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Untargeted Proteomics Profiling of Liver and Plasma in Fed and Fasted Liver-Specific Glycogen Storage Disease Type Ia (GSD Ia) Mice: Toward Potential Protein Biomarkers

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ABSTRACT

Glycogen storage disease type Ia (GSD Ia) is a rare autosomal recessive inherited disorder of carbohydrate metabolism, caused by a deficiency in glucose 6-phosphatase- α (G6PC1). Patients primarily suffer from failure to thrive, hepatomegaly, and severe fasting intolerance, biochemically characterized by hypoketotic, hypoglycemia, and hyperlipidemia. Because of clinical and biochemical heterogeneity, identifying biomarkers is imperative for prognosis and monitoring. An untargeted proteomics workflow was employed for identifying protein changes in liver and plasma from hepatocyte-specific *G6pc* knockout mice under fed and fasted conditions. This links the effect of hepatic G6Pase/G6pc deficiency to circulating protein biomarkers and allows assessment of the relationship with different clinical circumstances and long-term complications. In the liver, the main differences between hepatic GSD Ia mice versus controls were observed in proteins related to carbohydrate and lipid metabolism, proteasome, ribosome, NAD⁺ metabolism, and mitochondria. In GSD Ia mouse plasma, proteins were mainly down-regulated in the complement and coagulation cascades. Effects in hepatic GSD Ia mice were in general more pronounced under fasting conditions. Several potential biomarkers that showed significant alterations in both liver and plasma were identified. These include proteins involved in carbohydrate and lipid metabolism (e.g., UGP2, ALDOB, and FASN), complement and coagulation cascades (SERPINA1E, C8b, and MBL2), 20S proteasome subunits (PSMA4, PSMA7, and PSMB5), and the electron transport chain (SDHA). Their consistent changes observed in both the liver and circulation indicate their potential as circulating biomarkers reflecting liver condition. Together with their reported associations with liver diseases, we hypothesize that they could monitor hepatic complications.

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1 | Introduction

Glycogen storage disease type I (GSD I) is a rare metabolic disorder [1], caused by pathogenic variants in either the *G6PC1* gene (encoding the glucose 6-phosphatase- α enzyme, G6PC1, E.C. 3.1.3.9) (GSD type Ia [2], OMIM: #232200), or the *SLC37A4* gene (encoding the glucose-6-phosphate transporter, G6PT) (GSD type Ib [3], OMIM: #232220). GSD Ia accounts for the majority (about 80%–90% [4]) of GSD I patients. The deficiency of G6PC1 or G6PT reduces cytoplasmic glucose availability and ultimately blood glucose homeostasis in the circulation under fasting conditions [5–7].

Untreated GSD Ia patients display acute, life-threatening fasting hypoglycemia. Additionally, untreated GSD Ia patients present with hypoketosis [8, 9], hyperlactatemia, hyperuricemia, and hyperlipidemia [10]. A strict medically prescribed diet is the cornerstone of treatment. On the one hand, a continuous exogenous source of glucose is provided to compensate for deficient endogenous glucose production and maintain glucose homeostasis. On the other hand, substrate reduction in the form of dietary restriction of simple sugars (i.e., sucrose, lactose, and fructose) should prevent excessive glucose-6-phosphate accumulation. Together, the diet aims to reduce or prevent acute/chronic complications, while trying to preserve quality of life as much as possible [11, 12]. However, it does not always prevent long-term complications [13], such as growth restriction, abnormal intellectual development, anemia, osteopenia, kidney failure, non-alcoholic fatty liver disease (NAFLD), and especially hepatocellular adenoma (HCA) and carcinoma (HCC) [14]. In GSD Ia, 70%–80% of the patients over 25 develop HCA, with about 10% progressing to HCC [10, 14–16].

The mechanisms underlying long-term complications are incompletely understood. Large heterogeneity between individual GSD Ia patients exists and it is challenging to identify which patients are at risk of developing specific long-term complications. This requires suitable biomarkers for monitoring patients [2, 12, 17] which is in line with the top research priorities identified by the international priority-setting partnership for liver GSDs, in which patients, caregivers, and healthcare professionals emphasized the need for less burdensome and more reliable monitoring methods [18]. Proteins are attractive as biomarkers since they are longer-lived than metabolites [19, 20].

Preclinical studies have shown that many GSD Ia symptoms and complications are primarily driven by G6Pase/G6PC deficiency in hepatocytes, illustrating the relevance of liver-specific GSD Ia proteome profiling [21–23]. Cangelosi et al. conducted proteomics in GSD Ia mouse livers to explore metabolic reprogramming [21]. However, in clinical practice, the liver is a relatively inaccessible organ, and tissue collection requires invasive biopsies in patients. As blood sampling is performed routinely, the use of circulating protein biomarkers for GSD Ia potentially provides an adequate and valuable approach to monitoring and risk stratification in GSD Ia patients.

This study aimed to identify leads for potential non-invasive protein biomarkers in plasma for prognosis and monitoring. As

a secondary aim, it allowed us to explore the pathophysiological mechanism in hepatic GSD Ia focusing on the role of proteins in adaptations to the disease. We used liquid chromatography coupled with mass spectrometry (LC–MS) to perform liver and plasma proteomics in fed and fasted hepatocyte-specific *G6pc* knockout mice (*L-G6pc*^{−/−}) [23]. Since regular intake of uncooked cornstarch along with elevated levels of G6P keeps patients continuously in a fed state, the fed and fasted mice can be regarded as models of adequate and poor control of biochemical symptoms, respectively [17]. This approach allowed us to (i) assess the impact GSD Ia on the liver and plasma proteomes (ii) assess how GSD Ia affects the fasting response, and (iii) establish the overlap between hepatic and plasma proteome profiles to identify potential biomarkers in patient plasma for prognosis and monitoring.

2 | Materials and Methods

2.1 | Study Design

An untargeted proteomics workflow was established to identify and quantify protein groups of liver tissue and plasma from hepatic GSD Ia and wildtype control mice (Figure 1). The workflow was schematically summarized in the figure in three steps: sample collection and preparation (Figure 1A), data collection (Figure 1B), and data analysis (Figure 1C).

2.2 | Animals

Male adult (7–11 weeks) hepatocyte-specific Cas9-expressing mice [24] were housed individually with wood bedding, nesting material, and cardboard rolls in a light- and temperature-controlled facility with a 12 h light/12 h dark regime, and fed a standard laboratory chow diet ad libitum (RM1, Special Diet Services, UK). Anesthetized mice received retro-orbital injections of 1.0×10^{11} [11] vp three single-guide RNAs (sgRNAs) against exon 1 of *G6pc* (sgG6pc) using an adenoviral gene delivery system, or the adenoviral vector (pX459; Addgene plasmid #48139; Addgene, Watertown, MA) without any sgRNAs to generate hepatic GSD Ia or wildtype control mice, respectively. In the fasted group, mice were deprived of food for 6 h weekly during the light phase after sgRNA injection to confirm the presence of fasting hypoglycemia. Four to five weeks after viral injection, they were euthanized by cardiac puncture under isoflurane anesthesia for blood and tissue collection at 8:00 a.m., either in the fed state or after an overnight fast (9:30 p.m.–8:00 a.m.) during which drinking water was provided. Liver tissues were quickly excised, freeze-clamped, and stored at -80°C . Blood was centrifuged ($2500 \times g$ for 10 min at 4°C), and the plasma was stored at -80°C [22, 23]. Details of mouse group and their metabolic parameters in plasma (glucose, triglycerides, ALT and lactate) and liver (liver-to-body ratio, G6PC activity, glycogen, and triglycerides) were summarized in Table S1. The reduced hepatic G6PC activity in both fed and fasted GSD Ia mouse models confirmed their disease state. The lower plasma glucose, elevated lactate levels, increased liver-to-body weight ratio, and excessive hepatic glycogen accumulation indicated that the mice exhibited key features of GSD Ia patients, including hypoglycemia, hyperlactatemia,

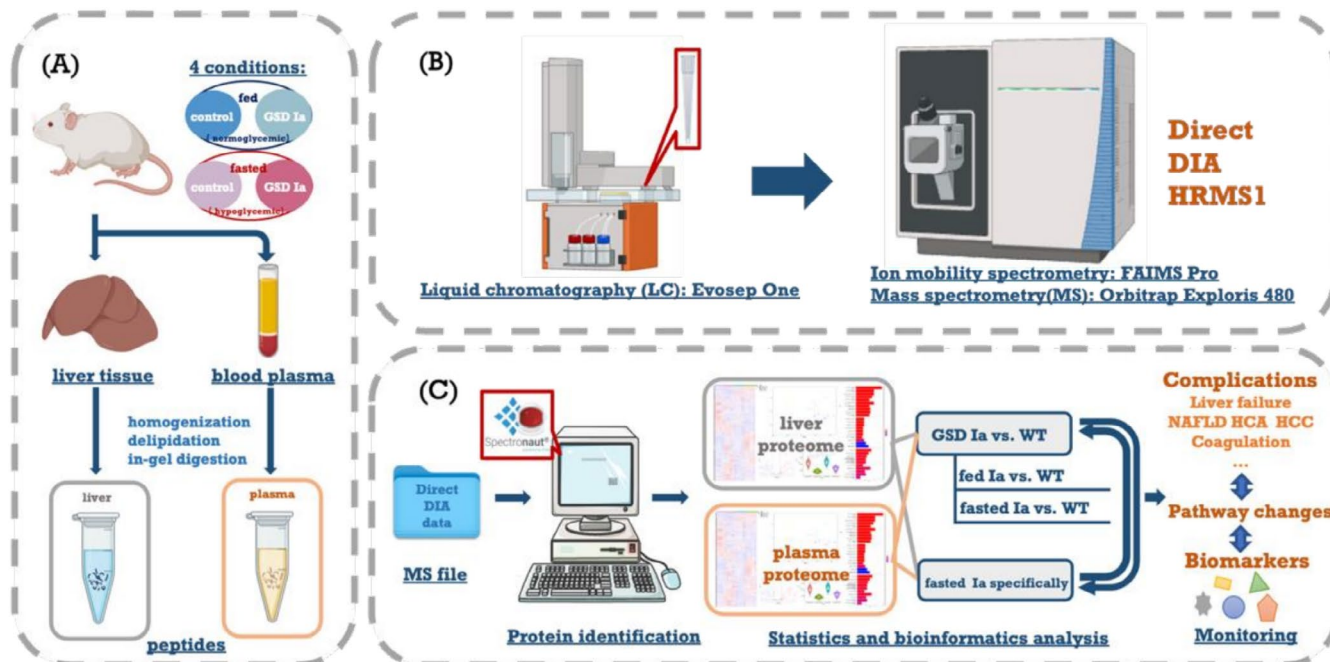


FIGURE 1 | Schematic representation of the untargeted proteomics workflow. (A) Sample collection and preparation. (B) Data collection: Untargeted proteomics using LC-FAIMS-MS. (C) Data analysis: Protein identification, quantification, and interpretation.

hepatomegaly, and glycogen storage in the liver. Liver adenomas and carcinomas were not macroscopically detected in these mice, in accordance with previous observations in our mouse studies that these on appear after 55 weeks of follow-up after injection [23].

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, The Netherlands) under permit number AVD10500202115288 and are in line with the Guide for the Care and Use of Laboratory Animals.

2.3 | Liver Homogenization

Forty milligrams of liver tissue was lysed in 10% (w/v%) RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 2 mM EDTA (pH 8.0), 50 mM sodium fluoride (NaF), 0.2 mM sodium orthovanadate (Na_3VO_4), and 10% 10× Complete protease inhibitor cocktail (Roche beating Diagnostics, Mannheim, Germany)) to prepare homogenates using a TissueLyser LT (Qiagen) at 50 Hz for 2 min. Lysates were subsequently stored on ice for at least 30 min and vortexed every 10 min, after which they were centrifuged at $2500 \times g$ for 10 min at 4°C . The supernatants were collected as liver homogenates.

2.4 | Determination of Protein Concentrations in Liver Homogenates

Total protein concentrations of liver homogenates were quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific),

and absorbance was measured at 540 nm on an EL800 microplate reader (Biotek).

2.5 | Sample Delipidation

One hundred and fifty microliters of liver homogenates and $30 \mu\text{L}$ plasma were delipidated using 4 mL MeOH and 4 mL cold diethyl ether (-20°C), followed by centrifugation for 30 min at $1500 \times g$ at 4°C . After discarding the supernatant, 4 mL cold diethyl ether was added to each sample and centrifuged for 30 min at $1500 \times g$ at 4°C . Samples were dried under N_2 after discarding the supernatant and then resuspended with $100 \mu\text{L}$ 1× lithium dodecyl sulfate (LDS) sample loading buffer (Abcam PH=8.5, diluted with RIPA buffer). After sonicating, vortexing, pipetting up and down several times, and centrifugation at $1500 \times g$ for 1 min, the supernatants were taken and diluted five times using 1× LDS sample buffer.

2.6 | Protein Digestion

In-gel digestion was done as described previously [25]. Briefly, in-gel digestion was done with $20 \mu\text{L}$ of delipidated samples using 300 ng trypsin after reduction with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide.

2.7 | Sample Loading Onto EvoTips

The eluted and dried peptide digests were resuspended in 0.1% FA at a concentration of $25 \text{ ng}/\mu\text{L}$ based on the protein concentration before delipidation. Twenty microliters of peptide solutions were loaded onto the Evtips (Evosep Aps, Odense C,

Denmark) following the sample loading protocol [26], for injection on the LC. Briefly, EvoTips were washed with 100 μ L 100% ACN and activated with 100 μ L 1-propanol, followed by 0.1% FA in water (Solvent A), and then 20 μ L peptides were loaded and washed with 20 μ L Solvent A (centrifuge at 700g for 60s at every step). Finally, 100 μ L Solvent A was loaded to keep the tips wet (centrifuge at 700g for 20s).

2.8 | Liquid Chromatography Separation

All samples were analyzed by the Evosep One HPLC (Evosep Biosystems, Odense C, Denmark) using a C18 column with length/ID/C18 bead size of 15cm/150 μ m/1.9 μ m (EV-1106, Evosep Biosystems). The pre-programmed gradient (30 samples per day, 0.5 μ L/min flow rate, 48 min cycle time, 44 min gradient length) was applied. The column temperature was maintained at 40°C by a column heater controller (Phoenix S&T, Chadds Ford, USA) and coupled online with an Orbitrap Exploris 480 equipped with a FAIMS Pro.

2.9 | Mass Spectrometric Analysis

Mass spectrometric analyses were performed on Orbitrap Exploris 480 (Thermo Scientific) using a 44-min HRMS1-DIA based-method [27] with the FAIMS Pro (Thermo Scientific) connected. The FAIMS Pro was set to a standard resolution with 3.8L/min total carrier gas flow and voltages switching between –45 and –60 CV. MS¹ and MS² data were acquired at a resolution of 120k and 15k, across a 400–1200 m/z and 400–1000 m/z scan range, and with normalized AGC targets of 300% and 1000%, respectively. MS¹ and MS² were acquired with a maximum inject time of 400ms. HRMS1-DIA settings included 3 full scans and 35 fragment scan events with 17 m/z isolation windows, 32% HCD collision energies, 40% RF lens, and 12 loop spectra.

2.10 | Data Analysis and Statistics

Raw MS data were processed in Spectronaut v.16 (SN16) using a library-free approach (directDIA) for quantification at MS¹ level, using background signal as the imputation strategy for the values that did not fulfill the FDR threshold. Data were aligned with mouse protein sequences based on the Mouse UniProt reviewed sequences (17021 entries). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [28] partner repository with the dataset identifier PXD054398. The gene names related to the proteins were used for visualization and their integrated peak areas were processed using RStudio. Missing values were imputed by the mean of the respective experimental group. If no group values were available, missing values were replaced by 0.1, representing the instrument detection limit. The data normalization and differential determination, including $\log_2^{\text{Fold Change}}$ ($\log_2^{\text{FC}}$) and false discovery rates (FDR) between two groups were analyzed using the “EdgeR” package in R [29]. In addition, principal component analysis (PCA), heatmap, volcano plots, and receiver operating characteristic (ROC) analysis were made using R packages “sjmisc,” “pheatmap,” “ggplot2,” and “pROC.” Significantly changed proteins were filtered by FDR

(≤ 0.05). KEGG pathways analysis was performed using DAVID Bioinformatics Resources (v2023q1).

3 | Results

3.1 | Untargeted Proteomics Workflow

We established a workflow for liver and plasma proteome profiling in fed and fasted hepatocyte-specific GSD Ia and wildtype mice (Figure 1). Liver tissue and plasma were obtained from adult, liver-specific GSD Ia mice at an early disease stage, that is, 4 weeks after hepatic *G6pc* editing. When taking all experimental groups together, we identified 3351 proteins in liver tissue and 475 proteins in plasma. The PCA visualization shows a clear separation of all groups based on nutritional status and genotype, in both liver (Figure 2A) and plasma (Figure 2E). In the comparison of fed GSD Ia mice with fed WT mice a total of 674 proteins were significantly changed (FDR < 0.05) in liver (Figure 2B) and 157 proteins in plasma (Figure 2F). For the fasted GSD Ia mice compared to fasted WT mice a total of 705 proteins were significantly changed in liver (Figure 2C) and 222 proteins in plasma (Figure 2G). The GSD Ia specific comparison between fed and fasted mice showed a total of 864 proteins in liver (Figure 2D) and 37 proteins in plasma (Figure 2H). The proteins with $\log_2^{\text{FC}}$, FDR values, and the annotations are summarized in Tables S2–S7.

3.2 | Protein Levels Related to Carbohydrate and Lipid Metabolism, Coagulation and Complement Cascades, and Proteasome Were Changed in Both Liver and Plasma of GSD Ia Versus WT Mice

To investigate the pathophysiological mechanisms of GSD Ia, we first compared WT to GSD Ia mice both in the fed and fasted condition. Next, we searched for common patterns between both feeding states.

Out of the top 30 FDR-ranked KEGG pathways differentially regulated between WT and GSD Ia in liver, 23 were shared between the fed and the fasted condition (Table S8). Additionally, 22 pathways were shared for the same comparison in plasma (Table S9). The complete list of enriched pathways and significantly changed proteins included in these pathways in each condition is presented in Tables S10–S13, respectively. After clustering pathways based on their core related metabolites (Tables S8–S9), the heatmaps of significantly changed proteins included in these top changed pathways of GSD Ia are shown in Figure 3.

KEGG pathways that were differentially regulated between GSD Ia and WT in both fed and fasted GSD Ia mouse liver included glycolysis/gluconeogenesis, pyruvate metabolism, fatty acid synthesis/elongation and fatty acid catabolism, and the pentose phosphate pathway (Table S8; #1–6). Of the 53 proteins that were differentially regulated between GSD Ia and WT in carbohydrate and lipid metabolism in both feeding states, 14 (26%) were downregulated, including G6PC, while 39 (74%) were upregulated (Figure 3A). Figure 4 shows the regulated proteins mapped on the pathways of carbohydrate and lipid metabolism. The knockout of G6PC in the liver

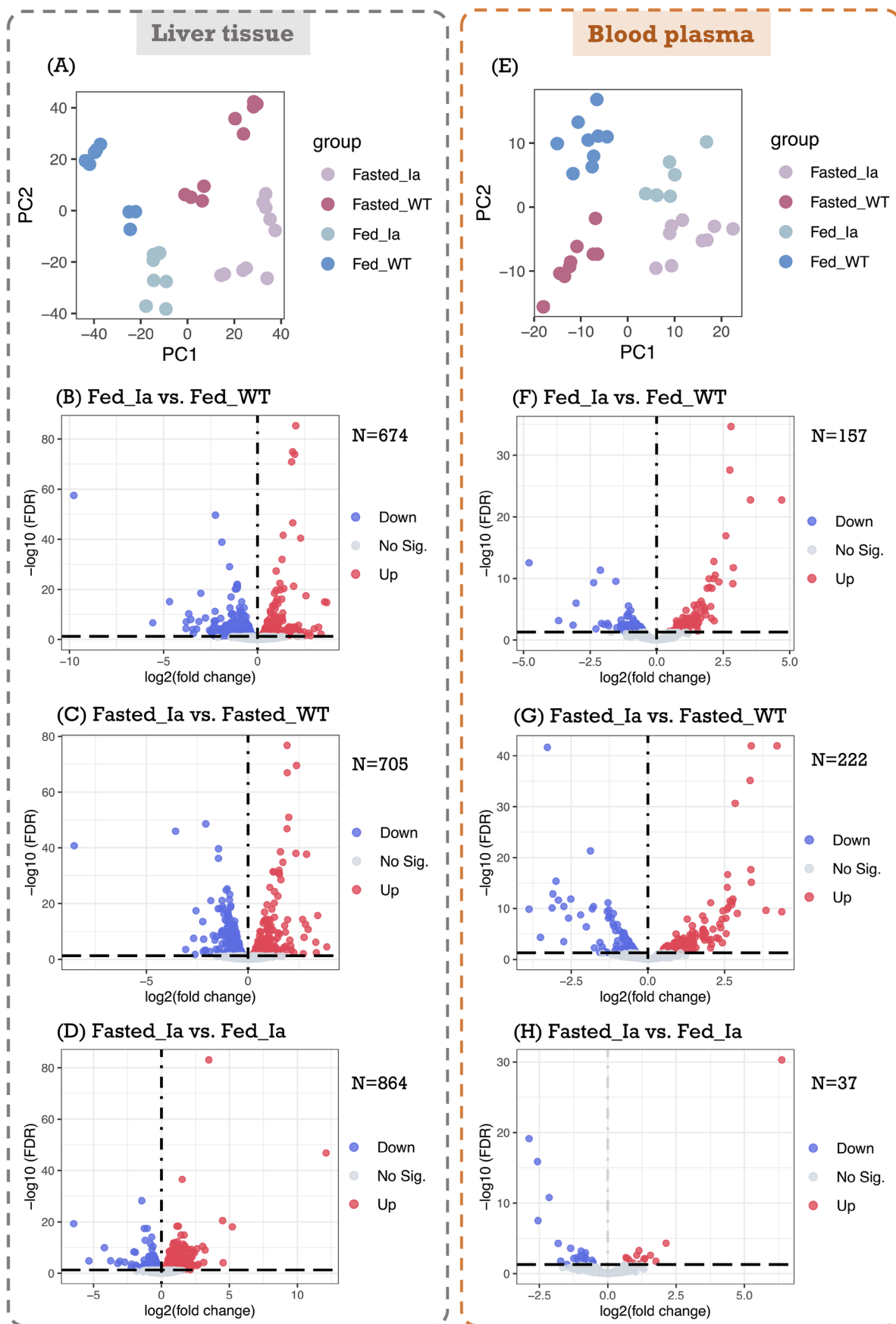


FIGURE 2 | Overview of proteomics data. (A, E) principal component analysis (PCA) plot of four conditions in liver tissue and plasma. (B–D, F–H) Volcano plots of statistically significant analysis on different liver tissue and plasma groups. “N” represents the number of significantly changed proteins. “No sig.” refers to proteins that show no significant changes.

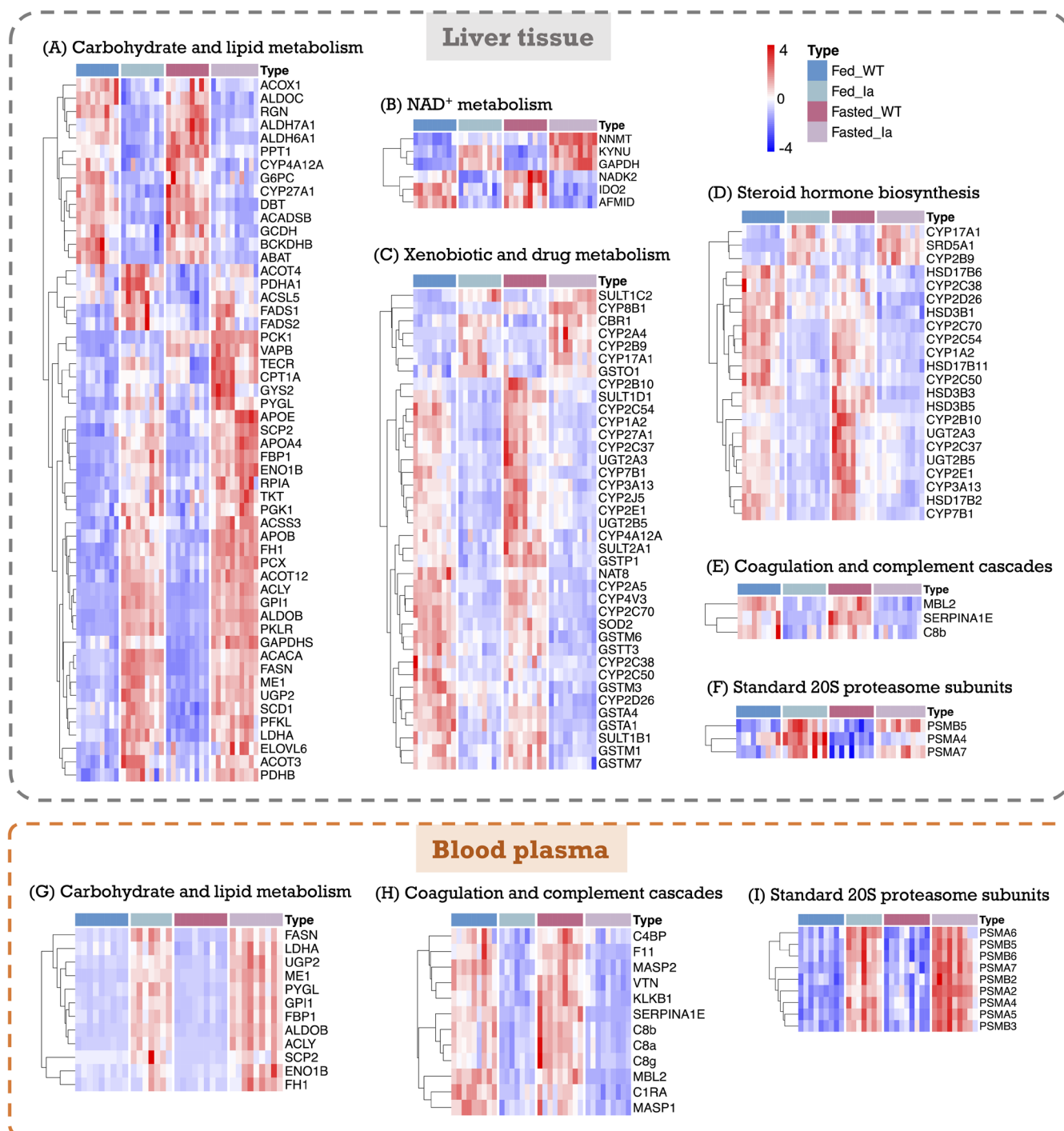


FIGURE 3 | Heatmaps showing z-score intensity of significantly changed proteins in both fed and fasted GSD Ia versus wildtype groups, quantified per liver and plasma samples in top changed pathways of GSD Ia. All four sample types were included to provide a visualization of all conditions for these significantly changed proteins. Z-scores were calculated per protein across all samples, such that each protein's abundance is standardized relative to its mean and standard deviation across the dataset.

resulted in a clear upregulation of almost all proteins involved in glycolysis/gluconeogenesis, glycogen synthesis and lipid synthesis.

Newly identified enriched pathways in hepatic GSD Ia mouse livers included nicotinamide adenine dinucleotide (NAD⁺) metabolism, drug and xenobiotic metabolism, chemical carcinogenesis, genotoxicity, and steroid hormone synthesis

(Figure 3B–D, Table S8; #7–9). In the NAD⁺ metabolism, three proteins were significantly down-regulated and three proteins up-regulated (Figures 3B and S1). Furthermore, levels of proteins involved in the metabolism of retinol, cofactors, and amino acids were also changed in GSD Ia mouse liver (Table S8; #10–12). In addition, the differential regulation of CYPs, UGTs, SULTs, GSTs, HSDs, and SRD5A1 indicates an altered detoxification response (Figures 3C and S2) and affected steroid

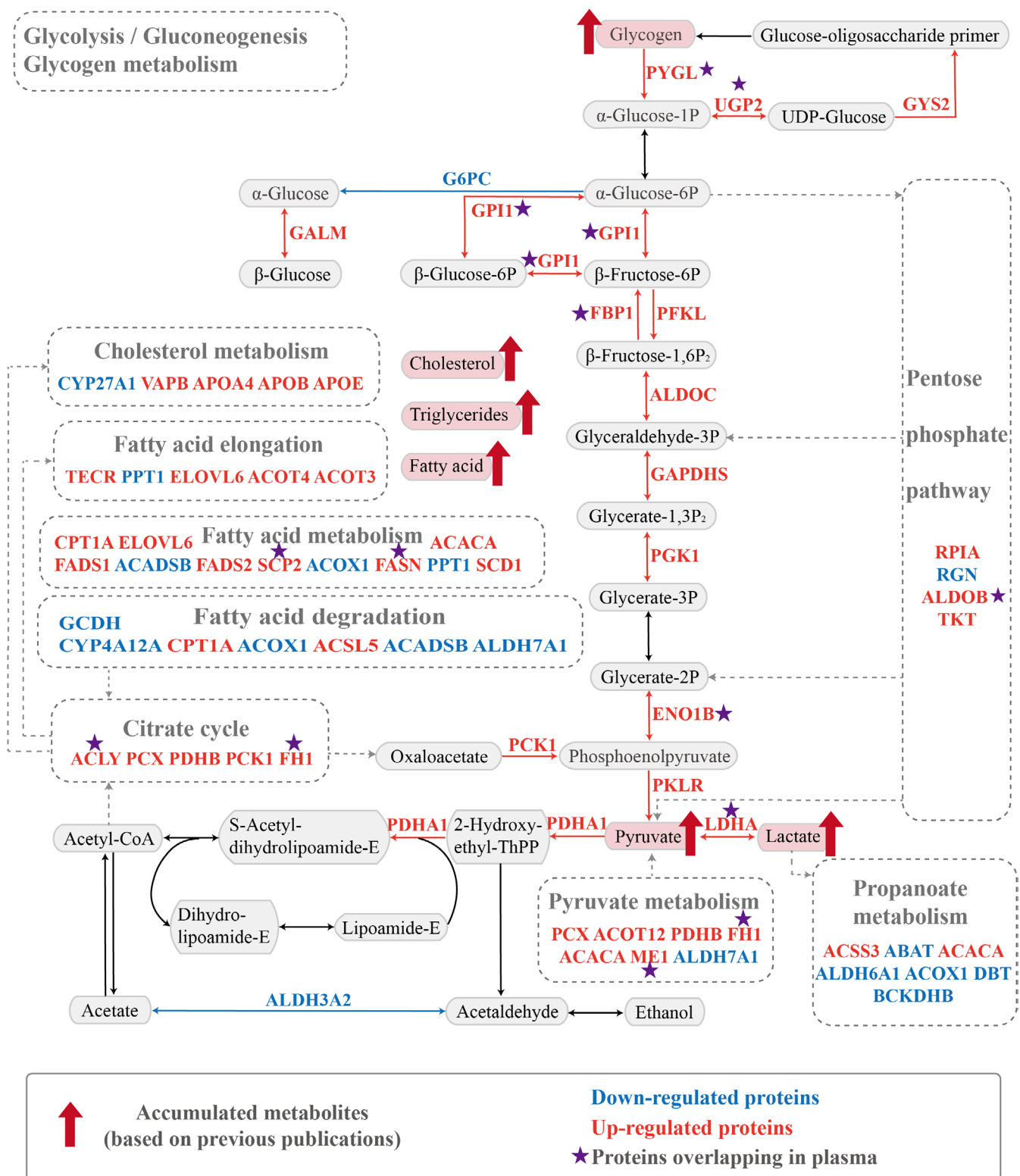


FIGURE 4 | Summary of the significantly changed proteins related to carbohydrate and lipid metabolism in GSD Ia mouse liver compared with wildtype, along with overlapping plasma proteins, within fed and fasted groups. The dotted arrow means some steps are omitted in between.

hormones (Figures 3D and S3) in GSD Ia, since they are the main enzymes in those pathways. Particularly in the metabolism of steroid hormones, about 86.4% of proteins (19 out of 22) were significantly decreased in GSD Ia mouse liver, and in the detoxification response, around 81.6% of proteins (31 out of 38)

were down-regulated. In addition, it is worth noting that the glutathione S-transferase family (GSTs) and superoxide dismutase (SOD2) were decreased, while peroxidase (CBR1) increased in GSD Ia mouse livers (Figure 3C), indicating specific regulation in parts of the detoxification pathways. The metabolism of

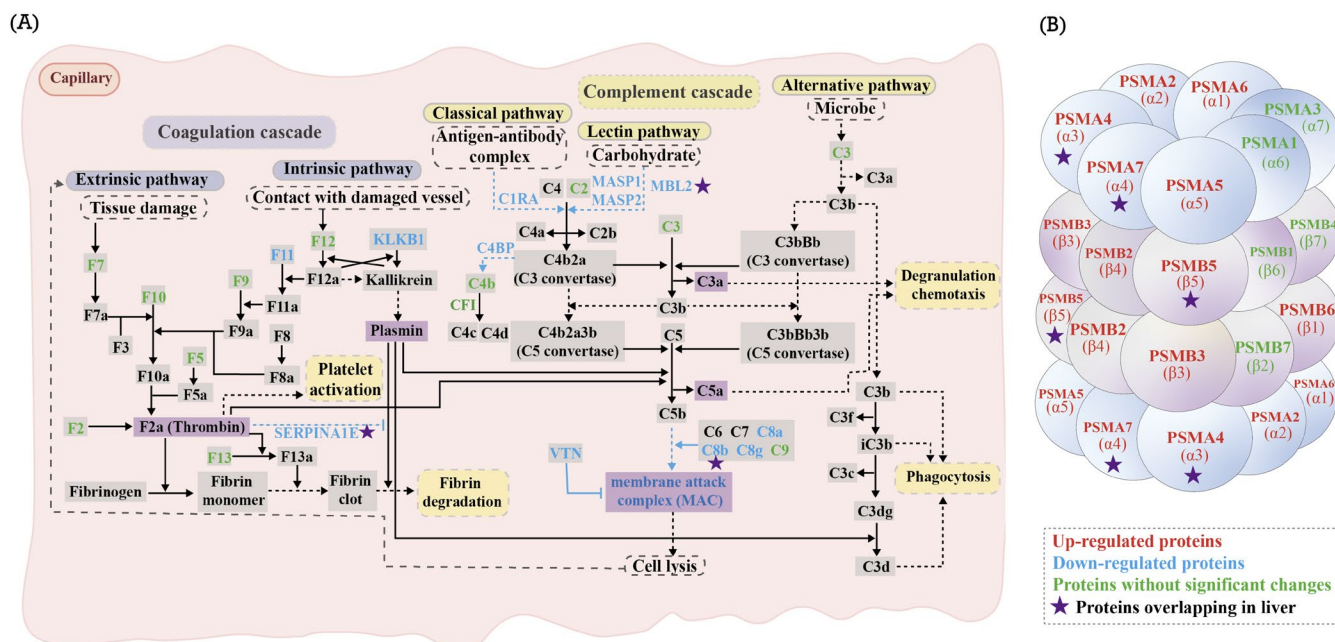


FIGURE 5 | Summary of the significantly changed proteins related to (A) coagulation and complement cascades and (B) standard 20S proteasome in fed and fasted GSD Ia mouse plasma compared with wildtype, as well as the overlapping proteins in liver. The dotted arrow means some steps are omitted in between.

xenobiotics via the cytochrome P450 (CYPs) pathway was also affected in GSD Ia mice plasma (Table S9; #5).

Proteins that are differentially regulated in both plasma and liver are potential biomarkers for the state of the liver, particularly if they are mechanistically involved in specific disease symptoms. We primarily focused on proteins with the same direction of regulation in liver and plasma. In carbohydrate and lipid metabolism, 12 proteins were up-regulated (FASN, LDHA, UGP2, ME1, PYGL, GPI1, FBP1, ALDOB, ACLY, SCP2, ENO1B, and FH1) in GSD Ia in both liver and plasma (Figures 3A,G and 4). In addition, differentially regulated proteins related to coagulation and complement cascades and proteasome also overlapped between GSD Ia liver and plasma (Figures 3E,F,H,I and 5). The coagulation and complement cascade pathway was the most differentially regulated pathway in GSD Ia plasma (Table S9; #1), with 12 proteins significantly decreased in this pathway (Figures 3H and 5A), and MBL2, SERPINA1E, and C8b similarly affected in the GSD Ia liver (Figures 3E and 5A). As for proteasome components (Table S9; #6), 9 subunits were up-regulated in GSD Ia (Figures 3I and 5B), with PSMB5, PSMA4, and PSMA7 overlapping between liver and plasma (Figures 3F and 5B).

In summary, the identified differential regulation results agree with the previously described accumulation of glycogen, pyruvate, lactate, cholesterol, triglycerides, and fatty acids in GSD Ia liver and blood [2, 10, 14]. It confirmed the previously reported increased carbohydrate and lipid metabolism-related proteins in GSD Ia mouse livers [21]. Additionally, we newly identified differential regulation in the metabolism of NAD⁺, xenobiotic, genotoxicity, steroid hormone, retinol, cofactors, and amino acids. Lastly, based on similar responses observed both in the liver and plasma proteome, we identified 18 proteins as potential biomarkers for hepatic GSD Ia.

3.3 | Fasting-Specific Differentially Regulated Proteins in Hepatic GSD Ia

To assess specific biological process alterations in hypoglycemic GSD Ia (Fasted_Ia) mouse livers and blood, we focused on fasting-specific differentially regulated proteins in hepatic GSD Ia. First, we selected the proteins that significantly changed because of fasting, which were proteins with FDR < 0.05 both for GSD Ia and WT respectively. Secondly, we looked at the proteins that are specific for fasting in GSD Ia by looking at proteins with differential regulation specific for GSD Ia (at least with 30% more increase or decrease compared to the general fasting effect in WT, Tables S14 and S15). The top 30 KEGG pathway analysis of these proteins were clustered based on potential symptoms in GSD Ia (Table S16, entire list Table S17). The heatmaps of the proteins clustered in related pathways were shown in Figure 6. KEGG pathways related to fatty acid oxidation (FAO), cholesterol metabolism, chemical carcinogenesis-ROS, electron transport chain (ETC), oxidative phosphorylation system (OXPHOS), and the peroxisome were among the more significantly regulated pathways in GSD Ia upon fasting (Table S14; #1). In these pathways, 8 proteins were specifically decreased and 37 increased in fasted GSD Ia mouse livers (Figure 6A). Among these the majority of the up-regulated proteins (21) are subunits of complexes I-IV (CX I-IV) of the ETC, while the results for the peroxisomal proteins show mixed effects of up- and down-regulation (Figure 7A). In addition, the shared component proteins of lipoproteins (APOC1, APOC3, APOE), and the proteins contributing to very-low-density lipoprotein (VLDL) metabolism (VDAC1, 2, 3, and TSPO) were more up-regulated in the fasted GSD Ia mice, compared with the other groups (Figures 6B and 7B). Furthermore, four ribosomal proteins were very significantly decreased in the fasted GSD Ia mouse livers while 1 protein was increased (Figure 6C). Part of the observed pathway changes had already been observed upon the GSD Ia

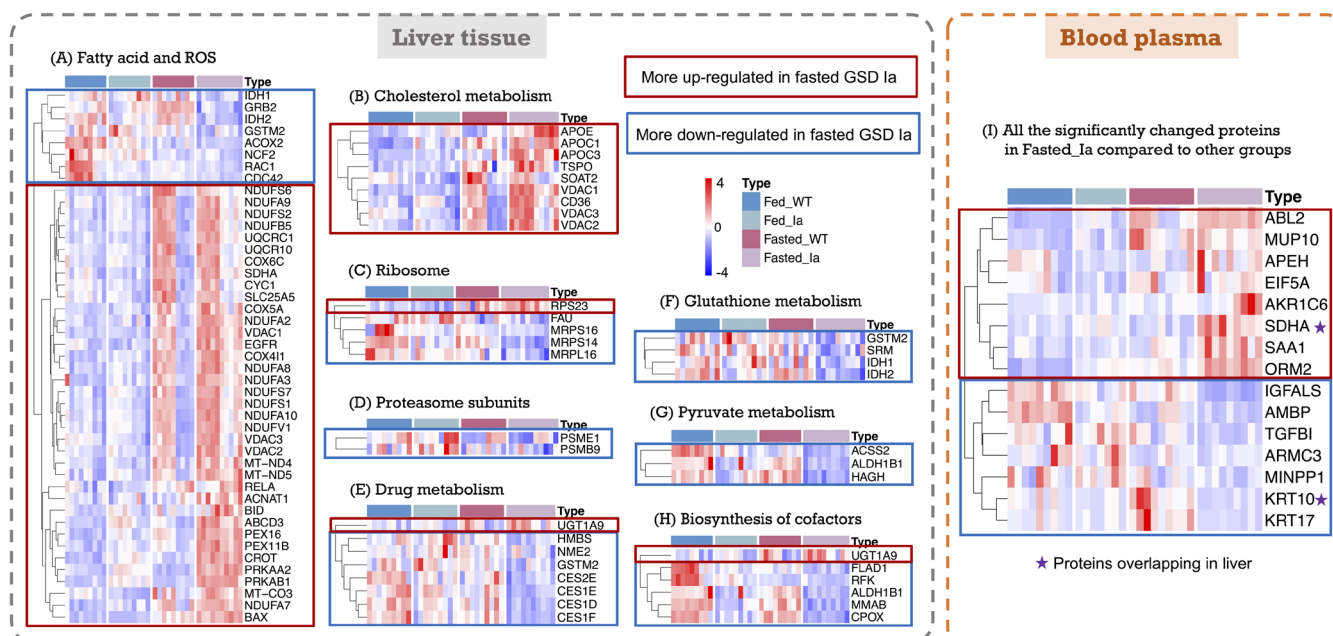


FIGURE 6 | Heatmaps showing z-score intensity of significantly changed proteins quantified per liver and plasma samples in different pathways, specifically in fasted GSD Ia mice. SDHA and KRT10 are overlapping proteins. However, since the detected peptides of mouse KRT10 overlap completely with the human keratin-10 protein (common sample handling contaminants), we did not consider this protein a potential biomarker. All four sample types were included to provide a visualization of all conditions for these significantly changed proteins. Z-scores were calculated per protein across all samples, such that each protein's abundance is standardized relative to its mean and standard deviation across the dataset.

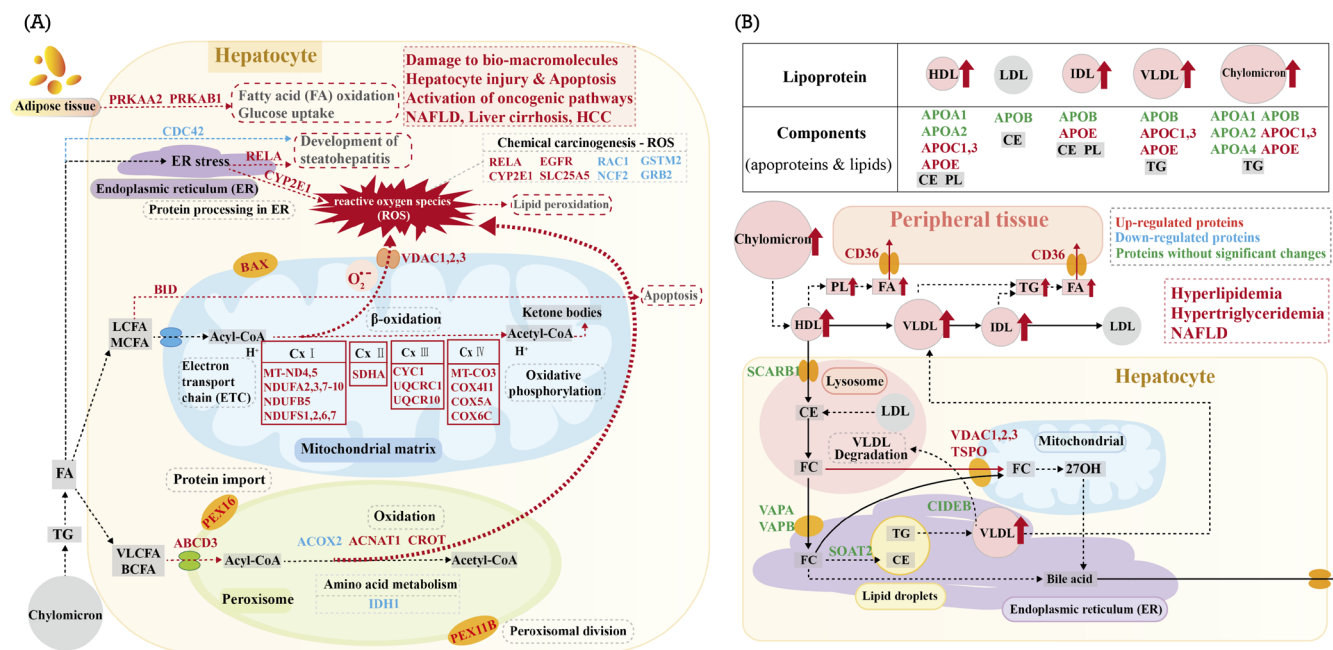


FIGURE 7 | Summary of the typical significantly changed proteins related to (A) fatty acid oxidation and chemical carcinogenesis—ROS and (B) cholesterol metabolism and lipoproteins in fasted GSD Ia mouse livers. 27OH, 27-hydroxycholesterol; BCFA, branched-chain fatty acids; CE, cholesteryl ester; FA, fatty acid; FC, free cholesterol; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCFA, long-chain fatty acid; MCFA, medium-chain fatty acid; PL, phospholipid; TG, triglyceride; VLCFA, very long-chain fatty acids; VLDL, very low-density lipoprotein.

versus wildtype (Table S8), which means these pathways had already changed in GSD Ia but were stronger in fasted GSD Ia. Specifically, this applies to proteins related to proteasome, drug metabolism, glutathione metabolism, pyruvate metabolism, and biosynthesis of cofactors (Figure 6C–H).

These findings suggested that not only proteins related to FAO, lipoprotein metabolism, and ROS production, but also ribosomal, proteasomal, and liver metabolism of drugs and other substances, proteins are important for the observed metabolic dysregulation specific for fasting in GSD Ia mouse livers.

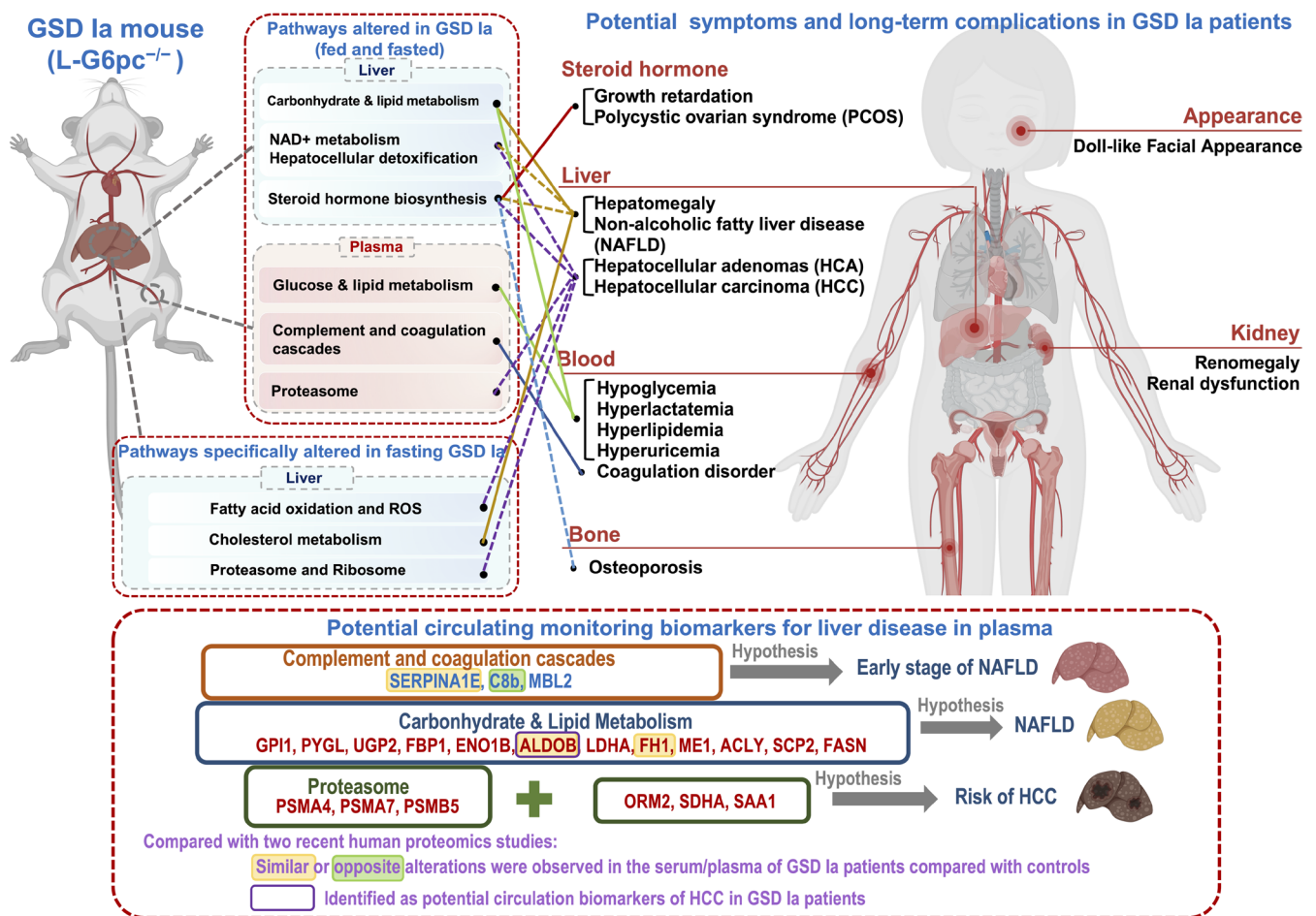


FIGURE 8 | Summary figure. Solid lines refer to direct links, whereas dotted lines refer to indirect impact. Given the multifactorial nature of long-term complications, the depicted relationships should be interpreted as exploratory and hypothesis-generating. Further studies are required to establish causality. The details of these links were summarized in Tables S8 and S9.

In plasma, only 15 proteins were differentially regulated specifically in the fasted GSD Ia mice (Figure 6I). These proteins were separated into two groups with eight up-regulated proteins and seven down-regulated proteins. Among them, two proteins responded the same as in the liver (SDHA and KRT10). SDHA was up-regulated in response to fasting specifically in the GSD Ia group (Figure 6A,I), though this effect was only just significant in the liver due to the variation observed in both fasting conditions. KRT10 was not further considered as the detected peptides of mouse KRT10 overlap completely with the human keratin-10 protein (a common sample handling contaminant) and can therefore not be annotated unambiguously to the mouse protein. Due to the limited number of overlapping differentially regulated proteins between the plasma and liver proteomics for metabolic control, we also looked broader to the potential of all eight up-regulated proteins for their potential as biomarkers. Based on the receiver operating characteristic (ROC) curve (Figure S4A), several of the up-regulated proteins also provide interesting targets to distinguish the metabolic control of the fasted GSD Ia mice. The area under the curve (AUC) values of ORM2 (0.991), SDHA (0.982), and SAA1 (0.951) indicate the best discriminatory performance. The use of all three proteins together as a biomarker panel, consisting of these three proteins, achieved an AUC of 1 in distinguishing fasted GSD Ia from

other groups (Figure S4B), demonstrating its potential for enhancing specificity and sensitivity in metabolic control screening for GSD Ia.

4 | Discussion

The current study is an unprecedented comprehensive proteomics analysis for mice with liver-specific G6Pase deficiency, including parallel screening of the liver tissue and plasma under fed and fasted conditions, to identify potential prognosis and monitoring biomarkers for the GSD Ia disease. The overview of these results and the relation to long-term complications are summarized comprehensively in Figure 8. These connections are based on observed proteomic alterations and existing literature and should be interpreted as potential mechanistic hypotheses rather than direct causal relationships, given the multifactorial nature of long-term complications in GSD I. Biomarkers are necessary to monitor the metabolic control in the disease and the risks for liver-specific long-term complications.

An ideal biomarker for GSD Ia should reliably reflect the underlying metabolic disturbances in the liver, correlate with the severity of hepatic complications, and be detectable in easily

accessible biofluids. In this respect, it is helpful that the liver regulates metabolic homeostasis and secretes various proteins that signal to distant tissues [27, 30]. Moreover, many of the plasma proteins are synthesized and secreted by the liver [31]. The mouse model benefits from a combined screening of the protein changes in GSD Ia as a readout of the state of the liver and how this is observed in the circulation as an easily accessible biofluid. Here we will further link the observed protein changes to previous literature about the various long-term complications that GSD Ia patients face with the ultimate aim of defining potential prognostic and monitoring therapy efficacy biomarkers that may be clinically meaningful.

The observed up-regulation of proteins involved in the glycolytic, lipid, and cholesterol metabolic pathways in the liver (Figure 4) is in agreement with a previous proteomics GSD Ia mouse study focusing on the liver in the fed state [21] and also with the known accumulation of glycogen, lactate, pyruvate, fatty acids, cholesterol and related compounds in GSD Ia mouse livers [10, 17]. Accumulation of triglycerides and cholesterol in the liver, has been described previously as key pathological features for NAFLD [32, 33].

Clinically, GSD Ia patients often exhibit symptoms of abnormal bleeding and difficulty in achieving hemostasis, indicative of coagulation disorders. The down-regulation of fibrin degradation pathway inhibitor SERPINA1E (Figure 5A) in the coagulation pathways that we observed could relate to these observed symptoms [14, 34]. In addition, our findings on the KEGG coagulation and complement cascade support the inflammation pathway changes in GSD Ia mouse livers reported by Cangelosi et al. [21] The down-regulation of C8a, C8b, and C8g (Figure 5A) may reduce the assembling of membrane attack complex (MAC), thereby suppressing cell lysis and inhibiting coagulation cascade activation [35, 36].

We also identified several new differentially regulated proteins and pathways. For instance, NNMT, a key enzyme in NAD⁺ metabolism (Figure S1), increased in GSD Ia mouse liver (Figure 3B). NNMT methylates nicotinamide, a metabolite of NAD nicotinamide, thereby promoting its excretion. The gene expression of NNMT is increased in the liver in hepatitis [37] and NAFLD patients [38, 39], and it could be a negative autophagy regulator in liver cancer [40]. Thus, we speculate that the increase of NNMT is one of the risk factors for liver failure in GSD Ia patients. Furthermore, based on our findings, 81.6% of proteins (31 out of 38) related to drug metabolism were decreased in hepatic GSD Ia mouse livers (Figures 3C,D, S2, and S3). This could point toward liver damage in GSD Ia mice as well, which aligns with the higher occurrence of liver complications, like liver tumors, in GSD Ia already at a young age [41–45].

For the potential GSD Ia monitoring biomarkers, we focused on the differentially regulated proteins that are shared between plasma and liver. The proteins that we identified in GSD Ia mice, related to carbohydrate and lipid metabolism (up-regulation of GPI1, PYGL, UGP2, FBP1, ENO1B, ALDOB, LDHA, FH1, ME1, ACLY, SCP2, and FASN), complement and coagulation cascades (down-regulation of SERPIN1E, C8b, and MBL2), with the potential of both monitoring and prognostic monitoring of early risk of NAFLD [46, 47] and liver fibrosis [48].

Additionally, we identified the 20S proteasome subunits (up-regulation of PSMA4, PSMA7, and PSMB5) as potential biomarkers. Elevated levels of 20S proteasome subunits in the circulatory system (c-proteasomes) have been observed in patients with hematological malignancies and HCC [49]. Henry et al. demonstrated that plasma proteasome levels are reliable early biomarkers for HCC, effectively distinguishing cirrhotic patients with HCC from those without and from controls, even with minimal tumor mass and with higher accuracy than the common clinical marker α -fetoprotein [50]. In our study, proteasome subunits were elevated in plasma despite the mice being in the early stages of GSD Ia without HCA or HCC. Studies have shown that *L-G6pc*^{-/-} mice developed HCA with diameters of 1–10 mm after 18 months, advancing to hepatocellular carcinoma (HCC), indicating a significant tumorigenesis risk in hepatic GSD Ia mice [23, 51, 52]. Therefore, we hypothesize that PSMA4, PSMA7, and PSMB5 have potential to assess HCC risk.

Since GSD I patients are at risk for hypoglycemia during prolonged fasting, we considered the fasted GSD Ia mice as a model for poor metabolic control. We identified a number of pathways that responded to fasting more strongly in GSD Ia mouse liver than in controls, including ETC, FAO, lipoproteins, proteasome, and ribosome proteins. First, 21 ETC proteins were increased in fasted GSD Ia (Figure 7A), suggesting potential alterations in mitochondrial function. In a comprehensive overview of mitochondrial dysfunction in different GSD types, Kumudesh et al. argued that GSD can lead to an oxidative burden, which in turn may impair mitochondrial integrity and exacerbate the metabolic aberrations observed in GSD [53]. Since mitochondrial dysfunction is closely linked to ROS production [23, 54, 55], further investigation is needed to determine if and how ROS production is affected in GSD Ia patients or mice. Second, the elevated levels of apolipoproteins (APOC1, APOC3, and APOE) in the liver of fasted GSD Ia mice (Figure 7B) align with previous findings that hyperlipidemia, hepatic steatosis, and increased VLDL-TG levels were exacerbated in fasted GSD Ia mice [17], since these apolipoproteins regulate the levels of triglycerides and cholesterol into the bloodstream [56].

For the identification of biomarkers related to the metabolic control of the disease, we focused on the circulating proteins that stood out in the fasted GSD Ia group. In a ROC curve, we showed that a combination of three circulating biomarkers (ORM2, SDHA, and SAA1) presented excellent sensitivity and specificity to distinguish the fasted GSD Ia group from the other groups (Figure S4B). Previous studies have shown that germline SDHA gene mutations are associated with mitochondrial complex II deficiency and multi-system metabolic disease [57]. Complex II dysfunction can increase mitochondrial reactive oxygen species (ROS) production, and accumulating evidence indicates that altered SDH function and ROS-mediated oxidative stress contribute to the development and progression of hepatocellular carcinoma [58, 59]. Additionally, elevated SAA1 and ORM2 are indicators for liver diseases (NAFLD, HCC, and non-alcoholic steatohepatitis) [60–63]. In conclusion, it will be interesting to assess if the combination of ORM2, SDHA, and SAA1 proteins can be used as a potential biomarker panel for monitoring metabolic control in GSD Ia patients.

In contrast to GSD Ia patients, who express the mutant G6Pase protein mostly in liver, kidney and intestine, this study employed a hepatocyte-specific *G6pc* knockout mouse. *G6PC1* in humans and *G6pc* in mice are not only expressed in the liver, but also in the intestine and kidneys [64, 65]. We, therefore, focused our analyses on the liver-specific complications, which are the most prominent complications observed in GSD Ia patients. Two recent studies on human serum/plasma from GSD Ia patients [66, 67] and general patterns concerning the increase of key enzymes involved in carbohydrate and specific proteins lipid metabolism were detected in all studies. The first human serum GSD Ia proteomics study identified ALDOB as one biomarker that was selected based on its high liver-tissue specificity and its correlation to the ALT and AST-levels [66], while the second study distinguished a subset of GSD Ia patients with hepatocellular adenomas or carcinomas, showing that ALDOB can distinguish even more specifically between patients with and without this liver-specific complication [67]. The mouse study shows that the identified upregulated apolipoproteins are strongly correlated to the fasting state, indicating an additional role of the metabolic state of the patients on top of the GSD Ia phenotype, which is not easily regulated in patients. Differences between the species were, for example, observed in the proteasomal proteins that were clearly altered in the mouse model, but were not significantly detected in either of the human studies, indicating a (liver-specific knockout) mouse effect. The combined details from these three studies will provide an excellent starting point for future finetuning of biomarkers for the prognosis and monitoring of GSD Ia specific biomarkers. Future studies are necessary to validate our findings in a longterm fashion in plasma or serum samples of GSD Ia patients, during regular care and after experimental therapies, such as AAV8 gene transfer (phase 1–2 completed: NCT03517085 [53, 68–71] and currently in phase 3: NCT05139316 [72]), mRNA therapy (phase 1–2: NCT05095727 [73, 74]), and base editing (phase 1–2: NCT06735755 [53, 75–77]).

In summary, in this comprehensive proteomics study we analyzed the liver and plasma of GSD Ia mice in both fed and fasted states. Most notably, the identification of potential biomarkers stands out as a critical outcome, offering a foundation for improved GSD Ia liver-specific long-term complications management. The primary limitation of this study is that the mouse models represent only the early stage of GSD Ia, with typical features of hypoglycemia, hyperlipidemia, and glycogen accumulation comparable to patients, but without liver tumor manifestation. Notably, a previous study from our group using the same GSD Ia mouse models reported liver tumor manifestation after 55 weeks of follow-up after injection [23]. Additionally, consistent with previous reports, this mouse model does not fully reflect all clinical features of GSD Ia [22, 23]. Although both fed and fasted GSD Ia mice exhibited significantly increased plasma lactate levels compared to wildtype controls, fed GSD Ia mice unexpectedly showed higher lactate levels than fasted GSD Ia mice (Table S1). This phenomenon has been described in liver-specific GSD Ia mice [17] and may relate to preserved extrahepatic G6Pase activity, including renal lactate clearance and endogenous glucose production during fasting. Furthermore, fasted GSD Ia mice did not display the expected exacerbation of the hepatic and plasma lipid phenotype. We speculate that this may be due to residual hepatic G6Pase activity in our cohort (Table S1). Notably, this may more closely resemble the human

condition, where residual hepatic G6Pase activity ranging from 0%–20% has been reported in patients with GSD Ia [53, 78, 79].

The second limitation is inherent to our choice to study the proteome using mass spectrometry, which is intrinsically biased toward the proteins/peptides with better properties for LC–MS detection. However, with the increased acceptance of mass spectrometry methods in clinical care, this will also provide a potential strength toward the future, where we would like to screen multiplexed panels of biomarkers for which the mass spectrometer is excellently suited. Additionally, we only look at the changes in protein abundances, while post-translational modifications could also be affected and these underlying changes are not addressed in these analyses. Protein glycosylation would be of special interest for GSD I since carbohydrate metabolism is altered in this disease.

A further limitation is that although potential biomarkers have been hypothesized for monitoring and prognosis of GSD Ia in this study, their clinical relevance requires further validation in patient cohorts. The next step will be to correlate the identified putative biomarkers in patients with long-term complications in GSD Ia patients or longitudinal studies on the development of long-term complications. If successful, this may enable non-invasive metabolic monitoring through accessible blood analysis of relatively stable molecules.

Author Contributions

Ruiqi Xiao: methodology, proteomics measurements, data analysis, manuscript drafting. **Hilda I. de Vries:** animal experimentation. **Aycha Bleeker:** sample preparation supervision. **Candelas Gross-Valle:** methodology, mouse metabolic parameter measurements, manuscript reviewing and editing. **Terry G. J. Derks:** conceptualization and clinical translation, manuscript reviewing and editing. **Justina C. Wolters, Maaïke H. Oosterveer, and Barbara M. Bakker:** conceptualization, project supervision, manuscript reviewing and editing. All authors reviewed the manuscript and approved of the final version.

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Ethics Statement

The data were obtained by experiment and data analysis. All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University

of Groningen (Groningen, The Netherlands) under permit number AVD10500202115288, and are in line with the Guide for the Care and Use of Laboratory Animals.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD054398.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **FIGURE S1:** Summary of the NAD⁺ metabolism changes in GSD Ia mice liver. The protein is highlighted in green as long as no significant change exists in either fed or fasted group. ACMS, α -amino- β -carboxymuconate ϵ -semialdehyde; MNAM, N1-methylnicotinamide; NAAD, nicotinic acid adenine dinucleotide; NAM, nicotinamide; NAMN, nicotinamide mononucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside. **FIGURE S2:** Xenobiotic metabolism changes in GSD Ia mice liver in the hepatocyte (taking the oxidation reaction as an example). ABCs, ATP binding cassette; CYPs, cytochrome P450; GA, glucuronic acid; GSH, glutathione; GSTs, Glutathione S-transferase; NATs, N-acetyltransferases; NTCP, sodium taurocholate cotransporting polypeptide; OATPs, organic anion transporting polypeptides; OATs, organic anion transporters; OH, hydroxyl; PAPs, phosphoadenosine-phosphosulfate; SLCs, solute carrier transporters; SULTs, sulfotransferases; UDPGA, uridine diphosphate-glucuronic acid; UGTs, UDP-glucuronosyltransferase. **FIGURE S3:** Summary of the hepatic steroid metabolizing enzymes in GSD Ia mouse livers of steroid hormone biosynthesis pathway. The primary steroid hormones are highlighted in yellow and other metabolites are in grey. The dotted arrow means some steps are omitted in between. **FIGURE S4:** Receiver operating characteristic (ROC) analysis of (A) 8 increased proteins (B) combined model with ORM2, SDHA, and SAA1 for distinguishing the Fasted_Ia group from other groups. The ROC curve was generated by plotting the true positive rate (sensitivity) against the false positive rate (1—specificity) at various threshold settings. The black diagonal line represents the performance of a random classifier with the area under the curve (AUC) equal to 0.5. **TABLE S1:** 1 Metabolic parameters of four mouse groups (Fed_WT, Fed_Ia, Fasted_WT, and Fasted_Ia) with statistics for the comparisons between WT and GSD Ia mice. 2. Metabolic parameters of four mouse groups (Fed_WT, Fed_Ia, Fasted_WT, and Fasted_Ia) with statistics for the comparisons between fed and fasted mice. **TABLE S2:** Significantly changed proteins of fed GSD Ia mice compared with wild-type ones in the liver (Fed_Ia vs.

Fed_WT). **TABLE S3:** Significantly changed proteins of fasted GSD Ia mice compared with wild-type ones in the liver (Fasted_Ia vs. Fasted_WT). **TABLE S4:** Significantly changed proteins of fasted GSD Ia mice compared with fed ones in the liver (Fasted_Ia vs. Fed_Ia). **TABLE S5:** Significantly changed proteins of fed GSD Ia mice compared with wild-type ones in the plasma (Fed_Ia vs. Fed_WT). **TABLE S6:** Significantly changed proteins of fasted GSD Ia mice compared with wild-type ones in the plasma (Fasted_Ia vs. Fasted_WT). **TABLE S7:** Significantly changed proteins of fasted GSD Ia mice compared with fed ones in the plasma (Fasted_Ia vs. Fed_Ia). **TABLE S8:** Summary of the biological processes and pathways significantly altered in GSD Ia mouse livers. **TABLE S9:** Summary of the biological processes and pathways significantly altered in GSD Ia mouse plasma. **TABLE S10:** Biological processes and pathways changes of fed GSD Ia mice compared with wild-type ones in the liver (Fed_Ia vs. Fed_WT). **TABLE S11:** Biological processes and pathways changes of fasted GSD Ia mice compared with wild-type ones in the liver (Fasted_Ia vs. Fasted_WT). **TABLE S12:** Biological processes and pathways changes of fed GSD Ia mice compared with wild-type ones in the plasma (Fed_Ia vs. Fed_WT). **TABLE S13:** Biological processes and pathways changes of fasted GSD Ia mice compared with wild-type ones in the plasma (Fasted_Ia vs. Fasted_WT). **TABLE S14:** Proteins with typical changes in fasted GSD Ia mouse livers. **TABLE S15:** Proteins with typical changes in fasted GSD Ia mouse plasma. **TABLE S16:** Summary of the biological processes and pathways significantly specifically altered in fasted GSD Ia mouse livers. **TABLE S17:** Biological processes and pathways changed specifically in fasted GSD Ia mouse livers.