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Quantification and Evaluation of Plasma-free Amino Acid Concentrations by LC-MS/MS After Total Gastrectomy

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Abstract: The effect of total gastrectomy (TG) on plasma free amino acid (PFAA) concentrations in patients with stage II gastric cancer was investigated in the study. Nineteen patients' plasma samples were collected before and three months post-gastrectomy, and PFAA levels were quantified using LC-MS/MS. For gradient elution of amino acids, the mobile phases (A: 3% formic acid-5% methanol-30 mM ammonium formate, B: acetonitrile) and a Hypersil C18 column (100 mm x 2.1 m, 1.9 µm) were used. The findings revealed substantial modifications in the profile of PFAA after TG. In particular, the concentrations of twenty amino acids increased significantly, including branched-chain amino acids, L-glutamate, L-alanine, L-methionine, glycine, L-cystine, and L-histidine. Conversely, L-arginine was also reduced statistically. These alterations in the PFAA profile indicate the favorable effects of TG on various physiological processes, such as enhanced immune function, improved tissue healing, and increased energy production. Investigating the effects of various surgical techniques on PFAA profiles is a promising approach for optimizing surgical procedures, improving metabolic function, increasing immunological responses, and improving overall quality of life. These findings highlight the significance of evaluating amino acid metabolism as an important part of treatment, given its potential to improve clinical outcomes and general well-being.

Keywords: Amino acids, Total gastrectomy, Gastric cancer, LC-MS/MS.

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1. INTRODUCTION

Radiation and chemotherapy are the most popular treatment options for gastric cancer. However, the disease's recurrence and high death rate following these therapies necessitated a variety of surgical resections. As a result, total and subtotal gastrectomy is frequently favored in gastric cancer (1,2).

Total gastrectomy (TG) is a surgical procedure that removes the entire stomach. As a result, the rate and amount of macronutrients such as amino acids, carbs, and lipids entering the systemic circulation vary (3,4). In addition, gastrectomy influences the release of numerous digestive enzymes (pepsin and pepsinogen) from the intestine. As a result, protein digestion and amino acid absorption are impaired. Insufficient intake of macronutrients disrupts biological reactions and metabolic functioning, causing malabsorption, symptoms, and mortality (5).

Eliminating the negative consequences of TG will both allow long-term survival and increase the quality of life of patients. Biochemical parameters to be used in monitoring the health status of patients after TG are guided in the development of new treatment strategies. In recent studies, parameters such as amino acids, metabolites, and trace elements have been investigated in detail to monitor diseases and treatments. Thanks to these studies, molecular mechanisms and biological processes leading to complications can be clearly revealed (6).

Amino acids contribute to immune function by activating T lymphocytes, B lymphocytes, and macrophages and by producing antibodies, cytokines, and other cytotoxic substances. They also play a key role in energy production, tissue repair, and development by providing protein synthesis (7). Previous clinical studies have shown that amino acid supplements can improve the clinical profile and reduce symptoms of various diseases. It is also known that dietary supplementation of certain amino acids to malnourished people improves immune status and thus reduces mortality. Monitoring amino acid profiles in biological fluids can both predict possible organ damage and symptoms and guide the development of different treatment strategies (6,8-12).

Maintaining the balance of plasma-free amino acids (PFAAs) is critical for the continuation of biological and physiological processes in individuals. Many previous studies have shown that gastrectomy affects the fecal microbiome and metabolome profiles over time (13). As can be seen, these studies reveal the effects of the surgical procedure on biological processes. Therefore, changes in amino acid metabolism and concentrations after TG should be taken into consideration. Because changes in the PFAA profile following TG may have profound effects on the nutritional status, weight gain, and general well-being of patients.

There are only two studies in the literature investigating the effects of TG and fundectomy on plasma amino acid concentrations. These studies were conducted only on male individuals and pigs (14,15). More comprehensive studies are needed to elucidate the relationship between TG and amino acid metabolism. Changes in PFAA concentrations following TG were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Thus, the study aimed to contribute to the understanding of changes in plasma amino acid profiles, metabolic processes, and their long-term effects after TG in patients with stage II gastric cancer.

2. MATERIALS AND METHODS

2.1. Study Design and Participants

Between May 2018 and May 2019, the study was conducted at the Intensive Care Unit of the Department of General Surgery at Atatürk University Research Hospital, including 19 patients with stage II gastric cancer and decided TG (9 females and 10 males, aged 37-81). The tumor histological type of the patients was moderately differentiated adenocarcinomas, and they were diagnosed with stage II stomach cancer according to the classification of malignant tumors (TNM). The study was conducted in Erzurum, Turkey. The study was approved by the Non-Interventional Clinical Trials Ethics Committee, Ataturk University, with ID: B.30.2.ATA.0.01.00/244. This study was conducted in accordance with the World Medical Association's Helsinki Declaration (2000). Prior to the surgery, patients were informed about the study, and written consent was obtained. Gastroscopy-enhanced computed tomography (CT) of the abdomen and pelvis, plain CT of the chest, and ultrasound scanning of the tumors were used for preoperative evaluation. Patient information and tumor characteristics of gastric cancer are presented in Table 1. Patients with poorly/advanced differentiated adenocarcinomas, gastric surgery, different cancer diseases other than stage II gastric cancer, or urgent surgery due to complications such as bleeding, perforation, or obstruction were excluded from the study. To ensure that diet did not affect the concentrations of amino acid in the plasma, a standard nutritional protocol was followed for 3 days before plasma samples were taken. Daily calorie and protein intakes were ensured to be ≥25 Kcal/kg and ≥1g/kg, respectively (16). Postoperative treatment protocols were similar for all patients.

Table 1: Demographic and clinical variables of patients.

Patient No	Gender/ Age (years)	Tumor Size (cm)	Depth of Invasion	Lymph Node Metastasis
1	F/59	<5	T2	N0
2	F/37	3.5×2.5×2	T3	N2
3	M/47	2.5×2.5×1.5	T3	N1
4	M/54	2.5	T3	N2
5	F/62	2.5×2×1	T3	N1
6	M/54	2×1.5	T3	N0
7	M/81	3×2×1.5	T3	N2
8	F/53	3	T3	N2
9	M/52	<3	T3	N2
10	F/65	2.5×2.5×1	T4	N2
11	M/75	6×5×2	T3	N2
12	M/51	6.5×5.5×1.5	T3	N0
13	F/47	4×1.5×0.7	T4	N0
14	F/65	3×2×1.5	T3	N0
15	M/54	2.5×2×1.5	T4	N2
16	M/58	4×2.5	T3	N2
17	F/48	4×1.5×1	T4	N0
18	F/64	<5	T2	N0
19	M/71	<5	T2	N0

2.2. LC-MS/MS Analysis of Plasma Amino Acid Levels

The concentrations of amino acids in plasma were determined by the validated LC-MS/MS method developed using the JASEM Amino Acids LC-MS/MS Analysis Kit (12). Following an overnight fasting period, blood samples were collected from the antecubital vein of all participants into blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, both before the operation and 3 months after the operation. To obtain plasma, the tubes were centrifuged at 3500 rpm for 10 minutes. Plasma samples were stored at -80°C until analysis.

After the samples were thawed at room temperature, they were vortexed for 30 seconds. 50 µL of plasma sample from both the before-surgery (BS) and after-surgery (AS) groups were placed in separate Eppendorf tubes. Subsequently, 50 µL of internal standard was added to each tube, followed by vortexing for 10 seconds.

Then, 700 µL of an amino acid solvent solution consisting of Mobile Phase A and Mobile Phase B in a 1:4 volume ratio was added to each tube. The samples were vortexed for 5 seconds and then centrifuged at 4000 rpm for five minutes at 4°C. The supernatant was filtered with 0.45 µm filters. The clear supernatant was decanted into an HPLC vial prior to injection.

The LC-MS/MS system (Agilent 6460 Triple Quadropol, USA) was utilized to quantify the samples. Amino acids were separated using a Hypersil C18 column (100 mm x 2.1 mm, 1.9 µm). The mobile phase (A: Formic Acid–Methanol–30 mM Ammonium Formate (3:5:92; v:v:v), B: Acetonitrile) was passing through the system in gradient elution mode and at a rate of 0.4 mL/min. The analysis time was 22 minutes. Chromatographic conditions and mass spectrometry settings, which enabled the separation and identification of amino acids, are provided in Tables 2 and 3, respectively.

The regression equations, correlation coefficients, linear ranges, and LOQ of the LC-MS/MS method are given in Table 4.

Table 2: Solvent Composition Schedule during the gradient elution for LC-MS/MS.

Flow: 0.7 mL/min	Change Solvent Composition	
Time	**A**	**B**
1.00 min	22.00 %	78.00 %
4.00 min	70.00 %	30.00 %
5.00 min	70.00 %	30.00 %
5.10 min	22.00 %	78.00 %
9.00 min	22.00 %	78.00 %

*A: Formic Acid – Methanol–30 mM Ammonium Formate (3:5:92; v:v:v), B: Acetonitrile

Table 3: Mass conditions.

Parameters	Value (+)	Value(-)
Gas Temp (°C)	150.00	150.00
Gas Flow (L/min)	11.00	11.00
Nebulizer (psi)	40.00	40.00
Sheath Gas Heater	375.00	375.00
Sheath Gas Flow	11.00	11.00
Capillary (V)	2000.00	0.00
VCharging	0.00	0.00
Injection Volume (µL)	1.00	
Ion Source	AJS ESI	
Ion Mode	Positive	

2.3. Metabolic Pathway Analysis

The metabolic pathways affected by the changing amino acid profile after TG were determined with MetaboAnalyst 4.0 Software. The number of amino acids involved in the affected metabolic pathways was also determined.

2.4. Data Analysis

The statistical analyses were performed using SPSS Statistics (IBM v.20, Chicago, IL, USA). Initially, the factor analysis was conducted on the cluster of data for normalization. The Non-paired Student's t-test was used for normally distributed data, and the non-parametric Mann-Whitney U-test was also used for non-normally distributed data. The statistical significance was set at $p < 0.05$. This statistical test allowed for the comparison of mean values between the two groups to determine if there were significant differences in amino acid levels before and after the surgical procedure.

For visualizing amino acids with significant differences among the groups, boxplot graphs were created using the ggpubr package in RStudio (v.

1.3.1093). The correlation graph was generated using the GGally package. The Spearman correlation matrix method was used to reveal the correlation between plasma concentrations of amino acids over time following TG (12,17).

Table 4: The regression equations, correlation coefficients, linear range, and LOQ of the LC-MS/MS method.

Amino Acids	Internal standard	Regression equations	R^2	Linear range (ng/mL)	LOQ (ng/mL)
1-methyl-L-histidine	-	y= 0.009171x - 0.017750	0.99937	1.0-350.0	1.0
3-amino isobutyric acid	-	y= 0.003340x + 0.001234	0.99732	0.1-110.0	0.1
3-methyl-L-histidine	3-methyl-L-histidine IS	y= 0.078033x - 0.410524	0.99922	5.0-350.0	5.0
Beta-alanine	-	y= 0.003194x - 0.004878	0.99605	1.5-50.0	1.5
Ethanolamine	-	y= 0.812943x - 5.866369	0.99768	5.0-270.0	5.0
Gamma-aminobutyric acid	-	y= 0.015012x + 0.001904	0.99956	0.01-110.0	0.01
Glycine	Glycine IS	y= 0.001123x - 0.040868	0.99847	50.0-1700.0	50.0
L-2-aminobutyric acid	-	y= 0.230656x - 0.502684	0.99567	2.5-80.0	2.5
L-alanine	L-alanine IS	y= 0.002418x - 0.067955	0.99813	25.0-1400.0	25.0
L-anserine	-	y= 0.008411x - 0.005699	0.99605	0.01-25.0	0.01
L-arginine	L-arginine IS	y= 0.010397x - 0.050441	0.99930	1.0-320.0	1.0
L-asparagine	L-asparagine IS	y= 0.013342x - 0.037875	0.99728	1.0-200.0	1.0
L-aspartic acid	L-aspartic acid IS	y= 0.023892x - 0.085002	0.99782	5.0-160.0	5.0
L-citrulline	L-citrulline IS	y= 0.013412x + 0.004663	0.99845	0.1-80.0	0.1
L-cystathionine	-	y= 0.012178x + 0.001751	0.99878	0.01-24.0	0.01
L-cystine	DL-Cystine IS	y= 0.008333x - 0.014697	0.99949	1.0-220.0	1.0
L-glutamic acid	L-Glutamic acid IS	y= 0.014644x + 0.171485	0.99881	20.0-1500.0	20.0
L-glutamine	L-glutamine IS	y= 0.004897x + 0.012509	0.99954	1.0-1400.0	1.0
L-histidine	-	y= 0.266900x – 2.381075	0.99968	1.0-1200.0	1.0
L-isoleucine	-	y= 0.001207x - 0.004704	0.99822	5.0-1000.0	5.0
L-leucine	L-leucine IS	y= 6.330595E-004x – 0.005242	0.99783	10.0-440.0	10.0
L-lysine	L-lysine IS	y= 0.018868x - 0.083803	0.99875	5.0-420.0	5.0
L-methionine	L-methionine IS	y= 0.021931x - 0.041218	0.99855	2.5-90.0	2.5
L-norvaline	-	y= 0.082804x + 0.030635	0.99684	0.05-22.0	0.05
L-ornithine	L-ornithine IS	y= 0.016225x - 0.077035	0.99804	2.5-340.0	2.5
L-phenylalanine	L-phenylalanine IS	y= 0.015304x - 0.148367	0.99808	10.0-480.0	10.0
L-proline	L-proline IS	y= 0.005904x - 0.111633	0.99804	10.0-900.0	10.0
L-serine	L-serine IS	y= 0.013875x - 0.225179	0.99866	10.0-900.0	10.0
L-threonine	L-threonine IS	y= 0.010328x + 0.016741	0.99787	1.0-500.0	1.0
L-tryptophan	L-tryptophan IS	y= 0.024814x - 0.086062	0.99747	2.5-150.0	2.5
L-tyrosine	L-tyrosine IS	y= 0.009429x - 0.053190	0.99464	5.0-260.0	5.0
L-valine	DL-Valine IS	y= 0.003244x - 0.031811	0.99781	5.0-750.0	5.0
Taurine	-	y= 1.04617E-004x - 6.482529E-005	0.99873	1.0-440.0	1.0
Trans-4-hydroxy L-proline	-	y= 0.001151x - 0.006470	0.98052	5.0-140.0	5.0

LR: Linear regression equations, R^2: Correlation coefficient

3. RESULTS

After TG, patients were put on a diet according to the postoperative treatment protocols. There was no significant difference between the patients' BMI values before and 3 months after TG (25.72 ± 0.69 vs. 24.87 ± 2.47, p = 0.059). Plasma-free amino acid concentrations of the patients were measured. Amino acids whose concentrations changed significantly were identified. The metabolic pathways in which these amino acids take part were determined.

3.1. LC-MS/MS Analyses Results: Alterations in Plasma Amino Acid Concentrations After Gastrectomy

To evaluate the impact of TG on the amino acid profile, a validated LC-MS/MS method was employed (12). This method was chosen due to its high precision, certainty, and accuracy, enabling the generation of reliable qualitative and quantitative data. Blood samples were collected from 19 individuals who participated in the study, both prior to the operation and three months after the operation. The LC-MS/MS analysis was conducted to measure the levels of 34 specific PFAAs, allowing for a comprehensive assessment of any potential changes in the amino acid profiles resulting from the surgical procedure. The chromatograms and spectrums of the BS and AS groups obtained from LC-MS/MS analysis are given in Figure 1a-d. Table 4 shows the linear regression equations and correlation coefficients for PFAAs. The obtained data were analyzed using linear regression equations. The concentrations of PFAA determined by LC-MS/MS are expressed as mean ± standard deviation in Table 5.

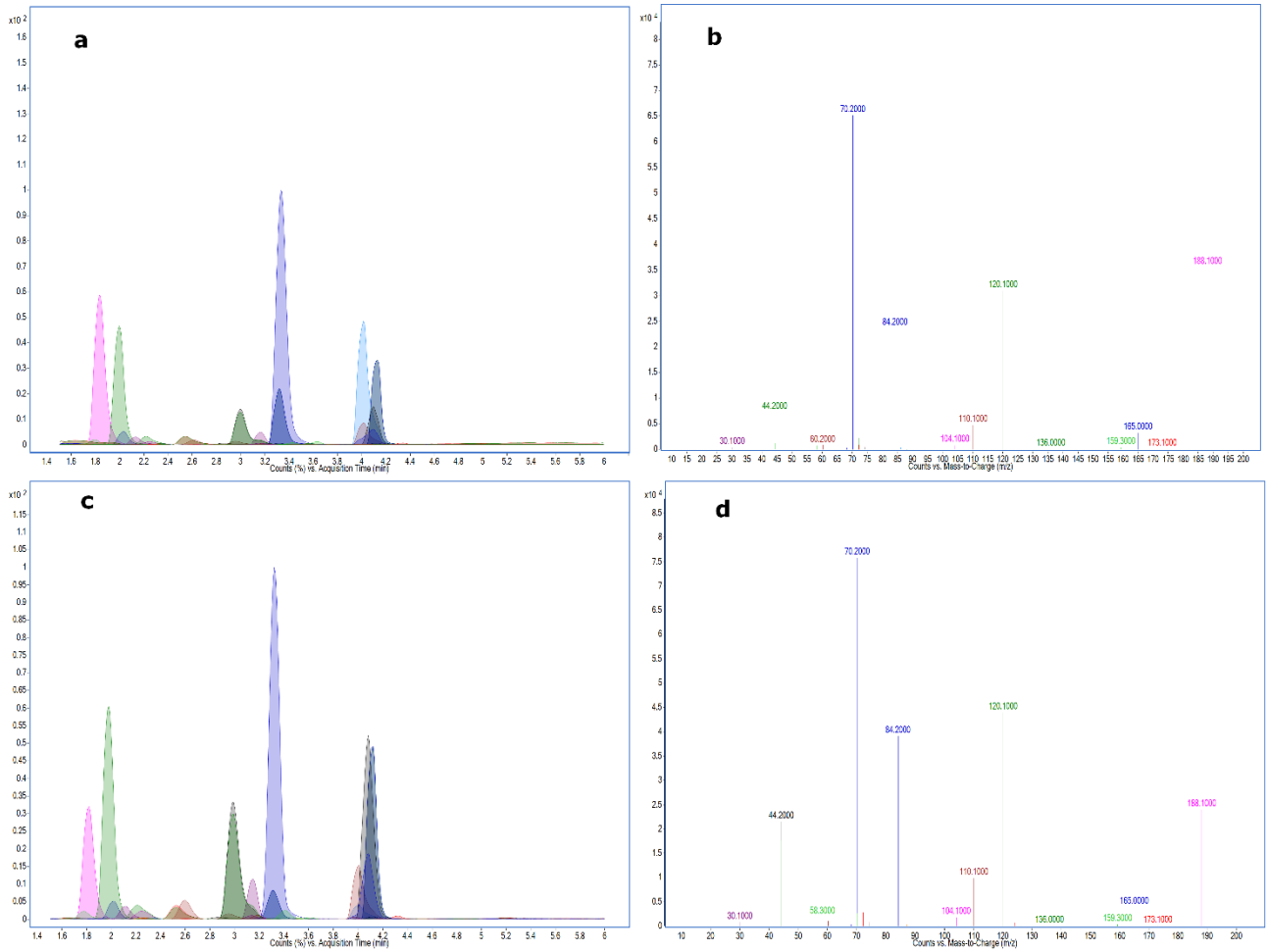

Figure 1: Typical chromatograms and spectrums obtained from LC-MS/MS: a) The chromatogram of the BS group, b) The mass spectrum of the BS group, c) The chromatogram of the AS group, b) The mass spectrum of the AS group.

Table 5: PFAA concentrations (ng/mL) of stage II gastric cancer patients before and after TG (n=19).

Amino acids	Group BS Mean±SD (ng/mL)	Group AS Mean±SD (ng/mL)	*p*-value*
1-methyl L-histidine	6.67 ±0.94	8.81 ±0.86	0.1502
3-aminoisobutyric acid	2.69±0.81	4.13±0.76	0.0052*
3-methyl L-histidine	9.40±0,24	10.54±0,19	0.0033*
Beta-alanine	3.98±0.40	4.69±0.61	0.0880
Ethanolamine	73.49±6.31	93.00±4.72	0.1685
Gamma-aminobutyric acid	0.14±0.26	0.34±0.19	0.0162*
Glycine	339.01±31.61	512.99±28.73	0.0021*
L-2-aminobutyric acid	32.66±3.76	33.65±1.28	0.8394
L-alanine	547.53±16.76	731.16±21.39	0.0058*
L-anserine	1.03±0,01	0.99±0,03	0.6848
L-arginine	155.11±0,26	93.32±0,58	4.1e-07*
L-asparagine	54.55±2.43	67.65±2.62	0.0063*
L-aspartic acid	39.80±1.93	31.59±2.46	0.1139
L-citrulline	52.98±9.42	41.41±4.76	0.3237
L-cystathionine	0.18±0.03	0.32±0.01	0.0196*
L-cystine	62.76±5.54	76.43±6.31	2.2e-06*
L-glutamic acid	477.70±34.43	1360.88±76.81	0.0037*
L-glutamine	818.56±62.43	683.19±38.16	0.5477
L-histidine	168.65±18.43	210.54±14.36	0.0169*
L-isoleucine	94.82±3.78	136.79±5.14	0.0001*
L-leucine	157.73±9.74	190.88±6.87	0.0302*
L-lysine	231.25±18.29	279.55 ±23.48	0.0135*
L-methionine	20.23±1.93	30.86±1.58	6.21e-07*
L-norvaline	0.56±0.03	0.42±0.02	0.0959
L-ornithine	110.07±19.15	175.97±11.74	0.0009*
L-phenylalanine	126.16±9.26	137.20±10.71	0.3582
L-proline	290.04±35.65	320.32±27.85	0.3512
L-serine	204.06±17.36	211.88±20.3	0.6096
L-threonine	163.58±14.16	199.33±13.11	0.0145*
L-tryptophan	65.28±1.78	66.24±2.41	0.7537
L-tyrosine	75.33±7.43	91.96±8.63	0.0065*
L-valine	202.98±19.59	255.64±24.51	0.0007*
Taurine	332.50±12.37	329.20±17.15	0.9199
Trans-4-hydroxy L-proline	13.30±1.72	16.15±2.09	0.0058*

*: $p<0.05$; BS: Before surgery, AS: After surgery

3.2. Non-paired Student's t-test Analysis

Through the utilization of the Non-paired Student's *t-test* analysis, significant differences were identified between the BS and AS groups in terms of specific amino acids. The analysis revealed that a total of 20 amino acids exhibited statistically significant changes ($p < 0.05$) in their plasma profiles across the two groups. Table 5 highlights the amino acids that exhibit notable variations in their concentrations subsequent to the surgical procedure. These findings suggest that the operation had a discernible impact on the levels of these specific amino acids, potentially reflecting underlying metabolic shifts or physiological adaptations associated with the surgery.

After a period of 3 months following TG, no statistically significant differences were observed in the plasma concentrations of L-tryptophan, taurine, L-phenylalanine, L-norvaline, ethanolamine, Beta-alanine, L-2-aminobutyric acid, L-aspartic acid, L-serine, and L-proline. These specific amino acids exhibited no marked alterations in their plasma levels, suggesting that their concentrations remained relatively stable during the postoperative period.

After TG, a notable alteration in the plasma concentrations of L-threonine, L-leucine, L-isoleucine, and L-valine was observed statistically. Following gastrectomy, the plasma levels of these specific amino acids exhibited a distinct increase (Figure 2a-d).

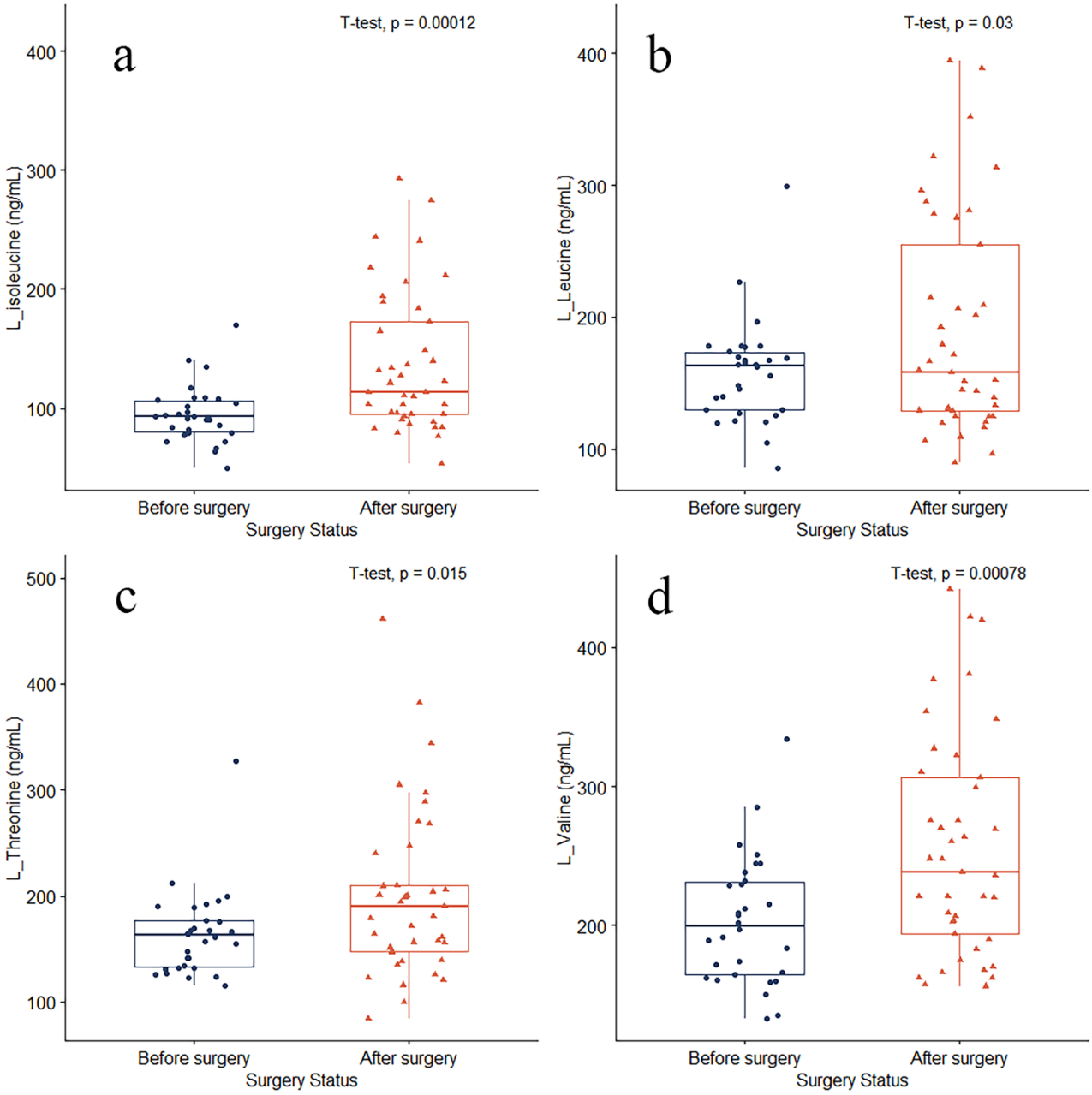

Figure 2: Box plots graphs showing differences in amino acid levels: a) L-isoleucine, b) L-leucine, c) L-threonine, d) L-valine.

After a period of 3 months following surgery, significant changes in plasma levels of L-ornithine, Trans-4-hydroxy L-proline, and L-methionine were observed, with a marked increase in their concentrations (Figure 3a-d). Conversely, the plasma level of L-arginine exhibited a remarkable decrease during the same timeframe (Figure 3d and Figure 4a-d).

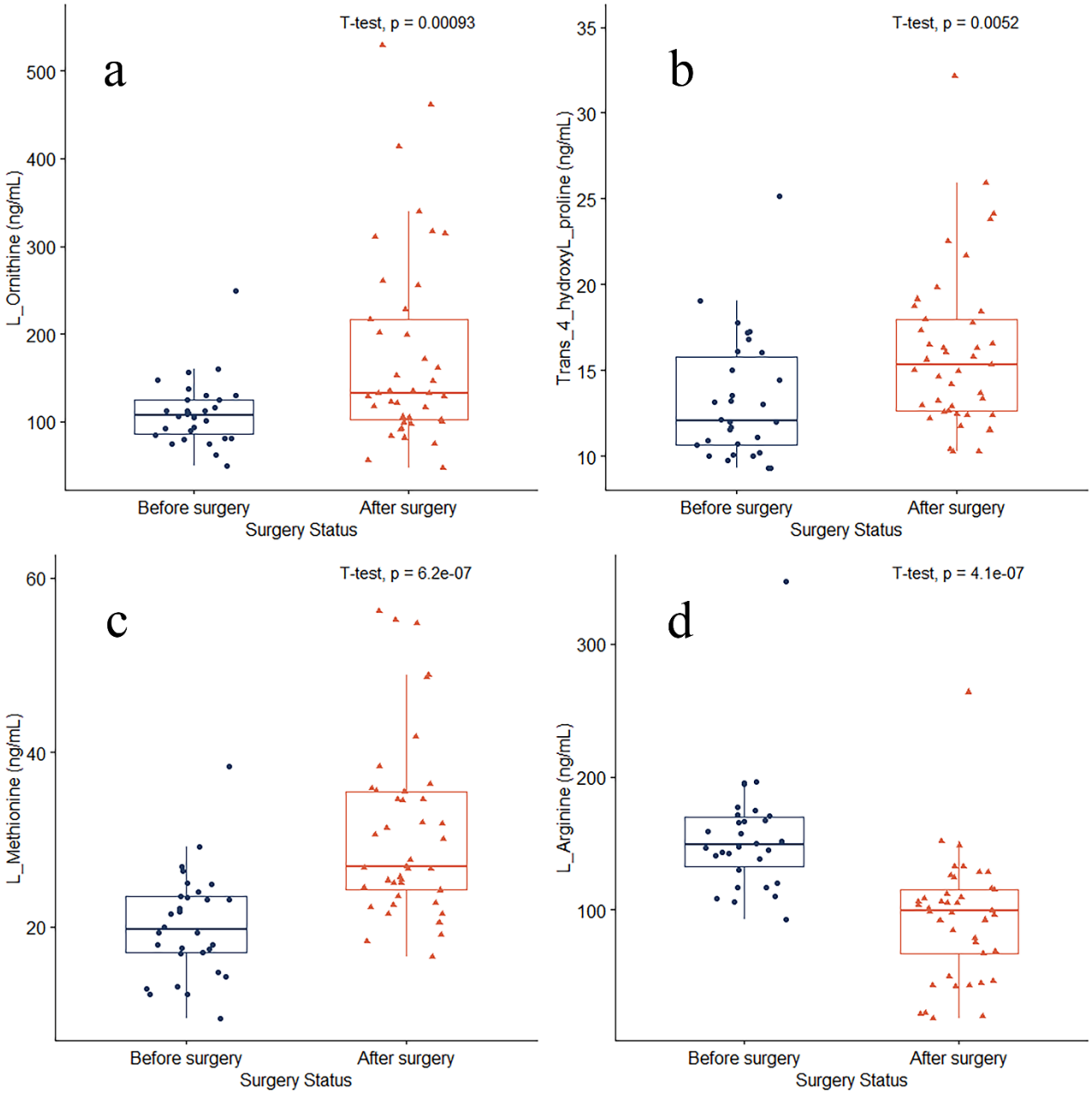

Figure 3: Box plots graphs showing differences in amino acid levels: a) L-ornithine, b) Trans-4-hydroxy L-proline, c) L-methionine, and d) L-arginine.

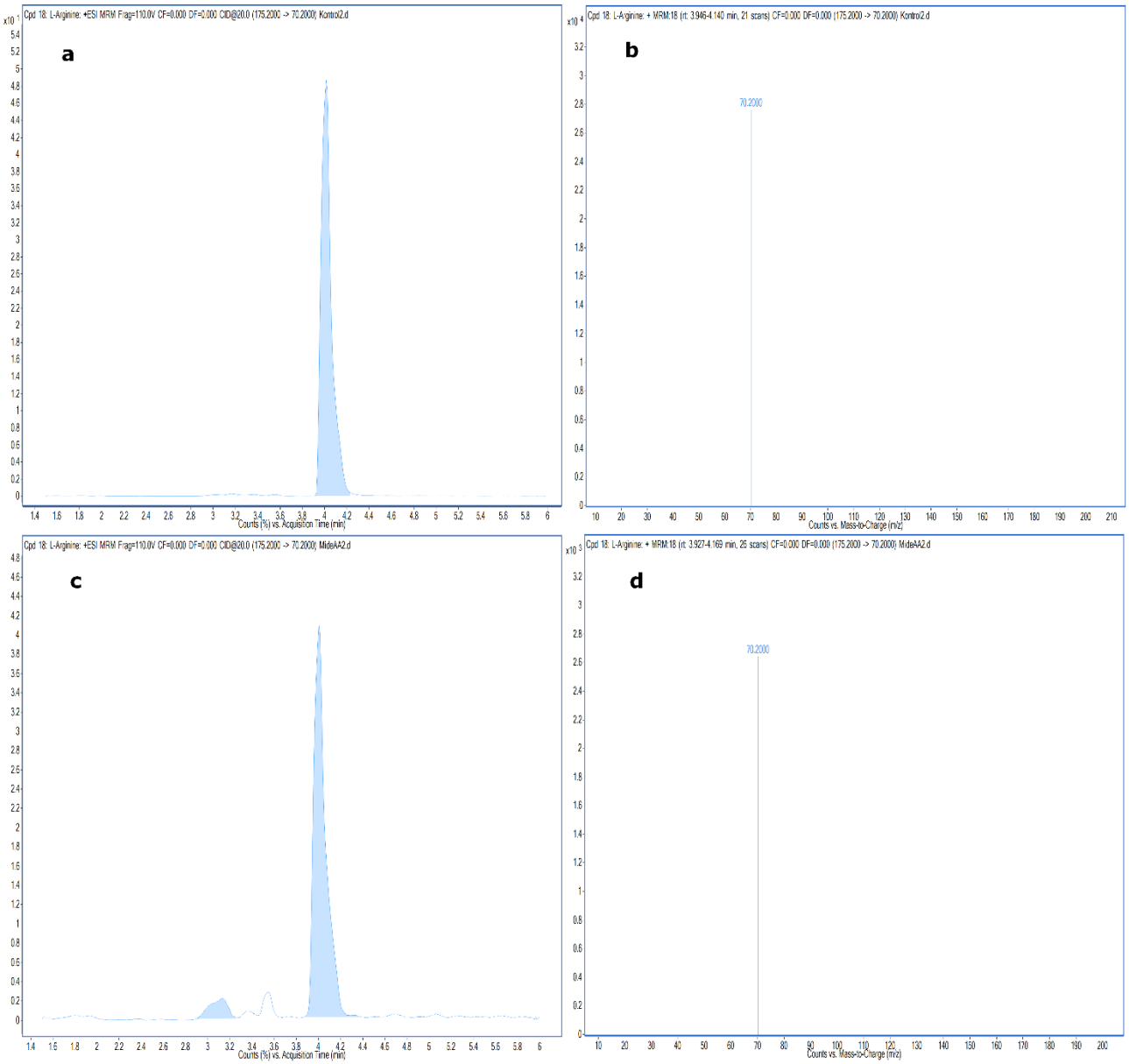

Figure 4: Typical chromatograms and product ion mass spectrums for L-arginine: a) The chromatogram BS of the group, b) the mass spectrum of BS the group, c) the chromatogram of AS the group, and d) the mass spectrum of AS group.

A comparative analysis of plasma amino acid levels between the BS and AS groups revealed significant differences. According to the *t*-test analysis results, plasma concentrations of L-asparagine, L-alanine, gamma-aminobutyric acid (4-aminobutanoate), and L-glutamic acid (L-glutamate) exhibited a noteworthy increase in the AS group compared to the BS group (Figure 5a-d).

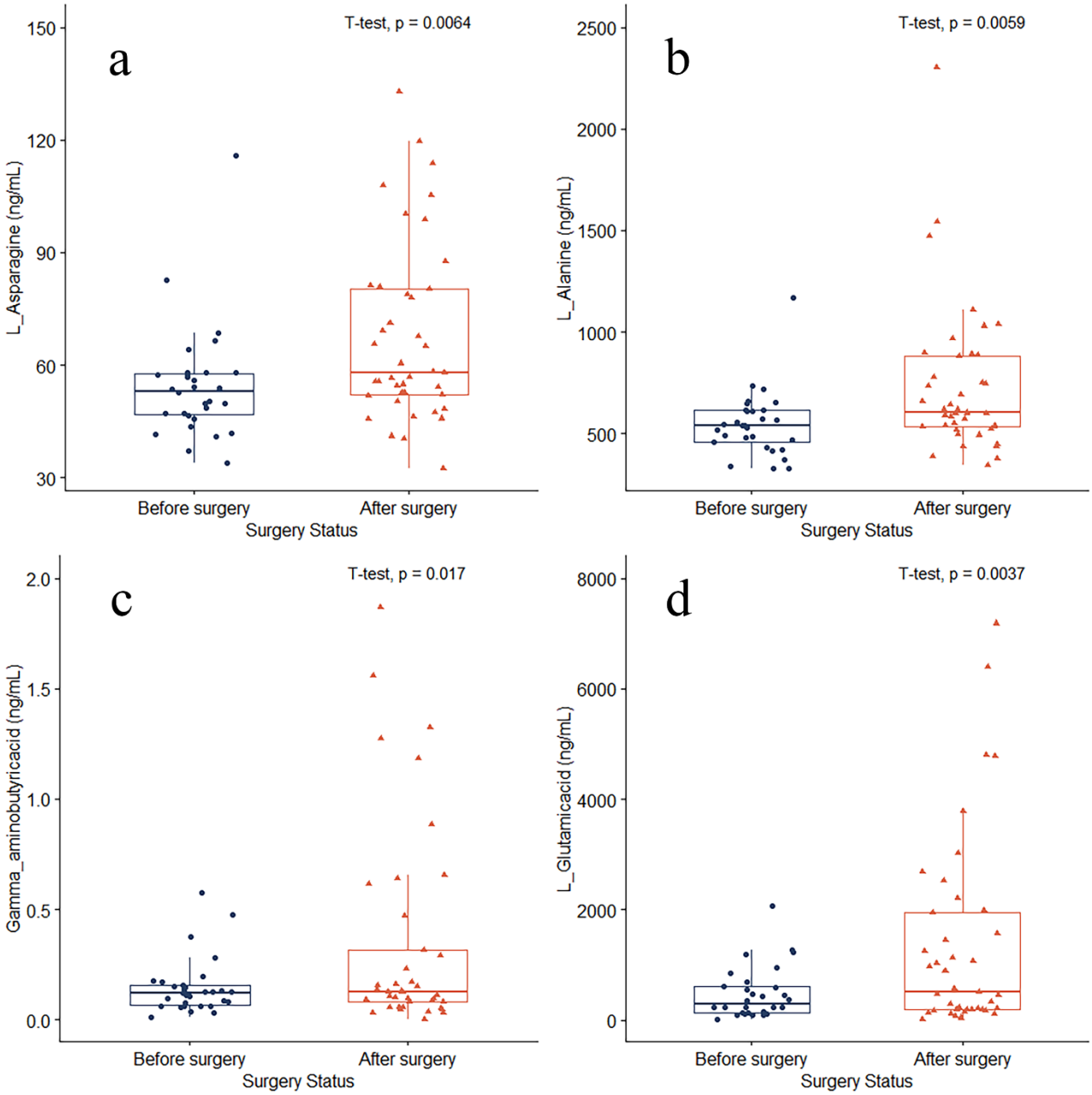

Figure 5: Box plots graphs showing differences in amino acid levels a) L-asparagine, b) L-alanine, c) gamma-aminobutyric acid, d) L-glutamic acid, e) L-histidine, f) 3-methyl-L-histidine, g) L-tyrosine, and h) 3-aminoisobutyric acid.

Among the amino acids analyzed, four demonstrated statistically significant differences in plasma concentrations following gastrectomy. These amino acids included L-histidine, 3-methyl-L-histidine, L-tyrosine, and 3-aminoisobutyric acid. Notably, the plasma levels of these amino acids exhibited a significant increase after a three-month period following the surgical procedure (Figure 6a-d).

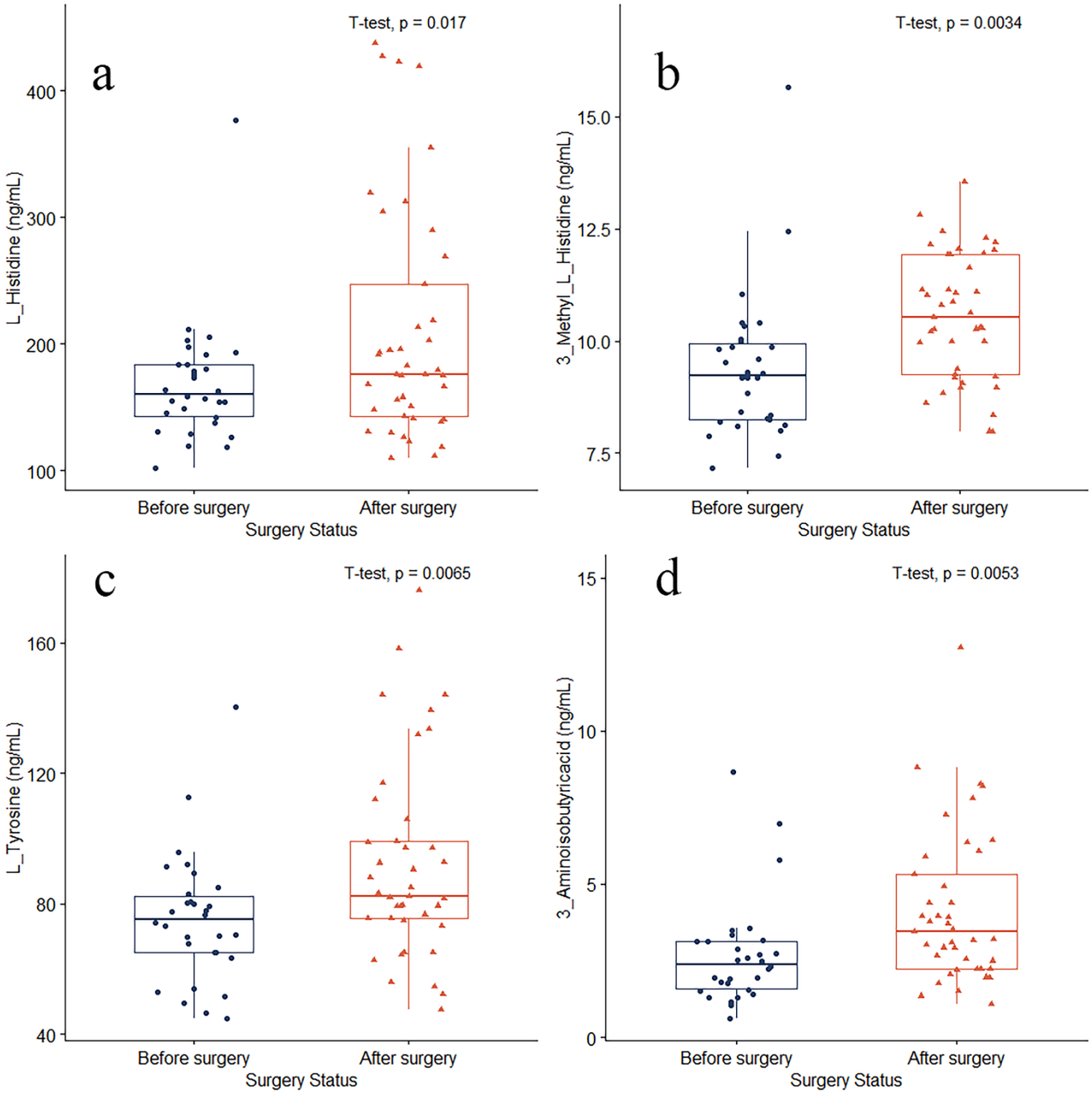

Figure 6: Box plots graphs showing differences in amino acid levels a) L-histidine, b) 3-methyl-L-histidine, c) L-tyrosine, and d) 3-aminoisobutyric acid.

Furthermore, significant changes were observed in plasma concentrations of glycine, L-cystathionine, L-cystine, and L-lysine following TG, particularly after a three-month duration. Notably, the plasma levels of these amino acids demonstrated a distinct increase, highlighting their altered metabolic profiles in response to the surgical intervention (Figure 7).

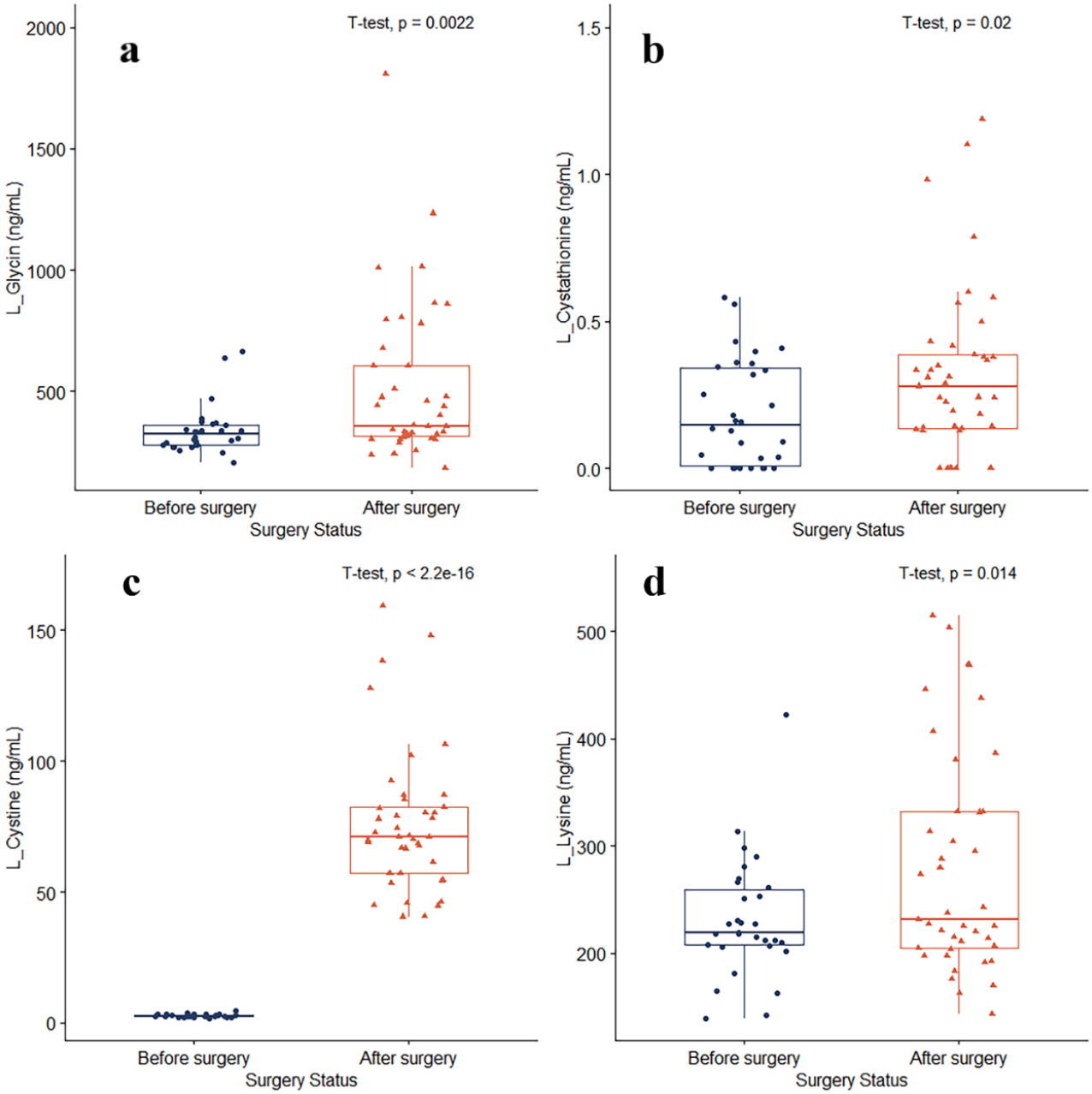

Figure 7: Box plots graphs showing differences in amino acid levels: a) glycine, b) L-cystathionine, c) L-cystine, and d) L-lysine.

3.3. PCA (Principal Component Analysis)

PCA was performed on 34 amino acid variables. Eight principal components with eigenvalues of 1 or above were extracted. The eight principal components explained 78.0% of the total variance. Table 6 shows the eigenvalues and variance ratios of the eight principal components. The first principal component had the largest eigenvalue (15.558) and explained 40.9% of the total variance. The second principal component had the second largest eigenvalue (3.492) and explained 9.9% of the total variance. The third principal component had the third largest eigenvalue (2.926) and explained 7.7% of the total variance. The remaining five principal components explained 19.5% of the total variance.

Table 7 shows the results of the Kaiser-Meyer-Olkin (KMO) test and the Bartlett test. The Kaiser-Meyer-Olkin Measure and Bartlett's test of sphericity were used to assess sampling adequacy. Kaiser-Meyer-Olkin Measure was 0.748, and Bartlett's test of sphericity was 306 (df 703; $p < 0.001$), suggesting acceptable sample adequacy.

Table 6: PCA results (Total Variance Explained).

Total Variance Explained									
Component	**Initial Eigenvalues**			**Extraction Sums of Squared Loadings**			**Rotation Sums of Squared Loadings**		
	Total	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**	**Total**	**% of Variance**	**Cumulative %**
1	15.558	40.943	40.943	15.558	40.943	40.943	14.784	38.905	38.905
2	3.492	9.189	50.131	3.492	9.189	50.131	2.785	7.330	46.235
3	2.926	7.701	57.832	2.926	7.701	57.832	2.343	6.166	52.401
4	2.440	6.421	64.253	2.440	6.421	64.253	2.040	5.369	57.770
5	1.604	4.221	68.474	1.604	4.221	68.474	2.037	5.360	63.130
6	1.300	3.420	71.894	1.300	3.420	71.894	2.037	5.359	68.490
7	1.227	3.228	75.123	1.227	3.228	75.123	1.868	4.916	73.406
8	1.100	2.894	78.017	1.100	2.894	78.017	1.752	4.611	78.017
9	0.965	2.540	80.557						
10	0.942	2.479	83.036						
11	0.808	2.127	85.163						
12	0.727	1.912	87.075						
13	0.653	1.718	88.793						
14	0.516	1.358	90.151						
15	0.478	1.259	91.410						
16	0.434	1.142	92.552						
17	0.393	1.034	93.586						
18	0.329	0.867	94.453						
19	0.314	0.827	95.280						
20	0.304	0.800	96.080						
21	0.246	0.646	96.727						
22	0.217	0.570	97.297						
23	0.195	0.513	97.810						
24	0.151	0.396	98.207						
25	0.131	0.345	98.551						
26	0.106	0.278	98.829						
27	0.085	0.225	99.054						
28	0.065	0.170	99.224						
29	0.059	0.156	99.380						
30	0.047	0.124	99.505						
31	0.045	0.117	99.622						
32	0.037	0.098	99.720						
33	0.031	0.081	99.801						
34	0.029	0.075	99.876						
35	0.020	0.053	99.930						
36	0.013	0.034	99.964						
37	0.008	0.021	99.985						
38	0.006	0.015	100.000						

Extraction Method: Principal Component Analysis.

Table 7: The Kaiser–Meyer–Olkin and Bartlett test results of PCA analysis.

KMO and Bartlett's Test		
Kaiser-Meyer-Olkin Measure of Sampling Adequacy.		0.748
Bartlett's Test of Sphericity	Approx. Chi-Square	3060.971
	df	703
	Sig.	0.000

3.4. Correlation Matrix

Utilizing correlation matrix (R, Spearman) analysis, the interrelationship among plasma concentrations of amino acids over time following TG was investigated. Notably, a strong positive correlation coefficient exceeding 0.8 was observed between the branched-chain amino acids (BCAAs) L-leucine, L-isoleucine, and L-valine. These amino acids demonstrated a concurrent increase in their plasma levels after surgery. Additionally, plasma L-histidine levels exhibited a similar pattern of increase alongside L-ornithine, L-leucine, and L-isoleucine. Conversely, a correlation coefficient greater than 0.8 was found between L-tyrosine levels and L-lysine, L-methionine, L-valine, and L-leucine. Furthermore, Spearman correlation matrix analysis revealed a strong positive correlation among plasma levels of L-ornithine, glycine, L-phenylalanine, and ethanolamine (Figure 8).

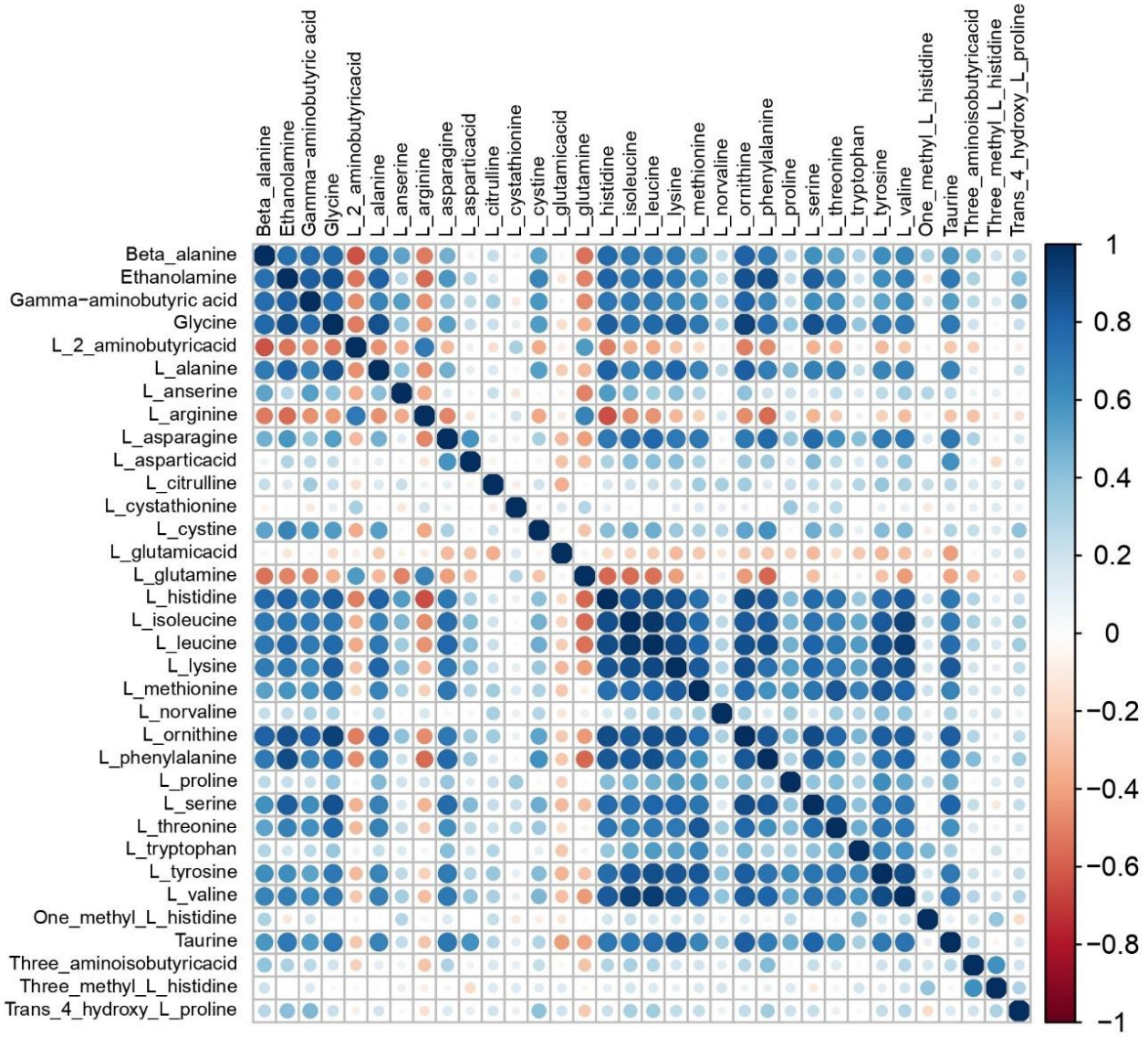

Figure 8: Spearman correlation matrix of plasma free amino acids levels. Positive and negative correlations are indicated by blue and red colors, respectively.

3.5. Metabolic Pathway Analysis

The metabolic pathways that the metabolic changes could impact were identified through pathway analysis. Using the MetaboAnalyst 4.0 software, the 20 amino acids listed in Table 8 were analyzed in detail to determine their metabolic pathways. Figure 9 displays the relevant pathways and their respective effect values. According to the data obtained from MetaboAnalyst 4.0, it was observed that 25 different metabolic pathways may be affected due to the significant difference observed in 20 amino acids. Amino acids that were significantly altered, involved in the pathways of which have -log p values higher than 2 were discussed below (Significantly changed amino acids / total metabolites in an individual pathway): 4/8 valine, leucine, and isoleucine biosynthesis, 5/36 arginine and proline metabolism, 4/28 alanine, aspartate and glutamate metabolism, 3/14 arginine biosynthesis, 3/16 histidine metabolism, 3/28 glutathione metabolism, 3/33 glycine, serine and threonine metabolism, 3/33 cysteine and methionine metabolism, 3/40 valine, leucine and isoleucine degradation, 2/15 butanoate metabolism, 1/4 phenylalanine, tyrosine and tryptophan biosynthesis.

Table 8: Metabolic pathway analysis of amino acids whose concentration changed significantly after TG.

Pathway Name	Match Status*	*p*	-log(*p*)
Valine, leucine, and isoleucine biosynthesis	4/8	1.0302E-6	5.9871
Arginine and proline metabolism	5/36	4.3297E-5	4.3635
Alanine, aspartate and glutamate metabolism	4/28	2.5837E-4	3.5878
Arginine biosynthesis	3/14	4.9883E-4	3.302
Histidine metabolism	3/16	7.5577E-4	3.1216
Glutathione metabolism	3/28	0.004033	2.3944
Glycine, serine and threonine metabolism	3/33	0.0064644	2.1895
Cysteine and methionine metabolism	3/33	0.0064644	2.1895
Valine, leucine and isoleucine degradation	3/40	0.011095	1.9549
Butanoate metabolism	2/15	0.013189	1.8798
Phenylalanine, tyrosine, and tryptophan biosynthesis	1/4	0.047432	1.3239

***** Significantly changed amino acids / total metabolites in an individual pathway.

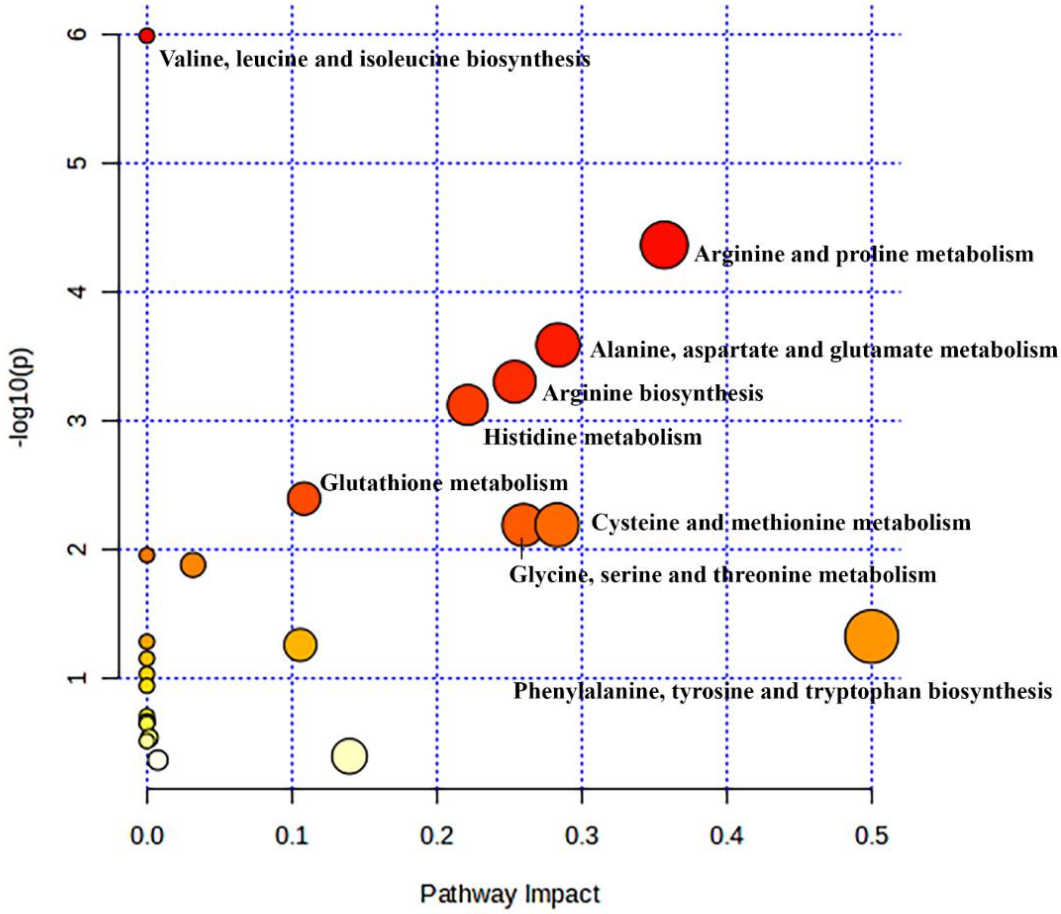

Figure 9: Impact analysis for pathways created by MetaboAnalyst online: main pathways altered in the BS group vs the AS group.

4. DISCUSSION

Understanding the interaction between amino acids and molecular pathways may be useful for optimizing pharmaceutical and surgical treatment methods and reducing mortality (18). The study provides important insights into the changes in amino acid profiles in patients with stage II gastric cancer after TG. The study suggests that negative symptoms (overall morbidity, ≥ grade 3 and local complications, intraabdominal infection, intestinal obstruction, abnormal food passage, disturbed nutrition intake, compromised digestion and absorption processes, and deficiencies in essential micronutrients) can alter PFAA levels in 3 months post-gastrectomy.

Understanding the specific alterations in PFAA profiles is crucial for several reasons. First, it can shed light on the underlying mechanisms responsible for postoperative nutritional deficiencies and metabolic changes, enabling the development of targeted interventions to mitigate these issues. Second, it may help identify potential biomarkers for

assessing the nutritional status and prognosis of gastric cancer patients postoperatively.

The results of the PCA analysis suggest that the eight principal components can be used to represent the 34 amino acid variables. The first principal component is likely to be related to the overall hydrophobicity of the amino acids. In contrast, the second principal component is expected to be associated with the overall polarity of the amino acids. The third principal component is likely to be related to the overall charge of the amino acids. The remaining five principal components are likely to be related to other properties of the amino acids. The PCA analysis provides a way to reduce the dimensionality of the 34 amino acid variables to eight principal components. This can be useful for further analysis of the data.

The study revealed a significant increase in plasma concentrations of L-leucine (p = 0.0302), L-isoleucine (p = 0.0001), and L-valine (p = 0.0007) after gastrectomy. In contrast to these results, Tatara et al. (15) did not observe a significant difference in plasma levels of L-leucine, L-isoleucine, and L-valine one year after TG in men (p>0.05). Another study revealed that long-term fundectomy statistically reduced plasma leucine and valine concentrations in pigs (14). Accordingly, it is seen that BCAAs concentrations, which increase due to the impaired function of the gastric-hypothalamic-pituitary axis in the first periods after TG, can decrease and reach normal levels within a year. It can be thought that, unlike TG, fundectomy may further reduce the plasma concentrations of these amino acids in the long term. The increase in BCAAs concentrations in the three months after TG may be a sign of pathology due to the increase in tissue repair in the surgical area, muscle catabolism, and prevention of trauma-induced cachexia (12,19,20). Additionally, the correlation matrix analysis found correlation values larger than 0.8 (R, Spearman) for leucine, isoleucine, and valine (Figure 8). The analysis showed that the plasma concentration of BCAA increased together for 3 months following TG. These findings suggested that the TG increases BCAA breakdown in muscles by affecting BCAA metabolism and highlight the need for clinical studies to determine whether BCAA supplementation can prevent organ damage in patients after TG.

Glutathione (GSH), synthesized using cysteine, glutamate, and glycine, is vital for activating T-lymphocytes and leukocytes and producing cytokines. Thereby it facilitates robust immune responses for the body in the face of immunological challenges. Maintaining adequate GSH concentrations is crucial for the immune system, less weight loss, regulation of gastrointestinal hormones, and inflammation (21,22). In this study, plasma concentrations of L-glutamic acid (glutamate) (p=0.0037), glycine (p=0.0021), and L-cystine (p=2.2e-06) increased significantly, while the decrease in L-glutamine level was not statistically significant. (Table 5) In a previous study in men, TG did not considerably alter L-glutamic acid, glycine, L-cystine, and L-glutamine plasma concentrations (15).

In contrast to the results, prolonged fundectomy decreased both glycine and glutamine concentrations (14). On the other hand, long-term fundectomy increased glutamate and cystine concentrations, which is parallel to the study. According to these results, it is estimated that the release of L-glutamate, glycine, and L-cystine from the gastric mucosa increases to reduce weight loss and inflammation in the first 3 months of TG. It can be thought that L-glutamine is also consumed for glutamate synthesis. In 1 year, the concentrations of these amino acids may have reached normal levels with the use of GSH synthesized from these amino acids, adequate nutrition, and the end of weight loss and inflammation. Consequently, supplementation with L-glutamine may be recommended to increase intracellular levels of these amino acids, thereby maximizing glutathione synthesis.

L-lysine plays a role in regulating nitric oxide generation from arginine and acts as a crucial precursor for energy production and protein synthesis. L-arginine serves as a precursor for synthesizing various essential amino acids, including nitric oxide, L-proline, L-ornithine, L-glutamate, and L-citrulline. In addition to its role as a precursor, L-arginine plays a critical role in tissue repair and wound healing processes, facilitating the regeneration and restoration of damaged tissues (20,23). In this study, at the end of 3 months, L-arginine (p=4.1e-07) concentration decreased statistically, while L-lysine (p=0.0135), L-ornithine (p=0.0009), and L-glutamate (p=0.0037) increased. In parallel with this study, fundectomy significantly decreased L-arginine levels and increased glutamate over a 1-year period. However, unlike the study, L-arginine, L-lysine, L-ornithine, and L-glutamate concentrations did not change significantly at the end of 1 year following TG. This reduction in the first period of TG may be due to the use of L-arginine in the regeneration of damaged tissues and protein synthesis.

Additionally, L-arginine may have decreased after TG to produce L-lysine, L-ornithine, and L-glutamate. It has been reported that plasma nitric oxide levels increase after gastrectomy (24). This may explain the increase in L-lysine production for the increased energy needs due to malnutrition after gastrectomy.

In the study, L-alanine, L-asparagine, L-histidine, 3-methyl-L-histidine, and L-methionine plasma levels increased significantly in the 3 months after gastrectomy (Table 5). In previous studies, while histidine and alanine did not change considerably in the first year after fundectomy, the plasma concentration of methionine decreased significantly, unlike our study. In another study, like this study, although an increase in alanine and histidine plasma concentrations was observed 1 year after TG, this change was not significant. L-asparagine has a suppressive effect on apoptosis (25). L-alanine and L-methionine improve the immune system (20,26,27). L-histidine is a crucial amino acid that

serves as a substrate for histamine synthesis in specific immune cells, including macrophages, platelets, dendritic cells, and T lymphocytes. 3-methyl-L-histidine is an amino acid that increases muscle and tissue destruction (28,29). Significant changes in these amino acids indicate that TG affects tissue regeneration and the immune system.

5. CONCLUSION

Total gastrectomy statistically changed the concentrations of 20 amino acids and affected 12 metabolic pathways. The effect of total gastrectomy on amino acid profiles may have some consequences for patients with stage II gastric cancer. First, the changes in amino acid profiles may lead to impaired metabolic functioning, which could increase the risk of complications such as malnutrition, anemia, and infection. Second, the changes in amino acid profiles may impair the immune response, which could increase the risk of cancer recurrence. Third, the changes in amino acid profiles may lead to fatigue, weakness, and other symptoms that can negatively impact the quality of life. However, contrary to these expectations, the findings support that total gastrectomy has a positive effect on the immune system, tissue repair, and energy production. It is conceivable that L-arginine supplementation after gastrectomy may prevent the risk of cancer recurrence, the possibility of infection, and malnutrition. By maintaining optimal levels of L-arginine, patients' immune function, tissue healing, and overall well-being can be improved after surgery. All these results need to be supported by new and more comprehensive studies. The study showed that assessing amino acid profiles may help improve metabolic functioning, immune response, and quality of life in these patients. Investigating the effects of different surgical approaches on amino acid metabolism can contribute to optimizing surgical strategies and improving long-term outcomes for patients.

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7. ETHICAL STATEMENT

Ethical approval was obtained from the Non-Interventional Clinical Trials Ethics Committee, Atatürk University, on March 29, 2024, under the permit number B.30.2.ATA.0.01.00/244.

8. DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

9. CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest to disclose.

10. REFERENCES

1. Goh YM, Gillespie C, Couper G, Paterson-Brown S. Quality of life after total and subtotal gastrectomy for gastric carcinoma. Surg [Internet]. 2015 Oct 1;13(5):267–70. Available from: <URL>.

2. Sexton RE, Al Hallak MN, Diab M, Azmi AS. Gastric cancer: A comprehensive review of current and future treatment strategies. Cancer Metastasis Rev [Internet]. 2020 Dec 7;39(4):1179–203. Available from: <URL>.

3. Svedlund J, Sullivan M, Liedman B, Lundell L. Long term consequences of gastrectomy forpatients' quality of life: The impact of reconstructive techniques. Am J Gastroenterol [Internet]. 1999 Feb;94(2):438–45. Available from: <URL>.

4. Schölmerich J. Postgastrectomy syndromes—diagnosis and treatment. Best Pract Res Clin Gastroenterol [Internet]. 2004 Oct 1;18(5):917–33. Available from: <URL>.

5. Poitou Bernert C, Ciangura C, Coupaye M, Czernichow S, Bouillot JL, Basdevant A. Nutritional deficiency after gastric bypass: Diagnosis, prevention and treatment. Diabetes Metab [Internet]. 2007 Feb 1;33(1):13–24. Available from: <URL>.

6. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med [Internet]. 2014 Oct 28;20(10):1193–8. Available from: <URL>.

7. Öztürk Er E, Erarpat S, Bodur S, Günkara ÖT, Özbek B, Bakırdere S. Accurate determination of amino acids by quadruple isotope dilution-reverse phase liquid Chromatography-Tandem mass spectrometry after derivatization with 2-Naphthoyl chloride. J Chromatogr A [Internet]. 2022 Mar 29;1667:462870. Available from: <URL>.

8. Mondanelli G, Iacono A, Carvalho A, Orabona C, Volpi C, Pallotta MT, et al. Amino acid metabolism as drug target in autoimmune diseases. Autoimmun Rev [Internet]. 2019 Apr 1;18(4):334–48. Available from: <URL>.

9. Rondanelli M, Opizzi A, Antoniello N, Boschi F, Iadarola P, Pasini E, et al. Effect of essential amino acid supplementation on quality of life, Amino acid profile and strength in institutionalized elderly patients. Clin Nutr [Internet]. 2011 Oct 1;30(5):571–7. Available from: <URL>.

10. Manig F, Kuhne K, von Neubeck C, Schwarzenbolz U, Yu Z, Kessler BM, et al. The why and how of amino acid analytics in cancer diagnostics and therapy. J Biotechnol [Internet]. 2017 Jan 20;242:30–54. Available from: <URL>.

11. Bi X, Henry CJ. Plasma-free amino acid profiles are predictors of cancer and diabetes development. Nutr Diabetes [Internet]. 2017 Mar 13;7(3):e249. Available from: <URL>.

12. Atila A, Alay H, Yaman ME, Akman TC, Cadirci E, Bayrak B, et al. The serum amino acid profile in COVID-19. Amino Acids [Internet]. 2021 Oct 4;53(10):1569–88. Available from: <URL>.

13. Erawijantari PP, Mizutani S, Shiroma H, Shiba S, Nakajima T, Sakamoto T, et al. Influence of gastrectomy for gastric cancer treatment on faecal microbiome and metabolome profiles. Gut [Internet]. 2020 Aug 1;69(8):1404–15. Available from: <URL>.

14. Tatara MR, Śliwa E, Krupski W, Worzakowska M. 3-Hydroxy-3-methylbutyrate administration diminishes fundectomy-induced osteopenia of the lumbar spine in pigs. Nutrition [Internet]. 2008 Jul 1;24(7–8):753–60. Available from: <URL>.

15. Tatara MR, Krupski W, Szpetnar M, Dąbrowski A, Bury P, Szabelska A, et al. Effects of total gastrectomy on plasma silicon and amino acid concentrations in men. Exp Biol Med [Internet]. 2015 Dec 2;240(12):1557–63. Available from: <URL>.

16. Aquilani R, Maestri R, Boselli M, Achilli MP, Arrigoni N, Bruni M, et al. The relationship between plasma amino acids and circulating albumin and haemoglobin in postabsorptive stroke patients. Clelland JD, editor. PLoS One [Internet]. 2019 Aug 14;14(8):e0219756. Available from: <URL>.

17. Package "ggpubr" type package title "ggplot2" based publication ready plots [Internet]. [cited 2024 May 18]. Available from: <URL>.

18. Gwin JA, Church DD, Wolfe RR, Ferrando AA, Pasiakos SM. Muscle Protein Synthesis and Whole-Body Protein Turnover Responses to Ingesting Essential Amino Acids, Intact Protein, and Protein-Containing Mixed Meals with Considerations for Energy Deficit. Nutrients [Internet]. 2020 Aug 15;12(8):2457. Available from: <URL>.

19. Holeček M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond) [Internet]. 2018 Dec 3;15(1):33. Available from: <URL>.

20. Wu G. Amino acids: Metabolism, functions, and nutrition. Amino Acids [Internet]. 2009 May 20;37(1):1–17. Available from: <URL>.

21. Wu G, Lupton JR, Turner ND, Fang YZ, Yang S. Glutathione metabolism and its implications for health. J Nutr [Internet]. 2004 Mar 1;134(3):489–92. Available from: <URL>.

22. Cheng X, Xu HD, Ran HH, Liang G, Wu FG. Glutathione-depleting nanomedicines for synergistic cancertherapy. ACS Nano [Internet]. 2021 May 25;15(5):8039–68. Available from: <URL>.

23. Kumar M, Ji B, Babaei P, Das P, Lappa D, Ramakrishnan G, et al. Gut microbiota dysbiosis is associated with malnutrition and reduced plasma amino acid levels: Lessons from genome-scale metabolic modeling. Metab Eng [Internet]. 2018 Sep 1;49:128–42. Available from: <URL>.

24. Nagahama S, Korenaga D, Honda M, Inutsuka S, Sugimachi K. Assessment of the intestinal permeability after a gastrectomy and the oral administration of anticancer drugs in rats: Nitric oxide release in response to gut injury. Surgery [Internet]. 2002 Jan 1;131(1):S92–7. Available from: <URL>.

25. Luise D, Chalvon-Demersay T, Correa F, Bosi P, Trevisi P. Review: A systematic review of the effects of functional amino acids on small intestine barrier function and immunity in piglets. animal [Internet]. 2023 Jun 1;17:100771. Available from: <URL>.

26. Shynkevych VI, Kolomiiets S V., Kaidashev IP. Effects of l-arginine and l-ornithine supplementations on the treatment of chronic periodontitis: A preliminary randomized short-term clinical trial. Heliyon [Internet]. 2021 Nov 1;7(11):e08353. Available from: <URL>.

27. O'Riordain M. Effect of glutamine on immune function in the surgical patient. Nutrition [Internet]. 1996 Dec 1;12(12):S82–4. Available from: <URL>.

28. Chinkes DL. Methods for measuring tissue protein breakdown rate in vivo. Curr Opin Clin Nutr Metab Care [Internet]. 2005 Sep;8(5):534–7. Available from: <URL>.

29. Demopoulos C, Antonopoulou S, Theoharides TC. COVID-19, microthromboses, inflammation, and platelet activating factor. BioFactors [Internet]. 2020 Nov 9;46(6):927–33. Available from: <URL>.