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Original Research

Investigating Serum Metabolite Profiles in Healthy Individuals with Hot-Wet and Cold-Dry Temperaments: A Comparative Study Using LC-MS Technique in the Context of Persian Medicine Theory

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Abstract

Persian Medicine (PM) categorizes individuals into distinct temperaments (Mizaj) based on their unique morphological, physiological, and psychological characteristics. However, the scientific community requires further evidence to validate these temperament classifications. This study aimed to compare the serum metabolite profiles of healthy individuals with hot-wet and cold-dry temperaments using liquid chromatography-mass spectrometry (LC-MS). A descriptive cross-sectional untargeted metabolomics investigation was conducted between 2022-2023, focusing on 20-25-year-old male participants exhibiting hot-wet and cold-dry temperaments. Blood samples were collected, and their serum metabolite profiles were analyzed using LC-MS. The obtained data were then compared between the two groups, using HMDB, METLIN, KEGG databases, and MetaboAnalyst web-based platform for pathway analysis. Among the 59 differentially expressed metabolites, 56 (94.9%) exhibited significantly higher abundance in individuals with hot-wet temperament compared to those with cold-dry temperament (fold-change ≥ 1.5, $p < 0.05$, FDR-corrected $q < 0.05$, VIP >1.0). Enrichment analysis based on KEGG revealed seven metabolic pathways with higher expression in the hot-wet temperament group compared to the cold-dry group, including the alanine, aspartate, and glutamate metabolic pathways, purine metabolism, arginine and proline metabolism, aminoacyl tRNA biosynthesis, citrate cycle (TCA cycle), the pentose phosphate pathway, and glycolysis and gluconeogenesis. This preliminary study highlights significant differences in the serum metabolic profiles of individuals with hot-wet temperament compared to those with cold-dry temperament, particularly in protein and energy metabolism pathways. These findings provide preliminary biochemical insights into the concept of temperament classification within PM. Further research involving larger and more diverse populations is needed to confirm and expand upon these observations.

Keywords: Metabolomics; Temperament; Mizaj; Persian medicine; Mass spectrometry

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Introduction

Persian Medicine (PM) is one of the oldest branches of traditional and complementary medicine, renowned worldwide through the contributions of distinguished scholars such as Rhazes (865–925 AD), Haly Abbas (949–982 AD), Avicenna (980–1037 AD) and Hakim Esmail Jorjani (1042–1137 AD), whose work had a significant impact on the medical knowledge of their time and continues to influence the field centuries later [1]. This medical tradition adopts a comprehensive and holistic approach to human health, considering various aspects of life and multiple factors that influence well-being. Additionally, a key characteristic of PM is its person-centered perspective, which emphasizes that each individual's lifestyle is unique and that treatments are tailored with completely personalized prescriptions [2].

In PM, Temperament or *Mizaj* is a pivotal concept that significantly influences the diagnosis and treatment of various health conditions. In the theoretical framework of temperament, an individual's characteristics are defined by the dominance of specific traits, which can be categorized into two pairs: warmth-coldness and wetness-dryness. The predominance of these qualities within an individual determines their unique temperament [3]. Based on PM, human temperament is believed to be unique and distinct for each individual. By considering the shared attributes among people, the concept of temperament is categorized into nine distinct groups. Among these, one group embodies a balanced temperament, characterized by equal presence of warmth-coldness and wetness-dryness. Furthermore, the classification includes four individual temperament types, namely cold, hot, wet, and dry, and four mixed temperament types, including hot-wet, hot-dry, cold-wet, and cold-dry [4].

From the perspective of PM, individuals with distinct temperaments exhibit variations in morphological, physiological, behavioral, and personality attributes. For instance, those with a hot temperament often possess heightened energy levels, increased muscle mass, and exhibit extroverted tendencies. Conversely, people with a cold temperament typically demonstrate the opposite characteristics. Furthermore, individuals exhibiting a wet temperament often display increased body volumes and higher fat content, along with a greater sleep requirement, in contrast to those with a dry temperament [5-7].

In recent years, the exploration of temperamental differences among individuals has gained significant attention in research. Preliminary studies have indicated disparities in the anthropometrical indices [8], autonomic system (sympathetic and parasympathetic), cytokine patterns [9], basal metabolic rates [10], certain physiological indices [11], body mass index [12], and blood groups [13] across individuals with distinct temperaments. In a recent study, it was further discovered that the proteomic content varies between those with hot-wet and cold-dry temperaments [14]. Furthermore, research has demonstrated variations in the genetic makeup of individuals exhibiting distinct temperaments [15,16].

However, the current body of scientific evidence remains insufficient to validate the classification of humans based on temperament, necessitating further investigation. This study aims to compare the serum metabolite profiles of healthy individuals with hot-wet and cold-dry temperaments using the liquid chromatography-mass spectrometry (LC-MS) technique, contributing to the ongoing pursuit of understanding the potential implications of temperament on human health and well-being.

Material and Methods

Participants

This study is a descriptive, cross-sectional, untargeted metabolomics investigation conducted in 2022-2023 among male participants aged 20–25 years residing in Tehran, Iran, with a focus on individuals exhibiting hot-wet and cold-dry temperaments. The selection of these two temperament groups was based on the premise of PM, which posits that individuals with hot-wet and cold-dry exhibit the most significant disparities in terms of hotness-coldness and wetness-dryness attributes.

Participants' temperaments were assessed using a validated questionnaire developed by Mojahedi et al., originally designed for individuals aged 20 to 40 years. The questionnaire has a Cronbach's alpha coefficient of 0.71, indicating acceptable internal consistency. It consists of 10 items: eight questions evaluate hotness versus coldness, and two questions assess wetness versus dryness. The tool has demonstrated acceptable sensitivity (65–97%) and specificity (52–76%) in identifying respective temperament groups [17], and it has been frequently cited in numerous studies by researchers. To ensure accurate temperament classification, two Persian Medicine specialists independently evaluated each participant's temperament, and their assessments were compared with the questionnaire results to verify consistency.

To ensure study validity, participants were carefully selected to include only completely healthy individuals. Health assessments were based on the absence of chronic (e.g., diabetes, hyperlipidemia, hypertension) and acute (e.g., colds, muscle cramps) diseases, and on participants not being obese or very thin. To maintain study accuracy, participants with temperament-related illnesses, as identified through history and examination, were excluded. Furthermore, participants with a history of smoking, alcohol consump-

tion, or those currently on medications were excluded from the study to ensure a more precise analysis.

Given the limited existing research on the relationship between temperament in the context of PM and metabolomics, as well as the preliminary nature of this study and its specific challenges—such as difficulties in sampling due to strict inclusion criteria and the high costs associated with metabolomic analyses—the sample size was set at 10 participants for this initial investigation. A convenience sampling approach was employed to facilitate access and maintain consistent conditions, thereby minimizing potential data bias. To control for variables known to influence temperament—such as age, gender, and place of residence—participants were selected from among men aged 20-25 years residing in religious schools in Tehran, Iran. The age range of 20-25 years was chosen because, from a PM perspective, this period marks the cessation of significant growth and fundamental changes in inherent characteristics that affect temperament. Additionally, individuals in this age group typically have healthier bodies compared to middle-aged and elderly populations.

Study protocol

In this study, participants were included based on the established criteria after receiving thorough explanations about the research and its procedures, particularly blood sampling. To minimize acute postprandial effects, participants were instructed to consume a light meal (low in fat and protein, excluding fast food and sugary beverages), the night before blood collection, and to fast for at least 12 hours (after 8:00 PM). At 8:00 AM, a 5 mL venous blood sample was collected from each participant by a professional laboratory technician while they were seated, using their right cubital area. Following blood collection, serum separation was achieved by centrifuging the samples at a relative centrifugal force (RCF) of 3,000 *g* for 10 minutes at 4°C. Subsequently, 1.5 mL of serum was transferred to 2-mL microtubes. The acquired serum was kept on ice in sterile, sealed containers and subsequently stored at -80°C for future LC-MS analysis, which involves liquid chromatography-mass spectrometry.

LC-MS/MS process

All serum samples were immediately flash-frozen in liquid nitrogen and stored at -80°C until analysis. Prior to LC-MS/MS processing, samples were thawed on ice and vortexed to ensure homogeneity. For metabolite extraction, 150 µL of serum was mixed with 150 µL of a cold 50:50 (v/v) water-acetonitrile solution. The mixture was vigorously vortexed for 30 seconds, followed by incubation at −20 °C for 10 minutes. Subsequently, samples were centrifuged at 15,000 *g* for 10 minutes at 4 °C. The supernatant was carefully transferred and used for LC-MS/MS analysis. Analysis was performed using a Waters Quattro micro API triple quadrupole mass spectrometer equipped with electrospray ionization (ESI). Chromatographic separation was carried out on an Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size). The mobile phase consisted of solvent A (water, + 0.1% formic acid) and solvent B (acetonitrile, + 0.1% formic acid). The gradient elution program began with 5% B for the first 15 minutes, increased to 95% B by 20 minutes, and was maintained until the end of the run.

Mass spectrometry parameters were set as follows:

- Capillary voltage of 3.2 kV
- Cone voltage of 30 V
- Extraction cone voltage of 3 V
- Drying gas)nitrogen): 200 L/h at 350°C
- Mass range: 50-1000 m/z.

Data acquisition was performed in both positive and negative ion modes to maximize metabolite coverage. Tandem mass spectrometry (MS/MS) scans were acquired for selected precursor ions showing significant differences, enabling structural characterization and pathway mapping. All chemicals and solvents were of LC-MS grade and purchased from Sigma-Aldrich (Burlington, MA, USA) to ensure reproducibility and accuracy.

Data processing and statistical analysis

The Raw data from the LC-MS/MS analysis was first processed using MassLynx v4.1 software to extract mass spectral features. Subsequently, data were converted into mzXML format using MSConvert GUI (64-bit) for compatibility with downstream analysis tools. Metabolomic data processing, including peak detection, alignment, and feature extraction, was conducted using the XCMS Online platform (Scripps Research Institute). This pipeline enabled the identification of reproducible molecular features based on accurate mass-to-charge ratio (m/z) and retention time (Figure 1). Multivariate statistical analysis was performed using Simca v14.1.0.2047 (64-bit) (MKS Umetrics, Malmö, Sweden). Both Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) were applied to explore inherent clustering patterns and identify discriminatory metabolites between hot-wet and cold-dry temperament groups. Ions with variable importance in projection (VIP) scores >1.0, $p < 0.05$ (Mann–Whitney U test with FDR correction), and fold change >1.5 were considered statistically significant (Figure 2). Metabolite annotations were performed by matching accurate mass-to-charge ratios (m/z ±5 ppm) and retention times against entries in public databases (HMDB, METLIN, KEGG, and MetaboAnalyst). Due to instrumental limitations of the Waters Quattro micro API (optimized for targeted SRM), tandem MS/

MS fragmentation data were not acquired; therefore, all reported metabolite identifications are classified as Level 3 under the Metabolomics Standards Initiative (MSI) [18]. Annotations were further filtered using statistical thresholds (VIP >1.0, FDR q < 0.05, fold-change ≥1.5) and biological relevance. Pathway enrichment analysis was carried out to determine overrepresented metabolic pathways associated with temperament differences. All statistical tests were two-sided, with *p* values < 0.05 considered statistically significant. Multiple testing corrections were applied using the False Discovery Rate (FDR) method. Data visualization included score plots, loading plots, and heatmap clustering to enhance interpretability.
This study employed an untargeted metabolomics strategy to maximize discovery potential and ensure comprehensive coverage of serum metabolite profiles.

Ethical consideration

This study's protocol received ethical approval from the local committee of Shahid Beheshti University of Medical Sciences, bearing the code IR.SBMU.RETECH.REC.1398.225. Prior to commencing the study, thorough explanations were provided to the participants regarding the research objectives and involved procedures. Informed consent was duly obtained from each subject, ensuring the confidentiality of their information, which would be used exclusively for this research.

Results

In this study, 400 temperament questionnaires were distributed to men aged 20-25, of whom 178 completed the questionnaire. However, 136 participants were excluded because they did not have hot-wet and cold-dry temperaments. After applying additional study entry criteria, such as health status and absence of temperament-related diseases, the sample size was further reduced to 42 individuals. Ultimately, 18 participants fulfilled all necessary criteria and joined the study. Of these, 10 attended the scheduled blood collection appointment. These 10 participants were then divided into two groups: five with hot-wet temperament and five with cold-dry temperament, both of whom successfully completed the blood sampling stage.

Metabolites identification

A total of 59 differentially expressed and annotated metabolites were identified between the two temperament groups. Of these, 56 (94.9%) exhibited significantly higher abundance in individuals with hot-wet temperament compared to those with cold-dry temperament. These findings reflect the relative enrichment of specific metabolic pathways in the hot-wet group, rather than comprehensive coverage of the entire serum metabolome. The remaining 3 metabolites (5.1%) were downregulated in the hot-wet group. (fold-change ≥1.5, $p < 0.05$, FDR-corrected q < 0.05, VIP >1.0).
A comprehensive list of differentially expressed metabolites is presented in Table 1, showcasing metabolites exhibiting distinct expression patterns between the two investigated temperament groups: hot-wet and cold-dry. In individuals with the hot-wet temperament, metabolites such as amines, amino acids, and peptides increased by 2 to 6 folds, carbohydrates increased by 2 to 3 folds, carbonyl compounds increased by 3 to 6 folds, ceramides increased 6 fold, diacylglycerols and fatty acid esters increased 3 to 4 folds, glycerophosphocholines increased 2 to 5 folds, glycerophosphoinositols increased 3 folds, phosphosphingolipids increased 2 folds, purine nucleosides increased 2 to 4 folds, purine nucleotide sugars increased 4 folds, purine ribonucleotides increased 2 to 4 folds, pyrimidine 2-deoxyribonucleoside increased 2 times, quinoline carboxylic acids increased 3 times, tricarboxylic acids increased 3 to 6 times, and triacylglycerol increased 4 to 6 times compared to cold-dry individuals.
In contrast, the expression of only three metabolites (about 5%) was lower in people with hot-wet temperament compared to those with cold-dry temperament. Specifically, 2, 3-Diphosphoglyceric acid decreased by 2.27 folds, palmitic acid decreased by 3.44 folds, and PS (18:0/18:0) decreased by 20.21 folds in hot-wet individuals compared to cold-dry individuals.
The table includes the following information: Column 2: The mass-to-charge ratio (m/z) is used to identify the mass of the compounds, with "m" representing the mass and "z" representing the number of charged ions. In mass spectrometry (MS), an electron is removed from molecules to generate charged ions. Consequently, m/z often corresponds to the mass-to-charge ratio. Column 4: This column contains the Human Metabolome Database (HMDB) codes for identified metabolites. For metabolite identification, we refer to the corresponding m/z values (Column 1) in HMDB. Column 5: Statistical differences in expression levels between the hot-wet and cold-dry temperament groups are represented by *p* values. Column 6: The fold change indicates the degree of difference in metabolite expression between the hot-wet group (H) and the cold-dry group (C). Column 7: The variation in metabolite levels between hot-wet and cold-dry individuals is reported as either an increase (up) or decrease (down) in expression. All fold-changes are reported as median ratios (hot-wet / cold-dry) based on normalized peak intensities. Statistical significance was determined by Mann–Whitney U test with FDR correction (q < 0.05). For illustrative purposes in the text, representative metabolites are presented as mean ± SD to reflect consistency across biological replicates (n=5 per group).

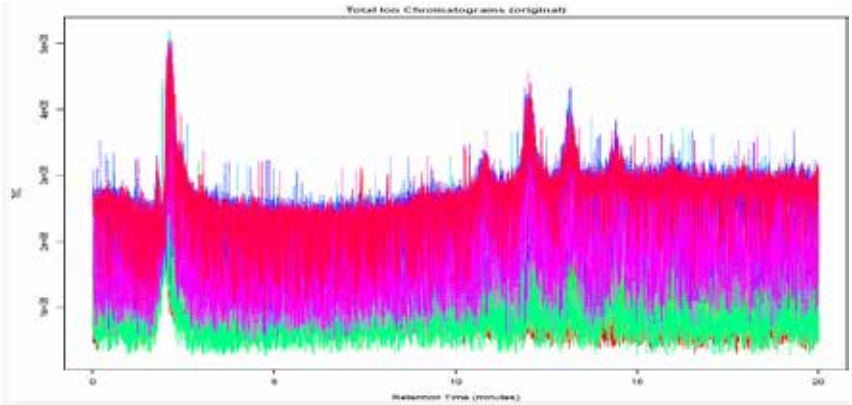

Figure 1. The LC-MS total ion chromatogram (TIC) from serum metabolites.

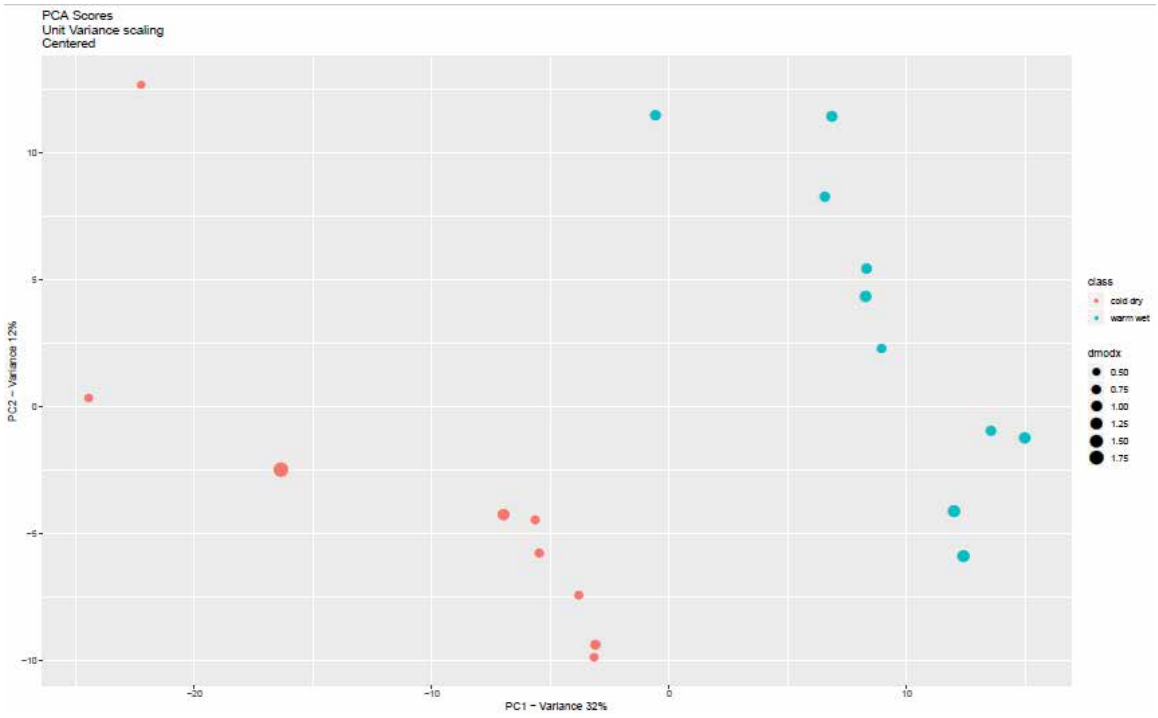

Figure 2. Principal component analysis (PCA) scores plot showing separate clusters for hot-wet and cold-dry temperaments.

Quantitative enrichment analysis (QEA) revealed significant metabolic pathway alterations between temperament groups (Figure 3). Using raw concentration data, QEA identified overrepresented metabolite sets associated with: alanine, aspartate, and glutamate metabolism; purine metabolism; arginine and proline metabolism; aminoacyl-tRNA biosynthesis; citrate cycle (TCA); pentose phosphate pathway; glycolysis/gluconeogenesis. Notably, the glycolysis/gluconeogenesis pathway showed the highest enrichment score and lowest p value ($p < 0.05$), indicating its central role in temperament-associated metabolic differences

Metabolic pathways

Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, pathway enrichment analysis was performed to identify overrepresented biological processes. Statistical significance was defined as $p < 0.05$ and at least two mapped metabolites per pathway. Seven metabolic pathways showed significant differences between hot-wet and cold-dry temperament groups (Table 2). No significant differences were observed in the remaining 18 pathways after multiple testing corrections. All statistical comparisons were performed using the Mann-Whitney U test, followed by False Discovery Rate (FDR) correction. Adjusted p values were calculated using the Holm-Bonferroni method, which is recommended for high-throughput datasets.

Discussion

Table 1. Metabolites exhibiting distinct expression patterns between the two investigated temperament groups:hot-wet and cold-dry.

Category	m/z med	Metabolite	HMDBID	*p* value	Fold change (H/C)	Up /down
Amines	235.366	Spermine	HMDB0001256	0.00023	3.36	UP
Amino acids, peptides, and analogues	177.1337	N-Acetylvaline	HMDB0011757	1.29E-06	3.6	UP
	176.1694. 176.1676, 176.1618	L-Proline	HMDB0000162	1.37E-06	4.2	UP
	619.2108	Argininosuccinic acid	HMDB0000052	2.34E-05	2.93	UP
	327.6825, 678.2468, 681.2675	Glutathione	HMDB0000125	2.60E-05	6.4	UP
	624.1688	Aspartylphenylalanine	HMDB0000706	2.68E-05	4.44	UP
	486.0178	Phosphocreatine	HMDB0001511	3.77E-05	3.08	UP
	485.0181	N-Acetyl-L-tyrosine	HMDB0000866	4.03E-05	2.82	UP
	503.0382, 544.0403	L-Cystine	HMDB0000192	5.94E-05	2.74	UP
	159.0525, 159.0509	L-Valine	HMDB0000883	0.00016	4.92	UP
	561.1141	O-Phosphotyrosine	HMDB0006049	0.00063	2.38	UP
	389.0528	N-Acetyl-L-aspartic acid	HMDB0000812	0.00077	2.93	UP
	436.1839	L-Tryptophan	HMDB0000929	0.00083	2.38	UP
	545.0269	O-Sulfotyrosine	HMDB0155722	0.00187	2.65	UP
	518.9868, 177.2296	L-Homocystine	HMDB0000676	0.00491	4.85	UP
	520.0355	N-Acetyltryptophan	HMDB0013713	0.00636	2.46	UP
Carbohydrates and carbohydrate conjugates	562.1172, 543.0979, 560.0942	Glucose 6-phosphate	HMDB0001401	4.85E-05	2.98	UP
	660.1323	N-Acetylneuraminic acid	HMDB0000230	0.00035	3.4	UP
	565.9951	Cytidine triphosphate	HMDB0000082	0.00057	3.17	UP
	144.9745	2,3-Diphosphoglyceric acid	HMDB0001294	0.00225	2.27	DOWN
	502.0428	D-Ribulose 5-phosphate	HMDB0000618	0.00248	2.21	UP
Carbonyl compounds	518.0338	N-Acetylkynurenine	HMDB0240342	0.00072	5.92	UP
	486.9692	Hydroxykynurenine	HMDB0000732	0.00077	3.56	UP
Ceramides	311.6466, 292.6010, 310.6554, 292.6150, 292.6185	Cer(d18:0/16:0)	HMDB0011760	2.51E-05	6.16	UP
	334.6625	Cer(d18:0/22:0)	HMDB0011765	6.53E-05	6.63	UP

Diacylglycerols	234.4145, 234.4120, 234.4459	DG(20:0/16:0/0:0)	HMDB0007359	2.06E-08	3.66	UP
	387.8658	DG(20:1(11Z)/18:0/0:0)	HMDB0007390	1.03E-06	3.48	UP
Fatty acid esters	117.7386, 117.7326, 505.0239	Hydroxypropionylcarnitine	HMDB0013125	9.78E-06	4.89	UP
	620.2089	3-Methylglutarylcarnitine	HMDB0000552	3.98E-05	3.03	UP
Fatty acids and conjugates	221.3135	Palmitic acid	HMDB0000220	0.00355	3.44	DOWN
Glycerophosphocholines	383.7716, 390.7860, 391.7804, 385.8204, 371.7508, 792.4569	PC (P-18:1(11Z)/P-16:0)	HMDB0011298	5.92E-09	5.45	UP
	428.9194, 406.7393	PC (O-18:1(9Z)/18:0)	HMDB0008036	9.49E-05	3.83	UP
	369.7615, 368.7665, 378.5918	PC(18:0/14:0)	HMDB0008031	1.00E-05	3.33	UP
	312.6279	LysoPC(22:0/0:0)	HMDB0010398	0.00012	5.71	UP
	676.3579	LysoPC(24:0/0:0)	HMDB0010405	0.00013	2.53	UP
	448.8481, 449.8646	PC(O-22:0/20:1(11Z))	HMDB0013447	0.0003	3.48	UP
	364.7634	PC(16:0/14:0)	HMDB0007965	0.00059	3.14	UP
	444.8614	PC(22:0/18:1(9Z))	HMDB0008532	0.0011	2.29	UP
	314.5275	LysoPC(24:1(15Z)/0:0)	HMDB0010406	0.00231	2.62	UP
	293.6292, 293.6387	PC(P-18:1(9Z)/24:0)	HMDB0011329	0.00333	3.03	UP
	399.8402, 442.7342	PC(P-18:0/20:0)	HMDB0011248	0.00367	3.79	UP
	370.7794	PC(O-16:0/16:1(9Z))	HMDB0013404	1.36E-06	4.01	UP
Glycerophosphoinositols	618.2035, 677.2593, 679.4215, 621.1315	LysoPI(0:0/18:0)	HMDB0061704	6.64E-06	3.13	UP
Glycerophosphoserines	415.7329	PS(18:0/18:0)	HMDB0012378	0.00552	20.21	DOWN
Phosphosphingolipids	372.8204	SM(d18:0/16:1(9Z))	HMDB0013464	0.00668	2.02	UP

Purine nucleosides	623.1703	N2,N2-Dimethylguanosine	HMDB0004824	6.46E-05	4.77	UP
	601.0725, 563.1468	1-Methyladenosine	HMDB0003331	0.0003	2.98	UP
	606.0395, 602.9825	1-Methylinosine	HMDB0002721	0.00051	3.42	UP
Purine nucleotide sugars	333.6324, 355.7330	NADH	HMDB0001487	9.47E-05	4.57	UP
Purine ribonucleotides	445.9402	Guanosine monophosphate	HMDB0001397	0.00015	2.62	UP
	565.0312, 602.1268, 505.8417	Guanosine triphosphate	HMDB0001273	0.00021	3.23	UP
	504.0361	Guanosine diphosphate	HMDB0001201	0.00041	2.66	UP
	488.1263, 426.8955, 427.9029	ADP	HMDB0001341	0.00073	4.56	UP
Pyrimidine 2'-deoxyribonucleosides	484.052	Deoxyuridine	HMDB0000012	0.00241	2.21	UP
Quinoline carboxylic acids	473.9138	8-hydroxykynurenic acid	HMDB0000881	0.00347	3.61	UP
Tricarboxylic acids and derivatives	386.8338	cis-Aconitic acid	HMDB0000072	3.99E-07	3.77	UP
	422.8328	Citric acid	HMDB0000094	0.00068	6.06	UP
Triacylglycerols	332.6426, 328.7227, 350.6621, 350.7619, 350.7683, 507.9256	TG(18:0/20:1(11Z)/22:0)	HMDB0044967	6.06E-05	6.61	UP
	499.9378, 446.8920, 506.9381	TG (16:0/20:1(11Z)/O-18:0)	HMDB0044164	0.00607	4.5	UP

From the perspective of PM, an individual's temperament serves as a characteristic that encompasses their genetic makeup, physiological functioning, and organ performance. This temperament plays a crucial role in determining the most suitable lifestyle for maintaining one's health. Furthermore, when an illness arises, the choice of treatment method and medications is tailored to an individual's temperament. For instance, in the case of the same disease, the appropriate medication for a person with a hot-wet temperament and a person with a cold-dry temperament may differ based on their respective temperament types [19].

In contemporary medicine, the concept of personalized healthcare has gained prominence. This approach incorporates cutting-edge knowledge from various fields, including genomics, proteomics, metabolomics, pharmacogenomics, and nutrigenomics. Personalized medicine emphasizes the importance of con-

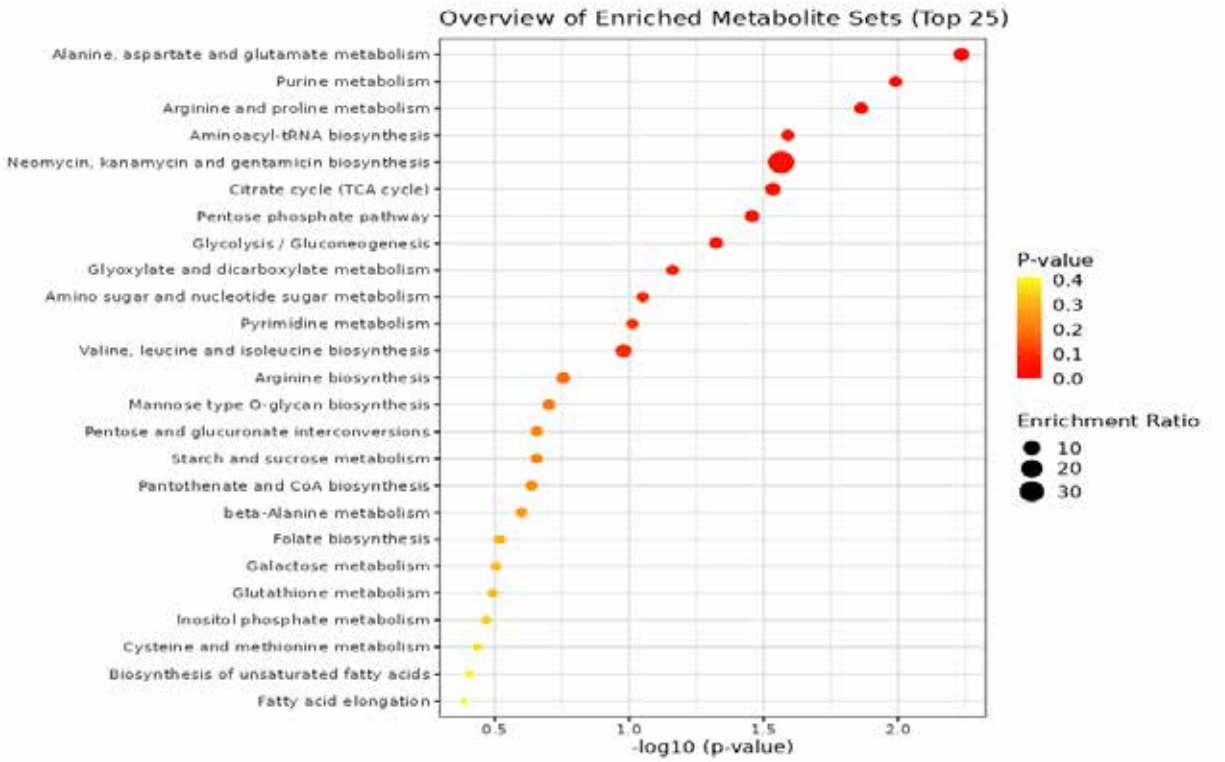

Figure 3. Quantitative Enrichment Analysis (QEA) of metabolite differences in hot-wet and cold-dry temperament groups: circle size and dimensions depict enrichment levels, with dark red circles representing lower p-values and thus higher statistical significance.

Table 2. The list of metabolic pathways with significant differences between hot-wet and cold-dry temperament groups.

Metabolic pathway	P-value
Alanine, Aspartate, Glutamate Metabolism	0.0058
Purine Metabolism	0.0102
Arginine and Proline Metabolism	0.0137
Aminoacyl-tRNA Biosynthesis	0.0257
Citrate Cycle (TCA Cycle)	0.0292
Pentose Phosphate Pathway	0.0349
Glycolysis/Gluconeogenesis	0.0475

sidering individual differences in the diagnosis and treatment of diseases, ensuring a more tailored and effective approach to patient care [20].

To the best of our knowledge, the investigation of serum metabolite profiles in individuals with hot-wet and cold-dry temperaments, as classified by PM, has not been previously explored. This study aimed to fill this gap by comparing the metabolic profiles of these temperaments using LC-MS analysis, which may contribute valuable insights to the field of traditional medicine research.

The results of this preliminary study showed differences in the metabolic profiles of the hot-wet and cold-dry temperament groups. When comparing individuals from the hot-wet temperament group to those in the cold-dry group, 94.9% of the measured metabolites in individuals with hot-wet temperament demonstrated an increase in expression; while only 5.1% of these metabolites showed a decrease. Furthermore, the expression levels of metabolic pathways associated with alanine, aspartate, and glutamine metabolism; purine metabolism; arginine and proline metabolism, aminoacyl-tRNA biosynthesis, citrate cycle (TCA cycle), pentose phosphate pathway, glycolysis, and gluconeogenesis were significantly higher in individuals with hot-wet temperament compared to those with cold-dry temperament.

In 2016, Rezadoost et al. investigated the differences between hot-wet and cold-dry temperaments from a proteomic perspective. Their proteomics analysis of healthy individuals with hot-wet and cold-dry temperaments revealed that some mitochondrial proteins exhibited distinct expression patterns. In their research, the activity of the pyruvate kinase enzyme was found to be higher in individuals with hot-wet temperament

compared to those with cold-dry temperament [14]. It is known that pyruvate kinase plays a crucial role in regulating energy metabolism and glycolysis in the body [21]. In our study, we observed that metabolites involved in cellular energy metabolism pathways, such as glycolysis, gluconeogenesis, the citrate cycle, and the pentose phosphate pathway, showed increased levels in hot-wet individuals. The findings of the present study are similar to Rezadoost's study. Our data reveal a metabolomic signature consistent with heightened activity in pathways of energy metabolism — including glycolysis, TCA cycle, and amino acid biosynthesis — in individuals classified as hot-wet by PM criteria.

In a study conducted by Mohammadi Farsani et al. in 2020, it was observed that the basal metabolic rate and thyroid hormone profile were higher in individuals with a hot temperament [10]. Given that one of the proposed hypotheses explaining the origin of hot temperament is its correlation with thyroid activity and hormone secretion [22], and considering recent findings suggesting a relationship between increased metabolism, protein synthesis, and thyroid hormone levels [23,24], the results from our study further validate and confirm the association between enhanced protein synthesis and energy metabolism in hot-wet individuals.

The distinct metabolite variations in hot-wet and cold-dry temperaments can inspire hypotheses, serving as the foundation for future research. This may enhance our understanding of how temperament influences disease occurrence and potentially uncover novel therapeutic approaches by incorporating temperament considerations. For instance, according to our research findings, L-tryptophan exhibits a 2.38-fold increase in the hot-wet group compared to the cold-dry group. Given that L-tryptophan is a metabolite that serves as a precursor to serotonin, a crucial molecule involved in various bodily functions such as mood, behavior, and cognition [25], this may help explain the behavioral and personality differences among individuals with distinct temperaments. In line with prior studies, the connection between temperament and serotonergic activity has been proposed [15]. In another instance, our research demonstrated that pathways associated with amino acid metabolism—such as those involving alanine, glutamine, proline, and arginine— were more active in individuals with a hot-wet temperament compared to those with a cold-dry temperament. Since previous studies have shown a direct correlation between the metabolic changes of these amino acids and an increased likelihood of diabetes [26,27], the hypothesis linking temperament and diabetes occurrence becomes more compelling. As a case–control study, the relationship between temperament and diabetes has been examined [28]. At the current time, these findings do not demonstrate that temperament causes metabolic differences, but rather that individuals assigned to these classical categories based on clinical observation display reproducible, pathway-specific metabolic signatures. These signatures provide a quantifiable foundation for future studies aiming to uncover potential biological correlates, including genetic, endocrine, or neuroautonomic mechanisms of temperament, as described in PM.

One of the notable strengths of this study lies in the meticulous selection of the samples. To ensure the reliability of the findings, the participants were enrolled only after confirming that they had no history of acute or chronic illnesses, abstained from smoking and alcohol consumption, and did not suffer from any diseases related to temperament according to PM. These evaluations were performed by assessment conducted by two Persian Medicine specialists, one of whom was a faculty member at a university in the field of Persian Medicine. Although this rigorous process extended the sampling duration, it significantly enhanced the accuracy of the study outcomes.

While our study identified 59 significant metabolites, it is important to note that untargeted metabolomics typically captures only a fraction of the estimated range of more than 10,000 to 20,000 metabolites present in human serum [29]. Therefore, our results represent a targeted discovery of differentially abundant metabolites, not a complete metabolomic profile.

The current study highlights the need for further exploration of metabolite profiles in individuals with different temperaments. Future investigations may consider examining additional metabolites, such as urinary metabolites, to expand our understanding of the metabolic distinctions between temperaments. To achieve a more accurate representation of the metabolomic differences among individuals with varying temperaments, it is crucial to conduct comprehensive comparisons involving all four temperament groups: hot-wet, hot-dry, cold-wet, and cold-dry. This comprehensive approach will contribute significantly to validating the concepts of temperament in PM and potentially uncover novel insights into human metabolism.

Limitations

This study has several important limitations. First, in this study, the sample size was small. Although selecting participants of the same gender, within a narrow age range, from the same city and living environment, and adhering to the inclusion criteria ensured homogeneity of the samples, it reduces its generalizability. Thus, future studies should involve larger cohorts, encompass diverse age groups, and include both genders to improve the generalizability of the findings.

Second, while classical PM associates hot-wet tem-

perament with increased body volume and muscle mass, and cold-dry temperament with leanness and low fat and muscle mass [30], we did not perform direct anthropometric measurements (e.g., BMI, DEXA, or bioimpedance). However, all participants were selected from identical institutional environments with standardized diets and physical activity patterns, and individuals with obesity or who appeared very thin based on visual assessment were excluded —thus minimizing potential confounding by body composition.

Additionally, a key limitation of this study is the lack of formal quantification of habitual dietary intake. Although participants consumed standardized institutional meals and received pre-sampling dietary instructions, individual variations in snack consumption, beverage habits (e.g., tea, coffee), or micronutrient intake could not be measured via food diaries or biomarkers. However, the extreme homogeneity of the individuals—including uniform living conditions, strict exclusion criteria, and absence of confounding lifestyle factors—provides strong internal validity for detecting temperament-associated metabolic signatures .While dietary factors may contribute to metabolic variation, the profound and consistent upregulation of core energy and biosynthetic pathways in hot-wet individuals — aligning with prior proteomic and metabolic rate data — suggests that temperament-associated physiology may dominate over short-term dietary influences in this tightly controlled cohort.

Third, due to instrumental constraints of the Quattro micro-API system, tandem MS/MS fragmentation data were not acquired, limiting metabolite annotations to Level 3 under MSI guidelines. Nevertheless, the high consistency of metabolic signatures across replicates and their alignment with prior proteomic and physiological findings support the biological plausibility of our results.

Finally, as an observational cross-sectional study, we identify metabolic associations rather than causal mechanisms. Future work should integrate longitudinal sampling, intervention trials, and multi-omics profiling to validate whether temperament drives metabolic phenotypes or vice versa.

Conclusion

The results of this preliminary study reveal substantial disparities in the serum metabolic profiles of individuals with hot-wet temperament compared to those with cold-dry temperament. These differences are particularly evident in the pathways of protein and energy metabolism, indicating a metabolomic signature consistent with heightened anabolic and catabolic flux in individuals classified as hot-wet. While these patterns align with classical descriptions of temperament, they represent correlative biomarkers rather than evidence of inherent physiological causation.

Furthermore, the metabolic pathways associated with alanine, aspartate, and glutamine, purine, arginine and proline, aminoacyl tRNA biosynthesis, citrate cycle (TCA cycle), pentose phosphate pathway, glycolysis, and gluconeogenesis also exhibit higher activity in the hot-wet group compared to the cold-dry group. To further validate and expand our understanding of the metabolic distinctions between individuals with different temperaments, additional research with a large number of participants focusing on a more comprehensive investigation of metabolites is essential.

Conflicts of Interest

The authors have declared that there are no conflicts of interest.

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