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## Original Research Article

# Predictive Ability of Serum Amino Acid Levels to Differentiate Fibromyalgia Patients from Healthy Subjects



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## Abstract

### Background

Fibromyalgia is a complex illness **AQ1** to diagnose and treat.

### Objectives

To evaluate a broad range of circulating free amino acid (AA) levels in fibromyalgia patients as **AQ2** well as the ability of the AAs to

differentiate fibromyalgia patients from healthy subjects.

## Design

We carried out a case-control study to evaluate AA levels in 62 patients with fibromyalgia **AQ3** and 78 healthy subjects. This study adheres to the STROBE guidelines.

## Methods

AAs content was assayed by HPLC in serum samples. The predictive value of AA levels in fibromyalgia **AQ4** was determined by receiver operating characteristic (ROC) curve and forward binary logistic regression analyses.

## Results

Fibromyalgia patients showed higher serum levels of aspartic acid, glutamic acid, aminoadipic acid, asparagine, histidine, 3-methyl-histidine, 5-methyl-histidine, glycine, threonine, taurine, tyrosine, valine, methionine, isoleucine, phenylalanine, leucine, ornithine, lysine, branched chain AAs (BCAAs), large neutral AAs, essential AAs (EAAs), non-essential AAs (NEAAs), basic AAs, EAAs/NEAAs ratio, phenylalanine/tyrosine ratio, and global arginine bioavailability ratio than the controls. Serum alanine levels were lower in patients than in controls. According to ROC analysis, most of these AAs may be good markers for differentiating individuals with fibromyalgia from healthy subjects. Results of logistic regression showed that the combination of glutamic acid, histidine, and alanine had the greatest predictive ability to diagnose fibromyalgia.

## Conclusions

Our results show an imbalance in serum levels of most AAs in patients with fibromyalgia, which suggest a metabolic disturbance. The determination of serum levels of these AAs may aid in the diagnosis of fibromyalgia, in combination with clinical data of the patient.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s40291-023-00677-8.

Overview

***Key Points***

| |
|---|
| We found altered serum levels of a broad range of amino acids in fibromyalgia patients. |
| Serum levels of many amino acids may be reliable parameters to facilitate the diagnosis of fibromyalgia. |
| Altered amino acids levels in fibromyalgia patients suggest a metabolic disturbance. |

# 1. Introduction

Fibromyalgia (FM) is a heterogeneous disorder characterized by chronic widespread pain. Other common symptoms include fatigue, sleep disturbances, and mood disorders. FM shows different phenotypes and disease expression in patients and even in one patient over time [1]. It is estimated to affect 2–8% of the worldwide population, with a higher prevalence among women [2]. FM is one of the most common causes of chronic widespread pain [3]. However, its physiopathology is still not fully understood, which currently makes FM a challenge in terms of diagnosis and treatment. FM can take up to 5 years to be correctly diagnosed [4] and, given the limited efficacy and adverse effects of the drugs used to treat this syndrome, the treatment of choice is multidimensional, consisting of pharmacological therapy, cognitive behavioral therapy, and others such as exercise or dietary interventions [5]. The main physiopathological hypothesis of FM includes the dysfunction of central and peripheral pain modulation systems [6], which implies altered levels of neurotransmitters such as catecholamines, serotonin [7], and several amino acids (AAs) such as glutamic acid or γ-aminobutyric acid (GABA) [6].

In addition to their main role for the synthesis of tissue proteins, AAs play important roles in neurotransmission, regulating gene expression, cell signalling, blood flow, nutrient transport and metabolism, anti-oxidative responses, hormone secretion and immune response [8]. Several AAs have been reported to play an important role in the aetiology of pain, mainly glutamic acid and GABA [9, 10]. A proposed mechanism of chronic pain is dysregulation between the main inhibitory (GABA) and excitatory (glutamic acid) neurotransmitters of the central nervous system (CNS) [10]. Reduced inhibitory neurotransmission may lead to chronic pain states, and both GABA-A and GABA-B receptors seem to be involved in the pathophysiology of chronic pain [11]. Several studies in animal models of chronic pain have shown that the development of allodynia and/or hyperalgesia are related to increased activity of glutamic acid receptors [9]. Moreover, AAs are key substrates for the formation of many important substances such as nitric oxide, catecholamines, serotonin, glutathione, creatine, thyroid hormones, melanin, melatonin, and heme [8]. Therefore, any alteration in their physiological levels could lead to a pathological state. Levels of free AAs are highly regulated through a continuous cycle of protein formation/degradation to maintain a physiological protein balance [12]. Free AAs are a minor fraction of the total AA concentration in the body. The levels of circulating free AAs reflect the net effect of all factors that affect the total flow of AAs in the body [13]. The determination of circulating free AA profile is promising as a biomarker and predictor of several pathologies including cancer and diabetes [14]. Circulating AA levels in patients diagnosed with FM have been previously examined in a few studies, but results are contradictory [13, 15, 16, 17, 18, 19, 20, 21].

Based on this background, it is essential to identify biomarkers that facilitate the diagnosis and treatment of FM, and that may also contribute to understanding of the physiopathology of this complex syndrome. Our aim was to evaluate a broad range of serum free AA

levels in FM patients and healthy controls. The ability of the AAs to differentiate individuals with FM from healthy subjects was also examined. Finally, correlations were assessed to determine whether serum free AA levels influence FM-related clinical parameters.

# 2. Material and Methods

## 2.1. Type of Study

This case-control study was carried out in accordance with the Declaration of Helsinki of the World Medical Association (WMA). The study was approved by the Ethics Committee of the University of Granada (Spain) (approval number: 1797-N-17). This study adheres to the STROBE guidelines (Online Supplementary Material (OSM) File 1).

## 2.2. Determination of Sample Size

Sample size was determined using Ene 3.0 software (GlaxoSmithKline, Rockbille, MA, USA). To achieve a power ($1 - \beta$ error) of 0.80, taking into account a significance level (α error) of 0.05 and based on the results of plasma valine levels in FM patients [ 15 ], it is necessary to include at least 16 subjects per experimental group in the study.

## 2.3. Participants

Sixty-two subjects with FM and 78 healthy subjects were enrolled in this study. Patients with FM were contacted via both AGRAFIM (Association of Fibromyalgia of Granada, Spain) and AFIXA (Association of Fibromyalgia of Jaén, Spain), and healthy controls were recruited from friends and relatives of the patients, from friends and colleagues of the healthy subjects, and from staff of the Faculty of Health Sciences (University of Granada, Spain). All the participants were over 18 years old. The patients had been previously diagnosed with FM by a professional rheumatologist of the Public Health System of Andalucía (Spain) and met the 2010 American College of Rheumatology (ACR) criteria for FM.

The exclusion criteria, for both patients and controls, were presence of any chronic disease (e.g., diabetes mellitus, cancer, hypertension, or cardiac, digestive, renal, pulmonary, hepatic or rheumatic disease), grade II obesity (body mass index (BMI) ≥ 35 kg/m$^2$), severe physical disability, psychiatric illness, previous history of surgery, pregnancy, or lactation, and treatment with vasoactive drugs, anticoagulants, corticosteroids, estrogens, or agonist/antagonist opioid receptors (morphine, tramadol, oxycodone, naltrexone, etc.). No participant was taking vitamin supplements.

## 2.4. Demographic and Clinical Data of Participants

Each participant provided demographic and clinical data by means of interviews and questionnaires carried out by the same specialist in our laboratories of the Faculty of Health Sciences of the Universities of Granada and Jaén between January and April 2022. All the questionnaires were completed by FM patients and by controls with the exception of the Revised Fibromyalgia Impact Questionnaire (FIQ-R), which was completed only by the patients. The severity of FM was assessed by the Spanish version of the FIQ-R, which has an internal consistency (Cronbach's alpha) of 0.91 [ 22 ]. The questionnaire consists of 21 items and the total score ranges from 0 to 100. Self-reported musculoskeletal pain was measured according to a visual analogue scale (VAS), with a score range from 0 to 100. This scale has shown a high sensibility and specificity in the FM population [ 23 ]. Fatigue was evaluated by the Spanish version of the Multidimensional Fatigue Inventory (MFI) [ 24 ], which consists of 20 items and has a potential score range from 20 to 100. The Spanish version of the MFI has shown high internal consistency, with a Cronbach's alpha of 0.93 [ 25 ]. The anxiety-related symptoms were evaluated using a Spanish version of the Beck Anxiety Inventory (BAI) [ 26 ], which has a Cronbach's alpha of 0.93 [ 27 ]. The BAI consists of 21 items and the score ranges from 0 to 63. Sleep quality was evaluated using the Spanish version of the Pittsburgh Sleep Quality Index (PSQI), which has an internal consistency (Cronbach's alpha) of 0.805 [ 28 ]. The questionnaire consists of 24 items and the total score varies in a range of 0–21. For all these questionnaires, higher values reflect worse symptomatology.

## 2.5. Blood Collection

Participants were instructed not to make physical efforts on the day of blood collection. To avoid potential circadian variations in the levels of the AAs, the blood samples were collected at the same time of day. Venous blood was taken after an overnight fast in the morning between 8 and 9 am. Samples were taken by the same practitioner from the antecubital vein into an anticoagulant-free tube after the participants completed the questionnaires mentioned above. Blood was allowed to clot for 30 min at room temperature. The tube was then centrifuged at 3500 rpm for 5 min at 4 °C to obtain serum samples.

## 2.6. Serum Amino Acid (AA) Level Determination

AAs content was assayed in May 2022 by high performance liquid chromatography (HPLC) coupled to a fluorescence detection system as we previously described [ 12 ]. Briefly, serum samples were deproteinated by ultrafiltration and the deproteinated serum was precolumn derivatized with OPA reagent (*o*-phthaldialdehyde in borate buffer pH 9.5 containing 3-mercaptopropionic acid) and injected through a refrigerated Perkin-Elmer Series 200 automatic sample injector into a 150 × 3.9 mm Waters Resolve 5µ C-18 column. The fluorescence detector (Perkin-Elmer Series 200a) was set at an excitation wavelength of 340 nm and an emission wavelength of 420 nm. Data was processed with the TotalChrom WorkStation version 6.3.1 software from Perkin-Elmer. Concentrations were expressed as picomoles of AA per microliter (pmoles/µL).

## 2.7. Calculations

Table 2 showsthe abbreviations for each AA measured in the present study. Val, Leu, and Ile were summed as branched-chain AAs (BCAAs). BCAAs plus the aromatic AAs Tyr and Phe were summed as large neutral AAs (LNAAs). BCAAs along with Phe, Met, Thr,

Lys, and His were summed as essential AAs (EAAs). Ala, Gly, Ser, Gln, Arg, Tau, Glu, Asn, Asp, Orn and Cit were summed as nonessential AAs (NEAAs). Arg, Orn, Lys and His were summed as basic AAs (BAAs).

The GABR ratio (global arginine bioavailability ratio) was calculated as the ratio between arginine levels and the sum of the levels of ornithine and citrulline [ 29 ]. The TSM ratio was calculated as the ratio between 100 times the taurine level and the product of the levels of serine and methionine. It is an indicator of the AAs involved in transmethylation processes, which are altered in diseases such as Alzheimer's disease [ 30 ] and psychoses [ 31 ].

### 2.8. Statistical Analysis

The statistical analysis of the data was performed using the statistical package IBM SPSS Statistics 24 for Windows (SPSS Inc, Chicago, IL, USA). Data for continuous variables were expressed as mean ± standard deviation (SD) and for categorical variables as frequency (%). Categorical variables were analyzed by the Chi-square test ($\chi^2$). For continuous variables, we performed the Kolmogorov–Smirnov test ($\alpha$-value = 0.05) and Levenne test ($\alpha$-value = 0.05) to test normality and homoscedasticity, respectively. Data that followed a normal distribution and the principle of homoscedasticity of variances were tested using an unpaired Student's *t* test to compare differences between means. To establish the degree of statistical significance in data that did not follow a normal distribution or the principle of homoscedasticity we applied the Mann–Whitney *U* test (all the variables except for BMI). To analyze the effects of medication on serum levels of AAs, a Kruskal–Wallis non-parametric hypothesis test analysis was performed after categorizing FM patients according to the medication they were taking into four groups: G1: no medication; G2: analgesics or anti-inflammatories drugs, G3: anxiolytics, antidepressants, or muscle relaxants, G4: any combination of the above (analgesics, anti-inflammatories, anxiolytics, antidepressants, or muscle relaxants).

To assess the relationships between variables, we used the Spearman's correlation coefficient as a nonparametric measure of rank correlation. We performed the Wald test for forward binary logistic regression to identify the combination of variables that has the greatest predictive ability in FM. For the receiver operating characteristic (ROC) analysis, we used MedCalc Statistical Software to calculate area under the curve (AUC), cutoff point, positive and negative predictive values (PV+ and PV−, respectively), sensitivity and specificity. The Youden index was used to determine the cutoff point of the variable in the ROC curve [ 32 ].

We set the level of statistical significance at $p < 0.05$.

## 3. Results

### 3.1. Participants

The demographic and clinical characteristics of the participants are shown in Table 1. A total of 91 patients diagnosed with FM and 89 healthy controls were interested in participating in the present study, but 29 patients and 11 controls were excluded on the basis of meeting any exclusion criterion. Finally, 62 patients with FM (59 women and three men) and 78 healthy subjects (70 women and eight men) were included in the study. There were no statistically significant differences between the study groups by age or BMI. The VAS, MFI, BAI, and PSQI scores of FM patients were significantly higher than those of the healthy controls (all $p < 0.001$). There were statistically significant differences for drug consumption between patients with FM and controls ($p < 0.001$). However, the results of the Kruskal–Wallis analysis to investigate the effects of medication on serum AA levels did not show statistically significant differences for the median values of any AA ($0.079 \geq p \leq 0.997$) among the groups of FM patients who were not taking medication ($n = 9$), who were consuming analgesics or anti-inflammatories ($n = 11$), who were taking anxiolytics or antidepressants or muscle relaxants ($n = 9$), and who were consuming any combination of the above medications ($n = 33$).

**Table 1**

Demographic and clinical data of patients with fibromyalgia (FM) and healthy controls

| | Controls ($n = 78$) | Patients with FM ($n = 62$) | *p* value |
|---|---|---|---|
| Age (years) | 55.38 ± 11.60 | 54.62 ± 7.08 | 0.648 |
| Sex (male/female) | 8 (10.3)/70 (89.7) | 3 (4.8)/59 (95.2) | – |
| BMI (kg/m$^2$) | 26.11 ± 4.29 | 27.29 ± 4.26 | 0.114 |
| FIQ-R | – | 70.03 ± 16.77 | – |
| VAS | 18.59 ± 23.25 | 72.46 ± 17.66 | < 0.001 |
| MFI | 45.54 ± 14.47 | 80.28 ± 9.87 | < 0.001 |
| BAI | 10.18 ± 9.74 | 32.85 ± 9.71 | < 0.001 |
| PSQI | 6.93 ± 4.04 | 15.44 ± 3.68 | < 0.001 |
| *Drugs* | | | |
| Analgesics or anti-inflammatories | 11 (14.1) | 11 (17.8) | < 0.001 |
| Anxiolytics or antidepressants or muscle relaxants | 6 (7.7) | 9 (14.5) | |
| Any combination of the above medications | 0 (0) | 33 (53.2) | |
| No medication | 61 (78.2) | 9 (14.5) | |
| Data were expressed as mean ± standard deviation (SD) or as n and frequency (%) in brackets | | | |
| *BMI* body mass index, *FIQ-R* Revised Fibromyalgia Impact Questionnaire, *VAS* visual analogue scale, *MFI* Multidimensional Fatigue Inventory, *BAI* Beck Anxiety Inventory, *PSQI* Pittsburgh Sleep Quality Index | | | |

## 3.2. Serum AA Levels

There were statistically significant differences in most of the AAs measured between patients with FM and healthy controls (Table 2). Patients with FM showed higher serum levels of aspartic acid, glutamic acid, aminoadipic acid, asparagine, histidine, 3-methyl-histidine, 5-methyl-histidine, glycine, threonine, taurine, tyrosine, valine, methionine, isoleucine, phenylalanine, leucine, ornithine, and lysine than the healthy subjects (all $p < 0.05$). Serum alanine levels were lower in patients with FM than in controls ($p < 0.001$). Differences between groups approached statistical significance for tryptophan and arginine levels ($p = 0.053$ and 0.054, respectively).

**Table 2**

Serum free amino acids in patients with fibromyalgia (FM) and healthy controls

| | Abbreviation | Controls ($n = 78$) | Patients with FM ($n = 62$) | $p$ value |
|---|---|---|---|---|
| Aspartic acid | Asp | 21.55 ± 3.07 | 32.50 ± 15.39 | < 0.001 |
| Glutamic acid | Glu | 37.20 ± 3.41 | 71.67 ± 32.08 | < 0.001 |
| Aminoadipic acid | Aad | 0.65 ± 0.35 | 1.23 ± 1.05 | 0.030 |
| Asparagine | Asn | 39.39 ± 2.42 | 52.16 ± 24.43 | 0.005 |
| Serine | Ser | 117.99 ± 8.13 | 137.13 ± 67.15 | 0.997 |
| Glutamine | Gln | 132.98 ± 5.80 | 154.77 ± 45.30 | 0.310 |
| Histidine | His | 28.72 ± 0.99 | 47.59 ± 24.14 | < 0.001 |
| 3-Methyl-histidine | 3-Met-His | 82.72 ± 13.36 | 137.65 ± 102.86 | 0.011 |
| 5-Methyl-histidine | 5-Met-His | 2.47 ± 0.99 | 3.71 ± 2.66 | 0.048 |
| Glycine | Gly | 53.01 ± 12.57 | 77.77 ± 37.61 | < 0.001 |
| Threonine | Thr | 92.76 ± 4.86 | 157.30 ± 93.79 | < 0.001 |
| Citrulline | Cit | 24.79 ± 5.11 | 26.60 ± 12.03 | 0.840 |
| Arginine | Arg | 61.65 ± 5.58 | 73.91 ± 26.66 | 0.054 |
| Alanine | Ala | 320.59 ± 18.34 | 234.85 ± 82.84 | < 0.001 |
| Taurine | Tau | 62.10 ± 12.49 | 82.97 ± 35.05 | < 0.001 |
| Tyrosine | Tyr | 56.46 ± 7.68 | 73.37 ± 33.79 | 0.018 |
| Carnosine | Car | 162.75 ± 35.47 | 175.86 ± 104.64 | 0.943 |
| Valine | Val | 188.17 ± 5.44 | 291.71 ± 162.43 | 0.002 |
| Methionine | Met | 21.08 ± 2.04 | 30.55 ± 16.86 | 0.030 |
| γ-Aminobutyric acid | GABA | 215.84 ± 38.63 | 209.71 ± 65.84 | 0.330 |
| Isoleucine | Ile | 47.84 ± 8.55 | 72.61 ± 38.95 | 0.001 |
| Phenylalanine | Phe | 55.18 ± 7.56 | 79.72 ± 31.38 | < 0.001 |
| Tryptophan | Trp | 71.97 ± 15.70 | 106.33 ± 76.70 | 0.053 |
| Leucine | Leu | 117.34 ± 23.43 | 190.55 ± 94.91 | < 0.001 |
| Ornithine | Orn | 362.57 ± 67.75 | 504.33 ± 284.57 | 0.003 |
| Lysine | Lys | 114.28±6.46 | 160.65 ± 88.27 | 0.046 |

Data are expressed as mean ± standard deviation (SD). Concentrations were expressed as picomoles of amino acid per microliter (pmoles/μL)

FM patients had higher values of BCAAs, LNAAs, EAAs, NEAAs, and BAAs than the controls (all $p < 0.001$; Table 3). EAAs/NEAAs ratio, Phe/Tyr ratio, and GABR ratio were higher in patients with FM in comparison to healthy subjects (all $p < 0.05$; Table 3).

**Table 3**

Groupings and ratios of amino acids in patients with fibromyalgia (FM) and healthy controls

| | Controls ($n = 78$) | Patients with FM ($n = 62$) | $p$ value |
|---|---|---|---|
| BCAAs | 353.35 ± 28.07 | 554.88 ± 251.40 | < 0.001 |
| LNAAs | 465.01 ± 31.58 | 707.97 ± 291.52 | < 0.001 |
| EAAs | 665.39 ± 29.18 | 1030.70 ± 424.87 | < 0.001 |
| NEAAs | 1233.88 ± 77.01 | 1448.71 ± 398.94 | < 0.001 |
| BAAs | 567.24 ± 68.55 | 786.49 ± 350.79 | < 0.001 |
| EAAs/NEAAs ratio | 0.54 ± 0.04 | 0.71 ± 0.25 | < 0.001 |
| Phe/Tyr ratio | 0.99 ± 0.18 | 1.24 ± 0.61 | 0.018 |

Data are expressed as mean ± standard deviation (SD). Concentrations of BCAAs, LNAAs, EAAs, NEAAs, and BAAs were expressed as picomoles of amino acid per microliter (pmoles/μL)

*BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine, *GABR ratio* global arginine bioavailability ratio

| | Controls ($n$ = 78) | Patients with FM ($n$ = 62) | $p$ value |
|---|---|---|---|
| TSM ratio | 2.54 ± 0.63 | 3.17 ± 2.77 | 0.725 |
| GABR ratio | 0.164 ± 0.03 | 0.166 ± 0.08 | 0.048 |
| Data are expressed as mean ± standard deviation (SD). Concentrations of BCAAs, LNAAs, EAAs, NEAAs, and BAAs were expressed as picomoles of amino acid per microliter (pmoles/μL) | | | |
| *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine, *GABR ratio* global arginine bioavailability ratio | | | |

## 3.3. Correlations Between Variables in Patients with Fibromyalgia (FM)

Table 4 shows correlations between serum AA levels and clinical manifestations in FM patients. The BAI score was positively correlated with threonine, 5-methyl-histidine, isoleucine, leucine, BCAAs, EAAs, and EAAs/NEAAs ratio in patients with FM (all $p < 0.05$). Several other correlations approached statistical significance ($p \leq 0.1$). The BAI score was positively correlated with asparagine, taurine, and LNAAs in FM patients. The MFI score was positively correlated with serine y GABR ratio, and the PSQI score was positively correlated with GABR ratio. Figure 1 shows correlations among serum levels of the different AAs in patients with FM.

**Table 4**

Correlations between serum amino acid levels and clinical parameters in patients with fibromyalgia

| | | VAS | FIQ-R | MFI | BAI | PSQI |
|---|---|---|---|---|---|---|
| Aspartic acid | $\rho$ | 0.097 | − 0.205 | − 0.161 | − 0.023 | − 0.005 |
| | $p$ | 0.458 | 0.134 | 0.215 | 0.858 | 0.970 |
| Glutamic acid | $\rho$ | 0.044 | − 0.031 | − 0.120 | 0.074 | − 0.205 |
| | $p$ | 0.734 | 0.812 | 0.358 | 0.573 | 0.134 |
| Aminoadipic acid | $\rho$ | 0.123 | − 0.052 | − 0.087 | 0.177 | − 0.038 |
| | $p$ | 0.345 | 0.692 | 0.503 | 0.173 | 0.781 |
| Asparagine | $\rho$ | − 0.053 | − 0.120 | 0.008 | 0.221 | − 0.099 |
| | $p$ | 0.686 | 0.360 | 0.951 | 0.087 | 0.470 |
| Serine | $\rho$ | − 0.057 | − 0.012 | 0.228 | 0.139 | 0.095 |
| | $p$ | 0.664 | 0.927 | 0.077 | 0.285 | 0.492 |
| Glutamine | $\rho$ | 0.110 | − 0.011 | − 0.124 | − 0.023 | 0.143 |
| | $p$ | 0.401 | 0.932 | 0.340 | 0.862 | 0.298 |
| Histidine | $\rho$ | − 0.161 | − 0.163 | − 0.013 | 0.129 | 0.067 |
| | $p$ | 0.214 | 0.213 | 0.923 | 0.321 | 0.625 |
| Glycine | $\rho$ | 0.058 | 0.078 | 0.082 | 0.121 | 0.011 |
| | $p$ | 0.658 | 0.555 | 0.527 | 0.355 | 0.939 |
| Threonine | $\rho$ | − 0.093 | − 0.047 | 0.139 | 0.316* | 0.211 |
| | $p$ | 0.474 | 0.723 | 0.287 | 0.013 | 0.123 |
| Citrulline | $\rho$ | 0.046 | − 0.083 | − 0.008 | 0.102 | 0.022 |
| | $p$ | 0.722 | 0.526 | 0.951 | 0.432 | 0.875 |
| Arginine | $\rho$ | 0.014 | − 0.003 | 0.049 | 0.051 | 0.089 |
| | $p$ | 0.913 | 0.981 | 0.709 | 0.695 | 0.517 |
| 5-Methyl-histidine | $\rho$ | 0.077 | 0.014 | − 0.039 | 0.294* | 0.185 |
| | $p$ | 0.561 | 0.917 | 0.770 | 0.024 | 0.186 |
| Alanine | $\rho$ | − 0.021 | − 0.023 | 0.148 | 0.039 | − 0.047 |
| | $p$ | 0.875 | 0.863 | 0.257 | 0.767 | 0.735 |
| Taurine | $\rho$ | 0.090 | 0.073 | − 0.113 | 0.243 | 0.102 |
| | $p$ | 0.492 | 0.581 | 0.385 | 0.059 | 0.459 |
| Tyrosine | $\rho$ | − 0.061 | − 0.067 | − 0.046 | 0.126 | 0.098 |
| | $p$ | 0.643 | 0.612 | 0.726 | 0.332 | 0.477 |
| 3-Methyl-histidine | $\rho$ | − 0.113 | 0.086 | − 0.081 | 0.157 | 0.119 |
| | $p$ | 0.387 | 0.512 | 0.533 | 0.225 | 0.388 |
| Carnosine | $\rho$ | − 0.063 | − 0.093 | 0.017 | 0.205 | − 0.064 |
| | $p$ | 0.629 | 0.480 | 0.899 | 0.113 | 0.640 |
| $\rho$ Spearman correlation coefficient, *VAS* visual analogue scale, *FIQ-R* Revised Fibromyalgia Impact Questionnaire, *MFI* Multidimensional Fatigue Inventory, *BAI* Beck Anxiety Inventory, *PSQI* Pittsburgh Sleep Quality Index, *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine, *GABR ratio* global arginine bioavailability ratio | | | | | | |
| *$p < 0.05$ | | | | | | |

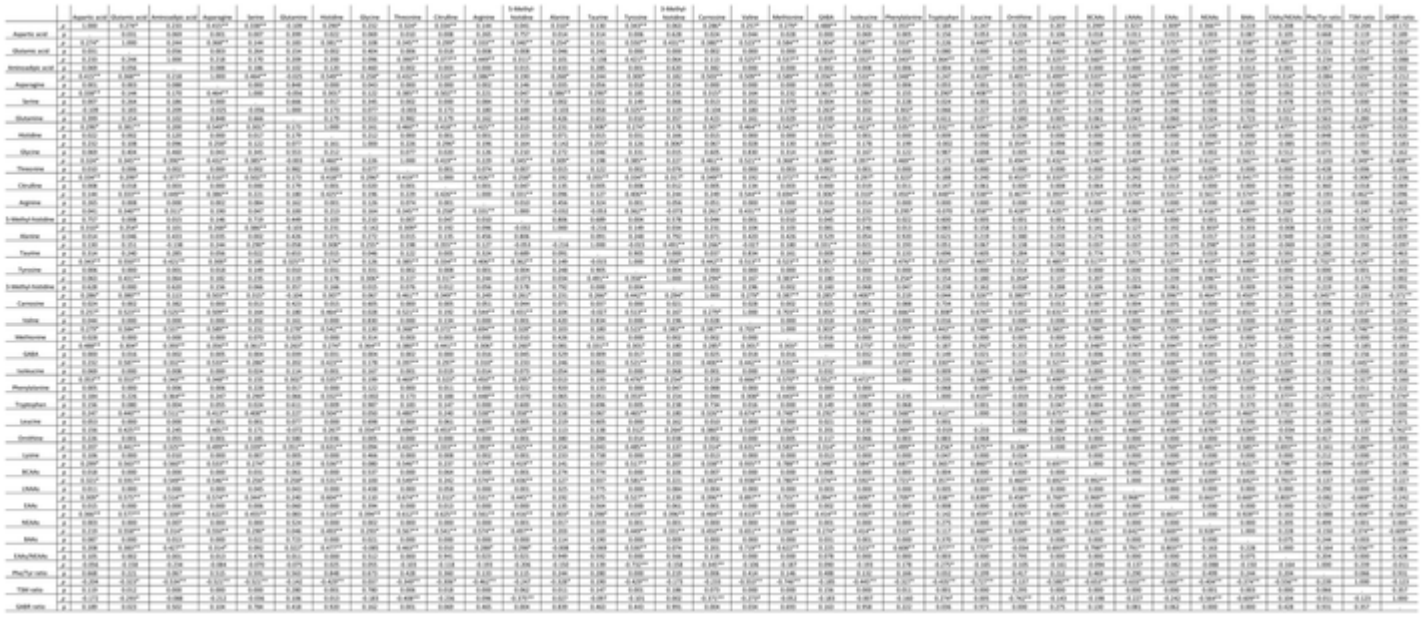
| | | VAS | FIQ-R | MFI | BAI | PSQI |
|---|---|---|---|---|---|---|
| Valine | $\rho$ | − 0.071 | − 0.018 | − 0.099 | 0.198 | 0.084 |
| | $p$ | 0.589 | 0.894 | 0.446 | 0.125 | 0.541 |
| Methionine | $\rho$ | 0.003 | − 0.149 | − 0.103 | 0.194 | − 0.046 |
| | $p$ | 0.981 | 0.255 | 0.428 | 0.134 | 0.737 |
| γ-Aminobutyric acid | $\rho$ | 0.167 | − 0.039 | − 0.128 | 0.100 | 0.153 |
| | $p$ | 0.199 | 0.768 | 0.327 | 0.443 | 0.264 |
| Isoleucine | $\rho$ | 0.006 | 0.149 | 0.178 | 0.316* | − 0.019 |
| | $p$ | 0.961 | 0.256 | 0.169 | 0.013 | 0.890 |
| Phenylalanine | $\rho$ | 0.073 | 0.033 | − 0.131 | 0.184 | 0.175 |
| | $p$ | 0.578 | 0.804 | 0.314 | 0.155 | 0.202 |
| Tryptophan | $\rho$ | − 0.008 | − 0.093 | 0.030 | − 0.018 | 0.011 |
| | $p$ | 0.950 | 0.485 | 0.822 | 0.890 | 0.937 |
| Leucine | $\rho$ | 0.095 | 0.033 | 0.072 | 0.303* | 0.124 |
| | $p$ | 0.466 | 0.801 | 0.580 | 0.018 | 0.365 |
| Ornithine | $\rho$ | − 0.016 | 0.011 | − 0.060 | 0.036 | − 0.024 |
| | $p$ | 0.900 | 0.934 | 0.647 | 0.785 | 0.864 |
| Lysine | $\rho$ | − 0.045 | − 0.082 | 0.022 | 0.144 | 0.119 |
| | $p$ | 0.728 | 0.532 | 0.869 | 0.269 | 0.389 |
| BCAAs | $\rho$ | 0.017 | 0.036 | − 0.029 | 0.257* | 0.088 |
| | $p$ | 0.897 | 0.784 | 0.824 | 0.045 | 0.521 |
| LNAAs | $\rho$ | 0.029 | 0.029 | − 0.050 | 0.245 | 0.085 |
| | $p$ | 0.823 | 0.824 | 0.703 | 0.057 | 0.539 |
| EAAs | $\rho$ | − 0.005 | 0.007 | 0.029 | 0.276* | 0.131 |
| | $p$ | 0.968 | 0.957 | 0.822 | 0.031 | 0.341 |
| NEAAs | $\rho$ | 0.029 | − 0.005 | − 0.044 | 0.138 | 0.022 |
| | $p$ | 0.827 | 0.970 | 0.737 | 0.290 | 0.872 |
| BAAs | $\rho$ | − 0.031 | − 0.028 | − 0.071 | 0.099 | 0.018 |
| | $p$ | 0.811 | 0.834 | 0.588 | 0.447 | 0.897 |
| EAAs/NEAAs ratio | $\rho$ | − 0.059 | − 0.005 | 0.012 | 0.274* | 0.182 |
| | $p$ | 0.654 | 0.970 | 0.926 | 0.032 | 0.183 |
| Phe/Tyr ratio | $\rho$ | 0.098 | 0.062 | − 0.033 | − 0.077 | − 0.014 |
| | $p$ | 0.454 | 0.638 | 0.803 | 0.557 | 0.920 |
| TSM ratio | $\rho$ | 0.110 | 0.187 | − 0.121 | − 0.181 | − 0.020 |
| | $p$ | 0.407 | 0.160 | 0.361 | 0.169 | 0.888 |
| GABR ratio | $\rho$ | − 0.137 | 0.083 | 0.222 | 0.091 | 0.228 |
| | $p$ | 0.300 | 0.533 | 0.090 | 0.494 | 0.100 |

$\rho$ Spearman correlation coefficient, *VAS* visual analogue scale, *FIQ-R* Revised Fibromyalgia Impact Questionnaire, *MFI* Multidimensional Fatigue Inventory, *BAI* Beck Anxiety Inventory, *PSQI* Pittsburgh Sleep Quality Index, *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine, *GABR ratio* global arginine bioavailability ratio

*$p < 0.05$

**Fig. 1**

Correlations between serum amino acid levels in patients with fibromyalgia (FM). $\rho$ Spearman correlation coefficient, *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine, *GABR ratio* global arginine bioavailability ratio. *$p < 0.05$, **$p < 0.01$



## 3.4. Predictive Ability of Serum AA Levels in FM

The predictive value of serum AA levels in FM was determined by both ROC analysis and forward binary logistic regression. Figure 2 shows the ROC curves for the parameters with a statistically significant AUC. Table 5 shows the AUC, *p* value, cutoff point, sensitivity, specificity, PV+, and PV− for these parameters. For example, glutamic acid showed the highest AUC value (0.877), with a cutoff point of 42.84 pmoles/μL, sensitivity of 77.42%, specificity of 100%, PV+ of 100, and PV− of 99.4 (Table 4). These data indicate that the probability of classifying a person with FM as such (serum glutamic acid levels > 42.84 pmoles/μL) is 100% and that more than 99% of persons with serum glutamic acid levels ≤ 42.84 pmoles/μL may be catalogued as healthy with high sensitivity and specificity. Therefore, serum glutamic acid levels > 42.84 pmoles/μL could be proposed as a diagnostic marker of having FM.

**Fig. 2**

Receiver operating characteristic (ROC) curves. *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine

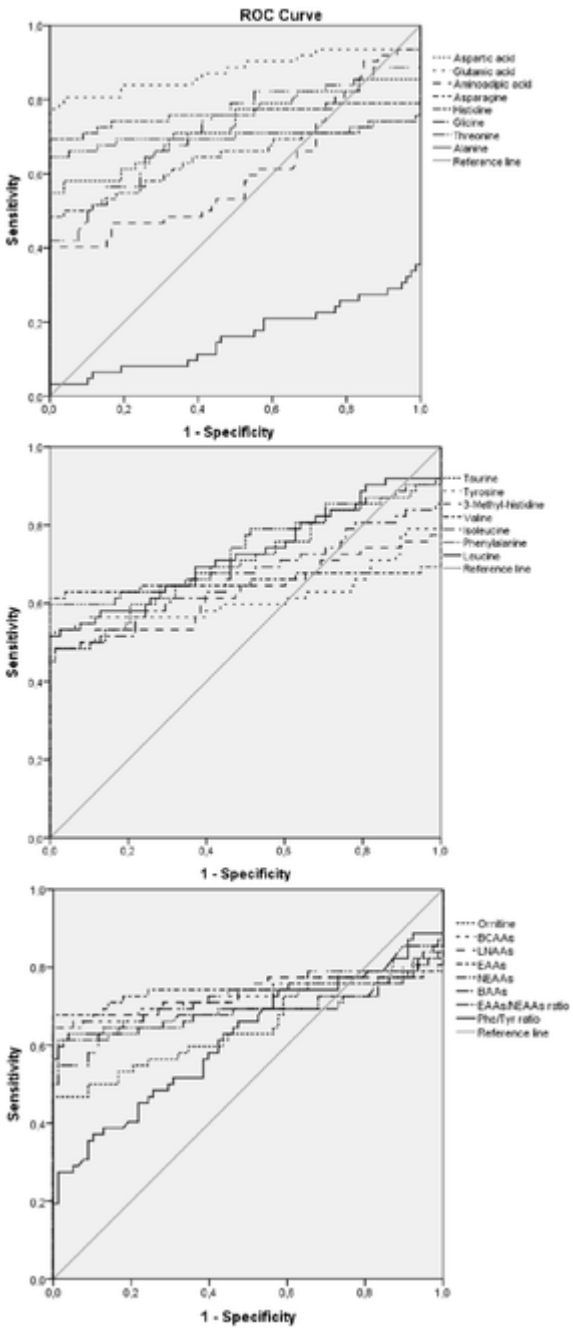



**Table 5**

Receiver operating characteristic (ROC) analysis

| | AUC | *p* value | Cutoff point | Sensitivity (%) | Specificity (%) | PV+ | PV− |
|---|---|---|---|---|---|---|---|
| *AUC* area under the curve, *PV+* positive predictive value, *PV−* negative predictive value, *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine | | | | | | | |

| | AUC | *p* value | Cutoff point | Sensitivity (%) | Specificity (%) | PV+ | PV− |
|---|---|---|---|---|---|---|---|
| Aspartic acid | 0.730 | < 0.0001 | > 27.2 | 54.84 | 100 | 100 | 98.9 |
| Glutamic acid | 0.877 | < 0.0001 | > 42.84 | 77.42 | 100 | 100 | 99.4 |
| Aminoadipic acid | 0.607 | 0.039 | > 1.32 | 40.32 | 100 | 100 | 98.6 |
| Asparagine | 0.639 | 0.012 | > 43.64 | 48.39 | 100 | 100 | 98.7 |
| Histidine | 0.758 | < 0.0001 | > 30.69 | 69.35 | 100 | 100 | 99.3 |
| Glicine | 0.722 | < 0.0001 | > 75.28 | 41.94 | 100 | 100 | 98.6 |
| Threonine | 0.701 | 0.0004 | > 101.46 | 64.52 | 100 | 100 | 99.1 |
| Alanine | 0.841 | < 0.0001 | ≤ 292.43 | 70.97 | 94.87 | 25.4 | 99.3 |
| Taurine | 0.704 | < 0.0001 | > 82.86 | 48.39 | 98.72 | 48.1 | 98.7 |
| Tyrosine | 0.617 | 0.042 | > 68.47 | 53.23 | 98.72 | 50.5 | 98.8 |
| 3-Methyl-histidine | 0.625 | 0.024 | > 103.63 | 48.39 | 98.72 | 48.1 | 98.7 |
| Valine | 0.655 | 0.008 | > 198 | 61.29 | 100 | 100 | 99.1 |
| Isoleucine | 0.665 | 0.001 | > 63.49 | 48.39 | 100 | 100 | 98.7 |
| Phenylalanine | 0.740 | < 0.0001 | > 67.77 | 59.68 | 100 | 100 | 99 |
| Leucine | 0.727 | < 0.0001 | > 165.29 | 51.61 | 100 | 100 | 98.8 |
| Ornithine | 0.647 | 0.005 | > 477.48 | 46.77 | 100 | 100 | 98.7 |
| BCAAs | 0.729 | < 0.0001 | > 418.69 | 64.52 | 100 | 100 | 99.1 |
| LNAAs | 0.734 | < 0.0001 | > 543.53 | 64.52 | 100 | 100 | 99.1 |
| EAAs | 0.740 | < 0.0001 | > 729.98 | 67.74 | 100 | 100 | 99.2 |
| NEAAs | 0.691 | 0.0005 | > 1368.65 | 61.29 | 97.44 | 37 | 99 |
| BAAs | 0.722 | < 0.0001 | > 687.82 | 54.84 | 98.72 | 51.3 | 98.9 |
| EAAs/NEAAs ratio | 0.698 | 0.0003 | > 0.62 | 61.29 | 98.72 | 54 | 99 |
| Phe/Tyr ratio | 0.616 | 0.022 | > 1.21 | 37.10 | 89.74 | 8.2 | 98.3 |

*AUC* area under the curve, *PV+* positive predictive value, *PV−* negative predictive value, *BCAAs* branched chain amino acids, *LNAAs* large neutral amino acids, *EAAs* essential amino acids, *NEAAs* non-essential amino acids, *BAAs* basic amino acids, *Phe* phenylalanine, *Tyr* tyrosine

The results of forward binary logistic regression have shown that the combination of glutamic acid, histidine, and alanine has the greatest predictive ability for differentiating individuals with FM from healthy subjects with a sensitivity of 95.6% (Nagelkerke's $R^2 = 0.923$; glutamic acid ($B = 0.233$; $p = 0.004$), histidine ($B = 0.245$; $p = 0.014$) and alanine ($B = -0.131$; $p = 0.003$)).

## 4. Discussion

The determination of the circulating free AA profile has been useful in understanding the physiopathology of several pathologies characterized by altered levels of certain AAs, such as Hartnup disease, familial iminoglycinuria, cystinosis, and the Fanconi syndrome [ 15 ]. The pathophysiological mechanisms underlying FM have not yet been clarified. We assessed the serum AA profile in patients with FM and in healthy subjects as well as the associations between AA levels and FM clinical features. To the best of our knowledge, we have measured, for the first time, several AAs in FM. Our results have shown significantly higher serum levels of most AAs in patients with FM than in the healthy controls. A recent systematic review has concluded that AA levels were altered in patients with FM in relation to healthy subjects [ 33 ], but results, including those of the present study, are contradictory [ 13, 15, 16, 17, 18, 19, 20, 21 ]. This is probably attributable to several factors, such as differences in the recruitment of patients, or the selected sample size. Regarding the latter, our study has the largest sample size of controls ($n = 78$) and the second largest sample size of patients with FM ($n = 62$) followed by that of Clos-Garcia et al. [ 19 ], which included 105 patients with FM. Therefore, our results would be very consistent at the statistical level. Also, compared to the previous studies, we measured the largest number of AAs. As for the recruitment of participants, patients with FM usually have many comorbidities, which could explain the diversity of results obtained in these studies. In this sense, levels of AAs in the bloodstream could be influenced by underlying health conditions [ 34, 35 ]. We consider that an advantage of our methodology, compared to previous studies, is a meticulous selection of the study population by establishing numerous exclusion criteria and thus reducing confounding factors that may influence circulating levels of AAs. While three studies took into account few exclusion criteria [ 16, 19, 21 ], others [ 13, 15, 18 ] and ours, established the presence of a major clinical condition other than FM as an exclusion criterion. We have established additional exclusion criteria, not mentioned in these previous studies, such as presence of grade II obesity [body mass index (BMI) ≥ 35 kg/m$^2$], severe physical disability, and lactation. Moreover, blood levels of AAs could be influenced by other factors such as medication, diet, and exercise [ 34, 35, 36, 37 ]. Our patients were taking pain-related medication and maintained their usual diet and physical activity but, unlike previous studies, we examined the effects of these factors on AA levels. Regarding medication consumption, Maes et al. [ 18 ] excluded patients who had taken different drugs in a given period of time, and none of their participants took psychotropic drugs for 1 year before the study. Bazzichi et al. [ 15 ] and Ruggiero et al. [ 13 ] reported that all FM patients abstained from taking medications for 2 weeks before blood sample collection. Menzies et al. [ 20 ] established as an exclusion criterion the systemic use of corticosteroids. Menzies et al. [ 20 ] and Hsu et al. [ 21 ] did not control for medications in either the FM or control group. Clos-Garcia et al. [ 19 ] reported that 70% of their patients used painkillers, 55% were taking antidepressants and benzodiazepines, and approximately 30%, antiepileptic drugs. In our case, an exclusion criterion, both for patients and controls, was treatment with vasoactive drugs, anticoagulants, corticosteroids, estrogens, or agonists/antagonists of opioid receptors. However, we did not ask patients to stop their pain-

related medication to participate in our study due to the consequences that this could have on their quality of life. Eleven of our FM patients were consuming analgesics or anti-inflammatory drugs, nine were taking anxiolytics or antidepressants or muscle relaxants, and 33 were consuming any combination of the above medications (analgesics, anti-inflammatories, anxiolytics, antidepressants, or muscle relaxants). Mostly, our FM patients were taking muscle relaxants, anxiolytics, or antidepressants as an adjunct to their pain medication for more effective pain control. However, our results have shown that medication consumption did not significantly affect the levels of AAs in patients with FM. On the other hand, although our study participants maintained their usual physical activity, they were instructed not to perform physical exertion the day of blood collection. Bazzichi et al. [ 15 ] also reported that all patients maintained their usual diet and physical activity. Menzies et al. [ 20 ] included as a limitation of the study not having collected data on dietary intake. Hsu et al. [ 21 ] did not control for dietary intake or recent exercise. Clos-García et al. [ 19 ] reported that half of FM patients reported some physical exercise. Since circulating AA levels can be influenced by diet, it is important to know the dietary protein intake of the study participants, whether the participants were taking vitamin supplements, and whether the blood samples were collected after fasting to eliminate confounding factors in the analyses. To the best of our knowledge, none of the previous works included information on dietary protein intake of the study participants. In our case, to find out if diet could affect our results, we assessed the usual protein dietary intake of our study participants using a semiquantitative interviewer-administered food-frequency questionnaire (FFQ), and we did not find significant differences in protein dietary intake between FM patients and healthy subjects (data not shown). Furthermore, our participants were not consuming vitamin supplements, which to our knowledge, has not been taken into account in any of the previous studies. In three works [ 13, 16, 18 ] and in ours, the participants fasted before blood collection, while two works mentioned as a limitation of the study not having taken it into account [ 20, 21 ]. Moreover, to avoid potential circadian variations in the levels of the AAs, we collected the blood samples at the same time of day, as did Maes et al. [ 18 ]. Finally, we assessed the level of physical activity that our participants performed in the last 7 days using the International Physical Activity Questionnaire (IPAQ). We did not find any significant correlation between serum levels of AAs and the level of physical activity in patients with FM, and we found only a positive correlation between the levels of GABA and physical activity ($p = 0.044$, Spearman coefficient = 0.259) in controls (data not shown). These results suggest that the practice of physical activity of our study participants in the last 7 days does not seem to affect serum levels of AAs.

Many AAs participate in neurotransmission, either as neurotransmitters (GABA, glycine or glutamic acid, among others), neurotransmitter precursors (phenylalanine, tyrosine or tryptophan, among others), or neuromodulators (alanine) [ 38 ]. There are AAs that have the ability to be neurotransmitters by themselves, either with an excitatory function (glutamic acid, aspartic acid), inhibitory function (GABA, glycine, taurine), or both functions (serine) [ 38 ]. Our results have shown significantly higher serum levels of aspartic acid, glutamic acid, glycine, and taurine in patients with FM than in controls, showing an imbalance in the metabolism of these neurotransmitters in FM. We did not find significant differences in the levels of GABA and serine between groups. Glutamic acid is the principal excitatory neurotransmitter in the CNS and is involved both in nociception and central sensitization. Aspartic acid is a structural homologue of glutamic acid and is generally considered as the secondary excitatory neurotransmitter in the CNS. Our results have shown a significant positive correlation between glutamic acid and aspartic acid in patients with FM (see Fig. 1 ). The main hypothesis on the physiopathology of FM includes altered CNS pathways that lead to amplified pain sensitivity [ 6 ]. In agreement with our results, previous studies found increased levels of glutamic acid [ 13, 19 ] and aspartic acid [ 13 ] in serum and of glutamic acid in cerebrospinal fluid (CSF) [ 39 ] in FM patients compared to controls. In this line, it has been reported that the abundance of bacteria of the genera Bifidobacterium and Lactobacillus (involved in the transformation of glutamic acid to GABA) was reduced in patients with FM, suggesting that this could contribute to elevated serum glutamic acid levels in these patients [ 19 ]. Additionally, positive correlations were found between glutamic acid levels and the number of tender points, the hallmark of FM, in these patients (in plasma [ 15 ] and CSF [ 39 ]). In the brain of FM patients, glutamic acid levels were correlated to severity of FM symptoms [ 40 ], and increased glutamic acid and glutamine levels were related to enhanced pain sensitivity [ 41 ]. In line, elevated levels of glutamic acid could increase the activation of glutamic acid receptors, which are linked to nociception [ 42 ]. In the present study, serum glutamine levels did not change between patients and controls. Glutamine is involved in the generation of glutamic acid, aspartic acid, and GABA. Coinciding with our data, GABA levels showed similar results in brain of patients with FM and in healthy subjects [ 43 ]. Contrarily to our results, previous studies comparing FM patients and healthy controls reported higher serum levels of glutamine [ 19 ], lower CSF levels of aspartic acid [ 44 ], and unchanged circulating glutamic acid levels in the patients versus controls [ 15, 16 ]. Taurine has an inhibitory effect on neurons by acting on GABA and glycine receptors [ 38 ]. We have found significant positive correlations between taurine with GABA and glycine in patients with FM (see Fig. 1 ). Coinciding with our results for taurine serum levels, elevated levels of taurine have been previously reported in serum [ 13 ], plasma [ 20 ], and urine [ 45 ] in patients diagnosed with FM versus controls. However, other studies showed lower taurine levels between groups [ 15, 16 ]. In the present study, the correlation between serum taurine levels and the BAI score, indicative of anxiety symptoms, approached statistical significance ($p = 0.059$) in FM patients. Previous studies concluded that elevated levels of taurine were related to unrestful sleep (plasma levels) [ 15 ], to tender point index, a measure of pain intensity (CSF levels) [ 44 ], and to pain and fatigue (urine levels) [ 45 ] in patients with FM. Glycine is the main inhibitory neurotransmitter in the spinal cord. Although our results of increased glycine levels in FM coincide with a previous study [ 13 ], others did not show changes in their levels between patients and controls [ 15, 16 ]. Elevated CSF levels of glycine were reportedly correlated to tender point index [ 44 ] in patients with FM. Alanine is a weaker agonist for glycine receptors. Alanine levels were lower in our patients with FM than in controls, in line with previous data [ 15, 16 ], but in disagreement with others [ 13 ]. It has been reported that FM patients with paraesthesia had lower serum alanine levels than those without paraesthesia [ 13 ]. In sum, we have found altered serum levels of glutamic acid, aspartic acid, glycine and taurine in patients with FM versus controls, which could affect the normal functions of these neurotransmitters in FM patients and be related to the pathophysiology of this syndrome.

It is well known that some AAs act as precursors of important neurotransmitter and neuromodulatory molecules (phenylalanine, tyrosine, tryptophan, methionine, lysine, asparagine, threonine, glutamine). The catecholamines are obtained from phenylalanine and tyrosine, and serotonin is obtained from tryptophan. We found that patients with FM had higher serum levels of tyrosine and phenylalanine than healthy

subjects, which could ultimately lead to an alteration in the metabolism of catecholamines. Our results agree with a previous work [13], but disagree with others [15,16,18]. Catecholamines are sympathetic neurotransmitters, which are implicated in stress responses. It has been suggested that patients with FM have an inadequate response to stressful situations when compared to healthy subjects due to the dysregulation of the sympathetic nervous system [46]. In accordance with the increased serum levels of tyrosine and phenylalanine found in our patients with FM, previous studies have reported higher levels of norepinephrine in these patients in comparison to controls [7,47,48]. Moreover, higher norepinephrine levels were associated with worse physical health status in FM patients [7]. In agreement, pain increased after norepinephrine injection in patients with FM in comparison with healthy subjects [49]. However, the results available in the literature regarding the levels of catecholamines are conflicting, showing increased, decreased, and unchanged levels of dopamine, norepinephrine, and epinephrine in subjects with FM versus controls [7,47,48,50]. Although we have not found statistically significant changes in tryptophan levels between groups, levels approached statistical significance ($p = 0.053$). Our results agree with previous studies [15,17,18,51], but disagree with others that reported significantly decreased levels of tryptophan in patients with FM [16,21]. Therefore, our result of unchanged tryptophan levels between groups does not support the hypothesis that a deficiency in serotonin may be related to the physiopathology of FM [6,7]. Methionine is the precursor to homocysteine, which is an excitatory neuromodulator. Our data showed increased circulating levels of methionine in FM patients, similar to previous results [13], but in disagreement with another authors [15,16]. Methionine restriction has been reported to prevent altered methionine/transmethylation metabolism, thereby decreasing DNA damage and carcinogenic processes, and possibly preventing arterial, neuropsychiatric, and neurodegenerative diseases [52]. Lysine, asparagine, and threonine are the precursors to glutamic acid, aspartic acid, and glycine, respectively. Lysine levels were significantly correlated to glutamic acid levels in our FM patients as well as asparagine with aspartic acid (see Fig. 1). The correlation between threonine and glycine approached statistical significance ($p = 0.077$). In the present study, all these AAs were upregulated in patients with FM, as are the neurotransmitters they are precursors to. Coinciding with our results, a previous study detected a significant increase in serum levels of threonine and Nε-methyl-L-lysine in patients with FM compared to controls [19]. However, other studies reported results contrary to ours [13,15,16]. Lysine, arginine, and glycine have also been reported to play a role in pain modulation [38], so their altered values in our patients with FM could contribute to the characteristic pain of these patients. In the present study, whereas serum threonine levels were significantly correlated to anxiety symptoms, the correlation between asparagine and the BAI score approached statistical significance ($p = 0.087$) in FM patients. In line, it has been reported that elevated CSF levels of asparagine were related to tender point index in patients diagnosed with FM plus another painful condition [44]. To conclude, the altered serum levels of these AAs (phenylalanine, tyrosine, tryptophan, methionine, lysine, asparagine, threonine, glutamine), precursors of several neurotransmitters and neuromodulatory molecules, in our patients with FM suggest metabolic disturbances that could be related to the physiopathological mechanisms underlying this complex syndrome.

Other AAs act as precursors of important molecules, such as nitric oxide (NO), which is obtained from arginine, and histamine, which is obtained from histidine. Although we have not found statistically significant changes in serum arginine levels between groups, in agreement with previous studies [13,15,16], levels approached statistical significance ($p = 0.054$), showing increased levels in FM patients. In this sense, higher serum levels of arginine have been found in patients with FM compared to controls [19]. We have also calculated the GABR ratio, an indicator of the global bioavailability of arginine, which has been reported to be more reliable than arginine levels [53]. Our results showed a higher GABR ratio in patients than in controls, suggesting a higher bioavailability of arginine in patients with FM, which may ultimately be reflected in increased NO levels. In line, significant increases in NO levels have been found in FM patients [54,55], although results of other studies were in disagreement [56,57]. NO is involved in vasodilation, inflammation, and neurotransmission processes, playing a complex role in pain modulation at both central and peripheral levels and contributing to the development of central sensitization in FM [58,59]. In agreement, elevated CSF levels of arginine have been related to tender point index in patients with FM plus another painful condition [44]. Arginine can be metabolized to produce other AAs, citrulline, or ornithine. In line, our results have shown significant correlations between arginine with citrulline and ornithine in patients with FM (see Fig. 1). Although our results have not shown significant differences in serum citrulline levels between groups, we have found higher levels of ornithine in patients with FM than in controls. Our results agree with previous results [13,19], but are in contradiction with others [16]. On the other hand, our FM patients had significantly higher serum levels of histidine and its derivatives (3-methyl-histidine and 5-methyl-histidine) than the healthy controls, indicating an alteration in the metabolism of these molecules and, probably, of histamine in these patients. To the best of our knowledge, this is the first work that analyzes serum 3-methyl-histidine and 5-methyl-histidine levels in FM. In addition, we have found, for the first time, significantly increased serum histidine levels in patients with FM, disagreeing with previous studies that found decreased [16,17] or unchanged [13,15] values. Histamine has been proposed to be involved in the physiopathology of FM after being released from mast cells, acting as a neurosensitizing molecule that contributes to pain [60]. Therefore, this hypothesis is in agreement with our results of increased levels of the histamine precursor in patients with FM. We have also found that serum 5-methyl-histidine levels were positively correlated with the BAI score in patients with FM, suggesting that the imbalance in their levels may be related to anxiety symptoms in these patients.

The aminoadipic acid is an oxidized derivative from lysine and is considered as a biomarker of protein oxidation [61]. Levels of aminoadipic acid were significantly correlated to lysine levels in our patients with FM (see Fig. 1). We have found increased levels of aminoadipic acid in patients with FM compared to controls. To our knowledge, this is the first work that analyzes the aminoadipic acid in FM patients. The aminoadipic acid is reported to increase oxidative stress-related parameters such as reactive oxygen species generation and oxidation of proteins and lipids in vitro [61]. The increased levels of aminoadipic acid in our patients are consistent with previous studies that found higher levels of oxidative stress in patients with FM than in the healthy controls, proposing that oxidative stress may have a role in the physiopathology of this syndrome [62,63].

Our results have also shown higher serum levels of BCAAs, LNAAs, EAAs, NEAAs, BAAs, EAAs/NEAAs ratio, and Phe/Tyr ratio in patients with FM than in healthy subjects. To the best of our knowledge, this is the first work that analyzes LNAA, BAA, Phe/Tyr ratio, EAA/NEAA ratio, TSM ratio, and GABR ratio in FM. The sum of isoleucine, leucine, and valine constitutes the branched-chain AAs (BCAAs). BCAAs play a role in the maintenance of glutamic acid at a relatively constant level [ 64 ]. In addition, BCAAs compete with aromatic AA transport, indirectly modulating synthesis of catecholamines, serotonin and histamine [ 65 ]. We have found higher serum levels of isoleucine, leucine, and valine, and consequently of BCAAs, in FM patients than in controls, which eventually may lead to an alteration in the metabolism of glutamic acid, catecholamines, and histamine in FM. BCAAs levels were positively correlated with levels of isoleucine, leucine, and valine in our patients with FM (see Fig. 1 ). Our results of increased BCAAs in FM are in agreement with previous authors [ 13 ], but in disagreement with others [ 15, 18 ]. Elevated circulating BCAA levels have also been correlated with severity of insulin resistance and altered glucose uptake [ 66 ]. It has been reported that FM patients showed a higher glycemic response to a glucose load than the healthy controls [ 67 ]. In line, FM is associated with an increased risk for both glucose metabolism disturbances and diabetes mellitus [ 67 ]. Therefore, our results support the hypothesis of a dysregulated metabolic status in FM. We have also found significantly increased values of essential and nonessential AAs (EAAs and NEAAs, respectively) in patients with FM versus controls, in disagreement with previous results [ 15 ]. The Phe/Tyr ratio and the EAAs/NEAAs ratio were calculated because they are typically lower when there is a deficit in dietary protein [ 68 ]. Our patients with FM showed significantly higher Phe/Tyr ratio and EAAs/NEAAs ratio than controls, which may suggest that FM patients could have a higher protein intake than controls, thereby explaining the increased levels of most AAs that we have found. However, as stated before, we did not find significant differences for protein intake between FM patients and healthy controls (data not shown). Therefore, the increased serum AAs levels that we identified in our patients with FM could not be explained by differences in protein intake, suggesting that they may be attributed to the disease itself. FM is commonly associated with fatigue, anxiety, and sleep disorders. In the present study, the BAI score was positively correlated with isoleucine, leucine, BCAAs, EAAs, and EAAs/NEAAs ratio in patients with FM. Several other correlations approached statistical significance ($p \leq 0.1$). That is, LNAAs were correlated with anxiety symptoms, and GABR ratio was correlated with fatigue and quality of sleep. The altered values of these ratios and groupings of AAs in FM patients as well as their correlations with anxiety, a common symptom of this syndrome, may support the hypothesis of a metabolic disturbance in FM.

The search for biomarkers for FM is currently a pressing need in biomedical research to facilitate both diagnosis and treatment, a problem that involves high health expenses worldwide [ 69 ]. The diagnosis of FM remains clinically based due to the absence of reliable biomarkers. In this regard, we have analyzed serum AAs levels in patients with FM and controls in order to investigate their potential predictive ability to differentiate subjects with FM from healthy subjects. According to ROC analysis, most of the parameters that showed statistically significant differences between groups may be good markers for differentiating individuals with FM from healthy subjects, glutamic acid being the one that showed the greatest predictive capacity (see Table 5 ). In addition, the results of forward binary logistic regression showed that the combination of glutamic acid, histidine, and alanine had the greatest predictive ability to diagnose FM. Therefore, our results could facilitate the diagnosis of FM, together with the clinical data of the patient. However, future studies should validate our results. In line, the determination of circulating AAs has shown promising results as biomarker and predictor of several pathologies [ 14 ].

A limitation of the present study is the small number of men enrolled, which may limit the generalizability of the results to men. Due to the higher prevalence of FM among middle-aged women than in men, it is difficult to find men with FM, hence the low number of men included in this study.

# 5. Conclusions

Our results show an imbalance in serum levels of most AAs in patients with FM, which could alter the many metabolic pathways in which these AAs participate, thereby leading to a pathological state. This altered AA homeostasis in patients with FM may be related to the dysregulation of both pain neurotransmission and the catecholaminergic system, to increased oxidative stress, and even to impaired glucose metabolism. Our results also show that the determination of serum levels of several AAs may aid in the diagnosis of FM, together with the clinical data of the patient. The BAI score was positively correlated with some AAs in FM patients, proposing that these AAs could become biomarkers of anxiety in these patients. These results could guide future adjuvant treatments for FM such as nutritional interventions to regulate serum AA homeostasis.

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## Declarations

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***Conflict of interest*** All authors declare that they have no conflict of interest.

***Availability of data and material*** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

***Ethics approval*** This case–control study was carried out in accordance with the Declaration of Helsinki of the World Medical Association (WMA). The study was approved by the Ethics Committee of the University of Granada (Spain) (approval number: 1797-N-17).

***Consent to participate*** All participants provided written informed consent and did not receive financial incentive.

***Consent for publication*** Not applicable.

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***Author contributions*** Alma Rus contributed to analysis and interpretation of results, drafted the manuscript, critically revised the manuscript, and gave final approval. José Alberto López-Sánchez contributed to analysis of results, critically revised the manuscript, and gave final approval. José Manuel Martínez-Martos and María Jesús Ramírez-Expósito performed the laboratory experiments, critically revised the manuscript, and gave final approval. Francisco Molina contributed to conception and data acquisition, critically revised the manuscript, and gave final approval. María Correa-Rodríguez and María Encarnación Aguilar-Ferrándiz contributed to conception and design, data acquisition, critically revised the manuscript, and gave final approval.

# Supplementary Information

Below is the link to the electronic supplementary material.

Supplementary file1 (DOC 99 kb)

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