From Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

SEX-DIFFERENT CONTROL OF HEPATIC METABOLISM IN RELATION TO INSULIN SENSITIVITY

Carolina Gustavsson

Karolinska
Institutet

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"Our greatest glory is not in never failing,
but in rising every time we fall"
- Confucius

Till
mamma,
Marcus och Andreas

ABSTRACT

The liver is a key metabolic organ. The liver has adapted to the different metabolic needs in men and women, and therefore responds in a sex-specific manner to various stimuli. Specific genes have recently been related to the development of hepatic insulin resistance (IR) and with our improved knowledge of sex differences in fuel metabolism, it may be postulated that the liver has a crucial role in sex-dependent development of IR. To improve the prevention and treatment of hepatic IR, a better understanding of the mechanisms behind sex-differentiated hepatic metabolism is needed. The general aim of this thesis was to investigate if there is a relationship between sex differences in hepatic fuel metabolism and development of IR.

Sex differences in hepatic fuel metabolism were characterized in healthy male and female rats (Paper I). Male rats showed higher ratios of insulin to glucagon levels, higher levels of hepatic glycogen, lower degree of hepatic AMPK phosphorylation, higher expression of hepatic gluconeogenic genes and higher hepatic glucose output, as compared to the females. Effects of short-term high-fat feeding on hepatic insulin sensitivity, gene expression, lipid metabolism and plasma lipids in healthy male rats were shown to depend on the lipid source (Paper II). Safflower oil-enriched diet increased hepatic β-oxidation, was beneficial in terms of circulating VLDL-TG, but led to reduced hepatic insulin sensitivity. Cocoa butter-enriched diet did not affect plasma total TG levels, VLDL-TG or hepatic insulin sensitivity. However, effects observed on hepatic gene expression indicated that prolonged cocoa butter feeding might lead to increased lipid synthesis, and concomitant lipotoxicity, inflammation, and IR. The role of hepatic sex differences in metabolic pathways in the development of glucose intolerance and IR was investigated using Zucker diabetic fatty (ZDF) rats (Paper III). It was shown that high-fat feeding in female ZDF rats lead to a more male-like hepatic phenotype, including reduced lipogenesis, increased FA oxidation and ROS production, while glucose intolerance and IR developed. Sex differences in hepatic metabolic control were also observed at the level of hepatic metabolites (Paper I and III). Metabolite profiles generated from hepatic perfusates from healthy rats using ^{1}NMR spectroscopy verified that male livers exported more glucose than females. Liver-derived lactate was also higher in males, and there was a trend towards higher levels of glycerol and glucogenic amino acids. Testosterone treatment in male ZDF rats reduced hepatic fat content but increased blood glucose levels, reduced glucose tolerance and increased circulating levels of TG-rich VLDL particles (Paper IV). Surprisingly, testosterone reduced STAT3 activity, a key mediator of leptin actions in liver and essential for hepatic insulin sensitivity.

Taken together, these findings suggest that the hepatic functions of female rats might contribute to a lower risk of developing lipid-induced oxidative stress and hepatic IR. Observations of key metabolic transcripts suggest that the capacity of females to retain lipogenesis and the secretion of VLDL-TG might be related to this. We speculate that this together with higher rates of FA oxidation and glucose production in males, might at least partly explain why males are more prone to develop insulin resistance, T2D and the metabolic syndrome.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

AA	Amino acids
ACC	Acetyl-CoA carboxylase
ACOX1	Acyl-coenzyme A oxidase 1
AMPK	AMP-activated protein kinase
Angptl4	Angiopoietin-like 4
AR	Androgen receptor
ARE	Androgen response element
ArKO	Aromatase knockout
ARKO	Androgen receptor knockout
CBD	Cocoa butter-enriched diet
CE-TOFMS	Capillary electrophoresis time-of-flight mass spectrometry
CHREBP	Carbohydrate-responsive element-binding protein
CoA	Coenzyme A
CPT-1a	Carnitine palmitoyltransferase 1a
CVD	Cardiovascular disease
CYP7A1	Cytochrome P450, family 7, subfamily A
E2	Estradiol
ER	Estrogen receptor
ERKO	Estrogen receptor knockout
FA	Fatty acid
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase
FDR	False discovery rate
FFA	Free fatty acid
FGF21	Fibroblast growth factor 21
FoxO	Forkhead box protein O
G6Pase	Glucose-6-phosphatase
GH	Growth hormone
GHR	Growth hormone receptor
GHRH	GH-releasing hormone
GLUT	Glucose transporters
GOT1	Glutamate oxaloacetate transaminase 1
GSH	Glutathione (red)
GSK3b	Glycogen synthase kinase 3b
GSSG	Glutathione (ox)
GST	Glutathione *S*-transferase
HDL	High density lipoprotein
HGO	Hepatic glucose output
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
HNF	Hepatocyte nuclear factor
HOMA-IR	Homeostasis Model of Assessment - Insulin Resistance
IGF	Insulin growth factor

IGFBP	Insulin growth factor binding protein
IR	Insulin resistance
IRE-BP1	Insulin response element-binding protein-1
IRS	Insulin receptor substrate
JAK2	Janus Kinase 2
LDL	Low density lipoprotein
LIRKO	Liver insulin receptor knockout
LMC	Low molecular weight compounds
LXR	Liver X receptor
MAO	Monoamine oxidase
MAPK	Mitogen-activated protein kinase
Mt1	Metallothionein
MUP	Major urinary protein
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non Alcoholic Steatohepatitis
NEFA	Non-esterified fatty acids
NMR	Nuclear magnetic resonance
NQO1	NAD(P)H:quinone oxidoreductase 1
Ob-R	Obese receptor
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1	PPARγ coactivator-1
PI3-kinase	Phosphatidylinositol 3-kinase
Pnpla3	Patatin-like phospholipase domain-containing protein 3 (Adiponutrin)
PPARα	Peroxisome proliferator-activated receptor α
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
SAM	Significance Analysis for Microarray
SCD	Stearoyl-CoA desaturase
SD	Sprague-Dawley
SFA	Saturated fatty acid
SOCS	Suppressor of cytokine signaling
SOD	Safflower oil-enriched diet
SREBP	Sterol regulatory element binding proteins
STAT	Signal transducer and activator of transcription
StD	Standard diet
T2D	Type 2 diabetes
TF	Transcription factor
TG	Triglyceride
TNF-α	Tumor necrosis factor α
VLDL	Very low density lipoprotein
ZLC	Zucker lean control

Abbreviations used only once are described where they appear in the text.

1 INTRODUCTION

Sex is established by the set of XX or XY chromosomes. This genotype is responsible for a sex-specific phenotype including the development of a sex-specific hormonal system. This sex-specific endocrine system includes the central nervous system (CNS), the gonads and several other tissues. The manifestation of a sex-specific endocrine system is not only dependent on the genotype - also environmental and epigenetic factors play a role. Amongst a variety of different sex-different functions, sex differences in metabolism have received attention. Studies on sex-different metabolism are of interest from different perspectives; in some situations e.g. pregnancy there is a need to alter metabolic functions and the incidence of several disorders that can be connected to metabolism have a sex-dependent distribution. Various aspects can be put on the development of sex differences in organisms. In this thesis we put the focus on the existence of sex differences manifested after puberty.

In this situation the sex hormones, androgens and estrogens, regulate functions of metabolically active tissues such as adipose, liver and skeletal muscle [1, 2]. For instance, the regulation of body fat distribution is influenced by changes in gonadal sex hormones, including the adipose-derived hormone leptin, via the hypothalmus [3]. In the liver, metabolism of steroid hormones and drugs has been shown to be sexually differentiated, mainly due to gonadal hormones causing differences in the growth hormone (GH) secretion pattern. Studies in rodents have indicated that sex differences in hepatic steroid metabolism may support a pregnant state when the liver is exposed to high continuous levels of steroid hormones [4]. Thus, sex differences may underlie the development of obesity and other related metabolic disorders like insulin resistance [3]. It is an on-going and important effort to better understand the functional significance of sex differences in metabolism and to put these in relation to disorders that can be coupled to metabolism. **S**ex differences are observed in many common disorders, such as type 2 diabetes (T2D) [5] and the metabolic syndrome [6, 7]. Thus, improved knowledge about the mechanisms contributing to these sex differences may have a substantial impact on the prevention, diagnosis and treatment of these diseases [8-10].

1.1 Sex differences in body composition and metabolism

It is well known that men and women have different body compositions. Men tend to have more skeletal muscle mass [11], different types of muscle fibers [12, 13] and heavier bones than women. Also, the fat depots differ

between the sexes. In general, women tend to have more fat distributed in both muscle and adipose tissue. Furthermore, when fat mass is increased and stored in excess, men are more likely to develop abdominal obesity with visceral fat around the digestive organs, whereas fertile women have a larger distribution of subcutaneous fat in the gluteal-femoral region [9, 14-16]. However, after menopause when estrogen levels decrease in women, these sex differences tend to diminish and a change in fat distribution occurs. A majority of postmenopausal women observe an increase in visceral fat [17-19].

Differences in body composition are presumably due to sex differences in metabolism. Out of the total fat content in a digested meal, men use 56% for energy production via fatty acid (FA) oxidation, 7% is stored in the lower body (subcutaneously) and 14% is stored in the upper parts of the body (visceral). In contrast, women use 41% of the digested fat for oxidation, 22% is stored in the lower body and 25% is stored in the upper body [20, 21].

An upper body/visceral fat distribution in obesity is strongly linked with the metabolic complications of obesity hence the development of metabolic disease. The delivery of excess FFA to the liver from systemic or visceral adipose tissue lipolysis will prevent the normal insulin mediated suppression of glucose output by the liver [22] hence, a reduction in hepatic insulin sensitivity.

1.1.1 Insulin resistance

Insulin resistance (IR) has a strong predictive value with respect to the development of T2D and together with decreased insulin production from β-cells of the pancreas it provides the pathophysiological background for T2D [23, 24]. Initially, β-cells compensate for IR by increasing insulin secretion, leading to hyperinsulinemia. However, as time goes by, the β-cell function becomes altered and fails to compensate for increasing IR and blood glucose levels increase and hyperglycemia develops [25]. Depending on the target tissue, IR has different definitions. Hepatic IR is defined as an impaired ability of insulin to suppress hepatic glucose production through gluconeogenesis or to inhibit glucose output from the liver. Peripheral IR is defined as inhibition of normal insulin stimulation of whole body glucose uptake, e.g. to promote glucose uptake in muscle [26].

IR is a central component in the so-called metabolic syndrome. The definition of the syndrome has been extensively discussed, but in 1998 the World Health Organisation (WHO) recommended a definition of the

metabolic syndrome that includes IR, fasting hyperglycemia, impaired glucose tolerance or T2D, hypertension and hyperlipidemia [27].

IR is reported to be more common in obese men compared to obese premenopausal women. Only within the past decades a great deal of effort has been dedicated to uncover the relevant physiological differences between the sexes that may affect the prevention, diagnosis and treatment of disorders such as T2D. In the case of T2D, it is not clear whether the disorder is more common in one of the sexes. Recent investigations indicate that there is a sex shift within late-onset diabetes. Fertile women seem to be more insulin sensitive than men and therefore protected against T2D. However, after menopause, women develop IR to a greater extent and are no longer protected against T2D and other metabolic problems [28, 29]. Also, T2D affects men and women differently. Reports from population studies conclude that diabetic women suffer more from lipid abnormalities than diabetic men. Women with diabetes also have an increased risk for developing and die from cardiovascular disease [30]. It is evident that sex and sex-related differences are important factors in the understanding of development of metabolic disease.

Interestingly, a number of studies have shown that the highest risk for development of the metabolic syndrome and diabetes occurs in adults who are born small and become overweight in early childhood. It is proposed that the environment surrounding the developing fetus in uterus may permanently influence long-term health and disease in the adult. Intrauterine growth restriction (IUGR), due either to maternal, placental, or genetic factors, may permanently alter the endocrine–metabolic status of the fetus [31]. A temporary IR state can promote survival for a short period of time if needed. However, it may facilitate the development of T2D and metabolic syndrome in adult life [32, 33]. This occurs especially when the intrauterine nutrient restriction is followed by a postnatal obesogenic environment. Furthermore, an energy-rich environment during fetal programming may also drive the development of excess abdominal fat and T2D in later life, demonstrating that both intrauterine nutrient restriction as well as intrauterine nutrient excessive supply may predispose for the development of adult diabetes.

T2D is known to be a multi-factorial disease and resulting from the interaction between specific genes and environmental factors such as diet and physical activity. Today, 27 gene variants have been confirmed to be associated with T2D [34]. Eight of these are expressed in the β-cells and known to be involved in insulin secretion and in the response to increased IR and obesity. One gene is involved in regulating insulin sensitivity, another in glucose transport and two are related to obesity. The functions of

the other genes are still unknown [35]. Interestingly, transcription factor 7-like 2 (*TCF7L2)*, a nuclear receptor for β-catenin, is identified as a risk factor for non-alcoholic fatty liver disease (NAFLD) and a predictor of hepatic IR. This polymorphism also modulates a fat-induced increase in circulating markers of hepatocyte apoptosis in non-alcoholic steatohepatitis (NASH) [36].

The latter finding of a gene variant associated with T2D and related to NAFLD and NASH, further strengthen the connection between hepatic metabolism, hepatic IR and T2D development. Together with our knowledge about sex differences in hepatic fuel metabolism, it may be postulated that the liver has a crucial role in a possible sex-specific development of IR. To improve the prevention and treatment of hepatic IR, a better understanding of the mechanisms behind the sex-specific hepatic metabolism (through investigation of pathways leading to the development of IR) is needed. Studies in human liver are limited since liver biopsies cannot be routinely taken due to the risk of complications. However, with the use of suitable animal models these investigations can still be performed.

1.2 The liver

The liver is a key metabolic organ. It is the primary organ affected by the nutrients and other substances which make the liver a major part of fuel metabolism. The main functions of the liver are storage of glycogen and release of glucose, ketone bodies and lipoproteins [37], i.e. maintenance of carbohydrate and lipid homeostasis. Other important functions of the liver include detoxification, immune response and storage of vitamins.

This key metabolic organ has adopted the different metabolic needs in men and women, and therefore responds in a sex-specific manner to various stimuli. The sexual dimorphism of the liver involving steroid and drug metabolism has been recognized for several decades [38, 39]. Early studies established that growth hormone (GH), acting via a sex-dependent pattern of pituitary hormone secretion, is a major player in establishing and maintaining the sexual dimorphism of hepatic gene transcription that emerges in rodents at puberty [40, 41]. These patterns are ultimately determined by neonatal exposure to testosterone, which imprints the male program of neuroendocrine control of the pulsatile pituitary GH secretion that is first seen at puberty and continues through adulthood [42]. If such an androgen re-programming does not occur, the secretion pattern will remain as the feminine pattern (continuous). The sex differences in GH secretion pattern are most marked in rats, but are also evident in mouse [43] and humans [44, 45]. During more

recent years, novel technologies have been used to describe hepatic sex differences – at least 20% of all hepatic genes have a sex-specific expression pattern [46].

1.2.1 Metabolism

1.2.1.1 Carbohydrates

Glucose is the carbohydrate that is most commonly utilized for energy production in mammals. The brain needs glucose continuously and too low concentrations of glucose in the blood can result in seizures and death. On the other hand, severely elevated levels of blood glucose can lead to tissue damage. Blood glucose levels are therefore carefully maintained (glucose homeostasis). This is accomplished by a strict hormonal system regulating glucose uptake by peripheral tissues and glucose production by the liver. After a meal, blood glucose levels increase and insulin plays a major role in keeping the blood glucose levels in balance, usually between 4-7 mM. The β-cells of the pancreas respond to increasing glucose levels by releasing insulin into the blood. During fasting, liver glycogen is broken down to glucose by glycogenolysis and released into the circulation. Following prolonged fasting, glycogen is depleted and there is an increased synthesis of glucose (gluconeogenesis) in the liver from amino acids and glycerol.

The key enzymes controlling the gluconeogenic pathway are phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). These enzymes are regulated by the substrates and hormones available at that time (mainly insulin, glucagon and glucocorticoids). In times of fasting, stress or exercise; glucagon and/or glucocorticoids induce these enzymes in order to stimulate hepatic glucose production and release. On the contrary, insulin inhibits gluconeogenesis by suppression of these enzymes. Overexpression of either PEPCK or G6Pase in animal models have shown to increase the rate of gluconeogenesis, resulting in hyperglycemia and IR [47]. In patients with T2D, the rate of hepatic gluconeogenesis is considerably increased compared with control subjects, thereby contributing to the characteristic fasting hyperglycemia in diabetes [48].

1.2.1.2 Lipids

There are five major groups of lipoproteins: chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL). Dietary fat is digested and absorbed by the intestine and released into the circulation as triglyceride-rich chylomicrons. Mature chylomicrons are formed in the intestinal mucosa following the absorption of the products of fat digestion.

These large lipoproteins enter the blood via the lymphatic ducts and are transported to peripheral tissues such as the adipose tissue for storage and the liver for break down. In a fasted state, lipolysis in the adipose tissue results in the release of non-esterified fatty acids (NEFA) which are transported and taken up by the liver and other tissues. In the liver, the NEFA will either be oxidized for energy production or re-esterified to form triglycerides (TG). The lipid oxidation takes place in the mitochondria or the peroxisomes. The energy produced by FA oxidation is one of the major energy sources for gluconeogenesis. In times of prolonged starvation, ketone bodies can be produced by FA oxidation and serve as fuel for the brain (instead of glucose). Key factors controlling FA oxidation are carnitine palmitoyltransferase I (CPT-1) and malonyl-coenzyme A (CoA). CPT-1 is an important regulator for the entry of FA into the mitochondria which is inhibited by malonyl-CoA, a substrate for lipogenesis. Re-esterification of NEFA produces TG which are stored in the liver. The triglycerides also serve as a component of VLDL, synthesized by the liver.
The liver also synthesizes FA and cholesterol. A key enzyme for FA synthesis is the acetyl-CoA carboxylase (ACC), which in response to insulin converts acetyl-CoA (involved in glycolysis and amino acid catabolism) to malonyl-CoA. Fatty acid synthase (FAS) condensates both acetyl-CoA and malonyl-CoA to form long chain fatty acids (LCFA). To form cholesterol, acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The rate-limiting enzyme HMG-CoA reductase then converts HMG-CoA into malonate that will be further processed into cholesterol [49, 50].

Triglycerides from both chylomicrons and VLDL are hydrolyzed by lipases that enable free fatty acids to be supplied to peripheral organs through membrane-associated FA transporters. There are at least five potential FA transporters identified, including fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP) and fatty acid transporter protein (FATP). Once in the cell most FAs are noncovalently bound to proteins like FABP and affecting several different metabolic processes. Some of these processes involve regulation of gene transcription leading to changes in cell growth and metabolism [48, 51, 52]. Previous studies have shown that the up-take of LCFA and their incorporation to TG are higher in female livers than in male [53]. These observations can be linked to the concentration of FAT/CD36 and FABPs, which are higher in female livers [54]. Also, female rats secrete more very-low-density-lipoproteins [55].

Transcription factors such as PPARα, LXR and SREBP-1c are also important regulators of lipid metabolism, controlled by different FAs and have been described to also be involved in the regulation of gluconeogenesis [56-58]. PPARα is activated by FAs released in the fasting state, leading to increased hepatic β-oxidation and gluconeogenesis [59]. Estrogens have been

shown to inhibit the actions of PPARα in obesity and lipid metabolism through its effects on PPARα-dependent regulation of its target genes [60].

1.2.1.3 Amino acids

Dietary amino acids (AA) are taken up by the liver via the portal vein, for the purposes of oxidative metabolism, protein synthesis and/or gluconeogenesis, they are not stored or excreted intact. The primary metabolic fate of AA is their conversion to glucose [61] as a substrate for energy production in the brain and peripheral tissues. Amino acids such as alanine and cysteine are all converted into pyruvate through different processes like transamination [62, 63]. Pyruvate serves as a substrate for gluconeogenesis, lipogenesis and ketogenesis. By transamination, amino groups are transferred to alpha-ketobutyrate to create glutamate and keto acids. Glutamate transports nitrogen to the urea cycle where it is oxidized and energy produced.

1.2.2 Sex- different response to metabolic stressors

1.2.2.1 Absorptive state

The absorptive state (postprandial) is the period during which ingested nutrients enter into the circulation and supply the body with energy. Glucose is the major energy source and some of it is converted to glycogen, stored in skeletal muscle and liver. In adipose tissue, glucose is transformed and stored as fat. However, a report shows that women have a higher rate of nutrients appearance than men after a meal and therefore show higher levels of plasma glucose. No differences in insulin or hepatic glucose production have been observed at this time point [64]. This might be explained by the greater muscle mass in men which is correlated to increased glucose uptake. In the same absorptive state, women have lower levels of plasma TG than men [65]. This sex-difference may be explained by the fact that adipose lipolysis after a meal is more suppressed in women, especially in the upper body subcutaneous depots. In the adipose tissue, insulin stimulates lipoprotein lipase (lipid uptake) and inhibits hormone sensitive lipase (lipolysis). Thus, women might be more insulin sensitive and thus have suppressed lipolysis at this time point. As mentioned above, men store more dietary fat in the upper body whereas women tend to have more in the lower body, thus resulting in a sex-specific body composition. Postprandial VLDL production is decreased in both men and women. However, the basal VLDL production is higher in women [10]. Most of the dietary amino acids are used in protein synthesis or oxidized and used for energy production.

1.2.2.2 Post-absorptive state

The post-absorptive state is the period during which the gastric intestinal (GI) tract is empty of nutrients and the body utilizes stored energy. The metabolic homeostasis at this state is entirely maintained by the interplay in uptake and release of metabolites in the different metabolic organs. The plasma levels of most metabolites are similar between the sexes. However, men and women seem to have different approaches to maintain the homeostasis. Women utilize more stored fat as energy source compare to men who primarily use carbohydrates and amino acids. After an overnight fast, women have higher levels of NEFA and ketone bodies than men [64] and the rate of VLDL production and secretion is also higher in females [10].

1.3 Hormonal regulation of hepatic gene expression

1.3.1 Steroid hormones

1.3.1.1 Estrogen

The female sex hormone estrogen is mainly synthesized in the ovaries from the granulose (theca) cells through the synthesis of androstenedione from cholestreol. The enzyme aromatase catalyzes the last step in the formation of estrogens from androgens [66]. There are three major forms of the hormone: estrone (E1), estradiol (E2), and estriol (E3). Estradiol (E2) is the most potent ligand and the predominant form in fertile women. A minor part of the total estrogen produced is synthesized at extragonadal sites such as the brain, liver, bone and adipose tissue [67]. However, in postmenopausal women and men, this amount of estrogen (mainly E1) can constitute a significant part of the total estrogen produced. Several studies show that the aromatization of androgens to estrogens is increased with age and obesity [68]. The aromatase-knockout (ArKO) mouse cannot produce estrogen and as a consequence, have increased intra-abdominal adipose tissue. Also, the ArKO mice develop a fatty liver phenotype which may be related to increased levels of cholesterol [69]. It has also been reported that estrogens increase the activity of cholesterol 7α-hydroxylase (Cyp7a1), the rate-limiting enzyme in bile acid synthesis, in rat hepatocytes [70]. Moreover, mice deficient in the oxysterol receptor LXR ($LXR^{-/-}$) fail to induce transcription of the Cyp7a1*,* and thereby also accumulate cholesterol in the liver [71]. Another study reports impairment in hepatic FA β-oxidation in an aromatase-deficient mouse model. Analyses revealed a decrease in both mRNA expression and activities of specific enzymes (medium chain acyl CoA dehydrogenase (MCAD), very long-chain acyl-CoA synthetases (VLACS), AOX and catalase) required in FA β-oxidation [72].

There are two estrogen receptors: ERα and ERβ [73, 74]. Ligand-activated ERs dimerize and bind directly to estrogen response elements (ERE) [75] or indirectly to other response elements [76]. Depending on the relative abundance and binding affinities of co-regulators, ERs can activate or inhibit target gene expression. Both male and female ERα knockout (ERαKO) mice develop IR and impaired glucose tolerance [77], similar to humans lacking ERα [78] or aromatase [79]. The IR in ERαKO mice is largely localized to the liver [80], including increased lipid content and hepatic glucose production. Genes involved in hepatic lipid biosynthesis are up-regulated, while genes involved in lipid transport are down-regulated in these animals. The hepatic gene expression of the leptin receptor is also lower while the adiponectin concentration was decreased in the ERαKO mice [80]. Interestingly, the expression of lipogenic genes was decreased in diabetic Ob/Ob mice after E2 treatment, supporting a connection between glucose tolerance and the expression of lipogenic genes in the liver [81, 82]. Also, in the livers of high-fat diet fed female mice treated with E2, reduced expression levels of the hepatic key lipogenic gene SCD1 was observed. This was accompanied by decreased hepatic TG content. Importantly, E2 decreased the hepatic expression of G6Pase [83]. Evidently, estrogens and ER play important roles in hepatic lipid and glucose homeostasis.

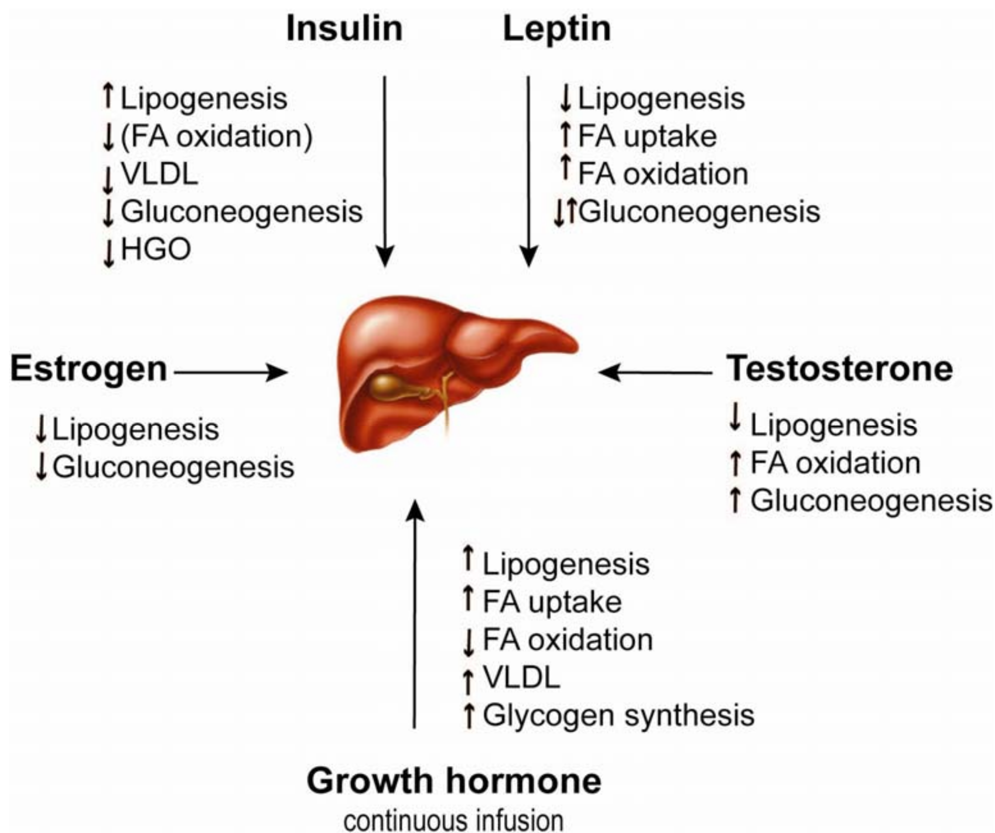

Figure 1. Overview of the actions of selected hormones on hepatic carbohydrate and lipid metabolism.

1.3.1.2 Testosterone

Testosterone (17-hydroxy-4-androsten-3-one) is one of the most prevalent androgens and belongs to the steroid hormone superfamily. Naturally synthesized testosterone is primarily produced from the Leydig cells of testes while a small amount comes from the adrenal glands. The secretion of the hormone is controlled by the hypothalamic-pituitary-testis axis (reproductive hormone axis) [84]. Secreted testosterone binds and activates the intracellular androgen receptor (AR), a member of the nuclear receptor superfamily that functions as a ligand-activated transcription factor [85]. Once activated, AR translocates into the nucleus where it dimerizes and regulate gene transcription either by direct binding to the androgen response element (ARE) on the target gene promoter, or indirect by interacting with other transcription factors [86].

As mentioned earlier, testosterone is important for the imprinting of the male endocrine program, including the pulsatile pituitary GH [42]. A recent study supports the notion that neonatal testosterone exposure contributes to the remodeling of the GH axis and defeminizing hepatic steroid-metabolism, emphasizing the indirect impact of testosterone on hepatic function. Testosterone treatment in newborn female mice results in imprinting of different GH-dependent and sex-specific hepatic genes. The male-predominant genes Cyp2d9 and MUP 1/2/6/8 were not masculinized after treatment, but two female-predominant genes, Cyp2b9 and Cyp2a4, were defeminized [87].

Hepatic AR-knockout (ARKO) male mice (H-AR$^{-/y}$), fed a high-fat-diet, developed hepatic steatosis and IR [88], suggesting that AR plays an important role in preventing such metabolic disorders. H-AR$^{-/y}$ mice show decreased expression of peroxisome proliferator-activated receptor α (PPARα), genes for fatty acid (FA) β-oxidation and FA oxidation rates, increased levels of sterol regulatory element binding protein 1c (SREBP1c) and genes for *de novo* lipid synthesis and hepatic content of TG. However, female ARKO mice do not have this phenotype [88]. Higher levels of estrogens in female animals and ERα-dependent effects on hepatic lipid metabolism might contribute to this sex-difference.

1.3.2 Peptide hormones

1.3.2.1 Growth hormone

Growth hormone (GH), also called Somatotropin, is a polypeptide mainly secreted from the somatotrophs within the anterior pituitary gland. The synthesis of the hormone is regulated by GH-releasing hormone (GHRH). The secretion of GH is regulated by the antagonists: GHRH and GH-releasing

inhibiting factor (somatostatin) [89]. However, the balance of these stimulating and inhibiting peptides is in turn affected by many physiological stimulators (e.g. exercise, nutrients, sleep and sex hormones) and inhibitors (e.g. glucocorticoids, IGF-1 and GH itself by negative feedback) [90-93]. The release of GH into the circulation is characterized by a pulsatile pattern which is sex-specific, especially in rats. In male rats, the GH secretion is more pronounced with distinct peaks every 3-5 hour intervals. In contrast, the GH secretion in female rats are continues with only small changes in plasma concentration [94, 95].

GH binds to its receptor (GHR) and promotes homodimerization of the receptor forming the GH-GHR complex which in turn associates the intracellular Janus Kinase 2 (JAK2). Two associated JAK2 molecules activate each other by trans-phosphorylation leading to phosphorylation of the GHR on multiple tyrosine residues providing docking sites for other signaling molecules of different pathways [96]. The most well studied intracellular pathway involves the signal transducers and activators of transcription (STATs).

One of the most prominent effects of GH is to promote postnatal longitudinal growth in bone [97]. Nevertheless, GH has also been shown to influence a wide range of other important physiological processes in the body. The hormone is known to impact the regulation of metabolism, cell differentiation and maintenance of the immune system [96, 98]. The liver was the first organ identified as a target for GH [96], but today we know that many organs express GHR. In muscle, GH promotes increase in muscle mass by stimulating protein synthesis and amino acid uptake [99]. The effects of GH in adipose tissue includes stimulation of lipolysis and inhibition of lipoprotein lipase activity [100]. Thus, GH is an anabolic hormone that reduces fat mass and increases lean muscle mass. The liver is also a major target for GH. The hormone has effects on hepatic glucose, lipid, amino acid and drug metabolism and the sex-specific secretion pattern has shown to have a great impact on the hepatic transcriptional regulation. Several transcription factors (TF) have been identified as the mediators of this regulation, leading to the GH-dependent sex-specific hepatic gene expression [101, 102]. These TFs include; STAT5b, HNF4α, HNF6 and HNF3β which all are involved in the regulation of the sex-specific CYP genes. In male liver, STAT5b is directly activated by the GH secretion pattern, which also activates the HNF4α. STAT5b binding sites have been found in the promoter of e.g. suppressor of cytokine signaling 2 (SOCS2) [103] and several male-specific CYP genes in rat, including Cyp2c12, Cyp2c11, Cyp2a2 and Cyp4a2. In female liver, HNF6 and HNF3β are increased by the continuous GH secretion pattern [104]. The binding of those two TFs to specific promoters have been shown to induce the expression of for example Cyp2c12. HNF4 and HNF3β have

been shown to regulate genes involved in glucose and lipid metabolism, hence the GH effects on metabolism could be via these TFs [105, 106]. In rats, at least 30% of hepatic sex differences are explained by the female-specific secretion of GH, through the induction of female-predominat transcripts and suppression of male-predominant [107]. Continuous administration of GH has been shown to increase hepatic expression of SREBP-1c and its downstream target genes [108, 109], as well as hepatic TG synthesis and VLDL secretion [110, 111]. Furthermore, PPARα which is male-predominant in rat liver has been shown to be suppressed by continuous GH infusion in hypophysectomized [112] or old [109] rats and by GH treatment in cultured rat hepatocytes [113].

Sex-dependent expression and GH regulation also characterizes other families of genes involved in steroid or xenobiotic metabolism, such as the sulfotransferases and class α and μ glutathione- *S*-transferases (GST) [114].

Animal model	**Investigated sex**	**Effects on hepatic metabolism**		**Reference**
		Lipid	**Carbohydrate**	
ERKO ($ER\alpha^{-}/^{-}$)	both	↑ Lipid content ↑ Lipogenesis	↑ HGO	Heine [115] Bryzgalova [80]
ArKO ($^{-}/^{-}$)	males	↑ Lipid content ↓ FA oxidation		Nemoto [72]
ARKO ($H\text{-}AR^{-/y}$)	males	↑ Lipid content ↑ Lipogenesis ↓ FA oxidation ↑ Lipogenesis		Lin [88]
LIRKO ($IR^{-}/^{-}$)	both		↑ HGO	Fisher [116] Michael [117]
GHRKO ($Ghr^{-}/^{-}$)	males	↑ Lipid content ↑ Lipogenesis ↓ Lipid output		Fan [118]
db/db ($Lep^{db/db}$)	both	↑ Lipogenesis		Tartaglia [119] Sahai [120]

Table 1. Effects of important hepatic hormone receptors on hepatic fuel metabolism in rodents.
Liver-specific knock-out: LIRKO, ARKO and GHR KO.
Whole-body knock-out: ERKO and ArKO.
Natural mutation: db/db.
Estrogen receptor α knock out (ERKO), Aromatase receptor knock out (ArKO), Androgen receptor knock out (ARKO), Liver-specific insulin receptor knock out (LIRKO), Growth hormone receptor-knock out (GHR KO).

1.3.2.2 Leptin

Leptin was first identified after cloning of the mouse and human Ob gene in 1994 by Friedman *et al* [121]. The hormone is one of several adipokines (e.g. TNF-α, IL-6, PAI-1, RBP4) and mainly secreted from white adipocytes. However, several studies show that leptin also can be synthesized in other tissues such as placenta, skeletal muscle and activated stellate cells [122].

Leptin acts primarily on the hypothalamus to modulate food intake, energy expenditure and whole-body energy balance [123, 124]. Originally, the function of leptin was thought to only involve prevention of obesity because leptin-deficient *ob/ob* mice and leptin-resistant *db/db* mice are obese. However, decreased levels of leptin are crucial for metabolic adaptation to starvation [125, 126]. Leptin is also important for the onset of puberty by accelerating the maturation of the reproductive axis [127]. The expression of leptin is known to be regulated by several different factors such as glucocorticoids [128-130], insulin [130] and fatty acids [131].

The OB receptor (OB-R) was cloned in 1995 by Tartaglia *et al* [119] and so far, the leptin receptor (OB-R) is known to be spliced into six isoforms (OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf) [132]. However, the OB-Rb appears to account for the majority of leptin actions [133, 134]. OB-Rb activates the JAK/STAT-signaling pathway (JAK2/STAT3) [135], affecting expression of hypothalamic neuropeptides, thus regulating food intake. The soluble isoform (OB-Re) is a serum leptin-binding protein. Leptin circulates as a free form or OB-Re-bound form, and the sum of the two is generally measured as the total leptin level [136].

In healthy humans, the circulating level of leptin is higher in women than in men [137]. This sex-difference can be explained by the differences in body composition and sex hormones. Women have more subcutaneous fat depots, which is known to produce more leptin, compared to men, who tend to have more visceral fat [138]. Leptin is significantly elevated in obese compared to normal weight humans and rodents [139-141]. Also, experimental data support the idea that testosterone exerts a negative influence on leptin levels in both humans and rodents. Low leptin levels are associated with androgenicity in non-obese men and women [142]. In obese subjects, elevated concentration of circulating leptin fails to promote weight loss. This phenomenon is referred to as “leptin resistance”, the mechanism is today not clear [143, 144]. Suppressor of cytokine signaling 3 (SOCS3) [145, 146] and an increase in serine phosphorylation of Jak2 have been shown to contribute to the blockade of leptin action [147]. In addition, the oxidative stress that is correlated to obesity has also recently been shown to be a contributing factor to leptin resistance [148].

In the liver, leptin is known to stimulate FA oxidation and prevent the accumulation of lipids by inhibiting the expression of SREBP-1 [149]. In ob/ob mice, leptin has also been shown to repress hepatic mRNA levels and enzymatic activity of SCD1, which is important in the biosynthesis of monounsaturated fatty acids. *ob/ob* mice with mutations in SCD1 had histologically normal livers with significantly reduced triglyceride storage and VLDL production [123]. A recent study show that leptin treatment in male diabetic ob/ob mice, induce the hepatic mRNA expression of insulin growth factor binding protein (IGFBP) 2 independently of its ability to alter body weight. This induction of IGFBP2 significantly improved the insulin sensitivity, glucose tolerance and hepatic glucose production [150], implicating a new therapeutic regime for T2D.

1.3.2.3 Insulin

In year 1921, scientists Banting and Best discovered insulin, a hormone produced and secreted from pancreatic β-cells of Langerhan´s islands in response to increased blood glucose after digestion of food [151]. Most cells of the body have insulin receptors attached to its surface to which insulin can bind and initiate an intracellular signalling cascade. Binding of insulin to its receptor results in the activation of two main pathways: (a) the phosphatidylinositol 3-kinase (PI3-kinase) pathway and (b) the mitogen-activated protein kinase (MAPK) cascade [48].

The insulin receptor is a heterodimeric receptor with intrinsic tyrosine kinase activity, which undergoes a conformational change in response to insulin binding. Upon ligand binding, the kinase is activated and phosphorylation of several tyrosine residues within the cytoplasmic domain of the receptor takes place. Activation and tyrosine phosphorylation of the receptor leads to the recruitment of several docking molecules including insulin receptor substrate proteins (IRS) 1-4. These interactions result in a spectrum of intracellular responses by activating downstream adaptor molecules such as p85 regulatory subunit of PI3-kinase and growth factor receptor-bound protein 2 (Grb2). The activation of PI3-kinase leads to phosphorylation of Akt (also konown as protein kinase B) at different sites (Ser, Thr). Insulin-dependent stimulation of e.g. glycogen synthesis occurs through Akt-dependent phosphorylation (inactivation) of glycogen synthase kinase 3 (GSK-3), an inhibitor of glycogen synthase, leading to enhanced glycogen synthase activity. This is one example of how this signaling pathway regulates glucose, lipid and protein turnover [48, 152-154]. Activation of the MAPK pathway results in the activation/phosphorylation of specific transcription factors regulating cell proliferation and differentiation [48]. Liver insulin receptor knockout (LIRKO) mice display severe hepatic IR and a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression [116, 117]. This observed

hepatic IR in the LIRKO mice leads to hyperinsulinemia and secondary extra-hepatic IR.

In liver, insulin stimulation results in enhanced utilization and storage of glucose as lipid and glycogen while repressing glucose release and synthesis by inhibiting the key enzymes for gluconeogenesis (PEPCK and G6Pase) [48] and VLDL production [155, 156]. The transcription factor SREBP is known to up-regulate genes involved in lipogenesis and down-regulate FA-oxidation in response to insulin [157, 158]. Other transcription factors like Insulin-response element binding protein-1 (IRE-BP1) and FoxO1 are regulated by insulin signaling via Akt-mediated phosphorylation. IRE-BP1 inhibits the expression of specific gluconeogenic genes and genes involved in FA oxidation. The transcription factor FoxO1 mediates negative effects of insulin [159] on hepatic gluconeogenesis.

Phosphorylation by Akt results in the sequestration of FoxO1 in the cytoplasm, leading to reduced expression of its target genes. The opposite is the case for IRE-BP1, where impaired Akt signaling leads to cytoplasmic sequestration and de-regulated gene expression of target genes [160]. Taken together, hepatic IR leads to decreased hepatic glucose uptake, increased VLDL production and increased glucose production and output.

1.4 Oxidative stress

Oxidative stress is caused by a disturbance in the balance between the antioxidant defense capacity of the body (reduced) and the production of reactive oxygen species (ROS) (increased). This imbalance is closely associated with a number of diseases including cancer, neurodegenerative diseases, cardiovascular diseases and diabetes [161].

Endogenous ROS can be produced from many systems in cells including the mitochondrial respiratory chain [162], the cytochrome P450s [163, 164], oxidative enzymes such as xanthine and monoamine oxidase (MAO), the NADPH oxidase complex [165], autooxidation of heme proteins such as ferrohemoglobin and myoglobin or substances such as catecholamines. In addition, ROS levels can be increased by environmental sources including radiation, UV light, smoke and chemicals [166].

The mitochondrial respiratory chain normally produces superoxide (O_2^-) and peroxide (O_2^{2-}) that are necessary for cellular functions. Excess superoxide is neutralized by endogenous antioxidants and antioxidant enzymes. However, superoxide can be converted to hydrogen peroxide (H_2O_2) which can produce

extremely reactive hydroxyl radicals (OH^-) which cannot be eliminated by an enzymatic reaction [162].

Because ROS production is a naturally occurring process, a variety of enzymatic and non-enzymatic mechanisms have evolved to protect cells against ROS (Yu 1994). Protective enzymes include the superoxide dismutases (SODs) that remove O_2^-; catalase and the GSH peroxidase system that remove H_2O_2; glutathione transferases that can remove reactive intermediates and lipid aldehydes, metallothioneins, heme oxygenase, thioredoxin that remove various ROS. The non-enzymatic protection includes low molecular weight antioxidants such as GSH itself, vitamin E, ascorbate (vitamin C), retinol (vitamin A), ubiquinone, uric acid and bilirubin [167, 168].

1.4.1 Obesity-induced diabetes

Several mechanisms have been proposed to cause oxidative stress and consequently the development of IR and T2D in animal models and humans [169]. An increase in the production of ROS has been associated with fat accumulation in the liver subsequently leading to non-alcoholic fatty liver disease (NAFLD) and hepatic IR [170], see figure 2 [171].

The exact mechanism for the increased ROS production in the liver is not clear but an increased uptake of NEFA by the hepatocytes might lead to mitochondrial β-oxidation overload which may exhaust the antioxidant defense (including GSH and glutathione transferases) and induce oxidative stress. Schulman and co-workers have demonstrated that intracellular accumulation of TG and FA intermediates in muscle and liver is associated with IR in humans [172] and that mitochondrial dysfunction leads to decreased FA β-oxidation and fat accumulation in skeletal muscle or liver [173]. Also, ROS have been shown to induce various signaling pathways involving FoxO1, MAPK, JAK/STAT, p53, phospholipase C and PI(3)K. For example, ROS-induced hepatic IR has been shown to be mediated via JNK [170], known to be activated by oxidative stress [174].

1.5 Metabolite profiling in metabolic disease

Metabolites are the building blocks for all other biochemical substrates and structures including genes, transcripts and proteins. Metabolites are defined as low molecular-weight compounds (LMC) and can either be organic or inorganic. The LMC are the reactants, intermediates or products of enzyme mediated biochemical reactions. General classifications of metabolites

involve molecular weight, polarity and structure or reaction similarity. The most frequently applied method is similarity where metabolites are classified according to chemical core structure (e.g. amino acids) or by involvement in the same metabolic pathway (e.g. glycolysis). In contrast to genomics, transcriptomics and proteomics, metabonomics is a newly developed technique that has been described as the recognition and measurement of the entire metabolic reaction of an organism in response to internal or external influences [175]. Metabolites are intermediates in signaling pathways that can regulate gene expression [176]. For example, FAs act as ligands for several of the PPAR nuclear receptors in the liver. Metabolite abundance reflects a biological response to exogenous and endogenous inputs, and when investigating pathways from genotype to phenotype, metabolites can provide a powerful complement to gene expression data and give novel insights into disease pathogenesis mechanisms (see figure 3). It is regarded as a diagnostic tool that is useful for metabolic classification of individuals. The great asset of this technique is that it involves the quantitative, noninvasive analysis of easily sampled human body fluids such as urine, blood and saliva. Analysis of tissue extracts is also possible.

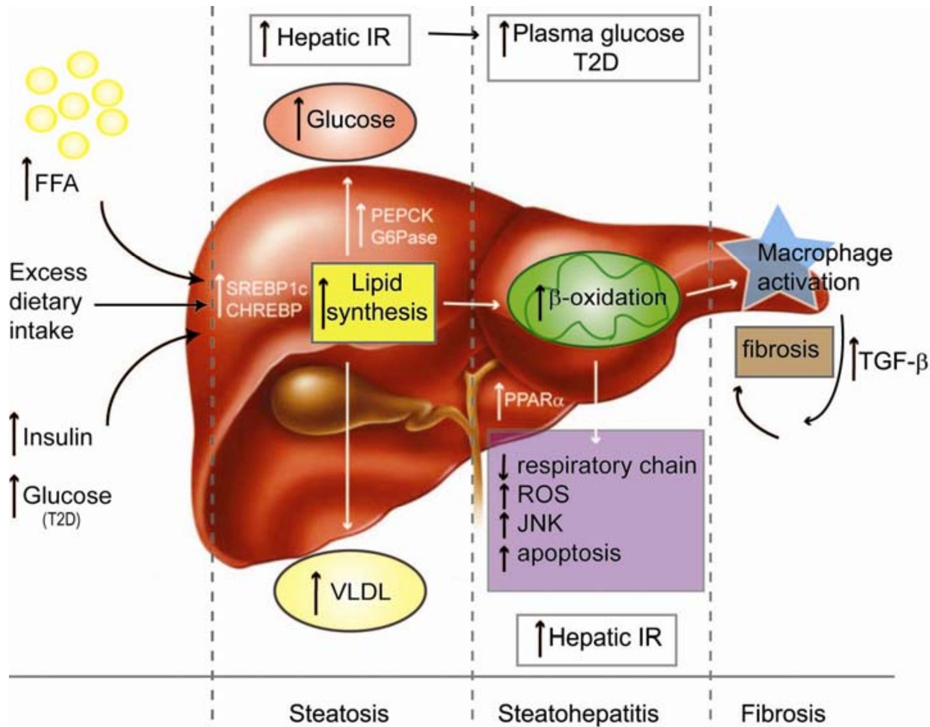

Figure 2. Proposed model for the development of hepatic IR in NAFLD.
Remodeled from Cusi, 2009 [171].

1.5.1 Serum metabolites

Measurement of blood glucose levels is a standard procedure in the diagnosis of metabolic disease such as T2D. Elevated levels may indicate hyperglycemia due to peripheral IR and impaired glucose uptake, or increased hepatic glucose output due to hepatic IR. Similarly, altered levels of other metabolites in plasma may reflect the metabolic profile of the whole-body or a specific tissue. During the last decade, metabolic profiling of body fluids has emerged as a research tool for the search of biomarkers which can potentially be used for prevention and/or to follow responses to therapeutic interventions. A recent study demonstrated the potential of such a technique. Plasma profiles of subjects with NAFLD and NASH, generated using an untargeted global metabolomic analysis, were used to identify disease-specific LMC patterns and candidate biomarkers [177].

1.5.2 Liver-derived metabolites

Metabolites derived from the liver may predict the metabolic state of the organ. Thus, liver-derived metabolites may be of importance for diagnosis of metabolic disorders related to the liver. For example, several reports link changes in the levels of hepatic oxidative stress and circulating amino acids to T2D. In a recent study, metabolite profiles of plasma demonstrates elevated levels of several amino acids such glutamate, lysine, tyrosine, and isoleucine in human subjects with steatosis and/or NASH [177]. The higher level of glutamate in plasma is speculated to be due to increased transamination of amino acids being degraded in the liver. The human serum metabolome has also been shown to depend on e.g. age and sex [178]. Whether age- or sex-related differences in hepatic metabolism can be revealed using metabolomics is yet to be determined.

Ophthalmate

The tri-peptide ophthalmate (glutamate-2-aminobutyrate-glycine) has been proposed as a biomarker for oxidative stress. By determining metabolic profiles through capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), Soga *et al.* detected global changes in metabolite levels in the liver and serum of acetaminophen (AAP)-treated male mice (analgesic). Significant increases in ophthalmate levels after treatment were observed. Because of its function as a sensitive indicator of hepatic glutathione (GSH) depletion, ophthalmate was identified as a biomarker that can reveal acute liver injury [179, 180]. Ongoing clinical investigations will determine the usefulness of measuring the concentration of plasma GSH and ophthalmate in patients with various liver diseases [180].

Betaine

Betaine is a well-studied metabolite known to protect against the development of hepatic steatosis in animal models [181]. Betaine has been shown to reduce toxic levels of homocysteine (Hcy), a marker for many diseases including cardiovascular disease (CVD) [182]. It has also been shown that supplementation of betaine to high-carbohydrate (sucrose) diet-fed male mice decreased hepatic fat accumulation. This was mediated by direct activation of the hepatic AMP-activated protein kinase (AMPK) system and subsequent inhibition of *de novo* lipogenesis in the liver [183]. An improvement of hepatic steatosis and IR due to betaine supplementation has also been reported in male mice fed a high-fat diet for 12 weeks [184]. In a long-term moderate high dietary fat model of NAFLD in mice, betaine treatment prevented and treated the condition of fatty liver. Betaine also significantly improved the hepatic IR by increasing the activation of IRS1, with resultant improvement in downstream signaling pathways [185]. Interestingly, the control of betaine metabolism has been shown to differ between male and female non-diabetic patients [186].

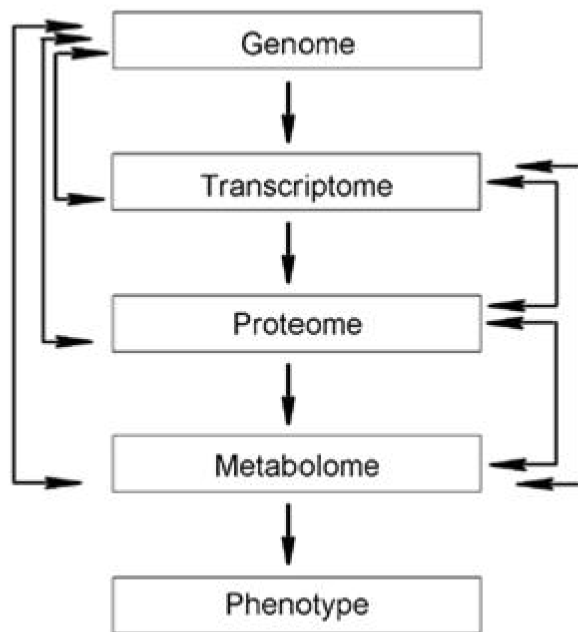

Figure 3. Omics organizational relationship.
General schematic of the "-omics" organisation where the flow is from genes to transcripts to proteins, with metabolites being the link to phenotype. Metabolite abundance reflects a biological response to stimuli and when investigating pathways from genotype to phenotype, metabolites can provide a powerful complement to gene expression data and give novel insights into disease pathogenesis mechanisms

2 AIMS

The general goal of this thesis was to investigate if there is a relationship between sex differences in hepatic fuel metabolism and the development of insulin resistance.

To achieve this goal, more specific aims were formulated:

A. To characterize sex differences in lipid and carbohydrate metabolism in rat liver.

B. To investigate the effects of short-term high-fat feeding on hepatic metabolism and insulin sensitivity in rats.

C. To investigate if differences in liver functions could provide clues to sex-different development of insulin resistance and glucose intolerance in Zucker diabetic fatty (ZDF) rats.

D. To determine the extent of which sex differences in hepatic gene expression are translated into sex-different levels of low molecular-weight compounds.

E. To evaluate the effects of testosterone treatment on glucose and lipid metabolism in male ZDF rats.

3 METHODS

This section gives a general commentary of some of the methods used in our studies presented in this thesis. More detailed descriptions of the separate methods can be found in the material and methods sections of paper I-IV.

3.1 Animals

The Zucker diabetic fatty (ZDF/Gmi™-fa) rat has become a widely used animal model for studying T2D. The Zucker diabetic fatty (ZDF) rats have a mutation in the *fa* gene which results in impaired leptin receptor signaling, obesity and IR. However, male and female rats of this strain show differences in the development of T2D. The males spontaneously develop hyperlipidemia and hyperglycemia by 8 weeks of age and diabetes by 12 weeks whereas the females only become diabetic when fed a diabetogenic (high-fat) diet [187]. The mechanistic reasons for this sex-different phenotype is not clear, thus further studies are requested. In paper III, we wanted to investigate the sex-different development of T2D, and therefore we used the ZDF rats as a model.

3.2 Transcript profiling

3.2.1 Microarray

The microarray methodology is used to determine transcript profiles. The microarray technique was developed in the late 1990s and is now an established method for gene expression analysis [188]. There are a number of different platforms for transcript profiling. The most common ones are spotted arrays and Affymetrix GeneChips® [189]. In this thesis, we used spotted arrays and the following description will therefore only describe the principles concerning that type of array. For paper I, II and III we used non-commercial microarrays manufactured at the Royal Institutet of Technology (KTH), whereas in paper IV we used the commercialized Agilent platform.

All experiments included in this thesis were standardized according to the Minimum Information About a Microarray Experiment (MIAME) [190].

3.2.1.1 Experimental design

Due to the cost, time and amount of data that the experiment will generate, one of the most important parts of a microarray experiment is to address a specific biological question. Depending on the biological question and what type of samples that will be used, there are different ways of designing the experiment in order to achieve the best results. The design of an experiment

from which one wants to generate a list of differently expressed genes comparing two treatments (e.g. untreated vs. treated) differ from the design of an experiment where the aim is to study different states in a disease development depending on the effect of time.

One of the features to consider while designing an experiment is the use of one- or dual-color arrays. One-color arrays will provide an absolute value of the expression of the analyzed sample. This provides the opportunity to more freely compare different groups and if desired, add more samples to the analysis later on. In one-color experiments, the use of dye Cy3 is mostly used due to its stability. A dual-color experiment results in a ratio between two samples that are hybridized together on the array, labeled with either Cy3 or Cy5 e.g. treated versus untreated. This approach is more limiting since new groups of samples cannot be added without altering the primary design. However, if you have a clear biological question and an isolated experiment, this approach can be more cost-efficient since two samples are analyzed at the same time. In all studies included in this thesis (paper I-IV) we used the dual-color approach. The design is also depending on the type of samples. Samples from a cell-line are known to be more homogenous compared to human and animal samples from e.g. tissues which often show high individual differences. There are different ways to compare the different sample groups (direct, indirect or loop design) in order to get the best comparison.

Biological replicates are important to minimize the influence of individual variations. Because of the large amount of transcripts that are being measured in each experiment, there will always be variations between individuals even if they are treated in the same way. Technical replicates are essential for minimizing the influence of sample preparation, labeling, hybridization or quantification. The minimum numbers of replicates that are required to except the statistical analysis outcome have to be considered for each individual experiment. Generally, the more replicates the better, but it has been shown that trustworthy results can be obtained with only a few biological replicates [191]. In this thesis, at least four biological replicates were analyzed.

3.2.1.2 RNA quality

The quality of the RNA samples should always be checked using a Bioanalyzer or with a method equal to that. The Bioanalyzer consider the 28S/18S ratio as well as the RNA Integrity Number (RIN) of the RNA sample [192]. The RIN had not been commercialized when we performed our first microarray experiments described in this thesis. For paper I-II, only the 28S/28S ratio (>1.8) was considered, whereas for the RNA samples used in paper III and IV, a RIN above 8 [193] was considered acceptable for further analysis.

3.2.1.3 Labeling and hybridization

Depending on the platform, different protocols for labeling and hybridization of the samples are available. In short, total RNA is amplified and incorporated with fluorescence-labeled nucleotides in a first strand cDNA or cRNA synthesis (depending on platform and protocol). The labeled samples are purified from unincorporated nucleotides and applied to the array for overnight hybridization. The hybridization normally takes place in a hybridization chamber put in a water bath or hybridization oven. After hybridization, the arrays are washed and scanned. Microarray scanners contain lasers that excite the fluorophores at different wavelengths. The scanning creates an image of the array which is used for further data analysis. See figure 4.

For paper I, II and III we used non-commercial (generic) microarrays manufactured at the Royal Institute of Technology (KTH). Therefore, we used a labeling and hybridization protocol suitable for this purpose. In paper II, the effect of different high-fat diets were studied in male SD rat livers. Samples from control animals (standard diet) were hybridized with samples from rats fed a high-fat diet. In paper I, we studied the sex differences in healthy male and female SD rats. Samples from male rat livers were hybridized with female samples on individual arrays in order to get the direct effect of sex on hepatic gene expression. In paper III, we investigated the sex-different development of IR in ZDF rats. To identify high-fat diet-induced changes, healthy females (fZDF) were hybridized together with high-fat diet induced diabetic (HF-fZDF) females. To find sex-dependent hepatic gene products that might be related to disease development, fZDF and diabetic males (mZDF) were hybridized together on each array. In paper IV, the microarray experiment was performed using the Agilent platform. The effect of testosterone treatment on hepatic gene expression was investigated in male lean and obese ZDF rats by hybridizing an individual untreated sample with TU-treated sample onto the array.

3.2.1.4 Data analysis and statistics

Processing of microarray data include annotation of spots, background correction, normalization and transformation of raw data into expression values.

For the arrays (KTH) used in paper I, II and III, image analysis was performed using the GenePix software (Axon Instruments). The fluorescence ratios of the detected spots were normalized with the Locally Weighted Scatter Plot Smoother (LOWESS) method using the statistical language R. Identification of differentially expressed genes was performed using the Significance Analysis for Microarray (SAM) software developed at the Stanford University [194]. For the arrays in paper IV, images were

annotated using the Feature Extraction software developed by Agilent Technologies. Further data analysis and identification of significantly expressed genes were performed with GeneSpring, also developed by Agilent Technologies.

A 5% false discovery rate (FDR) was used as a first cut-off. Genes with a greater than 1.5-fold increase or decrease were considered as being regulated, even though smaller changes in gene expression may also have important biological consequences [107]. The results are represented as the mean of at least three independent determinations.

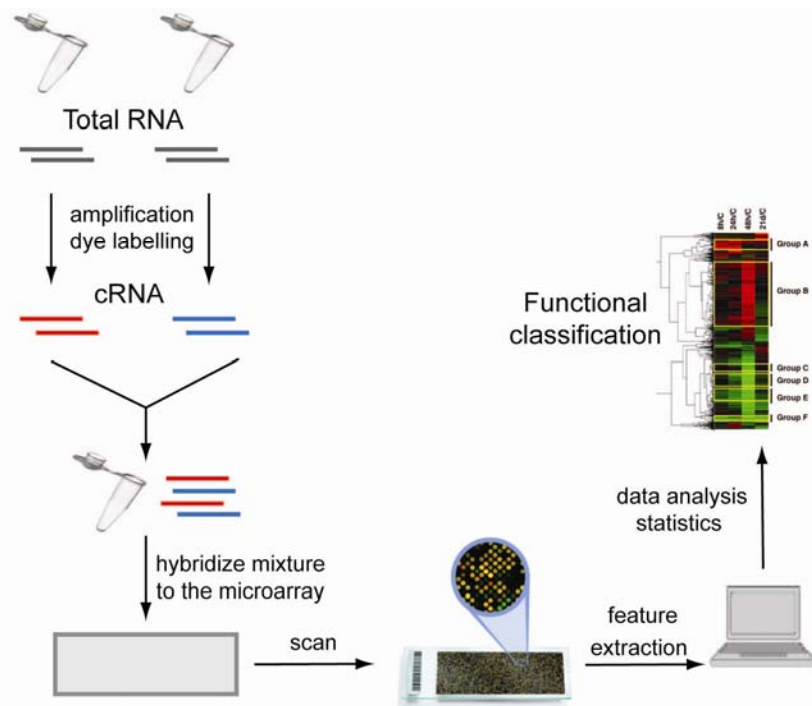

Figure 4. Flow chart for microarray analysis.
Total RNA is amplified and incorporated with fluorescence-labeled nucleotides in a first strand cRNA synthesis. The labeled samples are purified and applied to the array for hybridization. After hybridization, the arrays are washed and scanned. The scanning creates an image of the array which is used for further data analysis

3.2.2 Real-time quantitative PCR

There are different methods for validating the results achieved from microarray experiments. The most commonly used method today is real-time quantitative PCR. This method allows you to study the expression of a number of selected transcripts and compare to array data. Real-time PCR is more sensitive, specific and faster to perform compared to microarray.

Currently, four different types of real-time PCR chemistries are available; TaqMan® (Applied Biosystems), Molecular Beacons, Scorpions® and SYBR® Green (Molecular Probes). They all detect PCR products through the generation of a fluorescent signal. TaqMan probes, Molecular Beacons and Scorpions generate the fluorescence signal via the coupling of a fluorogenic dye molecule and a quencher to the same or different oligonucleotides. SYBR Green is a fluorogenic dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA. In this thesis, all of the microarray experiments were confirmed by selecting key genes for quantification by real-time PCR using the SYBR® Green technique.

In short, RNA is reversed-transcribed into cDNA. The SYBR Green dye binds to the double-stranded DNA, which enhances the fluorescence substantially, and the emission can be recorded after each cycle of amplification. In this thesis, the transcript level was determined by the cycle threshold, at which the amplification rate is switched from lagging phase to the exponential phase. The detected levels were normalized to the level of corresponding reference gene of each sample. In order to monitor the primer efficiencies, relative standard curves were constructed by dilution series of pooled cDNA representing all individual RNA samples included in the analysis. The obtained data were statistically analyzed by performing analysis of variance (ANOVA) followed by Fisher's post-hoc analysis and expressed as means ± SE.

3.3 Metabolite profiling

While findings in gene expression only reflect the genotype of a system in a transcriptional network, knowledge of the posttranslational alterations and alterations in metabolite levels reveal the actual phenotype. To understand the complete system of a cell, it is essential to link the expression of transcripts and proteins with cellular metabolites. Our ability to compare the transcript profiles with the metabolite profiles in paper I and III, provided new knowledge about hepatic sex differences in metabolism.

There are a number of different platforms used for metabolite profiling. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are the most widely used techniques for metabolite profiling although they differ in specificity and sensitivity. NMR can measure simultaneously all kinds of metabolites. However, NMR has limitations regarding sensitivity and should therefore only be applied to samples with high concentration of compounds. For enabling detection of compounds with low concentration, more sensitive methods should be considered such as MS. Usually, mass spectrometry is used to identify and quantify metabolites after separation by different techniques. The most established combinations of separation and quantification techniques are: gas chromatography/mass spectrometry (GC/MS), liquid chromatography/mass spectrometry (LC/MS), Fourier transform ion cyclotron resonance/mass spectrometry (FTICR-MS) and capillary electrophoresis/mass spectrometry (CE-MS) [179, 195, 196].

In this thesis, ^{1}H-NMR spectroscopy was used for metabolite profiling of liver perfusates (paper I), whereas CE-TOFMS was used to detect metabolites in liver extracts (paper III).

3.3.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is a nondestructive, high-throughput technique. It has been widely employed in chemistry, particularly for distinguishing isomers, as well as in molecular conformation and in studies of molecular dynamics. Recently, NMR spectroscopy methods have been used to identify metabolites through the interpretation of NMR spin relaxation times and determination of molecular diffusion coefficients. When placed in a strong magnetic field, each chemically distinct proton within a solution exhibits a unique chemical shift that varies according to the extract stereochemical environment that surrounds that proton. The intensity of this signal depends on the concentration of the proton, and hence of the metabolite, in the solution. Through assignment of each chemical shift to a metabolite and analysis of relative changes in signal intensity between disease and control samples, changes in metabolite concentration can be monitored simultaneously for a wide range of metabolites.

In paper I, we used ^{1}H-NMR spectroscopy to investigate the possibility that low molecular weight compounds might be leaving the liver (perfusate) in a sex-dependent manner in healthy SD rats.

3.3.1.1 Reference compounds

Reference compounds serve as internal controls for normalizing the technical variation between the samples. In paper I, Hexa deutero-4,4-Dimethyl-4-silapentane-1-ammonium trifluoroacetate (DSA) was used as an internal control, enabling absolute concentrations of metabolites to be determined.

3.3.1.2 NMR analysis

The NMR spectroscopic measurements were made on a Bruker 600 MHz instrument (Bruker BioSpin, Rheinstetten, Germany) operating at 600.23 MHz, equipped with a 5 mm inverse probe and a SampleJet sample changer. 1H-NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo sequence to attenuate broad signals arising from macromolecular components [197]. 512 transients were acquired into 64 K data points using a spectral width of 12019 Hz with a spin echo loop time of 76.8 ms and relaxation delay of 2.0 s with a total repetition time of 4.82 s. Suppression of the water resonance was achieved by presaturation during the relaxation delay and the spin-echo loop.

3.3.1.3 Data analysis and statistics

The time domain data were processed using a exponential window function with a line broadening factor of 0.3 Hz and zero-filled to yield a real spectrum of 64 K data points in the frequency domain after Fourier transformation. The spectra were manually phase corrected using Topspin 1.3 (Bruker Biospin) and imported into Chenomx NMR Suite 5.1 (Chenomx Inc., Edmonton, Canada) for further analysis. Spectra were baseline corrected and referenced to the DSA peak [198, 199]. Metabolite signals were identified and quantified using the metabolite library in the software [200]. The ^{1}H-NMR metabolite data with low quantification quality, normality could not be assumed and Friedman's non-parametric test was utilized to determine effect of insulin treatment or sex. The groups were also compared using Student's t-test. Differences between groups were considered significant at $p<0.05$.

3.3.2 Capillary electrophoresis – mass spectrometry

Capillary electrophoresis mass spectrometry (CE-MS) enables separation (CE) and quantification (MS) of low molecular-weight hydrophilic compounds such as amino acids and carbohydrates.

Separation by CE is accomplished by electrical force applied onto the ion, which in turn depends on the charge and on the flow resistance. At a low pH, the amino groups are protonated, and protons in general are the sole source of charge under these conditions. The position of each peptide in a

CE-separation can therefore be calculated with perfect accuracy if its mass and the number of basic amino acids are known [201]. Its main advantages are the robustness, the simple separating principle with high reproducibility. Furthermore, CE-MS enables reproducible and comparable analysis of highly complex samples.

In this thesis, we used CE-TOFMS to evaluate if the overlapping set of differentially expressed genes could be related to liver-derived intermediary metabolites that differentiate male and female ZDF rats regarding their susceptibility to develop diabetes, metabolite profiling on liver extracts was performed (paper III).

3.3.2.1 Reference compounds

In the paper III, L-methionine sulfone and D-camphor-10-sulfonic acid (CSA) were used as references for cations whereas 2-morpholinoethane-sulfonate (MES) served as reference for anions.

3.3.2.2 Extraction

Metabolites were extracted from liver by homogenization in methanol. The homogenate was mixed with Milli-Q water and chloroform (2:5) and centrifuged. The upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter to remove proteins. Finally, the filtrate was concentrated by centrifugation and dissolved in Milli-Q water containing reference compounds prior to CE-TOFMS analysis.

3.3.2.3 CE-TOFMS analysis

All CE-TOFMS experiments were performed using an Agilent CE capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany), an Agilent G3250AA LC/MSD TOF system (Agilent Technologies, Palo Alto, CA, USA), an Agilent1100 series binary HPLC pump, and the G1603A Agilent CE-MS adapter- and G1607A Agilent CE-ESI-MS sprayer kit. For system control and data acquisition we used the G2201AA Agilent ChemStation software for CE and the Analyst QS for Agilent TOFMS software. CE-MS/MS analyses for compound identification were performed on a Q-Star XL Hybrid LC-MS/MS System (Applied Biosystems, Foster City, CA. USA) connected to an Agilent CE instrument.

CE-TOFMS conditions for cationic metabolite analysis: Separations were carried out in a fused silica capillary (50 μm i.d. x 100 cm total length) filled with 1 M formic acid as the electrolyte [202]. Approximately 3 nl of sample solution were injected at 50 mbar for 3 s and 30kV of voltage was applied. The capillary temperature was maintained at 20°C and the sample tray was cooled below 5°C. Methanol-water (50% v/v) containing 0.5 μM reserpine was delivered as the sheath liquid at 10 μl/m. ESI-TOFMS was operated in

the positive ion mode and the capillary voltage was set at 4,000V. A flow rate of heated dry nitrogen gas (heater temperature 300°C) was maintained at 10 psig. In TOFMS, the fragmentor, skimmer, and Oct RFV voltage were set at 75V, 50V, and 125V, respectively. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards. The methanol adduct ion ([2MeOH+H]+, *m/z* 65.0597) and reserpine ($[M+H]^+$, *m/z* 609.2806) provided the lock mass for exact mass measurements. Exact mass data were acquired at a rate of 1.5 cycles/s over a 50-1,000 m/z range [179]. *CE-TOFMS conditions for anionic metabolite analysis:* A chemically coated with a cationic polymer-coated COSMO (+) capillary (Nacalai Tesque, Kyoto, Japan) was used as the separation capillary. A 50-mM ammonium acetate solution (pH 8.5) was used as electrolyte solution for CE separation [203]. Sample solution (30 nl) was injected at 50 mbar for 30 s and -30kV of voltage was applied. Ammonium acetate (5 mM) in 50% methanol-water (v/v) containing 0.1 μM Hexakis was delivered as the sheath liquid at 10 μl/m. ESI-TOFMS was conducted in the negative ion mode; the capillary voltage was set at 3,500V. For TOFMS, the fragmentor, skimmer, and Oct RFV voltage were set at 100V, 50V, and 200V, respectively. A flow rate of drying nitrogen gas (heater temperature 300 °C) was maintained at 7 L/min. Automatic recalibration of each acquired spectrum was performed using reference masses of reference standards ([^{13}C isotopic ion of deprotonated acetic acid dimer $(2CH_3COOH\text{-}H)]^-$, m/z 120.03841), and ([Hexakis + deprotonated acetic acid $(CH_3COOH\text{-}H)]^-$, m/z 680.03554). Exact mass data were acquired at a rate of 1.5 spectra/s over a 50-1000 m/z range. Other conditions were described in a previous report [203].

3.3.2.4 Data analysis and statistics

Processing of metabolite data includes identification of metabolites, baseline subtraction, dataset normalization and alignment, visualization on 2D plots (m/z and time) and detection of significant metabolites.

In our study presented in paper III, data analysis was performed using the proprietary software called MasterHands (Institute for Advanced Biosciences, Keio University, Japan) [204, 205]. Briefly, the peaks were detected from sliced electropherograms (m/z 0.02 width), and accurate m/z values were calculated by Gaussian curve fitting. The migration times of the detected peaks were next normalized by a time-warping function, whose numerical parameters were optimized by a simplex method with peak matching across multiple data sets based on dynamic programming techniques [206]. Any redundant features, such as isotopic peaks, fragments and adduct ions, were removed. Finally, the metabolites contained in the standard compounds were assigned to the remaining features by matching their m/z values and normalized migration times.

All data were subjected to analysis of variance (2-way ANOVA) followed by Fisher´s post hoc analysis and expressed as mean ± SE. Differences between groups were considered significant at $p<0.05$.

3.4 *In situ* liver perfusion

For analysing the amount of glucose produced by male and female livers, respectively, we performed *in situ* liver perfusion. This technique enables collection of metabolites that are produced by an intact organ such as the liver, in a living system. In paper I, we present data showing measurements of hepatic glucose output (HGO) and other identified metabolites detected in the perfusates using ^{1}H-NMR spectroscopy.

In short, the rats are anaesthetized and the intact liver is exposed. Non-circulating Krebs-Henseleit bicarbonate buffer is entered via the portal vein, as described previously [207]. For the aim of our studies, no gluconeogenic precursors should be present in the buffer and the perfusion pressure and flow rate kept constant. Samples are finally collected from the inferior caval vein, starting five min after perfusion initiation when the liver appears to be cleared from any remnants such as blood. In our study, hepatic glucose output was calculated using the mean glucose concentration in relation to flow rate and hepatic dry weight.

4 RESULTS

The results from the papers included in the thesis are summarized here, and described in more detail below:

A. Sex differences in hepatic fuel metabolism were characterized in healthy male and female rats. Male rats showed higher ratios of insulin to glucagon levels, higher levels of hepatic glycogen, lower degree of hepatic AMPK phosphorylation, higher expression of hepatic gluconeogenic genes and higher hepatic glucose output, as compared to the females.

B. Differences in effects of short-term high-fat feeding (SOD and CBD) were demonstrated on hepatic insulin sensitivity, gene expression, lipid metabolism and plasma lipids. Cocoa butter did not affect plasma total TG levels, VLDL-TG or hepatic insulin sensitivity. Safflower oil increased hepatic β-oxidation, was beneficial in terms of circulating VLDL-TG, but led to reduced hepatic insulin sensitivity. CBD showed effects on hepatic gene expression that might lead to increased lipid synthesis, lipotoxicity, inflammation and IR if the diet would be extended.

C. Sex differences in the development of hepatic IR were investigated using male and female ZDF rats as a model. Changes in hepatic gene expression revealed reduced hepatic lipogenesis, increased FA oxidation and metabolic stress in the high-fat diet fed females and diabetic male rats, compared to the healthy females.

D. Sex differences in hepatic metabolic control were translated and extended into sex-different hepatic metabolites. Metabolite profiles of hepatic perfusates from healthy rats using NMR verified that male livers contained more glucose than female perfusates. Liver-derived lactate was also higher in males, and there was a trend towards higher levels of glycerol and the glucogenic amino acids glutamine and glutamate in males.

E. Testosterone treatment in male diabetic ZDF rats reduced the hepatic fat content. Also, the treatment increased blood glucose levels, reduced glucose tolerance and increased circulating levels of TG-rich VLDL particles. Surprisingly, testosterone reduced STAT3 activity, a key mediator of leptin, essential for hepatic insulin sensitivity.

4.1 Sex-different hepatic glycogen content and glucose output in rats (Paper I)

To investigate underlying molecular mechanisms of possible sex-specific development of hepatic IR, we decided to first improve the knowledge of how healthy male and female rats differ regarding hepatic fuel (lipid and carbohydrate) metabolism. In paper I, we concluded that males had higher ratios of insulin to glucagon levels, higher levels of glycogen, lower degree of AMPK phosphorylation, higher expression of gluconeogenic genes and higher hepatic glucose output. Possibly, these sex differences reflect a higher ability for the healthy male rat liver to respond to increased energy demands.

Out of 27 649 gene probes represented on the microarrays, approximately 3 500 were detected in liver. Among these, 383 (11%) transcripts were expressed significantly higher in females whereas 399 (11%) transcripts were significantly higher in males (cut-off: 5% FDR and 1.5 fold change). In line with previous reports from our lab [107], the female-predominant genes are involved in hepatic uptake of long-chain fatty acids, synthesis of TG and assembly of VLDL particles. In addition, a male-predominant capacity for mitochondrial and peroxisomal FA oxidation was revealed. Also, the male rats had higher levels of gene products for glucose uptake and synthesis of glycogen compared to the females.

The normal metabolic state of an individual can be affected by different stressors such as fasting. To investigate how the metabolism of healthy male and female rats respond to this type of stress, they were fasted for either 4h (absorptive state) or 12h (post-absorptive state) and compared regarding hepatic gene expression. Interestingly, most gene products showed a greater sex-difference in the post-absorptive state, including higher levels of ACOX1, CPT-1a, GOT1, G6Pase and SCD1 in males.

Hepatic TG content, FA oxidation rate and ketone bodies in the blood of the fasted animals was determined in order to study the relation to the hepatic mRNA expression levels. However, no sex differences could be detected at these levels. The hepatic glycogen stores were measured in the animals showing that the males had higher levels of hepatic glycogen as compared to females.

Hepatic glucose output (HGO) was determined by *in situ* perfusion of the livers. Interestingly, glucose levels were higher in perfusates collected from male rats ($P<0.0001$), but there were no significant differences in HGO between 4 and 12h fasting. When blood glucose levels were determined at different durations of fasting, a significant decrease was observed after 12h but sex differences could not be detected at any time point. The discrepancy

between glucose levels in blood and liver perfusates might be explained by differences in glucose uptake by peripheral tissues.

To explore the possibility that other low molecular weight compounds might be leaving the liver in a sex-dependent manner, the perfusates were also analysed using ^{1}H-NMR spectroscopy. Quantitative analysis verified that perfusates generated from male livers contained more glucose, as compared to females ($P<0.01$). There were also significantly higher levels of lactate detected in male samples ($P<0.01$).

To test whether male and female livers respond equally well to insulin at the level of insulin receptor signaling, the responses to insulin were compared between male and female rats regarding Akt-phosphorylation (Ser473) and gene expression. No sex-difference could be observed in the degree of Akt-phosphorylation 40 min after insulin treatment, using an ELISA assay. The effect on blood glucose levels was the same in males (reduced from 6.1 ± 0.3 to 2.5 ± 0.3) and females (reduced from 5.8 ± 0.4 to 2.3 ± 0.4) 40 min after insulin treatment.

4.2 Cocoa butter and safflower oil elicit different effects on hepatic gene expression and lipid metabolism in rats (Paper II)

In paper II, we compared the effects of cocoa butter-enriched diet and safflower oil-enriched diet on hepatic gene expression, lipid metabolism, insulin sensitivity and plasma lipid profiles, in healthy male rats.

Cocoa butter-enriched high-fat feeding for 3 days increased hepatic triglyceride (TG) content and fatty acid (FA) oxidation rate to similar extents (60% and 70% respectively). No effects were observed on plasma total TG levels or TG-rich VLDL particles. In the safflower oil-fed animals, hepatic TG was increased to the same extent (60%) but FA oxidation enhanced to a greater extent (170%), as compared to the cocoa butter group. Plasma total TG and VLDL-TG were lowered in the oil-fed rats.

Using whole-genome rat oligo microarrays hepatic transcript profiles were generated reflecting lipid-mediated gene activities. Cocoa butter had a greater impact on gene expression than safflower oil, and most effects were dependent on the type of lipid. Rats fed cocoa butter diet had increased levels of several gene products from important biosynthetic pathways such as cholesterol and bile acid synthesis. Gene products involved in stress-mediated survival pathways within the liver seemed to be increased to similar extents by the two high-fat diets, although the effect on IGFBP-1

protein was higher with cocoa butter. SCD-1 and -2 were among the gene products being repressed by high-fat feeding, with safflower oil eliciting the greatest effect.

Only safflower oil-based high-fat feeding had a negative effect on hepatic insulin sensitivity, using the ratio between phosphorylated Akt and total Akt as a measure of insulin signalling capacity. These findings suggest that the two different types of fat used in this study, will affect hepatic gene expression, sterol metabolism, and insulin sensitivity differently.

4.3 Sex-dependent hepatic transcripts and metabolites in the development of glucose intolerance and insulin resistance in Zucker diabetic fatty rats (Paper III)

With the data concluded from the previous studies, in paper III we wanted to investigate if differences in liver functions could provide clues to why male Zucker diabetic fatty rats (mZDF) spontaneously develop hyperlipidemia, hyperglycemia and diabetes, whereas females (fZDF) only become diabetic when fed a diabetogenic high-fat diet (HF-fZDF).

By using whole-genome arrays, we first identified high-fat diet-induced changes in hepatic gene expression, comparing the non-diabetic females with the diabetic females (13-week-old fZDF and HF-fZDF). Out of 27 649 gene probes printed on the arrays, 94 were differentially expressed between the groups (using a 5% FDR and a cut-off at 1.5-fold difference). Functional annotation of the differentiated transcripts revealed that females developing IR and glucose intolerance have increased levels of gene products for FA oxidation (e.g. CPT-1a) and reduced levels of transcripts for lipid synthesis (e.g. ACC). Signs of increased oxidative stress included elevated mRNA levels of metallothionein (Mt1) and reduced levels of glutathione S-transferases (GST).

To find sex-dependent hepatic gene products that might be related to disease development, transcript profiling was performed comparing the non-diabetic female with the diabetic male rats (13-week-old fZDF and mZDF). This resulted in 168 differentially expressed hepatic transcripts, being either male- or female-predominant. Interestingly, among 94 fat-induced changes, 32 were sex-dependent. Overlapping differences (comparing the two sets of arrays) included several gene products for FA oxidation (male-predominant and induced by high-fat diet) and lipid synthesis (female-predominant and reduced by high-fat diet). Also, signs of increased oxidative stress, including elevated mRNA levels of Mt1 and reduced levels of GST were observed. Ten gene products with differential expression and important functions in

hepatic lipid metabolism or metabolic stress responses were confirmed through real time quantitative PCR.

To evaluate if the overlapping differentially expressed genes could be related to liver-derived intermediary metabolites, metabolite profiling on liver extracts were performed using a method based on capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS). Among 151 low molecular weight compounds (LMC) detected in liver extracts, 45 were found to be significantly different between HF-fZDF and fZDF, whereas 34 were different between mZDF and fZDF. In line with the findings in the transcript profiles, the observed metabolic changes indicate increased activity through catabolic pathways (FA oxidation and amino acid degradation) and reduced biosynthesis of GSH and its precursors within the livers of HF-fZDF.

These liver-derived molecular profiles indicated that males and females might respond differently to increased hepatic lipid infiltration. The effects of three days high-fat feeding on the expression of ten selected genes for lipid turnover and metabolic stress responses were investigated in healthy male and female Sprague Dawley (SD) rats. This short-term high-fat feeding has previously been reported to trigger a gene expression program for hepatic lipid disposal and cell survival in SD rats [208]. Of the investigated transcripts, NAD(P)H:quinone oxidoreductase 1 (NQO1) was significantly altered in response to high-fat feeding but only in the males. In contrast, Mt1 seemed to be more diet-induced in females, increasing the sex-different expression of this gene.

4.4 Testosterone–mediated effects on the liver differ between lean and obese Zucker rats (Paper IV)

In the fourth paper, we wanted to investigate the effects of testosterone treatment and further explore a role of testosterone on hepatic metabolism in male ZDF (fa/fa), using lean Zucker (fa/+) (ZLC) rats as a control. The use of testosterone as a possible treatment has been suggested for hypogonadal males with T2D and metabolic syndrome [209].

Three weeks of testosterone treatment significantly increased blood glucose and VLDL-TG levels in the obese ZDF rats and significantly reduced the glucose tolerance. Also, ZDF-specific effects were observed on hepatic lipid metabolism, with decreased hepatic TG content in response to testosterone.

To identify testosterone-regulated hepatic genes, and to compare the effects of testosterone in ZLC and ZDF rats, whole-genome rat oligo microarrays were used. 827 transcripts were altered in response to testosterone in lean,

and 412 were regulated in obese. Among those, only 64 transcripts were testosterone-regulated in both lean and obese, whereas 330 transcripts were differently regulated between the groups. Testosterone treatment in both lean and obese rats reduced the expression of genes for *de novo* lipid synthesis (IRE-BP1, ChREBP, SREBP1c, FAS, ACC1, Elovl6 and Adiponutrin), although the effects were more pronounced in the obese. Stimulatory effects of testosterone were more prominent in the lean rats, including IRS2, PEPCK and Angptl4. The obese state was associated with increased levels of IRS1, IRE-BP1, CPT-1a, SHP and FGF21, regardless of testosterone status. Lower rates of lipid synthesis together with enhanced capacity for fatty acid oxidation (CPT-1a) and bile acid synthesis (CYP7A1) might explain the lowering effect on hepatic TG observed in T-ZDF.

We also noted that hepatic STAT3 phosphorylation was reduced by testosterone in both groups. Interestingly, this effect on STAT3 could also be seen in cultured human hepatocytes, in the presence or absence of leptin.

Pathway	Gene	Paper I		Paper II			Paper III			Paper IV			
		SD		SD			ZDF			ZLC		ZDF	
		♀	♂	♂	♂	♂	♀	♀	♂	♂	♂+T	♂	♂+T
		Std		Std	SOD	CBD	Std	HFD	Std	Std			
Gluconeogenisis	PEPCK	-	↑				-	-	↑	-	↑	-	-
FA oxidation	CPT-1a	-	(↑)	-	↑	↑	-	↑	↑	-	-	-	-
Lipid synthesis	ACC						-	-	↓	-	-	-	↓
	FAS	-	↓							-	-	-	↓
	SCD1	-	↑	-	↓	↓							
	Pnpla3							↓	↓	-	-	-	↓

Table 2. Summary of observed key hepatic transcripts, paper I-IV. The table is based on the confirmed mRNA expression levels, as performed by real-time PCR, as described in paper I-IV.

Paper I: Effects of sex in 12h fasted rats. Arrows indicate ↑(induced) or ↓ (reduced) compared to the females (-).
Paper II: Effects of high-fat diets in male rats. Arrows indicate ↑(induced) or ↓ (reduced) compared to the StD fed (-) (controls).
Paper III: Effects of sex and/or diet. Arrows indicate ↑(induced) or ↓ (reduced) compared to the StD fed females (-) (fZDF).
Paper IV: Effects of testosterone treatment. Arrows indicate ↑(induced) or ↓ (reduced) compared to the ZLC (-) or ZDF (-) controls, respectively.

Male (♂), female (♀), Sprague Dawley (SD), Zucker lean control (ZLC), Zucker diabetic fatty (ZDF), standard diet (Std), safflower oil diet (SOD), cocoa butter diet (CBD), high-fat diet (HFD).

5 DISCUSSION

5.1 Sex differences in lipid and carbohydrate metabolism in rat liver

5.1.1 Carbohydrate metabolism

Gene expression

In paper I, we confirmed previous observations showing a male-predominate turn-over in hepatic carbohydrate metabolism [107] by performing microarray analysis. Higher levels of gene products for glucose uptake and synthesis of glycogen were observed in the healthy male rat livers compared to the females. Interestingly, these sex differences seemed to be enhanced upon prolonged fasting (post absorptive state, 12h fasting) in the mRNA expression of e.g. glutamate oxaloacetate transaminase 1 (GOT1) and the gluconeogenic enzymes PEPCK and G6Pase. For this reason, it could be speculated that the male are more efficient in producing glucose and glycogen.

In paper III, the impact of short-term high-fat diet (safflower oil) on carbohydrate turn-over was tested using PEPCK as a marker. The diet decreased the mRNA expression of PEPCK in both sexes. However, no significantly difference was observed by comparing the males and the females but there was a clear trend of a male-predominate expression, indicating that the males are more vulnerable to an increased lipid load/accumulation, resulting in increased hepatic glucose production/output.

In paper III, we wanted to expand our knowledge on hepatic sex differences in relations to IR and used the ZDF rat as a model for the study of disease development. We determined transcript profiles and classified the significant different gene products by their function. In line with the previous studies, PEPCK was confirmed to be significantly elevated in the diseased males (mZDF), no difference between the female groups were observed.

Hepatic glucose output and glycogen content

The hypothesis that healthy male rat livers produce more glucose compared to female was tested in paper I. Hepatic glucose output (HGO) was determined by *in situ* perfusion of the livers. Interestingly, glucose levels were higher in perfusates collected from male rats supporting our findings at the gene expression level. A sex-difference in hepatic glucose output has as far as we know not been described before, but there are reports on male-predominant hepatic glycogen content, G6Pase [210] and PEPCK activities [211]. In line with this, in paper I we could report a higher level of hepatic glycogen in the

male rats compared to the females. For future studies, it would be of interest to determine the HGO and glycogen content in the livers of the different groups of ZDF rats in order to investigate if these sex differences also may be a contributing factor the development of T2D in these rats.

PEPCK activity has been shown to be reduced by castration or estrogen treatment. Estrogens have also been shown to decrease the expression of PEPCK in virgin female rats [212] as well as G6Pase protein levels and enzyme activity in ob/ob mice [213]. Thus, estrogens have been suggested as an anti-hyperglycemic agent in this model of T2D. Accordingly, estrogen receptor α deficient (ERαKO) mice develop fatty liver, have higher fasting blood glucose, plasma insulin levels and impaired glucose tolerance [80]. Since estrogen levels are higher in fertile females than males, estrogen signaling seems to be essential for these hepatic sex differences. However, other sex-dependent hormones such as testosterone and growth hormone (GH) are likely to be of similar importance. As demonstrated in paper IV, testosterone treatment in the lean ZDF rats (but not the obese) induced the hepatic gene expression of PEPCK. This may indicate that testosterone stimulate glucose turnover through enhanced hepatic insulin sensitivity (IRS2 and Angptl4), leading to improved regulation of hepatic glucose and VLDL output in the lean rats.

Another interesting sex-difference was observed regarding plasma levels of insulin and glucagon in the healthy SD rats. The male rats had higher ratios of plasma insulin to glucagon levels, which might also contribute to higher glycogen content in males. In spite of this sex-difference in insulin to glucagon ratios, blood glucose concentrations were the same in male and female rats, which might be interpreted as male rats being less sensitive to insulin. It should also be noted that AMPK was Thr172-phosphorylated to lower degrees in male livers, indicative of higher activity through glucogenic pathways as compared to females. Whether a cause-effect relationship between higher insulin to glucagon ratios, greater capacity for glycogen synthesis and less AMPK activity exists in male rat livers, and whether this explains the greater HGO in male rat livers observed in this study, have to await further investigations.

There is an established link between gluconeogenesis and glycogen synthesis, meaning that a higher rate of glucose production from e.g. amino acids, lactate or glycerol will also lead to a greater capacity to synthesize glycogen. A similar pattern of mRNA expression was observed for the gluconeogenic enzymes GOT1, G6Pase and PEPCK, suggesting a common mechanism of regulation. Further experiments are required to determine whether this explains the male-predominant content of glycogen.

A sex-difference in hepatic glucose output has to our knowledge, not been reported before. Since men are more prone to use carbohydrates as energy source in a fasted state, it is logical that they have higher levels of HGO. Increased supply of substrates is necessary for increased HGO and an increase in gluconeogenesis is crucial for higher rates of HGO. However, in patients with decreased peripheral insulin sensitivity, this increased HGO in men may lead to hyperglycemia and exhaust the system, eventually impair the pancreatic β-cell function.

5.1.2 Lipid metabolism

Gene expression

In paper I, we accentuated previous observations showing a female-predominate turn-over in hepatic lipid mRNA expression [107]. As expected, higher levels of gene products for lipid uptake, lipid output and synthesis of lipids (TG and VLDL particles) were observed in the healthy female rat livers compared to the males. However, transcripts involved in lipid turn over and FA oxidation were more prominent in the male rat livers. As for the observed sex differences in the carbohydrate metabolism, these effects were enhanced after 12h fasting. As described in previous studies [107, 214], CD36 (fatty acid translocase) has been implicated as an important player in the context of hepatic lipid uptake and is female-predominant. CD36 is increased during situations of increased hepatic lipid content [107, 215] but reduced during starvation [214], indicating that it might be involved in anabolic actions. Whereas for the hepatic FA oxidation, acyl-coenzyme A oxidase 1 (ACOX1) and CPT-1a showed higher levels of mRNA expression in the males. These results were in line with the increased expression levels of genes for FA-oxidation in HF-fZDF and mZDF, as compared to fZDF (paper III).

In paper III, the impact of short-term high-fat diet (safflower oil) on lipid turn-over and FA oxidation were investigated using ACCα and CPT1-a as markers. As expected, the mRNA expression of ACCα was significantly higher in the livers of the female rats. However, the expression of CPT-1a was not shown to be significantly different comparing the males and the females but there was a clear trend of a male-predominate expression, in line with previous results. In line with this, female rats have previously been shown to have higher rates of hepatic FA uptake [53, 216], esterification [53], VLDL-TG formation and output [55] as compared to males. During situations of increased adipose lipolysis (e.g. fasting) and availability of NEFA in the blood stream, the liver will take up more NEFA which will lead to the activation of PPARα and thereby increased expression of gene products for FA oxidation [217] and ketogenesis [218]. Male rats have been shown to have higher levels of hepatic PPARα [219] and to be more

responsive to peroxisome proliferators than female rats [220, 221], including higher rates of FA oxidation. Our finding that fasting induced the PPARα-dependent genes ACOX and CPT-1a in males to a greater extent than in females might thus be explained by higher levels of PPARα in male liver. Testosterone treatment in the lean and obese ZDF rats reduced the expression of several key genes for *de novo* lipid synthesis (IRE-BP1, CHREBP, SREBP1c, FAS, ACC1, Elovl6 and Adiponutrin), although the effects were more pronounced in the obese (paper IV). This indicate that testosterone have a greater effect on *de novo* lipid synthesis in the obese ZDF. The latter might be a consequence of higher SHP mRNA levels in the obese state, since SHP has been shown to down-regulate expression of CHREBP and SREBP1c.

FA oxidation and TG content

In contrast to the observed differences at the transcript levels, we did not detect any sex-difference in the amount of hepatic TG content in the healthy SD rats (paper I). This might be explained by a compensational mechanism in the females with greater capacity for TG synthesis by having a greater VLDL-TG output compared to the males, as described in previous studies [55]. Also, reduction of genes encoding leptin have been reported in estrogen-treated obese female mice [83]. Thus, it might be speculated that leptin is involved in promoting TG output.

5.2 Effects of short-term high-fat feeding on hepatic insulin resistance in male rats

Short term high-fat feeding to rats using safflower oil as lipid source, has shown to result specifically in hepatic fat accumulation and has been used as a model to study the mechanisms underlying hepatic IR [222]. In contrast, studies based on cocoa butter as lipid source with the saturated fatty acid stearic acid as the main FA, have shown to have a neutral effect on the plasma lipid profiles [223]. Ingested cocoa has also been shown to reduce de novo lipid synthesis in rat liver [224]. In paper II, we investigated the effects of short-term (three days) high-fat feeding on the hepatic metabolism in male rats. The effects of two different types of diets were compared and in relation to a control standard diet (StD) (9% fat). The safflower oil-enriched diet (SOD) (52% fat) increased hepatic β-oxidation, was beneficial in terms of circulating VLDL-TG particles, but led to reduced hepatic insulin signaling. Whereas the cocoa butter-enriched diet (CBD) (52% fat) was neutral regarding serum lipids and hepatic insulin sensitivity. The effect of cocoa-butter was more extensive at the level of hepatic gene expression compared to the effects of safflower oil. The observation in the SOD rats showing an increase in hepatic β-oxidation but reduced hepatic insulin sensitivity can be

compared to the results in paper III where the diabetic mZDF rats showed a similar pattern. Increased FA oxidation rates are known to increase the hepatocellular levels of ROS and the risk of developing hepatic IR [170] thus, confirm our findings.

Hepatic lipid content

It is well established that fat accumulation in the liver leads to IR [225]. However, it is not clear how long time of fat accumulation it will take in order for those effects to be detected. In paper II, after only three days of HFD the level of TG was increased in the livers of the fat fed animals compared to the controls but no differences between the diets were identified. However, only the SOD fed rats showed elevated levels of hepatic cholesterol suggesting lipid-specific effects on hepatic lipid metabolism.

Plasma lipid profiles

The lipid-specific effects were also analyzed in plasma. Safflower oil decreased VLDL-TG particles and increased cholesterol-rich VLDL particles. At the same time, HDL cholesterol was reduced. No difference was observed between the male rats fed CBD and StD. The diet-specific effects on plasma lipids are in agreement with previous studies [226], and might partly be explained by differences in hepatic FA oxidation rates. The level of TG in plasma is most often related to VLDL production in the liver, and this in turn is dependent on whether FA are being used for FA oxidation or re-esterification [227]. The enhanced FA oxidation rate in the SOD-fed rats indicate that both de novo FA synthesis [228] and lipoprotein formation was reduced [229], leading to lowered levels of plasma TG. In comparison, female rat fed a SOD demonstrated even lower levels of VLDL-TG compared to the males (unpublished data). Additional studies are needed to further speculate about this sex-difference.

Hepatic gene expression profiles

SOD mediated an increase in the expression of important gene products involved in bile acid production and cholesterol excretion, CYP7A1 and ABCG5. The SOD-mediated increase in hepatic cholesterol might lead to liver X receptor (LXR) stimulation and thus explain the increased expression of CYP7A1 [230] and the sterol transporter ABCG5 [231]. This effect of safflower oil on gene products involved in hepatic cholesterol turnover has to our knowledge not been described before but might be related to the reduced expression of SCD-1 and capacity to export CE in VLDL particles in the SOD-fed rats. Common effects of the two diets on gene expression included increased levels of p8, DIG-1, IGFBP-1 and FGF21, as well as reduced levels of SCD-1 and SCD-2. This indicates that a lipid-dependent program for hepatic lipid disposal and cell survival was

induced, independently of the lipid source. The observation that cocoa butter exerted a similar degree of SCD-1 reduction as did safflower oil has to our knowledge not been described before. The mechanism behind this effect is as yet unknown, but data presented herein suggest that it is independent of reduced SREBP-1 maturation [232]. It has been suggested that inhibition of SCD-1 in the liver results in secretion of VLDL particles that are highly enriched in SFA-rich CE, giving rise to SFA-CE-rich LDL particles. Increased delivery of SFA to macrophages leads to accumulation of SFA, enhanced inflammatory cytokine secretion and a proinflammatory phenotype. In the present study, reduced expression of SCD-1, was in parallel with increased hepatic cholesterol, increased VLDL cholesterol and reduced HDL-cholesterol. Whether this effect would persist upon longer periods of high dietary intake of safflower oil is not known and should be addressed. The high-fat diet-mediated increase in FGF21 mRNA is interesting, since FGF21 has been shown to increase β-oxidation. FGF21, a hormone induced by fasting, is predominantly expressed in the liver and has beneficial effects on glucose homeostasis and insulin sensitivity both in rodents [233] and humans [234]. It has also been shown that FGF21 induces SOCS2, blocks GH-dependent STAT5b signaling and alters the expression of GH-regulated genes during starvation [235]. Among other GH-suppressed gene products [236], hepatic IGFBP-1 expression was found to be induced by FGF21 [235]. Our finding that FGF21 was induced by high-fat feeding in parallel with increased IGFBP-1 and increased FA oxidation is in line with the reports cited above, and adds to the picture of FGF21 as a sensor of FA availability, during starvation as well as during hepatic fatty infiltration.

Hepatic insulin sensitivity

Only a few studies have been reported reduced insulin sensitivity after only a short period of time of high-fat feeding. In paper II, we reported that male rats fed SOD for only three days had reduced hepatic insulin-mediated phosphorylation of Akt and GSK3β. These findings were in line with a previous report [222] in which blunted insulin-mediated suppression of hepatic glucose output (HGO) also was reported. HGO was not investigated in paper II but would be of interest for future investigations as well as other signaling molecules up-stream of Akt (e.g. IRS1 and IRS2). Interestingly, these effects on Akt phosphorylation was not detected in the rats fed CBD for three days. However, it cannot be ruled out that cocoa butter feeding would lead to the same detrimental effect if the diet would be prolonged. Interestingly, unpublished data from our group show that SOD does not affect the hepatic insulin-mediated activation of Akt in female rats.

5.3 Sex differences in the development of hepatic insulin resistance

It is well accepted that men are more prone to develop metabolic syndromes including IR and T2D than premenopausal women [6, 7, 237]. This observation can be linked to differences in body composition and sex hormones. However, the underlying molecular mechanisms are not entirely explained. One aspect of hormone-dependent differences between males and females comprise liver functions. Animal studies support a protective role of estrogen-mediated effects on the liver since both male and female ERαKO mice develop non-alcoholic fatty liver disease (NAFLD), hepatic IR and impaired glucose tolerance [80]. In this thesis, we used male and female Zucker diabetic fatty rats to further investigate possible mechanisms that may be involved in the sex-specific development of hepatic IR. In parallel, we studied sex differences in the livers of healthy Sprague-Dawley rats.

Blood glucose and insulin levels

As expected, the blood glucose and insulin levels increased with time in the mZDF rats, confirming the disease development in these animals thus, confirming the notion that fZDF rats are less susceptible to diabetes development [187, 238]. However, four weeks of high-fat (48% kcal fat) feeding will induce the development of marked hyperglycemia in fZDF, which leads to islet dysfunction and diabetes within the following four months [238]. Elevated levels of insulin indicate that the β-cells are still functioning, trying to repress the elevated blood glucose levels in these animals. With time, it is expected that the insulin production will shut down due to exhaustion of the system, as part of the consequences of T2D.

Hepatic fuel metabolism

As discussed earlier, at the transcriptional level, the most prominent sex differences in the disease development in the ZDF rats included FA oxidation (male-predominant) and lipid synthesis (female-predominant). This is in line with previous studies [107] as well as our own observations in the healthy SD rats (paper I).

Adiponutrin (Pnpla3) showed the greatest sex-difference among the gene products being confirmed to be female-predominant and reduced by high-fat feeding in paper III. In line with this, we observed a reduction of adiponutrin after testosterone treatment in the mZDF, described in paper IV. Adiponutrin is a predominantly liver-expressed transmembrane protein with phospholipase activity that is regulated by fasting and feeding. Although its putative role in metabolic disease development has only recently started to be explored, various genome-wide association studies have identified the adiponutrin gene to be associated with liver-related phenotypes [239-241].

Since it is up-regulated in response to feeding and down-regulated in the fasted state, a potential role for adiponutrin in lipid storage has been suggested [242-244]. Interestingly, genes of importance for *de novo* lipid synthesis (such as ACCα) were found to be co-regulated with adiponutrin in our studies. This finding is supported by reports showing an increased expression of the adiponutrin gene by the transcription factors SREBPs, PPARγ and ChREBP [242, 245].

Previous findings have shown a female-predominant efficiency of incorporation of hepatic FA into TG and VLDL particles and secretion of TG-enriched lipoprotein particles. Thus, this ability may also contribute to a reduced hepatocellular load of lipids in obese females (fZDF). Elevated levels of hepatic FA in the HF-fZDF and mZDF might be caused by saturation in this lipid-clearing system. This might in turn activate PPARα-dependent genes and hepatic FA oxidation, as well as reduce the actions through SREBP-dependent lipogenic pathways. Results obtained in paper III are in line with this and could be exemplified by higher expression levels of genes for FA-oxidation (such as CPT-1a) in HF-fZDF and mZDF, as compared to fZDF.

Interestingly, higher FA oxidation rates are known to increase the hepatocellular levels of ROS and the risk of developing hepatic IR [170]. We did not determine hepatic insulin sensitivity in. However, the expression of the insulin-induced gene 1 (Insig-1), a negative regulator of SREBP-maturation and lipogenesis [246], was significantly lower in the HF-fZDF and mZDF as compared to the fZDF. Interestingly, overexpression of hepatic Insig-1 or -2 has previously been shown to reduce lipogenesis in obese male ZDF rats [247].

Hepatic oxidative stress

By comparing the transcript profiles of the HF-fZDF and mZDF, it was indicated that the production of reactive oxygen spices (ROS) were increased compared to the fZDF (paper III). These observations included increased levels of metallothionein and angiotensinogen mRNA, both known to be induced by various forms of oxidative stress. Surprisingly, two GST isoenzymes (GSTa1 and GSTm2) as well as quinine oxidoreductase 1 (NQO1) were reduced in the insulin resistant rats (HF-fZD and/or mZDF). The reason for this is not known, but is likely to reduce the hepatic detoxification capacity. Metallothioneins are potent cellular antioxidants that play important roles in essential trace element homeostasis, metal detoxification, and in the protection against various injuries resulting from ROS [248], including the development of diabetes and its complications. Previous studies in fatty livers of obese ZDF rats have shown increased levels of malondialdehyde (a lipid peroxidation product) [249] and reduced

levels of GSH [250, 251], supporting the idea of increased hepatic ROS production and GSH consumption in this model.

5.4 Sex differences in hepatic metabolites

Hepatic perfusates of healthy SD rats

As discussed earlier, we concluded that healthy male rats have higher levels of hepatic glucose output compared to the females (paper I). This difference was detected in collected perfusates produced from *in situ* liver perfusion. With the use of ^{1}H-NMR spectroscopy analysis of the perfusates, we extended our search for metabolites produced by the liver of the 12h-fasted male and female rats. Using this approach, we identified glucose, lactate, glycerol, propionate, several amino acids and ketone bodies as being released from the liver. Further, quantitative analysis of the perfusates verified that male livers contained more glucose than females. Liver-derived lactate was also higher in males, and there was a trend towards higher levels of glycerol and the glucogenic amino acids glutamine and glutamate in males. These findings are in line with our previous observations of the mRNA expression of the key gluconeogenic enzymes, indicating that the male liver is more active in producing and exporting not only glucose but also metabolites important for glucose production. ^{1}H-NMR spectroscopy analysis on rat liver perfusates has to our knowledge not been performed before. With the use of this technique, we are able to support previous findings but also further contribute to the understanding of the complete system, thus sex differences in the healthy rat liver.

Hepatic extracts from ZDF rats

Overlapping differences in the transcript profiles included several gene products for FA oxidation (male-predominant) and lipid synthesis (female-predominant) as well as oxidative stress (paper III). To evaluate if these differentially expressed genes could be related to liver derived metabolites that differentiate male and female ZDF rats regarding their susceptibility to develop diabetes, metabolite profiling on liver lysates and plasma was performed by using CE-TOFMS. This method enables separation and quantification of low molecular weight hydrophilic compounds such as amino acids and carbohydrates [203]. The most differential pathway detected from the CE-TOFMS analysis involved metabolites related to Glutathione (GSH), an important antioxidant involved in the response to oxidative stress. The cell is constantly balancing between GSH (red) and the oxidative (ox) form of Glutathione (GSSG). Normally, GSH is the predominant form and exists in most liver cells, the GSSG content is less than 1% of GSH [252]. An imbalance may cause an increase in the levels of oxidative stress [253, 254]. Livers from HF-fZDF had significantly lower

levels of GSH as compared to fZDF, indicating a greater usage of GSH due to increased oxidative stress. Although mZDF and fZDF rats had almost similar GSH levels, many glutathione related metabolites or substrates (ophthalmate, 2-aminobutyrate, glycine, hypotaurine and serine) were lower in mZDF (as compared to fZDF). These results indicate that the capacity of GSH production was lower in male rats, and that GSH turnover was higher in females. Interestingly, major differences in ophthalmate levels were observed, higher levels in HF-fZDF, whereas mZDF had lower levels (as compared to fZDF). Ophthalmate (γ-Glu-2-aminobutyrate-Gly) has previously been shown to be synthesized through the same pathway as GSH [179]. The synthesis of Ophthalmate is produced from 2-aminobutyrate through γ- glutamylcysteine synthetase (GCS) and glutathione synthetase. GCS is the rate- limiting enzyme and feedback-inhibited by GSH, and thus GSH consumption leads to GCS activation, resulting in enhanced biosynthesis of ophthalmate. Since ophthalmate levels were shown to predict of the amount of GSH production [179], ophthalmate measurements provide valuable information about hepatic GSH turnover. Reduced levels of GSH in HF-fZDF, while both ophthalmate and its substrate 2-aminobutyrate were increased, indicate that although high-fat feeding in fZDF rats generates oxidative stress, the capacity to synthesize GSH might still be high due to sufficient amount of substrates (Cys, Glu, Gly, Ser etc). The substrates in GSH synthesis may be directly or metabolically provided from the high-fat diet, since GSH can be regenerated from GSSG using NADPH produced in the pentose phosphate pathway. These results indicate that the capacity of GSH production was higher in female rats, which at least partly could explain why females are less susceptible to oxidative stress.

5.5 Effects of testosterone treatment on hepatic functions in male ZDF rats

Whether hypogonadal men, with or without T2D, should be treated with testosterone is a question of great concern. There are reports on positive effects on adiposity, insulin sensitivity and total cholesterol [255]. However, since androgen administration lowers antiatherogenic HDL-cholesterol, testosterone treatment might enhance cardiovascular disease risk [256]. In paper IV, we used treated the male obese ZDF rat with testosterone and compared the effects on the liver to the lean control ZDF rat.

Insulin and glucose levels

As expected, the blood glucose levels were higher in the obese ZDF rats compared to the lean controls. However, the testosterone treatment increased the glucose levels even further. Thus, the glucose tolerance was reduced in

these animals, indicating reduced uptake by peripheral tissues or enhanced hepatic glucose output. By calculating a HOMA-IR index we could hint that the over-all body insulin sensitivity was not significantly altered by the testosterone treatment. However, further measurements of the hepatic insulin sensitivity should be conducted before concluding the worsening effects of testosterone in the liver.

Hepatic lipid content and serum lipid profiles

After the observed reduction in glucose homeostasis, an increase in hepatic lipid content would be expected. However, the diabetic male ZDF rats showed reduced hepatic fat content and increased circulating levels of VLDL-TG particles after testosterone treatment. Normalized hepatic lipid content could be beneficial for the diabetic state in the ZDF rats. But unless energy expenditure would be elevated to the same extent as the availability of fuel molecules in blood, maintained testosterone treatment over time would probably worsen the metabolic state in these rats.

Hepatic gene expression

To find an explanation for the ZDF-specific effects of testosterone, hepatic transcript profiles were generated and compared between lean and obese rats. In line with the results described above, most hepatic effects on gene expression were different between the groups. In the testosterone treated lean ZDF rats, a beneficial effect was observed on the expression of IRS2 and Angptl4, indicating improved insulin sensitivity thus, improved regulation of glucose and VLDL output. Angptl4 expression is induced in adipose tissue and liver during fasting, and acts as a powerful signal to prevent fat storage, stimulate fat mobilization [257, 258] and hepatic insulin sensitivity [259]. Serum levels of Angptl4 in human subjects have been shown to inversely correlate with plasma glucose concentrations and HOMA-IR [259]. Further studies involving measurements of circulating levels of Angptl4 protein in hypogonadal men before and after testosterone replacement would help to evaluate the importance of our finding in a human perspective.

In the obese ZDF rats, testosterone treatment had a great effect on *de novo* lipid synthesis, through inhibition of CHREBP and SREBP1c, and their target genes. This might partly be a consequence of higher SHP mRNA levels in the obese state, since SHP has been shown to down-regulate expression of CHREBP and SREBP1c. Lower rates of lipid synthesis together with enhanced capacity for FA oxidation (CPT1 and FGF21) and bile acid synthesis (CYP7A1) might explain the lowering effect on hepatic lipids observed in ZDF rats upon testosterone treatment. In addition, the transcription factor IRE-BP1 (insulin-response element binding protein-1), was significantly reduced by testosterone in the obese rats. If lower IRE-

BP1 mRNA levels would also lead to lower IRE-BP1 protein and capacity to respond to insulin, hepatic IR might develop [260, 261].

Hepatic STAT3 activity

Leptin treatment has been shown to improve IR in various animal models and to correct hyperglycemia and hyperinsulinemia in leptin-deficient animals and humans [262]. Hepatic leptin receptor signaling involves the activation of the transcription factor STAT3 by phosphorylation [263]. Hepatic STAT3 signaling has been shown to be essential for normal glucose homeostasis [264]. The degree of hepatic STAT3-phosphorylation was different between the groups of rats used in this study. To our surprise, testosterone seemed to reduce STAT3 activity in both lean and obese rats. This effect of testosterone was confirmed in vitro, using leptin-treated primary cultures of human hepatocytes. This suggests that testosterone has the ability to block hepatic actions of STAT3, a key mediator of leptin, essential for hepatic insulin sensitivity and normal glucose homeostasis. Consequently, liver-specific STAT3 KO mice have impaired glucose tolerance [264]. Here we demonstrate that testosterone is a potent inhibitor of hepatic STAT3-phosphorylation. It is interesting to note that administration of estrogen to ob/ob mice improves the diabetic state and that hepatic STAT3 levels are increased [81]. These findings suggest that androgens and estrogens are having counter-acting effects on liver metabolism and that leptin receptor signaling may be a converging pathway involved in this. Finally, since testosterone treatment aggravates the diabetic state in ZDF male rats, it is suggested that the leptin status should be considered in situations of androgen treatment.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

The general goal of this thesis was to investigate if there is a relationship between sex differences in hepatic fuel metabolism and development of insulin resistance. This was mainly studied at the levels of hepatic gene expression, since hormonal regulation of gene expression plays an important role in maintaining metabolic homeostasis, and sets the basis for sex differences in important biological functions. By using whole-genome microarrays, we were able to identify hepatic metabolic pathways that are sex-dependent, affected by high-fat feeding and/or related to diabetes development. One important question remaining to address is whether males and females are differently affected by high-fat feeding-induced hepatic insulin resistance. We have preliminary data showing that short-term high-fat (safflower oil-enriched diet) feeding does not reduce hepatic insulin sensitivity, as compared to males that do develop insulin resistance. This should be extended to other measurements of insulin sensitivity and lipid metabolism, as well as more long-term feeding protocols.

The studies in this thesis are of observational nature, but cause-effect relationships should be addressed in future studies to enable more definitive answers regarding molecular mechanisms involved in different situations of metabolic stress. However, by using all transcript profiles generated in this thesis it should be possible to find co-regulated genes of importance for sex-different development of diet-induced T2D. This would also enable identification of *cis*- and *trans*-acting factors of importance for this. The use of *in silico* promoter analysis of co-regulated genes have previously been used in the prediction of transcription factor binding sites in the genomic sequences [103].

In order to elucidate molecular sex basis for sex differences in transcription, Waxman *et al* recently used genome-wide *in vivo* mapping of transcriptional regulatory elements. By the use of a DNase hypersensitive method coupled to high-throughput sequencing, they were able to reveal sex differences in 1284 hypersensitive sites of mice livers [265]. Also, continuous GH infusion suppressed the majority of male-specific sites and induced a set of female-specific sites of these male livers, emphasizing the importance of extensive mapping of sex-different responses within the liver.

While findings in gene expression only reflect the genotype of a system in a transcriptional network, knowledge about alterations in proteins and metabolites reveal the actual phenotype. To understand the complete system

of a cell, it is essential to link expression of transcripts and proteins with cellular metabolites. Hence, a strength of this thesis is reflected by our ability to compare transcript profiles with metabolite profiles (paper I and III), providing a unique opportunity to explore common and essential response mechanisms in the search for sex differences in hepatic metabolism and insulin sensitivity. As an example, we observed sex differences in the expression of key enzymes for gluconeogenesis, indicating an increase of HGO in the male rats. By performing in situ liver perfusion, we could show that the males did have higher levels of glucose output, confirming the expression data. Further, by using ^{1}NMR spectroscopy other interesting findings was confirmed at the metabolic level. These results have to our knowledge not been observed before. This approach would be of interest to use for further investigations of sex differences in e.g. the response to high-fat diet.

It would have been of interest to have a more translational approach in the study designs, to link the observations in the animal models with the human situation. However, studies in human liver are limited since liver biopsies cannot be routinely taken. Also, the treatments and their effects on the liver that we set out to investigate demanded a closed and supervised system as an animal model provides. Thus, as a next step, it would be of interest to use primary hepatocytes from humans to investigate the effects of the hormones included in this thesis. The results could then be compared to the animal studies. Hopefully, this approach will provide more evidence for the sex-different hepatic metabolism and its relation to hepatic IR.

Taken together, these findings suggest that the hepatic functions of female rats might contribute to a lower risk of developing lipid-induced oxidative stress, and hepatic IR. Observations of key metabolic transcripts suggest that the capacity of females to retain lipogenesis and the secretion of VLDL-TG might be related to this. We speculate that this together with higher rates of FA oxidation and glucose production in males, might at least partly explain why males are more prone to develop insulin resistance, T2D and the metabolic syndrome.

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"Really great people make you feel that you too, can become great." - Mark Twain

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