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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 diagno-

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

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sis with respect to other diseases, like systemic lupus 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.

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

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%)	

[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

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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 $\pm 0.5\,m/z$. In order to minimize errors associated with sample dilution, the XIC peak value of each protein/peptide analyzed was correct with respect to the XIC peak value of the leucine enkephalin internal standard.

Percentages were calculated on the basis of the following XIC peak area *ratios*: oxidized cystatins (mono-oxidized plus di-oxidized) = 100 × (mono-oxidized + di-oxidized)/(mono-oxidized + di-oxidized + not oxidized), truncated statherin and P-C peptide = 100 × (total truncated proteoforms)/(total truncated proteoforms + entire proteoforms). See table S-1 for fragments under consideration.

2.5. Structural characterization of prolactin-inducible proteins by RP-HPLC high-resolution ESI-MS and MS/MS experiments

Characterization of Prolactin-Inducible Protein (PIP) proteoforms was performed by a top-down proteomic approach on the intact proteins present in the acidic soluble fraction of WS.

The experiments were carried out by an Ultimate 3000 RSLC Nano System HPLC apparatus (Thermo Fisher Scientific, CA) coupled to an LTQ Orbitrap Elite apparatus (Thermo Fisher Scientific, CA). The injection volume was 20 μL. The column was a Zorbax 300SB-C8 (3.5 μm particle diameter; 1.0 × 150 mm), and eluents were: (eluent A) 0.1% (*v*/v) aqueous formic acid (FA) and (eluent B) 0.1% (v/v) FA in acetonitrile-water 80/20. The gradient was: 0–2 min 5% B, 2–40 min from 5% to 70% B (linear), 40–45 min from 70% to 99% B, at a flow rate of 50 μL/min. MS and MS/MS spectra were collected in positive mode with the resolution of 60,000 (at 400 *m/z*). The acquisition range was from 350 to 2000 *m/z*. Tuning parameters: capillary temperature was 300 °C, and the source voltage 4.0 kV, S-Lens RF level 60%. In data-dependent acquisition mode the five most abundant ions were selected and fragmented by using higher energy collision dissociation (HCD), with 35% normalized collision energy for 30 ms, isolation width of 5 *m/z*, activation q of 0.25. HPLC-ESI-MS and MS/MS data were generated by Xcalibur 2.2 SP1.48 (Thermo Fisher Scientific, CA) using default parameters of the Xtract program for the deconvolution. Protein sequences and sites of covalent modifications were validated by manual inspection of the experimental fragmentation spectra against the theoretical ones generated by MS-Product software available at the ProteinProspector website (http://prospector.ucsf.edu/prospector/mshome.htm).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://www.ebi.ac.uk/pride) *via* the PRIDE [37] partner repository with the dataset identifier PXD009440. Username: reviewer08630@ebi.ac.uk; Password: mnbXmGlX.

2.6. Statistical analysis

Statistical analyses were performed with the software GraphPad Prism 6.0. Data distributions were tested for normality by *D'Agostino-Pearson* test. A comparison between MSPs and HCs was performed by using the following statistical tests depending on data distribution (normal or skewed), and variance (homogeneous or unequal): parametric *t-test* (variance homogeneous); *t-test* with *Welch* correction (normal distribution, variance unequal), and the nonparametric *Mann-Whitney U test* (skewed distribution, variance unequal). Results of *t-tests* analyses are graphically represented by the *Volcano* plot representing the $\log_2$ mean value of XIC peak areas from (MSPs/HCs) *ratio versus* the $\log_{10}$ *p*-value of statistical tests.

Proteins showing levels statistically different between MSPs and HCs were further analyzed to evidence possible effects due to treatment of MSPs. For this purpose the group of MSPs under disease-modifying therapies (Treated-MSPs: T-MSPs, $n = 32$), and the group of not treated patients (Untreated-MSPs: UT-MSPs, $n = 17$) were compared to HCs by *Kruskal-Wallis* test followed by *Dunn's* multiple comparisons test.

Outliers values, were always included in the analysis. However, the evaluation of their exclusion on the significance of the statistical tests was also performed. In all the tests, the p-value $< .05$ (two-tailed) was considered statistically significant.

3. Results

Top-down RP-HPLC-MS experiments were performed after treatment of WS with aqueous TFA solution to minimize protein degradation. Thus, the quali/quantitative investigation was performed only on the acidic-soluble fraction of salivary proteins/peptides. Table S-1 and table S-2 report the Swiss-Prot codes, the retention times, experimental and theoretical Mav, and the multiply charged ions used to selectively extract the XIC peak areas of 119 proteins/peptides. A total of 79 proteins/peptides derived from salivary gland secretion (Table S-1) [38]. They comprised acidic proline-rich proteins (aPRPs) (aPRP-1 type, aPRP-3 type, P-C peptide, Db-s, Db-f), statherins (phosphorylated and truncated derivatives), proline-rich P-B peptide, type 2 cystatins (phosphorylated, oxidized and truncated derivatives of S-type cystatins, cystatin C, cystatin D). Among the highly polymorphic basic proline-rich proteins (bPRPs), only P-J, IB1, P-F, and P-H peptides (codified by PRB2L allele) were considered [39]. α-Amylases and glycosylated bPRPs were excluded from this analysis since the great heterogeneity of their ESI-spectra did not allow their identification and quantification [40, 41].

The 40 proteins and peptides not secreted from salivary glands [42] (Table S-2) comprised type 1 cystatins (different proteoforms and acetylated derivatives of cystatin A, cystatin B and its oxidized derivatives), α-defensins (α-defensin 1–4), thymosins (Tβ4, Tβ4 sulfoxide and Tβ10), S100A proteins (S100A7 variant $E_{27} \rightarrow D$, S100A8 and its oxidized derivatives, S100A9 long (L) and short (S) isoforms, S100A12), two fragments of the polymeric Immunoglobulin Receptor (AVAD, fragment 610–648 and ASVD, fragment 623–648 of pIgR) [26], and antileukoproteinase (also secreted from parotid gland) [43]. Three proteoforms of prolactin-inducible proteins (PIP), characterized for the first time in the present study by high-resolution ESI-MS/MS top-down experiments, were also quantified.

3.1. Structural characterization of prolactin-inducible proteins (PIP) proteoforms

In the HPLC high-resolution ESI-MS profiles of both MSPs and HCs saliva three proteins with exp. monoisotopic ion $[M + H]^+$ at $13493.9 \pm 0.2\,m/z$, $13,696.9 \pm 0.2\,m/z$ (+203 Da, with respect to 13,493.9), and $13,843.1 \pm 0.2\,m/z$ (+147 Da, with respect to 13,696.9) eluting between 37.8 and 38.2 min, were detected (Fig. 1 panel A). The mass difference of 203 Da suggested that the protein with exp. monoisotopic $[M + H]^+$ at $13696.9 \pm 0.2\,m/z$ could correspond to the *N*-acetylhexosamine (theor. monoisotopic ion at 203.1 *m/z*) derivative of the $13,493.9 \pm 0.2\,m/z$ protein. On the other hand, the mass difference of 146 Da between the proteins with exp. monoisotopic $[M + H]^+$ at $13843.1 \pm 0.2\,m/z$ and $13,696.9 \pm 0.2\,m/z$ was in agreement with an additional deoxyhexose moiety (theor. monoisotopic ion at 146.1 *m/z*) (Fig. 1 panel B). Manual inspection of the high-resolution MS/MS spectra of the $[M + 10H]^{+10}$ ions at 1351.00, 1371.32 and 1386.11 *m/z* allowed to establish that the three proteins were different proteoforms of PIP with the N-terminal glutamine residue converted to pyro-glutamic acid and with two disulfide bonds, but not to assign the glycosylation site. The presence in the MS/MS spectra of low-molecular weight saccharide oxoniun ions at 204.087 and 138.106 *m/z* confirmed

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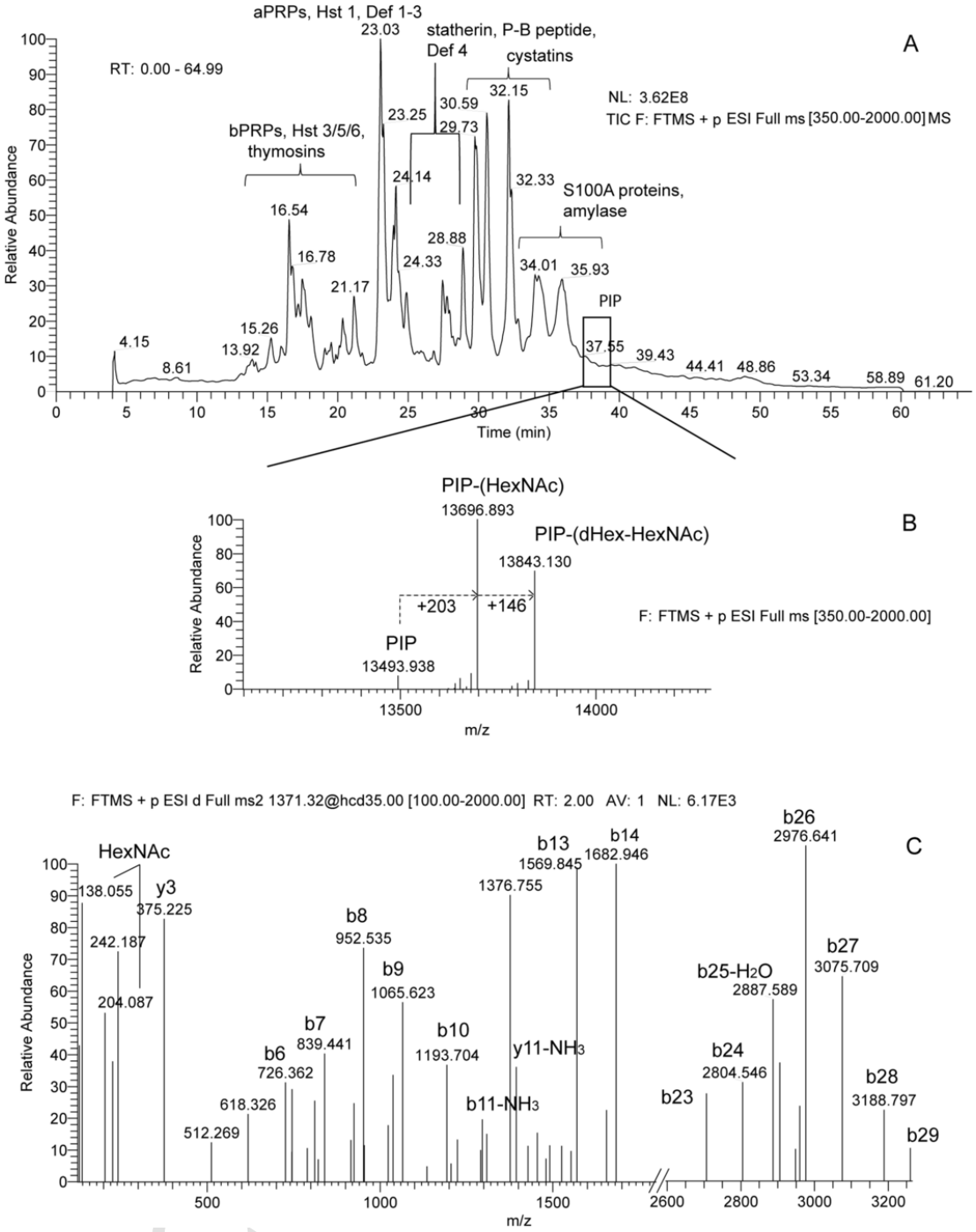

Fig. 1. HPLC-high resolution-ESI-MS characterization of PIP proteoforms. Representative HPLC-high resolution-ESI-MS TIC profile of the acidic soluble fraction of saliva from a MSPs with the elution range of several salivary proteins (panel A), boxed region contains PIP proteoforms. Deconvoluted high resolution MS spectra of three PIP proteoforms (values are monoisotopic monocharged $[M + H]^{+1}$) (panel B). Annotated HCD MS/MS spectrum (panel C) performed on the ion 1371.32 m/z ($[M + 10H]^{+10}$).

the presence of an *N*-acetylhexosamine moiety in the protein with $[M + H]^+$ at 13696.9 ± 0.2 *m/z* (Fig. 1 panel C) [44].

3.2. Protein quantification and statistical analysis

Table S-3 summarizes the results of the quantitative analyses, performed by comparing the XIC peak areas of the proteins/peptides measured in all the MSPs and HCs. *t-tests* results are shown in the *Volcano* plot ($\log_{10}$ *P*-values obtained by comparing the XIC peak area mean value of each protein/peptide measured in the two groups *vs.* $\log_2$ fold change MSPs /HCs XIC peak areas) of Fig. 2. Points above the red horizontal line represent proteins with *p*-values <.05 and points to the left and to the right of the two vertical dashed lines represent fold changes of (MSPs/HCs) mean values of XIC peak areas less than and >1.5, respectively.

Of the 119 proteins quantified, only 27 proteins with p-values <.05 showed a significant different level in the two groups and a ± 1.5 fold change of MSPs/HCs XIC peak areas (Table 2). The following 10 proteins showed lower salivary levels in MSPs with respect to HCs (Fig. 3): mono- and di-oxidized cystatin SN, mono- and di-oxidized cystatin S1,

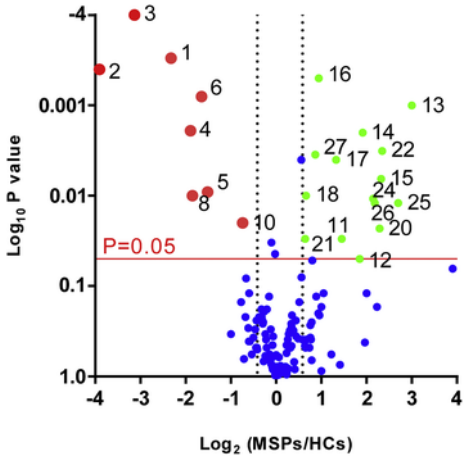

Fig. 2. Results of *t*-tests analyses on salivary proteins/peptides graphically represented by the Volcano plot. The log10 p-values (obtained by comparing the XIC peak area mean value of each protein/peptide measured in the two groups) is plotted against the $\log_2$ fold change of MSPs /HCs mean XIC peak areas. Red dots represent proteins/peptides with lower levels in saliva of MSPs with respect to HCs, and green dots those with higher levels. Labels on dots refer to protein numbers as reported in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cystatin SA and mono-oxidized cystatin SA, mono-phosphorylated statherin.

The 17 proteins showing higher levels in MSPs with respect to HCs were (Table 2, Figs. 4 and 5): Fr.1–14, Fr. 26–44, Fr. 36–44 of P-C peptide, SV1 fragment of statherin, ASVD peptide, two glycoforms of PIP, cystatin SN Des_{1-4}, cystatin SN $P_{11} \rightarrow L$ variant, cystatin A $T_{96} \rightarrow M$ variant [35], cystatin B and cystatin C, S100A7 $E_{27} \rightarrow D$ and S100A8-SNO and antileukoproteinase.

The percentage of the oxidized and truncated forms of the proteins differentially represented in the two groups was also calculated. It was found that the percentage of all the oxidized proteoforms of cystatins SN and SA was lower in MSPs with respect to HCs ($p = .0006$, and $p = .004$, respectively), whereas the percentage of all the oxidized proteoforms of cystatin S1 was not significantly different between the two groups. Regarding the truncation extent of salivary proteins in MSPs, we observed an increased percentage of fragmentation for statherin ($p = .006$), and P-C peptide (p = .004). Of the two peptides deriving from pIgR, only ASVD showed a significant higher level in MSPs with respect to HCs.

Among the three PIP proteoforms partially characterized in this study, the proteoform with $[M + H]^+$ at 13493.9 *m/z* was found at very low frequency both in MSPs and HCs (8/49 and 8/54, respectively). On the contrary, the two glycosylated proteoforms with $[M + H]^+$ at 13696.9 and 13,843.1 *m/z*, showed higher levels and frequency in MSPs with respect to HCs (Table 2).

Finally, antileukoproteinase protein, S100A7 $E_{27} \rightarrow D$ variant and S100A8-SNO showed higher levels in MSPs with respect to HCs ($p = .05$, 0.01, and 0.01, respectively) (Table 2).

3.3. Effect of therapies in proteins/peptides levels among MSPs

Panels of proteins with statistically different levels between MSPs and HCs groups were further analyzed to evidence possible variations between MSPs under disease-modifying therapies and the group of not treated patients (Table 3).

Kruskal-Wallis test evidenced that cystatin SN oxidation was lower in both T- MSPs and UT- MSPs with respect to HCs while for cystatins S1 and SA a significant lower level of oxidized derivatives was observed only for UT- MSPs with respect to HCs. On the contrary, statistical analysis showed a significant higher concentration in UT- MSPs with respect to HCs of two C-terminally truncated proteoforms of statherin (SV1 and Des-TF fragments) and two fragments of P-C peptide (Fr.1–14 and Fr. 26–44,) not evidenced for T- MSPs.

Table 2
List of peptides/proteins with significantly higher and lower levels in MSPs saliva samples compared to HCs.

#	Protein name	UniprotKB code	Entry name	Frequencies		*p* value	
				MSPs	HCs	Up-regulated	Down-regulated
1	Cystatin SN mono-ox	P01037	CYTN_HUMAN	8/49	26/54		0.0003
2	Cystatin SN di-ox	P01037	CYTN_HUMAN	2/49	16/54		0.0004
3	Cystatin SN-ox Tot			8/49	26/54		0.0001
4	Cystatin S1 mono-ox	P01036	CYTS_HUMAN	9/49	24/54		0.002
5	Cystatin S1 di-ox	P01036	CYTS_HUMAN	6/49	19/54		0.009
6	Cystatin S1-ox Tot			9/49	27/54		0.0008
7	Cystatin SA	P09228	CYTT_HUMAN	14/49	29/54		0.03
8	Cystatin SA mono-ox	P09228	CYTT_HUMAN	6/49	18/54		0.01
9	Cystatin SA Tot			14/49	29/54		0.04
10	Statherin 1P	P02808	STAT_HUMAN	29/49	42/54		0.02
11	Statherin SV1	P02808	STAT_HUMAN	49/49	52/54	0.03	
12	Antileukoproteinase	P03973	SLPI_HUMAN	17/49	16/54	0.05	
13	PIP pyro (dHex-HexNAc)	P12273	PIP_HUMAN	17/49	4/54	0.001	
14	PIP pyro (HexNAc)	P12273	PIP_HUMAN	20/49	8/54	0.002	
15	PIP Tot			23/49	15/54	0.006	
16	Peptide P-C Fr. 1–14	P02810	PRPC_HUMAN	47/49	51/54	0.0005	
17	Peptide P-C Fr. 26–44	P02810	PRPC_HUMAN	38/49	37/54	0.004	
18	Peptide P-C Fr. 36–44	P02810	PRPC_HUMAN	38/49	27/54	0.01	
19	Peptide P-C Fr Tot			49/49	27/54	0.05	
20	Cystatin SN Des_{1-4}	P01037	CYTN_HUMAN	22/49	14/54	0.02	
21	Cystatin SN (P → L)	P01037	CYTN_HUMAN	10/49	3/54	0.03	
22	Cystatin A ($T_{96} \rightarrow M$)	P01040	CYTA_HUMAN	14/49	5/54	0.003	
23	Cystatin B Tot	P04080	CYTB_HUMAN	49/49	54/54	0.004	
24	Cystatin C	P01034	CYTC_HUMAN	18/49	8/54	0.01	
25	S100A7($E_{27} \rightarrow D$)	P31151	S10A7_HUMAN	16/49	6/54	0.01	
26	S100A8-SNO	P05109	S10A8_HUMAN	13/49	4/54	0.01	
27	ASVD	P01833	PIGR_HUMAN	45/49	38/54	0.003	

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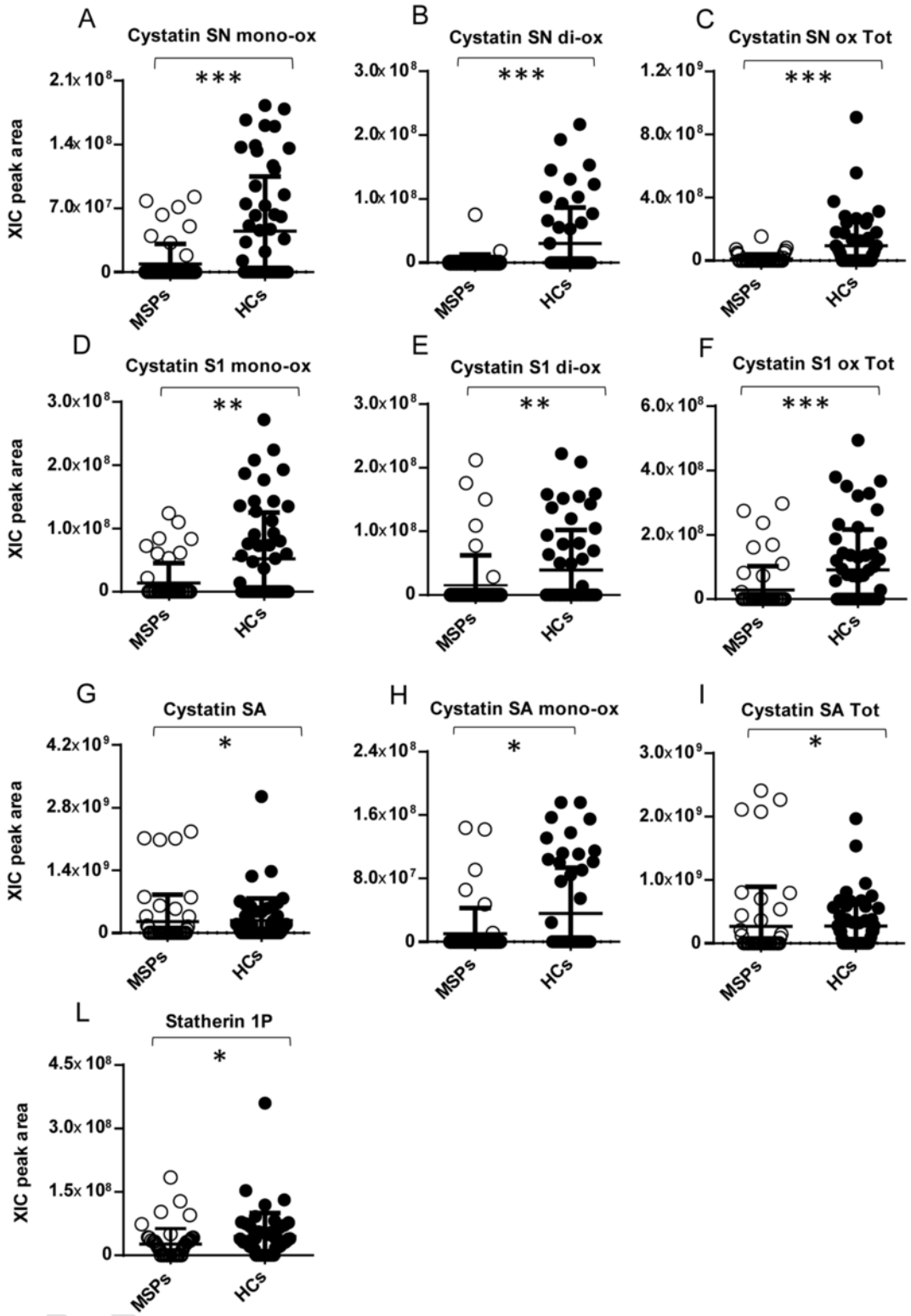

Fig. 3. Distribution of the XIC peak area values of salivary proteins showing lower levels in MSPs with respect to HCs. Mono- and di-oxidized cystatin SN (panels A–C), mono- and di-oxidized cystatin S1 (panels D–F), cystatin SA and mono-oxidized cystatin SA (panels G-I), mono-phosphorylated statherin (panel L). Statistically significant differences are indicated with asterisks: *($p < .05$), **($p \leq .01$), ***($p \leq .001$).

4. Discussion

To our knowledge, this is the first report showing the variation of the naturally occurring salivary proteome and peptidome of MSPs with respect to HCs explored by top-down proteomics. Among the 27 proteins/peptides showing a significant different level in MSPs with respect to HCs, cystatins were principally represented. Cystatins are a superfamily of intracellular and extracellular proteins with inhibitory activity toward cysteine proteinases, mainly represented by the largest

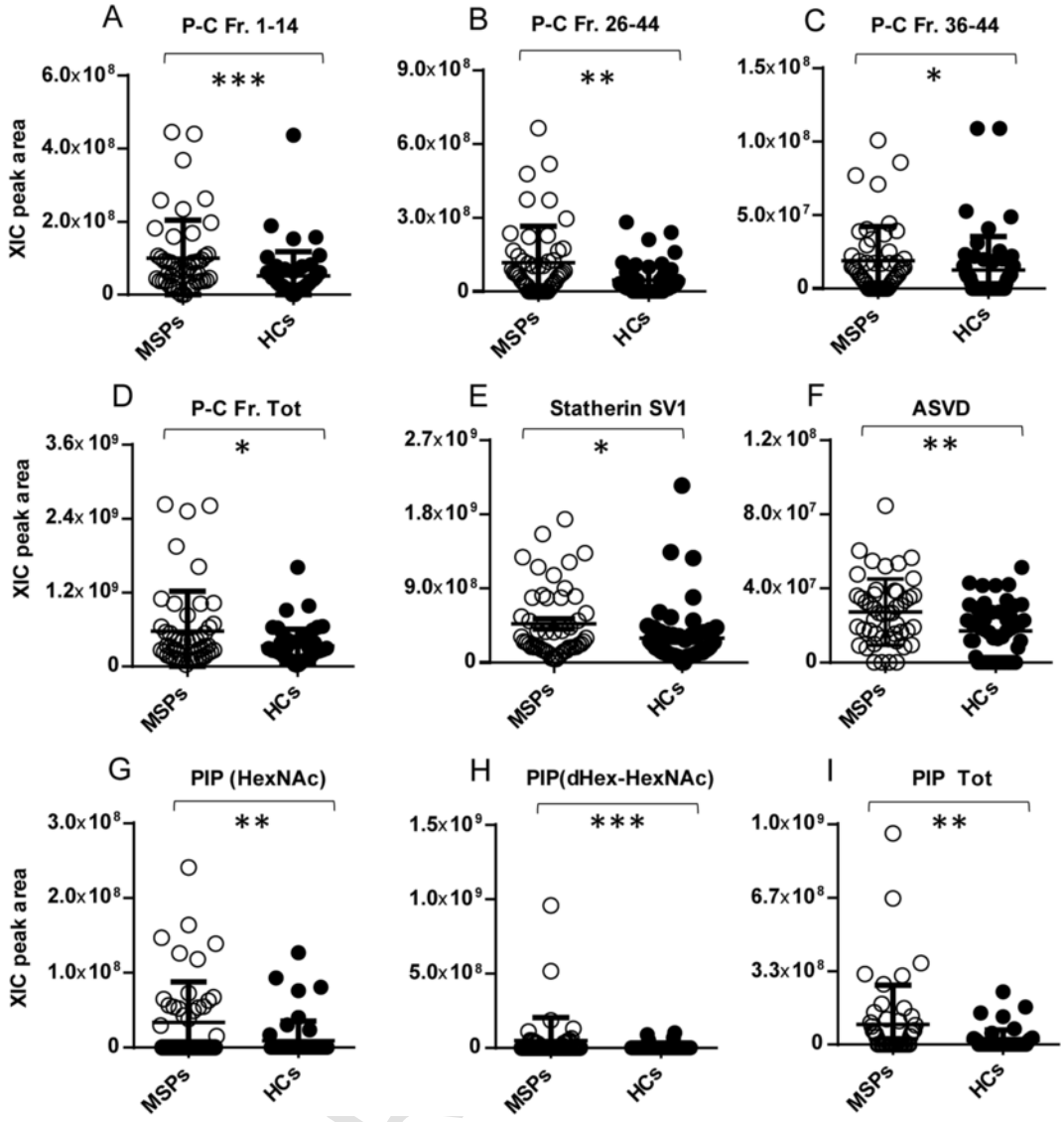

Fig. 4. Distribution of the XIC peak area values of salivary proteins showing higher levels in MSPs with respect to HCs. Fr.1–14, Fr. 26–44, Fr. 36–44 of peptide P-C (panels A–D), SV1 fragment of statherin (panel E), ASVD peptide (panel F), two glycoforms of PIP (panels G-I). Statistically significant differences are indicated with asterisks: *($p < .05$), **($p \leq .01$), ***($p \leq .001$).

cathepsin family [45]. Cathepsins were shown to play important roles in both processing and degradation of several neuronal proteins [46]. The involvement of cysteine cathepsins in neuronal cell death [47], and in several neurodegenerative diseases has been also demonstrated [48]. Indeed, imbalance between cysteine cathepsins, and their related inhibitors cystatins may lead to a variety of immune-mediated disorders [49–51], multiple sclerosis comprised [52]. Among cystatins, which usually show a restricted tissue distribution, cystatin B and cystatin C were found widely expressed in numerous tissues, including the brain. Cystatin B deficiency was found to be implicated in myoclonus epilepsy [53], whilst cystatin C levels were found lower in CSF of patients with amyotrophic lateral sclerosis with respect to both HCs and other neurological disease patients [54, 55]. Decreased levels of cystatin C in CSF have been also reported for other neurological disorders including multiple sclerosis, Alzheimer's, and Creutzfeldt-Jakob diseases [56]. The reduction of CSF cystatin C level in MSPs and in other neurodegenerative disorders suggested that cystatin C may play a role in modulating neuroinflammation, by exhibiting both neuroprotective and neurotoxic properties [57]. However, opposite results were obtained in leucocytes and serum of MSPs under treatment with disease-modifying drugs. Indeed, treatment of MSPs with interferon-β and methylprednisolone increased both the RNA expression level in peripheral blood leucocytes and the serum protein level of cystatin C [52]. Nonetheless, the increased level of cystatin B and cystatin C found in saliva of our MSPs with respect to HCs should not be reasonably related to the treatment, since cystatin B and cystatin C levels were found not statistically variated between treated- and untreated-MSPs.

The function for cystatin B in defending cerebellar granule neurons from oxidative stress has been also demonstrated [58]. The role of cystatin B in protecting neurons from oxidative stress, has been further supported by its interaction with SOD1, which is involved in the control of cellular redox state [59]. Oxidative stress is crucial for the multiple sclerosis pathogenesis playing a predominant role in demyelination development mainly associated with mitochondrial dysfunction, iron accumulation in the brain and oxidant/antioxidant balance [60]. The present study highlighted a reduced level of oxidation of S-type cystatins, which represent the larger portion of cystatins found in saliva. Recently, high levels of oxidative stress markers and lower antioxidant status, have been reported in saliva and plasma of MSPs under corticosteroid therapy [19]. However, different results were obtained in sev-

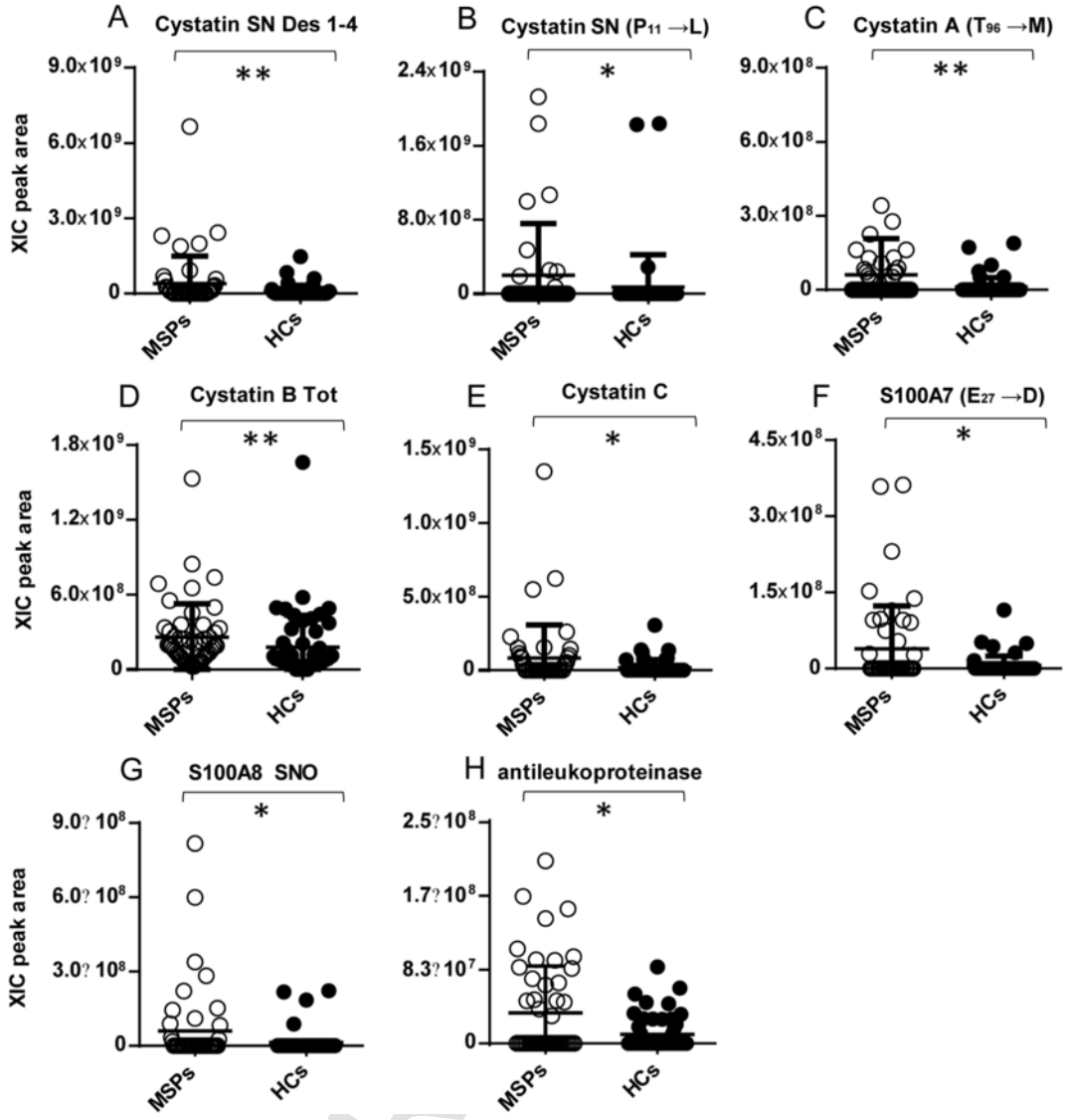

Fig. 5. Distribution of the XIC peak area values of salivary proteins showing higher levels in MSPs with respect to HCs. cystatin SN Des1–4, cystatin SN P11 → L variant (panels A–B), cystatin A T96 → M variant (panel C), cystatin B and cystatin C (panels D–E), S100A7 E27 → D and S100A8-SNO (panels F–D), and antileukoproteinase (panel H). Statistically significant differences are indicated with asterisks: *($p < .05$), **($p \leq .01$), ***($p \leq .001$).

eral studies focused on the immunotherapy role in oxidative stress, which highlighted a positive effect on antioxidant capacity evidenced by a reduction of oxidative stress markers [61]. Except for the 17 MSPs therapy free, 32 MSPs enrolled in the present study were under immunotherapy. On this regard, we speculate that the reduced level of oxidation we observed in S-type cystatin of MSPs, may be related to the use of immunomodulatory and immunosuppressive drugs, which have been shown to be activators of the intrinsic response mechanisms to oxidative stress [62, 63].

In contrast to cystatins B and C, little is known about the role of cystatin A with respect to neurodegeneration. Increased levels of cystatins A, found in the plaques of Alzheimer and Parkinson diseases, as well as in patients suffering from senile dementia, suggested that this protein is an amyloid constituent [64]. According to our results on saliva, Noben et al. [65] reported a statistically increased level of cystatin A in plasma of MSPs. The top-down proteomic approach applied in this study highlighted an higher frequency in MSPs of the cystatin A variant $T_{96} \rightarrow M$. Similarly, increased levels in MSPs were found for the variants of cystatin SN $P_{11} \rightarrow L$ and of S100A7 $E_{27} \rightarrow D$. Association between multiple sclerosis and polymorphic variants within different innate immunity genes has been largely documented [66, 67], and they have never been involved on clinical course, severity, or age at onset of the disease. Genetic association studies need to be performed to determine whether the variants determined in the present investigation are also in association with the multiple sclerosis disease.

The term of "multiple sclerosis degradome" has been proposed to encompass the set of proteases, inhibitors of protease and natural substrates involved in development and progression of multiple sclerosis [68]. On this regard, the present work showed in MSPs an increased level of two C-terminally truncated proteoforms of statherin (SV1 and des-TF fragments), three fragments of P-C peptide (Fr.1–14, Fr. 26–44, and Fr. 36–44) and the fragment-peptide ASVD deriving from pIgR. The occurrence in saliva of several naturally occurring fragments of statherin has been confirmed by mass spectrometry [69]; in particular, SV1 and statherin Des-TF fragments originate by the action of a carboxypeptidase [70]. Interestingly, the carboxypeptidase B2 was found to be upregulated in Experimental Autoimmune Encephalomyelitis (EAE)-rats [71], an animal model of the human CNS demyelinating diseases, including multiple sclerosis. Fragmentation of P-C peptide, occurring at XPQ↓G site, seems to be related to a glutamine endoprotease

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Table 3
Summary of proteins/peptides with different levels between treated-MSPs (T-MSPs), untreated-MSPs (UT-MSPs), and HCs.

Protein name	*Kruskal-Wallis* test p value	*Dunn's* multiple comparisons test			*t-Test*
		T-MSPs(n = 32) *vs* UT-MSPs(n = 17)	T-MSPs *vs* HCs(*n* = 54)	UT-MSPs *vs* HCs	MSPs(*n* = 49) *vs* HCs
Cystatin SN mono-ox	0.0003	ns	*↓	***↓	0.0003
Cystatin SN di-ox	0.002	ns	*↓	*↓	0.0004
Cystatin SN-ox tot	0.0002	ns	*↓	***↓	0.0001
Cystatin S1 mono-ox	0.002	ns	ns	**↓	0.002
Cystatin S1 di-ox	0.01	ns	ns	*↓	0.009
Cystatin S1-ox Tot	0.0007	ns	ns	***↓	0.0008
Cystatin SA mono-ox	ns	ns	ns	ns	0.01
Statherin 1P	0.046	ns	ns	*↓	0.02
Statherin SV1	0.0004	**↓	ns	***↑	0.03
Statherin Des TF	0.009	*↓	ns	**↑	ns
Antileukoproteinase	ns	ns	ns	ns	0.05
PIP pyro (dHex-HexNAc)	0.005	ns	**↑	ns	0.001
PIP pyro (HexNAc)	0.009	ns	*↑	*↑	0.002
Peptide P-C Fr. 1–14	0.0004	ns	ns	***↑	0.0003
Peptide P-C Fr. 26–44	0.0005	*↓	ns	***↑	0.0001
Cystatin SN Des_{1-4}	0.03	ns	ns	*↑	0.023
Cystatin A $T_{96} \rightarrow M$	0.01	ns	*↑	ns	0.003
Cystatin B	0.007	ns	**↑	ns	0.004
Cystatin C	0.003	ns	ns	**↑	0.01
S100A8-SNO	0.04	ns	ns	*↑	0.01
S100A7 $E_{27\rightarrow}D$	0.03	ns	ns	*↑	0.01
ASVD	0.003	ns	ns	**↑	0.003

of microbial origin [72]. Fragments 1–14 and 26–44 were found in Sjögren's syndrome patients [73], and associated with dental caries. Thus, these fragments may be a clue of an increased risk for dental caries, gingivitis and periodontitis in MSPs [74]. A strong negative correlation between dental caries and salivary phosphopeptides has been also previously reported [75], and it could be connected to the decreased level of monophosphorylated statherin that we observed in MSPs.

The fragment-peptide ASVD derives from AVAD peptide after a trypsin-like cleavage, and AVAD peptide, in turn, derives from a transmembrane sequence of pIgR after a cleavage which could be made by several proteases, including matrix metalloproteases [76]. The increased level of the ASVD peptide in MSPs, may be related to an increased degradation of pIgR by several proteases, even if it cannot be excluded that salivary pIgR itself may be increased in MSPs since a strong activation of immunoglobulin receptors in neural cells has been extensively reviewed in the major neurodegenerative conditions including Alzheimer's disease, Parkinson's disease, ischemic stroke and multiple sclerosis [77].

Interestingly, the increased levels of statherin C-terminal fragments, P-C peptide Fr.1–14 and 26–44, and fragment-peptide ASVD, were only determined in the untreated-MSPs, being the levels in treated-MSPs comparable to those observed in HCs. Indeed, several drugs currently used for multiple sclerosis management, *i.e* IFN-βs, exert their effect also by reducing the production of matrix metalloproteases [78]. On this regard, it cannot be excluded that also other drugs can exert a similar effect on different proteases present in the oral cavity or in the secretory granules.

The top-down approach used in this study allowed us to highlight an increased level of two different glycoforms of prolactin-inducible protein. PIP is a polypeptide, with a single glycosylation site, known by various names due to its versatile nature and function in human reproductive and immunological systems [79]. The glycosylation pattern of salivary PIP has been previously described, and it comprises different *N*-linked sugars mainly represented by complex di-antennary oligosaccharides with different degree of fucosylation [80]. The two PIP glycoforms detected at a different level in our MSPs with respect to HCs showed small glycan moiety which do not have features related with the common core structure of *N*-linked sugars. It cannot be excluded that these glycoforms result by an increased activity of oral glycosidase in MSPs, as well as we may speculate the presence of single O- or S-GlcNAcylation in the protein [81, 82]. Further studies are needed to understand the biological meaning of such altered glycosylation pattern, since deviations from normal protein glycosylation have been implicated in various diseases, including Alzheimer's disease, diabetes, cancer [83, 84], and altered branching of *N*-linked sugars has also been reported in multiple sclerosis [85].

Finally, the increased level of antileukoproteinase we observed in MSPs is in agreement with the recent findings of its involvement in the evolution or repair of EAE-lesions. Antileukoproteinase was found to be up-regulated >100-fold during EAE attack and incubation of adult neural stem cells with recombinant antileukoproteinase resulted in an increase of cell proliferation and of differentiation toward oligodendrocytes [86] suggesting a novel role for this protein in the promotion of tissue repair.

5. Conclusion

The differences observed between the salivary proteomic profile of MSPs and HCs is in agreement with current knowledge about the state of widespread inflammation caused by the disease. These differences, in fact, are mainly due to different levels of a set of proteins involved in inflammatory processes or in the immune response. However, some of the observed differences could be associated with the different disease-modifying therapies in MSPs. Further studies will have to be conducted to evaluate the level of salivary proteins in the different groups of MSPs according to the therapeutic treatment in place and clinical courses, in order to highlight possible alterations related not only to the different forms of the disease, but also to the level of disability of the patients, as suggestions of potential biomarkers of multiple sclerosis.

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Author contributions

Study conception and design: BM, AO; Acquisition of data: BL, FV, FI; Analysis and interpretation of data: BM, BL, AO; Provided the salivary samples of MSPs: EC, LL; Drafting of manuscript: BM; Critical revision: IM, MC, TC; All authors approved of the final version of the manuscript.

Conflict of interest disclosure

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.07.019.

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