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Hummel, Julia

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Dekan: Professor Dr. B. Pichler

1. Berichterstatter: Professor Dr. M. Heni
2. Berichterstatter: Professor Dr. Dr. P. Ruth
3. Berichterstatter: Professor Dr. S. Meyhöfer

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Abbreviations

ADA	American Diabetes Association
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
ATP	Adenosine 5′ Trisphosphate
BMI	Body Mass Index
CNS	Central Nervous System
CO_2	Carbon Dioxide
DNA	Deoxyribonucleic Acid
e.g.	*Exempli gratia* (for Example)
EGP	Endogenous Glucose Production
ER	Endoplasmatic Reticulum
Et al.	*Et altera* (and others)
FAD	Flavin Adenine Dinucleotide
FFA	Free Fatty Acids
FGF-21	Fibroblast Growth Factor-21
(f)MRI	(Functional) Magnetic Resonance Imaging
G-CSF	Granulocyte-colony Stimulating Factor
GIP	Glucose-dependent Insulinotropic Polypeptide
GLP-1 RA	Glucagon-like Peptide-1 Receptor Agonist
H	Hour(s)
HbA1c	Hemoglobin A1c
IL-6	Interleukin-6
IRS-1	Insulin Receptor Substrate-1
MCP-1	Monocyte Chemoattractant Protein-1
NAD	Nicotinamide Adenine Dinucleotide

NAFLD	Non-alcoholic Fatty Liver Disease
O_2	Oxygen
OECD	Organization for Economic Co-operation and Development
OGTT	Oral Glucose Tolerance Test
PI3K	Phosphatidylinositol-3-Kinase
PPAR-α	Peroxisome Proliferator-activated Receptor-α
R	Receptor
RER	Respiratory Exchange Ratio
ROS	Reactive Oxygen Species
RQ	Respiratory Quotient
SGLT2(i)	Sodium Glucose Cotransporter 2 (Inhibitor)
Suppl. Mat.	Supplementary Material
T2DM	Type 2 Diabetes Mellitus
TAG	Triacylglyceride
TNF-α	Tumor Necrosis Factor-alpha
V	Volume
VLDL	Very Low-density Lipoprotein
WHO	World Health Organization

1 Introduction

1.1 Definition, epidemiology and prevalence of obesity and type 2 diabetes

1.1.1 Obesity

The global number of individuals with overweight and obesity almost tripled during the last 50 years (World Health Organization, 2021). Excess weight increased especially in children and adolescents (Abarca-Gómez et al., 2017; Fanzo et al., 2018) as well as in low- and middle-income countries (Templin et al., 2019), making obesity not only a disease of the wealthy or the old. In 2016, 1.9 billion adults were overweight, representing 39 % of the world's population (World Health Organization, 2021). Of these, 650 million had obesity (World Health Organization, 2021). Reports from Germany show that more than half of the population is overweight or obese (Schienkiewitz et al., 2017).

The world health organization (WHO) defines overweight as body mass index (BMI) ≥ 25 and < 30 kg/m², while a BMI of 30 kg/m² or greater is termed as obesity (World Health Organization, 2000). Long-term imbalance between energy intake and expenditure is leading to excess weight gain (Hill et al., 2012). Obesogenic environmental factors as an unhealthy food system including highly processed, energy dense convenience food (Swinburn et al., 2011) accompanied by a shift to lower physical activity are supposed to be main drivers of this pandemic. Sedentary behavior is favored by technological advances at home and at work, including occupational work, transportation as well as activities in leisure time (Chooi et al., 2019; Ng and Popkin, 2012). Projections for 2030 estimate the prevalence to rise to 2.16 billion for overweight and 1.12 billion for obesity in the worldwide adult population (Kelly et al., 2008). Obesity therefore represents a major global health concern and is associated with increased morbidity and all-cause mortality (Berrington de Gonzalez et al., 2010). Since excessive fat accumulation affects numerous physiological processes, obesity was identified as major risk factor for a number of clinical conditions including type 2 diabetes (Singh et al., 2013), fatty liver disease (Li et al., 2016), cardiovascular diseases (Singh et al., 2013), musculoskeletal diseases (Anandacoomarasamy et al., 2008), Alzheimer´s disease (Anstey et al., 2011), depression (Jantaratnotai et al., 2017) and even some types of cancer (Lauby-Secretan et al., 2016). All of these obesity-related diseases adversely affect not only quality of life

(Fontaine and Barofsky, 2001), but also lead to markedly increased health care costs. Indeed, obesity-related diseases account for 8.4 % of total health expenditures of the OECD countries, positioning obesity as a serious economic burden (OECD, 2019).

1.1.2 Type 2 diabetes

Several factors, including a growing life expectancy, a rise in urbanization as well as a sedentary lifestyle with unhealthy diet and an excess of calories favor the development of type 2 diabetes mellitus (T2DM) (Tinajero and Malik, 2021). Over the last decades, the worldwide prevalence of diabetes accelerated dramatically from 108 million in 1980 to 422 million in 2014 (Zhou et al., 2016). In 2019, 463 million individuals were globally affected by diabetes corresponding to 9.3% of the population, of which 90 % had T2DM (Saeedi et al., 2019). This number is projected to rise to 578 million by 2030 and will reach 700 million by 2045 (Saeedi et al., 2019). Interestingly, a more rapid growth of diabetes cases was recorded in low- and middle- than in high-income countries (Saeedi et al., 2019; Tinajero and Malik, 2021). Aging is accompanied by an increasing prevalence with 19.9 % being diabetic between 65 and 79 years (Saeedi et al., 2019). Germany recorded 6.9 million T2DM cases in 2019/2020 with an estimated number of around 2 million unknown cases of diabetes (Jacobs and Rathmann, 2019).

For the diagnosis of T2DM at least one of three criteria must be fulfilled: hemoglobin A1c (HbA1c) ≥ 6.5%, fasting plasma glucose ≥126 mg/dl or 2-h plasma glucose ≥ 200 mg/dl in a 75-g oral glucose tolerance test (American Diabetes Association, 2021; Arzneimittelkommission Der Deutschen Apotheker et al., 2021; Schleicher et al., 2021).

Diabetes represents a large burden for the health systems. In 2010, T2DM was attributed to 10 % of total healthcare expenses in Germany (Jacobs et al., 2017). Diabetes is the major cause for kidney failure (Drawz and Rahman, 2015) and doubles the risk for cardiovascular diseases (Emerging Risk Factors Collaboration et al., 2010), which are among the leading causes of death (Lozano et al., 2012). Indeed, diabetes accounted for 4.2 million deaths in 2019 and is therefore linked to 1 in 9 deaths worldwide (Saeedi et al., 2020), placing the disease as major global threat.

1.2 Pathophysiology of obesity and type 2 diabetes

1.2.1 Obesity

1.2.1.1 Genetics, epigenetics and lifestyle

Obesity is a complex multifactorial chronic disease, caused by a long-term energy imbalance. Estimates from twin and adoption studies suggest a heritability of BMI ranging from 40 up to 70 % (Herrera et al., 2011). While monogenetic forms of obesity are rare, polygenic obesity got into focus (Pigeyre et al., 2016; Rohde et al., 2019). Currently, more than 300 genetic loci for single nucleotide polymorphisms have been identified to associate with BMI (Goodarzi, 2018). However, these genetic variants explain less than 5 % of BMI variation (Pigeyre et al., 2016). Further, epigenetic mechanisms are likely involved in the heritability of obesity (Pigeyre et al., 2016). Epigenetic modifications are chemical modulations of deoxyribonucleic acid (DNA) bases as well as changes of the protein complex surrounding the DNA (histones) that impact on gene function despite the DNA sequence remains unaffected (Al Aboud et al., 2021). As a result of these depicted reactions, gene expression is modified (mostly decreased but in some instances potentially also increased) (Al Aboud et al., 2021). Intrauterine and postnatal nutritional exposure are thought to have persistent effects on metabolic health later in life conveyed via epigenetic mechanisms (Oestreich and Moley, 2017). In addition to the genetic and epigenetic predisposition, several behavioral and environmental factors contribute to the regulation of energy homeostasis. Obesity, as a result of dysregulated energy metabolism, is promoted by excessive food intake and lack of physical activity (Swinburn et al., 2011).

1.2.1.2 Central regulation of energy homeostasis

The brain, especially the hypothalamus regulates energy homeostasis via food intake and energy expenditure (Myers and Olson, 2012). Specialized neurons receive signals from the periphery that inform the brain about nutrient supply of the organism (Timper and Brüning, 2017). Energy availability or shortage is e.g. conveyed via vagal afferents from digestive organs (Berthoud, 2008). Furthermore, circulating metabolites as glucose as well as hormones like leptin, ghrelin and insulin signal to the brain (Berthoud, 2008;

Schwartz and Porte, 2005). These signaling factors are released from stomach, adipose tissue, and pancreas, respectively (Sobrino Crespo et al., 2014; Valassi et al., 2008). Highly specialized neurons in the hypothalamus translate these signals into behavior and inhibit or stimulate eating (Vettor et al., 2002). Further, they regulate absorption and storage as well as mobilization of substrates via vagal efferents, the sympathetic nervous system and hormonal mechanisms (Berthoud, 2008). Though, obesity is associated with insulin resistance and leptin resistance of peripheral tissues as well as the brain, whereby the physiological functions of these hormones in energy homeostasis are disrupted (Myers et al., 2010). Besides these basic homeostatic mechanisms in the hypothalamus, eating behavior is further influenced by centrally regulated processes as taste perception, hedonic liking of food, reward response to food stimuli as well as cognition and decision making (Davidson et al., 2019). One theory suggests that processes of homeostatic control can be overwritten by reward-related response mechanisms to food (cues) favoring weight gain (Burger and Berner, 2014; Lutter and Nestler, 2009).

In addition to energy intake, the expenditure of energy is pivotal to maintain energy balance. Though hard to measure, energy expenditure by means of physical activity is assumed to be involved in obesity development (Blüher, 2019; Ng and Popkin, 2012). However, current evidence argues against an earlier supposed lower resting energy expenditure in persons with obesity compared to their normal weight counterparts, which would favor a positive energy balance (Carneiro et al., 2016a). Moreover, no differences of resting and activity-related energy expenditure between persons with normal weight and overweight have been found after adjusting for fat free mass (Oussaada et al., 2019). Thus, the relative contribution of energy expenditure to the development of obesity is still under debate.

1.2.1.3 Adipose tissue and mitochondrial function

The surplus of energy is predominantly stored as triacylglycerides (TAG) in white adipose tissue. Excessive fat deposition adversely affects metabolic processes and leads to an increase in adipocyte size and apoptosis (Strissel et al., 2007) with progressive immune-cell infiltration into adipose tissue (Harford et al., 2011). Altered adiponectin, adipokine and pro-inflammatory cytokine secretion by adipocytes and macrophages promote a state of chronic low-grade inflammation and foster the development of insulin

resistance (Harford et al., 2011). Subsequently, these secreted adipokines and pro-inflammatory cytokines induce so called oxidative stress (Fernández-Sánchez et al., 2011). In detail, oxidative stress is caused by highly reactive chemicals produced as byproducts from oxygen respiration. These reactive oxygen species (ROS) emerge from the mitochondrial oxidation of glucose and free fatty acids (FFA), and further metabolic processes within the cell (Bondia-Pons et al., 2012). In order to control ROS levels, an antioxidant defense system disarms these radicals (Birben et al., 2012). However, in the case of excess nutrient supply to adipose and non-adipose tissue cells, glucose and FFA can overload the mitochondria leading to a surplus of ROS production (Bondia-Pons et al., 2012). If ROS formation exceeds the cellular elimination capacity, these radicals accumulate and can oxidize e.g. lipids, proteins, DNA, or even cell structures which thereby lose their biological function (Birben et al., 2012). These mechanisms likely impair mitochondrial function and lead to a diminished oxidative capacity which might favor excessive fat accumulation (Bondia-Pons et al., 2012). Finally, oxidative stress further aggravates the inflammatory processes that are already present in obesity and in turn adversely affects insulin action in its target organs. Indeed, reduced insulin sensitivity was observed in persons with obesity (Kahn and Flier, 2000).

1.2.1.4 Body fat distribution

Since lipid handling and storage capacity of adipose tissue are impaired in obesity, the overflow energy can lead to ectopic fat deposition in the liver, muscle, and pancreas (Snel et al., 2012). Of notice, it appears that not every fat compartment is equally detrimental for future health (Ibrahim, 2010). It turned out that body fat distribution is pivotal for whole-body metabolism (Booth et al., 2014). Ectopic fat deposition e.g. in the liver as well as central obesity with visceral fat accumulation (Preis et al., 2010) are associated with abnormalities in metabolic functions, insulin resistance (Snel et al., 2012), and a markedly elevated risk for T2DM (Shuster et al., 2012). Increased hepatic release of very low-density lipoproteins (VLDL) due to lipid accumulation in the liver and diminished suppression of lipolysis by insulin in adipose tissue lead to elevated levels of FFA and TAG (Choi and Ginsberg, 2011; Ebbert and Jensen, 2013). Elevated concentrations of especially saturated FFA promote insulin resistance, and thereby contribute to the susceptibility to develop T2DM (Griffin et al., 1999).

1.2.2 Type 2 diabetes

T2DM is a multifactorial disease. Most likely, an interaction of genetic and epigenetic predisposition with environmental and lifestyle factors is involved in the etiology of this metabolic disorder (Blüher, 2019). As a polygenetic disease, roughly 18 % of T2DM risk is explained by known genetic variation in loci of T2DM genes (Mahajan et al., 2018). Especially genes which play a role in ß-cell function and integrity appear to be involved (Mahajan et al., 2018).

On a more mechanistic level, T2DM is caused by combined occurrence of insulin resistance and pancreatic ß-cell failure (Roden and Shulman, 2019; Stumvoll et al., 2005). This results in hyperglycemia, the common definition of diabetes. The earliest detectable pathogenic alteration typically is a diminished action of insulin in its classical target organs e.g. liver, adipose tissue and skeletal muscle (Galicia-Garcia et al., 2020). For some time, this can be compensated by increased insulin secretion from ß-cells to maintain normal glucose homeostasis (Cerf, 2013). Alterations in glucose metabolism are already seen several years prior to overt diabetes with a strong drop of insulin sensitivity starting 5 years before diagnosis (Tabák et al., 2009). To compensate for diminished insulin action, the secretion of insulin sharply increases 3-4 years prior to pathological glucose values, enabled potentially in part through enlargement of Langerhans islet size and rise of ß-cell number (Sachdeva and Stoffers, 2009). This state is characterized by hyperinsulinemia, which further promotes insulin resistance (Roden and Shulman, 2019), while glucose levels can still be kept within a physiological or prediabetic range. Fasting glucose gradually rises over time but start to strongly increase 3 years prior overt T2DM. The latter is also observed for 2-h glucose values after an oral glucose load (Tabák et al., 2009). Thus, T2DM is preceded by a phase of prediabetes with slightly elevated glucose levels still remaining under the thresholds for T2DM. If insulin secretory capabilities decrease rapidly in the 3 years preceding T2DM diagnosis due to ß-cell loss, glucose levels increase and convert to a diabetic state (Stumvoll et al., 2007; Tabák et al., 2009).

1.2.2.1 Insulin resistance

Several causative factors that contribute to insulin resistance are discussed (Yaribeygi et al., 2019). A strong correlation between insulin sensitivity and visceral adiposity is well

established (Macor et al., 1997). One proposed underlying mechanism linking visceral fat with insulin resistance describes how adipokines interfere with the insulin signaling pathway. FFA, pro-inflammatory cytokines (e.g. tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6)) and chemokines (e.g. monocyte chemoattractant protein-1 (MCP-1)) are released by adipose tissue, a large non-classical endocrine organ (Trayhurn, 2007; Vázquez-Vela et al., 2008). These factors reach insulin-sensitive tissues via the bloodstream where they induce cellular insulin resistance by promoting serine phosphorylation of insulin receptor substrates (Shulman, 2000). This serine phosphorylation augments activity of the classical insulin receptor, namely the insulin receptor substrate (IRS)-1 - Akt signaling cascade (Shulman, 2000). Another crucial factor appears to be adiponectin. This peptide hormone is released from adipose tissue (Scherer et al., 1995). It was characterized as an insulin-sensitizing and anti-inflammatory adipokine (Wang and Scherer, 2016). Its expression and release are reduced in expanded adipose tissue (Arita et al., 1999). This lack of adiponectin is thought to promote insulin resistance in liver and muscle (Gilcampos, 2004).

Besides the classical insulin sensitive organs, the brain has been characterized as insulin responsive (Ruud et al., 2017). In addition to the described effects on behavior above, insulin action in the brain is also involved in the regulation of peripheral metabolism and linked to body fat distribution (Kullmann et al., 2015a, 2020a, 2020b). Peripheral insulin reaches the brain, more specifically the hypothalamus where it initiates signals towards the periphery (Banks et al., 2012). This central regulation of peripheral metabolism was untangled in a number of studies in humans, showing brain insulin action to enhance whole-body insulin sensitivity (Heni et al., 2014), to promote insulin secretion (Heni et al., 2020) and to suppress hepatic endogenous glucose production (Dash et al., 2015; Heni et al., 2017). Though, diminished insulin responsiveness was detected in conditions like obesity and T2DM, a state termed brain insulin resistance (Heni et al., 2015). Decreased sensitivity of insulin in the brain adversely affects metabolic health and is moreover linked to an unfavorable body fat distribution with more fat surrounding the internal organs (Heni et al., 2015).

Besides visceral fat, emerging evidence supports a close link between ectopic fat accumulation and insulin resistance (Snel et al., 2012). Ectopic fat means the deposition

of triglycerides in non-adipose tissues (cells), which are typically not linked to adipose tissue storage (e.g. muscle and liver) (Snel et al., 2012). Especially excess lipid accumulation in the liver, termed as non-alcoholic fatty liver disease (NAFLD) was identified as predictor of T2DM (Lallukka and Yki-Järvinen, 2016). Since the liver represents the key organ for systemic metabolism, lipids within the parenchyma likely disturb the secretory properties (e.g. proteins, lipids, metabolites) of the liver (Watt et al., 2019). Especially an altered pattern of hepatokine secretion, which is involved in the regulation of lipid metabolism, inflammation and insulin sensitivity may adversely affect metabolism in other tissues (Watt et al., 2019).

1.2.2.2 Insulin secretion

Over the course of time, insulin hypersecretion is depleted, which likely corresponds to ß-cell dedifferentiation and ß-cell apoptosis (Stumvoll et al., 2007). *Post mortem* studies indicate a loss of 40-50 % of ß-cell mass in patients with T2DM (Butler et al., 2003). While acute rises in FFA and glucose physiologically stimulate insulin secretion, a chronic overload hinders insulin secretion/decreases ß-cell function and subsequently even induces ß-cell apoptosis (Rhodes, 2005). As underlying mechanisms for ß-cell apoptosis, an activation of Phosphatidylinositol-3-kinase (PI3K) (Eitel et al., 2003), the formation of ROS in substrate oxidation in ß-cells and endoplasmatic reticulum (ER) stress mediated signaling pathways are discussed (Donath and Halban, 2004).

Of note, increased intrapancreatic fat has been observed in patients with prediabetes (Ou et al., 2013) and T2DM (Gaborit et al., 2015). Adipocytes located next to the islets within the pancreatic parenchyma have the potential to adversely affect ß-cell function via their secreted factors (e.g. FFA, cytokines) (Quiclet et al., 2019; Rodríguez et al., 2015). Indeed, increased intrapancreatic fat content associates with diminished insulin secretion in prediabetes (Heni et al., 2010). First hints point towards a positive effect of pancreatic fat reduction on restoration of insulin secretion (Lim et al., 2011; Wagner et al., 2021a). Therefore, pancreatic lipids are likely involved in the development of T2DM and may serve as a potential target to improve insulin metabolism (Wagner et al., 2021a).

The observation that oral glucose intake evokes higher insulin secretion than isoglycemic intravenous administration in healthy persons is called “incretin effect” (Elrick et al.,

1964; Nauck and Meier, 2018). Incretins are peptides secreted from gut cells upon oral nutrient intake and play a major role in the stimulation of insulin secretion (Buchan et al., 1978; Eissele et al., 1992). Since the incretin effect is diminished or even absent in T2DM, insulin secretion is not sufficient to prevent hyperglycemia (Nauck and Meier, 2016).

1.3 Energy metabolism and substrate oxidation

In order to generate biochemical energy, humans rely on the oxidation of nutrients (lipids, carbohydrates, proteins). Major metabolic pathways for cellular energy generation are illustrated in figure 1.

Glucose serves as primary energy substrate, which is first catabolized into two molecules of pyruvate in a series of enzymatic catalyzed reactions in the cytoplasm, called glycolysis (Pilkis and Granner, 1992). Under aerobic conditions, pyruvate is transformed to acetyl CoA via oxidative decarboxylation which then undergoes complete oxidation to CO_2 in the citric cycle (Krebs, 1940; Maughan, 2009). These reactions allow the reduction of the cofactors nicotinamide adenine dinucleotide (NAD) and flavin adenine nucleotide (FAD), which deliver electrons for the respiratory chain (Maughan, 2009; Rigoulet et al., 2020). Electrons are transported along a chain of protein complexes in the inner mitochondrial membrane via redox reactions - evoking an electrochemical gradient that drives the formation of adenosine 5′ trisphosphate (ATP) (Bonora et al., 2012; Hatefi, 1985). Oxidation of glucose yields 36 ATP per mol and serves as carrier of biochemical energy (Ferrannini, 1988). These described intracellular processes take place in the mitochondria. In cells without mitochondria (e.g. erythrocytes) (Harvey and Kaneko, 1976) or during lack of O_2 (e.g. exercise) anaerobe glycolysis is the predominant pathway for energy generation (Skinner and Mclellan, 1980), where pyruvate is reduced to lactate.

Break down of TAG via lipolysis serves as further energy source (Wang et al., 2008). Emerging glyceride enters glycolysis, while FFA are transformed to acetyl CoA in a series of reactions called ß-oxidation which flows into citric cycle (Kennedy and Lehninger, 1949). The oxidation of the FFA palmitate yields 131 ATP (Ferrannini, 1988).

Last, proteins get catabolized into amino acids and are further degraded to enter glycolysis or citric cycle. Proteins play a minor role in energy metabolism and generate only 23 ATP per mol (Ferrannini, 1988).

In conditions like fasting, when glucose and insulin levels are low, or in states of insulin resistance, when glucose utilization is diminished, the formation of ketones is stimulated (Balasse and Féry, 1989). Ketogenesis in liver mitochondria originate mainly from the intermediate acetyl CoA of fatty acid oxidation (Puchalska and Crawford, 2021). Ketone bodies serve as energy source especially for the skeletal and cardiac muscle as well as the brain which is not able to oxidize FFA (Laffel, 1999).

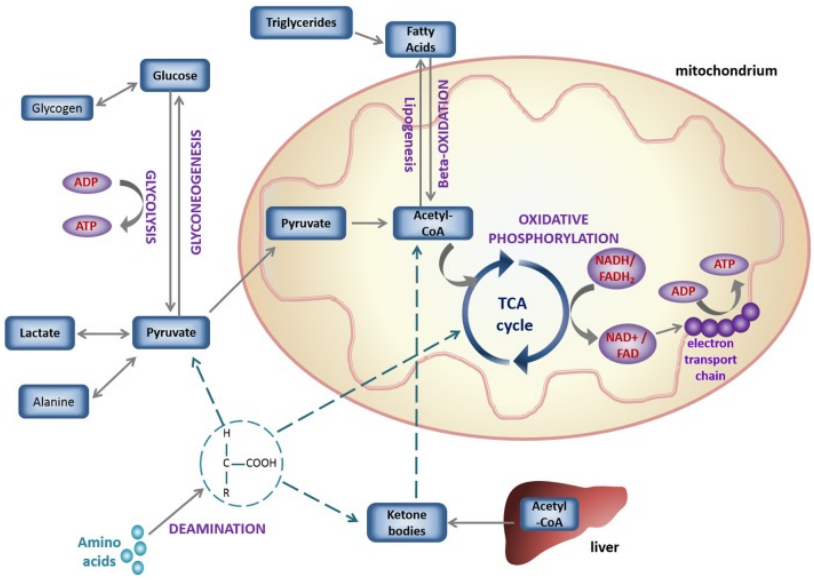

Figure 1: Major metabolic pathways for cellular energy generation
Reference: Malandraki-Miller et al., Front cardiovasc Med, 2018 (Malandraki-Miller et al., 2018)

1.3.1 Potential determinants of substrate oxidation

1.3.1.1 Insulin and glucagon

Major regulators of cellular nutrient availability for oxidation are insulin and glucagon (Qaid and Abdelrahman, 2016). High circulating glucose levels trigger the secretion of insulin from ß-cells of the pancreas (Suckale and Solimena, 2008). The binding of insulin to its receptor initiates an intracellular cascade, activating several lipid and protein kinases, that regulate glucose homeostasis and metabolism (Saltiel, 2021). Insulin facilitates cellular glucose uptake and promotes glycolysis as well as glycogen synthesis to lower blood glucose (Saltiel and Kahn, 2001). Insulin further affects lipid metabolism via suppression of lipolysis, stimulation of fat storage and reduction of FFA oxidation in

muscle and liver (Saltiel and Kahn, 2001). Since insulin promotes an anabolic state, the oxidation of glucose is favored while FFA oxidation becomes secondary.

When glucose and insulin concentrations fall, glucagon is secreted from pancreatic α-cells and potentially suppresses insulin secretion (Sutherland and De Duve, 1948). Glucagon stimulates glucose mobilization via hepatic breakdown of glycogen as well as gluconeogenesis in order to maintain normoglycemia (Qaid and Abdelrahman, 2016). This is further facilitated by diminished glucose uptake by tissues due to the lack of insulin in this fasting state (Qaid and Abdelrahman, 2016). In addition, glucagon promotes mobilization of FFA via increased lipolysis (Galsgaard et al., 2019; Keller and Shulman, 1979). Consequently, substrate oxidation is shifted towards FFA utilization with less oxidation of glucose (Habegger et al., 2010; Pégorier et al., 1989).

1.3.1.2 Proglucagon cleavage products

Beside glucagon, further proglucagon-derived peptide hormones are metabolically relevant (Rouillé et al., 1994). Proglucagon is processed in a cell-type-specific manner. The cleavage products glicentin (Raffort et al., 2017), glucagon-like peptide-1 (GLP-1) (Müller et al., 2019) and glucose-dependent insulinotropic polypeptide (GIP) (Holst, 2019) are secreted from intestinal cells in response to food intake (Eissele et al., 1992).

Though not thoroughly elucidated so far, actions of glicentin likely affect glucose metabolism via insulin production and secretion as well as intestine physiology as suggested by animal (Ohneda, 1987; Ohneda et al., 1995) and *in vitro* studies (Tomita et al., 2005).

The incretins GLP-1 and GIP are known to potentiate glucose-stimulated insulin secretion from pancreatic ß-cells and are therefore crucially involved in glucose homeostasis (Holst, 2019). Beyond this, GLP-1 and GIP have the potential to affect postprandial glucagon secretion (Holst, 2019; Vilsbøll et al., 2003). Moreover, GIP might be involved in the regulation of glucagon secretion during low glucose levels (Tura et al., 2019). However, the role of these proglucagon cleavage products in the fasting state have not been decoded so far, but preliminary data show a low but continuous basal secretion of GLP-1 and GIP (Seino et al., 2010). Observations from rodents indicate alterations in fasting glucose concentrations in GLP-1 and GIP receptor knockout mice (Tura et al.,

2019), while in a mouse model of hyperinsulinemia, fasting GLP-1 levels were increased (Lim et al., 2009). Studies in humans report increased basal GLP-1 levels in adolescents with obesity (Stinson et al., 2021), while reduced fasting levels of GLP-1 and glicentin were seen in adolescents with obesity and glucose intolerance (Manell et al., 2016). These findings give a hint for their potential relevance also in fasting metabolism. This is further emphasized by a previous investigation, suggesting that incretins may foster non-insulin dependent glucose disposal (Wagner et al., 2021b). In detail, in the presence of low basal insulin concentrations, glucose itself is able to promote its disposal, inhibit hepatic glucose production (Best et al., 1996) and even stimulate its cellular uptake (Galante et al., 1995). Since incretins have central signaling properties, they could also affect glucose metabolism (hepatic glucose output) via nervous system in a non-insulin dependent way, arguing for a potential role in fasting metabolism (Campbell and Drucker, 2013).

1.3.1.3 Other potential regulators of energy metabolism

Adipose tissue as metabolic organ is pivotal for whole-body energy metabolism (Trayhurn, 2007). In addition to its ability to store energy as fat, adipose tissue possesses endocrine functions (Vázquez-Vela et al., 2008). Secreted leptin is crucial for appetite inhibition (Obradovic et al., 2021) and promotion of lipid oxidation (Reidy and Weber, 2000). Concentrations of this hormone are increased in obesity (Maffei et al., 1995). Of note, not solely the total amount of fat but also body fat distribution may affect energy metabolism (Bjorntorp, 1991; Ibrahim, 2010). In contrast to subcutaneous fat beneath the skin, visceral adipose tissue located around internal organs is strongly linked to metabolic disturbances as insulin resistance and T2DM (Preis et al., 2010; Snel et al., 2012) with potential consequences for substrate oxidation. Though controversially discussed, a reduced ß-oxidation of FFA seems to be present in NAFLD (Perseghin et al., 2006).

Cortisol, originating from adrenal cortex follows a circadian rhythm and is also released in response to low glucose levels or in response to stress. The hormone promotes catabolic processes to foster gluconeogenesis in the liver with the aim to maintain stable energy supply via blood glucose (De Feo et al., 1989). Further, thyroid hormones play a role in the regulation of basal metabolism and thermogenesis as the interrelationship between thyroid function and weight regulation is well known (Reinehr, 2010). A further regulator of metabolic processes is the sympathetic nervous system as one part of the autonomic

nervous system (Carnagarin et al., 2018). Increased sympathetic nervous system activity and reduced reactivity are described in obesity (Guarino et al., 2017).

1.3.2 Disease-related alterations of energy metabolism

Several clinical conditions have been linked to changes in fuel partitioning. Higher carbohydrate oxidation was observed in persons with hypertension (Ferro et al., 2013) and subclinical carotid atherosclerosis (Montalcini et al., 2013). Further, abnormalities of substrate utilization are prevalent in the failing heart (Steggall et al., 2017) as well as in cancer (Ma et al., 2018), where an increased reliance on fat oxidation was reported in newly diagnosed cancer patients (Cao et al., 2010). These observations highlight the importance of substrate use for health and disease.

1.3.2.1 Alterations of energy metabolism in obesity and type 2 diabetes

Over the last decades, fuel selection was repeatedly reported to predispose for subsequent rise in BMI. Longitudinal observations revealed higher weight gain over the years when individuals have a lower fat relative to carbohydrate utilization after overnight fasting (Ellis et al., 2010; Marra et al., 2004; Seidell et al., 1992) as well as over 24 h (Zurlo et al., 1990). These observations were made in persons without (Ellis et al., 2010; Marra et al., 2004; Seidell et al., 1992) and with obesity (Schutz, 1995). Though, lipids are not primarily selected for energy generation, indicating a diminished capacity to oxidase fat (Serra et al., 2013; Simoneau et al., 1999). This could favor a further accumulation of lipids (Simoneau et al., 1999). In addition to its potential involvement in obesity development, an altered fuel selection is related to insulin sensitivity (Kelley and Mandarino, 2000; Petersen et al., 2004). A clinical study in persons with obesity revealed higher fat utilization in participants characterized as metabolically healthy compared to metabolically unhealthy persons with prevalent metabolic syndrome or T2DM (Pujia et al., 2016). In line, a low lipid oxidation was suggested as a predictor for metabolic syndrome and T2DM (Pujia et al., 2019).

Beside a lower ability to use fat as fuel in the fasting state, the upregulation of carbohydrate use in the postprandial phase seems to be blunted in case of insulin resistance (Corpeleijn et al., 2009; Galgani et al., 2008). At the end of the last century, Kelley and colleagues shaped the term of “metabolic flexibility”, meaning the ability of

the mitochondria or the organism to match fuel selection according to nutrient availability e.g. during conditions like fasting, feeding or exercise (Kelley and Mandarino, 2000; Kelley et al., 1999). The adaption of substrate oxidation to the fasting state with mainly lipid oxidation and to the fed state with insulin-stimulated suppression of lipid oxidation and predominant carbohydrate oxidation seems to be deteriorated (Corpeleijn et al., 2009; Galgani et al., 2008). This metabolic inflexibility was detected in conditions like T2DM and the metabolic syndrome (Smith et al., 2018).

As a consequence of deficient insulin levels and action during T2DM, the proper regulation of metabolic processes is disturbed. Cellular glucose uptake and oxidation are decreased while hepatic glucose production and output are upregulated (Roden and Shulman, 2019). Since amino acids are increasingly transformed to glucose precursors, protein synthesis is inhibited. The same holds true for lipids. Due to insulin resistance, insulin is not potent to suppress lipolysis leading to higher release of FFA into the bloodstream. During more severe (absolute) insulin deficiency as seen e.g. in type 1 diabetes, FFA are metabolized to ketone bodies (McGarry and Foster, 1972).

Moreover, higher levels of glucagon were observed in patients with T2DM in the fasting state (D'Alessio, 2011). This indicates a potential contribution of glucagon to hyperglycemia, mediated through an increased hepatic glucose production in the fasted state (D'Alessio, 2011). If glucagon levels differ between healthy individuals and persons with T2DM in the postprandial phase remains highly debated (Wagner et al., 2021b).

1.3.3 Assessment of energy metabolism

The method of indirect calorimetry is the gold standard to assess resting energy expenditure in humans (Ferrannini, 1988). This non-invasive, *in vivo* technique is also applied for the measurement of whole-body substrate utilization using an open circuit hood system to measure respiratory gas exchange (Matarese, 1997). The measurement is performed during rest in a room with indifference temperature. Subjects stay in a supine position and are instructed to stay awake but remain still. The ratio of consumed VO_2 and produced VCO_2 is termed as respiratory quotient (RQ), and is a read out for substrate oxidation rate (Ferrannini, 1988; Weir, 1949). In more detail, RQ refers to O_2 and CO_2 turnover on a cellular level, while the respiratory exchange ratio (RER) describes the

relation of VCO_2 and VO_2 in the exhaled air (Gupta et al., 2017). During rest, RQ and RER are interchangeable. Since the RQ is more frequently used in literature to describe substrate oxidation as assessed by indirect calorimetry, the term of RQ is used in this work. Of note, macronutrients are oxidized to water, carbon dioxide and heat (Jequier et al., 1987). Under the assumption, that the oxidation of each substrate (glucose, fat, protein) yields a specific ratio of O_2 consumption and CO_2 production, the RQ indicates the predominantly oxidized substrate for cellular energy generation (Jequier et al., 1987; McLean et al., 1987; Mtaweh et al., 2018). An RQ of 0.7 is an indicator for fat being metabolized, oxidation of protein yields an RQ of 0.8, while an RQ equal to 1 reflects pure carbohydrate utilization (Ferrannini, 1988; Weir, 1949). Physiologic values of the RQ range between 0.67 and 1.2 (Haugen et al., 2007). Conditions like lipogenesis from carbohydrates and overfeeding yield an RQ greater than 1, while during ketogenesis the RQ sometimes drops below 0.7 (Haugen et al., 2007; Matarese, 1997).

Protein oxidation can be measured via urinary nitrogen excretion but the contribution of proteins to heat production are relatively small and constant. Assuming a stable protein use and neglecting nitrogen excretion causes an error of 4 % in the assessment of energy metabolism (Gupta et al., 2017; Weir, 1949). This approach is commonly applied since the collection of 24-h urine is sometimes technically difficult and strongly depending on the participants (Ferrannini, 1988).

As indirect calorimetry represents a unique method to assess fasting substrate partitioning *in vivo* in a non-invasive manner, it is widely used in research and sometimes even in clinical practice (Delsoglio et al., 2019). Instead of using a hood system, indirect calorimetry in metabolic chambers enable the assessment of 24-h substrate oxidation to record changes over the day (Melanson et al., 2010). Though, solely net oxidation rate is recorded in indirect calorimetry neglecting metabolic interconversion. This could be addressed by means of stable isotope infusion and measuring disappearance rates (Kim et al., 2016). Highly artificial methods combining indirect calorimetry with tracer infusion or insulin clamp radioisotope turnover techniques (Kim et al., 2016; Simonson and DeFronzo, 1990) allow to distinguish between endogenous and exogenous substrate oxidation (Beylot, 2006; Gerrits and Labussière, 2015). Biosamples, e.g. obtained by skeletal muscle biopsies enable assessment of the respiration or mitochondrial ATP

synthesis with chemiluminescence of specific tissue cells *ex vivo* (Abdul-Ghani et al., 2009).

1.3.4 Modulation of energy metabolism

Acute adaptions of substrate oxidation in response to exercise or food intake were extensively studied (Egan and Zierath, 2013; Yu et al., 2021). Whether long-term changes in fasting substrate selection can achieve favorable effects for diseases like obesity or T2DM is still under investigation.

Available evidence suggests a beneficial effect of metabolically-acting drugs (e.g. ranolazine) for impaired mitochondrial energy generation in the failing heart (Steggall et al., 2017). In conditions like obesity, lifestyle intervention trials revealed opposing effects of dietary energy restriction (Coutinho et al., 2018) or modification (Goldenshluger et al., 2021) on fasting substrate oxidation. Endurance exercise training for 7 weeks in premenopausal women with overweight lowered the RQ (Barwell et al., 2009). Furthermore, changes in fuel use were observed in patients after bariatric surgery (Jabbour and Salman, 2021). Metabolic benefits in response to bariatric surgery are likely attributed to reduction of body fat mass and changed intestinal peptides (Laferrère, 2011).

In line, GLP-1 receptor agonists favored fat utilization in patients with diabetic dyslipidemia (Patel et al., 2014). Notably, further antidiabetic agents as metformin also have the potential to decrease fasting fat oxidation in healthy (Tokubuchi et al., 2017) as well as in patients with T2DM (Levin and Perlov, 1971). Though, Gormsen et al. reported no effect of metformin on fat oxidation (Gormsen et al., 2018).

Beside their known benefits for glycemic control, the drug class of sodium glucose cotransporter 2 inhibitors (SGLT2i) has shown positive effects on body weight, body fat distribution (Neeland et al., 2016), and cardiovascular health (Anker et al., 2021). This drug class exerts its action via inhibition of glucose reabsorption in the proximal tubule of the kidney leading to enhanced urinary glucose excretion (Ferrannini, 2017). Emerging evidence from studies in rodents support a role of this drug class also in lipid metabolism and regulation of lipid synthesis, transportation and FFA oxidation (Szekeres et al., 2021). Of note, first clinical trials indicate a switch towards FFA oxidation in patients with T2DM after administration of the SGLT2i dapagliflozin (Daniele et al., 2016). Since the

different compounds of this drug class vary between their selectivity for SGLT2 (Ferrannini, 2017) and therefore potentially exert different actions, further research is needed to elucidate these mechanisms and the potential of substrate oxidation modulation in persons with prediabetes and obesity.

1.4 Research questions

Substrate oxidation is potentially fundamental for long-term health. Therefore, this work aims to investigate fasting substrate oxidation, its determinants as well as its relationship to obesity and hyperglycemia in order to gain a better understanding of changes in energy metabolism in the pathogenesis of metabolic disorders. A further objective of this work is to explore the effects of a metabolically acting drug on substrate oxidation and its relation to intrapancreatic fat and insulin secretion as potential approach for prevention or therapy of metabolic disorders.

The following research questions are addressed in this thesis:

- What are the determinants of fasting substrate oxidation?
- Can fasting substrate oxidation be changed by the SGLT2i empagliflozin?
- Does pharmacological modulation of fasting substrate oxidation contribute to a reduction in intrapancreatic fat and an improvement in insulin secretion?

2 Results

2.1 1st Publication: "Free fatty acids, glicentin and glucose-dependent insulinotropic polypeptide as potential major determinants of fasting substrate oxidation."

Authors:

Julia Hummel, Louise Fritsche, Andreas Vosseler, Corinna Dannecker, Miriam Hoene, Konstantinos Kantartzis, Hans-Ulrich Häring, Norbert Stefan, Jürgen Machann, Andreas L. Birkenfeld, Cora Weigert, Robert Wagner, Andreas Peter, Andreas Fritsche, Martin Heni.

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Free fatty acids, glicentin and glucose-dependent insulinotropic polypeptide as potential major determinants of fasting substrate oxidation

Julia Hummel[1,2], Louise Fritsche[1,2], Andreas Vosseler[1,2,3], Corinna Dannecker[1,2], Miriam Hoene[4], Konstantinos Kantartzis[1,2,3], Hans-Ulrich Häring[1,2,3], Norbert Stefan[1,2,3], Jürgen Machann[1,2,5], Andreas L. Birkenfeld[1,2,3], Cora Weigert[1,2,4], Robert Wagner[1,2,3], Andreas Peter[1,2,4], Andreas Fritsche[1,2,3] & Martin Heni[1,2,3,4]✉

The selection of carbohydrates or fat to generate intracellular energy is thought to be crucial for long-term metabolic health. While most studies assess fuel selection after a metabolic challenge, the determinants of substrate oxidation in the fasted state remain largely unexplored. We therefore assessed the respiratory quotient by indirect calorimetry as a read-out for substrate oxidation following an overnight fast. This cross-sectional analysis consisted of 192 (92 women, 100 men) either lean or obese participants. Following an overnight fast, the respiratory quotient (RQ) was assessed, after which a 5-point 75-g oral glucose tolerance test was performed. Unlike glucose and insulin, fasting free fatty acids (FFA) correlated negatively with fasting RQ ($p < 0.0001$). Participants with high levels of the ketone body β-hydroxybutyric acid had significantly lower RQ values. Fasting levels of glucose-dependent insulinotropic polypeptide (GIP) and glicentin were positively associated with fasting RQ (all $p \leq 0.03$), whereas GLP-1 showed no significant association. Neither BMI, nor total body fat, nor body fat distribution correlated with fasting RQ. No relationship between the RQ and diabetes or the metabolic syndrome could be observed. In the fasting state, FFA concentrations were strongly linked to the preferentially oxidized substrate. Our data did not indicate any relationship between fasting substrate oxidation and metabolic diseases, including obesity, diabetes, and the metabolic syndrome. Since glicentin and GIP are linked to fuel selection in the fasting state, novel therapeutic approaches that target these hormones may have the potential to modulate substrate oxidation.

The selection of the predominant fuel source for intracellular energy generation is crucial for long-term health. Alterations are linked to a number of diseases, including obesity, diabetes and cardiovascular diseases[1–3]. The two major cellular energy substrates glucose and free fatty acids (FFA) are metabolized via glycolysis and beta oxidation, respectively, to generate acetyl-CoA. Acetyl-CoA is further metabolized and biochemical energy is generated in the mitochondria by a process known as oxidative phosphorylation[4].

The relative amount of substrate that is oxidized over a certain period of time can be assessed non-invasively by indirect calorimetry. The respiratory quotient (RQ)—as a ratio of exhaled carbon dioxide to consumed oxygen—indicates the preferentially oxidized substrate[5].

[1]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany. [2]German Center for Diabetes Research (DZD), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany. [3]Department of Internal Medicine, Division of Diabetology, Endocrinology and Nephrology, Eberhard Karls University Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany. [4]Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany. [5]Department of Radiology, Section on Experimental Radiology, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany. ✉email: martin.heni@med.uni-tuebingen.de

Several studies report that both fasting and 24-h-RQ are predictors for long-term weight gain. High fasting as well as average 24-h-RQ, which are indicative of predominant glucose oxidation relative to fatty acids, is linked to subsequent body fat accumulation[6–9]. The importance of altered substrate oxidation is further emphasized by the association shown between higher RQ and insulin resistance[10], hepatic steatosis[11], hypertension[12] and subclinical carotid atherosclerosis[13] in individuals with overweight/obesity.

Metabolic flexibility describes the ability to adapt substrate oxidation to substrate availability[14]. This was initially reported for the transition from the fasting to the fed state. Only later was the crucial switch of fuel oxidation in response to overnight fasting brought into focus[15]. To date, several clinical conditions with impaired metabolic flexibility have been identified. In patients with insulin-resistance, obesity and diabetes, diminished fat oxidation during nocturnal fasting and diminished postprandial upregulation of carbohydrate oxidation have been reported[1,16]. Despite the fact that key determinants of postprandial regulation have been addressed in a number of studies, the regulation of fasting substrate oxidation remains largely elusive. Since most individuals spend a substantial time of their day in a fasted state, the oxidized substrates following overnight fasting are probably crucial to maintain long-term health. Glucose concentrations decrease during fasting while FFA increases. We therefore hypothesize that the predominant cellular energy source after overnight fast are lipids. To test this hypothesis, and to detect disease-related alterations, we analyzed early morning fasting RQ and its´ relation to glucose and FFA in a cohort of humans, ranging from lean and healthy to obese patients with diabetes.

Results

In our participants, the median RQ was 0.85 (IQR: 0.79–0.9). No significant difference was found in RQ between men and women ($p = 0.06$, $p_{adj, age} = 0.2$). RQ was correlated to age, with lower values in older subjects ($p = 0.03$, $p_{adj, sex, BMI} = 0.02$; Fig. 1A). Neither BMI nor the total amount of adipose tissue was correlated to RQ (all $p \geq 0.06$; Figs. 1B and 2A, respectively). RQ was not correlated with any of the analyzed body fat compartments ($p \geq 0.1$; Fig. 2C,D, respectively) or with the amount of intrahepatic lipid accumulation ($p = 0.6$, $p_{adj, sex, age} = 0.3$; Fig. 2B).

We first tested whether substrate oxidation is linked to fasting substrate availability. Fasting glucose was not associated with RQ ($p = 0.2$, $p_{adj, sex, age} = 0.7$; Fig. 1C). By contrast, a significant negative correlation was found between FFA and RQ ($p < 0.0001$; Fig. 1D). This correlation remained significant after adjustment for sex and age ($p < 0.0001$). We also detected a significant negative association between ß-hydroxybutyric acid and RQ ($p = 0.02$, $p_{adj, sex, age} = 0.02$; Fig. 1E).

Since the impact of insulin is crucial for substrate availability, we further analyzed the relationship between insulin and substrate oxidation. Neither circulating insulin, nor whole-body insulin sensitivity, nor adipose tissue insulin sensitivity were related to RQ (all $p \geq 0.07$; Table 1).

We next analyzed the relationship of proglucagon cleavage products and GIP to RQ. Albeit there was no link for GLP-1 ($p = 0.08$, $p_{adj, sex, age} = 0.1$; Table 1), glucagon ($p = 0.03$; Fig. 1F), glicentin ($p = 0.02$; Fig. 1H) and GIP ($p = 0.008$; Fig. 1G) were positively associated with RQ. These associations remained significant after adjustment for sex and age ($p \leq 0.03$). Following adjustment for FFA, glucagon was no longer associated with RQ ($p_{adj, sex, age, FFA} = 0.09$), whereas the association of RQ and GIP as well as with glicentin remained significant ($p \leq 0.04$). Moreover, adjustment for fasting glucose did not alter the association of glucagon with RQ.

Neither thyroid-stimulating hormone nor serum cortisol was correlated to RQ (all $p \geq 0.2$; Table 1).

We further addressed the relationship between metabolic disorders and fasting substrate oxidation. RQ was comparable between participants with normal glucose tolerance, prediabetes or newly-diagnosed type 2 diabetes ($p = 0.6$, $p_{adj, sex, age} = 0.8$; Table 1). It was also comparable between participants with and without the metabolic syndrome ($p = 0.8$, $p_{adj, sex, age} = 0.4$; Table 1).

We next tested for potential interactions between weight groups (normal weight/overweight vs obese) and anthropometric/clinical parameters on RQ. These analyses revealed no statistically significant interactions for glucose and FFA, indicating that their relation to RQ is comparable between the two weight groups (Suppl. Table 1). Similar interaction analyses with glycemic categories and presence/absence of the metabolic syndrome also revealed no interactions between glucose or FFA on RQ (interaction with glycemic categories: $p_{(interaction\ glycemic\ cat \times glucose)} = 0.2$ and $p_{(interaction\ glycemic\ cat \times FFA)} = 0.8$; interaction with metabolic syndrome: $p_{(interaction\ met\ syndrome \times glucose)} = 0.5$ and $p_{(interaction\ met\ syndrome \times FFA)} = 0.1$).

No association was found between systolic or diastolic blood pressure and RQ (all $p \geq 0.6$). Since 53 of the 192 participants received antihypertensive medication, we repeated the analysis after excluding the former. Thereafter, blood pressure still did not correlate with RQ (all $p \geq 0.4$). However, the correlation between FFA and RQ remained significant ($p = 0.003$, $p_{adj, sex, age} = 0.005$).

We next analyzed resting energy expenditure. There was a negative correlation of age with resting energy expenditure (Suppl. Fig. 1A). Higher BMI was associated with higher resting energy expenditure (Suppl. Fig. 1B). While resting energy expenditure was positively associated with fasting glucagon (Suppl. Fig. 1F), this significance disappeared after adjustment for sex ($p = 0.7$).

Discussion

We investigated potential major metabolic determinants of substrate oxidation in the fasting state. An analysis of a wide range of participants, from lean and healthy to obese with diabetes, enabled us to identify FFA as a potential major determinant of substrate oxidation. Furthermore, glicentin, GIP and ketone bodies were additional independent determinants. By contrast, glycemia was not related to nutrient utilization. Surprisingly, no link could be established between the preferred oxidized substrate and metabolic diseases, including obesity, diabetes and the metabolic syndrome.

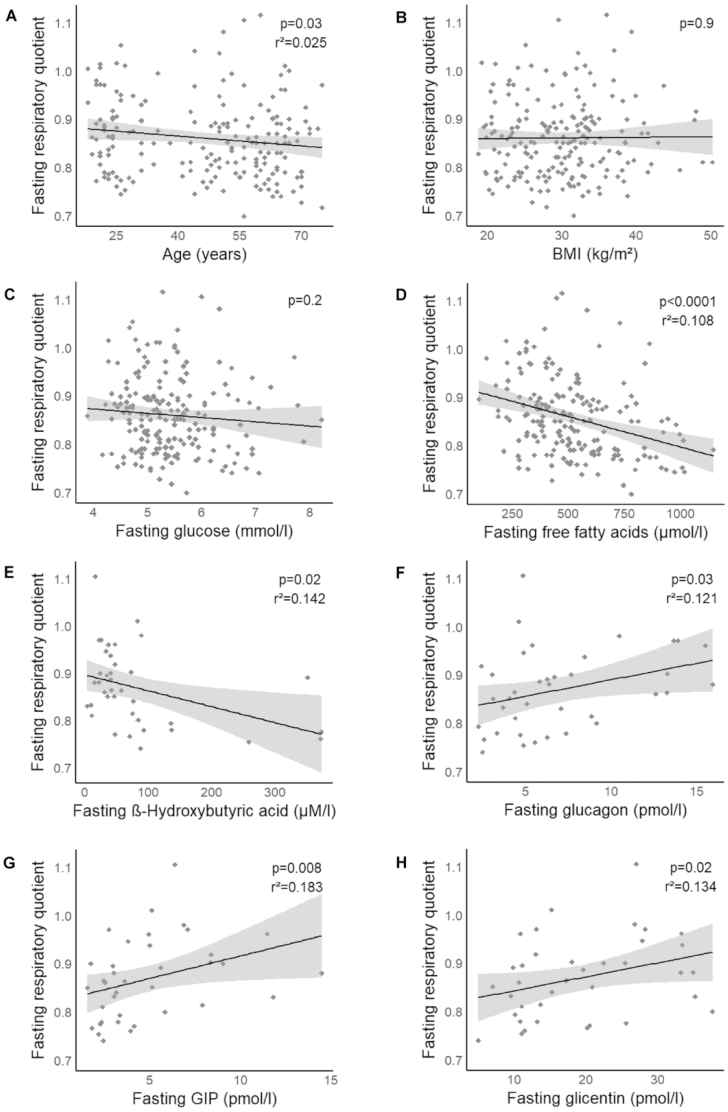

Figure 1. Association of respiratory quotient (RQ) and its´potential determinants. RQ was negatively associated with age (**A**), with lower RQ values with increasing age. No correlation between RQ and BMI (**B**) or plasma glucose (**C**) was detected. Free fatty acids (**D**) as well as the ketone body ß-hydroxybutyric acid (**E**) were significantly correlated to RQ. Glucagon (**F**), GIP (**G**) and glicentin (**H**) were positively associated to RQ. Data are presented as scatterplots with linear regression lines and 95% CI. *p* values were taken from linear regression analyses.

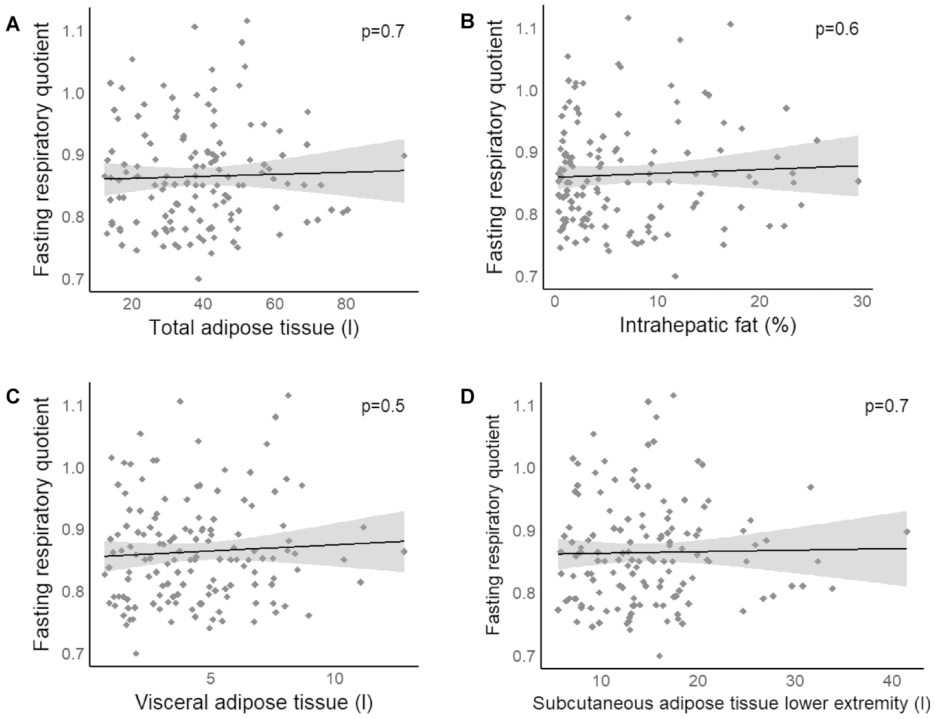

Figure 2. Association of respiratory quotient (RQ) and body fat distribution determined by whole-body MRI and liver MRS. Total adipose tissue (**A**), intrahepatic fat content (**B**), neither visceral nor subcutaneous adipose tissue (**C**, **D**) was correlated to RQ. Data are presented as scatterplots with linear regression lines and 95% CI. *p* values are derived from linear regression analyses.

In contrast to our hypothesis, the predominant cellular energy source before breakfast in the morning was not solely FFA, but a mixture of substrates with large intra-individual differences (and a RQ range of 0.7–1.1). This tallies well with previous findings in a number of populations[17].

Aging has been reported to result in a shift from fat to glucose oxidation in some[18,19] but not all previous trials[20]. Our analysis detected higher rates of fat oxidation in older persons. Age-related insulin resistance has been proposed to be one contributing factor[21]. However, since we did not detect any association between insulin sensitivity and RQ, our results do not support this hypothesis. Since substrate oxidation takes place in the mitochondria, age-related alterations in mitochondrial function[22], together with rising FFA concentrations, could govern shifts in substrate oxidation towards lipids during aging. Results on differences in substrate utilization between sexes are inconsistent[6,12,18,20,23]. We did not detect any significant differences in fasting RQ.

Glucose is a major energy source, and a sufficient supply is ensured even in the fasting state. While more ATP is generated from lipids, cellular energy formation from glucose is more efficient in terms of oxygen consumption[24], and some cell types largely depend on glucose. In the muscle, there is a competition between glucose and FFA as the major energy source[25]. However, there is no link between circulating glucose and substrate utilization. While carbohydrate availability has been proposed as a determinant[26], our current results argue against the hypothesis that substrate oxidation is purely the result of substrate availability.

In contrast to glucose, circulating FFA are robustly linked to cellular substrate oxidation, independent of the tested confounders. When abundantly available or experimentally elevated[27], FFA tend to be oxidized. This tallies with earlier reports[28,29] and is in accordance with Randle's hypothesis that fatty acids suppress glucose oxidation in skeletal muscle[25]. Indeed, fatty acid oxidation is directly linked to circulating FFA concentrations[30]. Of note, the regulation of substrate oxidation by FFA was independent of insulin, insulin-sensitivity and of the amount of lipids stored in adipose tissue. This might be due to direct intracellular regulation, since fatty acids and their metabolic intermediates are not only energy sources but also possess signaling properties[31,32].

N = 192	Median (IQR)/n	*p* value (unadj./adj.*)	Stand. β (standard error)
Sex		0.06/0.2#	− 0.092 (0.0069)
Male	100		
Female	92		
Age (years)	51 (27–62)	**0.03/0.02**##	− 0.188 (0.017)
Body mass index (kg/m²)	30.1 (24.7–33.7)	0.9/0.06	0.162 (0.036)
Blood pressure[a]			
Systolic (mmHg)	137 (127–145)	0.6/0.97	− 0.003 (0.065)
Diastolic (mmHg)	89 (80–96)	0.7/0.6	0.044 (0.001)
Heart rate (bpm)[a]	69 (63–78)	0.6/0.7	− 0.029 (0.041)
Metabolic syndrome[b]		0.8/0.4	− 0.071 (0.007)
Yes	87		
No	100		
Glycemic category		0.6/0.8	
Normal glucose tolerance	107		
Prediabetes	75		
Diabetes mellitus (newly diagnosed, treatment naïve)	10		
Indirect calorimetry			
Fasting respiratory quotient	0.85 (0.79–0.90)		
Resting energy expenditure (kcal)	2008 (1746–2275)	0.3/0.9	0.009 (0.000)
Body composition			
Total adipose tissue, MR-derived (l)[c]	37.9 (26.5–47.3)	0.7/0.1	0.184 (0.022)
Subcutaneous adipose tissue lower extremity, MR-derived (l)[c]	13.7 (9.7–17.6)	0.7/0.1	0.161 (0.025)
Visceral adipose tissue, MR-derived (l)[d]	4.2 (2.3–6.0)	0.5/0.1	0.180 (0.017)
Intrahepatic fat, MRS-derived (%)[e]	4.1 (1.4–9.7)	0.6/0.3	0.110 (0.008)
Glycemia			
HbA1c (mmol/mol)[f]/HbA1c (%)[f]	37 (34–40)/5.5 (5.3–5.8)	0.9/0.3	0.090 (0.064)
Fasting glucose (mmol/l)[g]	5.3 (4.9–5.8)	0.2/0.7	− 0.033 (0.056)
Fasting insulin (pmol/l)[g]	70 (45–109)	0.5/0.1	0.122 (0.011)
Fasting C-peptide (pmol/l)[h]	516 (363–711)	0.9/0.2	0.115 (0.016)
Disposition index[i]	1243 (684–2061)	0.1/0.6	0.040 (0.009)
Insulin sensitvity index (OGTT-derived)[j]	9.6 (5.6–15.2)	0.8/0.1	− 0.119 (0.011)
Adipo-IR (mmol/l * pmol/l)[h]	36.0 (19.2–57.4)	0.07/0.3	− 0.082 (0.009)
Plasma lipids			
Free fatty acids (µmol/l)[h]	498 (375–633)	**< 0.0001/< 0.0001**	− 0.305 (0.016)
Triglycerides (mg/dl)[i]	97 (74–137)	0.8/0.4	0.073 (0.015)
Cholesterol (mg/dl)[i]	190 (158–227)	0.6/0.4	0.081 (0.000)
LDL-Cholesterol (mg/dl)[i]	121 (95–150)	0.4/0.7	0.032 (0.025)
HDL-Cholesterol (mg/dl)[i]	52 (43–62)	0.9/0.4	0.069 (0.029)
Lipoprotein (a) (mg/dl)[i]	12 (7–39)	0.3/0.3	− 0.082 (0.006)
Proglucagon cleavage products[k]			
Insulin/Glucagon ratio	15.5 (12.7–20.4)	0.9/0.97	0.008 (0.030)
Glucagon (pmol/l)	5.67 (4.27–8.91)	**0.03/0.03**	0.421 (0.032)
Glicentin (pmol/l)	17.28 (11.11–26.90)	**0.02/0.03**	0.365 (0.031)
Glucagon-like peptide 1 (pmol/l)	3.39 (2.22–4.25)	0.08/0.1	0.284 (0.021)
Glucose-dependent insulinotropic polypeptide (pmol/l)	3.82 (2.52–6.64)	**0.008/0.01**	0.474 (0.028)
Others			
ß-Hydroxybutyric acid (µM/l)[k]	47.2 (30.2–85.4)	**0.02/0.02**	− 0.382 (0.015)
Thyroid-stimulating hormone (mU/l)[i]	1.82 (1.19–2.75)	0.3/0.7	0.026 (0.009)
C-reactive protein (mg/dl)[j]	0.12 (0.03–0.35)	0.7/0.1	0.121 (0.005)
Morning cortisol, serum (nmol/l)[i]	387 (297–508)	0.5/0.2	− 0.104 (0.019)

Table 1. Associations with respiratory quotient (RQ). *MR* magnetic resonance, *MRS* magnetic resonance spectroscopy, *HbA1c* hemoglobin A1c, *OGTT* oral glucose tolerance test, *Adipo-IR* adipose tissue insulin resistance index, *LDL-Cholesterol* low-density lipoprotein cholesterol, *HDL-Cholesterol*: high-density lipoprotein cholesterol. *Adjusted for sex and age; # adjusted for age; ## adjusted for sex and BMI. Standardized ß are from multivariate linear regression models. [a]: n = 190; [b]: n = 187; [c]: n = 137; [d]: n = 139; [e]: n = 140; [f]: n = 178; [g]: n = 191; [h]: n = 181; [i]: n = 179; [j]: n = 184; [k]: n = 38. $p < 0.05$ are printed in bold.

Unlike prior reports[14,29,33], we found no connection between fasting insulin or insulin sensitivity and substrate oxidation. Inclusion of participants with different glycemic categories cannot explain these differences, as glycemic status did not influence our detected relations. Insulin is a potent suppressor of lipolysis and thereby inhibits FFA supply. Concurrently, it stimulates cellular glucose uptake and could thereby promote glucose oxidation. However, these functions occur in the postprandial state, during which insulin concentrations rise rapidly. Theoretically, in the fasting state, insulin should not determine substrate oxidation. This could explain why we did not detect any links to RQ, while other studies which included postprandial periods found such contributions of insulin.

Some organs, including the brain, are unable to utilize fatty acids as their sole energy source. These organs rely on ketone bodies, formed from fatty acids in the liver, as an alternative energy source[34]. In the current study, we assessed ß-hydroxybutyric acid which is, quantitatively speaking, the major ketone body. The link of ketone bodies with lower RQ is plausible since their formation depends on fatty acid oxidation in the liver. Furthermore, the utilization of ketone bodies also results in a low RQ. Physiologic ketone body production has recently been linked to beneficial effects on heart, brain and muscle[35]. High spontaneous ketones were even found to be predictive of long-term reduced risk for diabetes[36].

Further novel observations are the correlations between substrate oxidation and GIP as well as with glicentin. Upon food intake, the incretins GIP and glicentin are secreted from duodenal cells and subsequently modulate energy metabolism[37,38]. Their regulation in the fasting state is largely unknown. We observed lower rates of fat oxidation in persons with higher fasting levels of circulating GIP and glicentin. Our data are in line with animal models in which GIP signaling decreased fat oxidation via skeletal muscle and adipose tissue[39] and was crucial in cellular lipid handling[40]. How glicentin contributes to substrate oxidation is still unknown and further experimental studies are required to determine the significance of our findings.

The detected association of glucagon and RQ was presumably mediated by FFA, which is probably due to glucagon's stimulatory effect on FFA turnover[41]. We assume that glucagon reduces the availability of FFA since the latter serve as precursors for gluconeogenesis. This could explain the observed increased rates of glucose oxidation in persons with high circulating glucagon levels.

Several trials reported a relationship between fat mass and lipid oxidation[33,42,43]. As visceral fat associates with insulin resistance and lower fat oxidation[44,45], whereby subcutaneous fat is linked to higher fat oxidation[46,47], we quantified both. However, fuel selection was associated with neither BMI, nor fat mass, nor body fat distribution. This is in agreement with Ferro et al., who likewise detected no link between fasting RQ and body fat content[12]. Our data therefore do not support the hypothesis of reduced lipid oxidation as a cause or consequence of obesity. This "low fat oxidation hypothesis" was discussed in a recent meta-analysis which came to the conclusion that there is no convincing experimental evidence to support this hypothesis[17].

While one study reported lower fasting RQ in diabetes[10], neither we nor two earlier trials[12,20] detected such a difference to persons with normal glucose tolerance. Thus, fasting substrate oxidation does not appear to make any major contribution to body weight, body fat distribution, or metabolic diseases.

Since our current work is a cross-sectional analysis, the data do not permit us to test the prospective value of RQ. Furthermore, some associations might only be present in specific subgroups that were not available in sufficiently large numbers to warrant analysis in our study. The fasting concentrations of some of the analyzed hormones are rather low and their physiological relevance in the fasting state has not been extensively studied so far (e.g. incretins).

Taken together, we detected FFA as a potential major determinant of fasting substrate oxidation and possible contributions of glicentin and GIP as well as of ketone bodies. Since the preferred oxidized substrate was not linked to metabolic diseases, fuel selection in the fasting state probably does not make any major contribution to obesity, diabetes, or the metabolic syndrome. Upcoming pharmacological approaches target GIP signaling[48,49]. Our results indicate that they have the potential to modulate preferred substrate oxidation.

Materials and methods

Data acquisition was performed at the University Hospital of Tübingen between October 2016 and August 2020, as part of ongoing clinical studies (NCT02991365, NCT03590561, NCT03615209, NCT03227484, NCT03525002, and NCT04052399). The study protocols were approved by the local ethics committee (Ethics Committee of the Medical Faculty of the Eberhard Karls University and the University Hospital Tübingen) and all experiments were conducted in accordance with the relevant guidelines and regulations. Further, all participants provided informed written consent.

Study design and participants. One hundred and ninety-six participants from six ongoing trials were included in this cross-sectional analysis. We selected all participants, for whom data from indirect calorimetry in the fasting state were available, which was performed at the beginning of each study. Four participants were excluded from data analysis due to implausible indirect calorimetry or laboratory measurements. Participants had a median age of 51 years (IQR: 28–62), the majority were overweight or obese (median (IQR): BMI: 30.1 kg/m^2 (25.0–33.6)), and had a median HbA1c of 5.5% (5.3–5.8). 53 participants took antihypertensive medication. Patient characteristics are reported in Table 1 and Supplementary Table 2.

A medical history was recorded and a physical examination was performed. Following an overnight fast and an indirect calorimetry, all participants were subjected to a 5-point, 75-g oral glucose tolerance test (OGTT) to assess insulin sensitivity, insulin secretion and glycemic categories. Normal glucose tolerance was found in 107 participants, 75 had prediabetes, and in 10 patients, type 2 diabetes was diagnosed by way of this OGTT. None of the participants took any medication interfering with glucose metabolism.

Laboratory measurements and calculations. Venous blood samples were taken before, as well as 30, 60, 90 and 120 min after glucose administration. Serum insulin and C-peptide were determined by an immunoassay on an ADVIA Centaur system (Siemens Healthineers, Eschborn, Germany). Glucose and triglycerides, together with total, HDL, and LDL lipoprotein cholesterol levels were assessed using the ADVIA XPT clinical chemical system (Siemens Healthineers, Eschborn, Germany). Plasma concentrations of total FFA were measured with an enzymatic method (WAKO Chemicals, Neuss, Germany) on the latter instrument. Glycated hemoglobin (HbA1c) measurements were carried out with the Tosoh A1c analyzer HLC-723G8 (Tosoh Bioscience GmbH, Griesheim, Germany).

Whole-body insulin sensitivity was determined using Matsuda Index[50]. One unusually high value for Matsuda Index (> 5 SD above median) was excluded from data analysis. Insulin resistance of adipose tissue at fasting was estimated by Adipo-IR Index (adipose tissue insulin resistance index)[51]. Insulin secretion was assessed by Disposition Index[52].

Metabolic syndrome was classified in accordance with the criteria of the International Diabetes Federation (IDF)[53], while glucose tolerance was in accordance with the criteria of the American Diabetes Association (ADA)[54].

In a subset of 38 participants, fasting glucagon, GLP-1, GIP, and glicentin were measured by commercial immunoassays (Mercodia, Uppsala, Sweden). Fasting β-hydroxybutyric acid was measured by an enzymatic 3-hydroxybutyrate dehydrogenase-based Ketone Body Assay (Sigma-Aldrich, St. Louis, MO, USA).

Indirect calorimetry. Indirect calorimetry was performed in the morning after an overnight fast of at least 10 h abstinence from food, nicotine and caffeine.

The exhaled air was analyzed with Vyntus CPX (Carefusion, Hoechberg, Germany). Total average energy expenditure in kilocalories (kcal) was calculated using the modified Weir´s equation[55].

During substrate oxidation, oxygen (VO_2) is consumed and carbon dioxide (VCO_2) is produced. The ratio of VCO_2 and VO_2 in the exhaled air represents the respiratory quotient. It is dependent on the relative amounts of oxidized substrates and reflects substrate utilization. Physiologically, the RQ ranges between 0.67 and 1.2[56,57]. Under stable protein utilization, an RQ equal to 1 indicates preferential glucose metabolism, while an RQ of 0.7 reflects predominantly lipid oxidation.

Body fat distribution and intrahepatic fat. Quantification of whole-body adipose tissue and intrahepatic fat was performed on the same day prior to indirect calorimetry by magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (^{1}H-MRS). A 3T whole-body imager (Magnetom Vida, Siemens Healthineers, Erlangen, Germany), as described previously[58], was used. Segmentation of adipose tissue was performed by an automatic procedure based on fuzzy clustering[59]. Intrahepatic fat was quantified by localized ^{1}H-MRS from the posterior part of segment 7 by applying a single-voxel STEAM technique with a short echo time (TE = 10 ms). Integrals of methylene/methyl resonances (lipids) and water were assessed and intrahepatic fat was determined in percent by calculating the ratio of lipids and water + lipids.

Statistics. Statistical analysis was carried out using JMP 14.0 (SAS Institute, Cary, NC, USA). Prior to analysis, skewed variables were log transformed. Correlations were tested by multivariate linear regression analysis with adjustments for sex and age. Data are presented as median with IQR. p values < 0.05 were considered as statistically significant.

Data availability

The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

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Author contributions
J.H. researched and analyzed data and drafted the manuscript. L.F., A.V., C.D., K.K., J.M. and R.W. researched data. M.Ho., H.U.H., N.S., A.B., C.W. and A.F. contributed to the discussion and interpretation of the results. A.P. supervised the laboratory measurements and interpreted the results. M.He. supervised the project and drafted the manuscript. All authors contributed to the discussion and approved the final manuscript prior to submission.

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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.H.

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2.2 2nd Publication: "Empagliflozin improves insulin sensitivity of the hypothalamus in humans with prediabetes: a randomized, double-blind, placebo-controlled, phase 2 trial."

Authors:

Stephanie Kullmann*, **Julia Hummel***, Robert Wagner, Corinna Dannecker, Andreas Vosseler, Louise Fritsche, Ralf Veit, Konstantinos Kantartzis, Jürgen Machann, Andreas L. Birkenfeld, Norbert Stefan, Hans-Ulrich Häring, Andreas Peter, Hubert Preissl, Andreas Fritsche, Martin Heni.

*authors contributed equally

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Diabetes Care 1

Empagliflozin Improves Insulin Sensitivity of the Hypothalamus in Humans With Prediabetes: A Randomized, Double-Blind, Placebo-Controlled, Phase 2 Trial

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Stephanie Kullmann,[1,2] Julia Hummel,[1,2] Robert Wagner,[1,2,3] Corinna Dannecker,[1,2] Andreas Vosseler,[1,2,3] Louise Fritsche,[1,2] Ralf Veit,[1,2] Konstantinos Kantartzis,[1,2] Jürgen Machann,[1,2,4] Andreas L. Birkenfeld,[1,2,3] Norbert Stefan,[1,2,3] Hans-Ulrich Häring,[1,2,3] Andreas Peter,[1,2,5] Hubert Preissl,[1,2,3,6,7] Andreas Fritsche,[1,2,3] and Martin Heni[1,2,3,5]

OBJECTIVE

Insulin action in the human brain reduces food intake, improves whole-body insulin sensitivity, and modulates body fat mass and its distribution. Obesity and type 2 diabetes are often associated with brain insulin resistance, resulting in impaired brain-derived modulation of peripheral metabolism. So far, no pharmacological treatment for brain insulin resistance has been established. Since sodium–glucose cotransporter 2 (SGLT2) inhibitors lower glucose levels and modulate energy metabolism, we hypothesized that SGLT2 inhibition may be a pharmacological approach to reverse brain insulin resistance.

RESEARCH DESIGN AND METHODS

In this randomized, double-blind, placebo-controlled clinical trial, 40 patients (mean ± SD; age 60 ± 9 years; BMI 31.5 ± 3.8 kg/m^2) with prediabetes were randomized to receive 25 mg empagliflozin every day or placebo. Before and after 8 weeks of treatment, brain insulin sensitivity was assessed by functional MRI combined with intranasal administration of insulin to the brain.

RESULTS

We identified a significant interaction between time and treatment in the hypothalamic response to insulin. Post hoc analyses revealed that only empagliflozin-treated patients experienced increased hypothalamic insulin responsiveness. Hypothalamic insulin action significantly mediated the empagliflozin-induced decrease in fasting glucose and liver fat.

CONCLUSIONS

Our results corroborate insulin resistance of the hypothalamus in humans with prediabetes. Treatment with empagliflozin for 8 weeks was able to restore hypothalamic insulin sensitivity, a favorable response that could contribute to the beneficial effects of SGLT2 inhibitors. Our findings position SGLT2 inhibition as the first pharmacological approach to reverse brain insulin resistance, with potential benefits for adiposity and whole-body metabolism.

Over the last 15 years, the human brain has been identified as an important insulin-sensitive organ (1,2). Insulin action in the brain modulates eating behavior, body

[1]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, Tübingen, Germany
[2]German Center for Diabetes Research, Neuherberg, Germany
[3]Division of Diabetology, Endocrinology and Nephrology, Department of Internal Medicine, Eberhard Karls University Tübingen, Tübingen, Germany
[4]Department of Diagnostic and Interventional Radiology, Section of Experimental Radiology, Eberhard Karls University Tübingen, Tübingen, Germany
[5]Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, Eberhard Karls University Tübingen, Tübingen, Germany
[6]Institute of Pharmaceutical Sciences, Department of Pharmacy and Biochemistry, Interfaculty Center for Pharmacogenomics and Pharma Research at the Eberhard Karls University Tübingen, Tübingen, Germany
[7]Institute for Diabetes and Obesity, Helmholtz Diabetes Center, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany

Corresponding author: Martin Heni, martin.heni@med.uni-tuebingen.de

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S.K. and J.H. contributed equally to this work.

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weight, and body fat content (1). Moreover, it improves peripheral metabolism by enhancing whole-body insulin sensitivity (1,3), suppressing hepatic glucose production (3,4), and stimulating pancreatic insulin secretion (5). Pioneering studies in mice revealed that selective disruption of neural insulin receptors, particularly in the hypothalamus, induced an obese phenotype with increased body fat and peripheral insulin resistance (6). Restoring insulin action in the hypothalamus, in contrast, prevented diabetes (7). The tremendous significance of hypothalamus insulin action for peripheral metabolism was first shown in rodent models, in which insulin signaling in the hypothalamus was discovered to control hepatic glucose production (8). In line with experimental evidence from animals (2), insulin action in the human hypothalamus appears to be central for these brain-derived effects on the periphery (3,5). Many studies on insulin action in the human brain combined brain imaging with the administration of insulin as nasal spray. This route of administration enables insulin delivery into the brain, while only tiny amounts are absorbed into the bloodstream (9). Thus, intranasal insulin enables selective stimulation of brain insulin action. In this study, we used the nasal insulin dose that showed robust effects on regional brain activity in two previous dose-response trials (10,11). A reduction in hypothalamic activity in response to intranasal insulin indicates insulin sensitivity (12–14).

Of note, a substantial number of people display reduced, or even absent, brain responses to insulin, a condition termed brain insulin resistance (1). While obesity is the most prominent condition linked to insulin resistance of the brain, a number of additional associated risk factors have been identified (1,2). Impaired hypothalamic insulin action, based on failure to reduce hypothalamus activity in response to central insulin (12,14), hinders signals to the periphery (1,2,14) and thereby predisposes for whole-body insulin resistance (3) and insufficient postprandial insulin secretion (5). Furthermore, brain insulin resistance is linked to long-term weight gain and unhealthy body fat distribution (14).

For the treatment of brain insulin resistance, several approaches are discussed (1). Among the pharmacological candidates are sodium–glucose cotransporter 2 (SGLT2) inhibitors. This class of substances has been developed for the treatment of diabetes, as inhibition of SGLT2 promotes glucose excretion through the urine, thereby lowering blood glucose (15). Large clinical trials with SGLT2 inhibitors demonstrated their ability to improve morbidity and reduce mortality (15), with benefits even in patients without diabetes (15). Among others, an increase of glucagon (16–18) or ketone body concentrations (16) in the circulation has been proposed as a potential mechanism explaining their beneficial effects (19,20).

Comparable to humans, brain insulin action is reduced in rodents with high-fat diet–induced obesity (2). Administration of the SGLT2 inhibitor dapagliflozin to high-fat diet–fed rats restored brain insulin signaling, improved whole-body metabolism, and prevented cognitive decline (21). One proposed pathomechanism for brain insulin resistance is hypothalamic subclinical inflammation. In fact, canagliflozin, another SGLT2 inhibitor, was able to revert inflammation in the hypothalamus of mice fed a high-fat diet (22). At least some of the beneficial effects of this class of substances on the entire body appear to rely on intact brain-periphery cross talk via the parasympathetic nervous system. Vagotomy in obese mice attenuated body weight reduction in response to the SGLT2 inhibitor tofogliflozin (23), indicating that the therapeutic effect at least partially depends on the intact brain -periphery communication via the vagus nerve. Thus, there is evidence that SGLT2 inhibition is able to suppress inflammation and revert insulin resistance in the hypothalamus of obese rodents. Furthermore, an intact communication between the brain and the periphery appears to be necessary for some of the beneficial effects of these substances. Though, the potential of SGLT2 inhibitors to treat brain insulin resistance in humans is still unknown.

In this randomized, controlled, phase 2 trial, we therefore tested effects of the potent SGLT2 inhibitor empagliflozin on brain insulin action in overweight and obese patients with prediabetes.

RESEARCH DESIGN AND METHODS

This randomized, placebo-controlled, double-blind, phase 2 trial was conducted at the University Hospital of Tübingen between July 2017 and October 2019. The protocol was approved by the local ethics committee and conducted according to the Declaration of Helsinki and the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use Good Clinical Practice guidelines. This study was preregistered at ClinicalTrials.gov under number NCT03227484 and at the European Union Clinical Trials Register under EudraCT2016–003477-18 (https://eudract.ema.europa.eu/).

Study Design and Patients

Forty-two patients were randomized to receive either 25 mg empagliflozin or placebo, once daily, for 8 weeks (Supplementary Fig. 1) using a block randomization with a block size of 4. Patient characteristics are presented in Tables 1 and 2. Power considerations and sample size determination are presented as Supplementary Material 6. Before enrollment, patients provided informed written consent. Prior to randomization, a medical history was obtained, and a physical examination as well as a 75-g oral glucose tolerance test (OGTT) were performed. Detailed inclusion and exclusion criteria are given in Supplementary Table 5. In short, patients between 30 and 75 years of age had to be overweight or obese (BMI $\geq$25 and $\leq$40 kg/m^2) and show impaired fasting glucose and/or glucose tolerance (Table 2), according to the American Diabetes Association criteria (24). Detailed phenotyping was conducted before and after drug intake (for details, see Supplementary Fig. 2). On the days of study visits, patients took any concomitant medication (and the study drug at the end of study visit) only after completion of the respective examinations. Further, they were instructed to refrain from exercise and alcohol intake the day prior to each study visit and to refrain from smoking on the days of examinations. Depression was ruled out by Beck Depression Inventory II. Patients completed food diaries on 7 consecutive days before and at the end of treatment. Diet composition was estimated with a validated software using 4 days with complete data out of the 7-day food diary (DGE-PC 3.0; German Nutrition Society, Bonn, Germany). A questionnaire addressing a subjective feeling of hunger was assessed before nasal spray application

Table 1—Outcomes

	Placebo (n = 21)		Empagliflozin (n = 19)		
	Before	After 8 weeks	Before	After 8 weeks	$P_{(MANOVA)}$
Blood pressure					
Systolic (mmHg)	144 ± 12	145 ± 17	141 ± 17	137 ± 17	0.5
Diastolic (mmHg)	93 ± 10	91 ± 10	88 ± 10	88 ± 10	0.5
Heart rate (bpm)	67 ± 9	67 ± 11	76 ± 11	75 ± 8	0.6
Glycemia					
HbA_{1c} (%)	5.74 ± 0.30	5.70 ± 0.32	5.76 ± 0.28	5.69 ± 0.29	0.7
HbA_{1c} (mmol/mol)	39.3 ± 3.3	38.7 ± 3.4	39.4 ± 3.1	38.6 ± 3.3	0.7
Fasting glucose (mmol/L)	5.88 ± 0.66	5.64 ± 0.57	6.09 ± 0.43	5.60 ± 0.46	0.031
2-h glucose (mmol/L)	8.07 ± 1.68	7.85 ± 1.90	7.66 ± 1.35	7.77 ± 1.72	0.6
Area under the glucose curve (mmol/L)	18.49 ± 3.05	18.15 ± 3.23	18.63 ± 3.07	17.73 ± 2.83	0.4
HOMA-IR	5.1 ± 3.2	4.2 ± 2.6	5.1 ± 2.5	4.3 ± 2.3	0.8
Matsuda insulin sensitivity index (OGTT-derived)	6.7 ± 3.7	7.5 ± 4.4	6.8 ± 3.1	7.8 ± 3.9	0.8
NEFA insulin sensitivity index (OGTT-derived)	2.53 ± 1.02	2.75 ± 1.10	2.66 ± 1.05[a]	2.72 ± 0.92	0.2
Body composition					
BMI (kg/m^2)	30.75 ± 3.32	30.61 ± 3.29	32.33 ± 4.15	31.90 ± 4.16	0.1
Total adipose tissue, MR-derived (L)	37.9 ± 9.3	38.1 ± 9.2	41.9 ± 10	41.2 ± 9.6	0.021
Subcutaneous adipose tissue, lower extremity, MR-derived (L)	13.2 ± 3.6	13.2 ± 3.4	14.5 ± 3.3	14.2 ± 3.2	0.1
Visceral adipose tissue, MR-derived (L)	6.1 ± 2.4	6.2 ± 2.4	5.6 ± 2.5	5.7 ± 2.5	0.5
Intrahepatic fat, MRS-derived (%)	9.9 ± 7.0	11.2 ± 7.6	9.7 ± 6.9[a]	9.1 ± 6.6[a]	0.005
Indirect calorimetry					
Resting energy expenditure (kcal)	1,862 ± 316[b]	1,782 ± 291[b]	1,834 ± 420	1,849 ± 413	0.3[c]
RQ	0.94 ± 0.09[b]	0.99 ± 0.15[b]	0.95 ± 0.10	0.91 ± 0.07	0.026

Data are means ± SD. A repeated-measures ANOVA was performed to investigate treatment × time interactions. [a]n = 18. [b]n = 20. [c]Adjusted for sex.

(functional MRI measurement day). Subjective feeling of hunger was rated using a visual analog scale from 0 to 10 (0 = not hungry at all; 10 = very hungry).

Functional MRI

Data Acquisition

Brain imaging was conducted in a 3T whole-body Siemens scanner (MAGNETOM Prisma; Siemens Healthineers, Erlangen, Germany) with a 20-channel head coil. The time course of this measurement is depicted in Supplementary Fig. 3. Whole-brain cerebral blood flow (CBF) was recorded at each visit before and 30 min after nasal insulin spray application. A total of 160 units of regular human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) were administered as nasal spray (8 puffs containing 10 units in each nostril over 4 min). In addition, high-resolution T1-weighted anatomical images were obtained.

Table 2—Patient characteristics

	Placebo (n = 21)	Empagliflozin (n = 19)
Sex		
Men	11	5
Women	10	14
Age (years)	62.5 ± 7.7	57.2 ± 9.9
BMI (kg/m^2)	30.8 ± 3.3	32.3 ± 4.2
Waist-to-hip ratio	0.95 ± 0.06	0.93 ± 0.06[a]
Glycemic category (IFG/IGT/IFG+IGT)	5/6/10	10/1/8
Smoker (yes/no)	3/18	1/18
Antihypertensive drugs (yes/no)	14/7	9/10
Lipid-lowering drugs (yes/no)	3/18	3/16
BDI score	3.7 ± 2.3	6.6 ± 5.6

Data are means ± SD unless otherwise indicated. BDI, Beck Depression Inventory II; IFG, impaired fasting glucose; IGT, impaired glucose tolerance. [a]n = 18.

To acquire CBF, a quantitative measure of brain activity, pulsed arterial spin labeling measurements, was obtained with a PICORE-Q2TIPS (proximal inversion with control for off-resonance effects—quantitative imaging of perfusion by using a single subtraction) sequence by using a frequency offset corrected inversion pulse and echo planar imaging readout for acquisition. A total of 16 axial slices with a slice thickness of 4.5 mm (0.90-mm gap) were acquired in ascending order. Each measurement consisted of 79 alternating tag and control images with the following imaging parameters: inversion time (TI), TI1 = 700 ms, TI2 = 1,800 ms; repetition time = 3,000 ms; echo time = 13 ms; in-plane resolution = 3 × 3 mm^2; field of view = 192 mm; matrix size 64 × 64; and flip angle = 90°, resulting in a total scan time of 4:02 min. The first image of the series (M0) was measured before the preparation scans and used to estimate the equilibrium magnetization of the blood (M0B) for absolute CBF quantification.

ASL Image Processing

Image preprocessing was performed by using the ASLtbx with SPM12 (Wellcome Trust Centre for Neuroimaging). Functional images were motion corrected, coregistered to the individual anatomical image, and smoothed (full width at half maximum: 6 mm). Perfusion images were generated by calculating the control-tag differences by using surround subtraction. For accurate CBF quantification (mL × 100 g^{-1} × min^{-1}), we used a unique M0 value extracted from a region of interest in the cerebrospinal fluid. For absolute perfusion quantification, the general kinetic model was applied. Possible outliers were cleaned using a slice-wise procedure based on priors. The high-resolution T1-weighted image was normalized in Montreal Neurological Institute space (1 × 1 × 1 mm) using SPM12's unified segmentation normalization, and the resulting parameter file was used with the individual coregistered CBF maps in normalized space (3 × 3 × 3 mm). A brain mask was used to exclude extracranial voxels in the normalized CBF images. Image quality was visually inspected; patients showing strong artifacts were excluded from the analyses (n = 3).

Whole-Body Metabolism

Before and after treatment, patients underwent a 75-g OGTT in a seated or lying position after an overnight fast (Accu-Check Dextrose O.G-T.; Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Venous blood samples were obtained before as well as 30, 60, 90, and 120 min post–glucose ingestion. Plasma nonesterified fatty acid (NEFA) concentrations (enzymatic method; WAKO Chemicals, Neuss, Germany) as well as clinical chemical parameters were measured on the ADVIA Clinical Chemistry XPT System. Serum insulin and C-peptide were measured on the ADVIA Centaur and erythropoietin on the Immulite immunoassay systems (all from Siemens Healthineers). Glycated hemoglobin (HbA_{1c}) measurements were performed using the Tosoh A1c analyzer HLC-723G8 (Tosoh Bioscience GmbH, Griesheim, Germany). All of these measurements have been performed in an accredited diagnostic laboratory.

Glucagon was measured at all five time points of the OGTTs using a commercial immunoassay (Mercodia, Uppsala, Sweden). Fasting β-hydroxybutyric acid was quantified using an enzymatic 3-hydroxybutyrate dehydrogenase–based Ketone Body Assay Kit (Sigma-Aldrich, St. Louis, MO).

Areas under the curve were calculated according to the trapezoid rule. Whole-body insulin sensitivity was assessed as HOMA of insulin resistance (HOMA-IR) in the fasting state and Matsuda index as well as NEFA index during the OGTT.

While we planned to address heart rate variability, technical recording errors prevented sufficient analyses of these data.

Investigators were blinded for the urine glucose excretion data.

Intrahepatic Fat and Body Fat Distribution

Intrahepatic fat content and body fat distribution were assessed by ^{1}H-MRS and MRI as described before (25). Briefly, intrahepatic fat content was quantified from a volume of interest of 3 × 3 × 2 cm^3, which was placed in the posterior part of segment seven in the liver with subjects being in expiration during data acquisition. Intrahepatic fat is given by the integral of methylene plus methyl resonances (fat) divided by water plus fat in percentage of total signal. Axial T1-weighted whole-body MRI was performed providing quantification of different adipose tissue compartments along the axis of the body.

Indirect Calorimetry

The exhaled air was analyzed for at least 15 min after bed rest prior to measurement with Vyntus CPX (CareFusion, Hoechberg, Germany) after an overnight fast to assess resting energy expenditure and the respiratory quotient (RQ). An RQ of 1 indicates preferential glucose metabolism, whereas an RQ of 0.7 reflects predominant lipid oxidation.

Statistical Analyses

All analyses were performed in the intention-to-treat population, including all patients, for whom baseline and 8-week data were available. Unless otherwise stated, data are presented as mean ± SD. A P value of $\leq$0.05 was considered to indicate statistical significance.

Analysis of the Primary Outcome Brain Insulin Action Using CBF

The prespecified primary outcome was absolute CBF changes in response to intranasal insulin from before (pre-) to after the 8-week intervention. To this end, CBF maps of each patient were corrected for baseline measurements to determine the effect of central insulin action before (ΔCBF_{pre} = $CBF_{fMRI\text{-}2}$ − $CBF_{fMRI\text{-}1}$) and after the intervention ($\Delta CBF_{post\text{-}8\text{-}wk}$ = $CBF_{fMRI\text{-}2}$ − $CBF_{fMRI\text{-}1}$) (see Supplementary Fig. 3). Whole-brain analyses were performed using a voxel-wise approach in SPM12. ΔCBF maps of each measurement day of each patient were entered into a flexible factorial design to determine the interaction of treatment × time on central insulin action. A statistical threshold of $P < 0.001$ uncorrected and a $P < 0.05$ family-wise error (FWE) corrected for multiple comparisons at a cluster level was applied. Additionally, small volume correction was performed for the hypothalamus, the striatum, and the hippocampus, as they are a priori regions of interest. The masks were based on the *wfu* pick atlas (https://fmri.wfubmc.edu/software/PickAtlas).

Additionally, we extracted CBF values of significant clusters to perform post hoc analyses in SPSS (version 27; SPSS Inc).

Analyses of Secondary Outcomes

Statistical analyses of the secondary outcome data were conducted using JMP 14 (SAS Institute, Cary, NC). Treatment × time interactions were tested by repeated-measures ANOVA. Correlations were assessed by multiple linear regression analyses.

Mediation analyses of the relationship among treatment group (binary variable), fasting glucose levels, intrahepatic fat, urinary glucose, and hypothalamus insulin response ($\Delta CBF_{post\text{-}8\text{-}wk}$) at follow-up were performed using PROCESS version 3.3 procedure in SPSS (www.afhayes.com). The relationship between a predictor (x) and an outcome variable (y) can be explained by their relationship to a third variable (the mediator [m]). The predictor (x) predicts the mediator (m) through the path denoted by path *a*. The mediator (m) predicts the outcome (y) through the outcome denoted by path *b*. The relationship between predictor and outcome controlling for the mediator in the model is denoted as direct effect (i.e., path c'). The total effect is the effect of the predictor on the outcome when the mediator is not present in the model (i.e., path c). The effect of

mediation is investigated through the indirect effect, which is a cross product of *a* and *b*. The mediation model included treatment group (binary variable) as the predictor (x), fasting glucose or urinary glucose or intrahepatic fat at follow-up as the outcome variable (y), and hypothalamus insulin response at follow-up as the mediator (m). The significance of the mediation analysis (i.e., indirect effect *ab*) was estimated based on a bias-corrected bootstrap CI (95% CI, 10,000 bootstrap samples). If the CI does not contain zero, then the "true effect" size is different from "no effect," indicating a significant mediation.

Data and Resource Availability

The data generated during the current study are not publicly available due to the data containing information that could compromise research participant privacy/consent.

Results

Of the 42 randomized patients, 40 completed the trial with 2 dropouts in the empagliflozin group (Supplementary Fig. 1). Retrospective measurement of urinary glucose concentrations ensured drug adherence in patients randomized to empagliflozin (Supplementary Fig. 4). Among the routine clinical chemistry measurements, only differences in alkaline phosphatase, CRP, magnesium, and uric acid were detected (Supplementary Table 4). A number of adverse events were recorded in both treatment groups (empagliflozin, n = 18; placebo, n = 12) (Supplementary Table 1). No severe adverse events occurred during the conduct of this trial.

For the prespecified primary outcome, we analyzed the impact of empagliflozin versus placebo on insulin responsiveness of the brain by combining functional MRI with nasal insulin. Whole-brain analysis revealed a significant time × treatment interaction solely in the hypothalamus [$F(1,35)$ = 13.18; $P < 0.001$, uncorrected; P_{FWE} = 0.03, small-volume corrected] (Fig. 1*A* and *B*). This remained significant after adjustment for only BMI as well as for BMI, sex, and age. Post hoc analyses detected no differences between groups prior to treatment ($P > 0.05$), though the insulin response was different between the two treatment arms after 8 weeks of treatment [$T(32)$ = 2.46; P = 0.019]. Within-group comparisons revealed a significant change in hypothalamic insulin response in the empagliflozin group [$T(14)$ = 2.2; P = 0.04], while no significant change was observed in the placebo group ($P > 0.05$) (Fig. 1*B*).

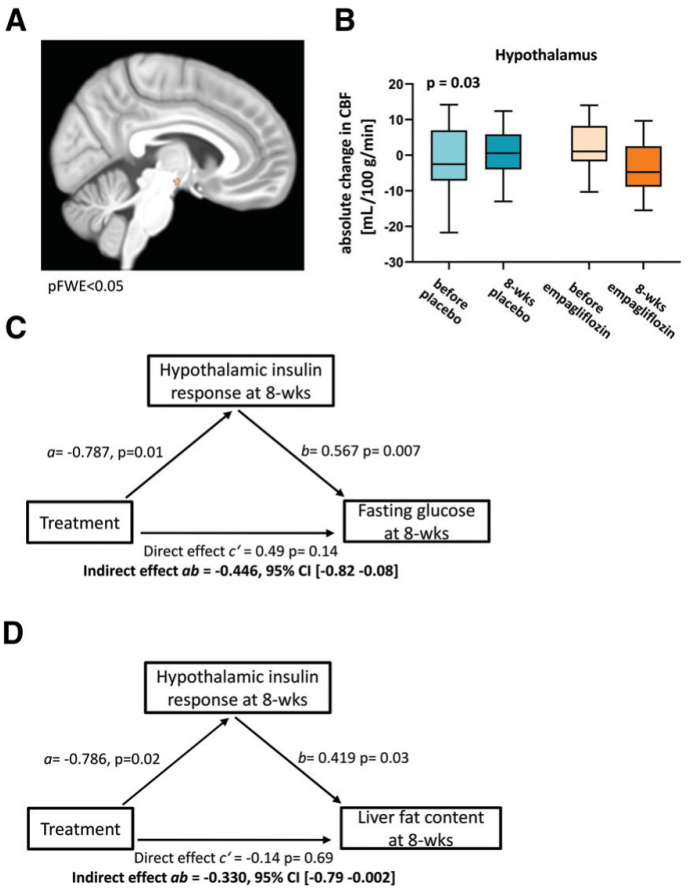

Figure 1—Primary outcome: impact of empagliflozin treatment on insulin action in the brain. *A*: Interaction of treatment × time on insulin responsiveness of the brain. Whole-brain analysis revealed a significant cluster in the hypothalamus, as indicated by the color-coded F map ($P_{FWE} < 0.05$, small-volume corrected). *B*: Insulin response in regional CBF of the hypothalamus was extracted. Presented are box plots with whiskers indicating 1.5 interquartile range. *P* value is for treatment × time interaction that was tested by repeated-measures ANOVA. Model of treatment group (empagliflozin vs. placebo) as a predictor of fasting glucose (*C*) and liver fat content (*D*) after 8 weeks of treatment mediated by improved hypothalamic insulin responsiveness. Path coefficients and corresponding *P* values are shown next to arrows; path *a* indicates the relationship between treatment and hypothalamic insulin action after treatment; path *b* indicates the relationship between the hypothalamic insulin response and fasting glucose levels or liver fat content at the end of treatment; path *ab* indicates the indirect effect of treatment on fasting glucose or liver fat via hypothalamic insulin response; and path *c'* indicates the direct effect of treatment on fasting glucose or liver fat.

Subjective feeling of hunger in the fasted state changed significantly different between the two treatment groups [time × treatment interaction: $F(1,35)$ = 6.4; P = 0.016]. This remained significant after adjustment for BMI, sex, and age. Within-group comparisons revealed a significant decrease in hunger rating in the empagliflozin group [$T(17)$ = 2.1; P = 0.05], while no significant change was observed in the placebo group ($P > 0.05$) (Supplementary Fig. 5).

By contrast, estimates for whole-body insulin sensitivity did not change differently between groups, neither in the fasting state (HOMA-IR, P = 0.8) (Table 1) nor

during the OGTT (Matsuda index, $P = 0.8$; NEFA index, $P = 0.2$) (Table 1). Empagliflozin significantly reduced fasting blood glucose by 0.49 ± 0.39 mmol/L [$F(1,38) = 0.13$; $P = 0.03$] (Fig. 2*A* and Table 1). Glucose during the OGTT and 2-h after the OGTT and HbA_{1c} did not change differently between groups ($P \geq 0.7$) (Fig. 2*B* and Table 1).

Fasting glucagon, glucagon kinetics during the OGTT, erythropoietin, as well as fasting β-hydroxybutyric acid did not significantly differ between treatments ($P \geq 0.1$) (Supplementary Table 3).

Body weight and BMI did not significantly change ($P \geq 0.1$) (Fig. 2*C* and Table 1), though there was a decrease in the total amount of adipose tissue mass upon empagliflozin treatment [$F(1,38) = 0.16$; $P = 0.02$] (Fig. 2*D* and Table 1). Neither visceral nor subcutaneous fat content changed differently between groups ($P \geq 0.1$) (Table 1). While energy expenditure was unaltered ($P = 0.3$, adjusted sex) (Table 1), RQ decreased in the patients who received empagliflozin, indicating preferred lipid oxidation [$F(1,37) = 0.15$; $P = 0.026$] (Table 1). Total caloric intake as well as intake of the major macronutrients remained unaltered during treatment ($P \geq 0.06$) (Fig. 2*F* and Supplementary Table 2).

The majority of our patients (25 of 40) had fatty liver disease with a liver fat content >5.56%. There was a time × treatment interaction on intrahepatic lipids [$F(1,36) = 0.25$; $P = 0.005$] (Fig. 2*E* and Table 1) with an absolute 1.0 ± 2.5% reduction in liver fat content in patients treated with empagliflozin and an absolute 1.2 ± 2.1% increase in intrahepatic lipids in the placebo group. This difference between groups remained significant after adjustment for baseline BMI [$F(1,35) = 0.25$; $P = 0.0053$] or change in BMI [$F(1,35) = 0.16$; $P = 0.0222$].

Mediation analyses were performed to test whether hypothalamic insulin sensitivity served as a mediator between treatment (independent variable) and fasting glucose or liver fat (dependent variable). This analysis revealed a significant negative indirect effect of empagliflozin treatment on fasting glucose and liver fat content in the follow-up visit via hypothalamic insulin response (standardized indirect effect on fasting glucose $ab = -0.446$, 95% bootstrap CI −0.829 to −0.096; standardized indirect effect on liver fat $ab = -0.330$, 95% bootstrap CI −0.797 to −0.008) (Fig. 1*C* and *D*). Alternative models using glucose and liver fat content as mediators did not result in a significant mediation effect between treatment and hypothalamic insulin response. Thus, the increase in insulin action in the hypothalamus in response to empagliflozin significantly mediated the decreases in liver fat content and fasting glucose levels in the empagliflozin group. No such mediation was observed in the baseline visit. Further alternative models included urinary glucose as a possible mediator between treatment (independent variable) and fasting glucose or liver fat (dependent variable). No significant indirect effect was observed.

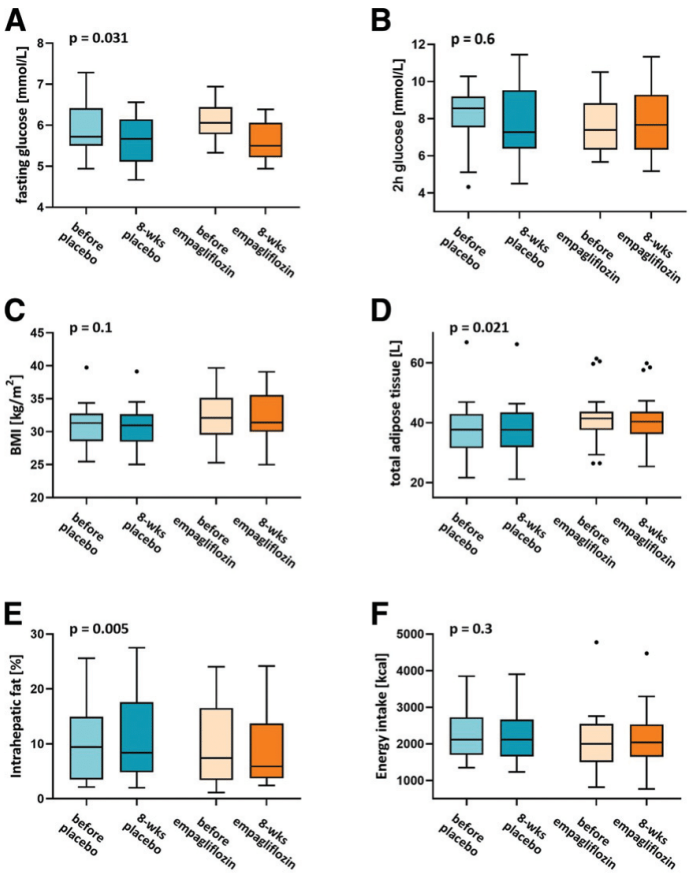

Figure 2—Impact of empagliflozin vs. placebo on key secondary outcomes. *A*: The course of fasting plasma glucose was different between the two treatment groups. No significant interaction between treatment × time was detected for 2 h glucose during the OGTT (*B*) or BMI (*C*). The course of total adipose tissue content (*D*) and liver fat content (*E*) was significantly different between treatment groups. Caloric energy intake did not change during the study (*F*). Presented are box plots with whiskers indicating 1.5 interquartile range. *P* values are for treatment × time interactions that were tested by repeated-measures ANOVA.

Conclusions

We investigated the effect of SGLT2 inhibition on brain insulin sensitivity in patients with prediabetes. While central insulin application had no effect on hypothalamic activity prior to treatment, empagliflozin treatment significantly enhanced hypothalamic insulin action. In contrast, no effects on estimates of whole-body insulin sensitivity were detected. Reduction in fasting glucose and liver fat content

in response to empagliflozin treatment was mediated via improved hypothalamic insulin responsiveness. Despite having no effect on body weight, empagliflozin reduced whole-body fat content without changing food intake.

As predicted, patients with prediabetes do not adequately respond to intranasal insulin application, failing to reduce regional blood flow in the hypothalamus. This corroborates insulin resistance of the human hypothalamus in people with prediabetes, a condition previously described in normal glucose-tolerant individuals with visceral obesity (1,12,14).

Whole-brain analysis detected significant changes in insulin action in the brain in response to empagliflozin in one specific brain area: the hypothalamus. Empagliflozin introduced hypothalamic insulin responsiveness similarly, as previously detected in healthy lean people (12,14). Hence, our results demonstrate that SGLT2 inhibition is able to improve insulin responsiveness of the hypothalamus in humans with prediabetes and obesity. Our data are strengthened by previous findings in rodents, which reported improved brain insulin sensitivity upon SGLT2 inhibition (21). Our results support the hypothesis that insulin resistance of the human brain is not a fixed trait but represents a treatable condition with potential benefits for a number of diseases well beyond metabolism (1,26). Of notice, the observed improvement in insulin sensitivity was likely specific to the brain, as we did not detect effects on estimates for peripheral insulin sensitivity that were reached by other antidiabetic drugs in even shorter periods (27). Our data are well in line with previous trials with SGLT2 inhibitors in similar patients, in whom no improvement in whole-body insulin sensitivity was detected (28,29). More important, the brain response to insulin after empagliflozin treatment was independent of body weight, which by itself is closely linked to insulin sensitivity of at least some brain areas (1).

The capability of empagliflozin to reduce excessive fat accumulation in the liver has been reported before (28,30), making this substance and other SGLT2 inhibitors with comparable effects promising candidates for the treatment of fatty liver disease. Most of the patients in our study fulfilled the diagnostic criteria for fatty liver disease, and we confirm a clinically relevant reduction of liver fat content upon empagliflozin treatment. Of note, this response was most likely not due to weight loss, but was mediated via improved hypothalamic insulin action, suggesting a potential neural contribution. Indeed, insulin action in the hypothalamus regulates liver metabolism in rodents via the vagus nerve (2). In humans, intranasal insulin was reported to improve hepatic energy metabolism with a rapid reduction in liver fat content (31). This response was blunted in type 2 diabetes (31), presumably due to brain insulin resistance (1). Our results suggest that the reversal of hypothalamic insulin resistance by empagliflozin might restore brain-derived regulation of liver metabolism with a subsequent reduction of liver fat content.

Our current analyses indicate that brain-derived mechanism could contribute to the isolated reduction in fasting blood glucose that we observed in patients treated with empagliflozin and that was previously reported in other trials in prediabetes and recent-onset diabetes (28,29). Hepatic endogenous glucose production (EGP) is the major determinant of fasting glucose concentrations (32). Reduction of liver fat content in response to SGLT2 inhibition was reported to be accompanied by lower EGP in some (33), but not all previous trials (28,34). Though, some trials even reported increased EGP in the fasting state upon SGLT2 inhibitor treatment as a compensatory mechanism in the face of increased glucose excretion (16,18,35). This was, however, attenuated after a few weeks (19). Of note, brain insulin action was shown to modulate EGP, both in rodents (2) and humans (3,4). Yet, this is thought to be most important in the postprandial state when systemic insulin concentrations are high, while no major effects were detected in the fasting state (36). The duration of treatment in our study might not have been long enough to restore all postprandial brain-derived mechanisms that modulate postprandial metabolism in the periphery (1), as we did not detect significant improvements in glucose tolerance or HbA_{1c}.

One interesting observation is the reduction of total adipose tissue mass, without changes in body weight or body fat distribution. For empagliflozin, this has not been reported before. It is unlikely that brain insulin's regulation of eating behavior (1) is involved, as our patients reported no changes in total energy intake or macronutrient composition of their diet. It might either be due to the limited sample size and the short duration of treatment that could have hindered detection of smaller effects or due to a preferential breakdown of adipose tissue. The latter hypothesis is supported by our analysis of whole-body substrate oxidation. In line with previous results (16,35), our data indicate a shift from glucose toward lipid oxidation in the empagliflozin-treated group. This effect of empagliflozin is of major clinical importance, as it likely contributes to the ketoacidosis risk associated with SGLT2 inhibitors (37). Though, the underlying mechanism is still not fully clear. While basic studies indicate that brain insulin action regulates lipolysis (2,38), the relevance of this for humans is under debate (1). Our current analyses do not support a major contribution of improved hypothalamic insulin responsiveness to the shift in substrate oxidation in the current study.

As we detected improved hypothalamic insulin responsiveness, the underlying mechanism is of great interest. While animal studies uncovered structural and functional changes in this area upon SGLT2 inhibitor treatment (21,22), the detailed mechanisms are still unclear. Of note, SGLT2 is not only expressed in the kidney (19), but the protein is also detectable in the human brain (39). Some findings in animals suggest direct effects in the brain (40). Hence, inhibition of this transporter could reduce glucotoxicity in brain cells. This might subsequently diminish proinflammatory signals and restore insulin responsiveness (41,42). However, there is no proof that empagliflozin is transported across the blood–brain barrier (40), making direct effects of the drug unlikely.

SGLT2 inhibitors have previously been reported to increase glucagon (16–18,35) and ketone body (16,35) concentrations in the circulation. As both substances have known effects in the hypothalamus (43,44), we measured both. However, neither fasting glucagon, nor glucagon during the OGTT, nor β-hydroxybutyric

acid significantly changed upon treatment. They are, therefore, most likely not involved in the beneficial effects of empagliflozin in the hypothalamus. As most previous results have been obtained in patients with overt diabetes (17,18), a difference in the glycemic status might explain unaltered levels in our patients. Indeed, a previous study in subjects with prediabetes also did not detect changes in glucagon (29). Furthermore, studies that reported increasing glucagon levels used assays with higher potential cross-reactivity with other hormones, while our and other studies with more specific measurements could not detect this response (45).

We hypothesize that the autonomic nervous system is a major metabolic regulator. SGLT2 inhibitors were reported to modulate activity of the autonomic nervous system with a shift from sympathetic toward parasympathetic tone (20). The hypothalamus receives autonomic inputs, integrates them with further information, and generates outputs toward the autonomic nervous system. Indeed, realignments in the central nervous system are believed to underlie changes in autonomic tone in response to empagliflozin treatment (46). Hence, empagliflozin might exert its effects in the hypothalamus via neuronal pathways rather than the bloodstream. Further studies are needed to uncover the detailed mechanisms by which SGLT2 inhibitors improve insulin sensitivity of the hypothalamus.

A limitation of our study is the uneven distribution of sexes among treatment groups, as we did not randomize in a sex-stratified manner. Thus, we cannot analyze sex-specific effects that have been reported for brain insulin (1) or exclude an influence on our results. However, the lack of major sex differences in the safety or efficiency of SGLT2 inhibitors (47) argues against major sex differences in the response to empagliflozin. While we included overweight and obese persons with prediabetes to ensure brain insulin resistance in our patients, our findings must not necessarily be translatable to other patient populations. β-Hydroxybutyric acid was measured in the morning when drug effects were potentially weakened. Thus, we cannot exclude effects during other times of the day. Furthermore, limited statistical power might have hindered detection of smaller treatment effects (e.g., in peripheral insulin sensitivity that was also not quantified by hyperinsulinemic-euglycemic glucose clamp). While our mediation analyses suggest a central role of insulin action in the hypothalamus for peripheral effects of empagliflozin, this does not exclude major direct peripheral effects. The complex interplay between insulin action directly in peripheral organs and insulin-induced signals from the brain (and possible effects of pharmacological treatment on this relationship) clearly needs further study.

In summary, we detected restored hypothalamic insulin sensitivity upon treatment with empagliflozin in people with prediabetes. Mediation analyses indicate that this effect could contribute to the observed reduction in liver fat content and fasting blood glucose, which are major risk factors for diabetes and cardiovascular complications. Therefore, improved brain insulin responsiveness might have contributed to the beneficial effects of empagliflozin in large clinical trials that demonstrated a relevant reduction of morbidity and mortality (15). Our current findings reveal that brain insulin resistance is a condition that is treatable by pharmacological interventions with potential benefits for adiposity and whole-body metabolism.

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Duality of Interest. S.K. reports lecture fees from Novo Nordisk outside of the current work. A.L.B. reports lecture fees from AstraZeneca, Boehringer Ingelheim, and Novo Nordisk and participated in advisory boards of Boehringer Ingelheim, AstraZeneca, and Novo Nordisk outside of the current work. N.S. was and is consulting and lecturing for Allergan, AstraZeneca, Boehringer Ingelheim, Gilead, Genkyotex, Intercept Pharmaceuticals, Merck Sharp & Dohme, Novartis, Novo Nordisk, Pfizer, and Sanofi; he conducted clinical studies with support from AstraZeneca, Boehringer Ingelheim, Sanofi, DSM Nutritional Products, and Roche Diagnostics outside of the current work. A.F. reports lecture fees from Sanofi, Merck Sharp & Dohme, and AstraZeneca; he participated in advisory boards of Boehringer Ingelheim, Sanofi, Novo Nordisk, and Eli Lilly and Company outside of the current work. M.H. reports an independent research grant from Boehringer Ingelheim to the University Hospital of Tübingen for this study; a research grant from Sanofi to the University Hospital of Tübingen outside of the current work; consulting for Boehringer Ingelheim; and lecture fees from Sanofi, Novo Nordisk, Eli Lilly and Company, and Merck Sharp & Dohme. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. S.K., J.H., C.D., and R.V. researched and analyzed data. R.W., A.V., L.F., K.K., J.M., and A.P. researched data. A.L.B., N.S., H.-U.H., H.P., and A.F. contributed to the design of the trial and discussed data. M.H. researched data, supervised the project, and drafted the manuscript together with S.K. and J.H. All authors contributed to discussion and approved the final version of the manuscript prior to submission. M.H. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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2.3 3rd Publication: "Eight weeks of empagliflozin does not affect pancreatic fat content and insulin secretion in people with prediabetes."

Authors:

Julia Hummel, Jürgen Machann, Corinna Dannecker, Stephanie Kullmann, Andreas L. Birkenfeld, Hans-Ulrich Häring, Andreas Peter, Andreas Fritsche, Robert Wagner, Martin Heni.

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Eight weeks of empagliflozin does not affect pancreatic fat content and insulin secretion in people with prediabetes

Short running title: No effect of empagliflozin on intrapancreatic fat

Julia Hummel[1,2], Jürgen Machann[1,2,3], Corinna Dannecker[1,2], Stephanie Kullmann[1,2,4], Andreas L. Birkenfeld[1,2,4], Hans-Ulrich Häring[1,2,4], Andreas Peter[1,2,5], Andreas Fritsche[1,2,4], Robert Wagner[1,2,4], Martin Heni[1,2,4,5]

1. Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
2. German Center for Diabetes Research (DZD), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
3. Department of Radiology, Section on Experimental Radiology, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany
4. Department of Internal Medicine, Division of Diabetology, Endocrinology and Nephrology, Eberhard Karls University Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
5. Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany

Corresponding author
Prof. Martin Heni, MD
University Hospital Tübingen, Internal Medicine IV
Otfried-Müller-Str. 10
72076 Tübingen
Germany
Email: martin.heni@med.uni-tuebingen.de
Phone: +49 7071 2982714
ORCID ID: 0000-0002-8462-3832

Background

Fat accumulation within the pancreatic parenchyma is known to be a risk factor for a number of conditions, including pancreatic cancer and chronic pancreatitis[1]. There is also growing evidence that the accumulation of pancreatic fat may contribute to the pathogenesis of diabetes[1,2]. Adipocytes within the pancreas could impair insulin secretion via secreted factors[1,3]. Some studies reported that fatty pancreas is linked to reduced insulin secretion, especially in persons with prediabetes, while other trials did not detect any such associations[1]. Previous findings of the DiRECT lifestyle intervention trial emphasize that pancreatic fat reduction plays a crucial role in the improvement of ß-cell function and remission of type 2 diabetes by hypocaloric diet[4]. Pharmacological approaches for reducing fat in the pancreas are, however, still lacking. The substance class of sodium/glucose cotransporter 2 (SGLT2) inhibitors can lower ectopic fat accumulation in the liver and shift energy metabolism towards fat oxidation[5]. We have now tested the ability of empagliflozin to reduce pancreatic fat content in overweight and obese persons with prediabetes.

Methods

The study protocol of this double-blind, placebo-controlled randomized trial was approved by the local ethics committee. Participants provided informed written consent prior to enrollment. Forty participants with prediabetes (according to ADA's OGTT criteria) were 1:1 randomized, receiving 25 mg empagliflozin qd or placebo for 8 weeks (fig. 1A). Detailed methods and patient characteristics were reported previously together with the primary endpoint[6]. In short, we observed improved insulin sensitivity of the hypothalamus in response to empagliflozin. This was accompanied by lowering of fasting glucose and reduction of liver fat[6].

Before and after treatment, pancreatic fat accumulation was quantified by magnetic resonance imaging (3T Magnetom Vida, Siemens Healthcare, Germany) following an overnight fast (fig. 1B). An 18-channel body-array receiver coil was placed on the upper abdomen of the participants, which were placed in a supine position. Localizer imaging was subsequently performed by applying single breath-hold gradient echo imaging technique in all three spatial directions (10s) for planning of the chemical shift encoded Dixon sequence, enabling quantification of proton density fat fraction (PDFF). A 3D multi-echo CSE sequence covering the abdominal organs, entire liver and pancreas was applied. Detailed sequence parameters: matrix size 160x132, field-of-view 380x314 mm, partition thickness 3 mm with 80 partitions in total. Repetition time TR = 8.9 ms, six echoes with echo times TE = 1.09, 2.46, 3.69, 4.92, 6.15 and 7.38 ms, a low excitation angle of 4° to minimize T1-bias, acceleration by Caipirinha, factor 2 in both, phase-encoding and slice encoding directions, bandwidth 1080 Hz/pixel, acquisition time TA = 16 s in breath-hold (expiration). Image reconstruction for calculation of PDFF-maps was performed inline on the console of the scanner using a multistep fitting algorithm as

described[7,8], correcting for microscopic magnetic field inhomogeneities by correction for R2* of water and fat and the spectral components of fat[9]. In this PDFF-map, signal intensity values directly reflect PDFF of the respective tissue in percent.

Reproducibility and sensitivity of pancreatic fat measurement were recently reported[10], and showed that the applied technique is highly reproducible with coefficients of variation of less than 1% in pancreatic subregions (head, body, tail), and capable of detecting changes in the low range of PDFF.

Following an overnight fast, participants were also subjected to 5-point 75 g oral glucose tolerance tests (OGTT) to assess insulin sensitivity (Matsuda Index) and insulin secretion prior to and after treatment (fig. 1B). Insulin secretion was assessed by oral Disposition Index as well as the areas under the C-peptide curves divided by the areas under the glucose curves during the first 30 minutes of the OGTT ($AUC\text{-}C\text{-}peptide_{0\text{-}30}/AUC\text{-}glucose_{0\text{-}30}$) as described previously[11].

Statistical analyses were performed in JMP14. Changes within groups were compared by two-tailed paired t-tests, and treatment x time interactions were tested by repeated measures ANOVA. P values < 0.05 were considered statistically significant.

Results

Mean pancreatic fat content did not change in either treatment group (both $p \geq 0.2$; before treatment: empagliflozin group 7.1 ± 4.6 %, placebo group 10.3 ± 9.1; after treatment: empagliflozin group 6.2 ± 4.2 %, placebo group 10.5 ± 8.5 % [mean±SD], fig. 2A), and the course of pancreatic fat content was comparable between treatments (p=0.2, fig. 2A & 2C).

Insulin secretion, as assessed by Disposition Index (before treatment: empagliflozin group 731 ± 455 AU, placebo group 837 ± 596 AU; after treatment: empagliflozin group 765 ± 366 AU, placebo group 1073 ± 1040 AU, fig. 2B & 2D) and $AUC\text{-}C\text{-}peptide_{0\text{-}30}/AUC\text{-}glucose_{0\text{-}30}$ (adjusted for insulin sensitivity; before treatment: empagliflozin group 162 ± 50 AU, placebo group 166 ± 56 AU; after treatment: empagliflozin group 168 ± 35 AU, placebo group: 179 ± 65 AU) did not differ between treatments (p=0.3 and p=0.7, respectively). While empagliflozin treatment lowered fasting glucose significantly[6], neither C-peptide nor glucose curves differed significantly after treatment (fig. 2E & 2F).

Conclusions

In contrast to dietary energy restriction or exercise[1,4,12], empagliflozin did not reduce pancreatic fat in overweight and obese persons with prediabetes in our study. This implies that the beneficial effects of empagliflozin on systemic metabolism and general health[5] do not depend on alterations in pancreatic fat. While we detected a reduction in liver fat and fasting glucose[6],

our 8-week treatment with empagliflozin might have been not long enough to detect alterations in pancreatic fat, since we did not observe any changes in body weight either[6]. However, GLP-1 receptor agonist treatment for six months, with significant but mild weight loss, was not able to reduce pancreatic fat content in patients with type 2 diabetes either[1].

Improving ß-cell function is thought to be pivotal for the prevention and remission of type 2 diabetes. The DiRECT lifestyle intervention trial detected a recovery of ß-cell function as the key determinant of type 2 diabetes remission[4]. Of note, ß-cell recovery was dependent on pancreatic fat reduction[4]. Since empagliflozin was unable to significantly reduce pancreatic fat in our trial, it comes as no surprise that ß-cell function did not improve in our participants with prediabetes. In overt diabetes, ß-cell function is also compromised by glucotoxicity, that is typically not present in prediabetes. Via their glucose lowering effect, SGLT2 inhibitors reduce glucotoxicity and can thereby improve insulin secretion in patients with overt diabetes[5]. Although the glycemic benefits of SGLT2 inhibitors have already been well established in diabetes, data from large randomized trials suggest that these benefits are inconsistent in prediabetes[13,14].

The uneven distribution of sexes among treatment groups posed a restriction to our work. Further, the fact that the duration of the study was limited to 8 weeks meant that possible long-term effects of empagliflozin on pancreatic fat may have been overlooked. However, effects on liver fat content and fasting glucose were detectable in our trial[6], which was sufficiently powered to detect the reduction of pancreatic fat as previously reported from the DiRECT trial[4] (G*Power, α=0.05, Power=0.9). The response of pancreatic fat to empagliflozin treatment has, however, not been reported in overt type 2 diabetes so far. Insulin secretion (and insulin sensitivity) was not assessed via gold standard glucose clamps but estimated from OGTTs.

Taken together, this randomized, controlled trial did not detect any effects of empagliflozin on pancreatic fat content and insulin secretion in persons with prediabetes. Further options for pancreatic fat reduction therefore need to be evaluated.

Author Contributions

JH, JM and CD researched and analyzed data, SK, AP and RW researched data, ALB contributed to the discussion, HUH and AF contributed to the design of the trial and discussed data. MH researched data and supervised the project. All authors contributed to discussion and approved the final version of the manuscript prior to submission.

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Disclosure Statement

Outside of the current work, SK reports lecture fees from Novo Nordisk.

Outside of the current work, ALB reports lecture fees paid to the University of Tübingen from AstraZeneca, Boehringer Ingelheim, Novo Nordisk. He participated in advisory boards of Boehringer Ingelheim, Astra Zeneca and Novo Nordisk. Fees were paid to the University of Tübingen.

Outside of the current work, AF reports lecture fees from Sanofi, Merck Sharp & Dohme and AstraZeneca. He participated in advisory boards of Boehringer Ingelheim, Sanofi, Novo Nordisk and Lilly.

MH reports an independent research grant from Boehringer Ingelheim to the University Hospital Tübingen for this study. Outside of the current work, MH reports a research grant from Sanofi (to the University Hospital Tübingen), advisory board for Boehringer Ingelheim, lecture fees from Boehringer Ingelheim, Novo Nordisk, and Amryt.

All other authors have nothing to declare.

Guarantor's statement

Prof. Martin Heni is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Data Availability Statement

The data generated during the current study are not publicly available due to the fact that they contain information that could compromise research participant privacy/consent.

Registration

This study was pre-registered at Clinicaltrials.gov as trial number NCT03227484 and at The European Union Clinical Trials Register as EudraCT number 2016-003477-18.

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Figure legends

Figure 1 - Study outline

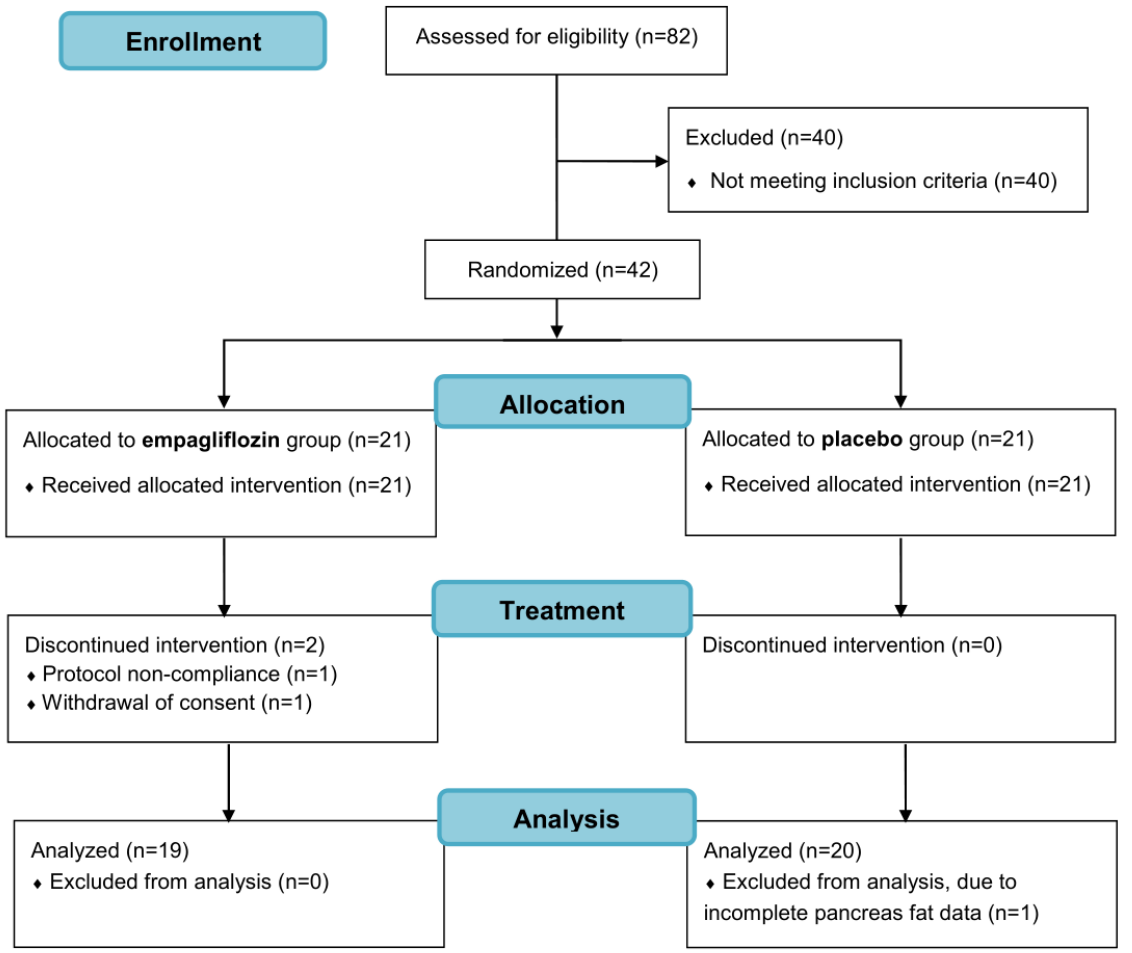
(**A**) CONSORT flow diagram (adapted from [6]). (**B**) Study outline for the assessment of insulin secretion and pancreatic fat content (adapted from [6]).

Figure 2 - Impact of empagliflozin versus placebo on pancreatic fat content, insulin secretion and glycemia

Fat contents were quantified from MRI for the caput, corpus and cauda of the pancreas and a mean fat content was calculated for each participant. (**A, C**) The majority of the participants had a substantial amount of pancreatic fat which did not change upon treatment. (**B, D**) OGTT-derived Disposition Index did not change during the study. Following treatment, neither glucose (**E**) nor C-peptide curves (**F**) differed significantly between groups. Depicted are: (**A, B**) box plots with whiskers indicating 1.5 IQR, individual data points (**C, D**), and means ± SD (**E, F**). P-values are derived from repeated measures ANOVA, testing treatment x time interactions (n=39 for pancreatic fat; n=40 for insulin secretion [**A, B**]) or two-tailed t-tests for the areas under the curve (**E, F**).

Figure 1

A



B

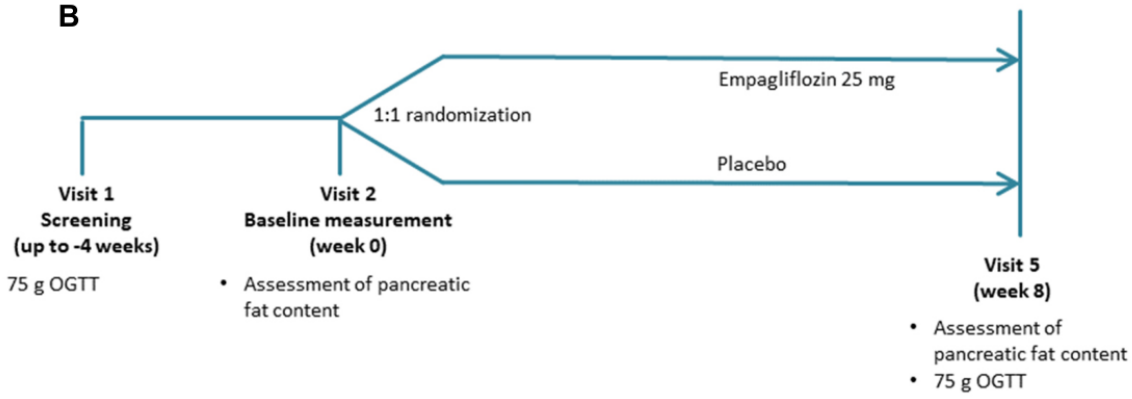
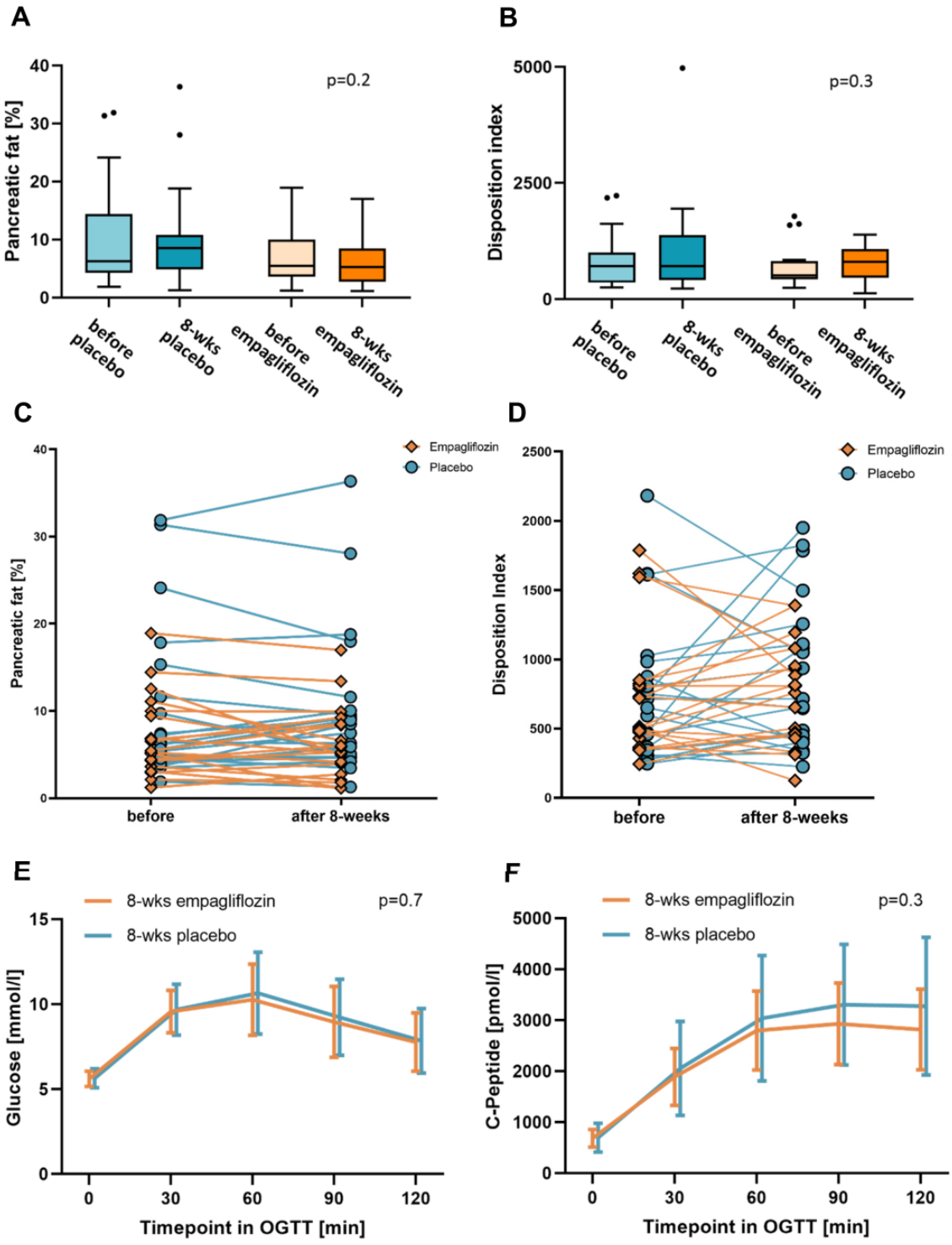


Figure 2



3 Discussion

This thesis focused on fasting substrate oxidation and elucidated its potential determinants as well as pharmacological modulation with the SGLT2i empagliflozin. Whether a change in fasting substrate oxidation in response to SGLT2 inhibition contributes to changes in intrapancreatic fat and insulin secretion was further evaluated. The findings of this research were summarized in three original research papers which are embedded in this thesis (see 2).

First, we explored factors that potentially affect fasting substrate oxidation. Therefore, a cross-sectional analysis was performed (see 2.1). The latter included 192 persons with a wide range of clinical and metabolic parameters ranging from lean and healthy to obese persons with diabetes. This analysis revealed FFA to be independently correlated with the preferentially oxidized substrate. Likewise to FFA, high levels of ß-hydroxybutyrate were linked to predominant fat oxidation. In contrast to GLP-1, GIP and glicentin were uncovered as independent determinants of substrate preferences in a sub-group of persons with prediabetes. A relationship between insulin and whole-body insulin sensitivity with fuel utilization could not be detected in this work. Moreover, BMI, total fat mass as well as body fat distribution including intrahepatic lipid content were not related to substrate oxidation. There were no differences between normal glucose tolerance, prediabetes and newly-diagnosed T2DM in regard to the preferred substrate. Likewise, no relationship between the metabolic syndrome and substrate oxidation could be established.
In the context of this thesis, a randomized controlled trial including 40 persons with overweight or obesity and prediabetes were analyzed to elucidate the potential of pharmacologic modulation of nutrient use (see 2.2). Altered substrate oxidation with a pronounced utilization of FFA was apparent after treatment with SGLT2i empagliflozin. This pharmacologic modulation of substrate oxidation by empagliflozin did neither affect intrapancreatic fat content nor pancreatic insulin secretion (see 2.3).

These summarized results are discussed in the following sub-chapters.

3.1 Determinants of fasting substrate oxidation

The analyzed potential determinants for fasting substrate oxidation FFA, glucose, ß-hydroxybutyrate, insulin and insulin sensitivity, age as well as sex were already

extensively discussed in the manuscript presented above (see 2.1; (Hummel et al., 2021)). Detailed conclusions in the context of available literature are moreover found in the manuscript in regard to the results of BMI as well as body fat distribution and metabolic diseases as obesity, diabetes and the metabolic syndrome (see 2.1, (Hummel et al., 2021)).

Our work revealed predominant fat oxidation in case of low levels of glucagon in a population with overweight or obesity and prediabetes. This is in contrast to current textbook knowledge, where glucagon is classically thought to promote ß-oxidation (Cryer, 2012; McGarry and Foster, 1980). Of note, this previous assumption is based on data from studies with highly experimental conditions. Hence, actions of glucagon may differ under basal, physiologic conditions *in vivo*.

Moreover, glucagon is heavily involved in lipid handling in the liver (Galsgaard et al., 2019). In accordance, our detected link between glucagon and substrate oxidation was mediated through FFA (Hummel et al., 2021). It may therefore be possible that glucagon shifts FFA preferably to gluconeogenesis which are consequently not available for oxidation. To ultimately clarify the complex interplay between glucagon, GLP-1, FFA, glucose and insulin for the regulation of substrate oxidation, further studies and appropriate experiments are needed.

Unlike GLP-1, higher levels of GIP and glicentin were correlated with predominant carbohydrate oxidation. Of notice, the antidiabetic drug class of GLP-1 receptor agonists (GLP-1 RA) which are approved for the treatment of T2DM (Croom and McCormack, 2009) were reported to affect substrate oxidation. Liraglutide decreased 24-h RQ, indicating an overall higher fat utilization (van Can et al., 2014) and improved metabolic flexibility in the transition to the postprandial phase (Anholm et al., 2019). While the known actions of GLP-1 in response to food intake include stimulation of insulin secretion and suppression of glucagon release (Holst, 2019), its role in the fasting state is less understood. In contrast to the postprandial phase, insulin levels are lower and glucagon levels are increased in a fasting situation (Boyle et al., 1989), representing a completely different metabolic milieu. Therefore, insulinotropic effects of GLP-1 seem not to play a major role in basal conditions. Our results during fasting conditions are comparable to those of research in adults with overweight or obesity, where no link between fasting GLP-1 levels and the choice of oxidized substrates was detected

(Poggiogalle et al., 2018). In contrast to these findings, higher fasting GLP-1 levels were however reported to associate with an increased fat oxidation in a glucose tolerant population with a wide ranging BMI from 18.5-50 kg/m² (Pannacciulli et al., 2006). It was suggested that this observed relationship in normal glucose tolerant individuals could be conveyed via sympathetic nervous system activation which enhances lipolysis and FFA oxidation. Central administration of GLP-1 was shown to activate autonomic nervous system that in turn promotes sympathetic tone (McLean et al., 2021; Yamamoto et al., 2002, 2003). These heterogenous results might be explained by the different glucose tolerance states of the study populations, since the link between GLP-1 and substrate oxidation was solely seen in glucose tolerant subjects (Pannacciulli et al., 2006). It can therefore be hypothesized that the described mechanism via sympathetic nervous system is impaired in persons with glucose intolerance which were studied in this thesis. Moreover, the interpretation of this inconsistent results has to take the markedly higher GLP-1 concentrations in the study of Pannacciulli et al. into account. This might be due to cross-reactivity of the assay with other pro-glucagon cleavage products that were a well-known problem in older GLP-1 immunoassays. Thus, the discrepancy might be explained by improvements in assay technology over the last years, meaning a lower cross-reactivity of current assays measuring incretins (Bak et al., 2014) that were applied in our study. Moreover, adolescents with obesity and impaired glucose tolerance were reported to have lower fasting GLP-1 levels compared to persons with normal glucose tolerance (Manell et al., 2016), which could be a further explanation for higher GLP-1 levels in glucose tolerant individuals in the study of Pannacciulli et al..

Compared to GLP-1, GIP gained less attention as a therapeutic target so far. In addition to its role in glucose homeostasis (it promotes insulin secretion in response to food intake), GIP exerts further metabolically relevant actions (Holst, 2019; Thondam et al., 2020). Data from studies in rodents are consistent with those in humans, demonstrating GIP to promote re-esterification of FFA, to increase TAG storage in adipose tissue, to decrease plasma FFA and to lower the oxidation of FFA (Thondam et al., 2020). In accordance with these findings, a lower reliance on fat oxidation during high fasting levels of GIP was observed within this thesis (Hummel et al., 2021). Of note, we studied participants with prediabetes who had diminished insulin sensitivity. Current findings of this work confirm the results of an insulin resistant mouse model lacking GIP receptor

(Zhou et al., 2005). These mice had an overall decreased RQ indicating a shift to fat oxidation in liver and skeletal muscle along with a preserved fat storage (Zhou et al., 2005). GIP receptors are not solely located in the pancreas, but also expressed in other tissues, including adipose tissue and the brain (Usdin et al., 1993). Therefore, changes of substrate oxidation in the skeletal muscle of GIP receptor knockout mice can be suspected to be mediated via central nervous system (Pfeiffer and Keyhani-Nejad, 2018).

The role of GIP during basal fasting conditions has not been well described so far, which impedes a direct comparison of literature with the results of this work. However, data of GIP infusion experiments might help when interpreting our current findings, since the effect of GIP on insulin secretion in humans was reported to depend on actual glucose levels. When glucose was low, GIP had only marginal effects on insulin secretion but rather increased glucagon secretion, while GIP infusion markedly increased insulin secretion and suppressed glucagon during hyperglycemia (Christensen et al., 2011). This argues for the relevance of GIP action during the fasting state where the incretin could function in an insulin-independent manner. A further hint towards its role in fasting metabolism are the observed differences of basal GIP levels between healthy individuals and persons with T2DM, in whom GIP is increased (Alssema et al., 2013).

Albeit GLP-1 and glicentin are likewise secreted by intestinal L-cells, the physiologic role of glicentin is still unclear and even its receptors remain unknown (Perakakis and Mantzoros, 2020). An affinity to GLP-1 and -2, GIP or glucagon receptors is considered to be plausible (Perakakis and Mantzoros, 2020; Raffort et al., 2017). Beside the described insulinotropic actions of glicentin (Ohneda et al., 1995), the hormone was detected in the brain of rats (Jin et al., 1988), making a central action possible. Underlining its relevance in obesity, first data show lower fasting glicentin levels in persons with severe obesity (Raffort et al., 2018), while the concentrations are increased after bariatric surgery (Poitou et al., 2018; Raffort et al., 2017). In adolescents with obesity, fasting levels of glicentin were lower in those with impaired glucose tolerance compared to normal glucose tolerance, which can be interpreted as a sign of early metabolic deterioration (Manell et al., 2016). Current data of this thesis obtained in persons with overweight or obesity and prediabetes indicate that in the presence of high levels of glicentin, carbohydrates are

preferentially oxidized over fat. Though, a pursuing discussion is restricted due to the lack of data concerning glicentin and substrate oxidation.

Since the discovery of a reduced or even missing incretin effect in patients with T2DM (Elrick et al., 1964; Nauck and Meier, 2016) and the detection of weight and glucose lowering features of GLP-1 receptor agonists (Christensen et al., 2009), incretins gained growing attention over the last years. Moreover, first hints for differences in fasting levels of GLP-1, GIP and glicentin were reported in the literature for obesity, impaired glucose tolerance and T2DM (Chia et al., 2014; Manell et al., 2016; Raffort et al., 2017). Preclinical trials testing GIP agonists as well as GIP antagonists to induce weight loss and improve whole-body metabolism were successful (Killion et al., 2020; Widenmaier et al., 2010). Synergistic effects were achieved when they were combined with GLP-1 RA in rodents and humans (Frias et al., 2018; Killion et al., 2018). Recently, first clinical data on effects of GLP-1R/GIPR co-agonists revealed an improvement of β-cell function and insulin sensitivity in T2DM (Thomas et al., 2021). The co-agonism of GLP-1 and GIP represents a promising new therapeutic option for both T2DM and obesity (Frías, 2020). This underlines the importance of incretins as upcoming targets in diabetes research and emphasizes the clinical relevance of our findings that low fasting levels of GIP and glicentin are linked to preferred fat oxidation.

3.2 Pharmacologic modulation of fasting substrate oxidation

In order to analyze the second research question of this thesis, a population of 40 adults with overweight or obesity and prediabetes were enrolled in a randomized controlled, investigator-initiated trial. Participants had a mean BMI of 31.5 ± 3.8 kg/m² and were 60 ± 9 years old. Since persons with first impairments of glucose metabolism face a high risk to progress to T2DM, they represent a population of opportunity for interventions in order to prevent further metabolic deteriorations.

As part of this thesis, a randomized controlled trial was analyzed, where empagliflozin treatment over 8 weeks resulted in a decreased RQ, reflecting a change of substrate use towards increased reliance on fat oxidation (Kullmann et al., 2021). The SGLT2 inhibitor empagliflozin elevates renal glucose excretion and thereby reduces blood glucose levels (Vallon, 2015). This pharmacologic modulation of substrate oxidation was accompanied

by further metabolic improvements in empagliflozin-treated persons. Fasting glucose levels, liver fat content as well as total adipose tissue were reduced after the 8-weeks intervention (Kullmann et al., 2021). In addition to a corroboration of brain insulin resistance in persons with prediabetes, restoration of hypothalamic insulin sensitivity was achieved in response to SGLT2 inhibition (Kullmann et al., 2021).

Interestingly, this improvement of brain insulin responsiveness mediated the amelioration of fasting glucose and liver fat. Accumulating evidence demonstrates brain insulin action to suppress endogenous glucose production (EGP) (Dash et al., 2015; Heni et al., 2017; Könner et al., 2007; Obici et al., 2002b). Since the latter is central for fasting glucose concentrations (Féry, 1994), the improved hypothalamic insulin sensitivity upon empagliflozin may has the potential to convey the positive effects on fasting glucose levels. However, this inhibition of EGP was solely shown under postprandial conditions with elevated circulating insulin levels, while the effect during fasting remains unknown. Moreover, contradictory results from trials with SGLT2i were reported, reaching from reduced EGP (Cusi et al., 2019) over unaffected (Kahl et al., 2020) to increased EGP to compensate for glucose loss (Ferrannini et al., 2016). These divergent results suggest that further factors could interact with the effects of SGLT2i treatment on EGP. Additional interacting signals might include signals originating in the brain. Therefore, further research is needed to elucidate the relationship between SGLT2i, brain insulin action and glucose metabolism.

In addition to EGP, first reports indicate that brain insulin action could be involved in the regulation of lipolysis (in adipose tissue) of rodents (Koch et al., 2008; Scherer et al., 2011). Moreover, injection of insulin into the preoptic area of the hypothalamus results in enhanced fat utilization in mice (Sanchez-Alavez et al., 2009). Thus, the improved brain insulin sensitivity upon SGLT2 inhibition could have affected liver fat mobilization and fuel oxidation in our study. However, according to the performed mediation analyses in the scope of this work (see 2.2), the change in substrate oxidation was likely not conveyed via the brain. Further, emerging evidence supports the hypothesis that SGLT2 inhibition affects the autonomic nervous system (Scheen, 2019; Spallone and Valensi, 2021), which in turn regulates lipolysis (Geerling et al., 2014) with possible implications of fat oxidation. This mechanism could have affected liver fat reduction and substrate

oxidation. However, we did not assess autonomic outflow to the periphery and therefore this hypothesis remains elusive.

Corresponding to the results of the prior presented manuscript (see 2.1 and 3.1), glucagon and FFA could have modulated the altered fuel selection in response to empagliflozin treatment (Hummel et al., 2021). A potential increase of glucagon in response to reduced fasting glucose levels could promote lipolysis, while a potential oversupply of FFA in peripheral tissues could subsequently promote their oxidation (Galsgaard et al., 2019). Alternatively, glucagon could be involved in the shift of FFA to gluconeogenesis or hepatic ß-oxidation (see 3.1) (Galsgaard et al., 2019). Though, neither fasting values of FFA, nor those of glucagon were modified in empagliflozin-treated persons in our trial (Kullmann et al., 2021). They are therefore most likely not involved in the observed increased rates of fat oxidation upon empagliflozin treatment. According to the literature, insulin as well as whole-body insulin sensitivity could explain changes in substrate use (Kelley and Mandarino, 2000; Thiebaud et al., 1982). However, both were not changed by our empagliflozin treatment (Kullmann et al., 2021), arguing against a possible contribution to the switch of the utilized fuel.

Multifaceted health benefits of SGLT2i emerged over the last years (Bonora et al., 2020; Zelniker et al., 2019). Beyond glucose control, pleiotropic effects on cardiovascular and renal health were uncovered in large clinical trials (Wanner et al., 2016; Zinman et al., 2015). The underlying mechanisms are still unclear. Discussed mechanisms include effects on diuresis as well as the enhanced lipolysis and ketogenesis in response to SGLT2 inhibition (Verma and McMurray, 2018). Ketone bodies are superior to FFA in regard to substrate efficiency (Mudaliar et al., 2016). However, ß-hydroxybutyrate levels, representing the quantitively primary ketone body, were not affected by empagliflozin in the trial of this thesis (Kullmann et al., 2021). As revealed in the first manuscript of this thesis (see. 2.1 and 3.1), high levels of ß-hydroxybutyrate correspond to predominant oxidation of fat (Hummel et al., 2021). Consequently, ketones seem not to contribute to the drop in RQ seen after empagliflozin treatment in the current trial.

A previously upcoming hypothesis stated, that SGLT2 inhibition, known to elicit a state of mild caloric restriction due to increased glucose excretion, could favor restoration of diurnal rhythm and therefore exert its diverse health benefits (Esterline et al., 2018). Both,

in persons with obesity as well as insulin-resistance, the adaption of substrate oxidation to fasting and feeding is believed to be disrupted (Smith et al., 2018). Recently, it was reported that SGLT2i treatment led to more pronounced fat oxidation during daytime as well as nighttime with a larger day-to-nighttime RQ difference (Op den Kamp et al., 2021). The authors concluded an improved metabolic transition from the fed to the fasted state, which could favor the restoration of the diurnal metabolic rhythm (Op den Kamp et al., 2021).

Evidence from studies inducing caloric restriction indicate benefits for cardiovascular and metabolic health (Cioffi et al., 2018; Patterson and Sears, 2017). Moreover, periods of energy deficit shifted substrate oxidation to fat use (Most and Redman, 2020) and revealed ameliorated metabolic flexibility (Johnson et al., 2016). In conclusion, beneficial effects of SGLT2i may be conveyed via an activation of several molecular pathways induced by energy shortage, which are crucial to preserve intact cellular function and are linked to healthy metabolism (Esterline et al., 2018; Op den Kamp et al., 2021). Of note, glucose loss was not yet compensated by a change in total caloric intake or macronutrient composition in the presented trial (Kullmann et al., 2021). This leads to the assumption that a possible mild calorie deficit was apparent, which however was too small to induce significant weight reductions. The relatively short study duration of 8 weeks made it possible to detect metabolic benefits independent of weight loss.

Since trials on SGLT2 inhibition in prediabetes are scarce, we compared our results to findings from overt T2DM. Following intake of the SGLT2i dapagliflozin, fat utilization was upregulated in the fasted state as well as during nighttime (Daniele et al., 2016; Ferrannini et al., 2014; Op den Kamp et al., 2021). In terms of substrate oxidation, SGLT2 inhibition revealed consistent results in T2DM and in persons with prediabetes analyzed in this work. However, these studies differ in respect to weight reduction, as well as in effects on fasting FFA, fasting insulin and insulin sensitivity outcomes (Daniele et al., 2016; Ferrannini et al., 2014; Op den Kamp et al., 2021), suggesting further factors to be involved in the mediation of SGLT2i effects on substrate use. Furthermore, SGLT2i may have direct cellular effects interfering with lipid metabolism. Results from rodents make a mechanistic link between SGLT2 inhibition and substrate oxidation possible. The SGLT2 inhibitor canagliflozin was shown to upregulate the peroxisome proliferator-

activated receptor-alpha (PPAR-α) in an obese animal model (Wei et al., 2020). As a nuclear receptor protein, PPAR-α acts as transcription factor regulating the expression of several genes involved in hepatic lipid metabolism (Bocher et al., 2006). Its central role in the upregulation of genes that control ketogenesis and ß-oxidation could explain a decreased RQ after SGLT2 inhibition (Ji et al., 2017). In addition, SGLT2i empagliflozin enhanced the activation of AMP-activated protein kinase carboxylase (AMPK) in skeletal muscle and elevated the levels of fibroblast growth factor-21 (FGF-21) in liver and plasma of obese mice (Xu et al., 2017). FGF-21 was characterized as hepatokine that regulates lipolysis in white adipose tissue (Inagaki et al., 2007) and promotes hepatic ß-oxidation (Badman et al., 2007). The enzyme AMPK triggers catabolic pathways in order to preserve energy, therefore shifting fatty acids from lipid synthesis to ß-oxidation (Hardie, 2008; Ruderman et al., 2003; Velasco et al., 1997). These molecular mechanisms may represent a direct link between SGLT2 inhibition and enhanced FFA oxidation. Besides SGLT2i, metformin represents a further anti-diabetic drug with the potential to modulate substrate oxidation (Malin and Kashyap, 2014). Although not investigated in detail so far, mechanisms of metformin action interfere with the mitochondrial respiratory chain and likewise to SGLT2i activate AMPK (Zhang et al., 2009). Despite this stated mechanistic rationale, data from human studies are conflicting. Some trials reported no change in (fasting) fuel utilization in T2DM (Avignon et al., 2000; Gormsen et al., 2018; Stumvoll et al., 1995) and healthy persons (Fruehwald-Schultes et al., 2002), while others observed an enhanced fat utilization in healthy individuals and T2DM (Tokubuchi et al., 2017).

Beside SGLT2i and metformin, further potential modulators of fuel partitioning are found within other anti-diabetics e.g. GLP-1 RA. The GLP-1 RA liraglutide was reported to decrease 24 h substrate use (van Can et al., 2014), while no change in fasting RQ could be detected in response to semaglutide in persons with obesity (Blundell et al., 2017). Upcoming pharmacological approaches even target several receptors in parallel. First data suggest promising results for the treatment of metabolic diseases (Ambery et al., 2018). For example, GLP-1/glucagon dual receptor agonist shifted nutrient partitioning to preferential fat oxidation in preclinical models (Patel et al., 2014; Pocai et al., 2009). This alteration was accompanied by weight loss and reductions in hepatic liver content (Pocai et al., 2009).

The shift towards fatty acid oxidation likely contributes to favorable effects on liver fat accumulation. Data from preclinical models are consistent with those of clinical trials describing a reduction of intrahepatic lipids in response to SGLT2i treatment in T2DM (Raj et al., 2019). Evidence from *in vitro* studies analyzing human adipose tissue biopsies elucidated improvements in insulin resistance and lipid deposition in response to an experimentally-induced shift towards enhanced fat utilization in e.g. adipocytes (Malandrino et al., 2015). Since there was no beneficial effect on whole-body insulin sensitivity after 8 weeks of empagliflozin treatment in our current trial, improvements in liver fat seem to be independent of insulin action and weight reductions in the current work (Kullmann et al., 2021). Due to the link between ectopic fat and excess body weight (Verkouter et al., 2019), a decrease of BMI typically has favorable effects on lipid deposition (Dubé et al., 2011). However, data of this thesis revealed no change in BMI after 8 weeks of empagliflozin intake (Kullmann et al., 2021). This is in line with an article of Kuchay et al., where liver fat reduction upon empagliflozin administration was independent of changes in body weight in T2DM (Kuchay et al., 2018), excluding body weight reduction as main driver of liver fat loss in this context. Moreover, preliminary evidence from rodents indicate intracellular lipid accumulation by inhibiting ß-oxidation *in vivo* (Dobbins et al., 2001; Ravussin and Smith, 2006; Yokono et al., 2014). This observation places the choice of oxidized substrates as potential mediator for the reduction of excessive fat deposition. How this could have affected pancreatic fat content in our trial is discussed in the following section.

3.3 Pharmacologic modulated fasting substrate oxidation: implications on intrapancreatic fat content and insulin secretion

The importance of ectopic fat in metabolic deteriorations is further emphasized by findings from the DiRECT trial (Taylor et al., 2018). In this study, diabetes remission was achieved when ß-cell function recovered. This process depends on reductions in intrahepatic as well as pancreatic fat (Taylor et al., 2018). As stated above, SGLT2i treatment typically lowers body fat mass as well as ectopic fat accumulation (Ferrannini, 2010; Schork et al., 2019; Shao et al., 2020). This holds true especially for intrahepatic fat in persons with T2DM (Shao et al., 2020), while the effect on pancreatic fat has not been studied yet. We hypothesized that the observed shift from carbohydrate to fat

oxidation may also have the potential to mobilize lipids from this fat depot. Indeed, findings from an *in vitro* study support this hypothesis. Experimentally promoted fat oxidation demonstrated benefits for insulin sensitivity and lipid deposition in human adipocytes (Malandrino et al., 2015), which is of relevance since adipocytes represent the major location of fat within the pancreas (Wagner et al., 2021a). Moreover, preliminary evidence from rodents indicate a reduced fat mass by shifting energy utilization from carbohydrates towards fatty acids under SGLT2 inhibition (Yokono et al., 2014), placing the choice of substrates as potential mediator for the reduction of excessive fat deposition.

Data of the current work indicate that the tested intake of empagliflozin for 8 weeks was not potent enough to achieve pancreatic fat reduction (Hummel et al., unpublished data, submitted, march 2022). In accordance, a previously published trial in T2DM (n=22) reported no change in pancreatic fat upon any of the five tested SGLT2i treatments (Horii et al., 2021). However, a subgroup of 11 patients with extensive fat accumulation in the pancreas reduced their pancreatic fat content after SGLT2 inhibition (Horii et al., 2021; Kim et al., 2014). Despite the participants reduced their body weight after SGLT2i treatment, the observed weight reduction did not correlate with the loss of pancreatic fat in the subgroup of patients with fatty pancreas (Horii et al., 2021). Though, the study design was retrospective and pancreatic fat was assessed by abdominal computed tomography scans, which solely exert moderate validity (Horii et al., 2021; Wagner et al., 2021a). Further anti-diabetic drugs were tested to target intrapancreatic lipids in T2DM. In agreement with the results of SGLT2i in our current work, administration of GLP-1 RA did not affect fat accumulation in the pancreas in several trials, despite all these trials reported significant but mild weight loss (Dutour et al., 2016; Kuchay et al., 2020; Vanderheiden et al., 2016). In accordance with results of this thesis on SGLT2i, Vanderheiden et al. detected a liver fat reduction upon GLP-1 RA treatment, without changes in pancreatic fat (Vanderheiden et al., 2016). These findings were confirmed by data from bariatric surgery, where fat reduction in the pancreas was independent of liver fat loss (Gaborit et al., 2015), arguing for a tissue-specific regulation of ectopic fat storage. This leads to the assumption of a differentially influenced substrate oxidation in different tissues.

Prior reports observed a link between pancreatic fat and reduced insulin secretion, especially in prediabetes (Heni et al., 2010; Yokota et al., 2012). In disagreement with the results of this thesis, one trail reported improved ß-cell function subsequent to a 2-weeks empagliflozin intervention in persons with impaired fasting glucose (Abdul-Ghani et al., 2017). This trial quantified insulin secretion by highly-controlled hyperglycemic clamp technique (Abdul-Ghani et al., 2017) that might have higher sensitivity than an OGTT, which was applied in our trial. Thus, the applied method for quantifying insulin secretion in this thesis might have not detected smaller changes. Though, the authors of the hyperglycemic clamp trial did not measure pancreatic fat and their results were limited by a small sample size (n=16) and a missing control group (Abdul-Ghani et al., 2017).

While not reported for pharmacologic approaches so far, there are reports of improved ß-cell function after pancreatic fat loss in response to bariatric surgery (Gaborit et al., 2015; Honka et al., 2015a), exercise (Heiskanen et al., 2018b; Solomon et al., 2013) or low calorie diets (Lim et al., 2011; Steven et al., 2016; Wagner et al., 2021a). Since Heiskanen and colleagues especially highlighted pancreatic fat reductions in persons with high lipid content in the pancreas at baseline (>6.2%) (Heiskanen et al., 2018b), participants of this thesis might had too little fat in pancreatic parenchyma to achieve major effects (Hummel et al., unpublished data, submitted, march 2022). However, a separate analysis with a cut-off point of 6.2% might be underpowered due to the sample size of 40. Even though, the mean pancreatic fat content in our trial was comparable to that in participants of Lim et al. with T2DM (Lim et al., 2011). In these, marked weight reduction and glycemic improvements were achieved in addition to decreased pancreatic fat content (Hummel et al., unpublished data, submitted, march 2022; Lim et al., 2011). While this suggests a role of weight loss in pancreatic fat reduction, another trial reported decreased pancreatic fat despite stable body weight (Heiskanen et al., 2018b). Interestingly, this decline of pancreatic fat was regardless of baseline glucose metabolism and ranged from healthy over prediabetic to diabetic states (Heiskanen et al., 2018b). Contradictory, weight loss due to bariatric surgery was solely accompanied by reductions in intrapancreatic lipids in patients with T2DM, while pancreatic fat content remained the same in persons with normal glucose tolerance although they lost a similar amount of body fat (Steven et al., 2016). These results point towards metabolic conditions as key factor for pancreatic fat reduction rather than sole weight loss.

Though, data are inconsistent presumably due to diverse study populations and applied experimental approaches. Most important, the lack of studies analyzing pancreatic fat hinders to gain a complete picture which warrants further investigations of this fat compartment.

3.4 Strengths, limitations and future directions of the research concept

The analysis of potential determinants of fasting substrate oxidation is limited by the cross-sectional correlative design. This approach can only be hypothesis generating but can never prove causality. Though, the large sample size of 192 subjects and the rigorous phenotyping with highly controlled pre-analytic and analytic conditions strengthens the scientific evidence of the findings. The heterogenous study population ranging from lean, healthy to obese persons with glucose intolerance allows to examine substrate oxidation independent of metabolic condition in a real-world setting. This population enables to study the link between substrate oxidation and different glycemic categories and provides the opportunity for a separate analysis stratified for weight groups (see appendix suppl. mat. 1st publication). The results concerning T2DM have to be considered with caution due to the small sample size (n=10). Moreover, this sub-group of persons with T2DM is rather specific, since they were newly diagnosed with T2DM and therefore were treatment naïve, which limits the comparability with patients with longtime T2DM.

The focus of this thesis was to untangle fasting substrate oxidation in unstimulated conditions. Next, it would be of interest to assess links of fasting RQ and 24h RQ and their shared determinants. A further limitation is the absence of longitudinal data, which has the potential to foster the understanding of the link between pathophysiologic deteriorations of e.g. glycemia and alterations of the utilized fuel.

Beside analyzing the large cohort with 192 subjects, proglucagon cleavage products were solely assessed in a smaller subgroup of 38 persons with prediabetes. Therefore, careful interpretation is necessary and the results of the thesis concerning proglucagon cleavage products have to be confirmed in larger cohorts and ideally in randomized controlled trials. Nevertheless, to my knowledge this analysis is one of the first uncovering a relationship between fasting GIP and glicentin to fasting substrate oxidation in persons with prediabetes. A further point to be considered is that glicentin and GLP-1 are thought

to be secreted in equal amounts, but solely glicentin was related to fuel utilization. This might be due to the rather limited sample size, which may has hindered detection of smaller effects. Considering the molecular similarity of the proglucagon cleavage products, the quantification is technically challenging. The availability of a recently commercialized ELISA kit allowed us their quantification with high specificity. The manufacturer reports no cross-reactivity between the proglucagon products (Manell et al., 2016; Perakakis and Mantzoros, 2020). Moreover, all measurements of proglucagon-cleavage products within this work were inside the assay range of the respective immunoassay, i.e. above the lowest calibrator.

The strength of this thesis lies in the choice of a randomized, controlled, double-blind study design to test pharmacologic modulation of RQ as well as implications on pancreatic fat and insulin secretion that has the strongest empirical evidence. Considered as the gold standard in clinical research, the prospective design allows statements about causality (Hariton and Locascio, 2018). Moreover, blinding of participants as well as study personnel minimizes a potential performance and assessment bias. The study was performed in a real-world scenario with concomitant medication and comorbidities, frequently seen in this group of persons. However, chronic diseases (e.g. cancer, cardiovascular diseases, liver disease, impaired kidney function), which may affect the results were excluded. Since solely persons with overweight or obesity and prediabetes were studied, the findings exert compromised generalizability to the overall population due to the applied eligibility criteria. Moreover, the study design does not allow to distinguish between the effects in persons with solely excess fat mass or solely hyperglycemia and their separate contribution to changes in RQ. Further research should uncover, whether the effects of SGLT2 inhibition on substrate preference are independent of blood glucose and excess weight. Importantly, the potential and detriments of this approach as preventive strategy to impede metabolic deteriorations should be deciphered in future studies.

The random allocation to treatment groups resulted in an uneven sex distribution between groups, as stratification was not performed in a sex-dependent fashion. This might have affected the results and therefore represents a limitation of this work.

Performance of indirect calorimetry provides information about whole-body substrate oxidation. However, this technique does not allow to distinguish between substrate use of different tissues e.g. liver, muscle or adipose tissue. Collection of muscle and adipose tissue biopsies could foster the understanding of tissue partitioning to empagliflozin-induced changes in the choice of oxidized substrate. Despite, too comprehensive for the frame of a thesis, the understanding of fluctuations of substrate use over 24h or during postprandial conditions using a combination of clamp and tracer infusion technique could uncover the changes in metabolism, contributing to altered substrate oxidation.

Notably, the presented work is not able to clarify, if the observed change in substrate oxidation contributed to the loss of liver fat. On a more artificial level, assessment of lipolysis during the post-absorptive phase or hepatic and pancreatic fatty acid uptake would have been possible by applying e.g. a tracer dilution technique. Collection of liver biopsies would provide the opportunity to study hepatic substrate oxidation. Though, this method is impeded by safety and ethical concerns in humans and is therefore more appropriate for animal models. To elucidate the detailed metabolic pathways of ectopic fat loss, further research is needed. Assessment of the expression and activity of key molecules in main metabolic tissues, which are relevant for energy metabolism especially for glucose and fatty acid oxidation are of relevance to clarify this research question.

Since pancreatic fat is likely increased in T2DM (Wagner et al., 2021a), additional studies could test whether the effect of SGLT2i on intrapancreatic lipids is more potent in a diabetic population with longer treatment duration. Despite pancreatic fat content was quantified by MRI, which is recognized as the most reliable technique for pancreatic fat assessment (Wagner et al., 2021a), data interpretation is restricted due to the absence of a generally accepted threshold of pancreatic steatosis. Representing a minor limitation of the study concept, the assessment of insulin sensitivity and secretion was not performed by means of glucose clamp technique, that represents the gold standard and would have been more sensitive to smaller effects. However, standardized performance of OGTT technique provides a good estimate for whole-body insulin sensitivity and secretion with high reproducibility of this OGTT-derived measures (Hudak et al., 2021). In addition, the performance of OGTT is more feasible, less resource consuming and much more convenient for the study participants.

3.5 Conclusions

As demonstrated in the presented work, FFA, GIP and glicentin are potentially involved in cellular fuel selection in humans. Especially the uncovered link between GIP and glicentin with the preferred oxidized substrate put the spotlight on upcoming pharmacological approaches targeting incretins. Emphasizing the relevance of our findings, preclinical studies that targeted GIP signaling suggest benefits for glucose metabolism and body weight. The therapeutic value of GLP-1R/GIPR co-agonists is already evaluated in clinical studies. To clarify the clinical value of GIP and glicentin to modulate substrate oxidation, further clinical trials are needed to assess whether potential changes in substrate oxidation contribute to the seen benefits for whole-body metabolism and weight loss.

This work provides novel findings on the effect of SGLT2i treatment on fasting substrate oxidation in persons with prediabetes. The results suggest that the benefits of SGLT2 inhibition also include increased fat oxidation which may foster the reduction in excessive liver fat content. The choice of the mainly oxidized substrate in persons with prediabetes represents a promising target for the prevention of metabolic diseases. The SGLT2 inhibition in our trial might have hindered changes in substrate oxidation. As a result, cellular dysfunction and cellular fat accumulation would be prevented. Our current findings suggest that the positive metabolic effects of SGLT2i were independent of pancreatic fat and insulin secretion. The empagliflozin-induced change in whole-body fasting substrate oxidation did likely not affect pancreatic lipids. This indicates tissue-specific mechanisms for ectopic fat loss. Therefore, assessing tissue-specific alterations of fuel utilization is of great interest for future research. Nevertheless, pancreatic fat and insulin secretion still remain interesting candidates for the prevention of diabetes, as underscored by the DiRECT trial, where diabetes remission was achieved through hypocaloric diet in persons who reduced pancreatic fat (Taylor et al., 2018).

In future projects, different drugs or lifestyle interventions should be tested as novel modulators of substrate oxidation and ectopic fat with the goal of preventing or even treating metabolic diseases. Moreover, one important topic for future studies should be potential differences between sub-groups. Indeed, recent research stratified prediabetes (Wagner et al., 2021c) and diabetes (Ahlqvist et al., 2018) based on their different

pathophysiological phenotypes. It is likely that these sub-phenotypes are also different in substrate oxidation, which needs to be clarified in subsequent analyses. The ultimate goal of this line of work is the development of tailored treatment regimens on the way towards precision medicine in prediabetes and diabetes.

4 Summary

4.1 English summary

Alterations in substrate oxidation are a potential contributor to the pathogenesis of metabolic diseases as they might foster ectopic lipid accumulation. Longitudinal studies identified predominant carbohydrate oxidation to predispose for subsequent weight gain. However, determinants of fasting substrate oxidation remain elusive and evidence for substrate oxidation as a potential pharmacologic target is lacking.

To investigate alterations in energy metabolism that can be involved in the pathogenesis of metabolic diseases, this research project aimed to elucidate determinants of fasting substrate oxidation. This thesis furthermore tested whether the latter can be pharmacologically modulated by the sodium glucose cotransporter 2 inhibitor (SGLT2i) empagliflozin to identify therapeutic approaches for altered fuel use. Moreover, implications of modulated fasting substrate oxidation on intrapancreatic fat and insulin secretion were evaluated with the aim to detect potential approaches for the prevention of type 2 diabetes (T2DM). These research questions were addressed in three publications embedded in this thesis.

In order to elucidate determining factors of substrate oxidation in the fasted state, a cross-sectional analysis was performed, including 192 individuals with a wide range of BMI as well as different glycemic categories. Following the assessment of fasting respiratory quotient ($RQ=VCO_2/VO_2$) by indirect calorimetry as a measure of substrate oxidation, participants underwent a 5-point 75 g oral glucose tolerance test (OGTT). The latter allows to estimate insulin sensitivity and determine the glycemic status beside quantification of several clinical parameters from basal blood. In the fasting state, high free fatty acid (FFA) concentrations were strongly linked to a low RQ, indicative of predominant fat oxidation. Participants with high levels of the ketone body β-hydroxybutyric acid had significantly lower RQ values, while glucose and insulin levels were not correlated to RQ. Unlike glucagon-like-peptide 1 (GLP-1), fasting levels of glucose-dependent insulinotropic polypeptide (GIP) and glicentin associated positively with fasting RQ. There was neither a correlation between BMI nor the total amount or the

allocation of body fat compartments with fasting RQ. Hyperglycemia, insulin sensitivity or the metabolic syndrome were not related to RQ.

In a double-blind, placebo-controlled randomized trial, 40 participants with prediabetes were treated with empagliflozin or placebo once daily for 8 weeks. Subsequent to overnight fasting, indirect calorimetry as well as a 75 g OGTT were performed before and after treatment. Body fat compartments including lipids in the liver as well as intrapancreatic fat were quantified using magnetic resonance imaging (MRI). A combination of intranasal insulin application with functional MRI was used to determine hypothalamic insulin sensitivity. Empagliflozin reduced fasting RQ, lowered fasting glucose as well as liver fat content and increased hypothalamic insulin sensitivity. Pancreatic fat content as well as insulin secretion remained unaffected upon empagliflozin treatment.

Data of this work support the role of FFA as independent determinants of fuel selection, while metabolic disorders were not linked to substrate preferences. This work gained hints for glicentin and GIP to be involved in fuel choice in the fasting state, representing a promising pharmaceutic target to modulate substrate oxidation with possible implications on whole-body metabolism. Upcoming therapeutic approaches in the preclinical as well as clinical phase targeting GIP receptor highlight the relevance of our results.

Beside the empagliflozin-induced switch towards predominant fat use, intrahepatic lipids were diminished upon SGLT2 inhibition. This demonstrates the potential of pharmacologic modulation of substrate utilization. Since we detected such changes already in prediabetes, substrate oxidation is an interesting target for the development of preventive strategies of metabolic diseases as T2DM. Discussed underlying mechanisms for the empagliflozin-induced change in substrate oxidation are a restoration of diurnal rhythm by caloric restriction, centrally mediated effects and direct cellular effects of SGLT2i interfering with lipid metabolism. Beside SGLT2i, further antidiabetic drugs such as GLP-1 receptor agonists and metformin may have the potential to modulate substrate oxidation. Therefore, approaches to modulate substrate oxidation should be further evaluated and optimized in future studies. Since the observed increase in fat oxidation was neither accompanied by reductions in pancreatic fat nor by enhanced

insulin secretion, this work argues for tissue-specific regulation in substrate oxidation and fat mobilization which should be untangled in future studies. It is however possible that prolongation of treatment duration or a population with higher pancreatic fat content might have led to different results. Since lifestyle intervention trials achieved pancreatic fat reduction, approaches to reduce fat in the pancreas remain a promising strategy to improve insulin secretion for the prevention or therapy of T2DM. Future research should put a spotlight on tailored medicine and find out which sub-groups of persons with prediabetes and T2DM could especially profit from such therapies that target substrate oxidation and reduce ectopic fat deposition.

4.2 German summary - Zusammenfassung

Veränderungen in der Substratoxidation sind ein potenzieller Faktor in der Pathogenese metabolischer Erkrankungen. Hierbei könnte die ektope Akkumulation von Fett eine wichtige Rolle spielen. In Längsschnittstudien wurde eine vorherrschende Kohlenhydrat-Oxidation als Prädiktor für eine spätere Gewichtszunahme identifiziert. Faktoren, die die basale Substratoxidation bestimmen sind jedoch bisher weitgehend unklar. Ob sich die Substratoxidation als potentielles Ziel für pharmakologische Ansätze eignet ist zudem nicht geklärt.

Um das Wissen über Veränderungen im Energiestoffwechsel bei der Entstehung von Stoffwechselerkrankungen zu verbessern, wurden in dieser Arbeit die Determinanten der Nüchtern-Substratoxidation analysiert. Darüber hinaus wurde untersucht, ob diese durch den SGLT2-Hemmer (Natrium Glukose Cotransporter 2) Empagliflozin pharmakologisch moduliert werden kann, um so therapeutische Ansätze zur Modulation der Substratoxidation zu identifizieren. Darüber hinaus wurden die Auswirkungen der veränderten Nüchtern-Substratoxidation auf das Pankreasfett und die Insulinsekretion untersucht, um mögliche Ansätze zur Prävention von Typ 2 Diabetes (T2DM) zu finden. Diese Forschungsfragen wurden in drei Veröffentlichungen adressiert, die Bestandteil dieser Arbeit sind.

Es wurde eine Querschnittsanalyse durchgeführt, um Determinanten der Substratoxidation im Nüchternzustand zu untersuchen. Hierzu wurden Daten von 192 Personen mit einem breiten BMI-Spektrum sowie verschiedenen glykämischen

Kategorien untersucht. Nach der Bestimmung des Respiratorischen Quotienten ($RQ=VCO_2/VO_2$) mittels indirekter Kalorimetrie, als Maß für die Substratoxidation, wurden die Teilnehmer einem 5-Punkt 75 g oralen Glukosetoleranztest (OGTT) unterzogen. Letzterer ermöglicht neben der Quantifizierung verschiedener klinischer Parameter, die Einschätzung der Insulinsensitivität und die Bestimmung der Glukosetoleranz. Im Nüchternzustand waren hohe Konzentrationen an freien Fettsäuren (FFA) stark mit einem niedrigen RQ assoziiert, was auf eine vorherrschende Fettoxidation hinweist. Personen mit hohen Spiegeln des Ketonkörpers β-Hydroxybuttersäure hatten signifikant niedrigere RQ-Werte, während Glukose- und Insulinspiegel nicht mit dem RQ korrelierten. Im Gegensatz zu GLP-1 korrelierten Glukoseabhängiges Insulinotropes Polypeptid (GIP) und Glicentin positiv mit dem RQ im Nüchternzustand. Es gab weder eine Korrelation zwischen dem BMI noch der Gesamt-Fettmasse oder der Verteilung der Körperfettkompartimente mit dem Nüchtern-RQ. Hyperglykämie, Insulinsensitivität oder das Metabolische Syndrom standen nicht mit dem RQ in Zusammenhang.

In einer weiteren Untersuchung, die als doppel-blinde, Placebo-kontrollierte, randomisierte Studie angelegt war, erhielten 40 Personen mit Prädiabetes für 8 Wochen einmal täglich Empagliflozin oder Placebo. Vor und nach der Behandlung wurde eine indirekte Kalorimetrie sowie ein 75 g OGTT durchgeführt. Die Körperfettkompartimente einschließlich Leberfett und Pankreasfett wurden mit Hilfe von Magnetresonanztomographie (MRT) quantifiziert. Eine Kombination aus intranasaler Insulinapplikation und funktionellem MRT wurde zur Bestimmung der hypothalamischen Insulinsensitivität eingesetzt. Empagliflozin reduzierte den Nüchtern-RQ, senkte den Nüchtern-Glukosespiegel sowie den Leberfettgehalt und erhöhte die hypothalamische Insulinempfindlichkeit. Der Fettgehalt des Pankreas sowie die Insulinsekretion wurden durch die Behandlung mit Empagliflozin nicht beeinflusst.

Die Daten dieser Arbeit stützen die Rolle von FFA als unabhängige Determinanten, während Stoffwechselstörungen nicht mit Substratpräferenzen in Verbindung standen. Diese Arbeit liefert Hinweise darauf, dass Glicentin und GIP an der Auswahl des Energiesubstrats im Nüchternzustand beteiligt sind, was ein vielversprechendes pharmazeutisches Ziel darstellt, um die Substratoxidation mit möglichen Vorteilen für

den Ganzkörperstoffwechsel zu modulieren. Neue therapeutische Ansätze, die auf den GIP-Rezeptor abzielen, sind in der präklinischen sowie klinischen Phase und unterstreichen die Relevanz unserer Ergebnisse.

Neben der durch Empagliflozin induzierten Umstellung auf eine überwiegende Fettverwertung wurden der Leberfettgehalt durch die SGLT2-Hemmung verringert, was das klinische Potenzial einer pharmakologischen Modulation der Substratoxidation unterstreicht. Da die Veränderungen in unserer Studie bereits bei Prädiabetes beobachtet wurden, ist die Substratoxidation ein interessantes Ziel für die Entwicklung von Präventionsstrategien für metabolische Erkrankungen wie T2DM. Diskutierte zugrunde liegende Mechanismen für die Veränderung der Substratoxidation durch Empagliflozin sind eine Wiederherstellung des Tagesrhythmus durch Kalorienrestriktion, zentral vermittelte Wirkungen und direkte zelluläre Wirkungen von SGLT2-Hemmung, die in den Lipidstoffwechsel eingreifen. Neben SGLT2-Hemmern wurde festgestellt, dass weitere Antidiabetika wie GLP-1-Rezeptoragonisten und Metformin möglicherweise die Substratoxidation modulieren. Daher sollten in zukünftigen Studien Ansätze zur Modulation der Substratoxidation weiter getestet und optimiert werden. Da die beobachtete Zunahme der Fettoxidation weder mit einer Verringerung des Pankreasfetts noch mit einer erhöhten Insulinsekretion einherging, spricht diese Arbeit für gewebespezifische Regulationsmechanismen der Substratoxidation und Fettmobilisierung, die in zukünftigen Studien untersucht werden sollten. Trotzdem ist es möglich, dass eine Verlängerung der Behandlungsdauer oder eine Studienpopulation mit höherem Pankreasfettgehalt zu anderen Ergebnissen geführt hätte. Da Lebensstil-Interventionsstudien eine Reduktion des Pankreasfettes erreicht haben, bleiben Ansätze zur Reduktion von Fett im Pankreas eine vielversprechende Strategie zur Verbesserung der Insulinsekretion in Bezug auf die Prävention oder Therapie von T2DM. Zukünftige Forschung sollte einen Fokus auf individualisierte Medizin richten und herausfinden, welche Subgruppen von Personen mit Prädiabetes und T2DM besonders von Therapien zur Modulation von Substratoxidation und Fettreduktion in ektopen Geweben profitieren könnten.

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Declaration

Herewith I, Julia Hummel, declare, that I wrote the thesis presented for the degree of "Dr. sc. hum.", which is entitled

„Determinants and pharmacologic modulation of fasting substrate oxidation in humans"

by myself (without external help), and that I did not use any sources other than those I have indicated. I also assure that this thesis has not been submitted for any other doctoral procedure or degree and that this doctoral procedure was not preceded by any finally failed doctoral procedures. I swear upon oath that these statements are true and that I have not concealed anything, and that I have made them to the best of my knowledge and belief. My contribution and those of the other authors to the publications which are part of this thesis have been explicitly indicated below.

Tübingen, the 02.06.2023

Date *Signature (Julia Hummel)*

Contributions

This work was carried out in the University Hospital of Tübingen, Medical Clinic IV at the Clinical Study Center of Diabetology under the supervision of Prof. Dr. med. Martin Heni.

I played a major role in the preparation and execution of the experiments, and the data analysis, presentation and interpretation are by own work as specified below. The following section lists the contributions to all included publications. This list is equal to the author contributions listed within each article. All co-authors agreed to the below listed itemization of contributions (see appendix).

Chapter 2.1

1st Publication: "Free fatty acids, glicentin and glucose-dependent insulinotropic polypeptide as potential major determinants of fasting substrate oxidation."

Julia Hummel, Louise Fritsche, Andreas Vosseler, Corinna Dannecker, Miriam Hoene, Konstantinos Kantartzis, Hans-Ulrich Häring, Norbert Stefan, Jürgen Machann, Andreas L. Birkenfeld, Cora Weigert, Robert Wagner, Andreas Peter, Andreas Fritsche, Martin Heni

Published in Scientific Reports

I developed the research concept of this study with the support of Prof. Martin Heni. The studies underlying this work were designed by Prof. Martin Heni, Prof. Hans-Ulrich Häring, Prof. Norbert Stefan and Prof. Andreas Fritsche. Dr. Corinna Dannecker supported the submission as well as the coordination of the studies.

I was supported by PD Konstantinos Kantartzis (physical examinations and medical care of participants) as well as by study nurses in the care of participants during the experiments (anthropometric measurements, blood extractions, blood pressure measurements, blood glucose measurements, documentation in the case report forms). The indirect calorimetry measurements were carried out by me in cooperation with Andreas Vosseler and Dr. Corinna Dannecker. I was responsible for the study coordination, participant recruitment as well as data acquisition and management with support of Dr. Louise Fritsche and Dr. Corinna Dannecker. The MRI examinations were

carried out under the direction of PD Jürgen Machann at the department of Radiology at the University of Tübingen, who provided the respective data. The performance and data provision of the biochemical blood analyzes took place under the direction of Prof. Andreas Peter in the department of Diagnostic Laboratory Medicine at the University Hospital of Tübingen.

I carried out the statistical evaluation after consultation with Prof. Martin Heni and Prof. Robert Wagner. I also created all presented graphs and tables.

I certify that I have independently written the first draft of the manuscript (following the guidance of Prof. Martin Heni) and that I did not use any sources other than those I have indicated.

Prof. Andreas L. Birkenfeld, Prof. Norbert Stefan, Dr. Louise Fritsche, Prof. Cora Weigert, Prof. Andreas Fritsche, Dr. Miriam Hoene and Prof. Robert Wagner made suggestions for the discussion and proofread the manuscript.

All authors have read the final version of the manuscript and have given their consent for publication. All authors agreed to the contributions to the publications of this thesis (see appendix).

Chapter 2.2

2nd Publication: "Empagliflozin improves insulin sensitivity of the hypothalamus in humans with prediabetes: a randomized, double-blind, placebo-controlled, phase 2 trial."

Stephanie Kullmann*, **Julia Hummel***, Robert Wagner, Corinna Dannecker, Andreas Vosseler, Louise Fritsche, Ralf Veit, Konstantinos Kantartzis, Jürgen Machann, Andreas L. Birkenfeld, Norbert Stefan, Hans-Ulrich Häring, Andreas Peter, Hubert Preissl, Andreas Fritsche, Martin Heni *authors contributed equally

Published in Diabetes Care

The study was designed by Prof. Martin Heni in collaboration with Prof. Hans-Ulrich Häring, Prof. Andreas Fritsche and PD Stephanie Kullmann. Dr. Corinna Dannecker supported the submission as well as coordination of the study.

I was supported by PD Konstantinos Kantartzis (physical examinations and medical care of participants) as well as by study nurses in the performance of study visits of the participants during the experiments (anthropometric measurements, blood extractions, blood pressure measurements, blood glucose measurements, documentation in the case report forms). The indirect calorimetry measurements were carried out by me in cooperation with Andreas Vosseler and Dr. Corinna Dannecker. I took over the recruitment of participants, study coordination as well as data acquisition and data management with the support of Dr. Louise Fritsche and Dr. Corinna Dannecker.

The MRI examinations were carried out under the direction of PD Jürgen Machann at the department of Radiology at the University of Tübingen, who provided the respective data. The performance and data provision of the biochemical blood analyzes took place under the direction of Prof. Andreas Peter in the department of Diagnostic Laboratory Medicine at the University Hospital of Tübingen.

PD Stephanie Kullmann and PD Ralf Veit prepared and made available the functional MRI data under the direction of Prof. Hubert Preißl.

The statistical analysis regarding the anthropometric as well as metabolic data as well as the data of the indirect calorimetry was carried out independently by me after consultation with Prof. Martin Heni and Prof. Robert Wagner. I also created the graphs (Fig. 1A and Fig. 2) and all tables in the manuscript and the supplementary material. The statistical evaluation of the functional MRI data and the creation of Fig. 1A and Fig. 1C-D was carried out by PD Stephanie Kullmann.

I certify that I have independently written the first draft of the manuscript together with PD Stephanie Kullmann (following the guidance of Prof. Dr. Martin Heni) and that I did not use any sources other than those I have indicated. Prof. Andreas L. Birkenfeld, Prof. Norbert Stefan, PD Stephanie Kullmann, Prof. Andreas Fritsche, Prof. Hubert Preissl and Prof. Robert Wagner made suggestions for discussion and edited the manuscript. All authors have read the final version of the manuscript and have given their consent for publication. All authors agreed to the contributions to the publications of this thesis (see appendix).

Chapter 2.3

3rd Publication: "Eight weeks of empagliflozin does not affect pancreatic fat content and insulin secretion in people with prediabetes."

Julia Hummel, Jürgen Machann, Corinna Dannecker, Stephanie Kullmann, Andreas L. Birkenfeld, Hans-Ulrich Häring, Andreas Peter, Andreas Fritsche, Robert Wagner, Martin Heni

At the time of submission of the doctoral thesis: *Submitted to Diabetes, Obesity and Metabolism.*

At the time of printing of the doctoral thesis: *Published in Diabetes Obes Metab. 2022 Aug;24(8):1661-1666. doi: 10.1111/dom.14733. Epub 2022 Jun 6.*

The study was designed by Prof. Martin Heni in collaboration with Prof. Hans-Ulrich Häring and Prof. Andreas Fritsche. Dr. Corinna Dannecker supported the submission as well as coordination of the study.

I was supported by PD Konstantinos Kantartzis (physical examinations and medical care of participants) as well as by study nurses in the performance of study visits of the participants during the experiments. I took over the recruitment of participants, study coordination as well as data acquisition and data management with the support of Dr. Corinna Dannecker. The MRI examinations were carried out under the direction of PD Jürgen Machann at the department of Radiology at the University of Tübingen, who provided the respective data. The performance and data provision of the biochemical blood analyzes took place under the direction of Prof. Andreas Peter in the department of Diagnostic Laboratory Medicine at the University Hospital of Tübingen.

The statistical evaluation and the creation of the graphs were carried out independently by me after consultation with Prof. Martin Heni and Prof. Robert Wagner.

I certify that I have independently written the first draft of the manuscript (following the guidance of Prof. Dr. Martin Heni) and that I have not used any sources other than those I have indicated.

Prof. Andreas L. Birkenfeld, PD Stephanie Kullmann, Prof. Andreas Fritsche and Prof. Robert Wagner made suggestions for discussion and proofread the manuscript.

All authors have read the final version of the manuscript and have given their consent for publication. All authors agreed to the contributions to the publications of this thesis (see appendix).

Tübingen, the 02.06.2023

Date *Signature (Julia Hummel)*

Acknowledgement / Danksagung

Diese Promotionsarbeit konnte erst durch das Mitwirken zahlreicher Personen gelingen, welchen ich hier meinen herzlichsten Dank aussprechen möchte. Mein ganz besonderer Dank gilt dabei meinem Betreuer und Doktorvater **Prof. Dr. med. Martin Heni,** der mir neben einer hervorragenden fachlichen Betreuung seine Begeisterung für die Welt der Wissenschaft vermittelt hat.

Ein lieber Dank geht an meine Mentorin **Dr. rer. nat. Louise Fritsche,** die mich herzlich in Ihr Team aufgenommen hat und mir stets mit Rat und Tat zur Seite stand.

Des Weiteren möchte ich **Prof. Dr. med. Andreas L. Birkenfeld** und **Prof. Dr. med. Hans-Ulrich Häring** meinen Dank für die Möglichkeit in der Inneren Medizin IV meine Promotionsarbeit anzufertigen, vielmals danken. Der konstruktiven Zusammenarbeit und dem stets freundlichen Arbeitsklima in der Abteilung gilt meine große Wertschätzung.

Ein herzlicher Dank geht an **PD Dr. med. Konstantinos Kantartzis, Ines Wagener, Eva-Maria Stehle, Alexandra Eberle, Anja Dessecker, Andreas Vosseler, Susanne Wegner, Dorothee Neuscheler, Henrike Peuker** und **Kerstin Kernn** aus dem Team der „Studienzentrale Diabetologie“, die mich bei den Vorbereitungen, der Probensammlung, der Durchführung der Messungen, der Probandenbetreuung und der Probenaufbereitung tatkräftig unterstützt haben. Für die mithilfe bei der Planung und Organisation der klinischen Studien und Datenaufbereitung möchte ich mich herzlich bei **Vanessa Hartmann, Dr. sc. hum. Corinna Dannecker, Dr. sc. hum. Vera Valenta, Dr. rer. nat. Sabine Eckstein und Elisabeth Schrempf** bedanken. Die Datenaufbereitung der Ernährungsprotokolle hat **Sabine Kümmerle** freundlicherweise übernommen, wofür ich ihr hiermit herzlich danke.

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Bei **Prof. Dr. med. Robert Wagner** und **Prof. Dr. med. Andreas Fritsche** möchte ich mich für die Beteiligung bei der Studienplanung, Hinweise zur Datenauswertung und die hilfreichen Anregungen zur Diskussion herzlich bedanken.

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Für die Durchführung der MRT Messungen bedanke ich mich herzlich bei **Karin Waneck** und **Maike Borutta. PD Dr. sc. hum. Jürgen Machann, PD Dr. rer. nat. Stephanie Kullmann** sowie **Prof. Dr. rer. nat. Hubert Preißl** möchte ich in diesem Zusammenhang neben der Datenaufbereitung und -bereitstellung für die hilfreichen Anregungen zur Diskussion der Ergebnisse vielmals danken.

Ein weiterer Dank gilt **Prof. Dr. rer. nat. Cora Weigert** und **PD Dr. rer. nat. Miriam Hoene** für das Mitwirken an der konstruktiven Diskussion der Ergebnisse.

Meine **Freunde** und mein Partner **Valentin** haben stets für genügend Ablenkung, persönliche Unterstützung und schöne Auszeiten am Wochenende gesorgt – Danke dafür! Natürlich wäre diese Promotionsarbeit nicht ohne die bedingungslose Unterstützung meiner **Familie** möglich gewesen, wofür ich unendlich dankbar bin.

Appendix

Declaration of contributions: 1st Publication (see 2.1)

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Faculty of Medicine

PhD Program in Doctoral Procedures Dr.sc.hum

Declaration of contributions:

Herewith I, Julia Hummel declare, that I have contributed to the major part of the following publication:

"Free fatty acids, glicentin and glucose-dependent insulinotropic polypeptide as potential major determinants of fasting substrate oxidation."

The authors contributed to the publications as indicated in the following table (indicated in %):

	Doctoral Candid.	Author 2	Author 3	Author 4	Author 5	Author 6	Author 7	Author 8	Author 9	Author 10	Author 11	Author 12	Author 13	Author 14	Corr Auth
Contribution	J. Hummel	L.Fritsche	A. Vosseler	C. Dannecker	M. Hoene	K. Kantartzis	H-U. Häring	N. Stefan	J. Machann	A.L. Birkenfeld	C. Weigert	R. Wagner	A. Peter	A. Fritsche	M. H
Research concept	50						5								45
Selection of methods	50								5				5	5	35
Recruitment of patients	60	10	15	15											
Data acquisition: Indirect calorimetry, OGTT, anthropometrics	60		20	10		10									
Data acquisition: Physical examination						90						10			
Data acquisition: Clinical chemistry													100		
Data acquisition: MRI								10	90						
Data analysis	85											5			10
Interpretation of results	45				3					3	3	4		2	40
Preparation of Manuscript	100														
Editing of Manuscript		5			5	5				5		10		5	65

OGTT=oral glucose tolerance test, MRI=magnetic resonance imaging

1

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Faculty of Medicine

Signature of the doctoral candidate:

[redacted]

As supervisor, I agree with the declarations by the candidate:

[redacted]

As corresponding author, I agree with the declarations by the candidate:

[redacted]

As co-authors, we agree to the declarations above:

[redacted]

[Signature Author 2]

[redacted]

[Signature Author 3]

[redacted]

[Signature Author 4]

[redacted]

[Signature Author 5]

[redacted]

[Signature Author 6]

[redacted]

[Signature Author 7]

[redacted]

[Signature Author 8]

[redacted]

[Signature Author 9]

[redacted]

[Signature Author 10]

[redacted]

[Signature Author 11]

[redacted]

[Signature Author 13]

[redacted]

[Signature Author 12]

[redacted]

[Signature Author 14]

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TÜBINGEN

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ribution	S. Kullmann*	J. Hummel*	R. Wagner	C. Dannecker	A. Vosseler	L. Fritsche	R. Veit	K. Kantartzis	J. Machann	AL. Birkenfeld	N. Stefan	H-U. Häring	A. Peter	H. Preissl	A. Fritsche	M. Heni
earch concept	25											5		5		65
ction of methods	30			5					5				5	5	5	45
uitment of nts		55		15	15	15										
acquisition: T, indirect rimetry, ropometrics		55		10	25			10								
acquisition: ctional MRI	50						40							10		
acquisition: MRI									90		10					
acquisition: sical examination			10					90								
acquisition: cal chemistry													100			
analysis: abolic data		90														10
analysis l data	80						15									5
pretation of lts	25	20	4							3				3		45
paration of nuscript	50	50														
ing of Manuscript			5						5	5	5		5	5	5	65

*authors contributed equally, MRI=magnetic resonance imaging, OGTT=oral glucose tolerance test

EBERHARD KARLS
UNIVERSITÄT
TÜBINGEN

Faculty of Medicine

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[redacted] [Signature Author 5]

[redacted] [Signature Author 6]

[redacted] [Signature Author 7]

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[redacted] [Signature Author 10]

[redacted] [Signature Author 11]

[redacted] [Signature Author 12]

[redacted] [Signature Author 13]

[redacted] [Signature Author 14]

[redacted] [Signature Author 15]

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TÜBINGEN

Faculty of Medicine

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Herewith I, Julia Hummel declare, that I have contributed to the major part of the following publication:

"Empagliflozin does not affect pancreatic fat content and insulin secretion in humans with prediabetes."

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	J. Hummel	J. Machann	C. Dannecker	S. Kullmann	A.L. Birkenfeld	H-U. Häring	A. Peter	A. Fritsche	R. Wagner	M. Heni
Research concept				25		5				70
Selection of methods		5	5				5	5		80
Recruitment of patients	60		40							
Data acquisition: OGTT	60		30						10	
Data acquisition: MRI		100								
Data acquisition: clinical chemistry							100			
Data analysis	95									5
Interpretation of results	30								5	65
Preparation of Manuscript	100									
Editing of Manuscript					5		5			90

OGTT=oral glucose tolerance test, MRI=magnetic resonance imaging

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UNIVERSITÄT
TÜBINGEN

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[redacted]

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[redacted]
[Signature Author 2]

[redacted]
[Signature Author 3]

[redacted]
[Signature Author 4]

[redacted]
[Signature Author 5]

[redacted]
[Signature Author 6]

[redacted]
[Signature Author 7]

[redacted]
[Signature Author 8]

[redacted]
[Signature Author 9]

Supplementary Material 1st Publication (see 2.1)

Supplementary material

Free fatty acids, glicentin and glucose-dependent insulinotropic polypeptide as potential major determinants of fasting substrate oxidation

Julia Hummel[1,2], Louise Fritsche[1,2], Andreas Vosseler[1-3], Corinna Dannecker[1,2], Miriam Hoene[4], Konstantinos Kantartzis[1-3], Hans-Ulrich Häring[1-3], Norbert Stefan[1-3], Jürgen Machann[1,2,5], Andreas L. Birkenfeld[1-3], Cora Weigert[1,2,4,] Robert Wagner[1-3], Andreas Peter[1,2,4], Andreas Fritsche[1-3], Martin Heni[1-4]

1. Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center
1. Munich at the University of Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
2. German Center for Diabetes Research (DZD), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
3. Department of Internal Medicine, Division of Diabetology, Endocrinology and Nephrology, Eberhard Karls University Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
4. Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany
5. Department of Radiology, Section on Experimental Radiology, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany

Supplementary table 1: Associations with respiratory quotient (RQ) stratified by weight group.

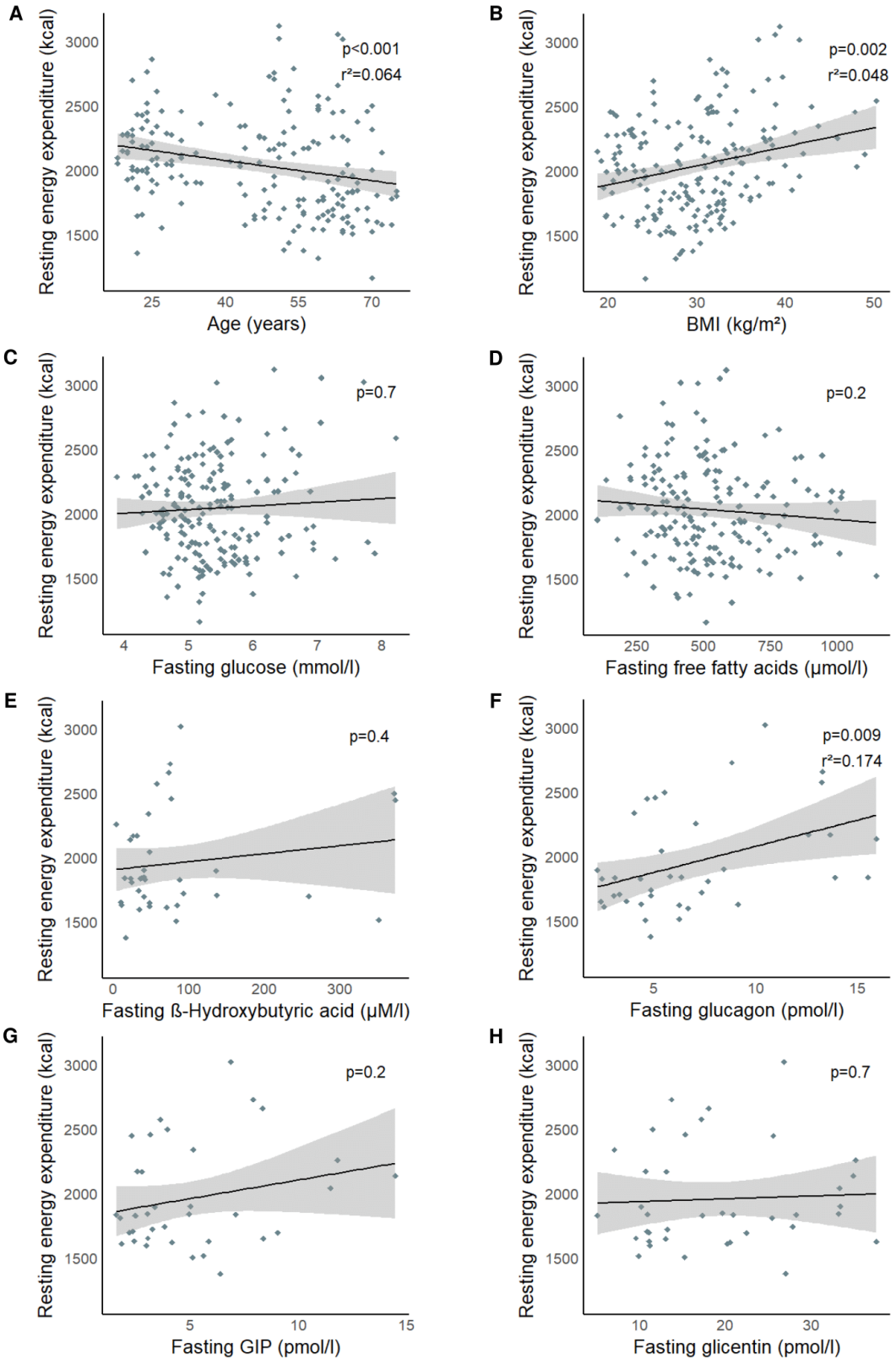
N = 192	**Lean/Overweight** (BMI < 30 kg/m²) N=95			**Obese** (BMI ≥ 30 kg/m²) N=97			
	Median (IQR) / n	**p value** (unadj./adj.*)	**Stand. β** (standard error)	**Median (IQR) / n**	**p value** (unadj./adj.*)	**Stand. β** (standard error)	**p**interaction
Sex		0.2 / 0.5#	-0.078 (0.012)		0.1 / 0.06#	-0.191 (0.010)	0.9
Male	65			62			
Female	30			35			
Age (years)	31 (23 - 61)	0.1 / 0.4##	-0.113 (0.022)	54 (44 - 62)	**0.03** / **0.02**##	-0.245 (0.031)	0.3
Body mass index (kg/m²)	24.6 (21.9 - 27.8)	0.7 / 0.3	0.141 (0.099)	33.6 (32.0 - 37.9)	0.6 / 0.7	0.042 (0.077)	0.5
Blood pressure							
Systolic (mmHg)	132 (125 – 142)[a]	0.2 / 0.1	0.173 (0.101)	140 (133 – 148)	0.07 / 0.1	-0.178 (0.087)	**0.03**
Diastolic (mmHg)	84 (77 – 90)[a]	1.0 / 0.6	0.064 (0.001)	91 (85 – 100)	0.5 / 0.9	-0.016 (0.001)	0.6
Heart rate (bpm)	66 (61 – 75)[a]	0.2 / 0.2	-0.152 (0.059)	72 (65 – 81)	0.7 / 0.7	0.039 (0.060)	0.2
Metabolic syndrome		1.0 / 0.4	-0.096 (0.013)		0.7 / 0.7	0.038 (0.010)	0.8
Yes	19[a]			68[b]			
No	74[a]			26[b]			
Glycemic category		0.7 / 0.2			0.1 / 0.2		0.2
Normal glucose tolerance	66			41			
Prediabetes	28			47			
Diabetes mellitus (newly diagnosed, treatment naïve)	1			9			
Indirect calorimetry							
Fasting respiratory quotient	0.85 (0.79 – 0.90)			0.85 (0.80 – 0.90)			
Resting energy expenditure (kcal)	1925 (1649 – 2211)	0.3 / 1.0	-0.004 (0.073)	2108 (1839 – 2454)[c]	0.9 / **0.03**	-0.310 (0.071)	0.4

Body composition							
Total adipose tissue, MR-derived (l)	23.6 (16.4 – 30.9)[d]	0.8 / 0.9	-0.040 (0.058)	44.5 (41.1 – 55.7)[e]	0.8 / 0.8	0.027 (0.046)	0.7
Subcutaneous adipose tissue lower extremity, MR-derived (l)	9.2 (7.5 – 12.8)[d]	0.9 / 0.9	0.025 (0.057)	16.9 (14.1 – 20.1)[e]	0.9 / 1.0	-0.000 (0.040)	0.9
Visceral adipose tissue, MR-derived (l)	2.1 (1.5 – 3.5)f	0.95 / 0.8	0.049 (0.031)	5.1 (4.2 – 7.4)e	0.7 / 1.0	-0.003 (0.034)	0.8
Intrahepatic fat, MRS-derived (%)	1.4 (0.8 – 3.1)[g]	0.6 / 0.6	0.075 (0.013)	7.1 (3.4 – 14.2)[h]	0.9 / 0.8	-0.023 (0.011)	0.7
Glycemia							
HbA1c (mmol/mol) / HbA1c (%)	36 (33 – 38)[i] 5.4 (5.2 – 5.7)[i]	0.9 / 0.2	0.160 (0.190)	39 (36 – 42)[j] 5.7 (5.4 – 6)[j]	0.7 / 1.0	-0.000 (0.131)	0.8
Fasting glucose (mmol/l)	5.1 (4.7 – 5.5)[b]	0.8 / 0.3	0.137 (0.093)	5.6 (5.1 – 6.2)[k]	**0.04** / **0.04**	-0.124 (0.072)	0.1
Fasting insulin (pmol/l)	53 (36 – 70)[b]	0.8 / 0.5	0.082 (0.019)	99 (70 – 144)[k]	0.4 / 0.7	0.043 (0.017)	0.7
Fasting C-peptide (pmol/l)	394 (270 – 573)[a]	0.5 / 0.1	0.185 (0.024)	666 (501 – 853)[j]	0.9 / 0.8	-0.025 (0.026)	0.6
Disposition index	1751 (953 – 2735)[j]	0.6 / 0.8	-0.026 (0.014)	1001 (579 – 1470)[l]	0.09 / 0.2	0.146 (0.012)	0.4
Insulin sensitvity index (OGTT-derived)	14.4 (10.2 – 19.2)[m]	0.8 / 0.3	-0.130 (0.021)	6.3 (4.4 – 9.2)[n]	0.7 / 0.9	-0.012 (0.017)	1.0
Adipo-IR (mmol/l*pmol/l)	22.4 (14.5 – 39.5)[a]	0.09 / 0.2	-0.135 (0.014)	49.9 (32.9 – 79.7)[j]	0.3 / 0.3	-0.108 (0.014)	0.7
Plasma lipids							
Free fatty acids (µmol/l)	464 (319 – 654)[a]	**0.005** / **0.01**	-0.269 (0.020)	511 (407 – 632)[j]	**<0.001** / **<0.001**	-0.370 (0.028)	0.1
Triglycerides (mg/dl)	73 (54 – 88)[o]	0.9 / 0.6	0.051 (0.025)	108 (82 – 141)[e]	0.6 / 0.7	0.050 (0.022)	0.8
Cholesterol (mg/dl)	172 (147 – 206)[l]	0.6 / 0.5	0.104 (0.000)	210 (177 – 234)[j]	0.9 / 0.7	0.039 (0.000)	0.8
LDL-Cholesterol (mg/dl)	104 (83 – 131)[l]	0.7 / 0.6	0.062 (0.034)	141 (114 – 165)[j]	0.4 / 0.7	-0.051 (0.043)	0.6
HDL-Cholesterol (mg/dl)	55 (44 – 67)[l]	0.7 / 0.7	0.039 (0.042)	49 (43 – 59)[j]	0.5 / 0.1	0.168 (0.045)	0.4

Lipoprotein (a) (mg/dl)	11 (6 – 42)[l]	0.4 / 0.4	-0.096 (0.009)	14 (7 – 32)[j]	0.5 / 0.5	-0.067 (0.010)	1.0
Others							
Thyroid-stimulating hormone (mU/l)	2.0 (1.4 – 3.1)[l]	0.7 / 0.2	-0.14 (0.016)	1.7 (1.1 – 2.4)[j]	0.1 / 0.2	0.137 (0.011)	0.2
C-reactive protein (mg/dl)	0.04 (0.01 – 0.15)[l]	0.6 / 0.2	0.141 (0.007)	0.26 (0.11 – 0.56)[j]	0.8 / 0.9	0.020 (0.008)	0.9
Morning cortisol, serum (nmol/l)	413 (336 – 524)[l]	0.7 / 1.0	0.005 (0.027)	368 (277.8 – 488.8)[j]	0.2 / 0.06	-0.210 (0.026)	0.2

*Adjusted for sex and age; # adjusted for age; ## adjusted for sex. Standardized ß are from multivariate linear regression models. a: n=93; b: n =94; c: n=96; d: n=57; e: n=80, f: n=59; g: n=61; h: n=79; i: n=90; j: n=88; k: n=79; l: n=91; m: n=89; n: n=95; o: n= 71. MR: magnetic resonance; MRS: magnetic resonance spectroscopy; HbA1c: hemoglobin A1c; OGTT: oral glucose tolerance test; Adipo-IR: adipose tissue insulin resistance index; LDL-Cholesterol: low-density lipoprotein cholesterol; HDL-Cholesterol: high-density lipoprotein cholesterol.

Supplementary figure 1



Supplementary figure 1 - Association resting energy expenditure and its´ potential determinants
Resting energy expenditure was negatively associated with age **(A)**, with lower resting energy expenditure with increasing age. There was a positive association of resting energy expenditure and BMI **(B).** Resting energy expenditure was neither significantly correlated with plasma glucose **(C)** nor with free fatty acids **(D)** nor with the ketone body ß-hydroxybutyric acid **(E)**. While glucagon was positively associated with resting energy expenditure **(F)**, this association was not independent of sex (p after adjustment for sex 0.7). Neither GIP **(G)** nor glicentin **(H)** were associated with resting energy expenditure. Data are presented as scatterplots with linear regression lines and 95% CI. P-values were taken from linear regression analyses.

Supplementary table 2: Patient characteristics

	Overall cohort (n=192)	Sub-group with incretin measurements (n=38)
	Median (IQR) / n	
Sex		
Male	100	16
Female	92	22
Age (years)	51 (27 – 62)	62 (56 – 66)
Body mass index (kg/m^2)	30.1 (24.7 – 33.7)	31.5 (28.7 – 33.3)
Total adipose tissue, MR-derived (l)	37.9 (26.5 – 47.3)[a]	39.8 (32.2 – 42.9)
HbA1c (mmol/mol)/ HbA1c (%)	37 (34 – 40)[b] 5.5 (5.3 – 5.8)[b]	39 (37 – 41) 5.7 (5.5 – 5.9)
Fasting glucose (mmol/l)	5.3 (4.9 – 5.8)[c]	5.9 (5.6 – 6.5)
Fasting insulin (pmol/l)	70 (45 – 109)[c]	91 (62 – 147)
Insulin sensitvity index (OGTT-derived)	9.6 (5.6 – 15.2)[d]	5.9 (4.0 – 8.8)
Free fatty acids (µmol/l)	498 (375 – 633)[e]	519 (430 – 651)
Triglycerides (mg/dl)	97 (74 – 137)[f]	98 (73 – 139)[g]
Fasting respiratory quotient	0.85 (0.79 – 0.90)	0.86 (0.80 – 0.92)

Data are presented as median (IQR); a: n=137; b: n =178; c: n=191; d: n=184; e: n=181; f: n=179; g: n=34. MR: magnetic resonance; HbA1c: hemoglobin A1c; OGTT: oral glucose tolerance test.

Supplementary Material 2nd Publication (see 2.2)

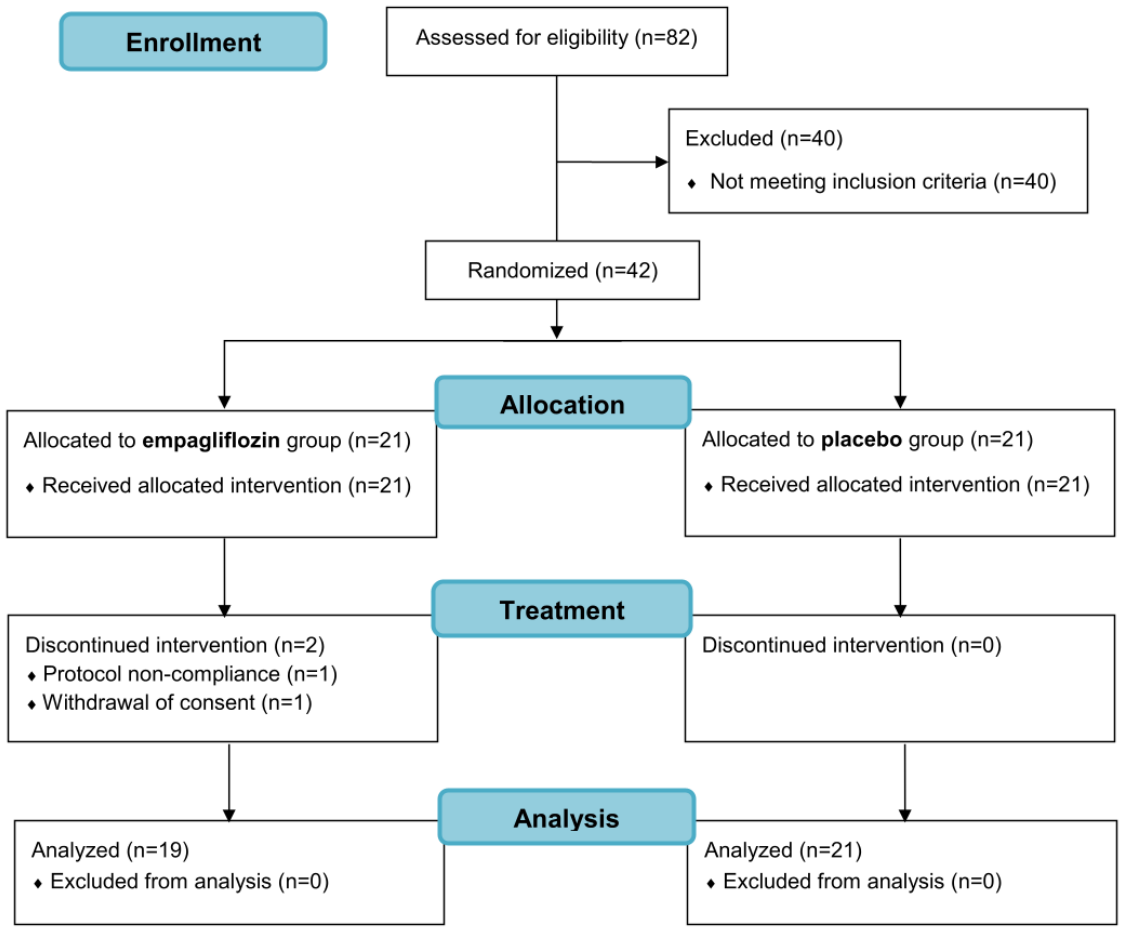
Supplementary material

Empagliflozin improves insulin sensitivity of the hypothalamus in humans with prediabetes: a randomized, double-blind, placebo-controlled, phase 2 trial

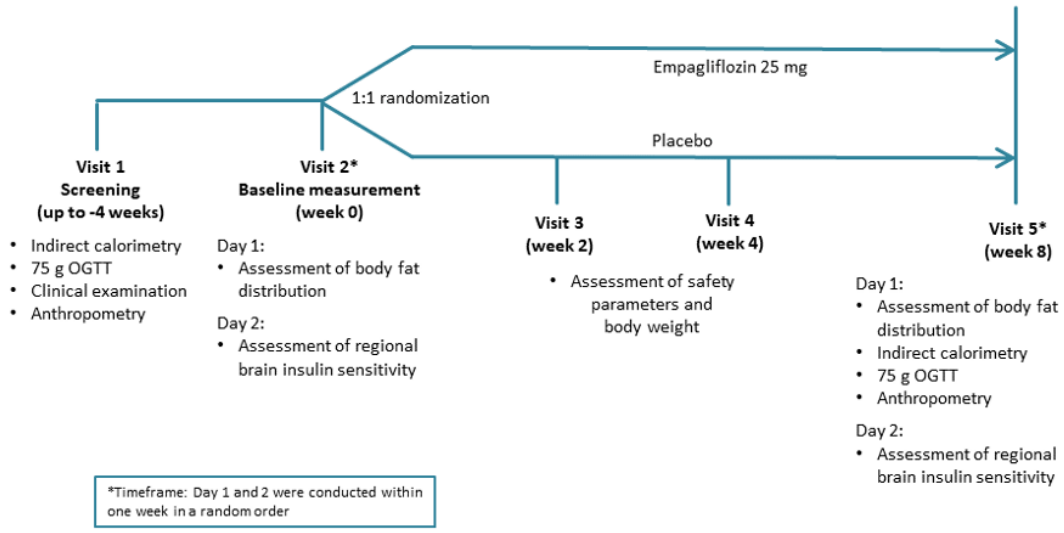
Stephanie Kullmann, PhD[1,2]*, Julia Hummel, M. sc.[1,2]*, Robert Wagner, Prof.[1,2,3], Corinna Dannecker, PhD[1,2], Andreas Vosseler, M. sc.[1,2,3], Louise Fritsche, PhD[1,2], Ralf Veit, PhD[1,2], Konstantinos Kantartzis, MD[1,2], Jürgen Machann, Prof.[1,2,4], Andreas L. Birkenfeld, Prof.[1,2,3], Norbert Stefan, Prof.[1,2,3], Hans-Ulrich Häring, Prof.[1,2,3], Andreas Peter, Prof.[1,2,5], Hubert Preissl, Prof.[1,2,3,6,7], Andreas Fritsche, Prof.[1,2,3], Martin Heni, Prof.[1,2,3,5]

1. Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
2. German Center for Diabetes Research (DZD), Ingolstädter Landstraße 1, 85764 Neuherberg, Germany
3. Department of Internal Medicine, Division of Diabetology, Endocrinology and Nephrology, Eberhard Karls University Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany
4. Department of Radiology, Section on Experimental Radiology, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany
5. Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, Eberhard Karls University Tübingen, Hoppe-Seyler-Str. 3, 72076 Tübingen, Germany
6. Institute of Pharmaceutical Sciences, Department of Pharmacy and Biochemistry; Interfaculty Centre for Pharmacogenomics and Pharma Research at the Eberhard Karls University Tübingen, Tübingen, Germany
7. Institute for Diabetes and Obesity, Helmholtz Diabetes Center, Helmholtz Zentrum München, German Research Center for Environmental Health (GmbH), Neuherberg, Germany

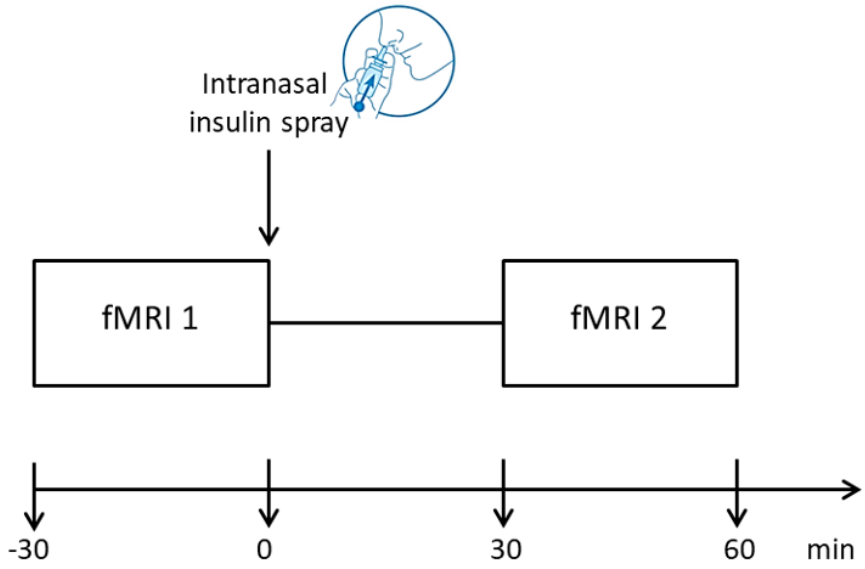
Supplementary figure 1: CONSORT flow diagram



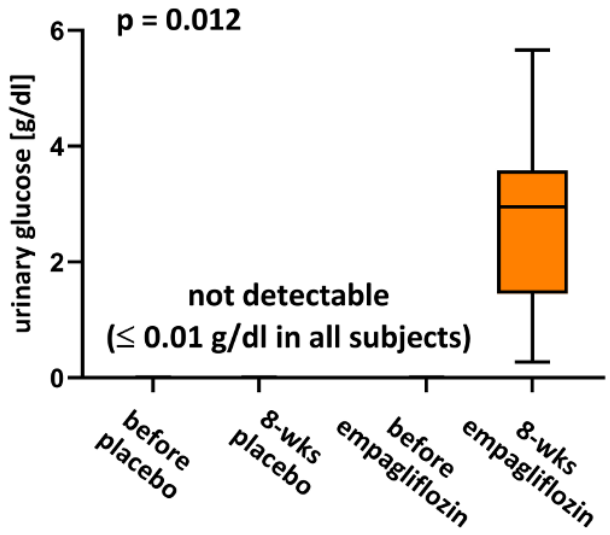
Supplementary figure 2: Study outline



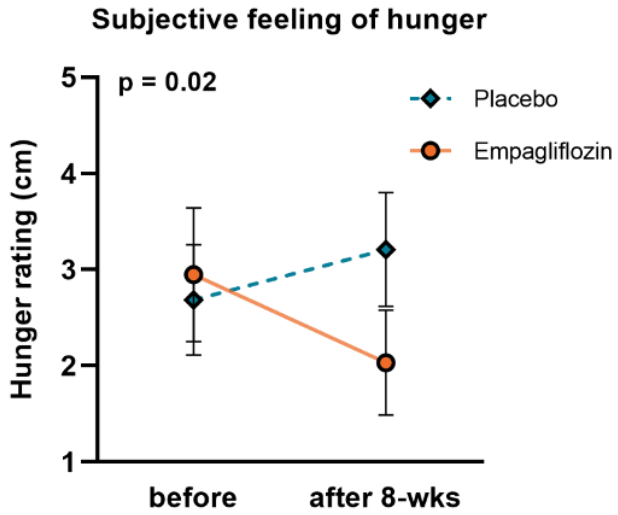
Supplementary figure 3:



Supplementary figure 4: Urinary glucose



Supplementary figure 5: Subjective feeling of hunger



Presented are hunger ratings based on the visual analogue scale (VAS) in the fasting state. There was a significant treatment by time interaction (F(1,35)=6.4, p=0.02). Presented are means ± SEM.

Supplementary table 1: Classification und number of adverse events (AE) according to MedDRA LLT coding

Adverse events (MedDRA LLT)	**Placebo** (n=21)	**Empagliflozin** (n=19)
Fall	1	1
Fibrinous bronchitis	0	1
Pruritus	0	1
Gonitis	0	1
Cold	1	2
Stomach pain	0	1
Diarrhoea	0	1
Urine leukocyte esterase positive	0	1
Pain in lumbar spine	1	0
Aching pain in hands, forearms, elbows	1	0
Creatine kinase increased	1	0
Burning micturition	0	2
Deafness left ear	1	0
Gum pain	0	1
Acute nasopharyngitis (common cold)	0	1
Rash	0	1
Muscle pain	0	1
Tooth pain	1	0
Presyncope	1	0
Urinary tract infection	2	1
Cyst of kidney	1	0
Stye	0	1
Back pain	0	1
Hematoma	1	0
Total Number of AEs	**12**	**18**

Supplementary table 2: Daily intake of nutrients in the empagliflozin and the placebo group before and at the end of treatment.

	Placebo (n=16)		**Empagliflozin** (n=17)		
	Before	**After 8 weeks**	**Before**	**After 8 weeks**	**p**(Manova)
Caloric intake (kcal)	2342 ± 699	2220 ± 732	2078 ± 875	2165 ± 891	0.3/0.2*
Fat (g)	103 ± 37	96.7 ± 40	90 ± 39	90 ± 39	0.7
Carbohydrates (g)	233 ± 69	219 ± 80	204 ± 86	221 ± 113	0.2
Protein (g)	85 ± 26	81 ± 26	77 ± 30	82 ± 36	0.5
Fibers (g)	22.7 ± 7.1	20 ± 1.7	21.2 ± 8.8	22.2 ± 8.9	0.057

Data is given as mean ± SD. A repeated measures ANOVA was performed to investigate treatment by time interaction. *adjusted for sex. 33 subjects completed food diaries on seven consecutive days before and at the end of treatment. Diet composition was estimated with validated software, using four days with complete data out of the seven-day diary (DGE-PC 3.0; Deutsche Gesellschaft für Ernährung, Bonn, Germany).

Supplementary table 3: ß-Hydroxybutyric acid, glucagon, and erythropoetin before and after treatment in both study arms.

	Placebo (n=21)		**Empagliflozin** (n=19)		
	Before	**After 8 weeks**	**Before**	**After 8 weeks**	**$p_{(Manova)}$**
ß-Hydroxybutyric acid (µM/l)	79.8 ± 102.8	95.7 ± 100.9	79.9 ± 87.4	75.9 ± 76.7	0.1
Fasting glucagon (pmol/l)	7.07 ± 4.59	6.90 ± 4.28	6.88 ± 3.09	6.42 ± 3.72	0.7
Area under the glucagon curve during the OGTT (pmol/l)	7.29 ± 3.13[a]	8.31 ± 4.46[b]	6.34 ± 2.21[c]	6.66 ± 2.28[d]	0.3
Erythropoetin (mU/ml)	8.85 ± 5.40[c]	8.17 ± 3.41	10.84 ± 7.74[d]	12.17 ± 8.78	0.8

Data are given as mean ± SD. A repeated measures ANOVA was performed to investigate treatment by time interactions. a: n=14, b: n=15, c: n=16, d: n=18. OGTT: oral glucose tolerance test.

Supplementary table 4: Clinical chemistry

	Placebo (n=21)		**Empagiflozin** (n=19)		
	Before	**After 8 weeks**	**Before**	**After 8 weeks**	**p**(Manova)
Liver enzymes					
Aspartate aminotransferase (U/l)	22.2 ± 12.6	23.4 ±12.5	18.3 ± 8.9	16.8 ± 6.2	0.4
Alanine aminotransferase (U/l)	31.4 ± 19.5	31.3 ± 19.1	30.1 ± 16.5	27.1 ± 12.2	0.5
γ-Glutamyltransferase (U/l)	30.3 ± 21.0	29.8 ± 21.0	33.7 ± 19.0	31.0 ± 19.0	0.4
Alkaline phosphatase (U/l)	70.7 ± 17.9[a]	66.6 ± 16.0	70.2 ± 17.1	72.6 ± 16.6	**0.025**
Serum lipids					
Fasting non-esterified fatty acids (µmol/l)	591 ± 239	571 ± 217	532 ± 162	605 ± 187	0.1
Area under the curve of non-esterified fatty acid during OGTT (µmol/l)	504 ± 170	437 ± 140	439 ± 120[b]	520 ± 167	**0.004**
Triglycerides (mg/dl)	125.0 ± 52.9	126.9 ± 59.8	113.4 ± 60.6	118.8 ± 74.2	0.8
Cholesterol (mg/dl)	210 ± 40	204 ± 44	211 ± 40	212 ± 46	0.4
LDL-Cholesterol (mg/dl)	139 ± 35	137 ± 43	141 ± 43	144 ± 46	0.5
HDL-Cholesterol (mg/dl)	52 ± 14	52 ± 16	55 ± 12	56 ± 16	0.9
Kidney and urine					
Plasma creatinine (mg/dl)	0.80 ± 0.17	0.80 ± 0.13	0.70 ± 0.13	0.70 ± 0.14	0.6
GFR-MDRD (ml/min/1.73 m^2)	90.56 ± 24.45	87.59 ± 16.82	97.13 ± 19.80	98.38 ± 24.92	0.6
GFR-CKD-EPI (ml/min/1.73 m^2)	85.52 ± 5.19	85.57 ± 6.58	89.11 ± 2.81	88.89 ± 3.70	0.9
Urinary protein-to-creatinine ratio (mg/g)	83.3 ± 67.6[c]	80.6 ± 60.2	75.1 ± 46.6[b]	120.5 ± 104.5[b]	0.4
Urinary albumin-to-creatinine ratio (mg/g)	14.6 ± 14.1[c]	15.4 ± 12.2	15.1 ± 13.7[b]	22.2 ± 20.5[b]	0.3
Urinary urea-to-creatinine ratio (g/g)	14.3 ± 3.9[c]	15.2 ± 3.4	14.2 ± 4.8[b]	15.9 ± 6.1[b]	0.9
Urinary glucose (g/dl)	0.01 ± 0.0[d]	0.01 ± 0.0	0.01 ± 0.0a	2.6 ± 1.6	0.011
Electrolytes					
Potassium (mmol/l)	4.08 ± 0.29	4.11 ± 0.33	4.02 ± 0.23	4.11 ± 0.18	0.5
Sodium (mmol/l)	140.43 ± 1.91	140.38 ± 1.77	139.95 ± 2.17	140.32 ± 1.67	0.5
Chloride (mmol/l)	106.48 ± 2.27	106.86 ± 2.06	106.74 ± 2.64	107.42 ± 2.36	0.7

Magnesium (mmol/l)	0.86 ± 0.06	0.84 ± 0.05	0.83 ± 0.06	0.88 ± 0.05	**0.0002**
Phosphorus (mmol/l)	1.07 ± 0.22	1.07 ± 0.20	1.06 ± 0.14	1.08 ± 0.15	0.6
Further blood parameters					
C-reactive protein (mg/dl)	0.34 ± 0.52	0.19 ± 0.25	0.47 ± 0.49	0.62 ± 0.75	**0.009**
Uric acid (mg/dl)	6.8 ± 1.2	6.6 ± 1.1	5.4 ± 0.9	4.1 ± 0.9	**0.0095**
Morning cortisol, serum (nmol/l)	371 ± 115	348 ± 93	434 ± 137	383 ± 137	0.7
Fasting insulin (pmol/l)	114 ± 62	100 ± 58	111 ± 51	103 ± 50	0.6
Fasting C-Peptide (pmol/l)	708 ± 270	698 ± 282	727 ± 206	685 ± 173	0.5

Data are given as mean ± SD. A repeated measures ANOVA was performed to investigate treatment by time interactions. a: n=17, b: n=18, c: n=20, d: n=15. OGTT: oral glucose tolerance test, LDL: low-density lipoprotein cholesterol, HDL: high-density lipoprotein cholesterol, GFR-MDRD: glomerular filtration rate-modification of diet in renal disease, GFR-CKD-EPI: glomerular filtration rate-chronic kidney disease epidemiology collaboration.

Supplementary table 5: Inclusion and exclusion criteria

Inclusion Criteria	Subjects meeting all of the following criteria will be considered for admission to the trial: • Must be between 30 and 75 years at the time of signing the informed consent. • Fasting blood glucose between 100 and 125 mg/dl and/or 2-hour post load glucose between 140 and 199 mg/dl during a 75 g oral glucose tolerance test (ADA criteria for prediabetes). • Body mass index (BMI) between 25 and 40 kg/m². • Understand and voluntarily sign an informed consent document prior to any study related assessments/procedures. • Ability to adhere to the study visit schedule and other protocol requirements. • Females of childbearing potential (FCBP[1]) must agree o to utilize two reliable forms of contraception simultaneously or practice complete abstinence from heterosexual contact for at least 28 days before starting study drug, while participating in the study (including dose interruptions), and for at least 28 days after study treatment discontinuation and must agree to pregnancy testing during this timeframe o to abstain from breastfeeding during study participation and 28 days after study drug discontinuation. • Males must agree o to use a latex condom during any sexual contact with FCBP while participating in the study and for 28 days following discontinuation from this study, even if he has undergone a successful vasectomy o to refrain from donating semen or sperm while participating in this study and for 28 days after discontinuation from this study treatment. • All subjects must agree to refrain from donating blood while on study drug and for 28 days after discontinuation from this study treatment. • All subjects must agree not to share medication.

	[1]A female of childbearing potential is a sexually mature woman who: 1) has not undergone a hysterectomy or bilateral oophorectomy; or 2) has not been naturally postmenopausal for at least 24 consecutive months (i.e., who has had menses at any time in the preceding 24 consecutive months).
Exclusion Criteria	Subjects presenting with any of the following criteria will not be included in the trial: • Women during pregnancy and lactation. • History of hypersensitivity to the investigational medicinal product or to any drug with similar chemical structure or to any excipient present in the pharmaceutical form of the investigational medicinal products. This includes empagliflozin, placebo and human insulin. • Participation in other clinical trials or observation period of competing trials up to 30 days prior to this study. • Diabetes mellitus • Known malformation of the central nervous system • Persons working nightshift • Treatment with glucose lowering drugs, drugs with central nervous actions or systemic steroid therapy • Any relevant (according to investigator's judgment) cardiovascular disease, e.g. myocardial infarction, acute coronary syndrome, unstable angina pectoris, PTCA, heart failure (NYHA II-IV), stroke or transient ischemic attack (TIA), within 12 months prior to screening. • Indication of liver disease, as per medical history or defined by serum levels of either Alanine Aminotransferase (ALT [SGPT]), Aspartate Aminotransferase (AST [SGOT]), or alkaline phosphatase above 3 x upper limit of normal (ULN) as determined during screening. • Alcohol abuse, defined as more than 20 gr/day • Impaired renal function, defined as estimated Glomerular Filtration Rate (eGFR) ≤ 60 ml/min (MDRD formula) as determined during screening. • Known structural and functional urogenital abnormalities, that predispose for urogenital infections. • Subjects with a haemoglobin (Hb) ≤ 11.5 g/dl (for males) and Hb ≤ 10.5 g/dl (for females) at screening. • Bariatric surgery within the past two years and other gastrointestinal surgeries that induce chronic malabsorption within the last 5 years.

	• Medical history of cancer (except for basal cell carcinoma) and/or treatment for cancer within the last 5 years. • Treatment with anti-obesity drugs 3 months prior to informed consent or any other treatment at the time of screening (i.e. surgery, aggressive diet regimen, etc.) leading to unstable body weight. • Known autoimmune disease (except autoimmune disease of the thyroid gland) or chronic inflammatory condition. • Claustrophobia • Any other clinically significant major organ system disease at screening such as relevant gastrointestinal, neurologic, psychiatric, endocrine (i.e. pancreatic), hematologic, malignant, infection or other major systemic diseases making implementation of the protocol or interpretation of the study results difficult. • Presence of any contraindication for the conduct of an MRI investigation, such as cardiac pacemakers, ferromagnetic haemostatic clips in the central nervous system, metallic splinters in the eye, ferromagnetic or electronically operated active devices like automatic cardioverter defibrillators, cochlear implants, insulin pumps and nerve stimulators, prosthetic heart valves etc. • Refusal to get informed of unexpected detected pathological MRI findings • Any other clinical condition that would jeopardize subjects' safety while participating in this clinical trial.

Supplementary material 6: Sample size and power considerations

We designed the trial to study a continuous response variable (brain response to intranasal insulin). Based on previous studies with fMRI and intranasal insulin delivery, we hypothesize that the response will be normally distributed with a standard deviation of 20%. If the true difference is 20%, we will need to study 17 subjects for each treatment group to be able to reject the null hypothesis that the follow-up versus baseline measurements are equal with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. We plan to include three more subjects in each group who could replace possible dropouts. Thus, 40 subjects are needed.