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Original Research

Integrating metabolomics into obesity care: towards biomarker-guided pharmacy practice

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Abstract

Background: Obesity is a complex and prevalent global health issue strongly associated with chronic diseases. Early diagnosis and precise monitoring remain challenging due to the complex metabolic dysregulation underlying obesity. Metabolomics provides a powerful tool to investigate biochemical changes and discover novel diagnostic biomarkers. Objectives: This study used untargeted metabolomics of human plasma samples to identify distinct plasma metabolite profiles and altered metabolic pathways in overweight and obese individuals. The ultimate goal is to improve obesity management through early diagnosis and personalized treatments. **Methods:** A total of 74 Jordanian participants were recruited and categorized into normal-weight (n=29), overweight (n=17), and obese (n=28) groups based on BMI and metabolic parameters. Plasma samples were analyzed using ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC-ESI-QTOF-MS). Data processing and statistical analysis were performed using MetaboScape and MetaboAnalyst 5.0. Group comparisons were evaluated using t-tests, ANOVA, and multivariate models, and pathway enrichment analysis was conducted to determine altered metabolic pathways. **Results:** A total of 82 metabolites were identified, with 26 showing significant differences between groups. In the overweight group, pantothenic acid and L-proline were elevated, while phenylacetaldehyde and glycerophosphocholine were decreased. The obese group exhibited increased levels of L-leucine, L-tryptophan, phenylalanine, and tyrosine, and reduced levels of 2,3-diaminopropionic acid and phenylacetaldehyde. Key altered pathways included pantothenate and CoA biosynthesis, beta-alanine metabolism, phenylalanine and tyrosine metabolism, and beta-oxidation of long-chain fatty acids. **Conclusions:** The study revealed significant novel metabolic disturbances associated with overweight and obesity, highlighting potential diagnostic biomarkers and perturbed metabolic pathways. These findings provide valuable insights into the molecular underpinnings of obesity, underscore the potential of metabolomics in advancing personalized approaches for managing obesity, and warrant further validation in larger, diverse populations to assess their diagnostic and clinical relevance.

Keywords: Obesity, Overweight, Metabolomics, Metabolites, Metabolic pathways

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INTRODUCTION

In 1997, the World Health Organization (WHO) officially recognized obesity as a significant public health challenge and a widespread global epidemic. Obesity is a condition marked by an abnormal rise in body weight relative to height, primarily resulting from the accumulation of excess fat and metabolic dysregulation. The typical method for diagnosing obesity involves assessing the body mass index (BMI), a calculation derived from the ratio of body weight (in kilograms) to the square of height (in meters squared). This BMI calculation enables physicians to categorize individuals from being overweight to morbidly obese. Generally, an individual with a BMI of 25 kg/m^2 or higher is classified as overweight, while a BMI of 30 kg/m^2 or higher falls into the obesity category[1]. Over the past four decades, the prevalence of obesity has undergone a substantial surge on a global scale. If this upward trajectory persists, a majority of the adult population worldwide is projected to be either overweight or obese by the year 2030[2]. This phenomenon is influenced by various factors, encompassing genetic, metabolic, behavioral, and environmental elements[3]. Obesity poses a significant risk to public health, leading to premature mortality and constituting a substantial portion of the global burden of non-communicable diseases. This includes conditions such as type 2 diabetes, cardiovascular disease, hypertension, and specific forms of cancer[4].

Recently, Omics technologies, including metabolomics, proteomics, genomics, and transcriptomics, have become the method for diagnosing diseases in their early stages[5,6]. Metabolomics is characterized as a technological tool designed to identify and quantify alterations in the patterns and concentrations of small molecular weight metabolites (< 1500 Da), which are small intermediates or end products of metabolic reactions catalyzed by several enzymes within cells, tissues, organs, systems, or entire organisms in response to genetic variations or physiological and pathological states[7]. Biomarker innovative research to detect and characterize biomolecules in complex matrices with high sensitivity and selectivity has been at the forefront of mass spectrometry (MS) in combination with liquid chromatography (LC) and gas chromatography (GC) separation techniques, enhancing comprehension of underlying mechanisms and potential diagnostic indicators for several diseases[8].

In the context of obesity, metabolomics serves as a valuable tool for assessing molecular-level alterations of metabolites in obese or overweight subjects compared with normal-weight individuals, facilitating the identification of distinctive biomarkers linked to BMI and obesity. Leveraging metabolomics investigations can enhance the efficacy and precision of clinical practices and research involving obese individuals. This approach enables a more targeted exploration of particular metabolites and critical pathways, offering valuable insights for the treatment and potential prevention of obesity and its associated severe complications[9].

This study examines the metabolomes of individuals with normal-weight and those with overweight or obesity using ultra-high performance liquid chromatography, electrospray ionization, quadrupole time of flight, and mass spectrometry (UHPLC-ESI-QTOF-MS) in a Jordanian cross-sectional study. The advanced analytical platform (UHPLC-ESI-QTOF-MS) is employed to identify biomarkers linked to obesity and complications related to associated diseases. Our research aims to unveil biomarkers that could facilitate early disease diagnosis related to obesity, exploring their connection to metabolic pathways for a deeper understanding of obesity's molecular-level pathophysiology. This cutting-edge MS technology platform provides an advanced capability for identifying and profiling metabolites, even in challenging sample conditions.

MATERIALS AND METHODS

Population and Study Design

A cross-sectional research study was conducted in Jordan from June to December 2017 in the Diabetes and Endocrinology Outpatient Clinics at the National Center for Diabetes Endocrinology and Genetics (NCDEG), University Hospital (JUH). Study participants were recruited following approvals obtained from the Scientific Research Committee at the School of Pharmacy at the University of Jordan, and the clinical Institutional Review Board (IRB) Committees affiliated with NCDEG JUH (101675/9/SM80/2016/1439). All procedures involving human participants in this study adhered to the ethical standards set by the institutional research committee and aligned with the principles of the Helsinki Declaration.

The participants' ages ranged from 18 to 75 years. The study population was categorized into three groups based on participants' BMI values, glycemic parameters, and the presence of at least two components of metabolic syndrome (MetS), along with central obesity, along with central obesity, per the definition specified by the International Diabetes Federation (IDF) [10]. Recruiters were divided into three groups:

1. Group 1 (Normal-weight individuals as control): Normoglycemic (with HbA1c<5.7% or FPG <100 mg/dL) and lean with 19.5< BMI kg\m² < 25.
2. Group 2 (Overweight individuals): Non-diabetic overweight subjects of BMI ≥25 kg/m² having three or more of the MetS components as delineated by the International Diabetes Federation (IDF) [10].
3. Group 3 (Obese individuals): Non-diabetic obese subjects of BMI ≥ 30 kg/m² having three or more of the MetS components as delineated by the IDF[10].

Collection of Samples

A total of 74 individuals were recruited for the study. Plasma samples were collected from 29 healthy individuals with normal-weight, 17 overweight, and 28 obese individuals. Plasma was obtained after collecting blood samples into heparinized tubes, followed by centrifugation for 5 min (14,000 rpm). Plasma samples were stored at –80 °C and shipped to the Research Institute, University of Sharjah, for further analysis.

Preparation of the Samples for Metabolomics Extraction

Upon aliquoting the samples into 100 µL Eppendorf tubes, 300 µL of methanol (sourced from Wunstorfer Strasse, Seelze, Germany) was introduced. The tubes were thoroughly mixed with a vortex mixer and were subsequently incubated at -20 °C for 2 hours. After this period, the samples were vortexed again and centrifuged for 15 minutes at 14,000 rpm. The resulting supernatant underwent evaporation at 35–40 °C.

A quality control (QC) sample was prepared to guarantee the analysis's consistency and reliability by combining an equal volume (10 µL) from each sample. This QC sample was injected into the system after every 9-10 samples to evaluate the analysis's reproducibility. Before injection, the extracted samples were reconstituted in 250 µL of 0.1% formic acid in deionized water using Honeywell's LC-MS CHROMASOLV in Wunstorfer Strasse, Seelze, Germany. After sample preparation, the supernatant underwent filtration for subsequent LC-MS/MS analysis. This filtration utilized a hydrophilic nylon syringe filter with a pore size of 0.45 µm. The filtered sample was meticulously collected within a specialized insert positioned inside LC glass vials, ensuring its integrity for further analysis[11].

Ultra-High-Performance Liquid Chromatography Coupled to Electrospray Ionization and Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-ESI-QTOF-MS)

The LC-MS/MS analysis utilized an advanced ultra-high-performance liquid chromatography system (UHPLC) provided by Bruker Daltonik GmbH in Bremen, Germany. This system included vital components such as a quadrupole time-of-flight mass spectrometer (QTOF), an electrospray ionization (ESI) source, a solvent delivery system pump (HPG 1300), an autosampler, and a thermostat column compartment. Operating on the Windows 10 Enterprise 2016 LTSB operating system, the system employed Bruker Compass HyStar 5.0 SR1 Patch1 (5.0.37.1), Compass 4.1 for timsTOF Series, and otofControl Version 6.2 software for data acquisition.

The analysis employed mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). The gradient program was: 0–2 min, 99% A: 1% B; 2–17 min, 99–1% A: 1–99% B; 17–20 min, 99% B: 1% A. The flow rate was fixed at 0.25 ml/min. Subsequently, 20–20.1 min 99% B to 99% A; 20.1–28.5 min, 99% A: 1% B at 0.35 ml/min flow rate; 28.5–30 min, 99% A: 1% B at 0.25 ml/min. The flow rate was maintained at a constant value throughout the analysis. The sample, in the form of a 10 µL aliquot, was injected into a Hamilton® Intensity Solo 2 C18 column (2.1 mm × 100 mm, 1.8 µm) for separation. The column oven temperature was set to 35 °C. For each injection, the parameters of the ESI source were configured as follows: The capillary voltage was adjusted to 4500 V, the flow rate of the drying gas was set at 10.0 l/min with a temperature of 220 °C, and the nebulizer pressure was held steady at 2.2 bar. In the MS2 acquisition phase, the collision energy stepping spanned from 100 to 250%, maintaining a constant value of 20 eV and an end plate offset of 500 V[12].

To perform the external calibration process, sodium formate served as the calibrant. The acquisition process was divided into two segments: the auto MS scan segment, spanning from 0 to 0.3 minutes, and the auto MS/MS segment, encompassing fragmentation, lasting from 0.3 to 30 minutes. Both segments were executed in the positive mode at 12 Hz. The automatic in-run mass scan range covered 20 to 1300 m/z, with a precursor ion width of ±0.5. Three precursors were chosen per cycle with a cycle time of 0.5 seconds, and the threshold was established at 400 counts. Active exclusion was initiated after three spectra and lifted after 0.2 minutes. Positive ion mode is chosen for its ability to ionize a wide variety of metabolites, providing higher sensitivity in detecting many metabolite types, especially those with basic functional groups. This enhances the identification of low-abundance compounds and ensures broader coverage of the metabolome.

Data Processing and Analysis

The acquired data underwent analysis through MetaboScape® 4.0 software (Bruker Daltonics, Billerica, MA, USA). For the processed data, the T-ReX 2D/3D workflow employed bucketing parameters that included an intensity threshold of 1000, a peak length spanning 7 spectra, and the use of peak area for quantification. Mass spectra calibration was executed within the 0-0.3-minute range, utilizing features from a minimum of 50 to 148 samples. The auto MS/MS scan followed the average method, with a retention time range from 0.3 to 25 minutes and a mass range of 50 to 1000 m/z. The LC-QTOF analysis involved duplicate samples obtained from 74 participants across all groups. After merging these samples, a dataset comprising 3763 unique features was generated. The identification of metabolites was accomplished by aligning the MS/MS spectra and retention time with the HMBD 4.0 database, meticulously

crafted to address the specific needs of the metabolomics community. Following filtration using MetaboScape®, a comprehensive set of 85 distinct metabolites was chosen. The peak intensities of each metabolite were employed to construct the quantitative data matrix. Only metabolites demonstrating statistical significance, with a *p-value* of less than 0.05* and documented in the human metabolome database 4.0 (HMDB), were incorporated into the metabolite datasets. The online website HMDB (https://hmdb.ca/metabolites/HMDB0059911) was used to filter the human metabolites. Following HMDB filtration, 82 unique metabolites remained. The metabolite datasets were exported as CSV files and subsequently imported into the MetaboAnalyst 5.0 software—a comprehensive metabolomics data analysis platform created by McGill University in Montreal, QC, Canada. For sample classification, the sparse partial least squares-discriminant analysis (sPLS-DA) method in MetaboAnalyst was employed. This method was chosen because it offers clearer group separation, enables focused identification of key metabolites that contribute most to group differences through feature selection, manages high-dimensional data more effectively, and enhances overall interpretability. These strengths are particularly important in metabolomics studies involving plasma groups. This process aimed to minimize the rate of false positives, and corrections for multiple hypothesis testing were applied using the false discovery rate (FDR) approach. The identification of significantly altered metabolites in the overweight or obese group, as opposed to the standard weight group, was accomplished through a two-tailed independent Student's t-test. This led to the creation of a volcano plot, visually representing the statistical significance and fold change (p<0.05*, FC=1.25), highlighting the dysregulation of cellular metabolites for each condition. Furthermore, a one-way analysis of variance (ANOVA) was applied for a comprehensive comparison across multiple groups, encompassing normal-weight, overweight, and obese groups. The threshold for significance was p<0.05*. Functional Enrichments were constructed using Metaboanalyst (https://www.metaboanalyst.ca). Additionally, MetaboAnalyst 5.0 was utilized for the enrichment of metabolite sets and pathway analysis. A Venn diagram was generated using (http://bioinformatics.psb.ugent.be/webtools/Venn/).

RESULTS

Participant and Blood Sample Characteristics

The study included a total of 74 participants, all of whom were exclusively of Jordanian nationality, with 28 males (37.84%) and 46 females (62.16%). The average age of all study participants was calculated at 40.61 ± 4.1 years, revealing a statistically significant age distinction between the overweight or obese groups and the normal-weight group (p < 0.001***), as delineated in Supplementary Table 1. A comprehensive examination of various health parameters revealed that the overweight and obese groups exhibited notably elevated levels of diastolic blood pressure (DBP) (p < 0.001***), systolic blood pressure (SBP) (p < 0.001***), triglycerides (p < 0.001***), low-density lipoprotein cholesterol (LDL-C) (p < 0.001***), and glycosylated hemoglobin (HbA1C%) (p < 0.001***). Simultaneously, they displayed lower levels of high-density lipoprotein cholesterol (HDL-C) (p < 0.001***) compared to the normal-weight group, as outlined in Supplementary Table 1.

Metabolic Changes

A total of 82 metabolites were identified, with 26 exhibiting significant differences (p<0.05) among the three groups analyzed using the ANOVA test. The sPLS-DA model used group labels (e.g., normal weight, overweight, obese) to guide the analysis, demonstrating successful partial separation of the dataset obtained from the three groups, overcoming some challenges attributed to the dataset's complexity and high dimensionality, as illustrated in Figure 1A. However, the sPLS-DA model demonstrated a distinct separation with minimal overlap between each pair of groups when compared to the normal-weight group, indicating significant dissimilarities, as illustrated in Figure 1B and Figure 1C.

A Venn diagram showed an overlap of four metabolites that exhibited differential abundance in overweight and obese individuals compared to those with normal weight, namely, pantothenic acid, L-proline, phenylacetaldehyde, and glycerophosphocholine. Interestingly, it is noteworthy that there were 16 metabolites exclusively exhibiting dysregulation in the obese group, as depicted in Supplementary Figure 1**.** These include L-acetylcarnitine, L-Leucine, isovalerylcarnitine, hippuric acid, heptadecanoic acid, DL-2-aminooctanoic acid, cortisol, L-serine, m-coumaric acid, phenol, L-tyrosine, 5-hydroxy, L-tryptophan, L-kynurenine, 2,3-Diaminopropionic acid, L-phenylalanine, and uric acid (Supplementary Table 2).

The MetaboAnalyst 5.0 software was employed for conducting a two-tailed independent Student's t-test and assessing the fold change between pairs of groups (p<0.05*, FC=1.25). The comparative analysis was conducted in the following directions: Overweight/Normal-weight; Obese/Normal-weight. Four of the 82 identified metabolites exhibited significant differences when comparing the normal-weight group with the overweight group (2 increased vs. 2 decreased) as detailed in Table 1. Specifically, pantothenic acid and L-proline showed increased levels in the overweight group, whereas phenylacetaldehyde and glycerophosphocholine were notably decreased in the overweight group compared to the normal-weight group, as illustrated in Figure 2.

The results of the t-test analysis indicate that among the 82 analyzed metabolites, 24 displayed a statistically significant difference (p < 0.05*). These significant metabolites comprised 15 increased and 9 decreased metabolites, as outlined in Table 2. It is worth noting that the obese group showed significantly elevated levels of certain metabolites, including L-proline, L-leucine, L-tryptophan, phenylalanine and tyrosine, and others, as illustrated in Figure 3. Conversely, the obese group demonstrated significantly lower levels of metabolites such as 2,3-Diaminopropionic acid, phenylacetaldehyde, uric acid, and others in comparison to the normal-weight group, as illustrated in Figure 3.

Functional Analysis Pathway Changes

We employed MetaboAnalyst 5.0 (https://www.metaboanalyst.

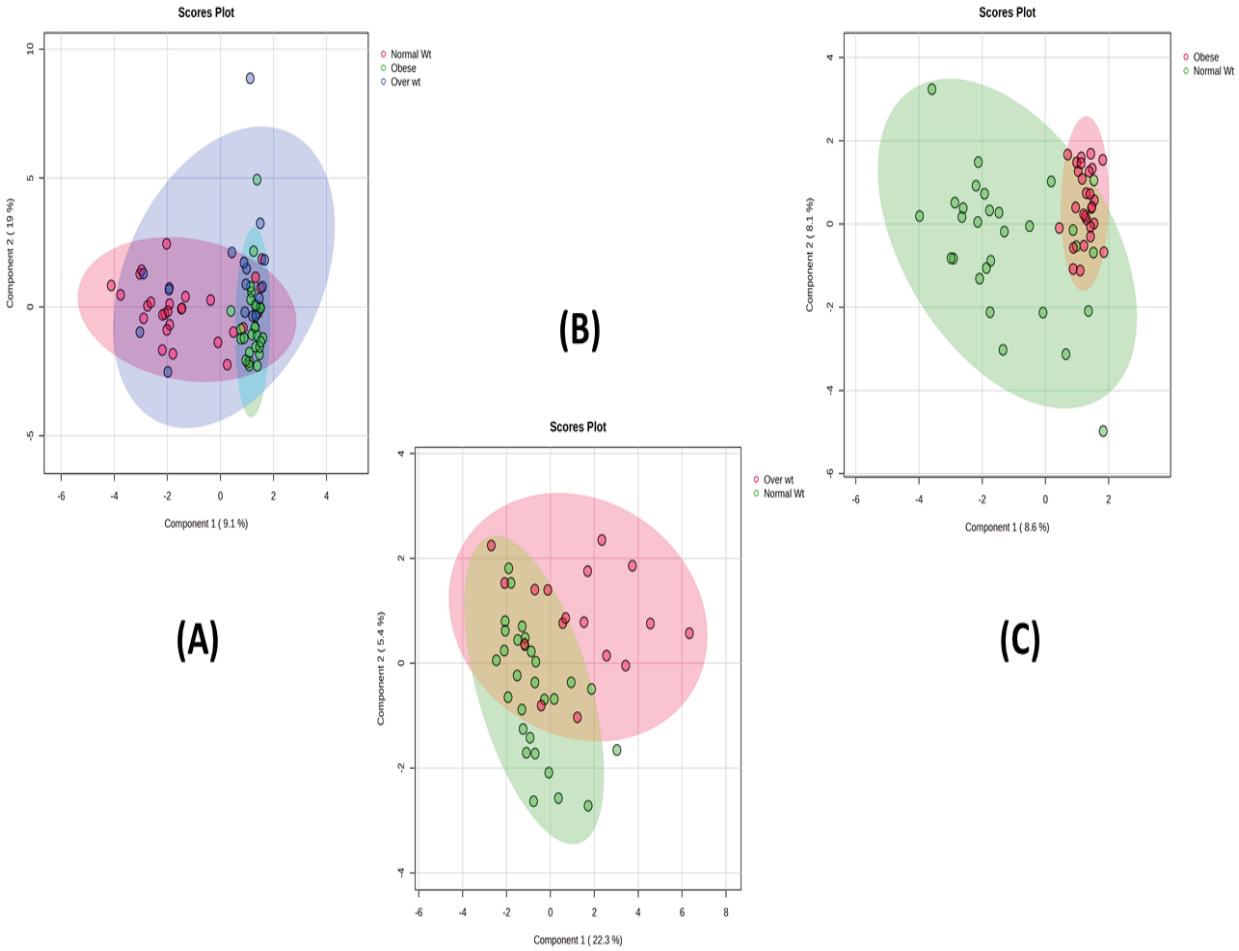

Figure 1. The sparse partial least squares-discriminant analysis (sPLS-DA) for all groups; Group 1: normal-weight; Group 2: overweight; Group 3: obese. (A) sPLS-DA model for all three groups. (B) sPLS-DA model for Groups 1 and 2. (C) sPLS-DA model for Groups 1 and 3.

Table 1. Metabolites with significant differences between the overweight and the normal-weight groups.

Metabolite name	**t.stat**	***P*.value**	**FDR**	**Fold Change**
Phenylacetaldehyde	-3.6872	0.00042	0.017764	0.60972
Pantothenic acid	3.7738	0.000433	0.017764	1.5645
L-Proline	3.6184	0.000794	0.021694	1.9523
Glycerophosphocholine	-3.2507	0.001697	0.034782	0.77455

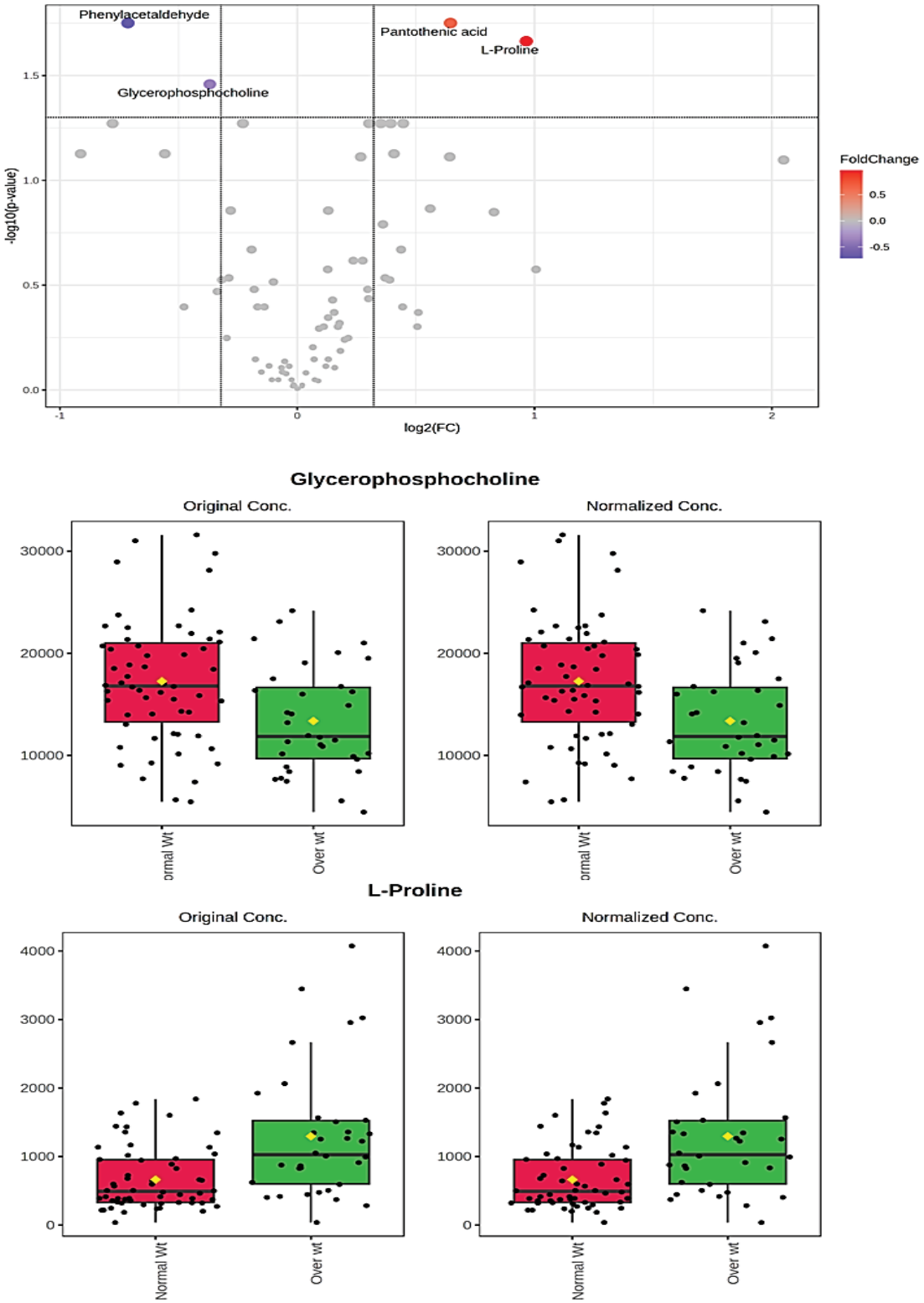

Figure 2. Volcano plots showing metabolites that were significantly altered in the overweight group compared to the normal-weight group and box plots for selected significant metabolites.

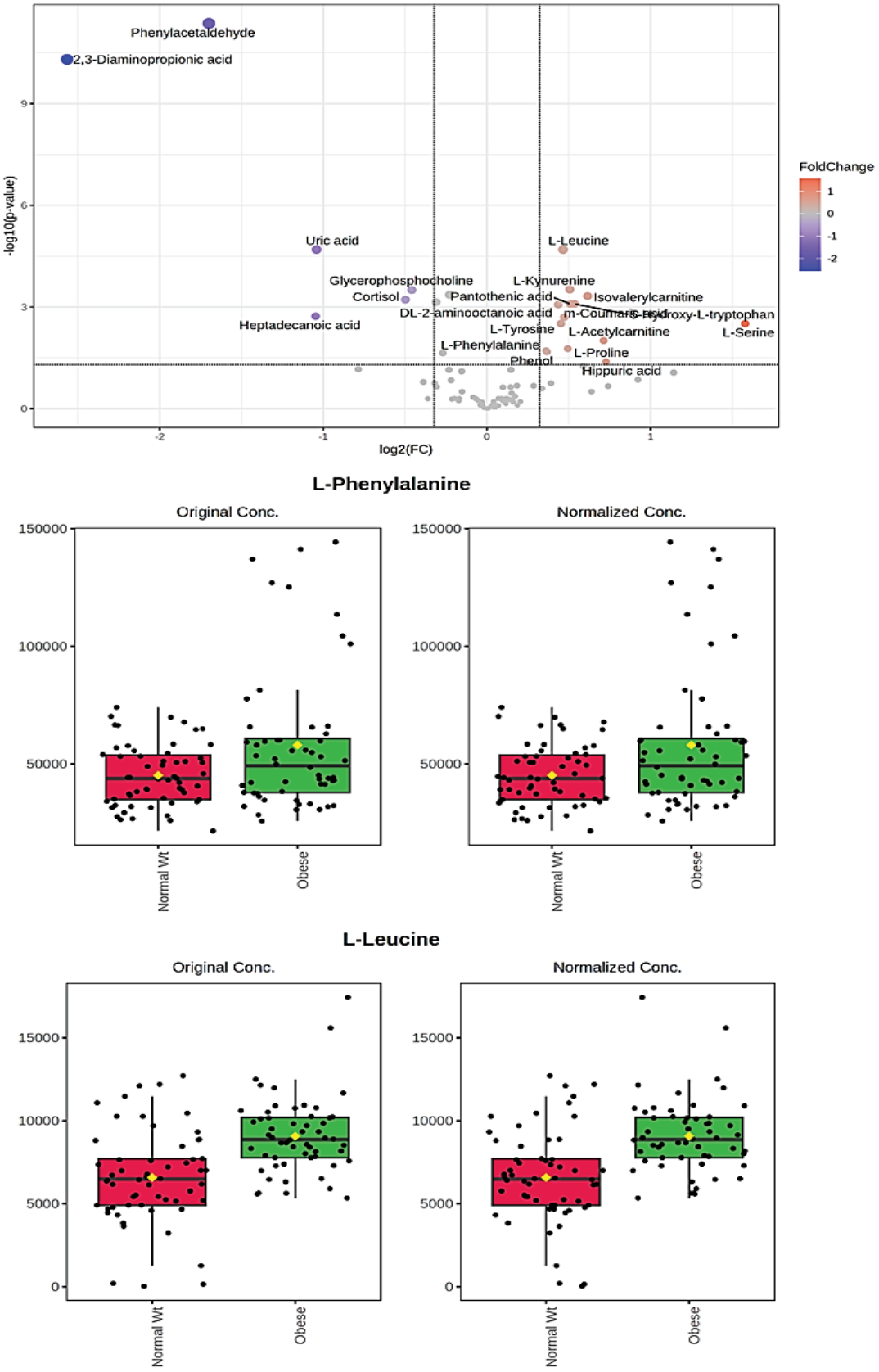

Figure 3. Volcano plots showing metabolites that were significantly altered in the obese group compared to the normal-weight group and box plots for selected significant metabolites.

Table 2. Metabolites with significant differences between the obese and the normal-weight groups.

Metabolite name	t.stat	p.value	FDR	Fold Change
Phenylacetaldehyde	-9.3706	$p < 0.001$***	4.33E-12	0.30786
2,3-Diaminopropionic acid	-9.05	$p < 0.001$***	4.96E-11	0.16887
L-Leucine	5.2265	$p < 0.001$***	2.03E-05	1.3803
Uric acid	-5.1978	$p < 0.001$***	2.03E-05	0.48579
L-Kynurenine	4.5255	$p < 0.001$***	0.000304	1.4202
Glycerophosphocholine	-4.4216	$p < 0.001$***	0.000315	0.72738
Urea	-4.354	$p < 0.001$***	0.000437	0.85326
Isovalerylcarnitine	4.2692	$p < 0.001$***	0.000475	1.5315
Cortisol	-4.1614	$p < 0.001$***	0.000606	0.70794
5-Methylcytidine	-4.0719	$p < 0.001$***	0.00072	0.80617
Pantothenic acid	4.0191	0.00011	0.000812	1.4241
5-Hydroxy-L-tryptophan	3.9989	0.000119	0.000812	1.4494
DL-2-aminooctanoic acid	4.0266	0.000134	0.000844	1.3527
Heptadecanoic acid	-3.7518	0.00032	0.001873	0.4838
m-Coumaric acid	3.676	0.000369	0.002016	1.3871
L-Tyrosine	3.5297	0.000612	0.003079	1.3672
L-Serine	3.5756	0.000638	0.003079	2.9851
L-Acetylcarnitine	3.1523	0.002151	0.009798	1.6395
L-Proline	2.946	0.003942	0.017015	1.4084
L-Phenylalanine	2.9113	0.004751	0.019477	1.2842
Phenol	2.8358	0.005426	0.021188	1.2893
2,4-Diaminobutyric acid	-2.8025	0.00613	0.022847	0.82921
Hippuric acid	2.5812	0.011608	0.041386	1.6558
4-Methoxyphenylacetic acid	2.5469	0.012276	0.041944	NA

ca/MetaboAnalyst/, Accessed on 27 November 2023) for pathway analysis to investigate dysregulated metabolites in two crucial comparisons: normal-weight versus overweight and normal-weight versus obese groups. The identified metabolites were cross-referenced with the HMDB, PubChem, and KEGG databases. Our focus in enrichment pathway analysis was to pinpoint metabolic pathways where the metabolite profiles of overweight and obese groups significantly differed from those of the normal-weight group. The pathway analysis results unveiled notable differences in several metabolic pathways between the overweight and obese groups compared to the normal-weight group. Specifically, pantothenate and CoA biosynthesis, and beta-alanine metabolism were notably modified in the overweight vs normal-weight groups. Conversely, phenylalanine and tyrosine metabolism, homocysteine degradation, D-arginine and D-ornithine metabolism, phosphatidylethanolamine biosynthesis, arginine and proline metabolism, thyroid hormone synthesis, tryptophan metabolism, and beta-oxidation of very long-chain fatty acids exhibited significant alterations in the obese vs. normal-weight groups. Visual representations and a summary of these identified metabolic pathways are provided in Supplementary Figure 2 and Figure 4.

Metabolomics data are deposited in the Metabolomics Workbench with (datatrack_id:4549 study_id:ST003026). The DOI for this project (PR001880) is: http://dx.doi.org/10.21228/M8Q72K

DISCUSSION

Impact of Metabolic Dysregulation on Biomarker Signatures of Obesity

This work aimed to study the metabolic profile of plasma samples obtained from overweight and obese individuals. Our statistical analysis revealed significant differences in 26 metabolites between the overweight/and or obese vs. the control group. Illustrated in the Venn diagram are four metabolites with significant differences shared between the overweight and obese groups, showcasing a pattern of 2 increased and 2 decreased metabolites (Supplementary Figure 1**)**. Specifically, there was an observed increased level of pantothenic acid and L-proline. At the same time, phenylacetaldehyde and glycerophosphocholine exhibited a noteworthy reduction in comparison to the control group, as depicted in Supplementary Table 2. Obesity, characterized by an excess of body fat, emerges as a major contributor to cardiovascular disease (CVD) risk. It

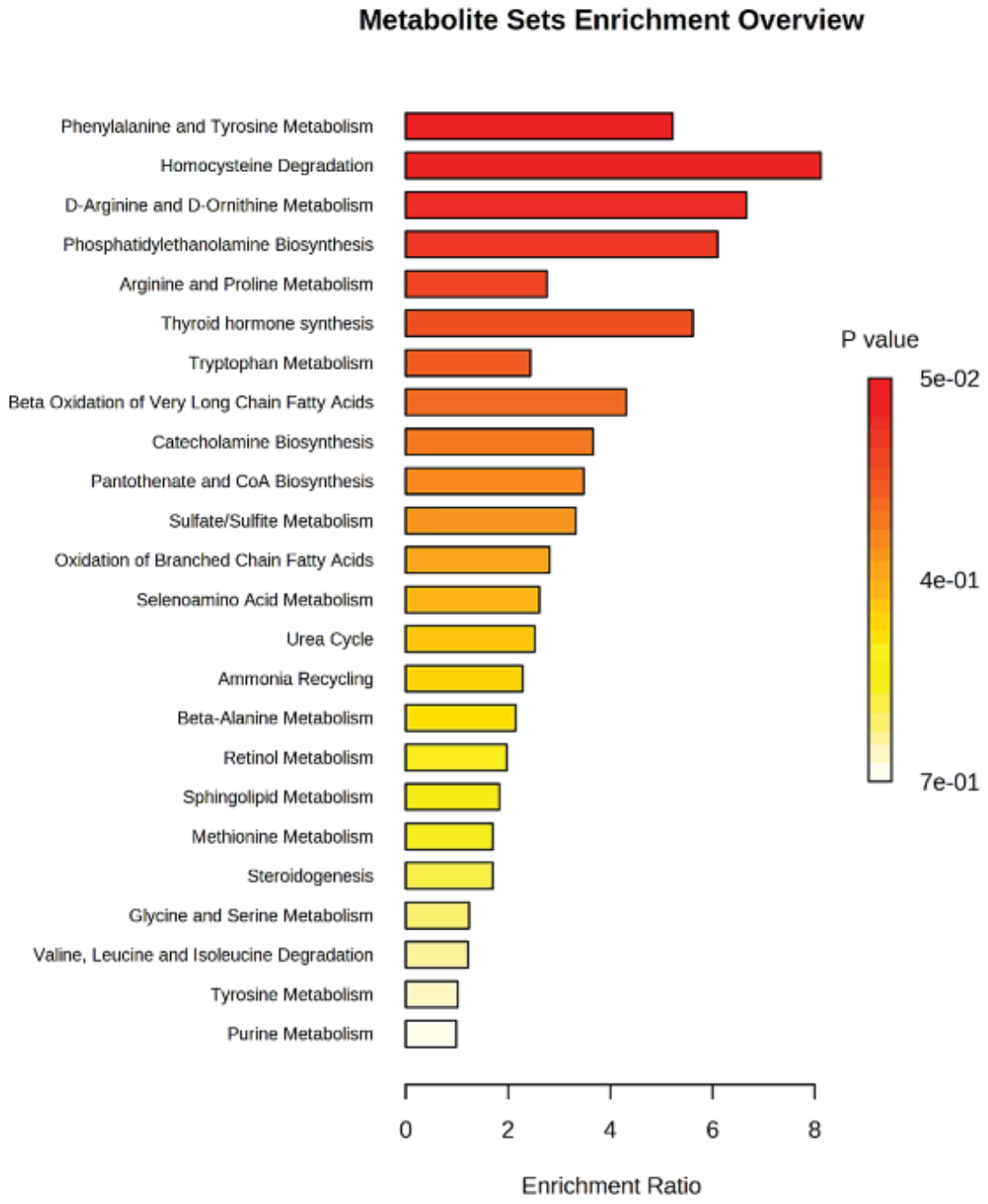

Figure 4. Enrichment Pathway analysis of the obese group vs the normal-weight group.

fosters the development of cardiovascular issues through the endocrine and paracrine effects of expanding adipose tissue. A notable consequence of obesity on cardiovascular health is atherogenic dyslipidemia. This condition is typically evaluated by considering commonly measured and recently discovered low-abundance lipids circulating in the blood. Among these lipids are glycerophosphocholine (GPC) metabolites, which hold the potential to enhance the prediction of CVD outcomes[13]. In a previous cohort study, targeted LC-ESI-MS was employed to identify and quantify novel lipid species, such as GPC, through targeted serum lipidomics. The study investigated the potential associations between these GPCs and classical risk factors for CVD, including excess visceral fat (VF), elevated blood pressure, insulin resistance, and atherogenic dyslipidemia. The researchers successfully identified novel GPCs strongly linked to multiple CVD risk factors. These GPCs have the potential to

serve as sensitive indicators of obesity-related CVD risk[14]. In another cohort study, scientists investigated the association between adolescent urinary metabolic signatures and BMI z-scores to explore the metabolic mechanisms underlying childhood obesity. Their findings showed that obesity in adolescents is linked to alterations in amino acid metabolism, and they mentioned that the identified metabolites may serve as potential biomarkers for the metabolic consequences of obesity, such as cardiovascular disease, stroke, diabetes, and cancer[15] Given the frequent coexistence of CVD in individuals with obesity during advanced stages and recognizing the potential of GPCs as novel biomarkers for preclinical CVD and obesity, our study hypothesized that plasma samples from overweight and obese individuals may exhibit GPC dysregulation (Figure 2, Figure 3, and Supplementary Table 2) similar to the previously mentioned study. We also believe that understanding these cardiometabolic effects is crucial for addressing the risks associated with obesity and developing effective strategies for preventing cardiovascular issues related to excess weight.

In addition, L-proline in our study was increased in the overweight and obese groups compared to the normal-weight group (Figure 2, Figure 3, and Supplementary Table 2). Proline, categorized as a non-essential amino acid, is essential for preserving cellular redox balance and for the structure and function of proteins. It is obtained from dietary sources, produced internally within cells, and released from protein structures[16]. Elevated levels of proline in plasma, observed in individuals with obesity, type 2 diabetes, and insulin resistance, may indicate the early activation of proline biosynthetic enzymes[17]. Numerous studies have examined the relationship between the progression of obesity and disturbances in proline metabolism. Analysis of data from the Obesity Research targeted metabolomics study for Mexican children reveals that an elevated proline metabolomic amino acid signature is not solely linked to obesity, insulin resistance, and lipid concentrations but also acts as an autonomous risk factor and predictor for subsequent hypertriglyceridemia[18]. These findings align with a previous study linking specific amino acids to obesity and insulin resistance in both adults and children[19]. Comparable results have been noted in adults with obesity and hyperlipidemia, indicating a significant elevation in the serum levels of proline[20]. Taken together, heightened proline levels in the blood could emerge as a potential independent predictor for recognizing obese individuals at risk of metabolic disorders.

In contrast, the Venn diagram (Supplementary Figure 1 and Supplementary Table 2) revealed 16 metabolites with distinctive differences exclusive to the obese group. In our observations, we identified significant changes in the level of phenylalanine, tyrosine, L-leucine, and others in obese individuals compared to those with normal body weight, as depicted in Table 2.

The metabolites phenylalanine and tyrosine were increased in obese individuals (Figure 3), indicating the likelihood of being a potential biomarker for obesity. Research consistently demonstrates notable alterations in phenylalanine and tyrosine levels, a hydroxylation product of phenylalanine metabolism, in individuals with obesity. These essential amino acids are primarily metabolized in the liver[21]. This observed increase aligns with findings from various studies investigating amino acid biomarkers in obesity, though the underlying mechanisms remain incompletely understood[22]. The rise in plasma phenylalanine and tyrosine levels could be attributed to various factors. One hypothesis suggests that heightened circulating levels of branched-chain amino acids (BCAAs) compete with aromatic amino acids for uptake into tissues through the shared large neutral amino acid transporter (LAT1)[23]. Another plausible explanation links the elevation to increasing liver dysfunction, a recognized risk factor for fatty liver disease in obesity, resulting in decreased phenylalanine and tyrosine metabolism and subsequent elevation in plasma levels[24]. The elevation of tyrosine with obesity lacks a clear explanation, indicating that the metabolic signatures of obese individuals evolve complexly[21].

Additionally, our results indicate an elevation in L-leucine levels in the obese group vs. the normal-weight group (Figure 3). In a previous study examining the metabolomes of individuals under normal and obese conditions, significant alterations in metabolites and associated metabolic pathways linked to energy balance and mitochondrial function were revealed. Notably, they found that BCAA, including isoleucine and valine, exhibited distinct expression levels between the two groups, with elevated BCAA levels suggested as potential indicators of diabetes incidence. Furthermore, they emphasized that the accumulation of downstream products in the BCAA metabolism pathway contributed to mitochondrial dysfunction. Evidence of irregular mitochondrial morphology was observed in obese and diabetic individuals. The investigators from this prior study concluded that the overall results emphasize the vital significance of preserving typical mitochondrial function to prevent or reduce the risk of obesity and diabetes. This insight illuminates the potential influence of BCAA in regulating the abnormal metabolic conditions linked with obesity[25]. Our results align with this, demonstrating a substantial increase in leucine levels, as shown in Figure 3, and highlighting the heightened risk of diabetes associated with obesity.

Insights into Obese Individuals' Altered Metabolic Pathways

Obesity is characterized by an imbalance wherein the body's energy intake surpasses its expenditure[26]. Over recent decades, there has been a comprehensive exploration of the pathophysiology of obesity, uncovering an escalating involvement of various signal transduction pathways in this complex condition. This imbalance significantly disrupts the plasma metabolome, contributing to a profound perturbation. Previous research has successfully identified distinct metabolic signatures associated with obesity, particularly emphasizing heightened levels of branched-chain and aromatic amino acids[27].

Phenylalanine and Tyrosine Metabolism

The phenylalanine and tyrosine metabolic pathways (Figure 5) include a crucial series of biochemical reactions with substantial implications for obesity and overall metabolic

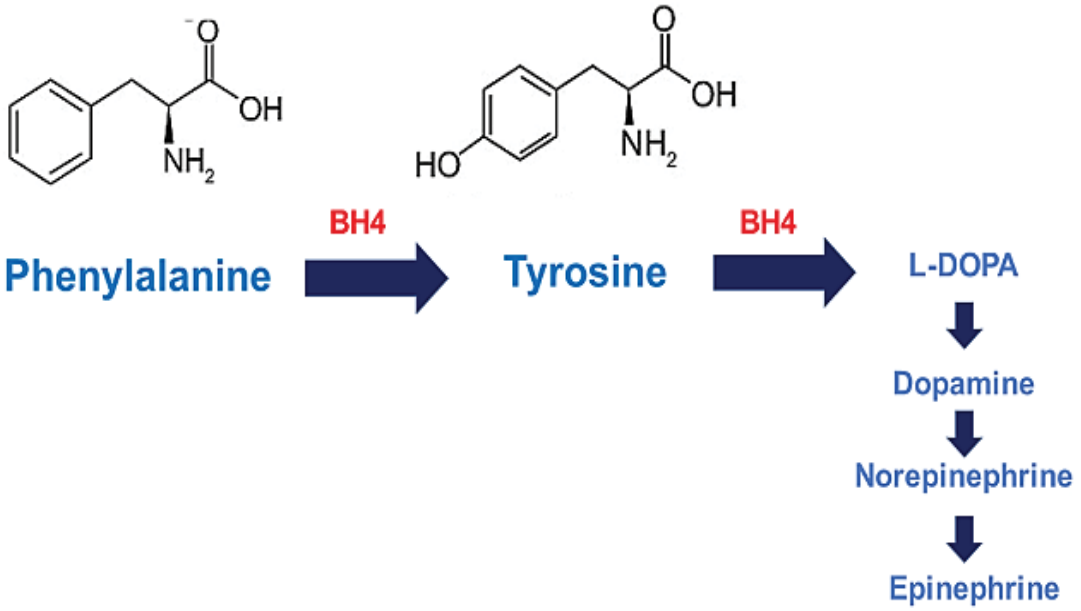

Figure 5. Phenylalanine and Tyrosine Metabolism pathway. Abbreviations: BH4: tetrahydrobiopterin.

health. Phenylalanine undergoes a pivotal transformation into tyrosine, facilitated by the enzyme phenylalanine hydroxylase. During this conversion, tetrahydrobiopterin (BH4) functions as a cofactor for phenylalanine 4-hydroxylase, playing a crucial role in the process. Afterward, when enriched with BH4, tyrosine becomes a fundamental component for synthesizing vital catecholamines, including neurotransmitters like dopamine, norepinephrine, and epinephrine. The subsequent transformation of tyrosine into L-DOPA by tyrosine 3-hydroxylase also necessitates BH4 as a cofactor, emphasizing its importance in neurotransmitter synthesis[28].

In the context of obesity, any disturbances in this complex metabolic pathway can lead to changes in phenylalanine and tyrosine levels. These disruptions, in turn, can potentially affect the synthesis of neurotransmitters crucial for various physiological processes. Numerous previous studies have identified a correlation between modifications in the operation of the sympathetic nervous system (SNS) and the initiation and persistence of both obesity and insulin resistance, underscoring the significant role of the SNS in regulating metabolic conditions in individuals with obesity[24]. The prior investigation demonstrated that any alteration in the catecholamine response could potentially impact the sensitivity of α- and β-adrenoceptors in adipose tissue, leading to a decrease in lipolysis and an increase in fat storage[29]. Additionally, changes in phenylalanine and tyrosine levels may influence appetite regulation, energy expenditure, and broader metabolic functions, contributing to the complexities associated with obesity, as evidenced by a previous study[30].

Our Pathway analysis assigns the highest impact score to phenylalanine and tyrosine metabolism, specifically through the increasing level of phenylalanine and tyrosine (as depicted in Figure 4). This implies that liver function plays a complex role in regulating metabolism in obesity, likely via the phenylalanine and tyrosine metabolism pathway. It highlights the significance of liver dysfunction in the metabolic dynamics of obese individuals, underscoring the critical role the liver plays in their metabolism, similar to that shown in prior investigations [24]. Additional research is essential for elucidating the complex mechanisms through which disturbances in the phenylalanine and tyrosine metabolic pathway and BH4 availability impact outcomes associated with obesity. In summary, our study highlights the considerable impact of phenylalanine and tyrosine metabolism on obesity and overall metabolic health, showcasing the intricate involvement of impaired liver function in the metabolic processes of individuals with obesity.

Tryptophan Metabolism

Numerous metabolites warrant in-depth exploration for their implications in the metabolic status associated with obesity. Notably, in our study, elevated concentrations of tryptophan and its metabolites, particularly kynurenine (Kyn), have been observed in individuals with obesity (Figure 3), displaying a correlation with BMI in comparison to those with normal-weight. Our results align with other study results[31]. Interestingly, abnormalities in the Kyn pathway have been observed in individuals with both obesity and insulin resistance[32]. Furthermore, elevated levels of both tryptophan and Kyn have been observed in patients with diabetic retinopathy as a complication of obesity[33].

In our study, the functional enrichment analysis revealed significant disruptions in the tryptophan metabolic pathway when comparing the obese group with the normal-weight group (Figure 4). Disturbances in amino acid metabolism, such as in the tryptophan metabolic pathway, are frequently observed in individuals with obesity. A prominent feature is

the increased presence of plasma Kyn, a metabolite derived from tryptophan. However, the primary origin of Kyn and its specific impact on obesity remain incompletely understood. Investigators in prior research suggested that the primary contributor to elevated Kyn levels is the increased activity of indoleamine 2,3-dioxygenase 1 (IDO1) in adipocytes, highlighting the central role of adipocytes in kynurenine metabolism. Mechanistically, kyn disrupts lipid balance in adipocytes by activating the aryl hydrocarbon receptor (AhR)/Signal transducer and activator of the transcription 3/ interleukin-6 signaling pathway[34]. Various studies discovered that the increased abundance of these amino acids, including tryptophan and kynurenine, might indicate immune activation or low-grade systemic inflammation, possibly linked to increased IDO enzyme activity[35]. The augmented activity of IDO appears to be intricately linked to the advancement of obesity. This connection can be elucidated through the involvement of dysregulated tryptophan, facilitated by IDO, in enhancing the supply of this amino acid for serotonin synthesis. This may influence the equilibrium of neurotransmitters, contributing to mood disruptions, depression, and compromised satiety, ultimately resulting in elevated caloric intake and the onset of obesity[36]. Serotonin undergoes a dual-phase synthesis process (Figure 6), initiated with a hydroxylation step dependent on BH4, facilitated by tryptophan hydroxylase (THB). Subsequently, a decarboxylation process is performed by DOPA decarboxylase[37]. Elevations in tryptophan levels result in an augmented serotonin content in the brain. It is crucial to note that the availability of BH4, an essential cofactor for tryptophan 5-hydroxylase in this pathway, plays a critical role in regulating serotonin synthesis [38]. Remarkably, our investigation revealed an elevation in tryptophan levels (Figure 3) upon examining plasma samples from obese individuals, indicating a substantial modification in tryptophan metabolism (Figure 4). Consequently, we hypothesized that these observations might contribute to increased caloric consumption and the development of obesity through excessive serotonin synthesis. These results underscore the crucial importance of managing serotonin synthesis as a preventive and controlling measure against obesity, offering valuable insights into the intricate interactions of biochemical pathways that influence metabolic well-being.

Beta Oxidation of Very Long Chain Fatty Acids

The beta-oxidation of very long-chain fatty acids was significantly perturbed when comparing the obese group with the normal-weight group (Figure 4). Fatty acids are favored as a primary energy source over glucose, resulting in an increased rate of fatty acid oxidation and a simultaneous decline in both glycolysis and glucose oxidation. This metabolic shift is particularly prominent in conditions such as obesity and insulin resistance, with a notable rise in fatty acid beta-oxidation and a corresponding decrease in glucose oxidation rates[39]. Previous research has shown that excessive beta-oxidation of fatty acids in individuals with obesity and diabetes can negatively impact cardiac function. This was evidenced in young women, where the combination of obesity and insulin resistance led to alterations in myocardial substrate metabolism and efficiency[40]. Furthermore, in another study, researchers observed heightened myocardial fatty acid metabolism in individuals with type I diabetes. This was characterized by increased beta oxidation of fatty acids and a diminished utilization of myocardial glucose[41]. In our results, beta-oxidation of very long-chain fatty acids was highly affected in obese individuals,

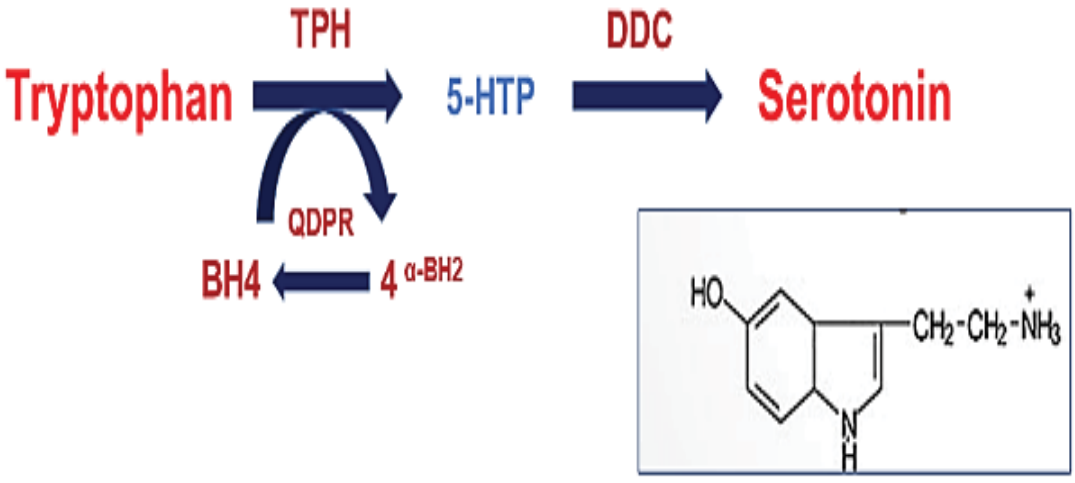

Figure 6. Pathway for serotonin synthesis from tryptophan. Abbreviations: THP: tryptophan hydroxylase; QDPR: quinoid dihydropteridine reductase (more commonly called dihydropteridine reductase); 4α-BH2: 4α-dihydrobiopterin; BH4: tetrahydrobiopterin; 5-HTP: 5-hydroxytryptophan; DDC: DOPA decarboxylase.

as mentioned above, and this metabolic imbalance contributes significantly to cardiovascular complications, diabetes, and insulin resistance observed in individuals with obesity.

Building on Established Research While Unveiling Novel Metabolomic Patterns in obesity

Our findings further enrich the expanding body of research on metabolomic alterations associated with obesity and complications related to obesity. A variety of metabolomics studies have investigated the range of metabolites and pathways involved in these conditions, consistently identifying key biomarkers and metabolic disturbances. This study identified several metabolites and metabolomic pathways that have been previously implicated in obesity, such as L-leucine and L-tyrosine. These findings are in agreement with earlier metabolomics research, including a previous study which recruited perimenopausal Chinese women some with obesity and some without to investigate serum metabolic profiles using untargeted metabolomics with UHPLC-QTOF/MS[42], which reported elevated levels of L-leucine, L-valine, and L-tyrosine in individuals with obesity and domination of D-arginine and D-ornithine metabolism in obese group. However, in contrast, we observed no significant changes in L-valine, suggesting potential population-specific metabolic variations or differences in study design. Our findings also highlighted several metabolomic pathways that have been previously linked to hypertension and obesity. These results are consistent with a prior metabolomics study aimed at analyzing the differences in metabolic composition associated with obesity and hypertension using untargeted UPLC-MS metabolomic techniques[43]. Notably, this study similarly observed significant alterations in tryptophan biosynthesis, as well as in the metabolism of phenylalanine and tyrosine in obese individuals.

Moreover, our study revealed several metabolites that have been established in earlier research as being related to obesity, with one or more abnormal metabolic indexes as metabolic abnormal obesity. These findings correspond with prior metabolomics investigations that aimed to examine the metabolic composition differences associated with abnormal obesity using both LC-MS and GC-MS[25], which similarly reported elevated levels of L-kynurenine and decreased levels of glycerophosphocholine. Such consistency across metabolomics studies underscores the robustness of metabolites such as L-leucine, L-tyrosine, L-kynurenine, glycerophosphocholine, and pathways involving D-arginine and D-ornithine metabolism, tryptophan biosynthesis, as well as phenylalanine and tyrosine metabolism as key markers of metabolic disturbances associated with obesity.

In contrast, our study revealed a significant reduction in phenylacetaldehyde, as illustrated in Figure 3, which we propose as a novel biomarker for obesity. This finding represents a noteworthy contribution to the field, as no prior obesity metabolomics studies have established a connection between phenylacetaldehyde and obesity. Our data reveal substantial alterations in this metabolite, leading us to hypothesize that it could play a role in the early diagnosis and monitoring of obesity-related disorders. Nonetheless, further research is essential to validate this hypothesis and to thoroughly understand its biological role. Phenylacetaldehyde is a human metabolite synthesized by microorganisms like *Escherichia coli* through different key processes[44]. In a prior study, researchers conducted a twelve-week intervention to explore the anti-obesity effects of yak bone collagen hydrolysates in mice fed a high-fat diet. Obesity-related phenotypes were assessed, and fecal samples were analyzed using gene sequencing and untargeted metabolomics. The results showed that this supplementation significantly improved obesity-related phenotypes, particularly at medium and high doses. In addition, phenylacetaldehyde levels were elevated in the medium and high dose groups compared to the high-fat control group. Further metabolic pathway enrichment analysis revealed that phenylalanine metabolism and arginine biosynthesis were the primary pathways affected by the intervention[45]. These findings are consistent with our own observations of decreased phenylacetaldehyde levels and the enrichment of similar metabolic pathways, particularly phenylalanine metabolism and arginine biosynthesis, further reinforcing their connection to obesity. We propose that phenylacetaldehyde may serve as a novel biomarker for obesity, although its precise role and mechanism of action remain unclear. While further research is necessary to fully understand its involvement, this discovery underscores the potential significance of phenylacetaldehyde in our study and opens new avenues for investigating its role in obesity.

Study Limitations and Future Research Directions

Building on the foundational insights gained from this study, our future research will focus on expanding both the scope and depth of our investigation. We plan to increase the sample size in subsequent studies, allowing for greater statistical power and a more comprehensive understanding of metabolic variations. Additionally, we will recruit a diverse cohort to ensure that our findings are broadly applicable and reflective of varied populations. A key advancement in our future work will be the transition from untargeted to targeted metabolomics. This will allow us to validate the most promising biomarkers identified in this study and provide a more focused and detailed analysis. By focusing on specific metabolic pathways, we intend to translate these findings into practical clinical applications, guiding personalized treatment strategies. Ultimately, our research aims to contribute significantly to the growing field of precision medicine by offering a metabolomics-based approach to identifying novel therapeutic targets and developing individualized treatment plans that are tailored to the unique metabolic profiles of patients.

Towards Clinical Translation of Biomarkers: Implications for Early Diagnosis and Monitoring Therapeutic Efficacy

To fully understand the clinical potential of the identified biomarkers, future research should prioritize key areas of translational relevance. Although this study highlights promising metabolic biomarkers associated with obesity, further validation in diverse populations is essential to confirm their clinical utility. Larger, multi-center studies are needed to establish the robustness and reproducibility of these findings.

Incorporating biomarkers shown to be significantly altered in this study, such as phenylalanine, tyrosine, proline, and leucine, into diagnostic panels could enhance the early identification of obesity and related metabolic risks. Beyond diagnosis, these biomarkers hold promises for guiding personalized treatment approaches by monitoring disease progression and evaluating therapeutic response, thus supporting more dynamic and responsive care models. For successful integration into clinical pharmacy practice, a structured translational pathway must be established. This includes the development of cost-effective, standardized assays that are compatible with routine clinical workflows, such as immunoassay or LC-MS/MS-based platforms. Pharmacists, particularly in clinical and community settings, can play a key role in implementing biomarker-guided care by interpreting results, optimizing pharmacotherapy, and providing patient education. Integration into electronic health records and decision-support tools would further enhance the application of these biomarkers in risk stratification and treatment planning. Interdisciplinary collaboration among pharmacists, clinicians, and health policymakers will be crucial to evaluating the feasibility, cost-effectiveness, and impact of biomarker implementation in real-world settings. Ultimately, the adoption of these biomarkers into pharmacy practice could significantly improve the early detection and individualized management of obesity, aligning with the evolving role of pharmacists in delivering precision health interventions.

Implications for Pharmacy Practice

This study identifies several circulating metabolites significantly associated with obesity, including phenylalanine, tyrosine, proline, and leucine. These findings have the potential to transform how pharmacists contribute to the early detection, monitoring, and management of obesity and its related complications. Pharmacists can play a central role in translating these biomarkers into clinical decision-making by integrating biomarker testing into medication therapy management (MTM) programs and chronic disease management services[46]. With the growing availability of point-of-care testing and laboratory diagnostics in pharmacy settings, biomarker-guided screening may become a routine part of community and ambulatory care practice. This enables early identification of at-risk patients, therapeutic response monitoring, and personalized health counseling delivery. Furthermore, integrating metabolomic data into electronic health records and clinical decision-support tools allows pharmacists to contribute more effectively to team-based care and precision medicine initiatives[47,48]. Such advancements align with the evolving role of pharmacists in preventive healthcare and chronic disease management.

CONCLUSION

Our study investigated metabolites and metabolic pathways in overweight and obese individuals within a Jordanian cross-sectional study. Four of the 82 identified metabolites showed significant differences when comparing the overweight group with the normal-weight group. Specifically, pantothenic acid and L-proline exhibited increased levels in the overweight group, while phenylacetaldehyde and glycerophosphocholine were notably decreased compared to the control group. In contrast, the obese group displayed significantly elevated levels of specific metabolites, including L-proline, L-leucine, L-tryptophan, phenylalanine, and tyrosine. Conversely, the obese group demonstrated significantly lower levels of metabolites such as 2,3-diaminopropionic acid, phenylacetaldehyde, and uric acid, among others. Moreover, significant alterations were observed in the obese group in metabolic pathways such as phenylalanine and tyrosine metabolism, tryptophan metabolism, beta-oxidation of very long-chain fatty acids, and others. Understanding sophisticated metabolic relationships provides insight into the complex factors impacting the metabolic landscape in obese individuals. This understanding enriches our understanding of the molecular mechanisms involved and provides opportunities to explore targeted interventions for managing or preventing obesity by modulating altered metabolomic pathways.

AUTHORS CONTRIBUTION

Conceptualization and study design, A.M.A., M.H.S., and Y.A.H.; Sample collection, V.K., N.B., B.A.; methodology, M.H.S., Y.B., and F.M.A.; Analysis and interpretation of data, F.M.A., H.M.A., and B.M.S.; writing—original draft preparation, B.M.S. and F.M.A; writing—review and editing, A.M.A., F.M.A., B.M.S., A.G., N.C.S., H.M.A, W.E., M.A.Y.A., A.Y.A., K.H.A, E.A., V.K., N.R.B., B.A., Y.B., M.H.S.

CONFLICTS OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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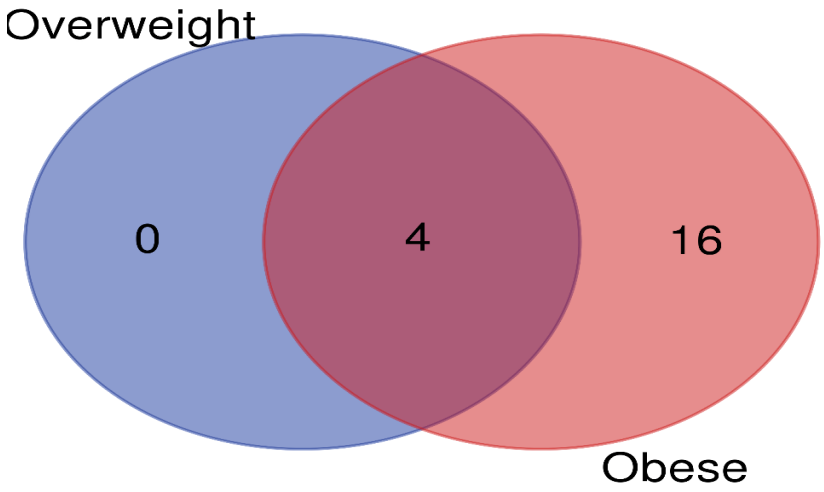
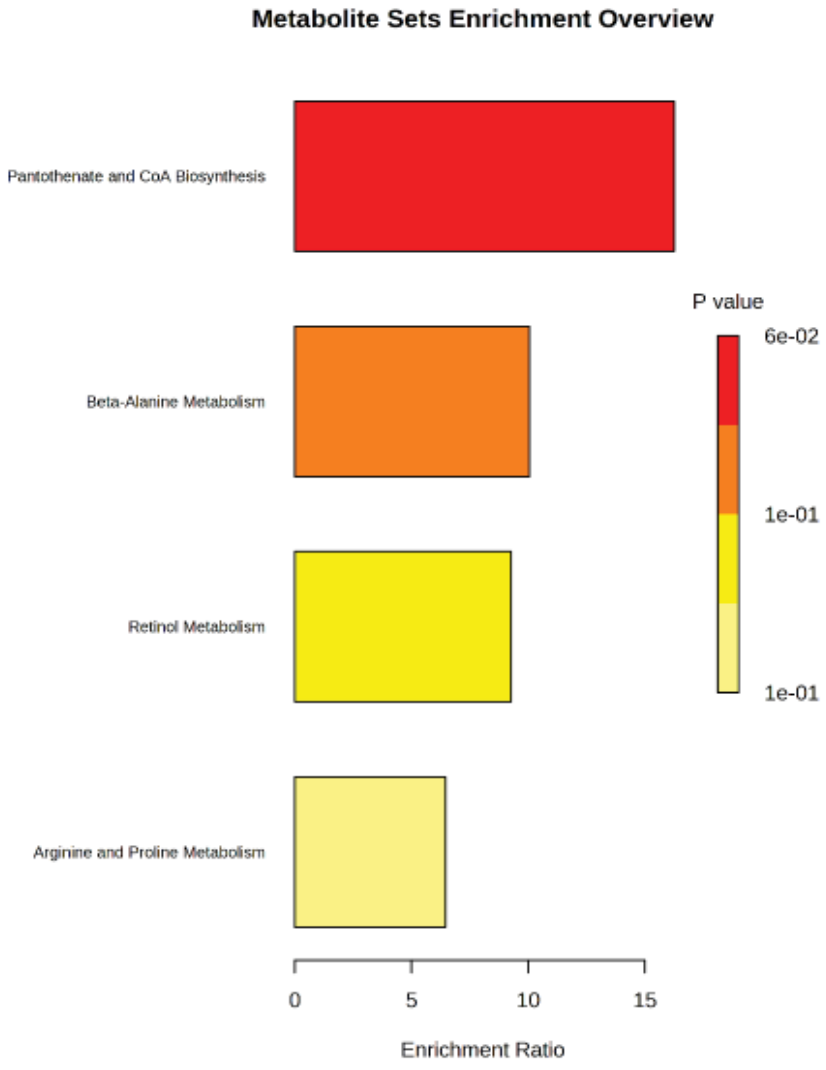


Supplementary Figure 1. Venn diagram comparing metabolites identified in overweight and obese groups compared to normal-weight group.



Supplementary Figure 2. Enrichment Pathway analysis of the overweight group vs. the normal-weight group.