

Amino acids levels as a potential biomarker in myasthenia gravis

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Folia Neuropathol 2022; 60 (1): 122-127

DOI: <https://doi.org/10.5114/fn.2022.114053>

Abstract

The search for new diagnostic and therapeutic approaches for myasthenia gravis (MG) is highly desirable. Therefore, the aim of the present study is to assess the profile of non-essential amino acids in the serum of MG patients. We evaluated the serum levels of non-essential amino acids in MG patients ($n = 10$) and control subjects (CS, $n = 10$) using high-performance liquid chromatography (HPLC) method and assuming a two-fold concentration difference between the groups as significant. Serum levels of aspartic acid and glutamic acid in MG patients were significantly higher than in CS. There were no significant differences in mean serum levels of glycine, proline, alanine, serine, cysteine, arginine and tyrosine between MG patients and CS. The results indicate the need for further research to assess the role of non-essential amino acids in MG. Moreover, our preliminary results suggest that the metabolism of some amino acids seems to be changed in patients with MG. Therefore, we conclude that amino acids profiling might be helpful in searching for putative biomarkers of the central nervous system diseases such as myasthenia gravis.

Key words: metabolomics, amino acid profiling, myasthenia gravis, diagnosis, biomarker.

Introduction

Myasthenia gravis (MG) is a chronic autoimmune disorder caused by antibodies against postsynaptic membrane proteins at the neuromuscular junction receptors of striated skeletal muscles. Dysfunction of neuromuscular transmission results in fatigable muscle weakness, which can be restricted to ocular muscles (ocular MG) or involve the extra-ocular muscles (generalized MG). Due to clinical features mimicking stroke, motor neuron disease or inflammatory neuropathies, an early and accurate diagnosis of MG remains a big challenge [2,7,8]. The diagnosis of MG is established on the basis of clinical symptoms, electrophysiologic studies, detection of specific antibodies and response to treatment [1]. The type of autoantibodies found is

closely related to the distribution of muscle involvement, the age of first symptoms, the severity of the disease, and resistance to immunotherapy. Importantly, the serum autoantibody levels do not reliably predict the disease course [7]. Moreover, autoantibodies against the nicotinic acetylcholine receptor (AChR) are found in about 80% of patients with generalized MG and in 50% of ocular MG patients. In around 5% of patients with generalized MG, antibodies towards muscle-specific tyrosine kinase (MuSK) are detected. Thus, approximately 15% of patients with generalized MG and 50% of ocular MG subjects remain seronegative both to AChR and MuSK [6,18,22]. In all seronegative MG patients, electrophysiological testing is required for confirmation of neuromuscular junction disturbances.

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Noteworthy, the sensitivity of repetitive nerve stimulation in generalized MG and in MG with antibodies to MuSK is about 80% and 50%, respectively [13,16]. Furthermore, the fluctuation of clinical symptoms may delay the proper diagnosis and treatment [4]. Therefore, there is a strong need to find a specific biomarker for MG identification. Recently, metabolomic profiling has been shown to be capable of distinguishing patients with seropositive MG from those without and elevated serum κ free light chain levels have been reported as a potential biomarker for double seronegative disease type [3,23]. The aim of the present study is to assess the profile of non-essential amino acids in the serum of MG patients with different disease severity.

Material and methods

Chemicals and materials

AccQ Fluor Reagent Kit (Waters), amino acid standards (Waters), internal standard α -aminobutyric acid (Sigma Aldrich) acetonitrile and methanol (Sigma Aldrich). Deionized water, purified with Direct-QUV (Millipore, France) system was used for all aqueous solutions.

Instrumentation

HPLC Shimadzu combined with DAD detector and RF-20A XS fluorescence detector (Shimadzu, Kyoto, Japan).

Sample collection and preparation

Blood samples were collected from the patients after overnight fasting. Preparation of serum for chromatographic analysis included deproteinization and derivatization of the sample. For the serum deproteinization, Phree Phospholipid Removal Solutions, with a solid phase extraction kit, were used (acetonitrile with 1% formic acid, internal standard [IS] and serum loaded to the cartridge). The serum samples prepared were subjected to a derivatization procedure in accordance with the Waters AccQ Tag method. The course of the derivatization procedure was the following: taking deproteinized serum, adding reagent # 1 (AccQ Fluor Borate Buffer), adding reagent # 2 (reconstituted AccQ Fluor Reagent), leaving the mixture for one minute at room temperature, placing the mixture in a thermoblock (55°C, 10 minutes). Finally, derivatized samples were anal-

ysed using high-performance liquid chromatography with fluorescence detection.

Chromatographic method

Chromatographic analysis, which was aimed at measuring the amino acid concentration in samples, was carried out using the high-performance liquid chromatography (HPLC) with fluorescence detection using AccQ Tag column (Waters). The final parameters of the chromatographic system consist of: 150 mm \times 3.9 mm chromatographic column, AccQ Tag (Waters), fluorescence detection: ex/em 250/395, mobile phase A: AccQ Tag Eluent A (Buffer), mobile phase B: Acetonitrile, mobile phase C: Water, gradient elution, flow: 1 ml/min, injection volume: 5 μ l, analysis time: 35 minutes. Chromatographic method with fluorescence detection was optimized and validated with the use of standard solutions of 18 amino acids 1) aspartic acid (Asp), 2) serine (Ser), 3) glutamic acid (Glu), 4) glycine (Gly), 5) histidine (His), 6) NH₃, 7) arginine (Arg), 8) threonine (Thr), 9) alanine (Ala), 10) proline (Pro), 11) cysteine (Cys), 12) tyrosine (Tyr), 13) valine (Val), 14) methionine (Met), 15) lysine (Lys), 16) isoleucine (Ile), 17) leucine (Leu), and 18) phenylalanine (Phe). The quantitative analysis was performed with the internal standard α -aminobutyric acid, added during serum deproteinization using Phree Phospholipid Removal Solutions columns with a solid phase extraction kit.

Characteristics of study participants

Ten women consulted in a neurological outpatient clinic with a diagnosis of MG, with a mean age of 47.9 \pm 10.5 were enrolled in the study. MG was diagnosed based on clinical features (fluctuating fatigable weakness of ocular and/or extraocular muscles) and at least one of the following criteria: positive test for AChR or MuSK autoantibodies, electrophysiological studies confirming postsynaptic neuromuscular junction dysfunction (repetitive stimulation and/or single fibre electromyography), and clinical response to cholinesterase inhibitors. The mean disease duration was 9.6 \pm 5.7 years. With regard to the clinical type of the disease, all patients were classified as generalized MG. Autoantibodies against AChR were found in 7 subjects and the remaining 3 patients were double-seronegative (negative test for anti-AChR and anti-MuSK antibodies). The thymic abnormalities were detected in 4 patients. No patient was diagnosed with thymoma.

The distribution of individuals in relation to the Myasthenia Gravis Foundation of America classification was: class IIA – 4 patients, class IIB – 1 patient, class IIIA – 3 patients, class IIIB – 1 patient, and class IVA – 1 patient [10]. All of investigated MG patients used pyridostigmine bromide, 9 steroids, and 4 immunosuppressive therapies. Ten age-matched women (mean age: 47.4 ± 11.1 years) without disorders of the central and peripheral nervous system served as control subjects (CS). The study protocol was approved

by the Bioethics Committee of Ludwik Rydygier Collegium Medicum (KB 135/2019), and written informed consent was obtained from all participants.

Results and discussion

Examples of the chromatograms presenting separation of amino acid standards as well as presenting separation of amino acid compounds in plasma were presented in Figure 1A, B, respectively. The mean serum levels of Asp and Glu were significantly

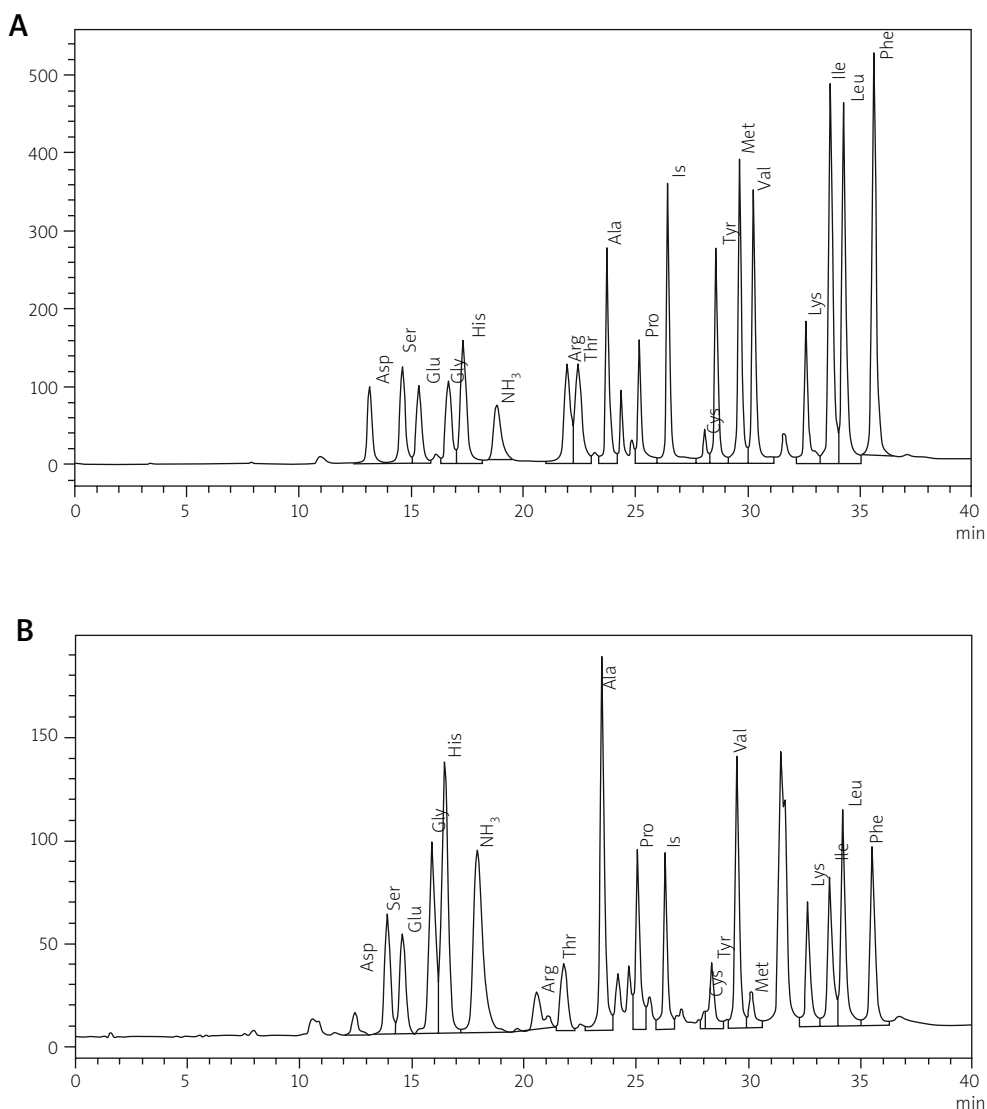


Fig. 1. Chromatogram presenting separation of amino acid standards (A) and presenting separation of amino acids in plasma (B). The order of peak elution: 1. Asp, 2. Ser, 3. Glu, 4. Gly, 5. His, 6. NH₃, 7. Arg, 8. Thr, 9. Ala, 10. Pro, 11. Cys, 12. Tyr, 13. Val, 14. Met, 15. Lys, 16. Ile, 17. Leu, and 18. Phe.

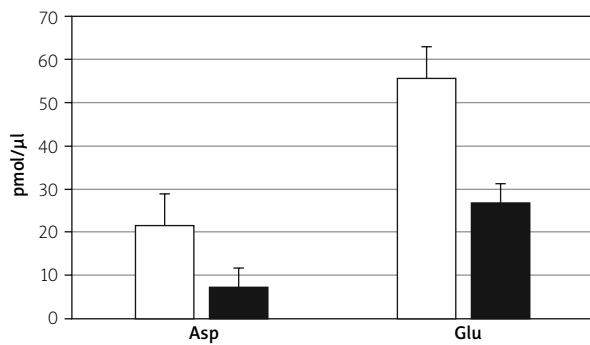


Fig. 2. Serum levels of endogenous amino acids in myasthenia gravis (MG) patients (□) vs. control subjects (CS) without any disease of the nervous system (■) for which plasma concentrations were significantly higher in MG subjects compared to CS. Values are means (\pm SD) of three individual experiments.

higher in MG subjects compared to CS (Fig. 2). There were no statistically significant differences in the average plasma concentrations of Ala, Ser, Gly, Arg, Cys, Pro and Tyr between MG patients and CS (Fig. 3).

There are no biomarkers that reliably identify MG and its clinical course. The available diagnostic tests can be false negative, and diagnosing particularly seronegative MG remains a big challenge [7]. Importantly, the available MG biomarkers are determined from blood samples, which confirms the peripheral nature of the autoimmune response in this disease entity. Therefore, profiling of serum amino acids for new biomarkers of MG may be warranted. Amino acids as the basic building blocks of proteins can play an important role in the formation and functioning of the neuromuscular junction [25]. Moreover, the infusions of amino acid-enriched solutions are recognized as a valuable source of energy in the skeletal muscles, and their effect on accelerating the regeneration of the neuromuscular junction has been proven [19]. Furthermore, the main immunogenic region of the muscle nicotinic AChR is formed by the amino acids mapped to residues alpha 67-76 of the α -subunits [7,21]. In the present study, we found significant differences in serum levels of two non-essential amino acids in MG patients compared to CS. The plasma concentrations of Asp and Glu were higher in MG participants, while there were no significant differences in plasma concentrations of the other seven non-essential amino acids (Ala, Ser, Gly, Arg, Cys, Pro and Tyr) between patients with MG and CS. Interestingly, the difference

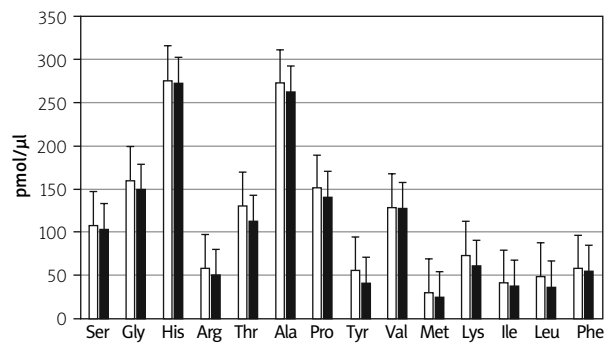


Fig. 3. Serum levels of endogenous amino acids in myasthenia gravis (MG) patients (□) vs. control subjects (CS) without any disease of the nervous system (■) for which plasma concentrations were not significantly different between the compared groups. Values are means (\pm SD) of three individual experiments.

concerned only excitatory amino acids. Non-essential amino acids can be synthesized endogenously and remain crucial for acetylcholine synthesis, modulation of immune system responses as well as regulation of antioxidative reactions [9,24]. Due to the lack of data on the serum non-essential amino acid levels in MG patients, the interpretation of the obtained results is hindered. Noteworthy, some reports suggest that glutamate may participate in modulating cholinergic transmission and plastic changes at the skeletal neuromuscular junction of vertebrates [5,15]. Likewise, the animal study of multiple sclerosis (MS) metabolomics conducted in experimental autoimmune encephalomyelitis (EAE) confirmed that at the peak of the disease, glutamine was significantly increased. However this animal model does not reflect all aspects of MS patients [17]. Although glutamine is identified as a biomarker in multiple sclerosis patients, it should be emphasized that multiple sclerosis specific biomarkers including glutamine still show inconsistent metabolite concentration due to multiple sclerosis heterogeneity as well as technical and chemometric limitations [11]. Currently the knowledge of unique metabolomic fingerprint in autoimmune diseases, for instance multiple sclerosis, rheumatoid arthritis, inflammatory bowel diseases, autoimmune diabetes etc. may be employed in the diagnosis, treatment, and detection mechanisms of diseases. Many authors reported that it is more and more possible to distinguish patients suffering from autoimmune diseases from healthy individuals with the use of metabolic

profiling coupled with chemometric analysis [11]. Changes in serum or plasma amino acid concentrations have been described in a number of disorders. For instance in the study by Smolenska *et al.*, elevated concentrations of arginine, acidic and aromatic amino acids with a simultaneous lower concentration of lysine were reported for rheumatoid arthritis (RA) compared to healthy subjects. What is more, correlations of amino acid levels with clinical indices and type of treatment were found in abovementioned study [20]. Also other studies indicate lower levels of histidine, methionine, asparagine and threonine whereas higher levels of glutamic acid in RA versus controls [14]. Amino acids and products of metabolic processes determine the effector function of T cells (regulators, instigators of inflammation or effectors of cytotoxicity) and that is why may be linked to autoimmunity and related pathology. Amino acids influence immune response, because they are essential for the generation of building blocks needed for cell proliferation, the generation of energy by controlling metabolic pathways, the control of epigenetic pathways, the production of phospholipids and the control of oxidative stress [12]. Despite presenting the preliminary results, the authors are aware of significant study limitations. First, it should be emphasized that the investigated group consisted of women only. Second, considering the relatively small number of participants, which is the main limitation of our study, further research should assess the serum non-essential amino acid levels in a larger cohort of MG subjects.

In conclusion, we have shown that levels of aspartic acid and glutamic acid were significantly higher in MG patients than in CS. These results show that amino acids may be involved in myasthenia gravis neurodegeneration mechanisms as well as may be potential biomarkers in MG patients' diagnosis. However, considering the multifactorial, heterogenous and complex nature of this disease, validation on a larger study sample in further research is required before application into diagnostic practice.

Research involving human participants and/or animals

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Bioethics Committee of Ludwik Rydygier Collegium Medicum (KB 135/2019).

Informed consent

Informed consent was obtained from all individual participants included in the study.

Disclosure

The authors report no conflict of interest.

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