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PROTEOMIC (HPLC-ESI-MS) STUDY OF SALIVARY PEPTIDES AND PROTEINS IN PATIENTS WITH SJÖGREN'S SYNDROME, BEFORE AND AFTER PILOCARPINE

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SOMMARIO

OBIETTIVI. Studiare l'effetto della Pilocarpina sulla composizione proteica salivare in soggetti affetti da sindrome di Sjögren primitiva (pSS) e comparare il profilo proteico dei soggetti affetti da pSS con soggetti sani di controllo e pazienti affetti da sindrome di Sjögren secondaria (sSS). E' stata inoltre ricercata la presenza di immunopeptidi, come le defensine e le timosine, allo scopo di descriverne l'eventuale presenza e l'abbondanza relativa nella saliva di soggetti affetti da SS e di valutarne il possibile ruolo come biomarker di malattia e/o di infiammazione.

METODI. Sono stati analizzati campioni di saliva di 9 pazienti affetti da pSS, 9 sSS e 10 soggetti sani mediante High Performance Liquid Chromatography e Mass Spectrometer-Electrospray Ionization Source. In 6 pazienti con pSS sono stati raccolti campioni di saliva anche dopo 30, 60 minuti e 24 ore dall'assunzione di 5 mg di Pilocarpina.

RISULTATI. Nei campioni basali, più del 50% delle proteine salivari di origine ghiandolare analizzate risultavano non rilevabili all'indagine spettrometrica o mostravano livelli significativamente più bassi nei pazienti con pSS rispetto ai soggetti sani. I pazienti con sSS mostravano un profilo di proteine salivari intermedio tra i pazienti con pSS ed i soggetti di controllo. Circa un terzo delle proteine meno rappresentate nei pazienti con pSS al basale risultavano rilevabili con frequenza simile ai controlli dopo 60 minuti dall'assunzione di Pilocarpina. Tutte le proteine con livelli significativamente più bassi al basale rispetto ai controlli raggiungevano livelli simili ai soggetti sani dopo 30 minuti dall'assunzione di Pilocarpina. La migliore risposta al farmaco è stata osservata tra le proteine di origine parotidea. I pazienti con pSS erano inoltre caratterizzati da alti livelli di α-defensina 1 e dalla presenza di β-defensina 2, peptidi di origine neutrofilica ed epiteliale rispettivamente, con funzioni antimicrobiche. Mentre la β-timosina 4, peptide strutturale con proprietà antinfiammatorie e riparatrici, risultava rilevabile in quasi tutti i soggetti sani e nei pazienti, la β-timosina 10, è stata riscontrata nella maggior parte dei pazienti con pSS e in un terzo dei soggetti con sSS; non era invece evidenziabile in nessuno dei soggetti sani.

CONCLUSIONI. Il nostro studio ha dimostrato come la Pilocarpina, farmaco secretagogo agonista colinergico, che si riteneva aumentare solo la quota fluida della secrezione salivare, sia invece in grado di ripristinare parzialmente i livelli ed il numero delle proteine salivari ridotte in corso di sindrome di Sjögren. L' α-defensina 1, la β-defensina 2 e la β-timosina 10 potrebbero essere considerati biomarker di infiammazione orale nei pazienti con pSS.

INTRODUCTION

SJŐGREN'S SYNDROME AND PILOCARPINE TREATMENT

Saliva is a complex fluid composed of a variety of electrolytes, metabolites, nucleotides, polynucleotides and proteins; it plays an important role in the maintenance of oral health (1). The rate of salivary protein secretion is controlled mainly by noradrenalin that is released from the sympathetic terminals and acts through the β-adrenergic receptors, while the rate of fluid and electrolyte secretion is controlled by acetylcholine, released from the parasympathetic terminals and acting through the muscarinic cholinergic receptors (2). A large number of systemic agents has been proposed as secretagogues, but only a few have shown consistent salivary secretion enhancing properties in well-designed trials. Among cholinergic agonists, pilocarpine is the most effective for protein secretion in rat (3), having also a mild β-adrenergic stimulating properties, but a few data have been reported in humans. Pilocarpine has been shown to improve symptoms of oral dryness and to increase salivary output in patients with primary Sjögren's syndrome (pSS) (4), a chronic autoimmune disorder of the exocrine glands with associated lymphocytic infiltrates and consequent dryness of mouth and eyes (5). Saliva composition in pSS patients has been found to be different from normal subjects (6). However the pattern of salivary gland proteins in patients with pSS is not completely defined with regard to its composition, mainly in relation to low-molecular-weight components as acidic and basic proline-rich proteins (PRPs), statherins and cystatins, as well as defensins, which are immunopeptides of epithelial and neutrophilic origin, and thymosins, G-actin-sequestering peptides with immuno-regulatory properties. In particular there are no data concerning the effects of pilocarpine on salivary protein profile in pSS patients. Moreover there are no studies on the possible differences in salivary protein profile between pSS and Sjögren's syndrome associated to other rheumatic diseases (sSS).

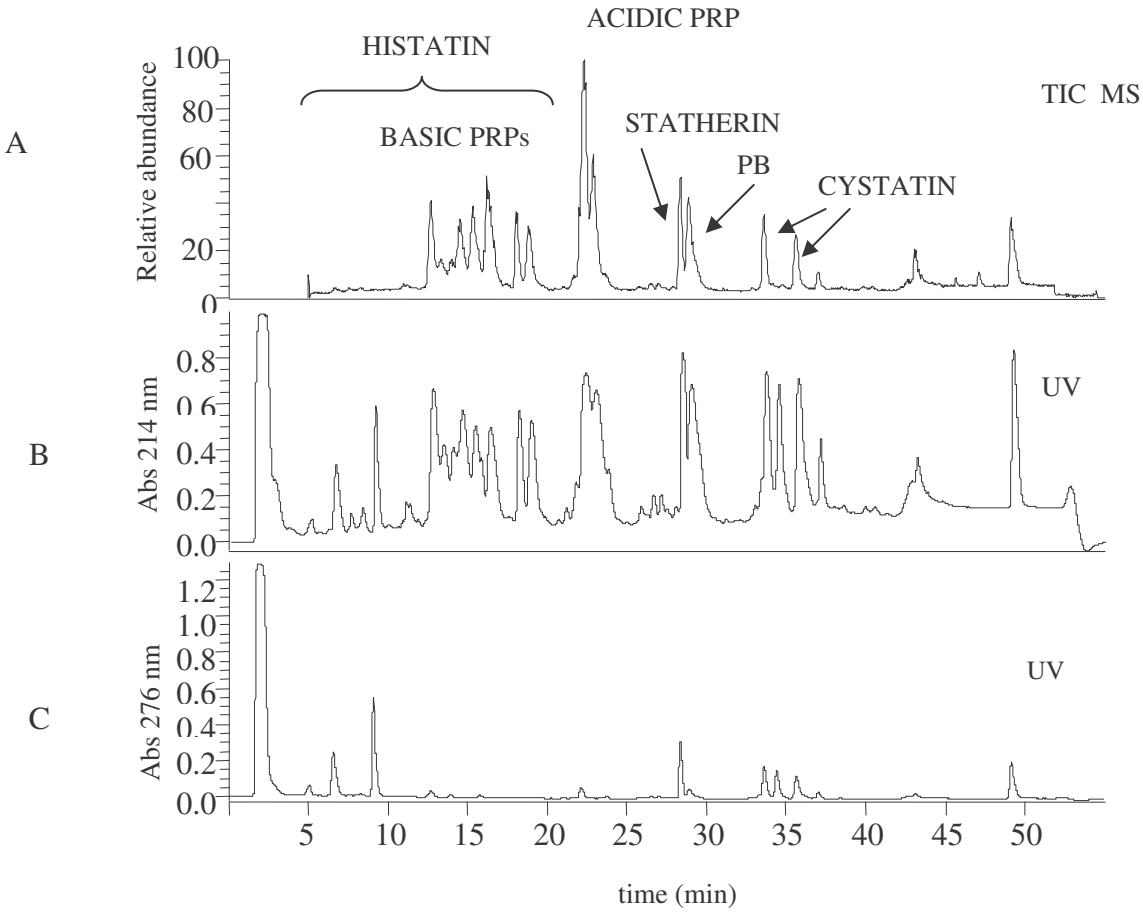
SALIVARY PEPTIDES

The table 1 shows the main salivary proteins classes and their origin.

Table 1. Main salivary proteins classes and their origin.

Salivary gland proteins	Parotid glands	Submandibular/sublingual glands
Lysozyme	+	+
Salivary Cystatins	-	+
Statherin	+	+
Histatins	+	+
Acidic PRPs	+	+
Basic PRPs	+	-

Figure 1



The pattern of salivary gland proteins in health and disease is not completely defined with regard to its composition, mainly in relation to these low-molecular-weight components.

Prolyn rich proteins (PRPs) are the main components in human saliva (table 2 and 3). They are classified in basic, acidic and glycosilated. Since their role is not well understood it seems that these proteins are involved in the metabolism of calcium in the mouth (7').

Table 2: acidic PRPs (Swiss Prot code P02810) found through HPLC-ESI MS.

Protein		Mean theoric mass [M]	Mean experimental mass [M]
PRP-2 (PRP-1, PIF-s)	di-P.	15514 (15515)	15515 ±2
PRP-2 (PPR-1, PIF-s)	tri- P.	15594 (15595)	15595 ±2
PRP-2 (PPR-1, PIF-s)	mono- P.	15434 (15435)	15434 ±2
Db-s	di- P.	17633	17633 ±3
Db-s	tri- P.	17713	17713 ±3
Db-s	mono- P.	17553	17553 ±2
Pa monomer	di- P.	15462	15462 ±2
Pa dimer	tetra- P.	30922	30922 ±3
Pa dimer	tri- P.	30842	30842 ±3
Pa dimer	di- P.	30762	30762 ±3
Pa dimer des Gln 150	tetra- P.	30794	30793 ±2
PRP-4 (PRP-3, PIF-f)	di- P.	11161 (11162)	11162 ±2
PRP-4 (PRP-3, PIF-f)	mono- P.	11081 (11082)	11081 ±3
PRP-4 (PRP-3, PIF-f)	non- P.	11001 (11002)	11002 ±1
PRP-4 (PRP-3, PIF-f) des Arg 106	di- P.	11005 (11006)	11006 ±1
Db-f	di- P.	13280	13280 ±2
Db-f	mono- P	13200	13200 ±2
PC peptide		4371	4371 ±1

Table 3: basic PRPs found through RP-HPLC-ESI MS

Peptide	Swiss-Prot code	Mean theoric mass [M]	Mean experimental mass [M]
P-C (IB-8b)	P02810	4371	4371
P-F (IB-8c)	P02812	5843 (0)	5843
P-J (IB-6 fr. 1-62)	P04280	5945 (1)	5944
P-E (IB-9)	P02811	6024 (0)	6024
P-D (IB-5)	P02813	6950 (1)	6950
P-H (IB-4) (IB-6 fr. 63-118)	P04280	5590 (0)	5590
IB-8a	-	11894 (1)	11898
IB-6	P04280	11517 (2)	11519
IB-1	P04281	9593 (0)	9593
II-2	-	7609 (1)	7608
IB-1 non-P	P04281	9513 (1)	9512
II-2 non-P	-	7529 (1)	7528
IB-1 des Arg 96	P04281	9437 (0)	9437
II-2 des Arg 75	-	7453 (1)	7452
P-B	P02814	5793 (1)	5793
Fragm P-B (fr.8-57)	P02814	5215 (1)	5215

Cystatins are composed by 115-120 amino acid, with molecular weight of 13-14 kDa and 2 sulphidrilic bridges. They have inhibitor activity against cystein (8'). Group S only has a glandular origin (table 4).

Table4: Cystatins found through HPLC-ESI MS.

Protein	Swiss-Prot code	Mean theoric mass [M]	Mean experimental mass [M]
Cystatin S	P01036	14188.7	14187
Cystatin S1	"	14268.7	14267
Cystatin S2	"	14348.7	14348
Cystatin SN	P01037	14316.0	14315
Cystatin SA	P09228	14350.0	14345
Cystatin C	P01034	13347.1	13345
Cystatin D	P28325	13858.6	13848
Cystatin A	P01040	11006.5	11006

DEFENSINS

Defensins are a family of small cationic proteins, 3000-4000 Da, involved not only in the innate immune system against infectious microbes, but also in adaptive immunity (9-11), inflammation and wound repair (12). The human defensin family is subdivided into α and β-defensins on the basis of the spatial distribution of the cysteine residues and connectivity of disulfide-bonds (13). α-defensins 1-4 were isolated from neutrophil cells and 5-6 are present in secretory granules of the intestinal Paneth's cells and in epithelial cells of the female genital tract (14). α-defensins 1-3 constitute the most abundant proteins of azurophil granules composing 30-50 % of the protein content (15). α-defensin-4 is two orders of magnitude less abundant than 1-3 (16). α-defensins 1-3 genes are constitutively expressed in neutrophils and there is no evidence that factors other than gene copy number influence the expression of α-defensins 1-3 (17). The expression of α-defensins in cells other than neutrophils has been reported in buccal epithelial cells of subjects with oral pathology (18) and in the striated duct cells of the submandibular glands (19). High performance liquid chromatography and mass spectrometry have shown the presence of α-defensins 1-3 in normal subjects saliva (20), moreover our group has recently found the presence of low levels of α-defensin-4 in gingival crevicolar fluid (GCF) of normal subjects (21) (table 5).GCF is one of the main sources of oral α-defensins. In normal subjects GCF concentration of α-defensins is approximately from five to fifteen times greater than that one determined in whole saliva.

Human β-defensins 1-4 have been described in some detail, but many additional human β-defensins genes exist and their functions remain to be explored (22). β-defensins 1-4 are expressed by many epithelial cells, especially in oral tissues (23,24). Oral keratinocytes but not fibroblasts express β-defensins 1-2 (25), moreover β-defensins-1 and 2 have also been detected in salivary glands and ducts (26,27). β-defensin-1 is constitutively and heterogeneously expressed in epithelial cells and only poorly induced by cytokines (28), while β-defensins-2 and 3 expression can be induced by a local cytokine imbalance with a predominance of Th1 cytokines as TNFα, IL 1β and IL 6 for β-defensin-2 and INFγ for β-defensin-3 as in oral inflammatory diseases or by infectious

agents by LPS (29). However significant individual heterogeneity in β-defensin gene expression has been reported (30, 31) as result of genetic polymorphisms or number of defensin gene copies (32), multiple regulatory pathways and mediators involved in their expression (33), different grade of tissue atrophy (34) or the replacing of the innate or natural defence by mechanisms of the acquired immune response in chronically inflamed tissues (35). In fact some studies have suggested increased expression of β-defensins-2 and 3 in tissues with inflammation (36, 37), whereas other investigators have shown decreased expression in inflamed oral tissue samples (38). Kawasaki S et al reported a significantly up-regulation of β-defensin-2 gene but not of other β-defensins genes in conjunctival epithelial cells from pSS patients (39).

Table 5: α-defensins found trough RP-HPLC-ESI-MS

peptide	Mean theoric mass [M]	Mean experimental mass [M]
α-Defensin 2	3371.0 (P59665/P59666)	3371.8
α-Defensin 1	3442.1 (P59665)	3442.6
α-Defensin 3	3486.1 (P59666)	3486.9
α-Defensin 4	3709.4 (P12838)	3709.5

THYMOSINS

β-thymosins are a family of peptides with a molecular mass of about 5 kDa and with a sequence of 40-44 amino acid residues. All β-thymosins, so far isolated, are acetylated at the N terminus; they are soluble and stable in diluted acid and are cpmosed by almost all polar amino acids. The name thymosin derives from the first isolation of these peptides from calf thymus, while the α, β and γ letters divide thymosins according to their different isoelectric points (below 5.0, between 5.0 and 7.0, above 7.0, respectively) (40). So far, more than 15 β-thymosins have been described and many more have been detected in cDNA library (40). In mammalian tissues thymosin β_4 ($T\beta_4$) is usually the main peptide, representing about 70-80% of the total β-thymosin content.

$T\beta_4$ is an ubiquitous peptide with very interesting functions. Initially $T\beta_4$ was embraced as a G-actin sequestring peptide, but biochemical and overexpression studies showed that $T\beta_4$ exerts its intracellular action not only as a monomer-buffering protein but also indirectly via activation and transcription of signalling molecules that alter the actin cytoskeleton. Thus, manipulation of the actin cytoskeleton can induce transcriptional, translational, and post-translational changes in crucial proteins within the actin regulatory network (41).

Some of its functions require full-length $T\beta_4$ or $T\beta_4$ fragments containing the actin-binding motif (N terminus), while others appear to be mediated through the N-terminal AcSDKP peptide that does not bind actin (41). $T\beta_4$ promotes cell migration and adhesion dynamics directly modulating actin polymerization/ depolymerization but also forming a functional complex with PINCH and ILK, from integrin network, that activates the prosurvival kinase Akt (42). $T\beta_4$ treatment or overexpression decreases the amount of E-cadherin, which stabilizes cell-cell adhesion and inhibits motility, mechanism involved in malignant transformation and differentiation (43).

$T\beta_4$'s properties to adopt a secondary structure after interaction with other proteins may allow it to bind multiple unrelated partners. This would explain why how such a small peptide can have such a wide spectrum of biological effects in vivo (44). Moreover tandem beta-thymosin motif containing

proteins can bind actin filament promoting actin assembly rather than preventing actin polymerization (45). TB4 itself acts as a monomer-sequestering protein at low concentrations and binds actin filaments at high concentrations (46). However, when TB4 increases acutely, it tends to depolymerize actin filaments, this suggests that TB4 act primarily as an actin-sequestering protein shifting the monomer:polymer equilibrium toward depolymerization (47).

Recently it has also become clear that $T\beta_4$ has an important extracellular role in the repair and remodelling of tissues and organ systems. $T\beta_4$ plays a role in regulation and differentiation of T lymphocytes (48) and can inhibit the migration of macrophages (49). Moreover, $T\beta_4$ exerts biological effects on hypothalamus and pituitary gland (50). $T\beta_4$ induces matrix metalloproteinases, modifies the rate of spreading of endothelial cells on matrix components (51,52), stimulates the migration of human umbilical vein endothelial cells (53), it is involved in the repair of damaged cornea (54,55), induces hair growth by activating hair follicle stem cells (56), it might be involved in the development and repair of damages of the heart and brain (57,58). The molecular mechanism underlying these effects are until now unknown, as well as the mechanism of release from cells. When $T\beta_4$ is released in the extracellular space by blood platelets it can be cross-linked by factor XIIIa either to fibrin or fibrinogen, preferentially to the αC-domain, by other tissue transglutaminases to collagen and actin (59). This provides a mechanism to increase the local concentration of $T\beta_4$ at sites of clot formation and tissue damage, where it may contribute to wound healing, angiogenesis, and inflammatory responses.

$T\beta_4$ sulfoxide is the product of oxidation of the methionyl residue at position 6. This oxidation increases the dissociation constant about 20-fold. Because cells produce peroxides (e.g. H2O2) during their metabolism, it has been hypothesized that this mechanism could regulate $T\beta_4$ actin-binding activity lowering its affinity to G-actin (40). Moreover $T\beta_4$ sulfoxide seems to have anti-inflammatory properties and can inhibit neutrophil chemotaxis (60). In lymphoid tissue $T\beta_4$ gene

also encodes a longer splice variant carrying an additional 98-pair exon at 5', which was found to be expressed in murin skin T γ/δ cells and showed anti-inflammatory activities (61).

$T\beta_4$ and $T\beta_{10}$ bind actin with similar affinity (62) but $T\beta_{10}$ is less abundant in normal tissue. However $T\beta_{10}$ is greater expressed in preneoplastic and neoplastic tissues (63) and in activated lymphocytes (64). $T\beta_4$ has been recently detected in human whole saliva and tears (65), while $T\beta_4$ sulfoxide and $T\beta_{10}$ have never been described in human saliva.

Our group found that $T\beta_4$ is almost always detectable in whole saliva, while its sulfoxide was sporadically and $T\beta_{10}$ was rarely detectable. The relative abundance of $T\beta_4$ in salivary secretions from parotid and submandibular/sublingual gland is often undetectable, when compared to the abundances observed in whole saliva. Analysis of crevicular gengival fluid reveals high relative abundances of Tβ4, its sulfoxide and Tβ10, being all them always detectable. Tβ4 is always the most abundant in crevicular fluid. These results indicate that gengival plexus is a relevant source for oral $T\beta_4$ and $T\beta_{10}$ (66).

$T\beta_4$ has been detected in human whole saliva and tears by immunological technique (65). Commercial available immunological tests cross-react with sulphoxide thymosins, while RP-HPLC allow to distinguish nonoxided and oxided thymosins, because the oxidation of a methionyl residue decreases the elution time. Moreover splice-variant can also be detected by RP-HPLC. There are no study reporting the presence of $T\beta_4$, $T\beta_4$ sulfoxide or thymosin β_{10} ($T\beta_{10}$) in saliva of patients with Sjögren's syndrome.

OBJECTIVES

The aims of this work have been to investigate the effect of pilocarpine on salivary peptide and protein profile in pSS patients, in order to analyze the differences between patients before and after pilocarpine assumption and healthy controls. Moreover the salivary profile in patients with pSS was also investigated.

The presence and levels of defensins and thymosins were also investigated in order to characterize their possible role as biomarker of disease or inflammation.

PATIENTS AND METHODS

Patients were enrolled at the Rheumatology Clinic, Catholic University, Rome. After obtaining the informed consent, saliva specimens were collected from:

9 pSS patients (all females, mean age 55.8 ± 13, mean duration of disease 7.9 ± 4.2),

9 sSS patients (3 with Systemic Sclerosis (SSc), 3 with Systemic Lupus Erythematous (SLE) and 3 with Rheumatoid Arthritis (RA); all females, mean age 48.5 ± 16.4, mean duration of disease 5.7 ± 5.1)

10 healthy subjects who did not show any sign of Sjögren's syndrome (SS) symptoms and were negative for immunologic and serologic tests and matched for sex and age.

Saliva specimens were also collected in 6 pSS patients after 30, 60 minutes and 24 hours from 5 mg pilocarpine intake. All patients were diagnosed according to the revised international classification criteria for SS (67). Whole saliva was collected using a very soft plastic aspirator between 2 and 4 pm in order to reduce concentration variability connected to circadian rhythms of secretion. The collection was performed at least 30 min after any food or beverage admission and tooth brushing. An acidic solution (0.2% TFA) was immediately added in ice bath to all collected salivary samples in 1:1 v/v ratio and the solution centrifuged at 8000 g at 4° C for 5 min. After discharge of precipitate the acidic solution was analysed by High Performance Liquid Chromatography coupled with Mass Spectrometer equipped with Electrospray Ionization Source (HPLC-ESI-MS) within 30 min from collection.

The HPLC-ESI-MS apparatus was a ThermoFinnigan (San Jose, CA, USA) Surveyor HPLC connected by a T splitter to a PDA diode-array detector and to a Xcalibur LCQ Deca XP Plus mass spectrometer. The mass spectrometer was equipped with an electrospray ion (ESI) source. The chromatographic column was a Vydac (Hesperia, CA, USA) C_8 column, with 5 μm particle diameter (column dimensions 150x2.1 mm). The following solutions were utilized for the reversed-phase chromatography: (eluent A) 0.056% (v/v) aqueous TFA and (eluent B) 0.050% (v/v) TFA in acetonitrile-water 80/20 (v/v). The gradient applied was linear from 0 to 55% of B in 40 min, at a

flow rate of 0.30 mL/min. The T splitter addressed a flow-rate of about 0.20 mL/min towards the diode array detector and a flow-rate of about 0.10 mL/min towards the ESI source. The diode array detector was set at the two wavelengths of 214 and 276 nm. Mass spectra were collected every 3 millisecond in the positive ion mode. MS spray voltage was 4.50 kV and the capillary temperature was 220° C. All common chemicals and reagents for the HPLC-MS analysis were of analytical grade and were purchased from Farmitalia-Carlo Erba, (Milan, Italy), Merck (Damstadt, Germany) and Sigma Aldrich (St. Louis, MI, USA).

DATA ANALYSIS

Deconvolution of average ESI mass spectra was automatically performed either by the software provided with the Deca-XP instrument (Bioworks Browser) or by MagTran 1.0 software (Zhang and Marshall, 1998). Experimental mass values obtained from the analysis were compared with average theroretical values available from the Swiss Prot and EMBL data banks (http://www.expasy.org/tools; http://www.embl-heidelberg.de).

The relative amount of the different salivary proteins was approximately computed by performing a multiple eXtracted Ion Current (XIC) strategy for any protein. The XIC procedure for a protein was based on the extraction from the Total Ion Current (TIC) profile to three m/z values selected among the most relevant, provided that these did not overlap with m/z values of nearly eluting proteins. For the peptides with a mass comprised in the detection range of the ESI-MS apparatus (300-2000), the mono-isotopic m/z values of either the mono-charged or of the bi-charged ion or both was chosen for the XIC procedure. Taking into account that constant analytical conditions were used for each sample, the numerical value corresponding to the integrated peak area of the XIC strategy was used for the statistical analysis and for rough estimation of relative changes in protein amount between two states.

Experimental Mass values were compared with average theoretical values available at the Swiss-Prot data bank (http://us.expasy.org/tools), where α-defensins 1-3 can be found with the codes P59665-P59666 and α-defensin 4 with the code P12838. Average mass values of α-defensins are the following: α-defensin 1, 3442.1; α-defensin 2, 3371.0; α-defensin 3, 3486.1; α-defensin 4, 3709.4 Da. Relative abundances of α-defensins were evaluated by considering the area of the XIC peaks, revealed in the total chromatographic TIC by selecting the following double-, triple- and tetra-charged ions: α-defensin 1, 1722.1, 1148.4, 861.5 (± 0.5); α-defensin 2, 1686.5, 1124.7, 843.8 (± 0.5); α-defensin 3, 1744.1, 1163.0, 872.5 (± 0.5); α-defensin 4, 1855.7, 1237.5, 928.4 (± 0.5). The area of the XIC peaks under similar experimental conditions is linearly related to the peptide concentration, as verified using standards of defensin 1 and 2. Elution time of α-defensins 1-3 was coincident at 23.7 (± 0.7) min, while α-defensin 4 eluted as a separate peak at 27.4 (± 0.7) min.

Experimental Mass values were compared with average theoretical values available at the Swiss-Prot data bank, where $T\beta_4$ and $T\beta_{10}$ have the P62328 and P63313 codes, respectively. Relative abundances of β thymosins were evaluated by considering the area of the XIC peaks, revealed in the total chromatographic TIC by selecting the following charged ions: $T\beta_4$, $[M+5H]^{5+}$ = 993.8 m/z ; $[M+4H]^{4+}$ = 1241.9 m/z ; $[M+3H]^{3+}$ = 1655.5 m/z ($M_{average}$: 4963.5). $T\beta_{10}$, $[M+5H]^{5+}$ = 988.3 m/z ; $[M+4H]^{4+}$ = 1235.1 m/z ; $[M+3H]^{3+}$ = 1646.5 m/z ($M_{average}$: 4936.5).

Data statistical treatment was carried out by SPSS 13.0 (SPSS. Chicago. IL-USA). Categorical and quantitative variables were respectively described as numbers, percentage (%) and mean ± standard deviation (SD). Mann-Whitney's test and Wilcoxon's test were used to compare continuous variable. Categorical variables were analysed using χ^2 test or Fisher's test, depending on sample size restrictions.

RESULTS

Analysis of frequency and levels of salivary gland proteins in pSS patients vs healthy controls

The principal salivary gland protein classes and their origin are listed in table 1. In the 9 pSS patients, 28 (43.8%) of the 64 proteins analyzed were significantly less detectable than in the 10 healthy controls (Tables 6-11). Especially salivary cystatins were less frequently found in pSS patients; only the cystatin A was found in a similar percentage in patients and in controls according to its not glandular origin. Only one peptide had a detection frequency higher in pSS patients than in controls: the PB Des^{1-4} peptide was found in 6/9 (60 %) of patients with pSS vs 1/10 (10 %) of controls (p=0.017).

Among proteins whose detection frequency was similar between pSS patients and controls, 8 (12.5%: IB-1, II-2 PRP-1, PRP-3, statherin, PC, P-B peptide) were less abundant in pSS patients than in controls (Tables 6-11).

Then 27 (56.3%) of the proteins analyzed were not detectable or showed lower levels in pSS patients than in controls.

Table 6. Basic PRPs levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Basic PRPs						
P-F	16±27 (10//10)	3.7±6.3 (5/9)	2.4±3.7 (4/9*)	8.4±7.0 (4/6°)	5.0±3.5 (5/6°)	4.4±3.6 (5/6°)
P-J	13±20 (10/10)	4.9±8.2 (5/9)	2.9±4.2 (5/9)	11±7.4† (5/6)	6.7±7.5† (5/6)	4.9±3.5 (5/6)
P-E	nv (1/10)	1.6±3.4 (4/9)	nv (1/9)	5.8±9.0 (2/6)	nv (1/6)	nv (1/6)
P-D	9.5±16 (8/10)	13±20 (8/9)	9.2±13 (8/9)	27±21† (6/6)	25±29† (6/6)	13±9.5 (6/6)
P-H	17±25 (10/10)	5.6±7.8 (7/9)	4.7±5.6 (7/9)	14±11 (5/6)	11±11† (6/6)	7.6±5.7 (5/6)
IB-8a	12±19 (6/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
IB-6	nv (1/10)	4.3±6.6 (5/9*)	nv (0/9)	3.8±5.8 (2/6)	nv (1/6)	nv (1/6)
IB-1	29±41 (10/10)	11±17* (8/9)	3.3±5.3* (6/9)	14±14‡ (6/6)	12±13 (6/6)	6.9±5.5 (5/6)
II-2	67±13 (10/10)	14±22 (7/9)	5.8±9.2* (7/9)	33±28‡ (6/6)	26±19 (6/6)	14±8.3 (6/6)
IB-1 DesR	1.6±2.8 (7/10)	nv (1/9*)	0.9±2.5 (2/9*)	2.1±4.7 (2/6)	2.3±4.9 (2/6)	0.1±2.7 (1/6)
II-2 DesR	2.8±4.1 (10/10)	3.5±4.9 (5/9)	3.9±4.1 (7/9)	2.3±5.9 (5/6)	3.2±6.3 (5/6)	3.1±3.9 (5/6)
IB-7	0.07±0.1 (2/10)	nv (0/9)	nv (0/9)	nv (0/6)	nv (0/6)	nv (0/6)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Table 7. Acidic PRPs proteins levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Acidic PRPs						
PRP-1 3P	1.0±1.3 (10/10)	3.0±7.5 (5/9)	0.2±0.5 (3/9*)	0.4±0.6 (3/6)	0.3±0.4 (2/6)	0.4±0.5 (3/6)
PRP-1	29±26 (10/10)	11±16 (7/9)	1.7±2.3* (6/9)	3.5±2.3‡ (5/6)	4.8±2.8† (6/6)	3.0±3.5 (6/6)
PRP-1 1P	2.9±2.1 (9/10)	2.8±6.6 (7/9)	0.3±0.4 (3/9*)	0.8±0.8 (5/6°)	0.9±0.5 (6/6°)	0.5±0.7 (5/6°)
PRP-3	17±18 (10/10)	15±16 (9/9)	1.5±2.1* (6/9)	4.6±3.6† (5/6)	5.9±5.0‡ (6/6)	3.2±3.1† (5/6)
PRP-3 1P	1.8±1.3 (10/10)	1.4±1.5 (8/9)	0.3±0.4 (4/9*)	0.6±0.5 (5/6°)	5.9±12 (6/6°)	0.5±0.5 (4/6°)
PRP-3 0P	0.06±0.08 (5/10)	0.9±1.5 (5/9)	0.4±0.8 (3/9)	0.7±0.9 (3/6)	0.8±0.6† (5/6)	0.5±0.7 (3/6)
PC	32±29 (10/10)	13±17 (7/9)	7.8±9.5* (9/9)	26±19‡ (6/6)	29±22‡ (6/6)	15±10 (6/6)
PC desQ	0.4±0.4 (9/10)	0.2±0.3 (4/9*)	0.4±0.5 (5/9)	0.8±1.1 (4/6)	1.0±1.4 (4/6)	0.3±0.4 (3/6)
PC desPQ	0.8±0.9 (9/10)	nv (1/9*)	0.5±1.1 (2/9*)	0.6±1.0 (3/6°)	nv (1/6)	0.5±0.8 (2/6)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Table 8. Sthaterin levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Statherin						
Stath	45±26 (10/10)	16±23* (8/9)	5.8±13* (7/9)	12±13 (5/6)	13±16‡ (5/6)	12±22 (5/6)
Stath 1P	0.9±0.7 (10/10)	0.3±0.5 (3/9*)	0.4±0.5 (5/9)	0.3±0.2 (4/6)	0.2±0.3 (3/6)	0.2±0.3 (2/6)
Stath 0P	0.03±0.06 (3/10)	0.3±0.4 (5/9)	0.3±0.3 (5/9)	0.09±0.1 (3/6)	nv (1/6)	nv (1/6)
SV1	2.3±2.3 (10/10)	2.8±3.4 (8/9)	1.5±2.2 (7/9)	2.3±3.0 (6/6)	1.7±1.3 (6/6)	2.9±4.8 (6/6)
Stath DesTF	1.2±1.3 (10/10)	0.8±1.1 (7/9)	0.6±0.8 (6/9)	0.5±0.7 (3/6)	2.6±3.5 (4/6)	0.7±0.8 (4/6)
Stath DesD1	0.9±0.8 (10/10)	0.3±0.4 (6/9)	0.8±0.2 (4/9*)	0.3±0.3 (3/6)	0.5±0.7 (3/6)	0.5±0.7 (3/6)
SV2	0.4±0.3 (9/10)	0.09±0.2 (2/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
SV3	nv (1/10)	nv (0/9)	nv (0/9)	nv (0/6)	nv (0/6)	nv (0/6)
Stath Des^{1-9}	1.4±1.0 (10/10)	0.9±1.5 (5/9)	0.2±0.4 (3/9*)	0.6±0.8 (3/6)	0.4±0.3 (4/6°)	0.5±0.7 (3/6)
Stath Des^{1-10}	0.4±0.3 (10/10)	0.6±0.7 (6/9)	0.3±0.7 (3/9*)	0.6±0.8 (3/6)	0.6±0.8 (4/6°)	0.6±0.8 (3/6)
Stath Des^{1-13}	0.3±0.2 (10/10)	0.4±0.4 (6/9)	0.3±0.4 (5/9)	0.3±0.4 (3/6)	0.5±0.5 (4/6°)	0.3±0.4 (3/6)
P-B peptide	24±15 (10/10)	18±20 (8/9)	8.9±11* (9/9)	14±12‡ (6/6)	9.6±20 (6/6)	12±16 (6/6)
P-B Des^{1-5}	0.8±0.9 (9/10)	1.2±1.8 (7/9)	2.4±3.1 (7/9)	1.4±1.8 (5/6)	3.2±4.5 (5/6)	1.9±3.4 (4/6)
P-B Des^{1-4}	nv (1/10)	0.6±1.1 (3/9*)	0.9±1.1 (6/9*)	0.8±0.8 (5/6)	1.1±1.7 (4/6)	0.8±1.2 (4/6)
P-B Des^{1-7}	2.1±1.4 (9/10)	3.7±3.9 (8/9)	1.6±2.3 (7/9)	3.5±2.8 (6/6)	5.1±4.1 (6/6)	3.5±3.3 (5/6)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Table 9. Cystatins levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Cystatins						
Cyst A	1.0±1.2 (10/10)	1.7±1.8 (9/9)	5.0±7.3*(8/9)	2.3±2.9 (5/6)	1.7±1.8 (4/6)	2.0±2.4 (4/6)
Cyst C	0.7±0.4 (9/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
Cyst D	nv (0/10)	nv (0/9)	nv (0/9)	nv (0/6)	nv (0/6)	nv (0/6)
Cyst S	0.5±0.4 (10/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
Cyst S1	5.9±4.6 (10/10)	3.9±6.1 (5/9)	1.9±3.2 (4/9*)	3.4±5.4 (3/6)	4.5±3.0 (3/6)	4.8±1.3 (3/6)
Cyst S2	2.7±2.5 (10/10)	nv (0/9*)	nv (1/9*)	nv (1/6)	4.6±4.1 (2/6)	5.4±3.2 (2/6)
Cyst SA	2.7±4.5 (10/10)	nv (0/9*)	nv (1/9*)	nv (1/6)	nv (1)	nv (1/6)
Cyst SN	18±21 (10/10)	nv (0/9*)	2.8±4.0 (4/9*)	4.7±6.4 (3/6)	5.3±1.4 (3/6)	5.4±3.0 (3/6)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Table 10. Histatins levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Histastins						
Hist 1	11±26 (9/10)	4.6±7.5 (8/9)	1.1 ±2.0* (5/9)	1.6±1.6 (5/6)	2.2±2.5 (5/6)	4.0±8.3 (4/6)
Hist 2	1.3±1.3 (9/10)	0.5±1.3 (3/9*)	nv (1/9*)	nv (1/6)	0.4±0.6 (3/6)	0.4±0.8 (2/6)
Hist 3	4.1±5.7 (7/10)	nv (1/9*)	nv (1/9*)	nv (1/6)	0.8±1.3 (2/6)	2.4±5.2 (2/6)
Hist 4	0.3±0.3 (10/10)	0.7±2.0 (2/9*)	nv (0/9*)	nv (1/6)	nv (0/6)	nv (0/6)
Hist 5	8.5±8.6 (9/10)	2.8±4.3 (7/9)	0.6±0.9 (4/9*)	0.7±1.1 (3/6)	1.5±1.7 (3/6)	2.6±4.3 (3/6)
Hist 6	2.2±2.3 (9/10)	1.0±1.5 (5/9)	0.2±0.2 (4/9*)	0.3±0.4 (3/6)	0.3±0.5 (3/6)	0.7 ±1.6 (2/6)
Hist 7	0.5±0.6 (10/10)	nv (1/9*)	nv (0/9*)	nv (1/6)	nv (1/6)	nv (0/6)
Hist 8	0.4±0.2 (9/10)	0.3±0.5 (5/9)	0.1±0.1 (2/9*)	0.1±0.1 (3/6)	0.1±0.2 (3/6)	0.03±0.1 (1/6)
Hist 9	0.1±0.2 (7/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
Hist 11	0.08±0.1 (10/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)
Hist 12	0.1±0.1 (9/10)	nv (0/9*)	nv (0/9*)	nv (0/6)	nv (0/6)	nv (0/6)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Table 11. Immune peptides levels/frequency in controls, sSS and pSS before and after pilocarpine

	Controls(10)	sSS(9)	pSS(9) T0	pSS(6) T30'	pSS(6) T60'	pSS(6) T24 h
Lysozime	0.5±0.3 (10/10)	nv (1/9*)	0.3±0.6 (2/9*)	0.6±0.6 (4/6°)	0.6±0.7 (3/6)	0.3±0.4 (2/6)
Defensins						
α-1	1.6±1.5 (9/10)	6.0±9.0 (8/9)	13±14* (9/9)	11±12 (6/6)	13±12 (6/6)	10±12 (6/6)
α- 2	1.3±1.3 (8/10)	4.8±7.8 (8/9)	9.8±9.3 (9/9)	7.5±9.0 (6/6)	8.9±9.3 (6/6)	7.5±9.4 (6/6)
α- 3	0.7±0.9 (8/10)	4.6±9.1 (8/9)	8.5±9.4 (7/9)	5.6±9.8 (5/6)	6.1±9.3 (6/6)	6.0±11 (6/6)
α- 4	0.3±0.4 (9/10)	1.4±2.4 (7/9)	2.6±3.4 (6/9)	2.0±2.7 (4/6)	2.4±2.7 (4/6)	1.4±1.8 (4/6)
β- 1	nv (0/10)	nv (0/9)	nv (0/9)	nv (0/6)	nv (0/6)	nv (0/6)
β- 2	nv (0/10)	0.1±0.4 (3/9)	1.9±4.6 (6/9*)	0.5±0.7 (3/6)	0.4±0.6 (2/6)	0.5±0.5 (3/6)
β-thymosins						
β- 4	0.9±0.7 (8/10)	2.9±4.8 (7/9)	3.0±2.7 (8/9)	2.0±1.7 (5/6)	4.2±5.2 (5/6)	2.1±2.0 (5/6)
β- 10	nv (0/10)	0.2±0.3 (3/9*)	0.1±0.1 (6/9*)	0.7±0.1 (2/6*)	0.1±0.3 (2/6*)	0.2±0.2 (4/6*)

Levels expressed in Extracted Ion Current area x 10^8 and detection frequency in brackets; pSS: primary Sjögren's syndrome patients; sSS: secondary Sjögren's syndrome patients; nv: not valuable; *p<0.05 versus controls; °detection frequency significantly increased; †p<0.05 versus T0; ‡ p<0.05 versus T0 and p>0.05 versus controls

Protein profile after pilocarpine assumption

In the 6 pSS patients who took 5 mg of pilocarpine, 5 proteins after 30 minutes after pilocarpine, 6 after 60 minutes showed a significant increase in detection frequency in pSS patients than in basal specimen; 3 proteins were detectable even after 24 hours from pilocarpine (Tables 6-11 and figure 2).

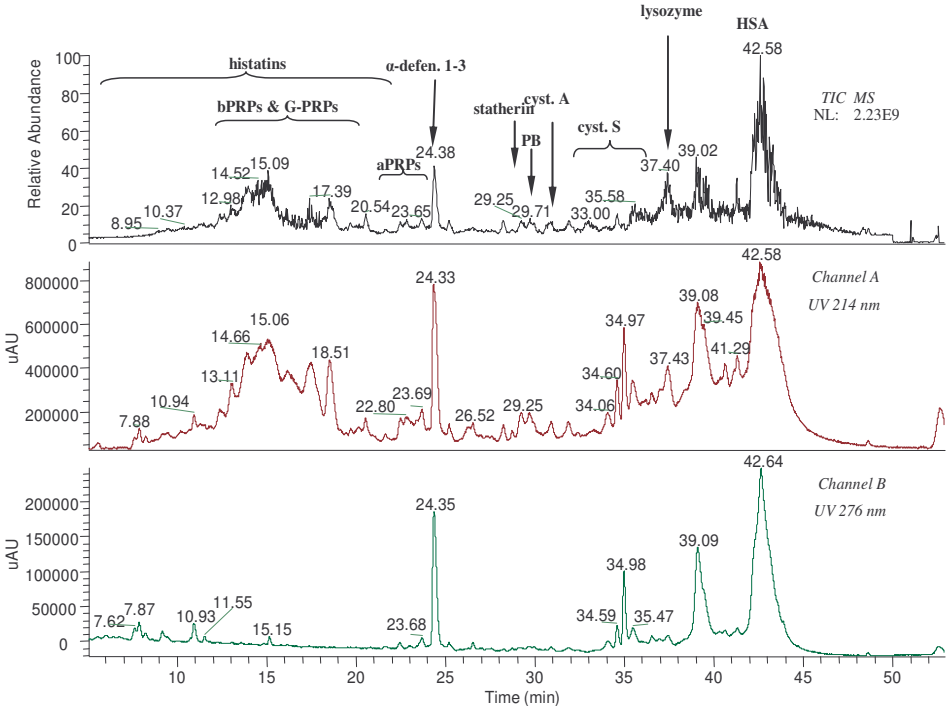
After 30 and 60 minutes from pilocarpine assumption, the levels of 8 proteins increased significantly compared to basal specimens (Tables 6-11); 7 of these proteins were significantly less abundant at baseline than in controls and reached levels comparable to controls: IB-1, II-2, PRP-1 PC and P-B peptide at 30 minutes and PRP-3 and statherin at 60 minutes after treatment with pilocarpine (Tables 6-11 and figure 2).

Then, the secretion of 14 (21.9%) species among the proteins undetectable or with lower frequency of detection in basal pSS saliva seems to be stimulated by pilocarpine. Moreover pilocarpine showed to be able to restore levels similar to normal of the proteins with lower levels at basal specimens.

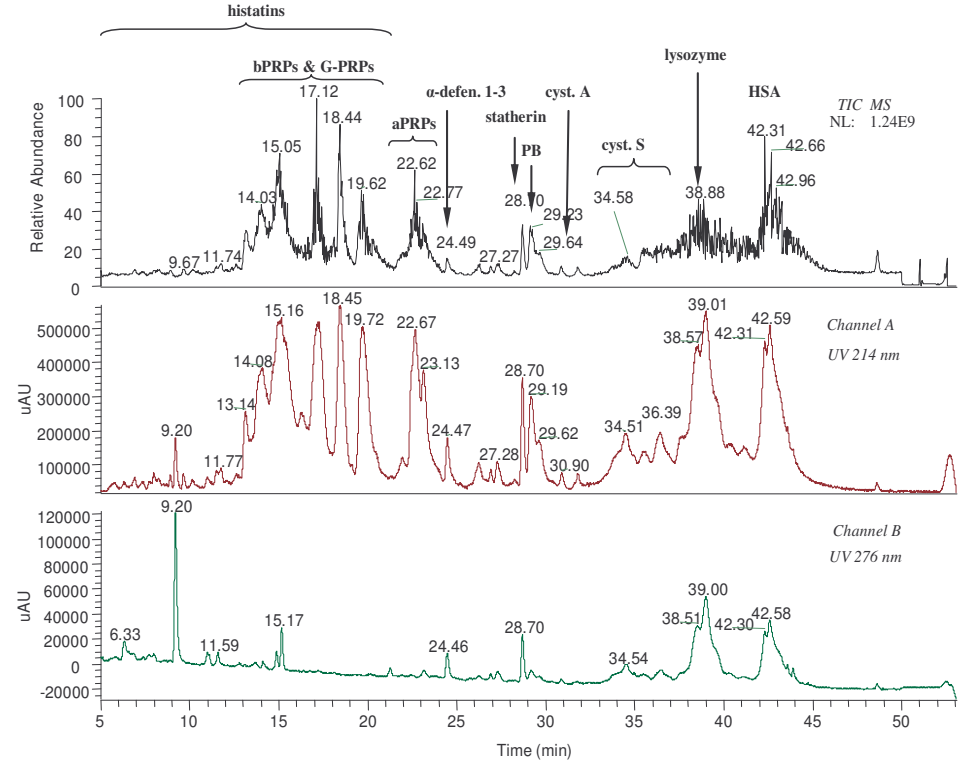
The salivary cystatins proteins were the class of proteins less modified by pilocarpine. In fact the number of patients in which cystatins were detectable was similar before and after the drug somministration (Table 9). Basic and acidic PRPs and statherin group showed the best response to pilocarpine (Tables 6 and 7).

Figure 2

Before pilocarpine



After pilocarpine



Analysis of the frequency and levels of salivary gland proteins in sSS patients vs pSS patients and healthy controls

No significant differences between pSS and sSS patients in protein detection frequency were observed, with the exception of IB-6 which was more frequently detected in sSS patients (Table 6). IB-6 was present in 5/9 (55.6 %) sSS patients and in none of pSS patients. In particular IB-6 was detectable in all sSS patients with RA and in 2 sSS patients with SLE but in none of the sSS patients with SSc. P-B Des$^{1-4}$, which was found in 6/9 (66.7 %) pSS patients, resulted detectable in 3/9 (33.3 %) sSS patients (p=ns) (Table 8).

Only two proteins (PRP-3 mono-Phos, PRP-3) resulted significantly less abundant in pSS than in sSS (Table 7).

In sSS patients 20 (31.3 %) proteins showed a lower detection frequency than in controls (Tables 6-11). IB-6 was significantly more represented in sSS patients (5/9) than in controls (1/10). As in pSS patients, the salivary cystatins were less frequently found in sSS patients than in controls. Instead the acidic PRPs showed a similar frequency in sSS patients than in controls. Only 2 proteins (4.5 % of proteins), statherin and IB-1, resulted significantly less abundant in sSS than in controls (Tables 6 and 8).

Defensins

No differences were found in α-defensins 1-4 detection frequency between pSS, sSS patients and controls (Table 11). However α-defensin-1 amounts was significantly higher in pSS patients than in controls (Table 11). The sSS patients had an intermediate α-defensin-1 amount between pSS patients and controls. After pilocarpine assumption α-defensin-1 levels became similar to controls at 30, 60 minutes and 24 hours (Table 11).

β-defensin-1 was not detectable in patients and in controls. β-defensin-2 was found in 6/9 (66.7 %) of pSS patients, in 3/9 (33.3 %) of sSS patients (2 SSc and 1 SLE) and in none of the controls (Table 11)). The 6 patients having β-defensin-2 detectable, had increased α-defensin-1 levels than the 3 patients without β-defensin-2 detectable (data not shown, p=ns).

Thymosins

TB4 was found in 8 (88.9%) of the patients with pSS, in 7 (77.8%) of the patients with sSS and in 8 (80%) of the normal subjects (Table 11).

TB10 was found in 6 (66.7%) of the patients with pSS, in 3 (33.3%) of the patients with sSS and in none of the normal subjects (Table 11).

DISCUSSION

Recent studies suggest that xerostomia and xerophtalmia in pSS are due to functional inhibition of autonomic neurotransmission to lacrimal and salivary glands, rather than to infiltration and destruction of the glands by lymphocytes, because there is a poor correlation between degree of glandular destruction or focus score and degree of dysfunction (6, 68). A reduction in acetylcholine sensitivity in acinar cells from pSS patients (69) with a consequent up-regulation of M3R (68), the most important cholinergic receptors on salivary gland cells, has been reported. Moreover anti-muscarinic receptor antibodies have been demonstrated in the serum of patients with pSS. These autoantibodies can inhibit parasympathetic neurotransmission (70) and the trafficking to the apical membrane of aquaporin 5, a water-channel protein which levels are increased by pilocarpine (71). Thus the study of pilocarpine effect on salivary gland function could be interesting for the comprehension of the pathogenesis of xerostomia in pSS patients. In fact, since pilocarpine has shown to increase salivary gland fluid secretion, our study found that pilocarpine can also increase the amount and the number of salivary proteins detectable in pSS patients.

More than 50 % of salivary gland proteins analyzed in pSS patients were not detectable or showed lower levels than in healthy controls. About 20% of the proteins less represented in pSS patients reached a frequency similar to controls after 60 minutes from pilocarpine and 3 of these proteins were found in patients saliva even 24 hours later. All but one of the proteins with lower levels than in healthy controls before pilocarpine reached levels comparable to controls, most of them after 30-60 minutes.

These data suggest that pilocarpine could partially overcome the dysfunction in salivary glands affected by SS. Further studies will be necessary in order to confirm this hypothesis and to study the effects of chronic pilocarpine treatment. In fact a recent study showed that the chronic stimulation of membrane-bound M3R by circulating anti-M3R autoantibodies or pilocarpine esposition can result in receptor desensitization (72,73).

The salivary cystatins group (cystatin S, S1, S2, SN) showed to be the less represented class in pSS and sSS patients and showed the worst response to pilocarpine. While cystatin A showed a detection frequency similar to normal subjects, according to its not glandular origin, cystatin C, which levels were found increased in pSS patients parotid saliva in a recent SELDI-TOF-MS based study (6), was not detectable in any pSS or sSS patients. Since salivary cystatins had a principal submandibular/sublingual origin, these data suggest that submandibular and sublingual glands may be less susceptible to pilocarpine effect. Statherin fragments and the most part of acidic and basic PRPs, which showed a lower detection frequency in pSS patients, reached a similar frequency with respect to controls after 60 minutes from pilocarpine treatment. Statherin and all the other species less abundant at basal specimens reached levels not significantly different than healthy controls after pilocarpine somministration.

The basic PRPs are of parotid origin while the acidic PRPs and statherin come from parotid, submandibular and sublingual glands. These data, together with the insufficient response to pilocarpine of the cystatins class, might suggest that parotid glands are the most sensitive to pilocarpine.

P-B des1-4 and IB-6 seem to characterize pSS and sSS patients, respectively. However, larger studies should confirm these data. sSS patients showed a saliva proteins profile intermediate between pSS patients and controls. In fact the salivary cystatins were less frequently found in sSS patients as in pSS patients while the acidic PRPs were similar to controls in frequency.

Although acidic PRPs and statherin role has not been completely understood, it seems related to oral calcium metabolism and to oral bacterial flora. Basic PRPs, together with acidic PRPs and statherin, seem to be involved in the formation of the protein net interacting with buccal epithelium and with dental enamel. Indeed the higher incidence of caries and parodontal diseases in pSS patients could be due to the reduction in those protein classes and may benefit from therapies which can restore a normal protein profile as pilocarpine has partially shown to do.

On the other hand our study showed higher amount of the imunopeptide defensins, which are not of glandular origin. Our study shows for the first time the presence of α-defensins 1-4 in pSS and sSS patients saliva with significantly higher α-defensin-1 amount in pSS patients. The increased amount of α-defensin-1 in pSS patients with respect to healthy subjects could be the result of the periodontal diseases which are common in pSS patients, thus suggesting a possible role of α-defensin-1 as a marker of oral inflammation in pSS patients. The reduction in α-defensins amount after pilocarpine is likely due to the increased fluid secretion with consequent dilution and relative reduction in defensins concentration.

Moreover our study demonstrates that β-defensin-2 is detectable in the majority (66.7 %) of pSS patients and in none of the healthy subjects thus suggesting that β-defensin-2 can be considered an inflammatory marker in pSS patients maybe reflecting high levels of cytokines such as IL 1, IL 6 and TNFα (74,75). Moreover this data is supported by the fact that pSS patients which had β-defensin-2 detectable tended to have increased α-defensin-1 amounts. sSS patients had α-defensin-1 levels intermediate between pSS and controls and only in 33.3 % of cases had β-defensin-2 detectable maybe because the oral involvement is milder in sSS than in pSS patients.

Tβ-4, a G-actin sequestering peptide with anti-inflammatory and repairing properties, has been found detectable in all the patients and normal subjects, while Tβ-10 has been found to be detectable in the majority of pSS patients, in 1/3 of sSS patients and in none of the healthy subjects.

In conclusion pSS patients are characterized by a different frequency and different amounts of salivary gland proteins compared to healthy subjects. Pilocarpine showed to restore the amounts and partially the number of the proteins decreased in pSS patients with the parotid gland proteins having the best response to the drug assumption. High α-defensin-1 amounts and the presence of β-defensin-2 and Tβ-10 in pSS whole saliva could be markers of inflammation in Sjögren's syndrome. Moreover sSS patients showed an intermediate protein profile between pSS and healthy subjects. All these data should be of help when considering the possible analysis of secretagogue function and the composition of salivary substitutes.

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