
Evaluation of salivary and plasma microRNA expression in patients with Sjögren's syndrome, and correlations with clinical and ultrasonographic outcomes

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ABSTRACT

Objective. To correlate the expression of microRNAs (miRNAs) 146a/b, 16, the 17-92 cluster and 181a in salivary and plasma samples taken from primary Sjögren's syndrome (pSS) patients with clinical, laboratory and ultrasound findings.

Methods. Plasma and salivary samples were collected from 28 patients with pSS according to 2012 ACR and/or 2016 ACR/EULAR criteria (27 females, mean age 64.4±10.1 years, mean disease duration 10.7±6.9 years), and from 23 healthy subjects used as controls. The following patient data were recorded: ESSDAI and ESSPRI scores, anti-SSA and anti-SSB antibody status and laboratory data, Schirmer's test, ultrasound scores of the four major salivary glands according to Cornec *et al.*, and concomitant treatments. The retro-transcribed and quantified miRNAs were: miR16-5p, miR17-5p, miR18a-5p, miR19a-5p, miR19b-1-5p, miR20a, miR92-5p, miR146a-5p, miR146b-5p, miR181a-5p.

Results. SS patients had higher expression of salivary miR146a than gender- and age-matched controls ($p=0.01$). Spearman's regression analysis revealed that salivary miR146b was significantly more expressed in the patients with worse ESSPRI scores ($p=0.02$), whereas salivary miR17 and 146b and plasma miR17 expression was lower in the patients with higher ultrasound scores (respectively $p=0.01$, $p=0.01$ and $p=0.04$). Salivary miR18a expression was significantly increased in the patients who were anti-La/SSB positive ($p=0.04$). Neither salivary nor plasma miRNAs correlated with disease duration or concomitant therapies.

Conclusion. Our data show that salivary mi146a may represent a marker of

the disease, and that the expression of salivary miR17, 18a and 146b may be altered in patients with pSS, and associated with worse ultrasound and ESSPRI scores and anti-La/SSB positivity.

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease characterised by inflammation and dysfunctioning of exocrine glands (primarily the salivary and lachrymal glands), although the inflammatory process can affect any organ (1). Almost all of the patients suffer from benign but disabling manifestations such as cutaneous and mucosal dryness, pain and fatigue, and 20-40% of them are affected by severe systemic manifestations.

The syndrome can present alone (primary Sjögren's syndrome, pSS) or be part of an underlying connective tissue disease, typically rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) (secondary Sjögren's syndrome, sSS) (2). Although its aetiopathogenesis is unknown, it has been clearly established that glandular epithelial cells are the targets of the autoimmune response (3). Autoantibodies reacting against the Ro/SSA and La/SSB intracellular ribonucleoproteins are the hallmarks of the syndrome, but a biopsy of the labial salivary glands is often essential for diagnosis (4). Schirmer's test for eye involvement and ultrasound (US) examination of the major salivary glands are reliable means of monitoring the course of the disease (5-7), and scoring methods such as the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) and the EULAR Sjögren's Syndrome Patient Reported Index (ESSPRI) allow a rapid and standardised evaluation of disease

activity as they are also capable of recording extra-glandular manifestations (8, 9).

A further non-invasive means of following the steps of the inflammatory cascade in pSS patients is the availability of salivary biomarkers. Saliva contains a number of organic and inorganic elements whose secretion depends on local and systemic stimuli. Chronic salivary gland inflammation alters the composition of saliva and various studies reported an increased expression of cytokines, beta-2 microglobulin as well as anti-Ro/SSA and anti-La/SSB antibodies in the saliva of SS patients (10). MicroRNAs (miRNAs) are a group of small RNAs, 21-25 nucleotides in length, that are involved in the regulation of a wide variety of cellular and physiological processes, including cell proliferation and differentiation, apoptosis, ontogenesis, metabolism, angiogenesis and inflammation. Furthermore, the fundamental role of miRNAs in the development of immune cells, as well as in the regulation of immune responses, suggests their importance in autoimmunity (11). They modulate messenger RNAs (mRNAs) translation by binding to complementary sequences generally located in the 3' untranslated region (UTR) of target transcripts, controlling the expression of about 60% of protein-coding genes (12). At the same time, some studies have also evidenced that miRNAs may bind the 5'UTR of some genes and promote their expression (13). In addition, complementary sequences to miRNAs have been detected on long non-coding RNAs, circular RNAs and pseudogene RNAs, whose pairing may prevent the interaction with the mRNA of coding genes, thus fomenting the competitive endogenous hypothesis (14). Dysregulated miRNA expression has been observed in the peripheral blood mononuclear cells (PBMCs) and salivary gland biopsies of pSS patients, with miRNA146a/b, 16, the 17-92 cluster and 181a being aberrantly expressed (15). Human saliva miRNAs have recently been used diagnostically: for example, Yoshizawa *et al.* found that they may regulate cell proliferation and apoptosis in oral cancer, thus suggesting their potential use

as biomarkers of early-stage disease (16). The authors provided a detailed method for isolating and profiling salivary miRNAs that can potentially be used to study salivary miRNAs in systemic autoimmune diseases.

Our was a single-centre, interventional, case-control, cross-sectional study aiming to assess the role of salivary miRNAs as biomarkers of pSS.

We aimed to correlate the expression of miRNAs 146a/b, 16, the 17-92 cluster and 181a in salivary and plasma samples taken from pSS patients with their laboratory data, ESSDAI, ESSPRI, and US scores. In addition, we correlated the salivary and plasma expression of the same miRNAs obtained from the pSS cohort with that observed in a group of healthy controls.

Materials and methods

Patients

The study involved 28 consecutive patients diagnosed as having pSS on the basis of the 2012 American College of Rheumatology (ACR) criteria and/or ACR/EULAR 2016 criteria (17, 18). All of the patients completed the self-administered ESSPRI questionnaire and were evaluated by an expert physician who assessed disease activity using the ESSDAI questionnaire. Furthermore, patients underwent Schirmer's test and US of the four major salivary glands using an ESAOTE My Lab 70 with a linear 7.5-10.0 MHz probe, with the images being scored according to Cornec *et al.* (7). A record was also made of their laboratory data, including anti-SSA or anti-SSB antibody status, erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels, as well as their SS treatment, including corticosteroids (allowed at a maximum dose of 10 mg/day of prednisone) and hydroxychloroquine (200-400 mg/day), and co-morbidities.

The control group consisted of 23 age-matched healthy subjects selected among the patients' physicians, nurses and relatives, who were previously screened for the inclusion and exclusion criteria by means of a questionnaire. The exclusion criteria for both patients and controls were concomitant oral infections, inflammatory or neo-

plastic conditions (*e.g.* stomatitis, aphthosis, pyorrhea, dental infections and oral cancer), other connective tissue diseases, chronic autoimmune arthritis, hepatitis, haematological disorders, sarcoidosis or any other medical condition that could lead to sSS.

Ethics

The study protocol was approved by our local Ethics Committee (no. 36896/2017 of 14 November 2017), and all of the participants gave their informed consent to take part in the study. All the procedures were conducted in accordance to Helsinki Declaration.

Laboratory evaluations

• Saliva and plasma processing

All the subjects were asked to refrain from eating, drinking, smoking and oral hygiene procedures for at least two hours before providing an unstimulated whole salivary sample in a sterile tube, after rinsing their mouths with distilled drinking water. At the same time, 10 mL of whole blood was collected by means of venipuncture into two 5 mL vacutainer tubes containing EDTA (Becton Dickinson, NJ, USA). The procedure of blood and saliva collecting was conducted at the same setting by the same operator in a timeframe from 9.00 A.M. to 6.00 P.M., with the 80% of samples obtained in the morning (until 12 A.M.).

The saliva samples were centrifuged at 4000 x g for 10 minutes, and the supernatant was transferred to a new tube (avoiding transfer of the pellet) with an equal volume of DNA/RNA Shield (Zymo Research, Irvine, California) in order to protect total RNA from RNase, and then immediately extracted. Plasma samples derived from whole blood centrifuged at 2300 x g for 10 minutes were stored at -80°C until RNA extraction. In order to protect the RNA from degradation, 200 µL of DNA/RNA Shield were added to each 200 µL of plasma.

• RNA extraction and purification

RNA was extracted using a Quick-RNA MiniPrep Plus kit (Zymo Research, Irvine, California). In accordance with the manufacturer's instructions, 400 µL of the processed saliva or plasma

samples were lysed with RNA Lysis Buffer (1:1), and the lysed samples were transferred to a Spin-Away filter in a collection tube and centrifuged at 13,000 x g for one minute in order to remove genomic DNA. One volume of ethanol was added to the sample flow-through, and the mixture was transferred to a Zymo-Spin III CG column in a collection tube and centrifuged. Finally, the samples were treated with DNase I and incubated for 15 minutes at room temperature.

After three washes with RNA wash buffer to remove any unspecific binding, the miRNAs were eluted in 60 µL of DNase/RNase-free water.

• MiRNA reverse transcription (RT)

Reverse transcription was conducted using a miRCURY LNA Universal RT miRNA PCR kit (Exiqon, Vedbaek, Denmark) containing, 2 µL of 5x reaction buffer, 1 µL of enzyme RT mix, 0.5 µL of synthetic RNA spike-ins, and 2.5 µL of H₂O plus 4 µL of extracted miRNAs. The samples were incubated at 42°C for 60 minutes, after which the enzyme was inactivated at 95°C for five minutes. The cDNAs were stored at -20°C until PCR quantification.

• Real-time polymerase chain reaction (PCR)

In order to quantify miRNA expression, the samples were diluted 1:40 with DNase/RNase-free water, and analysed by real-time PCR using the Exi-LENT SYBR® Green Master Mix (Exiqon, Vedbaek, Denmark) and a CFX Connect™ real-time PCR system (BIO RAD, Hercules, CA). The thermal profile was: 95°C for 10 minutes for polymerase activation-denaturation, followed by 45 cycles of amplification (95°C for 10 sec; 60°C for 1 min). The analysed miRNAs were: miR16-5p, miR17-5p, miR18a-5p, miR19a-5p, miR19b-1-5p, miR20a, miR92-5p, miR146a-5p, miR146b-5p, and miR181a-5p. The undetermined raw CT (Cycle Threshold) was set to 35 cycles. The expression data were normalised to miR-93 and miR-191, which are stable and constitutively expressed in fluids such as serum and plasma (19). The relative expression levels of miRNAs

were calculated using the comparative $\Delta\Delta C_t$ method as previously described (20). The fold changes in miRNAs were calculated by the equation $2^{-\Delta\Delta C_t}$. All the analyses were run in duplicate.

Statistics

The data were reported as mean values \pm standard deviation (SD) or median values with interquartile (IQ) and range depending on whether their distribution was Gaussian or not. Data distribution was assessed by means of Kolmogorov-Smirnov test for normality. The salivary and plasmatic miRNAs of patients and controls were correlated using parametric (Student's *t*-test) or non-parametric (Wilcoxon test) analysis according to Gaussian or non-Gaussian distribution of the data. Spearman's test was used in order to evaluate any associations between the miRNAs and the quantitative variables, and a two-tailed Mann-Whitney *U* test for unpaired samples was used to analyse miRNAs' expression in relation to the qualitative variables. Fisher's exact test was used for the comparison of nominal data. A *p*-value of <0.05 was considered statistically significant. All of the statistical analyses were made using SPSS 23 statistical software.

Results

Twenty-eight patients (27 women, mean age 64.4 \pm 10.1 years, mean disease duration 10.7 \pm 6.9 years) and 23 age-matched healthy controls (15 women, mean age 57.0 \pm 5.2 years) were consecutively enrolled in November and December 2017. Anti-Ro/SSA and anti-La/SSB antibodies were detected in respectively 18 (64.2%) and 8 patients (28.5%); rheumatoid factor in 8 (28.5%) and anti-nuclear antibodies (ANAs) in 26 (92.8%) patients. Median CRP level was 1.9 mg/L, (IQ 2.2, range 0.6-52.0), and mean \pm SD ESR was 28.0 \pm 17.8 mm in the first hour. The median ESSDAI and ESSPRI scores were respectively 1.0 (IQ 2, range 0-8) and 23 (IQ 10, range 6-28), which pointed out a low severity of symptoms. Only four patients had in fact an ESSDAI score \geq 5, and could be considered as affected by a moderately active disease, according to Seror *et al.*

Table I. Demographic characteristics of the 28 pSS patients.

Variable	Value
Age, years (mean \pm SD)	64.4 \pm 10.1
Gender, female (n°, %)	27, 96.4%
Disease duration (mean \pm SD)	10.7 \pm 6.9
Anti-nuclear antibodies (n°, %)	26, 92.8%
Anti-Ro/SSA positivity (n°, %)	18, 64.2%
Anti-La/SSB positivity (n°, %)	8, 28.5%
RF positivity (n°, %)	8, 28.5%
ESR, mm 1 st hour (mean \pm SD)	28.0 \pm 17.8
CRP, mg/L (median, IQ)	1.9, 2.2
ESSDAI (median, IQ)	1.0, 2
ESSPRI (median, IQ)	23, 10
Schirmer's test (median, IQ)	4.5, 6
Ultrasound score (mean \pm SD)	4.3 \pm 3.3
Hydroxychloroquine 200-400 mg/day (n°, %)	11, 39.2%
Prednisone 2.5-5 mg/day (n°, %)	7, 25%

(21). Specifically, 3 patients (10.7%) had a glandular involvement alone, 12 (42.8%) an extra-glandular involvement alone and 13 (46.4%) had both glandular and extraglandular involvement. Extraglandular involvement was articular in 19 cases, nervous in 4 cases, constitutional in 1 case, cutaneous in 6 cases, pulmonary in 4 cases, muscular in 2 cases, lympho-nodal in 2 cases, and haematologic in 2 cases. The median Schirmer's test score (the sum of the millimetres of lachrymal imbibition per eye) was 4.5 (IQ 6, range 0-32), and the mean \pm SD US score 4.3 \pm 3.3. Eleven patients underwent a biopsy of the minor salivary glands, which was positive in 4 cases (36.3%). None of the patients had a sialometry performed.

The demographic characteristics of the patient cohort are summarised in Table I.

There was a significant difference between the two groups in terms of gender, being males more numerous in the control group (*p*=0.006; Fisher's exact test). Therefore, we also performed a subanalysis considering only the female patients and controls, although, according to a study on healthy subjects, gender seems not to influence the expression of salivary miRNAs (22), and no data are available on SS patients.

Table II shows the expression of plasma and salivary miRNAs in the patients and controls reported as mean (\pm SD) or median (IQ and range) fold-

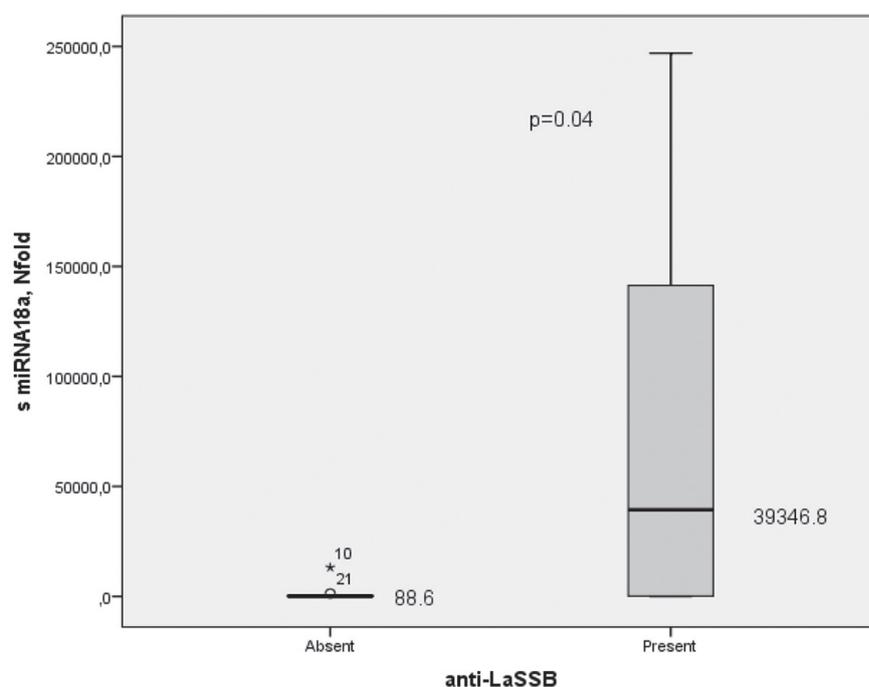
Table II. Plasma and salivary miRNA expression in patients and controls. Mean values \pm SD or median values, IQ and range (Kolmogorov-Smirnov test).

miRNA	Patient plasma samples (n-fold)	Control plasma samples (n-fold)	Difference in plasma expression	Patient saliva samples (n-fold)	Control saliva samples (n-fold)	Difference in salivary expression
miR16	86.4; IQ 57.8 (41.3-269.4)	94.8 \pm 38.0	Yes ($p=0.04$)	135.8 \pm 38.5	134.0 \pm 52.2	No
miR17	70.1; IQ 79.0 (14.8-1484.0)	89.1 \pm 51.3	No	187.2 \pm 135.4	106.4; IQ 138.3 (0-5493)	No
miR18a	109.1; IQ 90.0 (28.7-534.8)	127.4 \pm 40.6	No	120.5; IQ 947.7 (11.3-141.3)	85.0; IQ 165.7 (0-11104.5)	No
miR20a	125.0 \pm 53.1	121.4 \pm 25.7	No	146.9 \pm 56.5	129.6; IQ 173.5 (41.8-583.6)	No
miR146a	107.9; IQ 44.3 (32.9-240.0)	146.9 \pm 57.4	No	193.1 \pm 173.9	75.1; IQ 80.1 (0-714.8)	No
miR146b	1071.3; IQ 1554.0 (424.6-24136.0)	1161.6 \pm 952.3	No	156.9; IQ 317.2 (31.5-1333.8)	271.4; IQ 579.3 (0-1993.9)	No
miR181a	204.7 \pm 65.1	161.2 \pm 70.5	Yes ($p=0.02$)	83.2 \pm 37.5	81.7 \pm 32.7	No

change (N-fold): miR19a-5p, miR19b-1-5p and miR92-5p were undetectable using our laboratory procedures, and therefore excluded from the analyses. Considering the overall cohort, we did not find any significant difference in salivary miRNA expression between patients and controls. Plasma miR16 and 181a were significantly more expressed in the patients than in controls (respectively $p=0.04$ and $p=0.02$; two-tailed Wilcoxon test and Student's t test for unpaired samples). When selecting only female patients and controls, we found a higher expression of salivary miR146a in patients than in controls (193.1 ± 173.9 vs. 57.7 ± 38.4 N-fold, $p=0.01$), whereas no difference was found in the expression of the remaining salivary and plasma miRNAs.

The plasma and salivary miRNA expression was not significantly similar in the patients and controls, thus supporting the view that local miRNA production in the oral cavity presides over specific tasks. MiR16, 17 and 20a were more expressed and miR146b and 181a less expressed in the salivary samples than in the plasma samples of both patients and controls. In comparison with their plasma counterparts, salivary miR18a and 146a were more expressed in the patients, but less expressed in the controls.

In the SS group, salivary miR181a and 146a expression was significantly increased among the older subjects

**Fig. 1.** Salivary miRNA18a expression by anti-La/SSB positivity in patients with primary Sjögren's syndrome.

($p=0.01$ and $p=0.04$; Spearman's test), whereas the older subjects in the control group showed the greater expression of salivary miR16 ($p=0.006$) and 20a ($p=0.02$). Spearman's regression analysis showed that salivary miR146b was significantly more expressed in the patients with worse ESSPRI scores ($p=0.02$), whereas plasma and salivary miR17 and salivary miR146b expression was lower in the patients with higher US scores ($p=0.04$, $p=0.01$, and

$p=0.01$). Plasma expression of miR18a was increased in the patients showing less lachrymal production at Schirmer's test ($p=0.01$), whereas plasma expression of miR17 was reduced in the patients with higher ESR values ($p=0.01$). Salivary miR18a expression was significantly increased in the patients who were anti-La/SSB positive ($p=0.04$; Mann-Whitney U -test for unpaired samples) (Fig. 1). Salivary and plasma miRNAs did not correlate with disease dura-

Table III. Significant associations between salivary miRNAs and patient characteristics.

Salivary miRNA	Variables	Significance
↑ miRNA181a	↑ Age	<i>p</i> =0.01
↑ miRNA146a		<i>p</i> =0.04
↑ miRNA146b	↑ ESSPRI	<i>p</i> =0.02
↓ miRNA17	↑ Ultrasound scores	<i>p</i> =0.01
↓ miRNA146b		<i>p</i> =0.01
↑ miRNA18a	Anti-La/SSB positivity	<i>p</i> =0.04

SD: standard deviation; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein; RF: rheumatoid factor; IQ: interquartile; ESSDAI: EULAR Sjögren's Syndrome Disease Activity Index; ESSPRI: EULAR Sjögren's Syndrome Patient Reported Index; n-fold: target miRNA expression/control miRNA expression.

tion, which was significantly associated with worse ESSDAI scores (*p*=0.004), and the use of prednisone and hydroxychloroquine was not significantly associated with miRNA expression or the other variables.

Table III summarises the significant associations between salivary miRNAs and the clinical, laboratory and US variables.

Discussion

It has been shown that impaired epigenetic control is involved in the pathogenesis of many autoimmune diseases, including pSS. The main epigenetic mechanisms repressing gene expression are histone deacetylation, 5'—C—phosphate—G—3' (CpG) methylation and the formation of miRNA/mRNA complexes. MiRNAs are short non-coding RNAs that control many biological processes, including cell survival, metabolism and inflammation, and are responsible for the epigenetic regulation of about 60% of protein-coding genes either by silencing or promoting mRNA expression (12, 13, 23). In addition, it has been demonstrated that long non-coding RNAs, circular RNAs and pseudogene RNAs share a complementary sequence to many miRNAs, thus adding a further means of controlling gene expression by competing with mRNAs (14). Unbalanced miRNA expression contributes to the inflammatory cascade of many systemic inflammatory diseases, and may be measured in tissues and secreted fluids.

The evaluation of salivary miRNAs obtained by means of an easy and non-invasive procedure may be useful in monitoring and predicting the course of pSS. In this study, we proposed a method of extracting miRNAs from salivary fluid which, although being still expensive and poorly standardised, may be an alternative means of directly assaying salivary gland dysfunction. In addition, we evaluated the associations between salivary miRNAs and disease activity as measured by means of the ESSPRI and ESSDAI questionnaires, laboratory data, Schirmer's test and US findings. Results showed that miRNA expression was differently regulated in the salivary and plasma samples of both patients and controls, that salivary miR146a was more expressed in female SS patients than in female controls, and that salivary miR17, 18a and 146b expression in the pSS patients was associated with anti-La/SSB positivity and worse US and ESSPRI scores. These findings confirm an unbalanced epigenetic signature in pSS patients that is characterised by tissue specificity and may encourage the evaluation of samples other than plasma as a source of disease biomarkers.

It has been previously demonstrated that SS patients have an aberrant epigenetic background. Some authors have found the presence of demethylated sequences next to pro-inflammatory genes in the genome of cells extracted from labial gland biopsies (24), and recent studies have revealed the hypomethylation of some regions in the inactive chromosome X, which may explain the higher prevalence of pSS among females (25).

As miRNAs play a central role in orchestrating the immune response, various studies have evaluated their expression in patients with malignancies and systemic autoimmune diseases. The hyperexpression of miR146a, 16 and 155 in the blood and synovial fluid of RA patients has been associated with the likelihood of having the disease and greater disease activity (26, 27). It has also been shown that miRNA expression is unbalanced in patients with SLE, SS and systemic sclerosis (28). Some studies have found simi-

lar expression of a number of miRNAs (including the over-expression of miR146a, 16 and 21) in the PBMCs of both SLE and SS patients, which suggests that the two diseases may share pathogenic mechanisms such as the activation of B-lymphocytes (29). Conversely, others have shown that the expression of miR34b-3p, 4701-5p, 609, 300, 3162-3p and 877-3p in CD14⁺ monocytes is different in SS and SLE patients as a result of a differential control of the mitogen-activated protein kinase (MAPK) and transforming growth factor-beta (TGF-β) pathways (30). Finally, a recent study has reported an altered miRNA expression in the B and T lymphocytes of SS patients (31). These divergent data indicate that the expression of miRNAs is tissue and cell dependent, and highly variable in patients with systemic autoimmune diseases characterised by the involvement of multiple anatomical districts. It has also been demonstrated that infections may generate viral miRNAs in infected cells that alter their functions: for example, the salivary glands of SS patients may host Epstein Barr virus (EBV), a putative inducer of B cell lymphomas, and it has been reported that viral miR181a may be over-expressed in infected B lymphocytes and involved in B-cell proliferation, thus providing a hypothetical link between SS and the risk of developing a lymphoproliferative disorder (32).

The salivary glands are the primary sites of inflammation in pSS patients, and various studies have shown that about 94 miRNAs extracted from the salivary gland tissue and PBMCs of SS patients are dysregulated and differently expressed, including miR146a/b, 16, the 17-92 cluster and 181a (15). It has been found that miR181a and 16 are down-regulated in the labial salivary glands of SS patients and their levels of expression are inversely correlated with the severity of histological findings (33). The anomalous expression of miRNAs in SS patients seems to be involved in Ro/SSA and La/SSB epigenetic modulation: according to some authors, miR181a extracted from the minor salivary glands and PBMCs of pSS patients is associated with the

Ro52/tripartite motif-containing protein 21 (TRIM21) and La/SSB mRNA expression (34). Moreover, it has been found that miR146a is significantly up-regulated in the PBMCs and salivary glands of SS patients (35, 36). Alevizos *et al.* have shown that miRNA expression profiles is not only useful in distinguishing the glands of SS patients and controls, but they also identified subsets of SS patients with low- or high-level inflammation (37). The miR17-92 cluster has been associated with specific types of lymphocytes and lymphocytic diseases, and is down-regulated in patients with high salivary gland biopsy scores, and up-regulated in patients with lymphoproliferative diseases (11, 38).

Our patients had higher plasma but not salivary miR16 and 181a levels than controls but these results were not confirmed when only female subjects were analysed. Increased PBMC miR181a expression has previously been found in SS patients (39), and the lack of a parallel increase in salivary samples may be explained on the basis of a tissue-specific epigenetic regulation, and is in line with the findings of a previous study demonstrating the hypo-expression of miR181a in salivary glands (33).

Salivary miRNA146a expression was more pronounced in female SS patients than in controls and salivary miR146b but not miR146a expression levels inversely correlated with the patients' US scores and directly with their ESSPRI scores. Moreover, both blood and salivary miR17 expression inversely correlated with US-revealed glandular damage. These observations raise a number of considerations. The different behaviour of miR146a and 146b, which have previously been clustered together, may be related to changes in their molecular structure, coding chromosomes and biological functions (40). In particular, miR146b is encoded on chromosome 10, induced by interferon-gamma (IFN- γ), platelet-derived growth factor-beta (PDGF- β) or TGF- β , and seems to prevent inflammation, while promoting fibrosis and repair. MiR146a, which is encoded on chromosome 5, seems to be more involved in the acute phase of inflammatory processes. Induced

by other cytokines (*e.g.* interleukin-1 (IL-1), it counteracts the activation of the nuclear factor kappa light chain enhancer of activated B cells (NFkB) and interferes with co-stimulatory signals (41). The increased expression of miR146a in salivary samples of SS patients than in those of gender-matched controls may represent the clue of a buccal inflammatory process. On the other hand, the reduced expression of salivary miR146b in patients with more damaged salivary glands may underlie chronic inflammation, thus leading to worse US scores. Nevertheless, only eleven patients had a minor salivary gland biopsy performed, showing an inflammatory infiltrate in four cases, despite a glandular involvement clinically and instrumentally detected in 16 patients. Unless the usefulness of minor salivary gland biopsy in diagnosis and prognosis of SS, several false positive or negative cases may actually occur, due to concomitant inflammation sustained by other causes, advanced age or insufficient number of salivary glands included (42). Therefore, following these limits and in consideration of the invasiveness of the procedure, some authors have proposed the assessment of the autoantibody status as a preferable way of evaluating SS disease activity in daily clinical practice compared to minor salivary gland biopsy (43).

The reduced production of miR17, observed in patients with US-documented advanced glandular disease, may be related to the inhibitory effect of miR17 on the tumour necrosis factor-alpha (TNF- α) signalling cascade, as observed in patients with RA (44). Whereas, the increased plasma expression of miR18a in patients with less lacrimal production as well as the association between salivary miR18a and anti-La/SSB antibodies, may be due to its central role in the differentiation of T-helper 17 lymphocytes (45). Finally, the absence of a significant association between salivary miR18a and Schirmer's test scores may be related to differences in the regulation of miRNA expression at different production sites (in this case, differences between the lacrimal and salivary districts).

It has been reported that miR155 is up-regulated and miR19a and miR20a down-regulated in an experimental model of SS (46), but our clinical, laboratory and instrumental findings did not reveal any variation in salivary or plasma miR20a expression. We also obtained similar results when testing miR181a, which has been associated with the presence of anti-Ro/SSA and anti-La/SSB antibodies in previous studies (33).

The main limitations of our study are the small number of patients and controls, an unbalanced pSS population (27 females *vs.* 1 male) which was gender-mismatched with controls, the collection of blood and saliva sample in a variable time frame and the lack of standardised laboratory methodologies. We extracted miRNAs from the a-cellular component of biological fluids (plasma and saliva), and there is some risk of early deterioration. It has recently been found that evaluating the miRNAs contained in secreted exosomes significantly improves the sensitivity of the test and allows the detection of higher amounts of miRNAs in biological fluids such as saliva (47); this may be a stronger means of assessing miRNAs in human fluids in the future.

Conclusions

Our data show that salivary mi146a may represent a marker of the disease, and that the expression of salivary miR17, 18a and 146b is altered in pSS patients, and associated with worse US and ESSPRI scores and anti-La/SSB positivity. The demonstration of an imbalance in the expression of miRNAs in specific anatomical districts in SS patients and their central role in chronic inflammation may pave the way for the development of a new class of anti-sense oligonucleotide drugs that could be locally administered in order to regulate miRNA expression by means of base-pairing complementation (48). However, given our small number of patients and controls and the poorly standardised methodology, further studies are needed to clarify the role of salivary miRNAs as biomarkers of SS or targets of future medications.

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