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Original Article,

Amino Acid Profile Identified In Plasma Has Predictive and Diagnostic Value in Heart Failure Patients

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Abstract:

Background: Metabolic disruptions have effects on the pathogenesis and progression of heart failure (HF). Because of these disruptions are remarkable in HF patients, identity of new biomarkers is necessary to facilitate the determination of HF. In the present study we planned to evaluate the relationship between plasma amino acids and HF.
Materials and methods: 40 patients with cardiomyopathy (CMP) and 40 healthy controls were included in this cross-sectional prospective study. A profile consisting of 26 amino acids was measured by liquid chromatography and mass spectrometry method (LC-MS) from blood samples taken from fasting plasma.
Results: 5 amino acids (asparagine, beta-alanine, phenylalanine, tyrosine, and isoleucine) levels were higher in CMP patients. Glutamine, isoleucine, and glycine could effectively distinguish CMP and healthy controls. Isoleucine and beta-alanine may be potential biomarkers for CMP. Isoleucine/alloisoleucine, histidine/isoleucine, aspartic acid/isoleucine, and glycine/phenylalanine could predict CMP patients. Three differential pathways (phenylalanine metabolism, tyrosine metabolism, and phenylalanine, tyrosine, tryptophan biosynthesis) were found to be the underlying molecular metabolisms of HF.
Conclusion: The present study demonstrating that fasting plasma amino acids were closely associated with HF. Monitoring these amino acids with LC-MS could help the diagnosis and predicting HF, and provide new diagnostic goals and curative practices.

Keywords: cardiomyopathy, heart failure, amino acid, LC-MS, biomarker, metabolomics

Introduction:

Cardiomyopathy (CMP) is an important cause of mortality and morbidity resulting from deterioration of systolic and diastolic functions in the left ventricle, which occurs as a result of heart muscle damage [1]. Clinically, CMP is defined as ischemic and non-ischemic. Ischemic CMP is developed secondary due to a decrease of energy-dependent cardiomyocytes in blood flow, causes myocyte loss, resulting in myocardial scarring in the left ventricle. Conversely, Non-ischemic CMP is characterized by the enlargement of left ventricle, which is unrelated to coronary artery disease, and there is global myocyte involvement. Its molecular mechanism has not been fully clarified [2, 3].

Metabolomics are small particles present in a cell, tissue, or organism, resulting from the metabolic processes occurring in both physiologic and pathologic conditions [4]. Previous studies have shown that metabolic deformities determined in plasma are associated with results of heart failure (HF) patients and the importance of metabolomics profile in the pathophysiology, diagnosis, and prognosis of heart failure has been increasingly understood [5-9].

Metabolomics such as amino acids and their metabolites play a crucial role in many metabolic pathways, protein synthesis, regulation, and energy metabolism [10]. This has made it interesting to compare amino acid profiles between individuals with various diseases and

healthy individuals. Previously, studies showed that amino acids play a principal role in the pathology and physiology of cardiovascular system [11]. However, studies investigating the profile of plasma amino acids in the patients with HF are insufficient. Therefore, we aimed to determine the profile of plasma amino acids in the patients with HF compared to healthy controls.

Materials and Methods:

Study population

In this cross-sectional prospective study, 40 consecutive patients between the age of 25 and 75 years with CMP and 40 healthy individuals between the age of 22 and 60 years were included between the date of December 2020 and September 2021. Patients who met the diagnostic criteria for Framingham heart failure were included as inclusion criteria [12]. All CMP patients were under the optimal HF medical treatment and all the patients were in the decompensated period at the blood sampling time. Patients with peripartum CMP, alcoholic CMP, CMP related to metabolic disease, hyperthyroidism, amyloidosis, diabetes mellitus, neuromuscular disorders, toxic CMP, rheumatoid arthritis, and systemic lupus erythematosus were excluded from the study. Additionally, patients with severe valvular heart disease, and chronic kidney and renal failure did not enroll in the study. The study was designed and conducted with the approval of the Harran University Medicine Faculty ethics review commission and in accordance with the declaration of Helsinki. Informed consent forms were obtained from both patients and control groups.

Sample collection and metabolomics methods:

After the samples were obtained from the patient and healthy groups, their transfer, centrifugation (at 3500 rpm for 10 mins), and segregation were completed in one hour to avoid any factor that could affect plasma metabolite values prior to analysis. Samples taken from the participants were stored at -80 degrees as far as analysis.

We revealed the amino acid profiles of the patients with the liquid chromatography-mass spectrometry (LC-MS) method, which consists of liquid chromatography and mass spectrometry components. Amino acid levels were measured using commercially available kits (LCMS/MS, Jasem, Turkey). The calibration curves were prepared for a subset of the profiled analyses by serial dilution in stock pooled plasma using stable isotope-labeled reference compounds. LC-MS analyses were performed via protein precipitation with the addition of 50 µL sera to 700 µL reagent-1 containing an appropriate volume of mixed internal standards. The samples were taken to the centrifuge device and their plasmas were separated. After the preparation according to this protocol, 3 mL of the mixed samples was loaded into amino acid analytical column using the Shimadzu HPLC system. The analysis of the sample whose flow rate was set as 0.7 mL/min was completed at 7.5 minutes. Mass spectrometric detections were carried out in a positive ionization mode with an ESIequipped Shimadzu-8045 triple quadrupole tandem mass spectrometer (Shimadzu, Japan). The mass detector parameters were designated as follows; gas temperature 150°C, gas flow 10 L/min, nebulizer pressure 40 psi, and +2000-volt capillary voltage. Eventually, the sera amino acid level was expressed as µmol/L.

Statistical analysis:

Student's t-test or Mann-Whitney U test was used to compare amino acid profile between patient and control groups. Principal component analysis (PCA) model was used to determine the quality, homogeneity, and dominance tendencies of the separations of the patient and control groups in terms of amino acid profile. The partial least squares discriminant analysis (PLS-DA) model was used to determine the metabolites that separated the patient and control groups, and the variable importance in the protection (VIP) model was used to determine the important variables that differed with this method. Also, to determine the metabolic pathways for patient group pathway analysis was performed. All these statistical analyze were made in the MetaboAnalyst 5.0 program at http://www.metaboanalyst.ca/. The amino acid distributions of the patient and control groups were also shown with a heat map with the using of Heat map package (R version 3.6). Receiver operating characteristic (ROC) analysis was used to determine the optimal sensitivity and specificity cut-off values of amino acid levels for CMP. The Prism-GraphPad 8.0 program was used to perform statistical analysis. A p value less than 0.05 was considered statistically significant.

Results:

The levels of Asparagine, beta-alanine, phenylalanine, tyrosine, and isoleucine were significantly higher in CMP patients compared with the healthy controls (HC) (Table-1, Figure-1).

Table-1. Quantitative analysis of serum amino acids composition of the study groups (mean ± SD).

Amino Acid (µmol/L)	Abbreviations	Cardiomyopathy (CMP, n:40)	Healthy Control (HC, n:40)	p value
Alanine	Ala	428.31±202.99	420.82±245.86	0.859
Arginine	Arg	78.79±38.49	63.95±42.49	0.057
Asparagine	Asn	58.04±31.11	46.91±22.83	**0.029**
Aspartic Acid	Asp	44.02±22.14	53.88±35.80	0.116
Beta Alanine	B-Ala	5.86±3.11	3.88±2.74	**0.001**
Citrulline	Cit	39.51±27.13	38.56±22.81	0.849
Glutamine	Gln	825.54±304.91	893.31±499.48	0.434
Glutamic Acid	Glu	243.04±133.43	226.77±137.60	0.534
Glycine	Gly	337.12±167.99	361.37±209.80	0.495
Histidine	His	66.93±32.49	76.31±45.00	0.245
Hydroxyproline	Hyp	9.20±4.40	8.17±4.50	0.233
Lysine	Lys	211.12±100.31	203.09±121.34	0.701
Methionine	Met	25.96±13.95	25.91±15.36	0.985
Ornithine	Orn	275.12±154.99	293.12±189.33	0.579
Phenylalanine	Phe	98.23±43.41	81.15±44.88	**0.047**
Proline	Pro	236.12±115.51	224.76±120.16	0.617
Serine	Ser	141.64±66.01	135.99±75.13	0.674
Taurine	Tau	71.56±37.15	68.04±26.93	0.595
Threonine	Thr	133.99±76.77	124.73±71.84	0.526
Tryptophan	Trp	56.79±26.62	49.34±30.05	0.169
Tyrosine	Tyr	80.40±40.51	62.03±34.93	**0.016**
Valine	Val	257.53±116.59	239.97±139.82	0.469
Sarcosine	Sar	28.56±13.87	27.41±15.70	0.682
Leucine	Leu	213.95±103.93	193.56±106.69	0.317
Isoleucine	Ile	131.25±66.42	84.41±44.38	**<0.001**
Alloisoleucine	Allo-Ile	0.80±0.35	0.82±0.44	0.797
Branched Chain Amino Acids	BCAAs	174.56±86.32	170.48±90.74	0.612

The comparison of amino acid concentrations between the groups was performed by students' *t* test.
p <0.05 means statistically significance

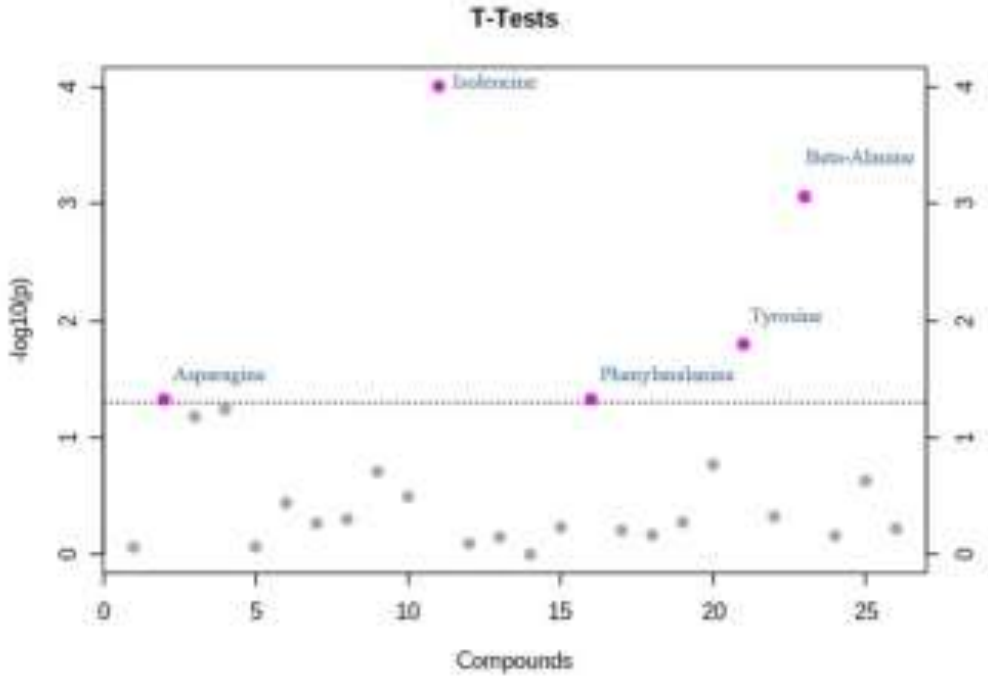

Figure-1. Demonstration of amino acids with significant differences between groups by Student's *t*-test. Amino acids that differ significantly between groups are shown in pink, and those that do not differ significantly are shown in gray.

PCA and PLS-DA analyze the CMP patients and healthy group

Firstly, PCA was performed to visualize the simple distribution and to show the overall difference in the 26 amino acids between CMP patients and healthy controls (Figures 2A and 2B). According to PC 1, 2, and 3; these three PC accounted for 53.1%, 31.3%, and 4.2% of the alteration in the data, respectively. A partial aggregation and discrimination was observed between the groups according to the numerical results of those amino acids. To maximize differences between the patient and healthy groups, PLS-DA analysis was carried out. As a result of the analysis, a better aggregation compared to PCA was shown in the 2D and 3D dimensional score graphs (Figures 2C and 2D).

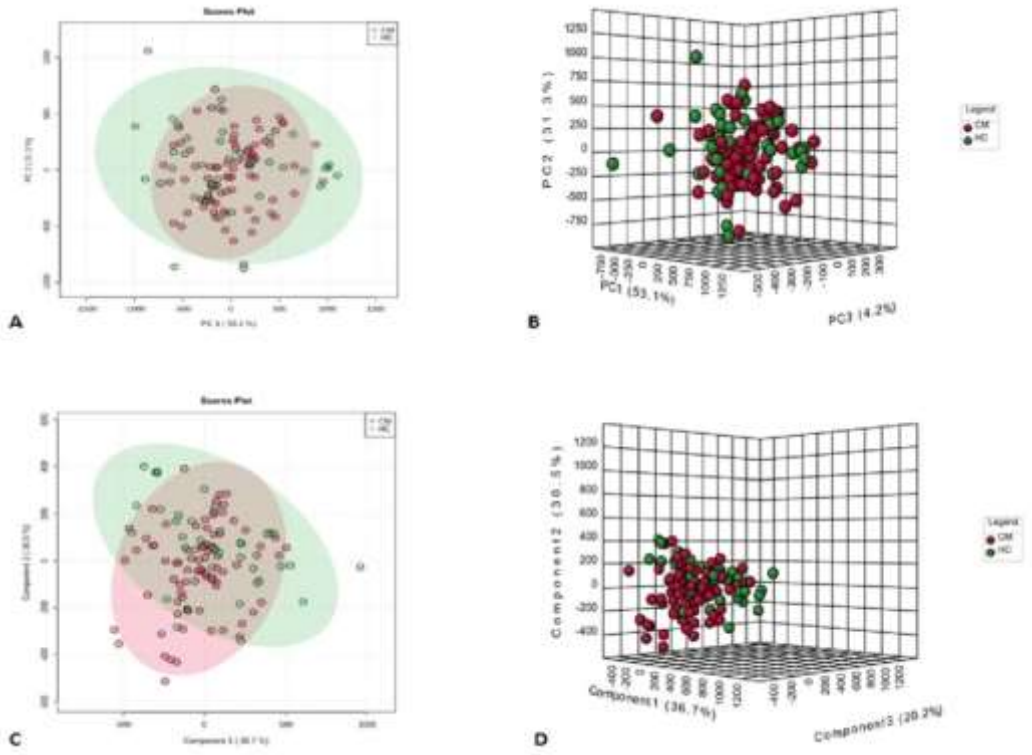

Figure-2. PCA and PLS-DA analyses of 26 amino acids, the red dots represent CMP patients, the green dots represent control group. 2D (A) and 3D (B) score graphs of PCA. 2D (C) and 3D (D) score graphs of PLS-DA.

Abbreviations:PCA: principal component analysis; PLS-DA: partial least squares discriminant analysis; CM: cardiomyopathy; HC: healthy control; D: dimensional

Screening for Differentiated Amino Acids

VIP analysis generated from PLS-DA models was performed to show the amino acids that differentiate CMP patients from healthy controls. The VIP plot ranked amino acids according to their discriminating power (Figure-3A). Glutamine, isoleucine, and glycine were determined as the first three amino acids with the highest VIP score. The higher the VIP score, the more it contributes to the separation of the groups. A heat map was created to visualize amino acid changes between groups (Figure-3B). No significant clustering was observed between the groups. But for some amino acids (e.g. isoleucine) color differences were observed in the heat map. In addition, a Volcano plot was drawn to detect differentiated amino acids (Figure-3C). The fold-change threshold was set as 1.5, and the p value was set as <0.05 for all amino acids. As a result of the analysis, 2 amino acids (isoleucine and beta-alanine) that contributed to the differentiation of the groups were determined. Isoleucine and beta-alanine may be potential biomarkers for CMP as shown in the Volcano graph (Figure-3C).

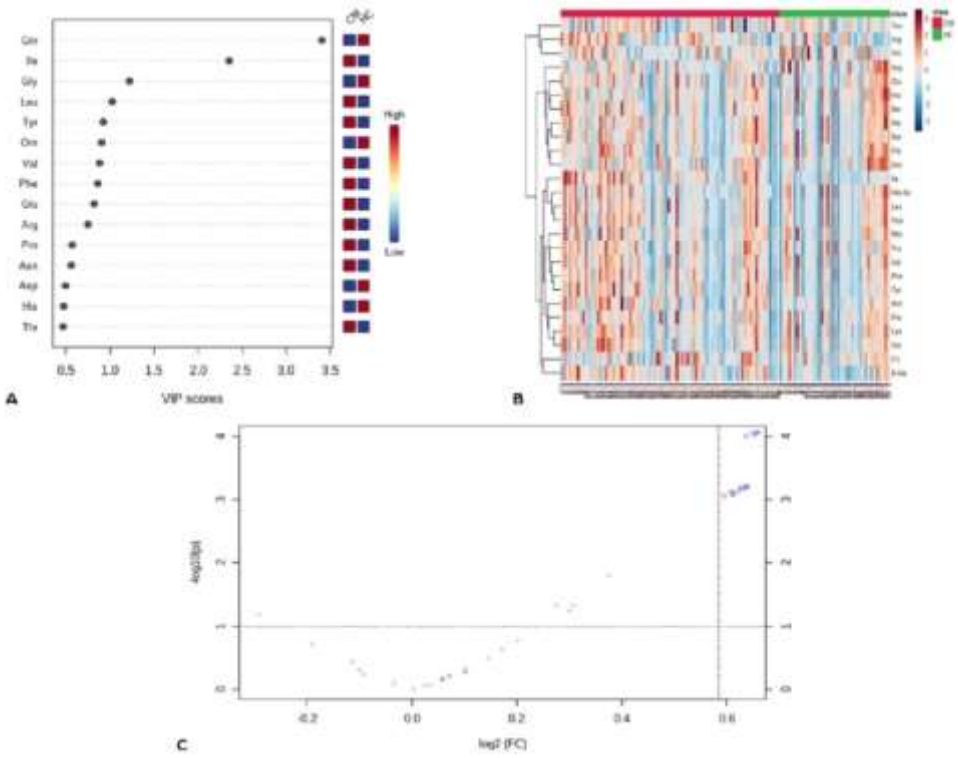

Figure-3. VIP plot: amino acids identified in decreasing order of importance (A). Heatmap shows the amino acid patterns in the groups (B). Volcano plot of differentiated amino acids (C).

Abbreviations: VIP: variable importance in the projection; CM: cardiomyopathy; HC: healthy control

Biomarkers for CMP candidates

ROC analysis was performed to detect potential predictors for CMP. With the univariate ROC analysis, four amino acids with the highest AUC (area under curve) cut-off value, sensitivity, specificity, positive odds ratio, and negative odds ratio are shown in Table-2. Amino acid markers with the highest AUC values isoleucine/alloisoleucine, histidine/isoleucine, aspartic acid/isoleucine, and glycine/phenylalanine have a strong predictive value for CMP. Boxplot and ROC curves of these markers are shown in Figure-4.

Table-2. ROC analysis results of potential biomarkers for CMP

Metabolites	Cut-off Value	AUC	Sensitivity (%)	Specificity (%)	Positive Likelihood Ratio	Negative Likelihood Ratio
Ile/Alo-Ile	6.78	0.898	0.8	0.9	8.5	0.166
His/Ile	-0.462	0.802	0.8	0.7	3.23	0.203
Asp/Ile	-1.23	0.796	0.9	0.7	2.91	0.178
Gly/Phe	1.92	0.767	0.8	0.7	2.58	0.321

Abbreviations: ROC: receiver operating characteristic; AUC: area under curve; CMP: cardiomyopathy

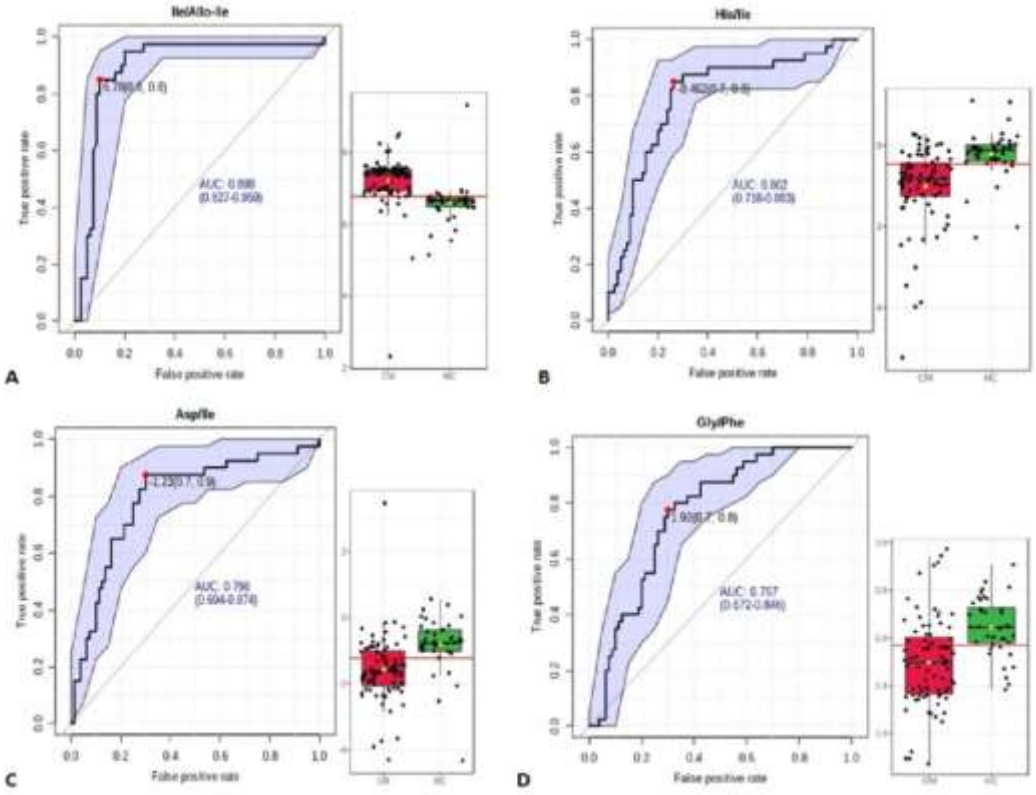

Figure-4. ROC curve of the 4 amino acids with the highest AUC. Isoleucine/alloisoleucine (A), histidine/isoleucine (B), aspartic acid/isoleucine (C), glycine/Phenylalanine (D).

Abbreviations: ROC: receiver operating characteristic; AUC: area under curve

Metabolic pathway analysis

We performed pathway analysis with MetaboAnalyst 5.0 to investigate which metabolic pathways were significantly altered in CMP. Pathway analysis revealed significant impairment of 3 pathways (a: Phenylalanine metabolism, b: Tyrosine metabolism, and c: Phenylalanine, tyrosine, and tryptophan biosynthesis) in patients with CMP (impact value>0.1, $p<0.05$). Metabolites with an impact value greater than 0.1 are shown in Table-3 and Figure-5.

Table-3. Impaired metabolic pathways in the cardiomyopathy

Pathway Name	Impact value	p value
Phenylalanine, tyrosine and tryptophan biosynthesis	1.0	**0.025**
Alanine, aspartate and glutamate metabolism	0.534	0.511
D-Glutamine and D-glutamate metabolism	0.5	0.389
Arginine biosynthesis	0.482	0.437
Taurine and hypotaurine metabolism	0.428	0.595
Beta-Alanine metabolism	0.399	0.111
Arginine and proline metabolism	0.393	0.662
Phenylalanine metabolism	0.357	**0.025**
Glycine, serine and threonine metabolism	0.338	0.517
Histidine metabolism	0.221	0.454
Tryptophan metabolism	0.143	0.169
Tyrosine metabolism	0.139	**0.015**
Glutathione metabolism	0.108	0.635
Glyoxylate and dicarboxylate metabolism	0.105	0.445
Cysteine and methionine metabolism	0.104	0.985

p <0.05 means statistically significance

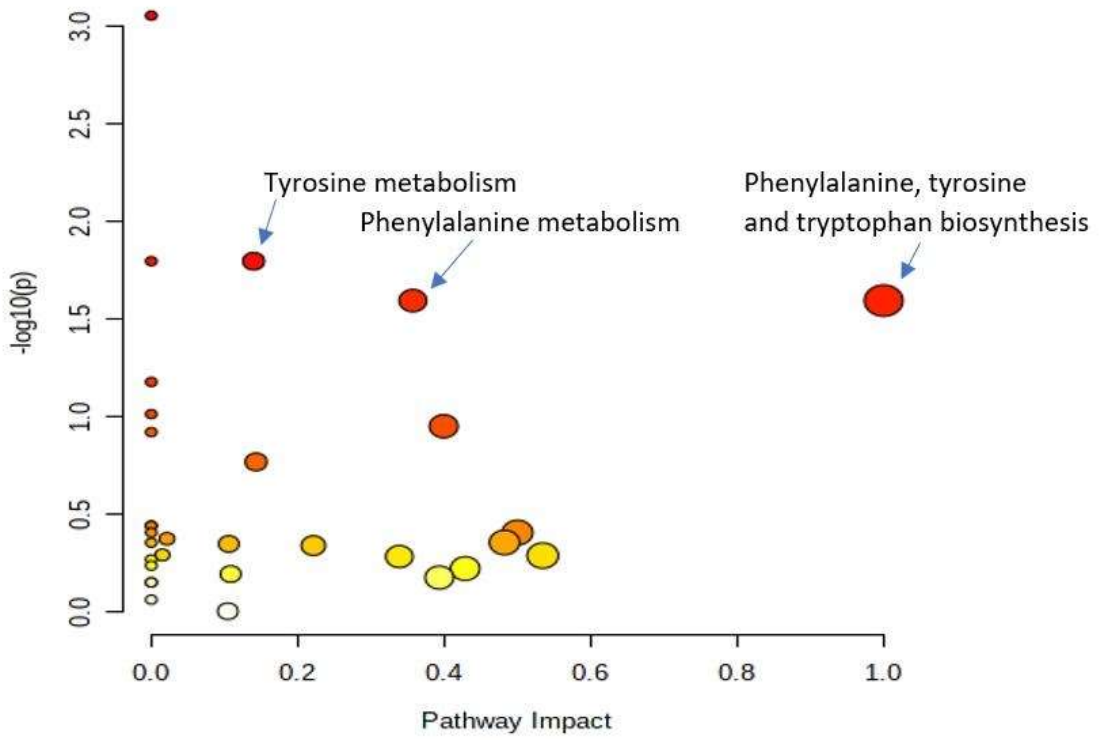

Figure-5. The color gradient shows the importance of the road ranked by p value (Yellow: higher p values and red: lower p values), and the circle size indicates the road impact score (The larger the circle, the higher the impact score).

Discussion:

In this study, we compared an amino acid profile that contains 26 amino acids between CMP patients and healthy controls. We demonstrated five significant findings. Asparagine, beta-alanine, phenylalanine, tyrosine, and isoleucine levels were higher in the CMP patients. Glutamine, isoleucine, and glycine were the amino acids that contribute the most to the differentiation of the two groups. Isoleucine/alloisoleucine, histidine/isoleucine, aspartic acid/isoleucine, and glycine/phenylalanine had a great value in predicting CMP. Isoleucine and beta-alanine may be potential biomarkers for CMP. Pathway analysis showed three different pathways that degrade cardiomyopathy, including phenylalanine metabolism, tyrosine metabolism, and phenylalanine, tyrosine, and tryptophan biosynthesis.

The basis of HF, in which myocardial damage results in systolic and diastolic ventricular dysfunction, is the disturbances in energy metabolism developed as a result of ischemia and hypoxia in myocardial substrates [13-16]. Metabolomics describing small molecular metabolites such as lipids, organic acids, nucleic acids and amino acids plays an important role in the metabolic state and pathophysiological changes of our biological system. The amount of metabolomics in the blood can provide information about chronic diseases and HF. There are studies showing that it is associated with cardiovascular diseases and cardiovascular events [17-20]. In our study, it was found to be higher in HF patients with amino acid profile.

Phenylalanine is one of the predecessor amino acids that form the structure of catecholamine (dopamine, epinephrine, and norepinephrine) that are effective in the pathophysiology of HF. The increased stress response as a result of decreased cardiac output due to HF causes an increase in the secretion of catecholamine [21-23]. High phenylalanine concentrations were found in previous studies in patients with HF, demonstrating that higher phenylalanine concentrations might be associated with poor cardiac functions [5, 21, 23, 24]. Nitric oxide (NO) has a substantial role in HF pathogenesis [25]. Induction of NO synthase-2 results in NO uncoupling and concurrent tetrahydrobiopterin depletion in the myocardium contributes to HF in animal models and human subjects [25, 26]. Coherent with this, the accumulation of phenylalanine and tyrosine in HF patients is indicative of tetrahydrobiopterin depletion. This occurs in parallel with a significant decrease in

arginine levels, which may disturb NO production and lead to cardiac dysfunction [27].

In the present study, a high concentration of isoleucine was shown in patients with HF, which was consistent with another previous study finding [28]. Additionally, Xue Gong et al [23] determined the ideal metabolites of 105 patients with dilated cardiomyopathy and found that isoleucine was the determinant in parallel with our results [23]. Hypoxia-induced inhibition of the citrate cycle seen in HF may lead to the use of isoleucine as energy compensation [22, 28]. By the aspect of all these, isoleucine, which is necessary for metabolic signaling pathways and protein synthesis, can also be an alternative energy source. This may be a useful compensatory mechanism in HF patients [22].

Asparagine plays an important role in immune function and raises the expression and activity of ornithine decarboxylase for polyamine synthesis in thymocyte [29]. In a previous study, asparagine and phenylalanine concentrations were increased in chronic obstructive pulmonary disease and pancreatic cancer patients, as we observed in patients with HF [30]. In the patient groups in this study, asparagine was associated with cachexia and immune modulators. The underlying reason for our findings of increased asparagine in HF patients may be the same mechanisms. Further studies are needed in this regard.

It was observed in a recent study that a metabolite profile with the inclusion of amino acids had a more important prognostic value than BNP in terms of HF mortality and re-hospitalization [31]. Similar to this study, we obtained significant results. Isoleucine and beta-alanine were the potential biomarkers for CMP. Glutamine, isoleucine, and glycine had the highest significance in discrimination between HF patients and healthy controls. According to ROC analysis, the metabolites Isoleucine/alloisoleucine, histidine/isoleucine, aspartic acid/isoleucine, and glycine/phenylalanine had a sensitivity of around 80% and a specificity of over 70% in predicting HF with the highest AUC. Metabolic pathway analysis of our study showed that three different pathways were the underlying molecular mechanisms of the HF.

We think that the measurement of amino acid levels by LC-MS is an easy and powerful method for clinicians to complete. Only a small amount of blood sample is needed and data takes only a few minutes to obtain. Therefore, it can be a simple method in the follow-up and treatment of cardiovascular diseases. With the using of this targeted, quantitative method, we successfully identified a group of circulating amino acids that significantly increased in the HF group when compared to heathy controls.

Our study has some limitations. Firstly, we used only one-time-point samples instead of samples collected from a time course. Thus the identified metabolic changes in HF patients cannot be firmly confirmed as effective early diagnostic tools to monitor heart conditions. Second, the etiological data of the HF were not included in our study. For example, no distinction was made between dilated CMP and ischemic CMP. New studies are necessary for which disease-specific metabolites are studied. Finally, our study was a single-center and the sample size had to be larger. For this reason, further researches in larger populations are needed to explore the mechanisms of the effects of these metabolomics on HF.

Conclusion:

HF is associated with a variety of abnormalities in amino acid profiles in multiple metabolic pathways and the subsequent occurrence of a complex metabolic storm. Apart from the understanding of pathogenesis, the profile of amino acids provides a more sensitive and better evaluation of HF. In addition, the invention of new biomarkers with predictive and diagnostic value in terms of HF, such as amino acids, which we obtained as a result of our study, may provide us the chance of a rapid diagnosis of HF. Studies like ours may contribute to finding specific markers that can be used to diagnose and follow up HF with simple blood tests such as BNP in the future.

Conflict of interest: No conflict of interest is declared.

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