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Serum and liver proteome in women with morbid obesity and metabolic dysfunction-associated steatotic liver disease

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Abstract

Objectives: This study aimed to characterize proteome in serum and liver samples from women with morbid obesity (MO) and metabolic dysfunction-associated steatotic liver disease (MASLD) to identify proteins and metabolic pathways.

Methods: HPLC-MS/MS proteomics was performed on serum and liver samples from 174 women with MO, classified by hepatic histology into three group: normal liver (NL, n=44), simple steatosis (SS, n=66) and metabolic dysfunction-associated steatohepatitis (MASH, n=64). All MASH cases presented mild-moderate hepatic inflammation without fibrosis.

Results: Serum proteomics identified 257 proteins. In MASLD group, we found most increased levels (log2FC>1.6) of fructose-bisphosphate aldolase, clusterin and collectin-10, and most decreased levels (log2FC < −1.6) of Adiponectin and sex hormone-binding globulin compared with NL group. These proteins were mainly linked to three complement cascade pathways: regulation (33.33 %), initiation (33.33 %) and activation (33.33 %). Liver proteomics identified 2,081 proteins. Comparative liver proteomics between MASLD, SS or MASH groups and NL did not reveal significant differences. However, comparison between MASH and SS identified 72 significantly upregulated and 84 downregulated proteins. Biological processes enrichment analysis of these protein groups revealed that these proteins were mainly involved in these pathways: amino acid metabolism (31.25 %), antimicrobial defense (20 %) and fatty acid metabolism (17.5 %) and others.

Conclusions: This study identifies serum and liver proteome that could be associated with MASLD and MASH. The study of these proteins and their associated metabolic pathways may be useful for physiopathological research. These findings warrant further validation in independent and diverse cohorts.

Keywords: obesity; liver; proteomics; metabolic dysfunction-associated steatotic liver disease (MASLD); metabolic dysfunction-associated steatohepatitis (MASH)

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) was recently termed to define the presence of hepatic steatosis in conjunction with one metabolic risk factor without alcohol consumption [1]. MASLD encompasses a range of liver conditions spanning from simple steatosis (SS) to the more severe form known as metabolic dysfunction-associated steatohepatitis (MASH), characterized by hepatocyte ballooning and lobular inflammation with or without liver fibrosis [2]. Approximately 20 % of MASLD patients have MASH, which can progress to cirrhosis or hepatocellular carcinoma (HCC) [3]. Furthermore, MASH is associated with an increased risk of cardiovascular disease, the primary cause of morbidity and mortality in patients with MASLD [4].

Proteomics is an omics technology that identifies and quantifies peptides and proteins in a biological sample, defining a disease phenotype. The study of proteomes plays an essential role in various research areas, including biomarker discovery, drug development and understanding disease mechanisms [5, 6]. To date, several proteomic analyses in human subjects have been previously published regarding MASLD and MASH [7]. Some studied identified proteins significantly altered in serum and liver samples of MASLD subjects compared to metabolically healthy controls [8, 9]. However, uncertainty remains regarding which

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proteins and metabolic pathways are involved in the pathophysiology of MASLD and MASH, highlighting the need for further proteomic studies on serum and liver samples in a considerable and homogeneous cohort.

In this context, we previously performed metabolomic and lipidomic analyses in serum samples of a cohort of women with morbid obesity (MO) and different hepatic histological diagnoses and found some lipid metabolites associated to MASLD and MASH [10, 11]. In this study, to advance this research, we aim to perform a proteomic analysis of serum and liver samples from a homogeneous cohort of women with MO and associated MASLD, to identify key proteins and metabolic pathways.

Materials and methods

Subjects

In this work, we recruited a homogeneous cohort of 174 women with morbid obesity (body mass index, BMI>40 kg/m^2), all of whom had available histological evaluation and surplus liver tissue obtained from liver biopsies that had been indicated for diagnostic purposes and performed at the time of bariatric surgery. Given that men and women differ in terms of metabolic parameters, we recruited only women for this study [12, 13]. This cohort was classified according to hepatic histological diagnosis into two groups: normal liver (NL) (n=44) and MASLD (n=130). The MASLD group was further subclassified into two subgroups: simple steatosis (SS) (n=66) and MASH (n=64). The MASH subgroup corresponds to the initial stages of the disease, characterized by mild to moderate inflammation without fibrosis.

The sample size was calculated accepting an alpha risk of 0.05 and a beta risk of less than 0.2 in a bilateral contrast. We needed a minimum of 68 cases (MASLD) and 34 controls (NL) to detect at least an odds ratio of 0.15. It is assumed that the exposure rate in the control group would be 0.333 (POISSON approximation, GRANMO sample size calculator v.7.04).

As exclusion criteria, we avoided patients who had an acute illness, acute or chronic inflammatory or infective diseases, or end-stage malignant disease; menopausal women and women receiving contraceptive treatment; women with an alcohol intake exceeding 20 g per day and recurrent smokers.

The Institut d'Investigació Sanitària Pere Virgili Ethics Committee approved this study (CEIm; 23c/2015) in accordance with the Declaration of Helsinki. Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

Anthropometric parameters and biochemical analyses

From each participant, we obtained anthropometric variables such as weight, height (BMI calculation) and waist-hip ratio. Additionally, specialized nurses collected fasting blood samples just before bariatric surgery using two types of tubes: (i) ethylenediaminetetraacetic acid (EDTA) tubes for plasma collection and (ii) tubes without anticoagulant for serum collection, both using a BD Vacutainer® system. EDTA tubes were centrifuged (3,500 rpm, 4 °C, 15 min) to obtain plasma, which was used for routine biochemical analyses. Blood collected in tubes without anticoagulant was allowed to clot, centrifuged under the same conditions, and the resulting serum was aliquoted for proteomic analyses. All plasma and serum samples were stored at −96 °C, ensuring long-term protein stability until analysis.

Biochemical variables measured using a conventional automated analyzer included levels of glucose, insulin, glycosylated hemoglobin A_{1c} (HbA_{1c}), total cholesterol, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) and alkaline phosphatase (ALP).

Histopathological analysis of the liver biopsies

During bariatric surgery, we collected liver samples in a tube with RNAlater solution (Qiagen, Hilden, Germany) at 4 °C and then preserved in a formaldehyde solution for histopathological diagnosis. Liver biopsies were obtained whenever liver alteration was suspected. Liver samples were scored and classified by an experienced hepatopathologist through eosin-hematoxylin staining according to Kleiner criteria [14] into NL (n=44), SS (n=66) and MASH (n=64). All cases of MASH corresponded to grade I-II with mild-moderate inflammation without the existence of fibrosis.

Serum proteome

Protein extraction and quantification for serum samples

Before proteomic analysis, the most abundant plasma proteins (albumin, immunoglobulin (Ig)G, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3 and transthyretin) were

depleted to increase the number of identified/quantified proteins. Ten microliters of each sample were passed twice through the Human-14 Multiple Affinity Removal Spin cartridge (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocol. Flow-through fractions were concentrated and buffer exchanged to about 100 µL of 6 M urea in 50 mM ammonium bicarbonate (ABC; Merck Life Science S.L.U, Madrid, Spain) using 5 K MWCO spin columns (5185–5991; Agilent Technologies, Santa Clara, CA, USA).

Protein digestion and peptide 11-plex TMT labelling

Twenty-five micrograms of total protein (quantified by Bradford's method) were reduced with 4 mM 1,4-dithiothreitol for 1 h at 37 °C and alkylated with 8 mM iodoacetamide for 30 min at 25 °C in the dark. Samples were then digested overnight (pH 8.0, 37 °C) with sequencing-grade Trypsin/Lys-C Protease Mix (ThermoFisher Scientific, Waltham, MA, USA) at an enzyme ratio of 1:50. Digestion was quenched by acidification with 1 % (v/v) formic acid, and peptides were desalted on Oasis HLB SPE columns (Waters, MA, USA) before TMT 11-plex labelling (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

To normalize all samples across different TMT-multiplexed batches, a pool containing all samples was labelled with the TMT-126 tag and included in each TMT batch. The different TMT 11-plex batches were desalted on Oasis HLB SPE columns before nanoLC-MS analysis.

NanoLC-(orbitrap)MS/MS analysis

Labelled and multiplexed peptides were loaded on a trap nano-column (75 µm I.D.; 1.5 cm length; 3 µm particle diameter, ThermoFisher Scientific, Waltham, MA, USA) and separated onto a C-18 reversed phase (RP) µPAC™ Neo HPLC column (180 µm bed; 50 cm length; 2.5 x 16 µm pillar diameter, ThermoFisher Scientific, Waltham, MA, USA) on a Vanquish Neo VN-S10 System (ThermoFisher Scientific, Waltham, MA, USA). Chromatographic separation was performed with a 180-min gradient using water LC-MS grade (0.1 % formic acid) and acetonitrile (0.1 % formic acid) as mobile phases at a flow rate of 300 nL/min.

Mass spectrometry (MS) analyses were performed on an Orbitrap Eclipse from ThermoFisher Scientific by an enhanced FT-resolution MS spectrum (R=60,000 FHMW) followed by data-dependent FT-MS/MS acquisition (R=50,000 FHMW, 30 % NCE HCD) from the ten most intense parent ions with a charge state acquisition from two to six and dynamic exclusion of 0.7 min.

Protein identification and quantification

Protein identification and quantification were performed using Proteome Discoverer software v.2.5 (ThermoFisher Scientific, Waltham, MA, USA) with the Mascot search engine (v2.8, Matrix Science) combining raw data files obtained from each plex. For protein identification, the workflow used the Mascot node combining *Homo sapiens* and contaminants databases, assuming trypsin digestion. The fragment ion mass tolerance assumed an error of 20 mmu for FT-MS/MS fragmentation mass and 10 ppm for FT-MS precursor ion mass. Oxidation of methionine and acetylation of the N-terminal were set as dynamic modifications, carbamidomethylation as a static modification, and TMT-6plex as a quantitation method. The false discovery rate (FDR) and protein probabilities were calculated by Percolator, with peptide identification set to a maximum of 1 %. Peptide quantitation data were retrieved from the 'Reporter ions quantifier' node in Proteome Discoverer, using the area of unique and razor peptides and total peptide amount as normalization. Peptide and protein results are expressed in abundance area and are dimensionless.

Liver proteome

Protein extraction and quantification for liver tissue samples

Liver tissue samples were homogenized by adding RIPA buffer (ThermoFisher Scientific, Waltham, MA, USA) and lysate using a BlueBender with 2 freeze-thaw cycles at −80 °C. Then samples were rotated 1 h and 30 min at 4 °C. Protein extractions were centrifuged at 16000 g for 20 min and the supernatants were collected for protein precipitation with the addition of 10 % TCA/acetone. Finally, protein pellets were resuspended in 6 M urea/50 mM ammonium bicarbonate (ABC; Merck Life Science S.L.U, Madrid, Spain) for digestion.

Protein digestion and peptide 11-plex TMT labelling

We followed the same methodology described above for serum samples.

High pH reverse phase fractionation and nanoLC-(orbitrap)MS/MS analysis

Labelled and multiplexed peptides were fractionated by High pH Reversed-Phase Peptide Fractionation Kit (ThermoFisher Scientific, Waltham, MA, USA) according to

manufacturer's protocol to increase protein identification through orthogonal peptide fractionation of complex peptide samples combined with low pH nanoLC-MS/MS analysis. Briefly, High pH Reversed-phase Peptide Fractionation Kit uses a sequential elution with eight different ratios of 0.1 % triethylamine (a high pH buffer) and acetonitrile. Then, the eluted fractions were evaporated to dryness on a SpeedVac and resuspended with water 0.1 % formic acid for direct nanoLC-MS/MS analysis.

Fractions of each TMT plex were loaded on a trap nanocolumn (75 µm I.D.; 1.5 cm length; 3 µm particle diameter, ThermoFisher Scientific, Waltham, MA, USA) and separated onto a C-18 reversed phase (RP) µPAC™ Neo HPLC column (180 µm Bed; 50 cm length; 2.5 ×16 µm pillar diameter, ThermoFisher Scientific, Waltham, MA, USA) on an Vanquish Neo VN-S10 System (ThermoFisher Scientific, Waltham, MA, USA). The chromatographic separation was performed with a 180 min gradient using Milli-Q water (0.1 % formic acid) and acetonitrile (0.1 % formic acid) as mobile phase at a flow rate of 300 nL/min.

Mass spectrometry analyses were performed following the same methodology described above for serum samples.

Protein identification and quantification

We followed the same methodology described above for serum samples.

Statistical analysis

The descriptive parameters were analyzed using the SPSS/PC+ for Windows statistical package (v27.0; SPSS, Chicago, IL, USA). The Kolmogorov-Smirnov test was employed to assess the distribution of variables. Variables were presented as the median and interquartile range. Comparative analyses were conducted using a nonparametric Mann-Whitney U test. p-Values <0.05 were considered statistically significant.

Regarding the serum proteomics data, only those proteins identified in>60 % of the samples in at least one of the experimental groups were considered. For these proteins, missing value estimation after data filtering was performed using the KNN algorithm (k-nearest neighbors) from Metaboanalyst v5.0 to carry out univariate statistical analysis.

Regarding the liver tissue proteomics data, only those proteins identified in 100 % of the samples were considered. This stringent criterion was applied to maximize robustness and avoid excessive missing values in a highly complex tissue proteome, which would have compromised downstream statistical and pathway analyses.

For statistics analyses of the proteomics data, a log base 2 data transformation was applied. One-way ANOVA with Tukey HSD Post hoc and Benjamini Hochberg FDR correction was performed between groups, with an adjusted p-value cut-off of <0.05 applied in each case. Additionally, gene enrichment analysis and clusters by Reactome classification (Pathways) were obtained using STRING and ClueGO plugins in Cytoscape software v3.10.2 using Benjamini Hochberg FDR correction, applying an adjusted p-value cut-off of <0.05.

Results

Subjects

We first classified our subjects according to their hepatic histology into NL (n=44) and MASLD (n=130), as shown in Table 1. In this case, subjects were comparable in terms of sex, age, BMI and waist-hip ratio. Subjects with MASLD presented higher levels of glucose, insulin, HbA_{1c}, triglycerides and hepatic transaminases than NL subjects. Then, we subclassified our MASLD cohort into SS (n=66) and MASH (n=64). We did not report significant differences between these two subgroups.

Table 1: Antropometric and basic biochemical parameters of the study cohort with morbid obesity compared between normal liver and metabolic-dysfunction associated steatotic liver disease.

Variables	NL (n=44)	MASLD (n=130)
Sex (%M/%W)	0/100	0/100
Age, years	41 (34–49)	47.7 (37–50)
BMI, kg/m^2	43.9 (41.2–49.3)	45.1 (43.2–49.9)
Waist-hip (m) ratio	0.88 (0.84–0.95)	0.92 (0.87–0.97)
Glucose, mg/dL	89 (79.5–100.7)	116 (91.7–146.2)[a]
Insulin, mUI/L	9.2 (5.7–12.8)	17.9 (10.8–31.2)[a]
HbA_{1c}, %	5.3 (5–5.7)	6.1 (5.4–7.8)[a]
Triglycerides, mg/dL	103 (76.5–135)	144.5 (112.7–201.7)[a]
Cholesterol, mg/dL	164 (139.5–199.5)	166.8 (148–189.6)
HDL-C, mg/dL	40 (32.6–50.2)	37 (33–45.2)
LDL-C, mg/dL	102.1 (76.6–126.9)	95.3 (80.6–114)
AST, UI/L	22.5 (19–41.7)	36 (24–52)[a]
ALT, UI/L	23.5 (16–44.5)	35.5 (26.2–57.5)[a]
GGT, UI/L	17 (12–25.5)	26 (17–48.2)[a]
ALP, UI/L	65 (53–76)	67.5 (57–79)

Data are expressed as the median and interquartile range. [a]Significant differences between NL and MASLD were considered when p-value <0.05 using the Mann-Whitney test. NL, normal liver; MASLD, metabolic dysfunction-associated steatotic liver disease; M, men; W; women; BMI, body mass index; HbA_{1c}, glycosylated hemoglobin A_{1c}; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; AST, aspartate-amino transferase; ALT, alanine-aminotransferase; GGT, gamma-glutamyl transferase; ALP, alkaline phosphatase.

Serum proteome

After proteomics analysis in serum samples, a total of 537 proteins were identified. Information regarding relative quantification and identification, such as Mascot score, protein FDR confidence and other details, are in Supplementary Material (Supplementary Table S1). Samples were filtered to include proteins present in>60 % of the samples in at least one experimental group, and KNN estimation was applied, resulting in 257 confident proteins.

We first examined which proteins differed in concentration and pattern between MO subjects with NL and MO subjects with MASLD. Women with MALSD presented significantly increased levels of 9 proteins and significantly decreased levels of 4 proteins compared to NL women (Table 2, Table S2). Specifically, we observed the most increased levels (log2FC>1.6) of fructose-bisphosphate aldolase (ALDOB) (2.05), clusterin (CLU) (1.73) and collectin-10 (COLEC10) (1.62), and the most decreased levels (log2FC < −1.6) of adiponectin (ADIPOQ) (−1.68) and sex hormone-binding globulin (SHBG) (−1.64).

Consequently, we evaluated specifically the serum proteome between women with NL and those women with SS. We reported 5 significantly increased proteins and 5 significantly decreased proteins in SS subjects compared to NL participants (Table 3, Table S3). In this case, we observed the most increased levels of ALDOB, pigment epithelium-derived factor (SERPINF1) and serotransferrin (TF), and the most decreased levels of gelsolin (GSN), ADIPOQ and SHBG.

Then, we compared the differential altered proteins between women with NL and MASH. We found 11 significantly increased proteins and only 2 decreased proteins in the MASH group compared to the NL group (Table 4, Table S4). Notably, we observed the most increased levels of ALDOB, CLU, COLEC10 and COLEC11, and the most decreased levels of ADIPOQ and SHBG.

To sum up, women with MASLD, SS and MASH, the three groups, showed most higher levels of ALDOB and most lower levels of ADIPOQ and SHBG compared to NL women. However, the three pathological groups also presented increased levels of SERPINF1 and vitronectin (VTN). On the one hand, both women with MASLD and SS also showed increased levels of serotransferrin (TF) and vitamin K dependent protein S (PROS1), and decreased levels of gelsolin (GSN) and complement factor D (CFD). On the other hand, both women with MASLD and MASH exhibited most increased levels of CLU and COLEC10 compared to NL women but also presented increased levels of COLEC11 and scavenger receptor cysteine-rich type 1 protein M130 (CD163).

Then, we compared the serum proteome between women with SS and women with MASH, but we did not report significantly differently altered proteins between these groups (data not shown).

Table 2: Serum proteins significantly upregulated or downregulated in metabolic-dysfunction associated liver disease compared to normal liver.

Upregulated in MASLD	
Coding protein name	**UniProt ID**
Fructose-bisphosphate aldolase (ALDOB)	A0A3B3IS80
Clusterin (CLU)	P10909
Collectin-10 (COLEC10)	Q9Y6Z7
Pigment epithelium-derived factor (SERPINF1)	P36955
Serotransferrin (TF)	P02787
Collectin-11 (COLEC11)	Q9BWP8
Vitronectin (VTN)	P04004
Scavenger receptor cysteine-rich type 1 protein M130 (CD163)	F5GZZ9
Vitamin K-dependent protein S (PROS1)	P07225
Downregulated in MASLD	
Coding protein name	**UniProt ID**
Adiponectin (ADIPOQ)	Q15848
Sex hormone-binding globulin (SHBG)	I3L145
Gelsolin (GSN)	P06396
Complement factor D (CFD)	P00746

Statistical differences were considered significant when the adjusted p-value was less than 0.05, as determined by the Student's t-test. Upregulated and downregulated proteins were identified by positive or negative log2 fold changes, respectively. Detailed statistical data from the comparisons are provided in Supplementary Table S2.

Table 3: Serum proteins significantly upregulated or downregulated in simple steatosis compared to normal liver.

Upregulated in SS	
Coding protein name	**UniProt ID**
Fructose-bisphosphate aldolase (ALDOB)	A0A3B3IS80
Pigment epithelium-derived factor (SERPINF1)	P36955
Serotransferrin (TF)	P02787
Vitronectin (VTN)	P04004
Vitamin K-dependent protein S (PROS1)	P07225
Downregulated in SS	
Coding protein name	**UniProt ID**
Gelsolin (GSN)	P06396
Adiponectin (ADIPOQ)	Q15848
Sex hormone-binding globulin (SHBG)	I3L145
Complement component C6 (C6)	P13671
Complement factor D (CFD)	P00746

Statistical differences were considered significant when the adjusted p-value was less than 0.05, as determined by the Student's t-test. Upregulated and downregulated proteins were identified by positive or negative log2 fold changes, respectively. Detailed statistical data from the comparisons are provided in Supplementary Table S3.

Table 4: Serum proteins significantly upregulated or downregulated in metabolic-dysfunction associated steatohepatitis compared to normal liver.

Upregulated in MASH	
Coding protein name	**UniProt ID**
Fructose-bisphosphate aldolase (ALDOB)	A0A3B3IS80
Clusterin (CLU)	P10909
Collectin-10 (COLEC10)	Q9Y6Z7
Collectin-11 (COLEC11)	Q9BWP8
Scavenger receptor cysteine-rich type 1 protein M130 (CD163)	F5GZZ9
Attractin (ATRN)	O75882
Pigment epithelium-derived factor (SERPINF1)	P36955
Vitronectin (VTN)	P04004
Complement factor H (CFH)	P08603
Thrombospondin-4 (THBS4)	P35443
Apolipoprotein A-IV (APOA4)	P06727
Downregulated in MASH	
Coding protein name	**UniProt ID**
Sex hormone-binding globulin (SHBG)	I3L145
Adiponectin (ADIPOQ)	Q15848

Statistical differences were considered significant when the adjusted p-value was less than 0.05, as determined by the Student's *t*-test. Upregulated and downregulated proteins were identified by positive or negative log2 fold changes, respectively. Detailed statistical data from the comparisons are provided in Supplementary Table S4.

To identify the pathways associated with the significant proteins in each comparison, we conducted a biological processes enrichment analysis (Reactome) of the metabolic pathways, organized by the percentage of proteins involved (Figure 1). For serum proteome, altered proteins significantly different between the NL and MASLD groups correspond completely to pathways related to complement cascade pathway, including regulation (33.33 %), initiation (33.33 %) and activation (33.33 %) of this process (Figure 1A). On the other hand, the comparison of proteome between NL and SS groups again correspond entirely to pathways from the complement cascade: regulation (50 %) and activation (50 %) (Figure 1B). Finally, the differently altered proteins between NL and MASH groups correspond in 50 % to complement cascade pathway (including activation (33.33 %) and initiation (16.67 %)), and also to response to elevated platelet cytosol calcium pathways (33.33 %) or integrin cell surface interactions (16.67 %) (Figure 1C).

Liver samples proteome

Subsequently, we performed a proteomics analysis on liver samples, identifying a total of 4,671 proteins. Relative quantification and identification details, including Mascot score and protein FDR confidence are provided in Table S5. After filtering proteins present in 100 % of the samples, 2081 proteins were confidently identified.

First, we aimed to determine which altered proteins differ in hepatic concentration between NL and MASLD subjects. However, no significant differences were found in this comparison (data not shown). Next, we evaluated the hepatic proteome between women with NL and those with SS. Similar to the previous comparison, we did not find significant differences in protein concentration (data not shown). When comparing the differential altered proteins between women with NL and MASH, once again, we did not find significant differences (data not shown).

Finally, we compared the hepatic proteome between women with SS and MASH. This analysis revealed 72 significantly upregulated and 84 downregulated proteins in the MASH group compared to the SS group (Table S6). Since there are no differences between SS and MASH compared to NL, we cannot identify differences between individual proteins, but we can analyze groups of proteins across metabolic pathways. Therefore, we performed another biological processes enrichment analysis (Reactome) in liver proteomics data. For liver proteome, altered proteins significantly different between the SS and MASH groups correspond primarily to pathways related to the metabolism of amino acids and derivatives (31.25 %), antimicrobial peptides (20 %) and fatty acid metabolism (17.5 %), among other, much less representative pathways such as phaseI-functionalization of compounds, hemostasis, apoptosis, neutrophil degranulation, binding to scavenger receptors, complement cascade regulation, peroxisome, metabolism of vitamins, global metabolism and amyloid fiber formation pathways (Figure 2).

Discussion

In this study, we evaluated the proteomics profile in samples of serum and liver tissue of a homogeneous cohort of women with MO and MASLD, classified according to liver histopathology.

In the circulating proteome analysis, we identified 257 proteins. MASLD subjects presented 13 altered proteins, with increased levels of 9 and decreased levels of 4, compared to NL individuals. Subjects with SS and MASH presented 10 and 13 deregulated proteins, respectively, compared to NL. In these last two comparisons, most of the deregulated proteins overlapped with those identified in the MASLD group. Notably, we observed most increased levels of ALDOB, CLU and COLEC10, and most decreased levels of ADIPOQ and SHBG.

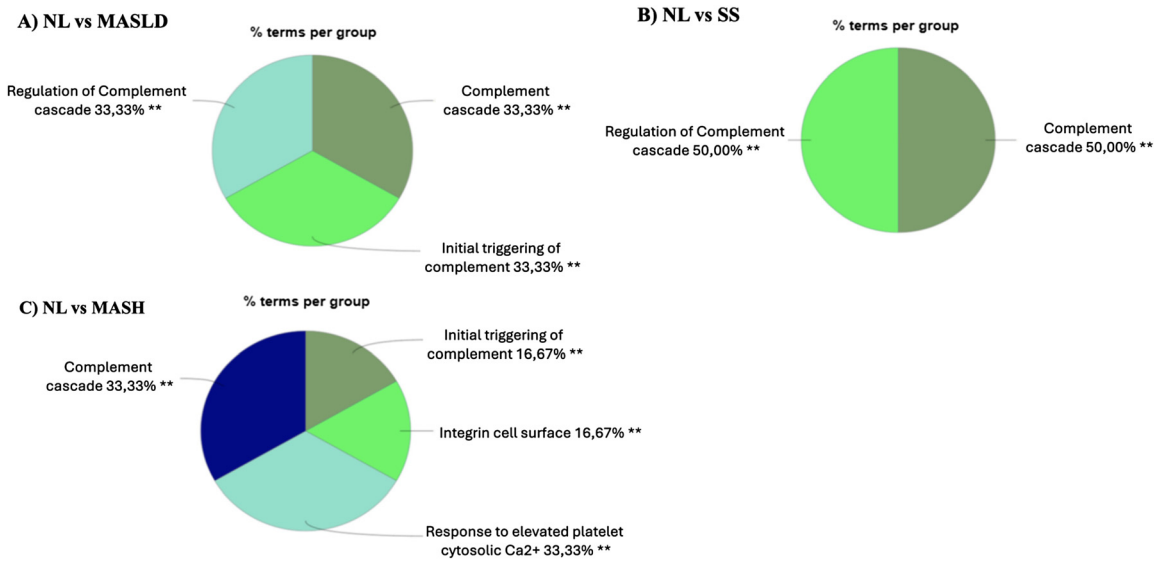

Figure 1: Overrepresentation analyses of the pathways (Reactome) most representative in serum samples between (A) normal liver and metabolic-dysfunction associated steatotic liver disease; (B) normal liver and simple steatosis; (C) normal liver and metabolic-dysfunction associated steatohepatitis.

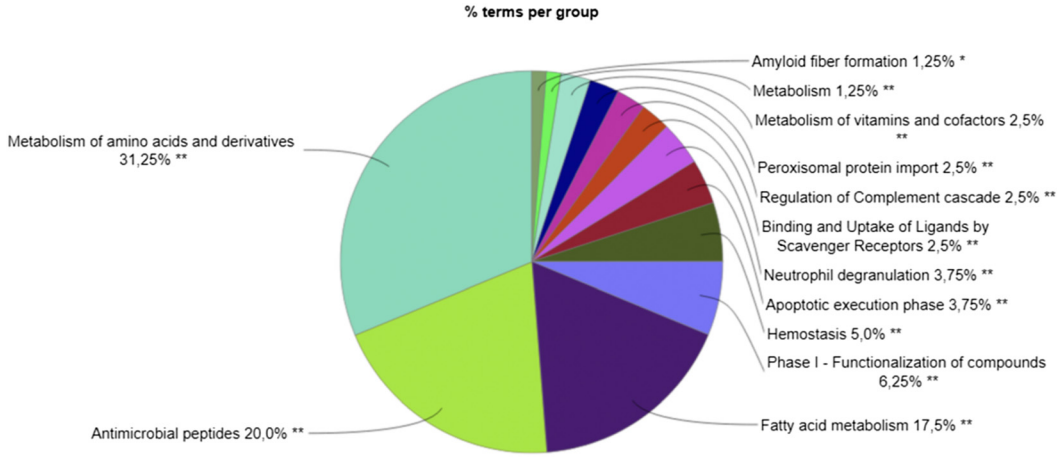

Figure 2: Overrepresentation analyses of the pathways (Reactome) most representative in liver samples between simple steatosis and metabolic-dysfunction associated steatohepatitis.

Next, we compared our findings with the literature. In a proteomics study on serum samples of 636 individuals with liver biopsy-confirmed MASH, authors aimed to identify protein signatures corresponding to key histological phenotypes such as steatosis, inflammation, ballooning and fibrosis [14]. Although our studies are not directly comparable, we concur with their finding that low levels of ADIPOQ are associated with inflammation without fibrosis. Moreover, they suggested that COLEC11 has a positive association with fibrosis, whereas we found increased levels of COLEC11 in MASLD and MASH groups compared to NL group. However, in our study, all MASH patients correspond to preliminary stages of inflammation without fibrosis. CLU and ALDOB, both previously linked to HCC [15, 16], were instead detected in preliminary and benign stages of MASH in our cohort, suggesting a different role in early disease. Finally, SHBG has been reported as a positive predictor of severe fibrosis in female MASLD patients at levels>10 ng/mL, with high specificity but modest sensitivity [17]; in contrast, we found relevant decreased levels of SHBG in our MASLD and MASH groups, and also, our MASH subjects did not present fibrosis.

In other three studies of proteomics in serum, there are coincident results with our findings. In the first, authors conducted a study on the general population, defining two groups: fatty liver (by ultrasound) and liver fibrosis (by

transient elastography) [18]. They found that thrombospondin-4 is directly associated with both hepatic conditions, although these findings were obtained in a cohort without histologically confirmed diagnosis. In contrast, our study revealed increased serum levels of this protein in MASH subjects, all of whom were in early stages of the disease and exhibited no evidence of fibrosis. In the second study, authors suggested that some proteins are associated with MASLD, such as VTN, which is associated to extracellular matrix responses to hepatic injury [19]. In this sense, we found increased levels of vitronectin in serum samples of MASLD and both subgroups, SS and MASH, compared to NL. Finally, the third study identified some proteins as candidate biomarkers of MASLD, including high levels of CLU, VTN, thrombospondin-4 and complement factor H [20]. We found increased levels of CLU and VTN in MASLD and MASH groups compared to NL group, and we also found increased levels of thrombospondin-4 and complement factor H in MASH. These findings reinforce the relevance of these molecules in MASLD pathogenesis.

After performing an enrichment analysis (Reactome) on the serum proteomics data, we confirmed that the groups of deregulated proteins identified in these comparisons are mainly involved in complement cascade processes. These proteins were primarily associated with three pathways of the complement system: regulation (33.33 %), initiation (33.33 %) and activation (33.33 %), which have already been linked to the pathogenesis of MASLD and MASH [21]. Future research is needed to validate the specific role of complement proteins in MASLD and MASH.

In the liver proteomics analysis, we identified 2081 proteins. We did not report significant differences in the proteome when comparing MASLD to NL groups or when comparing SS or MASH to NL. However, we found 72 proteins with increased levels and 84 proteins with decreased levels in the MASH group compared to the SS group. Since there are no differences between SS and MASH compared to NL, we cannot identify differences between individual proteins, but we can analyze groups of proteins across metabolic pathways.

Therefore, we performed another biological process enrichment analysis (Reactome) in liver proteomics data. This analysis indicated that proteins deregulated in MASH compared to SS are mostly related to the metabolism of amino acids and derivatives (31.25 %), antimicrobial peptides (20 %) and fatty acid metabolism (17.5 %), among other, much less representative pathways such as phaseI–functionalization of compounds, hemostasis, apoptosis, neutrophil degranulation, binding to scavenger receptors, complement cascade regulation, peroxisome, metabolism of vitamins, global metabolism and amyloid fiber formation pathways. It is well-known that amino acid metabolism, especially branched-chain amino acids and aromatic amino acids, is altered in MASLD and contributes to disease progression because it is strongly involved in lipid accumulation processes and inflammatory responses [22]. Additionally, altered fatty acid metabolism is a major factor in MASH, inducing lipotoxicity [23]. However, we cannot establish any relationship between the proteomic findings obtained in serum and liver tissue.

To sum up, our study supported by previous findings, reported some proteins in serum samples that could be relevant. Increased levels of CLU, COLEC11, VTN, complement factor H and thrombospondin-4, and decreased levels of ADIPOQ and SHBG, are aligned with other published reports. Moreover, these findings could be important for exploring potential pathogenic pathways involved in MASLD and MASH. In addition, after an enrichment analysis, we reported that most of altered proteins in serum samples between different hepatic histological groups are related to the complement cascade. In addition, we also found altered liver tissue proteins involved in the complement cascade. This finding is consistent with the role of the complement cascade in lipid metabolism and immune responses [24]. Activation of the innate immune system plays a key role in MASLD pathogenesis when the complement system recognizes danger signals such as tissue injury. Moreover, activation of the complement cascade is involved in disease progression and severity, including the clearance of apoptotic cells, liver inflammation, hepatic fibrosis and liver regeneration [25, 26].

This study has several limitations. First, it was conducted at a single center, which may limit the generalizability of the findings. Second, the sample size is moderate, particularly given the prevalence of the disease under study. Third, our results have not yet been validated using an independent routine diagnostic method. Therefore, the present study should be considered hypothesis-generating, and further validation in independent, multicentric cohorts is warranted.

Conclusions

In this proteomics study of a homogeneous cohort of women with MO and MASLD, we identified a group of several serum proteins altered in MASLD and MASH subjects compared to NL. We observed most significant increased levels of ALDOB, CLU and COLEC10, and most decreased levels of ADIPOQ and SHBG. The majority of these proteins are associated with the complement cascade mechanisms, highlighting this pathway as a potential key mediator of MASLD physiopathology.

Further studies in larger and diverse cohorts are warranted to validate these findings and to explore their contribution to the pathophysiological mechanisms of MASLD.

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Informed consent: Informed consent was obtained from all individuals included in this study, or their legal guardians or wards.

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