Comparison of Urine Proteome Between Obese and Normal Populations

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Abstract

Objective: To compare the urine proteome between obese and normal populations. Methods: Urine samples were collected from obese and normal populations and analyzed using label-free quantitative proteomics technology based on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differential proteins between the urine proteomes of obese and normal populations were screened for protein function and biological pathway analysis; the urine proteome of obese individuals was compared with that of the normal population, and common differential proteins were statistically analyzed for protein function and biological pathway analysis; reported obesity biomarkers were searched within the urine proteome of obese individuals. Results: Thirty-eight differential proteins could be identified in the urine proteome of obese populations compared with normal populations, some of which have been reported to be associated with metabolism and obesity, and the biological processes enriched by the differential proteins were also related to metabolic processes; comparison between obese individuals and normal populations enriched eight common differential proteins in the urine proteome, some of which have been reported to be associated with metabolism and obesity, and the biological processes enriched by the differential proteins were also related to metabolic processes; among the differential proteins in the urine proteome of obese individuals compared with normal populations, matches to reported obesity biomarkers could be found. Conclusion: The urine proteome can distinguish between normal individuals and obese individuals, with key proteins known to be associated with obesity and metabolism present among the differential proteins, and the differential proteins can be enriched in biological processes related to nutrition, metabolism, etc. The urine proteome has the potential to explore the mechanisms of obesity development and provide personalized treatment.

Full Text

Comparison of Urine Proteomes Between Obese and Normal-Weight Individuals

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Objective: To compare the urine proteomes between obese and normal-weight individuals.

Methods: Urine samples were collected from both obese and normal-weight subjects and analyzed using label-free quantitative proteomics via high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Differentially expressed proteins between the two groups were screened for functional and pathway analysis. Additionally, individual obese subjects were compared against the normal-weight cohort to identify commonly altered proteins, which were similarly analyzed for function and biological pathways. Reported obesity biomarkers were also searched within the urine proteomes of obese individuals.

Results: Thirty-eight differential proteins were identified in the urine proteome of obese individuals compared to normal-weight controls. Several of these proteins have been previously reported to be associated with metabolism and obesity, and the enriched biological processes of the differential proteins were also related to metabolic pathways. Comparison of individual obese subjects with the normal-weight group identified eight common differential proteins, some of which have been reported to be associated with metabolism and obesity, with enriched biological processes similarly linked to metabolic pathways. Among the differential proteins identified in obese individuals, matches were found with previously reported obesity biomarkers.

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Conclusions: The urine proteome can distinguish obese from normal-weight

individuals. The differential proteins include key proteins known to be associated with obesity and metabolism, and these proteins are enriched in biological processes related to nutrition and metabolism. Urine proteomics holds potential for investigating the mechanisms of obesity development and providing personalized therapeutic targets.

Keywords: Urine proteome; obesity; Body mass index

1. Introduction

Obesity has become a major global public health challenge, significantly increasing the risk of numerous chronic diseases including type 2 diabetes, coronary heart disease, and certain cancers. The pathogenesis of obesity is complex, resulting from interactions among genetic, nutritional, and metabolic factors. Although there is currently no clear consensus on terminology for classifying and characterizing obesity, it can be broadly categorized into four phenotypes: (1) normal weight obese (NWO); (2) metabolically obese normal weight (MONW); (3) metabolically healthy obese (MHO); and (4) metabolically unhealthy obese (MUO); (5) sarcopenic obese (SO) [1 2].

Currently, no significant biomarkers exist for distinguishing obesity and its subtypes. Body Mass Index (BMI), calculated as weight in kilograms divided by height in meters squared, remains the primary classification tool. According to Chinese BMI standards, underweight is defined as BMI < 18.5 kg/m^2, normal weight as $18.5 \leq$ BMI < 24 kg/m^2, overweight as $24 \leq$ BMI < 28 kg/m^2, and obesity as BMI ≥ 28 kg/m^2 [1 3 4]. However, BMI is an imperfect measure of abnormal body fat [1].

More sophisticated techniques such as magnetic resonance imaging are used to assess body fat distribution for better diagnosis of obesity subtypes, but these methods are not readily available in routine clinical practice and their cutoff values have not been established [1]. Simpler methods for rapidly identifying specific biomarkers that characterize the causes of obesity and provide targets for personalized treatment require further investigation. Urine, produced by renal filtration of blood to excrete metabolic waste, is not subject to homeostatic regulatory mechanisms and can therefore retain extremely subtle physiological changes [5]. Studies have demonstrated that the urine proteome can monitor disease biomarkers for conditions such as diabetes [6], Alzheimer's disease [7], depression [8], and autism [9], and can classify diseases including predicting chronic kidney disease [10] and distinguishing benign from malignant ovarian tumors [11]. However, urine proteomics has not yet been applied to identify biomarkers for understanding obesity pathogenesis and enabling personalized treatment. This study therefore investigates whether urine proteomics can reflect the causes of obesity, provide potential drug targets, and assist in personalized therapy through comparative analysis of obese and normal-weight populations.

2.1.1 Sample Collection

A total of 19 urine samples were collected from subjects at Beijing China-Japan Friendship Hospital. According to Chinese BMI reference standards, normal weight was defined as $18.5 < \text{BMI} < 23.9$ and obesity as $\text{BMI} > 28$. The cohort included 10 obese subjects (mean BMI = 35.79 kg/m^2) and 9 normal-weight subjects (mean BMI = 22.76 kg/m^2). This study utilized discarded samples from the clinical laboratory without involving any personal patient information. Ethics approval number: 2023-KY-126. Subject BMI values are shown in Table 1 .

2.1.2 Urine Sample Processing

Urine Protein Extraction: Urine samples were retrieved from -80°C storage and thawed at 4°C. Samples were centrifuged at 12,000 × g for 30 min at 4°C. Six milliliters of supernatant were collected, and 20 mM dithiothreitol (DTT, Sigma) was added, vortexed, and heated in a water bath at 37°C for 60 min, then cooled to room temperature. Fifty millimolar iodoacetamide (IAA, Sigma) was added, vortexed, and incubated at room temperature in darkness for 40 min. After the reaction, three volumes of pre-cooled absolute ethanol were added, gently mixed by inversion, and proteins were precipitated at -20°C for 24 h. The precipitated mixture was centrifuged at 12,000 × g for 30 min at 4°C, the supernatant was discarded, and ethanol was allowed to evaporate to dryness. The protein pellet was resuspended in lysis buffer (containing 8 mol/L urea, 2 mol/L thiourea, 25 mmol/L DTT, 50 mmol/L Tris), centrifuged at 12,000 × g for 30 min at 4°C, and the supernatant was transferred to a new 1.5 mL tube to obtain the urine proteins. Protein concentration was determined using the Bradford method.

Urine Protein Digestion: One hundred micrograms of urine protein were placed in a 1.5 mL tube, and 25 mmol/L NH_4HCO_3 solution was added to a final volume of 200 L. A 10 kDa ultrafiltration tube (Pall, Port Washington, NY, USA) was prepared by adding 200 L UA solution (8 mol/L urea, 0.1 mol/L Tris-HCl, pH 8.5) to the membrane and centrifuging at 14,000 × g for 5 min at 18°C; the lower filtrate was discarded and the wash was repeated once. The iodoacetamide-treated urine protein sample was added to the membrane and centrifuged at 14,000 × g for 30 min at 18°C, with the lower filtrate discarded, leaving urine proteins on the membrane. The membrane was washed with 200 L UA solution and centrifuged at 14,000 × g for 30 min at 18°C, repeated twice. The membrane was then washed with 25 mmol/L NH_4HCO_3 solution and centrifuged at 14,000 × g for 30 min at 18°C, repeated twice. Trypsin (Trypsin Gold, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for digestion at 37°C for 16 h. After digestion, the filtrate was

collected by centrifugation at 13,000 × g for 30 min at 4°C, yielding a peptide mixture. The peptide mixture was desalted using HLB solid-phase extraction columns (Waters, Milford, MA), lyophilized using a vacuum dryer, and stored at -20°C.

2.1.3 LC-MS/MS Tandem Mass Spectrometry Analysis

The lyophilized peptide mixture was dissolved in 0.1% formic acid, quantified using a BCA assay kit, and diluted to 0.5 g/ L. Six microliters from each sample were pooled and separated using a high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific). Ten fractions were collected by centrifugation, lyophilized using a vacuum dryer, and reconstituted in 0.1% formic acid. iRT reagent (Biognosys, Switzerland) was added to the ten fractions and all individual samples at a 10:1 sample-to-iRT volume ratio to calibrate the retention times of extracted peptide peaks.

The ten fractions were separated using an EASY-nLC1200 chromatography system (Thermo Fisher Scientific, USA), and the separated peptides were analyzed using an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA) in Data Dependent Acquisition (DDA) mode to generate ten raw files. These were imported into Proteome Discoverer software (version 2.0, Thermo Scientific) for library construction using the Swiss-iRT and Uniprot-Rat databases. Based on the library results, a DIA method with 39 variable windows was established for individual sample analysis. One microgram of peptides from each individual sample was separated using the EASY-nLC1200 system and analyzed by the Orbitrap Fusion Lumos Tribrid mass spectrometer in Data Independent Acquisition (DIA) mode using the newly established DIA method to generate raw files.

2.1.4 Label-Free DIA Quantitative Analysis

Individual sample raw files acquired in DIA mode were imported into Spectronaut Pulsar (Biognosys AG, Switzerland) software for analysis. Peptide abundance was calculated by summing the peak areas of fragment ions in MS^2 spectra, and protein abundance was calculated by summing the abundances of constituent peptides.

2.1.5 Data Analysis

Each sample was analyzed in three technical replicates, and average values were used for statistical analysis. Group comparisons between obese and normal-weight cohorts were performed to screen for differential proteins using the follow-

ing criteria: fold change (FC) ≥ 2 or ≤ 0.5, and two-tailed unpaired t-test $P < 0.01$. Additionally, one-to-many comparisons were conducted by comparing each individual obese sample against all nine normal-weight samples using criteria of FC ≥ 1.5 or ≤ 0.67 and $P < 0.055$. Common differential proteins present across all ten obese individuals were then identified. Differential proteins were functionally analyzed using the Uniprot database (https://www.uniprot.org/) and relevant literature was searched in PubMed (https://pubmed.ncbi.nlm.nih.gov).

3.1.1 Differential Proteins

Comparison of urine proteomes between obese and normal-weight groups identified 38 differential proteins meeting the criteria of FC ≥ 2 or ≤ 0.5 and two-tailed unpaired t-test $P < 0.01$. These differential proteins, sorted by fold change, are listed in Table 2 with their Uniprot IDs and protein names.

3.1.2 Differential Protein Functional Analysis

The 38 differential proteins were searched against the PubMed database for functional annotation. While several of the most significantly altered proteins have not yet been reported in association with obesity or diabetes, Insulin-like growth factor-binding protein 1 (IGFBP-1) showed a fold change of 0.286 ($P = 0.002$), indicating more than three-fold downregulation in the obese group relative to normal-weight controls. Numerous studies have demonstrated an inverse relationship between IGFBP-1 levels and BMI, waist-to-hip ratio, and fasting insulin levels [12 13].

IGFBP-1 levels are primarily regulated dynamically by insulin. Postprandial insulin secretion suppresses IGFBP-1 expression by inhibiting its upstream promoter, causing IGFBP-1 levels to decline rapidly. This reduction enhances the bioavailability of Insulin-like growth factor 1, potentiating its insulin-like effects [14 15 16]. The positive correlation between IGFBP-1 concentration and insulin sensitivity has been confirmed across diverse populations, including Europeans and Pakistanis [17], Asian Indians [18], healthy young adults [19], adults over 65 years [20], obese postmenopausal women [21], type 1 diabetes patients [22], and prepubertal children [12 23]. Consequently, IGFBP-1 is considered a potential marker of insulin sensitivity [24]. A study of 615 individuals identified IGFBP-1 concentration and its interaction with IGF-1 as important determinants in the development of glucose intolerance or diabetes [25]. Research on 355 Swedish men demonstrated that low fasting IGFBP-1 concentration could predict the development of abnormal glucose regulation, with some individuals showing a 40-fold increased risk of diabetes [26]. A 17-year follow-up study of 782 individuals found that low IGFBP-1 expression predicted type 2 diabetes onset [27], and an 8-year study of 240 women similarly associated IGFBP-1 with increased

diabetes risk [28].

Other differential proteins have also been linked to metabolism or obesity. Aconitate hydratase participates in the tricarboxylic acid cycle and carbohydrate metabolism, catalyzing the isomerization of citrate to isocitrate via cis-aconitate and controlling adipogenesis by mediating cellular ATP production [29]. Inhibins and activins regulate pituitary follicle-stimulating hormone secretion. Inhibin beta is involved in regulating hypothalamic and pituitary hormone secretion, gonadal hormone production, germ cell development and maturation, erythroid differentiation, insulin secretion, neuronal survival, embryonic axial development, and bone growth, with BMI serving as an important independent predictor of its levels [30].

While the most significantly altered proteins in the obese group have been associated with obesity or metabolism, those showing even greater changes have not yet been studied in this context, warranting further investigation to identify novel obesity biomarkers or potential drug targets.

3.1.3 Differential Protein Enrichment Biological Process Analysis

DAVID database analysis of differential proteins between obese and normal-weight groups enriched 24 biological processes with $P < 0.01$, as shown in Figure 1 [Figure 1: see original paper]. Notable metabolic processes included intracellular cholesterol transport, ATP metabolic process, bile acid metabolic process, cholesterol transport, and cholesterol metabolic process. Several processes related to gene expression and the nervous system showed even smaller P values. Telomere organization may be relevant to obesity, as telomere length is a robust marker of biological aging and accelerated telomere attrition has been observed in obese adults [31]. The remaining biological processes have not yet been linked to obesity or BMI, providing new research directions for understanding obesity pathogenesis.

3.2.1 Common Differential Proteins in Obese Individuals

Individual obese subjects were compared against the normal-weight cohort using criteria of FC ≥ 1.5 or ≤ 0.67 and two-tailed unpaired t-test $P < 0.05$ to identify common differential proteins across the obese group. Eight common differential proteins were identified, as listed in Table 3 . Notably, all differential proteins in individual obese subjects were downregulated relative to normal-weight controls, and except for Peptidyl-prolyl cis-trans isomerase-like 1 and Queuosine 5'-phosphate N-glycosylase/hydrolase, the remaining six proteins were expressed in the normal-weight group but absent in individual obese subjects.

3.2.2 Functional Analysis of Common Differential Proteins

Although the common differential proteins showed consistent and significant changes in the obese group, most have not been reported in association with obesity or diabetes. Coiled-coil domain-containing protein 194, in particular, has no annotated function or biological process in the Uniprot database. Nevertheless, some common differential proteins are obesity-related. Protocadherin beta exhibits calcium ion binding capacity and may be involved in establishing and maintaining specific neuronal populations in the brain. Protocadherin beta genes are expressed in the hypothalamus and exhibit rare variants with biological effects. A study of 30 extremely obese Caucasian adults (mean BMI = 51.1 kg/m^2) detected significant enrichment of such rare variants in peripheral blood leukocytes, a finding not observed in normal-BMI populations [32]. Similarly, rare mutations in this gene were significantly enriched in Korean obese children [33]. Detection of this protein's significant difference between obese (mean BMI = 35.79 kg/m^2) and normal-weight groups via urine proteomics demonstrates the sensitivity of this approach and its potential for identifying early obesity markers. Extremely obese populations also show enrichment of olfactory receptor gene mutations, which cooperate with protocadherin gene variants in extreme obesity [34].

These differential proteins exhibit significant and uniform changes in the obese group, yet remain understudied, meriting further investigation into their roles in obesity and metabolism to identify novel biomarkers or therapeutic targets.

3.2.3 Enrichment Analysis of Common Differential Proteins

DAVID database analysis of the eight common differential proteins identified across 8-10 obese individuals enriched 15 biological processes with $P < 0.05$, as shown in Figure 2 [Figure 2: see original paper]. Processes related to nutrition and metabolism included response to hypoxia (which exacerbates adipose tissue dysfunction and stimulates inflammatory molecule secretion, contributing to obesity [35]), ADP catabolic process, cellular response to nutrient levels, lipid transport, cholesterol metabolic process, and UDP-N-acetylglucosamine biosynthetic process. Adipose tissue UDP-N-acetylglucosamine correlates positively with BMI, and inhibiting its biosynthesis reduces glucose-stimulated leptin release in cultured adipocytes [36]. Dysregulated glucose metabolism associated with obesity, diabetes, or cancer correlates with increased enzyme levels in this pathway. Several gene expression and nervous system-related processes showed smaller P values. Telomere organization may be obesity-related, as telomere length is a robust marker of biological aging with accelerated attrition noted in obese adults [30]. The remaining biological processes have not been linked to

obesity or BMI, offering new research avenues for understanding obesity pathogenesis.

3.3 Individualized Analysis of Obese Subjects

Numerous obesity biomarkers have been previously identified [1 37 38]. Searching for these markers among differential proteins from individual obese subjects revealed that many could be detected through urine proteomics, with different biomarkers appearing in different individuals, as shown in Table 4 . This demonstrates the potential of urine proteomics to assist in developing personalized treatment strategies for obesity.

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4. Conclusion

Using LC-MS/MS-based label-free quantification, this study performed group comparisons of ten obese samples versus nine normal-weight samples, along with one-to-many individual analyses, to screen differential proteins and conduct functional and pathway analyses. Both group and individual analyses demonstrated that urine proteomics can distinguish obese from normal-weight individuals, with some differential proteins previously reported to be associated with obesity or metabolism. Individual analysis provides personalized clues for obese subjects, facilitating personalized medicine. This study highlights the potential of urine proteomics for investigating obesity mechanisms, identifying drug targets, and guiding personalized treatment strategies, warranting further exploration with larger sample sizes.

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Note: Figure translations are in progress. See original paper for figures.