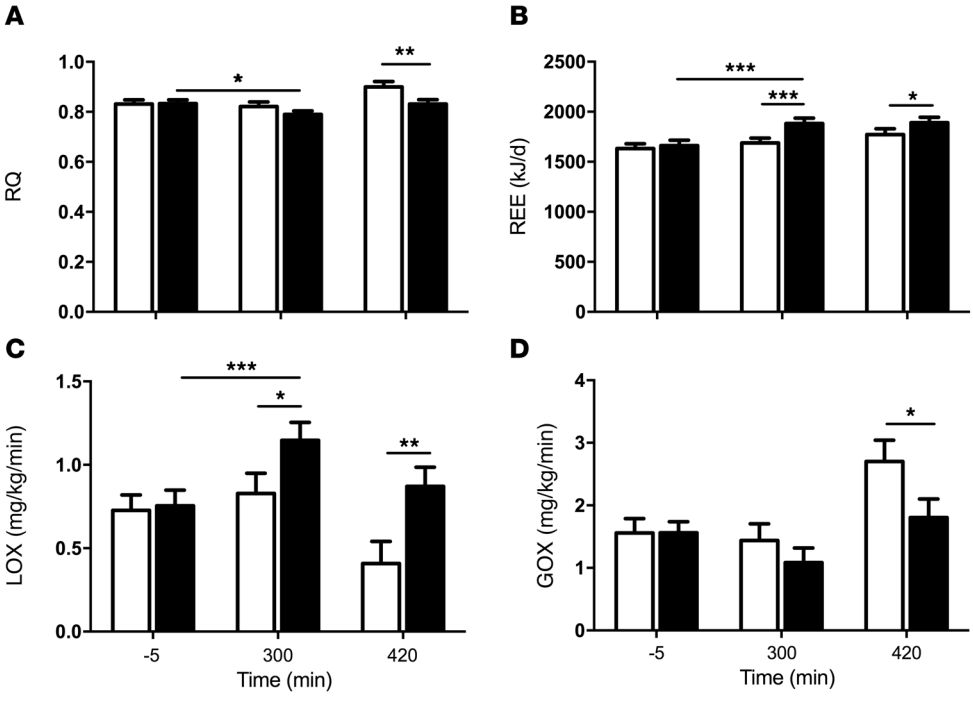

Figure 5. Time course of parameters of energy metabolism in lean, healthy volunteers after VCL and PO. VCL, white bars; PO, black bars. Parameters were obtained by performing an indirect calorimetry. The time points indicated are basal (–5 min), 300 minutes after intervention, and 420 minutes under insulin-stimulated conditions. Effects on RQ (**A**), REE (**B**), LOX (**C**), and GOX (**D**). Data represent the mean ± SEM. $n = 14$. $^{***}P < 0.001$, $^{**}P < 0.005$, and $^{*}P < 0.05$, by ANOVA.

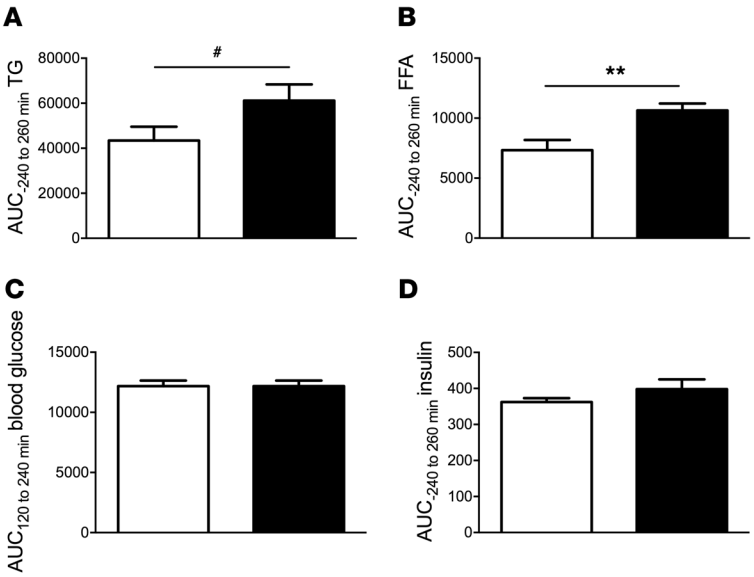

Figure 6. Circulating metabolites and hormones in mice. VCL (white bars) or PO (black bars) was administered via gavage to identical mouse cohorts at minute 0, after a 6-hour fast. Hyperinsulinemic-euglycemic clamp experiments were performed from 120 to 240 minutes. (**A**) The TG AUC tended to be higher after PO than after VCL. (**B**) The FFA AUC was increased after PO administration. Blood glucose (**C**) and insulin (**D**) levels were not different between groups. Data represent the mean ± SEM. n = 6–10. $^{**}P < 0.005$ and $^{\#}P = 0.08$, by 2-tailed t test.

because loss of function of this gene has been implicated in obesity, NAFLD, and diabetes in Bardet-Biedl syndrome (56). Of note, the descriptive nature of the transcriptome analyses and expression profiling carried out in our study does not allow final conclusions to be drawn as to causality. That is to say, given the results of this study, it is impossible to single out 1 gene, transcription factor, or pathway that is activated by acute exposure to PO and that can be causally linked to either insulin resistance or steatosis.

This study offers the advantages of the translational approach in mice and humans, the use of near-physiologic administration of saturated fat, and the comprehensive phenotyping of in vivo hepatic glucose and energy metabolism. This study's limitations include the relatively small number of humans and mice, which might have prevented the detection of discrete effects of an acute lipid challenge. Nevertheless, the results demonstrate marked metabolic and transcriptional changes associated with PO treatment, even in these small groups. Another caveat of this study is that the hepatic expression data obtained in mice are not necessarily transferable to humans, as we could not obtain liver specimens from our human participants for ethical reasons. The lack of proteomic analysis of the targets identified by Ingenuity software constitutes another limitation of this study. Each pathway and regulator necessitates a thorough study of their role in the pathogenesis of insulin resistance and NAFLD, a task that must be tackled in subsequent studies.

The practical implication of this work is that the PO challenge used in this study most likely resembles the effects of ingestion of a meal rich in saturated fat, e.g., an 8-slice pepperoni pizza, containing approximately 16.72 g of saturated fat/110 g (57) or a meal consisting of a 110-g cheeseburger and a large portion of French fries, containing 18–25 g and 7–14 g per 1,000 kcal of saturated fat, respectively (58). One such meal would probably be sufficient to induce transient insulin resistance and impair hepatic metabolism, which necessitates the activation of hepatic defense mechanisms. Other simultaneously ingested biomacromolecules would exacerbate this metabolic challenge (59). The amount and types of fatty acids and carbohydrates in one such meal are in contrast to the diet recommendations of the American Diabetes Association (ADA). In this diet, daily intake of saturated fatty acids should not exceed 10% of total caloric intake. Furthermore, intake of monounsaturated fatty acids and carbohydrates from vegetables, fruits, whole grains, and legumes is recommended (60). We presume that lean, healthy individuals are able to compensate adequately for excessive intake of saturated fatty acids, however, sustained and repeated exposure to such nutrients will ultimately lead to chronic insulin resistance and NAFLD. Recent studies have shown hepatic energy metabolism alterations and induction of insulin resistance in obese and lean patients after ingestion of a simple hypercaloric mixed meal containing 24 g of fat or a drink containing 1 g/kg BW of a mixture of palmitate and soybean oil (34, 61, 62). These results suggest that even lower doses of fatty acids are capable of inducing alterations similar to those observed with ingestion of pure PO.

In conclusion, the initial effects of ingestion of saturated fat include (a) augmented hepatic energy metabolism and lipid storage; (b) impaired hepatic insulin sensitivity, along with increased GNG flux; and (c) altered hepatic expression of genes regulating inflammatory and protective pathways, which predispose to and protect against the development of NAFLD.

Methods

Studies in humans

Volunteers. Fourteen lean, young male volunteers were enrolled in this randomized, controlled crossover trial (Figure 1 and Figure 9). Participants were recruited from March 2012 through December 2013. The sample size calculation was based on a 2-sided, paired t test, assuming a mean difference of EGP of 0.1 and a standard deviation of 0.11, resulting in a sample size of 12 to reach a power of 80%. The random allocation sequence was generated using SAS software (SAS Institute) by our in-house statistician. The possible order of treatments was randomly permuted in 2 blocks, with 1 extra block being generated to account for dropouts. Allocation was not concealed. Participants were enrolled and assigned to their treatment order by the study phy-

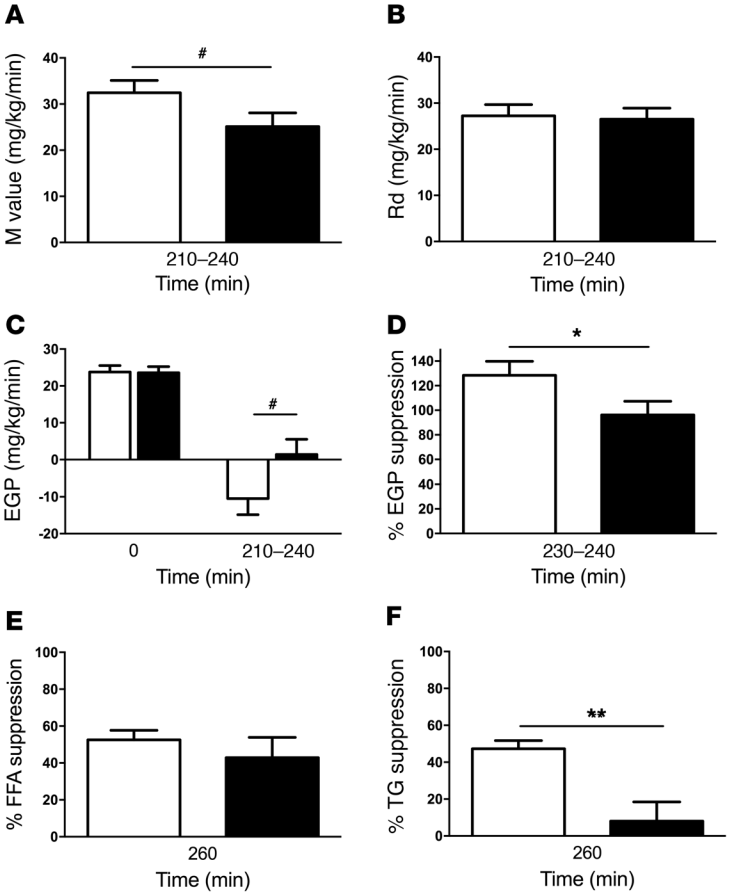

Figure 7. Parameters of insulin resistance in mice. VCL (white bars) or PO (black bars) was administered via gavage to identical mouse cohorts at minute 0, after a 6-hour fast. Hyperinsulinemic-euglycemic clamp experiments were performed from 120 to 240 minutes. The M value trended toward a reduction after PO (**A**), while the Rd was unchanged (**B**). EGP at basal (0 min) and at clamp steady state (210–240 min) (**C**). EGP suppression was impaired after PO (**D**). Insulin-induced FFA suppression (**E**) and TG suppression (**F**) are also shown. Data represent the mean ± SEM. n = 9–10. $^{**}P < 0.005$, $^{*}P < 0.05$, and $^{\#}P = 0.07$, by 2-tailed t test.

sician. Inclusion criteria were age between 20 and 40 years and a BMI between 20 and 25 kg/m^2. Exclusion criteria included dysglycemia, a family history of T2DM, acute, or chronic diseases, and the use of pharmacological agents known to affect insulin sensitivity, lipid metabolism, or immunological function. All participants underwent screening that included recording of clinical history, physical examination, bioimpedance assessment of lean body mass, routine laboratory tests, and a 75-g oral glucose tolerance test. Upon inclusion, participants were instructed to maintain their usual physical activity during the study period and to ingest a carbohydrate-rich diet for 3 days before each study day. They were then randomly assigned to 1 intervention and 8 weeks later to the other intervention. Basal hepatic ATP and HCL values from the study by Gancheva et al. were used as part of the control group data (63).

Experimental design. Participants arrived at the Clinical Research Center at 5 pm and received a standardized dinner containing approximately 684 kcal at 5:30 pm. At 8:00 pm, 10:00 pm, and 12:00 am, volunteers drank 1.6 g/kg body water of ^2H_2O (99.9%; Sigma-Aldrich), up to a total dose of 5 g/kg body water in order to assess GNG (19) (Figure 9). Body water was assumed to be 60% in all of our male study cohort participants (30). Starting at 5:00 am (defined as time point –180 minutes) of the next day, participants drank 200 ml of water containing 0.5% ^2H_2O every 60 minutes throughout the experiment to maintain an isotopic equilibrium of body water. At –180, 200, and 400 minutes, participants received an oral dose of acetaminophen (500 mg). For EGP calculation, participants received a 5-minute priming bolus (0.36 mg/kg BW/min × fasting plasma glucose [mg/dl]) of D-[6,6-$^2H_2$]glucose (99% enriched in ^2H glucose; Cambridge Isotope Laboratories) at –180 minutes, followed by a continuous infusion (0.036 mg/kg BW/min) (64). At zero time, participants drank either VCL or PO within 10 minutes. Patients with more than 70 kg BW drank 92 g, and those with less than 70 kg BW drank 80 g of PO (~1.18g/kg BW; Biopalm; Landkrone) (65). To facilitate ingestion, PO was heated to 60°C, mixed with 1.84 g or 1.6 g emulsifier (Glice, Texturas; Albert y Ferran Adria), 9 or 8 g sugar-free vanilla syrup (Torani), and 81.2 or 70.4 ml bottled still water, for a PO mix of 92 g and 80 g, respectively. Oil test drinks were stirred constantly and served hot. For VCL administration, PO was substituted with 173.2 ml or 150.4 ml bottled still water, respectively. At 360 minutes, a hyperinsulinemic-euglycemic clamp began (10-min insulin bolus: 40 U/hour; continuous insulin infusion: 40 mU/m^2/min; Insuman Rapid; Sanofi). Blood glucose concentration was adjusted to 90 mg/dl by adapting the glucose infusion rate (GIR) using 20% glucose (B. Braun AG) enriched with 2% D-[6,6-$^2H_2$]glucose, as described previously (19). Urine was sampled from –120 to 0 minutes, from 270 to 375 minutes, and from 390 to 510 minutes for the quantification of GNG and GLY. Blood was sampled at –60 and 360 minutes for assessment of GNG.

Indirect calorimetry. Indirect calorimetry (IC) was performed in the canopy mode using Vmax Encore 29n (CareFusion), as described previously (19), during baseline (at –170 min), intervention (at 200 min), and steady-state clamp conditions (at 450 min) for 20 minutes after a 10-minute adaptation period. RQ, REE, and substrate oxidation rates were calculated as reported previously (19). Nonoxidative glucose disposal was calculated from the difference between rates of glucose disappearance and carbohydrate oxidation.

Metabolites and hormones. Blood samples were immediately chilled, centrifuged, and the supernatants stored at either –20°C or –80°C until analysis. Venous blood glucose concentration was measured immediately using the glucose oxidase method (EKF Biosen C-Line glucose analyzer; EKF Diagnostics) (19). TG concentration was analyzed enzymatically on a Roche Cobas c 311 Analyzer (Roche Diagnostics). Serum chylomicron content was determined from the TG concentration in the first fraction of density-gradient ultracentrifugation (66). FFA were assayed enzymatically (Wako) using orlistat to prevent in vitro lipolysis (19). Serum C-peptide, insulin, and plasma glucagon levels were measured by radioimmunoassay (EMD Millipore). Cortisol levels in serum samples were measured by immunoassay using a Siemens Immulite 2000XPi Analyzer (67). GLP-1 and GIP were measured by ELISA (TECOmedical; EMD Millipore) (64). ELISA was used to measure plasma concentrations of IL-6, TNF-α, fetuin-A (all using Quantikine HS ELISA kits from R&D Systems), omentin, and chemerin (19) (both using ELISA kits from BioVendor). Intra-

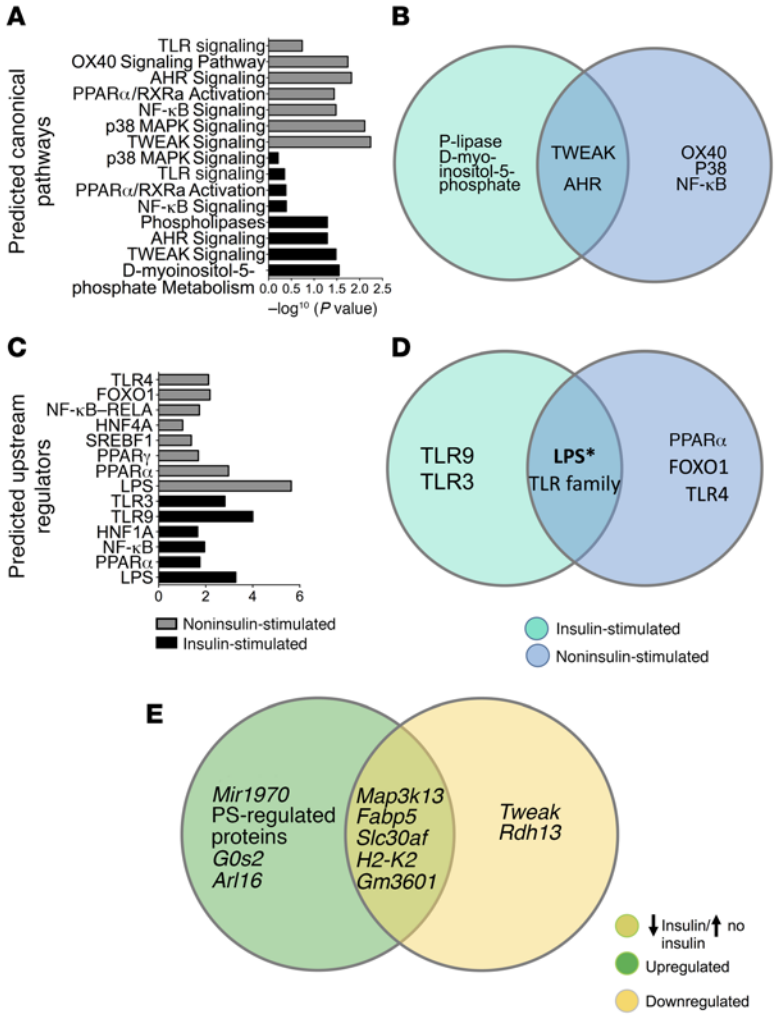

Figure 8. Transcriptome analysis after PO in murine hepatic tissue. Tissue was harvested from clamped and nonclamped murine cohorts. (**A**) Relevant canonical pathways predicted by Ingenuity software in hepatic samples from mice after PO under insulin- (black bars) and noninsulin-stimulated (gray bars) conditions. (**B**) Predicted canonical pathways with a *P* value of less than 0.05 during insulin-stimulated (turquoise) and noninsulin-stimulated conditions (blue). (**C**) Noteworthy predicted upstream regulators and (**D**) predicted upstream regulators with a *P* value of less than 0.01 (asterisk denotes an activation *Z* score above 1.9). (**E**) Genes that were upregulated in both cohorts (green), downregulated in both cohorts (yellow), and downregulated under insulin-stimulated, but upregulated under noninsulin-stimulated, conditions (green and yellow, respectively) after PO treatment, with a *P* value of less than 0.05, a fold change greater than 1.3, and an average expression in at least 1 group of greater than 4 (n = 9–10, 273, and 327 probe sets, respectively).

assay and interassay coefficients of variation (CV) for all cytokines were 2% to 7.2% and 4% to 14.4%, respectively.

Glucose and glucuronide ^2H enrichment measurements by ex vivo ^2H MRS. The positional enrichment of urinary acetaminophen glucuronide and plasma glucose, resulting from ingestion of ^2H_2O and acetaminophen at the level of glucose 6-phosphate (G6P), was assessed as previously described (30) to estimate the contributions of GNG and GLY to EGP. Plasma glucose was derivatized to monoacetone glucose (MAG), while urinary acetaminophen glucuronide was converted into 5-*O*-acetyl monoacetone glucuronic lactone (MAGLA) (68). When plasma glucose enrichment was inadequate, urinary glucuronide enrichment was analyzed instead, since both methods yield identical estimates of EGP contributions (68, 69). In total, 9 participants yielded sufficient data for NMR analysis, 5 of them for glucuronide. ^2H spectra were obtained with a Bruker Avance III HD 500 spectrometer equipped with a ^2H-selective 5-mm probe incorporating a ^{19}F lock channel. For MAGLA samples, 5,000–10,000 free-induction decays (FIDs) were collected. For MAG samples, 20,000–40,000 FIDs were collected. Positional ^2H enrichments of MAG and MAGLA derived from plasma glucose and urinary anion gap (AG) were determined using the methyl signals as an intramolecular standard (30). All spectra were analyzed using the curve-fitting routine supplied with the NUTS PC-based NMR Spectral Analysis Program (Acorn NMR).

Gas chromatography–mass spectrometry. Determination of atom percentage enrichment (APE) of ^2H in blood glucose was done after deproteinization and derivatization to the aldonitrile-pentaacetate. The analyses were performed on a Hewlett-Packard 6890 gas chromatograph equipped with a 25 m/0.25 mm/0.12 μm CPSil5CB capillary column (Chrompack; Varian) and interfaced with a Hewlett Packard 5975 mass selective detector. Selected ion monitoring was used to determine enrichments of fragments C3 to C6 (Supplemental Figure 1, A and B). Average mass units were 187 for endogenous glucose and 189 for D-[6,6-$^2H_2$]glucose as described previously (19).

MRS. All measurements were conducted with the volunteers lying in a supine position within a whole-body 3.0 T Achieva MRI machine (Philips Healthcare). Twelve volunteers were studied, including all participants for whom flux measurements were obtained. The effects of PO or VCL on HCL and hepatic ATP concentrations were assessed at baseline and 360 minutes after intervention. For hepatic ^1H MRS, a Q-body coil was used for shimming and HCL acquisition. Clinical T2-weighted turbo spin-echo (TSE) images were obtained in the transverse and coronal planes for localization and repositioning of the voxels used for HCL and ATP measurements. Respiratory-triggered ^1H spectra were acquired with a single-voxel (30 × 30 × 20 mm^3) stimulated echo acquisition mode (STEAM) sequence. The variables were as follow: repetition time (TR) 3,000 ms, echo time 10 ms, and signal averages 16. To accu-

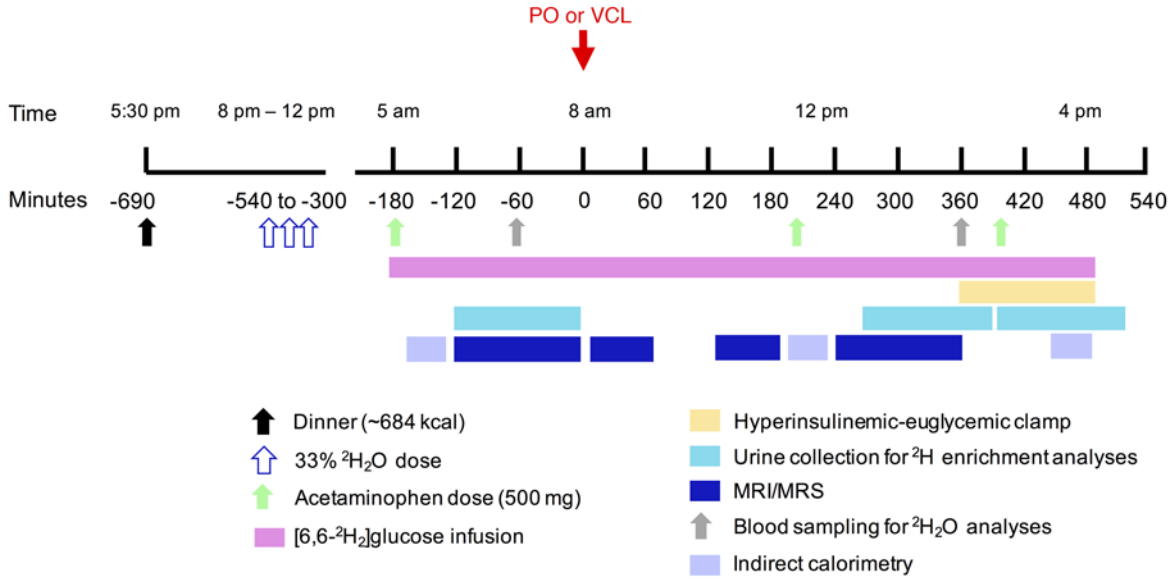

Figure 9. Human study design. Lean, healthy male adults randomly received either PO or VCL on 2 occasions. Hepatic metabolism was measured using in vivo ^{13}C, ^{31}P, ^1H and ex vivo ^2H MRS combined with ^2H_2O and acetaminophen ingestion before and during hyperinsulinemic-euglycemic clamps with D-[6,6-$^2H_2$]glucose–labeled 20% glucose infusion.

rately assess hepatic lipid volume, sets of non–water-suppressed and water-suppressed ^1H spectra were acquired, using a STEAM sequence (TR 3,000 ms, echo time 10 ms, signal averages 16 ms) and variable power and optimization relaxation (VAPOR) STEAM sequences (TR, echo time, and signal averages 3,000, 10, and 16 ms, respectively). Water and lipid peaks were fitted and quantified using the NUTS software package (Acorn NMR), and lipid was expressed as the summation of the methyl and methylene fat peaks relative to water content using the equations described in ref. 70. For hepatic ^{31}P MRS, a 14-cm ^{31}P circular surface coil (Philips Healthcare) was placed over the liver for the acquisition of hepatic ^{31}P spectra. Afterwards, ^{31}P-MRS proton-decoupled liver ATP measurements were conducted with a 3D image-selected in vivo spectroscopy (3D-ISIS) localized sequence (voxel: 60 × 60 × 60 mm^3, TR: 6,000 ms, averages: 128, decoupling: WALTZ-4 [wideband alternating-phase low-power technique for zero-residual splitting –4], time: 13 min). Absolute concentrations of γATP (CV = 9%) and Pi (CV = 7%) were quantified using the jMRUI v4.0 software package (EC Human Capital and Mobility Networks, France), as described previously (19). Liver volume measurements were made from the coronal plane T2-weighted TSE images. For liver glycogen measurements, ^{13}C spectra were obtained with a 7-cm dual-tuned $^{13}C/^1H$ coil (PulseTeq Ltd.) (68), via a proton-decoupled pulse acquisition sequence (TR: 230 ms; bandwidth: 8 KHz, averages: 2 × 4,000; data points: 256; decoupling: continuous wave; time: 30 min). Liver glycogen spectra were acquired with a block pulse (333 μs) that produced an Ernst angle at a distance of 35 mm. Coil loading was corrected via integration of the right-most peak of a ^{13}C-enriched sample of formic acid placed within the coil housing. Glycogen concentrations were determined from the integration of the C1-glycogen resonance (zero filling: 4k, effective line broadening: 20 Hz) after the addition of 2 scans and baseline correction within NUTS software (Acorn NMR Inc.). The glycogen signal was corrected for distance and quantified via aqueous glycogen phantom measurements of 70 and 140 mmol/l measured at a distances of 15 to 37 mm. The CV from repeated hepatic glycogen measurements was 6%.

Calculations. Total AUC were calculated using the trapezoidal method. FFA and TG suppression was calculated from 100 – (clamp FFA or TG concentrations × 100)/basal FFA or TG concentrations, respectively. Glycogen cycling, i.e., simultaneous fluxes through glycogen synthase (GS) and GP, was assessed. In order to measure glycogen cycling, isotopic tracer measurements of EGP, GNG, and GP fluxes must be supplemented by a measurement of GLYnet (in this case, ^{13}C MRS) (71). GLYnet was calculated from the linear regression of hepatic glycogen content at –2, 0, 2, and 4 hours of PO or VCL ingestion using the least mean squares method. Rates of GLYnet were normalized to liver volume and BW and are expressed in μmol/kg/min (61).

Whole-body glucose disposal (M value) was calculated from glucose infusion rates during the clamp steady state. The rates of EGP (in μmol/kg/min) were calculated by dividing the tracer infusion rate of D-[6,6-$^2H_2$]glucose times its enrichment to the hydrogens bound to carbon 6, by the mean percentage of enrichment of plasma D-[6,6-$^2H_2$]glucose and then subtracting the tracer infusion rate (19). To account for the incorporation of ^2H from ^2H_2O by GNG during the overnight fast, background D-[6,6-$^2H_2$]glucose was determined before administration of ^2H_2O as well as at –185 minutes on the day of the study. Consequently, for determination of basal EGP and EGP at the end of the intervention, the background D-[6,6-$^2H_2$]glucose enrichment from the –185-minute time point was used for calculations, whereas, during clamp conditions, with GNG being close to zero, the D-[6,6-$^2H_2$] value before administration of ^2H_2O was used. GNG (in μmol/kg/min) was calculated from the difference between EGP and GLYnet. The fractional GP flux contribution to EGP, i.e., the fraction of EGP originating from GLY, was calculated as 1 – (H5/H2); where H5/H2 is the ratio of glucuronide position 5 to position 2 enrichment from ^2H_2O, keeping in mind that glucose derived from GLY is enriched with ^2H in position 2, while glucose derived from GNG is enriched in positions 5 and 2. Absolute GP flux (in μmol/kg/min) was calculated from the equation: GP = EGP × (1 – H5/H2). Glycogen cycling was then calculated as: GP – GLYnet.

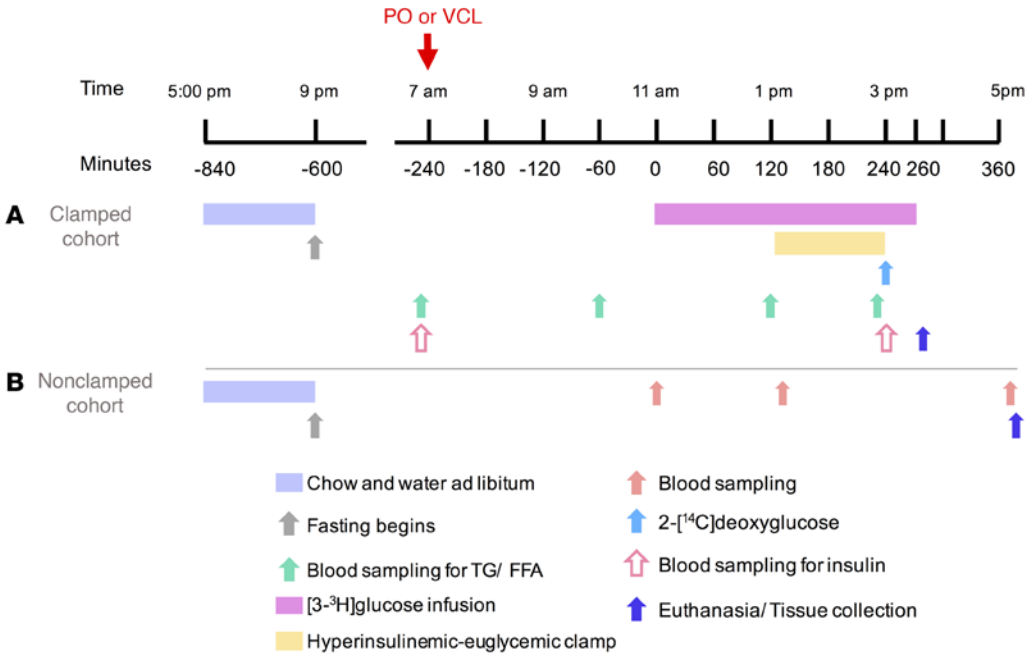

Figure 10. Mouse study design. Lean, adult male C57BL/6NTac mice were matched for BM and littermates and then divided into 2 cohorts. One cohort underwent hyperinsulinemic-euglycemic clamps after receiving either PO or vehicle via gavage (**A**), whereas another identical cohort underwent analysis of tissue and blood samples (**B**).

Studies in mice

Animals. Studies were conducted in lean, male, 14 week-old C57BL/6NTac mice (Taconic). Animals had ad libitum access to water and a standard chow diet. Mice were kept on a low-fat (LF) diet (13% of calories derived from fat, 17 kJ/g, Standard Diet 1310; Altromin), and were matched for body mass (BM) and littermates. BM and composition (MiniSpec LF50; Bruker Optics) were measured 1 day prior to the start of the experiment. Animals were bred and housed in a temperature- and humidity-controlled environment including a 12-hour light/12-hour dark cycle, in compliance with the Federation of European Laboratory Animal Science Associations protocols.

Experiments under insulin-stimulated conditions. A permanent jugular vein catheter was placed under ketamine/xylazine anesthesia into a cohort of 19 mice with the aforementioned characteristics. Six to seven days later, the mice were fasted for 10 hours and then received 2 g/kg BM PO or VCL via gavage. Six hours later, unrestrained, conscious mice underwent hyperinsulinemic-euglycemic clamps.

After 110 minutes of primed-continuous [3-^{3}H]glucose infusion (1.85 kBq/min), a blood sample was collected to determine plasma insulin, glucose, and [3-^{3}H]glucose concentrations for the calculation of basal EGP. A [3-^{3}H]glucose infusion (3.7 kBq/min) containing insulin (15 pmol/kg/min; HumulinR; Lilly) was started. Blood glucose concentrations were measured every 10 minutes and target glycemia established by adjusting the GIR. At minute 120, 2-[^{14}C]deoxyglucose (370 kBq) was injected intravenously to assess tissue-specific Rg rates. At the end of the experimental procedure, mice were euthanized by means of an intravenous ketamine/xylazine injection. Livers were collected, immediately snap-frozen in liquid nitrogen, and stored at –80°C. Blood was collected at culling, and plasma ^{3}H and ^{14}C radioactivity was determined in deproteinized plasma before and after ^3H_2O evaporation to estimate glycolysis rates. In hepatic lysates, 2-[^{14}C]deoxyglucose-6-phosphate was separated from 2-[^{14}C]deoxyglucose via ion-exchange columns (Poly-Prep AG1-X8; Bio-Rad) as previously described (72). Glucose uptake was calculated by multiplying the mean plasma glucose levels between 120 and 140 minutes of the clamp (mmol/ml) by 2-[^{14}C]deoxyglucose tissue content (dpm/100 g tissue), divided by the 2-[^{14}C]deoxyglucose plasma AUC in the same time frame. Radioisotopes were purchased from PerkinElmer and samples measured in an Ultima-Gold Scintillation Cocktail (TriCarb2910TR; PerkinElmer) (Figure 10). Whole-body glucose disposal (M value) was calculated from the tracer infusion rate, the specific activity of [3-^{3}H]glucose, and BW.

Biochemical analyses. Blood glucose concentrations were assessed using a Contour hand-held glucometer (Bayer Vital). Plasma TG levels were determined by a colorimetric assay (Cayman Chemical), and plasma FFA levels were assessed with the FFA-HR(2)-Test (Wako). Hepatic TG levels were measured in whole-liver homogenates biochemically with the BioVision Assay.

Experiments under noninsulin-stimulated conditions. C57BL/6NTac mice (n = 20) with characteristics identical to those described above were fasted for 10 hours and given 2 g/kg BM PO (Landkrone) or water (VCL) per gavage. Lateral tail vein blood samples were obtained prior to treatment and 2 hours afterward. Six hours after treatment, mice were euthanized with isoflurane, and a vena cava blood sample was collected and centrifuged at 4°C, and plasma aliquots were immediately frozen in liquid nitrogen. Liver was dissected and immediately snap-frozen in liquid nitrogen (Figure 10).

RNA isolation

Snap-frozen liver samples from both cohorts were processed after administration of PO. Total RNA was isolated using the mRNeasy Mini Kit (QIAGEN). The Agilent 2100 Bioanalyzer was used to assess

RNA quality, and only high-quality RNA (RNA integrity number [RIN] >7) was used for microarray analysis.

Expression profiling

Total RNA (~30 ng) was amplified using the Ovation PicoSL WTA System V2 in combination with the Encore Biotin Module (both from NuGEN). Amplified cDNA was hybridized on Affymetrix Mouse Gene 2.1 ST array plates containing approximately 35,000 probe sets. Staining and scanning (GeneChip Scanner 3000 7G; Affymetrix) were performed according to the Affymetrix Gene Titan expression protocol and modified according to NuGEN's Encore Biotin protocol.

Statistical transcriptome analysis

Expression Console software (version 1.3.0.187; Affymetrix) was used for quality control and to obtain annotated normalized robust multiarray average (RMA) gene-level data (standard settings included median polish and sketch-quantile normalization). Statistical analyses were performed using the statistical programming environment R implemented in CARMAweb (https://carmaweb.genome.tugraz.at/carma/). Genewise testing for differential expression was done using the limma *t* test, and a *P* value of less than 0.05 was set as the threshold to define sets of regulated genes. Filters for a fold change greater than 1.3 times and a linear average expression greater than 4 were applied. Pathway analyses were generated using the Ingenuity Pathway Analysis (QIAGEN; www.qiagen.com/ingenuity), where the overlapping *P* value identifies transcriptional regulators that can explain observed gene expression changes. The activation *Z* score helps infer activation states of predicted transcriptional regulators, with values of 2 or more indicating activation and values of –2 or less indicating inhibition.

General statistical analyses

Results are presented as the mean ± SEM and were compared using a 2-tailed Student *t* test or ANOVA adjusted for repeated measures, with Bonferroni's testing as appropriate. Calculations were performed using GraphPad Prism, version 6.02 (GraphPad Software). A *P* value of less than 0.05 was considered statistically significant, unless otherwise indicated.

Study approval

All participants provided written informed consent before inclusion in the study, which was performed according to the Declaration of Helsinki of 2013 and approved by the ethics board of the Heinrich Heine University Düsseldorf. All animal experiments were approved by the Upper Bavarian district government (AZ 55.2.1.54-2532-4-11).

Author contributions

MR initiated the investigation, led the clinical experiments and wrote, reviewed, and edited the manuscript. EÁH obtained and analyzed the data and wrote, edited, and reviewed the manuscript. SK obtained and analyzed the data, aided in designing the clinical study, and edited and reviewed the manuscript. AS obtained data and edited and reviewed the manuscript. PB designed the MRS study, obtained MRS data, and reviewed and edited the manuscript. EÁH, SK, AS, and PB contributed equally to this project. YK interpreted MRS data. BN researched the clinical data and reviewed and edited the manuscript. CB and FC performed derivatization experiments and enrichment analysis for ^{2}H-MRS. JGJ performed the ^{2}H-MRS analyses and reviewed and edited the manuscript. PN conducted analyses and reviewed and edited the manuscript. CH conducted laboratory analyses and reviewed and edited the manuscript. SN and JR supervised the mouse studies and edited and reviewed the manuscript. MI analyzed transcriptomics data and edited and reviewed the manuscript. JB and MHdA supervised transcriptome analyses and edited and reviewed the manuscript. All authors gave final approval of the version to be published.

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JCI insight

Monounsaturated fat rapidly induces hepatic gluconeogenesis and whole-body insulin resistance

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While saturated fat intake leads to insulin resistance and nonalcoholic fatty liver, Mediterranean-like diets enriched in monounsaturated fatty acids (MUFA) may have beneficial effects. This study examined effects of MUFA on tissue-specific insulin sensitivity and energy metabolism.

A randomized placebo-controlled cross-over study enrolled 16 glucose-tolerant volunteers to receive either oil (OIL, ~1.18 g/kg), rich in MUFA, or vehicle (VCL, water) on 2 occasions. Insulin sensitivity was assessed during preclamp and hyperinsulinemic-euglycemic clamp conditions. Ingestion of $^{2}H_2O$/acetaminophen was combined with [6,6-$^{2}H_2$]glucose infusion and in vivo $^{13}C/^{31}P/^{1}H$/ex vivo ^{2}H-magnet resonance spectroscopy to quantify hepatic glucose and energy fluxes.

OIL increased plasma triglycerides and oleic acid concentrations by 44% and 66% compared with VCL. Upon OIL intervention, preclamp hepatic and whole-body insulin sensitivity markedly decreased by 28% and 27%, respectively, along with 61% higher rates of hepatic gluconeogenesis and 32% lower rates of net glycogenolysis, while hepatic triglyceride and ATP concentrations did not differ from VCL. During insulin stimulation hepatic and whole-body insulin sensitivity were reduced by 21% and 25%, respectively, after OIL ingestion compared with that in controls.

A single MUFA-load suffices to induce insulin resistance but affects neither hepatic triglycerides […]

Monounsaturated fat rapidly induces hepatic gluconeogenesis and whole-body insulin resistance

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BACKGROUND. While saturated fat intake leads to insulin resistance and nonalcoholic fatty liver, Mediterranean-like diets enriched in monounsaturated fatty acids (MUFA) may have beneficial effects. This study examined effects of MUFA on tissue-specific insulin sensitivity and energy metabolism.

METHODS. A randomized placebo-controlled cross-over study enrolled 16 glucose-tolerant volunteers to receive either oil (OIL, ~1.18 g/kg), rich in MUFA, or vehicle (VCL, water) on 2 occasions. Insulin sensitivity was assessed during preclamp and hyperinsulinemic-euglycemic clamp conditions. Ingestion of $^{2}H_2O$/acetaminophen was combined with [6,6-$^{2}H_2$]glucose infusion and in vivo $^{13}C/^{31}P/^{1}H$/ex vivo ^{2}H-magnet resonance spectroscopy to quantify hepatic glucose and energy fluxes.

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CONCLUSION. A single MUFA-load suffices to induce insulin resistance but affects neither hepatic triglycerides nor energy-rich phosphates. These data indicate that amount of ingested fat, rather than its composition, primarily determines the development of acute insulin resistance.

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Introduction

There is an ongoing debate as to whether nutrient quality or quantity is mainly responsible for the effects on health or disease (1). Currently, energy-dense foods, rich in saturated fatty acids (SAFA), are considered the main culprits of the epidemic rise of obesity, type 2 diabetes mellitus (T2D), and nonalcoholic fatty liver disease (NAFLD) (1). In contrast, Mediterranean diet, which is rich in monounsaturated fatty acids (MUFA), may lower the risk of T2D, NAFLD (2) and cardiovascular disease (3).

Authorship note: TS and SK contributed equally to this work.

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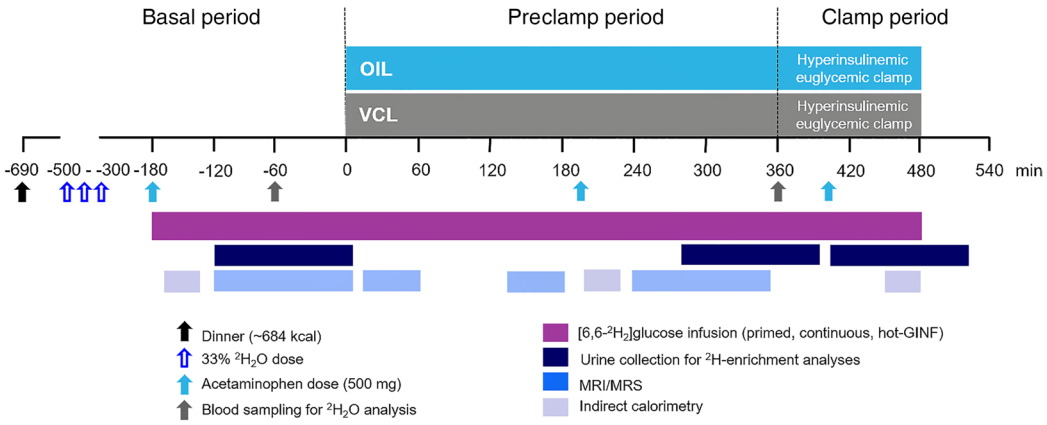

Figure 1. Study design. Participants randomly received either an oral dose of canola oil (OIL, blue) or an identical volume of water (vehicle [VCL]) on 2 occasions spaced by an 8-week period. Hepatic glucose and energy metabolism was measured by in vivo $^{13}C/^{31}P/^1H$ and ex vivo ^{2}H-magnetic resonance spectroscopy (MRS) combined with ^2H_2O and acetaminophen ingestion before and during hyperinsulinemic-euglycemic clamps, for which a "hot" glucose infusion (hot-GINF) protocol with [6,6-$^2H_2$]glucose was used.

Chronic high-fat diets cause hepatic triglyceride (TG) deposition and insulin resistance in humans (4). Of interest, fatty acid composition appears to play an important role for lipid-induced metabolic alterations, which is supported by the finding of higher liver TG content and insulin resistance with diets enriched in SAFA compared with those with MUFA, polyunsaturated fatty acids (PUFA), or simple sugars (4).

We recently demonstrated that a single oral SAFA-rich lipid load initiates hepatic insulin resistance (HEP-IR) and fat accumulation in healthy lean men (5), likely resulting from lipid-mediated inhibition of insulin signaling (6). This lipid load also raised hepatic gluconeogenesis (GNG), which is possibly due to lipid-induced allosteric stimulation of hepatic mitochondrial activity, as reported in rodent models (6). However, the acute effects of an identical amount of a MUFA-rich lipid load on hepatic glucose and energy metabolism still remain unclear (7). Moreover, the susceptibility to exogenous lipid-induced insulin resistance may differ between men and women, suggesting sex-specific metabolic differences upon unsaturated lipid administration (8).

Here, we therefore tested whether a MUFA-rich lipid load acutely induces insulin resistance in females and males by comparing the effects of a single oral dose of canola oil (OIL) versus placebo (vehicle [VCL]) on (a) tissue-specific insulin sensitivity during endogenous (preclamp) insulinemia and hyperinsulinemic-euglycemic clamp conditions as well as on (b) hepatic energy and glucose fluxes in healthy lean humans. To this end, we performed comprehensive real-time metabolic monitoring using stable isotopes and in vivo multinuclear and ex vivo ^{2}H-magnetic resonance (MR) techniques (Figure 1).

Results

OIL raises plasma TG and oleic acid concentrations during the preclamp period. Blood glucose and plasma insulin concentrations did not differ between OIL and VCL during both preclamp and clamp periods (Figure 1 and Figure 2, A and B). Total free fatty acids (FFA) were higher at +420 and +480 minutes (Figure 2C). During OIL intervention, plasma TG were higher compared with those during VCL from +120 minutes to +420 minutes (incremental area under the curve [iAUC] for TG, OIL vs. VCL, $P < 0.0001$; Figure 2D). At +360 minutes, plasma oleic acid was increased by 66% upon OIL intervention (+0 minutes to +360 minutes; $P = 0.017$), while other FFA concentrations did not change (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.134520DS1).

OIL does not acutely affect circulating hormones and cytokines. During the preclamp period, release of IL-18, calculated as iAUC for serum IL-18, was 54% higher after intervention with OIL compared with VCL ($P < 0.05$, Supplemental Table 2). The iAUCs of leptin, high-molecular-weight (HMW) adiponectin, IL-1ra, FABP4, FGF21, and cortisol were not different between interventions (Supplemental Table 2).

OIL results in whole-body and HEP-IR during preclamp and clamp conditions. During the preclamp period, whole-body resting energy expenditure (REE) was higher with OIL than with VCL (1657 ± 223 kcal/d vs. 1509 ± 203 kcal/d, $P = 0.0018$). Rates of whole-body lipid oxidation (LOX; OIL 0.9 ± 0.4 mg/kg/min vs. VCL 0.8 ± 0.4 mg/kg/min; $P = 0.306$) and glucose oxidation (GOX; OIL 1.8 ± 1.0 mg/kg/min vs. VCL 1.9

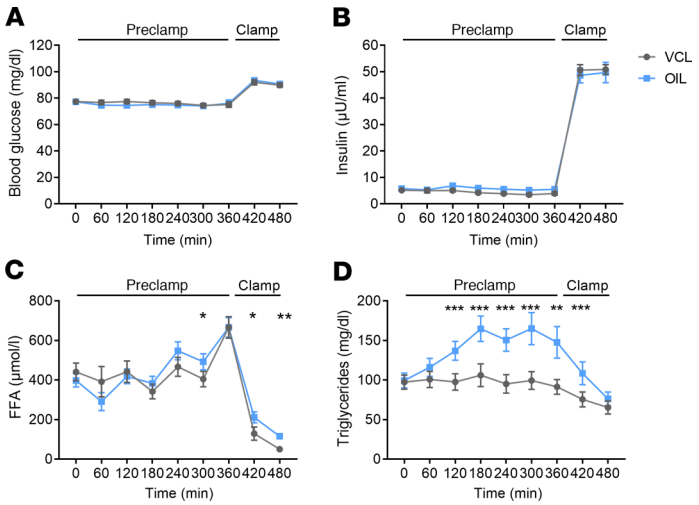

Figure 2. Time course of circulating metabolites and hormones. Blood glucose (**A**), plasma insulin (**B**), free fatty acids (FFA) (**C**), and TG (**D**) in healthy humans after canola oil (OIL, blue) or water (vehicle [VCL], gray) administration at 0 minutes. Data are shown as mean ± SEM. ANOVA was adjusted for repeated measures with Bonferroni's testing. n = 16; *P < 0.05 vs. CON; **P < 0.005 vs. CON; ***P < 0.001 vs. CON.

± 0.9 mg/kg/min; P = 0.899) were not different between the interventions. HEP-IR was 28% higher after OIL intervention than after VCL (P = 0.0037; Figure 3A).Whole-body glucose disposal (Rd), related to the ambient serum insulin concentrations, was 27% lower after OIL intervention than after VCL (P = 0.0043; Figure 3B).

During the clamp period, REE was comparable between OIL and VCL (1680 ± 244 kcal/d vs. 1608 ± 233 kcal/d, P = 0.697). Insulin-mediated EGP suppression was 21% (P = 0.0011) lower with OIL than with VCL (Figure 3C). Insulin-stimulated Rd was 18% lower after intervention with OIL compared with that after VCL (P = 0.011, Figure 3D). The reduction of Rd was mainly due to the 25% decrease in rates of GOX (OIL vs. VCL, P = 0.0072, Figure 3E) but not nonoxidative Rd (NOXGD; OIL 3.0 ± 0.3 mg/kg/min vs. VCL 3.0 ± 0.6 mg/kg/min; P = 0.139). LOX was 137% higher after OIL intervention (P = 0.022, Figure 3F).

OIL increases hepatic GNG but does not affect ATP nor the TG content. During the preclamp period, endogenous glucose production (EGP) was not different between the interventions (OIL vs. VCL, P = 0.585). However, the rate of GNG was 60% higher (OIL vs. VCL, P = 0.022), and the rates of net glycogenolysis (GLYnet) and glycogen phosphorylase (GP) flux were 47% (P = 0.0201) and 38% (P = 0.0082) lower, after OIL intervention than after VCL (Figure 4). Hepatic glycogen cycling was negligible under both conditions (P = 0.576; Figure 4). Hepatocellular lipid content was unchanged at preclamp conditions and comparable between groups (Table 1).

γ-Adenosine triphosphate (γATP) and inorganic phosphate (P_i) concentrations as well as γATP/P_i ratios were not different at –120 minutes (basal period) or at +240 (preclamp period) minutes between OIL and VCL (Table 1).

Effect of sex on the metabolic effects of OIL. A subanalysis of a possible interaction of sex with the effects of OIL during the preclamp and clamp period revealed no such effects on LOX (P = 0.156) nor GOX (P > 0.999) or on whole-body (preclamp period, Rd/insulin, P = 0.094; clamp period, Rd, P = 0.125) and hepatic (preclamp, P = 0.156 for HEP-IR; clamp period, P = 0.072 for percentage EGP suppression) insulin sensitivity.

Discussion

This study demonstrates that a single oral dose of oleic acid–rich OIL induces insulin resistance in skeletal muscle and liver during hyperinsulinemia but also already under preclamp insulinemia in whole body and liver. Additionally, OIL increased the rate of hepatic GNG and its contribution to EGP, but — in contrast to a former study on saturated fat intake — affected neither hepatic energy metabolism nor lipid deposition (5).

Effect of OIL on tissue-specific insulin resistance. The present study shows that a single MUFA-rich lipid drink reduces hepatic insulin sensitivity during preclamp and clamp conditions, as assessed from Hep-IR and EGP suppression, to a similar degree as a SAFA-rich lipid drink (5), whereas PUFA-enriched soy bean oil has no such effect (9). The impairment of hepatic insulin sensitivity during the preclamp period is reflected by the 61% higher rates of GNG, which is nominally similar to the 70% higher GNG

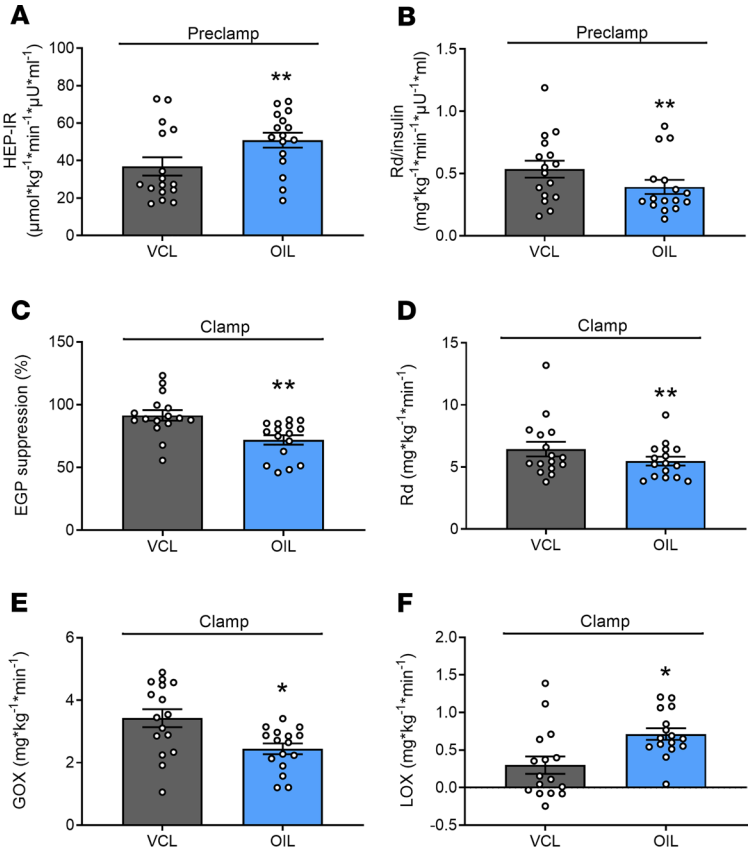

Figure 3. Whole-body glucose disposal and hepatic insulin sensitivity of the preclamp and clamp period. Insulin resistance of the liver (HEP-IR; **A**); rate of glucose disappearance (Rd) per serum insulin concentration (Rd/insulin; **B**) between +300 minutes and +360 minutes of the preclamp period; hepatic insulin sensitivity (EGP suppression; **C**); Rd (**D**); GOX (**E**); and LOX (**F**) between +420 minutes and +480 minutes of the clamp period in healthy humans after canola oil (OIL, blue) and water (vehicle [VCL], gray) ingestion at 0 minutes. Data are shown as mean ± SEM; cross-over test, $n = 16$. $^{**}P < 0.005$ vs. VCL; $^{*}P < 0.05$ GOX OIL vs. GOX VCL; $^{\#\#}P < 0.005$ NOXGD OIL vs. NOXGD VCL. GOX, glucose oxidation; LOX, lipid oxidation; EGP, endogenous glucose production.

observed upon SAFA-rich lipid loading (5). During clamp, reduced insulin action is presumed by the lower insulin-stimulated EGP suppression also reported for SAFA but not PUFA (5, 9). In the present study, the rise in GNG occurred along with reduced rates of glycogenolysis resulting in unchanged EGP. Likewise, in insulin-resistant states, such as obesity, increased rates of fasting GNG without changes in total EGP due to decreased hepatic glycogenosis were reported (10), along with impaired insulin-stimulated EGP suppression during clamp in nondiabetic humans (11). This indicates the operation of an autoregulatory mechanism limiting EGP in response to elevated GNG, previously demonstrated for other metabolic conditions (12).

Whole-body insulin sensitivity, which mainly reflects skeletal muscle insulin action, was equally impaired by 25% with OIL and an identical dose of SAFA-rich palm oil and PUFA-rich soy oil (5, 9). Moreover, Rd adjusted from prevailing insulin plasma levels was reduced during the preclamp period. Of note, a previous study found an increased insulin-to-glucose ratio after ingestion of SAFA-rich oil for 24 hours but not with unsaturated fatty acids compared with water control (13). However, this ratio was obtained from overweight and obese humans during hyperglycemic clamps, a method not considered a gold standard for measuring whole-body insulin sensitivity. In general, palmitate but not oleate is known to induce skeletal muscle insulin resistance (14). In this context, increasing MUFA and decreasing SAFA intake has been shown to improve insulin sensitivity measured by the insulin sensitivity index; however, this beneficial effect disappears at high-fat intake of >37% of daily energy intake (15). In addition, both oleate and palmitate are able to increase the serine phosphorylation of insulin receptor substrate-1 (16), suggesting activation of the diacylglycerol/protein kinase C pathway promoting insulin resistance. FFA

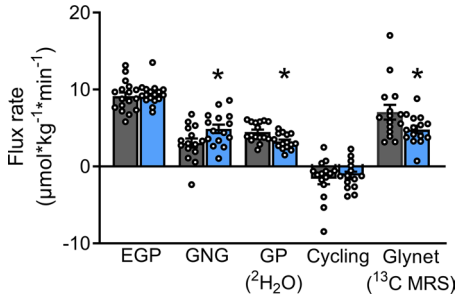

Figure 4. Hepatic glucose and glycogen fluxes between +15 minutes and +360 minutes of the preclamp period. Rates of gluconeogenesis (GNG), glycogen phosphorylase flux (GP), glycogen cycling (Cycling), and net glycogenolysis (GLYnet) were assessed using in vivo $^{13}C/^{31}P/^{1}H$ and ex vivo ^{2}H-MRS combined with $^{2}H_2O$/acetaminophen ingestion in humans after canola oil (OIL, blue) or water (vehicle, [VCL], gray) administration. Data are shown as mean ± SEM; cross-over test, $n = 16$; GP and cycling $n = 15$. $^{*}P < 0.05$ vs. CON.

may also act through c-jun N-terminal kinase and S6 kinase p70 (16) and oleate through increased incomplete β-oxidation (17). Taken together, these findings suggest that differential effects of fatty acid saturation critically depend on the ingested lipid quantity.

Effects of OIL on inflammatory markers and adipokines. The present study found no changes in circulating antiinflammatory cytokines IL-1ra and FGF21, in line with our previous studies on acute effects on SAFA (5) and PUFA, reporting constant levels of the classical inflammatory markers TNF-α and IL-6 after oil ingestion. Only, IL-18 levels (iAUC) were moderately increased after OIL, possibly reflecting metabolic adaptation rather than a proinflammatory response, as IL-18 has been shown to increase skeletal muscle LOX (18). The MUFA load did not effect circulating concentrations of leptin in accordance with unchanged plasma leptin concentrations after intravenous or oral lipid challenges in humans (19). In addition, HMW adiponectin and cortisol levels were not affected by the MUFA load, in line with unchanged levels after oral lipid challenge and during a PUFA-enriched infusion (5, 9).

Sex-specific differences in lipid handling. The present study found no effect of sex on the effects of OIL ingestion on rates of substrate oxidation or tissue-specific insulin sensitivity during the preclamp and clamp periods. Of note, a previous study observed sex-specific differences in muscle insulin sensitivity but not in EGP and HEP-IR (8). While the present data suggest no major sex-dependent difference in the susceptibility to acute lipid ingestion, they cannot exclude any such effects after long-term high-fat diets — maybe mediated by inflammatory pathways — as proposed for mice (20).

Metabolic and clinical relevance of MUFA in NAFLD and T2D. Current guidelines of the American Diabetes Association, European Association for Study of the Liver, European Association for Study of Diabetes, and European Association for Study of Obesity recommend a diet enriched in MUFA and a reduced intake of SAFA below 10% of total caloric intake as a treatment for T2D and NAFLD (21, 22). In the PREDIMED trial, which reported reduced cardiovascular and T2D risk with Mediterranean diet (3), daily fat intake was about 40% of total calorie intake, derived from sources rich in unsaturated fatty acids, mostly olive oil (50 g/d) and equivalent to 86 g daily fat at a total calorie intake of 2000 kcal/d. The amount of OIL of the study (~1.2 g/kg BW) resembles 1 meal rich in monounsaturated fat, such as 381 g pasta with pesto sauce containing about 80.1 g fat (59% MUFA; 24% PUFA, 17% SAFA) (https://www.fatsecret.com/calories-nutrition/generic/pasta-with-pesto-sauce?portionid=53757&portionamount=100.000). Our data suggest that the amount of fat intake per meal mainly determines acute insulin resistance, while its degree of saturation may be more relevant for hepatic energy metabolism and ectopic lipid storage. We did not detect higher hepatic concentrations of TG and phosphorus metabolites after OIL, whereas an acute rise in both TGs and ATP levels was found upon palm oil ingestion (5). Accordingly, a high-MUFA isocaloric diet for 8 weeks reduced liver fat content by 30% in patients with T2D, which was at least in part attributed to higher postprandial fatty acid β-oxidation, as measured from circulating β-hydroxybutyrate levels (23). In contrast to our previous study on the effects of SAFA (5), the present study found no differences in preclamp LOX but increased REE with OIL, which may contribute to favorable metabolic control and reduced NAFLD risk after MUFA intake (24). Furthermore, studies in mouse models with restricted MUFA supply and synthesis showed that oleate prevents from hepatic endoplasmic reticulum stress and inflammation (25). Nevertheless, randomized long-term controlled intervention studies are needed to clarify the effect of MUFA-enriched diets specifically on NAFLD.

Table 1. Hepatocellular lipids and phosphorus containing metabolites

Parameter	Intervention	Time	
		–120 minutes	+240 minutes
HCL (%H_2O)	CON	0.8 ± 0.1	0.8 ± 0.1
	RO	0.7 ± 0.1	0.8 ± 0.1
γATP (mmol/l)	CON	2.5 ± 0.2	2.6 ± 0.1
	RO	2.4 ± 0.2	2.6 ± 0.1
P_i (mmol/l)	CON	2.1 ± 0.2	2.3 ± 0.2
	RO	2.1 ± 0.2	2.2 ± 0.2
γATP/P_i	CON	1.2 ± 0.2	1.1 ± 0.1
	RO	1.1 ± 0.1	1.2 ± 0.1

Hepatocellular lipid (HCL), γ-adenosine triphosphate (γATP), and inorganic phosphate (P_i) contents as well as γATP/P_i ratios in healthy humans at –120 minutes before administration of canola oil (OIL) or water (vehicle [VCL]) and at +240 minutes (preclamp period). Data are shown as mean ± SEM, cross-over test, n = 16. RO, rapeseed oil.

Strengths and limitations. This study benefits from its design, which allows for direct real-time monitoring of hepatic metabolic fluxes in vivo by using 2 independent state-of-the-art techniques, as well as from the comparability with our previous study on saturated fat ingestion, due to an identical study design (5).

Limitations of this study include the application of a pure fat load, which allows for examination of lipid-mediated effects per se but does not necessarily reflect ingestion of mixed meals also containing carbohydrates and proteins. In addition, OIL does not exclusively contain MUFA but also contains about 5% SAFA and approximately 25% PUFA (26). Of note, only plasma concentrations of oleic acid, but not those of other fatty acid species, increased with OIL, pointing to the major contribution of oleic acid to the observed metabolic effects (Supplemental Table 1). While it is generally assumed that the prevailing fatty acid represents the fatty acid of relevance for the observed results (27), we cannot rule out contributions of the other fatty acids to the net metabolic effect. In addition, using water as the control intervention induces a difference in the total caloric load and can lead to other metabolic and endocrine alterations during fasting, which may result in effects independent of those of the ingested oil (28). Nevertheless, this approach has been used before in intervention studies on the metabolic effects of different dietary fats (5, 13). Finally, this study cannot provide data on the molecular mechanisms of action, as no liver biopsies were available due to ethical reasons.

In conclusion, the acute effects of OIL ingestion comprise (a) early decrease in hepatic insulin sensitivity but increased whole-body energy expenditure at fasting insulinemia, (b) increased hepatic gluconeogenic and lower glycogenolytic flux rates but unchanged hepatic TGs and energy-rich phosphates, and (c) hepatic and muscle insulin resistance during hyperinsulinemia. One may therefore speculate that a high-MUFA load may induce acute insulin resistance but without deleterious effects on hepatic lipid and energy metabolism, in contrast to the reported effects of SAFA.

Table 2. Participant anthropometrics and key blood parameters

Parameter	Mean ± SD or median (IQR)
n (males/females)	16 (10/6)
Age (yr)	25 ± 3
Body mass index (kg/m^2)	22 ± 1
Waist circumference (cm)	77 ± 5
Systolic blood pressure (mmHg)	121 ± 12
Diastolic blood pressure (mmHg)	75 ± 8
Fasting blood glucose (mg/dL)	79 ± 2
2-h post-OGTT blood glucose (mg/dL)	92 ± 28
Alanine aminotransferase (U/l)	22 ± 11
Aspartate aminotransferase (U/l)	27 ± 12

Healthy participant anthropometrics and key blood parameters before administration of rapeseed oil or water (control). Data are presented as mean ± SD or median (IQR), n = 16. IQR, interquartile range from first to third quartile.

Methods

Volunteers. Sixteen (10 male, 6 female) glucose-tolerant, lean, young volunteers were included into this randomized, cross-over, placebo-controlled study (ClinicalTrials.gov NCT01736202) (Table 2 and Supplemental Figure 1).

Experimental protocol. All volunteers underwent screening, including medical history and clinical examination, lean body mass assessment, routine laboratory tests, and a 75-g oral glucose tolerance test. This study follows up a previous study with identical design, except for the use of OIL instead of palm oil; the previous study reported data on the control intervention from 7 of 10 male participants (5). The inclusion and exclusion criteria were described in detail before (5). Females were examined between days 5 and 9 of their menstrual cycle. Briefly, all participants arrived at the study center at 5:30 pm, received a standardized dinner (~684 kcal) at 6:00 pm, and drank 3 portions of ^2H_2O (99.9%, MilliporeSigma), diluted to 33% with mineral water at 8:00 pm, 10:00 pm, and 12:00 am to yield a total dose of 5 g ^2H_2O per kg body water. On the next day, at 5:00 am (defined as time point –180 minutes; basal period, –180 minutes to 0 minutes), 2 intravenous catheters were inserted to contralateral forearm veins. Participants drank 200 mL ^2H_2O (0.5% in water) every 60 minutes to maintain isotopic equilibrium in body water. At –180, +200, and +400 minutes, participants ingested 500 mg acetaminophen. From –180 minutes, a bolus-continuous (0.036 mg/kg BW/min) infusion of [6,6-$^2H_2$]glucose (99% enriched in ^2H; Cambridge Isotope Laboratories) was administered. At 0 minutes, with start of the preclamp period (0 minutes to +360 minutes), participants received either OIL (63% oleic acid, 30% linoleic acid, 7% SAFA; Rapso, VOG AG, and Mazola, Peter Kölln GmbH & Co. KGaA), rich in MUFA, or VCL (water). Patients with more than 70 kg BW drank 92 g, and those with <70 kg BW drank 80 g OIL (~1.18 g/kg BW OIL) within 5–10 minutes (5). To yield a homogenous drink, OIL was heated to 60°C, mixed with 1.8 g or 1.6 g emulsifier (Glice, Texturas, Albert y Ferran Adria), 9 or 8 g sugar-free vanilla syrup (Torani), and 81.2 or 70.4 mL bottled still water, for 92 g and 80 g MUFA mix, respectively. To guarantee a stable emulsion, oil drinks were stirred constantly and served warm (40°C–45°C). The VCL drink was of equal composition but instead of OIL 173.2 mL or 150.4 mL bottled still water was used, respectively. The clamp period started at +360 minutes and continued until +480 minutes with a hyperinsulinemic-euglycemic clamp (40 mU/m2 body surface area/min; human regular insulin, Insuman Rapid, Sanofi). Blood glucose concentration was maintained at 90 mg/dL by adapting the glucose infusion rate using 20% glucose (B. Braun AG) enriched with 2% [6,6-$^2H_2$]glucose. Blood and urine samples were collected at timed intervals (Figure 1).

Indirect calorimetry. Indirect calorimetry (IC) was performed in the canopy mode using Vmax Encore 29n (CareFusion) during the basal (–180 to 0 minutes), preclamp (0 to +360 minutes), and steady-state clamp periods (+460 to +480 minutes; Figure 1) as described previously (5, 9) (Supplemental Table 3).

Analyses of metabolites and hormones. Whole-blood glucose, HbA_{1c}, serum TG, plasma FFA, plasma insulin, and cortisol were measured as previously described (5). Serum concentrations of IL-1 receptor antagonist (IL-1ra), leptin, FGF21, and fatty acid–binding protein 4 (FABP4) were determined using Quantikine ELISA kits from R&D Systems/Bio-Techne. Serum IL-18 was measured using the Human IL-18 ELISA kit from MBL. Serum concentrations of HMW adiponectin were assessed with the HMW adiponectin ELISA kit (ALPCO).

Gas chromatography–mass spectrometry. Measurement of atom percentage enrichment of [6,6-$^2H_2$]glucose in the blood glucose pool was performed on a Hewlett-Packard 6890 gas chromatograph equipped with a 25-m CPSil5CB capillary column (0.2 mm i.d., 0.12-μm film thickness; Chrompack/Varian) interfaced to a Hewlett Packard 5975 mass selective detector (5). Fatty acid spectra were analyzed as fatty acid methyl esters (FAMEs) using gas chromatography–mass spectrometry as previously described, with minor changes (29). Lipids were extracted from plasma after addition of internal standard using isopropyl alcohol/heptane/sulfuric acid (40:10:1). After separation by thin layer chromatography, extraction and derivatization to their corresponding methyl esters, FAMEs were analyzed on a Hewlett Packard 6890 gas chromatograph interfaced to a Hewlett Packard 5975 mass selective detector. Calibration curves of reference fatty acids were processed in parallel to tissue samples and were used for quantification of analytes.

Ex vivo ^2H nuclear MR spectroscopy. Positional ^2H enrichments in 5-O-acetyl monoacetone glucuronic lactone (MAGLA) derivatized from urinary acetaminophen glucuronide and in monoacetone glucose (MAG) derivatized from plasma glucose (30) were obtained with a Bruker Avance III HD 500 spectrometer equipped with a ^2H-selective 5-mm probe incorporating a ^{19}F lock channel and analyzed using the NUTS PC-based NMR spectral analysis program (Acorn NMR) as described previously (5, 31). Samples from all 16 participants yielded sufficient data for nuclear MR spectroscopy analysis.

In vivo $^{13}C/^{31}P/^{1}H$ MRS. Examinations were performed on participants in the supine position within a whole-body 3.0-T Achieva X-Series Philips scanner (Philips Healthcare) (5). Liver ^{1}H-decoupled ^{13}C spectra of glycogen were obtained with a 7-cm dual-tuned $^{13}C/^{1}H$ coil (PulseTeq Ltd.) at –120, +15, +130, and +300 minutes. Liver ^{31}P and ^{1}H MRS were performed at the basal (–120 minutes) and preclamp period (+240 minutes). For liver ATP and P_i concentrations, ^{31}P spectra were acquired with a 14-cm circular surface coil (Philips Healthcare) (5). ^{1}H spectra were obtained using the Q-body coil with a single-voxel stimulated echo acquisition mode sequence with and without water suppression by the chemical shift saturation technique and analyzed by jMRUI 4.0 and the AMARES algorithm (32, 33). Liver volumes were assessed from transverse T2-weighted turbo spin-echo images (5).

Calculations. During preclamp and clamp periods, whole-body Rd was obtained by the rate of glucose disappearance (Rd) from [6,6-$^{2}H_2$]glucose enrichments using Steele's steady-state equations (34) and divided by plasma insulin levels at the respective time points during the preclamp and clamp period (+300 to +360 minutes and +450 to +480 minutes). From indirect calorimetry, GOX rates (mg/kg/min) were calculated as follows: $([4.55 \times VCO_2] – [3.21 \times VO_2] \times 1.44) – 0.459(0.15 \times REE/16.74) \times 1000/(BW \times 1440)$, where VCO_2 and VO_2 are in ml/min, REE is in kJ/d, and BW is in kg. LOX (mg/kg/min) was calculated by the formula $([(1.67 \times VO_2) – (1.67 \times VCO_2) \times 1.44] – 0.307 \times POX) \times 1000/(BW \times 24 \times 60)$ (35), where VCO_2 and VO_2 are in ml/min, POX is in g/d, and BW is in kg (35). NOXGD was calculated as the difference between Rd and GOX. At between +300 minutes and +360 minutes in the preclamp period, HEP-IR was calculated as follows: EGP × mean insulin concentration (36). During insulin-stimulated conditions (clamp period), hepatic insulin sensitivity was assessed from EGP suppression, calculated as 100 – (mean clamp steady-state EGP concentrations × 100)/(basal EGP concentrations at 0 minutes) (Supplemental Table 3) (5).

The rate of GNG was calculated as the difference between EGP and GLYnet. GLYnet was derived from linear regression of hepatic glycogen concentrations — from ^{13}C MRS — over time using the least mean square method (5, 37). Fractional GP flux was calculated as 1-(H5)/(H2), where H5/H2 is the ratio of ^{1}H enrichment at carbon position 5 of glucuronide to that at position 2 after $^{2}H_2O$ ingestion (38). Absolute GP flux was calculated by multiplying fractional GP flux by EGP during the respective time period (38). Glycogen cycling, i.e., simultaneous fluxes through glycogen synthase and GP, was assessed by calculating the difference between GP and GLYnet (5, 39).

Total iAUCs for glucose, insulin, TG, FFA, and individual fatty acids, as well as for TG, IL-18, IL-1ra, FABP4, HMW adiponectin, and FGF21 during the preclamp period were calculated using the trapezoidal rule corrected for the respective AUC during the basal period (40).

Statistics. The power calculation was based on a 2-tailed *t* test, assuming a mean difference in EGP (clamp period) of 0.1 and a SD of 0.11 resulting in a sample size of $n = 16$ to reach a power of 92%. Results are presented as mean ± SEM or percentages. In crossover studies, differences between treatment effects were tested using the classical crossover test, which compares the outcome of intraindividual period differences between the sequence groups (41). For statistical analysis of time courses of distinct parameters, a mixed-model repeated-measures ANOVA was used, adjusted for basal values with Bonferroni's correction on PROC MIXED of SAS 9.3. Variables with skewed distributions were ln transformed before analysis. Statistical significance of differences was defined at $P < 0.05$. Calculations were performed using SAS version 9.4 (SAS Institute).

Study approval. All volunteers gave their written informed consent before inclusion into this study (ClinicalTrials.gov NCT01736202), which was performed according to the 2013 version of the Declaration of Helsinki and approved by the ethics committee of the medical faculty at Heinrich Heine University Düsseldorf.

Author contributions

MR initiated the investigation, lead the clinical experiments, and wrote, reviewed, and edited the manuscript. TS led the clinical experiments, obtained and analyzed the data, and wrote, edited, and reviewed the manuscript. SK obtained/analyzed data and aided in devising the clinical study design and wrote, edited, and reviewed the manuscript. JS and OPZ helped to conduct the study and reviewed the manuscript. FW conducted the MRS study and reviewed and edited the manuscript. CB performed derivatization experiments and enrichment analysis for ^{2}H-MRS and reviewed and edited the manuscript. JGJ performed derivatization experiments and enrichment analysis for ^{2}H-MRS and reviewed and edited the manuscript. DFM and CH conducted laboratory analyses and reviewed and edited the manuscript. PB performed statistical analysis and reviewed the manuscript. JHH supervised the MRS study and reviewed and edited the manuscript. All authors have given final approval of the version to be published.

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Empagliflozin Effectively Lowers Liver Fat Content in Well-Controlled Type 2 Diabetes: A Randomized, Double-Blind, Phase 4, Placebo-Controlled Trial

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OBJECTIVE

To evaluate whether the sodium–glucose cotransporter 2 inhibitor empagliflozin (EMPA) reduces liver fat content (LFC) in recent-onset and metabolically well-controlled type 2 diabetes (T2D).

RESEARCH DESIGN AND METHODS

Patients with T2D ($n = 84$) (HbA_{1c} 6.6 ± 0.5% [49 ± 10 mmol/mol], known disease duration 39 ± 27 months) were randomly assigned to 24 weeks of treatment with 25 mg daily EMPA or placebo. The primary end point was the difference of the change in LFC as measured with magnetic resonance methods from 0 (baseline) to 24 weeks between groups. Tissue-specific insulin sensitivity (secondary outcome) was assessed by two-step clamps using an isotope dilution technique. Exploratory analysis comprised circulating surrogate markers of insulin sensitivity and liver function. Statistical comparison was done by ANCOVA adjusted for respective baseline values, age, sex, and BMI.

RESULTS

EMPA treatment resulted in a placebo-corrected absolute change of −1.8% (95% CI −3.4, −0.2; $P = 0.02$) and relative change in LFC of −22% (−36, −7; $P = 0.009$) from baseline to end of treatment, corresponding to a 2.3-fold greater reduction. Weight loss occurred only with EMPA (placebo-corrected change −2.5 kg [−3.7, −1.4]; $P < 0.001$), while no placebo-corrected change in tissue-specific insulin sensitivity was observed. EMPA treatment also led to placebo-corrected changes in uric acid (−74 mol/L [−108, −42]; $P < 0.001$) and high-molecular-weight adiponectin (36% [16, 60]; $P < 0.001$) levels from 0 to 24 weeks.

CONCLUSIONS

EMPA effectively reduces hepatic fat in patients with T2D with excellent glycemic control and short known disease duration. Interestingly, EMPA also decreases circulating uric acid and raises adiponectin levels despite unchanged insulin sensitivity. EMPA could therefore contribute to the early treatment of nonalcoholic fatty liver disease in T2D.

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Patients with type 2 diabetes (T2D) are prone to develop nonalcoholic fatty liver disease (NAFLD) (1), and NAFLD itself is associated with a doubled risk of incident T2D (2). NAFLD associates not only with cardiovascular disease but also with diabetes-related chronic kidney disease and retinopathy (1). Moreover, patients with T2D are at a higher risk of progressing from steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, and cirrhosis (1).

Pronounced weight loss is effective for the treatment of NAFLD but difficult to achieve in many cases. Although well-known (glucagon-like peptide 1 receptor agonists, thiazolidinediones) and novel (e.g., pegbelfermin, elafibranor) compounds have demonstrated beneficial effects in patients with T2D and NAFLD, there is no accepted pharmacological treatment for these patients (3).

Sodium–glucose cotransporter 2 inhibitors (SGLT2is) not only improve glycemia by increasing urinary glucose excretion but also reduce body weight and blood pressure (4) and improve cardiovascular and renal outcomes (5). Some open-label and placebo-controlled studies have reported that SGLT2is may also alleviate NAFLD (6,7), while canagliflozin and dapagliflozin trended toward decreased liver fat content (LFC) compared with placebo (8,9). Body weight loss and glycated hemoglobin (HbA_{1c}) reduction may be mainly responsible for LFC reduction with canagliflozin (8,10), but empagliflozin (EMPA) could improve NAFLD independently of body weight and glycemia (7,11). Of note, SGLT2is ameliorated inflammation, oxidative stress, and dysregulated hormone secretion in preclinical studies (4). The current randomized, placebo-controlled clinical trial examined the effectiveness of EMPA on LFC reduction in patients with recent-onset, well-controlled T2D and explored its effects on tissue-specific insulin sensitivity.

RESEARCH DESIGN AND METHODS

Study Design

This randomized, parallel-group, double-blind, phase 4 trial was performed at five centers in Germany (Düsseldorf, Potsdam-Rehbrücke, Dresden, Tübingen, and Heidelberg) with a 1:1 allocation to treatment arms. The lead ethics committee of Heinrich-Heine University Düsseldorf approved all trial procedures.

Patients

The study population consisted of well-controlled patients with T2D with short known disease duration to exclude that the observed effects of EMPA were mainly driven by improvement of glycemic control. The rationale for this selection was the research question of whether SGLT2is would also be effective in early T2D, where effects on glycemia and changes in additional antihyperglycemic treatment during the intervention would be minimized. Participants were recruited by newspaper and Internet advertisements. Before inclusion, all patients gave written informed consent. Principal inclusion criteria were age 18–75 years, BMI <45 kg/m^2, known diabetes duration ≤ 7 years, HbA_{1c} of 6–8%, and no previous antihyperglycemic treatment or a 1-month washout period. Principal exclusion criteria included uncontrolled hyperglycemia at screening (fasting blood glucose [FBG] ≥ 240 mg/dL), liver disease other than NAFLD, previous thiazolidinedione treatment, and use of immunomodulatory, antiobesity, anti-NASH drugs. Full inclusion and exclusion criteria are listed in the Supplementary Data.

Randomization and Masking

All participants were randomized by a stratified computed randomization procedure to account for age, sex, and BMI to EMPA or placebo and were masked to the treatment assignment. The electronic master randomization list was only accessible to the assigned randomization list managers, and study sites received sealed opaque envelopes for unblinding in cases of emergency. Enrollment was performed at the respective site. Randomization and assignment to the double-blind study drug was done by central pharmacy personnel, who had access to the computer-generated randomization scheme. No open access to the code was available at study centers to monitors, statisticians, or sponsors' teams. Blinding of investigators and patients was achieved by providing EMPA and placebo tablets with identical appearance and packaging. Unblinding was performed after the final database lock.

Procedures

All procedures are summarized in the Supplementary Data. Eligibility of patients was assessed at screening and at the end of the 1-month washout period (for patients with previous antihyperglycemic treatment only). Participants received one individual dietary counseling before the baseline visit according to recommendations of the American Diabetes Association (12).

All baseline measures were performed before the first intake of study medication. From screening on, FBG levels were self-monitored daily with a glucose meter.

Enrolled patients were allocated to one treatment arm (EMPA 25 mg once daily or matching placebo orally; both from Boehringer Ingelheim, Ingelheim/Rhein, Germany) and returned to the study center at baseline; at weeks 1, 4, 8, 12, 16, 20, and 24 for efficacy and safety (including adverse events) assessments; and at 2 weeks after discontinuation of study medication.

Assessments of visceral adipose tissue (VAT) and subcutaneous adipose tissue (SCAT) volume and LFC, respectively, were performed at baseline and 12 and 24 weeks.

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See accompanying articles, pp. 275, 283, and 290.

LFC was assessed at each center using volume-selective proton MRS (^{1}H-MRS) using a stimulated echo acquisition mode (coefficient of variation [CV] 0.3–1.7%) as reported previously (13) or chemical shift–selective in-phase/opposed-phase imaging technique in one center (14). All measurements were performed in liver segment 7, and LFC was calculated as fat / (water + fat) * 100% by central reading.

SCAT (CV 1.5% [J.M., personal communication]) and VAT (CV 1.1% [15]) were measured using T1-weighted axial fast spin-echo (16) and quantified using an automated algorithm on the basis of fuzzy clustering and orthonormal snakes (15). Central reading was done at the Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at University of Tübingen by a spectroscopist blinded to patients' treatment allocation.

Two-step euglycemic insulin clamps with [6,6-$^2H_2$]glucose (17) were done to assess whole-body, mainly skeletal muscle, insulin sensitivity (M value, R_d) and adipose tissue (% suppression of free fatty acids [FFAs]) insulin sensitivity as well as parameters related to endogenous glucose production (EGP) (absolute EGP rates, % EGP suppression) during low and high insulinemia at baseline and week 24. Briefly, participants fasted overnight for at least 10 h and refrained from any exercise and alcohol for at least 24 h before the test. [6,6-$^2H_2$]glucose was given as primed-continuous intravenous infusion throughout the clamp. After 120 min, a primed (40 mU/m^2/min for 8 min) insulin intravenous infusion (Insuman Rapid; Sanofi, Paris, France) was given for the next 120 min at 20 mU/m^2/min (low insulin) and for the final 120 min at 40 mU/m^2/min (high insulin). A variable 20% glucose infusion enriched with [6,6-$^2H_2$]glucose was used to maintain blood glucose at ~90 mg/dL. The M value was calculated from glucose infusion rates during the last 20–30 min of both low- and high-insulin periods. Patients in whom steady-state conditions were not achieved were excluded from analysis. Because the study drug was discontinued at least 3 days before the clamps to account for the half-life of EMPA 25 mg (~10.7 h [8]), urinary glucose excretion was not measured.

Fasting hepatic insulin resistance (HIR) was calculated as fasting plasma insulin * basal EGP (8). Fasting adipose tissue insulin resistance was calculated as fasting FFA * fasting plasma insulin.

Daily energy intake was analyzed from 3-day food diaries, which were filled in by patients before each visit at the site using the Prodi system (Prodi 6.3.0.1 [Nbase 3.60]; Nutri-Science GmbH, Freiburg, Germany). Physical activity was assessed by Baecke index (18).

Glucose, insulin (hemolytic blood samples were excluded from analysis), C-peptide, and FFA concentrations were measured as previously described (19). Serum levels of cytokeratin 18 (CK18)-M30 and -M65, adiponectin, fibroblast growth factor 21 (FGF-21), tumor necrosis factor-α (TNF-α, interleukin-1 receptor antagonist (IL-1Ra), IL-6, and resistin were measured at baseline and after 12 and 24 weeks. IL-6 and TNF-α were measured with Quantikine High Sensitivity ELISA Kits (R&D Systems, Abington, U.K.), and IL-1Ra, FGF-21, and resistin were measured with Quantikine ELISA Kits (R&D Systems). High-molecular-weight (HMW) adiponectin was measured with the HMW and Total Adiponectin ELISA Kit (ALPCO, Salem, NH), and CK18-M30 (apoptosis-associated capase-cleaved keratin 18, K18Asp396, or M30 neoepitope) and CK18-M65 (soluble keratin K18) were measured using the M30 Apoptosense ELISA and M65 ELISA Kits (VLVbio, Nacka, Sweden).

Outcomes

The primary efficacy end point was defined as the difference in change of LFC (in %) between EMPA and placebo from baseline to 24 weeks of treatment. Secondary end points comprised the differences in changes of measures of whole-body/skeletal muscle (M value, R_d) and hepatic insulin sensitivity (HIR, insulin-stimulated EGP suppression, fasting EGP) measures between EMPA and placebo from baseline to 24 weeks. All assessments except LFC were exploratory. Safety was monitored by assessment of vital signs, physical examination, electrocardiogram, adverse events, and laboratory results (blood chemistry, hematological and coagulation parameters) at each visit.

Power Calculation

An ~3% reduction from baseline in body weight was observed for EMPA 25 mg in a phase 3 study with patients with T2D (20). In patients with T2D with a short disease duration and excellent glycemic control, an ~5% reduction in body weight corresponded to an ~7% reduction in LFC (19). Thus, the current study required a sample size of 30 patients/arm to detect a 4% absolute decrease in LFC from baseline with a pairwise comparison within a 95% CI, assuming an SD of 5.4% and a power of at least 80%. An estimated dropout rate of 15% resulted in 36 participants/arm.

Statistical Analyses

All analyses for efficacy parameters were performed in the intention-to-treat population, including all patients, of which at least the baseline and 12-week and/or 24-week LFC data were available. For patient characteristics, data are shown as mean with SD for normally distributed data and median with first and third quartiles for log-normally distributed parameters. Values of parameters at week 0 in both treatment arms are presented as means for normally distributed data and geometric means for log-normally distributed data with 95% CIs. Placebo-corrected changes from baseline to 24 weeks for normally distributed parameters are presented as absolute changes and for log-normally distributed data, as relative changes with corresponding 95% CIs adjusted for age, sex, BMI, and respective baseline parameter (least square means). Comparison of changes between treatments was done by an ANCOVA adjusted for age, sex, BMI, and the baseline value of the respective parameter. Calculations were performed with SAS 9.4 TS1M2 (SAS Institute, Cary, NC). No data monitoring committee was foreseen for this small-scale phase 4 trial.

RESULTS

Between 4 March 2016 and 1 February 2018, 84 patients were randomized to EMPA ($n = 42$) or placebo ($n = 42$) and received at least one dose of the study medication. Of all randomized patients, 65 (77%) completed the trial (Fig. 1).

Patient Characteristics

Baseline anthropometric and metabolic measures were all comparable between EMPA and placebo (Table 1). Physical activity and daily calorie intake neither differed at baseline nor changed from baseline to 24 weeks between the groups (data not shown).

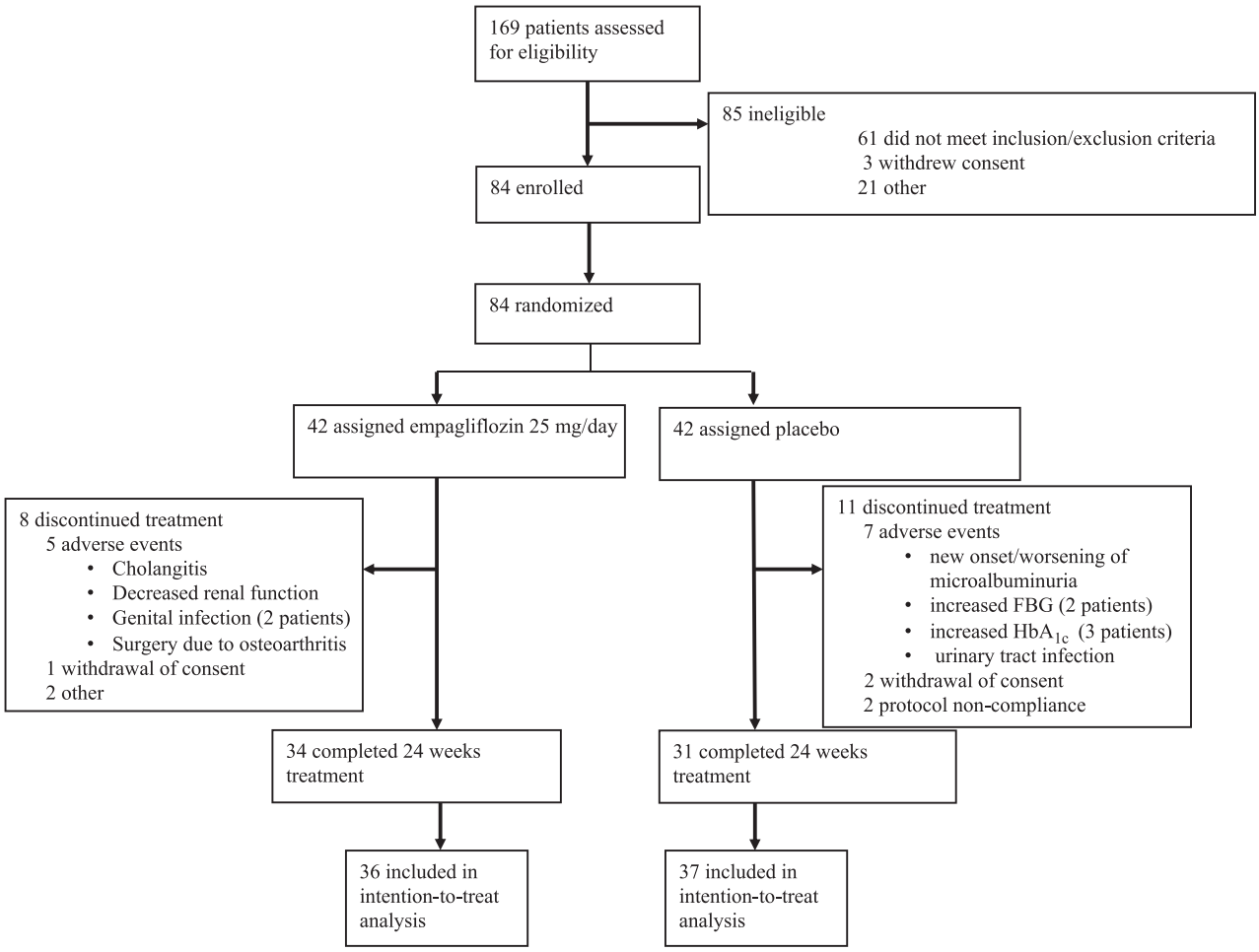

Figure 1—Trial profile.

Effect of EMPA on LFC

In the intention-to-treat population, 29 (81%) of 36 patients in the EMPA arm and 29 (78%) of 37 in the placebo arm had NAFLD at week 0. LFC was comparable between groups (EMPA 9.6% [95% CI 7.3, 12.7]; placebo 11.3% [8.6, 14.7]) and decreased in both groups already at week 12 (relative reduction: EMPA −21%, placebo −15%). At 24 weeks, a placebo-corrected absolute (−1.8% [−3.4, −0.2]; $P = 0.02$) and relative decrease in LFC (−22% [−36, −7]; $P = 0.009$) was observed, corresponding to a 2.3-fold higher relative reduction in EMPA (Fig. 2*A* and Supplementary Table 2). Further adjustment for change in body weight attenuated the difference in LFC reduction of EMPA and placebo (placebo-corrected decrease −6% [−23, 14]; $P = 0.50$).

Applying maximum likelihood methods to account for missing values for LFC at week 24 did not affect the results (data not shown). NAFLD resolution (LFC <5.56% [21]) occurred in 5 (20%) of 25 patients in the EMPA group and 2 (8%) of 24 patients in the placebo group at 24 weeks.

To examine the impact of the presence of NAFLD on EMPA-mediated reduction of LFC, an interaction term of treatment and NAFLD status (yes/no) was added to the model. Interaction of NAFLD status and treatment was not significant ($P = 0.94$).

The impact of sex on the EMPA-related reduction in LFC was examined by including an interaction term of treatment and sex in our model. There was a placebo-corrected decrease in LFC in males (−31% [95% CI −44, −14]; $P = 0.002$) but not in females (−1% [−28, 37]; $P = 0.96$). The test of interaction between sex and treatment did not achieve significance ($P = 0.075$).

Effect of EMPA on Skeletal Muscle and Hepatic and Adipose Tissue Insulin Sensitivity

During low-insulin clamp conditions, placebo-corrected whole-body/skeletal muscle R_d increased by 30% (95% CI 9, 55; $P = 0.005$) (Supplementary Table 1). However, there were no significant placebo-corrected changes in M value both at low (50% [0, 126]; $P = 0.05$) and high (12% [−12, 42]; $P = 0.36$) insulin with EMPA (Fig. 2*B* and Supplementary Table 1). Changes in HIR and insulin-mediated suppression of EGP at low- and high-insulin conditions were also comparable between groups (Fig. 2*C* and Supplementary Table 1). Likewise, changes in adipose tissue insulin resistance and insulin-stimulated FFA suppression at low- and high-insulin conditions did not differ between groups (Fig. 2*D* and Supplementary Table 1).

Effect of EMPA on Body Composition, Glycemia, and Lipidemia

EMPA resulted in a placebo-corrected weight loss of −2.5 kg (95% CI −3.7, −1.4; $P < 0.001$) at 24 weeks (Fig. 3*A* and Supplementary Table 2). The body weight reduction occurred in 31 (86%)

Table 1—Patient characteristics at week 0

	EMPA (n = 42)	Placebo (n = 42)
Sex		
Male	29 (69)	29 (69)
Female	13 (31)	13 (31)
Age (years)	62.7 ± 7.0	61.5 ± 10.0
Ethnicity		
Caucasian	42 (100)	41 (98)
Hispanic/Latino	0 (0)	1 (2)
BMI (kg/m^2)	32.1 ± 4.6	32.4 ± 4.2
Known diabetes duration (months)	36 ± 27	40 ± 27
Hepatic steatosis*	33 (79)	33 (79)
Concomitant medication		
Antihyperglycemic drugs#	28 (67)	26 (62)
Antihypertensive drugs	21 (50)	29 (69)
Lipid-lowering drugs	19 (45)	15 (36)
Glycemia		
HbA_{1c}		
%	6.8 ± 0.5	6.7 ± 0.7
mmol/mol	51 ± 6	50 ± 8
FBG (mmol/L)	7.5 ± 1.4	7.2 ± 1.3
Serum lipid concentrations		
Triglycerides (mg/dL)	159 (122; 202)	181 (103; 251)
HDL cholesterol (mg/dL)	50 ± 15	48 ± 10
LDL cholesterol (mg/dL)	133 ± 40	120 ± 30
Liver transaminases		
ALT (μmol/s/L)	0.54 (0.42; 0.80)	0.62 (0.42; 0.88)
AST (μmol/s/L)	0.42 (0.36; 0.49)	0.43 (0.37; 0.55)

Data are mean ± SD for normally distributed parameters, median (25%; 75%) for log-normally distributed parameters, or n (%). ALT, alanine aminotransferase; AST, aspartate aminotransferase. *LFC ≥5.56% measured by magnetic resonance–based methods. #Antihyperglycemic medication was stopped from at least 4 weeks before randomization until the end of the intervention period.

of 36 patients in the EMPA group and 18 (49%) of 37 patients in the placebo group. Weight loss of ≥5% occurred in 27% of patients on EMPA and in 16% on placebo. There were no placebo-corrected changes in VAT (−290 cm^3 [−694, 114]; P = 0.16) and SCAT (−2% [−10, 6]; P = 0.55) with EMPA. Of note, patients who underwent both VAT and SCAT measurements (n = 21 of 29) also exhibited a placebo-corrected decrease in body weight with EMPA (−2.6 kg [−4.0, −1.1]; $P < 0.001$).

EMPA led to a placebo-corrected change in FBG (−0.7 mmol/L [95% CI −1.3, −0.2]; $P = 0.01$) (Fig. 3*B*) but not in HbA_{1c} (Supplementary Table 2). Placebo-corrected changes in fasting insulin, C-peptide, and FFA levels did not reach significance (all $P > 0.2$) (Supplementary Table 2). Also, serum HDL and LDL cholesterol, serum total cholesterol, and plasma triglycerides were unaffected by EMPA treatment (data not shown).

Effect of EMPA on Adiponectin and Inflammation- and Liver-Related Parameters

Serum uric acid markedly decreased (placebo-corrected change −74 mol/L [95% CI −108, −42]; $P < 0.001$), and HMW adiponectin concentrations increased (placebo-corrected change 36% [16, 60]; $P < 0.001$) from 0 to 24 weeks (Fig. 3*C* and *D*). Placebo-corrected changes in IL-1Ra, TNF-α, IL-6, and FGF-21 did not differ between groups (all $P > 0.2$) (Supplementary Table 3).

Serum alanine aminotransferase and γ-glutamyl transferase were reduced with similar effect sizes in EMPA and placebo after 24 weeks (Supplementary Table 3). CK18-M30 and -M65 numerically decreased in the EMPA group, but no placebo-corrected changes were detectable (Supplementary Table 3).

CONCLUSIONS

This trial provides evidence that empagliflozin effectively reduces LFC compared with placebo but has no major effects on tissue-specific insulin sensitivity. Exploratory analyses revealed a marked decrease in serum uric acid and a rise in serum HMW adiponectin levels. Of interest, these effects occurred in the presence of moderate weight loss and despite only minor changes in glycemia in a cohort of metabolically well-controlled patients with T2D with a short disease duration.

Effects of SGLT2is on LFC and Body Weight

Recent randomized controlled trials demonstrated that SGLT2is can induce a reduction of LFC compared with baseline (6–9), but only one trial also reported a statistically significant effect on LFC compared with placebo (6). The magnitude of the reduction in LFC may depend on trial medication and design; duration of the intervention; cohort characteristics, such as NAFLD status, T2D duration, glycemic control, and sex distribution; and, finally, statistical power (22,23). The current study reports that EMPA leads to a nominally greater placebo-corrected decrease in LFC than dapagliflozin (6) but a slightly smaller decrease in change from baseline than canagliflozin (8). However, the absence of studies on dose dependency and head-to-head comparisons does not allow any conclusions about drug-specific effects at present. As indicated in other NAFLD trials (22,23), the guideline-based dietary counseling for all groups could have been responsible for the higher rates of LFC improvement observed in the placebo groups of this study and one previous (8) but not in other SGLT2i trials (6,9).

On the other hand, study duration may play a role as illustrated by the observation that alanine aminotransferase, as a crude surrogate marker of NAFLD, decreased only during the first 28 weeks of EMPA treatment (11). At the least, the nominally greater baseline-corrected decrease in LFC in the 24-week placebo-controlled trials (i.e., in one previous [8] and the current trial) than in the 8- and 12-week trials could support this contention (6,9).

As to cohort characteristics, the better metabolic control and shorter known diabetes duration compared with previous trials (6–9) and the possible inclusion of patients without NAFLD could have led to an underestimation of the efficacy of EMPA on LFC in our cohort (11). Indeed, incidence of NAFLD positively associates

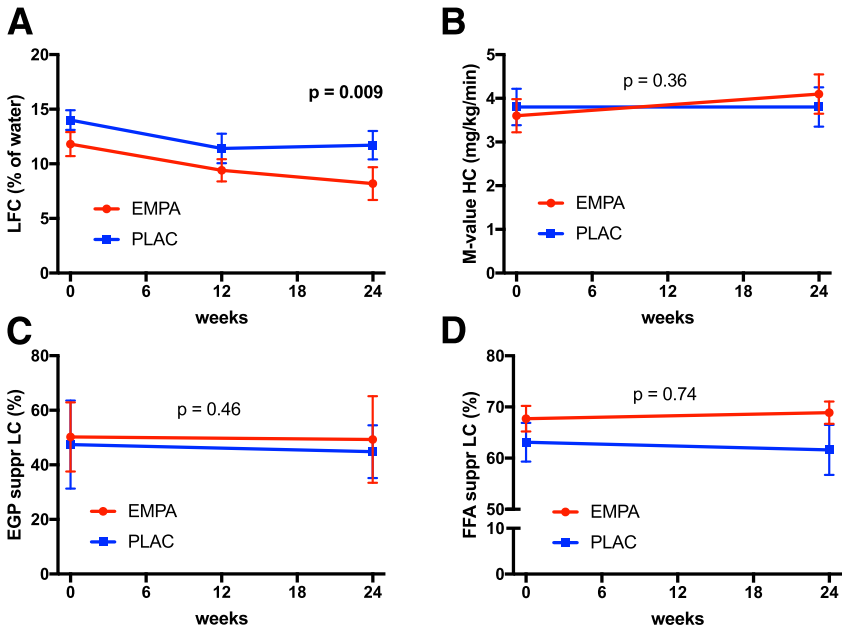

Figure 2—Effects of EMPA on LFC (*A*), whole-body insulin sensitivity (M value in high-insulin condition [40 IU/m^2 body surface area/min] [HC]) (*B*), hepatic insulin sensitivity as insulin-stimulated suppression of EGP under low-insulin conditions (20 IU/m^2 body surface area) (EGP suppr LC) (*C*), and adipose tissue insulin sensitivity as insulin-stimulated suppression of FFA under low-insulin conditions (FFA suppr LC) (*D*). Numbers of patients in EMPA and placebo (PLAC), respectively, of which week 0 and 24 data were obtained are as follows: 31 and 31 (*A*), 28 and 26 (*B*), 24 and 25 (*C*), and 27 and 27 (*D*). Unadjusted values of parameters are mean ± SEM. *P* values indicate significance level for PLAC-corrected EMPA effect and are based on ANCOVA with adjustment for age, sex, BMI, and the respective baseline parameter.

with higher HbA_{1c} and most likely also longer diabetes duration (24), and NAFLD frequency may affect the magnitude of LFC reduction in T2D (8,11).

Finally, this study found a placebo-corrected reduction of LFC in males but not in females, although the interaction of sex and treatment was not significant and the number of females small. Given that the percentage of males ranged from 60 to 81% in the previous randomized SGLT2i studies (6–9), sex-dependent differences in metabolic effects on LFC cannot be excluded. In this context, a recent study suggested sex differences in the effects of EMPA on arterial stiffness (25), whereas a post hoc analysis of the BI 10773 (Empagliflozin) Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients (EMPA-REG OUTCOME) did not detect any changes in outcomes between females and males (26).

This study shows that the changes in LFC occur in parallel to the decline in body weight during SGLT2i treatment. While significant reduction in LFC was considered to require weight loss of ≥5% (8), studies have indicated that even minor weight loss up to 5% can initiate a decrease in LFC by 33% (3,27). Because a body weight reduction of ≥5% was observed in only 27% of the EMPA group, the 34% decline in LFC underlines the role of minor weight loss for the effect of SGLT2is on LFC.

EMPA and Insulin Sensitivity

During low-insulin conditions, EMPA resulted in a borderline, but nonsignificant (M value) or significant (R_d) increase in whole-body glucose disposal. Under these conditions, R_d and M value represent the amount of glucose taken up not only by skeletal muscle but also by other organs like adipose tissue and the splanchnic bed (28). However, measures of adipose tissue (insulin-stimulated FFA suppression) or hepatic (insulin-stimulated EGP suppression) insulin sensitivity were not different between EMPA and placebo. Thus, the higher R_d could have resulted from EMPA-induced glucosuria, but study medication was stopped at least 3 days before the clamps (to account for the half-life of EMPA [~10.7 h for EMPA 25 mg (29)]), rendering urinary glucose loss unlikely. Moderate increases in R_d with SGLT2is have also been attributed to improvements in hyperglycemia and glucose toxicity (8,30). Thus, despite the (very) good glucometabolic control and rather short known diabetes duration, the minor decrease in fasting glycemia with EMPA could have contributed to the small increase in R_d during low-grade insulinemia. In contrast, the current study found no placebo-corrected effects of EMPA on R_d and M value during high-insulin clamps, which is in line with the trials on dapagliflozin (6) and canagliflozin (8). Under these conditions, R_d almost exclusively reflects insulin-stimulated skeletal muscle glucose uptake (28).

Interestingly, the decrease in LFC was not paralleled by improved hepatic insulin sensitivity, which is comparable to one dapagliflozin study (6) but in contrast to the canagliflozin trial (8). The latter study also reported lower HbA_{1c} and discussed reduction of glucotoxicity by canagliflozin as the cause (8). The absence of changes in HbA_{1c} in the current study supports this contention.

Previous studies demonstrated that the antihyperglycemic efficacy of SGLT2i is partly counteracted by a rise in EGP (31,32). This could result from a chronic SGLT2i-induced rise in plasma glucagon and decreased insulin concentrations. However, a recent clinical trial showed that canagliflozin still increases EGP when liraglutide prevents the changes in plasma insulin and glucagon levels (33). Similarly, hyperglucagonemia per se does not mediate the SGLT2i-induced increase in EGP (34). Of note, glycemia per se may regulate EGP in that a decrease in plasma glucose concentration can stimulate EGP independent of changes in plasma insulin and glucagon (35).

Finally, fasting FFA and insulin levels as well as adipose tissue insulin resistance were unchanged during this study. The previous placebo-controlled SGLT2i trials on LFC yielded contradictory results, showing elevated (8) or unchanged FFA levels (6,9) in the presence of decreased (8) or unchanged (6,9) fasting insulinemia. The previously reported SGLT2i-associated FFA elevation has been explained by glucosuria-induced relative hypoinsulinemia, which would reduce inhibition of lipolysis and tissue glucose uptake with a compensatory increase in lipid oxidation and hyperketonemia (4). In our cohort of patients with well-controlled, recent-onset T2D, EMPA did not decrease circulating insulin levels so that the ambient insulinemia

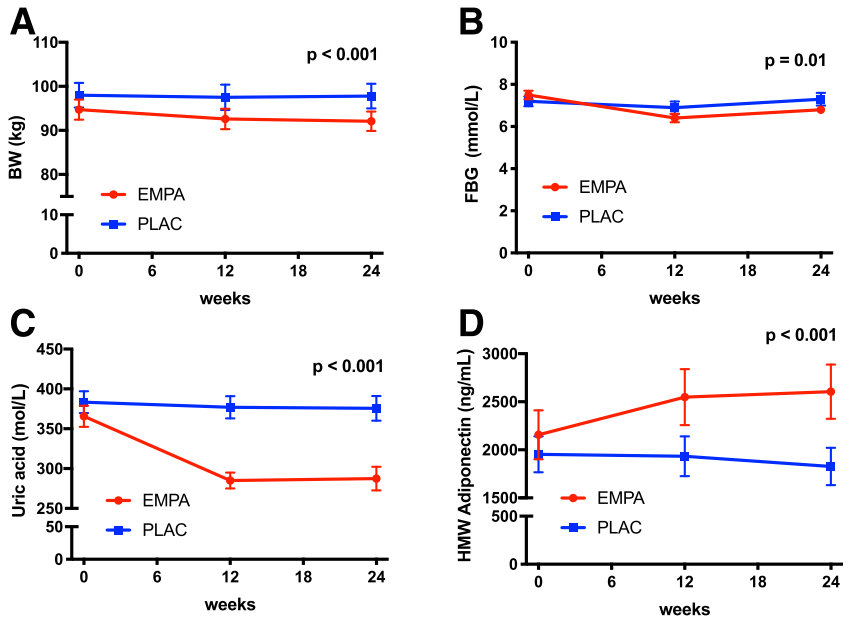

Figure 3—Effects of EMPA on body weight (BW) (*A*), FBG (*B*), serum uric acid levels (*C*), and serum HMW adiponectin levels (*D*). Comparison of changes in the respective parameters between treatment arms. Numbers of patients in EMPA and placebo (PLAC) groups, respectively, of which week 0 and 24 data were obtained are as follows: 32 and 32 (*A*), 31 and 31 (*B*), 31 and 31 (*C*), and 31 and 30 (*D*). Unadjusted values of parameters are mean ± SEM. *P* values indicate significance level for PLAC-corrected EMPA effect and are based on ANCOVA with adjustment for age, sex, BMI, and the respective baseline parameter.

might have sufficed to inhibit lipolysis as shown in humans without diabetes (4).

Exploratory Analyses of Circulating Parameters

A recent uncontrolled pilot study provided some evidence that EMPA treatment for 24 weeks could improve histological components of NASH and its resolution despite a mean reduction in BMI of only −0.7 kg/m^2 (36). The current trial did not observe placebo-corrected changes in circulating surrogate markers of liver injury, such as transaminases or CK18-M30 fragment. This is partly in line with some studies (6,7) but not in another that reported improvements in transaminases as well as CK18 fragments with dapagliflozin (9). The lack of an effect of EMPA could be due to the absence of NASH and fibrosis or masked by the greater decrease in LFC in the placebo group, which is a major trigger for reduction of these surrogate markers (27).

EMPA treatment markedly reduced serum uric acid and raised serum adiponectin concentrations. High uric acid levels trigger adipose tissue inflammation, insulin resistance, and hypoadiponectinemia (37). Of note, increased uric acid and decreased adiponectin levels associate with body weight; metabolic syndrome features, including T2D; and NAFLD (37–39).

Limitations

The patient cohort comprised exclusively metabolically well-controlled patients with T2D with short known disease duration with and without NAFLD. Thus, results cannot be necessarily extrapolated to the general population of patients with T2D, particularly to those with uncontrolled glycemia, longer disease duration, and more severe liver disease. On the other hand, this limitation represents a specific strength by showing that EMPA is effective in reducing LFC in the absence of major changes in glycemia. This trial provides no information about the efficacy and safety of EMPA in glucose-tolerant individuals with NAFLD, a collective at increased risk of T2D (2). Moreover, this study used detailed metabolic phenotyping with two-step euglycemic clamps but not mixed-meal tests, which would have allowed the assessment of postprandial β-cell function and metabolism, and did not include liver biopsies because of the expected early stages of NAFLD in these patients and the short duration of intervention. Finally, this study did not use multiple imputation to account for missing values but performed maximum likelihood methods for the primary end point (40).

In conclusion, this proof-of-concept trial shows that the SGLT2i EMPA decreases LFC in near-normoglycemic patients with recent-onset T2D with and without NAFLD. EMPA induced minor weight loss and no effect on tissue-specific insulin sensitivity. The marked decrease in serum uric acid and the rise in HMW adiponectin levels with EMPA treatment calls for further studies on the clinical relevance of these observations. Because future NAFLD treatment in T2D will require strategies that simultaneously address the different mechanisms underlying metabolic liver disease, EMPA could serve as a partner for such combination treatments because of its favorable effects on liver fat and body weight.

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Novo Nordisk, Servier Laboratories, Target Pharmasolutions, and Terra Firma. No other potential conflicts of interest relevant to this work were reported.

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ORIGINAL ARTICLE

WILEY

Effects of empagliflozin on markers of liver steatosis and fibrosis and their relationship to cardiorenal outcomes

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Abstract

Aims: Empagliflozin treatment reduced liver fat in small type 2 diabetes cohorts. This post-hoc study evaluated effects of empagliflozin on risk for non-alcoholic fatty liver disease-related steatosis and fibrosis, as well as the relationship between risk categories and cardiorenal outcomes in the randomized, placebo-controlled EMPA-REG OUTCOME trial.

Materials and methods: EMPA-REG OUTCOME treated 7020 people with type 2 diabetes and cardiovascular disease with 10/25 mg/day empagliflozin or placebo. For this analysis, the Dallas steatosis index, hepatic steatosis index, non-alcoholic fatty liver disease fibrosis score and Fibrosis-4 score were calculated to assess steatosis and fibrosis risk. Changes from baseline in scores were examined by mixed model repeated measures and their associations with cardiorenal outcomes and mortality by Cox regression.

Results: At baseline, 73% and 84% of participants had high steatosis risk by Dallas steatosis index and hepatic steatosis index, whereas 23% and 4% had a high risk of advanced fibrosis by non-alcoholic fatty liver disease fibrosis score and Fibrosis-4 score. Percentages of people at high steatosis risk slightly decreased with empagliflozin only, whereas empagliflozin did not improve percentages of individuals at high fibrosis risk over time compared with placebo. The high risk of advanced fibrosis at baseline related to higher risk for cardiovascular events. Effects of empagliflozin on cardiorenal and all-cause mortality outcomes were consistent across all risk groups.

Conclusions: Empagliflozin may reduce steatosis but not fibrosis risk in individuals with type 2 diabetes and cardiovascular disease. The improvements in cardiorenal outcomes and mortality associated with empagliflozin therapy appear to be independent of steatosis and fibrosis risk.

KEYWORDS
antidiabetic drug, clinical trial, liver, SGLT2 inhibitor

1 | INTRODUCTION

People with type 2 diabetes are prone to develop non-alcoholic fatty liver disease (NAFLD).[1] Type 2 diabetes further accelerates NAFLD progression from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis.[2] Moreover, NAFLD associates with an increased risk of cardiovascular diseases, including cardiomyopathy and certain cardiac arrhythmias, thereby contributing to the excess morbidity and mortality in people with both type 2 diabetes and NAFLD.[2]

Hepatic fibrosis is a strong predictor of NAFLD-related mortality[2] and clinically relevant advanced fibrosis stages, F3 and F4, can be present in up to 20% of persons with NAFLD and type 2 diabetes.[1] While the 'gold-standard' diagnosis of fibrosis still requires liver biopsy,[2] several imaging methods have been introduced including vibration-controlled transient elastography or magnetic resonance elastography. These methods are mostly restricted to use in specialized centres[3] as they require specific technical equipment, qualified and trained personal, making them difficult to perform in large multinational clinical trials involving hundreds of study sites. Thus, non-invasive indices calculated from demographic, anthropometric and laboratory parameters provide an opportunity to estimate the prevalence and effects of interventions on liver fibrosis in large cohorts.[3] In addition, hepatic steatosis, the primary criterium of NAFLD diagnosis, may be estimated by non-invasive indices.[4,5]

Sodium glucose co-transporter 2 inhibitors are associated with a modest weight loss and improved cardiovascular and renal outcomes.[6,7] Recent small-scale randomized controlled trials have shown that empagliflozin also improved hepatic steatosis and beneficial effects of empagliflozin on histological components including fibrosis were suggested from an uncontrolled pilot trial.[6] The underlying mechanisms remain largely unknown; however, apart from weight loss, they may even include improvement of adipose tissue function with amelioration of local inflammation and/or oxidative stress.[8,9]

The present study examined the effects of empagliflozin treatment in a large cohort of persons with type 2 diabetes with established cardiovascular disease (a) on indices of hepatic steatosis and fibrosis, (b) on glycaemia and body weight, as well as (c) on cardiorenal outcomes and all-cause mortality in groups at different steatosis and fibrosis risk. Finally, this analysis addressed the question whether baseline steatosis and fibrosis risk scores are associated with the incidence of cardiorenal events in this patient population. To this end, an exploratory post-hoc analysis was performed in the EMPA-REG OUTCOME study, previously showing lower rates of cardiovascular events and deaths from any cause with empagliflozin at a median observation time of 3.1 years.[7]

2 | MATERIALS AND METHODS

2.1 | Study design

The design of EMPA-REG OUTCOME has been previously decribed.[7] Adult individuals with type 2 diabetes and established cardiovascular disease were included; elevated liver enzymes >3× upper limit of normal were exclusion criteria (see Supporting Information for further details).

The main objective of this post-hoc analysis was to compare the effects of empagliflozin and placebo on Dallas steatosis index (DSI)[10] and hepatic steatosis index (HSI)[11] as well as on NAFLD fibrosis score (NFS) and Fibrosis-4 score (FIB-4) (see Supporting Information for calculations and cut-offs).

2.2 | Calculations and statistical analysis

The present post-hoc analyses of the randomized clinical trial EMPA-REG OUTCOME were performed on the modified intention-to-treat population, including all randomized participants, who received ≥1 dose of the study drug. The effects on risk scores as well as glycated haemoglobin (HbA1c) and weight were evaluated using a mixed-effect model repeated measurement model, which included baseline HbA1c and baseline of score (or weight) as linear covariates and their interaction with visit in addition to baseline estimated glomerular filtration rate (Modification of Diet in Renal Disease Study) category, geographical region and baseline body mass index category. Treatment, subgroup (if applicable) and visit were also entered as fixed effects as well as all two- and three-way interactions thereof. In addition, the model included a fixed categorical effect for 'time of randomization' to account for each patient's theoretical ability to 'reach' certain weeks in this study arising from the study design. Because of small group sizes, participants at baseline intermediate and high risk (based on FIB-4) were pooled for analysis of time courses of parameters in FIB-4 low and high fibrosis risk categories and HSI low and intermediate risk categories were combined for all subsequent analyses. All time to first event analyses were performed with multivariate Cox regression models that included terms for sex, baseline age, estimated glomerular filtration rate, body mass index, HbA1c, geographical region, subgroup, and treatment × subgroup interaction. Continuous baseline characteristics of fibrosis and steatosis risk groups are given as mean ± standard deviation, categorical variables as number and proportions. All other data are expressed as adjusted means (95% confidence interval) or adjusted means ± standard error. As this was a post-hoc exploratory study, no adjustments for multiple comparisons/outcomes were performed. Statistical significant differences were indicated at $p < .05$. Statistical analyses were performed with SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3 | RESULTS

3.1 | Baseline characteristics of the whole cohort and different steatosis and fibrosis risk groups

Of the 7020 participants who were treated with empagliflozin or placebo, 6927 and 7018 had data available to derive DSI and HSI, respectively, and 6970 and 6972, respectively, had data to derive NFS

TABLE 1 Baseline characteristics of DSI-, HSI-, NFS-, FIB-4-categorized steatosis and fibrosis risk groups

	Low risk	Intermediate	High risk
DSI			
Total n	253	1616	5058
Males, n (%)	201 (79.4)	1209 (74.8)	3546 (70.1)
Race, n (%)			
White	137 (54.2)	949 (58.7)	3920 (77.5)
Asian	69 (27.3)	509 (31.5)	928 (18.3)
Black/African American	46 (18.2)	150 (9.3)	156 (3.1)
Other	1 (0.4)	8 (0.5)	54 (1.1)
Age, years; mean ± SD	65.9 ± 9.0	65.1 ± 8.9	62.4 ± 8.4
Body mass index, kg/m^2; mean ± SD	23.9 ± 2.4	26.7 ± 3.9	32.2 ± 4.8
>10 years since T2D diabetes, n (%)	169 (66.8)	1055 (65.3)	2736 (54.1)
Glycated haemoglobin, %; mean ± SD	7.9 ± 0.9	8.0 ± 0.8	8.1 ± 0.9
Baseline insulin use, n (%)	102 (40.3)	715 (44.2)	2520 (49.8)
eGFR, ml/min/1.73 m^2; mean ± SD	79.3 ± 22.4	73.7 ± 21.3	73.9 ± 21.4
Hypertension, n (%)	199 (78.7)	1398 (86.5)	4738 (93.7)
CAD, n (%)	180 (71.1)	1222 (75.6)	3836 (75.8)
his			
Total n	89	1010	5919
Males, n (%)	66 (74.2)	803 (79.5)	4146 (70.0)
Race, n (%)			
White	27 (45.2)	470 (46.5)	4583 (77.4)
Asian	61 (68.5)	495 (49.0)	960 (16.2)
Black/African American	1 (1.1)	40 (4.0)	316 (5.3)
Other	0 (0)	5 (0.5)	59 (1.0)
Age, years; mean ± SD	67.2 ± 8.3	65.5 ± 8.7	62.7 ± 8.6
Body mass index, kg/m^2; mean ± SD	20.5 ± 1.7	24.2 ± 1.9	31.9 ± 4.7
>10 years since T2D diabetes, n (%)	57 (64.0)	645 (63.9)	3307 (55.9)
Glycated haemoglobin, %; mean ± SD	7.88 ± 0.94	7.94 ± 0.81	8.10 ± 0.85
Baseline insulin use, n (%)	29 (32.6)	359 (35.5)	2998 (50.7)
eGFR, mL/min/1.73 m^2; mean ± SD	73.4 ± 22.9	71.5 ± 20.5	74.5 ± 21.5
Hypertension, n (%)	71 (79.8)	862 (85.3)	5484 (92.7)
CAD, n (%)	59 (66.3)	760 (75.2)	4488 (75.8)
NFS			
Total n	809	4562	1599
Males, n (%)	493 (60.9)	3312 (72.6)	1175 (73.5)
Race, n (%)			
White	375 (46.4)	3278 (71.9)	1395 (87.2)
Asian	368 (45.5)	1015 (22.2)	119 (7.4)
Black/African American	55 (6.8)	231 (5.1)	69 (4.3)
Other	11 (1.3)	38 (0.8)	16 (1.0)
Age, years; mean ± SD	56.2 ± 8.4	62.6 ± 8.0	68.2 ± 7.4
Body mass index, kg/m^2; mean ± SD	27.04 ± 4.12	30.06 ± 4.74	34.07 ± 5.33
>10 years since T2D diabetes, n (%)	374 (46.2)	2541 (55.7)	1071 (67.0)
Glycated haemoglobin, %; mean ± SD	8.18 ± 0.87	8.07 ± 0.85	8.02 ± 0.84
Baseline insulin use, n (%)	316 (39.1)	2110 (46.3)	939 (58.7)
eGFR, mL/min/1.73 m^2; mean ± SD	80.5 ± 24.0	75.4 ± 21.0	66.9 ± 19.5

(Continues)

TABLE 1 (Continued)

	Low risk	Intermediate	High risk
Hypertension, n (%)	692 (85.5)	4162 (91.2)	1519 (95.0)
CAD, n (%)	512 (63.3)	3440 (75.4)	1319 (82.5)
FIB-4			
Total n	3505	3161	306
Males, n (%)	2334 (66.6)	2406 (76.1)	241 (78.8)
Race, n (%)			
White	2421 (69.1)	2384 (75.4)	244 (79.7)
Asian	811 (23.1)	636 (20.1)	56 (18.3)
Black/African American	236 (6.7)	114 (3.6)	5 (1.6)
Other	37 (1.1)	27 (0.8)	1 (0.3)
Age, years; means ± SD	59.5 ± 8.1	66.5 ± 7.5	69.8 ± 7.8
Body mass index, kg/m^2; mean ± SD	30.76 ± 5.28	30.51 ± 5.20	30.33 ± 5.44
>10 years since T2D diabetes, n (%)	1832 (52.3)	1945 (61.5)	210 (68.6)
Glycated haemoglobin, %; mean ± SD	8.15 ± 0.87	8.00 ± 0.82	7.96 ± 0.79
Baseline insulin use, n (%)	1670 (47.6)	1535 (48.6)	161 (52.6)
eGFR, mL/min/1.73 m^2; mean ± SD	77.9 ± 22.1	70.7 ± 19.9	64.8 ± 19.5
Hypertension, n (%)	3179 (90.7)	2920 (92.4)	276 (90.2)
CAD, n (%)	2487 (71.0)	2516 (79.6)	270 (88.2)

Abbreviations: CAD, coronary artery disease; DSI, Dallas steatosis index; eGFR, estimated glomerular filtration rate; FIB-4, Fibrosis-4; HSI, hepatic steatosis index; NFS, non-alcoholic fatty liver disease fibrosis score; T2D, type 2 diabetes.

and FIB-4 at baseline. According to the DSI, 5058 of 6927 (73%) participants were categorized at high steatosis risk at baseline, whereas for HSI, 5919 of 7018 (84%) participants were at high steatosis risk. Regarding fibrosis indices, 1599 of 6970 (23%) and 306 of 6972 (4%) participants were at risk of advanced fibrosis by NFS and FIB-4, respectively. Steatosis and fibrosis risk at baseline were further calculated by other established indices. Percentages of participants grouped at high steatosis risk were between 72% and 87%, whereas the percentage of participants at high risk of advanced fibrosis ranged from 1% to 69% (Table S1). Stratifying participants by NFS, FIB-4 and HSI, but not DSI, revealed more frequent insulin use and higher incidence of coronary artery disease in groups at high risk compared with those at low risk (Table 1).

3.2 | Empagliflozin-mediated changes of Dallas steatosis index, hepatic steatosis index, non-alcoholic fatty liver disease fibrosis score and Fibrosis-4 categories over time

At baseline, percentages of participants in the different DSI risk categories were comparable for empagliflozin and placebo. Over time, percentages remained largely unchanged for placebo whereas the percentage of participants at high steatosis risk dropped with empagliflozin from 73 at baseline to 66%, 67% and 67% at 52, 108 and 164 weeks, respectively (Figure 1A). The changes from baseline category to week 52 category and further on are visualized in Figure S1. In the empagliflozin group, more participants improved than worsened comparing baseline category to week 52 category, which was also observed for placebo although to a less extent. After week 52, this effect was attenuated in both groups.

Baseline percentages of participants in the different HSI risk groups were comparable for empagliflozin and placebo. Over time, numbers remained largely unchanged for placebo whereas the percentage of people at high steatosis risk dropped with empagliflozin from 84% at baseline to 77%, 78% and 77% at 52, 108 and 164 weeks, respectively (Figure 1B). Figure S2 shows that more participants improved than worsened compared with the baseline category with empagliflozin at 52 weeks and less prominent with placebo. After week 52, this effect was attenuated in both groups and no further improvements were observed thereafter in both groups.

Baseline percentages of participants in different NFS risk groups of advanced fibrosis were comparable for empagliflozin and placebo with only little variation throughout the course of the study (Figure 1C). After week 52, the percentage of people in the high-risk category was slightly increased in both groups Figure S3).

At baseline and thereafter, the percentages of participants in low-, intermediate- and high-FIB-4 risk groups were comparable for empagliflozin and placebo with only minimal changes over time (Figure 1D). After week 52, percentages of persons in the intermediate- and high-risk categories were increased in both groups (Figure S4).

Changes from baseline in the respective indices are depicted in Figure S5.

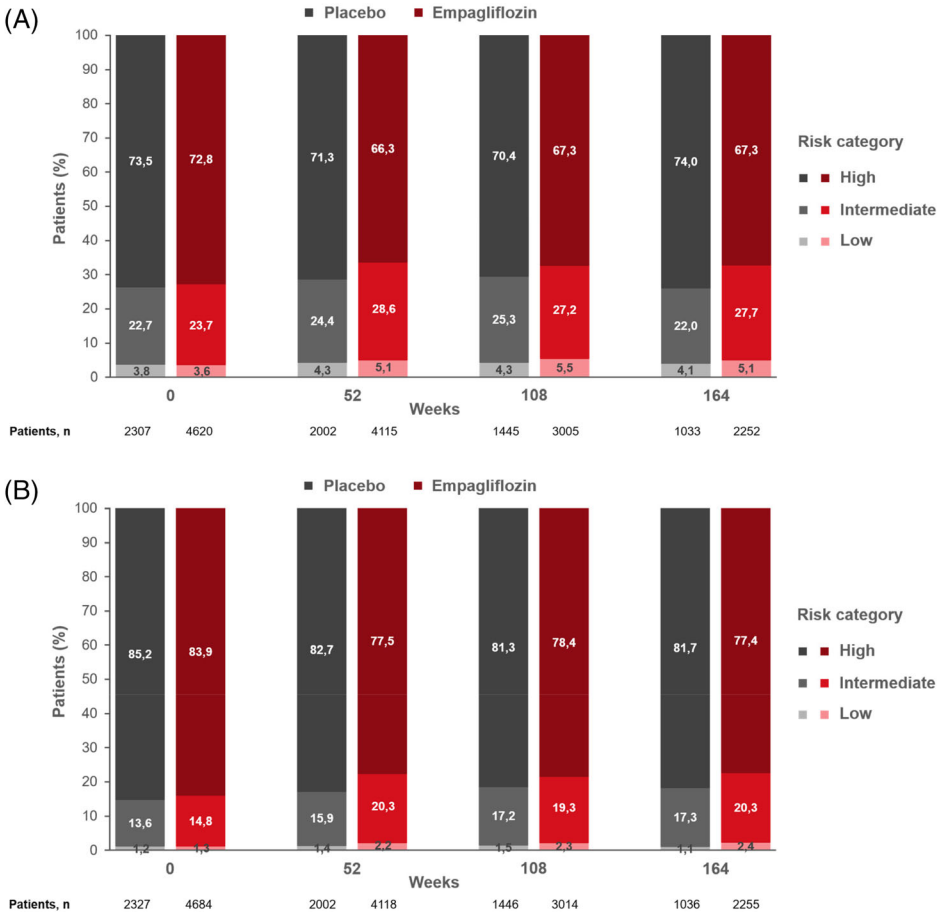


FIGURE 1 Percentage of patients in low/intermediate/high-risk categories by (A) Dallas steatosis index, (B) hepatic steatosis index, (C) non-alcoholic fatty liver disease fibrosis score and (D) Fibrosis-4 at 0, 52, 108 and 164 weeks of treatment

3.3 | Effects of empagliflozin on body weight and glycated haemoglobin in different risk categories

Across all derived steatosis and fibrosis risk groups, empagliflozin reduced body weight as compared with placebo at most time points (Figure S6). HbA1c decreased with empagliflozin compared with placebo at most time points in steatosis and at all time points in fibrosis risk groups (Figure S7).

3.4 | Effects of empagliflozin on biochemical parameters included in different indices

Serum triglyceride levels slightly increased over time in the low and intermediate DSI group and were unchanged in the high DSI group, without any differences between empagliflozin and placebo in any DSI category (Table S2).

Platelet counts decreased more with empagliflozin compared with placebo in the NFS and FIB-4 low-risk categories at 52, 108 and 164 weeks, but increased in the respective high-risk categories over time, with a more prominent decrease and less prominent rise in the empagliflozin group, respectively, at most time points (Table S3ab).

Albumin levels slightly increased after dosing with empagliflozin compared with placebo in NFS intermediate- and high-risk categories and remained higher at most time points and all categories (Table S4).

3.5 | Cardiorenal outcomes in fibrosis and steatosis risk groups

For DSI and HSI, highest placebo incidence rates for cardiovascular and all-cause mortality were found in the low steatosis risk category, with statistically significant differences compared with the high steatosis risk category (Figure 2A,B, Table S5a).

Using both NFS and FIB-4 for stratification of fibrosis risk, the groups at high risk of advanced fibrosis had substantially higher incidence rates of cardiovascular death, first hospitalization because of heart failure, first hospitalization for heart failure or cardiovascular death as well as all-cause mortality compared with the respective groups at low risk of advanced fibrosis (Figure 2C,D, Table S5a). However, incidence rates of new onset or worsening of nephropathy showed a similar albeit attenuated pattern across risk categories by all four scores.

A similar pattern was observed in the empagliflozin group, although the differences in relative risks in low- versus intermediate-

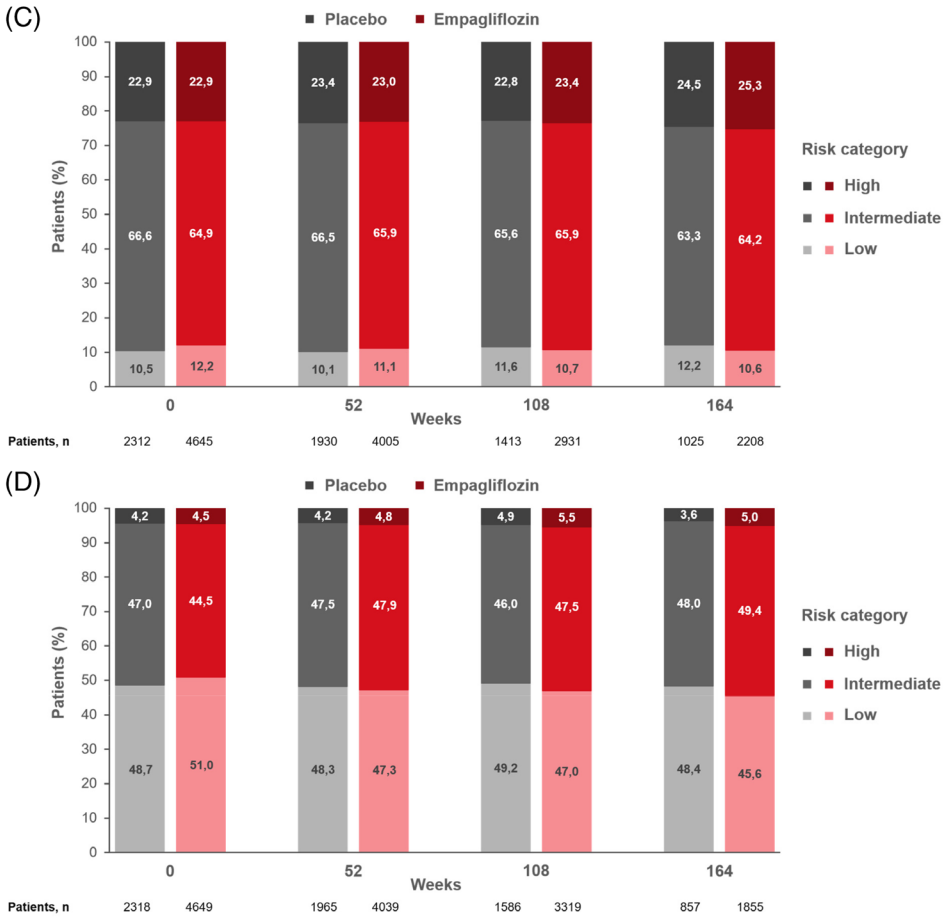


FIGURE 1 (Continued)

and high-risk categories did not reach statistical significance for most outcomes (Table S5b).

3.6 | Effects of empagliflozin on cardiorenal outcomes in groups at different risk of steatosis or fibrosis

Examining the treatment effects of empagliflozin versus placebo across the different steatosis and fibrosis risk groups showed consistent treatment effects on cardiorenal outcomes independent of steatosis or fibrosis category at baseline. Of note, for the combined endpoint of first hospitalization for heart failure or cardiovascular death, a quantitative interaction between DSI and treatment effect was observed, with all hazard ratios still reflecting a beneficial treatment effect, but of differing magnitude, for empagliflozin compared with placebo (Figure 2).

4 | DISCUSSION

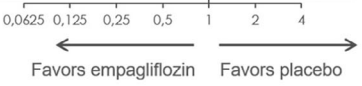
This post-hoc analysis of EMPA-REG OUTCOME suggests that empagliflozin (a) may improve steatosis risk as estimated by DSI and HSI, (b) does not reduce the risk of advanced fibrosis as calculated by NFS and FIB-4, (c) decreases glycaemia and body weight independent of steatosis and fibrosis risk, and (d) may confer consistent reduction of cardiorenal outcomes independent of steatosis and fibrosis risk category in people with type 2 diabetes when added to standard of care for about 3 years. Moreover, low- and high-risk fibrosis categories across all trial participants paralleled low and high risk for cardiovascular events and all-cause mortality.

Empagliflozin slightly reduced the percentages of participants in DSI and HSI high-risk categories over time when compared with placebo. In comparison, recent studies reported the effectiveness of 24-week empagliflozin treatment in the reduction of liver fat content, measured by ^{1}H magnetic resonance spectroscopy, when added to standard care or compared with placebo in individuals with type 2 diabetes.[8,12] However, fibrosis stage is the main prognostic factor in NAFLD.[13] An uncontrolled 24-week pilot trial provided first histological evidence that empagliflozin may reduce fibrosis in people with both type 2 diabetes and NASH.[14] A former analysis of EMPA-REG OUTCOME suggested improvements in serum transaminases [predominantly alanine aminotransferase (ALT)] with empagliflozin.[15] Of note, similar frequency and incidence of hepatic injury were reported for empagliflozin and placebo in clinical studies, but rare elevations in ALT and/or aspartate aminotransferase ≥5 times the upper normal

(A)

DSI

	Placebo		Empagliflozin				*p*-value for interaction
	n/N (%)	IR/1000 py	n/N (%)	IR/1000 py	HR (95% CI)	HR (95% CI)	
CV death							0.1145
No steatosis	11/88 (12.5)	43.8	7/165 (4.2)	14.6	0.32 (0.12, 0.81)		
Intermediate	43/523 (8.2)	28.9	43/1093 (3.9)	13.4	0.48 (0.31, 0.73)		
Steatosis	82/1696 (4.8)	16.5	117/3362 (3.5)	11.8	0.71 (0.54, 0.94)		
HHF							0.2641
No steatosis	3/88 (3.4)	12.5	3/165 (1.8)	6.4	NC		
Intermediate	23/523 (4.4)	15.9	25/1093 (2.3)	8.0	0.50 (0.28, 0.88)		
Steatosis	67/1696 (4.0)	13.9	96/3362 (2.9)	9.9	0.72 (0.53, 0.98)		
CV death or HHF*							0.0289
No steatosis	13/88 (14.8)	54.2	9/165	19.2	0.35 (0.15, 0.81)		
Intermediate	57/523 (10.9)	39.4	58/1093 (5.3)	18.6	0.47 (0.33, 0.68)		
Steatosis	125/1696 (7.4)	26.0	193/3362 (5.7)	20.0	0.77 (0.61, 0.96)		
All-cause mortality							0.4958
No steatosis	13/88 (14.8)	51.8	12/165 (7.3)	25.0	0.46 (0.21, 1.01)		
Intermediate	60/523 (11.5)	40.3	79/1093 (7.2)	24.6	0.62 (0.44, 0.87)		
Steatosis	120/1696 (7.1)	24.1	172/3362 (5.1)	17.3	0.72 (0.57, 0.91)		
Nephropathy							
No steatosis	15/74 (20.3)	81.5	18/143 (12.6)	48.5	0.52 (0.26, 1.04)		0.2332
Intermediate	74/451 (16.4)	64.8	130/956 (13.6)	50.9	0.77 (0.58, 1.02)		
Steatosis	292/1513 (19.3)	78.3	371/2962 (12.5)	47.0	0.58 (0.50, 0.68)		



(B)

HSI

	Placebo		Empagliflozin				*p*-value for interaction
	n/N (%)	IR/1000 py	n/N (%)	IR/1000 py	HR (95% CI)	HR (95% CI)	
CV death							0.0764
No / intermediate	35/345 (10.1)	35.2	32/754 (4.2)	14.3	0.42 (0.26, 0.68)		
Steatosis	102/1987 (5.1)	17.6	140/3932 (3.6)	12.1	0.69 (0.53, 0.88)		
HHF							0.7222
No / intermediate	10/345 (2.9)	10.3	16/754 (2.1)	7.3	0.75 (0.34, 1.65)		
Steatosis	85/1987 (4.3)	15.2	110/3932 (2.8)	9.8	0.64 (0.48, 0.85)		
CV death or HHF*							0.1292
No / intermediate	38/345 (11.0)	39.1	39/754 (5.2)	17.8	0.48 (0.30, 0.75)		
Steatosis	160/1987 (8.1)	28.6	226/3932 (5.7)	20.1	0.70 (0.57, 0.85)		
All-cause mortality							0.6115
No / intermediate	44/345 (12.8)	44.3	59/754 (7.8)	26.3	0.62 (0.42, 0.92)		
Steatosis	150/1987 (7.5)	25.9	210/3932 (5.3)	18.1	0.70 (0.57, 0.86)		
Nephropathy							0.6849
No / intermediate	68/298 (22.8)	92.0	95/641 (14.8)	54.9	0.57 (0.42, 0.78)		
Steatosis	320/1762 (18.2)	73.4	430/3483 (12.3)	46.4	0.62 (0.53, 0.71)		

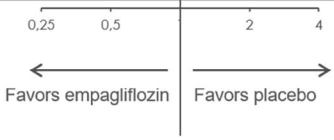
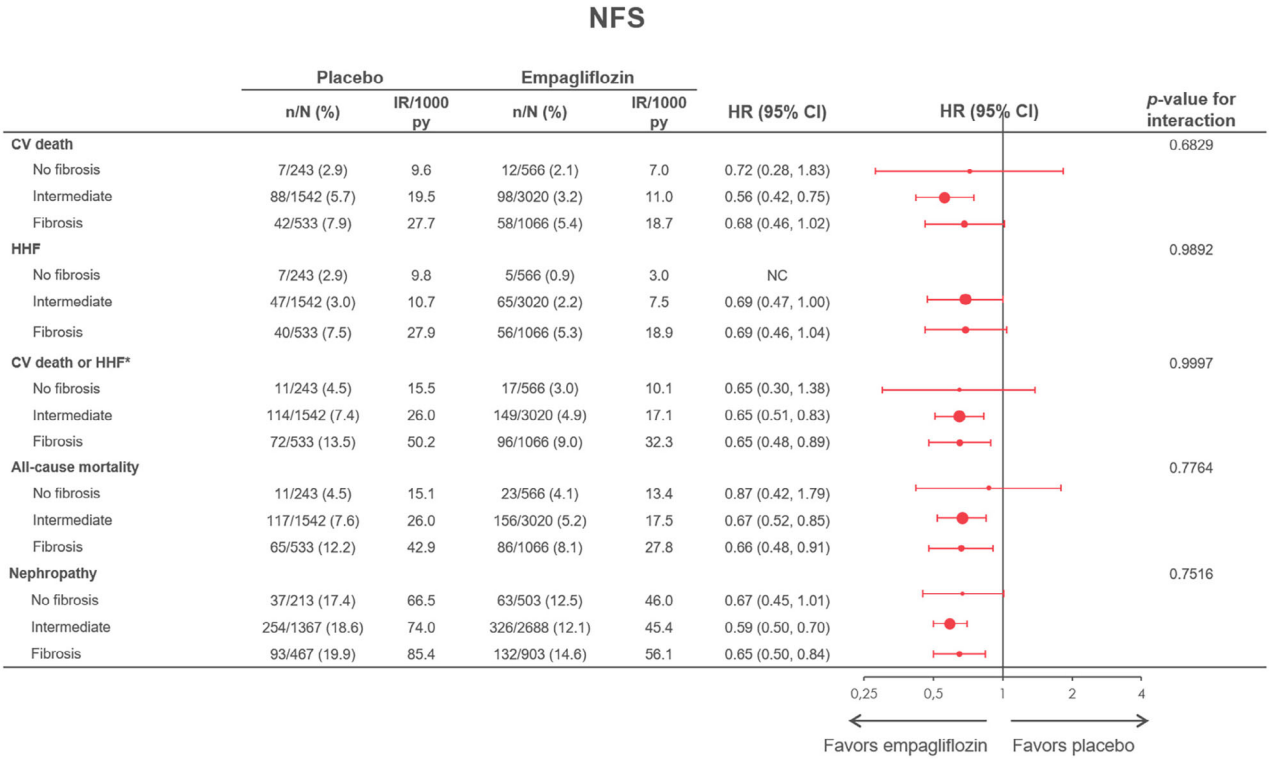


FIGURE 2 HR for empagliflozin versus placebo across different risk groups of (A) Dallas steatosis index (DSI), (B) hepatic steatosis index (HSI), (C) non-alcoholic fatty liver disease fibrosis score (NFS) and (D) Fibrosis-4 (FIB-4). The term "nephropathy" includes new onset or worsening of nephropathy. *Excluding fatal stroke. CI, confidence interval; CV, cardiovascular; HHF, hospitalization for heart failure; HR, hazard ratio; IR, incidence rates; py, patient years

limit were found to be more frequent with empagliflozin treatment.[16] In the current analysis, we found no reduction in the number or percentage of people grouped at high risk of advanced fibrosis by NFS and FIB-4 with empagliflozin. However, a recent post-hoc analysis of DURATION-8 reported a small decrease in the proportions of participants at high risk for fibrosis by NFS and FIB-4 from baseline to 28 weeks in individuals with type 2 diabetes.[17] Furthermore, a real-word data analysis from people with type 2 diabetes who switched to sodium glucose co-transporter 2 inhibitor treatment also found the number of cases classified as advanced fibrosis by FIB-4 reduced after

(C)



(D)

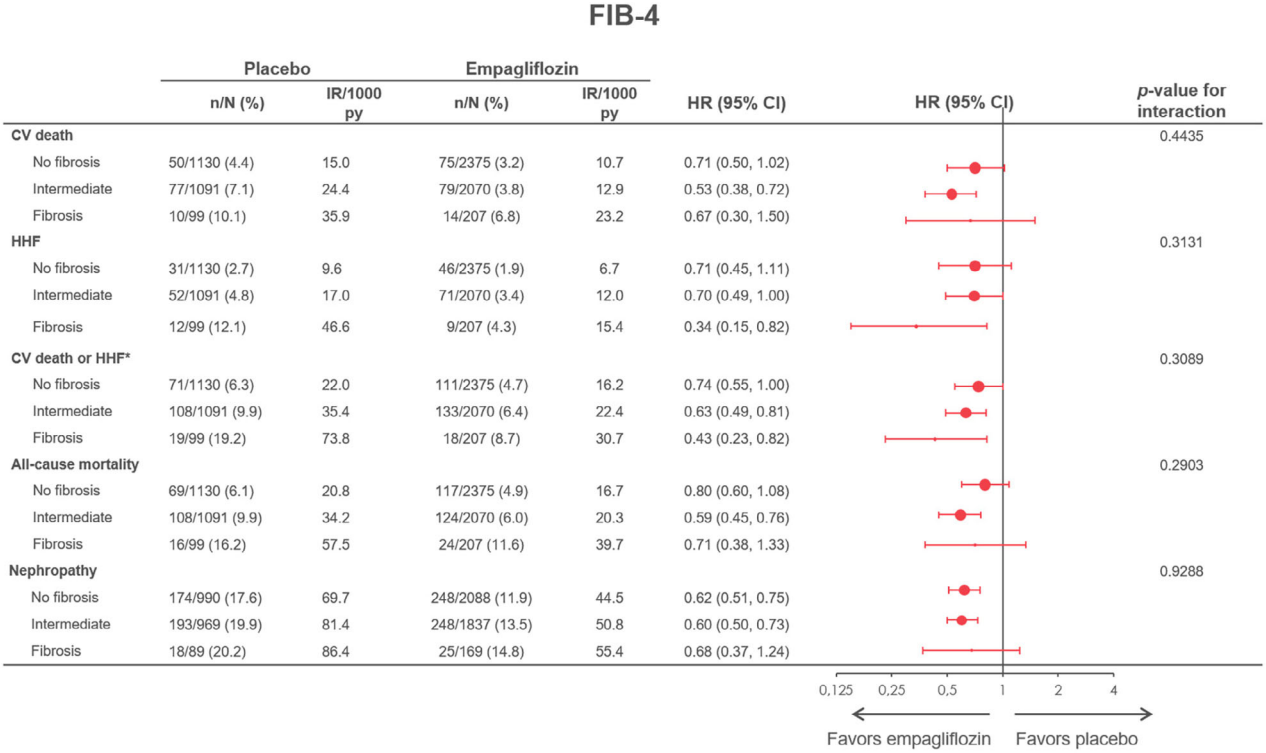


FIGURE 2 (Continued)

6 and 12 months.[18] At least in part, discrepancies in the different studies may be because of cohort characteristics. EMPA-REG OUTCOME included a type 2 diabetes collective with manifest cardiovascular disease, which often has progressive low-grade inflammation,[19,20] that may increase platelet count.[21] This effect may be counteracted by the previously reported anti-inflammatory effects of empagliflozin.[9] In EMPA-REG OUTCOME, platelet count, which is included in the denominator in FIB-4 and subtracted in NFS, was slightly lower with empagliflozin compared with placebo throughout the time of treatment and could contribute to the observed neutral

effect on fibrosis risk with empagliflozin. In contrast, serum albumin levels were higher with empagliflozin compared with placebo and may thereby contribute to lower NFS.

NAFLD closely associates with cardiorenal disease and risk is even increased in combined NAFLD and type 2 diabetes.[1] Patients grouped at high risk by either NFS or FIB-4 had higher placebo incidence rates of cardiovascular outcomes and all-cause mortality. While this observation may be expected, as age and/or serum albumin levels are included in the formulas,[22] several publications suggest that FIB-4 and/or NFS might be useful predictors of all-cause mortality in NAFLD with and without type 2 diabetes and thus help to improve risk stratification.[23-26] However, the relationship between risk scores and cardiorenal outcomes remains somewhat puzzling. Data from several cohorts as well as the present data show an association of FIB-4 and/or NFS high-risk categories with cardiovascular outcomes.[26,27] On the other hand, we did not observe an association of nephropathy with high-risk categories, in contrast to previous studies.[25,27] The differences in results may be at least partly because of cohort characteristics with different numbers of events. Moreover, smoking, baseline albuminuria, HbA1c, systolic blood pressure, glomerular filtration rate and haemoglobin concentrations were identified as determinants for progression of nephropathy in type 2 diabetes.[28] Thus, those risk factors may not be sufficiently reflected by fibrosis risk scores. Somewhat surprisingly, and although based on a low number of events, DSI and HSI identified individuals with low steatosis risk at highest incidence of cardiovascular and all-cause mortality endpoints. Of note, the degree of steatosis decreases during progression from advanced fibrosis to cirrhosis,[29] which might be an explanation of the observed results. Moreover, low body weight and thus (assumed) low liver fat as well as ALT values in the lower normal range were previously reported to associate with worse cardiovascular outcomes and mortality.[30] Of interest, advanced liver fibrosis (as measured by transient elastography) was identified as a risk marker and severe steatosis as a protective factor for cardiovascular complications and mortality in individuals with type 2 diabetes and NAFLD, which would support the present results.[31]

The present analysis further suggests that study participants in all steatosis and fibrosis risk categories profit similarly in relative terms from empagliflozin treatment in regard to cardiorenal outcome and all-cause mortality. Similarly, former analyses of the EMPA-REG OUTCOME study reported that cardiorenal treatment benefits with empagliflozin are consistent across age groups as well as underlying cardiovascular and heart failure risk.[32,33]

Major strengths of our analysis include the large number of enrolled participants, follow-up of more than 3 years, its placebo-controlled design and being the first to evaluate the effects of empagliflozin on risk of advanced fibrosis, metabolic parameters and cardiorenal outcomes dependent on steatosis and fibrosis category.

This analysis has also several limitations, as liver histology or imaging methods were not performed in the EMPA-REG OUTCOME. As with any clinical scoring system, the scores used herein merely imply the likelihood of but do not necessarily directly reflect the actual degree of steatosis or fibrosis. In addition, we cannot rule out other causes of fibrosis, as there was no defined assessment of alcohol intake and hepatitis serology foreseen in the trial protocol. This lack of data as well as the absence of records regarding liver-related clinical outcomes limits the interpretability and clinical significance of our data. However, as up to 20% of people with type 2 diabetes and NAFLD are predicted to have clinically relevant fibrosis,[1] NAFLD most likely represents the underlying cause in most cases.

In addition, the use of steatosis and fibrosis biomarkers has rarely been validated for assessment of longitudinal changes in these parameters in response to pharmacological treatment[34] and changes in the scores may also be driven by some components of the scores and not sufficiently reflect changes in liver tissue. Moreover, empagliflozin effects on hepatic fibrosis may not necessarily be detected by the indices even if present as one could assume that improvement of steatosis may also affect fibrosis in the long-term.[2,13] Of note, changes in NFS were previously associated with changes in liver fibrosis after 1 year of lifestyle intervention[35] suggesting a dynamic response to histological changes. Nevertheless, the current analyses do not support the beneficial effects of empagliflozin on hepatic fibrosis in type 2 diabetes.

In our post-hoc analyses, we did not adjust for multiple comparisons, as we considered them exploratory. Of note, this trial was not primarily powered to assess the cardiorenal effects of empagliflozin in different steatosis and fibrosis categories so that the differences in patient numbers of each risk category may limit interpretation of the obtained results. In addition, cohort size continuously dropped with time so that missing significances for scores between empagliflozin and placebo at later time points may rather result from loss of power than from time-dependent effects of empagliflozin itself.

NFS and FIB-4 were developed for assessment of fibrosis severity in NAFLD cohorts encompassing the whole clinical and histological liver disease spectrum. As our cohort was a preselected collective – because of the trial's inclusion and exclusion criteria,[7] liver-related findings from this study may be interpreted with caution and only adopt to patients with type 2 diabetes, cardiovascular disease and no or mild liver impairment.

Furthermore, several laboratory parameters were not routinely measured in the trial so that the steatosis markers recommended for NAFLD screening in the current European guidelines[2] (fatty liver index, NAFLD liver fat score) could not be calculated. However, we calculated HSI and DSI for assessing steatosis risk in our cohort.[10,11] As the major limitation of DSI, there is still only one external validation study for this index, which was not focused on for type 2 diabetes.[36]

Of note, most steatosis and fibrosis indices are considered imperfect markers, as their variability is high and their positive predictive value in terms of NAFLD and NASH seems limited.[4,27,37,38] Furthermore, they have not been specifically developed for individuals with type 2 diabetes. Several studies even suggest that non-invasive tests may perform less well when applied to individuals with type 2 diabetes[37] and many of them include type 2 diabetes as a risk factor in their formulas.[38] In our study and previous reports, steatosis and fibrosis scores identified different proportions of people at high risk for steatosis or advanced fibrosis[27] so that results derived from a single index need to be interpreted with caution. Nevertheless, the 2020 American[39,40] and European guidelines on clinical management of

NAFLD[2] recommend using surrogate markers of steatosis and fibrosis for screening of patients at high risk of NAFLD, including patients with type 2 diabetes.[2]

In conclusion, empagliflozin may improve steatosis (DSI, HSI) but not fibrosis (NFS, FIB-4) risk in patients with type 2 diabetes and pre-existing cardiovascular disease. High-risk categories of fibrosis were associated with higher incidence of cardiovascular events. Empagliflozin further seemed to improve cardiorenal outcomes across all steatosis and fibrosis categories. With a lack of large-scale, prospective randomized placebo-controlled clinical trials, including imaging or histology for assessment of steatosis and fibrosis, this study adds relevant information on the potential effects of empagliflozin on NAFLD, and on the cardiorenal effects of empagliflozin in varying risk groups of patients with type 2 diabetes. Nevertheless, future research, including liver histology and/or imaging, is needed to assess better the potential benefits of empagliflozin treatment on fibrosis and its importance for clinical practice.

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CONFLICT OF INTEREST

SK has received research grants from the German Center for Diabetes Research (DZD e.V.) and the German Diabetes Society (DDG). APO is employee of Boehringer Ingelheim. BZ has received research grants awarded to his institution from Boehringer Ingelheim, AstraZeneca, and Novo Nordisk, and honoraria from Janssen, Sanofi, Eli Lilly, Boehringer Ingelheim, Novo Nordisk, and Merck Sharp & Dohme. CW reports honoraria from AstraZeneca, Boehringer Ingelheim, Eli Lilly, Merck Sharp & Dohme, Bayer and Sanofi. ES is employee of mainanalytics, contracted by Boehringer Ingelheim. NS has consulted for Amgen, Astrazeneca, Boehringer Ingelheim, Eli-Lilly, Hanmi, MSD, Novartis, Novo-Nordisk, Pfizer and Sanofi and received grant support from Astrazeneca, Boehringer Ingelheim, and Roche Diagnostics. SEI has received honoraria for lectures, advisory work and/or clinical trial leadership from AstraZeneca, Boehringer Ingelheim, Novo Nordisk, Merck, Lexicon, Esperion, vTv Therapeutics, Abbott and Pfizer. MR has served on scientific advisory boards or received speaker's honoraria for Allergan, Boehringer-Ingelheim Pharma, Bristol-Myers Squibb, Eli Lilly, Fishawack Group, Gilead Sciences, Novartis Pharma, Intercept Pharma, Inventiva, Novo Nordisk, Target NASH. He has been also a consultant for Terra Firma and involved with clinical trial research for Boehringer Ingelheim, Danone Nutricia Research and Sanofi-Aventis.

DATA AVAILABILITY STATEMENT

The sponsor of the study (Boehringer Ingelheim) is committed to responsible sharing of clinical study reports, related clinical documents, and patient-level clinical study data. Researchers are invited to submit inquiries via the following website (https://trials.boehringer-ingelheim.com/).

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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