Identification of Differentially Expressed Proteins in Serum of Obese Patients by Isobaric Tags for Relative and Absolute Quantification (iTRAQ)-Coupled 2D LC-MS

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEF 1 **Ying Zhang**
BCDF 2 **Zhong Chen**
BCDF 2 **Yue He**
CDF 2 **Jianxia Wang**
BDF 2 **Minhui Jiang**
CF 2 **Jianjuan Xu**
CF 2 **Minghua Chen**
CG 2 **Yaling Feng**

1 Department of Obstetrics and Gynecology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui, P.R. China
2 Department of Obstetrics and Gynecology, Wuxi Maternal and Child Health Hospital Affiliated to Nanjing Medical University, Wuxi, Jiangsu, P.R. China

Corresponding Author:
Source of support:

Yaling Feng, e-mail: yalingf0817@163.com
This study was supported by grants from the Wuxi Kejiao Qiang Wei Key Medical Talent Project (ZDRC010), the Support Project of Women's Health Care Department of Key Subjects of Maternal and Child Health Care in Jiangsu (XK201703), the National Natural Science Foundation of China (81502813), and the Jiangsu Provincial Science and Technology Department of the Key Disease Standardized Diagnosis and Treatment Project (BE2015618)

Background: The aim of this study was to identify the differentially expressed proteins of obese patients compared with normal participants and to provide a potential target for future investigation of obesity.

Material/Methods: We enrolled 10 obese male adults and 10 matched normal subjects. Serum samples were collected to get total protein extraction, denaturation, deoxidation, and enzymatic hydrolysis. Differentially expressed proteins were distinguished with mass spectrometry after samples were labeled with iTRAQ.

Results: A total of 9622 differentially expressed peptides were identified, corresponding to 733 proteins; 118 proteins of these showed significant differential expression, with 15 upregulated and 103 downregulated.

Conclusions: iTRAQ is an effective technique to identify differentially expressed proteins in obese patients. The development of obesity is correlated with a series of complex elements and mutual effects. The proteins identified in this study may provide novel directions and targets for future pathological studies of obesity.

MeSH Keywords: 14-3-3 Proteins • Obesity, Abdominal • Serum Albumin, Bovine

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/924882

📄 1890   🗂 4   📊 3   📚 20

Background

Obesity refers to excessive accumulation and abnormal distribution of fat in the body, usually accompanied by weight gain. The World Health Organization (WHO) defines obesity as an abnormal or excessive accumulation of fat that can lead to health damage [1]. According to the survey of nutrition and health status of Chinese residents of 2002, the overweight rate and obesity rate of Chinese residents were 17.6% and 5.6% respectively. The sum of the 2 was 23.2%, which was close to a quarter of the total population. Obesity has become an important disease affecting the health of Chinese residents [2]. Obesity is a major risk factor for many diseases, such as hypertension, diabetes, dyslipidemia, coronary heart disease, myocardial infarction, stroke, and several types of cancer, including breast cancer. It has been recognized as the fifth most important major risk factor affecting health [3]. Some studies had found that ceruloplasmin, fibrinogen, and apolipoprotein A-IV were overexpressed in plasma of obese people; therefore, they might be potential protein markers of obesity [4,5]. In contrast, apolipoprotein A-1 and apolipoprotein E were lower in plasma of obese and overweight people compared with the normal population [5]. Some studies demonstrated that level of the thermogenic enzymes was higher while the level of lipid synthase was lower in obesity-resistant mice [6]. Cytochrome b-cl complex subunit 1, enol3, monoglyceride lipase, and glucose-6 phosphate dehydrogenase 1 participate in energy metabolism in mice, such as glycolysis and visceral adipose tissue formation; thus, they may also be involved in the molecular mechanism and metabolic pathway of obesity [7].

Proteomics is a recently-developed method to study the molecular mechanism of diseases. iTRAQ technology is a proteomic technology introduced by Applied Biosystems, Inc. in 2004, in which 4 or 8 samples under the same experimental conditions can be quantitatively studied simultaneously by using isotope labeling technology. iTRAQ reagents comprised heterotopic labeling molecules with molecular weight of 145 Da. The labeling molecules are made up of reporting group with molecular weights of 114, 115, 116, 11, 118, 119, and 121Da, equilibrium group, and a same polypeptide reaction group. The technique has very good accuracy and high repeatability. It can be used to analyze several different samples simultaneously. When combined with the chromatographic separation

system, it can be used to detect large-scale protein alterations by detecting microgram-level amounts of protein, thus providing large-scale quantitative information on proteins. Another advantage is its wide application, as it can assess any type of protein. Compared with gel electrophoresis, this technology can be used to identify large molecular weight proteins, extremely acidic proteins, and extremely basic proteins, as well as low solubility and even insoluble membrane proteins; thus, the range of proteins identified is greatly expanded [8].

In the present study, we used iTRAQ technique to identify the differentially expressed proteins of obese patients compared with normal participants, to explore the potential molecular mechanism of obesity.

Material and Methods

Participants and enrollment

Adults with BMI >28.0 kg/m^2 were categorized as obese, as specified in the Guidelines for the Prevention and Control of Overweight and Obesity in Adults of China promulgated and implemented by the Ministry of Health in 2002 [9]. We included 10 obese subjects and 10 normal subjects. Inclusion criteria of the obesity group were: male; age 18–44 years old; BMI ≥28.0 kg/m^2; no organic diseases; and normal blood pressure and serum glucose (Table 1). Matched controls were enrolled in a ratio of 1: 1. The inclusion criteria of the control group were: male; age 18–44 years old; 18.5 kg/m^2 ≤BMI <24.0 kg/m^2; no organic diseases; and normal blood pressure, serum glucose, serum lipid, and hemoglobin levels. All subjects signed the informed consent. The research was approved by the Ethics Committee of Wuxi Maternal and Child Health Hospital Affiliated to Nanjing Medical University.

Reagents and instrument

The system in the MARS.14 chromatographic column was obtained from Aglient Co.; the iTRAQ kit, QSTAR XL mass spectrometer, and Protein Pilot4.2 software were obtained from Applied Biosystems (USA); the liquid chromatograph (20AD) was obtained from Shimadzu Co. (Japan); Strong Cation Exchange Chromatography (2.1×100 mm, 5 um, 300 A) was obtained for the Nest Group Inc.

Table 1. Basic information of 20 participants (SBP – systolic blood pressure; DBP – diastolic blood pressure; Mean±SD).

Participants	Age (years old)	BMI (kg/m²)	SBP (mm Hg)	DBP (mmHg)	Glucose (mmol/L)
Obese	31.9±9.2	31.0±2.2	116.0±22.0	77.0±15.0	5.5±0.2
Control	30.2±10.5	21.5±1.5	115.0±24.0	76.0±17.0	5.2±0.1
P-value	NS	<0.0001	NS	NS	NS

NS – not significant.

System (USA); and the ZORBAX 300SB-C18 column (5 um, 300A, 0.1×150 mm) was obtained from Microm Co. (USA).

Sample collection and preparation

We collected 4 mL fasting venous blood and placed it in a tube. After being frozen on ice for 30 min, the sample was centrifuged under 2000 r/min for 10 min at 4°C. Supernatant was collected in 1.5-mL EP tubes before being stored at –80°C.

Removal of high-abundance proteins

High-abundance proteins were removed using the multiple affinity removal system human 14 chromatograph. The fractions collected were concentrated by freeze-drying.

ITRAQ labeling

One hundred ug samples of each group were added with reducing reagent, at 60°C for 1 h. Then, cysteine blocking reagent was added and placed at room temperature for 10 min. Trypsin was added and enzymatic hydrolysis was allowed at 37°C overnight. Then, samples were centrifuged to deposit, and iTRAQ labeling reagent was added to each sample. Reaction was allowed for 1 h at room temperature and 3 times the volume of super-pure water was added to dilute the labeling reagent.

Table 2. General information of identified proteins.

Category	Number
Differential peptides	9622
Differential expressed proteins	733
Up-regulated proteins with significance	15
Down-regulated proteins with significance	103
Total differential expressed proteins with significance	118

The sample of the obese group was labeled with iTRAQ 118 and samples of the normal group were labeled with iTRAQ 117. The labeled samples were mixed in a low-adsorption tube and underwent vacuum centrifugal evaporation drying, followed by storage at –80°C.

Strong cation exchange chromatography (SCX) and mass spectrometric analysis

We added 200 uL SCX buffer A to the labeled sample mixture, stratified by SCX. After stratification, samples were analyzed by nano liquid chromatography electron spray ionization mass spectrometry (nano-LC-ESI-MS/MS) system. The scanning range of paper and pen was 400–1800 m/z.

Table 3. List of all 15 up-regulated proteins.

Accession	Gene name	Description	E/C	P-value
Q6GMX3	IGL@	IGL@ protein	1.5083	0.003
P02741	CRP	C-reactive protein	1.4907	0.019
Q6N091	DKFZp686C02220	Putative uncharacterized protein	1.4597	0.003
Q6MZQ6	DKFZp686G11190	Putative uncharacterized protein	1.4309	0.011
Q6UWP8	SBSN	Suprabasin	1.4214	0.033
P18827	SDC1	Syndecan-1	1.3535	0.020
A0A075B7D0	IGHV1OR15-1	Protein IGHV1OR15-1	1.3198	0.031
Q96JD0		Amyloid lambda 6 light chain variable region SAR	1.3145	0.004
D5H3V7	HLA-A	MHC class I antigen	1.3037	0.030
B2RP22	AKAP6	A kinase (PRKA) anchor protein 6	1.2919	0.026
A0A0S2Z4L3	PROS1	Protein S isoform 2	1.2812	0.0003
Q76LX8	ADAMTS13	A disintegrin and metalloproteinase with thrombospondin motifs 13	1.2439	0.019
B4E1B3		cDNA FLJ53950, highly similar to Angiotensinogen	1.2352	0.035
Q92889	ERCC4	DNA repair endonuclease XPF	1.2254	0.033
P05155	SERPING1	Plasma protease C1 inhibitor	1.2104	0.032

Table 4. Top 15 down-regulated proteins, Apo-lipoproteins and energy metabolism related proteins.

Accession	Gene name	Description	E/C	P-value
Q9NNW7	TXNRD2	Thioredoxin reductase 2, mitochondrial	0.3468	0.029
Q9P1F3	ABRACL	Costars family protein ABRACL	0.4185	0.035
B2RNT7	KLHDC5	Kelch domain containing 5	0.4256	0.004
P0CG39	POTEJ	POTE ankyrin domain family member J	0.4603	0.013
Q15555	MAPRE2	Microtubule-associated protein RP/EB family member 2	0.4638	0.019
Q14019	COTL1	Coactosin-like protein	0.4792	0.031
P63104	YWHAZ	14-3-3 protein zeta/delta	0.4929	0.022
A0A024R1K7	YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	0.4943	0.021
A0A0K0K1I0	HEL-S-265	Epididymis secretory protein Li 265	0.5029	0.020
P23528	CFL1	Cofilin-1	0.5111	0.029
P01019	AGT	Angiotensinogen	0.5150	0.011
B4DW52		cDNA FLJ55253, highly similar to Actin, cytoplasmic 1	0.5315	0.040
Q9HBI1	PARVB	Beta-parvin	0.5428	0.015
V9HWH1	HEL57	Epididymis luminal protein 57	0.5474	0.012
P67936	TPM4	Tropomyosin alpha-4 chain	0.5494	0.030
Q7Z7Q0	APOB	APOB protein	0.62485	0.005
Q59HB3	APOB	Apolipoprotein B variant	0.6868	0.0254
O95445	APOM	Apolipoprotein M	0.827	0.0015
A0A0A0MTS2	GPI	Glucose-6-phosphate isomerase	0.831	0.048
Q6GTS8	PM20D1	N-fatty-acyl-amino acid synthase	0.7058	0.002
P00558	PGK1	Phosphoglycerate kinase 1	0.687	0.0276

Mass spectrometric data analysis

The data obtained with mass spectrometric analysis were retrieved by Protein Pilot 4.2 software, using the Paragon method. Protein identification confidence level was set as 95%, Prot Score >1.3, and a peptide above 95% matched peptides in the library. At the same time, a protein reverse library search was made. The FDR <5%, Ratio >1.2 or Ratio <0.8 was regarded as differential expression.

Gene Ontology (GO) functional analysis

All differentially expressed proteins identified by the approaches above were assigned their gene symbol via the PANTHER database (Protein Analysis through Evolutionary Relationships, *http://www.pantherdb.org/*). Protein classification was performed based on their functional annotations using GO for biological process, molecular function, and cellular component.

When more than 1 assignment was available, all of the functional annotations were considered in the results.

Data analysis and statistics

The data are presented as mean±SD and were assessed by *t* test. Differences with p values <0.05 were considered to be statistically significant.

Results

Age, BMI, blood pressure, and glucose of subjects

The BMI of obese group was significantly higher than that of the control group (Table 1). However, their age, systolic blood pressure, diastolic blood pressure, and glucose were normal and had no significant differences (p>0.05) (Table 1).
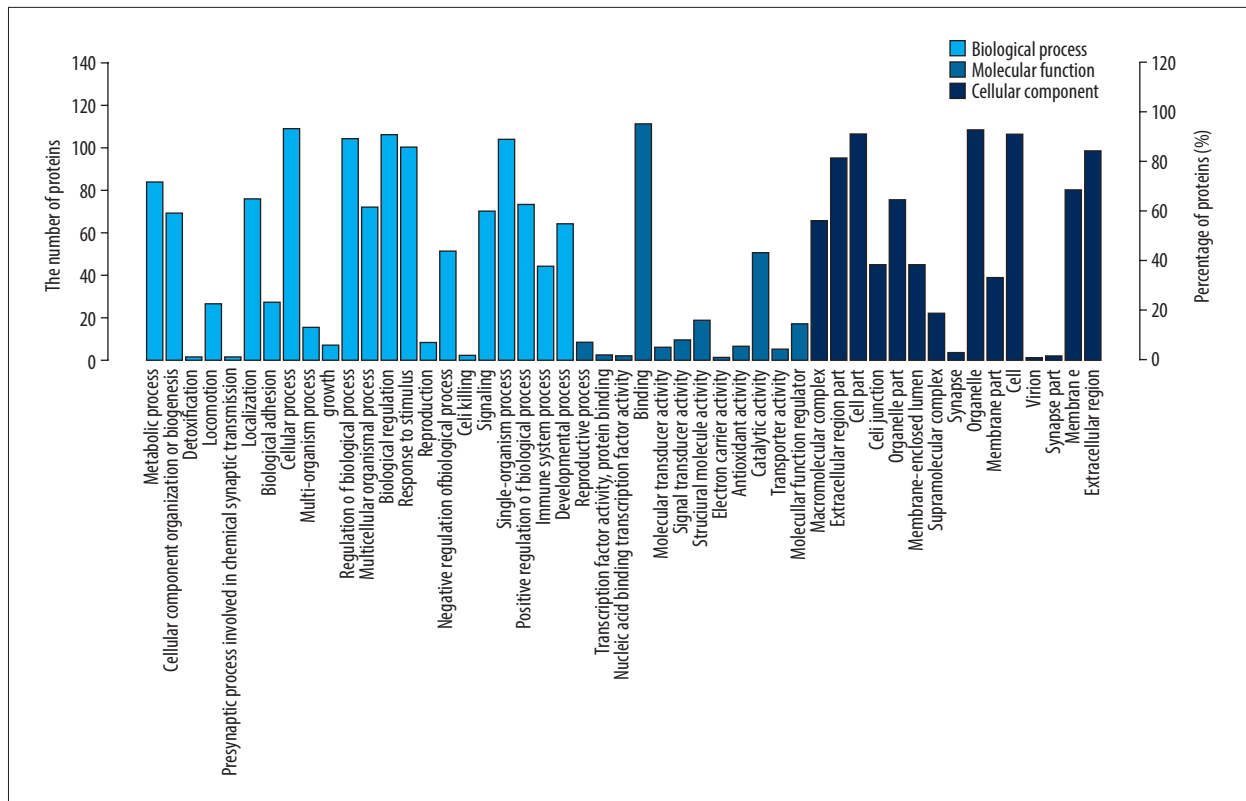
Figure 1. GO (Gene Ontology) functional annotation.

Basic data of identified proteins

A total of 9622 differential peptides were identified, corresponding to 733 proteins; 118 proteins showed significant differential expression, with 15 upregulated and 103 downregulated (Table 2). The upregulated proteins included IGL@ protein, C-reactive protein, Putative uncharacterized protein, and Suprabasin (Table 3). The downregulated proteins included Thioredoxin reductase 2, Costars family protein ABRACL, Kelch domain containing 5, and POTE ankyrin domain family member J (Table 4). The downregulated Apo-lipoproteins included apolipoprotein B variant, ApoB, and ApoM. The downregulated energy metabolism-related proteins included phosphoglycerate kinase 1, N-fatty-acyl-amino acid synthase, and glucose-6-phosphate isomerase.

Enrichment analysis of differentially expressed proteins

The results of Go functional annotation analysis indicated the percentage of differential expression of proteins involved in every biological procedure, molecular function, and cellular component (Figure 1). The items with high percentage of differential proteins were involved in included cellular processes, signaling process, binding, cell part, and organelles (Figure 1). Furthermore, the top 30 enriched GO terms indicated the significantly enriched GO terms involved by the differentially expression of proteins (Figure 2).

The significantly enriched GO terms included actin cytoskeleton, contractile fiber, myofibril, and platelet aggregation (Figure 2).

KEGG enrichment pathway analysis of differentially expressed proteins

The top 30 enriched pathways involved with differential proteins were demonstrated. These pathways included salmonella infection, tight junction, shigellosis, and regulation of actin cytoskeleton (Figure 3).

Discussion

The incidence of obesity in China is increasing rapidly. At present, more than one-half of males are overweight. The increase of abdominal obesity in men is fairly obvious, even in young males. It is necessary to investigate obesity to prevent and control the development and progression of obesity. In this study, we used iTRAQ technology to analyze the serum proteomics among obese male patients, and identified several obesity-related proteins, thereby providing a basis for further research on obesity.

According to the results of this study, the level of C-reactive protein was significantly higher in serum of obese patients, which
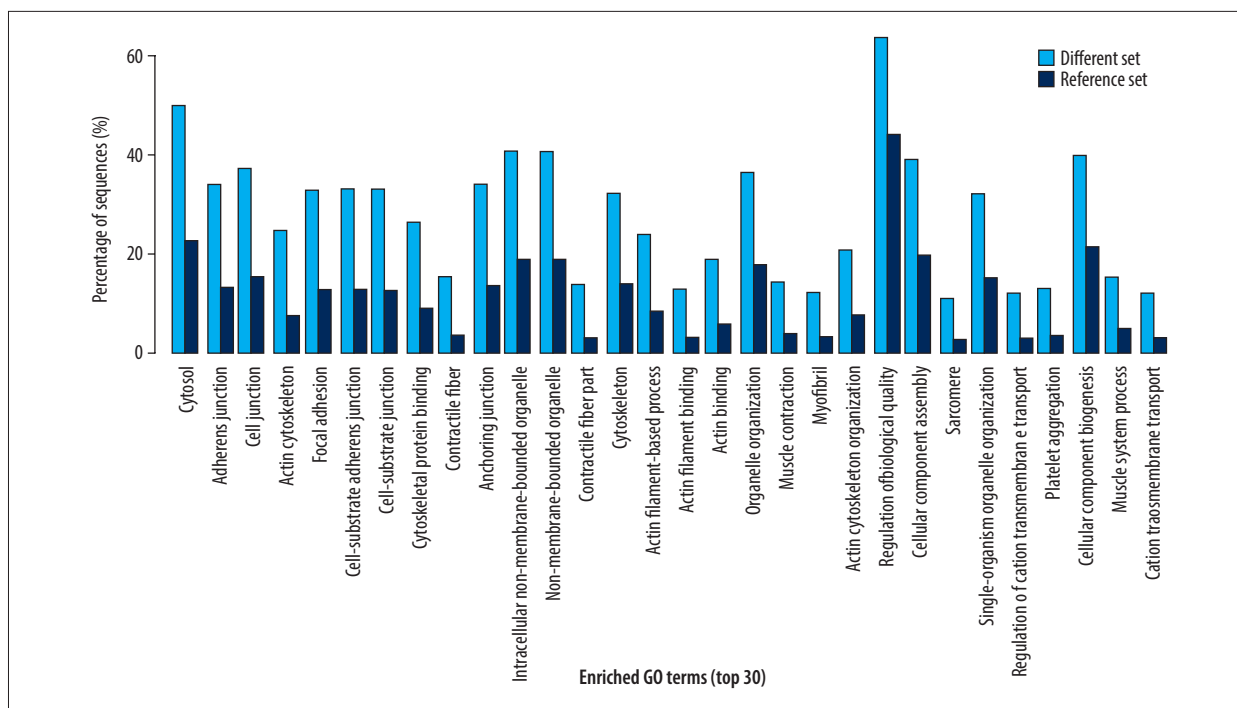
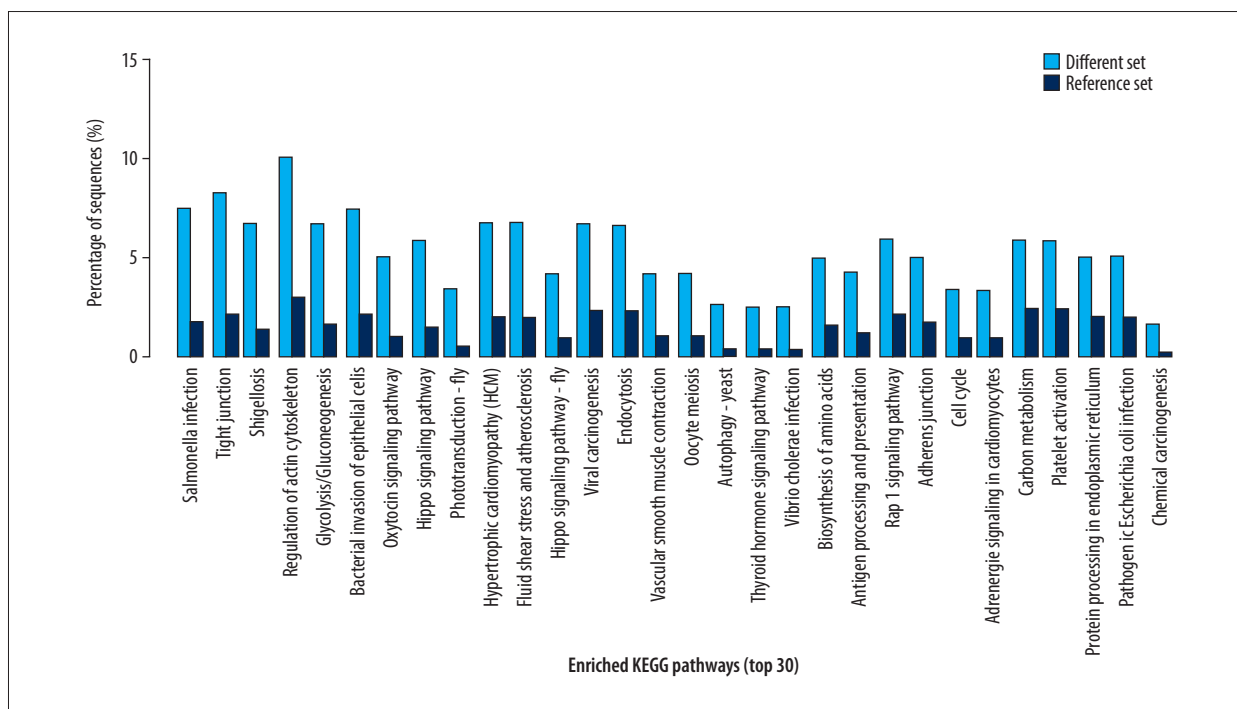**Figure 2.** Enriched GO terms (Top 30) analysis.

Figure 3. Top 30 enriched KEGG pathways.

was consistent with previous studies [10,11]. We found that upregulation of C-reactive protein promoted chronic inflammatory status. C-reactive protein is a pro-inflammation factor and can strongly predict the risk of cardiovascular events, since it can mediate cardiovascular impairment. Some studies have demonstrated that C-reactive protein in adipose cells can inhibit expression and secretion of adiponectin [10,11]. Adiponectin is a specific protein generated by adipose cells and is associated with glucose utilization, fatty acid oxidation, and energy consumption, thus playing an important role in obesity [12]. In addition, adiponectin is importantly involved in formation of arteriosclerosis plaque. It can inhibit inflammation reaction through various mechanisms, thereby preventing arteriosclerosis formation. However, more investigations are needed to explore the reverse association between C-reactive protein and adiponectin and its impact on obesity.

We found that the levels of apolipoprotein B variant, ApoB, and ApoM were significantly lower in obese patients. Relevant studies have demonstrated that ApoA1, Apo A-IV, and ApoB levels were downregulated and ApoC-III was upregulated in serum of obese patients. The ratio of ApoB/ApoA1 is tightly associated with obesity, which is consistent with that of previous studies [13–16]. Therefore, ApoB may be a potential marker of obesity. ApoM is a recently-discovered apolipoprotein that is a member of the lipoprotein superfamily. It mainly exists in high-density lipoprotein particles. ApoM is involved in pre-beta-HDL synthesis, promotes vascular protection function of HDL, and promotes insulin release [17]. It is regarded as being potentially protective against atherosclerosis, coronary disease,

and diabetes [18,19]. Similar to C-reactive protein, recent studies have found that ApoM expression was significantly regulated in some inflammation-related diseases such as hepatitis, sepsis, pneumonia, and arthritis [20]. Thus, apart from relying on the lipid transportation function of ApoM, the association between ApoM and obesity might be more complicated, and further investigations are needed to elucidate the mechanism involved.

Moreover, levels of phosphoglycerate kinase 1, N-fatty-acyl-amino acid synthase and glucose-6-phosphate isomerase, which are closely associated with energy metabolism, were significantly lower in obese patients. However, the levels of adiponectin and leptin were not assessed, partly due to the upregulation of C-reactive protein. Therefore, studies with larger sample sizes are needed in the future.

Conclusions

The development of obesity may involve a series of complex elements and mutual effects. Using the high-volume iTRAQ technique, we identified several proteins which were obviously regulated in serum of obese subjects. These proteins may provide novel directions and targets for future pathological studies of obesity.

Conflict of interest

None.

References:

1. Consensus of obesity prevention and treatment among Chinese adults. Chin J Endocrinol Metab, 2011; 27: 711–18

2. Wu Y, Ma G, Hu Y et al: [The current prevalence status of body overweight and obesity in China: Data from the China national nutrition and health survey.] Zhonghua Yu Fang Yi Xue Za Zhi, 2005; 39(5): 316–20

3. Mackay J, Mensah GA, Mendis S et al: The atlas of heart disease and stroke. Geneva: WHO, 2004; 5–6

4. Kim OY, Shin MJ, Moon J et al: Plasma ceruloplasmin as a biomarker for obesity: A proteomic approach. Clin Biochem, 2011; 44(5–6): 351–56

5. Galata Z, Moschonis G, Makridakis M et al: Plasma proteomic analysis in obese and overweight prepubertal children. Eur J Clin Invest, 2011; 41(12): 1275–83

6. Joo JI, Oh TS, Kim DH et al: Differential expression of adipose tissue proteins between obesity-susceptible and resistant rats fed a high-fat diet. Proteomics, 2011; 11(8): 1429–48

7. Xie WD, Wang H, Zhang JF et al: Proteomic profile of visceral adipose tissue between low-fat diet-fed obesity-resistant and obesity-prone C57BL/6 mice. Mol Med Rep, 2010; 3(6): 1047–52

8. Wei C, Lai M, Mo X: Screening serum biomarkers in gastric cancer by iTRAQ-coupled 2D-LC-MS/MS. J Prac Med, 2014; 30: 1154–57

9. Li L, Rao K, Kong L et al: Survey on nutrition and health status of Chinese residents in 2002. Chin J Epidemiol, 2005; 26(7): 478–84

10. Wang J, Yu W, Xu J et al: [Screening of protein markers on the plasma of obese young men.] Zhonghua Yu Fang Yi Xue Za Zhi, 2013; 47(2): 147–50

11. Gao P, Jiao Z, Ma J et al: Effects of C-reactive protein on regulation of adiponectin expression in human adipocytes. Journal of Harbin Medical University, 2011; 45: 27–29

12. Yamauchi T, Kamon J, Waki H et al: The fat-derived hormone adiponectin reverse insulin resistance associated with both lipotrophy and obesity. Nat Med, 2001; 7(8): 941–46

13. Li X, Hou Y, Wang Y et al: Analysis on levels of apolipoprotein, lipoprotein(a) and blood lipids in patients with adiposity. Journal of Tianjin Medical University, 2005; 11: 380–81

14. Zhang G, Wang Z, Huang H: Study on the clinical significance of overweight/obesity and diabetic patients serum apolipoprotein B and apolipoprotein A1 ratio. China Modern Doctor, 2013; 51: 54–56

15. Hui L, Gui-Fa XU, Gen-Sheng L et al: Relationship among Chinese serum apolipoprotein A-IV level, obesity and leptin level. Journal of Shangdong University, 2007; 45: 6–9

16. Suhong Wu, Bao-Jin C, Li-Wen Z et al: Study on the expression of Apo A5 in obese children and its correlation with adiponectin and fasting insulin. Journal of Clinical and Experimental Medicine, 2017; 16(5): 479–82

17. Ren K, Tang Z-L, Jiang Y et al: Apolipoprotein M. Clin Chim Acta, 2015; 446: 21–29

18. Ferrante M, Henckaerts L, Joossens M et al: New serological markers in inflammatory bowel disease are associated with complicated disease behavior. Gut, 2007; 56(10): 1394–403

19. Seow CH, Stempak JM, Xu W et al: Novel anti-glycan antibodies related to inflammatory bowel disease diagnosis and phenotype. Am J Gastroenterol, 2009; 104(6): 1426–34

20. Stephens NS, Stiffledeen J, Su X et al: Urinary NMR metabolomic profiles discriminate inflammatory bowel disease from healthy. J Crohns Colitis, 2013; 7(2): e42–48