Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Comparison of Plasma Exosome Proteomes Between Obese and Non-Obese Patients with Type 2 Diabetes Mellitus

Yanjun Wang, You Wu, Shuangzhu Yang, Yan Chen

Department of Endocrinology, The Second Hospital of Jilin University, Changchun, People's Republic of China

Correspondence: Yan Chen, Department of Endocrinology, The Second Hospital of Jilin University, Changchun, 130041, People's Republic of China, Tel +86 0431-81136436, Email cheny99@jlu.edu.cn

Purpose: Obesity is considered a promoter of type 2 diabetes mellitus (T2DM). However, the underlying mechanism remains unclear. This study aimed to identify plasma exosome differentially expressed proteins (DEPs) that are potentially involved in the development of obesity-related T2DM.

Methods: Exosomes were isolated from the plasma of obese and non-obese T2DM patients (n = 10 for each group). A label-free quantitative mass spectrometry analysis was applied to identify plasma exosome DEPs in obese patients compared with non-obese patients, followed by bioinformatics analysis including GO annotation, KEGG analysis, subcellular localization prediction, transcription factor analysis, and protein-protein interaction (PPI) prediction.

Results: We identified 2 significantly upregulated proteins (C9 and PON1) and 5 significantly downregulated proteins (HPX, A1BG, CFHR1, ANG, and CALM) in obese patients compared with those in non-obese patients. KEGG analysis demonstrated that the insulin signaling pathway was one of the pathways that significantly correlated with the DEPs. The DEPs were primarily localized in the extracellular space (5 out of 7). HMG-box and NF-Y beta might regulate the transcription of the DEPs. C9, PON1, HPX, and CFHR1 were present in the PPI network.

Conclusion: The plasma exosome DEPs are potentially responsible for the development of obesity-related T2DM possibly through the insulin signaling pathway and the interaction with other proteins. Our study may guide future research direction toward the pathogenesis of obesity-related T2DM.

Keywords: exosomes, proteomics, type 2 diabetes mellitus, obesity, mass spectrometry, insulin resistance

Introduction

Obesity has emerged as a leading global health concern as its prevalence has consistently increased in the past half century.[1] Obesity refers to the excessive fat accumulation in the body and increases the risk of developing many diseases, including diabetes mellitus, cardiovascular disease, hypertension, and hyperlipidemia.[2] Of these diseases, type 2 diabetes mellitus (T2DM) is most strongly associated with obesity. According to the WHO report in 2013, 44% of the T2DM burden is attributable to overweight and obesity.[3] A recent study has shown that overweight and obesity account for 85.8% of T2DM cases.[4] It has been expected that the prevalence of obesity-related diabetes will double to 300 million by 2025.[5] Thus, it is of great importance to understand the pathogenesis of obesity-related T2DM.

Insulin resistance is commonly linked to the development of obesity-related T2DM. In obese patients, a reduction in insulin signaling may result from elevated amounts of pro-inflammatory cytokines, mitochondrial dysfunction, endocrine dysregulation, impaired lipid metabolism, endoplasmic reticulum stress, and adipose tissue hypoxia.[6] A highly complex network of organ communication plays a key role in regulating insulin signaling, involving a complex scenario of crosstalk mediators such as nutrients and metabolites, extracellular vesicles, as well as peptides and proteins.[7] Approaches such as genomics, metabolomics, and proteomics have been used to identify biomarkers for early detection and management of obesity and T2DM.[8]

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Exosomes are a subtype of extracellular vesicles with a diameter of 30–150 nm, playing an important role in intercellular communication by transferring molecule cargoes, including DNA, RNA, proteins, and lipids, from the parental cells to the recipient cells.[9–11] Exosomes derived from different sources, such as adipose tissue, liver, and skeletal muscle, contribute to the development of insulin resistance in obesity.[12–14] Exosomes also serve as circulating mediators in T2DM through functional cargo.[15] Proteomics analysis of exosomes derived from adipocytes, urine, and plasma has been applied to identify the biomarkers and understand the pathogenesis of obesity- and diabetes-related diseases.[16–18] Since not all individuals with T2DM are overweight or obese, it is of great interest to compare the plasma exosome proteomes between obese and non-obese patients with T2DM for the identification of circulating exosome proteins that may facilitate the development of obesity-related T2DM. However, little research has been conducted for this comparison.

In this study, to identify the proteins that may contribute to the development of obesity-related T2DM through organ-organ communication, we, for the first time, compared the plasma exosome proteomes between obese and non-obese patients with T2DM. Our results may provide new information about the pathogenesis of obesity-related T2DM.

Materials and Methods

Sample Collection

A total of 10 obese T2DM patients and 10 non-obese T2DM patients who have visited the Department of Endocrinology at The Second Hospital of Jilin University (Jilin, China) between June 2021 and August 2021 were recruited. The criteria for obesity diagnosis was BMI $\geq$ 28 kg/m^2.[19] The exclusion criteria were: (1) patients with gestational diabetes and other secondary diabetes, (2) patients with hypothalamus, pituitary disease, Cushing's syndrome, or endocrine and metabolic diseases, and (3) patients who have used obesity-causing drugs. This study was approved by the Ethics Committee of The Second Hospital of Jilin University (2021 Research Review No. 194) and conducted according to the Declaration of Helsinki. Informed consent was obtained from each patient.

The whole blood (5 mL) was collected from the cubital vein of each subject into a tube containing anticoagulant EDTA. After a 30-min incubation at room temperature, the whole blood was centrifuged at 4000 g for 10 min at 4 °C. The upper plasma layer was collected and stored at −80°C until use.

Isolation of Plasma Exosomes

Plasma samples were quickly thawed at 37 °C, followed by centrifugation at 2000 *g* for 30 min at 4 °C. Samples were centrifuged again at 2000 *g* for 45 min at 4 °C. The supernatant was filtered through a 0.45 μm filter and ultracentrifuged at 100,000 g for 70 min at 4 °C using an ultracentrifuge device (CP100MX; Hitachi, Japan). The supernatant was discarded. The exosome pellet was resuspended in phosphate-buffered saline (PBS).

Transmission Electron Microscopy (TEM) Examination

TEM was used to examine the morphology of the exosomes. The samples were prepared as previously described.[20] In brief, 10 μL of exosome resuspension was loaded on the copper mesh for 1 min. The excess solution was removed using filter paper. Then, 10 μL of uranyl acetate was added dropwise to the copper mesh and precipitated for 1 min. The excess solution was removed using filter paper. After drying for a few minutes at room temperature, the exosomes were observed under a Hitachi HT-7700 microscope at 100 kV.

Nanoparticle Tracking Analysis

Particle size was measured using nanoparticle tracking analysis. Briefly, frozen exosome samples were thawed in a water bath at 25 °C, followed by incubation on ice. Samples were diluted with PBS and then analyzed using a nanoparticle tracking analyzer (ZetaVIEW S/N 20–602, Particle Metrix, Germany) and ZetaView software (version 8.05.14 SP7; Particle Metrix).

Proteomics Sample Preparation

The exosomes were lysed with SDT lysis buffer containing 4% SDS and 100 mM Tris-HCl (Sangon, Shanghai, China), sonicated, and centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatant was collected, and the protein concentration was measured using a bicinchoninic acid kit (Beyotime, Shanghai, China). The proteins were precipitated with cold trichloroacetic/acid acetone (1:9) (Sigma-Aldrich) for 4 h at − 20 °C, followed by centrifugation at 6000 *g* for 40 min. The protein pellet was washed with cold acetone three times and then dissolved in SDT buffer. A total of 50–200 μg of proteins for each sample were digested as previously described.[21] Briefly, the protein samples were digested with trypsin overnight at 37 °C. The resulting peptides were desalted using a C18 column (IonOpticks, Australia).

Mass Spectrometry (MS) Analysis

The peptides were analyzed on a nanoElute (Bruker, Bremen, Germany) coupled to a timsTOF Pro (Bruker, Bremen, Germany) equipped with a CaptiveSpray source. Peptides were separated on a 25 cm × 75 μm analytical column, with 1.6 μm C18 beads and a packed emitter tip (IonOpticks). The column temperature was maintained at 50 °C using an integrated column oven (Sonation GmbH, Germany). The column was equilibrated using 4 × column volumes before loading the sample in 100% buffer A (99.9% MilliQ water, 0.1% formic acid). Both steps were performed at 800 bar. Samples were separated at 300 nL/min using a linear gradient as follows: 2–22% buffer B for 75 min, 22–37% buffer B for 5 min, 37–80% buffer B for 5 min, hold in 80% buffer B for 5 min. The timsTOF Pro was operated in PASEF mode. The parameters were as follows: mass range 100 to 1700m/z, 1/K0 start 0.75 V·s/cm^2 and end 1.4 V·s/cm^2, ramp time 100 ms, lock duty cycle to 100%, capillary voltage 1500 V, dry gas 3 L/min, dry temperature 180 °C, PASEF settings, 10 MS/MS scans (total cycle time 1.16 sec), charge range 0–5, active exclusion for 0.5 min, scheduling target intensity 10,000, intensity threshold 2500, CID collision energy 20–59eV. For quality control, the MS data were analyzed using MaxQuant software version 1.6.17.0. Molecular weight distribution was examined. The repeatability of the samples was assessed by Pearson's correlation coefficient, principal component analysis, and relative standard deviation.

Identification of Differentially Expressed Proteins (DEPs)

To identify DEPs, MS data were searched against Uniprot_HomoSapiens_20387_20210928_9606_swissprot (http://www.uniprot.org). An initial search was set at a precursor mass window of 10 ppm. The search followed an enzymatic cleavage rule of Trypsin/P and allowed maximal two missed cleavage sites and a mass tolerance of 40 ppm for fragment ions. Carbamidomethylation of cysteines was defined as fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications. The cutoff of the global false discovery rate for peptide and protein identification was 0.01. Protein abundance was calculated based on label-free quantification intensity. Proteins which |Fold change| > 1.2 and *P*-value (Student's *t*-test) < 0.05 were considered DEPs.

Bioinformatics Analysis

DEPs were characterized by gene ontology (GO) functional annotation and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis. GO annotation was performed using the Blast2Go software (https://www.blast2go.com/).[22] Pathway analysis was performed using KEGG. The results of GO and KEGG enrichment assays were statistically analyzed using Fisher's exact test. Fisher's Exact Test was used to analyze and calculate the significance level of protein enrichment in each pathway, so as to identify significantly affected metabolic and signal transduction pathways. Subcellular localization was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/).[23] Domain annotation and enrichment analysis were conducted using InterProScan.[24] Transcription factor analysis was carried out using the AnimalTFDB 3.0 and PlantTFDB 5.0.[25,26] A protein-protein interaction (PPI) network was established using String (https://www.string-db.org/) and visualized using the Echart software.

Statistical Analysis

Data were analyzed using SPSS 22.0 (Armonk, NY, USA). The graphs were generated using Prism 7 (Graphpad, San Diego, CA, USA). Categorical data were described as percentages. The difference between the two groups was compared

using the one-way analysis of variance, followed by the *t*-test. A *P* value less than 0.05 was considered statistically significant.

Results

Isolation and Characterization of Exosomes from the Plasma of Patients

To compare the plasma exosome proteomes between obese and non-obese patients with T2DM, we collected the plasma exosome samples from the patients. The clinical characteristics of patients were summarized in Table 1. We observed significant differences in the height, weight, BMI, ALT, HDL-C, BUN, INS, HOMA-IR, and AHI between obese and non-obese patients, indicating that obese patients had greater impairment in lipid metabolism, liver and renal functions, and insulin resistance than non-obese patients. Under TEM, the exosomes isolated from the plasma samples appeared as spheres with clear and holonomic membranes (Figure 1A). The particle concentrations of samples 1 and 4 were 3.7×10^9 and 6.6×10^9 particles/mL, respectively. The diameters of the exosomes ranged from 30 to 200 nm (Figure 1B). These results suggest that we successfully obtained the exosomes from the plasma of patients.

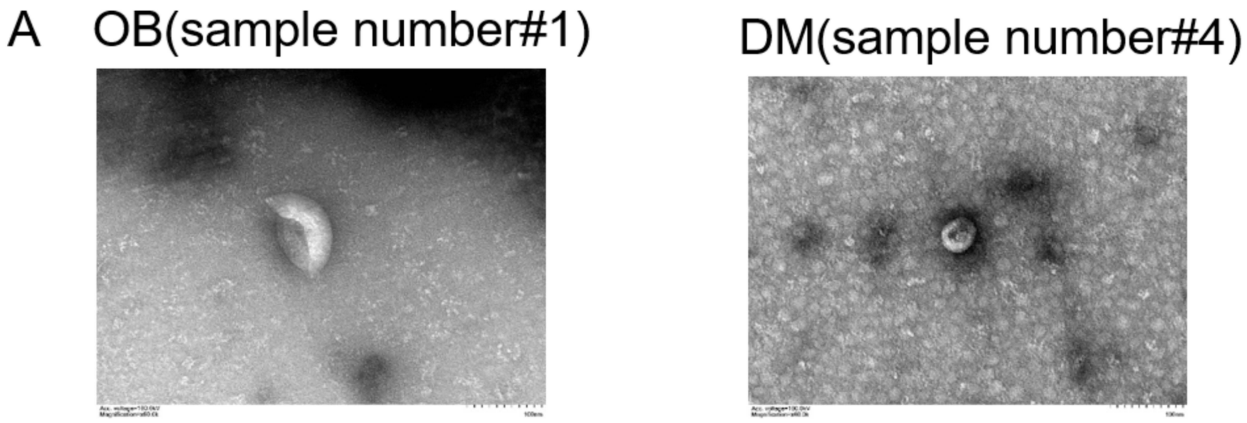
Identification of Plasma Exosome DEPs in Obese Patients with T2DM

To identify plasma exosome proteins involved in obesity-related T2DM, we performed a quantitative proteomics assay. We obtained 566,359 total spectrums, along with 69,563 matched spectrums, 5609 peptides, 5241 unique peptides, and 737 protein groups (Figure S1A). The molecular weight distribution of the proteins was shown in Figure S1B. The samples demonstrated good repeatability as evidenced by the results of Pearson's correlation coefficient, principal component analysis, and relative standard deviation (Figure S1C–E). With the threshold of |Fold change| > 1.2 and

Table 1 Clinical Characteristics of Patients

Variables	Non-Obese DM (n = 10)	Obese DM (n = 10)	P value
Age (years)	49.00± 5.16	69.29 ± 7.91	0.033
Gender			
Male (%)	5.00 (50.0)	6.00 (60.0)	0.673
Female (%)	5.00 (50.0)	4.00 (40.0)	
Duration (years)	0.99 ± 1.69	1.53 ± 1.40	0.447
Height (cm)	164.70± 7.03	172.40±8.32	0.032
Weight (kg)	63.50 ± 0.04	98.90± 20.17	0.000
BMI (kg/m2)	23.36 ±1.52	33.27 ± 5.45	0.000
ALT (U/L)	21.50± 9.56	77.00± 77.68	0.039
AST (U/L)	16.80±4.44	42.80± 37.39	0.056
TG (mmol/L)	2.38± 1.78	2.35± 1.00	0.967
TC (mmol/L)	5.84 ± 1.00	5.30 ± 0.83	0.206
HDL-C (mmol/L)	1.12± 0.13	0.93 ± 0.16	0.009
LDL-C (mmol/L)	3.23± 1.01	3.26± 0.60	0.941
BUN (mmol/L)	5.92 ±0.90	4.19± 1.00	0.001
Cr (μmol/L)	59.7 ± 17.11	65.90 ± 15.08	0.401
UACR (mg/mmol)	0.37 ± 0.38	0.70 ± 0.51	0.192
eGFR (mL/minl)	107.23 ±9.92	111.45± 18.11	0.529
FPG (mmol/L)	11.01 ± 2.82	9.68± 4.92	0.468
INS (mU/L)	11.11± 5.61	24.28± 11.50	0.004
HbA1C (%)	9.33± 1.62	8.96 ± 2.00	0.655
HOMA-IR	5.52± 3.41	9.33 ± 3.63	0.026
AHI	7.79± 3.48	18.83 ± 8.49	0.003

Abbreviations: DM, diabetes mellitus; BMI, body mass index; ALT, alanine aminotransferase aspartate; AST, aminotransferase; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; BUN, blood urea nitrogen; Cr, serum creatinine; UACR, urine albumin-to-creatinine ratio; eGFR, estimated glomerular filtration rate; FPG, fasting plasma glucose; INS, insulin; HbA1c, hemoglobin A1c; HOMA-IR, homeostasis model assessment of insulin resistance; AHI, the apnea–hypopnea index.



B Particle size and concentration distribution

Sample number	Average particle size (nm)	concentration (Particles/mL)
4	179.3	6.6E+9
1	186.7	3.7E+9

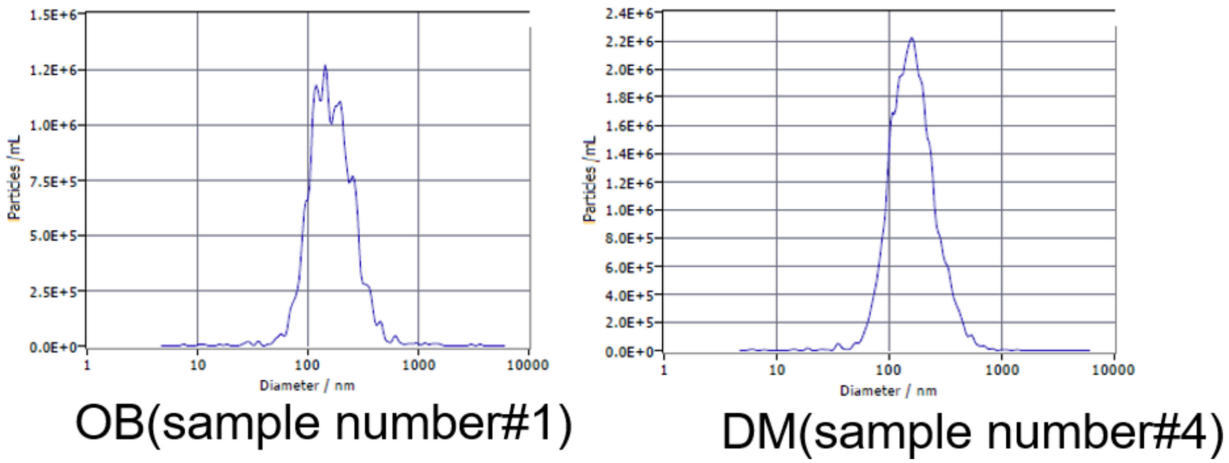


Figure 1 Characterization of plasma exosomes. Plasma exosomes were isolated from obese and non-obese patients with type 2 diabetes. (**A**) The morphology of plasm exosomes from sample 1 and sample 4 under a transmission electron microscope. Magnification 6000 ×. (**B**) Particle size was measured using nanoparticle tracking analysis. **Abbreviations**: OB, obese patients with type 2 diabetes; DM, non-obese patients with type 2 diabetes.

P-value < 0.05, we identified 2 significantly upregulated proteins (C9 and PON1) and 5 significantly downregulated proteins (HPX, A1BG, CFHR1, ANG, and CALM) in obese patients compared with those in non-obese patients (Figures 2A–C and 3, Table 2). These proteins may be involved in the development of T2DM in obese patients.

Bioinformatics Analysis

To characterize the functions of the DEPs, we performed GO and KEGG analyses. GO annotation revealed that the DEPs in plasma exosomes of obese patients were dominantly involved in the "membrane part", "basement membrane", and "heterotypic cell-cell adhesion" (Figure 4A). KEGG analysis demonstrated that the insulin signaling pathway was one of the pathways that significantly correlated with the DEPs in obese patients (Figure 4B). WoLF PSORT predicted that the

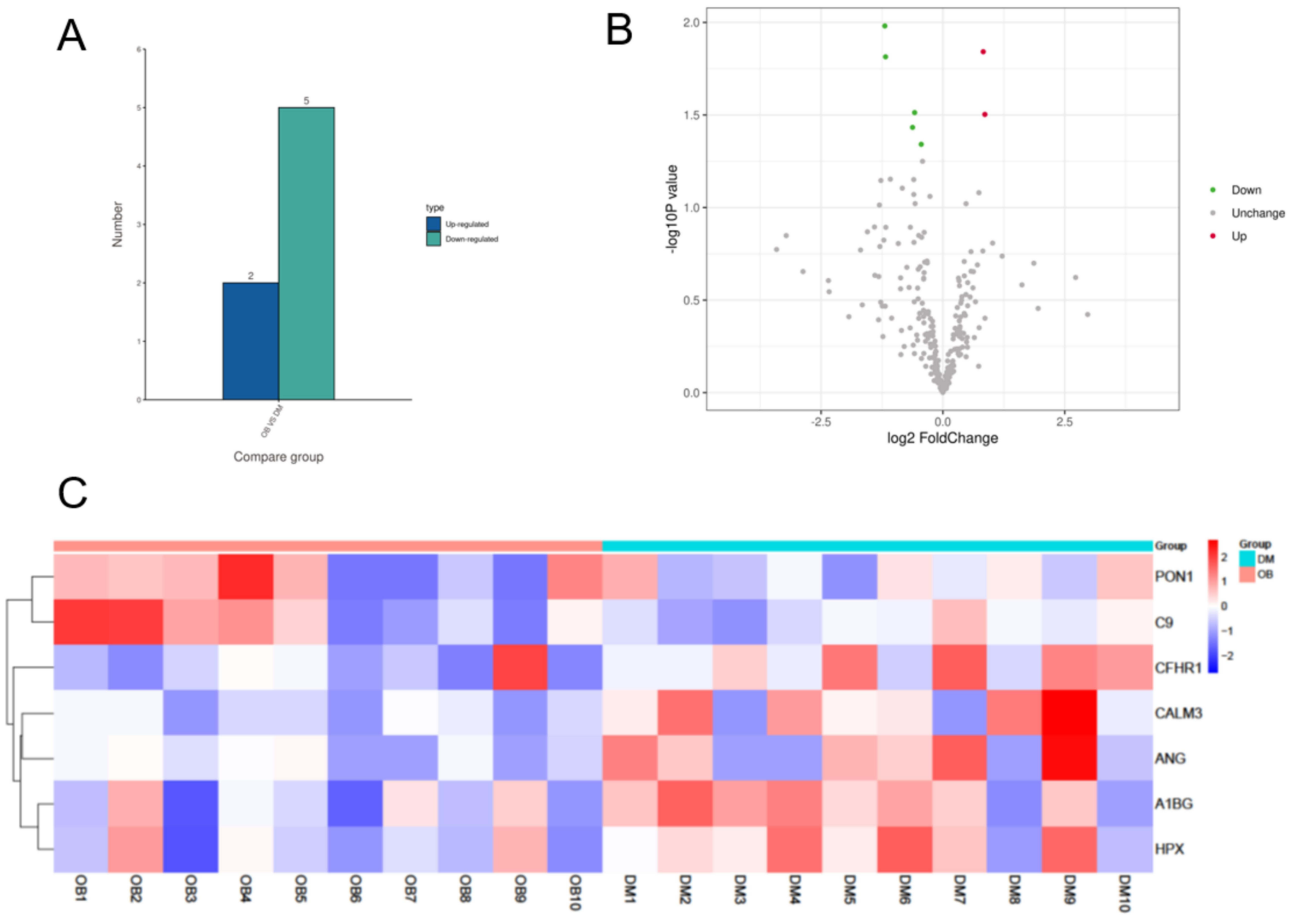


Figure 2 Identification of differentially expressed plasma exosome proteins. (**A** and **B**). A label-free quantitative mass spectrometry analysis was performed and identified 2 significantly upregulated proteins and 5 significantly downregulated proteins in obese patients with type 2 diabetes. (**C**). Heatmap of differentially expressed plasma exosome proteins.
Abbreviations: OB, obese patients with type 2 diabetes; DM, non-obese patients with type 2 diabetes.

DEPs were primarily localized in the extracellular space (71.4%; C9, HPX, A1BG, CFHR1, and ANG), followed by mitochondria (14.3%; PON1) and cytosol (14.3%; CALM3) (Figure 5A). This finding suggests that the plasma exosome DEPs are responsible for intercellular communication. Domain annotation and enrichment analysis characterized the protein families, such as the Ribonuclease A-like domain superfamily and alpha-1B-glycoprotein/leukocyte (Figure 5B). Transcription factor analysis predicted that HMG-box and NF-Y beta might regulate the transcription of the DEPs (Figure 6A). A PPI network illustrated the potential functional interaction among all identified proteins. We noticed that C9, PON1, HPX, and CFHR1 were present in the PPI network with interaction degrees of 7, 4, 4, and 2, respectively (Figure 6B, Table S1), suggesting that these proteins may play critical roles in the development of obesity-related T2DM.

Discussion

In this study, we identified 2 upregulated DEPs (C9 and PON1) and 5 downregulated DEPs (HPX, A1BG, CFHR1, ANG, and CALM) in plasma exosomes of obese T2DM patients compared with those of non-obese T2DM patients. Bioinformatics analysis suggested that the DEPs were related to the insulin signaling pathway, primarily localized in the extracellular space, and regulated by transcription factors HMG-box and NF-Y beta. Of these proteins, C9, PON1, HPX, and CFHR1 may play critical roles in the development of obese-related T2DM by interacting with other proteins. Our study provides new information about the pathogenesis of obese-related T2DM. Since many individuals with T2DM may have a long-term asymptomatic period of hyperglycemia before diagnosis,[27] the plasma exosome DEPs may serve as potential diagnostic markers for the early diagnosis of obese-related T2DM.

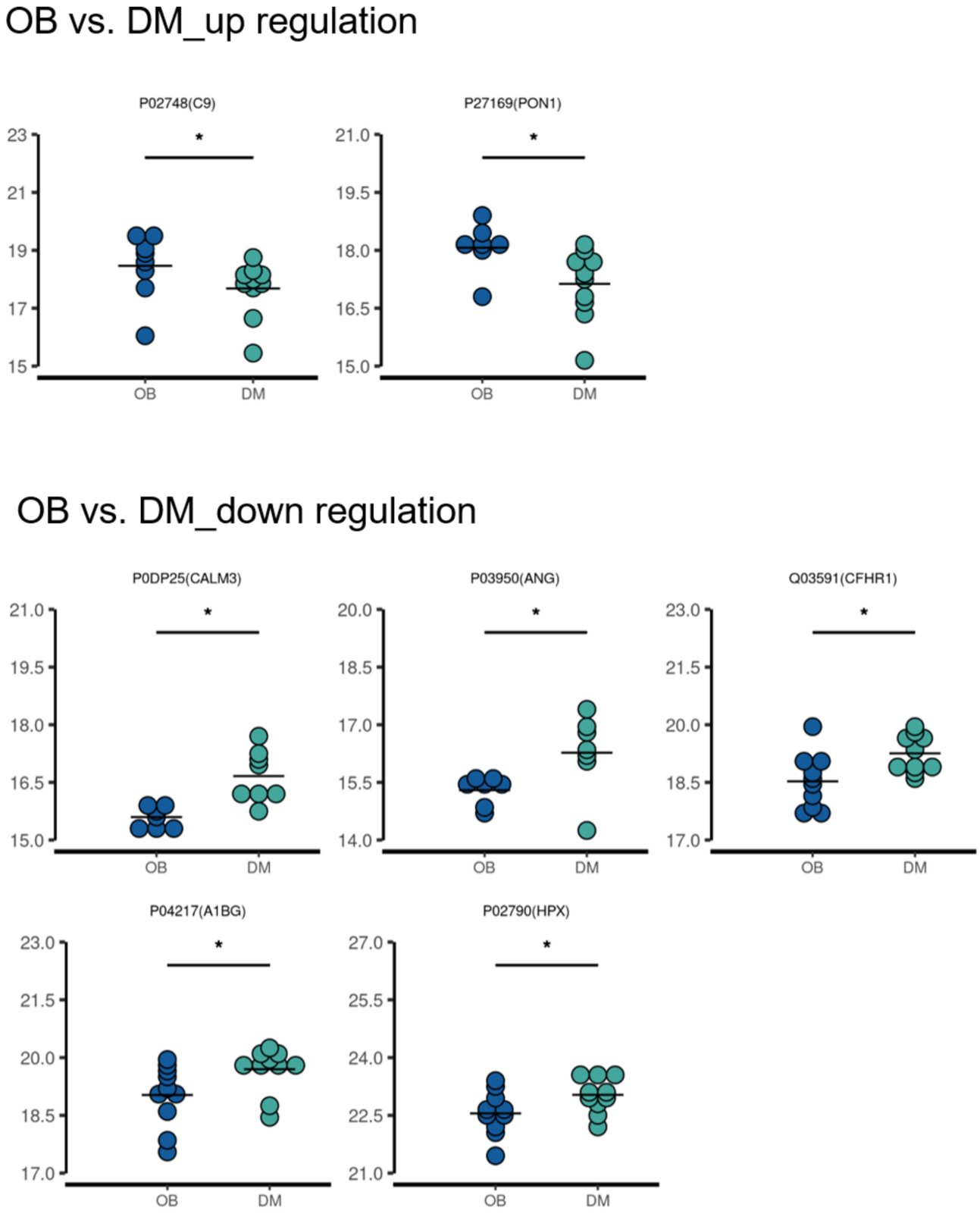


Figure 3 Comparison of the expression of differentially expressed plasma exosome proteins between obese and non-obese diabetic patients. *p<0.05.

Table 2 The Differentially Expressed Proteins in Obese Patients with Type 2 Diabetes

Protein	Regulation	Ratio of LFQ (OB/DM)	P value
C9	Up	1.815911592	0.031410504
PON1	Up	1.774121352	0.014398211
HPX	Down	0.734564827	0.045560876
A1BG	Down	0.668644594	0.030692879
CFHR1	Down	0.442142469	0.036910981
ANG	Down	0.649168087	0.015345186
CALM3; CALM2; CALM1	Down	0.437797365	0.010452527

Abbreviations: LFQ, label-free quantification; OB, obese patients with T2D; DM, diabetes mellitus.

Obesity and T2DM are closely linked to inflammation. The complement system is a central component of innate immunity and contributes substantially to inflammatory processes. Complement activation has been seen in metabolic disorders, including obesity, insulin resistance, and diabetic mellitus.Adipocytes are the main source of human factor D, play an important role in the activation of alternative complement pathways, further research shows that C3, factor B, etc. from the alternative complement pathway are expressed in adipose tissue, emphasizing the hypothesis that local complement activation can substantially affect adipose tissue. Further studies have found that the receptors for C3a (C3aR) and C5a (C5aR1 and C5aR2) have been shown to be expressed in lipocytes. Therefore, in addition to producing complement components, fat is also a potential target for complement action. C3adesArg, a product of the complement lysis process, promotes triglyceride synthesis in fat cells and increases plasma triglyceride levels, which may be part of the reason for the increased insulin resistance.[28] In this study, we found that complement C9 and complement factor H-related protein 1 (CFHR1) were significantly increased and decreased, respectively, in the circulating exosomes from obese T2DM patients compared with those from non-obese T2DM patients, suggesting that complement dysregulation plays a critical role in the development of obese-related T2DM. C9, together with C6 through C8, are essential components of the membrane attack complex (MAC) that mediates complement-induced tissue damage in complications of diabetes.[29] Obesity is associated with increased circulating complement C3,[30] and the cleavage of C3 promotes the recruitment of C9 in MAC formation.[31] Thus, the increased C9 in plasma exosomes may facilitate the development of T2DM in obese individuals by regulating inflammation.Thus, C9 may originate from the alternative activation pathway of complement, during which inflammation mediates the metabolic disorder of adipose tissue, especially C3.CFHR1 has been reported to regulate complement by blocking C5 convertase activity and competing with complement factor H for binding to C3b.[32,33] Therefore, CFHR1, as a secretory glycoprotein synthesized by the liver, plays a role in complement control. The significant decrease in CFHR1 in plasma exosomes in obese T2DM patients may be that adipose tissue affects the liver, affecting the synthesis of other CFH-related proteins such as CFHR1 by normal liver cells, so that the activation of the complement system is more dominant, resulting in immune imbalance and chronic low-grade inflammation, and studies have found that variants in the CFH gene are related to the risk of Chinese hypertension.[34] However, the direct role of CFHR1 in obesity and T2DM remains unexplored and needs further investigation.

Following C9, paraoxonase 1 (PON1) was the second most upregulated protein in obese patients with T2DM. PON1 possesses both paraoxonase and arylesterase activity and is physically associated with high-density lipoprotein (HDL). PON1 is expressed in the liver. After secretion into the blood, PON1 binds to HDL particles and is located in the subfraction containing apo A-I and clusterin, playing an important role in lipid metabolism by delaying or inhibiting the oxidation of both low-density lipoprotein and HDL particles.[35] Studies have shown that PON1 activity is significantly decreased in obese subjects compared with that in control subjects.[36,37] Serum PON1 activity is also significantly decreased in type 1 and type 2 diabetic individuals compared with that in healthy subjects.[38–40] In our study, PON1 was significantly increased in plasma exosomes in obese patients with T2DM compared with that in non-obese patients. We speculated that plasma exosomes may be involved in the degradation of circulating PON1 in obese patients, thus reducing the activity of PON1 in the blood of patients. It is also possible that the liver plays a role in regulating HDL-C

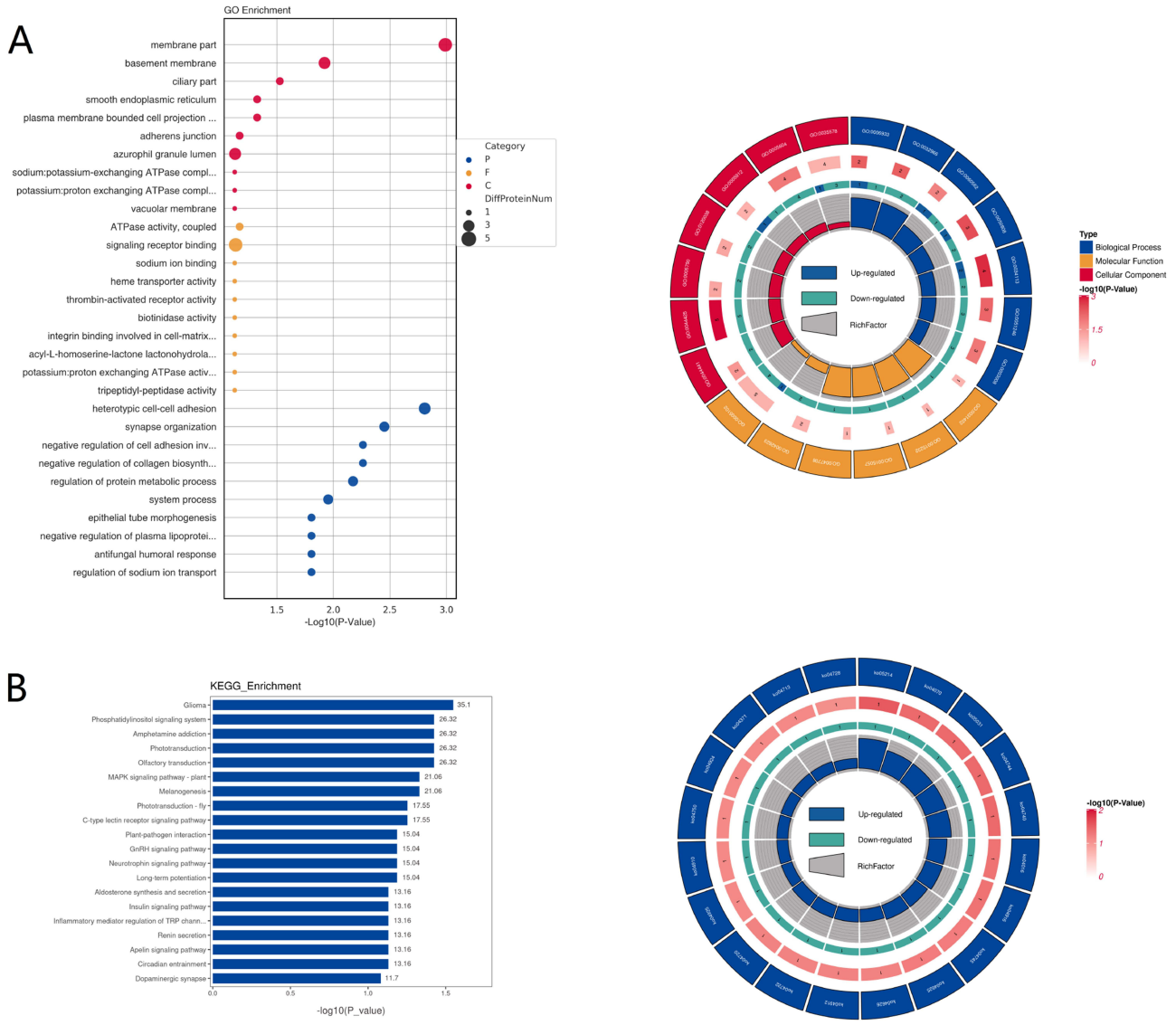


Figure 4 Gene ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) analysis. (**A**) Left: GO enrichment bubble chart. Right: GO enrichment circle diagram. The first circle represents the top 20 GO terms. The second circle represents the number and -log10 (P-value) of the differentially expressed proteins enriched in the corresponding GO term. The third circle indicates the number of upregulated or downregulated proteins. Blue represents upregulated protein. Green represents downregulated proteins. The fourth circle: rich factor value of each GO term. (**B**) Left: KO enrichment bar chart of top 20 pathways. Right: KO enrichment circle diagram. The first circle is the top 20 enriched pathways. The second circle represents the number of proteins in the corresponding pathway. The third circle indicates the number of upregulated or downregulated proteins. Blue represents upregulated proteins. Green represents downregulated proteins. The fourth circle indicates the rich factor value of each pathway.

by enhancing the synthesis of PON1, exerting a protective effect on the body and reducing the risk of cardiovascular disease.Interestingly, hemoglobin scavenger protein hemopexin (HPX) is essential for the inflammatory properties of HDL by binding to heme. HPX treatment may prevent heme and oxidative stress-mediated inflammatory conditions such as atherosclerosis.[41] HPX expression was significantly downregulated in obese patients compared with that in non-obese patients in our study, suggesting a dysregulated HDL metabolism in obese patients. Also, HPX was one of the hub genes in the PPI network, and we observed an HPX-PON1 interaction in the network. We speculate that HPX may interact with PON1 to regulate the properties of HDL in the development of obesity-related T2DM.

KEGG pathway analysis showed that the main ones associated with obese T2DM were phosphatidylinositol and insulin signaling pathways.Insulin resistance is linked to the development of obesity-related T2DM.[6] In this study, obese patients had significantly higher HOMA-IR than non-obese patients, suggesting insulin resistance in obese patients.

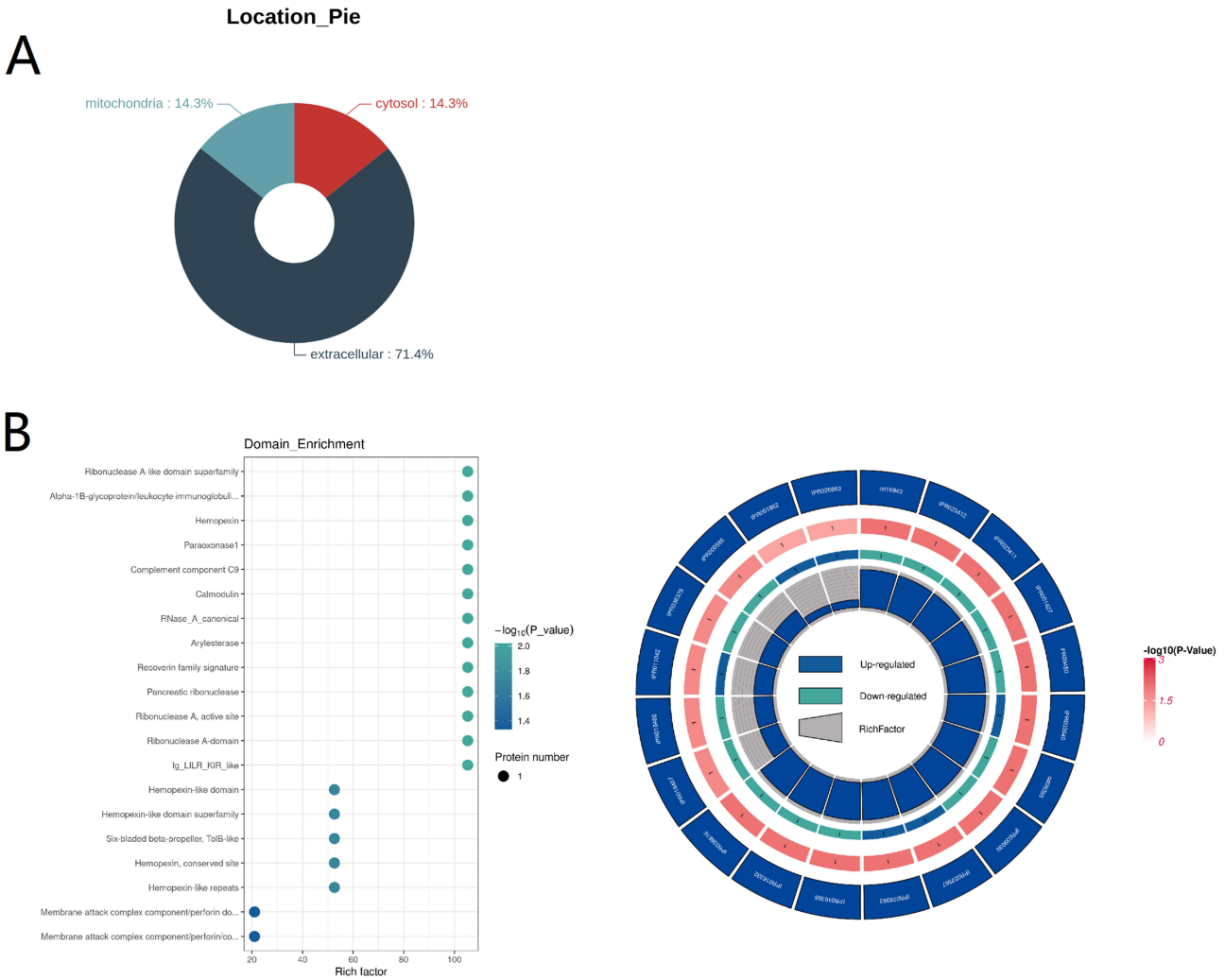


Figure 5 Characterization of the differentially expressed proteins. (**A**) Prediction of the subcellular localization of the differentially expressed proteins. (**B**) Left: Domain enrichment bubble chart. Right: Domain enrichment circle diagram. The first circle represents the top 20 enriched domains. The second circle reprensents the number of the proteins with the corresponding domain. The third circle represents the number of upregulated or downregulated proteins. Blue represents upregulated proteins. Green represents downregulated proteins. The fourth circle: rich factor value of each domain.

Macrophages of adipose tissue transition to pro-inflammatory M1 type under obese, secreting various inflammatory factors that have been shown to lead to obesity-related insulin resistance.[42] Insulin has a complex signal transduction pathway, and when insulin binds to receptors on the cell membrane, signal transduction is initiated, thereby activating the phosphatidylinositol pathway and the MAPK pathway, consistent with the results of the KEGG pathway.CALM was the most significantly downregulated protein in obese patients in our study. CALM inhibitors can block glucose transport induced by insulin or hypoxia in mouse skeletal muscle.[43,44] CALM also mediates the role of Ca^{2+} in insulin signaling in mammalian skeletal muscle.[45] These findings suggest that CALM is highly involved in insulin signaling, consistent with our data. In addition, Lin et al have demonstrated that the amount of brain serotonin in obese mice is dramatically higher than that in their lean counterparts and positively correlates to the amount of brain CALM in the lean mice, suggesting that CALM may regulate abnormal serotonin synthesis in obese mouse brain and contribute to the development of obesity.[46] In our study, the decreased level of plasma exosome CALM may imply its increase in the central nervous system. However, whether the abundance of brain CALM is increased in obese patients with T2DM needs further confirmation.In addition, studies have shown that CALM can affect the signal transduction of MAPK.[47]

We also found that A1BG and ANG proteins were significantly decreased in circulating exosomes of obese T2DM patients compared with those of non-obese T2DM patients. Iqbal et al have seen a significant increase in A1BG protein

A

Protein	TF_family
P09429	HMG_box
Q99880	NF-YB

B

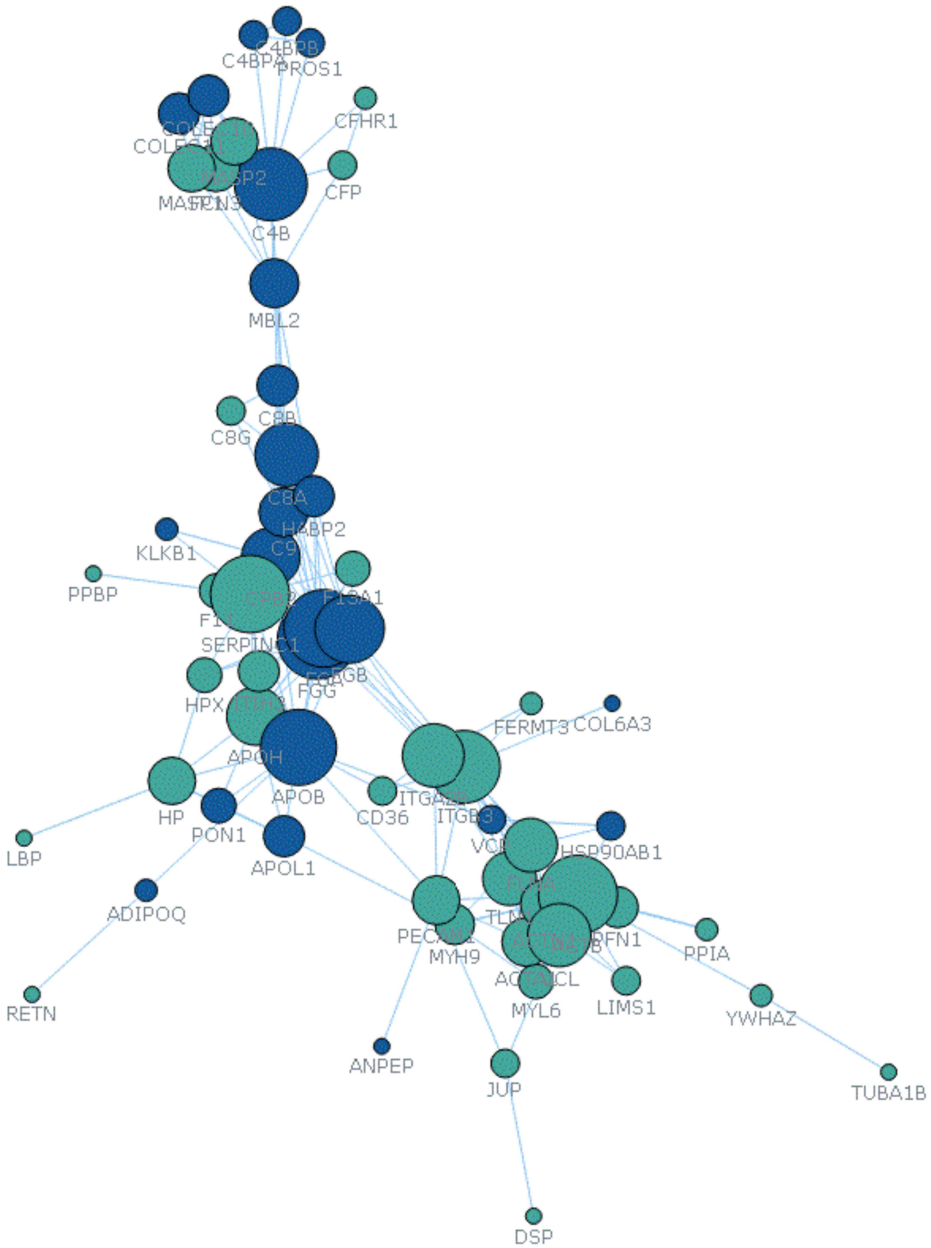


Figure 6 Transcription factor analysis and establishment of the protein-protein interaction network. (**A**) Transcription factor analysis identifies HMG-box and NF-Y beta may regulate the transcription of the differentially expressed proteins. (**B**) A protein-protein interaction network. Blue represents upregulated proteins. Green represents downregulated proteins.

levels in the plasma of T2DM patients 12 months after bariatric surgery.[48] van Bilsen1 et al have demonstrated that A1BG expression is significantly downregulated in the inflamed livers of obese mice compared with that of control mice.[49] These findings are consistent with ours, suggesting a potential association of A1BG with obesity. Dysregulation of angiogenesis contributes to diabetic microvascular complications, with excessive or defective angiogenesis observed in different tissues.[50] The adipose tissues in obese individuals are in a relative hypoxic state because the expansion of adipose tissues exceeds the ability of the vasculature to provide adequate oxygen and nutrients to all adipose cells, which in turn triggers inflammation in the adipose tissues and paves the way to insulin resistance and T2DM development.[51,52] The ANG downregulation observed in our study suggests an impaired angiogenic response in the adipose tissues that may cause T2DM onset and progression in obese individuals.[53] However, the role of ANG in the development of obesity-related T2DM remains unknown and needs further investigation.

C9, PON1, HPX, and CFHR1 are located in the core of the PPI, we speculate that exosome proteins produced by obese adipose tissue (including lipocytes, macrophages, endothelial cells, immune cells, etc.) cause adipose metabolism disorders by activating alternative complement pathways, which can be manifested as increased triglycerides synthesis, pro-inflammatory M1 like macrophage accumulation, and ultimately insulin resistance through inflammatory factors. In summary, obese patients with T2DM, these exosomes mainly from adipose tissue can be considered to be involved in the occurrence of insulin resistance, providing clues for understanding the T2DM mechanism associated with obesity, and can play a therapeutic role in the future through stem cell exosomes in this direction. However, this study has some limitations. First, the sample size is relatively small. The DEP alterations need to be validated in an independent cohort. Second, the roles of the DEPs in the development of obesity-related T2DM require further characterization.

Conclusion

In summary, we for the first time identified plasma exosome DEPs in obese diabetic patients compared with non-obese diabetic patients. Our findings may provide new information about the etiology of obese-related T2DM and potential diagnostic markers for the early diagnosis of obese-related T2DM.

Abbreviations

T2DM, type 2 diabetes mellitus; DEPs, differentially expressed proteins; PPI, protein-protein interaction; PBS, phosphate-buffered saline; TEM, Transmission electron microscopy; MS, Mass spectrometry; GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; CFHR1, complement factor H-related protein 1; MAC, membrane attack complex; HDL, high-density lipoprotein; HPX, hemopexin.

Data Sharing Statement

All data generated or analysed during this study are included in this published article [and its Supplementary Information Files].

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of The Second Hospital of Jilin University (2021 Research Review No. 194) and conducted according to the Declaration of Helsinki. Verbal informed consent was acceptable and approved by the Ethics Committee of The Second Hospital of Jilin University.

Author Contributions

YJ W and Y C carried out the studies, participated in collecting data, and drafted the manuscript. Y W and SZ Y performed the statistical analysis and participated in its design. All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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