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Investigation of the Predictive Value of Amino Acids for Tuberculous Meningitis, Aseptic Meningitis and Bacterial Meningitis

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ABSTRACT

In our study, we aimed to detect amino acid changes, if any, by comparing the levels of amino acids in cerebrospinal fluid (CSF) samples of patients with aseptic, bacterial, and tuberculous meningitis and control groups.
Patients diagnosed with aseptic meningitis (n=41), tuberculous meningitis (n=21), bacterial meningitis (n=41) and a control group consisting of 64 individuals with similar gender and age characteristics were included in the study. 2 mL of cerebrospinal fluid specimens were obtained from all patients and control group and stored at -80 °C until the study day. The amino acid measurements were performed using commercially available liquid chromatography-tandem mass spectrometry method (LC-MS / MS) kits.
When we investigated amino acids levels in all groups, the levels of 1- methyl histidine, alanine, asparagine, histidine, isoleucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline, sarcosine, threonine, tyrosine, valine in the aseptic, tuberculosis and bacterial meningitis groups were statistically higher than those of the control group. The levels of all amino acids except cystine, glutamic acid, homo-citrulline, and taurine in the patients with aseptic meningitis were statistically significantly higher than in the control group. The levels of all amino acids except homocitrulline, and tryptophan were statistically significantly higher in the patient group with tuberculous meningitis than in the control group. The levels of all amino acids except glutamic acid were statistically significantly higher in the patient group with bacterial meningitis than in the control group.

Keywords: LS-MS/MS, CNS INFECTION, AMINO ACIDS

Introduction

Meningitis is a severe infection of the protective membrane covering the brain and spinal cord caused by different pathogens such as viruses, bacteria, fungi or parasites (1). Meningitis can occur at any age, including babies, children, teenagers, and young adults (2). Aseptic meningitis is viral meningitis and has high prevalence than bacterial meningitis (3). Aseptic meningitis is characterized by increased lymphocytosis in the cerebrospinal fluid (CSF). It also includes confirmation of the absence of bacteria by gram staining, culture, and antigen tests in detecting aseptic meningitis (4). Although bacterial meningitis, another type of meningitis, is rarer than viral meningitis in the community, it has a high risk of fatal outcomes and severe neurological sequela, especially when diagnosis and antibiotic treatment are delayed (5-6). Tuberculosis (TB) is an infection caused by *Mycobacterium tuberculosis* and remains a global health problem (7). Tuberculous meningitis (TBM), on the other hand, can manifest as a symptom of TB, but can also occur concurrently with pulmonary or extrapulmonary infection sites (8). TBM symptoms include typical symptoms such as headache, fever, and stiff neck, but

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meningeal symptoms may not be present in the early stages of the disease. Cerebrospinal fluid (CSF) findings are important in TBM (9).

Amino acids are the building blocks of proteins that perform many functions in the body, such as defense, catalysis, and transport. Amino acids are not only the building blocks of proteins but also act as precursors in the synthesis of many molecules. Therefore, amino acid levels in body fluids are associated with many pathological conditions (10-12). Previous studies have examined amino acid levels in patients with meningitis. In a study conducted on patients with TBM and viral meningitis in 1981, the total amino acid level in CSF was determined and investigated by the high-pressure liquid chromatography method, and it was determined that the total amino acid concentration of patients with TBM increased significantly (13). In a study conducted in 1982, total amino acid concentrations were compared in patients with purulent meningitis and aseptic meningitis, and it was reported that total amino acid concentrations in patients with purulent meningitis were significantly higher than those in aseptic meningitis (14). In a study examining various neurochemical markers in CSF, increased aspartic acid, glutamic acid, gamma amino butyric acid, glycine and tryptophan were reported in patients with meningitis. In the same study, increased phenylalanine, arginine, and homocysteine levels were reported in patients with TBM. As it can be understood from these studies, there are prior changes in amino acid concentration in the CSF of the patients with meningitis (15).

In our study, we aimed to detect amino acid changes, if any, by comparing the levels of amino acids in CSF samples of patients with aseptic, bacterial, and TBM and control group using the liquid chromatography-tandem mass spectrometry method (LS-MS/MS), which is one of the most effective methods for amino acid measurement in body fluids. In addition, it was aimed to determine the amino acids in the amino acid profile and determine whether they have a predictive value that may be used in the differentiation of these meningitis types.

Material and Methods

Subjects and Specimens: Ethical approval of the study was obtained with the decision dated 05.10.2017, 10 sessions, number 05. Written and signed informed consent forms were obtained from all the volunteers in the patient groups and control group.

Patients diagnosed with aseptic meningitis (n=41), tuberculous meningitis (n=21), and bacterial meningitis (n=41) followed by the Infectious Diseases and Clinical Microbiology Clinic of Harran University Faculty of Medicine Training and Research Hospital were included in the study. Patients with chronic diseases such as diabetes, cancer, chronic obstructive pulmonary disease, cardiovascular diseases, renal failure, phenylketonuria and malnutrition were not included in the study.

A control group was formed from 64 individuals with similar gender and age characteristics. Patients who followed a special diet, had a chronic disease, and were hospitalized within the last six months were not included in the control group. Individuals in the control group were those who applied with the complaint of headache, had normal lumbar puncture and other examinations, and did not have any clinical and laboratory abnormalities.

2 mL of cerebrospinal fluid specimens were obtained from all the patients and the control group and stored at -80 °C until the study day.

Amino Acids Analysis: CSF samples taken from the patient and control groups were stored at -80 °C until to the measurement day. Amino acid measurements were performed using commercially available LC-MS / MS kits (Jasem LC-MS / MS amino acid analysis kit; SEM Laboratory Equipment Industry Marketing and Tarde, Istanbul, Turkey). Samples were prepared according to the protocol recommended by the manufacturer. Briefly, 50 µL of CSF was transferred to a sample vial, 700 µL of reagent-1 was added with 50 µL of the stable isotope-labeled internal standard mix, and the mixture was vortexed for 5 seconds. Basic organic buffer components prepared in propanol were added to balance the pH and enable the derivative reaction to occur more efficiently. At this stage, the precipitation of the proteins in the sample also occurred. Then, a chloroform/isooctane mixture containing 5% alkyl chloroformate as an active ingredient was added to the mixture and left at room temperature for 3 minutes. At this time, gas emissions will be observed due to carbon dioxide formed during the esterification reaction of alkyl chloroformate and amino acids. Derivatised amino acids are taken to the upper phase containing organic solvents by centrifugation. All samples were prepared according to the above procedures and injected into the LC-MS / MS system. 3 µL of

the sample prepared using the Shimadzu Nexera X2 HPLC system (Shimadzu North America, Columbia, MD, USA) was injected into the Jasem amino acid analytical column, and the instrument was set at 30 °C. The flow rate was adjusted to 0.7 mL/min, and sample analysis was completed in 7.5 minutes. Mass spectrometric detection was performed in positive ionisation mode with a Shimadzu 8040 triple quadrupole mass spectrometer (MS / MS - Shimadzu North America, Columbia, MD, USA). Mass detector parameters were as follows: Gas temperature 150 °C, gas flow 10 L/min, nebuliser pressure 40 psi and + 2,000 volts capillary.

Statistical Analysis: Statistical analyses of data were performed using SPSS version 22.0 (SPSS Inc.) and are presented as mean ± standard deviation (Sd) for results. Normality was assessed with the Shapiro-Wilk test and Histogram. We used Kruskal-Wallis with Temhane's T2 multiple comparative analysis of variance from Post-Hoc Tests for continuous variables.

For multivariate statistical analysis, data of the amino acids in groups were uploaded to the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) server. Principal Component Analysis (PCA) was used to detect individuals' clustering and dissociation tendencies in groups of the amino acids. Also, Partial Least Squares-Discriminant Analysis (PLS-DA) was performed to maximise differences between groups. A Variable Importance in the Projection (VIP) scores were calculated by calculating the amino acids contributing to the discrimination between the groups from the PLS-DA model. To visualise the concentrations of the amino acids we analysed in the groups, heat maps of the groups were created by hierarchical clustering. MetaboAnalyst's biomarker analysis module based on the Receiver Operating Characteristic (ROC) curve was used to identify potential biomarkers for diagnosing meningitis and differentiating different types of meningitis.

Results

There was no statistically significant difference between the patients and the control group in terms of gender and age (Table 1).

When we investigated the amino acids levels in all groups, the levels of 1- methyl histidine, alanine, asparagine, histidine, isoleucine, lysine, methionine, norvaline, ornithine, phenylalanine, proline, sarcosine, threonine, tyrosine, valine in the aseptic, TB and bacterial meningitis groups were statistically higher than those of the control group.

The levels of all amino acids except cystine, glutamic acid, homo-citrulline, and taurine in the patients with aseptic meningitis were statistically significantly higher than in the control group. The levels of all the amino acids except homocitrulline, and tryptophan were statistically significantly higher in the patient group with TBM than in the control group. The levels of all the amino acids except glutamic acid were statistically significantly higher in the patient group with bacterial meningitis than in the control group. All results were summarized in Table 2.

Separation of meningitis patients with PCA and PLS-DA Analysis: PCA analysis was performed to visualise the sample distribution in different types of meningitis groups and the outlines in the groups. Analysis results were given in two dimensions (2D) and three dimensions. PC-1 and PC-2 mean that there is a distinction between groups and account for 46.1% and 8.7% of the variation in the data, respectively. PCA analysis showed partial segregation and aggregation in the patient and control groups (Figure 1). In addition, PLS-DA was performed to maximise the differences between groups, and the results were presented in 2D and 3D (Figure 1). PLS-DA showed better segregation and aggregation between patient and control groups than PCA.

Screening of Differential Amino Acids: VIP plot was generated from PLS-DA models, ranking amino acids for their ability to discriminate meningitis patients from both controls and different types of meningitis patients. Among the 26 amino acids, glutamine (Gln), ornithine (Orn), and Sarcosine (Sar) are the first three amino acids with the highest VIP score (Figure 2). An increase in the VIP score indicates an increased contribution of that amino acid to intergroup separation.

The concentrations of the amino acids in the different types of meningitis groups and the control group were shown by a heat map (Figure 3). The amino acids are represented by rows and groups and individuals by columns. Blue tones indicate that the amino acid decreases in the individual sample, while brown tones show it increases. In addition, as the depth of tones increases, the level of increase and decrease also increases.

Biomarker Candidates for Meningitis and Types of Meningitis: Changes in the

Table 1: Demographic Data of The Patients and Control Groups

Demographic data		Control Group	Bacterial Meningitis	Aseptic Meningitis	Tuberculosis Menengitis	P value
Number of Patients (n)		64	41	41	21	-
Age (min-max)		19-60	21-62	20-59	20-61	0.723
BMI (kg/m2)		23.82±2.26	24.85±2.5	24.37±2.02	24.80±1.93	0.213
Gender	Female	18	15	12	7	0.894
	Male	46	26	29	14	

concentration of the amino acids suggested that it may have a predictive value for meningitis. As a result, a classical univariate ROC curve analysis was performed to identify potential biomarkers. As a result of the analysis, the cut-off value, AUC, sensitivity, specificity, and positive and negative likelihood ratio of the three amino acids with the highest AUC value (area under the ROC curve) in group comparisons were given (Table 3). According to the analysis result, valine, tyrosine, and proline may be potential biomarkers for discrimination between bacterial meningitis and the control group. Ethanolamine, ornithine, and 2-aminoisobutyric acid may have a potential for discrimination between aseptic meningitis and the control group. To discriminate between TBM and the control group, proline, alanine, and valine may be used as a biomarker. Some amino acids may also have the potential to be used as a biomarker in the differentiation of meningitis types. To discriminate between bacterial meningitis and aseptic meningitis, 2-aminoisobutyric acid, proline, and ethanolamine may be used as a biomarker. Serine, homocitrulline and tryptophan may be used discrimination between bacterial meningitis and TBM. In addition, tryptophan, ethanolamine and serine may be used discrimination between aseptic meningitis and TBM (Figure 4).

Discussion

One of the main findings of our study is that the amino acids are generally increased in the patients with meningitis. Similar results were found in previous studies. In the study by Corson et al., the total amino acid concentrations in the CSF samples of eleven viral meningitis and four TBM patients were determined, and they found that the total amino acid concentrations of the patients with TBM were higher than those of the patients with viral meningitis (13). Guerra-Romero et al., on the other hand, determined the levels of some amino acids in the CSF of the rabbits with pneumococcal meningitis and reported that glutamate, aspartate, glycine, taurine, and alanine amino acids were significantly increased in the infected rabbits (16). Qureshi et al. reported that aspartic acid, glutamic acid GABA, glycine, and tryptophan were increased both in the patients with aseptic meningitis and in the patients with TBM. The same study reported that phenylalanine increased only in the patients with TBM (15). In these studies, the sample size is low, and the methods used for amino acid measurements are high-pressure liquid chromatography and fluorometric methods. In our study, the sample size was relatively high, and our method was LC-MS/MS. LC-MS/MS is one of the most sensitive measurement methods available for amino acid measurement. Although the sample sizes and methods are different, the results of our study support previous studies. In a recent study by Mason et al., the amino acid profiles of 33 pediatric patients with TBM were compared with a healthy control group. The gas chromatography-mass spectrometry method, which is a very sensitive method for amino acid analysis, was used in this study. As a result of their study reported that five amino acids, namely alanine, asparagine, glycine, lysine, and proline, were significantly elevated in the TBM cases (17). The results of our study also support the results of previous studies.

We investigated the levels of the amino acids between meningitis groups too. The levels of alanine, asparagine, homocitrulline, proline, lysine, norvaline, and phenylalanine in the bacterial meningitis group were statistically significantly higher than those of the aseptic meningitis group. However, the level of gamma-aminobutyric acid in the bacterial meningitis group was significantly lower than those of the aseptic meningitis group. Haraldur Briem determined the total amino acid levels in the CSF of the patients with bacterial meningitis, aseptic meningitis, and the healthy control group and reported the total amino acid level of patients with bacterial meningitis was higher than patients with aseptic meningitis (18). The increase in the total amino acid concentration

Table 2: The comparison of All The Amino Acids Between The Groups

Amino acids	Group Mean±Sd (µmol/L)				Post-Hoc Tests (Tamhane's)
	Control Group (n:64)	Aseptic Menengitis (n:41)	Tuberculous menengitis (n:21)	Bacterial Menengitis (n:41)	
1- Methyl histidine (1-Mhs)	0.49±0.18	1.39±0.96	1.21±0.89	1.45±1.133	*,**,***
2-Aminobutyric acid (2-AIB)	0.82±0.59	2.68±1.13	1.74±1.33	1.71±1.39	*,**,***,+
Alanine (Ala)	49.8±24.9	132.6±78.6	205.1±153.7	210.9±158.8	*,**,***,+
Arginine (Arg)	23.1±6.81	48.7±27.3	25.69±15.9	44.9±23.3	*,**, ++,+++
Asparagine (Asn)	5.06±2.82	12.5±11.7	23.5±23.2	23.2±20.2	*,**,***,+
Cystine (Cys)	8.96±3.79	12.6±8.59	12.7±8.79	12.2±5.42	**
Ethanolamine (Eta)	9,443±3,409	31,693±15,149	14,701±10,072	21,157±11,061	*,***, +,++
Glutamic Acid (Glu)	51.1±24.1	34.7±20.9	56.9±42.5	40.1±29.1	*
Glutamine (Gln)	421.5±119.4	755.3±372.6	429.4±192.7	729.7±355.2	*,**, ++,+++
Histidine (His)	8.29±4.19	14.2±9.78	18.2±13.21	21.6±15.2	*,**,***
Homocitrulline (HCit)	1.52±0.75	1.64±0.79	1.28±0.75	2.49±1.43	**,+,+++
Isoleucine (Ile)	3.13±1.48	10.597±7.852	12.955±13.12	15.547±9.534	*,**,***
Lysine (Lys)	33.1±13.1	72.1±33.7	79.1±64.2	108.3±59.6	*,**,***,+
Methionine (Met)	2.52±1.73	7.45±6.27	10.4±8.54	9.18±6.17	*,**,***
Norvaline (Nva)	0.421±0.19	0.922±0.704	1.77±1.58	1.61±1.25	*,**,***,+
Ornithine (Orn)	22.1±6.96	85.3±32.2	43.7±30.2	78.2±38.1	*,**,***, ++, +++
Phenylalanine (Phe)	13.2±4.77	31.5±18.6	43.7±44.5	53.8±43.2	*,**,***,+
Proline (Pro)	2.38±1.74	17.2±13.3	46.3±43.1	34.916±24.233	*,**,***, +, ++
Sarcosine (Sar)	34.3±14.2	99.8±56.9	94.7±75.94	115.683±75.842	*,**,***
Serine (Ser)	25.6±10.6	53.2±23.6	27.6±12.2	69.7±44.3	*, **, ++, +++
Taurine (Tau)	32.6±19.8	39.2±21.2	43.1±30.8	56.2±38.8	**
Threonine (Thr)	31.4±14.2	68.9±37.1	78.1±61.8	72.2±44.1	*,**,***
Tryptophan (Trp)	1.94±0.88	5.17±3.67	1.42±1.24	6.74±4.67	*,**, ++, +++
Tyrosine (Tyr)	10.5±3.93	26.9±14.8	29.6±18.9	33.9±19.3	*,**,***
Valine (Val)	16.8±6.85	65.83±52.4	86.7±74.5	96.6±72.2	*,**,***
Gamma aminobuteyric acid (GABA)	0.41±0.21	2.31±1.86	0.65±0.52	1.69±1.56	*,**, ++,+++

Aseptic Meningitis – Control: *, Bacterial Meningitis - Control; **, Tuberculous Meningitis – Control:***, Aseptic Meningitis– Bacterial Meningitis; +, Aseptic Meningitis - Tuberculous Meningitis; ++,Bacterial Meningitis - Tuberculous Meningitis;+++

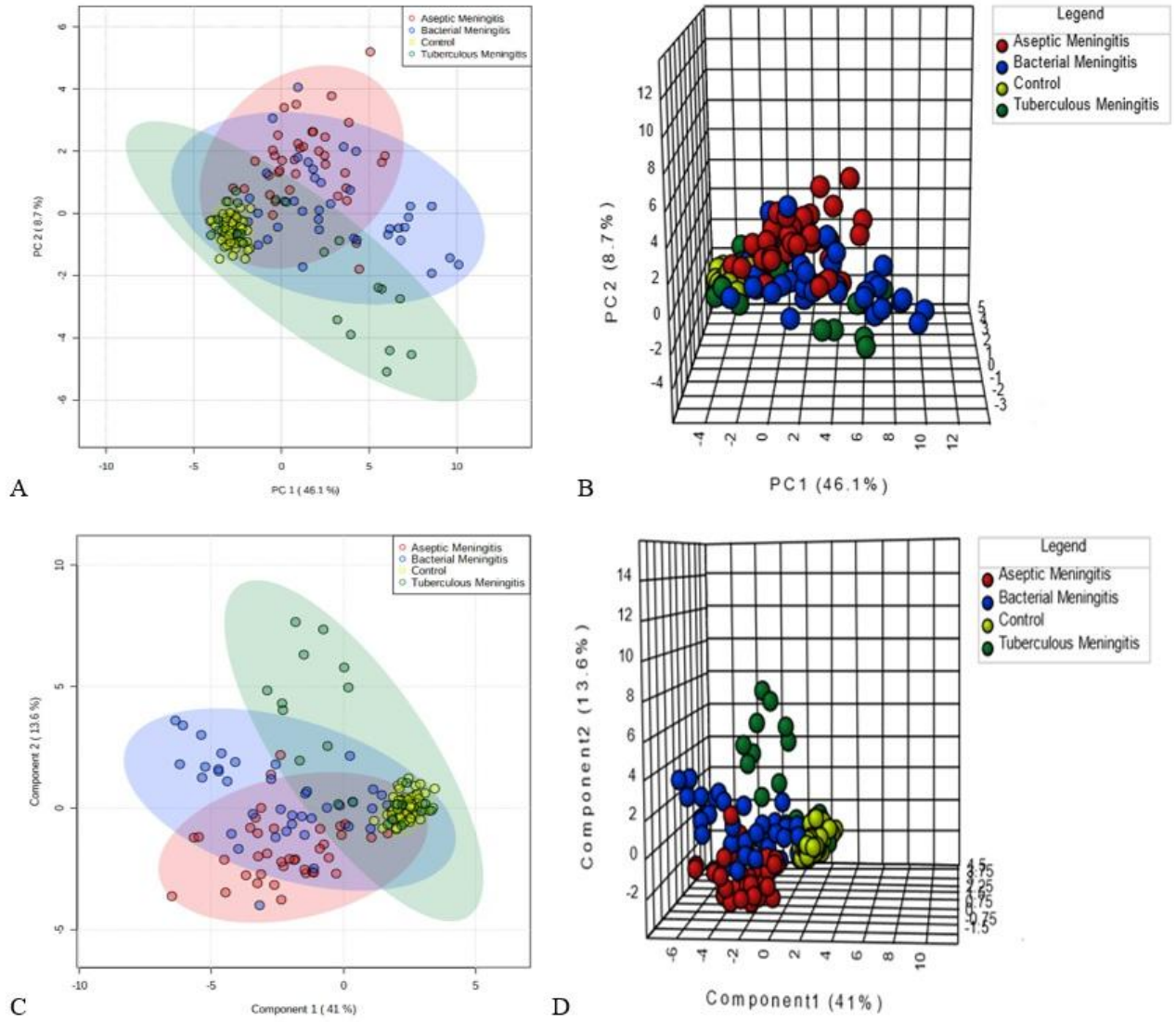

Fig. 1. The differentiation of different types of the meningitis patients and control groups according to the amino acids profile. 2D (A) and 3D (B) score graphs of PCA. PLS-DA's 2D (C) and 3D (D) score graphs

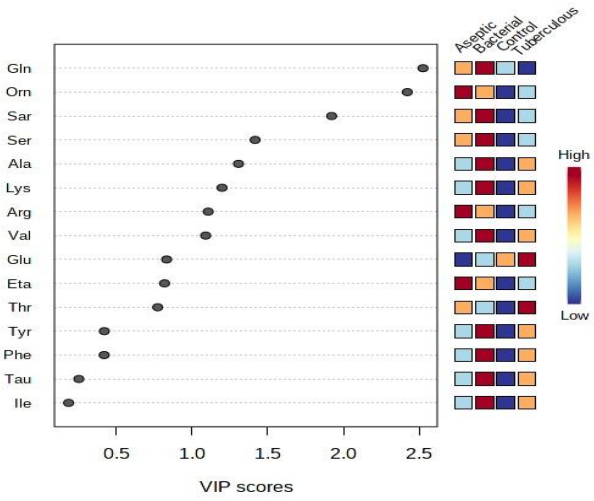

Fig.2. VIP table: Amino Acids In Ascending Order of Importance

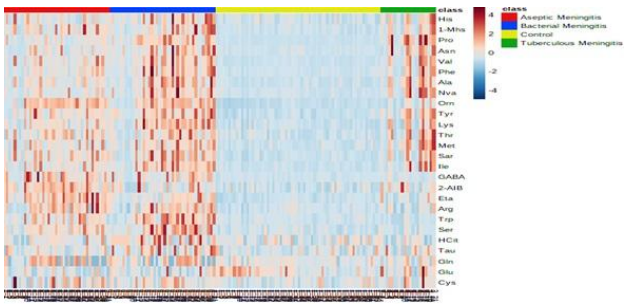

Fig. 3. Heat Map Showing The Concentrations of Amino Acids In Groups

may be the result of the amino acids being exposed as a result of protein degradation by the bacteria causing meningitis.

Increased lactate level has been reported in bacterial meningitis (19). On the other hand, alanine amino acid is closely related to lactate and shuttling systems (20). In our study, the alanine

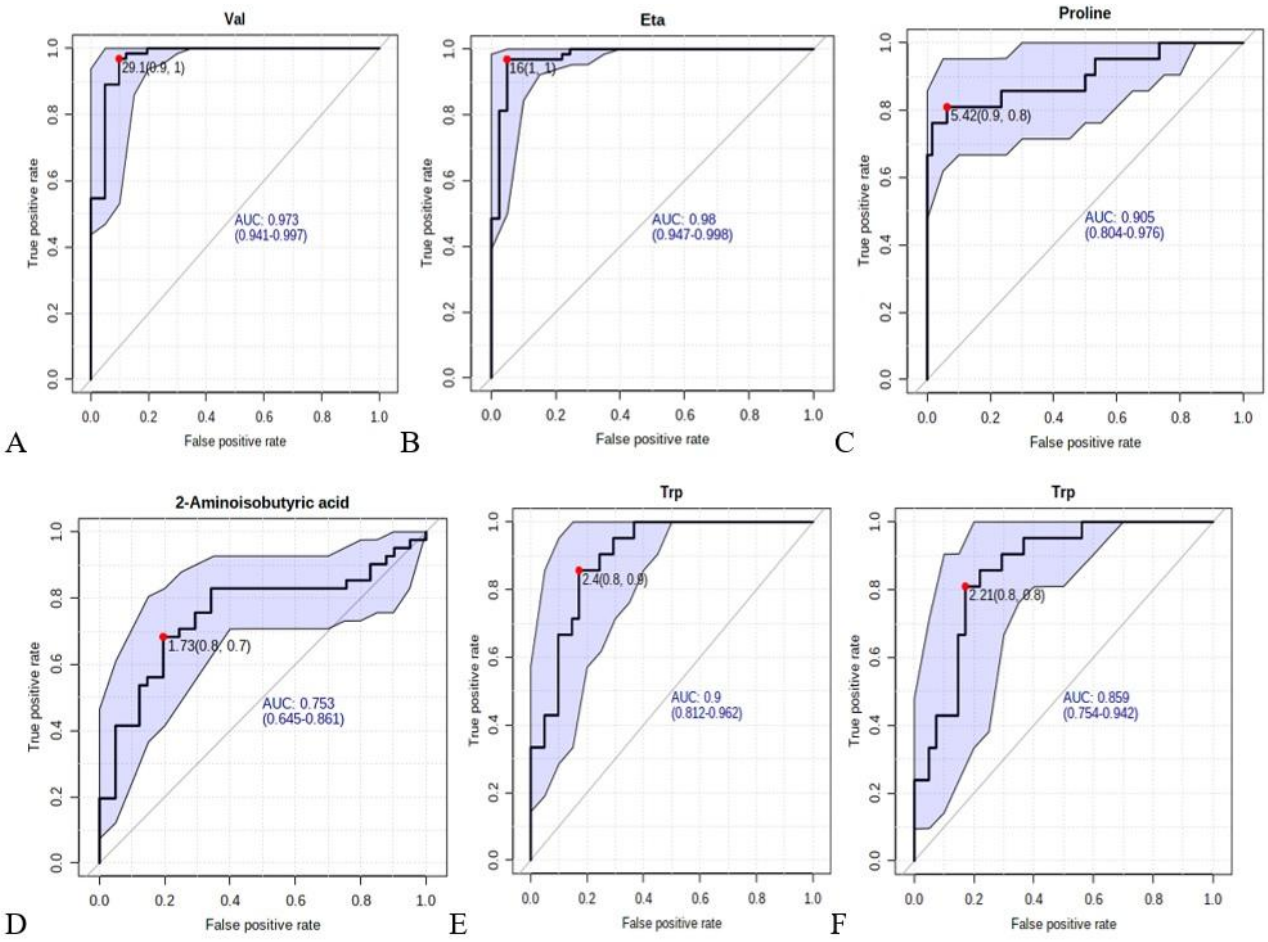

Fig. 4. ROC curve analysis results of the amino acids with the highest AUC value in the comparison between groups. *A; Bacterial Meningitis-Control, B; Aseptic Meningitis-Control, C; Tuberculous Meningitis-Control, D; Bacterial Meningitis-Aseptic Meningitis, E; Bacterial Meningitis-Tuberculous Meningitis, F; Aseptic Meningitis-Tuberculous Meningitis*

level was higher in all types of meningitis than in the control group, and the highest alanine level was detected in bacterial meningitis. This may be because the bacteria causing meningitis produce lactate as a result of anaerobic glycolysis. The high alanine concentration in CSF may be because it is a glucogenic amino acid. Another important amino acid is lysine. It has been reported that the levels of lysine increase in the CSF of the TBM patients are associated with mental retardation and other motor neuron diseases (21). In our study, lysine was elevated in all meningitis patients, and the highest lysine levels were detected in bacterial meningitis. In previous studies, it has been reported that nitrites are significantly increased in the patients with meningitis (15). Therefore, increased nitrogen excretion is expected in these patients. It is possible to expect that the levels of arginine, glutamine, and glutamic acid, which have an important role in nitrogen excretion, will change in these patients. The levels of these amino acids increased significantly in our study.

The increase in amino acid concentration in patients with meningitis and the higher increase in some amino acids in different types of meningitis suggests that these amino acids may have predictive value in differentiating meningitis types. In our study, the predictive values of these amino acids were also examined. There is no study in the literature examining the predictive values of amino acids for the differentiation of meningitis patients. Haraldur Briem examined the predictive value of the total amino acid level for the differentiation of bacterial meningitis and aseptic meningitis and emphasized that the total amino acid concentration had 91% sensitivity and 97% specificity (18). Mason et al. examined whether amino acids could be used to differentiate TBM from healthy control, and they performed PCA and PLS-DA analysis in their study. At the end of PCA and PLS-DA analyses, they reported that amino acids could be used to differentiate the two groups. They reported that 11 amino acids (alanine, alpha-aminobutyric acid, asparagine, glycine, hydroxylysine, lysine, ornithine, proline,

serine, threonine, and valine) were important differential metabolites, especially in the PLS-DA correlation load analysis (17). Similar results were obtained in our study. 2-aminoisobutyric acid, proline, and ethanolamine may have the potential to be useful in differentiating between bacterial meningitis and aseptic meningitis. Tryptophan, serine and homocitrulline may have the potential to be useful in distinguishing between bacterial meningitis and TBM. And the high predictive values of tryptophan, ethanolamine, and serine are remarkable in the differentiation of aseptic meningitis and TBM. It is noteworthy that proline, valine, and serine amino acids have distinctive features in our study and their study.

In this study, the amino acid levels were determined by highly sensitive LC-MS/MS method in CSF samples of patients diagnosed with aseptic meningitis, TBM, and bacterial meningitis. At the end of the study, it was determined that the amino acid levels were impaired in these patients and that some amino acids showed promise as a biomarker in the differentiation of meningitis types.

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Ethical Approval: Ethical approval of the study was obtained with the decision dated 05.10.2017, 10 sessions, number 05.

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