Secretagogue effect of PDE4 inhibitor apremilast on human salivary gland organoids obtained from primary Sjögren's syndrome patients

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Abstract

Objective

The aim of the study was to culture vital salivary gland organoids obtained through labial or parotid biopsy of primary Sjögren's syndrome (pSS) patients in order to evaluate their morphological and functional features in basal condition and after stimulation with Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) activator forskolin and phosphodiesterase 4 (PDE4) inhibitor apremilast, their in vitro regenerative capacity and the immune-histological resemblance with original tissue.

Methods

Salivary gland tissues from five pSS patients were processed to obtain vital organoids; swelling assay and cell proliferation tests were performed after forskolin and apremilast application. Immunochemistry evaluation on original salivary gland tissue and corresponding organoids was performed, and secretomics analysis was conducted to assess their functional status.

Results

After application of forskolin and apremilast, we observed organoid swelling after 30 minutes, compatible with a positive functional status and enhancement of saliva production. In 3 cases, apremilast induced organoid proliferation. All cases were positive for cytokeratin 14 (CK14) and most for cytokeratin 5 (CK5). All the cases were positive for amylase; its secretion, and thus functional status of organoids, was confirmed by its high concentration in the culture medium. A focal ductal differentiation was found in some cases, highlighted by epithelial membrane antigen (EMA) positivity. The more differentiated EMA positive areas were negative for the staminal marker CK14, showing a sort of "complementary staining".

Conclusion

Our data highlighted that differentiated cells and vital functional organoids that recapitulate the development of original salivary glands can be obtained from pSS epithelium. For the first time, the direct stimulating effect of PDE4 inhibitor apremilast on pSS human salivary gland organoids is reported, opening new perspectives on targeting oral dryness with drugs that combine secretagogue and immunomodulatory effects.

Key words

Sjögren's syndrome, dry mouth treatment, salivary gland organoids, swelling test, salivary glands secretomics, apremilast secretagogue effect

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Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease, the hallmark of which is the immune-mediated destruction of exocrine glands, mainly salivary and lacrimal glands, together with B-cell hyperactivation (1). These phenomena drive both the impairment of glandular function (sicca syndrome) and the highest risk of lymphoproliferative complications among autoimmune diseases in pSS patients [5–10%, predominantly mucosa-associated lymphoid tissue (MALT) lymphoma of the parotid gland] (2, 3).

The salivary gland epithelium components (acinar, striated and intercalated ductal, myoepithelial cells and progenitor stem cells) are key players in the development of sicca syndrome, through a complex interaction with a dysregulated immune system (4). Salivary gland progenitor cells (SGPCs) seem reduced in pSS, and the low differentiation capacity of the remaining components due to a premature senescence phenotype reiterates damage also on the surrounding epithelium (5). Saliva production relies on the interplay between epithelium and autonomic nervous system, and the related regulation of intracellular calcium and intracellular adenosine 3',5'-cyclic monophosphate (cAMP) concentrations. Interestingly, cAMP is responsible of the action of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is expressed on both ductal and acinar apical cell membrane in salivary glands and mediates saliva production (6, 7). An altered function and expression of CFTR has been postulated in sicca syndrome and pSS pathogenesis and its restoration might resolve disease symptoms in mice models (8, 9). Moreover, since phosphodiesterase 4 (PDE4) is involved in downregulating intracellular cAMP, PDE4 inhibitors action on stimulating salivary function through the related upregulation of CFTR has been recently evaluated in mice models (10). The therapeutic role of stimulating CFTR with drugs capable of inhibiting PDE4, such as apremilast, was also postulated in diseases such as cystic fibrosis (11). To date, scarce evidence of effective treatment of sicca syndrome and salivary gland function restoration is found in the literature. The concept of both targeting salivary gland inflammation and infiltration, and correcting aberrating epithelial biochemical mechanism through pro-epithelial therapies and patient tailored treatments might be an effective strategy. To this end, obtainment of functional salivary gland organoids might both shed light on disease pathogenesis and allow the testing of new epithelial active pharmacological agents. Some authors have already focused on mouse salivary gland stem cells and derived organoids respectively for treating radiation induced xerostomia and for orthotopic transplantation into parotid gland defective mice (12, 13). Pringle *et al*. evaluated regenerative potential of pSS SGPCs through the establishment of human salivary gland organoids (5).

In 2020, Yoshimoto *et al*. established a human salivary-gland derived organoid culture system and, in addition to determining that ALK signalling inhibition is necessary for organoid formation, they successfully induced an organoid swelling test, through the stimulation with carbachol, a non-selective cholinergic agonist, and forskolin (7beta-acetoxy-8, 13-epoxy-1a, 6β, 9a-trihydroxy-labd-14-en-11-one), an activator of CFTR (14).

Forskolin, in fact, acts on adenylyl cyclase to increase the level of intracellular cAMP that leads to the opening of CFTR ion channel followed by fluid and ion transport into the lumen of the organoids (15). A similar effect has been regarded also on intestinal organoid cells as *in vitro* model mimicking *in vivo* cystic fibrosis (16).

Recently, Yoon *et al*. defined long-term culture methods for all types of adult stem cell-based mouse and human salivary gland organoids (17). To date, no data have been found in the literature on the application of salivary gland organoids for the evaluation of new experimental drugs aimed specifically at the treatment of xerostomia in pSS patients. Therefore, based on all the aforementioned data, apremilast, a known immunomodulatory drug already used for the treatment of pso-

riatic arthritis and Behçet's disease, might be evaluated as a new possible secretagogue agent in pSS: in fact, its direct action as PDE4 inhibitor and the consequent cAMP dependent-CFTR activation on epithelial salivary gland cells might directly stimulate salivary gland function and possibly counteract dry mouth symptoms (10, 18).

The aims of this study were: the culture of vital salivary gland organoids obtained through both labial biopsy and open surgical or ultrasound-guided core needle parotid biopsy of pSS patients; the establishment of their *in vitro* regenerative capacity; the evaluation of their morphological structure in basal condition and after stimulation with forskolin (swelling assay), and their immunehistological and functional resemblance with the original tissue. Moreover, the effect of the application of apremilast as a secretagogue agent on salivary gland organoids has been investigated.

Methods

Patients selection, tissue isolation and culture of patient-derived salivary gland organoids

For this study, the tissue was harvested from 5 patients affected by pSS, who, between March 2021 and July 2022, underwent labial gland (LG) biopsy, open surgical or ultrasound-guided core needle biopsy (US-guided CNB) of the parotid gland (PG) for diagnostic purposes. Three out of five cases underwent US-guided CNB of PG due to chronic or recurrent glandular enlargement; 1/5 underwent open surgical PG biopsy due to chronic glandular swelling and 1/5 underwent LG biopsy for diagnostic purposes. Clinical, demographic, imaging and histological data were collected for all the patients.

Written informed consent was obtained from each patient in accordance with the Declaration of Helsinki and with local guidelines for good clinical practice. The study was conducted according to a protocol approved by the Regional Ethical Committee (CEUR-2017-Os-027- ASUIUD).

For organoid establishment, salivary glands were collected after surgery into DMEM/F12 (Sigma-Aldrich) plus antibiotics levofloxacin 100 ug/ml, Vancotex 25 ug/ml, gentamicin 200 ug/ml, Fungizone 5 ug/ml. The biopsies were mechanically dissociated into ~1-mm pieces, then digested with 1 mg/ml collagenase type II (GIBCO™) and 0.5 mg/ml hyaluronidase (Sigma-Aldrich) in DMEM/F12 without phenol red (Sigma-Aldrich) at 37°C for 1-2 h according to tissue size. After tissue digestion, DMEM/F12 media containing 10% FBS was added to the suspension to inactivate the enzymes. After washing in cold PBS, the organoids were centrifuged for 5 minutes at 1000 rpm. Then organoid pellet was suspended in 10 µl of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (GIBCO™) and seeded in prewarmed 24-well tissue culture plates. Plates were placed into a cell culture incubator at 37°C and 5% CO2 for 20–30 min to solidify the droplets and 450 uL of media was added to each well. Media consists of LWRN conditioned media containing Pen/Strep antibiotics (Sigma-Aldrich), Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), 1X N2 supplement (100X) (Thermo Fisher Scientific), 5 ng/ml EGF (PeproTech), 20 ng/ml FGF7 (PeproTech), 20 ng/ml FGF10 (PeproTech), 20 ng/ml Wnt3a (R&D Systems), 100 ng/ml R-spondin1 (PeproTech), 20 ng/ml human-Noggin (PeproTech), 1X B27 supplement (50X) (Thermo Fisher Scientific), and 10 µM Y-27632. The culture medium was changed every 2–3 days. During passaging and for the forskolin swelling assay, the organoid droplets were mixed with Accutase and placed in incubator at 37°C for a maximum of 5 min. The resulting cell clusters and single cells were washed and replated, following the protocol described above.

Forskolin swelling and cell viability assays on salivary gland organoids

Patient-derived salivary gland organoids were seeded in a flat-bottom 96-well culture plate in 2 µl Geltrex commonly containing 20–60 organoids and 100 µl culture medium. The organoids were allowed to grow overnight, and then treated for 30 min with 20 uM of forskolin as a positive control and 10 uM of apremilast (Medchem). The images were taken on Nikon Eclipse TS2R inverted microscope using a 2x objective. The swelling ratio between each experimental condition and control group was calculated by comparing the mean value for the experimental condition group divided by the mean value of the control group. The size of each organoid (V) was quantified using ImageJ (19). Cell proliferation was measured via the measurement of ATP levels using the CellTiter-Glo 3D reagent (Promega), according to the manufacturer's protocol. Luminescence was detected using Infinite 1000 Tecan reader.

Immunohistochemistry and secretomics

Salivary gland organoids were collected at day 3 and day 7 of culture, using cell recovery solution (Corning) to remove Matrigel and processed as previously described (20). Briefly, the organoids were fixed in 4% PFA solution for 20 minutes and, after two times cold PBS washing, embedded in 1% agarose. Organoids in agarose solution were paraffin-embedded following dehydration in alcohol and clarification in xylene. From paraffin blocks, 5 µm multiple serial sections were cut on a microtome. The first of each series of 7 sections was stained with haematoxylin and eosin by routine method and evaluated under a light microscope to judge the morphology and to choose the best levels for immunohistochemical stains. The following immunohistochemical markers were chosen in order to demonstrate various type of differentiation: α-amylase and aquaporin 5 (AQP5) for acinar differentiation, epithelial membrane antigen (EMA) for ductal differentiation, calponin for myoepithelial differentiation and cytokeratin 5 (CK5) for basal differentiation. Furthermore, cytokeratin 14 (CK14), c-Kit (CD119) and CD34 were applied as well-known markers of progenitor/stem cells in the salivary glands (21). All these markers were first tested on sections of healthy salivary glands before staining the organoids. They were also tested on the corresponding formalin-fixed paraffin-embedded tissue previously biopsied for histological diagnosis. Most of the primary antibodies were supplied prediluted by Roche and processed ac-

Table I. Demographic, clinical, imaging features and histological diagnosis of 5 pSS patients who underwent minor or major salivary gland biopsy.

Pt	Gender	Age	Glandular features	EG features	Objective tests	ANA	SSA	SSB	RF	Low C4	Cryos	MC	Hyper-gamma	US features	Procedure	Histological diagnosis
#1	M	66	Oral and ocular dryness; PG swelling	-	+	+	-	-	-	+	-	-	-	Omeract grade 3	Open PG biopsy	MALT lymphoma
#2	F	53	Oral and ocular dryness; PG swelling	-	+	+	+	+	+	-	-	+	+	Omeract grade 3	US-guided CNB	MALT lymphoma
#3	M	64	Oral and ocular dryness; PG swelling	-	+	+	+	+	+	-	-	-	+	Omeract grade 3	US-guided CNB	LESA
#4	F	28	Oral and ocular dryness; PG swelling	-	+	+	+	+	+	-	-	-	+	Omeract grade 3	US-guided CNB	LESA
#5	F	61	Oral and ocular dryness	TIN; marginal zone lymphoma (nodal)	+	+	-	-	-	-	+	-	-	Omeract grade 2	LG biopsy	LS

Pt: patient; PG: parotid gland; EG: extraglandular; TIN: tubulointerstitial nephritis; RF: rheumatoid factor; Cryos: cryoglobulinaemia; MC: serum monoclonal component; Hyper-gamma: hypergammaglobulinaemia; MALT: Mucosa-associated lymphoid tissue; LESA: lymphoepithelial sialadenitis; LS: lymphocytic sialadenitis; US: ultrasound; CNB: core needle biopsy; LG: labial gland.

cording to the manufacturer's protocol with proprietary reagents using Cell Conditioning 1 buffer (High pH) and heat-induced antigen retrieval, except for AQP5 (AbCam, clone EPR3747, 1:200 dilution), and for amylase (Santa Cruz, clone G-10, 1:500 dilution). All stainings were performed in a Ventana BenchMark ULTRA PLUS autostainer (Roche Diagnostics). The binding of the primary antibodies to their antigens was detected by the UltraView DAB IHC Detection Kit. Counterstaining was manually performed using Carazzi's H&E. The immunohistochemical stainings were evaluated independently by two experienced pathologists using a semiquantitative method estimating both the percentage of positive cells and the staining intensity. A case was considered positive when the marker stained at least 50% of the organoid cells, with any intensity; a case was considered negative when the marker did not stain any cell; a case was considered intermediate when it showed features between positive and negative definition. The differentiation of salivary gland organoids was evaluated through the identification of α-amylase 1B, a serous acinar cell marker of parotid glands indicator of salivary gland differentiation, along the experimental set (at 3 and 7 days). The organoids were cultured for 3 or 7 days, as previously described. The medium was changed every 3 days and conditioned for 48 h in advanced DMEM/F12 w/o FBS, before collection. This medium (secretome) (300 µL) was collected after 3 and 7 days. Proteins (approximately 100 µg) were digested (EasyPep™, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's protocol. The tryptic peptides were analysed in duplicate with LC-MS/MS, using a Q-Exactive Plus Hybrid Orbitrap mass spectrometer coupled to a Vanquish UHPLC system (Thermo Fisher Scientific). The relative protein amount across our samples was determined through label-free quantification (LFQ). LFQ and the database search was done with Proteome Discoverer software (v. 2.5.0.400; Thermo Fisher Scientific) using the Sequest search engine against the human database (UniProt release 2022_02). The differential abundance of α-amylase 1B, expressed as "abundance ratio 7 *versus* 3 days" [log2], was calculated as the ratio of the average LFQ intensities of α-amylase 1B observed in 7- and 3-day secretome samples.

Results

Clinical and demographic data

For all the patients, clinical, imaging and demographic data were collected, and are summarised in Table I. Of note, for 2/4 patients who underwent PG biopsy, the histological diagnosis was coherent with MALT lymphoma, in the other 2 cases with lymphoepithelial sialadenitis (LESA). In the remaining patient, the histological result of the LG biopsy was lymphocytic sialadenitis (LS), with a Focus Score of 0.75. In this patient, the coexistence of subjective and objective sicca, tubulo-interstitial nephritis, nodal marginal zone lymphoma, antinuclear antibodies and cryoglobulinaemia, OMERACT grade 2 at salivary gland ultrasonographic evaluation and histological features of lymphocytic sialadenitis with Focus Score 0.75 in labial glands were consistent with a clinical diagnosis of pSS.

Apremilast induces swelling and proliferation of patient-derived salivary gland organoids

To evaluate whether pSS patient-derived organoids could be an *in vitro* model for salivary gland diseases and dysfunctions, basing our hypothesis on the aforementioned literature, we firstly performed a swelling assay applying forskolin to induce swelling in our established salivary gland organoids via the activation of CFTR as a positive control, and then we evaluated and confronted the potential action of the PDE4 inhibitor apremilast as secretagogue agent. Figure 1 summarises the

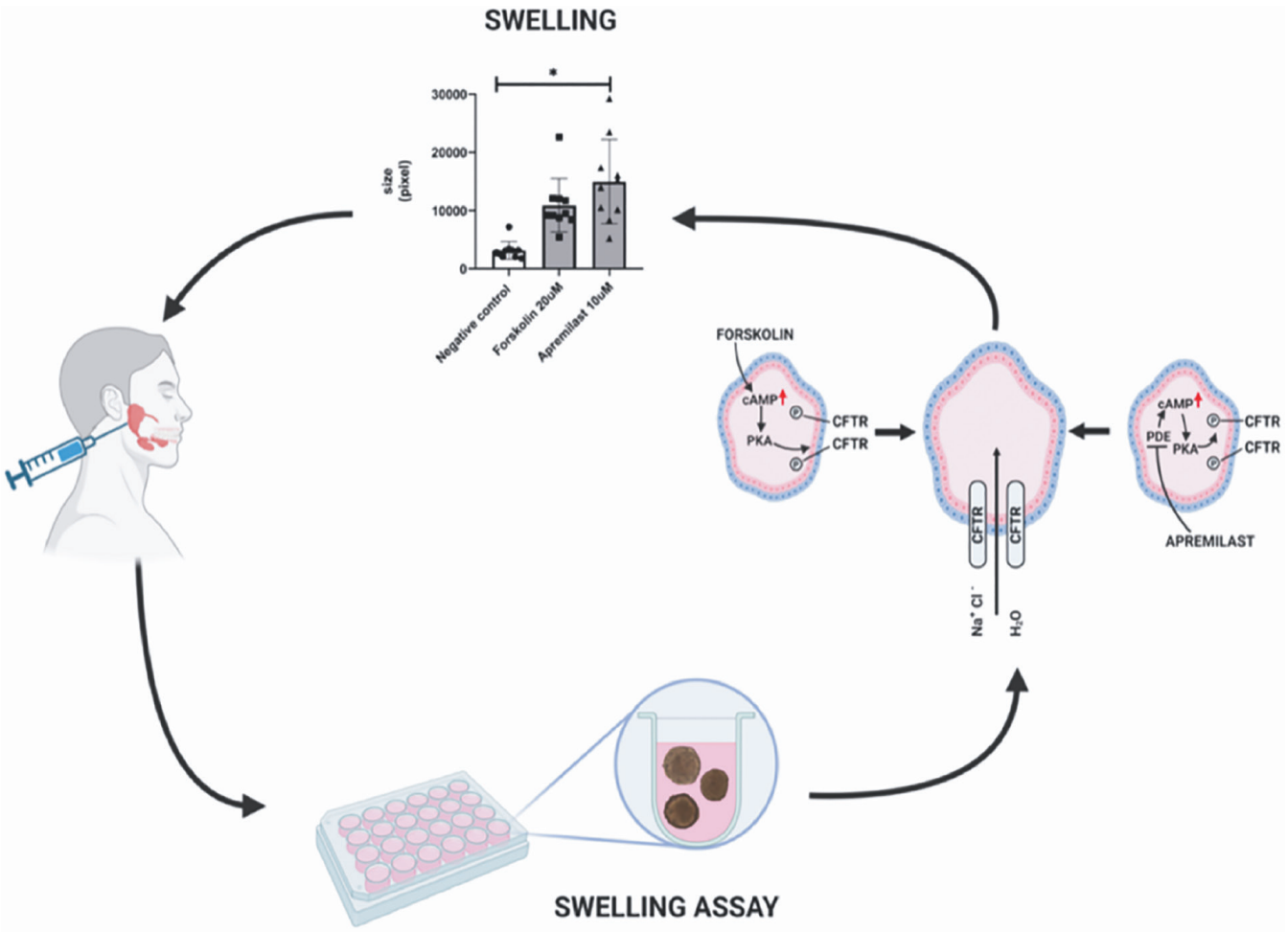

Fig. 1. Summarisation of technical and conceptual steps. The harvested salivary gland tissue is processed; a swelling assay with forskolin is performed on the obtained salivary gland organoids; apremilast, increasing intracellular cAMP, stimulates CFTR function and consequent organoid swelling.

technical and conceptual steps of this section. In the organoids treated respectively with forskolin and apremilast, we observed organoid swelling after 30 minutes, whereas DMSO-treated control organoids were unaffected. Finally, we examined the contribution of apremilast to the cell viability. In three patients, apremilast induced proliferation of pSS salivary gland organoids compared to control group (Fig. 2).

Immunohistochemistry and secretomics analysis show pSS organoids differentiation and resemblance to salivary tissue of origin

A total of 11 organoids derived from the 5 patients were immunohistochemically evaluated. Because of either technical issues or the paucity of the organoid cells, some of the markers were not evaluated in all the cases. In the normal salivary glands and in the tissue, samples collected from the 5 patients for histological diagnosis, α-amylase and AQP5 stained the acini, EMA stained mostly the ductal structures, calponin the myoepithelial cells and CK5 the basal cells. Myoepithelial and basal cells were both positive for CK14. No positive staining was found for c-Kit and CD34.

Figure 3 reports an immunohistochemical analysis representative of the obtained results. All the cases were strongly positive for CK14, a known marker of progenitor salivary gland cells; all the cases also showed at least some positivity for CK5, highlighting the immaturity of the organoid cells. Despite the immaturity of the cells, all the cases were positive for amylase. There was a greater expression of amylase compared to AQP5, probably because the latter, being a structural protein, is expressed later in the development of the glandular parenchyma. At least one focal ductal differentiation was found in the majority of the cases, highlighted by EMA positivity. Interestingly, the more differentiated EMA positive areas were negative for the staminal marker CK14, showing a sort of "complementary staining". Very few cells in only two cases were positive for the mesenchymal marker CD34, probably in relation to the epithelial-mesenchymal transition phenomenon.

The total results of the immunohistochemical analyses are listed in Table II. In order to analyse if during long-term culture of organoids (7 days) the cells acquired a more differentiated status, immunohistochemical analyses were performed. As highlighted in Figure 4, the expression of CK14 decreased and diffuse EMA positivity and a good CK5 positive layer of basal cells around ducts were observed.

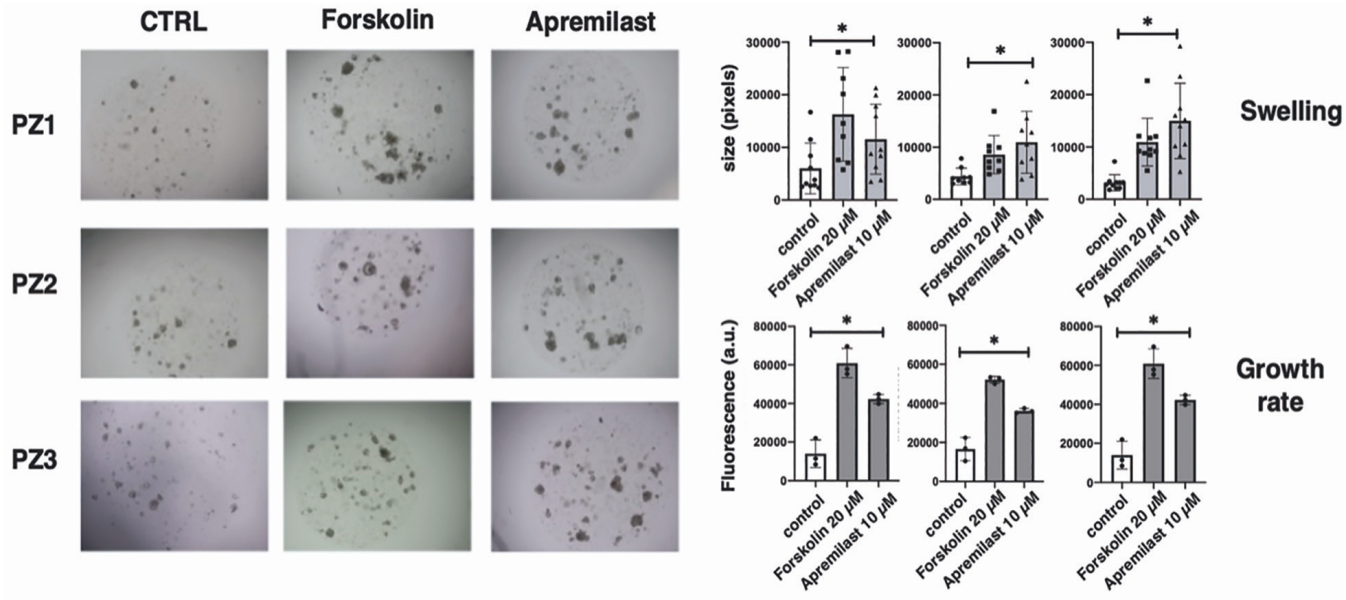

Fig. 2. Apremilast induces swelling of salivary gland organoids. The swelling of salivary organoids was induced by the activation of CFTR using forskolin and apremilast. Organoid swelling was observed after 30 minutes, whereas DMSO-treated control (CTRL) organoids were unaffected. Of note, apremilast increased the organoid growth rate compared to the control group. On the left, bright field images of three representative patients and on the right, the quantitative evaluations of the size and number of organoids.

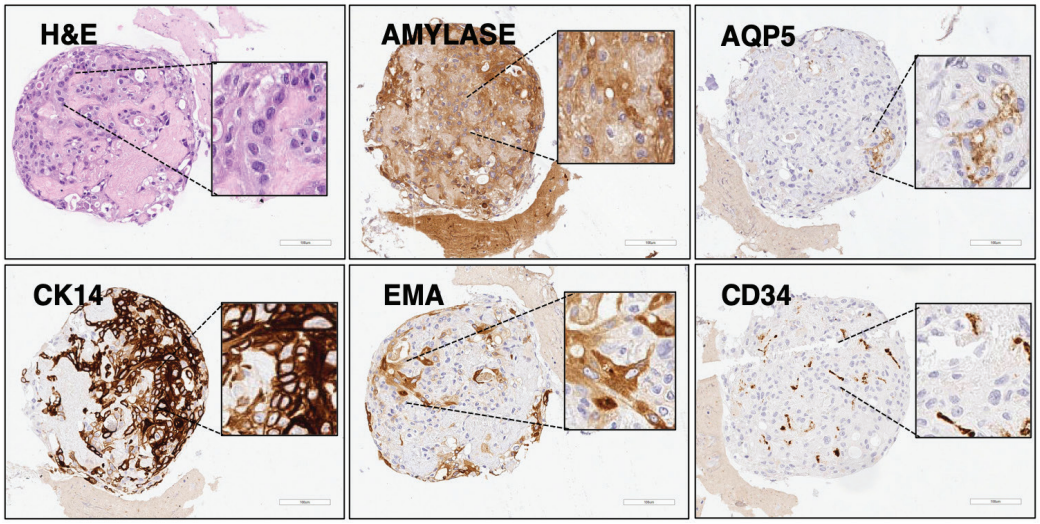

Fig. 3. Salivary gland organoids exhibit salivary gland markers. Haematoxylin and eosin-stained section of a representative organoid [Haematoxylin and eosin stain (H&E), original magnification 20X] and pictures showing immunohistochemical expression of some markers on the same cells; in particular amylase, AQP5, CK14, EMA and CD34. Note that the EMA positive cells are negative for CK14 and *vice versa*, showing a sort of complementary positivity, related to a different level of differentiation.

Multilayer digital pictures of short- (3 days) and long-term (7 days) culture of organoids showed that the levels of differentiation markers increase over time (Fig. 5). Overall, immunohisto-chemical staining and histological analysis showed the presence of both ductal, acinar and myoepithelial cells in the cultured organoids. To confirm the well differentiated phenotype of salivary organoids in long-term culture, α-amylase 1B was successfully identified in their secretomes (false discovery rate 1%). Its relative abundance was significantly higher (unpaired t-test $p<0.05$) at 7 days compared to 3 days (Table III).

Discussion

pSS is an autoimmune systemic disease, but oral and ocular dryness is the most frequent, and often the only symptom referred by patients. Dryness is a very disabling manifestation of pSS, however, treatment of sicca syndrome in pSS still remains an unmet need and still the object of hopeful research. It has been postulated that a thorough understanding of salivary gland epithelium contribution to disease pathogenesis and evolution might be fundamental in order to identify new possible and effective therapeutic targets (4). Moreover, researchers have focused on the pathogenetic role of the staminal salivary component in pSS in disease perpetuation (5). Human salivary gland organoids, being 3-D structures which mimic morphology and function of the original glands, might come to aid on these matters. In recent years, many authors have focused on the elaboration of lacrimal and salivary gland organoids, but no data are available on the use of human-derived organoids for drug testing systems in pSS-related salivary hypofunction (13, 17).

In this study, we successfully cultured vital organoids from five patients affected by pSS. Importantly, cultured

Table II. Results of the immunohistochemical markers assessed on organoids.

Markers	Positive	Intermediate	Negative
Amylase	7	4	0
AQP5	1	5	2
EMA	2	8	1
Calponin	1	2	6
CK5	5	5	0
CK14	5	0	0
c-Kit	0	1	4
CD34	0	2	4

Not all the markers have been evaluated in all cases because of either technical issues or paucity of the cells. Positive case: positive immunostaining in more than 50% of the cells, with any intensity. Negative case: none cell positive. Intermediate case: a case with features between positive and negative definition.

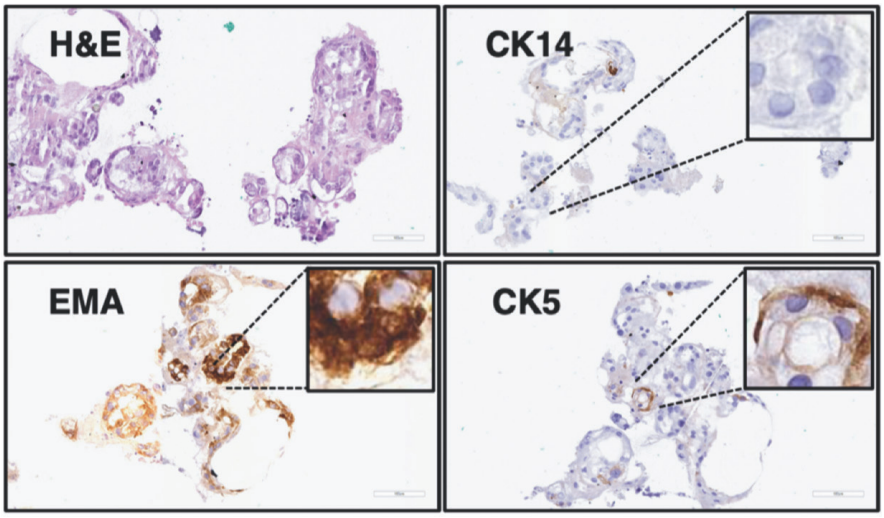

Fig. 4. Immunostaining on long cultured organoids. These organoids show a more differentiated morphology with clearly recognisable duct structures [Haematoxylin and eosin stain (H&E), original magnification 20X]; consistently with this histology, CK14 was mostly negative, EMA diffusely positive and CK5 showed a positive layer of basal cells around ducts.

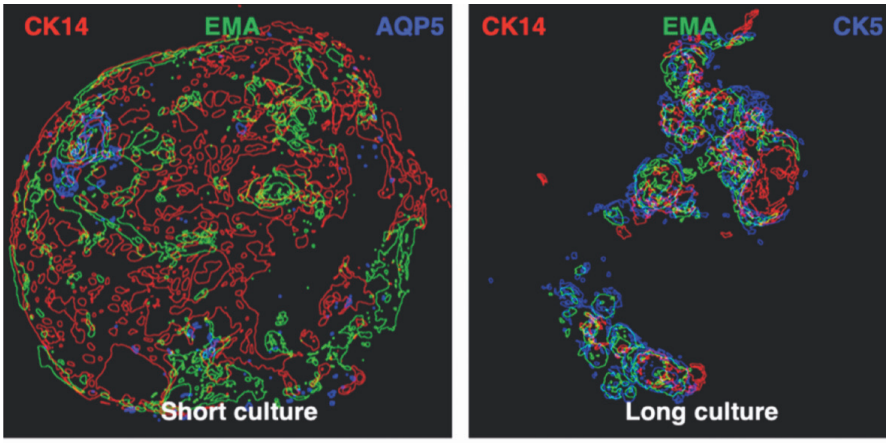

Fig. 5. Digital pictures showing a multi-layered positivity for three markers (CK14, EMA, AQP5 or CK5) in a short- or long-term culture organoids.
Note the less extent of CK14 red areas in the more differentiated organoids. The images were acquired with a slide scanner (Aperio AT2, Leica, Germany) at 40x magnification. For each organoid, sections were manually aligned by means of an interactive ImageJ script. Again with ImageJ, they were thresholded to select the positive areas, transformed into binary images, cleaned and outlined by means of mathematical morphology operations (closing, dilation, outline). Finally, to enhance the visibility of the outline, they were dilated three times. The 3 binary images were then merged into a single RGB image, where the red channel was taken from the CK14 image, the green channel from the EMA image, and the blue channel from either AQP5 or CK5.

vital organoids were derived both from parotid and minor salivary glands, supporting the presence of a staminal cellular component with regenerative potential in salivary gland epithelium from both sites. In fact, we demonstrated that from the epithelium of pSS patients, differentiated cells, that escape senescence, and organoids that recapitulate the development of the salivary glands can be obtained. Of note, we were able to culture vital organoids from tissue samples taken by different techniques, *i.e.* surgical or US-guided CNB (22). Another interesting point is that, differently from other methods described in the literature (12, 14), we were able to culture vital organoids without adding dexamethasone and hydrocortisone to our culture media, in order to avoid any additional potential immunosuppressant or metabolic effect on the cellular components. Moreover, we confirmed the epithelial origin of the organoids, through the detection of specific acinar, ductal, myoepithelial and staminal immunohistochemical markers, and their corresponding expression on the tissue of origin. α-amylase secretion by salivary gland organoids was observed, suggesting a functional salivary organoid formation. The increase in α-amylase secreted by long-term organoids (7 days) confirmed a higher degree of differentiation as compared to short-term ones (3 days), and we assume this might be due to a higher content of secretory granules (23). This is a crucial step to clearly demonstrate that organoids can recapitulate the same morphological and functional structure of the original tissue.

Interestingly, 4/5 cases were obtained from PG tissue, which is a more specific site of expression of pSS disease (24, 25). Yet, 2/4 cases of PG biopsy were obtained from patients carrying MALT lymphoma in that site and in 2/4 other patients a LESA.

Although no significant differences in expansion and differentiation between organoids derived from pSS patients at different lymphoproliferative stages were detected in this preliminary study, these results open the opportunity to study the activated epithelium of pSS when it interacts with the immune sys-

Table III. Identification and relative abundance of α-amylase 1B in salivary gland organoids (7 *vs*. 3 days).

Uniprot acc. nr.[a]	Protein description	Seq. coverage (%)[b]	Sequest score	Abundance ratio 7 *vs*. 3 days [log2]	Abundance ratio *p*-value
P0DTE7	α-amylase 1B, *Homo sapiens*	38	56.54	5.19	0.04

[a]UniProtKB accession number (https://www.uniprot.org/); [b]Seq. cov. %, percentage of protein sequence coverage.

tem at different grades of lymphoproliferation which you can observe in the course of pSS.

The study then focused on the evaluation of the epithelial function of these organoids in terms of salivary production. As described in the literature, forskolin, which stimulates the enzyme adenylate cyclase, was used as first swelling test, confirming the positive functional status of the epithelium compatible with an enhancement of saliva production, in line with previous demonstrations in organoids derived from other anatomical sites (26). In fact, an increase in cAMP activates CFTR channels, by phosphorylation of its regulatory (R) domain by cAMP-dependent protein kinase catalytic subunit (PKA). Upon stimulation with a cAMP-raising drug such as forskolin, CFTR activation has been clearly visualised in rectal organoids due to CFTR channel opening and transport of ions and fluids into the organoid lumen causing rapid organoid swelling (16).

Apremilast is a PDE4 inhibitor that works to reduce intracellular PDE4 activity. A reduction in PDE4 activity is thought to help reduce the overactive inflammation that characterises plaque psoriasis, psoriatic arthritis and mucocutaneous manifestations in Behçet's disease (18). Interestingly, it acts in a similar way as forskolin, by increasing the intracellular levels of cAMP. These are in fact tightly controlled by adenylyl cyclase, which promotes cAMP formation, and by cyclic nucleotide phosphodiesterases (PDEs), which are the only means of degrading cAMP, via enzymatic hydrolysis. PDE4, in particular, specifically hydrolyses cAMP rather than cGMP (18). The application of apremilast to the organoids provided very similar results on cellular swelling as forskolin, and thus on salivary function. Moreover, organoid proliferation was also stimulated by this PDE4 inhibitor. Interestingly, it has been reported that in a model of osteoarthritis, apremilast can suppress IL-17-induced cellular senescence (27), a mechanism possibly involved even in SS pathology (28). Our findings are supported by the results obtained by de Poel and colleagues in which PDE4 inhibitors, including apremilast, are potent CFTR function inducers in patient-derived rectal organoids (29).

By contrast, as expected by the absence of immune cells in the culture system, adding other immunomodulators in the culture media did not show the same positive results on these salivary models (data not shown). Thus, the effect of apremilast can be explained only by the biochemical effect on the intracellular levels of cAMP in the salivary organoids, rather than the anti-inflammatory effect that acts *in vivo* on the immune system cells. This original observation could open up a new prospective on the therapeutic rationale of the administration of apremilast in pSS. Indeed, apremilast might be studied in pSS patients specifically for its secretagogue action on salivary gland epithelium more than as a mere immunosuppressive agent. The good safety profile of apremilast makes it an intriguing therapeutic opportunity for dryness dominant phenotype of pSS. The evaluation of apremilast and other immunosuppressants in complex systems composed of co-cultures of both salivary gland epithelial cells and lymphocytes from the same patient is the next step of the research, thus testing both the anti-inflammatory and the biochemical effects, in order to verify the possible increasing value of combining two cellular targets simultaneously.

The limitations of this study include the small sample size and the heterogeneity of the tissue samples and of the procedures applied to obtain them. Although our study reported no differences between labial or parotid gland organoids obtained from pSS patients at different stages of disease, a wider sample size could extend our knowledge on the features of organoids obtained from different pSS phenotypes, and on the effect of apremilast, or other therapeutic agents, on them. The elaboration of organoids from both minor and major salivary glands in equal measures and from the same patient might shed more light on possible new targets of disease pathogenesis, and allow better definition of the yield of vital organoids obtained through different tissue sampling techniques. Moreover, the salivary organoids herein developed from pSS patients represent an incomplete model of the disorder, lacking the immune component of the autoimmune syndrome. To overcome these issues, our future agenda includes widening our sample size and the creation of complex co-cultures, to evaluate pSS microenvironment and its response to treatment in organoids derived from pSS patients at different stages of disease.

Conclusions

In conclusion, for the first time, cultured vital organoids were obtained from pSS patients in different lymphoproliferative stages of the disease and by different tissue sampling techniques, thus revealing the presence of stem cells in the activated epithelium of pSS. The direct stimulating effect of apremilast, as PDE4 inhibitor that is able to increase intracellular cAMP, on human pSS salivary gland organoids was originally reported, providing a new way to directly target dryness by acting on cAMP pathway even with drugs which combine a secretagogue action to immunomodulatory effects.

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