Fat metabolism in type 1 diabetes

REGNÉLL, SIMON

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Abstract

Type 1 diabetes is characterized by a lack of insulin production in the pancreatic beta cells. Insulin may affect liver fat content, and several previous publications have suggested that children with type 1 diabetes are at risk of fatty liver disease, which, in turn, is a risk factor for cardiovascular disease. The aim of this thesis was to explore aberrations of fat metabolism in type 1 diabetes.

In **paper I**, we used magnetic resonance imaging to study the hepatic fat fraction of children with type 1 diabetes. We found that the hepatic fat fraction of children with type 1 diabetes was lower than that of controls. Exploratory analyses indicated that the distribution of fat across Couinaud segments was different in children with diabetes compared to controls. We attributed this to a reduced effect of insulin in the livers of children with type 1 diabetes.

Based on these findings, we hypothesized that the fat fraction of the pancreas might be similarly affected due to a local lack of insulin. In **paper II**, we therefore studied the fat fraction and volume of the pancreas in the same children. We found no difference in pancreatic fat fraction between the groups, but the children with diabetes had lower pancreas volume. We found no correlation between diabetes duration and pancreas volume.

To further investigate the timing of changes in hepatic lipid metabolism in relation to the onset of type 1 diabetes, we longitudinally studied gene expression and serum metabolites in a rat model of type 1 diabetes in **paper III**. We found that there was a shift in hepatic lipid metabolism after the onset of hyperglycemia and that many of the lipid-regulating genes that changed their expression were influenced by insulin.

Overall, the thesis suggests that a lack of endogenous insulin production affects hepatic processes, including the accumulation of hepatic fat. It raises questions about further consequences of a lack of insulin in the liver in type 1 diabetes.

Populärvetenskaplig sammanfattning på svenska

Insulin påverkar kroppens omsättning av socker, protein och fett. Hos personer typ 1-diabetes slutar bukspottskörteln att tillverka insulin, vilket leder till att deras blodsocker blir för högt. Hos personer utan typ 1-diabetes tas det mesta av insulinet som kroppen tillverkar upp i levern. Eftersom insulin bidrar till att fett bildas förväntade vi oss att fetthalten i levern skulle spegla kroppens insulinproduktion. Publicerade undersökningar tydde även på att barn med typ 1-diabetes hade ökad risk för att få fettlever, men på grund av deras metodval var resultaten inte helt tillförlitliga. Därför undersökte vi i **artikel I** fetthalten i levern hos barn med typ 1-diabetes och barn utan typ 1-diabetes med magnetkamera, som mycket noggrant kan mäta fetthalten i kroppens organ. Undersökningen visade att barnen med typ 1-diabetes hade mindre fett i levern och att fettet hade en annorlunda fördelning i levern jämfört med barnen utan typ 1-diabetes. Vi tror att den lägre fetthalten hos barn med diabetes beror på att de får mindre insulin till levern än friska barn.

Resultaten gjorde att vi undrade om andra organ hos patienter typ 1-diabetes påverkas på ett liknande sätt. Eftersom bukspottskörteln också drabbas av insulinbrist resonerade vi att fetthalten även i detta organ kunde minska. Det är känt sedan tidigare att bukspottskörteln hos typ 1-diabetespatienter är mindre än hos andra, och en anledning till detta tros vara lokal brist på insulin. I **artikel II** undersökte vi bukspottskörtelns fetthalt och storlek hos samma barn som i **artikel I**. Vår undersökning visade att det inte fanns någon skillnad i fetthalt, men att barnen med typ 1-diabetes hade mindre bukspottskörtlar. Bukspottskörtelns volym berodde inte på hur länge barnen hade haft diabetes, vilket antyder att mycket av storleksminskningen sker innan patienternas insulinnivåer når en så pass låg nivå att de förlorar kontroll över sitt blodsocker.

I **artikel I** hade vi konstaterat att barn med typ 1-diabetes har mindre fett i levern än kontrollpersoner. Vi misstänkte att fettomsättningen förändrades under insjuknandet i typ 1-diabetes, då kroppens förmåga att tillverka insulin avtar. Detta är dock svårt att påvisa hos människor, eftersom man inte exakt nog kan avgöra när de kommer att insjukna i diabetes. Därför undersökte vi i **artikel III** hur genuttrycket i levern och kemikaliesammansättningen i blodet förändrades hos en slags råtta som på ett förutsägbart sätt drabbas av en motsvarighet till typ 1-diabetes. Vi analyserade prover från levern och blodet vid flera tillfällen före och minst ett tillfälle efter att råttorna hade insjuknat med diabetes. Våra fynd visade att levern i samband med att råttorna får diabetes ändrar sitt genuttryck så att mindre fett ansamlas. Många av generna som deltar i denna förändring påverkas av insulin.

Sammantaget är avhandlingens huvudfynd att barn med typ 1-diabetes har mindre fett i levern än personer utan diabetes och att detta främst beror på insulinbrist i levern. Detta väcker frågan om vilka andra konsekvenser som den lokala bristen på insulin i levern orsakar, vilket framtida studier får besvara.

List of papers

Papers included in this thesis

I. **Regnell SE**, Peterson P, Trinh L, Broberg P, Leander P, Lernmark Å, Månsson S, Elding Larsson H. Magnetic resonance imaging reveals altered distribution of hepatic fat in children with type 1 diabetes compared to controls. Metabolism. 2015;64(8):872-8

II. **Regnell SE**, Peterson P, Trinh L, Broberg P, Leander P, Lernmark Å, Månsson S, Elding Larsson H. Pancreas volume and fat fraction in children with Type 1 diabetes. Diabet Med. 2016;33(10):1374-9.

III. **Regnell SE**, Hessner MJ, Jia S, Åkesson L, Stenlund H, Moritz T, La Torre D, Lernmark Å. Longitudinal analysis of hepatic transcriptome and serum metabolome demonstrates altered lipid metabolism following the onset of hyperglycemia in spontaneously diabetic BioBreeding rats. Manuscript.

Papers not included in this thesis

I. **Regnell SE**, Lernmark Å. Hepatic steatosis in type 1 diabetes. Rev Diabet Stud. 2011;8(4):454-67.

II. **Regnell SE**. Cannabinoid 1 receptor in fatty liver. Hepatol Res. 2013;43(2):131-8.

III. **Regnell SE**, Lernmark Å. The environment and the origins of islet autoimmunity and Type 1 diabetes. Diabet Med. 2013;30(2):155-60.

IV. Cooper ME, **Regnell SE**. The hepatic cannabinoid 1 receptor as a modulator of hepatic energy state and food intake. Br J Clin Pharmacol. 2014;77(1):21-30.

V. **Regnell SE**, Lernmark Å. Early prediction of type 1 diabetes. Diabetologia. Accepted manuscript.

Abbreviations

ALAT	alanine transaminase
ASAT	aspartate transaminase
AUC	area under the curve
B	magnetic flux density
BBDP	BioBreeding diabetes-prone
BBDR	BioBreeding diabetes-resistant
BMI	body mass index
CBLB	casitas B-lineage lymphoma b
CT	computed tomography
FF	fat fraction
FLI	fatty liver index
GAD	glutamic acid decarboxylase
GC	group-specific component
GCKR	glukokinase regulator
GGT	gamma-glutamyltransferase
GLP-1	glucagon-like peptide-1
GPR	G protein-coupled receptor
HDL	high-density lipoprotein
HPLC	high-performance liquid chromatography
HSI	hepatic steatosis index
IA-2	islet antigen 2
IAA	insulin autoantibody
ICA	islet cell cytoplasmic autoantibody
ICSA	islet cell surface autoantibody
IDEAL	iterative decomposition of water and fat with echo asymmetry and least squares estimation
KDP	Komeda diabetes-prone
KND	Komeda non-diabetic
HLA	human leukocyte antigen
LCMS	liquid chromatography-mass spectrometry
LCP1	lymphocyte cystosolic protein 1
LDL	low-density lipoprotein
LETL	Long-Evans Tokushima lean
lyp	lymphopenia
LYPLAL1	lysophospholipase-like 1
M	magnetization/molecule
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MRS	magnetic resonance spectroscopy

MS	mass spectrometry
m/z	mass-charge ratio
NAFLD	nonalcoholic fatty liver disease
NAFLD-LFS	nonalcoholic fatty liver disease liver fat score
NAFPD	nonalcoholic fatty pancreas disease
NASH	nonalcoholic steatohepatitis
NCAN	neurocan
NMR	nuclear magnetic resonance
NOD	non-obese diabetic
PPAR	peroxisome proliferator-activated receptor
PPP1R3B	protein phosphatase 1, regulatory subunit 3B
PNPLA3	patatin-like phospholipase domain-containing protein 3
RF	radiofrequency
ROC	receiver operating characteristics
SNP	single nucleotide polymorphism
SREBP-1c	sterol regulatory-binding protein 1c
STZ	streptozotocin
T	tesla
THC	tetrahydrocannabinol
TM6SF2	transmembrane 6 superfamily 2
TR	repetition time
TE	echo time
VLDL	very low density lipoprotein
Wb	weber
ZnT8	zinc transporter 8
γ	gyromagnetic ratio
$\rho_{f,w}$,	fat or water signal, respectively
ω	precession rate

Aim and hypotheses

The overall aim of this thesis is to study aberrations of fat metabolism in type 1 diabetes, with a focus on fatty liver disease.

Paper I

Primary hypothesis: Hepatic fat fraction differs between children with type 1 diabetes and controls.

Exploratory outcomes:

- Distribution of hepatic fat across Couinaud segments in children with type 1 diabetes compared to controls
- Relation of the fat fraction of specific Couinaud segments with the total hepatic fat fraction.
- Correlations of anthropometric measurements and blood tests with hepatic fat fraction.

Paper II

Primary hypothesis: Pancreatic fat fraction differs between children with type 1 diabetes and controls.

Exploratory outcomes:

- Difference in pancreas volume between children with type 1 diabetes and controls.
- Difference in the fat fraction of a segment of m. erector spinae between children with type 1 diabetes and controls.
- Correlations of anthropometric measurements and blood tests with hepatic fat fraction.

Paper III

Primary hypothesis: Hepatic gene expression and serum metabolites differ in regard to lipid metabolism before and after diabetes onset.

Exploratory outcomes:

- Overlap of differentially expressed genes affecting lipid processes with differentially expressed genes regulated by insulin.
- Overlap of differentially expressed genes affecting lipid processes with differentially expressed genes regulated by glucose.
- Overlap between genes of metabolic processes and metabolites that are substrates in those metabolic processes.

Background

Type 1 diabetes

Type 1 diabetes is characterized by the progressive autoimmune destruction of pancreatic beta cells, which leads to a deficiency of insulin. Lack of insulin causes hyperglycemia with symptoms such as polydipsia, polyuria, polyphagia, and blurred vision. Untreated type 1 diabetes eventually leads to ketoacidosis and death. Although the clinical onset of the disease is often abrupt, type 1 diabetes can be preceded by demonstrable islet cell autoimmunity for up to several decades before insulin levels decline to the point at which the patient experiences symptoms (1).

Patients with type 1 diabetes are treated with exogenous insulin. Intensive treatment with insulin can maintain near-normoglycemia, whereas long-standing hyperglycemia increases the risk of complications such as retinopathy, neuropathy, and diabetic kidney disease (2). Due to the *glycemic legacy* or *metabolic memory* effect, periods of hyperglycemia permanently increase the risk of long-term diabetes complications, even if blood glucose later returns to normal levels (3).

There is substantial geographic variation in the prevalence of type 1 diabetes. Finland has the highest national rate of type 1 diabetes, with an incidence a hundredfold greater than for instance China (4). Type 1 diabetes represents about 10% of the cases of diabetes in Sweden and over 95% of diabetes in Swedish children. The incidence of type 1 diabetes in children increased from the introduction of

national registries in the 1970s until the early 2000s, particularly in younger age groups. However, there is evidence that this increased incidence of type 1 diabetes among younger children in Sweden has declined in recent years (5). Conversely, in countries with a lower incidence of type 1 diabetes, the increase in incidence has tended to accelerate (6).

Pathogenesis of type 1 diabetes

The causes of type 1 diabetes are still unclear, but both genetic and environmental factors are believed to contribute to its pathogenesis (7). The concordance of type 1 diabetes in monozygotic twins is 30-50%, yet only about 15% of newly diagnosed type 1 diabetes patients have a first-degree relative with the disease (8). The lifetime risk of developing type 1 diabetes among siblings of persons with the disease is about 7%, and slightly less than that of the children of a parent with type 1 diabetes (9).

Genome-wide association studies have identified over 40 genetic loci that affect the risk of type 1 diabetes (10). However, the human leukocyte antigen (HLA; also known as major histocompatibility complex, MHC) class I and II regions remain the greatest identified contributors to the genetic susceptibility to type 1 diabetes (11). HLA class II molecules are expressed in antigen-presenting cells such as dendritic cells, phagocytes, and B-cells. The HLA class II molecules induce an immune response against foreign antigens by presenting peptide-derived antigens to helper T-cells (12). 69% of persons who develop type 1 diabetes before adulthood have the DQ2 or DQ8 variants of HLA class II, compared to 15% of the general Swedish population (13). The DQ2/8 haplotype confers the highest risk of disease (7). HLA class I molecules display peptide fragments of non-self proteins from within the cell to cytotoxic T cells (12). Several HLA class I variants, including HLA-B*39, have been associated with type 1 diabetes (14).

Several environmental factors have been implicated in triggering islet cell autoimmunity and type 1 diabetes in the genetically susceptible (7). Exogenous influences might occur as early as during gestation; for instance, maternal enterovirus infection during pregnancy has been associated with a higher risk of type 1 diabetes among offspring (15). Further proposed environmental triggers of autoimmunity and progression to type 1 diabetes include virus infections, diet, weight, and the microbiome (7).

The mechanisms of specific beta cell destruction are not yet fully understood. The earliest sign of islet autoimmunity is usually the presence of islet autoantibodies, produced by B lymphocytes. Four major types of antibodies are generally recognized: antibodies against insulin (IAA), glutamic acid decarboxylase (GAD), islet antigen 2 (IA2), and zinc transporter 8 (ZnT8) (16, 17). 44-92% of type 1 diabetes patients are positive for IAA, 64-75% are positive for GAD, 61-77% for IA-2, and 61-80% for ZnT8. 96% of patients are positive for at least one of these four autoantibodies, and a number of further candidate autoantibodies have been identified (18). The presence of multiple autoantibodies predicts diabetes more strongly than any single autoantibody (19).

In persons who develop type 1 diabetes, islet cell autoimmunity is followed by beta cell killing. B cells are believed to participate in the immune response by presenting antigens to CD4+ and CD8+ T cells

(20). It is thought that cytotoxic T-cells, helper T-cells, natural killer cells, and macrophages contribute to the actual destruction of beta cells (21).

Beta cell destruction causes a deficiency of insulin. This ultimately leads to the absence of insulin action in its target tissues, including the liver, which is a major focus of **papers I** and **III**. The mechanisms of insulin secretion and insulin's interaction with the liver are therefore discussed below.

Insulin secretion

Insulin is produced and secreted by the beta cells of the islets of Langerhans in the pancreas. The initial precursor of insulin is preproinsulin, which has 110 amino acids arranged in a single chain, with a hydrophobic signal sequence at its N-terminal end (22). Removal of this signal sequence yields proinsulin. The signal sequence is degraded, while proinsulin is transported into maturing secretory vesicles. Proinsulin is then further processed, resulting in the removal of a peptide known as the connecting (C) peptide and the formation of mature insulin. In this mature, biologically active state, insulin consists of 51 amino acids arranged into two peptide chains, designated A and B, which are joined by two disulfide bonds (23). Equimolar amounts of insulin and C-peptide are present in the mature vesicles and are eventually secreted from the beta cells (24). As C-peptide is extracted to a lower extent in the liver than insulin, it is frequently used in clinical practice as a marker of insulin secretion (25). In **paper I**, we had hypothesized that liver fat could correlate with C-peptide, as we expected liver fat to reflect beta cell function.

Beta cells are stimulated to secrete insulin by a complex interplay of external and internal factors. These factors include carbohydrates, amino acids, fatty acids, hormones, and neurotransmitters of the autonomic nervous system (26-30). The most significant and extensively studied of these stimuli is glucose. Glucose-induced insulin secretion is believed to be mediated as follows: First, glucose is transported into beta cells through facilitated diffusion of GLUT2 glucose transporters. The intracellular glucose is then metabolized to ATP. The resulting elevation of the ATP/ADP ratio causes cell-surface ATP-sensitive K^+ channels to close, inducing cell membrane depolarization. The shift in electric potential causes cell-surface voltage-dependent Ca^{2+} channels to open, facilitating extracellular Ca^{2+} influx into the beta cell. The subsequent rise in cytosolic Ca^{2+} causes the fusion of the secretory granules with the cell membrane, resulting in the exocytosis of insulin, C-peptide, amylin and residual amounts of proinsulin (31). Figure 1 illustrates the basic intracellular events involved in glucose-stimulated insulin secretion from beta cells.

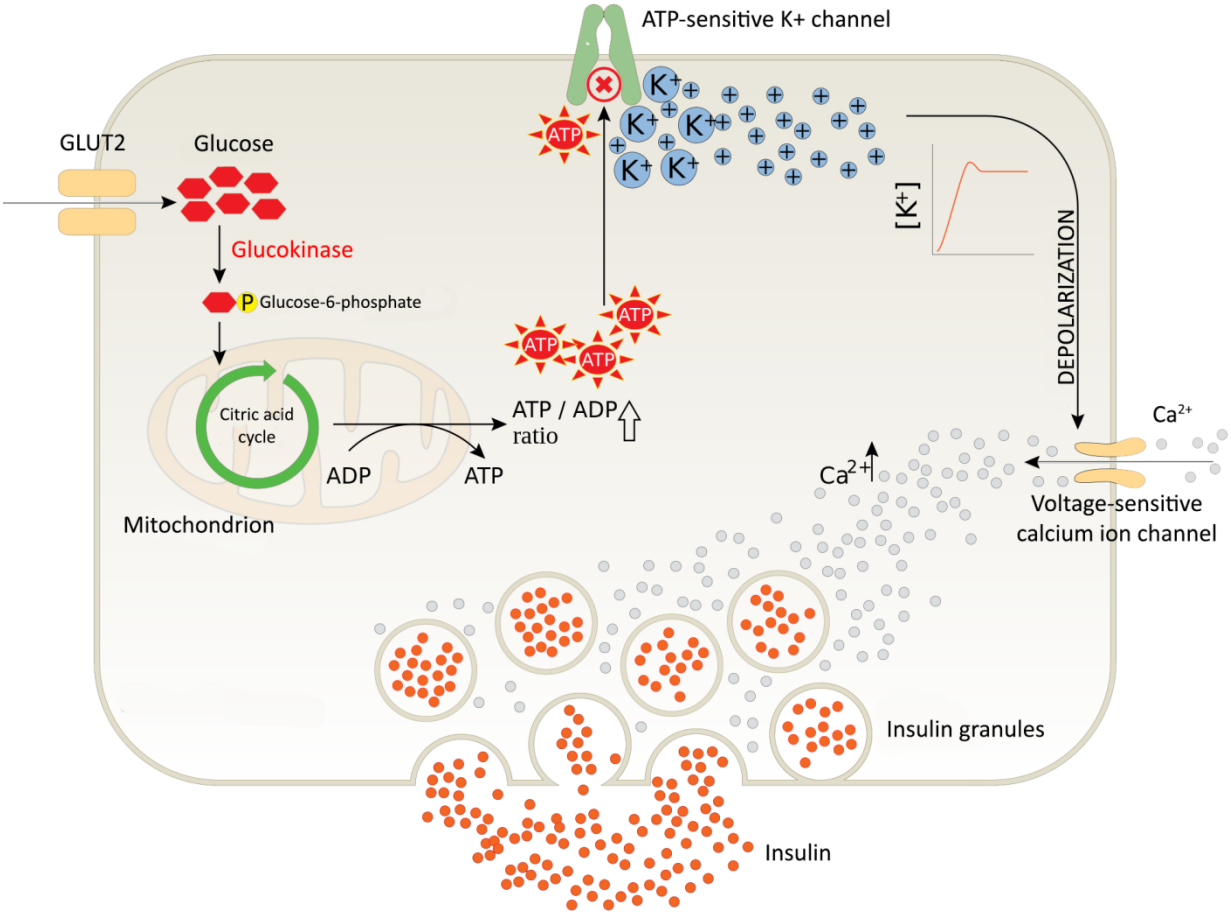

Figure 1. Basic intracellular events leading to glucose-stimulated insulin secretion from beta cells (32).

The release of insulin from beta cells is biphasic. An increase in glucose results in an initial, transient burst of insulin secretion that falls back to near-basal levels within ten minutes. The second phase involves a slower progression to maximal secretion levels, which persists throughout the duration of glucose exposure. This biphasic pattern is believed to be caused by the presence of two populations of insulin granules – the first phase stems from granules which are already docked at the cell membrane, whereas the second relies on recruitment from a pool of reserve granules from deeper within the cell (33).

Both basal and postprandial circulating insulin levels oscillate with a period of approximately five to ten minutes (34), contemporaneously with the calcium concentration of the beta cells (35, 36). The oscillations are well synchronized throughout entire islets because of the spread of electric potentials between adjacent cells through gap junctions (37, 38), as well as diffusible factors such as ATP (39). The periodicities of the approximately one million islets of the pancreas are in turn thought to be synchronized by autonomic ganglia (40). In addition to the aforementioned high-frequency oscillations in insulin concentration, low-frequency oscillations, with a period of 50 to 150 minutes have also been reported (41).

The arterial blood supply of the pancreas arises from the superior and inferior pancreaticoduodenal arteries and the splenic artery, which are divisions of the celiac trunk (42). The individual islet of Langerhans is supplied with blood by a central arteriole, the capillaries of which spread towards the islet's periphery. A core of beta cells is located upstream of the surrounding alpha cells, which leads to the former's insulin secretion affecting the latter (43). The capillaries of the pancreas's endocrine islets and exocrine lobules are continuous – after passing through the islets, the blood reaches a secondary network around the acinar cells (44). This connection between the endocrine and the exocrine pancreas underlies the hypothesis that the lack of insulin reaching the acinar cells in type 1 diabetes causes pancreatic atrophy, which is studied in **paper II**. The vessels containing venous blood eventually converge into the pancreatic and pancreaticoduodenal veins, which empty into the hepatic portal vein.

Insulin and the liver

Blood from the pancreas initially flows into the hepatic portal vein. Some 75% of the blood flow of the liver is supplied from the portal vein, with the remainder coming from the hepatic arteries (45). Blood from the portal vein, which in persons without type 1 diabetes contains insulin, mixes with blood from the hepatic artery in the sinusoids of the hepatic lobules. Blood in the sinusoids drains into the central veins of the hepatic lobules and finally leaves the liver through the hepatic veins (46).

Of the insulin that enters the liver, 50 to 80% of is cleared in first-pass transit (47). The fenestrated epithelium of hepatocytes allows proteins such as insulin to enter the space of Disse, from which the insulin can bind to insulin receptors or be absorbed by hepatocellular microvilli (48). Hepatocytes clear most of the insulin in the liver, while Kupffer cells only contribute to 15% of hepatic insulin degradation (49). The intracellular degradation of insulin is largely performed by insulin-degrading enzyme; the liver is the organ with the highest concentration of this enzyme (50).

The effects of insulin at its target cell begin by its binding to the transmembranous insulin receptor. The insulin receptor is a dimer consisting of two polypeptide α-subunits, which are each linked to a β-subunit. The extracellular α-subunits bind to the insulin molecule. Upon binding, they discontinue the inhibition of tyrosine kinase action of the transmembrane β-subunits (51, 52).

The β-subunits phosphorylate tyrosine residues on the intracellular enzymes. Its targets include insulin receptor substrate and Src homologous and collagen (Shc). Phosphorylation of these proteins causes further signaling via the phosphoinositide 3-kinase/Akt and Ras/MAP kinase pathways. Downstream effects of Akt promote glucose uptake, glycogen synthesis, and protein synthesis (53). Insulin also induces the expression of liver X receptor, which activates fatty acid synthase, steatoyl CoA desaturase 1, sterol regulatory element-binding protein 1c, and carbohydrate response element binding protein to promote lipogenesis (54, 55). Thus, insulin promotes fat accumulation in its target tissues; the macroscopic effects of the reduced action of insulin on hepatic fat were made clear in **paper I**.

Pancreatic morphology in type 1 diabetes

Although selective destruction of beta cells is the hallmark of type 1 diabetes, the overall morphology of the pancreas is also pathologically affected, which was the focus of **paper II**. Beta cells comprise only about 1% of the pancreatic volume (56), yet it was noted decades ago that patients with type 1 diabetes

have markedly lower pancreas volumes than healthy subjects (57). In adults with an average duration of type 1 diabetes of 13 years, the pancreatic volume, as measured with MRI, was 48% smaller than that of age-matched controls (58). In adults who had been diagnosed with type 1 diabetes during the previous six months, the MRI-determined pancreatic volume in relation to the body surface area was 31% lower than that of controls (59). An autopsy study found that type 1 diabetes-associated autoantibody-positive organ donors, who had a lower average C-peptide value than non-diabetic, autoantibody-negative donors, also had a lower pancreas weight (60). In children and adolescents, the size of the pancreas decreases in relation to age-matched controls with increasing duration of type 1 diabetes (61), although others have not found such a correlation in adults (62). It has further been noted that pancreatic atrophy is unrelated to glycemic control (63) and residual beta cell function (64).

The exocrine pancreas secretes over 20 types of enzymes through the pancreatic ducts into the duodenum. These include proteases, lipases, amylases, ribonucleases, and hydrolases (65). There is evidence that the reduced pancreas size in type 1 diabetes is associated with decreased exocrine function, as measured directly in the duodenum or with indirect markers such as fecal elastase and serum trypsin (66-68). As the pancreas has a large reserve capacity, 90% of acinar tissue typically needs to be lost before symptoms such as steatorrhea occur (69), and some have claimed that fecal elastin-1 is a poor marker of pancreatic insufficiency in type 1 diabetes patients (70). There is conflicting evidence about the relation between residual beta cell function and pancreatic insufficiency. Some studies have concluded that type 1-diabetic patients with residual insulin secretion produce more exocrine pancreatic enzymes than patients with an absolute insulin deficiency (71), whereas others have found no correlation between markers of exocrine pancreatic function and markers of residual beta cell function (71).

It has long been believed that diabetes-associated pancreatic atrophy and fibrosis is caused by the missing trophic paracrine effect of insulin (61). More recently, it has been suggested that pancreatic atrophy is a consequence of the long-standing inflammation associated with beta cell destruction (72). Histological analyses of exocrine pancreatic tissue from type 1 diabetes patients has revealed infiltration with neutrophils and eosinophils (73). Furthermore, several studies have shown that type 1 diabetes patients have higher serum levels of autoantibodies against exocrine pancreatic antigens than controls (74, 75). It has also been found that pancreatic acinar cells express some of the same antigens as beta cells (76).

In **paper I**, we found that children with type 1 diabetes had a lower hepatic fat fraction than controls. We suggested that this was caused by less insulin reaching the liver. Based on our finding, we hypothesized in **paper II** that the pancreas might be similarly affected, as we expected a reduced paracrine effect of insulin to reduce local lipogenesis. The pancreatic fat fraction in type 1 diabetes had not been studied before we performed our experiment. However, some studies of pancreatic fat have been performed in healthy and in type 2-diabetic persons.

Throughout childhood, the pancreatic fat volume increases while retaining the same proportion to the total growth of the pancreas, so that the ratio of pancreatic fat/parenchyma remains constant. In healthy adults, this ratio increases with age – more so in men than in women. Overweight persons have

more pancreatic fat than lean persons (77). Pancreatic fat content is positively correlated with liver fat. This relationship seems to be mediated by general obesity (78-80). Obese children with NAFLD who also have nonalcoholic fatty pancreas disease (NAFPD) tend to have more signs of hepatic inflammation and fibrosis than obese children with NAFLD but without NAFPD (81). Twice as much fat was found in the pancreata of type 2 diabetes patients as in those of age- and BMI-matched controls (about 20% compared to 10% of the organs' volume, respectively). In this study, pancreatic fat correlated negatively with indicators of beta cell function (82), although others have not found any such correlation (83). A more recent cohort study found no independent correlation between CT-determined fatty pancreas and the five-year incidence of type 2 diabetes after controlling for known risk factors (including fatty liver) (84).

Analogously with how NAFLD may progress to steatohepatitis and hepatocellular carcinoma (as discussed below), NAFPD may promote pancreatitis and pancreatic cancer (85). Extrapolating these data, one would expect a higher prevalence of pancreas cancer in populations with greater prevalence of NAFLD (86), and there is indeed evidence of a slightly increased risk of pancreatic cancer in obesity and type 2 diabetes (87, 88). However, there appears to be no increased risk of pancreas cancer in type 1 diabetes (89). Similar to how we in **paper I** found an uneven distribution of hepatic fat, there have been reports of pancreatic fat infiltration being more severe in the anterior aspect of the head of the pancreas than in the posterior aspect (90).

Rodent models of type 1 diabetes

Rodent models of type 1 diabetes have been frequently used to dissect the genetics, environment, and immunology of type 1 diabetes. The models can be divided into rodents that spontaneously develop autoimmune diabetes, and those which require an identified exogenous trigger to develop diabetes. See Table 1 for a summary of some of the most widely used rodent models of type 1 diabetes.

Non-obese diabetic (NOD) mice spontaneously develop diabetes from 10 weeks on, with a higher incidence in females than in males. NOD mice share many pathogenetic features with humans developing type 1 diabetes. The HLA region is the most important genetic determinant for their susceptibility to diabetes. NOD mice display many of the autoantibodies found in humans, including insulin, GAD, IA-2, and IA-2 β antibodies. The pancreas of NOD mice is infiltrated by dendritic cells, macrophages, neutrophils, and, later, lymphocytes (91).

BioBreeding diabetes-prone (BBDP) rats derive from a colony of rats which developed spontaneous hyperglycemia and ketoacidosis. At between 50 and 90 days of age, BBDP rats develop insulitis followed by destruction of beta cells and the rapid onset of hyperglycemia (92). In the BBDP rat, as in humans but in contrast to the NOD mouse, there is no significant leukocyte infiltration around the islets ("peri-insulitis") before progression to frank insulitis and diabetes (93). BBDP rats also develop complications of diabetes equivalent to those in humans, such as neuropathy, kidney disease, and vascular disease (94-96). BBDP rats also have a lower pancreas volume than diabetes-resistant (BBDR) rats before the onset of insulitis and diabetes (97), consistent with our findings in humans presented in **paper II**.

In addition to developing diabetes, BBDP rats are characterized by a severe reduction in both CD4+ and CD8+ T cells due to the cells' undergoing apoptosis within days of reaching the circulation (98, 99). Lymphopenia is required for the development of diabetes in these rats (100); this is entirely dissimilar to type 1 diabetes in humans, which is not associated with lymphopenia. Various immunosuppressive and immunomodulatory treatments can prevent diabetes in BBDP rats. These include thymectomy, tumor necrosis factor-α, lymphotoxin, interferon-α, interferon-γ, and anti-interferon-γ (101).

Introgression of the lymphopenia (*lyp*) gene interval from BB diabetes-prone rats onto the genetic background of BBDR rats resulted in a strain of rats designated BBDR.$^{lyp/lyp}$ (102). Breeding of the congenic DR.lyp rat lines produces Mendelian proportions of DR.$^{lyp/lyp}$ (25%), DR.$^{lyp/+}$ (50%), and DR.$^{+/+}$ (25%). DR.lyp/lyp rats are lymphopenic from birth and rapidly develop hyperglycemia at between 46 and 81 days, whereas DR.$^{lyp/+}$ and DR.$^{+/+}$ are have normal lymphocyte count and do not develop diabetes (103). In **paper III**, BBDR.$^{lyp/lyp}$ rats were used as models of type 1 diabetes, while BBDR.$^{lyp/+}$ and BBDR.$^{+/+}$ rats served as controls.

The Long-Evans Tokushima Lean (LETL) rat was the first rat model of spontaneous autoimmune diabetes without lymphopenia or sex-specificity (104). Subsequently, two substrains were established: the Komeda diabetes-prone (KDP) and the Komeda non-diabetic (KND) from the original inbred LETL rats. The cumulative frequency of diabetes in KDP rats is about 70%, and all rats have mild to severe insulitis at 120 to 220 days of age (105). In addition to mutations in HLA class II, a loss-of-function mutation in casitas B-lineage lymphoma b (*Cblb*), coding for a type of ubiquitin ligase, significantly contributes to the development of diabetes in KDP rats (106). However, variations in the *CBLB* gene have so far not been clearly linked to type 1 diabetes in humans (107).

The LEW.1AR1/Ztm-iddm rat arose through a spontaneous mutation in a congenic Lewis rat strain with a specific MHC haplotype (108). The prevalence of diabetes is about 20%, with onset of disease occuring at about 8 weeks of age.

In addition to rat models of spontaneous autoimmune diabetes, some environmental perturbants can induce diabetes in otherwise non-diabetic strains of rodents. For instance, diabetes can occur in diabetes-resistant BB rats exposed to immunomodulatory drugs or viral infections (109, 110). Other chemicals can bring about diabetes independently of the genetic background of the animal strain. An example is streptozotocin (STZ), which was originally identified as an antibiotic (111). The chemical was subsequently found to be selectively toxic towards beta cells. STZ has found infrequent clinical use for the treatment of rare islet cell tumors (112), but it is more widely used to induce beta cell failure in several animal models of type 1 diabetes (113). Several publications discussed in **paper III** have studied metabolomic changes in rodents after inducing diabetes with STZ.

Table 1. Comparison of human type 1 diabetes and rodent models of spontaneous autoimmune diabetes (91, 101, 114).

	Human	NOD mouse	BBDP rat	KDP rat	LEW rat
Lifetime incidence of autoimmune	0.3%	20% to 80%	100%	70%	20%

diabetes					
Age at onset	Infancy to adulthood	10 weeks	7 to 14 weeks	8 to 16 weeks	6 to 12 weeks
Ketoacidosis	Severe	Mild	Severe	Severe	Severe
Autoantibodies	GAD, IA-2, IAA, ZnT8	IAA, ICA, GAD	IAA, ICA, GAD	None known	None known
Dominant genetic influence	HLA	HLA	HLA	HLA, *Cblb*	HLA
Associated autoimmune diseases	Celiac disease, pernicious anemia, polyendocrine syndromes, thyroiditis, vitiligo	Thyroiditis	Thyroiditis	Adrenitis, hypophysitis, nephritis, thyroiditis	None known
Sex predilection	Possibly male > female after puberty	Female > male	None	None	None

Nonalcoholic fatty liver disease

Epidemiology of nonalcoholic fatty liver disease

NAFLD is defined as histological or radiological evidence of fat constituting more than 5% of the liver's weight in the absence of signficant alcohol consumption (30 g/day for men and 20 g/day for women) (115, 116). NAFLD is the most common chronic liver condition in the Western world (117); a magnetic resonance spectroscopy study identified NAFLD in 31% of a general US population (118), while a review of liver biopsies from Korean potential liver donors found > 5% fat in 51% of samples and > 30% fat in 10% of sampes (119).

NAFLD is usually associated with obesity, dyslipidemia, and insulin resistance (120), and an increasing incidence of NAFLD is believed to reflect an increase of obesity. NAFLD has also been associated with polycystic ovary syndrome, hypothyroidism, hypopituitarism, hypogonadism, hepatitis C, Wilson's disease, Reye's syndrome, HELLP syndrome, and drugs such as amiodarone, methotrexate, tamoxifen, and valproate (115, 116). Furthermore, some studies have implicated type 1 diabetes as a risk factor for NAFLD (121-124). This is one of the reasons we studied liver fat in children with type 1 diabetes in **paper I**.

Liver steatosis *per se* is normally considered a fairly benign condition, but it can progress to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and ultimately hepatocellular cancer (125-127). NASH was found in 2.2% of Korean potential liver donors (119). The term NAFLD encompasses the entire spectrum from benign hepatic steatosis to inflammation and cirrhosis (115).

NAFLD has been identified as an independent risk factor for overall mortality (128), although some studies have found that NASH, but not simple steatosis, is associated with increased mortality (129). A study of male Swedish army conscripts found that an increase of BMI with 1 kg/m^2 was associated with a

5% increased risk of severe liver disease during 38-year follow-up, after adjustment for known risk factors for liver disease and mortality (130). NASH has been associated with more severe insulin resistance and dyslipidemia in patients with type 2 diabetes (131).

There is no specific treatment for NAFLD, but there is evidence that lifestyle changes and some medicines can alleviate the condition. Weight loss, through a hypocaloric diet alone or in combination with increased physical exercise, can reduce hepatic steatosis and inflammation (132). There is also evidence that exercise without weight loss can reduce hepatic steatosis, although the effect on other aspects of liver histology has not been studied (133). Pioglitazone and vitamin E have moderate-grade evidence of improving NASH, whereas metformin and statins are not recommended by American gastroenterological guidelines (115). Pioglitazone redistributes fat from the liver to adipocytes (134), possibly by increasing adipocyte insulin sensitivity (131), while vitamin E is believed to reduce oxidative stress (135). Recently, the GLP-1 analogue liraglutide has been found to improve NASH (136).

Fatty liver disease correlates more strongly with insulin resistance than BMI does (137). In children, fat in the liver and in muscle is associated with insulin resistance (138). It has been suggested that preferential deposition of fat in skeletal muscle of type 1 diabetes patients increases insulin resistance (139), which is why we compared the muscular fat fraction of m. erector spinae in type 1-diabetic children and controls in **paper II**.

Pathophysiology of nonalcoholic fatty liver disease

NAFLD is characterized by the accumulation of triglycerides in the liver. Hepatic triglycerides are formed by the esterification of free fatty acids and glycerol. There are three potential sources of free fatty acids: (1) lipolysis of triglycerides in adipose tissue, (2) dietary fat, and (3) de novo lipogenesis within the liver. Free fatty acids in the liver can be used in three ways: (1) generating ATP through beta-oxidation in the mitochondria; (2) re-esterification with glycerol to triglycerides and storage in hepatocytes; and (3) export from the liver as very low density lipoproteins (VLDL). Hence, hepatic fat accumulation can occur as a consequence of increased fat synthesis, increased fat delivery, decreased fat export, and decreased fat oxidation.

In healthy persons, de novo lipogenesis contributes to less than 5% of hepatic triglycerides (140). In contrast, there is evidence that de novo lipogenesis is abnormally increased in NAFLD and insulin resistance (141). To establish the relative contribution of different sources of lipids to hepatic steatosis, Donnelly et al. injected NAFLD patients with multiple stable isotopes for four days before taking a liver biopsy. They found that approximately 60% of liver triglyceride content derived from free fatty acid influx from adipose tissue, 26% from de novo lipogenesis, and 15% from diet (142).

In patients with NAFLD, insulin suppresses adipocyte lipolysis less than in healthy persons, causing more free fatty acids to enter the blood (143). Hepatic mitochondria in NAFLD show structural abnormalities, decreased mitochondrial DNA, and impaired beta-oxidation (144). Furthermore, chronic hyperinsulinemia promotes hepatic expression of steatogenic transcription factors such as sterol regulatory-binding protein 1c (SREBP 1c) and decreases the export of lipids as VLDL (117). Taken together, this evidence suggests that NAFLD is caused by a combination of increased import of

adipocyte-derived fatty acids from the bloodstream, increased de novo lipogenesis, decreased beta oxidation, and decreased export of fatty acids as VLDL.

There is evidence that mitochondrial dysfunction, leading to reduced hepatic ATP levels, precedes NAFLD and hepatic insulin resistance (145). Reduced hepatic ATP has also been demonstrated in patients with type 1 diabetes (146).

Histology and pathophysiology of nonalcoholic steatohepatitis

Diagnosis of NASH currently requires a liver biopsy. Typical histologic features include (147)

- macrovesicular steatosis – hepatocytes containing large fat droplets that frequently displace the nucleus into the cell periphery;
- hepatocyte ballooning degeneration – hepatocytes augmenting during cell death; and
- inflammation – lymphocytic and granulocytic infiltration, typically most pronounced in the acinar zone 3 (furthest from the central vein).

Other histopathological features that may be observed on biopsy include (147-149)

- acidophil bodies – necrotic hepatocytes with dense cytoplasm and nucleus, surrounded by lymphocytes;
- Mallory–Denk bodies – aggregations of misfolded proteins that commonly seen near the nucleus of ballooned hepatocytes;
- glycogenated nuclei – glycogen accumulation in hepatocyte nuclei, which gives them a translucent appearance;
- iron deposition;
- megamitochondria – abnormally large mitochondria showing loss of cristae, multilamellar membranes, and paracrystalline inclusions;
- lobular lipogranulomas – nodules of lipids with granulomatous inflammation; and
- acinar zone 3 fibrosis.

Fibrosis is not required for the diagnosis of NASH; however, it predicts progression to advanced liver disease and death (150, 151). NASH in children can have a different morphology from that found in adults, with less hepatocyte ballooning and fewer Mallory-Denk bodies. It often displays macrovesicular, azonal hepatocellular steatosis; portal inflammation; and portal fibrosis (152). See Figure 2 for a comparison of the histology of NAFLD and NASH.

One theory of the pathogenesis of NASH suggests that the disease takes place in two steps. First, triglycerides are accumulated in the liver. Second, lipid peroxidation causes oxidative stress, which triggers the necroinflammatory changes seen histologically in NASH (153). Alternatively, it has been suggested that fatty acid-derived metabolites, rather than fatty acids themselves play a major role in the pathogenesis of NASH (154).

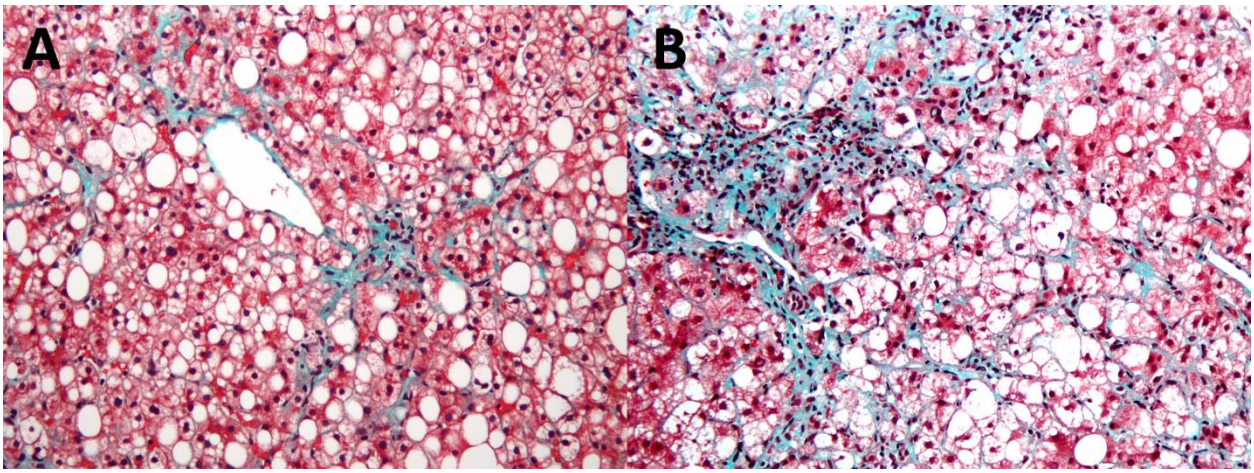

Figure 2. Histology of NAFLD (A) and NASH (B) in human livers stained with Masson's trichrome and Verhoeff stain (155, 156). Both display macrovesicular steatosis. Fibrosis (stained green) is more pronounced in NASH, which also features leukocyte infiltration, ballooning degeneration, and necrosis.

Nonalcoholic fatty liver disease in children

As mentioned, NAFLD is the most common chronic liver condition in the Western world, and this includes the pediatric population (157). It has been suggested that the progression of NAFLD in children often has a more rapid and severe course than in adults, with a higher risk of developing NASH (158, 159). Furthermore, the histopathology of pediatric and adult steatosis and NASH differ, although the significance of this is unclear (158).

In a Japanese cross-sectional study, fatty liver (determined by ultrasonography) was present in 2.6% of children between the ages of 4 and 12 (160). In this cohort, waist circumference was an independent risk factor for NAFLD (161). A more recent ultrasound study of Taiwanese children found NAFLD in 3% of normoweight children, 25% of overweight children, and 76% of obese children (162). In various other populations of obese children, ultrasonography-determined NAFLD has been found in between 12% and 77% of subjects (163, 164). An American autopsy study of 742 children who had died of unnatural causes found that 0.7% of children aged 2 to 4 and 17.3% of youths aged 15 to 19 had fatty liver. 38% of obese children had fatty liver (165). The prevalence of NASH among obese children has been estimated at up to 24%, with the severity of ultrasound findings being positively correlated to BMI, ALAT, insulin resistance and hypertriglyceridemia (166). A recent study of 24 obese adolescents found that 63% of them had NASH (167). The large differences in findings may be because of differences in methodology, definitions of obesity, and/or differences in the cohorts that were studied, such as genetics or lifestyle.

Mauriac syndrome is a rare complication of uncontrolled type 1 diabetes. It is characterized by growth failure, delayed puberty, Cushingoid features, and hepatomegaly, which can have both steatotic and glycogenic features on biopsy (168). A case report implicates a variant in the glycogen phosphorylase kinase complex, which catalyzes the first step of the breakdown of glycogen in the liver. Chronic hyperglycemia also promotes glycogen deposition (169).

The genetics of nonalcoholic fatty liver disease

The variations in hepatic fat fraction that we observed in healthy controls in **paper I** were presumably caused by both genetic and environmental influences. NAFLD has a significant genetic component – the heritability of hepatic steatosis has been estimated to be 39% after controlling factors such as age, sex, race, and body mass index (166). Polymorphisms in genes controlling lipid metabolism, pro-inflammatory cytokines, fibrotic mediators and oxidative stress may predispose individuals with NAFLD to developing NASH (170).

Several genome-wide analyses have identified patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), neurocan (*NCAN*), lysophospholipase-like 1 (*LYPLAL1*), protein phosphatase 1, regulatory subunit 3B (*PPP1R3B*), transmembrane 6 superfamily 2 (*TM6SF2*), and glukokinase regulator (*GCKR*) as being associated with NAFLD, steatohepatitis, and altered blood lipids (171-173). Group-specific component (*GC*, which codes for vitamin D-binding protein) and lymphocyte cystolic protein 1 (*LCP1*) were associated with NAFLD in adolescents (174). In addition, numerous genes have been identified as related to body mass index (BMI) (175), which affects liver fat.

PNPLA3 encodes the triglyceride lipase adiponutrin, which mediates triglyceride hydrolysis in adipocytes. *PNPLA3* contributes to NAFLD in children and adolescents (174). Variations of *PNPLA3* between ethnic groups contributes to susceptibility to NAFLD (172, 176-178), which is a major reason why Hispanics are more prone to the conditions (179). The effects of *PNPLA3* on serum liver enzymes and triglycerides seem to interact with abdominal fat and dietary intake (180). Variants of *PNPLA3* associated with an increased risk of hepatic steatosis, advanced liver disease, and hepatocellular carcinoma are however not associated with increased risk of diabetes and cardiovascular disease (181). Similarly, variants of *TM6SF2* associated with risk of steatosis, fibrosis, and hepatocellular carcinoma are associated with decreased risk of cardiovascular disease (181).

NCAN encodes the neurocan core protein, which is involved in cell adhesion. The mechanism of its potential contribution to NAFLD is currently unclear (182). Similarly, the biological function of *LYPLPAL1* is unclear, although it has been proposed that the gene product functions as a triglyceride lipase (183). *PPP1R3B* affects glycogen production (184). A variant in the *TM6SF2* gene impairs VLDL production (173).

GCKR is expressed predominately in the hepatocytes, where it codes for vitamin D-binding protein (VDBP). VDBP is the main carrier protein of vitamin D, low levels of which have been implicated in the development of obesity and diabetes and associated with NAFLD (174). More recently, vitamin D deficiency has been suggested to contribute directly to the pathogenesis of NAFLD (185).

Hepatic *GCKR* mRNA was found to be downregulated by 83% in adolescent subjects with NAFLD compared to controls. Possession of at least one copy of the variant C allele of single nucleotide polymorphism (SNP) rs222054 was associated with a 2.54-fold increased risk of NAFLD compared to the wild GG genotype (174).

In adolescents, *LCP1* gene expression was 300% higher in subjects with NAFLD compared to controls. Possession of at least one copy of the variant A allele of SNP rs7324845 was associated with a 3.29-fold

increased risk of NAFLD compared to the wildtype GG genotype (174). *LCP1* is mainly expressed in hematopoietic cells and is involved in leukocyte activation and tumor cell proliferation. So far there is not much evidence connecting its function directly to lipid homeostasis, so its association with NAFLD may be due to linkage disequilibrium (174).

Markers and diagnosis of fatty liver disease

In **papers I** and **II**, the fat fraction of the liver and the pancreas, respectively, were measured in children. We considered several diagnostic modalities before magnetic resonance imaging (MRI) was chosen. Furthermore, comparing our results to those of other research groups requires an understanding of the advantages and limitations of different diagnostic tools. Hence, the available approaches for measuring liver fat are discussed below.

Liver biopsy

Liver samples can be obtained through several methods: percutaneous biopsy, transjugular biopsy, laparoscopic biopsy, or fine-needle aspiration guided by ultrasonography or computed tomography (CT). Percutaneous biopsy is the most common technique. Although patients often find the procedure unpleasant, complications are rare, and percutaneous liver biopsies are routinely performed on an outpatient basis (186).

Liver biopsy is still considered the reference standard for diagnosing NAFLD. The disadvantages of liver biopsy include its invasive nature, potential sample variability between different parts of the liver, and intra- and interobserver variability (187).

Ultrasonography

Ultrasonography is cheap, fast, safe, and accessible. Hepatic steatosis appears as a diffuse increase in echogenicity – that is, brightness – caused by the reflection of ultrasound from the liver parenchyma (188). It has a high sensitivity and specificity compared to liver biopsy for moderate to severe fatty liver (189). Ultrasonography is therefore widely used to diagnose fatty liver disease.

However, ultrasonography also has limitations in determining liver steatosis. This modality has poor sensitivity for detecting fatty liver when the fatty infiltration is less than about 30% of hepatocytes (189), so it may underestimate the prevalence of less severe fatty liver. Conversely, some authors have suggested that ultrasonography may be unable to distinguish NAFLD from other liver pathologies like glycogenic hepatopathy and Wilson's disease, leading to possible misdiagnoses and exaggerating the occurrence of NAFLD (190). Sensitivity can also be poor in persons with BMI > 40 or severe NASH (167). In addition, there is substantial intra- and interobserver variability when assessing liver fat with ultrasonography, which decreases the reliability of the method's results (191), and there are no standards for ultrasonographically evaluating pediatric NAFLD (152).

Computed tomography

CT uses a rotating X-ray source and receiver to generate three-dimensional images of the body (192). Several quantitative CT indices have been used to assess hepatic steatosis. The two most frequently used are the absolute attenuation value of the liver and the liver-to-spleen difference in attenuation

(188). Overall, CT has been found to be accurate for diagnosing moderate-to-severe steatosis, but less so for mild steatosis (188).

Magnetic resonance methods

Magnetic resonance imaging and magnetic resonance spectroscopy provide more direct and accurate measures of hepatic fat than ultrasound or CT (193-195). Magnetic resonance techniques can accurately quantify even mild hepatic steatosis (196). Indeed, MRI has been found to outperform liver biopsy for the diagnosis of NAFLD (197), and it has been used extensively in children (198). The physics of magnetic resonance imaging is described in detail in the Methods section of this text.

Blood tests and anthropometry

Serum markers of liver damage, such as ASAT, ALAT, GGT, and ALP, are often used as markers of NAFLD (199). Liver biopsy studies of patients with persistently elevated liver enzyme levels and no viral serologic markers of chronic liver disease found NAFLD in 66% to 90% of cases (200-202). Further circulating compounds that have been used as biomarkers of NAFLD and NASH include cytokeratin 18 fragments, alpha 2-microglobulin, apolipoprotein A1, total bilirubin, hyaluronic acid, C-reactive protein, fibroblast growth factor-21, interleukin 1 receptor antagonist, adiponectin, and tumor necrosis factor-α (203).

However, there is also evidence against using liver enzymes as a proxy for NAFLD and for grading its severity. One study found that children with biopsy-confirmed liver fibrosis had ALAT levels that were only mildly elevated compared to those with more benign NAFLD (204). In adults, the entire histological spectrum of NAFLD and NASH was found in the livers of patients with ALAT in the normal range, with no significant difference compared to patients with raised ALAT (205). A German study of overweight, obese, and extremely obese children found elevated ALAT in 11% of the cohort (206). A comparison of this proportion with the studies using ultrasound to diagnose NAFLD suggests that using aminotransaminases as a proxy for NAFLD may risk underestimating its prevalence.

As radiological and histological methods for diagnosing NAFLD are expensive and time-consuming, several scores based on routine laboratory tests and anthropometric measurements have been proposed. The fatty liver index (FLI) is a score based on BMI, waist circumference, triglycerides, and GGT that predicts fatty liver disease (207). Persons with prediabetes who score highly on the FLI have a higher risk of progressing to type 2 diabetes (208). The hepatic steatosis index (HSI) uses ASAT, ALAT, BMI, sex, and the presence of diabetes. HSI was based on an Asian population; as Asians tend to have lower BMI and waist circumference than Europeans, the FLI was considered inappropriate in that population (209). Finally, the NAFLD liver fat score (NAFLD-LFS) is based on the presence of the metabolic syndrome (as defined by waist circumference, serum triglycerides, serum HDL, hypertension, and plasma glucose); the presence of type 2 diabetes; and ASAT, ALAT, and fasting insulin. The score was developed in a Finnish population of persons with and without type 2 diabetes (210).

An independent test of all three indices performed in 92 non-diabetic Europeans with hepatic fat fraction determined by MRS found that the diagnostic efficacy, as determined by the area under the receiver operating characteristic curve (ROC AUC) was lower than what had been in the original studies

(211). Presumably, this is due to different underlying characteristics of the populations that the indices have been developed and tested in. See Table 2 for a comparison of the three indices.

Table 2. Indices for estimating the risk of NAFLD based on routine laboratory and clinical measurements.

Index	**Laboratory measurements**	**Clinical measurements**	**Original population**	**ROC AUC** (211)
Fatty liver index	GGT, triglycerides	BMI, waist circumference	Italy	0.72
Hepatic steatosis index	ALAT, ASAT	BMI, diabetes status, sex	South Korea	0.79
NAFLD liver fat score	ALAT, ASAT, glucose, HDL, insulin, triglycerides	Diabetes status, hypertension	Finland	0.70

Methods

The major investigational technologies used in this thesis are MRI (**papers I** and **II**), transcriptomics (**paper III**), and metabolomics (**paper III**). The principles of these methods and significant findings related to diabetes that the methods have generated are discussed below. For details of how the techniques were used to investigate the hypotheses of each study, see the methods section of each corresponding paper.

Magnetic resonance imaging

In **papers I** and **II**, MRI was used to measure the volume and fat fraction of the liver and the pancreas in children with type 1 diabetes and controls.

Physical principles

MRI uses nuclear magnetic resonance (NMR) to produce images (212). The physical principles underlying NMR and MRI are discussed in the following section, beginning at the subatomic level and progressing to the macroscopic.

Many atomic nuclei have their own magnetic field (*magnetic moment*), with a north pole and a south pole. The most important nucleus for MRI is the hydrogen nucleus, which consists of a single proton. Similarly to the Earth, the protons *spin* (have angular momentum) around their magnetic axis. If there is no strong external magnetic field, the protons are randomly aligned (Figure 3A). The primary magnetic field of the MRI scanner makes the proton align itself either with or against (parallel or antiparallel to) the direction of the field (213). Most protons align themselves parallel to the magnetic field, as this represents a lower energy state than alignment against the direction of the field (Figure 3B). However, for quantum mechanical reasons, the protons cannot be aligned fully parallel to the magnetic field, but their magnetic moment remains at a fixed angle against the magnetic field, as shown in Figure 1C.

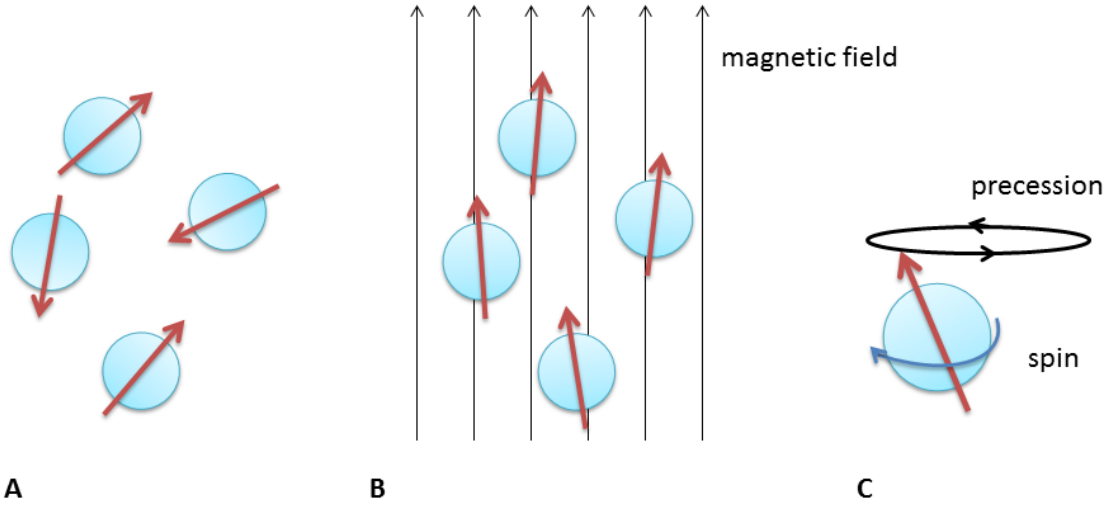

Figure 3. Magnetic properties of atomic nuclei. A. Random orientation of magnetic vectors. B. The magnetic vectors of the nuclei align when subjected to a magnetic field. C. Spin and precession of an atomic nucleus.

Due to the angle between their magnetic moment and the magnetic field, protons subjected to a magnetic field *precess*, which involves the vector of their magnetic moment rotating around the direction of the applied magnetic field (Figure 3C). The rate of precession is termed the Larmor frequency. It is proportional to the strength of the magnetic field and dependent on the nature of the nucleus. The rate of precession can be expressed by the following equation (214).

$$\omega = \gamma B_0$$

In this equation, ω is the rate of precession; γ is the gyromagnetic ratio, which is a nuclear-specific constant based on size, mass, and spin; and B_0 is the strength of the static magentic field. Hence, increasing the magnetic field strength will increase the rate of precession. When added together, the microscopic magnetic moments of all nuclei sum up to a net macrosopic magnetization, which, unlike the individual magnetic moments, is aligned perfectly parallel to the magnetic field.

In MRI, brief magnetic pulses generated by weaker, perpendicular gradient coils are superimposed on the static magnetic field. This causes some protons to shift into a high energy state. Additionally,the macroscopic magnetization vector is oriented in a transverse direction. The interaction of the RF pulsewith the nuclei is the resonance of nuclear magnetic resonance. Nuclear resonance is a brief, induced phenomenon, involving energy exchange between precessing spins and their environment; this contrasts with precession *per se*, which is spontaneous and unaccompanied by energy exchange. The vector in the transverse plane is called the transverse magnetization, which is registered as a signal by the MRI scanner (214).

Relaxation involves the macroscopic magnetization returning to its thermal equilibrium state after an RF pulse. Over time, interactions between nearby protons will cause a loss of phase coherence between the protons and therefore a decay of the transverse component of the macroscopic magnetization. This, in turn, causes the signal to decay. This is known as T2 relaxation, spin-spin relaxation, or transverse relaxation (Figure 4).

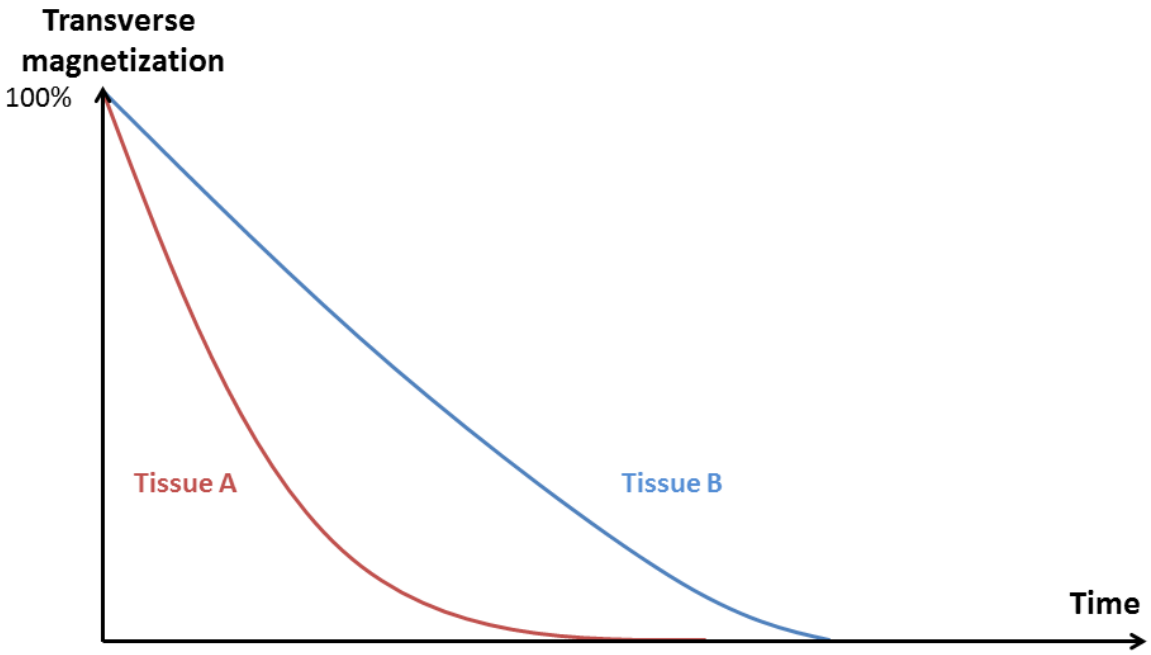

Figure 4. Tissue A has a shorter T2 than tissue B, as it more rapidly loses its transverse magnetization.

As the protons release the energy received from an RF pulse, the macroscopic magnetization regains its longitudinal component along the magnetic field. This is known as T1 relaxation. T1 relaxation is also known as spin-lattice relaxation or longitudinal relaxation. T1 relaxation can be described as the process in which the net magnetization (M) of a group of protons energized by a radiofrequency pulse returns to its original maximal value (M_0) that is parallel to B_0 (Figure 5). T2 relaxation is generally quicker than T1 relaxation.

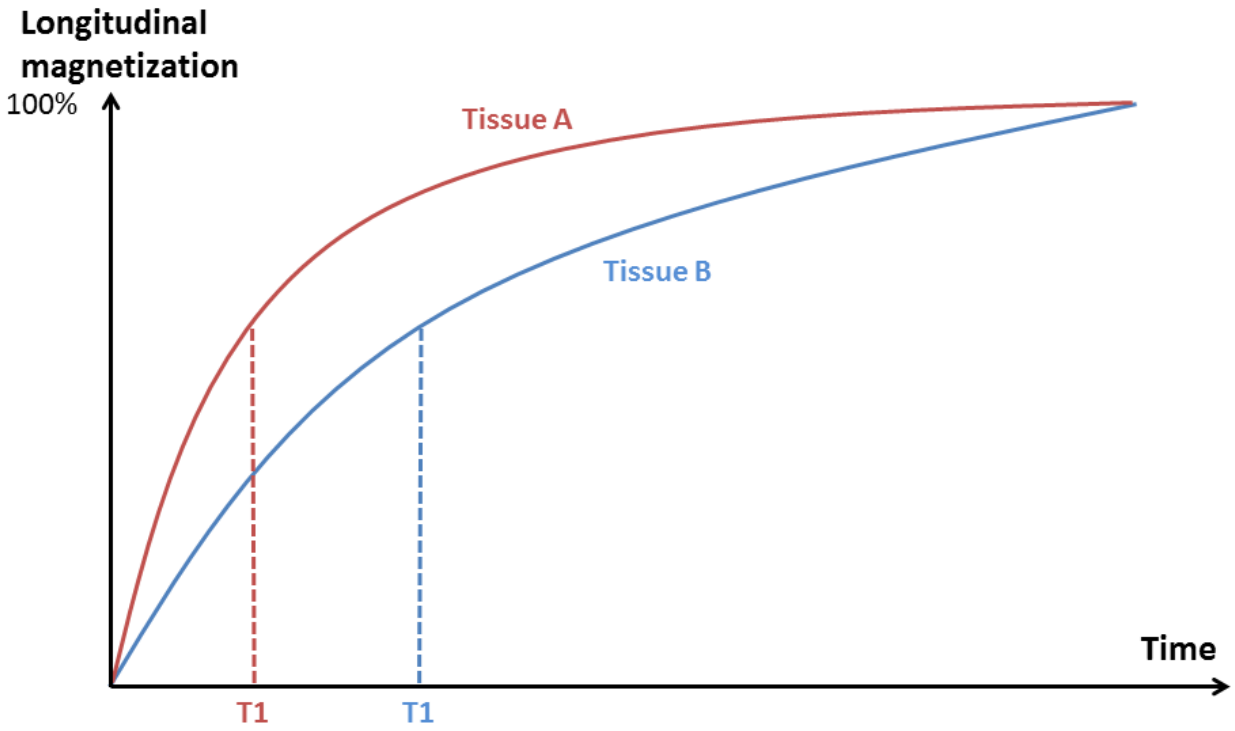

Figure 5. Tissue A has a shorter T1 than tissue B, as it more rapidly achieves longitudinal relaxation.

By applying a 180° RF pulse, the precession of the protons is reversed, so that protons with faster precession are ealier in phase than slower ones. Once the more rapidly precessing protons catch up with the slower ones, an *echo* is produced. The signal from the echo reaches a maximum intensity as the precession of protons becomes maximally synchronized, and then the signal once again decays as the precession of the protons goes out of phase from each other (214).

Hydrogen nuclei in different tissues or in different molecules have different local environments, which causes different T1 and T2 relaxation. Due to these differences in relaxation time, an MRI scanner can distinguish different types of tissues or types of molecules, such as water and fat.

These differences can be accentuated based on the *pulse sequence* employed. An MRI pulse sequence is a programmed set of RF pulses and changing magnetic gradients (215). A pulse sequence is defined by multiple variables, including

- Repetition time (TR): How frequently excition pulses are applied.
- Echo time (TE): The time between applying an excitation pulse and the peak of the measured signal.
- Flip angle: The amount of rotation of the net magnetization (M) when the radiofrequency pulse is applied.

Pulse sequences are often referred to by the dominant influence on the appearance of tissues. Examples of pulse sequences include

- T1 weighted. Fluid appears black and fat appears white. Pathological processes are often dark.
- T2 weighted. Fluid and fat appear white. Pathological processes are often bright.
- Proton density. Tissues with a higher density of hydrogen atoms produce a stronger signal. Fluid and fat both appear white, but since most tissues have similar proton density, images typically have poor contrast.
- Diffusion weighted. Relies on the Brownian motion of water to modulate the signal. In quantitative images of the diffusion constant, fluid appears white and fat appears black.

Fat quantification with magnetic resonance imaging

In 1984, Dixon described an MRI technique for water and fat separation (216). The technique is based on the fact that water protons and fat protons have slightly different Larmor frequencies (fat protons precessing 3.4 parts-per-million slower than water protons) (217). The original Dixon technique acquires two separate images – one with water and fat signals in phase and the other with the water and fat signals 180° out of phase. From these two images, a water-only image and a fat-only image can be generated, which allows water and fat quantification (218). The fat fraction can be regarded as the fat signal divided by the sum of the fat signal and the water signal:

$$FF = \frac{\rho_f}{\rho_f + \rho_w}$$

Later, the Dixon technique was improved by the acquisition of a third image, which enabled correction of inhomogeneities of the main magnetic field (219). In 2004, Reeder et al proposed the iterative decomposition of water and fat with echo asymmetry and least squares estimation (IDEAL) method, which is the basis for modern fat quantifcation with MRI (220). IDEAL is a further improvement of the Dixon technique, which allows for an arbitrary number of images to be used for the fat quantification. Typically, 3-6 images are used. Even more accurate fat quantification is possible when taking into account that the fat signal is actually composed of several different frequencies, as proposed by Yu et al (221). The technique by Yu was used for fat quantification in **papers I** and **II**.

Magnetic resonance imaging scanners and their use in medicine

The major components of an MRI system for clinical use are a magnet, gradient coils, and radiofrequency coils. A powerful magnet creates a homogeneous magnetic field. The strength (or *flux density*) of a magnetic field is measured in tesla (T), which is defined as one weber/square meter (Wb/m^2). One Wb, in turn, is the SI unit of magnetic flux (222). Clinical MRI is usually performed at 1.5 or 3 T, with higher flux density providing higher resolution.

There are three gradient coils, which cause linear spatial variations of the magnetic field. The magnetic gradients enable the MRI scanner to create cross-section images of a patient in arbitrary orientations (via so-called slice selection gradients). The gradient coils are named after the axis along which they act: x, y, and z. A slice selection gradient by the x-gradient coil produces sagittal images, by the y-gradient coronal images, and by the z-gradient axial images. The gradients are also responsible for encoding the NMR signal such that the signal can be reconstructed to usable images. The gradient coils cause the loud noises of the MRI machine.

RF coils send out the RF pulses used to excite the protons in the patient, and also receive the resulting magnetic resonance signals emitted from the protons. The RF-pulses is typically are typically sent by a large transmit coil built in to the main MRI apparatus. The receiving of the signal is usually handled by separate, local coils, designed to encapsulate specific body parts (223).

MRI is used in medicine for a broad range of purposes. Its advantages include not using ionizing radiation; being able to generate images in multiple planes without having to move the patient; and being able to apply advanced techniques such as diffusion, spectroscopy, and perfusion. Disadvantages include high cost and time consumption, as well as danger for patients with incompatible implants and foreign bodies (214).

Transcriptomics

Transcriptomics is the study of the transcriptome, which consists of all RNA in a cell, tissue, organ, or organism. This includes messenger RNA (mRNA), but can also include microRNA and transfer RNA. Although genes contain the information to code for RNA, only a small part of the genes are expressed in a given cell at a given time. The concentration of different types of mRNA varies based on the degree of gene expression and the rate of mRNA degradation. This, in turn, is influenced by intrinsic factors, such as the time in the cell cycle and the time of day, and extrinsic factors, such as paracrine or endocrine signaling (224). The transcriptome can thus be regarded as the step following the genome and preceding the proteome in cellular metabolic processes (Figure 6).

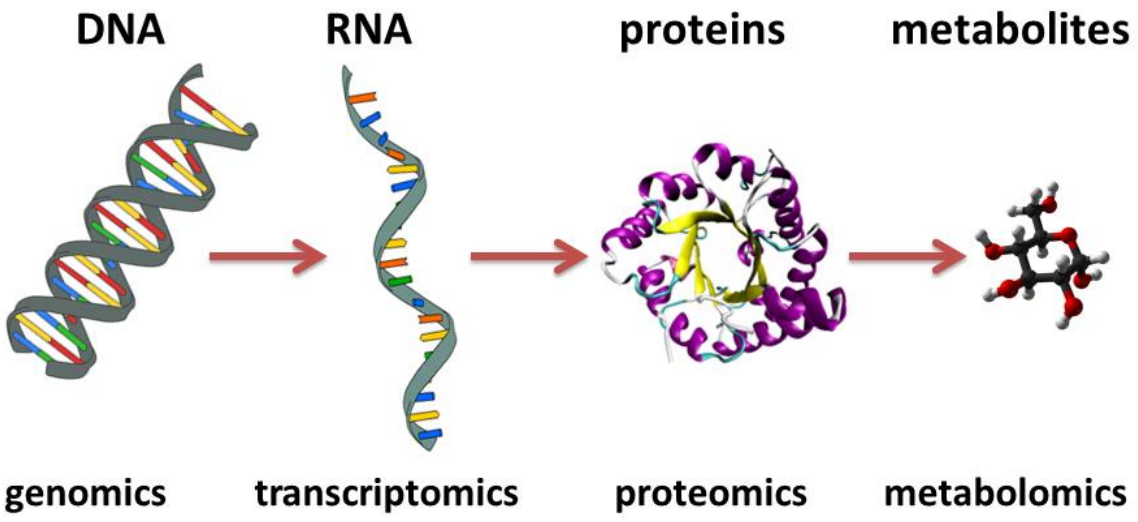

Figure 6. Cellular steps from genome to metabolome and the corresponding fields of study (225, 226).

Analysis of transcriptome data

Due to the amount of data collected, statistical analyses that compensate for multivariate testing are usually performed, such as by calculating the false discovery rate or Bonferroni correction (227). Drawing conclusions about the biological consequences of changes in gene expression observed in physiological or pathological states is often based on information from gene annotation databases. These databases provide information about for instance the function of genes, the location of genes within chromosomes, and the known cellular pathways that regulate gene expression (228).

Based on finding statistically significant differential expression of genes and knowledge about those genes through gene annotation, patterns can be found to determine the net effects of changes in gene expression. Gene ontology analysis can group differentially expressed genes into categories (229). Categories can be defined based on the genes'

- Involvement in biological processes, i.e. the net result of gene activity. This can be described with different levels of granularity – for example, a gene that is involved in *lipid metabolism* can be further subsumed under *lipid biosynthesis*, and still more precisely defined as relating to *oleate synthesis*.
- molecular function, i.e. the molecular interaction of the gene product, such as binding or phosphorylation; and
- cellular localization, i.e. which parts of the cell that the gene is expressed in.

By combining information about regulatory gene pathways with ontological exploration of the functions of the gene products, transcriptome analysis can be used to link how changes in gene expression cause changes in phenotype. In **paper III**, we used ontological analysis to study how changes in hepatic lipid metabolism overlapped with pathways known to be regulated by insulin.

Transcriptomics applied to type 1 diabetes

As applied to type 1 diabetes, transcriptomics methods have been used to study "serum signatures" of innate inflammation that distinguish persons with type 1 diabetes or persons at risk of type 1 diabetes from healthy controls and other persons with other diseases (230). Such studies shown promise for risk stratification of autoantibody positive individuals (231). It has been reported that a proinflammatory signature of gene expression is present in both patients with recent-onset type 1 diabetes and in high-risk individuals who later progress to diabetes. In the latter group, the gene expression signature preceded the appearance of autoantibodies (232, 233). These studies suggest that dysregulation of the innate immune system could be used as an early predictor of adaptive autoimmunity and type 1 diabetes.

Metabolomics

Metabolomics is the study of the metabolome, which consists of all metabolites in a cell, tissue, organ, or organism. Metabolites represent the intermediates and end products of cellular processes that begin with gene expression (Figure 6). Hence, it has been argued that information about metabolites can provide more information about a disease process than genomics alone; a change in the expression of a gene does not necessarily correlate directly with a variation in the activity level of a protein, whereas an alteration in a metabolite concentration does (234).

Methods for metabolomic analysis

Several analytical techniques can be employed to analyze metabolites. As the chemical composition of metabolites varies greatly, no one method can be used to analyze the entire metabolome. Among the most common techniques are mass spectroscopy, liquid chromatography, gas chromatography, and nuclear magnetic resonance. Often, several of these methods are combined for a single experiment

(235). In **paper III**, gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry were used to analyze the serum metabolite profiles of BB rats.

Mass spectrometry

Mass spectrometry has high sensitivity, with advanced platforms being able to detect metabolites in the femtomole range (236). In mass spectrometry, chemicals are ionized into gas, and the ions are sorted based on their mass-to-charge ratio. See Figure 7 for an overview of the parts of a mass spectrometry device.

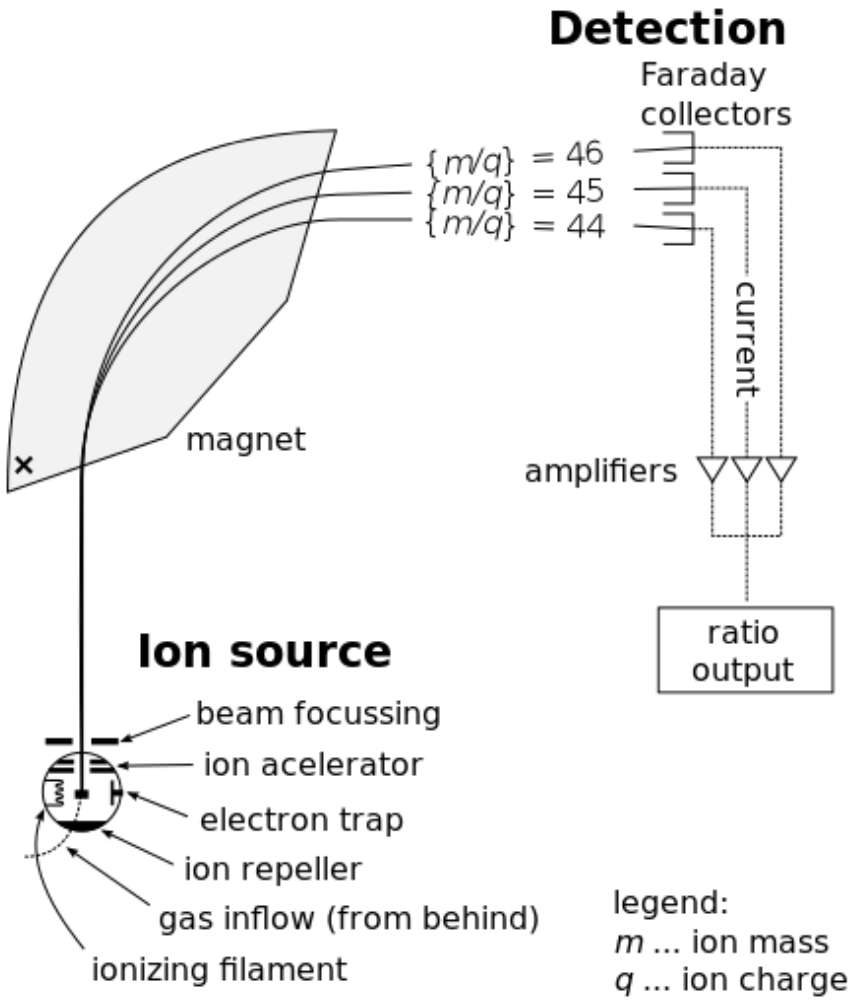

Figure 7. Main components of a mass spectrometer (image: public domain).

The analyte is ionized in the ion source. Several methods for ionization are available, including electron ionization and chemical ionization. Electron ionization involves bombarding the analyte with electrolytes, which causes the molecules to lose an electron and form an ion (237). This process can be described as the following reaction, in which M is the molecule being analyzed, e^- is an electrolyte, and M^+ is the resulting ion:

$$M + e^- = M^+ + 2e^-$$

In chemical ionization, ions stemming from the analyte are produced through the collision of the analyte with ions of a reagent gas. Commonly used gases include methane, ammonia, and isobutane. As chemical ionization is a lower-energy process than electron ionization, causing less fragmentation of the analyte, it typically provides less precise information about the mass of the analyte than electron ionization (238).

Ions of the analyte leave the ion source and are accelerated before entering the mass analyzer section of the mass spectrometry device. A magnetic field is applied to the ions, deflecting their path and causing them to be separated by their mass-charge ratio (m/z). Lighter ions are deflected more than heavy ones, and more positively charged ions are deflected more than less positively charged ones (239).

Finally, the ions reach the detector. The induced charge or current caused by the ions reaching the metal detector is recorded. Data are typically represented as a mass spectrogram, plotting the m/z ratio on the x axis against the signal intensity on the y axis.

Chromatography

Chromatography refers to a collection of laboratory techniques to separate mixtures of chemicals. The mixture is dissolved in a *mobile phase*, which is a fluid in liquid chromatography and a gas in gas chromatography (in cases in which the chemical of interest can be vaporized without decomposition). As different components of the sample interact differently with a *stationary phase* based on their chemical properties, they flow at different speeds and are separated (240).

In high-performance liquid chromatography (HPLC), the mobile phase is highly pressurized. Small volumes of sample material are added to the mobile phase, and the sample is carried into the HPLC column (the stationary phase). Upon exiting the stationary phase, the chemicals reach a detector, and the electric signal that is generated at the detector is displayed as a chromatogram (241).

Nuclear magnetic resonance

The principles of NMR spectroscopy are described above. NMR spectroscopy has the advantage of allowing direct analysis of metabolites in bodily fluids, cells, and intact tissues without the need to chemically extract analytes. Disadvantages include poor sensitivity, effects of pH, and the difficulty of deconvoluting and normalizing the spectra of complex metabolite mixtures in biological matrices like plasma, urine, or tissue extracts. Thus, despite NMR spectra being rich in information, the complexity of data and lack of sensitivity limit the amount of metabolites that can be profiled with currently available techniques (242).

Interpretation of metabolomics data

As methods for measuring metabolites are so sensitive, the results can vary substantially depending on many factors during sample collection and storage. Metabolites from samples from the same subject can vary depending on for instance when during the day that samples are collected, whether (and what) the subject has eaten recently, the medicines that the subject uses, the physical activity of the subject, and the anatomical location from which the sample was obtained. Furthermore, the way that samples are stored can alter their content of metabolites due to degradation and hemolysis (243).

A significant step during a metabolomics investigation is identifying the chemical composition of the measured metabolites. Compounds are usually identified by comparing the measured mass/charge ratios to those of known metabolites from databases (244). However, many of the data acquired during a typical metabolomics experiment consist of artifacts and uncharacterized metabolites (245).

As in transcriptomics, the interpretation of metabolomics data requires methods beyond the statistics commonly used in medical research. The datasets generated by metabolomics techniques are typically large, and the changes in metabolite patterns are often correlated, since their functions are biologically linked (246). Studies can be hypothesis-driven (targeted) or hypothesis-generating (untargeted). Often, studies have both a discovery and a validation cohort. As so many metabolites are studied in each sample, there is a high risk of false positives. This has led to the development of tools such as cluster analysis, partial least squares regression, pathway mapping, comparative overlays, and heatmaps for interpreting and visualizing the output of high-throughput metabolomics techniques (247).

Metabolomics applied to diabetes

It was noted almost half a century ago that obese persons have altered levels of certain circulating amino acids compared to normoweight persons (248). More recently, it has been shown that amino acid profiles can predict the progression of normoglycemic individuals to type 2 diabetes (249). Circulating branched chain amino acids are positively associated with the risk of type 2 diabetes (250). Ingestion of branched chain amino acids can acutely decrease insulin sensitivity in normoglycemic, obese women (251). In contrast, studies of type 2-diabetic patients who develop kidney disease had decreased levels of branched-chain amino acids (252, 253).

Reduced levels of the small amino acid glycine have been observed in normoglycemic individuals who later develop type 2 diabetes (254), in prediabetic persons (255), and in patients with type 2 diabetes (256). Genetic variants of glycine biosynthesis are associated with insulin resistance (257), implying a possible genetic basis for metabolomics findings. Metabolomic methods have also been used to investigate insulin secretion in beta cells (258, 259), the role of hepatic steatosis in insulin resistance (260), and potential novel therapeutic targets for type 2 diabetes (261). A model for predicting liver steatosis was developed using LCMS. The model includes one triglyceride and two phosphatidylcholines, and the authors reported a ROC AUC of 0.79 (262).

In type 1 diabetes, metabolomics techniques have been used to demonstrate that persons who progress to diabetes have different levels of certain lipids compared to persons who remain nondiabetic. There is evidence that these differences exist already *in utero*; altered lipid content of the umbilical cord may reflect a pathogenic pregnancy and an increased risk of developing type 1 diabetes at an earlier age. Cord-blood phosphatidylcholines and phosphatidylethanolamines were significantly decreased in children diagnosed with type 1 diabetes before 4 years of age (263).

In a longitudinal study, serum metabolite profiles were compared between 56 children who progressed to type 1 diabetes and 73 controls who remained nondiabetic and permanently autoantibody negative (264). Persons who developed diabetes had reduced serum levels of succinic acid and phosphatidylcholine at birth, reduced levels of triglycerides and antioxidant ether phospholipids

throughout the follow up, and increased levels of proinflammatory lysoPCs several months before seroconversion to autoantibody positivity. Diminished ketoleucine and elevated glutamic acid preceded the appearance of insulin and glutamic acid decarboxylase autoantibodies. Autoimmunity may thus be a relatively late response to the early metabolic disturbances. The lipid profiles in the progressors during the last visits before diagnosis of type 1 diabetes revealed no clear differences as compared with the profiles of matched nonprogressors, except for specific phospholipids which were similarly diminished as they had been at an early age and around the time of seroconversion to autoantibody positivity. The findings were subsequently validated with a different study population (265). The authors concluded that a reduction in choline-containing phospholipids in cord blood is associated with progression to T1D but not with development of beta cell autoimmunity.

In a further study, the metabolomic profiles of children who developed islet autoantibodies at the first (1-2 years of age) and second (8+ years of age) peak incidences were measured (266). There were differences in metabolite profiles that were dependent on age, islet autoantibody positivity, and the age of islet autoantibody development. Children with early autoimmunity had lower concentrations of methionine compared with children who develop islet antibodies late and with children who remain antibody-negative.

Since the exact clinical onset of type 1 diabetes in autoantibody-positive persons can currently not be predicted, the aforementioned studies for using metabolomics are limited to blood samples obtained during several years, often with a period of weeks to months intervening between the last blood sample and the clinical onset of type 1 diabetes. Hence, it is not known what metabolic changes take place shortly before the onset of type 1 diabetes. In **paper III** we the compared the metabolomic profiles of BBDR.$^{lyp/lyp}$, BBDR.$^{lyp/+}$, and BBDR.$^{+/+}$ rats before and after the onset of hyperglycemia in BBDR.$^{lyp/lyp}$ rats and at corresponding time points in rats with other genotypes.

In patients and rodent models of type 1 diabetes, several studies have shown differences in the metabolite profiles of diabetic as compared to nondiabetic participants (267-277). Some studies have investigated the metabolic effects of insulin deprivation, which, to a certain extent, mimics the onset of type 1 diabetes (267). Most of the identified metabolites that differed between rats before and after the onset of diabetes in **paper III** had previously been reported in these aforementioned publications, which makes it more plausible that they represent true positive results.

Metabolomics in clinical practice

So far, translational successes in using metabolomics in clinical practice have been limited to screening neonates for over 40 inborn errors of metabolism (278). Applying the methodology to other diseases is more challenging. Inborn errors of metabolism are usually characterized by an abnormally high accumulation of a single metabolite that is specific for the particular disease. Moreover, absolute quantification of multiple analytes in a module is not needed, since the screening only requires detection of differences in a single or a few metabolites in comparison to normal laboratory values (279). In most diseases, metabolite patterns are more diffusely altered, making diagnoses and prognoses more difficult.

In one example of a translational metabolomics effort for diabetes care, a panel of blood-based biomarkers was assessed using, among other methods, HPLC and MS. Based on these values, the risk of type 2 diabetes was determined in a cohort of overweight persons, of whom 55% were normoglycemic. Among the normoglycemic participants, 24% were identified as being at elevated risk of progression to type 2 diabetes. This information was presented to the patient classification results were presented to each patient and his or her physician, who was able to use the risk assessment in clinical assessment. Although a significant number of high-risk normoglycemic persons reduced their HbA1c and fasting glucose levels, the study is limited by the lack of a control group (280). Nevertheless, the study shows conceptually how metabolomics can be used in clinical practice.

Results and discussion

The major findings and implications of each paper are summarized below. For a more detailed account, see the full text of each paper.

Paper I

- Children with type 1 diabetes have a lower hepatic fat fraction compared to controls (1.8% versus 1.3%).
- The distribution of hepatic fat across Couinaud segments differs between children with type 1 diabetes and controls.
- We found no correlations between laboratory or anthropometric measurements and liver fat fraction.

Our results were unexpected, given that previous studies had suggested an increased prevalence of NAFLD in children with type 1 diabetes. At about the same time as our results were published, other groups also released data about MRI-determined fat fraction in patients with type 1 diabetes. Collectively, our results show that reduced fat fraction is apparent in both children and adults with type 1 diabetes.

Paper II

- Children with type 1 diabetes have reduced pancreatic volume compared to controls.
- We found no difference in pancreatic fat fraction between children with type 1 diabetes and controls.
- Pancreas volume did not correlate to diabetes duration after correcting for body surface area.

We did, however, find a correlation between pancreas size and units of insulin/kg body weight. We theorize that if reduced pancreas size in type 1 diabetes is caused by a lack of insulin in the pancreas, then exogenous insulin administration would decelerate the decline in size. This would be consistent with both our findings that insulin dosage, but not diabetes duration, was related to pancreas size.

Paper III

- Hepatic lipid metabolism changes at the onset of hyperglycemia in a rat model of type 1 diabetes.

- A significant number of differentially expressed lipid-related genes are regulated by insulin.
- Serum metabolite changes during the corresponding period are marked by a general increase in carbohydrates.

In this study we provided experimental evidence of the timing of changes in lipid metabolism in type 1 diabetes. We had hypothesized, based on our previous publications, that a lack of insulin in the liver caused reduced liver fat. Hence, we would expect a shift in lipid metabolism to coincide with the sudden hypoinsulinemia and hyperglycemia that characterized the rat model of type 1 diabetes we used. This is what we observed.

Conclusions and future perspectives

Overall, this thesis brings new insights to an otherwise poorly studied area of type 1 diabetes research. Its results harmonize well with subsequently published and currently ongoing studies. The thesis raises a number of new hypotheses and possibilities for further research about type 1 diabetes, insulin, and fat metabolism.

A subset of patients with type 1 diabetes retain clinically meaningful insulin production (measured as residual C-peptide), even years after the diagnosis of diabetes. Residual C-peptide production correlates with reduced long-term complications and reduced incidence of hypoglycemia (281). According to our hypothesis, these patients would also be expected to have a liver fat fraction more similar to that of the general population than patients with an absolute insulin deficiency. Our study was too small to find such correlations, but larger studies, perhaps in overweight adults in whom steatosis is expected to be more pronounced, could determine the significance of residual beta cell function for liver fat in type 1 diabetes.

Recent phase III studies of insulin analogues that preferentially target the liver (such as insulin peglispro) have shown that patients treated with these drugs exhibit higher levels of aminotransferases and hepatic fat than patients treated with regular insulin analogues (282, 283). This is what would be expected based on the results of this thesis. Clinical development of insulin peglispro has been discontinued (284), but it could prove to be a useful experimental tool in studying the effects of insulin in the liver.

Pancreatic atrophy and pancreatic exocrine insufficiency in type 1 diabetes remain insufficiently studied. It is currently unclear when the decline in pancreatic size begins in relation to the appearance of type 1 diabetes-associated autoantibodies, insulitis, and the clinical onset of type 1 diabetes. Characterizing the decline of pancreas size would require repeated measurements of the pancreas before and after the onset of type 1 diabetes. An ongoing study is using ultrasound and MRI to compare the pancreatic volumes of persons with recently diagnosed type 1 diabetes, persons with type 1 diabetes, persons with genetic risk of type 1 diabetes but no autoantibodies, and persons without known risk of type 1 diabetes (285). An alternative method of following pancreas size would be using a test of exocrine pancreatic function such as fecal elastase-1, which could more easily be studied in a larger population than

radiological examinations. If such studies are successful, pancreas size and/or exocrine function could be used as a predictor of the onset of type 1 diabetes.

Furthermore, if such studies confirm that pancreatic atrophy takes place before the onset of type 1 diabetes, strategies to prevent exocrine pancreatic insufficiency could be explored. If pancreatic atrophy is caused by insulin deficiency, then administering insulin before the loss of blood glucose control could be a plausible method. If pancreatic inflammation is the cause, then anti-inflammatory drugs could be administered. A number of trials attempting to prevent type 1 diabetes have been performed or are ongoing, some employing insulin or immunosuppressive regimens (286). It would be interesting to study whether pancreatic size and exocrine function are influenced by any of these preventative strategies.

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