RP-HPLC-ESI-IT Mass Spectrometry Reveals Significant Variations of the Human Salivary Protein Profile Associated with Predominantly Antibody Deficiencies

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ORIGINAL ARTICLE

RP-HPLC-ESI-IT Mass Spectrometry Reveals Significant Variations of the Human Salivary Protein Profile Associated with Predominantly Antibody Deficiencies

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Abstract

Purpose Present study is designed to discover potential salivary biomarkers associated with predominantly antibody deficiencies, which include a large spectrum of disorders sharing failure of antibody production, and B cell defects resulting in recurrent infections, autoimmune and inflammatory manifestations, and tumor susceptibility. Understanding and clinical classification of these syndromes is still challenging.

Methods We carried out a study of human saliva based on liquid chromatography-mass spectrometry measurements of intact protein mass values. Salivary protein profiles of patients ($n = 23$) and healthy controls ($n = 30$) were compared.

Results Patients exhibited lower abundance of α-defensins 1-4, cystatins S1 and S2, and higher abundance of glutathionylated cystatin B and cystatin SN than controls. Patients could be clustered in two groups on the basis of different levels of cystatin SN, S1 and S2, suggesting that these proteins may play different roles in the disease.

Conclusions Quantitative variations of these pro-inflammatory and antimicrobial peptides/proteins may be related to immunodeficiency and infectious condition of the patients. The high incidence of tumors in the group with the highest level of cystatin SN, which is recognized as tumoral marker, appeared an intriguing result deserving of future investigations. Data are available via ProteomeXchange with identifier PXD012688.

Keywords α-Defensins · cystatins · CVID · salivary proteomics · immunodeficiency

Abbreviations

PAD	Predominantly antibody deficiencies
CVID	Common variable immunodeficiency
UAD	Unclassified antibody deficiency
NK	Natural killer
SSG	glutathionylated
HC	Healthy controls
ESID	European Society for Immunodeficiency

Cristina Contini, Davide Firinu, Stefano Del Giacco and Tiziana Cabras contributed equally to this work.

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aPRPs	Acidic proline-rich proteins
Hst	Histatins
TBS-T	TBS with Tween-20

Introduction

Predominantly antibody deficiencies (PAD) constitute a heterogeneous group of disorders, with a large variability in clinical and immunological phenotypes [1, 2]. The major PAD without profound T cell deficiency encompasses (a) the common variable immunodeficiency (CVID), (b) agammaglobulinemia (e.g., X-linked agammaglobulinemia, autosomal recessive agammaglobulinemia), and (c) other subtypes of hypogammaglobulinemias [1, 2]. CVID is a group of disorders characterized by low or absent levels of IgG, IgA, and/or IgM in serum, B cell defects, leading to hypogammaglobulinemia and recurrent bacterial infections [1, 2]. Up to now, a specific laboratory test, which can lead to the diagnosis of CVID, does not exist, so diagnosis of CVID is based only on an exclusion criterion [3]. When not fulfilling CVID diagnostic criteria, subjects with recurrent infections, a marked decrease of at least one of total IgG, IgG1, IgG2, IgG3, IgM, or IgA, and insufficient IgG production to vaccines may be classified as unclassified antibody deficiency (UAD). Even if CVID is a rare disease, it is the most "common" primary immunodeficiency observed in the adult age. It usually appears in patients between 20 and 40 years of age with an average of 24 years of age [4], and it is defined "variable" because of its heterogeneity in the first clinical manifestation. Indeed, CVID patients can be divided mainly in two phenotypes that tend to be stable over time: one characterized predominantly by infectious events, and the other one by infections, autoimmune and inflammatory diseases [5]. Autoimmune syndromes can affect up to 30% of patients and the most common disease is immune thrombocytopenia purpura [6, 7]. Autoimmunity may seem a paradox in a condition of hypogammaglobulinemia, but, while specific response to antigens is impaired, autoantibodies and autoreactive B cells can be detected in serum [8]. Moreover, the impaired immune function appears to be linked to high malignancy risk in these patients [9]. Genetic defects are currently known only for a small percentage of patients [3], and it has also been suggested a polygenic nature of the disease [5]. Due to the low antibody production and inadequate response to vaccines, the B cells defect is clear [3], but T cells [10], dendritic cells [11], neutrophils [12], and natural killer (NK) cell [13] defects have also been described suggesting that both adaptive and innate immune responses and their synergic action are compromised in CVID. In addition, transcriptomic and epigenetic factors seem to be able to contribute to the disease [14, 15]. As underlined by Kienzler et al., a complete understanding of CVID is still a challenge and beyond genetics also, proteomics, metabolomics, and epigenetics can provide novel knowledge on the disease etiology [3]. Saliva is a biological fluid well suitable to be used in proteomic investigations to discover potential disease biomarkers, since its collection is inexpensive, safe, and noninvasive [16–18]. The protein composition of human saliva includes both proteins specific of the oral cavity, secreted by salivary glands, and proteins common to other tissues and bodily fluids. The last are released by salivary glands, leucocytes present in the gingival crevicular fluid, and epithelial cells of mucosa and glandular ducts. Moreover, several salivary proteins/peptides derive from the blood intracellularly through active transport or by a paracellular way through ultrafiltration at tight junctions between cells [19, 20]. Thus, exploring saliva as a diagnostic and prognostic fluid is particularly interesting. Furthermore, the dynamic range of proteins in saliva makes less challenging the detection of low abundant proteins with respect to plasma. For this reason, we investigated the salivary protein profile of PAD patients, with the aim to highlight qualitative and quantitative differences with respect to a healthy control group by applying a proteomic platform based on the high-performance liquid chromatography (HPLC) electrospray-ionization (ESI) ion-trap (IT) mass spectrometry (MS) analysis. This approach allows to obtain a profile of the naturally occurring salivary proteome/peptidome, to compare a limitless number of samples, and to perform a label-free quantification using the area of the eXtracted Ion Current (XIC) peak [21].

The investigation allowed to highlight significant different levels between PAD patients and healthy controls of eight proteins, out of more than the 60 analyzed, namely cystatins S1, S2, SN, B glutathionylated (B-SSG), α-defensins 1, 2, 3, and 4. Moreover, on the basis of cystatin S1, S2, and SN levels, it was possible to cluster the PAD patients in two groups. Interestingly, the patients with the highest level of cystatin SN, which is recognized as tumoral marker [22, 23], exhibited a high incidence of tumors.

Materials and Methods

Reagents and Instruments

All chemicals and reagents for MS analysis were purchased from Sigma-Aldrich (St. Louis, MO). HPLC low-resolution ESI-MS analyses were performed with a Surveyor HPLC system connected to a LCQ Advantage ESI-IT low-resolution mass spectrometer (ThermoFisher Scientific San Jose, CA). The chromatographic column was a reversed phase (RP) Vydac C8 (Hesperia, CA, USA) with 5-μm particle diameter (150 × 2.1 mm). HPLC high-resolution ESI-MS and MS/MS experiments were carried out using an Ultimate 3000 Micro HPLC apparatus (Dionex, Sunnyvale, CA, USA) equipped with a FLM-3000-Flow manager module and coupled to an LTQ Orbitrap Elite apparatus (ThermoFisher). The column was a Zorbax 300SB-C8 (3.5-μm particle diameter; 1.0 × 150 mm).

Study Subjects and Controls

Twenty-three PAD patients, 7 males and 16 females (mean ± standard deviation, 49.1 ± 17.1 years old), were enrolled from the Internal Medicine and Immunology outpatient clinic of the University of Cagliari. Thirty healthy controls (HC), 9 males and 21 females (mean ± standard deviation, 45.2 ± 13.2 years old), were enrolled as volunteers among the staff of the Department of Life and Environmental Sciences, University of Cagliari. The informed consent process was in agreement with the latest stipulations established by the Declaration of Helsinki. The local review boards approved the study. Ethical committee approval was requested as an extension of a previous approved observational study "analysis of clinical, laboratory and quality of life data of patients affected by primary immunodeficiency and C1-INH deficiency" (Dpt of Medical Sciences "M.Aresu", prot. Number 64330).

Clinical Data

Demographic and clinical features of the included patients are reported in Table 1. The 23 PAD patients were all under substitutive treatment with immunoglobulin (either intravenous or subcutaneous) except for the patients #1, #2, and #10. The patients were classified as CVID ($n = 10$), agammaglobulinemia ($n = 3$), and UAD (when not fulfilling CVID diagnostic criteria) ($n = 10$) based on the European Society for Immunodeficiency (*ESID*) and Pan-American Group for Immunodeficiency criteria (*ESID* registry-working definitions for clinical diagnosis of primary immunodeficiency diseases, available at the webpage https://esid.org/Working-Parties/Registry/Diagnosis-criteria). Patients with CVID and agammaglobulinemia were grouped for statistical analysis. All the PAD patients included in the study had a history recurrent infections occurring at paranasal sinuses, bronchial and pulmonary tissues, and/or the gastrointestinal tract. The mean age at onset was 28 ± 17 years. Besides the IgG levels at diagnosis, nine patients showed levels below the normal range of both IgA and IgM, three patients only of IgA and four of IgM. Table 1 reports both the clinical status at the time of sample collection and the clinical history of each patient. Sixteen patients exhibited autoimmune and inflammatory manifestations during their clinical history, and five had been previously affected by tumors (Online Resource, Table S1). All patients, which had previously diagnosed as having cancer, completed their surgery, chemotherapy, or other specific

Table 1 Demographic and clinical features of the patients included in the study. Not determined values are indicate with "n.d."

Patients	Age, gender	Substitutive treatment (Yes/No)	Classification	IgA mg/dl (nv. 40–350)	IgM mg/dl (nv. 50–300)	Age at onset	Associated diseases
#1	49 M	N	UAD	71	35	n.d.	A[a]
#2	45 F	N	UAD	140	61	42	A
#3	24 M	Y	Agammaglobulinemia	20	17	0.5	
#4	26 M	Y	Agammaglobulinemia	20	17	0.5	
#5	64 F	Y	UAD	177	89	45	AL[b]
#6	59 F	Y	UAD	27	26	39	A
#7	29 F	Y	CVID	0.7	21	6	A
#8	23 F	Y	CVID	6	27	4	AL
#9	75 F	Y	UAD	10	31	n.d.	I[c], T [d]
#10	47 F	N	UAD	6.67	400	40	A
#11	16 F	Y	Agammaglobulinemia	0.4	3	1	
#12	40 F	Y	CVID	6	107	19	A
#13	55 M	Y	CVID	23	61	23	I
#14	61 F	Y	UAD	170	66	35	A
#15	60 F	Y	CVID	450	35	51	A, T
#16	64 F	Y	UAD	116	100	40	A, AL
#17	71 F	Y	UAD	235	42	n.d.	A, T
#18	60 F	Y	CVID	200	20	47	A
#19	54 F	Y	CVID	n.d.	n.d.	41	
#20	60 F	Y	CVID	n.d.	n.d.	45	
#21	64 F	Y	CVID	2	15	34	I, T
#22	36 M	Y	CVID	30	44	28	A
#23	71 M	Y	UAD	2	41	40	A, T

[a] A = autoimmune disease; [b] AL = allergy; [c] I = inflammatory disease; [d] T = tumor

treatments since at least 2 years before the enrolment, and they were all free of metastatic diseases at the last available follow-up. PAD patients did not show periodontal diseases, dental caries, dry mouth, or other oral disorders. At the time of the saliva sampling, they had to be free of active or clinically overt (or recent) infectious episode or other acute illness, and none of them underwent long-term antibiotic prophylaxis.

Salivary Samples

Saliva sampling for those treated has been done on the day of immunoglobulin administration, prior to any other procedure or drug infusion or blood sampling. Unstimulated whole saliva samples were collected, according to the following standardized protocol: donors did not eat or drink at least 2 h before the collection, which was performed in the morning between 10:00 a.m. and 12:00 p.m., and they were invited to sit assuming a relaxed position and to swallow. Whole saliva was collected as it flowed into the anterior floor of the mouth with a soft plastic aspirator for less than 1 min and transferred to a plastic tube, cooled on ice, and immediately diluted in a 1:1 v/v ratio with a 0.2% aqueous solution of trifluoroacetic acid (TFA) containing 50 μM of leu-enkephalin as internal standard. We collected from 0.5 to 0.8 mL of whole saliva depending on the individual disposition. After this treatment, each sample was centrifuged at 20000*g* for 15 min at 4 °C. The acidic supernatant was separated from the precipitate and stored at − 80 °C until the RP-HPLC-ESI-MS analysis, which was performed as soon as possible and always before 2 weeks from the collection.

HPLC Low-Resolution MS Analysis and Quantification

The chromatographic separation was carried out using eluent A (0.056% TFA in water solution) and eluent B (acetonitrile/water 80:20 with 0.05% TFA). The gradient applied 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 0.1 ml/min. The injection volume was 30 μL. Mass spectra were collected every 3 ms in the *m/z* range 300–2000 in positive ion mode with a resolution of 6000. The MS spray voltage was 5.0 kV, and the capillary temperature was 255 °C. The MS total ion current (TIC) profiles were analyzed to selectively search and quantify the peptides/proteins reported in Online Resource, Table S2, which shows UniProt-KB codes, elution times, experimental and theoretical average mass values (Mav) of the proteins/peptides included in the study, comprised their PTMs derivatives. Table S2 reports also the multiply charged ions used for the eXtracted Ion Current (XIC) search, which were selected excluding values common to other closely eluting proteins. A window of ± 0.5 Da was used to extract XIC peaks. Mav, obtained by deconvolution of averaged ESI-MS spectra automatically performed by using MagTran 1.0 software [24], and elution times of proteins/peptides were compared with those determined under the same experimental conditions in our previous studies [25, 26]. Experimental Mav were also compared with the theoretical ones available at the UniProt-KB human data-bank (http://us.expasy.org/tools). Structural characterization of acidic proline-rich proteins (aPRPs) [27], histatins (Hst-1, Hst-3, Hst-3 1-24, Hst-3 1-25) [27], statherin [27], P-B peptide [27], thymosins β4 and β10 [28], cystatins A, B, C, D, and S-type [29–31], S100A8 [32, 33], S100A9 [32, 33], S100A7 [34], S100A12 [34], antileukoproteinase [34], and prolactin-inducible protein (PIP) [26], performed by both bottom-up and top-down proteomic platforms based on high-resolution MS/MS analysis, has been already described in our previous proteomic investigations of human saliva. Characterization of α-defensins 1, 2, 3, and 4 is described below. XIC peak areas were integrated by using the following peak parameters: baseline window 15, area noise factor 50, peak noise factor 50, peak height 15%, and tailing factor 1.5. Area of the XIC peaks is proportional to the protein concentration, and, under constant analytical conditions, it allows performing relative quantification of the same protein in different samples [35, 36]. Estimated percentage error of the XIC procedure was < 8%. Eventual dilution errors occurring during sample collection were corrected by correcting XIC peak areas of peptides/proteins with the XIC peak area of leu-enkephalin used as internal standard using the following equation:

Corrected Area of protein = Measured Area of protein × (Expected Area of leu-enkephalin 25 μM/Measured Area of leu-enkephalin). The corrected XIC peak areas were utilized for the statistical analysis and indicated in the results by omitting the term "corrected."

The expected XIC peak area of leu-enkephalin was determined by HPLC-ESI-MS analysis of the 0.2% aqueous solution of TFA containing 50 μM of leu-enkephalin (the same used to treat salivary samples) diluted 1:1 vol/vol with ultrapure water. The XIC peak of leu-enkephalin was evidenced by searching the monocharged ion at 556.64 *m/z* $[M + H]^+$.

Moreover, we determined total protein concentration in the acid soluble fractions of each salivary sample by the bicinconinic acid assay (MicroBCA™ protein assay kit, 0.5–20 μg/mL, ThermoFisher Scientific) in triplicate. The mean value of total protein concentration of each sample was used to normalize the corrected XIC peak areas of the peptides/proteins (corrected XIC peak area divided by the mean total protein concentration).

Characterization of α-Defensins by HPLC High-Resolution-ESI-MS and MS/MS Analyses

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. The injection volume was 20 μL. 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, 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 (ThermoFisher Scientific, CA) using default parameters of the Xtract program for the deconvolution. Protein sequences 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 presence of disulfide bridges in α-defensins 1-4 was considered in the computation of the theoretical *m/z* values of *b* and *y* fragment ions. 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 PXD012688.

Statistical Analysis

The software GraphPad Prism (version 5.0) was used to calculate means and standard deviations of protein XIC peak areas and to perform statistical analyses. Data distributions were tested for normality by D'Agostino-Pearson test. A comparison between PAD patients and controls and between the subgroups of patients was performed by the Mann-Whitney and unpaired *t* test with or without Welch's correction, depending on the data distribution (skewed or normal) and the variances (unequal or homogeneous). Statistical analyses were performed, in addition, by ANOVA one way, followed by Tukey post-test (95% confidence interval). Correlation analyses were performed with the Spearman or the Pearson tests based on the distribution of the data (skewed or normal). Outlier values were always included in the analysis. However, evaluation of their exclusion on the significance of the statistical tests was also performed. The statistical analysis was performed by using XIC peak areas both normalized and not normalized with respect to total protein concentration. The statistical analysis was considered significant when the *p* value was < 0.05 (two tailed).

Results

Comparison of the Salivary Proteome Between the Full Group of PAD Patients and Healthy Controls

Whole saliva collected from healthy subjects and patients was immediately mixed with the TFA acidic solution in order to obtain soluble fractions directly analyzable by HPLC-low-resolution-ESI-MS, and to avoid any possible degradation due to endogenous or exogenous oral proteases during sample handling. We cannot exclude a proteolysis eventually occurring before and/or during sample collection; however, it is worthwhile to observe that the levels of the main part of the proteins and peptides, such as their truncated proteoforms, were not significantly different in PAD patients with respect to the controls suggesting a similar protease activity in the two groups (Online Resource, Table S2). The acidic treatment caused the precipitation of several high molecular weight salivary proteins [38], which, therefore, could not be analyzed by RP-HPLC-ESI-MS. This study was particularly focused on selected proteins and peptides soluble in acid solution, as well as on their known proteoforms derived from phosphorylation, oxidation, proteolytic cleavage, and acetylation (Online Resource, Table S2). They include (i) proteins secreted by salivary glands, such as aPRPs, histatins, statherin, and P-B peptide; (ii) proteins secreted by both salivary glands and epithelial cells, such as cystatins C, D, and S-type, and antileukoproteinase; (iii) proteins released from epithelial cells or leucocytes, such as thymosins β4 and β10, α-defensins 1-4, cystatins A, B, S100A7, A8, A9, and A12, and prolactin-inducible protein. Figure 1 shows a typical RP-HPLC-low-resolution-ESI-MS TIC profile of the acid soluble fraction of a salivary sample from a PAD patient. The elution ranges of the several families of salivary proteins are indicated in the figure. We did not evaluate basic proline-rich proteins due to their high individual variability [39–41]. All the proteins/peptides reported in Online Resource, Table S2, were previously identified by high-resolution MS/MS analysis in proteomics investigation on human saliva [25–34], with the exception of α-defensins 1, 2, 3, and 4, which were characterized in this study (Online Resource, Table S3). All the proteins and peptides have been searched and quantified by XIC procedure, and the levels compared between PAD patients and healthy controls. Table S2 reports mean values of XIC peak areas of proteins and peptides not showing significant different levels in patients and controls. Among the 60 proteins/peptides included in the study, only 8 components exhibited different abundance in PAD patients with respect to healthy controls, namely α-defensins 1-4, and cystatins SN, S1, S2, and B-SSG (Tables S4-S5). The statistical analysis was performed also by utilizing the XIC peak areas normalized with respect to the total protein concentration determined on each sample. Since total protein concentration was not significantly different between PAD and controls (0.6 ± 0.3 μg/μL in PAD group, 0.5 ± 0.3 μg/μL in the control group, $p = 0.45$, Fig. 2), the results achieved by analyzing normalized and not-normalized XIC peak areas were very similar as shown in Tables S4-S5. The results of the statistical comparisons by considering the not-normalized data are shown in Figs. 3 and 4, reporting on the left the comparison between the entire

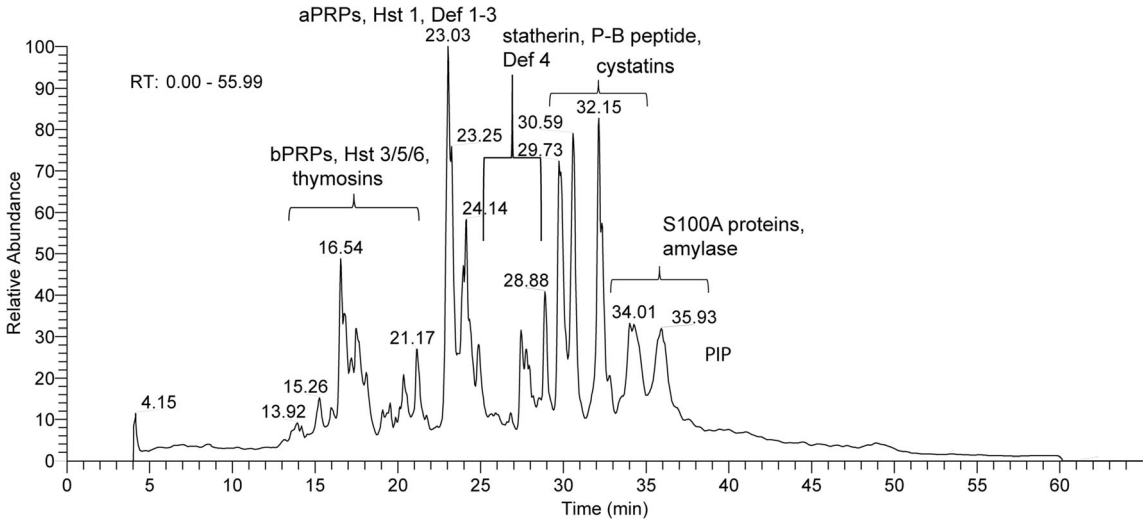

Fig. 1 Representative total ion current chromatographic profile obtained by RP-HPLC low-resolution-ESI-MS of the acid soluble fraction of a salivary sample from a PAD patient, the elution ranges of the several families of salivary proteins are indicated. Normalization level (NL) = 4.29E8

PAD group against controls, and on the right the comparison between the controls and the two subgroups of patients, CVID, and UAD. The statistical significance reported on the plots was obtained by the two-group comparison Mann-Whitney *t* test. Levels and frequencies of the α-defensins 1, 2, 3, and 4 were significantly lower in PAD patients when compared to healthy subjects, as evident from the plots of Fig. 3. The ANOVA analysis confirmed the result of α-defensin 1 and 2, providing p values < 0.0001 and < 0.05 respectively. Similarly, cystatin S1 and S2 exhibited a significant lower level in patients than in healthy controls (Fig. 4a, b), while the frequency was almost equal in the two groups. On the contrary, the cystatin SN and cystatin B-SSG exhibited significant higher levels in PAD patients than in healthy controls (Fig. 4c, d). Also in this case, the ANOVA analysis confirmed the significant different level of the two cystatins between PAD patients and healthy controls (p value < 0.0001 for both SN and B-SSG). Since the oxidized species of cystatins S1, S2, and SN showed a trend similar to the parent proteoforms, the sum of the XIC peak areas of non-oxidized plus oxidized forms was used for statistical analysis.

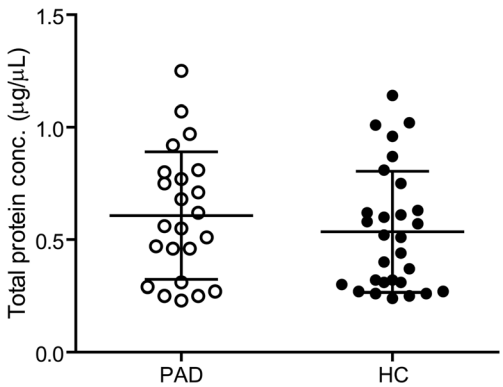

Fig. 2 Plot of distribution of the total protein concentration (µg/µL) measured in acid soluble fractions of salivary samples from PAD patients and healthy controls (HC)

Based on a classification of the PAD patients in CVID and UAD, a statistical analysis was also performed considering the two subgroups of patients, and by utilizing both not-normalized and normalized XIC peak areas (Table S5). When compared to the HC group, the CVID subgroup, exhibited a significant higher level of cystatins SN and B-SSG (Fig. 4c, d), and a significant lower level of cystatin S2 (Fig. 4b), and α-defensins 1, 2, and 4 (Fig. 3a, b, d). Conversely, the UAD subgroup did not show significant differences in the levels of cystatins and α-defensins with respect to neither HC subjects nor CVID subjects, even by excluding agammaglobulinemic patients (#3, #4, #11). All the statistical comparisons were also performed by excluding the three patients without substitutive treatment (#1, #2, and #10), but the results obtained were the same.

Correlation analysis between the XIC peak areas of the salivary proteins and IgA and IgM levels, and the age of onset did not provide significant results. Ten out of the 23 PAD patients, namely patient #1, #5, #12, #14, #15, #17, #19, #21, and #22, and #23, presented the highest XIC peak areas of cystatin SN (Fig. 4c). A correlation analysis performed considering cystatin SN and the other cystatins highlighted that the same ten patients exhibited also the highest levels of cystatins S1 and S2 (r^2 for the linear correlations were 0.63 and 0.54, respectively, and p values < 0.001 for both, Online Resource, Fig. S1). Among the patients with high levels of S-type cystatins, a prevalence of autoimmune associated diseases (seven out of ten) with respect to remaining patients (six out of 13) was found. Moreover, four out of the five PAD patients with a history of neoplasia exhibited very high levels of

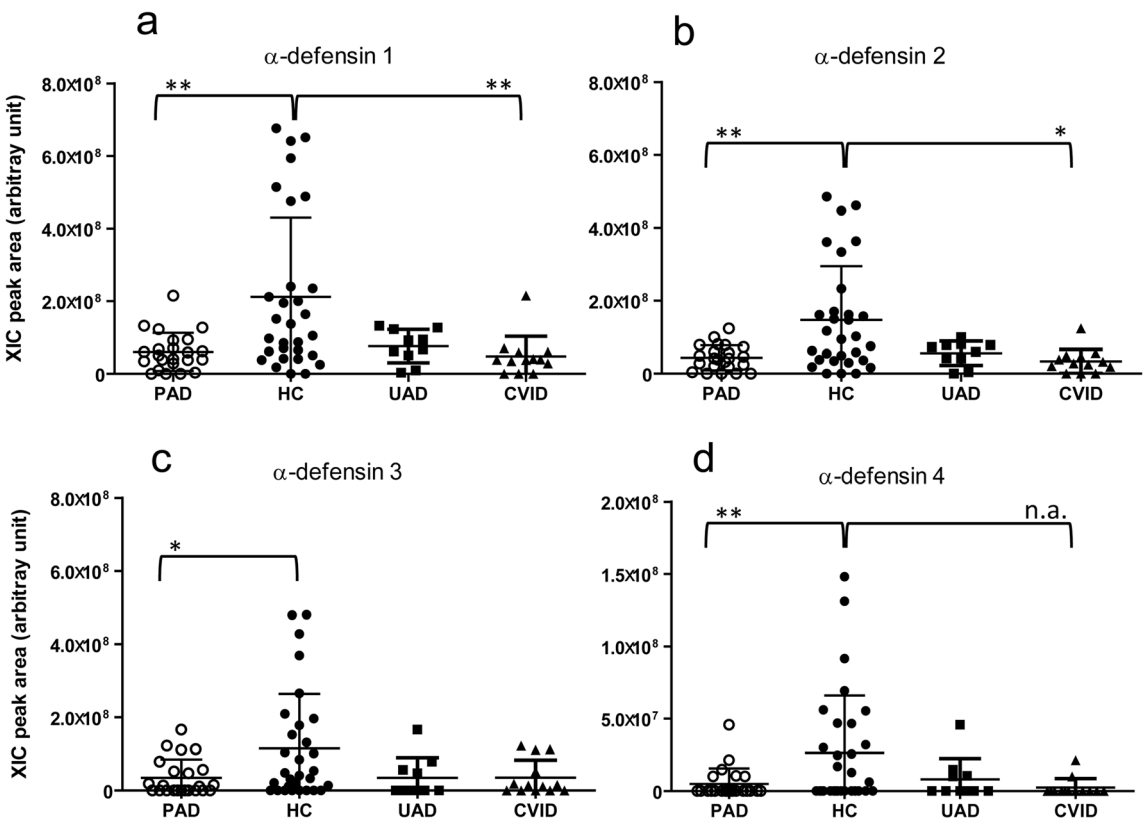

Fig. 3 Distributions of the XIC peak area values of α-defensins 1-4 (panels **a**–**d**), measured in the entire patient group (PAD), in healthy controls (HC), and in the two subgroups of PAD patients (CVID, and UAD). Asterisks indicate: * = p values < 0.05, ** = p value < 0.01. n.a. = not applicable statistical analysis (number of observation ≤ 2)

S-type cystatins (Table 1 and Online Resource, Table S1). Higher levels of cystatins were not linked to the disease classification, since five out of ten patients were CVID and five UAD.

Discussion

The aim of this study was to investigate possible significant variations of the salivary proteome of PAD patients with respect to a gender- and age-matched healthy control group useful in the identification of potential markers of the disease. In order to analyze the intact peptides/proteins present in the salivary samples, the protein fraction soluble in acid solution was investigated by HPLC-ESI-IT-MS allowing us to detect significant differences in the salivary proteome of patients and controls. It must be emphasized that, among more than 60 proteins and peptides analyzed in this study, only α-defensins 1-4, and cystatins SN, S1, S2, and B, showed significant different levels between the groups, and that the total protein concentration was the same in patients and controls, suggesting a specific relationship of these peptides/proteins with the disease.

α-Defensins

α-Defensins 1-4, secreted by neutrophil [42], are broad-spectrum antimicrobial peptides that participate to innate immunity [42, 43]. Their presence in human saliva is due to the neutrophil secretion into the gingival crevicular fluid [44]. Several bacterial, viral, and inflammatory signals trigger α-defensin secretion [45–47], not only by neutrophils but also by other cell populations, among them the NK cells, at level of several organs and bodily fluids [48]. Since previous studies reported defective functions and abnormality in the maturation of neutrophils [12] and NK cells [13] in CVID patients, it would be interesting to evaluate if the low levels and frequencies of salivary α-defensins 1-4 we observed mainly in the CVID subgroup may be associated with the status of recurrent infections typically observed in these patients. It appears interesting to underline that immunomodulatory functions were suggested for the α-defensins, as well as their involvement in the communication between the innate and adaptive immune systems [49]. Indeed, it was demonstrated that α-defensins can transfer from neutrophils to memory B cells to exert their bactericidal activity against *Streptococcus pneumoniae* [50]. Antibody deficient patients, in particular those with low IgA, are more prone to gastrointestinal infections and gut inflammation. Gastrointestinal infections, due to *Salmonella* spp.,

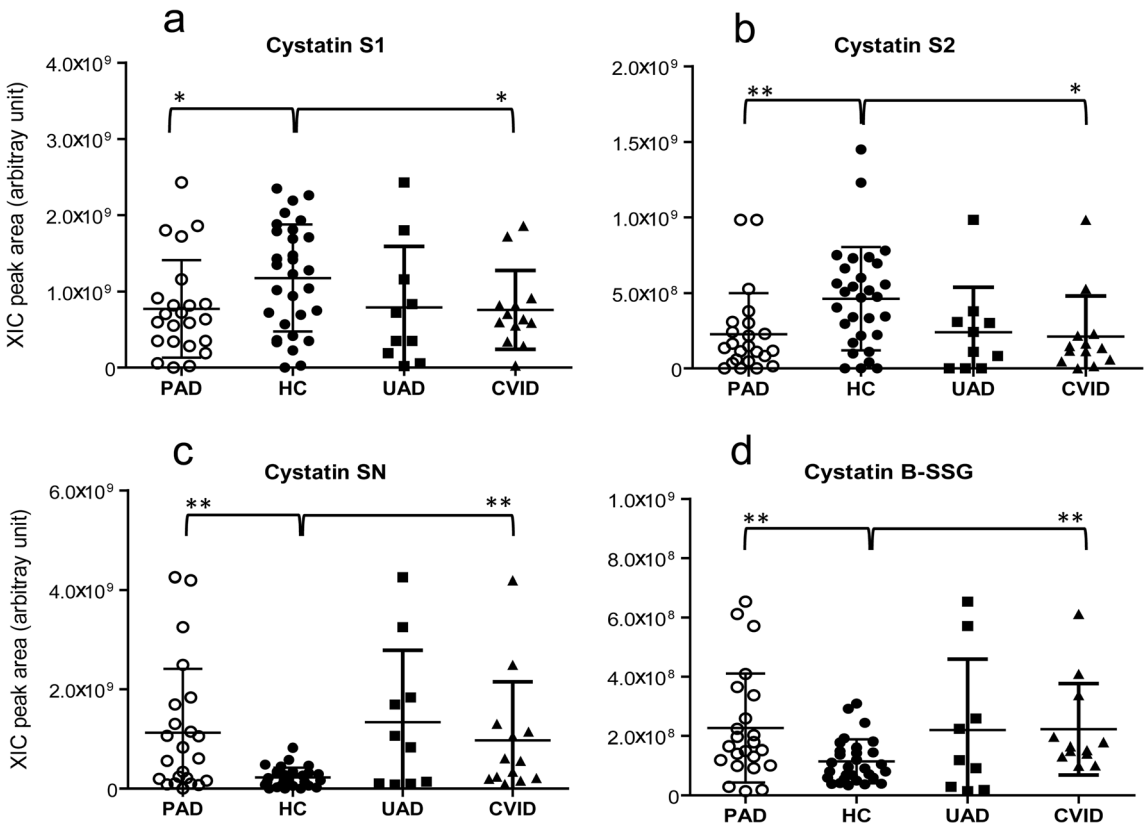

Fig. 4 Distributions of the XIC peak area values of salivary cystatins S1, S2, and SN (panels **a**–**c**) and cystatin B-SSG (panel **d**), measured in the entire patient group (PAD), in healthy controls (HC), and in the two subgroups of PAD patients (CVID, and UAD). Asterisks indicate: * = p values < 0.05, ** = p value < 0.01

Campylobacter spp, or *Giardia* [51], have been reported in about 20% of individuals affected by CVID and hypogammaglobulinemia. The immune/genetic defect causing hypogammaglobinemia exerts a complex effect on innate and adaptive immunity and it is known that it cannot be fully compensated by intravenous or subcutaneous immunoglobulin therapy [52]. Interestingly, it was demonstrated that a mouse model lacking α-defensins in small-intestine is prone to disease caused by oral viral infections, and that α-defensins are implicated as adjuvants in the generation of antibody response [53]. On the other hand, the low abundance of α-defensins could be associated to an inadequate inflammatory stimulation, specifically in the crossroad with the innate immune response at mucosal surfaces. Indeed, it was demonstrated that α-defensins stimulate cytokine production and regulate the expression of adhesion molecules in endothelial cells [54].

Cystatins SN, S1, S2, and B

The salivary protein profile also highlighted significant altered levels of cystatin B-SSG, SN, S1, and S2. It is important to underline that the more common cystatin proteoforms detectable in the acidic soluble fraction of saliva were included in this study, as reported in Table S2 (Online resource). Other proteoforms of salivary cystatins, variants and PTMs, have been identified in human saliva by HPLC-high-resolution MS/MS studies [30, 55], such as the cystatin SN variant carrying a substitution Pro11 → Leu [55]. This variant when present in saliva co-elutes with the main isoform under our experimental conditions [30]. We detected the main isoform of cystatin SN but not the cystatin SN (Pro11 → Leu) variant by checking the mass spectra of the salivary samples from the subjects included in this study. Cystatins S1, S2, SN, and B belong to superfamily of evolutionary related proteins whose main activity is the inhibition of cysteine proteases [56]. Cystatin B is an endogenous cathepsin inhibitor localized in the cytosol, mitochondria, and nucleus of different cell types and found also in extracellular fluids [25, 57, 58]. Cystatin B was also associated to the macrophage activation, apoptosis prevention [59], regulation of cell cycle entry [60], protection against oxidative stress of mitochondria [61], and neurons [62]. Cystatin B plays a role in the neurodegenerative and neuroinflammatory disorders [63, 64], and due to its neuroprotective role, the protein is considered part of the innate immunity. The ability of cystatin B to suppress the oxidative stress appears remarkable by considering that an enhanced oxidative stress was observed in CVID patients [65], suggesting that its elevated level found in our patients could be a clue of self-defense by the organism.

Salivary cystatins SN, S1, and S2 participate to the maintenance of the homeostasis and to the innate defense of the oral cavity against pathogens [55, 66], by inhibiting microbial cysteine proteinases [67] and suppressing some viral [68], bacterial [69], and fungal [70] infections. While cystatins S1 and S2 are specific proteins of the oral cavity, cystatin SN have also been found in other bodily fluids and organs [56]. It is known the anti-inflammatory role of cystatin SN, able to inhibit lysosomal cathepsins B and C implicated in the destruction of periodontal tissues [71]. The ability of cystatin SN to inhibit cathepsin action was associated also to the processes of tumor invasion and development [22]. The over-expression of cystatin SN has been associated with the development of lung cancer [72] and breast cancer [73], and it was individuated as a novel potential biomarker for pancreatic cancer [74] and colorectal cancer [23]. It was reported that patients with primary immunodeficiency manifest a high cancer risk [9, 75], and, interestingly, high levels of cystatin SN in our patients were associated with a high prevalence of autoimmune associated diseases and tumor incidence. Differently to cystatin SN, we observed a downregulation of cystatins S1 and S2, even if this alteration contributed, together the downregulation of α-defensins, to make weaker the oral innate defenses in our patients. To this purpose, it is important to emphasize that other peptides and proteins involved in the innate immune protection of the oral cavity were found at normal levels in our patients, such as histatins, and cystatins A and C, which could partially compensate the deficit of α-defensins and cystatins S1 and S2. Despite the limited number of subjects enrolled for this study, the quantitative differences observed appeared statistically robust and demonstrated that the HPLC-ESI-MS profiling of saliva can be a useful approach to highlight novel protein-disease association in PAD patients. However, a further investigation in a larger cohort of patients would be useful to confirm the present results and to better investigate the UAD group, which showed a greater variability.

Associated Data

The mass spectrometry proteomics data on α-defensins have been deposited to the ProteomeXchange Consortium (http://www.ebi.ac.uk/pride) via the PRIDE partner repository with the dataset identifier PXD012688.

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Authorship Contributions Study conception and design: TC, DF, SDG; cared for patients, extracted the clinical and lab data DF, FC, FaC, SDG; acquisition of data: CC, SS; analysis and interpretation of data: TC, CC, BM, AO; drafting of manuscript: BM, TC, DF, CC; critical revision: MC, IM, TC, FC. All authors read and worked on the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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