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Proteomics profiling of serum and liver in GSD Ia and Ib patients: insights into complication mechanisms and circulation biomarkers

Ruiqi Xiao[1,9], Candelas Gross-Valle[1,9], Albert Gerding[1,9], Adam M. Thorne[2,3], Maaike H. Oosterveer[1,4,5,9], Terry G. J. Derks[6,7,9], Vincent E. de Meijer[2,3], M. Rebecca Heiner-Fokkema[8,9], Barbara M. Bakker[1,7,9] and Justina C. Wolters[1,7,9*†]

Abstract

Background Glycogen Storage Disease (GSD) Types Ia and Ib are rare metabolic diseases caused by gene variants in *G6PC1* and *SLC37A4,* respectively. Although life-threatening fasting hypoglycemia can be controlled by a strict diet, patients often suffer from multiple metabolic abnormalities and severe long-term complications. However, the underlying mechanisms remain incompletely understood, and there is a lack of effective monitoring biomarkers. Therefore, the aims of this study are to investigate the pathological mechanisms of the disease and disease complications in GSD I and identify potential protein biomarkers.

Methods Comprehensive untargeted proteomics was performed on 18 GSD Ia and 8 GSD Ib sera samples from patients with 21 matched control sera, complemented by liver 3 GSD Ia samples and 1 GSD Ib sample from patient liver tissues, compared to 10 donor liver samples.

Results We identified 415 proteins in total. Significantly changed (FDR < 0.05) were observed in 158 (38%) proteins for GSD Ia vs Control, 116 (28%) for GSD Ib vs. Control, and 151 (36%) for GSD Ia vs. Ib. Pathway analysis revealed distinct alterations in serum/plasma, with 58, 32, and 29 significantly changed biological processes (FDR < 0.05) in these three comparisons, respectively. The coagulation pathway was the most significantly changed one in the GSD Ia patients. Immune response-associated proteins, especially immunoglobulins, were increased in GSD Ib specifically. Proteins related to liver injury, cholesterol, and amyloidosis were altered in two subtypes, though more pronounced in GSD Ia. Potential biomarkers with significant alterations both in the circulation and in the liver tissue were identified specifically for monitoring GSD I subtypes and prognosing liver deterioration, namely APOC1 and CD5L to distinguish between GSD Ia and Ib and ALDOB for the presence of hepatocellular carcinoma (HCC) in GSD Ia patients.

Conclusions These findings provide new insights into the differences between the two GSD I subtypes and the pathogenesis of GSD I-related complications, as well as highlighting the potential of protein circulating biomarkers for monitoring complication progression in GSD I and assessing HCC risk in GSD Ia patients.

†Justina C. Wolters Last author.

*Correspondence:
Justina C. Wolters
j.c.wolters@umcg.nl

Full list of author information is available at the end of the article

Keywords Glycogen storage disease type I, Proteomics, Complication mechanisms, Circulation biomarkers, Prognosis, Hepatocellular carcinoma

Background

Glycogen Storage Disease I (GSD I) is an inherited disease of carbohydrate metabolism [1]. GSD Ia (OMIM: #232200) and Ib (OMIM: #232200) are subtypes of GSD I, which are caused by gene variants in *G6PC1* (OMIM: *613742) [2] and *SLC37A4* (OMIM: *602671) [3], respectively, leading to dysfunction of the glucose 6-phosphatase enzyme (G6PC) or the glucose-6-phosphate transporter (G6PT).

GSD I patients suffer from severe fasting intolerance and hepatomegaly, and is biochemically characterized by hypoglycemia, hyperlipidemia, hyperlactatemia, and hyperuricemia [4, 5]. Although dietary management can help reduce part of these symptoms, patients are still at risk of acute and chronic complications, such as growth retardation, liver and kidney disease [5, 6]. Notably, in GSD Ia hepatocellular adenoma (HCA) [1] and carcinoma (HCC) [7] are more common, compared to GSD Ib. In addition, GSD Ib patients suffer from neutropenia and neutrophil dysfunction [8, 9], increasing the risks of infections and inflammatory bowel diseases [10]. Patients show significant clinical heterogeneity in the long-term complications, and the underlying mechanisms for these differences are only partially understood. Further understanding of these differences is crucial for treatment and monitoring, as well as for risk stratification of long-term complications [3, 11]. Several omics studies have been applied to gain a deeper understanding of GSD, which mainly have focused on GSD I (Table 1) [12–26]. In GSD I, metabolic dysregulation was observed in glucose, fatty acids, amino acids, creatine, purine/pyrimidine, and lipids, despite dietary intervention [19, 21, 24, 26]. On the glycosylation level, hypo-galactosylation and accumulation of high-mannose and hybrid type *N*-glycans was observed in GSD Ib [13, 20], and APOC3 hypo-sialylation was reported in both GSD Ia and Ib [16].

On the proteome level, a targeted study screening for 20 cytokines was performed on GSD I patient material, showing altered inflammatory signatures for both GSD Ia (reduced IL-4) and GSD Ib (elevated IL-10) [23]. More in-depth screening of the protein adaptations was performed specifically on GSD Ia [26]. This study revealed liver-specific protein alterations associated with liver damage and lipid dysregulation, with the identification of up-regulated aldolase B as a potential biomarker for liver injury [26]. Mouse studies on GSD Ia revealed extensive protein remodeling associated with metabolic reprogramming, including inflammatory pathway activation and M2 macrophage polarization, which potentially promote tumorigenesis [17]. However, these human studies did not include subjects with long-term complications. Current identification of biomarkers for long-term complications have so far only been achieved for HCA/HCC in GSD I by DNA and miRNA sequencing, identifying HNF1A (DNA) [14] and miR-130b (miRNA) [15] as potential biomarkers for HCA in GSD I. Identification of reliable tumor marker is essential since the classical biomarkers for HCA and HCC using imaging and serum markers, including alpha-fetoprotein (AFP) and carcino-embryonic antigen (CEA), which were initially suggested for GSD I by 2002 European guidelines [27]. However, in the more recent American College of Medical Genetics and Genomics (ACMG) guideline, AFP and CEA haven been shown to remain normal in GSD I patients with HCC [6]. Different groups have reported the protein des-gamma-carboxy prothrombin (DCP, or also called PIVKA-II) as a potential biomarker for HCC in GSD Ia more recently, but these observations are so far only described in four patients [28–30]. Underlining the importance for identification of reliable protein biomarkers in large(r) patients cohorts.

Therefore, we performed an explorative comprehensive proteomics profiling in serum and plasma of GSD Ia and Ib patients, with a subset of patients had already developed HCA and HCC. The aims of this study are understanding the mechanism of both GSD I subtypes and identifying prognostic biomarkers for GSD Ia and Ib patients, especially for HCC in GSD Ia patients.

Methods

Study participants

This study was conducted in accordance with the principles of the Declaration of Helsinki. For patients followed up at the University Medical Center Groningen (UMCG), the local Medical Ethics Committee determined that the Medical Research Involving Human Subjects Act did not apply to retrospective, non-interventional studies, and therefore, additional ethical approval was not required (METc 2019/119). The experimental protocols were reviewed and approved by the METc at UMCG, following the relevant guidelines and regulations outlined in protocol METc 2019/119.

This study enrolled blood (serum or plasma) and liver samples (Fig. 1A). Participant characteristics, including demographics, lipid profiles, liver markers, diet, supplements and medication regimes were summarized in Tables S1 and S2. Briefly, 18 GSD Ia serum or plasma samples were taken from 12 GSD Ia patients (age range 12–49 years; 6 females and 6 males; 9 non-HCA/HCC from 8 patients, 7 HCA from 3 patients, and 2 HCC from

Table 1 Summary of the omics study in GSD

First Author	GSD Subtype	Sample species	Sample Details	Omics Method	Key Findings
Baodong Sun, et al., 2009 [12]	GSD-Ia	Mouse	Liver (3 glucose treated, 3 non-glucose treated GSD Ia, 3 controls)	Proteomics	Elevated GAPDH in G6Pase-deficient hepatocytes promoted apoptosis, shown by increased TUNEL positivity and caspase-3 activation in GSD-Ia livers.
Bu'Hussain Hayee, et al., 2011 [13]	GSD-1b	Human	neutrophils (5 G6PC3-deficient, 2 GSD Ib patients)	Glycomics	Neutrophil hypo-galactosylation of *N*- and O-glycans in G6PC3 deficiency and GSD Ib patients.
Julien Calderaro, et al., 2013 [14]	GSD-Ia, GSD-Ib	Human	Liver (25 HCA and 7 non-HCA frozen samples from 14 GSD Ia, 1 Ib)	DNA sequencing	GSD-related HCA characterized by a lack of HNF1A inactivation. High frequency of b-catenin mutations could be related to the increased frequency of malignant transfor- mation in HCC.
Li-Ya Chiu, et al., 2014 [15]	GSD-Ia	Human	Liver (paired adenomas and normal liver tissues from 7 GSD Ia); Serum (6 GSD Ia, 20 controls)	miRNA sequencing	Distinct miRNA deregulation in HCA associated with GSD Ia, and miR-130b was a potential circulating biomarker for HCA.
Nina Ondruskova, et al., 2018 [16]	Types 0, Ia, non-Ia, III, IX	Human	serum (39 samples, 24 patients: 2 type 0, 7 Ia, 3 non-Ia, 7 III, 5 IX)	Glycomics	GSD types Ia and non-Ia exhibited mild APOC3 hyposialylation, whereas types III and IX displayed severe glycosylation defects.
Davide Cangelosi, et al., 2019 [17]	GSD Ia	Mouse	Liver (20 GSD Ia vs. 18WT)	Proteomics	Metabolic reprogramming, inflammation, and M2 macrophage polarization in GSD Ia mouse liver, potentially promoting tumorigenesis.
Roberta Resaz, et al., 2020 [18]	GSD Ia	Mouse	Plasma exosomes (45 GSD Ia mice vs. 18WT)	MicroRNAs	Altered exosomal microRNA expression in GSD Ia mice, linked to glucose metabolism, inflammation, and hepatocellular carcinoma.
Luciana Hannibal, et al., 2020 [19]	GSD Ia, GSD Ib, GSD III	Human	Fibroblasts (1 GSD Ia, 1 Ib, 3 III vs. 1 healthy)	Metabolomics	Distinct metabolic signatures in GSD subtypes, including altered energy metabolism, oxidative stress response, and creatine metabolism.
Bobby G. Ng, et al., 2021 [20]	GSD Ib	Human	Serum (7 GSD Ib), induced pluripotent stem cell (iPSC)-derived hepatocytes from fibroblasts (1 GSD Ib), and CRISPR-edited Huh7 cells)	Glycomics	Dominant *SLC37A4* mutation cause liver-specific congenital disorders of glycosylation (CDG) with coagulopathy, and accumulation of high mannose and hybrid type *N*-glycans in serum.
Tamara Mathis, et al., 2022 [21]	GSD Ia, GSD Ib	Human	Plasma (14 GSD I patients: 11 GSD Ia, 3 GSD Ib; 31 controls)	Metabolomics	Broad metabolic disturbances in plasma, including fuel metabolism, lipid metabolism, amino acid, methylation, urea cycle, and purine/pyrimidine metabolism.
Eran Tallis, et al., 2022 [22]	GSD Ib	Human	Plasma (1 GSD Ib patient, pre/post empagliflozin)	Metabolomics	Empagliflozin treatment reduces 1,5-anhydroglucitol, improves neutrophil function, and alleviates IBD symptoms in a GSD Ib patient.
Karina Colonetti, et al., 2023 [23]	GSD Ia, GSD Ib, GSD III/IX	Human	Plasma (16 GSD Ia, 6 Ib, 5 III/IX, 24 controls)	Multiplex luminex assay for 20 cytokines	Patients (GSD Ia/III/IX) presented reduced IL-4, MIP-1α/CCL3, et al. GSD Ib showed elevated G-CSF and IL-10. GSD III/IX had increased levels of IP-10/CXCL10. IP10/CXCL10 and MCP-1 were higher in GSD III/IX. GSD I patients with anemia presented higher levels of IL-4 and MIP-1α. Triglycerides correlated with cytokine levels.
Alessandro Rossi, et al., 2024 [24]	GSD Ia	Human	Serum (12 GSD Ia vs. 13 healthy and 7 hypercholesterolemia controls)	Targeted lipidomics	Total cholesterol and triglyceride values correlated with ceramide (Cer). Circulating Cer may allow for refined dietary assessment compared with traditional biomarkers.
Yuxuan Lu, et al., 2025 [25]	GSD II (Pompe)	Human	Muscle (6 late-onset Pompe vs. 4 controls)	Proteomics + Metabolomics	635 upregulated and 89 downregulated proteins; significant activation of mTOR, autophagy, and lysosome pathways; reduced L-arginine levels.
Francesca Pirozzi, et al., 2025 [26]	GSD Ia	Human	Serum (12 GSDIa vs. 12 controls)	Proteomics + Metabolomics	The altered levels of liver-specific proteins and choline-related metabolites in GSD Ia patients reveal liver damage and lipid dysregulation. ALDOB correlates with ALT and AST, supporting its potential as a biomarker for long-term monitoring of liver injury.

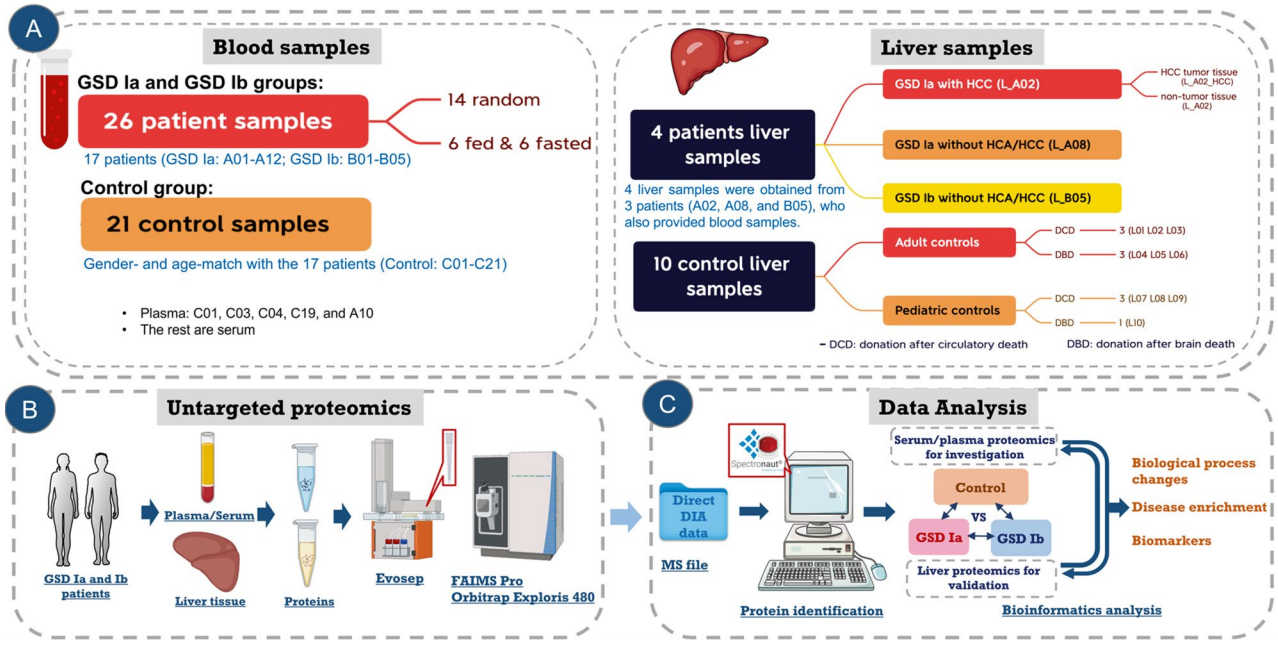

Fig. 1 Summary of samples, untargeted proteomics workflow, and data analysis

1 patient). 8 GSD Ib serum samples were taken from 5 GSD Ib patients (age range 11–19 years, 3 females and 2 males; non-HCA/HCC), as well as 21 serum or plasma samples from age- and sex-matched individuals (age range 10–50 years; 10 females and 11 males) without metabolic diseases. All retrospective blood samples in this study were serum, with the exception of A10, C01, C03, C04, and C19 were plasma. In addition, 4 liver samples (L_A02_HCC of tumor tissue, L_A02 of non-tumor tissue, L_A08, and L_B05) were from 2 GSD Ia (A02 and A08) and 1 GSD Ib (B05) patients, with 10 age- and sex-matched control liver samples (6 adult controls and 4 pediatric controls) from liver transplantation donors (Fig. 1A). Multiple samples from the same patient, collected at different times were labeled using 'a', 'b', and 'c' (e.g., A03a and A03b), which were collected from the same patient under different metabolic conditions (fed, fasted, and random) or under different times of disease progress, therefore representing distinct physiological states.

Untargeted proteomics workflow

An untargeted proteomics workflow was established to identify protein groups of serum/plasma and liver samples (Fig. 1B), followed by data analysis (Fig. 1C). Liver tissue proteomes were also included, providing a unique insight into the link between the liver and the circulation. The liver proteomics results were used to prioritize the identified potential plasma biomarkers by selecting proteins that show differential regulation in both sample types as the strongest indicators to assess the hepatic health status of these patients. The details of the sample preparation and specific HRMS1 mass spectrometric settings can be found in the Supplementary Methods section.

Data analysis and statistics

Raw MS data were processed in Spectronaut v0.19.4.241120.62635 using a library-free approach (directDIA) for quantification at MS1 level, using background signal as the imputation strategy. Data was aligned with protein sequences based on the Human UniProt reviewed sequences (20422 entries). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [31] partner (https://www.ebi.ac.uk/pride/archive) repository with the dataset identifier PXD066805. The gene names related to the proteins were used for visualization and their integrated peak areas were processed using RStudio. The proteins with missing values of more than 50% across all samples were removed from further analysis based on the published guidance [32]. The rest of the missing values were imputed by the mean of the respective experimental groups. If no group values were available, missing values were replaced by 0.1, representing the instrument detection limit. This approach avoids infinite fold-change values while preserving the overall data structure. The retrospective cohort included both plasma and serum samples, and some patients provided two or three blood samples at different time points, we performed separate analyses to compare these datasets (Supplementary Analysis 1). To ensure that the imputation strategy was appropriate, we also conducted parallel analyses using different imputation methods (Supplementary Analysis 2). The significantly changed proteins (SCPs) that overlapped

across Supplementary Analyses 1 and 2 were then used for biomarker identification, ensuring the robustness and reliability of the selected candidates (Supplementary Analysis 3). 'EdgeR' package in R Studio v0.4.2.2 [33] was used to normalize data and calculate $log2^{Fold\ Change}$ ($Log2^{FC}$) and false discovery rates (FDR) between groups. Briefly, MS1 intensities were log2-transformed and scaled to approximate integer values, followed by trimmed mean of M-values (TMM) normalization to adjust for differences in overall protein abundance across samples. Differential protein analysis was then performed using generalized linear models (GLMs) with negative binomial distribution, and p-values were adjusted for multiple testing using the Benjamini-Hochberg FDR procedure. The stability of the data and the effects of the imputation and normalization strategy was shown in Supplementary Figure S1. In addition, principal component analysis (PCA), heatmap, volcano plots, and barplots were made using R packages 'sjmisc', 'ggrepel', 'pheatmap', and 'ggplot2'. SCPs were filtered by FDR (≤0.05).

Gene Ontology (GO) analysis and DisGeNET disease enrichment analysis were performed using DAVID Bioinformatics Resources (v2024q2). In the disease enrichment results, we excluded the proteins related to dysfibrinogenemia because 5 plasma samples and 42 serum samples were employed, because a previous study described that the fibrinogen proteins are highly abundant in plasma, which could introduce bias with mixed sample types [34]. The GO and disease enrichment results were visualized as bar plots using the R package 'ggplot2'. For biomarkers identification, if there were at least two samples in each comparison group, R package 'pROC' was utilized to calculate the area under the curve (AUC) using the lower bound of the 95% confidence interval, and make the receiver operating characteristic (ROC) curves of proteins with AUC > 0.9 [35]. In the liver tissue, given that only one sample was available for GSD Ia with HCC and for the GSD Ib liver, proteins were considered potential biomarkers if their intensities were higher than 1.2 times the maximum or lower than 0.8 times the minimum observed in the reference group.

Results

GSD Ia and Ib show different serum/plasma protein patterns

An overview of untargeted proteomics in Fig. 2 shows the alterations in GSD I samples. 21 Controls, 18 GSD Ia (including 7 HCA, 2 HCC, and 9 without HCA/HCC), and 8 GSD Ib samples were grouped. A total of 415 proteins were identified and mapped on a heatmap to identify any potential clustering across all samples in Fig. 2A. As shown in Fig. 2B, principal component analysis (PCA) further revealed clear separation between groups, suggesting substantial global protein differences among GSD Ia, GSD Ib, and control samples. GSD Ia patients with HCA and HCC clustered together in Figs. 2B and S2B, suggesting additional specific changes in the proteome in relation to these long-term complications. The potential confounding factors, including age, gender, sample type, and feeding state (fed, fasted, random), did not show clustering in the PCA analyses in Figs. S2C-S2F. The robustness of the analyses were additionally evaluated by re-analyzing the data excluding the potential bias of dependent samples (Supplementary Analysis 1.1), plasma samples (Supplementary Analysis 1.2) and the evaluation of the imputation bias by using different imputation strategies (Supplementary Analysis 2).

The volcano plots show 158 (38%), 116 (28%), and 151 (36%) proteins were significantly changed in the GSD Ia vs. Control, GSD Ib vs. Control, and GSD Ia vs. Ib comparisons (Figs. 2C–2E and Tables S3–S5). Overall, these results provided a comprehensive overview and indicate clear differences in proteins not only between GSD I and controls, but also between the GSD Ia and Ib subtypes, and between non-HCA/HCC and HCA/HCA GSD Ia patients.

Biological processes related to immune response, blood coagulation, and complement activation are the primarily affected processes in serum/plasma of GSD Ia and Ib

To understand the context and biological relevance of these differences between the three groups, the SCPs in different comparisons (GSD Ia vs. Control, GSD Ib vs. Control, and GSD Ia vs. Ib) were clustered according to the Gene Ontology biological process (summarized in Tables S6–S8) to identify the main biological pathways that were altered in the circulation of the GSD I patients. Out of the top 10 p-value-ranked differentially regulated biological processes for each of the three comparisons between GSD Ia, GSD Ib, and control showed overlap in the immunoglobulin mediated immune response, blood coagulation, complement activation (classical pathways), and fibrinolysis in Fig. 3A. The immune response pathways showed the most significant alterations, with the top 3 altered pathways in both GSD Ib vs. Control (blue bars) and GSD Ia vs. Ib (yellow bars) being immune-related and showing considerable overlap. GSD Ia vs. Control (pink bars) showed differences in immunoglobulin mediated immune response, blood coagulation, and complement activation as the top 3 changed pathways. This suggests that these pathways are consistently affected in patients but exhibit subtype-specific variations in their alterations.

To visualize the specific proteins contributing to these pathway differences, we created chord diagrams of the top 3 enriched pathways for each comparison in Figs. 3B–3D. To prevent potential bias from the mixed plasma and serum samples, we only showed individual

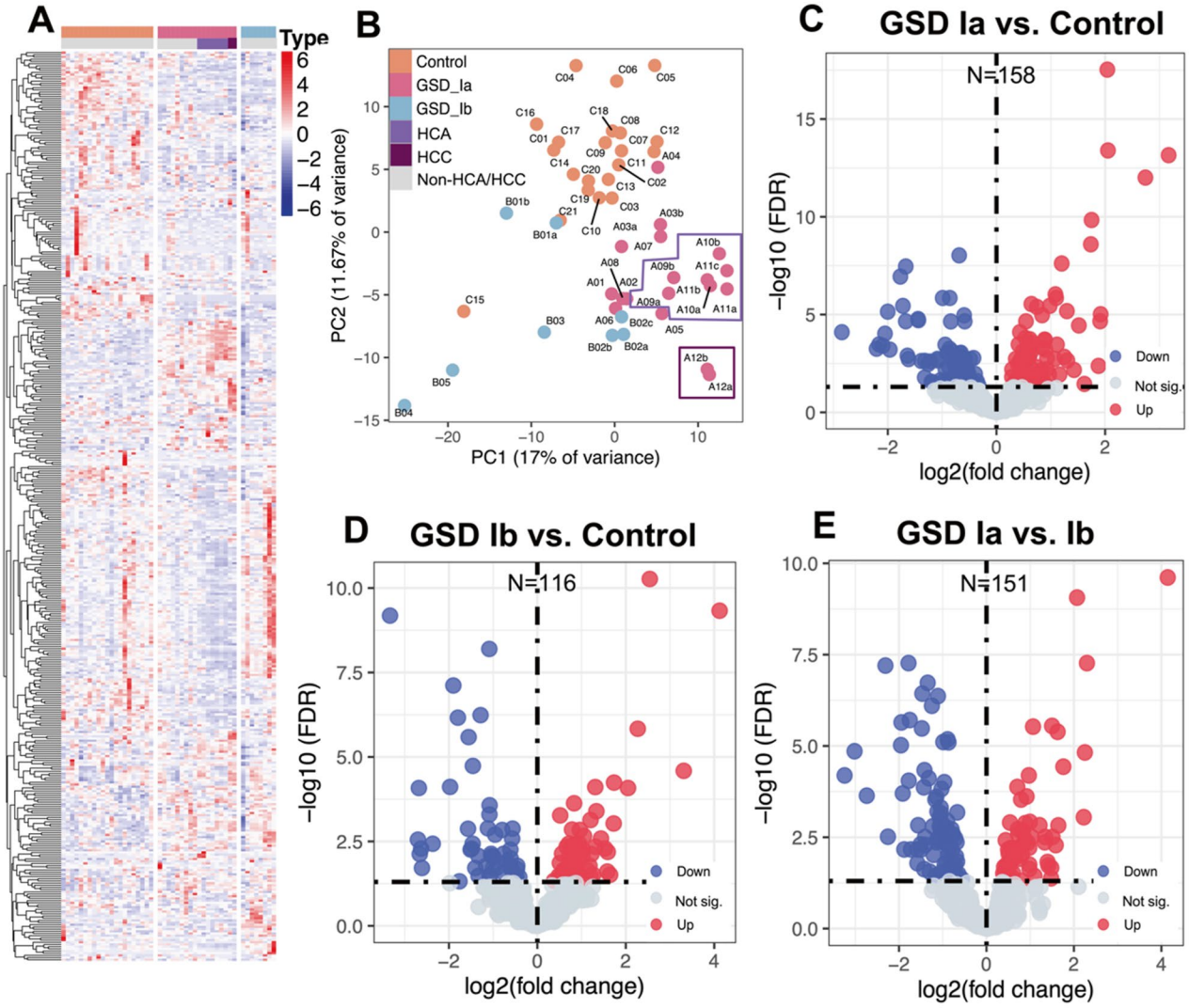

Fig. 2 Overview of serum/plasma proteomics analysis. (**A**) Heatmap showing the z-score of all detected proteins. (**B**) principal component analysis (PCA) plot illustrates the proteomic profile separation among groups. (**C**-**E**) volcano plots of differential protein between groups. Significantly up-regulated and down-regulated proteins (FDR < 0.05) are highlighted in red and blue, respectively. 'HCA' represents GSD ia patients with hepatocellular adenoma (HCA), and 'HCC' refers to those who initially had HCA and later progressed to hepatocellular carcinoma (HCC). 'N' refers to the number of significantly changed proteins (SCPs), including 'up' and 'down' proteins. 'Not sig.' refers to proteins that show no significant changes. Groups: C01–C21 were control samples; A01–A12 were GSD ia samples (A09a-A11b were GSD ia with HCA, A12a and A12b were GSD ia with HCC); B01–B05 were GSD ib samples. Multiple samples from the same patient, collected at different times were labeled using 'a', 'b', and 'c' (e.g., A03a and A03b)

proteins that were also identified upon exclusion of the plasma samples (Supplementary Analysis 1.2). In GSD Ia vs. Control, proteins were mainly up-regulated (marked with red boxes) in blood coagulation (12/18, 67%) and complement activation pathways (9/13, 69%), while in Fig. 3 Ball proteins were down-regulated (marked with blue boxes) in the immune response (14/14, 100%). Notably, as shown in Fig. 3B, 17 immunoglobulins (IGs) were decreased in GSD Ia, while 95% of the IGs were increased in GSD Ib in Fig. 3C (38/40, except IGHV1-69–2 and IGKV3D-7). This difference was also clear from the comparison between GSD Ia and Ib in Fig. 3D, where IGs (62/67) were also the proteins providing the largest differentiation between the two subtypes.

Taken together, the blood coagulation and complement activation pathways were the top 2 changes in GSD Ia, and the immune response pathway changed both in GSD Ia and Ib. Most of the immune response proteins were decreased in GSD Ia vs. Control, while increased in GSD Ib vs. Control, and we observed that these opposite effects provide the largest differentiation between GSD Ia vs. Ib. The top 3 altered biological processes in GSD Ia vs. Ib were all related to the immune response pathway. These differences in the types and trends of altered

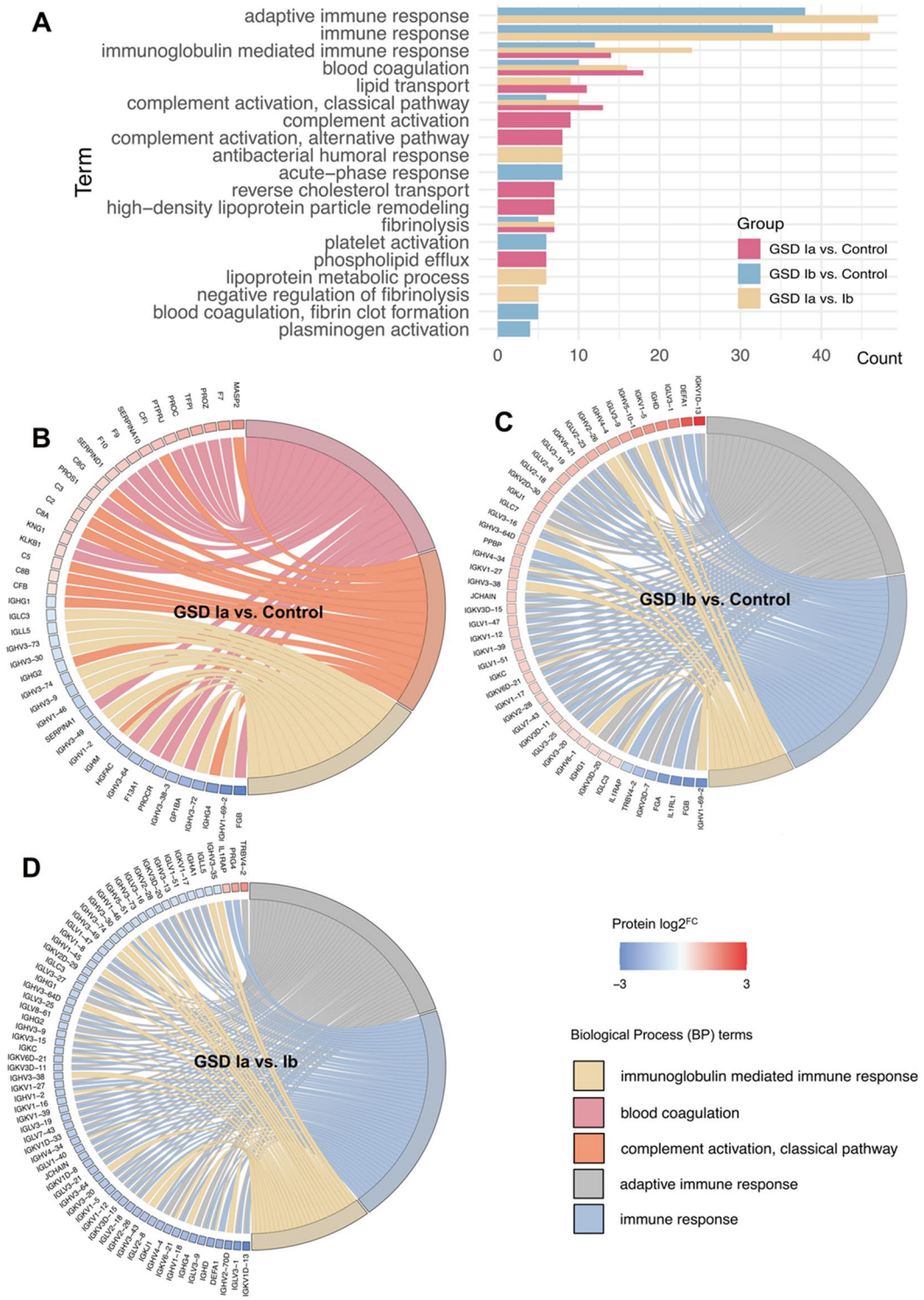

Fig. 3 Gene ontology (GO) biology process (BP) analysis of SCPs. (**A**) Bar plot showing the BP pathways enriched across SCPs in all three comparisons. The bar length reflects the number of SCPs associated with each pathway. (B-D) chord plots illustrate the top three enriched BP pathways for (**B**) GSD ia vs. Control, (**C**) GSD ib vs. Control, and (**D**) GSD ia vs. Ib. Each plot highlights the pathways and the specific SCPs contributing to enrichment, with connections representing the relationships between proteins and pathways

proteins between GSD Ia and Ib result in distinct coagulation and immune disruptions.

Significantly changed proteins in GSD I are primarily related to disease clusters: liver injury, thrombosis, complement and antibody deficiency, hypercholesterolemia and hyperlipoproteinemia, and amyloidosis

The context of proteins can alternatively be viewed from the predicted risks for disease complications, using a disease enrichment analysis (DisGeNET) on the serum/plasma SCPs from these three comparisons (GSD Ia vs. Control, GSD Ib vs. Control, and GSD Ia vs. Ib) to identify protein- and pathway-disease associations (listed per comparison in Tables S9–S11). The bar plots in Fig. 4A show the top 10 ranked disease clusters from the DisGeNET results, which were grouped in five overarching topics based on their function, as shown in Fig. 4B. Namely, liver injury, thrombosis, complement and antibody deficiency, hypercholesterolemia and hyperlipoproteinemia, and amyloidosis.

In the identified overarching disease clusters, proteins were divided into two groups in Fig. 4B, either as a specific effect for GSD Ia or GSD Ib or as a general effect observed in both GSD I subtypes. All the proteins with general effects in GSD Ia and Ib showed similar down-regulation when compared with controls. Notably, in the 'GSD Ia or GSD Ib specific effects' group, the majority of the proteins showed only significant up-regulation in GSD Ia in all these clusters, when compared to controls. In the 'General effects GSD I' group, F2, a key protein in blood clot formation, was decreased in both GSD Ia and Ib within the thrombosis-related cluster in Fig. 4, consistent with the altered blood coagulation pathway identified in both subtypes as shown in Fig. 3A. Interestingly, the apolipoproteins showed distinct mixed changes: APOA1 and APOA2 were decreased in both subtypes; APOA4 was decreased in GSD Ib; while APOC1, APOC2, APOC3, and APOE were increased in GSD Ia.

These analyses provided a focus on additional proteins involved in long-term complication processes, next to the immunoglobulin and coagulation pathways that were primarily identified in the GO clustering analyses.

Potential circulating prognostic biomarkers specific for GSD ia and GSD ib

To identify potential biomarkers, only the proteins that were overlapping between all the analyses, taking the various potential biases into account, were taken into consideration (Supplementary Analysis 3). We applied ROC curve analyses for all the significantly altered proteins in the different comparisons in serum/plasma and liver, as shown in Fig. S3 and the listing with confidence intervals in Table S12. In the serum/plasma, 6 potential protein biomarkers were found in GSD Ia vs. Control group (Fig. 5A), 9 in GSD Ib vs. Control (Fig. 5B), and 12 in GSD Ia vs. Ib (Fig. 5C).

Subsequently, we used the unique liver tissue proteomics data to identify which of these circulatory biomarker candidates also showed changes in the liver proteome. In the original analysis with all samples included, we identified several overlapping significantly changed proteins between the circulatory and liver proteome (Fig. S4). With the more stringent additional analyses on the potential circulatory biomarkers, we narrowed this selection to APOC1 and CD5L for the differentiation between GSD Ia and Ib (Fig. 5D). Information about this protein and direction of the change in the serum/plasma and liver proteome are summarized in Table 2.

ALDOB was identified as a potential prognostic biomarker in GSD Ia with HCC

The subset of GSD Ia patients with HCA and HCC provided us with the unique opportunity to identify prognostic biomarker candidates in the circulation for HCA/HCC. The ROC curves of proteins in these comparisons are shown in Fig. S5 and the listing of with confidence intervals can be found in Table S12. Two proteins were identified as potential prognostic markers for tumors in GSD Ia, as shown in Fig. 6A. They already show significant alterations when the HCA and HCC patients were combined as one group (HCA+HCC), compared to non-HCA/HCC GSD Ia and Controls. Focusing specifically on HCC (compared to all other samples) yielded a total of 32 proteins as potential biomarkers for monitoring of HCC in GSD Ia in serum/plasma, which is shown in Fig. 6B.

Subsequently, we used the unique liver tissue proteomics data to identify which of these potential circulatory biomarkers also showed changes in the liver proteome. In the original analysis with all samples included, we identified two overlapping significantly changed proteins between the circulatory and liver proteome (Fig. S6). The more stringent analysis highlighted one protein, namely ALDOB (up-regulated in both liver and serum/plasma HCC), as shown in Figs. 6A and 6C, identifying them as a potential key biomarker for HCC in GSD Ia.

In summary, the two identified biomarker candidates for GSD Ia vs. GSD Ib and the role of HCA/HCC in GSD Ia were listed in Table 2, including protein trends in serum/plasma and liver, functional categories, and descriptions. They are related to lipid metabolism and liver injury.

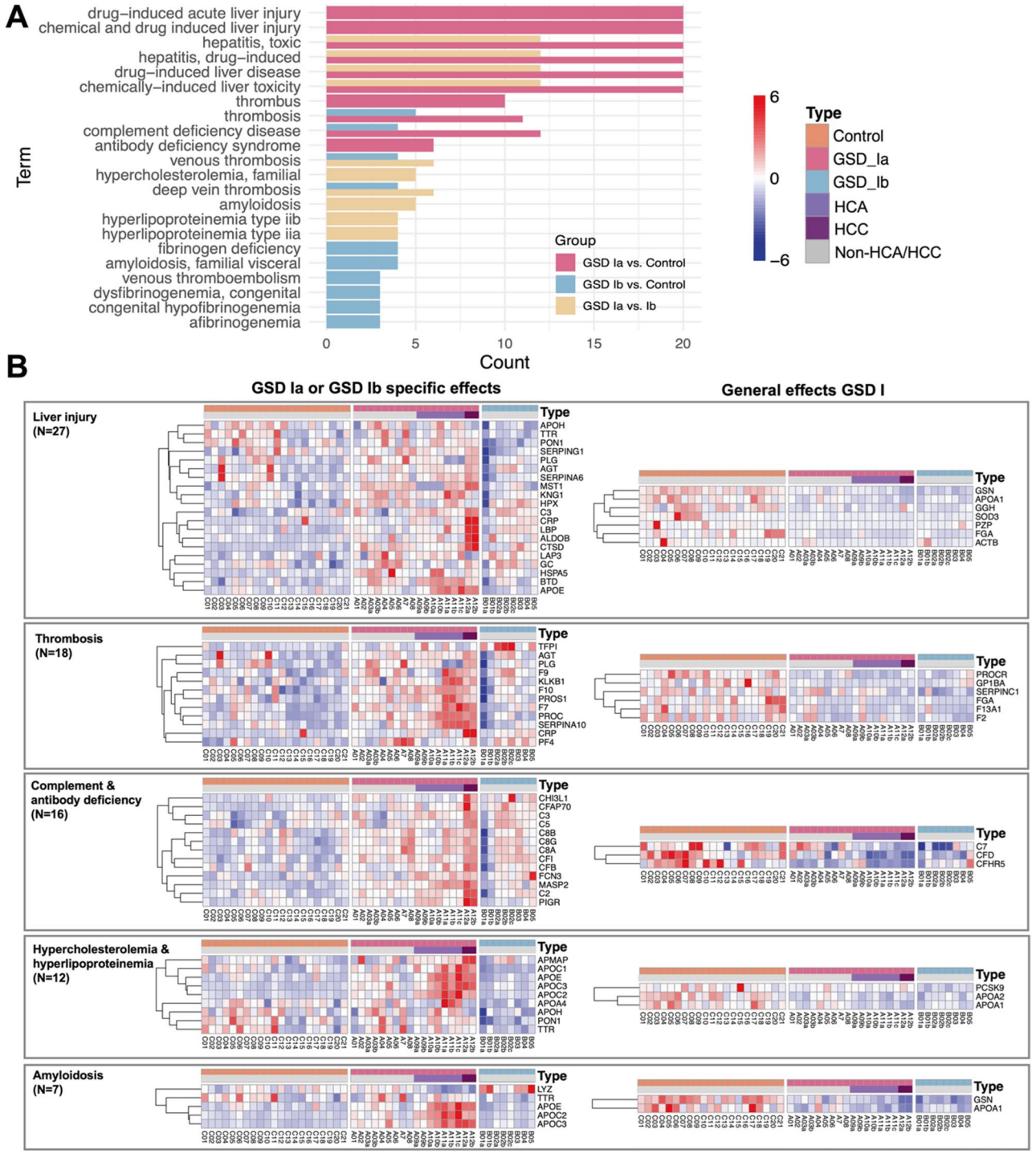

Fig. 4 Disease cluster analysis of SCPs in serum/plasma. (**A**) barplot showing the enrichment of SCPs in various diseases. (**B**) heatmaps represent the distribution of SCPs across different disease categories, as classified from panel A, highlighting the interesting proteins involved in each category. 'N' represents the number of SCPs. Groups: C01–C21 were control samples; A01–A12 were GSD ia samples (A09a-A11b were GSD ia with HCA, A12a and A12b were GSD ia with HCC); B01–B05 were GSD Ib samples

Discussion

Previous GSD omics studies, summarized in Table 1 [12–26], have characterized metabolic changes and identified some mRNA, metabolic, and protein biomarkers. Our research systematically analyzed the untargeted proteomics of samples from both GSD Ia and Ib patients in a relatively large cohort. Two elements made this study unique in design: the inclusion of patients who had

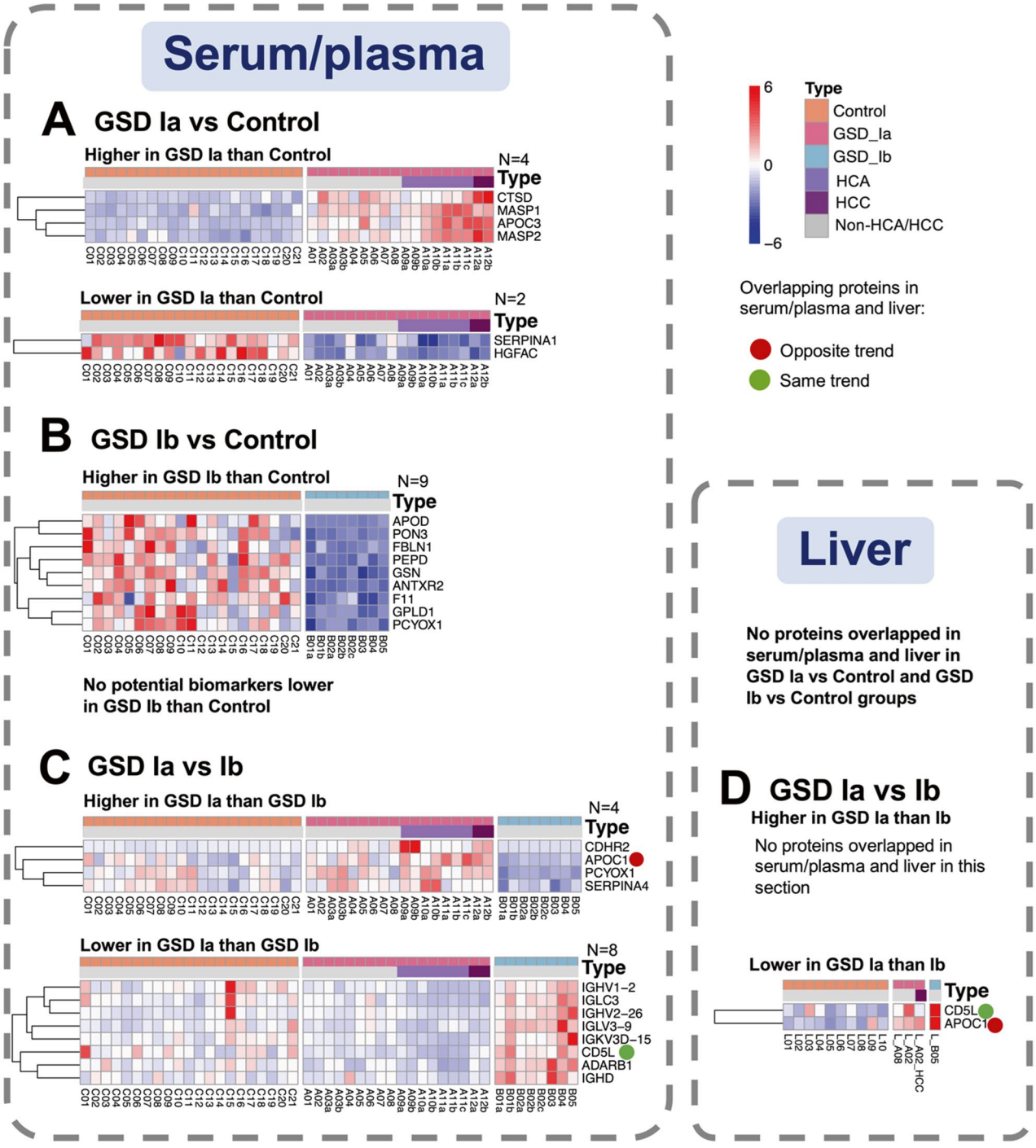

Fig. 5 Summary of potential biomarkers for GSD ia and ib. Heatmap showing the z-score of (**A**-**C**) potential protein biomarkers detected in serum/plasma samples, and (**D**) overlapping protein biomarkers in the liver. 'N' refers to the number of proteins. Groups: C01–C21 were control samples; A01–A12 were GSD ia samples (A09a-A11b were GSD ia with HCA, A12a and A12b were GSD ia with HCC); B01–B05 were GSD Ib samples

already developed HCA or HCC, and the correlation of circulating proteomics markers with the target tissue (i.e., the liver). Our results provide new insights into GSD Ia- and Ib-specific proteomic alterations and their possible links to biological processes and disease complications, providing leads for monitoring of the GSD I subtypes and prognostic biomarkers.

Pathway analyses revealed a significant enrichment of the blood coagulation pathway in GSD Ia, as shown in Fig. 3A. This finding aligns with the thrombosis disease cluster, as illustrated in Fig. 4B, in which a group

Table 2 Summary of the potential biomarkers of different comparisons. '↑' refers to increase, '↓' refers to decrease

Biomarkers for	Protein Name	Trend in serum/ plasma	Trend in liver	Functional Category	potential related complications in GSD I	Description
GSD Ia vs. GSD Ib	APOC1	↑	↓	Lipid metabolism	Hyperlipoproteinemia	Modulates lipoprotein metabolism, affects VLDL and LDL balance.
	CD5L	↓	↓	Immune regulation/ Lipid metabolism	Immune disorder, Hyperlipoproteinemia	Also known as AIM, regulates lipid metabolism and macrophage function.
Specific GSD Ia with HCC	ALDOB	↑	↑	Carbohydrate metabolism enzyme	Liver injury	Aldolase B, key enzyme in fructose metabolism, primarily expressed in the liver.

of thrombosis-associated proteins was significantly increased in GSD Ia compared with controls and GSD Ib. Separate analyses using only one sample per patient (OneSP) or only serum samples (Only Serum) confirmed this finding (Supplementary Analysis 1). This may help explain the clinical observations that GSD I patients commonly suffer from coagulation dysfunction and present with easy bruising, epistaxis, and excessive bleeding during surgery [36–39]. Additionally, a previous study in our group by La Rose et al. indicated that hepatocyte-specific glucose-6-phosphatase deficiency disturbs platelet aggregation and decreases blood monocytes upon fasting-induced hypoglycemia in GSD Ia mouse models [40]. In their study, the plasma levels of coagulation factors V (FV) and VIII (FVIII) were increased in GSD Ia mice in the fed and fasted conditions, while factor VII (FVII) was not affected. In line with their study, we validated that the G6PC deficiency disturbs coagulation in GSD I patients. In our study, 18 proteins involved in thrombosis were altered in GSD Ia and Ib patients, with 6 proteins decreased in both subtypes, especially the decrease of coagulation factor II (F2, also known as prothrombin). Prothrombin is the precursor of thrombin (F2a), the enzyme that provides the cleavage reaction to create the fibrin required for blood clot formation [41–43]. Thus, as a key factor in coagulation, the decrease of F2 could provide a monitoring biomarker for the risk of coagulation complications in GSD I patients. Notably, F2 represents the fully γ-carboxylated prothrombin, whereas PIVKA-II, elevated in the serum of four GSD Ia patients with HCC [28–30], corresponds to the undercarboxylated form. In our results, F2 was increased in GSD Ia patients without HCC rather than in those with HCC, suggesting that different forms of prothrombin might reflect distinct disease states. Whether a shift from the fully γ-carboxylated to the undercarboxylated form contributes to disease progression, particularly during HCC, remains unclear. However, when we only applied analysis to serum samples, F2 was not significantly changed (Supplementary Analysis 1). Further investigations with a larger sample size are needed in the light of existing knowledge on prothrombin metabolism. The additional number of up-regulated proteins related to thrombosis specific for GSD Ia could provide novel leads for the clinical observation that coagulation complications are more common in GSD Ia than Ib [44–46].

The top 3 altered biological processes in both GSD Ib, compared to controls, as well as GSD Ia were all related to immune responses, with the proteins being up-regulated in GSD Ib in comparison to the other two groups. In terms of clinical symptoms, GSD Ib patients specifically suffer from immune-related complications such as recurrent infection, chronic inflammation, and inflammatory bowel disease, due to neutrophil dysfunction [10, 47, 48]. In our study, a large number of IGs were increased in serum/plasma of GSD Ib patients, suggesting their infection and inflammation are more severe or they are continuously in a proinflammatory state, providing leads for the distinct immunological phenotype of GSD Ib patients [1, 10].

Our disease enrichment analysis identified interesting clusters related to liver injury, hypercholesterolemia and hyperlipoproteinemia, and amyloidosis as potential biomarkers for GSD I. These clusters contain a small subset of proteins being down-regulated in both GSD Ia and Ib, while a number of proteins are specificly up-regulated only in GSD Ia, which could provide leads to why GSD Ia tends to show more pronounced liver long-term complications than GSD Ib [10, 49, 50]. Notably, the observed protein changes, especially apolipoproteins related to hypercholesterolemia and hyperlipoproteinemia, is in line with previously describe alteration in lipid metabolites in GSD Ia and Ib patient plasms samples [21]. The consistent down-regulation of APOA1 and APOA2 in both subtypes reflected a shared impairment in the formation of high-density lipoprotein (HDL) and chylomicrons [51, 52], which may contribute to the dyslipidemia frequently reported in GSD I patients. The decrease of APOA4 in GSD Ib further indicated dyslipidemia, as APOA4 is essential for plasma chylomicron assembly [53]. It is known that GSD Ia patients have increased lipids (triglycerides and cholesterol) levels, consistent with observed elevation of APOC1, APOC2, APOC3, and APOE in GSD Ia, given these proteins and lipids are integral components of lipoproteins [54–57]. APOC2 activates lipoprotein lipase [58], which contribute to triglyceride clearance, whereas APOC3 and APOC1 inhibit it [59, 60]. APOE is a key ligand for remnant lipoprotein

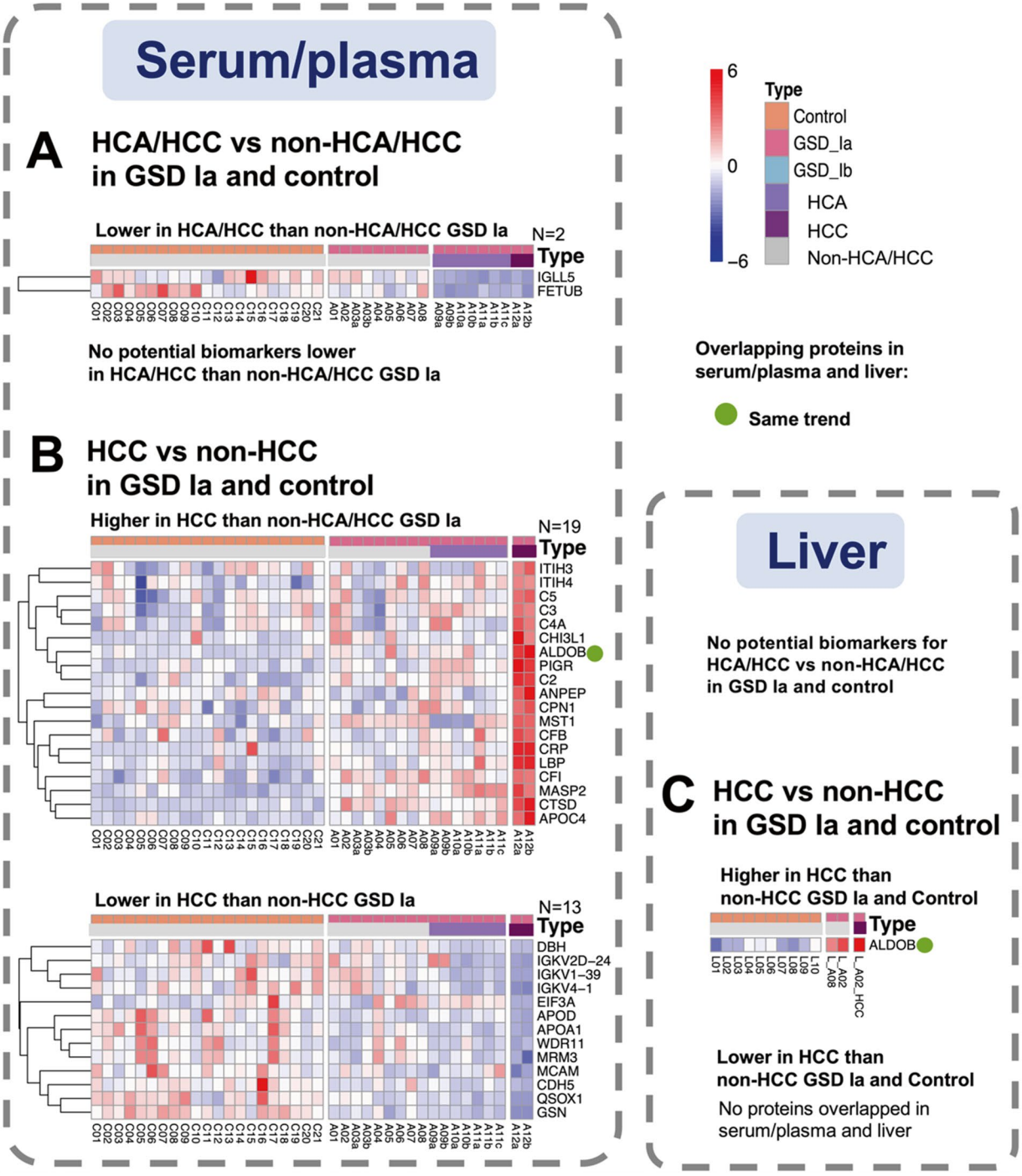

Fig. 6 Summary of potential biomarkers for HCA+HCC and HCC in serum/plasma and liver. Heatmap showing the z-score of (**A** and **B**) potential protein biomarkers detected in serum/plasma samples, and (**C**) overlapping protein biomarkers in the liver. 'N' refers to the number of proteins. Groups: C01–C21 were control samples; A01–A12 were GSD ia samples (A09a-A11b were GSD ia with HCA, A12a and A12b were GSD ia with HCC); B01–B05 were GSD Ib samples

clearance, may further reflect disordered lipid clearance and increased lipoprotein turnover [61]. Therefore, the complex effects of altered apolipoproteins may underlie the impaired lipid metabolism observed in GSD Ia and Ib. In particular, the increase of APOCs and APOE in GSD Ia could potentially reflect the more severe hyperlipidemia, aligning with results of mouse experiments [62] and clinical observations in patients [27, 63]. In addition, previous studies also indicated that the VLDL metabolism was impaired in hepatic-GSD Ia mouse model [64],

in agreement with the observed high VLDL level in GSD Ia patients [64], which we observed in the VLDL related APOE and APOC proteins.

ROC curve analyses provided us with a range of potential biomarkers, but the GSD I liver tissue data provided us with an additional selection criterium to define a shortlist of potential biomarkers with a clear link to the liver changes of these patients. As summarized in Table 2, this resulted in the shortlist of potential liver-linked biomarkers showing differential regulation not only in the circulation, but also in the liver for the various comparisons between GSD Ia, GSD Ib, and controls.

Applying strict filters on the selection for potential biomarkers based on the sensitivity analysis with different analysis methods (Supplementary analysis 3), did not retain significantly changed individual proteins overlapping between liver tissue and blood circulation for either GSD Ia or GSD Ib versus controls as potential biomarkers. Though the circulating proteins still provide valuable insights for further investigation. Especially the proteins which can be grouped into the pathways we clustered, such as immune response or complement and coagulation cascades (MASP1, MASP2, SERPINA1, F11, and GSN) [65–68] and lipid metabolism (APOC3, APOD, PON3, and PCYOX1) [59, 69–71]. In addition, APOC1 emerged as a robust discriminator between GSD Ia and GSD Ib, as it was identified as a potential biomarker in both blood and liver analyses. Given that APOC1 plays important roles in lipid transport, lipoprotein remodeling, and metabolic regulation [57, 62], its reproducible alteration may highlight subtype-specific disturbances in lipid-handling pathways. In addition, CD5L (also known as AIM) is a macrophage-derived secreted glycoprotein that contributes to immune regulation and intersects with lipid metabolic homeostasis, as evidenced by its transcriptional control via lipid-sensing nuclear receptors and roles in inflammatory processes and lipid handling [72, 73]. In this study, CD5L was significantly reduced in GSD Ia compared with GSD Ib, suggesting more pronounced dysregulation of immune–metabolic pathways in Ia.

Lastly, we also identified prognostic biomarkers for HCA and HCC in GSD Ia patients. Previous studies indicated 70–80% of GSD Ia patients would suffer from HCA over 25 years old, and 10% of them progress to HCC. [6, 53, 74] Thus, it is essential to identify circulating biomarkers for early detection and intervention of HCA and HCC in these patients. Due to the lack of liver material of GSD Ia patients with HCA we were not able to identify if these proteins directly linked to the liver-phenotype, but we identified two potential early prognostic biomarkers only in the blood circulation, that could serve as starting points for further validation.

For HCC, we had liver tissue material available from one patient to prioritize the selection, and we identified ALDOB (up-regulated) as the most interesting candidate in both blood circulation and liver proteomics. ALDOB, as a key enzyme in fructose metabolism, was identified up-regulated both in a GSD Ia mouse study [75] and was also identified as a potential biomarker for GSD Ia liver injury in the recently published serum proteomics screening on GSD Ia patients [26]. However, we show with the distinction between GSD Ia patients with and without HCC, which are most strongly detected in the case of long-term liver complications, further supporting the role as a potential prognostic biomarker specifically for GSD Ia specific liver injury. Notably, previous studies reported that ALDOB was decreased in HCC of (non-GSD) patients [76, 77], It is also not known how these observations relate to the strictly limited fructose intake [78] of the GSD I patients. We therefore see the opposite trend for ALDOB in GSD Ia compared to the general HCC patients. This suggests that metabolic changes in GSD Ia patients themselves may also affect the metabolism of liver cancer cells. This might be a unique metabolic adaptation in GSD Ia patients, which is further supported by the previously observed lack of responsiveness for the general HCC marker AFP [6]. Targeted measurements, such as targeted quantitative proteomics measurements or ELISA measurements, would provide subsequent validation to confirm the clinical significance and elucidate the precise role of ALDOB in GSD-related HCC and its potential as prognostic biomarker for liver-specific long-term complications.

However, several limitations should be acknowledged. First, despite the relatively large sample size, it is insufficient to capture the full heterogeneity of GSD I patients, especially the liver tissue samples. Second, GSD I patients follow strict diets, most notably taking some form of cornstarch every few hours. Their feeding states, along with other treatment regimes, comprise additional differentiation between the patients and controls, which could bias the observed differentially regulated proteins between the GSD I patients and the control group. Third, serum/plasma and liver samples were retrospectively collected, and probably not under the same conditions. Fourth, both serum and plasma samples were used, which may also have contributed to heterogeneity, although we did not see distinct clustering between both types as shown in Figure S2D and excluded the proteins known to be different between plasma and serum [37]. Supplementary Analyses 1–2, analyzed using different sample cohorts and imputation methods, provide additional support for our pathway and disease-cluster results. The biomarkers were determined based on SCPs consistently shared across multiple sample cohorts and imputation approaches, analyzed in Supplementary

Analysis 3. Altogether, the differential treatment regimens and sampling conditions may have contributed to heterogeneity in outcomes. Therefore, although potential biomarkers were identified in our study, their clinical applicability, stability, and specificity require further validation through large-scale and prospective studies.

Conclusions

In summary, this study was the first to employ untargeted proteomics on serum/plasma of both GSD Ia and GSD Ib patients and on liver tissue of the same patients (subset) compared to matched controls, providing valuable leads into the complications mechanisms and patient monitoring: (1) We first identified the pathways changes and disease enrichment for the monitoring of the GSD I subtypes. (2) We further focused the list of potential biomarkers from the pathway and disease cluster analyses for potential biomarkers showing differential regulation both in the circulation as well as in the liver, which were summarized in Table 2; From this list we identified specific subsets for the monitoring and prognostic biomarkers specific for GSD Ia vs. GSD Ib (APOC1 and CD5L) and HCC in GSD Ia (ALDOB).

Abbreviations

ABC	Ammonium bicarbonate
ACN	Acetonitrile
AUC	Area under the curve
CD163	Scavenger receptor cysteine-rich type 1 protein M130
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
F11	Coagulation factor XI
F2	Prothrombin
FA	Formic acid
FDR	False discovery rates
G6PC	Glucose 6-phosphatase
G6PT	Glucose 6-phosphate transporter
GO	Gene Ontology
GSD Ia	Glycogen Storage Disease type Ia
GSD Ib	Glycogen Storage Disease type Ib
HCA	Hepatocellular adenoma
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
IBD	Inflammatory bowel disease
LDS	Lithium dodecyl sulfate
NP-40	Nonidet *p*-40
PCA	Principal component analysis
PROC	Protein C
ROC	Receiver operating characteristic
UMCG	University Medical Center Groningen

Supplementary information

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Supplementary Analyses 1-3

Supplementary Figures S1-S6

Supplementary Methods

Supplementary Table S1_Blood samples details

Supplementary Table S2_Liver samples details

Supplementary Table S3_SCP in GSD Ia vs Control_blood

Supplementary Table S4_SCP in GSD Ib vs Control_blood

Supplementary Table S5_SCP in GSD Ia vs Ib_blood

Supplementary Table S6_BP in GSD Ia vs Control_blood

Supplementary Table S7_BP in GSD Ib vs Control_blood

Supplementary Table S8_BP in GSD Ia vs Ib_blood

Supplementary Table S9_DC in GSD Ia vs Control_blood

Supplementary Table S10_DC in GSD Ib vs Control_blood

Supplementary Table S11_DC in GSD Ia vs Ib_blood

Supplementary Table S12_ROC AUC with confidence intervals

Supplementary Analysis Table SA1_Imputed MinProb

Supplementary Analysis Table SA2_Imputed LeftCensor

Supplementary Table SA3_Imputed Bayesian

Supplementary Analysis Table SA4_Imputed Mean

Supplementary Analysis Table SA5_Imputed Variance

Supplementary Analysis Table SA6_overall overlap SPC

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Author contributions

Ruiqi Xiao: Methodology, Sample preparation, Proteomics measurements, Data analysis, Manuscript. Candelas G. Valle: Biology information of GSD Ia patients from clinical, Writing-reviewing and Editing. Albert Gerding: Help with methodology and sample preparation, Writing-reviewing and Editing. M. Rebecca Heiner-Fokkema: Provide some of the serum samples of the control group, Writing-reviewing and Editing. Adam M. Thorne and Vincent E. de Meijer: Provide liver samples of the control group, Writing-reviewing and Editing. Terry G.J. Derks: Biological information of GSD Ia patients from a clinical perspective, Writing-reviewing and Editing. Maaike H. Oosterveer, Barbara M. Bakker, and Justina C. Wolters: Conceptualization, Supervision, Writing-reviewing, and Editing.

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Data availability

Raw mass spectrometry data used for proteomic analysis are available through ProteomeXchange (https://www.ebi.ac.uk/pride/archive) with the dataset identifier PXD066805. The other data that supports the findings of this study are available in the supplementary materials.

Declarations

Ethics approval and consent to participate

The data were obtained by experiment and data analysis. The tenets of the Declaration of Helsinki were followed. The Medical Ethical Committee of the University Medical Center Groningen (UMCG) stated that for retrospective, non-interventional studies the Medical Research Involving Human Subjects

Act was not applicable and that further study approval by the Medical Ethical Committee was not required (METc 2019/119).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Laboratory of Pediatrics, University of Groningen, University Medical Center Groningen; Antonius Deusinglaan 1, Groningen, The Netherlands
[2]UMCG Comprehensive Transplant Center, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[3]Department of Liver Transplantation and HPB Surgery, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[4]Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[5]Therapy Accelerator for Rare Diseases, Radboud University Medical Center, Nijmegen, The Netherlands
[6]Beatrix Children's Hospital, Department of Pediatrics, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[7]United for Metabolic Diseases (UMD), Groningen, The Netherlands
[8]Laboratory of Metabolic Diseases, Department of Laboratory Medicine, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands
[9]UMCG Center of Expertise for Carbohydrate, Fatty Acid Oxidation and Ketone Bodies Disorders, University Medical Center Groningen, Groningen, the Netherlands

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