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Determination of Selected Amino Acids in Serum of Patients with Liver Disease

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. The determination of amino acids can be a reliable approach for extended diagnosis of liver diseases. This is because liver disease can be a cause of impaired amino acid metabolism. Therefore, a method for the determination of serum amino acids, applicable for clinical purposes, is necessary.
Objectives. The aim of this study was to find differences in the levels of selected amino acids between patients with liver disease and a control group.
Material and Methods. Samples of peripheral venous blood were obtained from a group of patients with liver disease (n = 131, 59 women at an average age of 60 years and 72 men at an average age of 52 years) and a control group (n = 105, 47 women at an average age of 62 years and 58 men at an average age of 58 years). Before the separation, the amino acids were derivatized with naphthalene-2,3-dicarboxaldehyde. For the separation, reverse phase column was used. The effluent was monitored with a fluorescence detector.
Results. There were significant differences in the concentrations of some amino acids between the patients and the control group, but also between women and men. Correlations between some amino acids and markers of liver blood tests and lipid metabolism were observed.
Conclusions. A simple, relatively rapid and selective HPLC method with fluorescence detection for the determination of selected amino acids in serum has been developed (**Adv Clin Exp Med 2016, 25, 6, 1227–1239**).

Key words: amino acids, HPLC with fluorescence detection, patients with liver disease.

Amino acids have various functions in the body. Serum amino acids are practically determined by the metabolism of the liver and skeletal muscle. In many papers, the levels of some amino acids, especially branched chain amino acids (BCAA; valine, leucine, isoleucine), the aromatic amino acids (phenylalanine and tyrosine) and methionine were different between patients with different liver diseases and a control group [1–7].

Fast and accurate determination of amino acids in the serum or plasma of patients with liver disease is essential for effective diagnosis and treatment monitoring. Techniques used to determine the serum or plasma amino acid levels include high-performance liquid chromatography (HPLC) [8, 9], gas chromatography [10], high-performance capillary electrophoresis [11], and tandem mass spectrometry [12, 13]. Although tandem mass spectrometry is very sensitive and specific, not all laboratories are equipped with such an expensive piece of equipment. One of the most popular methods for the determination of amino acids in serum or plasma is HPLC using pre-column or post-column derivatization with o-phthalaldehyde (OPA) [14], naphthalenedicarboxaldehyde (NDA) [15], dansyl chloride [16], 9-fluorenylmethyl chloroformate [17], 9-fluorenylmethoxycarbonyl chloride [18], 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate [19] or phenyl isothiocyanate [20].

The aim of this study was to find differences in the levels of selected amino acids between patients with different liver diseases, but with normal or slightly increased liver blood tests, and a control group.

Material and Methods

All amino acids, boric acid, potassium cyanide, NDA, sodium dihydrogen phosphate, sodium acetate, ammonium acetate, sodium hydroxide, hydrochloric acid, metaphosphoric acid, perchloric acid, trichloroacetic acid and 5-sulfosalicylic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA), and HPLC gradient grade methanol, ethanol and acetonitrile from Merck KGaA (Darmstadt, Germany). All the others chemicals were of analytical grade.

Chromatographic analysis was performed using a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-20AD solvent delivery system, SIL-20AC autosampler, CTO-20AC column oven, RF-20A fluorescence detector and CBM-20A system controller. The data was collected digitally using LCsolution software (v. 1.25).

Samples of peripheral venous blood were obtained from a group of patients with liver disease (n = 131, 59 women at an average age of 60 years and 72 men at an average age of 52 years) and a control group (n = 105, 47 women at an average age of 62 years and 58 men at an average age of 58 years) under standard conditions, from 7 to 8 a.m. after fasting. The blood was collected into plastic tubes with gel (Vacuette Detection Tube, No. 455077, Greiner Labortechnik Co., Kremsmünster, Austria). The serum was separated from the clot by centrifugation (1700 × g, 15 min, 8°C) and immediately stored at –80°C. Patients with liver disease were divided into the following 3 groups: group 1 (n = 27, 14 women and 13 men) with viral hepatitis; group 2 (n = 48, 15 women and 33 men) with alcoholic liver disease (alcoholic hepatitis); group 3 (n = 56, 30 women and 26 men) with other diseases of the liver (K730, chronic persistent hepatitis, not elsewhere classified; K760, fatty change of liver, not elsewhere classified). None of the participants of the control group had a serious or chronic disease. Written informed consent was obtained from all participants before starting the protocol and the Hospital Committee on Human Research (Regional Hospital of Pardubice, Czech Republic) approved the study. The group characteristics are shown in Table 1.

L-norleucine was used as an internal standard. It is not present in human serum and its chemical properties are quite similar. For the analysis of blood serum, 20 µL of the internal standard of norleucine solution in deionized water (≈ 900 µmol/L) was pipetted into a well-capped 1.5-mL polypropylene (PP) tube. 100 µL of serum was added and the content mixed vigorously on a vortex mixer for 60 s. 900 µL of cold ethanol was added and the solution was vortexed for 60 s, incubated (5 min, –20°C) and centrifuged (30 000 × g, 10 min, 4°C). 100 µL of the supernatant was transferred into a 1.5-mL well-capped amber glass vial. 300 µL of borate buffer (0.19 g of boric acid in 30 mL of deionized water; pH adjusted to 9.3 with 3 mol/L sodium hydroxide), 20 µL of a solution of potassium cyanide in deionized water (40 mmol/L), and 20 µL of a solution of 0.1% (m/v) NDA in ethanol were added, the mixture was vortexed for 60 s, incubated in the dark at 25°C for 15 min, then filtered through a nylon filter (pore size 0.20 µm, 4 mm diameter; Supelco, Bellefonte, PA, USA) and transferred into a 1.0-mL amber vial. The stock solutions of amino acids and norleucine (containing ≈ 5000 µmol/L of each) were prepared in deionized water (except tyrosine, which was prepared in 1 mmol/L hydrochloric acid) and stored at –80°C until used. The combined working solution of amino acids was prepared daily. It was diluted with deionized water to give a series of combined working standards. The prepared standards were subjected to the same procedure as described above for blood serum. For the recovery experiment, 10 µL of combined solution of individual amino acids at different concentrations was added to 90 µL of blood serum. The next steps were the same as for blood serum sample preparation.

The chromatographic analysis of the selected amino acids, after their derivatization with NDA to form relatively stable, highly fluorescent 1-cyano-2-substituted-benz[*f*]isoindole (CBI) derivatives, was accomplished using a gradient elution on a Discovery® Bio Wide Pore C18, 150 mm × 4.6 mm, 5 µm, analytical column, fitted with a Discovery® Bio Wide Pore C18, 20 mm × 4.6 mm, 5 µm, guard column (Supelco, Bellefonte, PA, USA) at 37°C. Mobile phase A was the mixture of 10 mmol/L of sodium dihydrogen phosphate-ethanol (80 : 20, v/v), pH 7.2 ± 0.1, and mobile phase B was the mixture of 10 mmol/L of sodium dihydrogen phosphate-ethanol (50 : 50, v/v), pH 7.2 ± 0.1. Prior to use, both phases were vacuum filtered and degassed ultrasonically. The gradient was applied in the following sequences: from 0 to 15 min – 10% to 20% B (linear gradient); 15 to 35 min – 20% to 100% B (linear gradient); 35 to 40 min – 100% B; and 40 to 45 min – 10% B. The flow rate was kept constant at 0.75 mL/min. Optimum response of the fluorescent derivative was observed when the excitation and emission wavelengths were set at 420 nm and 480 nm, respectively. The amount of amino acids was quantified from a peak area ratio of individual amino acid/internal standard using LC-solution chromatography software (Shimadzu, Kyoto, Japan). The concentrations of amino acids in the samples were determined from the calibration curve. The optimization of the derivatization

Table 1. Group characteristics

	Patients with liver disease			Control group			p-value (1 vs 3)	p-value (2 vs 4)
	women (1; n = 59)	**men (2; n = 72)**	**p-value (1 vs 2)**	**women (3; n = 47)**	**men (4; n = 58)**	**p-value (3 vs 4)**		
Age (years)	60 (14)	52 (21)		62 (22)	58 (24)			
Asp (μmol/L)	12.3 (4.6)	13.0 (6.2)	–	15.9 (4.5)	16.1 (9.6)	–	***	–
Aspn (μmol/L)	56.2 (10.7)	60.6 (11.8)	***	58.3 (11.2)	64.5 (13.3)	*	–	–
Glu (μmol/L)	65.8 (22.3)	76.7 (32.0)	**	48.7 (21.4)	62.6 (29.0)	*	***	**
Glun (μmol/L)	645.8 (101.9)	656.1 (124.0)	–	617.0 (98.1)	683.4 (109.5)	**	–	–
Asn (μmol/L)	44.7 (8.7)	47.0 (12.3)	**	41.6 (7.6)	46.6 (11.3)	**	–	–
His (μmol/L)	102.3 (17.1)	108.8 (25.0)	*	94.1 (22.8)	102.4 (23.4)	–	–	–
Gln (μmol/L)	584.0 (105.3)	575.2 (137.6)	–	563.9 (106.6)	617.9 (106.0)	*	–	*
Ser (μmol/L)	122.5 (35.6)	121.4 (30.6)	–	124.2 (38.0)	115.0 (30.3)	–	–	–
Arg (μmol/L)	51.6 (18.4)	52.7 (15.1)	–	63.6 (14.6)	62.4 (29.0)	–	***	–
Cit (μmol/L)	31.5 (10.1)	31.7 (11.8)	–	32.5 (16.3)	32.0 (15.7)	–	–	–
Gly (μmol/L)	239.7 (70.0)	228.0 (53.3)	–	282.1 (148.3)	271.1 (67.0)	*	*	–
Thr (μmol/L)	116.8 (30.8)	127.5 (39.9)	*	108.9 (42.4)	127.6 (47.5)	–	–	–
Tyr (μmol/L)	66.7 (21.2)	68.4 (25.7)	–	58.9 (18.0)	61.8 (18.1)	–	**	–
Tau (μmol/L)	137.0 (41.4)	132.1 (62.9)	–	120.0 (39.8)	133.7 (58.5)	–	–	–
Ala (μmol/L)	404.9 (152.3)	423.5 (177.0)	–	406.4 (165.4)	438.4 (136.5)	–	–	–
Htau (μmol/L)	3.0 (1.3)	3.6 (1.9)	*	3.3 (1.4)	4.1 (2.4)	*	–	–
2-AB (μmol/L)	18.1 (9.7)	19.6 (8.8)	–	16.6 (5.9)	20.6 (9.3)	*	–	–
Trp (μmol/L)	26.1 (10.7)	31.0 (10.4)	**	22.9 (9.9)	27.3 (8.6)	–	–	*
Met (μmol/L)	23.7 (6.4)	29.1 (8.5)	***	22.7 (3.9)	27.1 (6.6)	***	–	–
Val (μmol/L)	236.2 (61.8)	265.0 (86.8)	**	213.1 (54.2)	297.2 (60.4)	***	–	–
Phe (μmol/L)	67.4 (13.1)	74.3 (21.6)	***	67.4 (9.9)	78.5 (19.0)	***	–	–
Ile (μmol/L)	59.5 (24.5)	74.3 (24.2)	***	56.6 (12.6)	79.1 (26.3)	***	–	–
Leu (μmol/L)	119.4 (37.7)	144.0 (40.0)	***	124.0 (24.3)	154.1 (43.9)	***	–	–
BCAA/AAA	3.14 (0.71)	3.33 (0.86)	*	3.20 (0.53)	3.43 (0.91)	*	–	–
TC (mmol/L)	5.4 (1.7)	5.0 (1.2)	–					
TG (mmol/L)	1.00 (0.70)	1.00 (0.60)	–					
ALT (μkat/L)	0.40 (0.29)	0.51 (0.44)	–					
AST (μkat/L)	0.45 (0.31)	0.54 (0.34)	–					
ALP (μkat/L)	1.08 (0.54)	1.13 (0.50)	–					
GMT (μkat/L)	0.28 (0.58)	0.82 (1.44)	*					
TBIL (μmol/L)	12.0 (14.4)	16.2 (16.3)	–					

Data are expressed as median value with estimated interquartile range (IQR) in parentheses; statistical significance of a difference: *$p < 0.050$; **$p < 0.010$; ***$p < 0.001$ (Mann-Whitney rank sum test); BCAA – branched-chain amino acids (valine, leucine, isoleucine); AAA – aromatic amino acids (tyrosine, phenylalanine).

procedure (the optimum reaction time and concentrations of the components) to obtain the maximum CBI derivative formation at room temperature was examined. A maximum fluorescence was reached after 15 min with optimal composition of the derivatization reagent: 59 μg/mL of NDA and 153 μg/mL of potassium cyanide in 100 mmol/L borate buffer, pH 9.3. The mobile phase was optimized in order to obtain the best separation of the CBI derivatives of individual amino acids in the shortest time. The combined working solution of amino acids and blood serum was used for studying the mobile phase composition. Several mobile phases (namely different buffers containing ethanol) and gradients were assessed. The separation was optimized after studying the effect of ethanol concentration. The column temperature was changed from 20 to 45°C. The criteria used were the resolution, stability of fluorescence and analysis speed. To determine the same-day precision, the pooled blood serum sample was analyzed 10 times (10 samples of the same blood serum) in the same day under the same conditions. Similarly, data on the between-day precision was obtained using blood serum, analyzed on 10 different days. In the recovery experiment, the blood serum sample was spiked with a low, a medium, and a high concentration of the combined working solution of amino acids based on the dynamic range of the assay.

The fasting serum levels of total cholesterol (TC), triglycerides (TG), total bilirubin (TBIL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma-glutamyl transpeptidase (GGT) were determined by standard procedures using an automatic biochemistry analyzer, UniCel DxC 800 (Beckman Coulter, Pasadena, USA).

The data were analyzed using Sigmastat v. 3.5 (Systat Software Inc., Point Richmond, USA) and STATISTICA v. 12 (StatSoft CR s.r.o., Prague, Czech Republic). The data are presented as median and IQR (interquartile range). The differences between women and men and between the patients with liver disease and a control group were analyzed using the Mann-Whitney Rank Sum Test. A two-factor analysis of variance (ANOVA) was performed to investigate of changes in levels of serum amino acids as a function of age, gender and disease. *Post-hoc* comparisons were made using the Holm Sidak test, with alpha set at 0.05. The Holm Sidak test can be used for both pairwise comparisons and comparisons vs. a control group. It is more powerful than the Tukey and Bonferroni test, and, consequently, it is able to detect differences that these tests do not. Analyses of correlation were carried out using a Spearman rank test. Values were considered significant at $p < 0.050$.

Results

The following different precipitant reagents were added to blood serum and the standard solution of amino acids: MPA (10%), PCA (1 mol/L), SSA (10%), TCA (10%) and ethanol. Amino acids in blood serum precipitated with acids and ethanol were stable at 4°C for at least 24 h. Blood serum sample storage at 4°C can result in a decrease of asparagine and glutamine and an increase of glutamic and aspartic acids. These amino acids were only stable for up to 1 h in blood serum when stored at 4°C. NDA, in the presence of potassium cyanide, reacts with amino acids at room temperature forming CBI derivatives in a quick and simple reaction. The stability of CBI derivatives was more than 12 h. OPA, in the presence of thiols, reacts with amino acids similarly. However, one disadvantage is the instability of the isoindolic derivatives. We found that the isoindolic derivatives are stable for about half an hour, and we observed about a 30% decrease in fluorescence intensity (the excitation and emission wavelengths were set at 350 nm and 420 nm).

The described HPLC with fluorescence detection is able to detect and quantify 21 amino acids in serum within a total run period of 45 min. Highly fluorescent CBI derivatives of selected amino acids were separated on a reversed-phase column using a gradient system of ethanol and sodium dihydrogen phosphate. Using an eco-friendly mobile phase is preferred, because organic solvents such as acetonitrile and methanol are considered to be significant pollutants. According to our results, we can conclude that the presented method is highly robust.

The precision of the amino acid analyses and the spike recoveries for blood serum are summarized in Table 2. The coefficients of variation were < 10%. The calibration curves (10-point for determining analytical parameters and 6-point for routine analysis) were linear over the whole tested ranges. Calibration curve parameters obtained as an average of ten standard curves, linearity, limit of quantification (LOQ) and limit of detection (LOD) are given in Table 2.

One hundred and thirty one patients with liver disease were included in this study. The comparison of the patients with liver disease and the control group is given in Table 1. No significant differences in the concentrations of most of the serum amino acids were observed between the patients with liver disease and the control group. The levels of some amino acids (glutamine in men, arginine and glycine in women) were statistically significantly decreased, and tyrosine in women and tryptophan in men increased. In pa-

Table 2. Precision, recovery, the limit of quantification, the limit of detection and average parameters of 10 calibration curves for the method of high-performance liquid chromatography with fluorescence detection

AA	Intra-assay (n = 10) CV%	Inter-assay (n = 12) CV%	Recovery % (CV%)	LOQ µmol/L (fmol)	LOD µmol/L (fmol)	The working range of the calibration curve (µmol/L)	Mean slope (SD)	Intercept (SD) (µmol/L)	Correlation coefficient
Asp	4.2	9.7	97.3 (9.1)	0.7 (81)	0.2 (27)	0.7–12.3	0.0052 (0.0003)	0.0008 (0.0004)	0.9992
Glu	4.9	7.7	96.6 (8.7)	2.2 (240)	0.7 (79)	2.2–164.5	0.0058 (0.0002)	0.0018 (0.0012)	0.9998
Asn	2.5	5.4	103.8 (7.8)	6.9 (763)	2.3 (252)	6.9–127.7	0.0042 (0.0004)	0.0074 (0.0029)	0.9991
His	1.7	4.5	102.7 (7.8)	4.3 (475)	1.4 (157)	4.3–198.6	0.0040 (0.0003)	0.0031 (0.0017)	0.9997
Gln	1.5	3.1	99.7 (9.0)	19.1 (2123)	6.3 (700)	19.1–1246.0	0.0052 (0.0001)	0.0115 (0.0100)	0.9998
Ser	3.4	5.4	97.5 (7.9)	5.2 (579)	1.7 (191)	5.2–269.9	0.0054 (0.0001)	–0.0022 (0.0028)	0.9999
Arg	2.4	3.0	95.6 (8.2)	2.2 (246)	0.7 (81)	2.2–170.6	0.0056 (0.0001)	–0.0001 (0.0012)	0.9999
Cit	3.1	2.9	103.5 (8.0)	1.1 (122)	0.4 (40)	1.1–69.1	0.0060 (0.0001)	–0.0004 (0.0007)	0.9999
Gly	4.3	3.4	102.4 (7.5)	10.1 (1125)	3.3 (371)	10.1–689.2	0.0064 (0.0001)	–0.0063 (0.0064)	0.9999
Thr	2.5	4.1	101.5 (6.1)	7.6 (847)	2.5 (279)	7.6–310.3	0.0050 (0.0002)	0.0002 (0.0038)	0.9998
Tyr	1.0	3.8	103.2 (6.6)	3.5 (385)	1.1 (127)	3.5–139.7	0.0055 (0.0002)	0.0022 (0.0019)	0.9997
Tau	1.2	2.6	103.8 (6.1)	3.6 (405)	1.2 (134)	3.6–219.8	0.0058 (0.0001)	–0.0018 (0.0021)	0.9999
Ala	3.0	3.1	105.1 (7.4)	19.8 (2209)	6.5 (729)	19.8–874.4	0.0059 (0.0001)	0.0120 (0.0116)	0.9999
Htau	5.3	4.8	104.8 (6.2)	0.4 (42)	0.1 (14)	0.4–15.7	0.0045 (0.0001)	–0.0002 (0.0002)	0.9998
2-AB	1.5	4.1	103.2 (5.1)	1.1 (127)	0.4 (42)	1.1–65.4	0.0062 (0.0001)	–0.0002 (0.0007)	0.9999
Trp	6.7	9.5	92.1 (8.5)	3.9 (433)	1.3 (143)	3.9–112.1	0.0030 (0.0003)	0.0025 (0.0011)	0.9992
Met	2.2	3.8	101.1 (3.7)	1.1 (120)	0.4 (40)	1.1–74.7	0.0056 (0.0001)	–0.0002 (0.0006)	1.0000
Val	1.1	5.1	104.0 (5.2)	14.1 (1567)	4.6 (517)	14.1–500.9	0.0057 (0.0003)	0.0159 (0.0081)	0.9991
Phe	1.1	5.3	102.4 (5.5)	3.6 (404)	1.2 (133)	3.6–148.0	0.0054 (0.0002)	0.0044 (0.0020)	0.9993
Ile	1.2	3.9	104.2 (6.2)	3.0 (338)	1.0 (112)	3.0–174.3	0.0058 (0.0001)	0.0028 (0.0018)	0.9997
Leu	2.2	2.6	103.9 (6.3)	3.7 (413)	1.2 (136)	3.7–286.3	0.0062 (0.0001)	–0.0004 (0.0023)	1.0000

Limit of quantification (LOQ) and limit of detection (LOD) were calculated using the following equations: LOQ = 10 S_a/b; LOD = 3.3 S_a/b where S_a is standard deviation of the intercept and b is slope of the calibration curve; the x-intercept (in µmol/L) is the point at which the line crosses the x axis (where the y value equals 0).

tients with liver disease, there were also significantly decreased levels of aspartate in women and increased concentrations of glutamate in both women and men. We do not attach so much importance to these results, because increased levels of glutamic and aspartic acids can result in deamination of glutamine and asparagine during blood serum sample processing and storage. The typical changes, published in many studies, are increased concentrations of one or both of aromatic amino acids (AAA) tyrosine and phenylalanine together with methionine, and decreased concentrations of BCAA. This pattern was not confirmed in the patients with liver disease in the presented study. On the other hand, statistically significant differences in the levels of many amino acids were observed between women and men both in the patients with liver disease and the control group. These are primarily the amino acids phenylalanine, methionine, valine, isoleucine and leucine.

ANOVA was performed to examine the main effects of gender, age, and interaction (time x age) on the measured variables both in patients with liver disease and in the control group (Table 3). There was a significant main effect of gender in the ANOVA of the sum of glutamate and glutamine, methionine, valine, phenylalanine, isoleucine and leucine, and the ratio of BCAA/AAA in controls and glycine, tryptophan, valine, phenylalanine, isoleucine and leucine in patients.

Table 3. Summary table for the two factor analysis of variance (ANOVA) investigating the changes in levels of amino acids in patients with liver disease and a control group as a function of gender and age

Set	Factor	Patients with liver disease				Control group			
		F	p-value	power (1-β)	conclusion	F	p-value	power (1-β)	conclusion
Aspn (μmol/L)	gender	8.196	0.006	0.762	–	4.155	0.055	0.381	–
	age	0.602	0.944	0.050	–	1.110	0.408	0.077	–
	gender* age	0.597	0.947	0.050	–	1.355	0.253	0.158	–
Glun (μmol/L)	gender	0.694	0.408	0.050	–	21.946	< 0.001	0.996	significant
	age	1.378	0.132	0.289	–	1.667	0.133	0.289	–
	gender*age	1.445	0.105	0.338	–	1.928	0.077	0.411	–
His (μmol/L)	gender	8.476	0.005	0.779	–	2.417	0.136	0.193	–
	age	0.791	0.768	0.050	–	1.928	0.077	0.412	–
	gender*age	1.218	0.248	0.162	–	2.504	0.024	0.660	–
Ser (μmol/L)	gender	1.402	0.241	0.090	–	0.317	0.580	0.050	–
	age	1.889	0.016	0.698	–	1.686	0.127	0.298	–
	gender*age	1.415	0.119	0.313	–	1.603	0.152	0.261	–
Arg (μmol/L)	gender	0.535	0.467	0.050	–	1.572	0.224	0.105	–
	age	0.780	0.782	0.050	–	1.369	0.246	0.163	–
	gender*age	0.775	0.787	0.050	–	1.795	0.101	0.349	–
Cit (μmol/L)	gender	0.168	0.683	0.050	–	0.020	0.890	0.050	–
	age	0.742	0.825	0.050	–	0.546	0.904	0.050	–
	gender*age	0.769	0.795	0.050	–	0.792	0.692	0.050	
Gly (μmol/L)	gender	13.768	< 0.001	0.956	significant	3.102	0.093	0.268	–
	age	2.493	< 0.001	0.943	significant	0.971	0.524	0.050	–
	gender*age	1.663	0.042	0.526	–	1.433	0.215	0.188	–
Thr (μmol/L)	gender	2.992	0.089	0.268	–	1.844	0.190	0.133	–
	age	0.747	0.820	0.050	–	0.935	0.557	0.050	–
	gender*age	0.496	0.985	0.050	–	1.218	0.332	0.109	–
Tyr (μmol/L)	gender	1.756	0.190	0.128	–	5.871	0.025	0.551	–
	age	0.590	0.951	0.050	–	0.833	0.653	0.050	–
	gender*age	0.728	0.840	0.050	–	1.537	0.174	0.231	–
Tau (μmol/L)	gender	0.490	0.487	0.050	–	0.120	0.733	0.050	–
	age	0.879	0.653	0.050	–	1.243	0.317	0.117	–
	gender*age	1.006	0.481	0.052	–	1.482	0.195	0.208	–
Ala (μmol/L)	gender	2.569	0.114	0.219	–	0.229	0.638	0.050	–
	age	1.051	0.424	0.070	–	0.750	0.733	0.050	–
	gender*age	1.051	0.424	0.070	–	0.434	0.963	0.050	–
Htau (μmol/L)	gender	6.072	0.017	0.598	–	4.500	0.047	0.417	–
	age	1.394	0.129	0.295	–	1.488	0.192	0.211	–
	gender*age	1.535	0.073	0.415	–	1.549	0.170	0.237	–
2-AB (μmol/L)	gender	1.367	0.247	0.086	–	2.803	0.110	0.235	–
	age	0.843	0.701	0.050	–	0.961	0.533	0.050	–
	gender*age	1.303	0.183	0.222	–	1.201	0.344	0.104	–
Trp (μmol/L)	gender	12.706	< 0.001	0.938	significant	1.726	0.204	0.121	–
	age	0.942	0.566	0.050	–	1.005	0.494	0.051	–
	gender*age	1.173	0.289	0.133	–	1.656	0.136	0.284	–
Met (μmol/L)	gender	8.101	0.006	0.756	–	20.418	< 0.001	0.993	significant
	age	0.511	0.982	0.050	–	0.927	0.564	0.050	–
	gender*age	0.459	0.992	0.050	–	1.443	0.211	0.192	–

Table 3. Summary table for the two factor analysis of variance (ANOVA) investigating the changes in levels of amino acids in patients with liver disease and a control group as a function of gender and age – cont.

Set	Factor	Patients with liver disease				Control group			
		F	p-value	power (1-β)	conclusion	F	p-value	power (1-β)	conclusion
Val (μmol/L)	gender age gender*age	16.177 0.968 1.666	< 0.001 0.531 0.042	0.981 0.050 0.528	significant – –	77.802 2.362 3.787	< 0.001 0.032 0.002	1.000 0.605 0.941	significant – significant
Phe (μmol/L)	gender age gender*age	11.171 0.674 0.762	0.001 0.892 0.802	0.898 0.050 0.050	significant – –	41.913 3.824 2.958	< 0.001 0.002 0.010	1.000 0.949 0.803	significant significant significant
Ile (μmol/L)	gender age gender*age	25.248 0.772 1.613	< 0.001 0.791 0.052	0.999 0.050 0.483	significant – –	37.892 0.548 1.400	< 0.001 0.902 0.231	1.000 0.050 0.175	significant – –
Leu (μmol/L)	gender age gender*age	23.374 0.911 1.592	< 0.001 0.609 0.057	0.999 0.050 0.465	significant – –	65.287 1.948 3.514	< 0.001 0.074 0.004	1.000 0.421 0.909	significant – significant
BCAA/ AAA	gender Age Gender*Age	5.388 1.165 0.950	0.024 0.297 0.555	0.533 0.128 0.050	– – –	10.606 1.236 1.692	0.004 0.321 0.126	0.853 0.115 0.301	significant – –

Aspn – aspartic acid + asparagine; Glun – glutamic acid + glutamine; 2-AB – 2-aminobutyric acid; BCAA – branched-chain amino acids; AAA – aromatic amino acids (phenylalanine and tyrosine).

The effect of age in the ANOVA of phenylalanine in controls and glycine in patients was also observed. ANOVA was also performed to examine the main effects of gender, diagnosis, and interaction (gender x diagnosis) on the measured variables in patients with liver disease (Table 4). The patients with liver disease were divided into the following 3 groups: group 1 (viral hepatitis); group 2 (alcoholic liver disease); group 3 (other diseases of liver). An effect of the type of diagnosis in the ANOVA of methionine and ratio of BCAA/AAA was observed.

Analyses of correlation were carried out using a Spearman rank test (Fig. 1, 2). The degree of correlation is represented by the number of asterisks (*), a positive correlation is indicated with the symbol (+), a negative correlation with (–). There were significant correlations between individual amino acids both in patients with liver disease and controls. In the patients with liver disease, significant correlations between some amino acids and biochemical markers (TC, TG, ALT, AST, ALP, GMT, TBIL) were also observed.

Discussion

This method was developed for the determination of selected serum amino acids in patients with liver disease and indicates that disturbances in the metabolism of amino acids in patients with liver disease is imperceptible. The levels of some amino acids were statistically significantly decreased or increased, but only slightly. More significant differences in the levels of some amino acids were found between women and men. For this reason, it is necessary to compare amino acid levels between female patients and female controls as well as between male patients and male controls. Most publications refer to amino acid imbalances in patients with different liver diseases. Often, there are untreated patients with various types and degrees of liver diseases. We dealt only with patients treated in the Hepatology Clinic. These patients had the results of liver blood tests (ALT, AST, ALP, GMT, total bilirubin) and lipid metabolism (total cholesterol, triglycerides) normal or slightly increased.

Fischer and collaborators published that the plasma levels of BCAA are decreased and the AAA increased in patients with liver disease. These changes are caused by increased BCAA catabolism in skeletal muscle, whereas AAA catabolism in the failing liver is decreased [21]. Thus a decrease in the BCAA/AAA ratio, called a Fischer ratio, is observed in patients with liver disease. Our results indicate a disturbance in the metabolism of tyrosine in patients with liver disease, but only in women. In men, tyrosine levels were also increased, but not

Table 4. Summary for the two factor analysis of variance (ANOVA) investigating the changes in levels of amino acids in patients with liver disease as a function of gender and diagnosis (1, viral hepatitis; 2, alcoholic liver disease; 3, other diseases of liver)

Set	Factor	Patients with liver disease			
		f	p	power (1-β)	conclusion
Aspn (μmol/L)	gender	6.545	0.012	0.648	–
	diagnosis	4.019	0.020	0.570	–
	gender*diagnosis	2.625	0.076	0.325	–
Glun (μmol/L)	gender	0.076	0.783	0.050	–
	diagnosis	0.616	0.542	0.050	–
	gender*diagnosis	2.291	0.105	0.264	–
His (μmol/L)	gender	6.107	0.015	0.609	–
	diagnosis	0.867	0.423	0.423	–
	gender*diagnosis	1.830	0.165	0.165	–
Ser (μmol/L)	gender	1.831	0.178	0.137	–
	diagnosis	0.522	0.595	0.050	–
	gender*diagnosis	2.969	0.055	0.389	–
Arg (μmol/L)	gender	0.349	0.556	0.050	–
	diagnosis	4.318	0.015	0.615	–
	gender*diagnosis	0.818	0.444	0.050	–
Cit (μmol/L)	gender	0.605	0.438	0.050	–
	diagnosis	7.362	< 0.001	0.907	significant
	gender*diagnosis	1.639	0.198	0.149	–
Gly (μmol/L)	gender	5.729	0.018	0.573	–
	diagnosis	0.158	0.854	0.050	–
	gender*diagnosis	1.163	0.316	0.073	–
Thr (μmol/L)	gender	1.971	0.163	0.153	–
	diagnosis	1.654	0.195	0.151	–
	gender*diagnosis	0.488	0.615	0.050	–
Tyr (μmol/L)	gender	0.240	0.625	0.050	–
	diagnosis	4.792	0.010	0.682	–
	gender*diagnosis	1.607	0.205	0.143	–
Tau (μmol/L)	gender	1.222	0.271	0.071	–
	diagnosis	0.735	0.481	0.050	–
	gender*diagnosis	0.378	0.686	0.050	–
Ala (μmol/L)	gender	1.355	0.247	0.085	–
	diagnosis	1.208	0.302	0.079	–
	gender*diagnosis	0.445	0.642	0.050	–
Htau (μmol/L)	gender	7.449	0.007	0.720	–
	diagnosis	0.583	0.560	0.050	–
	gender*diagnosis	1.489	0.230	0.124	–
2-AB (μmol/L)	gender	0.752	0.388	0.050	–
	diagnosis	0.022	0.978	0.050	–
	gender*diagnosis	0.549	0.579	0.050	–
Trp (μmol/L)	gender	12.253	< 0.001	0.933	significant
	diagnosis	0.890	0.413	0.050	–
	gender*diagnosis	3.110	0.048	0.414	–
Met (μmol/L)	gender	5.296	0.023	0.530	–
	diagnosis	5.964	0.003	0.811	significant
	gender*diagnosis	2.940	0.057	0.383	–

Table 4. Summary for the two factor analysis of variance (ANOVA) investigating the changes in levels of amino acids in patients with liver disease as a function of gender and diagnosis (1, viral hepatitis; 2, alcoholic liver disease; 3, other diseases of liver) – cont.

Set	Factor	Patients with liver disease			
		f	p	power (1-β)	conclusion
Val (μmol/L)	gender	10.756	0.001	0.891	significant
	diagnosis	0.674	0.511	0.050	–
	gender*diagnosis	1.330	0.268	0.098	–
Phe (μmol/L)	gender	9.622	0.002	0.847	significant
	diagnosis	3.787	0.025	0.532	–
	gender*diagnosis	0.298	0.743	0.050	–
Ile (μmol/L)	gender	18.468	< 0.001	0.993	significant
	diagnosis	1.009	0.368	0.050	–
	gender*diagnosis	1.005	0.369	0.050	–
Leu (μmol/L)	gender	16.686	< 0.001	0.986	significant
	diagnosis	0.805	0.449	0.050	–
	gender*diagnosis	2.182	0.117	0.244	–
BCAA/AAA	gender	7.918	0.006	0.752	–
	diagnosis	7.169	0.001	0.897	significant
	gender*diagnosis	0.707	0.495	0.050	–

Aspn – aspartic acid + asparagine; Glun – glutamic acid + glutamine; 2-AB – 2-aminobutyric acid; BCAA – branched-chain amino acids; AAA – aromatic amino acids (phenylalanine and tyrosine).

statistically significantly. We found significant differences in AAA and BCAA concentrations between women and men, but not between the patients and the control group. This method was also used for the determination of serum amino acids in patients with type 2 diabetes mellitus [15]. The serum levels of BCAA were statistically significantly increased in patients. Therefore, patients with diabetes mellitus were excluded from the study. Insulin resistance results in increased proteolysis and BCAA levels are elevated [15, 22]. In skeletal muscle, BCAAs act as an essential donor of nitrogen in the BCAA transaminase reaction to form glutamate. Glutamate then reacts with ammonia to form glutamine. Hyperammonemia thus decreases BCAA levels and increases glutamine levels in serum [23]. We found increased levels of glutamate in patients with liver disease. Against all expectations, the levels of serum glutamine in male patients with liver disease were statistically significantly decreased. Examples of diseases in which the levels of AAA are increased and the levels of BCAA decreased are non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, cirrhosis, viral hepatitis, chronic hepatitis, biliary disease, primary biliary cirrhosis, sclerosing cholangitis and fulminant hepatic failure. Interesting results were obtained by Tietge et al. [3, 24]. Patients with cirrhosis had increased levels of plasma AAA and these levels correlated with the progression of the liver disease. After liver transplantation, the levels of plasma AAA were normalized. The levels of plasma BCAA were decreased and were unrelated to the disease stage. After liver transplantation, the levels of plasma BCAA remained subnormal although higher than before liver transplantation. The authors concluded that despite normal liver function, amino acid metabolism is only partially normalized after liver transplantation. After liver transplantation, a higher insulin production remains, insulin may thus contribute to the persistent alteration of muscular BCAA metabolism.

In addition to AAA and BCAA, disturbances in the metabolism of other amino acids can be observed in patients with liver disease. Morgan et al. [25] observed increased levels of plasma methionine in patients with chronic liver disease, and Tietge et al. [3] in patients with cirrhosis. After liver transplantation, the levels returned to normal [3]. Sato et al. [7] described mildly increased serum methionine in survivors of fulminant hepatic failure and extremely increased among non-survivors. They suggest that high levels of methionine may relate to higher mortality rates. In that case, the level of serum methionine may be a useful indicator in evaluating the prognosis of patients with acute fulminant hepatic failure. We found significant differences only in methionine levels between women and men.

Borg et al. [4] found decreased levels of serum tryptophan in patients with primary biliary cir-

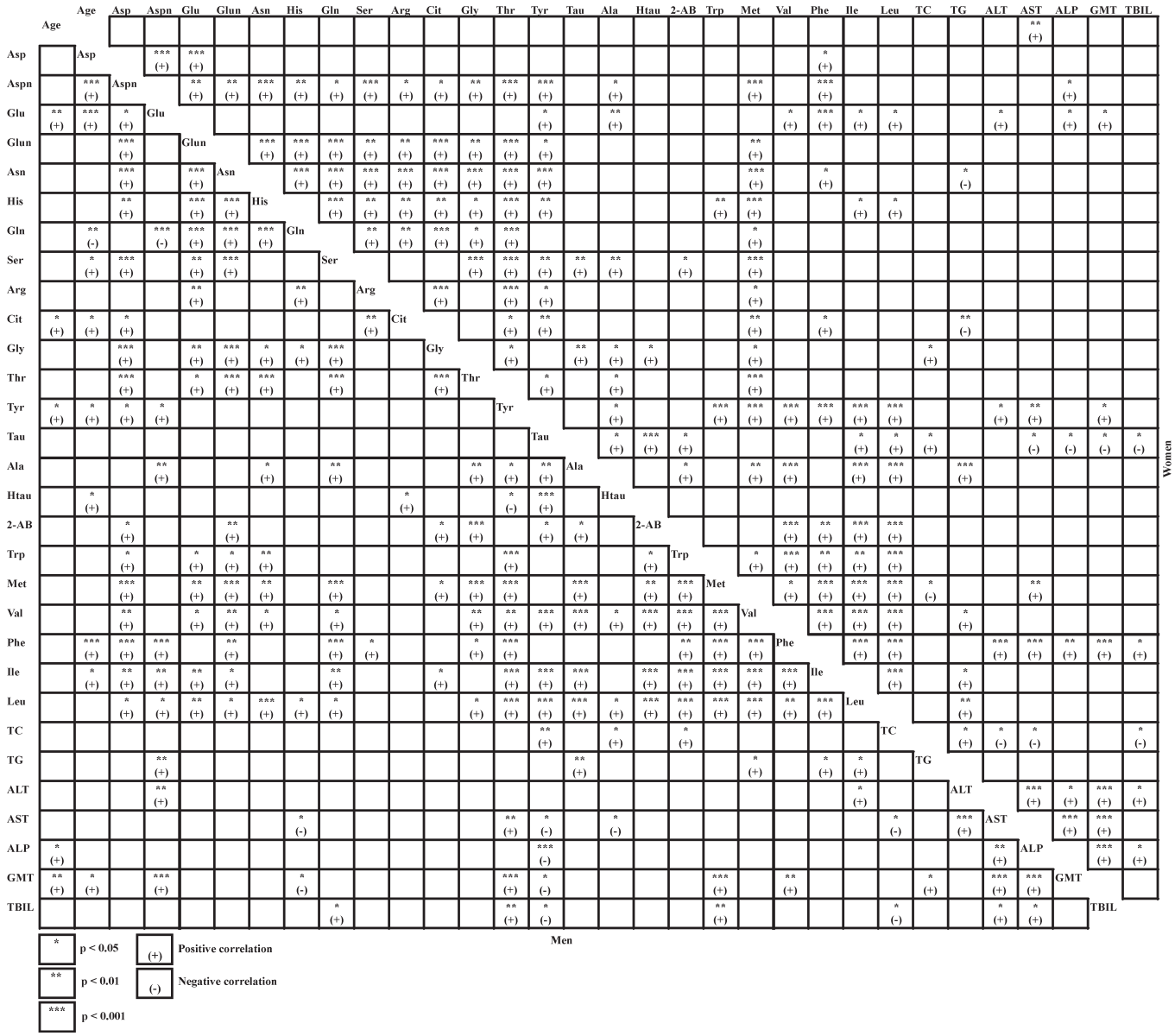

Fig. 1. The correlations between individual amino acids, markers of liver blood tests and markers of lipid metabolism calculated using a Spearman rank test in patients with different liver diseases

rhosis. Decreased concentrations of serum tryptophan might be explained by the utilization of tryptophan as a result of immune activation in biliary cirrhosis. In our study, the levels of serum tryptophan significantly differ between female and male patients and between male patients and male controls. Compared to the study by Borg et al., male patients had increased levels of serum tryptophan.

Most authors report that other amino acids probably have no relevance in the diagnosis of liver diseases. Tietge et al. [24] reported that 14 of the 18 amino acids measured were significantly altered in patients with cirrhosis and 11 of the 18 remained abnormal after liver transplantation compared to a control group.

At first glance, we may see more correlations between amino acids in patients with liver disease compared to a control group. In patients with liver disease especially, BCAA and AAA strongly correlate with each other. This is a very interesting result which may indicate a disturbance in the metabolism of amino acids in patients with liver disease while liver blood tests are relatively normal. We observed correlations between some amino acids and markers of liver blood tests and lipid metabolism. Positive correlations between tyrosine and AST, GMT, and TBIL, between phenylalanine and GMT in men, between tyrosine and ALT, AST, and GMT, and between phenylalanine and ALT, AST, ALP, GMT, and TBIL in women were observed. As has already been mentioned several times, serum levels of AAA are increased in patients with liver disease. Positive correlations between AAA and markers of liver blood tests con-

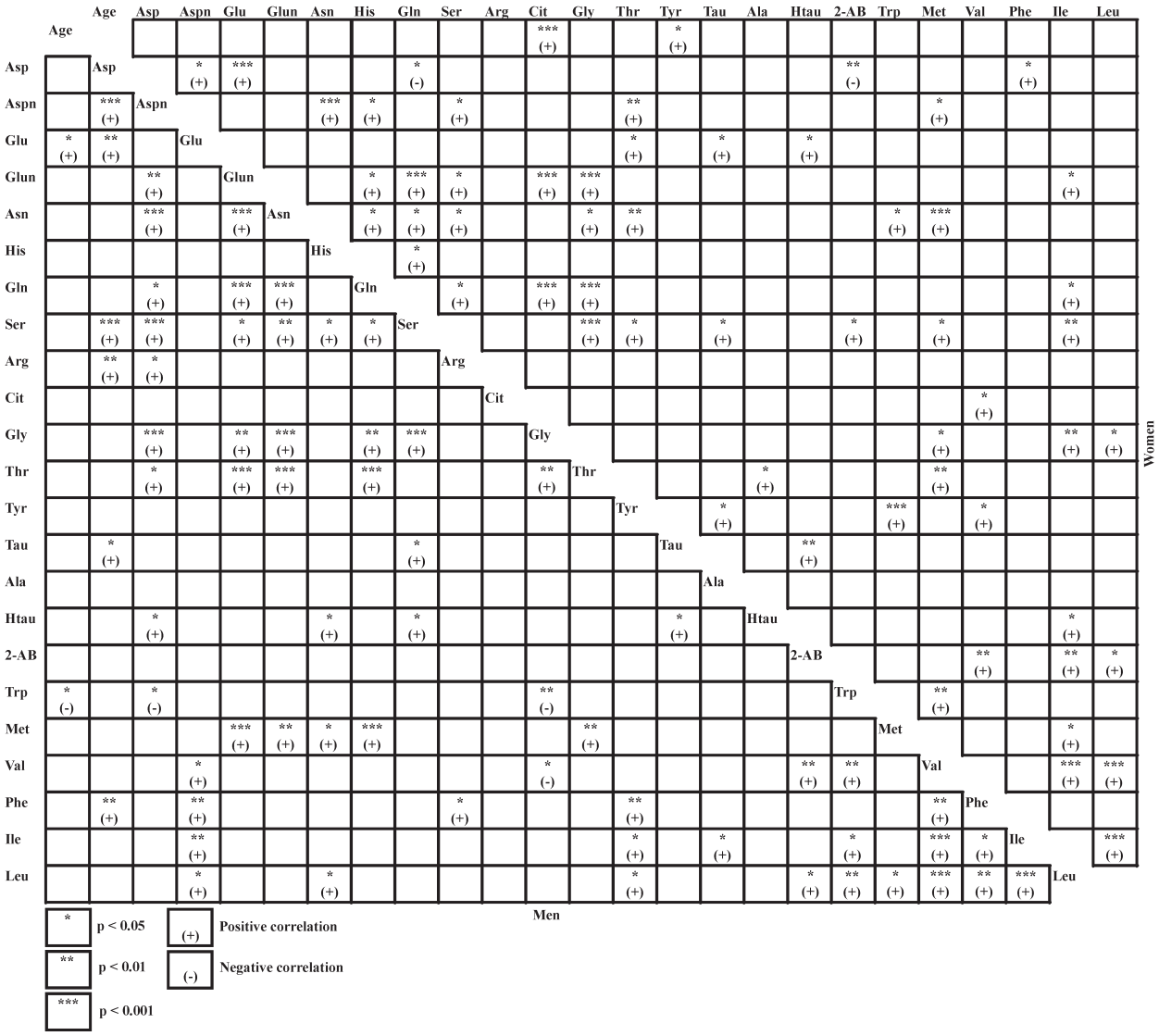

Fig. 2. The correlations between individual amino acids calculated using a Spearman rank test in a control group

firm this fact. Methionine also positively correlates with markers of liver blood tests (GMT and TBIL in men; AST in women). On the other hand, taurine negatively correlates with AST, ALP, GMT and TBIL in both women and men. Also worth mentioning is a positive correlation between BCAA and TG. It is known that BCAAs contribute to insulin resistance [22]. Insulin resistance may lead to an increase of serum TG.

In conclusion, significant differences in the metabolism of amino acids between patients with liver disease and a control group were observed, although not as much as in other papers. Because the amino acid levels depend on gender, it is necessary to compare the amino acid levels between female patients and female controls as well as between male patients and male controls. An interesting finding is that more correlations between amino acids in patients with liver disease were observed. It must also be noted that the method for the analysis of amino acids in serum must be sufficiently sensitive, precise, accurate and suitable for clinical testing purposes, meaning robust and inexpensive.

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