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Longitudinal assessment of clinical and inflammatory markers in patients with amyotrophic lateral sclerosis

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ABSTRACT

Objective: To evaluate potential associations between clinical features and inflammatory markers in patients with amyotrophic lateral sclerosis (ALS).

Methods: A consecutive series of 68 patients (39 males and 29 females) with sporadic ALS were subjected to a comprehensive clinical assessment and blood draw. A subset of these patients underwent a new assessment within 6–12 months after the baseline visit. In addition, a group of 62 subjects composed by age and sex-matched healthy subjects (38 males and 24 females) was enrolled in this study. Peripheral blood was drawn and plasma levels of chemokines and cytokines were measured by cytometric bead array and enzyme-linked immunosorbent assay.

Results: Our sample was composed by patients with ALS with an average age of 58 (± 12.3) years old and 3 (± 2.7) years of disease length at the baseline visit. Patients with ALS presented increased plasma levels of interleukin (IL)-6 and IL-8 in comparison with controls. After multivariate analysis, higher levels of IL-6 and lower levels of IL-2 were significantly associated with increased likelihood of ALS diagnosis. When evaluating the subset of patients assessed longitudinally, we did not find any significant difference in the levels of inflammatory markers between the two time points. Older age at ALS onset was the only factor associated with a faster rate of disease progression.

Conclusions: IL-6 levels could discriminate between ALS and controls and may be regarded as a potential biomarker of ALS diagnosis. An increase in IL-2 levels was associated with a protective effect on the odds of ALS diagnosis. Older age at ALS onset predicted a fast rate of disease progression.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by upper and lower motor neuron degeneration, with an average survival time of 3–5 years [1,2]. The main symptoms include progressive paresis of striated skeletal muscles, but non-motor symptoms such as pseudobulbar affect, apathy, cognitive impairment, depression and anxiety can also occur [3,4]. ALS incidence and prevalence range from 0.3–3.6 cases/100,000 persons/year and 1.0–11.3 cases/100,000 persons, respectively [5].

ALS may be sporadic (90–95% of the cases) or familial (5–10% of the cases). The pathogenesis of sporadic ALS is multifactorial and has not been fully elucidated. Several mechanisms seem to be involved in ALS pathophysiology, including glutamate-mediated excitotoxicity, neuroinflammation, disorganization of neurofilaments, mitochondrial dysfunction and impairment in the production and/or release of neurotrophic factors [6,7]. Neuroinflammation is characterized by microglial activation, astrogliosis and infiltration of peripheral immune cells such as monocytes and T cells in the central nervous system (CNS) [8]. Signs of inflammation and activation of the immune system have been reported in the blood, cerebrospinal fluid (CSF) and post-mortem brain samples of patients with ALS [9]. A positron emission tomography

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(PET) study using [^{18}F]-DPA-714 also found evidence of increased microglia activation in primary and supplementary motor areas and temporal cortex of patients with ALS [10]. Neuroinflammation seems to be a secondary event that contributes to neurodegeneration and therefore to disease progression [9,11,12].

Clinical markers such as older age, bulbar onset and a faster rate of disease progression were associated with worse outcomes in ALS [13,14]. Using both hypothesis and non-hypothesis driven approaches, a range of studies has searched for biomarkers of diagnosis, prognosis or related to pathophysiological pathways in ALS [15,16]. The potential of inflammatory molecules as diagnostic biomarkers of ALS has been explored. One study showed that a CSF biomarker panel composed by interleukin (IL)-6, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), IL-2 and IL-15 was capable of distinguishing patients with ALS from neurologically diseased controls with an accuracy of almost 90% [17]. Noteworthy, a recent meta-analysis confirmed that neurofilament levels are significantly elevated in the CSF of ALS patients in comparison with controls, and are negatively correlated with disease duration [18]. Regarding inflammation-related biomarkers for disease progression, higher plasma levels of ferritin and IL-2 have been associated with shorter survival [19], while increased serum levels of vascular endothelial growth factor (VEGF) predicted longer survival in ALS patients [20]. In addition, higher plasma levels of the chemokine CCL5/RANTES (an acronym for Regulated on Activation, Normal T Cell Expressed and Secreted) have been associated with shorter disease duration, while higher plasma levels of IL-10 with longer disease duration of ALS [21].

Valid biomarkers are necessary in clinical trials for new drugs, especially those dedicated to disease-modifying mechanisms. Besides providing pathophysiological insights, biomarkers may help in diagnosing the disease at early stages, when disease-modifying therapies are more effective. Biomarkers may also be very helpful in objectively monitoring disease progression and response to treatment. Despite the recent advances in the field of biomarkers for ALS, no biomarker was incorporated in the clinical practice to date. The available results of the studies conducted so far are limited, especially by their cross-sectional design. Accordingly, longitudinal studies are warranted to better clarify the role of inflammatory/immune mediators as ALS biomarkers, particularly markers of ALS progression. In this context, the current study was designed to investigate the potential of inflammation-related molecules as biomarkers of diagnosis and disease progression in ALS using both cross-sectional and longitudinal approaches. Our findings might be relevant to the understanding of ALS pathophysiology and to clarify which biomarkers have the potential to be incorporated in future trials and/or clinical practice.

2. Methods

2.1. Subjects and clinical evaluation

First, we performed a cross-sectional evaluation of 68 patients (39 males and 29 females) with probable or defined sporadic ALS diagnosed according to Awaji's criteria [22] (baseline group), in comparison with 62 age- and sex-matched healthy controls (38 males and 24 females). A subset of ALS patients ($N = 24$) was reassessed 6–12 months after the baseline visit (follow-up subgroup). The first and second assessments of the follow-up subgroup were named as T1 and T2, respectively. Forty-four out of the 68 patients were not reassessed due to different reasons: 9 refused to undergo a second assessment/blood draw; 2 did not have their blood drawn due to technical problems; 1 had active pneumonia at T2; 24 did not return to medical appointment due to severe illness or death; and 8 were first assessed close to the end of data collection.

Patients with ALS were consecutively recruited from the ALS outpatient clinic, University Hospital [*Universidade Federal de Minas Gerais* (UFMG), Belo Horizonte, Brazil] and Júlia Kubitschek Hospital, Belo Horizonte, Brazil. Controls were recruited from the local community and enrolled in the study if they did not present any past or current diagnosis of neurological disease. Participants were excluded if: i) they were younger than 18 years old; ii) they had a diagnosis of cancer or any inflammatory, infectious or autoimmune disease in activity within the three months prior to the study enrollment; iii) they had used corticosteroids, anti-inflammatories or antibiotics in the four weeks prior to the blood draw.

Patients with ALS were subjected to a clinical protocol that included questions about sociodemographic data, general clinical features, the ALS Functional Rating Scale Revised (ALSFRS-R) [23] and the Hospital Anxiety and Depression Scale (HAD) [24]. A score > 8 on HAD for depressive and anxiety symptoms indicated a probable case of clinically significant depression and anxiety, respectively [25]. The progression rate of the follow-up subgroup was calculated through the formula proposed by LU et al., 2016 [19]:

$$\Delta \text{ Score on ALSFRS} - \text{R (T2}-\text{T1)} \;/\text{Interval between clinical assessments (in months).}$$

All subjects provided written informed consent prior to the enrollment in the study. The Research Ethics Committees of both hospitals approved this study protocol, which was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

2.2. Biomarkers assessment

Ten milliliters of blood were drawn by venipuncture in vacuum tubes containing heparin (BD Vacutainer) on the same day of the clinical assessment. Plasma was then obtained within 2 h of the blood sampling. Samples were centrifuged at 1700*g* for 10 min, 4 °C. Plasma was collected and stored at −70 °C until assayed.

At the day of the biomarkers assessment, the samples were thawed and cytokines and chemokines were measured as routinely performed in our laboratory. Cytokines [IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF), interferon (IFN)-γ and IL-17] were measured by Cytometric Bead Array (CBA) using the Human Th1/Th2/Th17 Cytokines Kit (BD Biosciences, San Jose, CA, USA), following the manufacturer's instructions. Chemokines [CXCL10/induced protein 10 (IP-10), CCL2/monocyte chemoattractant protein-1 (MCP-1), CXCL9/monokine induced by interferon gamma (MIG) and CCL5/RANTES] were also evaluated by CBA using the Human Chemokine Kit (BD Biosciences). Briefly, the antibody-coated beads to capture each of the proteins to be assessed were mixed and incubated with the plasma samples and the Cytokine/Chemokine standard dilutions, plus phycoerythrin (PE)-conjugated antibodies (detection reagent). Each bead has a unique fluorescence intensity that resolves as a unique population on a flow cytometer. The detection reagent provides a fluorescent signal in proportion to the amount of bound analyte. After a 3 h-incubation, samples were washed and then acquired on the FACSCanto II flow cytometer (BD Biosciences). The instrument had been checked for sensitivity and overall performance with Cytometer Setup & Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v3.0 software (Soft Flow Inc., Pecs, Hungary). The analysis software identifies each bead population, generates a standard curve, and calculates the concentration of unknown samples.

Soluble tumor necrosis factor receptors 1 and 2 (sTNFR1 and sTNFR2) were measured by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (Dou Set R&D Systems, Minneapolis, MN, USA), which are briefly described as follows: first, the wells of a PVC microtiter plate were coated with the capture antibody specific to the analyzed protein. After overnight incubation at 4 °C, the plates were washed and the remaining protein-binding sites in the coated wells were blocked by adding bovine serum albumin (blocking buffer) for 2 h. The plates were washed and the