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Top-down proteomic profiling of human saliva in multiple sclerosis patients

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ABSTRACT

Multiple sclerosis is a chronic disease of the central nervous system characterized by inflammation, demyelination and neurodegeneration which is of undetermined origin. To date a single diagnostic test of multiple sclerosis does not exists and novel biomarkers are demanded for a more accurate and early diagnosis. In this study, we performed the quantitative analysis of 119 salivary peptides/proteins from 49 multiple sclerosis patients and 54 healthy controls by a mass spectrometry-based top-down proteomic approach. Statistical analysis evidenced different levels on 23 proteins: 8 proteins showed lower levels in multiple sclerosis patients with respect to controls and they were mono- and di-oxidized cystatin SN, mono- and di-oxidized cystatin S1, mono-oxidized cystatin SA and mono-phosphorylated statherin. 15 proteins showed higher levels in multiple sclerosis patients with respect to controls and they were antileukoproteinase, two proteoforms of Prolactin-Inducible Protein, P-C peptide (Fr.1–14, Fr. 26–44, and Fr. 36–44), SV1 fragment of statherin, cystatin SN Des_{1-4}, cystatin SN $P_{11} \rightarrow L$ variant, and cystatin A $T_{96} \rightarrow M$ variant. The differences observed between the salivary proteomic profile of patients suffering from multiple sclerosis and healthy subjects is consistent with the inflammatory condition and altered immune response typical of the pathology.

Data are available *via* ProteomeXchange with identifier PXD009440.

Significance: To date a single diagnostic test of multiple sclerosis does not exist, and diagnosis is based on multiple tests which mainly include the analysis of cerebrospinal fluid. However, the need for lumbar puncture makes the analysis of cerebrospinal fluid impractical for monitoring disease activity and response to treatment. The possible use of saliva as a diagnostic fluid for oral and systemic diseases has been largely investigated, but only marginally in multiple sclerosis compared to other body fluids. Our study demonstrates that the salivary proteome of multiple sclerosis patients differs considerably compared to that of sex and age matched healthy individuals and suggests that some differences might be associated with the different disease-modifying therapy used to treat multiple sclerosis patients.

1. Introduction

Multiple sclerosis is a chronic immune-mediated disease of the central nervous system (CNS) characterized by high heterogeneity in the pathologic [1], clinical [2], and radiologic features [3]. Currently, multiple sclerosis is view as a "simultaneous two-components" disease with different levels of inflammatory-demyelination and neurodegenerative damage, that early affect several regions of brain and spinal cord [4, 5]. Traditionally, multiple sclerosis has been classified in four clinical courses, including relapsing remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) [6]. Recently, these phenotypes have been re-examined principally based on disease activity [2]. To date a single diagnostic test of multiple sclerosis does not exist, and diagnosis is based on disease history, clinical evaluation, magnetic resonance imaging (MRI), and supplementary tests, which mainly include the analysis of cerebrospinal fluid (CSF) [7]. Although MRI shows a very powerful diagnostic sensitivity, a differential diagnosis with respect to other diseases, like systemic lupus

Abbreviations: MSPs, Multiple Sclerosis Patients; HCs, Healthy Controls

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erythematosus and Sjögren's syndrome, is sometimes difficult [8]. For these reasons, novel biomarkers, which can be used in combination with the current clinical findings, are necessary for a more accurate and early diagnosis, to predict prognosis, to monitor the disease and to understand the efficacy of treatment. In the last years mass spectrometry has been largely applied in biomarker discovery studies, and in particular a proteomic approach has been applied to investigate CSF in various neurodegenerative diseases [9] and in multiple sclerosis [10, 11]. CSF is the body fluid of choice for the proteomics-based studies in multiple sclerosis and other disorders affecting the CNS, since proteins/peptides released by the CNS, and reflecting the pathology, can be found in CSF [12–14]. However, the need for lumbar puncture makes CSF tests impractical for monitoring disease activity and response to treatment. The possible use of saliva as a diagnostic fluid for oral and systemic diseases has been largely investigated [15–19], but only marginally in multiple sclerosis compared to other body fluids (CSF, serum, blood, urine) [20].

It has been shown that saliva, as a mirror of oral and systemic health, provides valuable information because it contains not only proteins specifically secreted by the salivary glands [21], but also proteins deriving from the gingival crevicular fluid [22], others of oral microflora origin [23] and also plasmatic proteins transported from blood to saliva by both intra- and extracellular pathways. Several studies evidenced that various systemic disorders affected qualitatively and quantitatively the salivary proteome [24–27]. Saliva represents an attractive diagnostic fluid which can be collected noninvasively, easy to store, and inexpensive when compared to other bodily fluids utilized in clinical laboratories [28, 29].

Based on these considerations, the aim of the present study was to evidence by a top-down proteomic approach possible qualitative and/or quantitative differences of salivary proteins in patients with multiple sclerosis (MSPs) compared with healthy controls (HCs) as suggestion of potential salivary biomarkers of the disease.

2. Materials and methods

2.1. Ethics statements and subjects under study

The study included 49 subjects (24 males, 25 females) with multiple sclerosis diagnosed according to McDonald 2010 criteria [7], and recruited at the Multiple Sclerosis Center of the University of Cagliari. A first line therapy was reported for 13 patients, a second line therapy for 19 patients, while 17 patients were therapy free. In particular, 8 patients were treated with immunomodulatory drugs (interferon β 1a/1b, glatiramer acetate, dimethylfumarate), 7 with immunosuppressive drugs (fingolimod, azathioprine, teriflunomide), and 17 with monoclonal antibodies (alemtuzumab, ocrelizumab, natalizumab). 54 demographically and ethnically matched healthy controls were also recruited (22 males, 32 females). The patient's demographics (sex and age) and clinical data (disease course, disease duration, and disability level), evaluated using the Expanded Disability Status Scale (EDSS) [30], were taken together with Disease-Modifying Therapies. Clinical features of MSPs and demographic characteristics of both MSPs and HCs are shown in Table 1. The local ethics committee approved the study and all the participants signed the informed written consent and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Table 1
Demographic features of all subjects under this study and clinical data of MSPs.

	MSPs group	HCs group
	Total 49	Total 54
Sex (males)	24 (49%)	22 (41%)
Age (mean ± SD)	41 ± 12	40 ± 10
Disease duration (years mean ± SD)	11.5 ± 7.2	
Relapsing remitting (RR)	38 (78%)	
Primary progressive (PP)	6 (12%)	
Secondary progressive (SP)	5 (10%)	
EDSS (mean ± SD)	3.5 ± 1.1	
First line therapy	13 (26%)	
Second line therapy	19 (39%)	
No therapy	17 (35%)	

2.2. Samples collection

Resting whole saliva (WS) (from 0.2 to 1 mL) was collected according to a standard protocol optimized to preserve salivary proteins from proteolytic degradation, with a soft plastic aspirator at the basis of the tongue from 9 to 13 A.M. when salivary secretion is at a maximum [31]. Samples were collected at least 30 min after any food or beverage had been consumed and teeth had been cleaned. After collection, salivary samples were kept in an ice bath and immediately mixed with an equal volume of 0.2% 2,2,2-trifluoroacetic acid (*v*/v; TFA) containing 50 µM of leucine enkephalin as internal standard [32]. The acidic solution was centrifuged at 13400 x g for 10 min to remove the precipitate and the acidic soluble fraction of saliva was either immediately analyzed by HPLC-ESI-MS or stored at −80 °C until analysis.

2.3. RP-HPLC low-resolution ESI-MS analysis

Peptides and proteins search and quantification was made by reversed phase (RP)-HPLC low-resolution ESI-MS analysis of the acid soluble fraction (35 µL, corresponding to 17.5 µL of saliva) of WS. The analyses were carried out by a Surveyor HPLC system connected to a LCQ Advantage mass spectrometer (Thermo Fisher Scientific, CA). The mass spectrometer was equipped with an ESI source. The chromatographic column was a Vydac (Hesperia, CA) C8 column with 5 µm particle diameter (150 × 2.1 mm). The following solutions were used: (eluent A) 0.056% (*v*/v) aqueous TFA and (eluent B) 0.05% (v/v) TFA in acetonitrile/water 80/20. The gradient applied for the analysis of saliva was linear from 0 to 55% of B in 40 min, and from 55% to 100% of B in 10 min, at a flow rate of 0.10 mL/min toward the ESI source. During the first 5 min of separation, the eluate was diverted to waste to avoid instrument damage because of the high salt concentration. Mass spectra were collected every 3 ms in the *m/z* range 300–2000 in positive ion mode. The MS spray voltage was 5.0 kV and the capillary temperature was 260 °C. MS resolution was 6000. Deconvolution of averaged ESI-MS spectra was performed by MagTran 1.0 software [33].

2.4. Data analysis

Experimental average mass values (Mav) of salivary proteins and peptides already characterized in previous studies [26, 34, 35] were compared with theoretical average mass values available at Swiss-Prot Data Bank (http://www.uniprot.org/). The relative abundance of the salivary proteins was determined by measuring the area of RP-HPLC low-resolution ESI-MS eXtracted Ion Current (XIC) peaks, considered when the S/N *ratio* was at least 5. This value is linearly proportional to the peptide concentration and it can be used to monitor relative abundances, under constant analytical conditions [36]. The total protein concentration of the acidic soluble fraction of WS was measured in several MSPs and Controls samples, using the BCA assay, and it corresponded to 0.8 ± 0.5 mg/mL. We have established that with these concentration values the signal intensity of acidic PRPs (showing the most intense XIC peak areas) does not exceed the detector capacity. As far as it concerns the less concentrated proteins, we excluded from quantitation those showing XIC peaks area values lower than 1E6. To determine reliable XIC peak area, the choice of *m/z* values of the protein of interest is relevant, in particular in crowded chromatographic where ESI spectra belonging to other proteins might share ions with the same *m/z* value. The window for all these values was in a range of ±