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Plasma Amino Acids in NAFLD Patients with Obesity Are Associated with Steatosis and Fibrosis; Results from the MAST4HEALTH Study

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Abstract: Background & Aims: Non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) have been linked to changes in amino acid (AA) levels. The current observational study sought to investigate the relationship between plasma AA concentrations in a NAFLD population and MRI parameters reflecting inflammation and fibrosis, inflammatory and oxidative stress markers, and disease-related anthropometric and biochemical indicators. **Approach & Results:** Plasma AA levels were quantified with liquid chromatography in 97 NAFLD patients from the MAST4HEALTH study. Medical, anthropometric and lifestyle characteristics were collected and biochemical parameters, as well as inflammatory and oxidative stress biomarkers were measured. In total, males and subjects with higher MRI-proton density fat fraction (MRI-PDFF) exhibited higher plasma AA levels compared to females and subjects with lower PDFF respectively. Several associations of AAs with disease related markers were revealed, with the more prominent ones being those of aromatic amino acids with log-PDFF (beta: 1.190E-02, p-Value: 0.001) and log-ALT (beta: 7.55E-03, p-Value: 0.001), of branched amino acids with log-insulin (beta: 1.97E-03, p-Value: 1.16E-04) and of ethanolamine (beta: 0.036, p-Value: 3.65E-04) and L-ornithine (beta: 5.4E-04, p-Value: 0.021) with log-total antioxidant status (TAS). **Conclusions:** Plasma AA levels varied according to sex, BMI, and several MRI clinical factors. Furthermore, significant relationships were demonstrated between AA and several disease indicators, such as MRI parameters, biochemical and oxidative stress indices, showing the potential utility of AAs as diagnostic disease-related indicators activity.

Keywords: non-alcoholic fatty liver disease; magnetic resonance imaging; amino acids; metabolomics; inflammation

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is considered the leading cause of chronic liver disease in the world [1]. It represents a set of pathological conditions that range from simple hepatic steatosis (SS) or non-alcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH) and cirrhosis [2]. Primary NAFLD is now acknowledged as the hepatic manifestation of metabolic syndrome (MetS) [3,4]. Processes that are involved in the onset of SS and its transition to NASH remain not fully explored.

NAFLD is linked to pathological conditions such as hypertension, insulin resistance (IR) and type II diabetes (T2D); obesity and increased central adiposity are also strongly associated with metabolic liver disease. High rates of obesity and T2D lead to an ever-increasing number of patients with NASH [5]. Despite the efforts to uncover new treatment strategies for NASH, no pharmacological therapy has yet been approved. As there are no specific symptoms, the disease is usually diagnosed at later stages, when adjustments to risk factors and exploration of treatment options are not effective [6]. Liver biopsy remains the gold standard method for disease diagnosis, but it is invasive in nature with many limitations.

The identification of non- or minimally invasive biomarkers that can track the progression of the disease or help to assess response to therapeutic interventions is of upmost importance. Metabolomics have received a lot of scientific attention in recent years. As a result of the advent of metabolomics, scientists can now discover hundreds of metabolites that are implicated in several complex diseases. Given that urine or serum are the most commonly used samples for NAFLD testing, metabolomics is a valuable tool for assessing liver impairment. Numerous studies have addressed the alterations in metabolite profiles of patients with NAFLD [7–9] with amino acids (AAs) being a well explored group that is altered in different stages of the disease [10]. Although recently it has been suggested that plasma AA levels may be used as possible markers of disease severity as they have been associated with insulin resistance and protein catabolism [11], however not many studies address the relationship of AA levels with disease markers.

Therefore, the aim of the present study was to explore the association of plasma AAs concentrations in a NAFLD population with MRI parameters that reflect inflammation and fibrosis, inflammatory and oxidative stress markers, anthropometric and biochemical indices that are related to the disease.

2. Results

General characteristics of study participants

A total of 97 people, 69 of whom were males and 28 of whom were females, with a mean age of 49.04 ± 9.16, were included in the current analysis. Table 1 displays the descriptive characteristics of the population. ALT was found to be significantly higher in males than in females (p-Value: 0.001). Moreover, females had significantly higher AST/ALT ratio, total cholesterol, HDL, and LDL than males (AST/ALT ratio, p-Value: 0.004; total cholesterol, p-Value: 0.010; HDL, p-Value: 0.022; LDL, p-Value: 0.028).

Table 1. Anthropometric, demographic, lifestyle, MRI, and biochemical parameters in males and females.

Variables	N	Females (N: 28) (mean (SD))	Males (N: 69) (mean (SD))	p-Value
Age (years)	97	49.61 (7.67)	48.81 (9.74)	0.676
Smoking (Yes|No)	96	Yes: 7, No: 21	Yes: 14, No:54	0.839
BMI (kg/m²)	97	35.39 (5.19)	34.04 (4.06)	0.228
PAL (total MET-min/week)	91	3733.37 (5326.04)	3575.26 (5084.72)	0.452
FindRisk Score	96	14.21(3.35)	13.44 (3.94)	0.226
cT1 (ms)	94	874.27 (65.96)	879.5 (85.43)	0.82
PDFF (%)	95	12.89 (8.14)	18.06 (12.96)	0.058

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LIF *	94	2.25 (0.59)	2.27 (0.65)	0.902
AST (IU/L)	94	22.59 (8.15)	26.52 (12)	0.093
ALT (IU/L)	94	28.93 (14.97)	41.66 (21.29)	**0.001**
AST/ALT ratio	94	0.88 (0.32)	0.68 (0.17)	**0.004**
γ-gt (U/L)	96	62.04 (79.03)	52.77 (51.95)	0.28
Total cholesterol (mg/dL)	97	209.01 (33.5)	191.27 (38.03)	**0.010**
HDL (mg/dL)	97	48 (11.17)	43.1 (9.8)	**0.022**
LDL (mg/dL)	96	130.95 (30.35)	118.64 (36.23)	**0.028**
Triglycerides (mg/dI)	97	150.54 (76.47)	147.77 (60.83)	0.793
Glucose (mg/dL)	92	98.84 (10.35)	104.15 (17.21)	0.343
120 min-OGTT Glucose (mg/dL)	86	131.35 (38.08)	132.26 (51.45)	0.665
HOMA-IR	89	4.23 (2.43)	5.19 (2.65)	0.109
Insulin (μU/mL)	93	16.83 (10.18)	19.93 (9.6)	0.096

Note: * parametric variable. P-Value for comparison between females and males was obtained using t-test for parametric variables or Mann-Whitney U test for non-parametric variables, and the chi-square test for categorical variables. PAL: physical activity level; FindRisk Score: Finnish diabetic risk score; cT1: included iron-corrected; proton density fat fraction (PDFF); liver inflammation fibrosis score (LIF); AST: aspartate transaminase; ALT: alanine transaminase; AST/ALT ratio: AST to ALT ratio; γ-GT: γ-glutamyltransferase; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA-IR: homeostatic model assessment of insulin resistance.

AAs plasma levels across BMI, sex, PDFF and cT1 categories

BMI, PDFF and cT1 variables were dichotomized based on their median value (Table 2). Across BMI categories, mean cystine was lower in patients with BMI ≤ 35 kg/m2 compared to those with BMI > 35 kg/m2 (36.53 ± 16.23 vs. 45.03 ± 16.65) (p-Value: 0.010). The essential AAs (p-Value: < 0.001), BCAAs (p-Value: 3.06E-04), aromatic amino acids (AAAs) (18) (p-Value: 2.79E-04), sarcosine (p-Value: 0.035), cystine (p-Value: 0.047), ethanolamine (p-Value: < 0.001), 1-Me-L-histidine (p-Value: 0.037), 3-Me-L-histidine (p-Value: 0.027), L-alpha-aminoadipic acid (p-Value: 0.018), L-valine (p-Value: 0.020), L-methionine (p-Value: 0.007), L-tyrosine (p-Value: 0.015), L-isoleucine (p-Value: 3.42E-05), L-leucine (p-Value: 1.43E-06), L-phenylalanine (p-Value: 6.73E-06), and L-tryptophan (p-Value: 1.26E-05) were lower in females compared to males.

Within PDFF categories, the concentrations of essential (p-Value: 1.17E-04), and nonessential AAs (p-Value: 0.034), GSG index (11) (p-Value: 0.041), BCAAs (p-Value: 0.001), AAAs (p-Value: 7.89E-05), L-alanine (p-Value: 0.026), L-aspartic acid (p-Value: 0.011), L-threonine (p-Value: 0.027), L-glutamic acid (p-Value: 0.005), L-alpha-aminoadipic acid (p-Value: 0.009), L-proline (p-Value: 0.034), L-lysine (p-Value: 0.019), L-valine (p-Value: <0.001), L-Methionine (p-Value: 0.027), L-Tyrosine (p-Value: 1.25E-04), L-Isoleucine (p-Value: 1.52E-04), L-Leucine (p-Value: 0.016), L-Phenylalanine (p-Value: 4.61E-04), and L-Tryptophan (p-Value: 0.027) were lower in the PDFF ≤ 13.605% category compared to the PDFF > 13.605%. The PDFF ≤13.605 category had significantly higher values of D, L-beta-aminoisobutyric acid in comparison with PDFF > 13.605 (p-Value: 0.035). The L-threonine (p-Value: 0.01), L-lysine (p-Value: 0.016), L-phenylalanine (p-Value: 0.039) were lower in cT1 ≤ 873.2 ms than in cT1 > 873.2 ms.

Table 2. Plasma AAs levels in BMI, sex, PDFF and cT1 categories.

Amino Acids (AAs) μmoles/L	BMI		p-Value	Sex		p-Value
	≤35 mean (SD)	>35 mean (SD)		Females mean (SD)	Males mean (SD)	
Essential AAs	1089.59 (140.98)	1111.27 (185.45)	0.659	1043.46 (213.18)	1118.36 (121.84)	**<0.001**
Nonessential AAs	1636.14 (244.86)	1635.95 (189.46)	0.979	1622.8 (196.07)	1641.46 (239.64)	0.997
GSG index	15.54 (6.69)	19.47 (9.52)	0.060	14.82 (8.22)	17.66 (7.69)	0.059
BCAAs	492.81 (88.83)	520.79 (115.27)	0.164	465.66 (131.76)	516.8 (77.92)	**3.06E-04**
AAAs	139.74 (23.23)	140.89 (21.3)	0.851	129.13 (21.08)	144.58 (21.65)	**2.79E-04**
L-Alanine*	330.29 (55.71)	343.26 (63.07)	0.327	341.72 (61.02)	331.66 (57.27)	0.444
beta-Alanine*	7.82 (1.93)	7.32 (1.66)	0.210	7.33 (1.91)	7.79 (1.82)	0.273

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Sarcosine	3.61 (1.14)	4.07 (1.85)	0.905	3.31 (1.01)	3.94 (1.52)	**0.035**
Cystine	36.53 (16.23)	45.03 (16.65)	**0.010**	33.9 (15.8)	41.54 (16.76)	**0.047**
L-Serine	106.9 (100.65)	100.95 (57.73)	0.464	95.24 (21.33)	108.88 (104.07)	0.984
O-Phosphoethanolamine	1.58 (1.88)	1.57 (1.85)	0.872	1.51 (2.09)	1.6 (1.77)	0.481
Taurine	53.82 (19.49)	59.5 (23.54)	0.227	50.82 (15.08)	57.68 (22.72)	0.18
L-Asparagine	54.57 (8.21)	52.17 (8.91)	0.21	53.06 (9.68)	54.07 (8)	0.362
Hydroxy-L-Proline	12.16 (5.1)	14.57 (10.85)	0.768	13.27 (10.93)	12.82 (5.7)	0.237
Glycine	211.19 (69.61)	197.46 (54.3)	0.136	220.82 (58.46)	200.92 (67.01)	0.070
L-Glutamine*	582.38 (67.05)	568.72 (68.12)	0.350	576.05 (61.63)	578.61 (69.96)	0.866
Ethanolamine*	7.17 (1.1)	7.01 (0.97)	0.484	6.55 (1.01)	7.34 (1)	**<0.001**
L-Aspartic acid	3.09 (1.12)	3.47 (2.34)	0.727	3.24 (2.56)	3.21 (1.06)	0.068
L-Citruline	33.4 (7.38)	34.15 (8.87)	0.803	32.81 (9.82)	33.98 (6.98)	0.133
L-Threonine	123.57 (29.6)	119.36 (26.83)	0.459	123.84 (30.4)	121.51 (28.11)	0.877
L-Glutamic acid	45.64 (17.28)	52.96 (20.48)	0.09	42.92 (18.47)	50.14 (18.4)	0.078
L-Histidine*	82.61 (9.9)	81.28 (13.25)	0.583	79.91 (9.97)	83.09 (11.43)	0.201
1-Me-L-Histdine	8.71 (7.25)	8.76 (9.74)	0.517	7.39 (9.17)	9.27 (7.64)	**0.037**
3-Me-L-Histdine	4.02 (1.18)	4.11 (1.29)	0.92	3.67 (1.3)	4.2 (1.15)	**0.027**
gamma-Amino-butyric acid GABA	0.24 (0.18)	0.27 (0.22)	0.899	0.21 (0.13)	0.27 (0.21)	0.366
D,L-beta-Aminoisobutyric acid	1.5 (3.13)	0.98 (0.5)	0.33	0.87 (0.54)	1.51 (3.03)	0.054
D,L-alpha-Amino-n-butyric acid	20.29 (8.12)	19.67 (6.59)	0.973	18.79 (6.54)	20.61 (8)	0.496
L-alpha-Aminoadipic acid	1.93 (5.83)	1.47 (0.73)	0.384	1.12 (0.78)	2.04 (5.64)	**0.018**
L-Proline	188.34 (52.86)	194.02 (44.86)	0.468	183.79 (58.06)	192.82 (46.86)	0.231
L-Arginine*	67.26 (14.81)	73.01 (19.21)	0.108	67.82 (21.69)	69.71 (14.04)	0.613
L-Ornithine	86.98 (48.64)	78.6 (32.4)	0.199	77.47 (27.89)	86.95 (48.91)	0.277
L-Lysine	167.42 (26.22)	163.41 (36.11)	0.309	167.18 (36.58)	165.65 (26.73)	0.845
L-Valine	272.48 (48.67)	291.41 (58.75)	0.054	263.86 (65.68)	284.76 (45.55)	**0.020**
L-Methionine*	30.02 (7.33)	28.78 (6.61)	0.422	26.6 (7.42)	30.84 (6.62)	**0.007**
L-Tyrosine	77.19 (16.36)	77.92 (15.29)	0.803	72.05 (15.53)	79.62 (15.68)	**0.015**
L-Isoleucine	70.93 (18.41)	73.07 (26.46)	0.863	64.62 (30.77)	74.49 (15.34)	**3.42E-05**
L-Leucine	149.4 (26.47)	156.31 (35.31)	0.341	137.18 (39.8)	157.56 (22.18)	**1.43E-06**
L-Phenylalanine	62.55 (9.49)	62.97 (7.65)	0.779	57.09 (7.1)	64.96 (8.56)	**6.73E-06**
L-Tryptophan	63.35 (12.3)	61.66 (10.62)	0.487	55.36 (10.31)	65.81 (10.98)	**1.26E-05**

Note: * parametric variable. P-Value was obtained using t-test for parametric variables or Mann-Whitney U test for non-parametric variables, Essential AAs: Arginine + histidine + isoleucine + leucine + lysine + methionine + phenylalanine + threonine + tryptophan + valine, Nonessential AAs: Alanine + asparagine + aspartic acid + cysteine + glutamic acid + glutamine + glycine + proline + serine + tyrosine, GSG index: glutamate/(serine + glycine), BCAAs: Valine + Leucine + Isoleucine, AAAs: Tyrosine + Phenylalanine.

	PDFF (%)			cT1 (ms)		
Amino Acids (AAs) µmoles/L	**≤13.605 (median) mean (SD)**	**>13.605 (median) mean (SD)**	**p-Value**	**≤873.2 (median) mean (SD)**	**>873.2 (median) mean (SD)**	**p-Value**
Essential AAs	1039.21 (126.72)	1155.95 (164.75)	**1.17E-04**	1067.29 (134.53)	1124.76 (176.33)	0.077
Nonessential AAs	1589.73 (176.99)	1693.62 (255.93)	**0.034**	1603.23 (182.78)	1662.11 (266.7)	0.451
GSG index	15.02 (7.18)	18.6 (8.33)	**0.041**	15.67 (6.86)	18.03 (8.91)	0.291
BCAAs	469.14 (84.02)	535.61 (103.3)	**0.001**	495.41 (89.24)	508.26 (110.47)	0.646
AAAs	131.68 (18.74)	149.24 (22.69)	**7.892E-05**	134.99 (19.18)	144.94 (25.05)	0.127
L-Alanine *	322.48 (55.71)	348.97 (58.04)	**0.026**	321.61 (53.9)	344.21 (59.27)	0.056
beta-Alanine *	7.31 (1.79)	8.03 (1.88)	0.059	7.71 (1.77)	7.71 (1.87)	0.999
Sarcosine	3.72 (1.41)	3.84 (1.46)	0.364	3.74 (1.3)	3.83 (1.57)	0.952
Cystine	40.57 (16.45)	38.73 (17.14)	0.692	38.36 (15.41)	39.54 (17.93)	0.603
L-Serine	94.19 (19.54)	116.74 (123.84)	0.447	95.95 (19.44)	115.01 (125.59)	0.390
O-Phosphoethanolamine	1.59 (1.85)	1.6 (1.91)	0.942	1.39 (1.78)	1.77 (1.93)	0.29
Taurine	55.31 (19.14)	57.01 (22.68)	0.891	53.66 (19.94)	57.75 (22.43)	0.335
L-Asparagine	53.02 (9.28)	55.12 (7.17)	0.072	53.11 (10.11)	54.27 (6.7)	0.143
Hydroxy-L-Proline	12.09 (5.55)	14 (9.09)	0.322	12.85 (6.49)	12.99 (8.67)	0.787
Glycine	207.41 (55.26)	206.44 (74.96)	0.474	207.36 (54.54)	206.1 (76.42)	0.309
L-Glutamine *	573.82 (72.41)	585.24 (61.07)	0.408	575.93 (72.48)	576.84 (61.96)	0.948
Ethanolamine *	7.1 (1.12)	7.11 (1)	0.950	7.19 (1.09)	7.02 (1.05)	0.445
L-Aspartic acid	2.89 (1.2)	3.57 (1.92)	**0.011**	3.31 (2.09)	3.13 (1.06)	0.543
L-Citruline	33.58 (7.02)	33.97 (8.69)	0.885	33.31 (6.14)	33.33 (9.03)	0.668
L-Threonine	115.39 (21.42)	129.9 (33.04)	**0.027**	114.16 (21.03)	130.59 (33.58)	**0.01**
L-Glutamic acid	42.22 (16.52)	53.86 (19.09)	**0.005**	44.53 (15.69)	51.58 (21.06)	0.137
L-Histidine *	80.66 (8.85)	83.75 (12.98)	0.180	81.43 (10.18)	82.7 (12.24)	0.588

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1-Me-L-Histdine	7.44 (6.56)	9.96 (9.35)	0.322	7.87 (7.97)	9.64 (8.45)	0.210
3-Me-L-Histdine	3.87 (1.2)	4.2 (1.22)	0.200	4.08 (1.3)	4.04 (1.16)	0.94
gamma-Amino-butyric acid GABA	0.26 (0.18)	0.24 (0.21)	0.437	0.22 (0.15)	0.28 (0.21)	0.344
D,L-beta-Aminoisobutyric acid	1.75 (3.64)	0.92 (0.51)	**0.035**	1.65 (3.62)	0.96 (0.51)	0.146
D,L-alpha-Amino-n-butyric acid	19.81 (8.12)	20.32 (7.19)	0.804	20.54 (8.75)	19.65 (6.58)	0.857
L-alpha-Aminoadipic acid	1.14 (0.59)	2.42 (6.75)	**0.009**	2.24 (6.86)	1.35 (0.55)	0.589
L-Proline	181.09 (50.92)	201.55 (47.37)	**0.034**	188.46 (51.27)	191.53 (51.11)	0.619
L-Arginine *	68.82 (15.9)	70.07 (17.33)	0.714	67.34 (16.86)	70.09 (16.06)	0.420
L-Ornithine	76.55 (23.96)	92.57 (56.68)	0.107	78.1 (21.7)	89.2 (58.28)	0.857
L-Lysine	157.85 (26.04)	174.16 (31.58)	**0.019**	159.39 (28.13)	173.54 (30.07)	**0.016**
L-Valine	260.38 (48.33)	297.36 (51.62)	**<0.001**	275.5 (50.55)	282.1 (56.56)	0.537
L-Methionine *	27.98 (7.07)	31.2 (6.92)	**0.027**	28.65 (7.06)	30.32 (7.16)	0.258
L-Tyrosine	72.05 (13.46)	83.41 (16.17)	**1.25E-04**	74.61 (14.5)	79.9 (17.18)	0.151
L-Isoleucine	64.63 (13.8)	78.76 (25.23)	**1.52E-04**	69.45 (16.5)	73.82 (25.73)	0.557
L-Leucine	144.13 (25.62)	159.49 (32.12)	**0.016**	150.46 (26.75)	152.34 (33.29)	0.931
L-Phenylalanine	59.63 (7.2)	65.83 (9.51)	**4.61E-04**	60.38 (7.25)	65.04 (10.04)	**0.039**
L-Tryptophan	59.74 (10.56)	65.44 (12.15)	**0.027**	60.53 (8.84)	64.22 (13.32)	0.309

Note: * parametric variable. P-Value was obtained using t-test for parametric variables or Mann-Whitney U test for non-parametric variables, Essential AAs: Arginine + histidine + isoleucine + leucine + lysine + methionine + phenylalanine + threonine + tryptophan + valine, Nonessential AAs: Alanine + asparagine + aspartic acid + cysteine + glutamic acid + glutamine + glycine + proline + serine + tyrosine, GSG index: glutamate/(serine + glycine), BCAAs: Valine + Leucine + Isoleucine, AAAs: Tyrosine + Phenylalanine.

Associations of AAs with MRI parameters, inflammatory and oxidative stress markers, biochemicals parameters & anthropometrics

In the correlation analysis various statistically positive correlations were observed (Table S1). The AAAs, L-tyrosine and L-isoleucine exhibit positive correlation with PDFF. In addition, ethanolamine was positively correlated with hemoglobin levels (HGB) and total antioxidant status (TAS), and L-ornithine with TAS. The essential AAs, BCAAs, AAAs, L-proline, L-valine, L-isoleucine, L-leucine, and L-phenylalanine are positively correlated with insulin and HOMA-IR. Also, L-methionine is positively correlated with insulin and L-tyrosine with HOMA-IR. ALT is found in positive correlation with AAAs, L-phenylalanine, and L-tryptophan.

Then we applied linear regression models to explore the associations of statistically significant correlations (Table 3). The AAAs (beta: 1.190E-02, p-Value: 0.001), L-tyrosine (beta: 1.691E-02, p-Value: 1.33E-03), L-isoleucine (beta: 1.015E-02, p-Value: 0.006) were associated with increased values of log-PDFF in Model 5, after adjusting for age, sex, BMI, center of the study, smoking, PAL, and corresponding nutrient intake. Additionally, ethanolamine was associated with greater values of HGB (Model 4—beta: 3.542E-03, p-Value: 0.004) and log-TAS (Model 3—beta: 0.036, p-Value: 3.65E-04). Moreover, increased values of log-TAS were significantly associated with L-ornithine (Model 3—beta: 5.4E-04, p-Value: 0.021).

The AAAs (Model 5—beta: 7.55E-03, p-Value: 0.001) L-phenylalanine (Model 5—beta: 1.92E-02, p-Value: 0.001), and L-tryptophan (Model 5—beta: 1.60E-02, p-Value: 0.001) were associated with higher levels of log-ALT. The essential AAs, BCAAs, AAAs, L-proline, L-valine, L-isoleucine, L-leucine and L-phenylalanine were associated with higher values of log-insulin (Model 5—essential AAs, beta: 1.21E-03, p-Value: 4.45E-04; BCAAs, beta: 1.97E-03, p-Value: 1.16E-04; AAAs, beta: 8.25E-03, p-Value: 4.42E-04; L-proline, beta: 3.29E-03, p-Value: 0.002; L-valine, beta: 3.71E-03, p-Value: 6.92E-05; L-isoleucine, beta: 8.28E-03, p-Value: 3.5E-04; L-leucine, beta: 6.02E-03, p-Value: 5.22E-04; L-phenylalanine, beta: 2.24E-02, p-Value: 1.76E-04), and log-HOMA-IR (Model 5—essential AAs, beta: 1.37E-03, p-Value: 2.39E-04; BCAAs, beta: 2.22E-03, p-Value: 6.79E-05; AAAs, beta: 0.009, p-Value: 2.39E-04; L-proline, beta: 3.29E-03, p-Value: 0.004; L-valine, beta: 4.32E-03, p-Value: 1.96E-05; L-isoleucine, beta: 9.05E-03, p-Value: 3.19E-04; L-leucine, beta: 6.46E-03, p-Value: 6.32E-04; L-phenylalanine, beta: 2.44E-02, p-Value: 2.56E-04). The L-methionine and L-tyrosine are associated with increased values of log-insulin (L-methionine, Model 5- beta: 2.09E-02, p-Value: 0.009) and log-HOMA-IR (L-tyrosine, Model 5- beta: 1.2E-02, p-Value: 0.001), respectively.

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Table 3. The associations of AAs concentrations with log-PDFF, HGB, log-TAS, log-ALT, log-insulin, and log-HOMA-IR.

Amino Acids (AAs)	Model 1	Model 2	Model 3	Model 4	Model 5
	Beta (P-Value)	Beta (P-Value)	Beta (P-Value)	Beta (P-Value)	Beta (P-Value)
			Log-PDFF (%)		
AAAs (Tyrosine + Phenylalanine)	**0.013 (2.33E-05)**	**0.012 (2.00E-04)**	**0.012 (1.61e-04)**	**1.156E-02 (0.001)**	**1.190E-02 (0.001)**
L-Tyrosine	**0.018 (8.35E-05)**	**0.016 (3.58E-04)**	**0.017 (2.64E-04)**	**1.531E-02 (2.52E-03)**	**1.691e-02 (1.33E-03)**
L-Isoleucine	**0.011 (0.002)**	**0.009 (0.006)**	**0.009 (0.006)**	**9.819E-03 (0.006)**	**1.015E-02 (0.006)**
			HGB (g/mL)		
Ethanolamine	**0.005 (4.05E-05)**	**0.003 (0.007)**	**0.003 (0.007)**	**3.542E-03 (0.004)**	-
			Log-TAS (mmol/L)		
Ethanolamine	**0.046 (4.62E-06)**	**0.037 (1.96E-04)**	**0.036 (3.65E-04)**	1.501E-02 (0.095)	-
L-Ornithine	**6.48E-04 (0.009)**	**5.6E-04 (0.016)**	**5.4E-04 (0.021)**	2.959E-04 (0.128)	-
			Log-ALT (IU/L)		
AAAs (Tyrosine + Phenylalanine)	**9.6E-03 (1.32E-05)**	**8.4E-03 (1.74E-04)**	**8.2E-03 (2.12E-04)**	**6.63E-03 (2.76E-03)**	**7.55E-03 (0.001)**
L-Phenylalanine	**0.023 (3.15E-05)**	**0.019 (0.001)**	**0.019 (0.001)**	**1.79E-02 (0.001)**	**1.92E-02 (0.001)**
L-Tryptophan	**0.019 (5.18E-06)**	**0.016 (4.45E-04)**	**0.015 (6.7E-04)**	**1.28E-02 (0.004)**	**1.60E-02 (0.001)**
			Log-Insulin (μU/mL)		
Essential AAs	**0.001 (5.75E-05)**	**0.001 (1.8E-04)**	**0.001 (1.66E-04)**	**1.19E-03 (1.92E-04)**	**1.21E-03 (4.45E-04)**
BCAAs (Valine + Leucine + Isoleucine)	**0.002 (9.10E-06)**	**0.002 (4.69E-05)**	**0.002 (7.97E-05)**	**1.98E-03 (8.53E-05)**	**1.97E-03 (1.16E-04)**
AAAs (Tyrosine + Phenylalanine)	**0.009 (8.07E-05)**	**0.008 (2.86E-04)**	**0.009 (1.24E-04)**	**0.008 (8.7E-04)**	**8.25E-03 (4.42E-04)**
L-Proline	**0.004 (2.65E-04)**	**0.004 (3.94E-04)**	**0.004 (2.9E-04)**	**3.39E-03 (6.88E-04)**	**3.29E-03 (0.002)**
L-Valine	**0.004 (1.48E-05)**	**0.004 (5.28E-05)**	**0.004 (1.22E-04)**	**3.71E-03 (7.45E-05)**	**3.71E-03 (6.92E-05)**
L-Methionine	**0.023 (0.001)**	**0.021 (0.005)**	**0.024 (0.001)**	**0.022 (0.004)**	**2.09E-02 (0.009)**
L-Isoleucine	**0.009 (6.06E-05)**	**0.009 (2.57E-04)**	**0.009 (2.14E-04)**	**8.22E-03 (4.77E-04)**	**8.28E-03 (3.5E-04)**
L-Leucine	**0.007 (3.98E-05)**	**0.006 (2.6E-04)**	**0.006 (3.32E-04)**	**6.01E-03 (4.72E-04)**	**6.02E-03 (5.22E-04)**
L-Phenylalanine	**0.021 (1.73E-04)**	**0.02 (9.95E-04)**	**0.02 (7.33E-04)**	**2.03E-02 (6.62E-04)**	**2.24E-02 (1.76E-04)**
			Log-HOMA-IR		
Essential AAs	**0.001 (4.03E-05)**	**0.001 (1.44E-04)**	**0.001 (1.3E-04)**	**1.30E-03 (2.02E-04)**	**1.37E-03 (2.39E-04)**
BCAAs (Valine + Leucine + Isoleucine)	**0.003 (4.03E-06)**	**0.002 (1.53E-05)**	**0.002 (2.99E-05)**	**2.29E-03 (3.8E-05)**	**2.22E-03 (6.79E-05)**
AAAs (Tyrosine + Phenylalanine)	**9.8E-03 (4.95E-05)**	**0.009 (3.14E-04)**	**9.7E-03 (1E-04)**	**0.009 (6.87E-04)**	**0.009 (2.39E-04)**
L-Proline	**0.004 (7.46E-04)**	**0.004 (0.001)**	**0.004 (7.73E-04)**	**3.27E-03 (0.003)**	**3.29E-03 (0.004)**
L-Valine	**0.005 (4.03E-06)**	**0.004 (1.17E-05)**	**0.004 (3.42E-05)**	**4.4E-03 (2.1E-05)**	**4.32E-03 (1.96E-05)**
L-Tyrosine	**0.012**	**0.012**	**0.012**	**1.09E-02**	**1.2E-02**

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	(2.54E-04)	**(0.001)**	**(3.34E-04)**	**(0.003)**	**(0.001)**
L-Isoleucine	**0.01**	**9.9E-03**	**9.9E-03**	**9.27E-03**	**9.05E-03**
	(4.25E-05)	**(1.25E-04)**	**(9.83E-05)**	**(3.23E-04)**	**(3.19E-04)**
L-Leucine	**0.008**	**0.007**	**0.007**	**6.75E-03**	**6.46E-03**
	(4.34E-05)	**(2.01E-04)**	**(2.74E-04)**	**(4.31E-04)**	**(6.32E-04)**
L-Phenylalanine	**0.023**	**0.022**	**0.022**	**2.18E-02**	**2.44E-02**
	(2.27E-04)	**(0.002)**	**(8.79E-04)**	**(0.001)**	**(2.56E-04)**

The PDFF (%), TAS (mmol/L), ALT (IU/L), Insulin (μU/mL), and HOMA-IR were log-transformed due to the skewness of the distribution. Five adjustment sets were evaluated: Model 1: unadjusted; Model 2: age + sex; Model 3: age + sex + BMI; Model 4: age + sex + BMI + center of the study + smoking + PAL; Model 5: age + sex + BMI + center of the study + smoking + PAL + nutrient intake of the specific AA, for all AAs except ethanolamine and L-ornithine. Essential AAs: Arginine+ histidine+ isoleucine+ leucine+ lysine+ methionine+ phenylalanine+ threonine+ tryptophan+ valine, BCAAs: Valine + Leucine + Isoleucine, AAAs: Tyrosine + Phenylalanine. In all tests, a P value of < 0.05 was considered significant.

3. Discussion

In this study, different levels of several plasma AAs across sex, BMI, PDFF and cT1 categories were identified. Additionally, significant correlations were observed between several plasma AAs levels and PDFF, HGB, TAS, insulin, HOMA-IR, and ALT. For the significant correlations, linear regression models were performed, and statistically significant associations were detected.

According to our results and despite the known limitations for this analyte's measurement [13], lower levels of cystine were found in NAFLD patients with BMI ≤ 35 kg/m2 vs. BMI > 35 kg/m2. Studies have shown evidence of positive associations of cystine with obesity and NAFLD [14]. A clear association of BCAA with obesity has been reported [15]. However, in the study here all participants are obese (BMI>30 kg/m2 as inclusion criterion) which may explain why BCAA or also AAA levels are not higher in the BMI ≤ 35 kg/m2 vs. BMI > 35 kg/m2 group. Interestingly, our findings revealed that the concentration of the following AAs are higher in male NAFLD patients compared to female: essential AAs, BCAAs, AAAs, sarcosine, cystine, ethanolamine, 1-Me-L-histidine, 3-Me-L-histidine, L-alpha-aminoadipic acid, L-valine, L-methionine, L-tyrosine, L-isoleucine, L-leucine, L-phenylalanine, and L-tryptophan. The study of *Grzych* et al. [16] showed that males with NAFLD exhibit higher levels of three BCAAs: valine, isoleucine, and leucine than females, demonstrating that sex is a key element of different plasma BCAA concentrations. However, BCAA levels were reported to be associated with NAFLD status in females but not in males [16], motivating the hypothesis of estrogen mediates.

Overall, in patients with NAFLD circulating amino acids are increased to compensate for hepatic glucagon resistance within a vicious cycle identified as the liver-pancreas axis [17]. In NAFLD patients BCAAs positively correlate with each other [18].

Our findings show also elevated plasma levels of essential and nonessential AAs, GSG index, BCAAs, AAAs, L-alanine, L-aspartic acid, L-threonine, L-glutamic acid, L-alpha-aminoadipic acid, L-proline, L-lysine, L-valine, L-methionine, L-tyrosine, L-isoleucine, L-leucine, L-phenylalanine, and L-tryptophan in higher PDFF compared to lower PDFF category. Positive associations between plasma valine, isoleucine and leucine level, and intrahepatic lipid content have been previously reported [19]. Even in children with NAFLD, higher levels of plasma BCAAs were determined and correlated with MRI-PDFF [20]. Additionally, herein, lower values of D,L-beta-aminoisobutyric acid were found in higher PDFF compared to lower PDFF category. The levels of L-threonine, L-lysine, and L-phenylalanine are also significantly higher in higher cT1 than in lower cT1 category.

Recent studies proposed the GSG index, which incorporates three amino acids involved in glutathione formation, as a promising biomarker of NAFLD [11,21]. Its component glutamate has been found significantly higher in NASH patients with severe fibrosis in the study of *Ajaz* et al. [22], whereas glycine and serine have a negative association with steatosis grade [23]. Alanine, a nonessential AA, and valine and methionine, essential AAs, are implicated in the development of NASH [24]. Alanine being considered a key regulator of the liver-alpha cell axis [17]. Lysine was detected with higher levels in NAFLD vs. healthy controls, as well as in NAFLD patients with

hepatocellular ballooning grade 2 vs. healthy controls [11]. According to various research studies, elevated plasma BCAAs levels were identified in NAFLD patients. Patients with more severe liver impairment had higher values of BCAAs [11,25,26], which is also reflected in our results. The study of *Lake* et al. [27] revealed that serum leucine, isoleucine, and valine levels, which constitute BCAAs, were considerably elevated as steatosis progressed to NASH. This rise is linked to hepatic fat deposition in the initial stages of NAFLD. As observed in several studies, AAAs levels are higher in NASH and SS patients compared to controls [9,28]. Interestingly, patients with NASH are found with elevated serum levels of the AAAs tyrosine, phenylalanine, and tryptophan [29]. Noteworthy, phenylalanine was discovered to be higher in NAFLD, NASH and obesity; tyrosine was associated with insulin resistance and the NASH fibrotic stage; tryptophan was found higher in NASH vs. SS or controls and not in SS vs. controls, indicating its potential contribution to liver fibrosis or inflammation [9,11,15,29–31].

Interestingly, statistically significant associations of several AAs concentrations with log-PDFF, HGB, log-TAS, log-ALT, log-insulin, and log-HOMA-IR were detected. The AAAs, L-tyrosine, L-phenylalanine, and L-isoleucine were associated with increased values of log-PDFF in all models and as previously mentioned, are associated with more advanced stages of this disease, as shown by other research studies. Also, in our study, ethanolamine was positively associated with HGB and log-TAS. Importantly, individuals with NAFLD had greater levels of HGB, suggesting that HGB may have a therapeutic effect by serving as an antioxidant and mitigating the disease's detrimental consequences [32]. It's worth mentioning that TAS, an antioxidative stress marker, was shown to be elevated in NAFLD patients [33]. According to our findings, ethanolamine may be involved in increased HGB and TAS levels. Additionally, L-ornithine was associated with TAS. Exclusively located in the liver, ornithine together with citrulline, are critical metabolites in the urea cycle pathway. A global metabolomic study by *Ajaz* et al. [22] has identified the citrulline/ornithine ratio significantly reduced in NASH patients with severe fibrosis. It is worth mentioning that due to its antioxidative potential and its role in attenuation of lipid peroxidation by glutamine and glutathione and in regulation of hyperammonemia, L-ornithine L-aspartate agent has been considered an effective treatment approach in NAFLD [34].

The AAAs, L-phenylalanine and L-tryptophan were associated with greater log-ALT values. Obesity is related with higher serum concentration of phenylalanine, which is most likely due to liver dysfunction induced by hepatic steatosis [30]. The study of *Swierczynski* et al. [30] revealed that serum levels of phenylalanine are positively correlated with ALT levels in obese patients. Importantly, it was proposed through their study that poor liver function in these patients contributes to reduced phenylalanine metabolism resulting in a rise in serum concentration of phenylalanine, concluding that the concentration of serum phenylalanine in obese persons might be a noninvasive indicator of liver dysfunction linked with hepatic steatosis.

Higher levels of log-insulin and log-HOMA-IR were related to essential AAs, BCAAs, AAAs, L-proline, L-valine, L-isoleucine, L-leucine and L-phenylalanine. The L-methionine and L-tyrosine were associated with higher log-insulin and log-HOMA-IR values, respectively. In alliance with our results, it could be hypothesized that the aforementioned AAs may have a potential impact on insulin resistance mechanisms involved in NAFLD. The findings of other studies also strength our claim. Recent research studies [35–37] have suggested that high intake of BCAAs may cause insulin resistance and glucose intolerance. In addition to, numerous studies have highlighted their possible significance in the development and evolution of several pathological problems such as heart failure and metabolic diseases including diabetes and obesity [38]. Interestingly, the study of *Zhang* et al. [39] has revealed that the insulin resistance group of obese patients had higher serum levels of BCAAs and AAAs compared with no-insulin resistance group, and that BCAAs levels are associated with alleviation of insulin resistance after weight loss intervention. In addition, another study recently showed a strong association of BCAA levels and nutritional status, impaired glucose tolerance or T2D, and also demonstrated that BCAA levels and their response to, e.g., food intake, are stable over time, re-confirming their utility as potential biomarkers [40]. Furthermore, it is well known that elevated levels of BCAAs and AAAs are linked to a higher risk of hyperglycemia and diabetes

development, which are strongly associated with NAFLD. The findings of the study of *Liao* et al. [41] suggest that valine may be implicated in the etiology of T2D and may be linked to hypoglycemia treatment for T2D. Furthermore, a higher risk of T2D was linked to increased plasma concentrations of isoleucine, phenylalanine and tyrosine [42].

The limitation of our study is the small number of participants, the lack of biopsies for the staging of the disease and the lack of inclusion of a multiethnic population. However, the above limitations are counterbalanced using LiverMultiscan, a sensitive software with satisfying diagnostic accuracy, the adjustment of several confounders, such as the centre of the study, in the regression analysis and the application of a highly sensitive LC method.

4. Materials and Methods

Study Design and Patients

This is an observational study that used baseline data from a multicenter randomized double-blinded and placebo-controlled clinical trial (the MAST4HEALTH study [43], ClinicalTrials.gov Identifier: NCT03135873) that explored the effect of Mastiha supplementation on liver inflammation and fibrosis in patients with NAFLD. In total, 97 participants were recruited to three centers (the Department of Dietetics and Nutritional Science, Harokopio University, Athens, Greece (HUA), Consiglio Nazionale delle Ricerche Institute of Clinical Physiology, Milano section at Niguarda Hospital Italy, (CNR) and Faculty of Medicine, University of Novi Sad, Serbia (UNS)) as previously described project. The following inclusion and exclusion criteria were applied: a) males and females aged 18 to 67, b) a BMI of 30 kg/m2 or higher, and c) established NAFLD/NASH as defined by the LiverMultiScan magnetic resonance imaging (MRI) method (Perspectum Ltd., Oxford, UK, [44]). All centers obtained ethics committees approvals (HUA (Bioethics Committee 49/29-10-2015), CNR (Ethical Clearance by Commissione per l'Etica e l'Integrità nella Ricerca, February 2016) and Niguarda Hospital Ethics Committee 230-052017 (Comitato Etico Milano Area 3-11.05.2017), UNS (Faculty of Medicine Novi Sad, The Human Research Ethics Commission No. 01-39/58/1-27.06.2016)), and the trial was carried out in accordance with the rules of the Declaration of Helsinki and the Data Protection Act of 1998. Before being included in the study, all participants provided written informed consent.

Medical, anthropometric and lifestyle assessment

Detailed questionnaires on medical history and lifestyle were obtained. To estimate T2D risk, the Finnish diabetic risk score (FINDRISK) questionnaire was used. Questions pertain to age, BMI, waist circumference (WC), physical activity, vegetables and fruits consumption, hypertension, and personal and family history of hyperglycemia [45]. Physical activity level (PAL) was measured using the international physical activity questionnaire (IPAQ) [46], and metabolic equivalent task minutes per week (MET-min/week) was calculated using the IPAQ scoring system. Interviewers classified participants as smokers or nonsmokers based on their smoking status [47]. Body weight, height and waist circumference were measured, and body mass index (BMI) was computed by dividing weight (kg) by height (m)2. Waist and hip circumference were measured and waist to hip ratio (WHR) was computed. Nutritionist Pro™ (Axxya Systems) was used to assess the dietary intake based on 24-h recalls (three randomly selected days).

MRI parameters

MRI parameters (Magnetic Resonance Imaging Iron-corrected T1 (cT1), proton density fat fraction (PDFF), and liver inflammation fibrosis score (LIF), were derived from the use of LiverMultiscan software on the MRIs of the participants [44].

Blood collection

– Biochemical parameters

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Blood collection (25 mL) was performed during the baseline visit after an overnight fast and serum and plasma isolation was conducted after centrifugation (3000 rpm, 10 min) [43]. Serum was used for the measurement of liver enzymes (glutamyltransferase (g-GT), aspartate transaminase (AST), and alanine transaminase (ALT)), lipids (total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides (TG)), glucose, insulin [43]. Also, HOMA-IR was calculated as follows: fasting glucose (mg/dL) × (fasting insulin)/405 and 75-g of the 2 h oral glucose tolerance test (OGTT) was performed.

Plasma was stored at -80oc until further use for metabolomics analysis.

– Inflammation and oxidatives stress biomarkers

Total antioxidant status (TAS) (mmol/L) was determined in serum by Randox TAS kits (Randox Laboratories Ltd., Crumlin, UK) at Randox Clinical Laboratory Services (Antrim, UK). Superoxide Dismutase (SOD) activity was measured with the RANSOD kit (Randox Laboratories Ltd., Crumlin, UK), in erythrocyte pellet, on a Randox RX Series Analyser (Randox Laboratories Ltd., Crumlin, UK). IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, MCP-1, TNF-α, INF-γ, EGF and VEGF-A were quantified in serum with the Randox high sensitivity cytokine I multiplex array (Randox Laboratories Ltd., Crumlin, UK), in an Evidence Investigator analyser (Randox Laboratories Ltd., Crumlin, UK).

Plasma amino acid profiles

– Sample preparation and labelling with the aTRAQ® reagents

Sample preparation was based on amino acid derivatization using the aTRAQ® reagents (AB Sciex, MA, USA) as previously described [48]. In brief, 10 µL of 10% sulfosalicylic acid containing 400 pmol/µL of norleucine were added to 40 µL of plasma for protein precipitation. 10 µL of the supernatant were mixed with 40 µL of labelling buffer, containing 20 pmol/µL of norvaline. 10 µL of the supernatant were mixed with 5 µL of 121 aTRAQ® labelling reagent. Samples were incubated for 30 min at room temperature and finally 5 µL of hydroxylamine were added. Samples were dried using an Eppendorf vacufuge concentrator and reconstituted to 32 µL of 113 aTRAQ® internal standard diluted with 0.2% formic acid in water at an analogy of 1:1.

– Separation and detection

Liquid chromatography analysis was performed on an Acquity UPLC® system (Waters, MA, USA) equipped with a binary solvent pump. For detection, a TripleTOF® 5600+ mass spectrometer was employed (AB Sciex), equipped with a DuoSpray™ ion source operated in the positive ESI mode. Injection volume was set to 2 µL and separation was carried out on an Amino Acid Analyzer C18 Reversed Phase column (5 µm, 4.6 mm × 150 mm, AB Sciex), using a gradient comprising of water (Millipore Direct-Q 3 UV purification system, Millipore Sigma, MA, USA) and methanol (MS grade, J.T. Baker, NJ, USA) both containing 0.1% formic and 0.01% heptafluorobutyric acid. Column temperature was set to 50 °C and the flow rate was 0.8 mL/min. Analyte determination was based on a variable-window SWATH acquisition method. For the ESI source, temperature was set to 600 °C and ion spray voltage was 4500 V. Source gas and exhaust gas were both set to 60 psi and curtain gas was set to 30 psi. Data acquisition was performed using the Analyst® 1.7.1 software, while processing was achieved using the Sciex OS software platform.

Statistical analysis

The R programming language (R Foundation, Vienna, Austria) was used for data management and analysis. The variables are presented as mean ± standard deviation (SD). The Shapiro-Wilk test was performed to evaluate the variable distribution (normally distributed variables (parametric variables) (Shapiro–Wilk p-Value > 0.05)). The differences of variables were assessed using independent samples t-test for normally distributed (parametric) variables or Mann-Whitney U test for non-normally distributed (non-parametric) variables, and x-squared for categorical variables. Pearson's correlation coefficient for parametric variables or Spearman's rank correlation for non-parametric variables were estimated to determine the correlation between AAs concentrations and MRI parameters, inflammatory and oxidative stress markers, biochemicals parameters &

anthropometrics. Then for significant correlations we created linear regression models. Due to the skewness of the distribution, the PDFF, TAS, ALT, insulin and HOMA-IR were log transformed. Five adjustment sets were considered: Model 1—crude; Model 2—adjusted for age + sex; Model 3—adjusted for age + sex + BMI; and Model 4—adjusted for age + sex + BMI + PAL + smoking + center of the study; Model 5—adjusted for age + sex + BMI + PAL + smoking +center of the study + nutrient intake of the specific AA. In all tests, a p-Value<0.05 was deemed significant.

5. Conclusions

In conclusion, different plasma AA levels were observed according to sex and different MRI clinical variables. Also, several associations were presented between AA and different markers that reflect the disease, such as MRI parameters, biochemical and oxidative stress indices indicating a potential use of AAs as predictive markers of the disease activity.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Correlation analysis between Amino Acid Concentrations and MRI parameters, inflammation and oxidative stress biomarkers, biochemical parameters & anthropometrics. Pearson's correlation coefficient for normally distributed variables and Spearman's rank correlation for non-normally distributed variables. Note: *normally distributed variable. Correlation coefficient values are given. Yellow color indicates a statistically significant result of correlation analysis. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

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