



Serum amino acid profiling in differentiating clinical outcomes of multiple sclerosis

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ABSTRACT

Aim of the study. Amino acid metabolism is crucial for regulating immune responses and can be monitored in blood serum samples. This study aimed to analyse serum amino acid profiles in people with multiple sclerosis (pwMS), taking into account differences depending on the disease outcomes.

Clinical rationale for the study. Serum amino acid profiling is a promising, reproducible and minimally invasive technology, available at different stages of the disease, enabling the search for a specific biomarker to differentiate MS clinical outcomes.

Material and methods. The serum concentrations of 29 amino acids were determined using high-performance liquid chromatography mass spectrometry.

Results. A total of 121 pwMS (41 relapsing-remitting MS–RRMS; 55 secondary progressive MS – SPMS; and 25 primary progressive MS–RRMS) with a median Expanded Disability Status Scale (EDSS) score of 6 and 53 healthy controls (HCs) were included. We found significantly higher serum total amino acids concentrations in pwMS compared to HCs. Serum concentrations of arginine, 1-methyl-L-histidine and proline were higher in pwMS, while circulating citrulline, α-aminobutyric acid and tryptophan were lower in pwMS. We observed significant differences in serum total amino acids concentrations depending on MS type, with the highest level in the PPMS group and the lowest in the RRMS group. We found significantly higher serum levels of beta-aminoisobutyric acid in PPMS patients compared to those with RRMS and SPMS, and significantly higher serum levels of aspartic acid in PPMS patients compared to RRMS patients. From visual inspection, no trend was observed in total amino acids concentration with respect to the EDSS score. When analysing serum total amino acids concentration in pwMS with EDSS ≤ 5 compared to those with EDSS > 5, no significant differences were found.

Conclusions and clinical implications. Amino acid metabolism is altered in pwMS and depends on the clinical type of the disease. Further studies are needed to determine whether serum metabolomic profiling of amino acids may have an application in the search for clinical phenotype-specific MS biomarkers.

Key words: metabolomics, amino acid, multiple sclerosis, disability, biomarker

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Received: 10.05.2023 Accepted: 18.07.2023 Early publication date: 01.08.2023

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Introduction

Multiple sclerosis (MS) is an acquired, chronic disease caused by autoreactive T and B lymphocytes targeting the central nervous system (CNS) antigens, leading to the primary destruction of myelin sheaths with subsequent axonal degeneration and neuronal loss [1, 2]. MS is an unpredictable and highly heterogeneous disease, and remains the most common non-traumatic cause of permanent disability in young adults [2, 3].

Typically, MS begins with an initial relapsing-remitting course (RRMS), mediated by adaptive and innate immune responses, and characterised by the formation of acute inflammatory demyelinating areas in the CNS. Over the next 15–20 years, RRMS usually evolves into a progressive phase of the disease with underlying diffuse neurodegenerative activity, resulting in steadily increasing neurological dysfunction, defined as secondary progressive MS (SPMS) [1, 4–6]. About 15% of patients experience a gradual disability worsening from the disease onset; this is classified as primary progressive MS (PPMS) [3]. Notably, the progressive types of MS are mainly driven by a heightened innate immune mechanism within the CNS [2, 7]. Both innate and adaptive immune systems are modulated by chemical signalling networks and depend on an adequate supply of amino acids necessary to maintain basal metabolism as well as protein molecules synthesis [8, 9].

Metabolomic profiling of amino acids is a promising method of elucidating underlying mechanisms and the search for biomarkers, as well as providing data on treatment strategies of both autoimmune and neurodegenerative diseases [10–13]. Depending on the possibility of endogenous synthesis, amino acids can be classified as: essential [lysine (LYS), histidine (HIS), threonine (THR), phenylalanine (PHE), tryptophan (TRP), methionine (MET), leucine (LEU), isoleucine (ILE), valine (VAL)]; conditionally essential [glutamine (GLN), arginine (ARG), cysteine (CYS), glycine (GLY), proline (PRO) and tyrosine (TYR)]; or non-essential [glutamate (GLU), alanine (ALA), serine (SER), asparagine (ASN), and aspartic acid (ASP)] [14]. Previous studies have reported changes in amino acid profiles in serum and cerebrospinal fluid (CSF) of pwMS. However, these studies were often restricted to relatively small patient groups, limited arrays of amino acids (e.g. excitatory or non-essential), and an assessment of various biological samples derived from patients with different MS types [15–17].

Currently, MS is diagnosed based on clinical features, magnetic resonance imaging (MRI) and the presence of a molecular biomarker in the form of oligoclonal bands (OCB) in the CSF [2, 3]. Although CSF analysis is a recognised diagnostic tool for assessing the inflammatory profile of the CNS, its collection can be associated with several inconveniences [15]. Therefore, biomarkers of MS available in serum are increasingly being sought because blood sampling is more accessible, less invasive, and more reproducible at any MS stage compared to routine CSF collection [18]. There is a strong

need to discover new biochemical markers in the blood serum that may be useful in MS diagnosis and monitoring clinical outcomes of the disease [19].

The aim of this study was to analyse the profile of 29 amino acids in the serum of pwMS, taking into account differences depending on the disease outcomes.

Clinical rationale for the study

MS is characterised by unpredictable and highly heterogeneous clinical outcomes. There are major differences between the relapsing-remitting and progressive MS types, especially in terms of underlying pathogenesis, treatment response, and disability progression. The differences existing between MS types must be taken into account when choosing the appropriate disease-modifying therapies (DMTs). However, they are poorly reflected in commonly available diagnostic methods.

There is therefore a strong need to identify biomarkers that facilitate an early and accurate diagnosis of MS as well as differentiation between disease types. Amino acid metabolism is involved in the modulation of autoimmune responses and can be monitored in CSF and blood samples. Serum amino acid profiling is a promising, reproducible and minimally invasive technology, available at different stages of the disease, enabling the search for a specific biomarker to distinguish MS clinical outcomes.

Material and methods

Participants and study design

We conducted a cross-sectional study. The study population consisted of 121 pwMS (80 females) and 53 healthy controls (HCs). PwMS patients were recruited from the Neurology Outpatient Clinic at Sanitas (Bydgoszcz, Poland) and the MS Rehabilitation Centre (Borne Sulinowo, Poland). HCs had no evidence of central or peripheral nervous system disorders and were recruited from the local community of Bydgoszcz. Inclusion criteria for pwMS were a diagnosis of MS according to the revised 2017 McDonald criteria and age 18 years or older [3]. Exclusion criteria for pwMS were steroid treatment and/or disease relapse within three months preceding the blood draws. A relapse was defined as the presence of new, or the recurrence of previous, MS-related symptoms lasting more than 24 hours in the absence of infection and/or fever [3, 20].

The following clinical and demographic data were obtained from available medical records: age, sex, MS type, disease duration, disability status, and the use of DMTs. Patient disability was determined based on the Expanded Disability Status Scale (EDSS) [21]. MS clinical phenotypes were classified as RRMS, SPMS, or PPMS according to the Lublin and Reingold classification [22]. Blood samples (5 ml) were drawn at 7–9 a.m. after an overnight fast and stored at -80°C until metabolomics analysis. The study participants were instructed to maintain their current dietary habits and to avoid supplementation with

products that could increase protein intake within seven days preceding blood sample collection.

Chemicals and reagents

Amino acids standards at a concentration of 200 nmol/mL and the derivatisation reagents were included in the EZ:faast™ LC-MS Free Amino Acid kit. The EZ:faast™ amino acid analysis kit was obtained from Phenomenex, Inc. (Torrance, CA, USA). High-performance liquid chromatography (HPLC) grade methanol was obtained from Merck (Darmstadt, Germany). Ammonium acetate and formic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Water was deionised and purified using a Milli-Q system (Millipore, Bedford, MA, USA) and used to prepare all aqueous solutions.

Instrumentation and conditions

The HPLC system consisted of a binary Nexera XR LC-20 AD pump (Shimadzu, Kyoto, Japan) and a Nexera XR SIL-20AC autosampler (Shimadzu, Kyoto, Japan). The chromatography was performed with an EZ:faast amino acid analysis-mass spectrometry column (250 × 3.0 mm, 4 μm). The column temperature was kept at 35°C while the autosampler was maintained at 4°C. A binary gradient elution was carried out with mobile phases A (10mM ammonium formate in water) and B (10mM ammonium formate in methanol). The flow rate was 0.25 mL/min, and the injection volume was 1 μL.

Triple quadrupole tandem mass spectrometric detection was conducted on an LCMS-8045 Mass Spectrometer (Shimadzu, Kyoto, Japan). Electrospray ionisation (ESI) mass spectrometry was performed in the positive mode. Multiple reacting monitoring was used for quantification by monitoring ion transition of amino acids. LabSolutions software was used for instrument control and quantification.

Stock solutions, standards and quality control samples

A mixed stock solution of amino acids at a concentration of 100 nmol/mL was prepared using LC-MS grade water and stored for a maximum of three months at -20°C. For quantitation purposes, the stock solution was diluted in water to prepare a working range of solutions for calibration from 0.01 to 75 nmol/mL.

The kit contains as internal standard an amino acid mixture of homoarginine (HARG), methionine-d3 (MET-d3) and homophenylalanine (HPHE). The concentration in calibrators and samples was set to 200 nmol/L. The ratio between the area below the peak of the internal standard and the area below the peak of the analyte in the chromatogram was calculated. The concentration of the analyte was calculated using the slope of a calibration curve and the determined ratio.

Sample preparation

An EZ:faast amino acid analysis kit was used for serum sample preparation, and the preparation steps were as

described in the user manual. The EZ:faast amino acid analysis procedure consists of a solid phase extraction step followed by a derivatisation. The solid phase extraction is performed via a sorbent-packed tip that binds amino acids while allowing interfering compounds to flow through. Amino acids on sorbent are then extruded into the sample vial and quickly derivatised with reagent at room temperature in an aqueous solution. Derivatised amino acids concomitantly migrate to the organic layer for additional separation from interfering compounds. The organic layer is then removed, evaporated, and re-dissolved in an aqueous mobile phase and analysed on a LC/MS system.

Ethics

This study complied with the principles of the 1964 Declaration of Helsinki, and was approved by the Bioethical Committee of Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun (KB 135/2019). All subjects participated voluntarily and provided written informed consent to store their data.

Statistical analysis

The Kruskal-Wallis test was used to assess differences between continuous variables distribution (age, concentration) in relation to MS types. Post-hoc analysis was further used to explore differences between means while controlling the family error rate. Considering non-normally distributed data, differences between two groups were evaluated using Mann-Whitney U test. Bonferroni correction was used to adjust for multiple testing. Pearson's correlation was used to study the linear relationship between amino acids. All analyses and figures were done in Python [Van Rossum, G., & Drake, F. L. (2009). Python 3 Reference Manual, Scotts Valley, CA, USA: CreateSpace].

Results

The clinical and demographic characteristics of the study population are presented in Table 1. We found differences in age and sex distribution between pwMS and HCs ($p = 0.001$ and $p = 0.01$, respectively). Age differences were shown between patients with different MS types ($p < 0.001$).

We found higher serum total amino acids concentration in pwMS compared to HCs. At $\alpha = 0.05$ we observed significant differences in serum concentrations of some amino acids between pwMS and HCs, i.e. GLN ($p = 0.036$), ARG ($p = 0.0001$), citrulline (CIT) ($p = 0.0001$), 1-methyl-L-histidine (1MHIS) ($p = 0.0004$), 4-hydroxyproline (HYP) ($p = 0.047$), α -aminobutyric acid (ABA) ($p = 0.0004$), PRO ($p = 0.00005$), VAL ($p = 0.01$), TRP ($p = 0.0004$), LEU ($p = 0.036$), PHE ($p = 0.032$), and cystine (C-C) ($p = 0.041$). After Bonferroni correction (corrected p -value = 0.0017), the concentrations of ARG, CIT, 1MHIS, ABA, PRO, and TRP remained significant. Serum concentrations of ARG, 1MHIS and PRO were higher in pwMS

Table 1. Demographic and clinical data of study participants

	MS	HCs
Number of subjects (n)	121	53
Sex (male/female)	41/80	30/23
Age, years	52.5 ± 11.6	58.4 ± 12.8
Disease duration (years) [range]	16 ± 8.4	n.a.
Median EDSS score (IQR)	6 (4.0–6.5)	n.a.
MS type n (%)		
RRMS	41 (34%)	
SPMS	55 (45%)	
PPMS	25 (21%)	
Type of DMT n (%)		
Interferon-beta	2 (1.5%)	
Glatiramer acetate	1 (0.6%)	
Dimethyl fumarate	4 (3%)	
Mitoxantrone	4 (3%)	
Natalizumab	2 (1.2%)	
Fingolimod	1 (0.6%)	

MS — multiple sclerosis; RRMS — relapsing-remitting multiple sclerosis; SPMS — secondary progressive multiple sclerosis; PPMS — primary progressive multiple sclerosis; EDSS — Expanded Disability Status Scale; DMT — disease-modifying therapy; IQR — interquartile range

compared to the controls. Serum levels of CIT, ABA and TRP were lower in pwMS compared to HCs (Fig. 1A). Differences in serum concentrations of ARG, CIT, 1MHIS, ABA, PRO, and TRP between pwMS and HCs remained significant after dividing the compared groups by gender and applying multiple testing correction (Bonferroni corrected p-value = 0,0083). We found significant differences in serum total amino acids concentration depending on MS type, with the highest level in the PPMS group and the lowest in the RRMS group (PPMS vs. RRMS $p = 0.0001$, SPMS vs. RRMS $p = 0.011$ and PPMS vs. SPMS $p = 0.04$). Post-hoc analysis revealed significantly higher concentrations of beta-aminoisobutyric acid (BAIB) in PPMS patients compared to RRMS and SPMS patients ($p = 0.033$ for both comparisons) and significantly higher serum levels of ASP in PPMS patients compared to RRMS patients ($p = 0.028$). In terms of BAIB concentrations, several outlying points were observed (Fig. 1B). We did not reveal significant differences regarding serum levels of other analysed amino acids between pwMS and HCs or between patients with different MS types.

The visual inspection of serum amino acid concentrations in a function of age revealed a slightly positive trend for C-C concentrations in pwMS and a slightly negative trend for SER concentrations in HCs (Fig. 2A). We observed some slightly positive trends between MS duration and serum levels of SER, HYP, GLY and PRO, and a slightly negative trend for TYR levels (Fig. 2B). From visual inspection, no trend was observed in total amino acids concentration with respect to the EDSS score (Fig. 2C). When analysing serum total amino acids concentration in patients with EDSS < 5 compared to those with EDSS ≥ 5, no significant differences were found ($p > 0.05$).

Discussion

This study aimed to compare profiles of 29 amino acids in serum samples from pwMS and HCs, taking into account differences depending on the disease outcomes. We found higher serum total amino acids concentrations in pwMS compared to HCs. Furthermore, serum concentrations of ARG, 1MHIS and PRO were higher in pwMS, while circulating CIT, ABA and TRP were lower in pwMS compared to the controls. We found clinical phenotype-dependent differences in serum total amino acids concentrations, with the highest values in PPMS, followed by SPMS, and then RRMS. PPMS patients had significantly higher serum levels of BAIB than RRMS and SPMS patients, as well as significantly higher serum levels of ASP compared to those with RRMS.

It has been shown that amino acid metabolism is involved in the modulation of immune mechanisms [9]. Activated immune cells require increased access to amino acids and therefore its depletion may weaken the autoimmune responses [23]. A growing body of evidence suggests that pwMS have an altered amino acid metabolism with decreased catalytic activity resulting in increased secretion of pro-inflammatory cytokines, metabotoxins production, and a reduction in Treg cell numbers [11, 23]. As patients in our group were advised to maintain their current dietary habits and to avoid protein-rich meals within seven days preceding blood sample collection, impaired metabolism may underlie the observed elevated serum total amino acids concentration. Notably, we found significantly lower ABA levels in the serum of pwMS compared to HCs, which argues against increased protein catabolism or malnutrition as potential causes [24].

We demonstrated significant differences in serum total amino acids concentration between patients with different MS clinical phenotypes, with the highest values in the progressive MS types. Therefore, our results may indicate the potential utility of such assays in accurate identifying progressive MS types as well as in highlighting the potential role of amino acids metabolism in the mechanisms underlying disease progression. Fitzgerald et al. showed greater overall metabolomic dysfunction in pwMS compared to HCs, with lower circulating lactate-related metabolites of aromatic amino acids. These differences remained consistent after excluding pwMS for whom treatment status was missing, restricting the study sample only to patients on any DMTs, and including only progressive MS patients or only those who were not on a DMT at the time of blood collection [11]. The assumptions made by Fitzgerald et al. regarding progressive MS types indicate an altered amino acid metabolism in these patients, which was also reflected in our group [11].

Several amino acids have emerged as key biological regulators of immune responses. Among these, ARG and TRP deserve special attention. Interestingly, enzymes that catabolise TRP [tryptophan dehydrogenase (TDO) and two isoenzymes of indoleamine 2,3-dioxygenase (IDO 1, IDO2)] and ARG [two

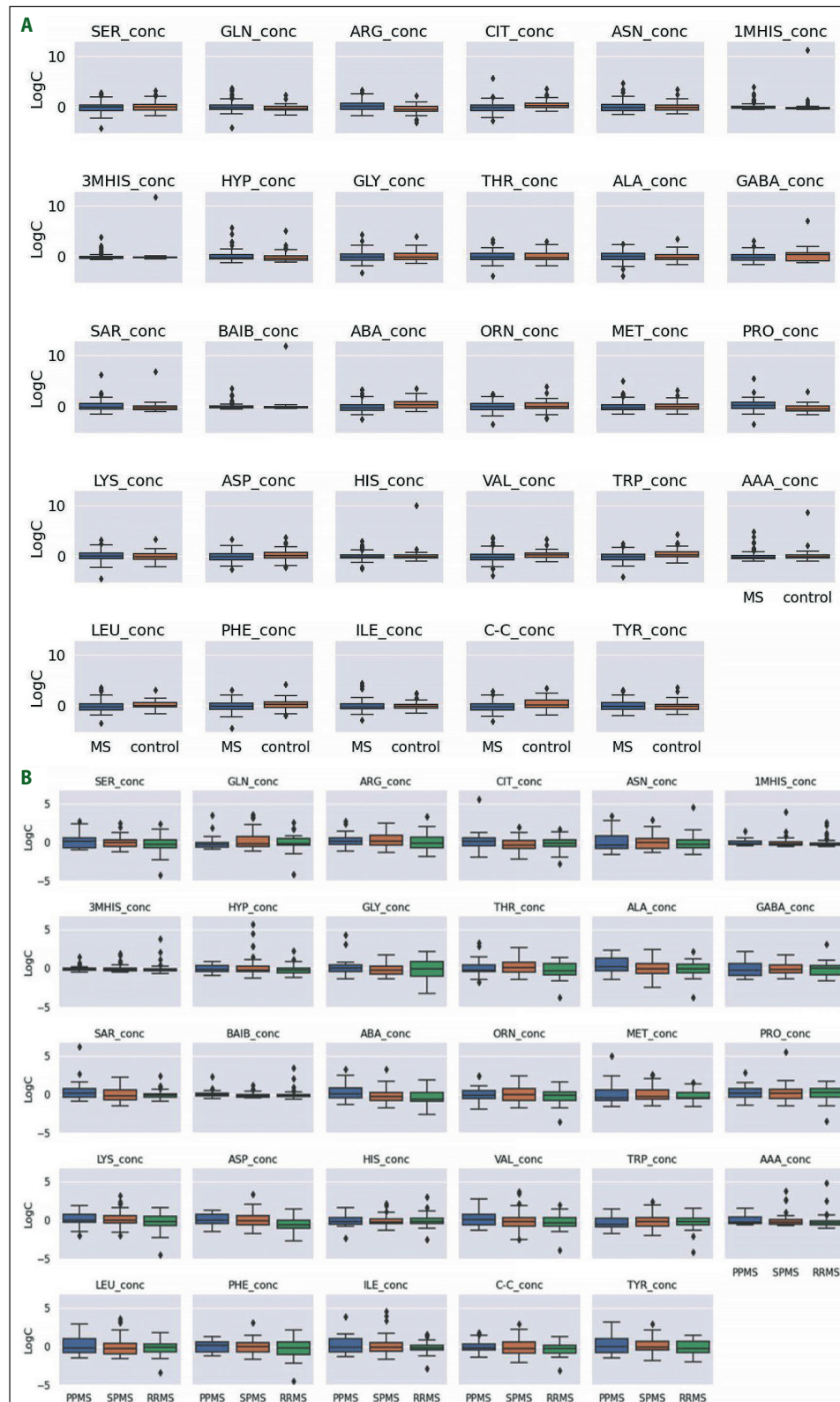


Figure 1. Centred and standardised concentrations of 29 amino acids in MS patients and controls (A), different MS types (B). Black dots represent outlying observations. MS – multiple sclerosis; RRMS – relapsing-remitting multiple sclerosis; SPMS – secondary progressive multiple sclerosis; PPMS – primary progressive multiple sclerosis; Conc – concentration; BAIB – beta-aminoisobutyric acid; ASP – aspartic acid; ARG – arginine; SER – serine; GLN – glutamine; CIT – citrulline; ASN – asparagine; 1MHIS – 1-methyl-L-histidine; 3MHIS – 3-methyl-L-histidine; HYP – 4-hydroxyproline; GLY – glycine; THR – threonine; ALA – alanine; GABA – gamma-aminobutyric acid; SAR – sarcosine; ABA – α -aminobutyric acid; ORN – ornithine; MET – methionine; PRO – proline; LYS – lysine; HIS – histidine; VAL – valine; TRP – tryptophan; AAA – α -aminoadipic acid; LEU – leucine; PHE – phenylalanine; ILE – isoleucine; C-C – cystine; TYR – tyrosine

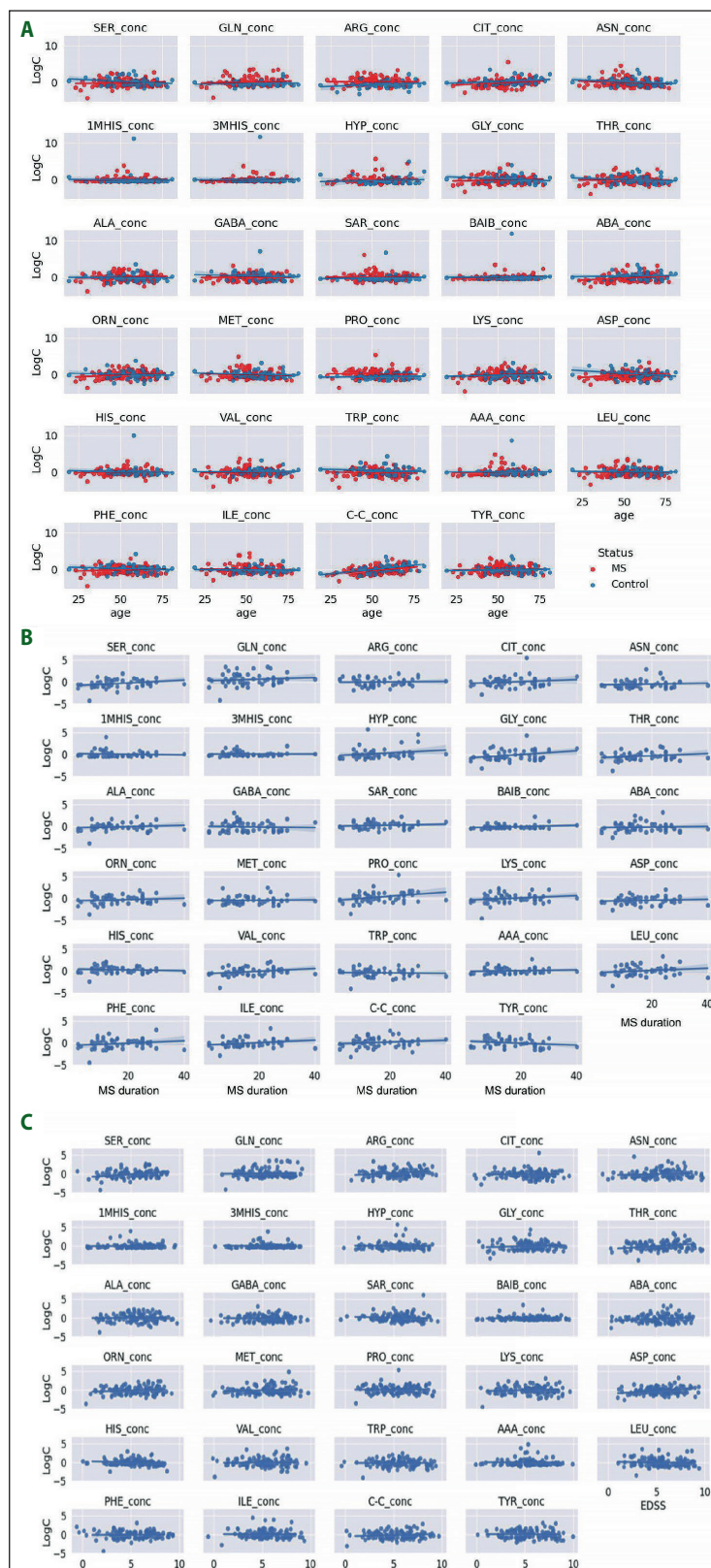


Figure 2. Centred and standardised concentrations of 29 amino acids in relation to age (A), MS duration (B) and EDSS (C). Red and blue lines represent linear regression lines. MS – multiple sclerosis; EDSS – Expanded Disability Status Scale; Conc – concentration; BAIB – beta-aminoisobutyric acid; ASP – aspartic acid; ARG – arginine; SER – serine; GLN – glutamine; CIT – citrulline; ASN – asparagine; 1MHIS – 1-methyl-L-histidine; 3MHIS – 3-methyl-L-histidine; HYP – 4-hydroxyproline; GLY – glycine; THR – threonine; ALA – alanine; GABA – gamma-amino-butyric acid; SAR – sarcosine; ABA – α -aminobutyric acid; ORN – ornithine; MET – methionine; PRO – proline; LYS – lysine; HIS – histidine; VAL – valine; TRP – tryptophan; AAA – α -amino adipic acid; LEU – leucine; PHE – phenylalanine; ILE – isoleucine; C-C – cystine; TYR – tyrosine

arginase isoforms (ARG1, ARG2) and inducible nitric oxide synthase (iNOS)] have potential immunosuppressive properties [25–27]. In our study, analyses using individual amino acids showed significant differences in serum levels of TRP, ARG and two amino acids involved in ARG metabolism (PRO and CIT) between pwMS and HCs. ARG is a semi-essential amino acid that can be derived from diet, protein breakdown or synthesised from CIT. The catabolism of ARG occurs via multiple pathways, including degradation by iNOS to nitric oxide (NO) and CIT as well as arginase-mediated degradation leading to the formation of PRO, urea, ornithine and polyamines.

Thus, the higher serum concentrations of ARG and PRO with lower concentration of CIT found in our patients may indicate a shift in ARG metabolism towards decreased degradation by iNOS. This alteration of ARG metabolism may have protective effects on pwMS as polyamines and PRO are important for tissue repair, while the excessive production of NO causes nitroxidative stress and contributes to neurodegeneration in MS [28, 29, 30]. Furthermore, we showed a slightly positive trend for serum PRO concentration in relation to disease duration, which may confirm the significant role of altered ARG metabolism at different stages of the disease.

Conversely, serum TRP concentrations were significantly reduced in pwMS compared to HCs and a slightly negative trend of TRP levels was observed in relation to the disease duration. In line with our findings, decreased TRP levels in plasma obtained from pwMS have been previously reported [31, 32]. Recent studies have shown that activation of the kynurenine pathway (KP), responsible for more than 95% of TRP degradation, plays a key role in MS pathogenesis by modulating cell-mediated immune responses and may be associated with disease progression [33, 34]. Lim et al. found decreased serum TRP concentrations and a significantly higher kynurenine/tryptophan ratio in pwMS compared to HCs, and this was more pronounced in those with progressive disease types, suggesting that abnormalities in the KP may be associated with the conversion from early-mild stage to progressive MS forms, which is consistent with our findings [35].

The dominance of mechanisms mediated by excitatory amino acids may contribute to neurodegeneration within the CNS through excitotoxic injury of neurons and glial cells [17]. Murgia et al. identified ALA, ASP, and GLU metabolism among the most altered pathways in serum samples between patients with PPMS and RRMS [17]. In our study, patients with PPMS had higher serum ASP concentrations compared to RRMS patients. In this context, our findings are in line with the results reported by Murgia et al., and confirm a shift towards excitatory amino acids dominance in the progressive types of MS [17].

In the present study, patients with PPMS were characterised by higher serum BAIBA levels compared to those with SPMS and RRMS. BAIBA has been categorised as a myokine produced and secreted by skeletal myocytes during physical activity [36]. Recent studies have shown that BAIBA can inhibit

hypothalamic inflammation and reverse the inflammatory processes in animal models of diet-induced obesity, protecting against vascular inflammation by enhancing the gene expression of the antioxidants and mitochondrial biogenesis-related molecules in humans. Therefore, the antiatherogenic properties of BAIBA could explain the beneficial effects of exercise on obesity and vascular endothelial function [37, 38]. So far, the impact of elevated serum BAIBA concentrations on MS pathogenesis and disability progression has not been reported. In our study, the increase in BAIBA concentrations in the serum of PPMS patients may have resulted from more intensive physical rehabilitation in this group compared to patients with an initial relapsing-remitting disease onset. Future studies are needed to address the potential role of BAIBA in MS, taking into account the intensity of physical rehabilitation.

Although we adopted dietary restrictions prior to blood sampling, we did not consider differences in the frequency and intensity of physical rehabilitation as potential factors influencing serum amino acid concentrations, which is a limitation of our study. Another limitation is a relatively large number of patients with progressive MS types, which made it possible to specify the profile of amino acids involved in neurodegenerative mechanisms, although this translates into overrepresentation of this group of patients and demographic heterogeneity of the entire study cohort.

Notably, age differences between the analysed groups found in our study were also observed in previous reports evaluating the metabolic alterations in MS and may confirm the overall heterogeneity of the disease [11, 17, 39]. So far, studies evaluating age- and sex-related differences in serum amino acid concentrations have used different methodologies, their results have been inconsistent, and they have not identified specific metabolic patterns [40–44]. These issues remain insufficiently addressed in relation to pwMS as well. In our study, the observed differences in serum concentrations of ARG, CIT, 1MHIS, ABA, PRO, and TRP between pwMS and HC remained significant after dividing the compared groups by gender. Furthermore, no linear trends (positive or negative) with ageing were found for the serum concentrations of the above-mentioned amino acids in pwMS and HCs.

With respect to MS type, the majority of our patients had a progressive MS course (66%) with reduced ambulatory ability (median EDSS score was 6, interquartile range 4.0–6.5), and 14 (11.5%) of them used DMTs. The lack of assessment of demyelinating lesions on magnetic resonance imaging and the low percentage of pwMS using DMTs can be considered as further limitations of our study. However, the negligible exposure of patients to DMTs at the time of blood sample collection may more reliably represent the changes in amino acid metabolism underlying the pathogenesis of various MS types. Finally, an undoubted limitation of this study is the lack of a prospective assessment of changes in plasma amino acid profiles in pwMS, including clinical phenotype-dependent differences.

Clinical implications/future directions

Our study revealed different patterns of serum amino acid profiles between pwMS and HCs as well as between patients with various disease types. The demonstrated differences may result from the participation of amino acids in immune responses, neurodegeneration processes and construction of muscle proteins. Therefore, serum amino acids may be considered as potential molecular biomarkers of MS, components of individualised therapeutic agents, or laboratory indicators to monitor the intensity of physical rehabilitation.

Future studies on amino acid profiling in MS involving a larger group of patients with different disease types are needed, taking into account the type of DMTs used, the profile of amino acids that best discriminates RRMS from progressive MS types and HCs, as well as the prospective evaluation of blood samples.

Conflicts of interest: *None.*

Funding: *None.*

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