Salivary extracellular vesicles *versus* whole saliva: new perspectives for the identification of proteomic biomarkers in Sjögren's syndrome

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ABSTRACT

In the era of personalised medicine new biomarkers are required to early diagnose Sjögren's syndrome (SS), to define different disease subsets and to direct patients' clinical management and therapeutic intervention. In the last few years, several efforts have evaluated saliva proteome to detect and monitor primary SS. Although clinically valuable, these studies presented some limitations that have partially prevented the use of salivary biomarkers in clinical practice. Nowadays, proteomic of extracellular vesicle (EV) represents an emerging and promising field in the discovery of -omic biomarkers for pSS. EV is a relatively new term that includes exosomes, microvesicles and apoptotic body. EVs are packed with proteins, growth factors, cytokines, bioactive lipids, but also nucleic acids and in particular: mRNA, microRNA, long non-coding RNA, tRNA and rRNA. Therefore, they may represent a useful source for diagnostic, prognostic and therapeutic biomarkers in several conditions. In this review we will specifically focus on EV proteomics as a tool for the identification of novel biomarkers for pSS. In the first part we focused on the state of the art of the studies on proteomics in SS existing in the literature. In the second part we provided a definition of EV with an update on biological sample collection and processing for EV proteomic studies. Finally, we summarised the state of the art of EV -omics in SS highlighting the potential advantages of this novel approach compared to the overall traditional concept of analysing the proteome of blood or saliva.

Introduction

Primary Sjögren's syndrome (pSS) is a complex autoimmune disease characterised by a progressive hypo-function of the salivary and lachrymal glands, frequently associated to a variety of extra-glandular manifestations, including lymphoproliferative disorders (1-4). Despite the progress achieved, several unmet needs are still present in the diagnosis, phenotype stratification and therapy of the disease (5-8). The urgent need for biomarker development in pSS has been promoted by several factors (9, 10). First, pSS patients despite apparently similar in their clinical presentation, may have a different long-term outcome and conventional biomarkers are generally not sufficiently predictive of clinical outcome (2, 3, 6, 11-13). Second, novel biological agents are in the pipeline for pSS and new biological drivers are crucial for their appropriate utilisation as no single therapeutic intervention is suitable for each individual patient (12, 14, 15). Currently, huge efforts are ongoing to identify novel biomarkers able to improve SS patients profiling and daily management (9, 16). A number of studies have analysed the proteome of saliva, tears and blood highlighting qualitative and quantitative differences between SS patients and healthy controls (17-23). However, some important pitfalls came out in the attempt of translating these preliminary results into clinical practice. Among the others, the inter-subject variability in biological fluid composition and the presence of high abundant proteins that may unmask pivotal but less represented proteins. In this scenario the possibility of exploring better preserved extracellular vesicles (EVs) has represented a possible answer in the search for disease specific biomarkers. Indeed, EVs have various biological functions involved in different processes such as inflammation, immune signalling, an-

giogenesis and tissue repair and their involvement in pathogenesis of autoimmune diseases represents a new area of research.

In this review, therefore we outlined the state of the art of proteomic studies on whole saliva and on EV in SS. In the first part we will summarise the state of the art of salivary proteomics in pSS. Then we will move to EV definition highlighting some practical aspects on how to collect, process and generally perform proteomic studies on EV. Finally, in the second part we will specifically discuss the recent literature on EV proteomics in pSS outlining the potential advantages of this emerging approach over the existing literature.

Salivary proteomics in pSS: state of the art

The concept of oral diagnostics has recently become increasingly important since saliva has appeared as a promising source for biomarkers not only for oral disorders but also for distal tissues and organs (24-30). Saliva has several advantages over blood sampling considering that it could be collected non-invasively, easily and less expensively and is less complex than blood in its composition. A further advantage in pSS diagnostics is that saliva may mirror directly salivary glands inflammation and damage, thus reflecting the involvement of pSS target organs.

To date, a number of proteomic studies have been conducted in whole saliva of pSS patients mostly in comparison with healthy volunteers or subjects with non-immune mediated sicca symptoms; despite heterogeneous these studies have described a distinctive profile for pSS salivary biomarkers thus suggesting that pSS-related tissue damage, inflammation and immune response may be reflected in saliva. (20-22, 31-40) Some of the most significant studies in pSS have been summarised in Table I. Overall, the vast majority of the data have highlighted that pSS salivary proteome is characterised by a decreased expression of acinary proteins physiologically involved in oral mucosa healing and protection, lubrification, digestion, sense of taste and dental mineralisation. By contrast, a number of inflammatory proteins including S-100 proteins have been described as over-expressed as well as immune-related molecules (*i.e.* immunoglobulins, beta-2 microglobulin, IL-1 family proteins). Some of the above mentioned proteins have been also correlated with salivary flow and minor salivary focus score in order to verify whether these proteins may be utilised in patient stratification and in monitoring response to therapy (21, 35, 38-42). From this perspective, Cystatin S, Gross cystic disease fluid protein-15(GCDFP-15)/prolactin-inducible protein (PIP) and Mucin 7 have been particularly associated with pSS-related salivary flow impairment (39, 41, 43). Moreover, according to Delaleu *et al.* (38), IL1 system was a dominant pro-inflammatory component associated with hyposalivation whereas, on the other hand, pregnancy-associated plasma protein A, thrombospondin 1 and peptide YY might recapitulate the presence or absence of tertiary lymphoid organisation for almost 94% of pSS patients. Finally, in a patient with pSS and MALT parotid lymphoma, investigating the correspondence between proteomic results, salivary proteomic profile and clinical response, we observed several qualitative and quantitative modifications in the salivary expression of a number of proteins, including immunoglobulin J chain, Ig kappa chain C region, alpha-1-antitrypsin, haptoglobin and Ig alpha-1 chain C region, thus suggesting that salivary proteomics may be also applied to the study of lymphoproliferation in pSS.

Despite these results, working with whole saliva is not free from potential pitfalls. An important point to consider is represented by the inter- and intra-subject variability in salivary flow rate and composition in relation to time/modality of sampling, subjects' food intake, aging and comorbidities. Other aspects we have to take into account include contamination, and possible sample inadequacy in patients with severe xerostomia. Finally, another, crucial issue is the presence of high abundance proteins (*i.e.* mucins, albumin, immunoglobulins) that might preclude the identification of putative low-abundant biomarkers. Deutsch *et al.* (44) have shown that a triple depletion of amylase, albumin, and IgG followed by a demethylation MS analysis allowed the quantification of at least 40 proteins that were not detectable with other proteomics approaches. Similarly, Cecchettini *et al.* used a nano-HPLC-SWATH-MS approach for the analysis of saliva proteome of different pSS subsets preceded by immunoaffinity chromatography to remove albumin and IgG and found 203 differentially expressed proteins in pSS patients with respect to controls.

Indeed, salivary proteomics has provided a major contribution to the identification of novel pSS biomarkers. However, in the next future there will be the crucial need of promoting advanced developments in omics techniques in order to provide novel possibilities for salivomics

At the same time, the work hypothesis of implementing proteomic analysis of extracellular vescicles in saliva seems particularly interesting and might probably help to identify more preserved protein biomarkers.

Extracellular vesicles: a promising source for proteomic biomarkers

Cell communication is of pivotal importance to control metabolic processes, to regulate growth and differentiation, to coordinate and integrate cell and tissue functioning and also to accelerate pathology changes and progression. It is exerted by several mechanisms such as direct contact or secretion of soluble substances. It is in the last decade that researchers and clinicians focused their attention on the release of extracellular vesicles (EVs) and the interest of the scientific community on this topic is demonstrated by the huge amount of articles published in PubMed (in 2018 the search of *extracellular vesicles* led to around 15,000 results). All cells produce and discharge membrane vesicles that are not simply casually dispersed "dust", as they were defined when they were described for the first time (45), but indeed they act as well-organised "post office", delivering complex chemical messages over long distances (46). EVs are packed with proteins, growth factors, cytokines (47), bioactive lipids (48), but also nucleic acids and in par-

Table I.

Authors	Methods	Proteins OVER-expressed	Proteins DOWN-expressed
Ryu (2006) (31)	SELDI-TOF-MS/ 2D-DIGE	Beta-2 microglobulin; Lactoferrin; Ig k light chain; Polymeric Ig Rec; lysozyme C; cystatin C	Proline-rich proteins; salivary alpha-amylase; carbonic anhydrase VI
Giusti (2007) (32)	2DE/MALDI-TOF-MS	Actin; FABP-E; Leukocyte elastase inhibitor; GST; Calgranulin B; Cyclophilin A; Lipocalin-1; PEBP; IGC protein; Zn alpha2 glycoprotein	Salivary alpha-amylase; Cystatin SN; Keratin 6L; GCDFP-15/PIP; Cystatin S; Cystatin D; Cystatin C; Carbonic anhydrase VI
HU (2007) (33)	2DE/LC-MS/MS	Calgranulin B, Psoriasin, FABP-E, IGHG protein; Ig gamma 1-chain-C region; IGHM protein; alpha enolase; Fragment amylase	Carbonic anhydrase VI; Polymeric IgR; Lysozime C; PIP, Von Ebner's, Cystatin C,Cystatin SN, Cystatin D; Cystatin S; Cystatin SA; Fructose-bisphosphonate aldolase A; Ig gamma-1-chain-C- region; Carbonic anhydrase I and II; Caspase 14; Ig-kappa-chain-C-region; Beta2 microglobulin; actin; serum albumin
Peluso (2007) (35)	HPLC-ESI	Defensin	Acid and Basic proline-rich proteins; statherin, Cystatins C,D, S,SA, SN; Hystatins
Fleissig (2009) (36)	2DE/ESI	Albumin; Actin; Ig-gamma chain C region; Calgranulin A and B	Polymeric-Ig R; Vitamin D binding protein; Salivary amylase
Baldini (2011) (34)	2DE/MALDI-TOF-MS	Alpha enolase, IGKC, psoriasin, Calgranulin B, FABP-E, beta-2 microglobulin	Carbonic anhydrase VI; PIP; alpha-amylase; G3PDH; Cystatin SN; SPLUNC-2
Deutsch (2015) (37)	LC-MS/MS	CEA-related cell adhesion molecule 1 precursor Histone H2B type 1-B MMP-9 precursor Chitotriosidase-1 precursor Leucocyte elastase precursor Profilin-1, Keratin, type I cytoskeletal 13, MPO precursor	Carbonic anhydrase I; Bactericidal/permeability-increasing protein-like 1 precursor
Delaleu (2015) (38)	Multiplexing antibody - based sandwich immunoassays	60 biomarkers related to immunity, chemotaxis and immune cell differentiation	Fibroblast growth factor 4
Delaleu (2016)(21)	Multiplexing antibody - based sandwich immunoassays	pregnancy-associated plasma protein A, thrombospondin 1 and peptide YY	
Aqrawi (2017) (17)	LC-MS/MS	LCN2, CALM, GRN, SIRPA, LSP1, APMAP, CPNE!, PRDX3	
Cecchettini (2019) (40)	SWATH-MS	S100s, IL-1Ra, IL-36-gamma, plastin2 Myeloperoxidase, Azurocidin, Lysozyme, Bactericidal fold-containing family B member 1, Cathepsin B, Cathepsin D	(GCDFP-15/PIP proline-rich proteins, cystatins

ticular: mRNA (49, 50), microRNA(24, 51-53), long non-coding RNA (54), tRNA and rRNA. In brief, they are able to transfer genetic information, thus contributing to change cell phenotype of the receiving cells. Specific proteins characterise their membranes, some of these proteins are legacy and memory of the vesicle origin, others are necessary for a precise sorting to target cells. Almost all cell types release vesicles and these have been classified according to their biosynthesis, dimension and morphology, but a precise standardisation and characterisation is still lacking. Extracellular vesicle is a relatively new term that includes exosomes, microvesicles (MV) and apoptotic body (AB) (55). Exosomes are the most well-known and studied class of EVs. They are the smallest and most homogeneous population of vesicles, with a diameter ranging between 40 and 100 nm and a characteristic cup-shape morphology when observed under the transmission electron microscopy. They are formed by inward budding of the multivesicular body (MVB) membrane and their biogenesis involves the endosomal sorting complex required for transport (ESCRT) pathway (56, 57), tumour susceptibility gene 101 protein (TSG101) (58), ALG-2- interacting protein X (ALIX)(59), and small GTPases such as Rab7a and Rab27b(60). Exosomes are liberated into the extracellular space following fusion of MVBs with the cell membrane, regulated by Rab27A, Rab11, and Rab31(61).

MVs are vesicles larger than 150 nm and heterogeneity, both in size and composition, is their most peculiar feature. They are also named ectosomes, shedding vesicles, microparticles, exosome-like vesicles, nanoparticles and this redundance causes confusion. They are produced by outward budding of plasma membrane where lipid microdomains and regulatory proteins such as ADP-ribosylation factor 6 (ARF6) are involved in the shedding mechanism (62). In comparison with exosomes, MVs are larger vesicles ranging from 100 to 1000 nm in diameter and their molecular composition is highly variable and not well defined.

Apoptotic bodies are blebs released by cells undergoing apoptosis, their diameter varies from 50 to 5000 nm (63) and this means that the smaller than 1000 nm in diameter are collected together with the MVs, at least when EVs are isolated by centrifugation methods, the most broadly used.

Once released extracellularly, EVs reach their target cells delivering their content through different mechanisms of interaction. They can relate directly via membrane receptors, vesicle membrane can

fuse with the plasma membrane of the recipient cell or they can be internalised by endocytosis or phagocytosis (64).

Many are the biological functions proposed for EVs, some of the effects they cause in the recipient cells are beneficial others are detrimental and harmful. Intercellular cross-talk and signalling are highlighted by many reviews (65-67) and we have already mentioned the importance of horizontal genetic information transfer. Extracellular RNA is protected inside the vesicles from degradation (68) and the exchange of proteins and lipids is assisted. miRNAs that are packaged into EVs regulate genes involved in differentiation, proliferation and apoptosis and for this it has been said that EVs exhibit pleiotropic effects (48). The first documented function for exosomes was in immune response and immune surveillance (69) and in fact EVs are involved in immune stimulation, inflammation and autoimmunity (70). Recent studies showed how stem cell-derived EVs transfer morphogens and differentiation factors thus playing crucial roles during embryonic development and regeneration of injured adult tissues (71). On the other hand, pathological and activated cells increase the rate of EVs release and it has been found that they have detrimental, "bad" effects on the surrounding tissues and environment. EVs released from vascular cells seem to have roles in calcification (72, 73). Tumour cells influence recipient cells promoting a hospitable micro-environment toward cancer growth. Oncogenic molecules can be transferred to healthy cells through EVs and invasion and metastasis is promoted by EV-mediated extracellular matrix remodelling (74). More recently, a critical role has been revised for EVs in mediating communication among neurons and microglia (66). Exosomes have been shown to contain disease-associated cargos such as neurodegenerative associated peptides (75), prions (76), alpha-synuclein (77). For this reason they have been marked as spreaders of neurodegenerative proteins. On the other hand, several reports suggested a beneficial effect to the clearance of protein aggregates, underlining complex, multiple and controversial roles (78).

It has been observed that cells improve EV and particularly MVs release if stimulated by chemical signals or in pathological conditions such as atherosclerosis, diabetes, hypercholesterolaemia or hypertension, in brief during cardiovascular and metabolic diseases. Among the release stimuli examined, there are high concentration of cholesterol (79), chemotherapy (80), smoke (81), shear stress (82), hypoxia (83).

In order to be studied, EVs have to be collected, isolated and purified, characterised and eventually their content analysed by proteomics, lipidomics, transcriptomics and genomics strategies. Collection is not a big problem, since they are present in almost all body fluids, including blood, saliva, urine, semen, milk, bile, sweat, amniotic fluid, ascites and cerebrospinal fluid and they are also released from cultured cells into the medium. The isolation and purification phases are more complicated, and different techniques are available: they exploit the differences in size and surface markers, all of them presenting pros and cons. The most commonly used method is the differential centrifugation and ultracentrifugation. This procedure is efficient, but also long and with a low throughput, thus not simply applicable in the clinical setting (84).

Ultrafiltration is fast and easy and can concentrate EVs up to 240-fold, but it ends up with low pure samples, small volumes are a limitation and vesicles can be trapped in the filter pores (85, 86).

Low purity is also a problem when commercial PEG-based precipitation kits are used, although they are rapid and inexpensive (87). Size exclusion chromatography is quick and has a good reproducibility too, but membrane-free macromolecular aggregates can contaminate the vesicles (88, 89).

Compared with other methods, affinity capture (antibody based) is the one that guarantees the highest purity, but its limit is the cost and besides the isolated vesicles might loose their functionality (90).

Protein content of EVs has been studied and extensively catalogued using proteomics technology and in particular MS-based analyses (91). The elective strategy has worldwide the shotgun, bottom-up proteomics in which proteins are extracted from a biological source and enzymatically digested into peptides that are separated by liquid chromatography and analysed by mass spectrometry (92). Protein quantification can be obtained using different methods (93) and these strategies allowed the identification of a huge amount of proteins.

Many proteins are inheritance of the EV origin, thus often being found in EVs regardless of the releasing cell, such as Rab GTPase, SNAREs, Annexins, Flotillin (involved in membrane transport and fusion), Alix and Tsg101 (MVB formation) (94). Tetraspanins, such as CD9, CD63, CD81, CD82, adhesion molecules (Integrins), antigen presentation molecules (95), heat shock proteins (HSP70, HSP90) are also commonly found, and they could play a role in protein sorting to the exosomes (96). Some of these proteins have been indicated as exosomal markers, but it is becoming clear that these molecules are enriched in exosomes, but they are not specific since they have been identified in MVs too. Only CD81 might be considered as an exosomal marker (97).

MVs contain a distinct population of proteins, including matrix metalloproteinases (MMPs) (98), glycoproteins, integrins, *e.g.* Mac-1 (99), receptors and cytoskeletal components such as α-actin and α-actinin-4 (61).

The interest of scientific community on EVs is demonstrated by the creation of three data repositories to collect not only proteomic, but also lipidomic, and transcriptomic EV data: ExoCarta (www.exocarta.org) (100), Vesiclepedia (www.microvesicles.org) (101), EVpedia (www.evpedia.info) (102).

In the clinical setting, researchers look at EVs as potential source of biomarkers, since the molecular characteristics of MVs and their cellular origins could reflect the progress of the disease and monitor the effects of treatments. They could represent powerful tools for diagnosis, prognosis and could provide information on recurrence and chemoresistance. Moreover, they could be seen as a molecular signature observable in body fluids, with the great advantage

that the sampling would be non-invasive compared with solid biopsies.

In various cardiometabolic diseases EVs were exploited as novel biomarkers for pathologic conditions, such as inflammation, endothelial dysfunction, or angiogenesis (103).

Tumour-derived EVs are studied as promising biomarkers to monitor cancer progression and efforts were focused on the search of unique tumour EV biomarkers (74).

More recently, researchers concentrated on the possible use of EVs as therapeutics, due to their ability to transfer molecules to distinct cell targets, becoming mediators of signalling. This has been observed for embryonic stem cell MVs (104), but it is also well-known that MVs from cancer cells contribute to the horizontal propagation of oncogenes among subsets of cancer cells (105) and macrophage MVs shuttle microRNAs into breast cancer cells (106). From these and other studies the idea came out of using EVs as efficient and selective drug delivery vehicles, able to overcome natural barriers, to precisely reach specific target and to protect their cargo from degradation (107-109).

Among the body fluids, saliva is an attractive sample and has been demonstrated as a useful tool for the detection of different diseases, not only oral, such as oral cancer (110), but also for systemic diseases, breast, pancreatic and lung cancer (24, 111-113).

Despite these interesting premises, few studies have been accomplished on salivary EVs. Gonzalez-Begne *et al*. identified 491 proteins in the exosome fraction of human parotid saliva using the shotgun approach (114). Xiao *et al*. identified 63 proteins in salivary microvesicles by gel electrophoresis with liquid chromatography-mass spectrometry (115).

Notwithstanding the extraordinary expansion of EVs research, there still are issues and challenges that have to be faced. A first problem relates to the terminology used that is confused and unclear and need to be refined, and a general consensus has also to be reached. A fundamental issue is to standardise EV isolation and collection methods and to agree on isolation/purification protocols for EV subtypes; the storing conditions are not precisely defined. Little is known about the physiological role of EVs, about their half-lives in tissues and organs and even if the packaging of the cargo is a selective or a random process (61).

Extracellular vescicles in SS: a source for new -omic biomarkers

Increasing levels of circulating EVs have been associated with various autoimmune diseases, including not only SS (116, 117), but also systemic lupus erythematosus (SLE) (118), rheumatoid arthritis (RA) (119) and systemic sclerosis (SSc) (120). Indeed, apoptotic bodies represent an exclusive source for autoantigens in autoimmunity and considering that considerable body of evidence indicates dysregulation of apoptosis as one of the most critical issues in pathogenesis of autoimmune systemic diseases (121), the content of apoptotic bodies may unreveal novel biomarkers for systemic disorders. The idea of analysing extracellular vesicles in pSS started few years ago with the analysis of salivary exosomes and the characterisation of their miRNA repertoire.

More specifically, in the last ten years, quite a number of authors have intensively studied the expression of small non-coding RNA molecules (miRNAs) in salivary exosomes (23, 53, 122, 123) as additional disease biomarkers beyond miRNA detected in salivary glands, monocytes and lymphocytes (42, 52, 124-130). In particular, Gallo *et al*. (131) described the ebv-miR-BART13-3p showing that it could be transferred from B cells to salivary epithelial cells through exosomes and recapitulate its functional effects on targets stromal interacting molecule 1 (STIM1), and calcium signalling. Recently, Driedonks *et al*. (132) have also shown the presence of Y-RNAs mid-sized extracellular RNAs associated with EV that has been implicated in cellular processes such as DNA replication and RNA quality control.

Over the years, a great interest has also arisen in analysing the proteome of EVs in autoimmunity to foster the identification of specific disease biomarkers.

Recently, proteomics of salivary extracellular vesicles have been characterised by Aqrawi *et al*. (17). The authors included in their study 27 SS patients and 32 healthy controls. EVs were isolated from stimulated whole saliva using size-exclusion chromatography and from tear fluid eluted from Schirmer strips. Nanoparticle tracking analysis was conducted on joint fractions from the saliva and tears to determine size distribution and concentration of EVs. Further EV characterisation was performed by immunoaffinity capture of CD9-positive EVs using magnetic beads, detected by flow cytometry. The proteome of saliva, tear fluid, and EVs of both saliva and tear fluid from SS patients and controls were examined by digestion of the proteins with trypsin, analysis of the proteins by LC-MS, identification of the proteins using Mascot database searches and further data analysis using Scaffold to find quantitative differences. The five upregulated proteins that deviated most in biological replicates between patients with SS and controls and that were detected in EVs from whole saliva were adipocyte plasma membrane associated protein (APMAP), guanine nucleotide-binding protein subunit alpha-13 (GNA13), WD repeat-containing protein 1 (WDR1), tyrosine-protein phosphatase nonreceptor type substrate 1 (SIRPA), and lymphocyte-specific protein 1 (LSP1). APMAP is a pivotal enzyme in adipocyte differentiation. Moreover, GNA13 is a G-protein that consequently plays a role in transmembrane signalling, while WDR1 is a regulatory protein involved in the disassembly of actin filaments. Interestingly, SIRPA is a glycoprotein present in innate immunity, particularly in the regulation of NK cells and dendritic cell inhibition. LSP1 is an actin-binding protein also involved in innate immunity, specifically neutrophil activation, and chemotaxis. As far as tear EVs was related, copine (CPNE1) and CALM were expressed more in the patient group. CPNE1 is a calcium-dependent phospholipid-binding protein involved in TNF-α receptor signalling, inflammation and apoptosis, whereas CALM is a calcium-binding protein that plays a role in intracellular signalling. Although previous proteomic

studies have highlighted the relevance of inflammatory and immune-response associated proteins as SS biomarkers, these results strengthened the concept of activation of the innate immune system and adipocyte differentiation in SS pathogenesis implementing the existing knowledge on potential non-invasive diagnostic biomarkers that can help to increase pSS diagnostic accuracy, and can also be useful when monitoring disease progression and search for disease pathogenetic pathways.

Conclusions

In conclusion, over the last ten years the available literature has increasingly demonstrated the value of salivary proteomics in identifying reliable biomarkers for several systemic diseases including tumour and psychiatric diseases. Particularly, mass spectrometry of whole saliva still represents undoubtedly an established technique for biomarkers search in autoimmunity and cancers and it remains a cornerstone in this field. On the other hand novel literature has recently shown the possibility to extract and characterise EVs from saliva thus expanding the possible application of proteomic analysis to this biological fluid that can be collected in a non-invasive manner by simple, safe and stress-free procedures. Notably, from a proteomic perspective, EVs isolation might minimise the variability of the composition of whole saliva and the highly abundant proteins, fostering a sub-proteome that has a high likelihood of being informative for disease detection. Combining conventional salivary proteomics with novel findings derived from EV proteomics and advanced bioinformatics promises to blow up a new area of research, particularly helpful in clarifying pSS pathogenetic pathways and identifying novel therapeutic targets. More specifically, it is likely that the concomitant analysis of whole saliva and EVs miRNAs and proteins may provide a comprehensive picture of the disease mechanisms offering the possibility of identifying novel specific biomarkers that could be easily translated into clinical settings and, above all an effective tool for monitoring disease progression and response to therapies.

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