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Research Article

Sex-based estimation of biological variation in plasma-free amino acid concentrations among healthy adults

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ABSTRACT

Introduction: Free amino acid (FAA) analysis plays a crucial role in diagnosing and monitoring inborn errors of metabolism, assessing nutritional status, and identifying metabolic imbalances associated with various diseases. This study aimed to provide updated biological variation (BV) data to support the reliable clinical application of FAA concentrations in plasma samples, utilizing LC-MS/MS.

Materials and methods: Venous blood was collected from 22 healthy Turkish adults (9 men and 13 women) over approximately nine weeks. Plasma FAAs were measured in duplicate. BV estimates with 95 % confidence intervals were determined using nested ANOVA for the entire study group and sex-stratified subgroups, following analysis of outliers, normality, steady-state conditions, and variance homogeneity.

Results: Within-subject variation (CV_I) and between-subject variation (CV_G) estimates ranged from 9.5 % to 32.5 % and 8.6 % to 50.0 %, respectively. The estimated CV_I values for essential amino acids were significantly lower than those for non-essential amino acids ($P = 0.03$). For most plasma FAAs, no significant differences in CV_I (except for alanine, arginine, glutamic acid, and threonine) or CV_G were observed between sexes. However, differences in the indices of individuality were noted between men and women for some plasma FAAs.

Conclusions: This Biological Variation Data Critical Appraisal Checklist-compliant study provides the first updated BV data for plasma FAAs. The significant variation observed in CV_I estimates is hypothesized to result from differences in the metabolic regulation of essential versus non-essential amino acids. The sex-stratified indices obtained in this study will aid in the appropriate application of population-based reference intervals for plasma FAA assessment.

Introduction

Amino acid analysis is essential for evaluating nutritional status and diagnosing liver, kidney, and muscle dysfunction. Beyond its well-established role in identifying and monitoring inborn metabolic disorders, free amino acid (FAA) profiles also serve as valuable tools for the

Abbreviations: APSs, analytical performance specifications; B_{APS}, analytical performance specification for bias; BIVAC, Biological Variation Data Critical Appraisal Checklist; BMI, body mass index; BV, Biological variation; CI, confidence interval; CK, creatine kinase; CRP, C-reactive protein; CV_A, analytical variation; CV_{APS}, analytical performance specification for analytical imprecision; CV_G, between-subject variation; CV_I, within-subject variation; EFLM WG-BV, European Federation of Laboratory Medicine Working Group on Biological Variation; EFLM, European Federation of Laboratory Medicine; ERNDIM, European Research Network for the evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism; EuBIVAS, European Biological Variation Study; FAA, Free amino acids; GGT, gamma-glutamyl transferase; II, index of individuality; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NHSP, the number of samples required to estimate the homeostatic set points; popRI, population-based reference interval; prRI, personalized reference interval; RCV, reference change value; RP-HPLC, reverse-phase high-pressure liquid chromatography; TEa_{APS}, analytical performance specification for total allowable error.

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early detection of diseases such as diabetes and cancer. Precise measurement of amino acid concentrations is critical for these clinical applications [1].

Biological variation (BV) data—including within-subject variation (CV_I), between-subject variation (CV_G), the reference change value (RCV), and the index of individuality (II)—provide essential parameters for interpreting amino acid analysis results [2]. CV_I reflects fluctuations in an analyte's concentration around a homeostatic set point under steady-state conditions, while CV_G represents variability between the homeostatic set points of different healthy individuals [2]. The RCV, derived from analytical variation (CV_A) and CV_I, aids in interpreting differences between consecutive measurements [3]. The II helps determine whether a population-based reference interval (popRI), personalized reference interval (prRI), or RCV is most appropriate for test result interpretation [2–4]. Additionally, BV data inform the development of analytical performance specifications (APSs) [5].

Reliable BV data are essential for all these applications. To ensure high-quality data, the European Federation of Clinical Chemistry and Laboratory Medicine Working Group on Biological Variation (EFLM WG-BV) conducted the European Biological Variation Study (EuBIVAS) using methodological procedures aligned with the Biological Variation Data Critical Appraisal Checklist (BIVAC) and state-of-the-art analytical techniques [6]. Ongoing evaluations assess BV studies for consistency with the online BV database and BIVAC scoring to determine eligibility for *meta*-analyses [7].

FAA analysis can be performed using various methods. Recent technological advancements and growing expertise have led to the widespread adoption of liquid chromatography-tandem mass spectrometry (LC-MS/MS) [1,8,9]. However, documented BV data for FAAs using LC-MS/MS are scarce, and few BV studies have focused on blood amino acids. A study published in 2010 reported the BV of plasma FAAs using reverse-phase high-pressure liquid chromatography (RP-HPLC) [10]. Conducted before the publication of BIVAC, the study had several limitations, including the lack of replicate analysis for all samples, which affected outlier identification and the presentation of confidence intervals for BV estimates.

More recently, a study assessed the BV of FAAs in serum samples using LC-MS/MS [11]. Both serum and plasma can be used for quantitative blood amino acid analysis; however, the concentration of many amino acids in serum is reportedly higher than in plasma, likely due to amino acid release from erythrocytes, leukocytes, or platelets during coagulation, potentially leading to falsely elevated results in serum [12]. Furthermore, the American College of Medical Genetics and Genomics technical standard for amino acid laboratory analysis recommends fasting plasma as the preferred sample to avoid misinterpretation, particularly in disorders related to amino acid synthesis that present with low concentrations of certain amino acids [1].

In this study, we aimed to determine the BV of FAAs in plasma samples using LC-MS/MS while, for the first time, adhering to the BIVAC criteria.

Materials and methods

Study participants and samples

The study was conducted between January and March 2022 at Afyonkarahisar Health Sciences University Hospital, Afyonkarahisar, Turkiye. Initially, 23 healthy volunteers (10 men and 13 women; age range, 18–50 years) were recruited. One male volunteer was excluded due to non-compliance, as indicated by elevated C-reactive protein (CRP) levels in subsequent weeks, resulting in a final cohort of 22 participants. Inclusion and exclusion criteria were based on a previous study by the EFLM EuBIVAS [13]. Accordingly, individuals with diabetes mellitus, dyslipidemia, a history of chronic kidney or liver disease, pregnancy, breastfeeding, or amino acid metabolism disorders were not included.

A series of tests—including glucose, alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), creatine kinase (CK), creatinine, CRP, total cholesterol, triglycerides, total protein, albumin, and hemolysis index—were conducted on samples collected during the first visit. In subsequent weeks, triglycerides, ALT, CK, CRP, and hemolysis index were analyzed and assessed using reference intervals provided by the manufacturer. Volunteers whose test results fell outside the reference values were excluded from the study.

Participants were monitored weekly regarding their health status, infections, dietary habits, and medication use. They were advised to maintain their normal lifestyle, abstain from alcohol, avoid medications, vitamins, or dietary supplements, refrain from dietary restrictions, and limit strenuous physical activity throughout the study period.

Serum samples collected during the first visit were tested for glucose, ALT, GGT, CK, creatinine, CRP, total cholesterol, triglycerides, total protein, albumin, and hemolysis index using a cobas c 702 automated analyzer (Roche Diagnostics, Mannheim, Germany). Over an average of nine weeks, triglyceride, ALT, CK, and CRP levels, along with the hemolysis index, were analyzed weekly and assessed using reference intervals provided by the manufacturer.

The study protocol was approved by the Ethics Committee of Afyonkarahisar Health Sciences University Hospital, Afyonkarahisar, Turkiye (approval No.: 2022/4), and informed consent was obtained from all participants.

Venous blood samples were collected on the same day each week. Four participants (two women and two men) attended all 10 weeks, 15 participants (10 women and five men) attended nine weeks, and three participants (one woman and two men) attended eight weeks. Samples were collected between 08:30 and 10:00 a.m. following an eight-hour fast. To minimize preanalytical variation, all samples were drawn by the same phlebotomist.

Samples were collected in EDTA tubes (3 mL; Becton Dickinson, Franklin Lakes, NJ, USA) and immediately centrifuged at 1,500 × *g* for 10 min. Plasma samples were aliquoted and stored at –80 °C until February 2023, for a maximum of 13 months post-collection, at which point the analyses were conducted.

Plasma-FAA measurement

Plasma FAAs were measured using LC-MS/MS (Agilent Technologies, Santa Clara, CA, USA) with a CE-IVD certified and validated LC-MS/MS amino acid analysis kit (Jasem, Istanbul, Türkiye) and an Agilent 6465 Ultivo Triple Quadrupole Mass Spectrometer equipped with an electrospray ionization source. Chromatographic separation was performed using gradient elution: 4.0 min at 84 % mobile phase B, decreasing to 30 % from 4.0 to 5.0 min, held at 30 % from 5.0 to 6.5 min, and returning to 84 % for re-equilibration from 6.6 to 10.0 min. The analytical column temperature was maintained at 10 °C, and the autosampler at 8 °C. The injection volume was 3 µL. Mass detection was carried out in positive-ion multiple-reaction monitoring mode.

The mass spectrometer settings were as follows: drying gas temperature, 150 °C; drying gas flow, 10 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 400 °C; sheath gas flow, 10 L/min; capillary voltage, 2,000 V (+). Plasma samples were processed following the kit's sample preparation protocol: 50 µL of the sample was pipetted into a tube, 50 µL of the internal standard was added, the tube was agitated for five seconds, 700 µL of reagent 1 was added, and the vial was centrifuged at 3,600 × *g* for five minutes. The supernatant was then transferred into a vial for injection into the LC-MS/MS system.

All samples were randomly analyzed in duplicate in a continuous batch over three days. Twenty-five amino acids were analyzed. Samples were run subsequent to daily internal QC assessments, which were considered acceptable if results fell within the defined concentration ranges in the package insert. Furthermore, amino acids were routinely evaluated by the ERNDIM external quality assurance program.

Statistical analysis

Statistical analysis was performed using the freely available BV calculation tool BioVar (https://turcosa.shinyapps.io/biovar/)[14]. The BV data were analyzed according to the guidelines by Bartlett *et al.* [15], the pipeline by Braga and Panteghini [16], and the updated checklist by Aarsand *et al.* [6]. The BV analysis was structured into the following steps:

Detecting Outliers: Outlier detection was conducted at three levels. Cochran's C test was used to identify outliers within duplicate measurements obtained from participants and within the variance of the means of measurements among individuals [16]. Outliers among participants' mean values were identified using the Dixon–Reed criterion.

Controlling Normality Assumption: Following the removal of outliers, the normal distribution assumption for all individuals was verified using the Shapiro–Wilk test.

Testing Homogeneity Assumptions: Bartlett's test was used to assess the homogeneity of CV_I and CV_A. If the homogeneity assumption was not met, Cochran's C test was applied. If homogeneity was still not achieved, results violating the homogeneous distribution were excluded until the assumption was met.

Checking Steady-State Condition: The participants' steady-state condition was assessed using linear regression analysis of mean measurements obtained during each week of blood collection against their corresponding point numbers. Participants were considered in a steady state if the 95 % confidence interval (CI) of the regression line slope encompassed zero.

Performing Analysis of Variance: BV estimates (CV_I and CV_G) and CV_A were calculated for the entire group and sex-stratified subgroups using nested ANOVA [17]. The 95 % CIs of BV estimates were computed using the formulas reported by Burdick and Graybill [18]. BV estimates were established for the entire group and for sex-stratified subgroups. If the 95 % CIs of subgroup BV estimates overlapped, it was determined that there was no significant difference between sexes. Student's *t*-test was used to compare the means of the two groups. Additionally, the significance levels of the estimated CV_I values of essential and non-essential amino acids were evaluated, with $P < 0.05$ indicating statistical significance.

Applications of biological variation

The APSs, asymmetrical RCVs, IIs, and the number of samples required to calculate the homeostatic set point (NHSP) were calculated employing Microsoft Excel.

When a significant difference was observed between CV_I of men and women, the lower value was chosen for APS calculation. If mean concentrations were significantly different between genders, the lower of the two CV_Gs was applied in the APS estimation [19]. APS values for desirable imprecision (CV_{APS}), bias (B_{APS}), and total allowable error (TEa_{APS}) were calculated using the following formulas [2]:

$$CV_{APS} < 0.5\ CV_I$$

$$B_{APS} < 0.250(CV_I^2 + CV_G^2)^{1/2}$$

$$TEa_{APS} = 1.65(0.5\ CV_I) + 0.250(CV_I^2 + CV_G^2)^{1/2}$$

RCVs for both an increase and a decrease in the measurand were determined using the log-normal approach reported by Fokkema, *et al.* [20]

$$RCV = 100\% \times \exp[\pm z_\alpha \times 2^{1/2} \times (CV_{lnA}^2 + CV_{lnI}^2)^{1/2} - 1]$$

where CV_{ln} refers to the ln-transformed data ($\ln[1 + CV^2]^{1/2}$), the CV_A values were obtained from replicate measurement results, and z_α has a value of 1.65 (probability level, 95 %). The CV_I value reported in the 2014 edition of "Desirable Specifications for imprecision, inaccuracy, and total allowable error, calculated from data on within-subject and between-subject biologic variation" (hereafter referred to as "the 2014 online database"; https://www.westgard.com/biodatabase1.htm#1) was used along with the CV_A value assessed in our study to calculate both RCVs for each amino acid.

The II was deduced from the BV components using Fraser's formula [2], expressed as CV_I/CV_G.

NHSP was calculated as follows [2]:

$$NHSP = \left(Z \times (CV_A^2 + CV_I^2)^{1/2}/D\right)^2$$

where $Z = 1.96$ ($P < 0.05$) and D is the desired percentage closeness to the homeostatic set point (set at 20 %, 10 %, and 5 %).The power of the study design in estimating CV_I was established by considering factors such as the number of replicates, samples, and participants, along with the ratio of CV_A to CV_I, which was introduced by Røraas, *et al.* [21]. In the current study, a power target level of 80 % and above is considered acceptable for accurate determination.

Results

The final population used to derive the BV of plasma FAAs comprised 22 participants (13 women and 9 men). The median age of the women was 30 years (range, 18–41 years), and for men, it was 28 years (range, 25–50 years). The median body mass index (BMI) for the entire study group was 24 kg/m^2 (range, 18–31 kg/m^2). Demographic data and measurement results are presented in Supplemental Data Table 1.

BV estimates were calculated for 22 out of 25 amino acids. After the exclusion of outliers, the number of cysteine results was insufficient (N = 8 participants) for BV estimation. The results for glycine and aspartate were excluded due to high CV_A values (54.1 % and 56.9 %, respectively). For nine amino acids (alanine, arginine, asparagine, glutamine, methionine, lysine, isoleucine, histidine, and taurine), all participants (N = 22) were included in the calculations. For other measurands, a lower number of participants were included, with a minimum of 16 for proline. In sex-stratified groups, a minimum of six men were included for leucine, proline, and tryptophan, while a minimum of 10 women were included for proline. Detailed information on excluded data is provided in Supplemental Data Table 2.

The normality assumption was verified using the Shapiro–Wilk test ($P > 0.05$ for all amino acids). The data were homogeneous for CV_A according to Bartlett's test ($P > 0.05$) and for CV_I according to Cochran's test ($P > 0.05$). In the steady-state analysis, the regression line slope included zero at the 95 % confidence interval, indicating that participants were in a stable condition.

Plasma concentrations of most amino acids, except asparagine, serine, glutamine, lysine, taurine, threonine, and 2-aminobutyric acid, were significantly higher in men than in women (Supplemental Data Fig. 1). CV_I values ranged from 9.5 % (histidine) to 32.5 % (*trans*-4-hydroxyproline). Except for alanine, arginine, glutamic acid, and threonine, CV_I values did not differ between sexes. CV_G values ranged from 8.6 % (phenylalanine) to 50.0 % (glutamic acid), with no significant sex differences observed (Table 1).

II values for all amino acids are presented in Table 1. II values for glutamic acid, glutamine, isoleucine, serine, proline, and tyrosine were ≤0.6, whereas those for lysine and *trans*-4-hydroxyproline were ≥1.4. II values for other amino acids ranged between 0.7 and 1.2. Sex-specific differences in II values were observed for some plasma FAAs (Table 1). Arginine, methionine, and threonine had slightly higher II values in females, whereas phenylalanine, ornithine, taurine, and valine had significantly higher II values in males.

Asymmetrical RCVs, APSs, and NHSPs for each amino acid are presented in Table 2. Except for glutamic acid, tryptophan, and taurine, RCVs in decreasing and increasing directions were similar to those obtained using CV_I values from the online 2014 BV database. The number

Table 1
Biological variation data of PFAAs with 95 % CIs, II, and historical BV database.

Amino acid, μmol/L	Group	Number of participants	Total number of results	Reference interval	Mean, μmol/L (95 % CI)	CV_A (95 % CI), %	CV_I (95 % CI), %	CV_G (95 % CI), %	II	Historical BV database‡ CV_I %, CV_G %
Alanine	All	22	394	200–579	439.2 (437.4 – 441.1)	1.1 (1.0 – 1.2)	17.7 (16.0 – 19.8)§	19.2 (14.3 – 28.1)	0.9	14.7, 55.8
	F	13	232		397.3 (394.6 – 400.0)		20.3 (17.9 – 23.5)	17.8 (11.8 – 30.7)	1.1	
	M	9	162		499.8 ‡ (496.3 – 503.4)		**14.7 (12.6 – 17.6)**	**13.0 (7.9 – 26.1)**	1.1	
Alfa (2)-Aminobutyric acid	All	21	374	9–37	8.1 (8.0 – 8.1)	5.0 (4.6 – 5.6)	**23.0 (20.7 – 25.9)**	**22.4 (16.4 – 33.4)**	1.0	24.7, 32.3
	F	13	232		7.7 (7.6 – 7.8)		25.7 (22.6 – 29.9)	24.4 (16.3 – 41.8)	1.1	
	M	8	142		8.7 (8.6 – 8.8)		18.9 (16.0 – 23.0)	19.0 (11.5 – 40.2)	1.0	
Arginine	All	22	398	32–120	70.6 (70.3 – 70.9)	1.1 (1.0 – 1.3)	21.9 (19.8 – 24.4)§	21.4 (15.8 – 31.4)	1.0	19.3, 34.1
	F	13	236		64.7 (64.3 – 65.1)		25.5 (22.5 – 29.5)	**16.0 (9.7 – 28.7)**	1.6	
	M	9	162		79.2‡ (78.2 – 80.1)		**17.5 (15.0 – 20.9)**	21.9 (14.1 – 43.0)	0.8	
Asparagine	All	22	364	37–92	45.0 (44.9 – 45.2)	1.2 (1.1 – 1.3)	**14.2 (12.8 – 15.9)**	**16.5 (12.2 – 24.7)**	0.9	12.3, 28
	F	13	202		42.3 (42.0 – 42.5)		15.8 (13.7 – 18.5)	13.0 (8.2 – 23.9)	1.2	
	M	9	162		48.4 (48.0 – 48.9)		12.4 (10.7 – 14.8)	17.43 (11.4 – 34.1)	0.7	
Citrulline	All	20	388	17–46	27.4 (27.2 – 27.5)	2.9 (2.6 – 3.3)	**9.8 (8.7 – 11.2)**	14.7 (10.7 – 23.2)	0.7	21.4, 43.9
	F	11	200		26.3 (26.1 – 26.4)		9.9 (8.6 – 11.8)	15.6 (10.4 – 28.9)	0.6	
	M	8	138		29.2‡ (28.9 – 29.5)		9.6 (7.9 – 12.1)	**12.1 (7.1 – 30.5)**	0.8	
Glutamic Acid	All	19	338	13–113	54.5 (53.9 – 55.2)	0.9 (0.8 – 1.0)	16.9 (15.2 – 19.0)§	50.0 (37.6 – 74.2)	0.3	46.4, 79.9
	F	12	220		44.4 (43.5 – 45.2)		20.2 (17.8 – 23.5)	50.3 (35.3 – 85.9)	0.4	
	M	7	118		72.0‡ (70.1 – 73.9)		**13.3 (11.2 – 16.5)**	**38.4 (24.5 – 84.9)**	0.4	
Glutamine	All	22	398	371–957	586.4 (584.1 – 588.6)	0.6 (0.5 – 0.6)	**10.1 (9.1 – 11.3)**	**17.7 (13.5 – 25.5)**	0.6	12.1, 22.0
	F	13	236		560.1 (556.6 – 563.7)		11.1 (9.8 – 12.9)	17.4 (12.2 – 29.1)	0.6	
	M	9	162		624.3 (618.7 – 629.8)		8.7 (7.5 – 10.4)	17.1 (11.3 – 33.1)	0.5	
Histidine	All	22	398	39–123	82.8 (82.6 – 83.0)	0.9 (0.8 – 1.0)	**9.5 (8.6 – 10.6)**	9.3 (6.9 – 13.7)	1.0	9.7, 27.2
	F	13	236		79.9 (79.7 – 80.2)		9.5 (8.4 – 11.0)	9.1 (6.1 – 15.6)	1.0	
	M	9	162		87.0‡ (86.6 – 87.4)		9.5 (8.1 – 11.3)	**7.7 (4.6 – 15.6)**	1.2	
Isoleucine	All	22	390	36–107	73.4 (73.1 – 73.8)	1.5 (1.4 – 1.7)	**12.7 (11.5 – 14.2)**	21.3 (16.1 – 30.7)	0.6	15.5, 45.5
	F	13	232		64.8 (64.4 – 65.3)		12.7 (11.2 – 14.7)	17.9 (12.4 – 30.0)	0.7	
	M	9	158		85.8‡(85.2 – 86.5)		12.5 (10.7 – 15.0)	**14.2 (9.0 – 28.0)**	0.9	
Leucine	All	18	316	68–183	131.0 (130.5 – 131.6)	1.0 (0.9 – 1.1)	**10.5 (9.4 – 11.9)**	15.6 (11.5 – 23.7)	0.7	14.8, 44.0
	F	12	212		121.3 (120.7 – 121.9)		11.7 (10.2 – 13.6)	12.0 (8.0 – 21.0)	1.0	
	M	6	104		150.5‡ (149.1 – 151.8)		8.5 (7.0 – 10.7)	**11.1 (6.6 – 28.1)**	0.8	
Lysine	All	22	398	103–255	151.6 (151.2 – 152.0)	1.4 (1.3 – 1.5)	**14.5 (13.1 – 16.2)**	**10.6 (7.5 – 16.0)**	1.4	11.5, 38.2
	F	13	236		146.9 (146.3 – 147.5)		14.6 (12.8 – 16.9)	11.2 (7.2 – 19.5)	1.3	
	M	9	162		158.4 (157.6 – 159.2)		14.3 (12.3 – 17.2)	8.8 (4.6 – 18.5)	1.6	
Methionine	All	22	398	4–44	26.5 (26.5 – 26.6)	1.5 (1.4 – 1.2)	**13.7 (12.4 – 15.3)**	13.0 (9.6 – 19.2)	1.1	14.7, 43.4
	F	13	236		24.6 (24.5 – 24.7)		14.3 (12.6 – 16.6)	**8.0 (4.6 – 14.6)**	1.8	
	M	9	162						1.1	

(*continued on next page*)

Table 1 (*continued*)

Amino acid, μmol/L	Group	Number of participants	Total number of results	Reference interval	Mean, μmol/L (95 % CI)	CV_A (95 % CI), %	CV_I (95 % CI), %	CV_G (95 % CI), %	II	Historical BV database‡ CV_I %, CV_G %
					29.3‡ (29.1 – 29.5)		13.0 (11.1 – 15.5)	11.4 (6.9 – 23.0)		
Ornithine	All	21	372	38–130	67.1 (66.9 – 67.4)	1.1 (1.0 – 1.3)	**19.7 (17.8 – 22.1)**	18.3 (13.3 – 27.3)	1.1	18.4, 54.9
	F	13	234		62.6 (62.2 – 63.1)		20.8 (18.3 – 24.1)	19.4 (13.0 – 33.3)	1.1	
	M	8	138		74.5‡ (73.9 – 75.1)		18.2 (15.5 – 22.1)	**12.0 (6.2 – 26.7)**	1.5	
Phenylalanine	All	20	360	35–80	46.2 (46.1 – 46.3)	1.6 (1.4 – 1.7)	**10.4 (9.3 – 11.7)**	8.6 (6.1 – 13.1)	1.2	9.5, 40.6
	F	13	234		44.8 (44.7 – 45.0)		10.6 (9.4 – 12.3)	8.8 (5.8 – 15.3)	1.2	
	M	7	124		48.8‡ (48.6 – 49.0)		9.9 (8.4 – 12.2)	**5.3 (2.1 – 13.5)**	1.9	
Proline	All	16	288	97–368	205.0 (203.6 – 206.5)	1.0 (0.9 – 1.2)	**14.6 (13.0 – 16.6)**	24.4 (17.7 – 38.2)	0.6	17.0, 104.4
	F	10	182		182.1 (180.4 – 183.9)		16.0(13.9 – 18.9)	20.4 (13.5 – 36.1)	0.8	
	M	6	106		243.3‡ (239.5 – 247.1)		12.8 (10.7 – 16.1)	**19.5 (11.7 – 48.8)**	0.7	
Serine	All	19	342	63–187	96.7 (96.1 – 97.3)	2.7 (2.4 – 3.0)	**15.9 (14.2 – 17.9)**	**25.4 (18.9 – 38.0)**	0.6	12.8, 42.8
	F	12	218		97.2 (96.2 – 98.3)		17.0 (14.8 – 19.8)	27.3 (18.9 – 47.0)	0.6	
	M	7	124		95.7 (94.2 – 97.2)		13.7 (11.5 – 16.8)	23.9 (14.9 – 53.2)	0.6	
Taurine	All	22	392	42–156	40.2 (40.1 – 40.4)	1.6 (1.4 – 1.8)	**11.1 (10.0 – 12.4)**	**15.5 (11.7 – 22.5)**	0.7	30.6, 44.0
	F	13	230		38.6 (38.3 – 38.9)		11.3 (9.9 – 13.1)	19.2 (13.5 – 32.1)	0.6	
	M	9	162		42.6 (42.4 – 42.8)		10.7 (9.2 – 12.8)	7.3 (4.1 – 15.2)	1.5	
Threonine	All	21	380	85–231	129.8 (129.3 – 130.3)	1.5 (1.4 – 1.7)	17.7 (16.0 – 19.8)§	**16.0 (11.6 – 23.9)**	1.1	17.9, 33.1
	F	12	218		124.5 (123.7 – 125.3)		21.2 (18.6 – 24.7)	15.4 (9.7 – 27.9)	1.4	
	M	9	162		136.8 (135.6 – 137.9)		**12.6 (10.8 – 15.0)**	16.1 (10.4 – 31.5)	0.8	
Trans-4-hydroxyproline	All	21	384	4–29	14.6 (14.6 – 14.7)	3.2 (2.9 – 3.6)	**32.5 (29.3 – 36.4)**	21.3 (14.7 – 32.4)	1.5	34.5, 56.7
	F	13	230		13.2 (13.1 – 13.4)		34.9 (30.7 – 40.5)	20.0 (11.6 – 36.5)	1.8	
	M	8	154		16.6‡(16.5 – 16.8)		29.6 (25.4 – 35.7)	**16.6 (7.9 – 35.8)**	1.8	
Tryptophan	All	18	324	29–77	61.8 (61.6 – 62.0)	1.1 (1.0 – 1.2)	**10.3 (9.3 – 11.7)**	13.5 (9.8 – 20.5)	0.8	22.7, 152.6
	F	12	218		57.9 (57.6 – 58.2)		11.6 (10.1 – 13.5)	11.0 (7.3 – 19.4)	1.0	
	M	6	106		69.6‡ (69.1 – 70.1)		8.2 (6.8 – 10.3)	**8.7 (5.0 – 22.2)**	0.9	
Tyrosine	All	19	342	31–90	61.2 (60.8 – 61.5)	1.2 (1.1 – 1.4)	**11.8 (10.6 – 13.3)**	22.0 (16.4 – 32.8)	0.5	10.5, 61
	F	12	218		55.3 (54.9 – 55.7)		12.6 (11.0 – 14.6)	**18.0 (12.4 – 31.1)**	0.7	
	M	7	124		71.2‡ (70.3 – 72.1)		10.8 (9.1 – 13.3)	18.6 (11.6 – 41.4)	0.6	
Valine	All	18	326	136–309	224.8 (223.9 – 225.7)	1.3 (1.2 – 1.5)	**10.6 (9.5 – 12.0)**	15.4 (11.3 – 23.4)	0.7	10.6, 40.1
	F	11	202		205.6 (204.3 – 206.8)		11.5 (10.0 – 13.4)	14.5 (9.8 – 26.1)	0.8	
	M	7	124		255.2‡ (254.2 – 256.2)		9.5 (8.0 – 11.8)	**4.8 (1.6 – 12.4)**	2.0	

†Results in bold indicate estimates used to calculate APSs. For APS calculation, when the mean values of the sex groups differed significantly, the lowest CV_G estimate was used, and when the CV_I values did not overlap between the sexes, the lowest CV_I value was used.
‡Corte, *et al.* (online 2014 biological variation database, https://www.westgard.com/biodatabase1.htm#1).
‡Indicates a significant difference between women and men in terms of mean values ($P < 0.05$).
§Indicates a significant difference between women and men in terms of CV_I ($P < 0.05$).
PFAAs, plasma-free amino acids; CI, confidence interval; BV, biological variation; All, all participants; F, female; M, male; CV_A, analytical variation; CV_I, within-subject variation; CV_G, between-subject variation; II, index of individuality.

Table 2
Desirable analytical performance specifications, RCV, and NHSP for PFAAs.

Analyte	CV_{APS}, %	B_{APS}, %	TEa_{APS}, %	RCV, % decrease/ increase	RCV†, % decrease/increase	NHSP 20 %	NHSP 10 %	NHSP 5 %	NHSP Corte, *et al.* [11] 20 %
Alanine	7.3	4.9	17.0	–33.7/50.7	–28.8/40.5	4	13	49	2
Alfa (2)-aminobutyric acid	11.5	8.0	27.0	–41.9/72.1	–43.8/78.0	6	22	86	7
Arginine	8.7	5.9	20.3	–36.7/65.8	–35.9/55.9	5	19	74	4
Asparagine	7.1	5.4	17.1	–28.1/39.1	–24.8/33.0	2	8	31	10
Citrulline	4.9	3.9	12.0	–21.1/26.8	–39.1/64.1	1	4	16	5
Glutamic Acid	6.7	10.1	21.1	–32.4/47.9	–64.1/178.5	3	11	44	21
Glutamine	5.0	5.1	13.4	–21.0/26.5	–24.4/32.3	1	4	16	2
Histidine	4.8	3.1	10.9	–19.9/24.9	–20.2/25.3	1	4	14	1
Isoleucine	6.4	4.8	15.3	–25.8/34.7	–30.2/43.2	2	7	26	3
Leucine	5.2	3.8	12.4	–21.7/27.7	–29.0/40.8	2	5	17	2
Lysine	7.2	4.5	16.4	–28.7/40.2	–23.5/30.7	3	9	33	1
Methionine	6.9	4.0	15.3	–27.4/37.7	–28.9/40.6	2	8	30	2
Ornithine	9.9	5.8	22.0	–36.6/57.8	–34.6/52.8	4	15	60	3
Phenylalanine	5.2	2.9	11.5	–21.7/27.6	–20.0/25.0	2	5	17	1
Proline	7.3	6.1	18.1	–28.8/40.4	–32.4/48.0	3	9	33	3
Serine	7.9	7.5	20.6	–31.2/45.3	–26.1/35.3	3	10	40	2
Taurine	5.5	4.8	13.9	–22.9/29.7	–50.1/100.3	2	5	20	9
Threonine	6.3	5.1	15.4	–33.7/50.8	–33.9/51.2	4	13	49	3
Trans-4-hydroxyproline	16.2	9.1	35.9	–52.4/110.1	–54.2/118.4	11	41	164	12
Tryptophan	5.2	3.4	11.9	–21.5/27.3	–40.6/68.3	2	5	17	6
Tyrosine	5.9	5.4	15.2	–24.2/31.9	–21.7/27.7	2	6	22	1
Valine	5.3	2.9	11.7	–22.0/28.1	–21.9/28.0	2	5	18	1

†Reference change values assessed with the same method using within-subject variation estimates from the online 2014 BV database and analytical variations from duplicate analysis of our samples.
CV_{APS}, analytical performance specification for analytical imprecision; B_{APS}, analytical performance specification for bias; TEa_{APS}, analytical performance specification for total allowable error; RCV, reference change values; NHSP, the number of samples required to estimate the homeostatic set points; PFAAs, plasma-free amino acids.

of samples required to assess homeostatic set points within 20 % accuracy ranged from 1 to 4 for most amino acids, except for arginine, 2-aminobutyric acid, and *trans*-4-hydroxyproline (Table 2).

Discussion

This study presents BV data on plasma FAAs based on LC-MS/MS, following methodological procedures aligned with BIVAC standards. In addition to estimates for the entire study group, sex-stratified estimates with 95 % CIs are provided. The 2014 BV database [22] included only one study reporting BV estimates for plasma FAAs, which were not sex-stratified—likely due to a limited number of participants—and did not include CIs for CV_I and CV_G. Additionally, the current online BV database of the EFLM does not contain BV estimates for plasma FAAs [7]. A recent study by Coşkun *et al.* [11] met BIVAC inclusion criteria and adhered to the statistical approaches recommended by EuBIVAS [7]. In that study, amino acid analysis was conducted using serum samples, with BV estimates calculated separately for men and women.

In our study, CV_I values did not differ between sexes except for alanine, arginine, glutamic acid, and threonine. In contrast, Coşkun *et al.* [11] found that for most amino acids, the 95 % CIs of CV_I values did not overlap between sexes, indicating sex-based differences in CV_I estimates, except for aspartic acid, citrulline, and phenylalanine. This discrepancy may be attributable to age differences between female participants in both studies. In Coşkun *et al.* [11], the median age of the women was 23, whereas in our study it was 30. Previous research has shown that plasma amino acid concentrations can vary with age [23]. Sample matrix-related differences have also been observed in blood-free amino acid concentrations, and BIVAC recommends documenting the type of sample material used [6]. However, the extent to which sample matrix differences influence BV parameters remains unclear.

For 14 amino acids, CV_I values derived from our full study group were lower than those reported by Corte *et al.* [10]. The CV_I estimates for essential amino acids were significantly lower than those for non-essential amino acids ($P = 0.03$), aligning with previously reported findings [10,11]. Non-essential amino acids are more susceptible to changes in physiological conditions [24,25], suggesting that differences in metabolic regulation may explain this trend [10]. CV_I values for non-essential amino acids were higher than those in the 2014 database, except for glutamine and proline. However, CV_I values for essential amino acids were lower than those reported by Corte *et al.* [10], except for phenylalanine and lysine.

Differences in study populations and experimental designs may account for these variations. Additionally, dietary habits, cultural differences, socioeconomic status, and the composition of animal- and plant-based foods in the diet can influence plasma FAA concentrations. Our study analyzed plasma samples from 22 healthy adults (13 women and 9 men), collected weekly over an average period of nine weeks. All samples were processed in duplicate during continuous analysis. In contrast, Corte *et al.* [10] examined plasma samples from 11 healthy adults (8 women and 3 men) collected once a week over five weeks using RP-HPLC, with only the first sample from each participant analyzed in duplicate. Furthermore, that study had limitations related to BIVAC grading.

Assessment of CV_G values in amino acid subgroups revealed no sex-related differences, consistent with recent findings [11], except for arginine, which exhibited a higher CV_G in men. Our estimations showed that phenylalanine had the lowest CV_G, lower even than CV_I. This supports the recommendation to use diagnostic cutoff values for classifying hyperphenylalaninemia due to the weak individuality of phenylalanine.

Amino acid analysis is recognized for its high accuracy and precision in detecting subtle alterations linked to organ dysfunction [1]. However, only two studies have focused on analytical performance goals for blood amino acids [10,11]. The first strategic conference of the European Federation of Clinical Chemistry and Laboratory Medicine proposed three models for defining analytical performance goals: the clinical outcome-based model, the BV-based model, and the state-of-the-art-based model [26,27]. While deriving APSs from clinical outcomes is considered ideal, this approach is only feasible for phenylalanine and tyrosine [28]. Due to the scarcity of relevant studies or the availability of data primarily from specific clinical settings, the general applicability of this approach in laboratories is limited. The BV-based model offers broader applicability but requires data from high-quality studies. In this study, BV data were generated following rigorous procedures

recommended by the EFLM WG-BV [7]. The CV_A obtained from duplicate measurements met the APS for imprecision, defined as half the CV_I.

Additionally, statistical power was assessed according to Røraas *et al.* [21]. The power of the overall study was 0.11 (range, 0.05–0.29), while sex-stratified estimates yielded values of 0.10 (range, 0.04–0.29) for women and 0.12 (range, 0.06–0.30) for men, indicating sufficient statistical power. A power threshold of 80 % or higher is generally considered adequate for accurately determining CV_I values. In the study by Røraas et al. [21], if the CV_A/CV_I ratio equals 1, the statistical power of detecting within-subject biological variation in samples collected over two weeks from 10 participants is 67 %. To increase this beyond 80 %, three replicates of two-week samples or four weeks of sample collection are necessary. If the CV_A/CV_I ratio is less than 1, the statistical power is considered 100 %. This study included 22 participants with a minimum follow-up of eight weeks, and the CV_A/CV_I ratio for each analyte was at most 0.30, ensuring full statistical power (100 %). Despite having only nine male participants, the eight-week follow-up period and CV_A/CV_I ratios ranging between 0.06 and 0.30 suggest full power for the male subgroup. Consequently, the desirable imprecision, bias, and total error goals provided for plasma FAAs (Table 2) may contribute to laboratory quality management.

The BV-derived II serves as a crucial indicator for evaluating the utility of reference intervals [2]. If the II is $\leq$0.6, conventional reference intervals have limited utility in identifying unusual results for an individual [2]. Conversely, if the II is $\geq$1.4, conventional reference intervals are appropriate for interpreting test results [2]. Intermediate II values constitute the gray zone [29], where population-based reference intervals should be used with caution. Further analysis of sex-stratified IIs revealed sex-specific differences in plasma FAAs. Based on these findings, sex-specific indices of individuality are recommended for accurately assessing the relevance of population-based reference intervals for plasma FAAs. Specifically, conventional reference intervals are advised for arginine, methionine, and threonine in women and phenylalanine, ornithine, taurine, and valine in men. These variations in biological variation and II values between sexes may be attributed to fundamental physiological differences. For instance, sex differences in gut microbiota composition can influence amino acid digestion and absorption. Several studies have demonstrated microbial composition differences between men and women [30]. Additionally, discrepancies in total muscle and fat composition and circulating hormone levels may contribute to sex-based II variations [31].

RCV has been suggested as a useful metric for detecting significant changes between consecutive test results with potential clinical implications [2]. Fokkema *et al.* [20] introduced an asymmetric RCV calculation method, yielding distinct RCV values for decreasing and increasing changes to account for skewed distributions of laboratory measurands [17]. The EFLM Biological Variation Database has adopted the asymmetrical RCV approach [7]. In this study, histidine exhibited the lowest asymmetrical RCV (−19.9 %/+24.9 %), while *trans*-4-hydroxyproline had the highest (−52.4 %/+110.1 %).

The homeostatic set point represents the physiological value around which a measurand naturally fluctuates. Replicate measurements are typically required to achieve an estimate within a predetermined deviation from its true value [2]. In this study, the number of samples required to assess an individual's homeostatic set point within 20 % accuracy ranged from one to four for most amino acids. For amino acids with pronounced individuality, the NHSP values may facilitate personalized reference interval calculations [4,32].

One limitation of this study is the inclusion of only adults. BV parameters are known to be influenced by age [2]. Carobene *et al.* [33] reported age-dependent variations in BV for certain analytes. Plasma FAA concentrations fluctuate with age, and age-specific reference intervals have been recommended [1,34]. Given that BV parameters for plasma FAAs may differ in children, these findings cannot be extrapolated to pediatric populations. Ethical considerations prevented the inclusion of children in this long-term study involving weekly blood sampling, although plasma FAA analysis has significant clinical relevance for adults.

Another limitation is the lack of ethnic diversity in the study population. Metabolomics research has identified substantial differences in amino acid metabolism across ethnic groups [35]. However, Lawton *et al.* [36] reported minimal effects of ethnicity on plasma FAA concentrations among healthy Europeans, African Americans, and Hispanics. A study comparing plasma FAA profiles among Korean, Chinese, and Japanese populations in East Asia found no significant differences [37]. Future research incorporating diverse populations is necessary to examine the influence of sociodemographic factors and dietary habits on BV components of plasma FAAs.

In conclusion, this BIVAC-compliant study provides updated BV data for plasma FAAs. No significant sex differences were observed in CV_I and CV_G estimates for most plasma FAAs. However, sex differences in CV_I estimates were noted for alanine, arginine, glutamic acid, and threonine. These findings suggest that sex-stratified indices of individuality are necessary for accurately assessing the relevance of population-based reference intervals for plasma FAAs.

Ethics statement

The study protocol was approved by the Ethics Committee of Afyonkarahisar Health Sciences University Hospital, Afyonkarahisar, Turkiye (approval No.: 2022/4), and informed consent was obtained from all participants.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2025.04.010.

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