Obesity, ectopic lipids, and insulin resistance

Tissue-specific defects in nutrient handling

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CHAPTER 11

Summary, general discussion, and future perspectives
SUMMARY

The studies described in this thesis address 3 major topics in obesity research. In **PARTS 1 to 3**, we feature work on clinical, nutritional, and molecular aspects of human insulin resistance, respectively. In the clinical studies, we focused on the identification of insulin resistance in high-risk patients and the contribution of insulin target tissues to the clinical phenotype. In the nutritional and molecular studies, we focused on the mechanisms that may cause insulin resistance in these tissues. We aimed to translate findings from basic science/animal research and explore the therapeutic potential of emerging metabolic pathways as novel targets for human insulin resistance and type 2 diabetes.

**PART 1: Clinical assessment of tissue-specific insulin resistance**

Insulin resistance is the major contributor to the cardiometabolic complications of obesity including type 2 diabetes, dyslipidemia, non-alcoholic fatty liver disease (NAFLD), and cardiovascular disease [32,125]. Early identification and subsequent treatment of high-risk insulin-resistant patients may favorably influence the development and/or progression of these metabolic complications [127], but current clinical practice lacks simple tools for the recognition of insulin resistance. In **Chapter 2**, we used the distribution of hyperinsulinemic-euglycemic clamp results in healthy subjects to propose a novel, rational definition of whole-body insulin resistance. We then determined if we could predict insulin resistance from readily-available clinical parameters and showed that insulin-resistant obese subjects can be identified from fasting plasma insulin >74 pmol/l with good sensitivity and specificity. This makes fasting plasma insulin a potentially useful marker of insulin resistance in obesity, facilitating future scientific and clinical identification of insulin resistance.

Resistance to insulin can occur, independently or simultaneously, in all insulin target tissues. In this regard, adipose tissue insulin resistance is an important feature of obesity-related metabolic disease [76]. Increased adipose tissue lipolysis results in the inappropriate release of free fatty acids (FFAs) and lipotoxicity in the liver, muscle, and other organs [159-161]. Assessment of lipolysis by metabolic tracer methods is labor-intensive and expensive. In **Chapter 3**, we demonstrate that several simplified index methods can accurately estimate adipose tissue insulin sensitivity in humans. We show that the best index methods rely on the assessment of plasma glycerol or FFA concentrations during hyperinsulinemic-euglycemic clamp experiments. Indices derived from a single fasting blood sample, such as the Adipo-IR index (that is, the product of fasting plasma insulin and FFA concentrations), have lower precision, but their simplicity makes them more suitable for large-scale epidemiology and clinical settings.

It is well established that insulin resistance occurs early in the pathogenesis of type 2 diabetes [433]. In fact, diabetes patients are characterized by insulin resistance in the liver, adipose tissue, muscle, and other tissues, and these defects may all contribute to hyperglycemia [200]. However, the relative contribution of tissue-specific defects to its pathogenesis is only partially elucidated. Humans with impaired fasting glucose (IFG) have fasting glucose levels in the prediabetes range and increased risk of future dia-
betes development [198,199]. Other prediabetes categories include impaired glucose tolerance (IGT) or combined IFG/IGT [185]. In Chapter 4, we performed tissue-specific insulin sensitivity measurements to determine the contribution of basal endogenous glucose production (EGP), and hepatic, adipose tissue, and peripheral insulin resistance to the pathogenesis of IFG in obese humans. We demonstrate that obese adults with IFG are characterized by distinctly impaired insulin action in the liver, but not in adipose tissue or muscle. In contrast, others have shown that IGT is associated with muscle, but not liver, insulin resistance [43,203], suggesting that the site of the defect determines the clinical phenotype. Likewise, in Chapter 5, we demonstrate that morbidly obese men have lower hepatic, but not adipose tissue or muscle, insulin sensitivity than equally-as-obese women. This suggests that hepatic insulin resistance may contribute to the higher global prevalence of diabetes in obese men [220]. Given the differences in underlying pathophysiology [52], patients in different prediabetes categories will likely benefit from different interventions. A better understanding of the biology underlying this gender difference may also reveal novel targets for the prevention of hepatic insulin resistance.

PART 2: Nutritional factors

In vivo nutrient fluxes (that is, into, in-between, and out of) metabolically-active tissues are major mechanistic determinants of insulin action. In recent years, fructose has emerged as a particularly challenging nutrient: its consumption is epidemiologically linked with many features of the metabolic syndrome [104,105,107,252,344], and its unique hepatic metabolism may predispose to metabolic disease [304]. The studies described in Chapters 6 to 8 were designed to evaluate the (adverse) metabolic effects of acute and long-term fructose consumption. To determine the effect of fructose consumption on insulin sensitivity in humans, we first performed a systematic review of the scientific literature. As described in Chapter 6, we identified 46 controlled comparisons in 1005 normal-weight and overweight or obese subjects. Overall, the pooled results from these diet-intervention trials indicate that short-term fructose consumption, in isocaloric exchange for control carbohydrates or in hypercaloric supplementation to a weight-maintenance control diet, promotes the development of hepatic insulin resistance in normal-weight nondiabetic adults. Fructose consumption did not induce peripheral or muscle insulin resistance. These findings may have been limited by the small sample size and short duration of included trials, but strengthen the growing body of evidence that causally links fructose to metabolic complications, in particular hepatic insulin resistance.

Chapter 7 describes an effort to determine the molecular basis for some of the concerning clinical observations regarding fructose consumption. High fructose consumption may be linked to the accumulation of ectopic lipids and development of insulin resistance due to, in part, its ability to activate the lipogenesis and gluconeogenesis pathways in the liver. We hypothesized that fructose stimulates a stronger hepatic lipogenesis response than glucose and obese humans with hepatic lipid accumulation may be more susceptible to this lipogenic effect of fructose. To test this, we recruited obese participants without hepatic steatosis or with NAFLD. These groups were carefully
matched for most baseline and clinical parameters, but subjects with NAFLD had higher fasting triglyceride levels and insulin resistance in the liver, adipose tissue, and muscle, indicating that NAFLD is part of a systemic metabolic phenotype. Administration of 75 g of fructose, but not glucose, acutely raised circulating triglyceride levels in both groups. Fructose also produced an increase in plasma glucose levels in subjects with NAFLD. We then obtained liver and adipose tissue biopsies ~2.5 h after fructose or glucose administration and measured fructose- and glucose-stimulated mRNA expression of metabolic genes. Fructose, but not glucose, ingestion was associated with an increase in hepatic expression of the transcription factor carbohydrate response element-binding protein (ChREBP) β and its gluconeogenesis and lipogenesis target genes. Notably, fructose-induced gene expression patterns did not meaningfully differ between obese subjects with or without NAFLD. In contrast, glucose-stimulated adipose tissue expression of glucose transporter (GLUT) 4 and fatty acid synthase (FASN) were reduced in subjects with NAFLD, suggesting that glucose disposal and nutrient storage in adipose tissue is impaired. Adipose tissue GLUT4 expression was a strong predictor of whole-body insulin resistance. These results support mechanisms by which fructose consumption, through hepatic ChREBP activation, and adipose tissue dysfunction, through impaired lipogenesis, may contribute to hepatic steatosis and insulin resistance.

Fibroblast growth factor 21 (FGF21) is a novel metabolic hormone with alleged beneficial effects on whole-body carbohydrate, lipid, and energy metabolism [346-348]. It has also been suggested to mediate an adaptive response to fructose ingestion by regulating the hepatic gene programs involved in fructose metabolism [434], but less is known about this paradigm in humans. We present our investigation of fructose-FGF21 biology in obese humans in Chapter 8. Fructose ingestion acutely and transiently stimulated an increase in circulating FGF21 levels. To our knowledge, this is the only known hormonal response to fructose ingestion, suggesting that FGF21 may be involved in post-absorptive fructose handling. In these obese subjects, an exaggerated response was associated with poor metabolic health including insulin resistance, warranting further examination of the role of FGF21 in fructose-related metabolic disease.

Obesity predisposes to vitamin D deficiency [374,375,377,391,392], whereas vitamin D has been suggested to exert beneficial effects on insulin sensitivity [373,380]. However, few studies have investigated the active vitamin D metabolite, and the interpretation of many previous findings is complicated by the confounding effect of obesity on both vitamin D status and insulin sensitivity. We determined 25-hydroxycholecalciferol [25(OH)D], 1,25-dihydroxycholecalciferol [1,25(OH)2D], and tissue-specific parameters of insulin action in obese women (Chapter 9). Obesity was associated with lower levels of circulating 25(OH)D, but not with 1,25(OH)2D. In addition, we show that neither vitamin D metabolite is associated with insulin sensitivity when groups are well-matched for obesity and adiposity, suggesting that vitamin D does not play a major role in obesity-related insulin resistance.
PART 3: Mechanism of hepatic insulin resistance
Ectopic hepatic lipid accumulation (that is, in the context of NAFLD) is emerging as an important cause of hepatic insulin resistance and type 2 diabetes [59], but the pathophysiological mechanisms that are key for human hepatic insulin resistance are only partially unraveled. Chapter 10 describes a translational study that was designed to determine the relationship between hepatic lipid species and insulin resistance, and to examine the molecular mechanisms that link them. Using hyperinsulinemic-euglycemic clamp and metabolic tracer techniques in a large number of obese subjects, we show that the presence of hepatic steatosis is associated with insulin resistance in liver, adipose tissue, and muscle. However, we found that hepatic steatosis was not strictly sufficient or necessary for hepatic insulin resistance, thereby indicating that other factors must be responsible for NAFLD-associated hepatic insulin resistance. To examine the molecular mechanisms, we collected liver biopsies from a subset of these subjects. Recent rodent studies have identified hepatic diacylglycerol (DAG) accumulation and DAG-mediated activation of protein kinase C (PKC) as an important underlying mechanism for hepatic insulin resistance. In accordance, we demonstrate that DAGs in the hepatocellular cytosol, but not DAGs in the cell membrane or ceramides, are increased in subjects with hepatic insulin resistance. Moreover, cytosolic DAG accumulation was strongly associated with hepatic PKCε activation as reflected by its translocation from the cytosol to the membrane. These data offer translational support for the DAG-PKCε hypothesis for lipid-induced hepatic insulin resistance in NAFLD, and support the therapeutic potential of this signaling axis as novel target for hepatic insulin resistance and/or type 2 diabetes.

GENERAL DISCUSSION

An average adult consumes approximately 1 million kcal per year. When energy intake exceeds expenditure by only 1%, body weight would increase by 1-2 kg every year [435], yet most humans are able to maintain stable body weight. These calories can be consumed in 1, 3, or 6 meals a day and as carbohydrate, protein, fat, or other nutrients, yet blood sugar levels and intracellular energy stores are maintained within acceptable limits [27]. To accomplish such miraculous metabolic flexibility, humans (and other mammals) have evolved highly-efficient, highly-dynamic nutrient storage and release systems [21]. We estimate that 100-200 g of glucose is produced by the liver and 300-400 g of triglycerides are synthesized and hydrolyzed in adipose tissue every day [230]. With fluxes like these, it is not hard to imagine that a small defect, a minimal supply-demand imbalance, may have vast consequences over the course of 5, 10, or 20 years. Long-term metabolic health is a remarkable feat of nature.

Obesity is caused by an imbalance between energy intake and energy expenditure [436]. It is, in a sense, a disease of aberrant nutrient and energy fluxes. Nutrient availability is normally linked to behavioral and metabolic responses that decrease further food intake and increase energy expenditure [436]. Defects in these feedback mechanisms likely contribute to obesity [437]. For a comprehensive review of the central and peripheral mechanisms that underlie disturbed feeding behavior in obesity, we refer
to the available literature [438-442]. Nevertheless, overexposure to nutrients under hypercaloric feeding conditions places chronic metabolic stress on nutrient storage and release pathways, which drives metabolic dysregulation [21]. The studies in this thesis have focused on these nutrient fluxes and pathways in obesity-related metabolic disease and insulin resistance.

Insulin resistance is the link between obesity and type 2 diabetes: obesity causes insulin resistance, and insulin resistance is 1 of 2 pathophysiological hallmarks of type 2 diabetes, the other being β cell failure [32,84]. It is present long before clinically overt hyperglycemia becomes apparent [35] and, even in the absence of hyperglycemia, an independent risk factor for cardiovascular disease [126]. Weight loss effectively reverses obesity-related insulin resistance [100] and may even cure type 2 diabetes [443]. Unfortunately, lifestyle-based weight loss strategies are notoriously ineffective in the long term [10]. Surgical (bariatric) interventions may be effective for sustained weight loss [15,16], but have obvious downsides. Besides, genetically or environmentally-predisposed normal-weight people may also develop insulin resistance and type 2 diabetes [19], but will not benefit from novel weight-loss therapies. Consequently, we are in clear need of other strategies to prevent and/or treat insulin resistance and type 2 diabetes. A better appreciation of the clinical presentations and mechanisms of insulin resistance will undoubtedly facilitate the identifications of novel preventive and/or therapeutic targets as well as the patients who may benefit from novel interventions.

That insulin resistance plays a central role in the pathogenesis of prediabetes, type 2 diabetes, and cardiovascular disease has been known, more or less, since the late 1930s [444]. Surprisingly, scientific progress over the past 8 decades has not pushed the assessment of insulin sensitivity into clinical practice. The diagnostic work-up in cardiovascular risk management commonly includes measurements of blood pressure, glycemia, and dyslipidemia; diabetes management is focused around glycemic targets [185]. This, we believe, is a missed opportunity: addition of a measurement of insulin sensitivity to the diagnostic work-up may help to identify high-risk (obese) patients with insulin resistance, even if they do not meet current criteria for (pre)diabetes (yet), and therapies that enhance insulin sensitivity may require follow-up measurements of insulin sensitivity to evaluate their efficacy. Although direct measurement of insulin action requires expensive and labor-intensive methods, such as the hyperinsulinemic-euglycemic clamp method, insulin effects in the liver, muscle, and adipose tissue can also be estimated from simple surrogate measurements. In Chapter 2, we demonstrate that the fasting plasma insulin concentration is a reliable tool to identify obese subjects with peripheral insulin resistance. Fasting plasma glucose concentrations, in contrast, provided no information on peripheral insulin sensitivity in these subjects. This observation has an important clinical implication: it indicates that obese patients with normal fasting glucose levels can still have severe muscle insulin resistance with high risk of diabetes development and benefit from early preventive action. A negative correlation between whole-body insulin sensitivity and fasting plasma insulin levels has been shown before [147], but we are the first to demonstrate that fasting plasma insulin also has diagnostic accuracy to detect insulin resistance in obese humans. In accordance with consensus
guidelines for defining reference ranges in clinical chemistry [139,140], we defined normal peripheral insulin sensitivity as insulin-stimulated glucose uptake within the reference range for this outcome in healthy, non-obese subjects. Following this approach, we found that 88% of obese subjects had below-normal peripheral insulin sensitivity, illustrating how big a problem insulin resistance is in modern society. Previously, a lifestyle-intervention program and metformin treatment both reduced the incidence of diabetes in prediabetes patients [127]. Now we are in need of trials that assess whether insulin-resistant, but nondiabetic patients also benefit from such interventions. Fasting plasma insulin may be used to identify these patients.

It is important to note that this diagnostic tool was able to detect changes in insulin-mediated glucose uptake, which primarily reflects muscle insulin resistance [35,361]. Patients may also present with insulin resistance in other tissues, and tissue-specific defects in insulin action may require the development of tissue-specific therapies. Hyperinsulinemic patients will likely benefit from muscle-focused therapies (Chapter 2), whereas patients with IFG, who are characterized by hepatic insulin resistance (Chapter 4), will likely benefit more from liver-focused therapies.

While insulin has long been known to inhibit the hydrolysis of triglycerides in adipocytes [158], the assessment of this effect in humans has been limited to small-scale research settings due to the complex nature of the measurement method [175]. Physiologically, insulin action facilitates nutrient storage in adipose tissue under postprandial conditions, but insulin-resistant adipose tissue is less responsive to insulin’s antilipolytic action. Elevated release of FFA from adipose tissue has been hypothesized to contribute to ectopic lipid accumulation and insulin resistance in the liver and muscle [159-161]. To advance our knowledge of these mechanisms, reliable quantification of adipose tissue insulin sensitivity in humans is essential. In Chapter 3, we performed metabolic tracer experiments to determine the rate of lipolysis under fasting and hyperinsulinemic conditions in obese subjects. In these subjects, insulin-mediated suppression of lipolysis ranged from 4-85%, indicating that there is substantial variation in adipose tissue insulin sensitivity. To facilitate future research in this field, we validated 7 indices of adipose tissue insulin resistance against the metabolic tracer-determined reference method. We identified 5 previously unvalidated fasting blood sample-based indices from the literature [171-174,179-182] and composed new 2 indices on the basis of insulin-adipose tissue physiology [177]. Using correlation analysis, Bland-Altman concordance analysis, and receiver-operator characteristic (ROC) curve analysis, we demonstrate that several simplified index methods can reliably quantify adipose tissue insulin sensitivity. Insulin-mediated suppressions of plasma glycerol and FFA concentrations, assessed during hyperinsulinemic-euglycemic clamp conditions, had the best accuracy and precision. Fasting indices, including the Adipo-IR index and the fasting plasma insulin-glycerol product, had lower precision, but their simplicity, combined with their acceptable sensitivity and specificity, makes them most suitable for large-scale trials and clinical practice. Validation of simple diagnostic tools in Chapters 2 and 3 is an important step towards the widespread assessment of muscle and adipose tissue insulin sensitivity in research and clinical settings.
Impaired glucose disposal and increased adipose tissue lipolysis are clinical presentations of insulin resistance in 2 target tissues. From an etiological point of view, however, these tissue-specific fluxes are also major contributors to systemic metabolic demise. When glucose uptake is reduced, it may be diverted into hepatic de novo lipogenesis (DNL) [431]. Lipolysis-derived FFAs and glycerol also provide substrate for hepatic gluconeogenesis and ectopic lipid accumulation [75,159]. More and more, it is becoming apparent that metabolic tissues, through nutrient fluxes and signaling molecules, are strongly interconnected. Dysfunction in 1 of these tissues affects metabolic functions in the others. In addition, much like endogenous glucose and FFAs, exogenous nutrients have also been hypothesized to directly (that is, body weight-independent) affect ectopic lipid accumulation and insulin resistance. In fact, the role of nutritional factors in the development of insulin resistance and related metabolic disorders is an active research interest of many groups [100,445-451].

Fructose has been 1 of the main simple sugars in human diets for centuries [303,343]. In recent decades, however, fructose consumption has increased dramatically [249]. Particular concerns have been raised regarding its alleged role in the current epidemics of obesity and related disorders [19,104]. These concerns are supported, but not consistently proven, by several lines of evidence. Firstly, most human cells do not metabolize ingested fructose directly. Although fructose and glucose are both hexose monosaccharides with energy values of 3.75 kcal/g, they are metabolized quite differently. Ingested glucose is primarily taken up by peripheral tissues. Fructose is preferentially metabolized by the liver, where it may be processed for ATP production, glycogen synthesis, gluconeogenesis, and/or lipogenesis [304]. On a cellular level, fructose may thus promote glucose production and hepatic lipid accumulation, processes which are associated with the development of hepatic insulin resistance [306]. Secondly, fructose-fed animals consistently display the metabolic syndrome phenotype [108-110]. Thirdly, high intake of fructose is epidemiologically associated with many features of the metabolic syndrome [104,105,107,252,344]. Finally, some human diet-intervention trials suggest that fructose promotes visceral adiposity, increased DNL, dyslipidemia, and hepatic insulin resistance [286,294,314], but other trials have not been able to confirm these adverse effects of fructose when it is consumed in more-realistic doses [112]. Unfortunately, translational data of cellular fructose metabolism in humans is limited, and the interpretation of human intervention-trial data is complicated, because the overconsumption of caloric sweeteners, such as fructose, also causes weight gain [106], which has evident health implications. In Chapter 6, we systematically reviewed the available evidence and addressed the latter issue by analyzing isocaloric and hypercaloric intervention trials separately. Although we were limited by the small number of available trials and participants, this was the first study – to our knowledge – to provide aggregate human evidence to support suspicions that fructose consumption is worse than consumption of other carbohydrates with respect to hepatic insulin resistance. Our pooled results also indicate that fructose consumption does not promote peripheral or muscle insulin resistance per se in human subjects, which is consistent with the concept that fructose is primarily metabolized in the liver [304].
The study presented in Chapter 7 was designed to explore the mechanistic links between fructose consumption and metabolic disease, in particular hepatic steatosis and insulin resistance, in humans. Throughout this thesis, we place an emphasis on interconnected nutrient fluxes in pathophysiology. Our findings on human fructose biology are consistent with this working model of obesity-related insulin resistance. We demonstrate that the presence of NAFLD, characterized by severe buildup of triglycerides and other lipid species in hepatocytes, is associated with glucose intolerance, and hepatic, adipose tissue, and muscle insulin resistance. Surprisingly, this whole-body metabolic phenotype was not associated with major differences in fructose metabolism: in both control subjects and subjects with NAFLD, the administration of fructose, but not glucose, stimulated the expression of gluconeogenesis and DNL genes. In accordance, fructose, but not glucose, ingestion raised plasma triglyceride levels after 4-5 h. This timeframe is consistent with the possibility that fructose stimulates triglyceride levels through its ability to acutely activate hepatic DNL, although this needs further examination using enzymatic activity and/or flux measurements. In rodents, fructose-mediated stimulation of hepatic gluconeogenesis and lipogenesis is dependent on the activation of ChREBPα and subsequent upregulation of ChREBPβ [37]. In our study, fructose administration to both obese control subjects and obese subjects with NAFLD was associated with increased hepatic ChREBPβ expression, suggesting that ChREBPβ may play an important regulatory role in human fructose handling. In subjects with NAFLD, both fructose and glucose ingestion was associated with increased hepatic ChREBPβ expression, but only fructose seemed to also regulate the expression of metabolic target genes: transcriptional regulation of metabolic target genes by ChREBPβ may be dependent on the presence of specific intrahepatic carbohydrate metabolites. Fructose may thus act as DNL substrate and nutritional regulator of hepatic DNL genes, illustrating how its consumption may contribute to hepatic lipid accumulation and hypertri-glyceridemia, even if subjects with NAFLD are not inherently more susceptible to the lipogenic effects of a single fructose dose.

These results also translationally support a mechanism by which fructose, through ChREBP activation, drives hepatic glucose production (from gluconeogenesis) independently of hepatic insulin signaling, thereby contributing to hepatic insulin resistance [37]. In the context of obesity and/or type 2 diabetes, however, hepatic insulin resistance is characterized by an apparent paradox: insulin is unable to appropriately decrease EGP, yet continues to promote lipogenesis and hepatic lipid accumulation/steatosis [33,37,452]. Extensive previous investigations have tried to find a point in the intrahepatic insulin signaling cascade where insulin regulation of glucose and lipid metabolism may branch, but this concept remains controversial [37]. A carbohydrate-driven mechanism of glucose production and lipogenesis may provide an alternative solution to the paradox. Sugar is substrate for DNL and an activator of transcription factors, including ChREBP and sterol regulatory element binding protein (SREBP) 1c, that regulate the hepatic lipogenesis pathway [87]. Fructose, in particular, is almost completely extracted from portal blood upon first-pass through the liver [304], maximizing intrahepatic substrate availability and ChREBP activation. The data in Chapter 7 support these lipogenic properties of fructose. In addition, triglyceride synthesis in rat liver varies with
FFA availability (that is, in the circulation), but is not affected by insulin resistance or insulin receptor antisense knockdown [159]. In humans, the majority of lipids in livers of NAFLD subjects also derives from circulating FFAs [53]. Our studies, consistent with previous reports [56], confirm that obese, insulin-resistant humans are characterized by increased FFA availability from adipose tissue lipolysis, suggesting that adipose tissue insulin resistance may contribute to hepatic lipid accumulation independent of direct hepatic insulin signaling. Together, these substrate-driven mechanisms may (continue to) promote hepatic lipogenesis despite resistance to direct hepatic insulin signaling. Moreover, if insulin resistance in adipose tissue predisposes individuals to hepatic lipid accumulation, then it may be clinically meaningful to identify patients with adipose tissue insulin resistance, using 1 of the indices described in Chapter 3, and screen for the presence of liver fat in those at risk for NAFLD. This is especially relevant given the clinical burden of NAFLD, with increased risk for liver cirrhosis and liver failure [405].

The relationship between liver fat content and insulin resistance is complex. Hepatic lipid accumulation in the context of NAFLD is often associated with hepatic insulin resistance [69-71,407-412], but these conditions can also be dissociated [64,417,420]. Our data also demonstrate that, although hepatic steatosis may be associated with hepatic insulin resistance on a group level, intrahepatic triglycerides are not strictly sufficient or necessary for the development of hepatic insulin resistance in obese humans (Chapter 10). It appears that the accumulation of triglycerides within inert lipid droplets does not impair hepatic insulin signaling [74,422]. Instead, emerging evidence points to the accumulation of DAG, another lipid species, as an important cause of lipid-mediated hepatic insulin resistance and type 2 diabetes. An increase in hepatic DAG content activates PKCε in genetic or diet-induced obese animal models [60,72,73]. Upon activation, PKCε translocates from the cytosol to the cell membrane, where it phosphorylates the insulin receptor (INSR) and inhibits INSR kinase activity, thereby blocking downstream insulin signaling [414]. More recently, it was shown that the sequestration of DAGs within hepatic lipid droplets protected mice from DAG-mediated PKCε activation [74], suggesting that for DAGs, too, the intracellular distribution determines the biological effects. Hepatic DAG content was correlated with markers of hepatic insulin resistance in 2 previous clinical studies [418,419], but the DAG-PKCε model of hepatic insulin resistance has not been studied in relation to clamp-determined insulin sensitivity. We now demonstrate that DAG accumulation in the hepatic cytosol, but not DAG accumulation in the cell membrane, is increased in humans with impaired insulin-mediated suppression of EGP. Note that DAGs in lipid droplets was not associated with hepatic insulin resistance in mice [74], but we measured DAGs in the entire cytosolic fraction, which may, in part, explain these differences. Obese subjects with hepatic insulin resistance also displayed strongly increased hepatic PKCε activation as reflected by its translocation from the cytosol to the cell membrane. Translation of the DAG-PKCε hypothesis to humans in Chapter 10 supports the development of interventions that target this pathway as novel therapies for the prevention and/or treatment of insulin resistance and type 2 diabetes. Data in Chapters 4 and 5 suggest that people with IFG and obese men, populations that are both characterized by hepatic insulin resistance and increased diabetes risk, are among those who may profit from such interventions.
Ceramides are a family of sphingolipids. Studies have suggested that an increase in hepatic ceramide content or synthesis is the main contributor to lipid-mediated hepatic insulin resistance [415-417], but hepatic ceramide content and hepatic insulin resistance were unrelated in our study (Chapter 10). Others have also reported this dissociation [418,419,429]. In addition, the mechanism by which ceramides may cause impaired insulin signaling is less clear. Previous studies in our lab have shown that intramuscular ceramides do not play a major role in FFA- or glucocorticoid-mediated muscle insulin resistance [453,454]. We now provide evidence to suggest that hepatic ceramides are not the major mediator of hepatic insulin resistance.

In this thesis, we show that fructose consumption and hepatic DAG-mediated activation may both contribute to increased hepatic glucose production and hepatic insulin resistance. There is also evidence to suggest that fructose itself may promote DAG accumulation. Short-term high-fructose feeding in rodents increases hepatic DAG content [337-339] and PKCε activation [340]. Thus, the DAG-PKCε model of hepatic insulin resistance may be 1 mechanism linking fructose consumption to hepatic insulin resistance, although fructose may cause hepatic insulin resistance through additional mechanisms [315]. We are not aware of any human intervention trials that examined hepatic DAG accumulation upon fructose administration, but eagerly await human translation of this paradigm.

Some nuances with respect to the work in this thesis should be made. An important limitation to some of our studies is the observational research design. We acknowledge that cross-sectional studies do not support conclusions on causality. Nevertheless, early translation of basic science and animal discoveries to humans is important, because preclinical studies do not always predict human outcomes [122,123]. Although not perfect, observational data may point to the most relevant or most promising targets for human disease. Here, we have focused on nutrient storage and release pathways in the pathogenesis of obesity-related metabolic disease, but this working model should be appreciated in light of additional mechanisms of insulin resistance [32]. Systemic and tissue-specific regulation of nutrient fluxes may be affected by countless factors, including, but not limited to, inflammatory markers and immune cells [79,455], gut microbes and microbial metabolites [94], gut hormones [95], mitochondrial and endoplasmic reticulum function [96], the brain [210,456], and adipokines such as adiponectin [90] and retinol binding protein 4 [84]. Some of these mechanisms/pathways have shown promise in humans; others have yet to be translated. Nevertheless, given the complexity of human metabolism and insulin resistance, it is unlikely that interventional modulation of a single contributing factor will induce major long-term beneficial effects in humans. Going forward, we advocate an integral approach that takes individual variants and multiple contributing factors into account. In that regard, a systems biology approach may prove to be key.

Finally, with reference to the overall aims of this thesis (Chapter 1), we conclude that insulin resistance presents in many ways, depending on the tissues and cell types that are affected. Conditions, including obesity and fructose overconsumption, that impair
adipose tissue nutrient storage, increase adipose tissue lipolysis, or promote hepatic lipid accumulation give rise to insulin resistance and metabolic disease. Many questions regarding the etiology of these interconnected nutrient flux defects remain to be answered, but these and other data support the development of interventions that promote healthy adipose tissue and decrease ectopic lipid accumulation for the prevention and treatment of insulin resistance and type 2 diabetes.

**FUTURE PERSPECTIVES**

Our findings provide ample leads for future research. The observations described in this thesis illustrate that insulin resistance is no single disease entity. It is a complex, heterogeneous, multifactorial, and dynamic interplay between nutrients, metabolic pathways, tissues, and organs. Genetics and epigenetics also contribute to the metabolic phenotype [457]. This poses a particular challenge to establishing a unifying model for all metabolic defects associated with insulin resistance, but can also be considered an opportunity. Metabolic pathways in the liver, adipose tissue, muscle, and other tissues are all excellent targets for novel therapies. In rodents, selectively promoting adipose glucose uptake [88], restoring DNL in white adipose tissue [320], or reverting adipose tissue inflammation-induced lipolysis [75] have shown to be promising strategies for improving whole-body insulin sensitivity and metabolic health. In liver and muscle, sequestration of harmful lipid species, such as DAG, within lipid droplets protects mice from lipid-induced insulin resistance [74,458]. Others have focused on the stimulation of lipid oxidation in these tissues to prevent or treat ectopic lipid accumulation [459]. These encouraging preclinical results illustrate that selective interventions in 1 of the interconnected nutrient handling pathways may have important systemic effects. However, with research funding ever decreasing and the number of people living with diabetes doubling or tripling in the next few decades [98], we need to ensure that scientific effort is directed at those targets most relevant for human metabolic disease. Clinical studies, like the ones described in this thesis, may provide guidance.

We and others consistently find that adipose tissue dysfunction is a central feature of NAFLD and insulin resistance in humans. Subjects with NAFLD have impaired adipose tissue glucose uptake and lipogenesis and increased lipolysis (Chapter 7). This promotes the diversion of excess nutrients elsewhere and is associated with ectopic lipid accumulation, the accumulation of harmful DAG species, and hepatic and muscle insulin resistance (Chapter 10). On the basis of these observations, we propose that interventions that promote nutrient storage in healthy adipose tissue and reduce their ectopic accumulation will be most effective in preventing or reverting the underlying pathophysiology of insulin resistance and type 2 diabetes in obese humans. Currently, NAFLD is primarily managed by diet and lifestyle recommendations. Both caloric restriction and exercise effectively reduce liver fat, but most patients fail to adhere to such lifestyle regimes in the long term [460]. Pharmacological treatment with insulin sensitizers may improve histological features of steatosis, inflammation, and/or ballooning, but their effect is limited and they are currently only recommended for patients with biopsy-proven non-alcoholic steatohepatitis (NASH) [461]. Several clinical trials of
novel agents for the treatment of NAFLD/NASH are underway, but none of these - to our knowledge - mechanistically target the major pathways (that is, hepatic DNL and hepatic lipid synthesis from peripheral substrate) that are key to the pathogenesis. Metformin is an oral hypoglycemic agent that primarily targets hepatic glucose production/hepatic insulin resistance [215]. This drug is effective in the management of glycemia in type 2 diabetes, but does not revert the underlying pathophysiology and is, therefore, not a cure. We are hopeful that our expanding knowledge of the mechanisms underlying these disorders will lead to the development of more-targeted interventions. Until then, bariatric surgery is the only effective treatment to reverse hepatic steatosis and ameliorate glucose and lipid metabolism in a clinically meaningful way.

From a public health perspective, fructose consumption may prove to be a strategic interest. Chapter 6 demonstrates that the consumption of a high-fructose diet promotes hepatic insulin resistance in nondiabetic humans. As shown in Chapter 7, this may be due to the ability of fructose to acutely promote hepatic lipogenesis and glucose production. Together, these human data raise the intriguing hypothesis that a reduction of fructose consumption has a beneficial impact on the development of hepatic insulin resistance, the incidence of type 2 diabetes, and, by extension, public health. Nowadays, fructose makes up a substantial portion of the Western diet, averaging 49 g/day in the USA [250]. Low-fructose sweeteners, such as glucose, maltose, or rice-malt syrup, are commercially available. To best guide the public, policy makers, and the food and beverage industry on the use of dietary sweeteners, we are in need of large, well-controlled trials to test if low-fructose substitutes are a healthier alternative.

In Chapter 8, we show that fructose ingestion is associated with an acute increase in circulating levels of FGF21. This is the only known - to our knowledge - hormonal response to fructose ingestion, suggesting that FGF21 may mediate an adaptive response to fructose ingestion. In rodents, fructose-mediated FGF21 secretion is dependent on hepatic ChREBP activation and FGF21 action is required for normal hepatic fructose metabolism [434]. The role of FGF21 in human fructose biology is currently unknown. Recombinant FGF21 therapy consistently improves metabolic health in animals [346-348], but obese animals are resistant to the beneficial effects of exogenous FGF21 [354]. In accordance, we found that high levels of FGF21 were associated with obesity and insulin resistance, consistent with the possibility that obese humans are resistant to FGF21’s adaptive role in fructose metabolism. Notably, fructose administration to healthy humans is associated with a decrease in plasma FFA levels, suggesting suppression of adipose tissue lipolysis, but the signaling mechanism is unknown [369]. It will be of interest to determine if FGF21 mediates this antilipolytic effect of fructose. Early trials of FGF21 analogs show promising effects on glucose, lipid, and energy metabolism [462]. When these agents become available, we suggest their effect on lipolysis is also investigated.

The most immediate challenge to widespread assessment of insulin sensitivity in primary and secondary care settings is the fact that insulin assays vary from laboratory to laboratory. For diagnostic tools, like the ones we validated in Chapters 2 and 3, to be reliable, each laboratory will have to carefully calibrate their assay and/or validate their
own set of reference values. Standardization of insulin assays will greatly help universal clinical application of insulin sensitivity (or secretion) cutoff values. The American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD), and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) established an Insulin Standardization Workgroup back in 2004. This workgroup has released recommendations on insulin measurement methods, assay specificity, sample collection, and calibration [154,155,463]. Unfortunately, a standardized insulin assay has yet to be introduced.

Obesity, ectopic lipids, and insulin resistance present another challenge to clinicians: patients with similar clinical phenotypes may have different underlying pathophysiology, and patients with similar pathophysiological features may display different symptoms. For instance, insulin suppresses breakdown of glycogen into glucose via direct hepatic insulin signaling, whereas the mechanism by which insulin suppresses gluconeogenesis is mainly through a reduction in substrate availability by suppressing adipose tissue lipolysis [75]. Defects in both pathways drive hepatic glucose production and hepatic insulin resistance, yet the mechanisms are different. In another example, hepatic lipid accumulation in the context of NAFLD is often associated with DAG accumulation and insulin resistance, whereas hepatic lipid accumulation in patients with familial hypobetalipoproteinemia or the I148M gene variant in PNPLA3 is not associated with DAG accumulation or insulin resistance [64,417]. No 2 patients are alike. If we want to comprehensively improve care for patients with metabolic disease, we need to move towards personalized diagnostic and treatment strategies, including an assessment of individual pathophysiological factors and interventions that target these factors.