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Plasma citrulline levels are increased in patients with multiple sclerosis

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ABSTRACT

Background: Multiple sclerosis is an inflammatory demyelinating disorder of the central nervous system (CNS). Myelin basic protein (MBP), which is one of the main compounds of CNS myelin, appears to be hypercitrullinated in the brain of patients with MS. We hypothesized that MS is associated with an increased release of citrulline from the brain.

Methods: Twenty-five patients with MS, 25 controls without neurological disease (CwND) and 25 subjects with non-MS cerebral white matter lesions were included in this study. Groups were matched for age and gender. Clinical MS disability measures were recorded by means of Expanded Disability Status Scale (EDSS) scores and Multiple Sclerosis Severity Scores (MSSS). Citrulline was assessed in plasma obtained from an antecubital peripheral vein (PV) in all participants. Additional internal jugular vein (IJV) samples were examined in 10 patients with MS and 10 CwND. Twelve patients with MS underwent brain magnetic resonance imaging to determine total brain and T2 fluid-attenuated inversion recovery lesion volume.

Results: Median [IQR] PV citrulline levels were increased in patients with MS (50.47 [86.61] μM), as compared to CwND (33.58 [43.65] μM, $P = 0.042$) and subjects with non-MS cerebral white matter lesions (32.41 [28.86] μM, $P = 0.006$). Citrulline IJV levels and IJV/PV ratios were comparable between patients with MS and CwND. No significant correlations were found between PV citrulline levels and any of the clinical, nor radiological, disease measures.

Conclusion: PV plasma levels of citrulline are elevated in patients with MS but this does not seem to result from an augmented release from the brain. Increased plasma citrulline may be a promising new biomarker in MS but the origin and significance need to be further elucidated.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating and degenerative disease of the central nervous system (CNS) affecting over 2 million people worldwide [1]. Myelin covers most axons in the brain and spinal cord to form a protective lipid- and protein-rich sheath which, among other functions, facilitates nerve conduction and provides trophic support. Myelin basic protein (MBP) is one of the most abundant proteins in CNS myelin [2]. The exact underlying pathophysiology of MS remains obscure but demyelination involves T-cell mediated inflammatory responses directed against MBP [3].

The pool of MBP in the human brain is very diverse. The coding gene is located on chromosome 18 but alternative exon splicing leads to the expression of multiple isoforms, varying in length and molecular weight [4]. Subsequently, MBP can undergo several posttranslational modification steps, one of which is the conversion of arginine to citrulline residues by peptidylarginine deiminase (PAD) enzymes [5]. A 18.5 kD (170 amino acid residues) uncitrullinated protein, termed MBP-C1, is the dominant subtype in mature CNS myelin of healthy individuals. Myelin isolated from the brain of subjects with MS appears to contain a higher proportion of MBP-C8, a variant in which 6 out of 19 arginine residues are converted to citrulline, as compared to control samples [6,7], and one study using ^{1}H-magnetic spectroscopy suggested that, as a consequence, total citrulline concentrations are elevated in

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cerebral white matter [8]. MBP hypercitrullination in MS is most likely mediated by increased PAD2 and 4 enzymatic activity and decreases the net positive charge of the protein, resulting in an impaired capacity to interact with negatively charged lipid compounds. These processes potentially lead to a less compact structure of the myelin sheath and increased susceptibility to proteolytic degradation [5].

Reliable body fluid biomarkers for MS diagnosis and treatment response monitoring are currently still lacking. Plasma citrulline levels have never been investigated within this context. In this study, we hypothesized that demyelination in MS leads to an increased release of citrulline from the brain to the circulation.

2. Methods

2.1. Study design and main objectives

We performed a prospective exploratory study at the Universitair Ziekenhuis (UZ) Brussel (Brussels, Belgium) and the Onze-Lieve Vrouw Ziekenhuis (OLVZ) Aalst (Aalst, Belgium) to investigate peripheral vein (PV) and internal jugular vein (IJV) plasma levels of citrulline in patients with MS and control subjects without neurologic disease (CwND). Plasma arginine, nitric oxide (NO) and glutamine levels were contemporaneously assessed since these agents are closely related to the metabolism of citrulline [9]. The difference in PV plasma citrulline levels between both groups was selected as the primary endpoint of this study. Significant alterations in patients with MS were verified against a second control group of individuals with non-MS cerebral white matter lesions to increase specificity. The study was approved by the ethics committees of both centers and conformed to the Declaration of Helskini principles. All participants were recruited from April 2011 to March 2014, and provided a written informed consent.

2.2. Subjects and blood sampling

Twenty-five patients with MS (relapsing-remitting: 12; secondary progressive: 10; primary progressive: 3), according to the 2010 revised McDonald criteria [10], 25 CwND and 25 subjects with non-MS cerebral white matter laesions (ischaemic leukoencephalopathy: 19; migraine: 2; antiphospholipid syndrome: 1; post-chemotherapy: 1; idiopathic: 1) were included in this study. All groups were *a priori* matched for age and gender. Individuals known with chronic kidney disease were excluded. Eleven MS patients received immunomodulatory drugs (interferon β-1a: 7; interferon β-1b: 2; glatiramere acetate: 1; natalizumab: 1). All MS patients were clinically stable without evidence of an exacerbation within 3 months prior to inclusion. The diagnosis of non-MS cerebral white matter laesions was based on a historic brain MRI, available in the medical records of these individuals, demonstrating at least 3 punctate or 2 confluent areas of T2-weighted hyperintensity in the cerebral periventricular and/or subcortical white matter. All respective brain scans were reviewed by the investigators and declared as non-suggestive of MS.

Participants fasted for a minimum of 8 h before blood was drawn from an antecubital PV. All blood samples were collected between 8 and 10 am. Recruitment took place at the UZ Brussel for 15/25 patients with MS (neurology department), 25/25 CwND (healthy volunteers and aenesthesiology department, see below) and 25/25 subjects with non-MS cerebral white matter laesions (neurology department). In 10 patients with MS and 10 CwND an additional blood sample was collected from the right IJV, simultaneously with the PV blood draw. These patients with MS (10/25) were recruited at the Department of Cardiovascular and Thoracic Surgery of the OLVZ Aalst, where they were actively seeking treatment with dilatation angioplasty, for so-called chronic cerebrospinal venous insufficiency [11,12], on their own initiative and specific request. The investigators were never involved in this therapeutic decision process. MS diagnosis was confirmed based on clinical grounds and access to the patient's medical records. The respective CwND (10/25) were individuals who underwent central venous catheter placement prior to scheduled cardiac surgery or pacemaker placement at the UZ Brussel. All endovascular procedures were performed by the same experienced cardiovascular surgeon in the patients with MS, or aenesthesiologist in the controls. Correct IJV localization was radiographically controlled in every case.

Blood samples were centrifuged and plasma was stored at −80 °C as soon as possible. Plasma citrulline, arginine and glutamine levels were determined by high-performance liquid chromatography. NO is an unstable gas which is rapidly oxidized to its breakdown products (NOx) nitrite and nitrate [13], and can therefore only be indirectly assessed. NOx levels were measured colorimetrically after reduction of nitrate to nitrite by nitrate reductase with a commercially available kit (Enzo Life Sciences®; Zandhoven, Belgium). Plasma samples obtained at the OLVZ Aalst were transported to the laboratory of the UZ Brussel on dry ice by car and, as a result, rapid storage at −80 °C was not always feasible. Because NOx still have a relatively short half-life in plasma and stability of measurement has only been demonstrated for delayed processing up to 48 h [14,15], we excluded the OLVZ Aalst samples from the NOx analysis.

2.3. Magnetic resonance imaging

MS patients recruited at the UZ Brussel (15/25) underwent additional brain MRI in supine position using a 3 T machine (Philips Achieva; Best, The Netherlands). The protocol contained, among others, two 3D sequences: a magnetization prepared 3D T1 TurboFLASH (TR 7.8 ms; TE 3.8 ms; TI, 970 ms; flip angle 8°, 240 FOV) and a fat-saturated 3D FLAIR (TR 8000 ms, TE 260 ms, 240 FOV). We used MSmetrix® software (Icometrix; Leuven, Belgium - coveted CE mark and FDA approved) to measure FLAIR laesion load, whole brain and grey matter volumes [16]. Volumetric analyses could not be performed in 3 patients due to technical issues.

2.4. Disability and disease progression in MS

Clinical disability was measured with the Expanded Disability Status Scale (EDSS) in all patients with MS [17]. Rate of progression of clinical disability was measured with the Multiple Sclerosis Severity Score (MSSS) [18]. This score is based on the combination of EDSS score and disease duration. Both EDSS scores and MSSS range from 0 to 10. Higher scores indicate, respectively, more disability and faster progression of disability.

2.5. Statistical analysis

All statistical analyses were performed with SPSS® (Version 24.0, IBM; Amonk, NY USA). PV plasma citrulline, arginine and glutamine values from the total cohort of 75 subjects did not pass the Kolmogorov-Smirnov test for normal distribution and therefore we expressed them as median [IQR]. Amino acid levels obtained in IJV plasma were presented likewise for reasons of uniformity. All other ordinal and continuous data showed a normal distribution and were presented as mean ± SD. Group differences were assessed using Mann-Whitney *U* tests. Spearman's rank correlation was used for correlation analyses. All reported *P* values are two-tailed and declared statistically significant at the 0.05 level.

3. Results

Demographics, clinical data and PV plasma results of all participants are presented in Table 1. Citrulline PV levels were significantly increased in patients with MS, as compared to CwND ($P = 0.042$) and individuals with non-MS white matter laesions ($P = 0.006$). Arginine PV levels were decreased in patients with MS, as compared to CwND, but this difference did not reach statistical significance ($P = 0.128$). In