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Sjögren's Syndrome: The Proteomic Approaches

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1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by epithelial cell destruction and by peri-epithelial B and T lymphocytic infiltration of multiple organ targets, and particularly of the exocrine glands. Salivary and lachrymal glands are emblematically involved, with dry mouth (xerostomia) and dry eyes (xerophtalmia) representing the clinical hallmarks of the disease. Moreover, despite the dominance of T cells in the glandular lesions, B cell activation plays a very prominent role as demonstrated by the presence of serum hypergammaglobulinemia, by the occurrence of a wide spectrum of autoantibodies (i.e., antinuclear antibodies, anti-Ro/SSA and anti-La/SSB antibodies, and Rheumatoid factor) and, in some cases, by the development of B cell lymphomas. High-throughput mass spectrometry approaches coupled with different separation techniques have been applied to several human rheumatic diseases in order to discover biomarkers and therapeutic targets by studying the proteome of biological fluids. We will describe our results obtained up to now on the proteomic analysis of whole saliva, particularly on how to distinguish primary and secondary SS manifestations. Moreover, we will report on the state of the art of proteomic studies of other biological fluids and of parotid gland tissues, focusing on the potentiality of proteomic applications in defining a panel of biomarkers useful in the diagnosis and therapy strategy of SS.

2. Clinical aspects

SS is a chronic inflammatory disease characterised by an autoimmune exocrinopathy of the lachrymal and salivary glands due to lymphocytic infiltrations. SS typically presents as dry eyes (xerophthalmia) and dry mouth (xerostomia). This process can manifest either as the independent phenomenon of primary SS or as a secondary when found in the context of another autoimmune process, most commonly rheumatoid arthritis, systemic lupus erythematosus or systemic sclerosis (Ramos-Casals et al., 2005a; Kassan & Moutsopoulos, 2004). Given the overlap of SS with many other rheumatic disorders, it is sometimes difficult to determine whether a clinical manifestation is a consequence of only SS or is due to one of its overlapping disorders.

2.1 Incidence and causes of Sjögren's syndrome

With a population prevalence ranging from 0.5 to 3%, SS appears to be a rather common disease (Binard et al., 2007). SS can develop at any age, but is most common in elderly people. Onset typically occurs in the fourth to fifth decade of life. It is frequent in women, who account for 9 out of 10 cases. The cause of SS remains unknown, but there is growing scientific support for genetic (inherited) and environmental factors. The presence of activated salivary gland epithelial cells expressing Major Histocompatibility Complex class II molecules and the identification of inherited susceptibility markers suggest that environmental or endogenous antigens trigger a self-perpetuating inflammatory response in susceptible individuals. Viruses are possible candidates for environmental triggers since Sjögren-like syndromes are seen in patients infected with HIV, hepatitis C and HTLV-1.

Damage and/or cell death due to viral infection or other causes may provide triggering antigens to Toll-like receptors in or on dendritic or epithelial cells, which, by recognising pathogen-associated patterns, are activated and begin producing cytokines, chemokines, and adhesion molecules. As T and B lymphocytes migrate into the gland, they themselves become activated by dendritic and epithelial cells, thereafter acting as antigen-presenting cells (Fox, 2005). Expressed antigens include SSA/Ro, SSB/La, alpha-fodrin and beta-fodrin, or cholinergic muscarinic receptors (Gottenberg et al., 2003). Recent studies suggest that the disease process of SS has a neuroendocrine component. Proinflammatory cytokines released by epithelial cells and lymphocytes may impair neural release of acetylcholine. In addition, Bolstad and colleagues (Bolstad et al., 2003) have focused on the role of apoptotic mechanisms in the pathogenesis of primary SS. A defect in Fas-mediated apoptosis, which is necessary for down-regulation of the immune response, can result in a chronic inflammatory destruction of the salivary gland, resembling SS.

2.2 Symptoms of Sjögren's syndrome

Symptoms of SS can involve the glands and /or other organs of the body (extra glandular manifestations). Glandular or exocrine manifestations of SS result from the periepithelial lymphocytic infiltration of the salivary and lacrimal glands. Inflammation of the salivary glands can lead to mouth dryness, swallowing difficulties, dental decay, cavities, gum disease, mouth sores and swelling, and stones and/or infection of the parotid gland. Dry lips often accompany the mouth dryness. Extraglandular problems in SS include joint pain or inflammation, Raynaud's phenomenon, lung inflammation, lymph node enlargement, and kidney, nerve and muscle disease. A rare serious complication of SS is inflammation of the blood vessels (vasculitis), which can damage body tissues supplied by these vessels. A common disease that is occasionally associated with SS is autoimmune thyroiditis (Hashimoto's thyroiditis), while a small percentage of patients with SS develop cancer of the lymph glands (lymphoma).

2.3 Diagnosis of Sjögren's syndrome and classification criteria

At present, the diagnosis of SS is based upon the combination of several clinical, serological, histological, and instrumental elements suggestive of both exocrine gland involvement and of typical laboratory abnormalities (antibodies anti-Ro/SSA and La/SSB). From a practical point of view, the diagnosis can be made according to the "American-European Consensus

Group Revised Classification Criteria, which were published in 2002 (Vitali et al., 2002) and revised in 2010 (Seror et al., 2010). Before their elaboration, there were several different concomitant criteria sets, varying in their emphasis, mostly on laboratory tests, on clinical features of dry eye and dry mouth, or on both. At that time, there was no uniform agreement on the diagnosis of primary SS, with substantial confusion in research publications and clinical-trial reports. The Revised Criteria exhibit approximately 95% sensitivity and specificity for SS, and due to their high specificity and sensitivity, they can be used as diagnostic criteria. They encompass the presence of subjective and objective sicca manifestations, antibodies to Ro/SS-A and La/SS-B antigens, and characteristic histopathologic findings in minor salivary glands with an average of 50 or more lymphocytes (focus) per 4 mmq of minor salivary gland samples. Of the 6 given criteria, 4 must be present to establish a diagnosis of SS, with 1 of the 4 being an objective measurement (i.e., by histopathologic examination or antibody screening) (Vitali et al., 2002). In their present state, the Classification Criteria are insufficient to make a clear diagnosis, and a certain proportion of patients may be misclassified, particularly in the early stages of the disorder, when the typical signs and symptoms are often lacking or are not entirely expressed. On the other hand, early diagnosis is crucial in avoiding destructive processes that frequently lead to a poor quality of life and early invalidity (Gran, 2002). Moreover, there is quite a weak correlation between clinical symptoms and the exocrinopathy measurements, and the assessment of organ involvement is currently limited to general markers of inflammation or organ function and needs profound improvement (Hay et al., 1998). Finally, no specific predictive factors of flares, disease relapses or disease outcomes have been described yet, even if unfavourable predictors have been thoroughly investigated, especially for lymphoproliferative disorders, which are the most serious complication in patients with SS (Gran, 2002; Manganelli et al., 2006; Voulgarelis et al., 1999).

2.4 Extra-glandular Sjögren's syndrome involvement

SS involves primarily the exocrine glands. Extraglandular involvement falls into two general categories. Peri-epithelial infiltrative processes include interstitial nephritis, liver involvement, and bronchiolitis, and generally follow a benign course. Extra-epithelial extra-glandular involvement in SS is related to B-cell hyper-reactivity, hyper-gammaglobulinemia, and immune complex formation, and includes palpable purpura, glomerulonephritis, and peripheral neuropathy. These latter manifestations occur later in the course of SS and are associated with a higher risk of transformation to lymphoma (Tzioufas & Voulgarelis, 2007). The incidence of systemic vasculitis manifestation in SS is approximately 5-10% of patients with SS (Ramos-Casals et al., 2005b). Skin involvement mainly manifests in the form of vasculitis cutaneous purpura. These lesions are clinically identical to those found in patients affected by systemic lupus erythematosus. In addition, Raynaud's phenomenon is a vascular condition with an incidence of 13% in patients with SS (Bayetto & Logan, 2010).

3. Applicability of proteomic to study rheumatic diseases

Proteomic approaches are expanding our ability to determinate changes in protein expression, and the technology used has rapidly evolved over the last decade allowing for more accurate quantitation of the differentially expressed proteins (Vanarsa & Mohan,

2010). In rheumatology, the application of proteomic in the search for potential biomarkers of the disease has produced a high number of reports concerning different diseases such as rheumatoid arthritis, systemic lupus erythematosus, ankylosing spondylitis, osteoarthritis, 333systemic sclerosis, and SS. Depending on the nature of the rheumatic disease, the choice of samples include saliva, serum, synovial fluid, urine, blood cells, cell lines (chondrocytes, synoviocytes, fibroblasts) or tissues (parotid glands, articular tissue, cartilage). Moreover, new applications have been found such as cerebro-spinal fluid in multiple sclerosis, peritoneal dialysate and haemodialysis fluid, broncoalveolar lavage fluid in interstitial lung disease. The aims have been to add new information about the disease pathogenesis and to identify protein biomarkers for non-invasive diagnosis, staging, and monitoring. A list of proteomic studies performed in rheumatic diseases in the last ten years is shown in table 1. Together, these studies underline the potentiality and applicability of proteomic in the study of rheumatic diseases. Unfortunately, there have not been any studies so far that have identified a panel of biomarkers with high specificity and sensitivity able to diagnose and predict rheumatic diseases.

Rheumatic diseases	References	Samples	Proteomic approach
Osteoartritis	De Ceuninck et al., 2005	cartilage	2DE/tandem MS
	Ruiz-Romero et al., 2005	chondrocytes	2DE/MALDI-TOF
	Gobezie et al., 2007	synovial fluid	1DE/electrospray ionization tandem MS (LC-ESI-MS)
	Wu et al., 2007	cartilage	2DE/nano-LC-tandem MS
	Guo et al., 2008	cartilage	2DE/linear ion trap-Fourier transform ion cyclotron resonance mass spectrometry
	Lambrecht et al., 2008	cartilage	2DE/tandem MS
	Rosenthal et al., 2011	cartilage	nano-LC-tandem MS
	de Seny et al., 2011	serum	SELDI-TOF-MS
	Ma et al., 2011	cartilage	2DDIGE/MALDI/TOF/MS
Spondyloarthritis	Tilleman et al., 2005	synovium	2DE/MALDI-TOF-ESI, tandem MS
	Wright et al., 2009	monocytes	2-DE/MALDI-TOF-MS
	Liu et al., 2007	serum	ESI-Q-TOF MS/MS
	Li et al., 2010	serum	2-DE/MALDI-TOF-MS
	Li et al., 2010	peripheral blood mononuclear cells	2-DE/MALDI-TOF-MS
Rheumatoid Arthritis	Sinz et al., 2002	serum, synovial fluid	2DE/MS

Rheumatic diseases	References	Samples	Proteomic approach
	Liao et al., 2004	serum, synovial fluid	LC/MS/MS
	Drynda et al., 2004	serum, synovial fluid	2DE/MS
	de Seny et al., 2005	serum	SELDI-TOF-MS
	Hueber et al., 2005	serum	antigen-microarrays
	Kim et al., 2006	synovial fluid	2DE/MALDI-TOF
	Matsuo et al., 2006	synovium	2DE/MALDI-TOF
	Schulz et al., 2007	peripheral blood mononuclear cells	2DE/MALDI-TOF
	Hueber et al., 2007	serum	antigen- microarrays
	de Seny et al., 2008	serum	SELDI-TOF-MS
	Zheng et al., 2009	plasma	capillary reversed-phase – HPLC/ion trap-FT-MS
	Chang et al., 2009	synovial tissues	2DE/MALDI-TOF
	Bo et al., 2009	synovial fibroblasts	2DE/MALDI-TOF-MS
	Giusti et al., 2010	saliva	2DE/MALDI-TOF-MS
	Li et al., 2010	serum	2DE/MALDI-TOF-MS
	Baillet et al., 2010	synovial fluid	SELDI-TOF-MS
	Matsuo et al., 2011	synoviocytes	phoshoproteomic
Wegener's Granulomatosis	Stone et al., 2005	serum	SELDI-TOF-MS
Systemic Sclerosis	Fietta et al., 2006	bronchoalveolar fluid	2DE/MALDI-TOF LC/MS/MS
	Giusti et al., 2007	saliva	2DE/MALDI-TOF
	Aden et al., 2008	skin	2DE /MALDI-TOF
	Scambi et al., 2010	serum	2DE/MALDI-TOF-MS
+ *Lupus erithematosus systemic*	Carlsson et al., 2011	serum	antibody microarray
Lupus erithematosus systemic	Pavon et al., 2006	plasma	2DE/MALDI-TOF-MS
	Mosley et al., 2006	urine	SELDI-TOF-MS

Rheumatic diseases	References	Samples	Proteomic approach
	Zhang et al., 2008	urine	SELDI-TOF-MS
	Dai et al., 2008	peripheral blood mononuclear cells	2DE/MALDI-TOF-MS
	Dai et al., 2010	serum	magnetic beads-based weak cation exchange chromatography/ MALDI-TOF-MS
	Lood et al., 2010	platelets	antigen- microarrays
	Wang et al., 2010	peripheral blood mononuclear cells	Isobaric tagging for relative and absolute protein quantification (iTRAQ)-multiple chromatographic fractionation and tandem mass spectrometry
Sjogren'Syndrome	Tomosugi et al., 2005	tears	SELDI-TOF-MS
	Ryu et al., 2006	parotid saliva	SELDI-TOF-MS/2D-DIGE
	Giusti et al., 2007	whole saliva	2DE/MALDI-TOF
	Stea et al., 2007	parotid glands	SDS-PAGE
	Peluso et al., 2007	whole saliva	HPLC-ESI/MS
	Hu et al., 2007b	whole saliva	2DE/MALDI-TOF-MS/LC-MS/MS
	Fleissig et al., 2009	whole saliva	2DE/MALDI-TOF-MS
	Hjelmervik et al., 2009	minor salivary glands	LC-ESI/MS-MS; 2DE/MALDI-TOF-MS
	Hu et al.2011	whole Saliva	protein microarrays
Fybromialgia	Bazzichi et al., 2009	whole saliva	2DE/MALDI-TOF
+ *Chronic Fatigue Syndrome*	Baraniuk et al., 2005	cerebrospinal fluid	capillary chromatography, quadrupole-time-of-flight mass spectrometry

Table 1. Proteomics in Rheumatology

4. Proteomic and Sjogren's syndrome

The majority of proteomic studies concerning SS chose saliva as the biological fluid (6 papers), and only a limited number used tears (1 paper) or salivary gland tissue (3 paper).

4.1 Tears

Histological and functional changes of the lachrymal gland might be reflected in proteomic patterns in tear fluids. In SS, a reduced production of aqueous tear was clarified when examined by the Schirmer test. Reduction of tear film stability as shown by the tear film break-up time test seems to be responsible for a disturbance of the quality of the mucus layer composition. However, there was no screening test for the changes in quality of tear components, which should accurately reflect the physiologic state of the lachrymal gland and the level of its function. The first proteomic trial for carrying out a determination of the disease biomarkers in tear fluid for SS was performed by Tomosugi and co-workers (Tomosugi et al., 2005). The authors, using surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry, identified 10 potential novel proteins that differed between SS patients and control subjects. Seven were down regulated, and three correlated significantly with SS scores and epithelial damage of the ocular surface. Although these investigators have not yet identified the proteins, this study clearly demonstrates how such techniques can be applied in identifying specific protein profiles involved in the pathophysiological processes associated with SS.

4.2 Parotid glands tissue

Proteomic analysis has been applied not only to the study of salivary and lachrymal fluids, but also to the study of gland tissues because SS directly affects the glands and because autoantibodies characterising SS (anti-Ro/SSA and anti-La/SSB) are produced mainly in these affected tissues. Parotid gland extracts of SS patients were then analysed by combining conventional immunological methods (2DE and immunoblot) with mass spectrometry in order to evaluate modifications of known autoantigens (i.e., La/SSB), and in order to determine other targets of the autoimmune response in the parotid glands of SS patients. In the work by Stea and co-workers (Stea et al., 2007), in order to identify the isoforms of La/SSB in parotid glands of SS patients, an immunoblot with purified anti-La antibodies was performed after 2DE of parotid gland extracts from two SS patients. An extract from a human salivary gland epithelial cell line and a parotid gland extract from a patient with mixed parotid tumour were used as controls. The results of the study revealed that SS salivary glands contained high levels of post-translationally modified La/SSB autoantigen, degraded from 48 kDa to 34 kDa. The 48 kDa form of the protein was faintly recognised, in contrast to normal controls. Moreover, only five distinct La/SSB isoforms were detected in SS patients' specimens, in contrast to seven isoforms in controls. Finally, a new potential autoantigen was identified in the parotid glands of SS. A protein at around 45 kDa was recognised as a target of autoantibodies by the SS sera. This protein was identified as human actin by combining conventional immunological methods and mass spectrometry. Moreover, Hjelmervik and colleagues (Hjelmervik et al., 2009) conducted a large-scale mapping of the minor salivary gland proteome, applying two complementary methods: the LC-ESI-MS/MS and 2DE. The main objective of their work was to achieve a large-scale delineation of the minor salivary gland proteome in samples from both SS patients and non-SS controls. Heat shock proteins, mucins, carbonic anhydrases, enolase, vimentin, and cyclophillin B were among the proteins identified. Six proteins were exclusively identified in SS patients with respect the controls in particular alpha defensin 1 and calmodulin. A

system biology approach has been used by Hu and co-workers (Hu et al., 2009) to study parotid gland tissue samples obtained from patients with primary SS, from patients with SS /MALT lymphoma, and from subjects without primary SS. The tissue samples were assessed by gene-expression microarray profiling and proteomics analysis. The authors defined a panel of 8 candidate genes for distinguishing primary SS/MALT lymphoma from primary SS. Among the 115 proteins showing >3-fold elevated levels, 20 proteins were up-regulated in primary SS parotid gland tissue samples as compared with non– primary SS control and primary SS/MALT lymphoma parotid gland tissue samples. Twenty-five proteins were up regulated in both primary SS and primary SS/MALT lymphoma samples as compared with non–primary SS control samples, and 70 proteins were up-regulated in primary SS/MALT lymphoma samples as compared with both non–primary SS control and primary SS samples. From a functional point of view, the proteins overexpressed in SS were related to the immune/defence response, apoptosis, cell-cell adhesion, and anti-oxidative stress, whereas many of the proteins with high expression in primary SS/MALT lymphoma were related to signal transduction, gene regulation, apoptosis, the immune response, and oxidative stress.

4.3 Saliva

Human saliva contains a large number of proteins and peptides, which have several important biological functions and potentially reflect both oral and systemic health conditions. Compared to blood, saliva possesses a smaller amount of proteins with a minor risk of non-specific interference. Saliva is an attractive medium for proteomic analysis for many different reasons. One of its major advantages is that salivary fluid can be obtained by using a non-invasive, simple, safe, and stress-free procedure that can be applied to large groups of subjects. The simple nature of saliva collection allows for repetition and multiple collection of saliva useful in early diagnosis, monitoring disease progression or treatment responses. Finally, this fluid undoubtedly reflects the salivary gland involvement that characterises SS disease (primary and secondary), which directly involves the oral cavity (Streckfus et al., 2007; Hu et al., 2007a).

Six studies have been performed in SS, and they are quite different in their principal goals as well as in their general methodologies (Ryu et al., 2006; Giusti et al., 2007; Hu et al., 2007b; Peluso et al., 2007; Flesseig et al., 2009; Hu et al., 2011). Whole saliva or individual glands saliva have been examined, and samples were collected both in stimulated and unstimulated conditions. Moreover, differences were present in salivary protein preparation and separation. However, although the collection protocol was different, many common biomarkers for SS have been found from the five different papers such as actin, Ig gamma-1 chain C region, beta-2 microglobulin, salivary amylase, carbonic anhydrase VI, prolactin inducible protein, calgranulins A and B, and fatty acid binding protein. Table 2 reports the proteomic studies performed up to now in saliva, distinguishing the source of this biofluid, and the type of proteomic approach. A list of potential biomarkers defined by these studies is also shown.

First, we will report the results obtained in other studies, and then in the following paragraph we will discuss our findings.

Study	Samples/Patients	Methods	Proteins differentially expressed
Ryu et al., 2006	stimulated parotid saliva/ primary Sjogren's syndrome	SELDI-TOF-MS/2D-DIGE	β2-microglobulin, lactoferrin, Ig light chain, l, polymeric Ig receptor (PIGR), lysozyme C, cystatin C, proline-rich proteins (PRPs), α-amylase, carbonic anhydrase VI
Giusti et al., 2007	unstimulated whole saliva primary Sjogren's syndrome	2DE/MALDI-TOF	carbonic anhydrase VI, cystatin S, cystatin C, cystatin D, calgranulin B, cyclophillin A, lipocalin-1, phosphatidylethanolammine-binding protein (PEPB), IgkC protein, zinc-α-glycoprotein, fatty acid binding protein (FABP), ACTB, β-actin fragment, leukocyte elastase inhibitor, glutathione-S-transferase, α-amylase precursor, cystatin SN precursor, keratin 6L, prolactine-inducible protein precursor
Peluso et al., 2007	unstimulated whole saliva primary and secondary Sjogren's syndrome	HPLC-ESI-MS	acidic and basic proline-rich proteins (PRPs), statherins, histatins, and cystatins, α-defensin 1, β-defensin 2, statherins
Hu et al.,2007b	Stimulated whole saliva/ parotid, submandibular, sublingual saliva primary Sjogren's syndrome	2DE/MALDI-TOF-MS/LC-MS-MS	carbonic anhydrase VI, polymeric immunoglobulin receptor, lysozime C, prolactin inducible protein, Von Ebner's gland protein, cystatin C, cystatin SN, cystatin D, cystatin S, cystatin SA, calgranulin A, calgranulin B, psoriasin, hemoglobin β-chain, hemoglobin α-1-globin chain, fatty acid binding protein epidermal, Ig-γ-1 chain C, Ig μ chain C region (IGHM), α-enolase, salivary α-amylase, fructose-biphosphate aldolase A, carbonic anhydrase I, carbonic anhydrase II, caspase 14, β2-microglobulin, actin, serum albumin
Fleissig et al.,2009	unstimulated whole saliva primary and secondary Sjogren's syndrome	2DE/ESI-MS-MS	calgranulin B, calgranulin A, Ig-γ-1 chain C, β-actin, serum albumin, keratine type I cytoskeletal, α-actin-1, α-amylase, vitamin D, polymeric-immunoglobulin receptor

Study	Samples/Patients	Methods	Proteins differentially expressed
Hu S et al., 2011	unstimulated whole saliva primary Sjogren's syndrome	protein microarrays	24 saliva autoantibodies: Bcl2 modifying factor, cardiolipin, chromosome X orf56, hypothetical protein DKFZp761G2113, Jun dimerization protein p21SNFT, La/SS-B, Lectin galactoside binding soluble 3, Lectin galactoside binding soluble 7, Megacaryocite-associated tyrosine Kinase, melanoma antigen family B 4, mesenchyme homeobox 1, NEFA-interacting nuclear protein, olfactory receptor family 6 N2, outer dense fiber of sperm tails 2, plasma membrane proteolipid, protein kinase C, ribosomal protein S6 kinase, Ro52/SS-A, SERPINA 3, small inducible cytokine subfamily E1, testis specific 10, TAO kinase 3, transglutaminase, Unfrac whole histone

Table 2. Saliva and Proteomic studies of Sjogren's syndrome.

The pilot study of proteomic applied to SS saliva was performed in 2006 by Ryu and co-workers. Using SELDI-TOF and 2D difference gel electrophoresis (2D-DIGE), they analysed stimulated parotid saliva from five healthy volunteers, 41 primary SS patients, and 20 non-SS subjects, including 15 non-SS subjects with complaints of xerostomia who, nonetheless, did not meet the diagnostic criteria for SS. Combining these two approaches, the authors focused their attention on ten differentially expressed proteins and, in particular, they identified significant increases of β-2 microglobulin, lactoferrin, Ig κ-light chain, polymeric Ig receptor, lysozyme C, and cystatin C. They also found in the patient group a reduction of amylase, carbonic anhydrase VI and of two presumed proline-rich proteins. Moreover, they found no association between the focus score and any biomarker. Lactoferrin and β2-microglobulin showed the greatest increases, but because their levels have been reported to increase also in other inflammatory diseases affecting salivary glands, the authors related these proteins to aspecific salivary gland inflammatory activity. More intriguingly, the increased levels of Ig κ-light chains were explained by the authors as being related to the increase in the intra-glandular immunoglobulin synthesis of the disease. Finally, the decrease of the two proline-rich proteins and α-amylase were ascribed to acinar parenchymal damage, while the reduction in carbonic anhydrase VI was reported as in line with a recent report on its decreased gene expression in SS minor gland biopsies.

Next, a profile of potential salivary proteomic and genomic biomarkers for SS was depicted by Hu and co-workers (Hu et al., 2007b). Sixteen WS proteins were found to be down-regulated, and 25 WS proteins were found to be up-regulated in SS patients compared with matched healthy control subjects. Moreover, using gene chip followed by real time PCR

analysis of whole saliva, Hu and co-workers revealed factors such as interferon (IFN) and IFN-inducible protein G1P2 specifically expressed in SS patients. One of the important findings of this study was that many up-regulated genes were involved in the IFN pathway, suggesting the involvement of viral infection in SS pathogenesis.

Peluso and colleagues (Peluso et al., 2007) analysed the differences in the salivary protein profiles of primary SS and secondary SS patients, and of control subjects using HPLC-ESI-MS. The authors collected whole saliva specimens from 9 primary SS patients, 9 secondary SS patients (3 Rheumatoid Arthritis-2° SS; 3 systemic sclerosis-2° SS; 3 systemic lupus erythematosus-2° SS), and 10 healthy controls, and they analysed the levels and frequencies of 62 proteins. The analysis focused mainly on low molecular weight proteins represented by acid and basic proline-rich proteins, statherins, histatins, cystatins, lysozyme, and defensins. In the second part of the study, the authors examined the effect of pilocarpine on the salivary peptide and protein profiles in a subgroup of 6 primary SS patients. They found that the basic and acid proline-rich proteins and the statherins had the best response to the pilocarpine treatment, while the salivary cystatin and histatin protein classes were modified less. In the comparison between primary and secondary SS salivary profiles, the researchers outlined that patients with secondary SS showed a protein profile that was intermediate between that of the primary SS patients and the healthy subjects. In particular, salivary cystatins (C, S, S2, SA, and SN) and histatins (2, 3, 4, 7, 9, 11 and 12) were less frequently identifiable in primary and secondary SS patients versus controls. On the other hand, 3 proteins (IB-6, P-B Des1-4, and α-defensin 2) were identifiable in a significantly higher percentage of secondary SS patients than in the controls. In particular, α-defensin 2 was found in 6 of the primary SS patients, in 3 of the 9 secondary SS patients, and in none of the controls. Finally, IB-1 and statherin showed significantly lower levels in secondary SS than in the controls.

Additional information was reported by Flesseig and colleagues (Flesseig et al., 2009), who in a preliminary individual saliva sample analysis showed that SS patients (six SS patients as well as one symptomatic subject not fulfilling the criteria completely, and one who had developed follicular lymphoma) exhibited two patterns of protein expression with an indirect relation to the clinical serological or histological severity of disease.

Recently, Hu and co-workers (Hu et al., 2011) have demonstrated the potential of the high-throughput protein microarray approach in the discovery of autoantibody biomarkers for the non-invasive diagnosis of SS. Saliva autoantibodies present in patients with SS or systemic lupus erythematosus and healthy control subjects were profiled with protein microarrays. After comparison with controls (systemic lupus erythematosus and healthy subjects), statistical analysis of the microarray data revealed 24 autoantibody biomarkers that could differentiate SS from both control groups. A validation of four of these autoantibodies (anti-SSA, anti-SSB, anti-transglutamine, anti-histone) was performed using commercial ELISA kits. Although these are known autoantibodies in SS, they were usually tested in serum samples. The authors suggest that testing these autoantibodies in saliva may be valuable for the diagnosis of SS. Therefore, up to now a wide spectrum of proteins has been identified that might include both "true" disease biomarkers, as well as specific markers of tissue damage (i.e., actin) or inflammation (calgranulins). Therefore, we can hypothesise that further studies might shed some light on this aspect.

4.3.1 Our results

In 2005, we began to study rheumatic diseases using a proteomic approach. In our studies, whole saliva was chosen as the biological fluid to discover specific disease biomarkers for primary and secondary manifestations of SS and also of other correlated rheumatic diseases such as systemic sclerosis (Giusti et al., 2007; Baldini et al., 2008), fybromialgia (Bazzichi et al., 2009) and rheumatoid arthritis (Giusti et al., 2010). In our work on SS, we analysed the whole saliva of 12 primary SS patients in comparison with 12 healthy controls by using quantitative 2DE experiments combined with MALDI-TOF-MS for protein identification (Giusti et al., 2007). In particular, in this study, by comparing the SS with control classes, we found that 4 proteins were unique to the control samples (carbonic anhydrase VI, cystatins S, C precursor and cystatin D), and 6 proteins were unique to the SS samples (calgranulin B, cyclophilin A, lipocalin-1 precursor, phosphatidyl ethanolammine binding protein, Ig kappa chain C region (IGKC), protein and Zinc-α-2 glycoprotein precursor). Moreover, in evaluating the mean ± SD of the percentage of the volume of each single protein of the analytical (not synthetic) gels, the authors also discovered that 10 protein spots were up-regulated with > 2-fold changes (fatty acid binding protein (E-FABP), ACTB protein, α-actin fragment, leukocyte elastase inhibitor, glutathione-S-transferase (GST), and 5 unidentified proteins). On the other hand, 4 were down regulated (α-amylase precursor, cystatin SN precursor, keratin 6L, prolactin-inducible protein precursor) in SS patients compared with controls. These results confirmed the decrease of some of the typical acinar proteins and the increase of many inflammatory proteins. Moreover, they outlined the relevance of proteins not previously described i.e., PIP, keratin 6L, and lipocalin, as markers of acinar damage and oral environment alteration. This study was the basis for further investigations aimed at characterising possible differences in salivary protein profiles in patients who have connective tissue diseases associated with secondary SS. Therefore, we extended the study to refine the diagnostic power of a panel of candidate salivary biomarkers described in SS with respect to both healthy volunteers and pathological controls (sicca syndrome). Moreover, the aim of the study was also to explore the biological and pathogenetic functions of the putative salivary proteomic biomarkers, both in the local exocrinopathy and in the systemic inflammatory autoimmune systemic processes of SS. Our preliminary results, to be published, suggest that novel, non-invasively-collected salivary proteomic biomarkers might be helpful in an early and accurate characterisation of primary and secondary SS. In addition, some of the secondary SS identified biomarkers apparently reflected not only the SS component, but also the concomitant systemic autoimmune disorders, shedding new light on the potential diagnostic role of saliva in autoimmune diseases irrespectively of salivary gland involvement.

The capacity of whole saliva to reflect systemic conditions was also suggested from the preliminary unpublished results of a case study on the salivary proteome of non-Hodgkin' lymphomas. We observed that clinical and functional changes of the salivary glands driven by autoimmune and lymphoproliferative processes might be reflected in patients' whole saliva proteins, and that there was a specific correspondence between clinical improvement and proteomic changes of the salivary peptide complex. These observations indicate the potential usefulness of proteomic analysis in discovering not only diagnostic but also prognostic and therapeutic biomarkers for patients with primary SS and non-Hodgkin's

lymphomas. Therefore, we speculate that during the follow-up of patients with lymphomas, proteomic analysis might be able to use the salivary biomarkers as early predictors of treatment response. From the perspective of the research, the analysis of biomarker signatures in saliva could also help to clarify the pathogenetic pathways underlying lymphoproliferation in SS, leading to the development of new methods in early diagnosis and curative therapies.

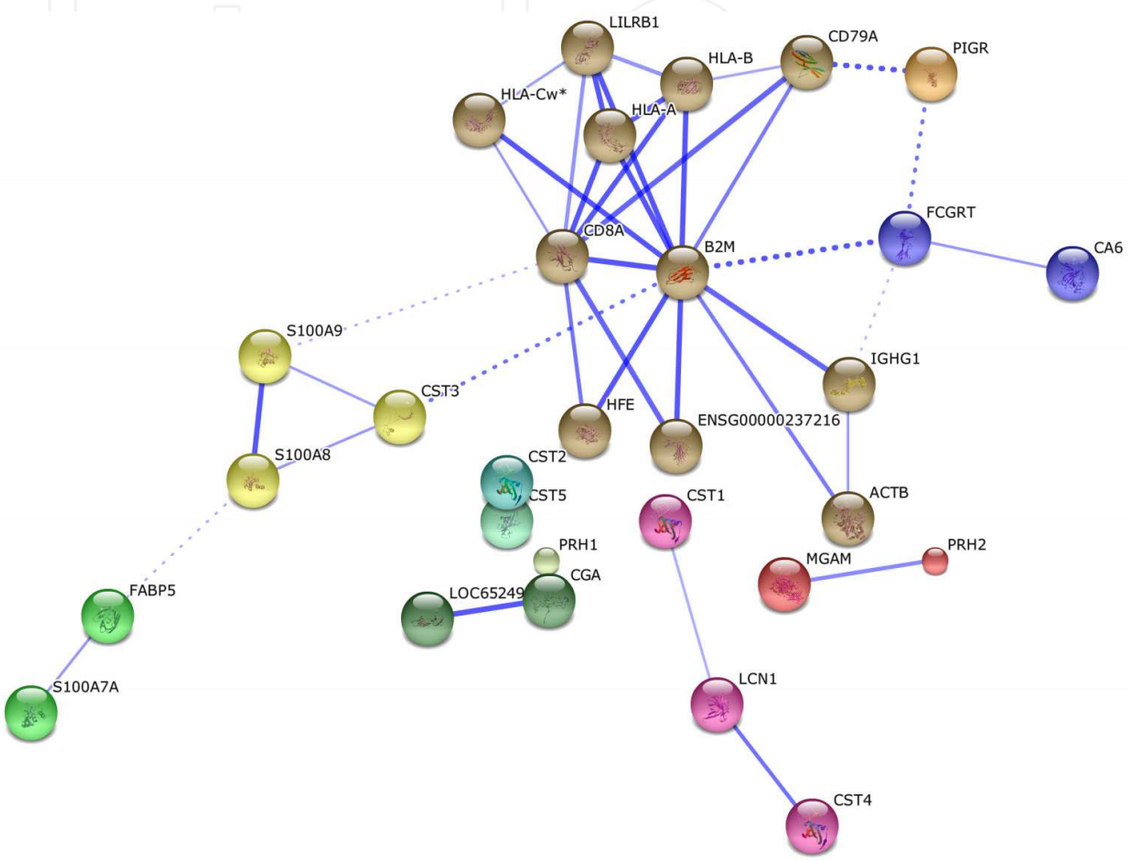

Fig. 1. Protein-protein interaction network of regulated pSS-associated proteins identified in at least two proteomic studies found differentially expressed in saliva. The STRING tool (http://string-db.org/) was used making the network with the following proteins: β-2-microglobulin, polymeric Ig receptor (PIGR), salivary acidic proline-rich phosphoprotein 1 (PRH1) and 2 (PRH2), α-amylase, carbonic anhydrase VI, Cystatin SA (CST2), Cystatin SN (CST1), Cystatin C (CST3), Cystatin S (CST4), Cystatin D (CST5), LCN1, Calgranulin A (S100A8), Calgranulin B (S100A9), Psoriasin (S100A7), Fatty acid-binding protein, epidermal (FABP5), IGKC (LOC652493), Ig γ-1 chain C region (IGHG1), β-Actin (ACTB), Fc fragment of IgG, receptor, transporter (FCGRT), leukocyte immunoglobulin-like receptor (LILRB1), HLA class I histocompatibility antigen, alpha chain G (ENSG00000237216) , major histocompatibility complex class IA (HLA-A) major histocompatibility complex class IB (HLA-B) and glycoprotein hormones, alpha polypeptide (CGA). In the figure is shown the potential interaction with additional proteins with score values ranging from 0.993 to 0.999. The different clusters are indicated by the same colour. The thickness of the connecting lines indicates the level of confidence.

5. Conclusions

SS lacks any true diagnostic criteria for primary and secondary manifestations as well as a set of activity criteria. The risk of misdiagnosis is still quite high, and it highlights the need for a more definitive set of tests and criteria to classify these patients. One possible solution is the proteomic approach, which might represent a promising tool to explore biomarkers for diagnostic aims. Until now, the studies performed have been carried out on parotid biopsies, tears, and saliva. Saliva represents an attractive medium for proteomic analysis because its composition is not complex, and it reflects more accurately the current state of the organism at any moment. Moreover, it presents many logistical advantages because the collection is not invasive, and may be repeated for monitoring over time. However, the identification of true biomarkers of primary and secondary SS is still in its infancy. The results obtained from different studies have not yet defined a conclusive panel of biomarkers useful in diagnostic purposes. Nonetheless, some conclusions can be drawn: SS patients showed a decrease of proteins of glandular origins and an increase of inflammatory proteins, while the salivary profile of secondary SS is intermediate between that of primary SS patients and healthy subjects. Figure 1 shows a representative interactive network obtained by STRING analysis among the proteins found differentially expressed in the proteomic studies performed in saliva. In addition to identifying proteins, we enlarged the network to obtain a large interactive network with more nodes. Interestingly, the figure shows that β-2 microglobulin, which is the invariant chain of the Major Histocompatibility Complex class I molecules, considered as a marker of B cell activation, is the key node of the main cluster.

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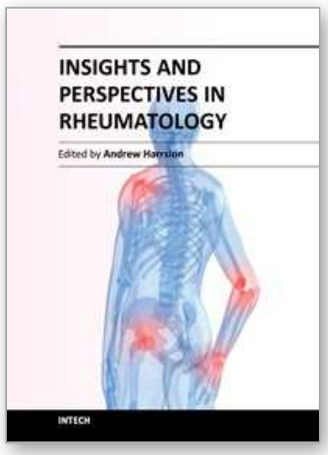

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This book offers a range of perspectives on pathogenesis, clinical features and treatment of different rheumatic diseases, with a particular focus on some of the interesting aspects of Sjögren's syndrome. It contains detailed and thorough reviews by international experts, with a diverse range of academic backgrounds. It will also serve as a useful source of information for anyone with a passive interest in rheumatology, from the genetic and molecular level, through to the psychological impact of pain and disability.

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