Glycogen storage disease type Ia

Glycogen Storage Disease type Ia (GSD Ia) (MIM#232200) is a rare, autosomal-recessive inborn error of metabolism (IEM), with an incidence of 1 in 100,000 and caused by mutations in the gene encoding for the catalytic subunit of glucose-6-phosphatase ($G6PC1$, G6Pase-α) (1). $G6PC1$ is an enzyme that converts glucose 6-phosphate (G6P) into glucose in hepatocytes, kidney cells, and enterocytes. A related disorder is GSD Ib (MIM#232220), which is caused by mutations in glucose-6-phosphate transporter ($SLC37A4$, $G6PT1$) (1). $SLC37A4$ transports G6P over the endoplasmic reticulum (ER) membrane into the ER lumen. The work in this thesis focuses on GSD Ia.

Biochemical symptoms and complications

GSD Ia represents ±80% of GSD I cases and is biochemically characterized by (fasting) hypoglycemia, hyperlipidemia, hyperlactatemia, and hyperuricemia (2), which are largely caused by hepatocyte-specific impairment of G6PC activity (3) (Fig. 1). In addition, GSD Ia patients display a severe hepatic phenotype, characterized by hepatomegaly due to the accumulation of G6P, which drives the synthesis of glycogen and lipids. These symptoms, together with an accompanying catabolic state due to low insulin levels, calorie insufficiency, and/or possible malabsorption of nutrients due to intestinal problems (4), result in growth retardation, also referred to as ‘failure-to-thrive’. After the introduction of dietary therapy in the 1970s/80s (see also below), the mortality has been drastically reduced. This resulted in a better management of biochemical symptoms but led to the appearance of long-term complications, such as osteoporosis, gout, renal disease, and hepatocellular adenomas (HCA) with the risk of hepatocellular carcinoma (HCC) development. Thus, reduced G6PC1 activity in liver causes metabolic imbalance, which eventually leads to liver tumor development.

Therapy

The introduction of dietary therapy in the 1970/1980s, more specifically dietary management by continuous gastric drip feeding and subsequently uncooked cornstarch, helped to prevent episodes of hypoglycemia and resulted in a drastic reduction in mortality (5,6). The effectiveness of this therapy in preventing abnormalities, such as hypoglycemia or hypertriglyceridemia, is also referred to as ‘metabolic control’. Although this dietary management can help in controlling the metabolic abnormalities, the underlying genetic defect is not corrected. Therefore, symptoms and complications, such as hepatomegaly, renal disease, and HCA/HCC still occur (1). In order to deal with long-term complications such as renal disease or HCA/HCC, transplantation of liver
and/or kidney is an alternative, whereas liver resection can be performed in case tumors are not diffuse (7–10). Currently, gene therapy has proven effective in mouse and dog models for GSD Ia, with the first clinical trials in humans ongoing (11–20). However, as it will likely still take time before this can be fully implemented in patient care and will not be suitable for all patients, a better understanding of mechanisms underlying liver tumor development and potential treatments is warranted.

**Urgent questions: clinical heterogeneity and tumor-initiating processes**

Despite advances in dietary therapy, development of liver tumors still represents a major long-term complication in GSD Ia patients. This complication is hepatocyte-borne (3), starts to emerge mostly in the second decade of life, and affects 60-70% of all patients by the age of 30 years (7). Importantly, patients typically show multiple HCAs across the entire liver with different molecular profiles (7,21,22). Research has identified chromosomal (gain of chromosome 6p and loss of 6q) and genetic (reduced expression of IGF2R and LATS1) alterations, activation of tumor-promoting pathways (NRF2, mTORC1, β-catenin, and YAP signaling), and deranged mitochondrial and autophagy function as potential mechanisms contributing to tumorigenesis in GSD Ia (23–27). Although the molecular mechanisms underlying initiation and progression of liver tumors in GSD Ia remain largely unresolved, severely
high plasma triglyceride (TG) levels (hypertriglyceridemia), which mark poor metabolic control (7,28,29), are positively associated with an increased risk for liver tumor development in GSD Ia patients (30–33). Despite this association, considerable variability in plasma TG levels between individual GSD Ia patients has been reported (30,34), which is presumably caused by a combination of genetic and environmental factors (34). The current lack of insight into phenotypic heterogeneity in symptoms and complications, as well as the mechanisms of liver tumor formation, poses major challenges to optimal healthcare for GSD Ia patients. This was recently highlighted in the top research priorities identified by the international liver GSD priority setting partnership (35). A better understanding of the mechanisms behind phenotypic heterogeneity and liver tumor formation, as well as of the connection between metabolic imbalance and long-term complications, are crucial to identify patients more at risk for long-term complications, to improve patient care, and to prevent long-term complications.

**Potential interactions between metabolic imbalance and tumour initiation**

As explained above, identifying the potential molecular connections between metabolic imbalance (a direct consequence of impaired G6PC1 activity) and tumor-initiating processes (which may explain increased risk for HCA/HCC in patients) is crucial to improve GSD Ia patient care. This metabolic imbalance, driven by intracellular accumulation of G6P, favors a metabolic profile that closely resembles the metabolic adaptations by cancer cells. This is also referred to as the Warburg effect, and is characterized by, for example, increased glycolytic and pentose phosphate pathway (PPP) activity, increased lactate production despite sufficient oxygen supply, and generation of macromolecules, which can be used as building blocks for new cells and thus can facilitate proliferation (36,37). Along the same hypothesis, it was suggested that a combination of increased glycolysis and PPP could lead to increased synthesis of uric acid, fatty acids and triglycerides, as well as increased build-up of lactate and pyruvate. This could eventually lead to alterations in ATP/ADP and NADH/NAD⁺ ratios that may support cell growth and proliferation, maintain cellular redox balance, and thus favor tumorigenesis (38). Although, these processes likely explain how metabolic rewiring in GSD Ia supports tumor progression, they cannot explain tumor initiation.

Some early potential links between metabolic imbalance and tumor initiation, in general or in GSD Ia, have also been postulated. For example, it is suggested that the combination of increased hepatic fatty acid uptake from plasma with
increased hepatic lipogenesis likely results in increased fatty acid omega-oxidation and increased peroxisomal \(\beta\)-oxidation and hydrogen peroxide generation, which contribute to oxidative stress, mitochondrial dysfunction, and subsequently liver injury and tumorigenesis (39). A similar explanation for tumorigenesis was hypothesized by Cho et al., who argue that SIRT1 downregulation underlies mitochondrial dysfunction, and that the resulting oxidative DNA damage may contribute to HCA/HCC development (27). In addition, a potential role for impaired autophagy contributing to tumorigenesis was suggested (26,40).

**Nutrient sensors**
Interestingly, the processes highlighted above, such as fatty acid metabolism, lipogenesis, mitochondrial function, and autophagy can all be integrated through the action of nutrient sensors. Nutrient sensors are proteins that control cellular metabolism to maintain energy and metabolic homeostasis, and are responsive to changes in availability of nutrients and energy. They respond to these changes by acting as cellular switches to tweak metabolic fluxes by impacting on the activities of enzymes, signal transduction pathways, as well as gene and protein expression. Notably, many nutrient sensors also harbor cellular functions other than regulation of metabolism, such as protection against oxidative stress and DNA damage (41–44). As the activities of nutrient sensors are often altered in both metabolic diseases and cancer, they may provide an interesting potential link between metabolic imbalance and tumor formation in GSD Ia.

**ChREBP**
Carbohydrate Response Element Binding Protein (ChREBP, also known as MLXIPL, MONDOB, or WBSCR14), a member of the Mondo protein family, is the key glucose-sensitive transcription factor in hepatocytes (45–48). It forms a heterodimer with its binding partner Max-Like Protein X (MLX) and binds to carbohydrate response elements (ChoREs) in the promoter of its target genes to regulate transcription. The histone demethylase Plant Homeodomain Finger 2 (PHF2) acts as a transcriptional co-activator of ChREBP by erasing H3K9me2 methyl marks on the promoters of ChREBP-regulated genes (49).

ChREBP consists of two isoforms: \(\alpha\) and \(\beta\) (51,52). ChREBP\(\alpha\) consists of two nuclear export signals (NES1 and NES2), a nuclear import signal (NLS), a proline-rich region, a glucose-response activation conserved element (GRACE), a low-glucose inhibitory domain (LID), and a DNA-binding basic helix-loop-helix/Zip domain and a Zip-like domain that serve as site
for heterodimerization with MLX (Fig. 2A). The GRACE accounts for ChREBP transactivation activity and is suggested to contain a binding site for glucose-derived metabolites such as G6P (see also Regulation of ChREBP activity below) (53). The LID represses GRACE transactivation activity by intramolecular inhibition under conditions of low levels of intracellular glucose-derived metabolites, such as G6P, xylulose-5-phosphate (X5P), and fructose-2,6-bisphosphate (F2,6P2) (Fig. 2B) (50). This also results in a protein conformation that is inactive and facilitates interactions with nuclear shuttling factors, such as 14-3-3 proteins and CRM1, causing ChREBPα to remain in the cytosol (54).

Figure 2. Schematic overview of ChREBP protein structure and regulation of its activity. (A) ChREBPα consists of two nuclear export signals (NES1 and NES2), a nuclear import signal (NLS), a proline-rich region, a glucose-response activation conserved element (GRACE), a low-glucose inhibitory domain (LID), and a basic helix-loop-helix/Zip domain and a Zip-like domain that serve as site for heterodimerization with MLX. (B) Under low intracellular levels of glucose-derived molecules, LID represses GRACE activity by intramolecular inhibition, which also causes protein phosphorylation and a conformation that promotes cytosolic retention. (C) Under high intracellular levels of glucose-derived molecules, the intramolecular inhibition of GRACE by LID is released (possibly through direct binding of glucose-derived metabolites to GRACE), resulting in protein acetylation and a conformational change that facilitates translocation to the nucleus to promote transcription of its target genes, such as ChREBPβ. ChREBPβ is a shorter isoform which is transcribed from an alternative promotor, lacks a LID, is exclusively localized in the nucleus and shows a much higher transactivation activity compared to ChREBPα. Figure based on (50).

Upon rising intracellular glucose levels, the intramolecular inhibition of GRACE by LID is released (possibly through direct binding of glucose-derived
metabolites to GRACE), resulting in a conformational change that facilitates translocation to the nucleus and heterodimerization with MLX to promote transcription of its target genes, such as ChREBPβ (Fig. 2C). ChREBPβ (Fig. 2C) is a shorter isoform which is transcribed from an alternative promoter. It lacks a LID and consequently is exclusively localized in the nucleus and shows a much higher transactivation activity compared to ChREBPα. As ChREBPα can regulate transcription of ChREBPβ, mRNA levels of ChREBPβ and ChREBP-target genes is considered a proxy of ChREBP activity (52,55). Hepatic ChREBP is hyperactivated in hepatic GSD Ia (26,56–58).

**Regulation of ChREBP activity**

ChREBP is activated by intracellular glucose metabolism via multiple mechanisms (Fig. 3). First, it can be activated via X5P - protein phosphatase 2A (PP2A)-mediated nuclear translocation and dephosphorylation (59). Further research showed that G6P may be more important for ChREBP activation, by promoting a ChREBP conformational change that facilitates interaction with co-factors and nuclear translocation (60,61). Next to X5P and G6P, also fructose-2,6-bisphosphate has been implicated in regulation of ChREBP activity (62). The insulin-mediated activation of ChREBP is also mediated through changes in intracellular glucose metabolism (47,50,61). Next to dephosphorylation, ChREBP activity (and degradation) is also regulated by acetylation and O-GlcNAcylation via the histone acetyltransferase P300 and

Figure 3. Schematic overview of pathways activating ChREBP in response to intracellular glucose signaling. ChREBP activation is regulated via glucose-derived metabolites, such as glucose 6-phosphate (G6P), xylulose-5-phosphate (X5P), and fructose-2,6-bisphosphate (F2,6P2), and glucose-dependent posttranslational modifications, such as dephosphorylation, O-GlcNAcylation, and acetylation. GlcNH₂-6P: glucosamine 6-phosphate.
O-linked N-acetylglucosamine transferase (OGT) (63). ChREBP activity can also be repressed in different ways. First, ChREBP cytosolic retention is regulated via phosphorylation by glucagon-dependent activation of protein kinase A (PKA) (64,65). Next, ChREBP nuclear localization is inhibited by increased ChREBP stabilization with 14-3-3 proteins that cause cytosolic retention, mediated by metabolites such as AMP and ketone bodies (66,67). Finally, ChREBP transactivation capacity is inhibited by decreasing its promoter-binding ability via phosphorylation by the central cellular energy sensor AMP-activated protein kinase (AMPK) (68–70).

**ChREBP and tumorigenesis**

Hepatocytic targets of ChREBP include the hepatokines FGF21 and HGFAC (71,72) as well as genes encoding for enzymes involved in glycolysis, de novo lipogenesis (DNL), the pentose phosphate pathway (PPP), very-low density lipoprotein (VLDL) assembly, and bile acid and branched chain amino acid metabolism (45–47,73–76). Interestingly, glycolysis, DNL, PPP, VLDL assembly, and bile acid metabolism are all enhanced in GSD Ia liver (7,25,56–58,74,77,78). In addition, induction of these pathways represents a typical hallmark of many cancer cells (79), and supports proliferation and growth through macromolecule synthesis, ATP production, and enhanced oxidative defense (80). In line with this, increased ChREBP expression has been linked to the increased incidence and prognosis of several types of cancer, including hepatocellular carcinoma (HCC) (81–87). More specifically, ChREBP knockout mice are protected against AKT or AKT/c-Met-driven HCC development in an oncogene-specific manner, and ChREBP loss inhibits growth of oncogenic β-catenin/YAP-driven hepatoblastomas (84,88). Next to this, ChREBP was shown to promote proliferation, inhibit apoptosis, and elevate glycolysis of HCC cells in vitro (87). Moreover, RNA interference-mediated inhibition of ChREBP expression was shown to redirect glucose metabolism from aerobic glycolysis to oxidative phosphorylation, decrease synthesis of lipids and nucleotides, reduce tumorigenic potential of xenografts in nude mice, and inhibit cell proliferation in vitro through oxidative stress-induced, p53-mediated cell cycle arrest (89). Furthermore, ChREBP deficient hepatocytes show impaired proliferation rates during liver repopulation in vivo (88). Finally, based on its target pathways, ChREBP may serve as a potential target to reduce NAFLD, which is interesting as NAFLD predisposes to HCA/HCC development (90). Thus, ChREBP serves as a competence factor for cell growth and liver tumor development. It therefore represents an interesting nutrient sensor that may serve as a connection between GSD Ia-associated metabolic imbalance and increased tumorigenic risk.
SIRT1
Sirtuin 1 (SIRT1) is a nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase (in the nucleus) and protein deacetylase (in the cytosol) that plays a key regulatory role in hepatic metabolism. SIRT1 is part of a family of seven mammalian sirtuins (SIRT1-7), which differ in their N and C termini and which likely have different biological functions due to differences in enzymatic activity, binding partners, substrate, subcellular localization, and expression patterns (41,43). Next to serving as a histone deacetylase in the nucleus, SIRT1 can also bind to and deacetylate other proteins, such as peroxisome proliferator-activated receptor gamma (PPARγ), PPARα, PPAR gamma coactivator 1 alpha (PGC-1α) and the forkhead box, subgroup O (FOXO) transcription factors (43). By doing so, SIRT1 is able to regulate metabolic processes such as mitochondrial biogenesis, gluconeogenesis, and fatty acid oxidation, and serves to protect against cellular oxidative stress and DNA damage (41–43). In addition, it was shown that SIRT1 serves as a crucial factor for the induction of beneficial effects of calorie restriction (43,91).

Regulation of SIRT1 activity
As NAD⁺ is a co-factor of SIRT1, its availability impacts on SIRT1 activity. This is evidenced by findings that NAD⁺ depletion, for example as a result of nutrient deprivation, decreases SIRT1 activity, and that supplementation of NAD⁺ precursors can increase SIRT1 activity (92–94). In line with this, SIRT1 activity is inhibited by NADH and NAM, a product of SIRT1-mediated deacetylation reactions. In addition, SIRT1 activity can be regulated by nicotinamide phosphoribosyltransferase (NAMPT), the enzyme that mediates conversion of NAM to NMN in the NAD⁺ salvage pathway (93,95–97). The regulatory effects of the nutrient sensor AMPK on SIRT1 are likely mediated via regulation of NAD⁺ levels and NAMPT (43,98,99). SIRT1 in its turn can regulate AMPK by activating the AMPK activator LKB1 (100). Next to regulation by these metabolites and proteins, SIRT1 activity may furthermore depend on its subcellular localization; it is found to localize in nucleus and cytosol, interact with proteins in both fractions, and have both nuclear localization and nuclear export signals (43). Interestingly, in cancer cells SIRT1 is found to be localized in the cytoplasm (101). In addition, SIRT1 is responsive to a wide range of stimuli, including daylight (related to its function in the circadian clock), cell stress, and calorie restriction, which effects are mediated on the transcriptional level by several transcription factors, such as p53, FOXO3, and c-Myc, and microRNAs (43). Finally, SIRT1 activity is regulated by various small molecules, such as the inhibitors splitomycin, EX-527, sirtinol, or sirtinol analogs, or by activators such as resveratrol,
SRT1720, SRT2183, and analogues of NAM (43,102–106).

**SIRT1 and tumorigenesis**

While much of the research on SIRT1 initially focused on its role during ageing and ageing-associated disease processes, it also plays a major role in cancer. SIRT1 accommodates genome maintenance and cell division to metabolic signals (107). For example, SIRT1 is recruited to DNA double strand breaks in response to DNA damage (108,109). In addition, it functions in DNA repair pathways by promoting homologous recombination (HR), non-homologous end joining (NHEJ), and nucleotide excision repair (NER) (110–114). This suggests that SIRT1 may serve as a tumor suppressor, which is for example evidenced by *in vivo* studies showing that reduced SIRT1 activity leads to (liver) tumorigenesis (109,115,116). However, the role of SIRT1 in cancer is context-dependent, likely because SIRT activity is subject to regulatory hierarchy, as reviewed in (43). For example, both increased and decreased SIRT1 expression have been associated with numerous types of cancer (101), including HCC (115,117–119). On the one hand, SIRT1 may serve as an oncogene, as its expression is increased in many types of cancer, as it can inhibit the transactivation capacity of the tumor suppressor p53, and as it can prevent apoptosis and senescence (by regulating p53, Ku70 and FOXO3) and increase cellular proliferation (101,120–126). In addition, SIRT1 can be negatively regulated by the tumor suppressors hypermethylated in cancer 1 (HIC1) and the inhibitor deleted in breast cancer 1 (DBC1) (121,124,127,128). On the other hand, the same review discusses how SIRT1 could also be viewed as a tumor suppressor, as it can stimulate cell death, decrease stability of the proto-oncogenic transcription factor c-Myc, and prevent or decelerate tumorigenesis in animal models, next to its roles in DNA damage repair pathways and maintenance of genome stability (109,129–131). Thus, the exact functions of SIRT1 in proliferation and cancer are likely model-, time-, tissue- and disease-specific.

As discussed above, increased and decreased SIRT1 expression have also been associated with HCC. More specifically, Choi *et al.* (118) found that SIRT1 expression was significantly elevated in human HCC tissue compared to non-tumor tissue, with SIRT1 overexpression found in 56% of HCC tissues, and siRNA-mediated SIRT1 silencing induced cell growth arrest in HCC cell lines. A similar percentage of SIRT1 overexpression in human HCC tissues (55%) was found by Chen *et al.* (117), who also found that SIRT1 overexpression (which was not attributable to increased mRNA levels) promoted tumorigenesis and resistance to doxorubicin, a chemotherapeutic agent, and sorafenib, a protein
kinase inhibitor often used as therapeutic agent for HCC. Similarly, Portmann et al. (119) found overexpression of SIRT1 in human HCC tissues and HCC cell lines, and found that shRNA-mediated knockdown and pharmacological inhibition of SIRT1 activity resulted in impaired proliferation, increased expression of differentiation markers, and cellular senescence. Moreover, they showed in an orthotopic xenograft model that SIRT1 knockdown resulted in a decreased number of animals developing tumors, and that pharmacological inhibition of SIRT1 activity resulted in an overall lower tumor burden. Although these three studies indicate a liver tumor-promoting role for SIRT1, Herranz et al. (115) showed that transgenic mice overexpressing SIRT1 are less susceptible to liver cancer and have improved hepatic protection against metabolic damage and DNA damage in a model that combined a single injection of the hepatic-specific carcinogen diethylnitrosamine (DEN) with continuous exposure to a high-fat diet (HFD). Thus, these findings suggest that the role of SIRT1 in liver cancer is complex and potentially model-, time-, and context-dependent. As SIRT1 expression and activity are decreased in hepatic GSD Ia (26,27), and because of its functions in metabolism and HCC, it represents an interesting nutrient sensor that may mediate GSD Ia-associated metabolic imbalance and increased tumorigenic risk.

Scope and outline of this thesis
GSD Ia is characterized by metabolic imbalance due to G6P accumulation, which drives glycogen accumulation, NAFLD, hepatomegaly, and liver tumor development. Research in mouse models for GSD Ia indicates that liver tumor development, the most prominent long-term complication of the disease, can be attributed to metabolic derangement resulting from G6PC deficiency specifically in hepatocytes (3). Although it is evident that this favors a metabolic profile that supports tumor progression, the mechanisms underlying tumor initiation, especially the role of nutrient sensors ChREBP and SIRT1, remain unresolved. Poor metabolic control and accompanying changes in plasma TG levels (7,28,29) are associated with increased risk for tumor development in GSD Ia patients (30–33). However, plasma TG levels vary considerably between patients (30,34), and incomplete insight into the consequences of this phenotypic heterogeneity in symptoms and complications hampers investigation of mechanisms underlying GSD Ia-associated tumorigenesis (35). To address these issues, we used in Chapter 2 CRISPR/Cas9-mediated somatic gene editing as a novel approach to generate a series of mice mimicking the phenotypic heterogeneity in GSD Ia patients and to study gene-gene interactions, which could serve as a valuable tool to study the contribution of other genes to GSD Ia-associated tumor initiation.
In Chapter 3, we investigated the consequences of ChREBP normalization on liver disease progression and tumorigenic risk in hepatic GSD Ia by using a short-hairpin RNA to reduce hepatic ChREBP expression in livers of Tamoxifen-inducible hepatocyte-specific G6pc knockout (L-G6pc⁻/⁻) mice. In Chapter 4, we studied the consequences of impaired SIRT1 function on liver disease progression in GSD Ia by employing transgenic Sirt1 overexpression and/or NAD⁺ precursor supplementation in L-G6pc⁻/⁻ mice as strategies to boost SIRT1 activity. Finally, in Chapter 5 we discussed the findings described in this thesis and put them in a broader perspective.
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General Introduction


