N-glycoproteomic analysis of Ginsenoside Rb1 on a hyperlipidemia rat model

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N-glycoproteomic analysis of Ginsenoside Rb1 on a hyperlipidemia rat model

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Abstract

Background: Ginsenoside Rb1, known as Renshen in traditional Chinese medicine, is one of the major bioactive saponins isolated from Panax ginseng C.A.Mey. N-glycosylation is the most common type of post-translational modification in cells. The widespread localization of N-glycosylated proteins (N-glycoproteins) between extracellular spaces and on the cell surfaces give them unique advantages as disease biomarkers and drug targets. Previous study found that Ginsenoside Rb1 could potentially play a preventive role in hyperlipidemia. This study aims to reveal the hypolipidemic effect at the protein modification level.

Methods: 24 male SD rats were randomly devided into 3 groups: control group (CON), hight fat diet group (HFD) and Ginsenoside Rb1 group (Rb1). Both HFD and Rb1 groups were fed with high-fat diet for 12 weeks. The Rb1 group started intragastric administering Ginsenoside Rb1 200 mg • kg^{-1} • d^{-1} at 5^{th} week for 8 weeks, while the CON and HFD group the same amount of normal saline for the same

amount of time. Lipid levels and liver histology were assayed to evaluate the effects of Ginsenoside Rb1 intake on hyperlipidemia rats. Furthermore, the workflow by combination of isotope TMT labeling, HILIC enrichment, and high-resolution LC-MS/MS analysis were employed to exploring the mechanisms of regulation role in hyperlipidemia rats.

Results: The histopathologic characteristics and biochemical data shows that Ginsenoside Rb1 exhibited regulative effects on hyperlipidemia rats. After being analyzed by N-glycoproteomic, 98 differential N-glycosylation sites on 53 glycoproteins between 2 comparison groups (HFD: CON, Rb1: HFD) were identified. Analyses of N-glycosylation sites distribution found thatalbumin (Alb) and Serpinc1 were most heavily modified with 6 N-glycosylation sites changed in this work. GO enrichment analysis showed that differential modified glycoproteins were involved in inflammatory response, cellular iron ion homeostasis and positive regulation of cholesterol efflux etc. biosynthetic process. Complement and coagulation cascades was the most significant enriched in the KEGG pathway enrichment analysis.

Conclusions: This study presents a comprehensive analysis of a new set of N-glycoproteins which are altered by Ginsenoside Rb1 and offers some valuable clues for novel mechanistic insights into the ragulative mechanism of Ginsenoside Rb1. Results from N-glycoproteomic suggest that to suppress hyperlipidemia, Rb1 may regulates N-glycosylation of Alb, Serpinc1, PON1, Lrp1, Cp and THBS1, as well as differentially modified glycoproteins in complement and coagulation cascades, which in turn improve the imbalance of lipid homeostasis.

Keywords: N-glycoproteomic; Ginsenoside Rb1; hyperlipidemia; high fat diet

Background

Dyslipidemia is a disease characterized by the overreach of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG) and lower of high-density lipoprotein cholesterol (HDL-C) compare to normal [1]. Worldwide, the incidence of dyslipidemia is 34% to 60%[2, 3]. Dyslipidemia is a risk factor for cardiovascular disease, type 2 diabetes, and other diseases, for which is the leading

cause of disease death around the world[4-6]. Even though hypolipidemic medications can lower blood lipid levels, they are constrained because of the absence of safety[7]. There is an urgent need to develop an effective and safer drug for the prevention and treatment of hyperlipidemia.

Ginsenoside Rb1 (Rb1) is one of the major bioactive saponins isolated from Panax ginseng C.A.Mey, and is known as Renshen in traditional Chinese medicine. Numerous studies have indicated that Ginsenoside Rb1 possesses a variety of biological activities, including, but not limited to, anti-Diabetic, anti-aging, anti-depressant, and myocardial protection[8-11]. In a previous study, Rb1 treatment reduced TC, TG, and LDL-C levels in apoE$^{-/-}$ mice fed with a high fat-diet[12]. Recent animals and cell models show that Rb1 has anti-atherosclerosis and anti-obesity effects. For example, Rb1 enhances atherosclerotic plaque stability by improving autophagy and lipid metabolism in macrophage foam cells[13], Rb1 improves leptin sensitivity in the prefrontal cortex in obese mice[14]. Our previous study found that Rb1 could potentially play a preventive role in hyperlipidemia[15]. However, the effect of Rb1 on N-glycosylation of plasma proteins in hyperlipidemia rats has not been studied.

Recent studies show that protein glycosylation plays an important role in maintaining lipid homeostasis[16-18]. Protein glycosylation is one of the most common post-translational processes of proteins, and more than 50% of mammalian proteins are glycosylated[19]. Plasma proteins are mostly modified by glycans. Furthermore, since plasma proteins are derived from tissues and organs, their properties are affected by the physiological or pathological conditions of various tissues and organs, indicating that plasma proteins and their glycans are good targets for monitoring healthy conditions[19]. Attributed to the structural variation of glycans, N-glycoproteins play important roles in cell recognition, Signal transduction, maintenance of plasma protein stability, and other biological processes[21]. With the continuous improvement and smarter use of liquid chromatography and tandem mass spectrometry (LC-MS/MS) instruments, as well as a wider selection of specialized software, the next milestone in glybiometric analysis, one of the last frontiers of

proteomics, is imminent[22].

In this study, N-glycoproteomic quantification based on a workflow by combination of isotope TMT labeling, HILIC enrichment, and high-resolution LC-MS/MS were applied to investigate the effect of dyslipidemia on N-glycosylation of plasma protein and regulative mechanism of Ginsenoside Rb1 on high fat diet induced hyperlipidemia rats.

Methods

Animals and sample

The Ethics Committee of Liaoning University of Traditional Chinese Medicine approved and supervised the research protocol (Approval number 2019022). 24 Rats (Liaoning Changsheng bio-technology Co., Ltd.) were divided into 3 groups, including the Control group (CON), Hight fat diet group (HFD), and Ginsenoside Rb1group (Rb1). The rats in the Control group were fed with a regular balanced diet, while those in HFD and Rb1 were fed with high-fat diet (10% lard, 1% cholesterol, 0.5% sodium cholic acid, sulfur 0.2% methyl oxygen pyrimidine, 5% sucrose and 83.3% common feed). After feeding for 4 weeks, the rats in Rb1 received Ginsenoside Rb1 (Xi 'an tianfeng biotechnology co. LTD) 200 mg • kg^{-1} • d^{-1} by intragastric administration for 8 weeks, while those in the CON and HFD received the same amount of normal saline for the same amount of time.

The rats were sacrificed after 10% chloral hydrate anesthesia at the end of 12th week. Blood from the abdominal aorta was collected and placed in an anticoagulant tube. After standing for 30 min at room temperature, the blood was centrifuged at 3,500 r/min for 25 min. The serum and plasma were collected and stored at -80℃. After all rats were sacrificed, small cuboids around 5 × 5 × 2-3 mm were cut out from the middle part of the right liver lobe were fixed with 4% paraformaldehyde solution while others were preserved at -80℃ to subsequent analyses.

Lipid measurement

The levels of TG, LDL-C, TC, and HDL-C in blood samples were determined by automatic TBA-120FR biochemical analyzer (Toshiba Corporation, Tokyo, Japan).

Liver histological examination

Hepatic tissues were fixed with 4% paraformaldehyde solution for 24 h for liver histological examination. Then the hepatic tissues embedded with paraffin were stained with hematoxylin and eosin (H&E), using standard techniques. In order to observe the accumulation of lipids in liver tissue more intuitively. Frozen sections of liver tissue (6 μ m for each section) were generated and stained with Oil Red O-hematoxylin. The samples were washed with 50% ethanol, stained with Oil Red O for 8 min, differentiated with 50% ethanol, rinsed with running water, and install with glycerin glue. The slices are examined and photographs are taken using an optical microscope attached to a digital CCD camera.

Protein extraction

The sample was removed from -80℃, centrifuged for 10 minutes at 4℃at 12000 g, and cell fragments were removed. The supernatant was transferred to a new centrifuge tube. The Kit produced by Bio-rad company was used to remove high protein enrichment according to the instructions of ProteoMiner™ Protein Enrichment Small Capacity Kit (Bio-rad). The BCA kit was used to determine the protein concentration.

Trypsin digestion

For digestion, the protein solution was reduced with 5 mm dithiothreitol for 30 min at 56 ℃ and alkylated with 11 mm iodoacetamide for 15 min at room temperature in darkness. The protein sample was then diluted by adding 100 mm TEAB to urea concentration less than 2M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight at 37 ℃ and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion.

TMT labeling

After trypsin digestion, peptide was desalted by Strata X C18 SPE column (Phenomenex) and vacuum-dried. Peptide was reconstituted in 0.5 M TEAB and processed according to the manufacturer’s protocol for TMT kit. Briefly, one unit of TMT reagent were thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted and dried by vacuum centrifugation.

HPLC fractionation

The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 (5 m particle size, 4.6 mm inner diameter, 250 mm long).

Affinity enrichment

The peptides were dissolved in a 40 L enrichment buffer solution (80% acetonitrile /1% trifluoroacetic acid) and then transferred to a supernatant HILIC microcolumn and centrifuged at 4000 g for about 15 min. Then the hydrophilic microcolumn was washed 3 times with enrichment buffer. Then 10% acetonitrile eluent was used to deglycopeptide, the eluent was collected and vacuum frozen. After being drained, the mixture was redissolved in 50 mm ammonium bicarbonate buffer dissolved in 50 L dioxyethylene water, and 2 L PNGase F glycosidic enzyme was added. The enzyme was digested overnight at 37 ℃ . Finally, according to the C18 ZipTips instruction, salt was removed, and the supply liquid and mass were analyzed after vacuum freezing and drying.

LC-MS/MS analysis

The peptides were dissolved in liquid chromatography (HPLC) mobile phase A and then separated using an EASY-NLC 1000 ULTRA high-performance liquid system. Mobile phase A was an aqueous solution containing 0.1% formic acid and 2% acetonitrile. Mobile phase B is an aqueous solution containing 0.1% formic acid and 90% acetonitrile. Liquid phase gradient setting: 0-24 min, 10%~25%B; 24-32 min, 25%~35%B; 32-36 min, 35%~80%B; 36-40 min, 80%B, flow rate maintained at 400 nL/min. The peptides were separated by an ULTRA high-performance liquid system and injected into an NSI ion source for ionization and then analyzed by Q Exactive Plus mass spectrometry. The ion source voltage was set to 2.0kV, and the peptide parent ion and its secondary fragments were detected and analyzed by using high resolution Orbitrap. The scanning range of primary mass spectrometry was set as 350-1800 m/z, and the scanning resolution was set as 70,000. The scanning range of the secondary mass spectrometry was fixed at 100 m/z, and the resolution of the secondary mass spectrometry was set at 35,000. In the data acquisition mode, a

data-dependent scanning program was used, that is, the first 20 peptide parent ions with the highest signal intensity were selected successively into the HCD collision pool after the first-stage scanning, and the fragmentation energy of 30% was used for fragmentation, and secondary mass spectrometry analysis was also performed in turn. In order to improve the effective utilization of the mass spectrum, automatic gain control is set to 5E4, the signal threshold is set to 10000 IONS /s, maximum injection time is set to 100 ms, and dynamic exclusion time of tandem mass scan is set to 30 seconds to avoid repeated scan of the parent ions.

Database search and mass spectrometry quality control test

Secondary mass spectrometry data were retrieved using Maxquant (V1.5.2.8). The retrieval parameters were set to Rattus_norvegicus (29955 sequences), an inverse library was added to calculate the false positive rate due to random matching, and a common contamination library was added to the database to eliminate the influence of contaminated proteins in the identification results. The enzyme digestion method was set as Trypsin/P; The number of missing bits was set to 2; The minimum length of the peptide segment was set as 7 amino acid residues. The maximum modification number of peptide segment was set as 5. The mass error tolerance of the primary parent ion of First search and Main Search was set as 20 PPM and 5 PPM respectively, and the mass error tolerance of the secondary fragment ion was 0.02 Da. The alkylation of cysteine was fixed, and the variable modification was methionine oxidation, n-terminal acetylation, deamidation, and deamidation (18O) of aspartate. The quantitative method was set as TMT-10plex, and the PROTEIN identification and PSM identification FDR were set as 1%.

Bioinformatics methods

Bioinformatics analysis were performed to construct annotations and for differentially modified proteins. Gene Ontology (GO) annotation proteome was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to annotate protein pathway. Firstly, using KEGG online service tools KAAS to annotated protein's KEGG database description. Then mapping the annotation result on the KEGG pathway database using KEGG

online service tools KEGG mapper. P values were used to test the reliability of the analysis.

Statistical analysis

Data are presented as the mean ± SD. Differences among the groups were evaluated with the ANOVA test for multiple groups using Prism GraphPad software (San Diego, CA, USA). P-values below 0.05 were considered statistically significant.

Results

Ginsenoside Rb1 alleviated serum lipid level and liver lipid deposition in hyperlipidemia rats

To evaluate whether Rb1 ragulates dyslipidemia by a high-fat diet, the levels of serum lipid were monitored in this study. Comparing to CON, HFD showed a marked increase in TC, TG, LDL-C level and decrease in HDL-C level (p<0.01), while Rb1 significant decreased the levels of TC, TG, LDL-C and increased HDL-C level compared to HFD (p<0.01) (Figure. 1). H&E and Oil red O staining showed that treatment with Ginsenosiduate Rb1marked attened HFD-iduced lipid deposition in liver which is consistent with the result of serum lipid (Figure. 2). All of these results suggested that Rb1 can improve the lipid metabolism disorder in hyperlipidemia rats caused by high fat diet.

Quantitative analysis of N-glycoproteome in hyperlipidemia rat plasma

In order to explore the potential molecular mechanisms of Rb1 in hyperlipidemia, N-glycoproteome method was performed for detecting differential modified proteins at N-glycosylation scale in each group (CON, HFD and Rb1). We identified a total of 603.0 glycation modification sites on 251.0 glycoproteins, among which 576.0 sites on 241.0 glycoproteins had quantitative information (Supporting Information Table 1). Further, 244 (up-regulated: 174, down-regulated: 70) differential N-glycosylation sites in HFD: CON and 135 (up-regulated: 88, down-regulated: 47) differential N-glycosylation sites in Rb1: HFD were identified respectively (Table 1, Supporting Information Table 2). Volcano plot and clustering analysist showed the details of 98 differential N-glycosylation sites (Figure 3A and 3B) on 53 glycoproteins (Figure 3C

239 and 4D) among 2 comparison groups (Supporting Information Table 3). These
240 co-altered glyproteins may be important to elucidate the mechanism by which Rb1
241 improves hyperlipidemia. Overall, our data suggest that the plasma protein
242 N-glycosylation of hyperlipidemia rats was significantly affected by Rb1.
243
244 **Table 1.** The quantity of differentially expressed glyproteins and sites identified in
245 experiments
246

Sample pairs	N-glycosylation Sites	
	HFD: CON	Rb1: HFD
Identified	603	
Quantified	576	
UP-regulated	174	88
Down-regulated	70	47
Total difference	244	135

247
248 **N-glycosylation sites distribution analyses**
249 It should be indicated that most of differential modified glycoproteins in this work,
250 which account for 51% of all glycoproteins, only one site could be found. However,
251 there are still 21, 11, 17% of glycoproteins identified with two, three, and more than
252 three sites (Fig. 4A). Serpinc1 and Alb were most heavily N-glycosylated with 6
253 N-glycosylation sites identified in this work. Besides, Tf, Cpb2, Lifr, Cp, Map1 and
254 Itih4 were also identified with more than 4 N-glycosylation sites. The N-glycosylation
255 sequence motif was analyzed as shown in Fig.4B and Supporting Information Table 4.
256
257 **GO and KEGG functional enrichment analyses**
258 48 of the 53 differential modified glycoproteins were clearly identified. To know the
259 functional classification of the 48 glycoproteins in the overlaps of HFD: CON and

Rb1: HFD, GO enrichment analysis was performed. The results were shown in Figure 5 and Supporting Information Table 5. In terms of biological process, it is obviously that majority of glycoproteins distributed in the extracellular space (77%), extracellular exosome (77%), blood microparticle (47.9%) and extracellular region (31.25%). It is highly consisted with property of glycoproteins, showing the high confident of the glycoproteome identified in this work. while a few of glycoproteins were localized in membrane attack complex (3%), cell (5%), extracellular matrix (5%), other organism cell (2%), platelet alpha granule (2%), high-density lipoprotein particle (2%) (Figure 5B). Paraoxonase 1 (PON1) is a blood microparticle and also a high-density lipoprotein particle, which is closely related to cholesterol metabolism. Serine-type endopeptidase inhibitor activity, serine-type endopeptidase activity, protease binding, endopeptidase inhibitor activity, glycoprotein binding etc. were enriched in the molecular function aspect (Figure 5C). In terms of biological process, negative regulation of endopeptidase activity, complement activation, classical pathway, blood coagulation, acute-phase response, fibrinolysis, proteolysis, negative regulation of fibrinolysis, innate immune response, inflammatory response, cellular iron ion homeostasis etc. were mainly enriched. In addition, and positive regulation of cholesterol efflux were also enriched into biological processes (Figure 5D). PON1 and LDL receptor-related protein1 (Lrp1) were differential modified glycoproteins in positive regulation of cholesterol efflux process. Compared with CON, the modification levels of PON1 in sites Asn 41 was up-regulated and of Lrp1 in sites Asn 3090 and Asn 115 were down regulated in HFD, and Rb1 can reverse the above differential modification levels. It suggests that N-glycosylation of PON1 and LRP1 is an important mechanism of Rb1 regulating lipid metabolism disorder. Mass spectrograms of PON1 and LRP1 are shown in Figure 6.

Furthermore, to analyze biological pathways that respond to ragulative effect of Rb1 on hyperlipemia, 48 differential modified glycoproteins were investigated using the KEGG database. The KEGG pathway analysis of the quantitatively changed proteins undergoes glycosylation showed one vital pathway-Complement and coagulation cascades (Figure 7A, Supporting Information Table 6). It is found that there were 41sites of 20 proteins changed in this pathway (Figure. 7B).

Discussion

Although our previous study has provided evidence of the therapeutical effect of Rb1 on hyperlipidemia in mice[15]. The mechanism of Rb1 on the modification level of plasma proteins in hyperlipidemia model has not been explored. Rb1 is a kind of glycoside compound, which may be related to protein glycosylation modification. Glycosylation is the most abundant and complex protein modification, and can have a great structural and functional effect on the conjugate. With the development of technology, glycosylation has gradually become the target of finding disease biomarkers for early diagnosis. Additionally, biofluids such as plasma, serum or saliva are of great use in this regard, as they are easily accessible and can provide relevant glycosylation information[23]. Thus, on the basis of confirming the therapeutical effect of Rb1 on hyperlipidemia rats, we further employed the N-glycoproteome of plasma of hyperlipidemia rats to better understand the underlying mechanisms of Rb1 formula in a system-based strategy. For this purpose, we established a quantification workflow for N-glycoproteome by the combination of TMT labeling and HILIC-based enrichment. Our study was the first to reveal aberrant N-glycosylation of plasma glycoproteins in hyperlipidemia rats induced by high fat diet and the intervention mechanism of Rb1, which determined 53 differentially modified proteins and 98 sites.

According to the data, we found that the modification sites of the protein Alb and Serpinc1 are the most altered. Alb is considered as one of the main carriers of fatty acids in the blood and is involved in the transport of long-chain fatty acids from the blood to the cells and mitochondria[24]. Alb binding with fatty acids can reduce the cytotoxicity of fatty acids and reduce the damage of fatty acids to cells. In the present study, the glycosylation level of 6 modification sites (Asn123, Asn291, Asn266, Asn249, Asn415, Asn82) of Alb was down-regulated in HFD. These changes may affect the binding ability of ALB to fatty acids and thus affect lipid homeostasis. Serpinc1 is also known as C1-INH, the main factor that controls classical pathway activation and also play a role in the proteolysis. Studies have found a linear relationship between C1-INH and chronic inflammation, endothelial dysfunction, and

cardiovascular disease[25]. It has been reported that C1-INH can reduce ApoE$^{-\backslash-}$ mice serum TG level and limits neointimal plaque formation and inflammation[26]. In this study, high-fat diet significantly down regulated N-glycosylation modification level of Asn130, Asn225, Asn129, Asn188, Asn168, Asn220 of C1-INH, which may have an impact on the function of C1-INH. Surprisingly, Rb1 was able to reverse the down-regulation of these 12 sites, suggesting that Rb1 may play a therapeutic role by influencing the glycosylation of Alb and Serpinc1.

Besides, we predicted the function of glycoproteins by performance of bioinformatic analysis. According to GO enrichment analysis results, it was found that three biological processes positive regulation of cholesterol efflux, cellular iron ion homeostasis and inflammatory response were closely related to lipid homeostasis. PON1 and Lrp1 were important proteins involved inpositive regulation of cholesterol efflux, their glycosylation level plays an important role in maintaining lipid homeostasis[27, 28]. PON1 is an antioxidant and anti-inflammatory glycoprotein from the paraoxonases family. It is mainly expressed in the liver and secreted to the bloodstream, where it binds to HDL-C. It can be an important determinant of HDL-C dysfunctionality[29]. The glycosylation of PON1 protein affects the normal function of PON1 protein and HDL-C. Marsillach et al have found that serum of patients with chronic liver disease and fatty liver patients carry a large amount of inactive PON1 in the HDL of the liver, and these PON1 proteins were highly glycosylated[30], which coincided with our study. In our study, it was found that the N-glycosylation level of PON1 protein in plasma of hyperlipidemic rats was up-regulated, and Ginsenoside can improve the high glycosylation level of PON1. Lrp1 is a multifunctional cell surface receptor and with diverse physiological roles, ranging from cellular uptake of lipoproteins and other cargo by endocytosis to sensor of the extracellular environment and integrator of a wide range of signaling mechanisms[28]. Lrp1 is also a member of the low density lipoprotein receptor family. All members of the LDLR family contain multiple N-linked glycosylation sites and are post-translationally modified by N-linked glycosylation. Petra May et al suggested a role for differential and tissue-specific glycosylation as a physiological switch that modulates the diverse

biological functions of these receptors in a cell-type specific manner[31]. In our study, it was found that the N-glycosylation level of Lrp1 in plasma of hyperlipidemic rats was dwon-regulated, and Rb1 can improve the low glycosylation level of Lrp1. Cellular iron ion homeostasis interacts with lipid homeostasis[32, 33]. Physiological interaction of iron and lipid obstructs iron efflux and accelerates the lipid accumulation in macrophages during foam cell formation[34]. Ceruloplasmin (Cp) was significantly changed in the three comparison groups. Compared with CON, the glycosylation levels of Asn956, Asn756, Asn710 and Asn396 in HFD were significantly down-regulated, and the glycosylation levels of the 4 sites were reversed after Rb1 treatment. Cp has ferroxidase activity and is an important factor in cellular iron efflux. It has reported that Cp suppresses ferroptosis by regulating iron homeostasis[35]and was found to be downregulated in atherosclerotic plaques. Patients with central obesity have characteristically higher Cp serum levels, and that Cp concentrations were strongly correlated with serum TG and TC levels[36]. Akira et al found 7 N-glycosylation sites of ceruloplasin in human plasma[37]. This study might be a valuable supplement of CP glycoform at the level of experimental animals. In inflammatory response, we found a adipokine-Thrombospondin 1 (THBS1)[38]. THBS1 is an extracellular matrix protein that interacts with a wide array of ligands including CD36 molecule[39]. A recent study found that THBS1 may play a role in inhibiting lipid oversynthesis by acting on CD36 receptors[40]. In these paper, we found Rb1 can reverse the upregulated glycosylation level of THBS1 in plasma of hyperlipidemia rats. Whether Rb1 affects the binding of THBS1 to the receptor by regulating N-glycosylation modification and thus regulates lipid homeostasis is a question that we need to continue to consider.

KEGG pathway analyses showed that pathway-complement and coagulation cascades was the most significant enrichment pathway. The complement system is a part of the innate immune system and can be activated in inflammatory conditions. Hyperlipidemia itself increases the expression of some inflammatory factors[41] and also increases the risk of inflammatory diseases such as periodontitis and acute

pancreatitis[24, 42]. Argilés even found that administrating IL-1 β to rats caused hyperlipidemia[43], suggesting that innate immune system and hyperlipidemia could influence each other. It has also reported that dyslipidemia activates the coagulation system[44]. In hyperlipidemia, the variation in lipid and lipoprotein levels can upset the balance between pro- and anticoagulant pathways, as well as provide a surface for the activation of procoagulation enzymatic complexes. In fact, the inflammatory and procoagulant reactions caused by hyperlipidemia are the key factors leading to the development of atherosclerosis. Therefore, our finding that the changes of protein N-glycosylation of proteins in complement and coagulation cascades pathway may be an important mechanism of hyperlipidemia causing inflammation and procoagulant response, and the reversal of some modifications by Rb1 suggests that it can improve these adverse effects of hyperlipidemia on organism.

Limitations

The limitation of our study is the deficiency of N-glycosylated antibody for further investigation.

Conclusions

This study presents a comprehensive analysis of a new set of N-glycoproteins which are altered by Rb1 and offers some valuable clues for novel mechanistic insights into the ragulative mechanism of Rb1. Results from N-glycoproteomic suggest that to suppress hyperlipidemia, Rb1 may regulates N-glycosylation of Alb, Serpinc1, PON1, Lrp1, Cp and THBS1, as well as differentially modified glycoproteins in complement and coagulation cascades, which in turn improve the imbalance of lipid homeostasis (Fig. 8).

Figure Legends

Figure.1 Effects of Rb1 on the serum levels of TC, TG, LDL-C and HDL-C in hyperlipidemia rats (n=8). CON: control group, HFD: high fat diet group, Rb1: HFD + Rb1 group. Data are presented as the mean ± standard deviation. $^{**}P<0.01$ vs. CON. $^{\#\#}P<0.01$ vs. HFD.

Figure.2 Effects of Rb1 on histopathological examination by H&E and Oil red O

(200×) in hyperlipidemia rats. CON: control group, HFD: high fat diet group, Rb1: HFD + Rb1 group.

Figure.3 Differential N-glycosylation sites and modified glycoproteins. (A) Venn-diagram of differential N-glycosylation sites. (B) Heatmap of differential N-glycosylation sites. (C) Venn-diagram of differential modified glycoproteins. (D) Heatmap of differential modified glycoproteins.

Figure.4 Analyses of N-glycosylation sites distribution and amino acids motifs of the glycoproteome data. (A) Distribution of single and multiple N-glycosylation. (B) Heat map of motif enrichment of amino acids upstream and downstream of glycosylation modification sites. Red indicates significant enrichment of the amino acid near the modification site, while green indicates significant reduction of the amino acid near the modification site.

Figure.5 GO analysis of differentially N-glycosylated modified proteins. (A) GO enrichment cycle diagram. All enriched term with p-value less than 0.05.(B) GO annotation in cellular components. (C) GO annotation in biological processes (D) GO annotation in molecular functions. Left Y-axis represented the enriched term; X-axis indicated gene percent.

Figure.6 Mass spectrogram of PON1 and LRP1. (A) Mass spectrogram of PON1 (Asn 41). (B) Mass spectrogram of LRP1(Asn 3090). (C) Mass spectrogram of LRP1(Asn 115).

Figure.7 KEGG analysis of differentially N-glycosylated modified proteins. (A) KEGG pathway clusting analysis for differentially N-glycosylated modified sites. Group1: Rb1 vs HFD ∩ HFD vs CON, Group2: (HFD vs CON)\ (Rb1 vs HFD), Group3: (Rb1 vs HFD)\ (HFD vs CON). (B) Pathway-Complement and coagulation cascades by the KEGG pathway analysis. The proteins in blue represent are altered in N-glycosylation modification.

Figure.8 The possible mechanism of Ginsenoside Rb1 regulating N-glycosylation of plasma protein in hyperlipidemia rats. The red and blue arrows indicate changes in the glycosylation modification level, red represents changes in the HFD group and blue represents changes in the Rb1 group.

Supplementary information

Additional file: **Table S1.** MS identified information. **Table S2.** Differentially expressed statistics. **Table S3.** Site venn results. **Table S4.** N.motif.model. **Table S5.** GO analysis. **Table S6.** KEGG pathway enrichment.

Abbreviations

N-glycoproteins: N-glycosylated proteins

CON: control group

HFD: hight fat diet group

Rb1: Ginsenoside Rb1 group

Alb: albumin

TC: total cholesterol

LDL-C: low-density lipoprotein cholesterol

TG: triglycerides

HDL-C: lower of high-density lipoprotein cholesterol

H&E: hematoxylin and eosi

Gene Ontology: GO

Kyoto Encyclopedia of Genes and Genomes: KEGG

PON1: Paraoxonase 1

Lrp1: LDL receptor-related protein1

Cp: Ceruloplasmin

THBS1: Thrombospondin 1

Declarations

Ethics approval and consent to participate

The Ethics Committee of Liaoning University of Traditional Chinese Medicine approved and supervised the research protocol (Approval number 2019022).

Consent to publish

Not applicable.

Availability of data and materials

The datasets used in our study are available from the corresponding author on reasonable request.

Competing Interests

The authors declared no conflicts of interest.

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Authors' Contributions

Ma Yixin contributed equally with Ning Shunyu, and is the co-first author of this article. The authors Ma Yixin and Ning Shunyu were responsible for writing articles, SN was responsible for experimental design, Chen Si and Leng Xue were responsible for experimental operations, Jia Lianqun and Yang Guanlin guided the design of the experiments and was responsible for modifications of the paper.

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Reference

[1]. Karr, S., Epidemiology and management of hyperlipidemia. Am J Manag Care, 2017. 23(9 Suppl): p. S139-S148.

[2]. Toth, P.P., D. Potter and E.E. Ming, Prevalence of lipid abnormalities in the United States: the National Health and Nutrition Examination Survey 2003-2006. J Clin Lipidol, 2012. 6(4): p. 325-30.

[3]. Pan, L., et al., The prevalence, awareness, treatment and control of

dyslipidemia among adults in China. Atherosclerosis, 2016. 248: p. 2-9.

[4]. Talayero, B.G. and F.M. Sacks, The role of triglycerides in atherosclerosis. Curr Cardiol Rep, 2011. 13(6): p. 544-52.

[5]. Iqbal, J., et al., Metabolic Syndrome, Dyslipidemia and Regulation of Lipoprotein Metabolism. Curr Diabetes Rev, 2018. 14(5): p. 427-433.

[6]. El-Tantawy, W.H. and A. Temraz, Natural products for controlling hyperlipidemia: review. Arch Physiol Biochem, 2019. 125(2): p. 128-135.

[7]. El-Ganainy, S.O., et al., A novel investigation of statins myotoxic mechanism: effect of atorvastatin on respiratory muscles in hypoxic environment. Toxicol Lett, 2019. 305: p. 58-64.

[8]. Zhou, P., et al., Ginsenoside Rb1 as an Anti-Diabetic Agent and Its Underlying Mechanism Analysis. Cells, 2019. 8(3).

[9]. Cheng, Y., L.H. Shen and J.T. Zhang, Anti-amnestic and anti-aging effects of ginsenoside Rg1 and Rb1 and its mechanism of action. Acta Pharmacol Sin, 2005. 26(2): p. 143-9.

[10]. Wang, G.L., et al., Monoaminergic and aminoacidergic receptors are involved in the antidepressant-like effect of ginsenoside Rb1 in mouse hippocampus (CA3) and prefrontal cortex. Brain Res, 2018. 1699: p. 44-53.

[11]. Zheng, Q., et al., Ginsenoside Rb1 for Myocardial Ischemia/Reperfusion Injury: Preclinical Evidence and Possible Mechanisms. Oxid Med Cell Longev, 2017. 2017: p. 6313625.

[12]. Zhou, P., et al., Inhibitory Effects of Ginsenoside Rb1 on Early Atherosclerosis in ApoE-/- Mice via Inhibition of Apoptosis and Enhancing Autophagy. Molecules, 2018. 23(11).

[13]. Qiao, L., et al., Ginsenoside Rb1 Enhances Atherosclerotic Plaque Stability by Improving Autophagy and Lipid Metabolism in Macrophage Foam Cells. Front Pharmacol, 2017. 8: p. 727.

[14]. Wu, Y., et al., Ginsenoside Rb1 improves leptin sensitivity in the prefrontal cortex in obese mice. CNS Neurosci Ther, 2018. 24(2): p. 98-107.

[15]. Lianqun, J., et al., Comprehensive multiomics analysis of the effect of

530 ginsenoside Rb1 on hyperlipidemia. Aging (Albany NY), 2021. 13.
531 [16]. van den Boogert, M., et al., N-Glycosylation Defects in Humans Lower
532 Low-Density Lipoprotein Cholesterol Through Increased Low-Density Lipoprotein
533 Receptor Expression. Circulation, 2019. 140(4): p. 280-292.
534 [17]. Ke, L.Y., et al., Increased APOE glycosylation plays a key role in the
535 atherogenicity of L5 low-density lipoprotein. FASEB J, 2020. 34(7): p. 9802-9813.
536 [18]. William, J.A., et al., Crosstalk between protein N-glycosylation and lipid
537 metabolism in Saccharomyces cerevisiae. Sci Rep, 2019. 9(1): p. 14485.
538 [19]. Dai, Z., et al., Lectin-based glycoproteomics to explore and analyze
539 hepatocellular carcinoma-related glycoprotein markers. Electrophoresis, 2009. 30(17):
540 p. 2957-66.
541 [20]. Miura, Y., et al., Change in N-Glycosylation of Plasma Proteins in Japanese
542 Semisupercentenarians. PLoS One, 2015. 10(11): p. e0142645.
543 [21]. Ohtsubo, K. and J.D. Marth, Glycosylation in cellular mechanisms of health
544 and disease. Cell, 2006. 126(5): p. 855-67.
545 [22]. Zhang, S., et al., Quantification of N-glycosylation site occupancy status
546 based on labeling/label-free strategies with LC-MS/MS. Talanta, 2017. 170: p.
547 509-513.
548 [23]. Mammadova-Bach, E., et al., Platelets and Defective N-Glycosylation. Int J
549 Mol Sci, 2020. 21(16).
550 [24]. Guo, C. and H.X. Zhou, Fatty Acids Compete with Abeta in Binding to
551 Serum Albumin by Quenching Its Conformational Flexibility. Biophys J, 2019. 116(2):
552 p. 248-257.
553 [25]. Hertle, E., et al., Classical Pathway of Complement Activation: Longitudinal
554 Associations of C1q and C1-INH With Cardiovascular Outcomes: The CODAM
555 Study (Cohort on Diabetes and Atherosclerosis Maastricht)-Brief Report. Arterioscler
556 Thromb Vasc Biol, 2018. 38(5): p. 1242-1244.
557 [26]. Shagdarsuren, E., et al., C1-esterase inhibitor protects against neointima
558 formation after arterial injury in atherosclerosis-prone mice. Circulation, 2008.
559 117(1): p. 70-8.

560 [27]. Berrougui, H., S. Loued and A. Khalil, Purified human paraoxonase-1
561 interacts with plasma membrane lipid rafts and mediates cholesterol efflux from
562 macrophages. Free Radic Biol Med, 2012. 52(8): p. 1372-81.
563 [28]. Xian, X., et al., LRP1 integrates murine macrophage cholesterol homeostasis
564 and inflammatory responses in atherosclerosis. Elife, 2017. 6.
565 [29]. Variji, A., et al., The combined utility of myeloperoxidase (MPO) and
566 paraoxonase 1 (PON1) as two important HDL-associated enzymes in coronary artery
567 disease: Which has a stronger predictive role? Atherosclerosis, 2019. 280: p. 7-13.
568 [30]. Marsillach, J., et al., Decreased paraoxonase-1 activity is associated with
569 alterations of high-density lipoprotein particles in chronic liver impairment. Lipids
570 Health Dis, 2010. 9: p. 46.
571 [31]. May, P., et al., Differential glycosylation regulates processing of lipoprotein
572 receptors by gamma-secretase. J Biol Chem, 2003. 278(39): p. 37386-92.
573 [32]. Li, D. and Y. Li, The interaction between ferroptosis and lipid metabolism in
574 cancer. Signal Transduct Target Ther, 2020. 5(1): p. 108.
575 [33]. Forcina, G.C. and S.J. Dixon, GPX4 at the Crossroads of Lipid Homeostasis
576 and Ferroptosis. Proteomics, 2019. 19(18): p. e1800311.
577 [34]. Wang, Q., et al., Iron Together with Lipid Downregulates Protein Levels of
578 Ceruloplasmin in Macrophages Associated with Rapid Foam Cell Formation. J
579 Atheroscler Thromb, 2016. 23(10): p. 1201-1211.
580 [35]. Shang, Y., et al., Ceruloplasmin suppresses ferroptosis by regulating iron
581 homeostasis in hepatocellular carcinoma cells. Cell Signal, 2020. 72: p. 109633.
582 [36]. Cignarelli, M., et al., Relationship of obesity and body fat distribution with
583 ceruloplasmin serum levels. Int J Obes Relat Metab Disord, 1996. 20(9): p. 809-13.
584 [37]. Harazono, A., et al., Site-specific N-glycosylation analysis of human plasma
585 ceruloplasmin using liquid chromatography with electrospray ionization tandem mass
586 spectrometry. Anal Biochem, 2006. 348(2): p. 259-68.
587 [38]. Varma, V., et al., Thrombospondin-1 is an adipokine associated with obesity,
588 adipose inflammation, and insulin resistance. Diabetes, 2008. 57(2): p. 432-9.
589 [39]. Asch, A.S., et al., Isolation of the thrombospondin membrane receptor. J Clin

590 Invest, 1987. 79(4): p. 1054-61.
591 [40]. Bai, J., et al., Thrombospondin 1 improves hepatic steatosis in diet-induced
592 insulin-resistant mice and is associated with hepatic fat content in humans.
593 EBioMedicine, 2020. 57: p. 102849.
594 [41]. Papapanagiotou, A., et al., Novel Inflammatory Markers in Hyperlipidemia:
595 Clinical Implications. Curr Med Chem, 2015. 22(23): p. 2727-43.
596 [42]. Zhou, X., et al., Interrelationship between diabetes and periodontitis: role of
597 hyperlipidemia. Arch Oral Biol, 2015. 60(4): p. 667-74.
598 [43]. Argiles, J.M., et al., Interleukin-1 and lipid metabolism in the rat. Biochem J,
599 1989. 259(3): p. 673-8.
600 [44]. Karpe, F., Mechanisms of postprandial hyperlipidaemia--remnants and
601 coronary artery disease. Diabet Med, 1997. 14 Suppl 3: p. S60-6.

Figures

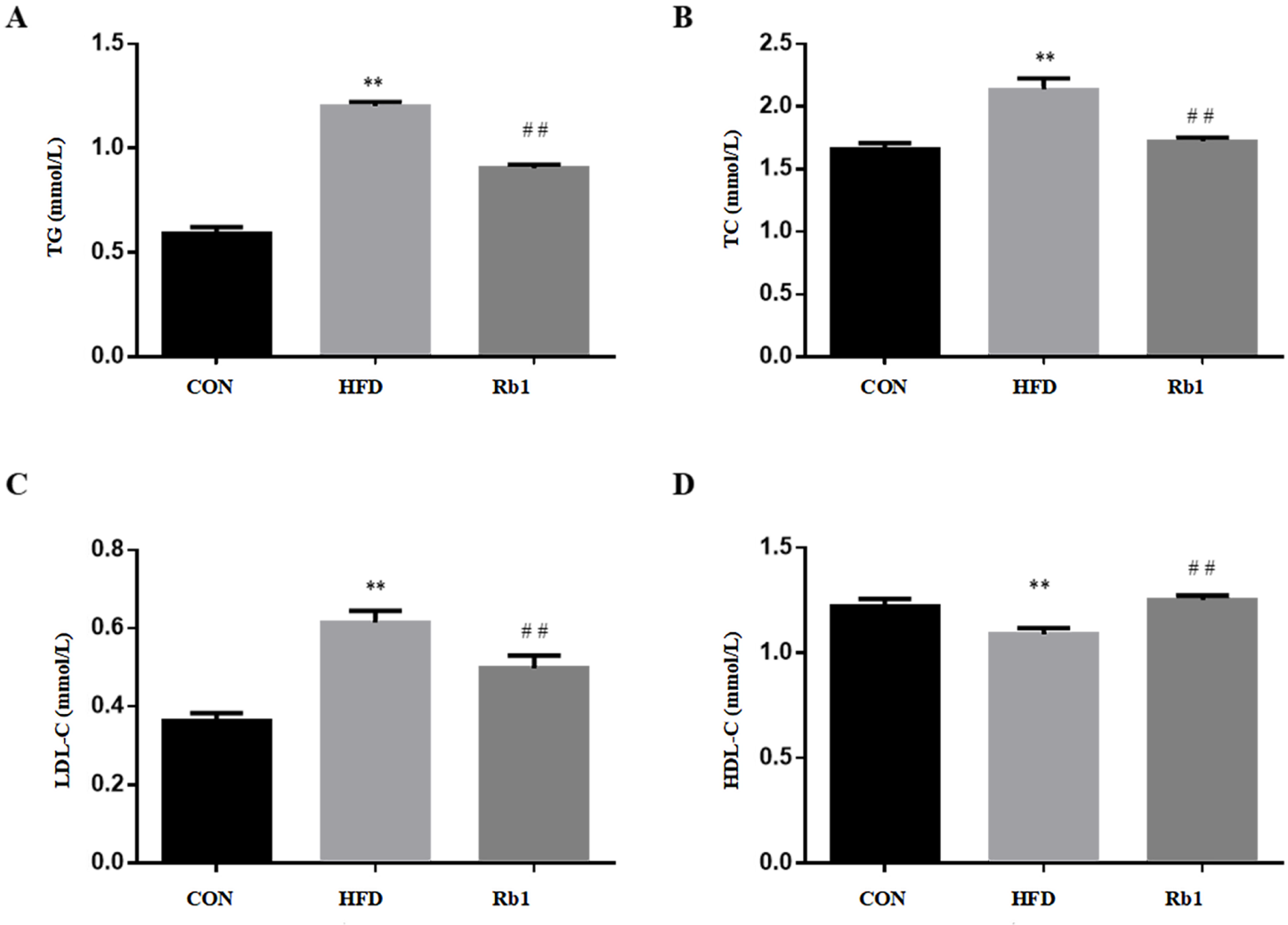

Figure 1

Effects of Rb1 on the serum levels of TC, TG, LDL-C and HDL-C in hyperlipidemia rats (n=8). CON: control group, HFD: high fat diet group, Rb1: HFD + Rb1 group. Data are presented as the mean ± standard deviation. **P<0.01 vs. CON. ##P<0.01 vs. HFD.

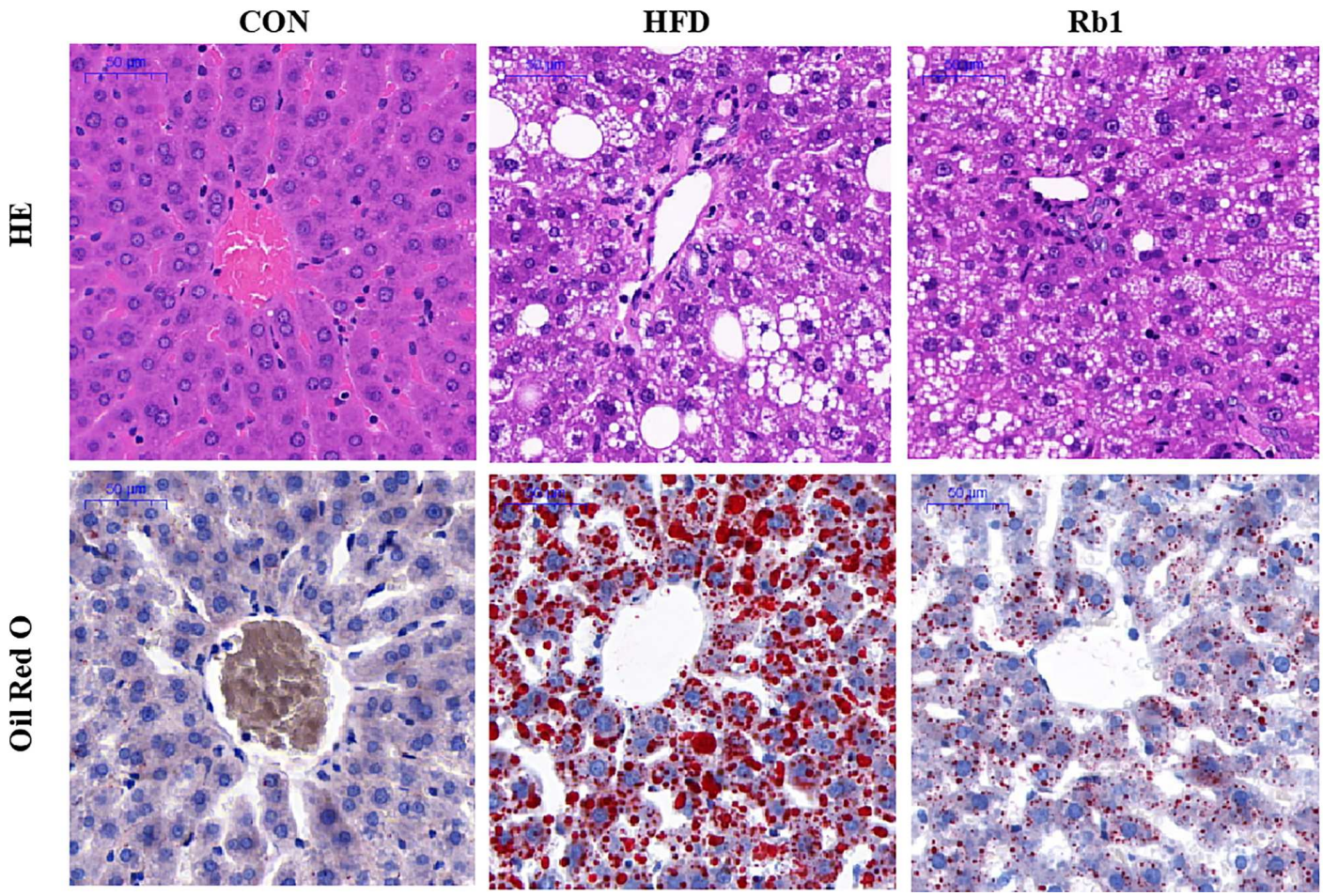

Figure 2

Effects of Rb1 on histopathological examination by H&E and Oil red O (200×) in hyperlipidemia rats. CON: control group, HFD: high fat diet group, Rb1: HFD + Rb1 group.

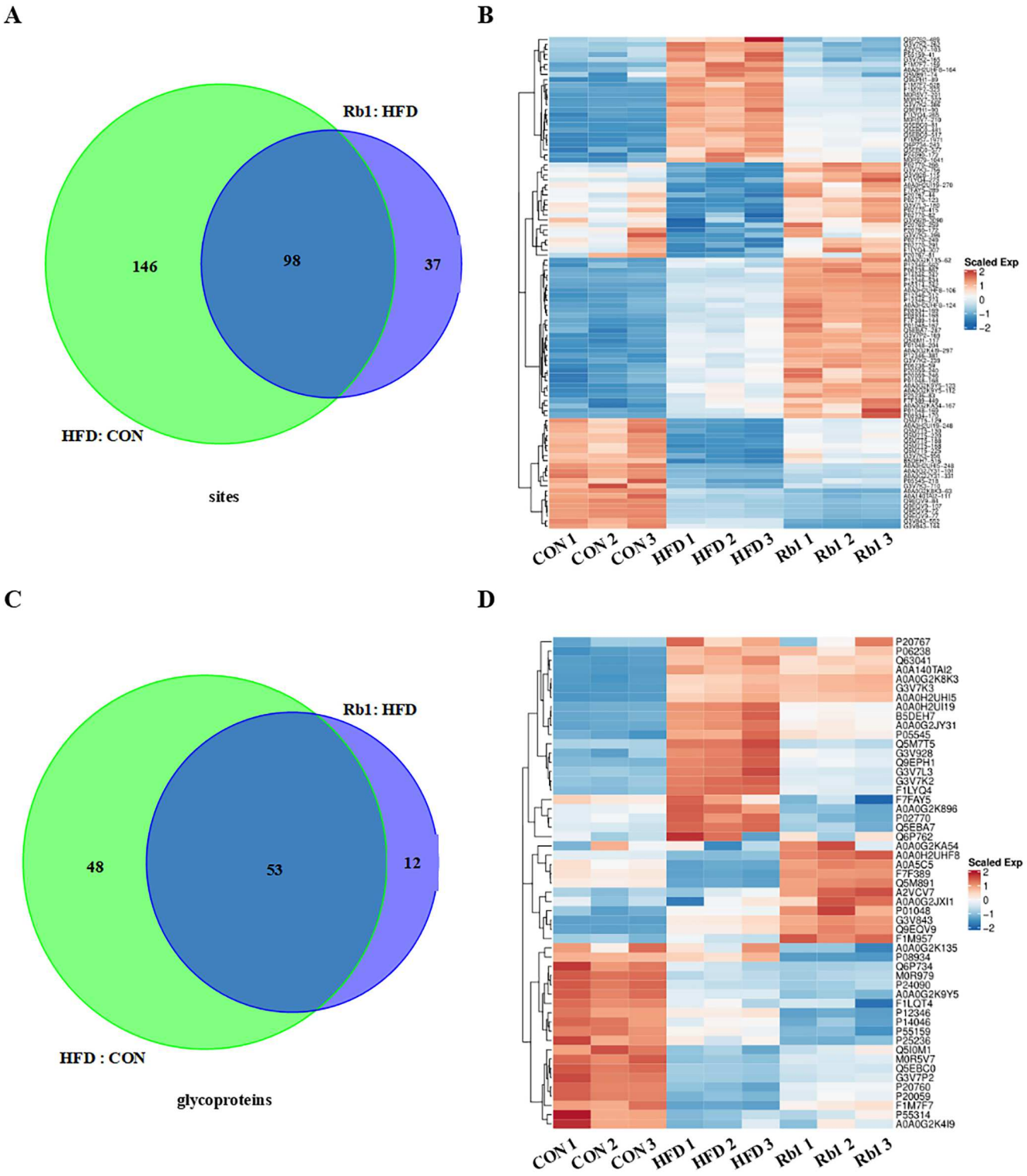

Figure 3

Differential N-glycosylation sites and modified glycoproteins. (A) Venn-diagram of differential N-glycosylation sites. (B) Heatmap of differential N-glycosylation sites. (C) Venn-diagram of differential modified glycoproteins. (D) Heatmap of differential modified glycoproteins.

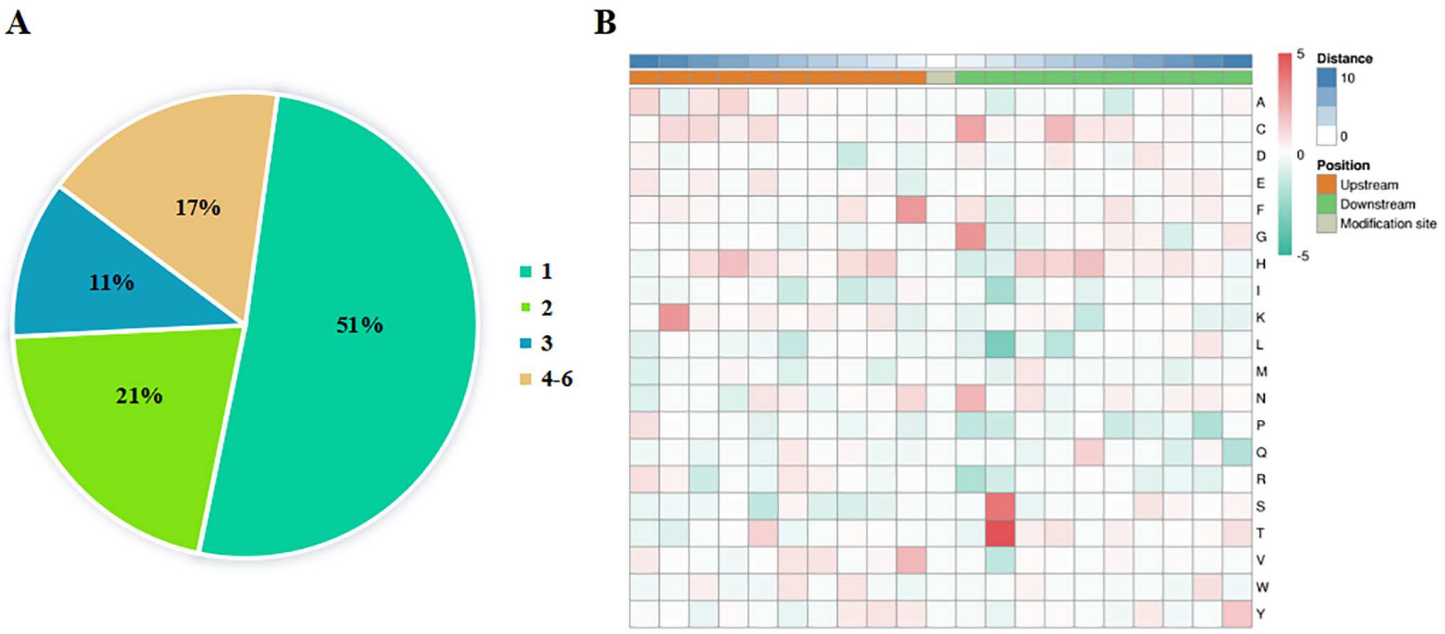

Figure 4

Analyses of N-glycosylation sites distribution and amino acids motifs of the glycoproteome data. (A) Distribution of single and multiple N-glycosylation. (B) Heat map of motif enrichment of amino acids upstream and downstream of glycosylation modification sites. Red indicates significant enrichment of the amino acid near the modification site, while green indicates significant reduction of the amino acid near the modification site.

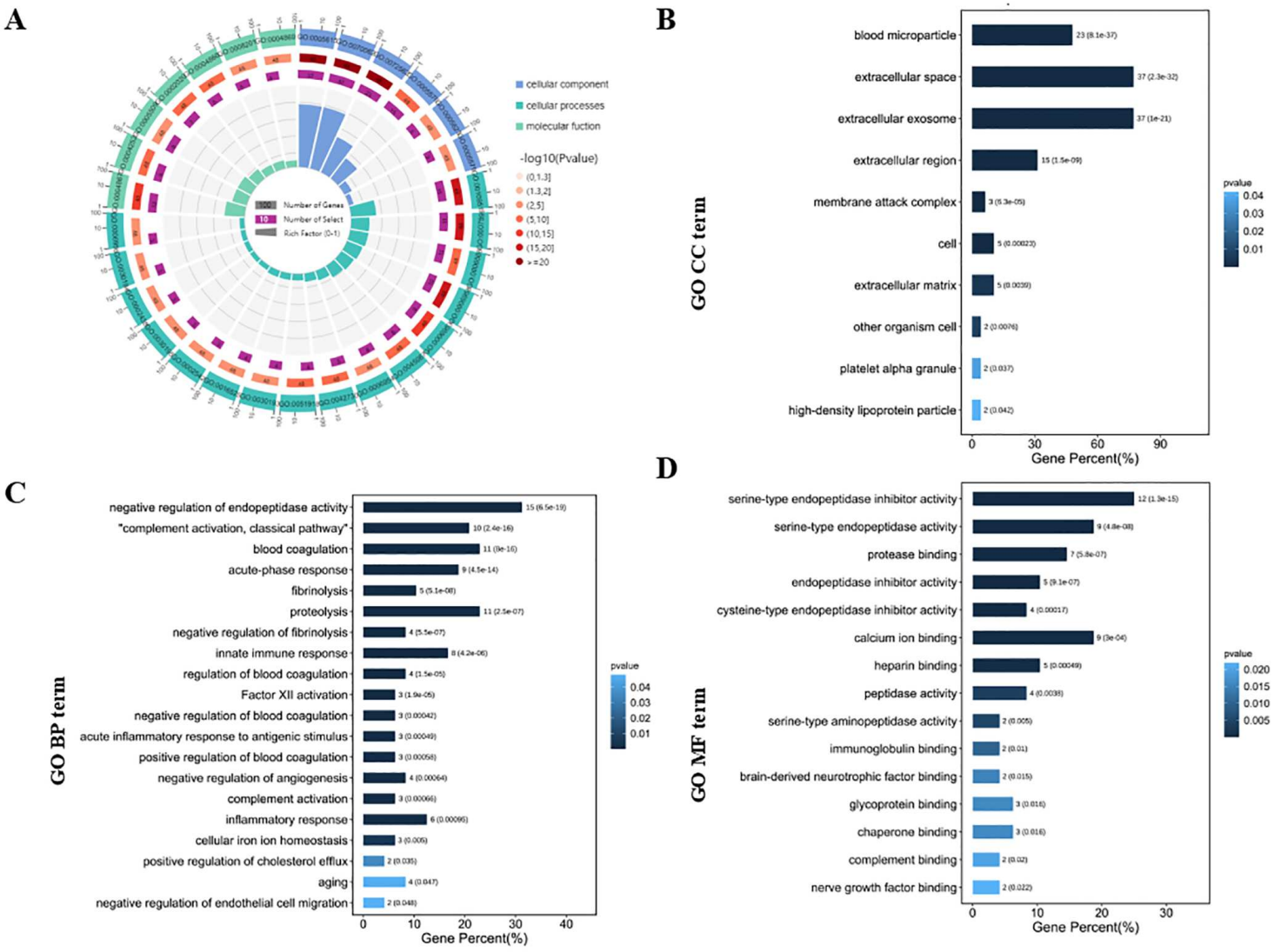

Figure 5

GO analysis of differentially N-glycosylated modified proteins. (A) GO enrichment cycle diagram. All enriched term with p-value less than 0.05.(B) GO annotation in cellular components. (C) GO annotation in biological processes (D) GO annotation in molecular functions. Left Y-axis represented the enriched term; X-axis indicated gene percent.

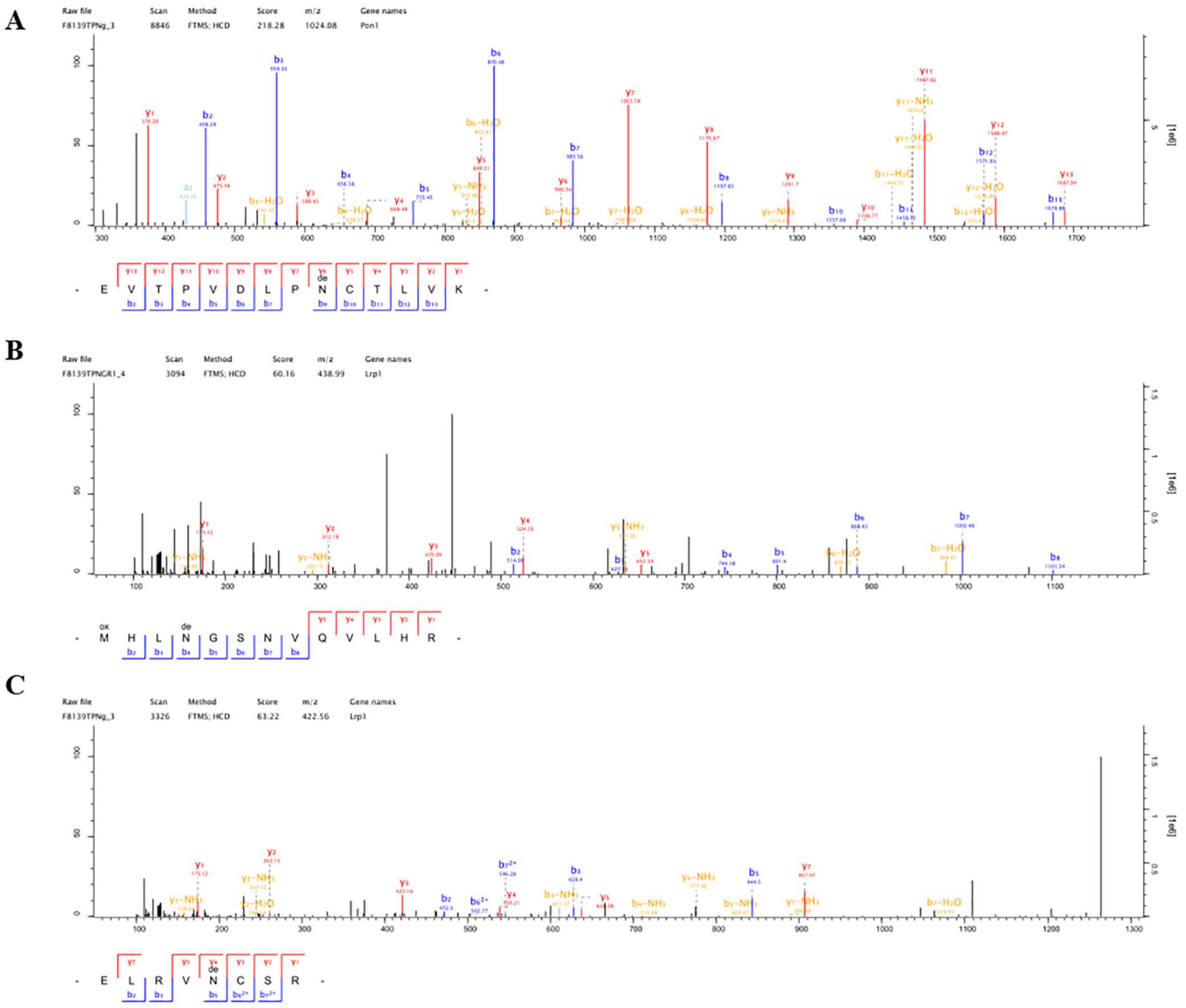

Figure 6

Mass spectrogram of PON1 and LRP1. (A) Mass spectrogram of PON1 (Asn 41). (B) Mass spectrogram of LRP1(Asn 3090). (C) Mass spectrogram of LRP1(Asn 115).

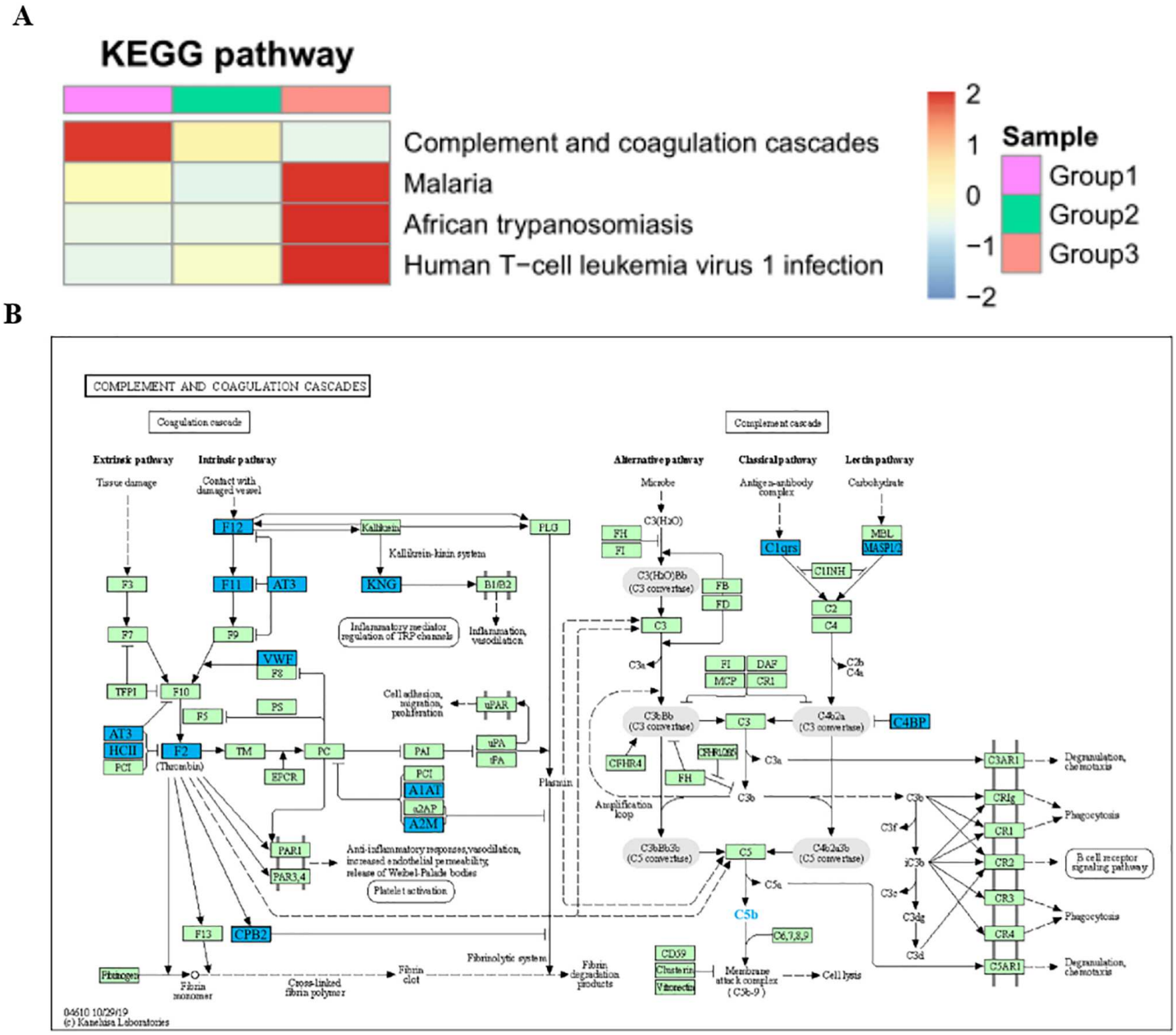

Figure 7

KEGG analysis of differentially N-glycosylated modified proteins. (A) KEGG pathway clusting analysis for differentially N-glycosylated modified sites. Group1: Rb1 vs HFD HFD vs CON, Group2: (HFD vs CON) (Rb1 vs HFD), Group3: (Rb1 vs HFD) (HFD vs CON). (B) Pathway-Complement and coagulation cascades by the KEGG pathway analysis. The proteins in blue represent are altered in N-glycosylation modification.

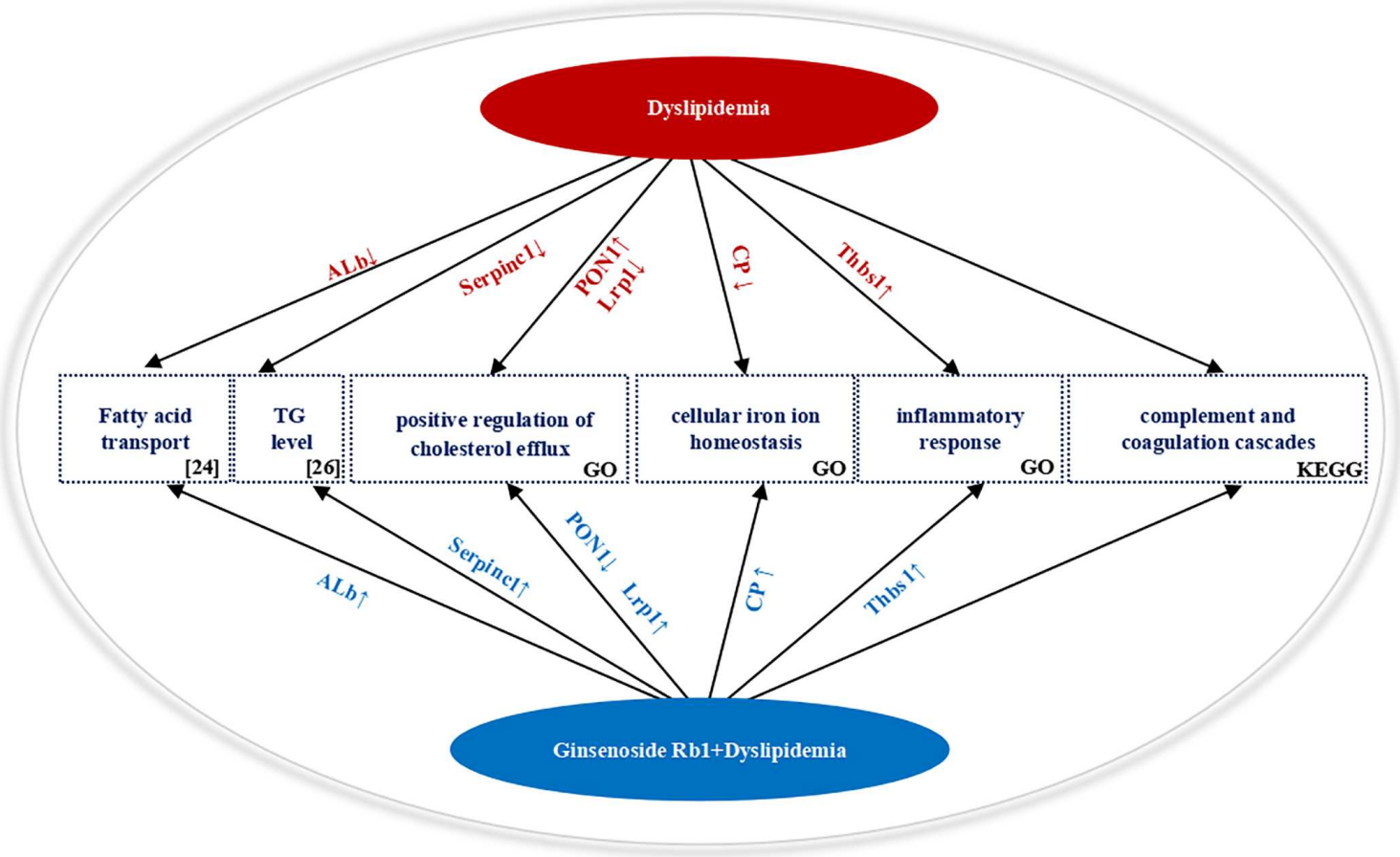

Figure 8

The possible mechanism of Ginsenoside Rb1 regulating N-glycosylation of plasma protein in hyperlipidemia rats. The red and blue arrows indicate changes in the glycosylation modification level, red represents changes in the HFD group and blue represents changes in the Rb1 group.

Supplementary Files

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- SupportingInformationTable1.xlsx
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- SupportingInformationTable3.xlsx
- SupportingInformationTable4.xlsx
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