# Reactivity of human labial glands in response to cevimeline treatment

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ABSTRACT

Among the pathologies affecting the salivary glands, the Sjögren's syndrome (SS), an autoimmune disease, causes progressive destruction of the glandular tissue. The effect of SS is particularly evident on the labial glands and the morphological analysis of these minor glands is considered useful for diagnosis. Cevimeline hydrochloride (SNI), a selective muscarinic agonist drug, is one of the elective treatments for the hyposalivation of SS, acting not only on major salivary glands, but also on labial glands since their secretion is primarily under parasympathetic control. As the data on the effects of SNI in SS treatment are scarce, this study aimed to describe the morphology of human labial glands treated with SNI using light, transmission, and high-resolution scanning electron microscopy. Moreover, a morphometric analysis was applied to the light and transmission electron microscopy micrographs to obtain data that were then compared with analogous data collected on the control and carbachol-treated labial glands. Following SNI administration, the mucous tubules exhibited enlarged lumina, which were filled with a dense product of secretion. Occasionally, small broken debris of the cells were retrieved into the lumen. In the mucous secretory cells, some mucous droplets fused to form a large vacuole-like structure. Similarly, the seromucous acini showed both dilated lumina and canaliculi. These structural changes were confirmed through morphometric analysis and a milder action of SNI than carbachol on labial parenchyma was observed. The study confirmed that SNI also evoked secretion on labial glands and that its effect is more physiologic than that of the pan-muscarinic agonists.

Keywords: human labial glands; cevimeline; ultrastructural morphology; electron microscopy.

## INTRODUCTION

Minor salivary glands (MSGs), located in the oral submucosa between the muscle fibers, are represented by labial, buccal, palatal, lingual, and retromolar glands. MSG salivary secretion, despite representing less than 10% of the total volume of saliva secreted (Humphrey and Williamson, 2001; Wang et al., 2015), ensures the lubrication of the mouth during eating and speaking (Eliasson and Carlén, 2010) and regulates the perceptions of taste stimuli. Furthermore, the labial gland secretion plays an important role in oral mucosa homeostasis owing to its abundance in antimicrobial, antibacterial, and anti-inflammatory components (Crawford et al., 1975; Ferguson, 1999; Isola M. et al., 2010, 2011, 2013).

The histologic structure of the labial glands consists of two types of secretory portions: elongated mucous tubules with cells full of large mucous droplets (most abundant); and roundish seromucous acini, whose cells display many secretory granules (less frequent). Transmission electron microscopy (TEM) showed that the lumina were located in the center of the tubules and acini. Moreover, in the seromucous acini, lumina branches, called intercellular canaliculi, are visible between the secretory cells. Both of them have an important role in secretion and are full of numerous microvilli. Occasionally, a ribbon of seromucous cells borders the mucous tubules, constituting the so-called Giannuzzi demilunes (Riva and Testa Riva, 1998; Riva et al., 2002). The ductal system comprises intercalated (directly forms the secretory portion), intralobular (collecting saliva from the previous), and excretory (in which the previous flows into) duct opening directly into the oral mucosa.

Labial gland secretion is primarily under parasympathetic control (Wang et al., 2015) through cholinergic transmission, mediated prevalently by M3 (Beroukas et al., 2002; Riva et al., 2002; Ryberg et al., 2008), but also regulated by M1 (Mei et al., 1990), M5, and M4 muscarinic receptors located on the secretory cells (Ryberg et al., 2008). Recently, it was demonstrated that similar mucous and seromucous cells in the human major sublingual gland may respond with secretion not only to parasympathetic activity, but also to sympathetic activity (Loy et al., 2020).

Nowadays, pathologies affecting the salivary glands have markedly attracted scientific interest, regarding, investigations on their morphology, protein identification, mechanism of secretion, and the action of new drugs on salivary glands physiology.

Pilocarpine and cevimeline (SNI) are two parasympathomimetic secretagogues in the market for the treatment of dry mouth arising because of radiotherapy to the head and neck, or Sjögren’s

syndrome (SS). SS is an autoimmune disease associated with the progressive destruction of the parenchyma of salivary and lacrimal glands (Yamada et al., 2007), also affecting nasal mucosa and olfaction (Gobeljić et al., 2020). SNI is an azaspirodecan derivative, an analog of acetylcholine that acts selectively on muscarinic M1- and M3-receptors (showing a higher affinity for M3). Its pharmacological activity increases saliva and tears secretion (Fife et al., 2002). SNI has been used in several drugs in the treatment of dry mouth, such as Evoxac$^{TM}$. In contrast, pilocarpine is a pan-muscarinic receptor agonist, exerting higher side effects than SNI, including headache, nausea, and mild gastrointestinal pain. (Fife et al., 2002; Petrone et al., 2002; Ono et al., 2004; Yamada et al., 2007; Braga et al., 2009; Brimhall et al., 2013, Lovelace et al., 2014). Among the side effects, sweating is observed in both cases but less so in SNI. Sweating after pilocarpine use has become so problematic in dry mouth-patients that they interrupt their treatment with pilocarpine. Moreover, SNI has no affinity for M2 cardiac receptor at variance to pilocarpine (Fife et al., 2002).

The effect of SNI on the morphology of human MSGs has not been clarified yet.

Owing to their easy access, labial glands located on the inner surface of the lips may be biopsied and used in the diagnosis of Sjögren´s disease and experimental studies (Vitali et al., 2002; Tavoni et al., 2012; Guellec et al., 2013; Fisher et al., 2017). In the present study, human labial glands exposed to SNI, carbachol (Cch, another pan-muscarinic agonist), or untreated ones were examined using light (LM), TEM, and high-resolution scanning electron microscopy (HRSEM) (Loy et al., 2014). Morphometrical analysis of LM and TEM images were conducted and the results compared.

## MATERIAL AND METHODS

Samples of human labial glands were obtained from eight male patients aged between 49 and 72 years. Informed consent was obtained from each participant and the study was approved by the local ethical committee (ASL 8, Cagliari, Italy). All tissues were obtained from non-irradiated patients undergoing surgery for the removal of tumors of the orofacial region and were found to be normal, as assessed using LM.

Few reviews are available covering the morphological changes in the labial glands and production of saliva during aging (Scott and Path, 1986; Xu et al., 2018). The variability in data

observed in elderly subjects show the importance to differentiate between younger and older participants or establish specific age ranges. In our study, we focused on the older adults (50–70 years) in whom labial glands were observed to be morphologically homogeneous and functionally active (Scott and Path, 1986).

*In vitro* treatment

Fragments of each sample were immediately cut into 1 $mm^3$ pieces and placed into an oxygenated inorganic medium (composed of 123 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 16 mM Tris, and 5 mM D-glucose at pH 7.4) containing 10 µM cevimeline hydrochloride, known as (±)-cis-2-methylspilo [1,3-oxathiolane-5,3´-quinuclidine] hydrochloride hemihydrate or SNI-2011, or 10 µM Cch, at 37°C for 30 min (Loy et al., 2014). Some experiments were performed by adding 20 µM atropine, a muscarinic antagonist, 5 min before stimulation with SNI. Specimens incubated for 35 min in the same medium without the drugs were used as controls. The concentrations of the drugs were chosen in agreement with our previous studies (Testa Riva et al., 2006). SNI was supplied by Snow BrandMilk (Tokyo, Japan). Cch and atropine were purchased from Sigma (St. Louis, MO, USA).

TEM analysis

For morphological examination using TEM, the treated and control samples were fixed in a mixture of 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 2 h, embedded in Epoxy resin, and treated as reported in our previous study (Loy et al., 2015; Isola et al., 2019). The micrographs were observed with a JEOL 100S TEM operated at 80 kV and with a Jeol JEM 1400 Plus operated at 80 kV. Semithin sections were stained for LM with Azure II/Toluidine blue and examined in a Leica DMR HC.

HRSEM analysis

For HRSEM analysis, labial fragments were fixed in a mixture of 0.2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature for 15 min and then subjected to analysis using the $OsO_4$ maceration method, with few modifications (Riva et al., 1993, 2007; Isola et al. 2010, 2013; Lilliu et al., 2015). Occasionally, the NaOH maceration method was applied to better preserve the muscle cells (Riva et al., 2000). To view the inner

morphology of the secretory cells, including plasmalemma and organelles, we used a rotating agitator during rinsing and maceration; the rotating agitator allows the removal of most of the soluble proteins embedded in the cytoplasm. The micrographs were observed using a Hitachi S4000 FEG HRSEM operated at 15–20 kV. The images were obtained using Quartz PCI (Quartz imaging, Vancouver, Canada).

Morphometry

By an unbiased approach, thirty images at different magnifications were used for morphometrical evaluation (104 measurements in toto).

For the LM images of the mucous tubules, we measured the areas of the tubules, inner lumina, area occupied by mucous droplets (OMD), and that free from the droplets (FMD, i.e., the cytoplasm on the basal part of the cell), as shown in Figure 6A.

To establish which tubules were secreting, we calculated the ratio between OMD and FMD (called “Droplets Secretion” or DS); we expected an increasing number in case of secretion. Moreover, we calculated the ratio between the area of the tubule and the matching lumen area (called “Lumen Enlargement” or LE); we expected a decreasing number when secretion occurred. Median values of DS and LE ratio (0.48 and 87, respectively), obtained for the control samples, were considered as reference values to indicate no secretion.

For TEM images, we focused on the mucous droplets, which appeared fused in the stimulated samples. As shown in Figure 6B, for each mucous cell, we determined the ratio of the calculated area occupied by the droplets to that occupied only by fused droplets (FD).

The above-described parameters were also determined for the LM and TEM micrographs of labial glands treated with Cch, which were collected in a previous study (Riva et al., 2002). In choosing the samples to evaluate, we used the same age range used in the present paper.

As the data did not follow a Gaussian curve, statistical differences were calculated using the Mann-Whitney U test. Statistical analyses were performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California, USA) and Excel 365. The data were collected from LM and TEM images using ImageJ 1.53e (Wayne Rasband & coll., NIH, USA).

## RESULTS

This study aimed to describe the morphological modifications that occur in labial glands after SNI stimulations. Based on our previous studies (Loy et al., 2014, 2015; Riva et al., 1990, 1993, 2000, 2002, 2003, 2007, 2011), we used LM to have a panoramic view of the samples and TEM and HRSEM to study the ultrastructure of the secretory cells. Particularly, HRSEM permitted us to visualize the three-dimensional organizations of the cells and their organelles.

### *Unstimulated labial gland morphology*

The ultrastructure of the unstimulated human labial glands was previously described (Riva et al., 2002). In brief, using LM (Figures 1A–D) and TEM (Figures 1E–F), it was observed that the parenchyma of the untreated labial gland consisted of branched elongated mucous tubules and few seromucous acini, both surrounded by ribbon-like interdigitated processes of myoepithelial cells (Figures 1B and F). The mucous tubules were characterized by cells that appeared completely occupied by numerous large clear droplets (Figures 1A, C, D, and E); typically, the nucleus and other cellular organelles were confined near the basal and lateral membrane, respectively. In seromucous acini (Figures 1B, C, D, and F) and demilunes (Figure 1A), the cells showed secretory granules preferentially placed at the apical and central portion of the cytoplasm. Rough endoplasmic reticulum and Golgi apparatus were well represented in the seromucous cells (Figure 1F). The acinar lumen and intercellular canaliculi, full of numerous microvilli, were clearly observable using TEM (Figure 1F).

The HRSEM-fixed samples, combined with the osmium maceration technique, enabled us to visualize the three-dimensional cytoarchitecture of the tubules (Figure 2). The myoepithelial cells wrap the tubular secretory portion of the whole length (Figure 2A). In the mucous cells, numerous ovoidal droplets occupied majority of the cytoplasm (Figures 2B and C). As in TEM images, some organelles and a nucleus were visible near the lateral and basal membranes, respectively. The morphology of the seromucous cells matched that already described by TEM (Figure 2C). The Golgi apparatus, mitochondria, and other intracellular organelles were observed, including the cellular plasmalemma and its specializations (Figure 2C). As observed in the major salivary glands, the cytoplasmic side of the intercellular canaliculi membrane showed numerous

holes, representing the bases of microvilli deprived of their cytoskeleton, and among the holes, few microbuds 80–150 nm in diameter were discernible (Figure 2D).

*Cevimeline treatment elicited signs of secretion*

For the labial secretory parenchyma, SNI treatment evoked clear morphological changes. Using LM, it was observed that the stimulated mucous tubules exhibited enlarged lumina, which were filled with a dense mucous secretion (Figure 3A). Most cells displayed a consistent reduction of the apical portion, similar to that observed in an apocrine secretion. Cytoplasmic debris could be observed in the lumen. The seromucous acini displayed both dilated lumina and canaliculi (Figure 3B). In the seromucous cells, partial depletion of the secretory granules, located primarily in the apical region, was evident (Figure 3B).

The morphological changes observed by LM in the labial parenchyma were confirmed by TEM observations (Figures 3C–F). In the mucous cells, pictures of some droplets, fusing to form a large vacuole-like structure, were clearly seen (Figures 3C–E). Cellular organelles were also observed (Figure 3C inset). Moreover, some images showed a large portion of the apical cell floating in the lumen, suggestive of the process of apocrine secretion (Figures 3C–D). In the seromucous acini, the lumen and intercellular canaliculi were evident (Figure 3F), even if signs of secretion were less dramatic, such as the appearance of few empty granules. In these cells, numerous coated pits on the inner face of the plasmalemma of the seromucous cells were also observed (Figure 3F Inset).

Using HRSEM, both the mucous tubules and the seromucous acini or demilunes exhibited the morphological changes already described (Figure 4). The three-dimensional observation enabled us to appreciate the mucous flux in the intercalated duct, and often, the small broken debris of secretory cells in the lumen of the dilated tubules (Figures 4A–B).

Figure 6 shows the morphometrical data obtained from the LM and TEM images. Median values for the lumen areas in the control, SNI-, and Cch-treated samples were 20, 588.40, and 1744 $\mu m^2$, respectively. Statistically significant differences between the control and SNI- ($p<0.001$) or Cch- ($p<0.01$) treated samples were observed. The data confirmed that the lumina were dilated after SNI treatment, but not as much as after Cch stimulation.

Moreover, median values of the DS ratio were 0.48, 0.81, and 1.27 in the control, SNI- and Cch-treated samples, respectively. Stimulation with SNI or Cch induced significantly different DS

ratios as compared with the controls ($p<0.001$ or $p<0.01$, respectively). These data indicate that the fraction of the cells with mucous droplets dramatically decreased after Cch or SNI administration; that is secretion occurred.

The LE ratios resulted in median values of 87.0, 5.6, and 3.5 in the control, SNI- and Cch-treated samples, respectively. The SNI or Cch samples had significantly different LE ratios as compared with the controls (both $p<0.01$). The LE ratio quantified the number of tubules with a dilated lumen.

Median values of the FD ratio were 0.16, 0.68, and 0.37 in the control, SNI- and Cch-treated samples, respectively. The SNI- and Cch-treated samples displayed significant differences when compared with the controls ($p<0.001$ and $p<0.05$, respectively). The FD ratio represents the fraction of the fused granules out of the total granules inside the mucous cell. Cch values are lower than SNI values, because exocytosis was so massive in the Cch-treated samples, such that only a few granules were left to be evaluated.

*Atropine inhibits the morphological changes due to cevimeline stimulation*

When labial gland samples were treated with atropine, the pan-muscarinic antagonist, before administration of cevimeline (or A+SNI), the secretory parenchyma showed the same morphology as in the control samples. That is, no images of dilated lumen and canaliculi or vesicle fusion were observed in the seromucous acini using LM (Figure 5A inset), TEM (Figure 5A), and HRSEM (Figure 5B).

The same was evident for Cch administration to labial gland specimens that were pretreated with atropine, as reported by Riva et al. (2002).

The morphometrical data showed a significant difference ($p<0.0001$) between the SNI- and A+SNI-treated samples with respect to lumen enlargement. The data confirmed that the lumina were dilated after SNI treatment, which was different from those pretreated with atropine.

The median of the DS ratios was 0.35 in the A+SNI-treated samples. The DS ratios of A+SNI-treated samples were significantly different ($p<0.001$) from those after SNI treatment, while no significant difference was obtained comparing A+SNI treatment to the control.

The median of the LE ratios was 73.9 in the A+SNI-treated samples and this was significantly different from that of the SNI-treated ones ($p<0.001$). No significant difference was obtained comparing the A+SNI treatment to the control.

Lastly, the median of the FD ratios was 0.10 in the A+SNI-treated samples. The samples treated with A+SNI showed a significant difference ($p<0.001$) when compared with those treated with SNI, while no significant difference was obtained comparing A+SNI treatment to the control.
All these morphometrical data, reported in Figure 6, confirm the action of SNI through muscarinic receptors since their inhibition after atropine administration inhibited the secretory processes in the labial glands.

## DISCUSSION

The current investigation demonstrates that remarkable morphological changes occurred after SNI treatment in the secretory parenchyma of the labial glands. In the mucous tubules, enlargement of the lumina, loss of mucous droplets, and size reduction in the mucous cells were observed. In the seromucous acini, dilated lumina, and canaliculi, partial loss of secretory granules were observed, as well as the appearance of coated pits on the inner face of the plasmalemma.
As reported in our previous studies, the above-mentioned morphological changes are to be interpreted as clear images of salivary secretion (Riva et al., 2003, 2007; Loy et al., 2012, 2014).
Using TEM, it was noted that serous secretion is associated with widening of the lumina and canaliculi, the secretory granules fused with the apical membrane and the discharge of their content, and numerous small vesicles appeared anchored to the apical plasmalemma. The latter may be interpreted as invaginating recycling vesicles when bearing a coating (the so-called coated pits), and when not, as a small secretory vesicle belonging to the minor secretion that fuses with the plasmalemma. Using HRSEM, these two types of vesicles were indistinguishable and called microbuds. The mucous acini of the labial glands also have the peculiarity of fusing many of their large granules with one another to produce a sort of channel that discharge the mucous into the lumen (Riva et al., 1990, 2002), similar to what happens in the mucous goblet cells (Specian and Neutra, 1980). The occurrence of cellular debris and remnants of secretory granule shells and the shortening of the height of the cells after secretion, suggest that in the mucous cells of the labial glands, secretion might also occur by an apocrine process, as previously reported (Tandler et al., 1969). Apocrine secretion requires the detachment and retrieval in the lumen of portions of the apical cells that contain cellular organelles, intact

secretory granules, and large proteins that cannot follow regular exocytosis because they do not contain a signal peptide that directs them (Gesase and Satoh, 2003; Farkas 2015, Fullwood et al., 2019). The apocrine process usually involves protrusions bulging from the plasma membrane; however, sometimes no protrusions are formed. The latter process, termed "non-protrusion forming apocrine secretion" involves the detachment of the apical portion of the cell following fusion of the secretory vesicles, leaving behind a crater in the upper portion of the cell (Gesase and Satoh, 2003, Gesase 2007). Since we did not detect cells with the bulging apex in the mucous acini of the labial glands, but encountered both signs of apocrine secretion (shortened cells and cell fragments in the lumina), we can assume that in the mucous labial glands, the latter could be present.

The morphometrical analysis confirmed all our observations on mucous cells using LM, TEM, and HRSEM as it showed that secretion occurred after SNI treatment, but it was not as dramatic as after Cch administration. Specifically, we demonstrated that mucous droplets were discharged into the lumen (DS ratio) and that the caliber of the lumina increased (LE ratio) after SNI administration. Moreover, we observed that the fraction of fused mucous granules increased (FD ratio) after SNI treatment. Almost all these factors were significantly higher in the Cch-treated samples, but were also enhanced after SNI treatment, meaning that Cch evoked a dramatically massive secretion from the labial glands.

The *in vitro* technique applied in this study enabled us to expose fragments of the labial glands directly to the drugs, avoiding any other physiological control: from the nervous system (Ekström, 1989) *via* adrenergic receptors stimulation (Ishikawa et al., 2000) but also from hormones or local neuroendocrine regulation (Loy et al., 2012). Therefore, the morphological changes herein described were induced directly by the secretory effect of SNI. As already reported, this drug may act principally on M3 and M1 receptors inducing saliva secretion (Ishikawa et al., 2000; Fox et al., 2001; Fife et al., 2002). Furthermore, M5 receptors could be a target of SNI, but not M2 and M4 receptors (Ryberg et al., 2008).

Inhibition of M receptors by atropine eliminated the effect of SNI. Some other drugs can be used for this purpose: a selective inhibitor of calmodulin kinase II blocked SNI-induced amylase secretion, suggesting that this enzyme was involved in exocytosis processes triggered by this drug (Yuan et al., 2003). Moreover, the myosin light chain kinase inhibitor and nitro-L-arginine methylester hydrochloride (L-NAME), a selective inhibitor of neuronal nitric oxide synthase,

were used in pancreatic cells to inhibit amylase secretion in response to SNI. Phospholipase C inhibitors could also inhibit SNI-induced amylase secretion in rat parotid acinar cells (Yuan et al., 2003).

The morphological changes induced by SNI are similar to those induced by Cch, a pan-muscarinic agonist, as described in our previous study (Riva et al., 2002). It should be noted that the massive discharge of mucous droplets and the images of enlarged and irregular lumina observed after Cch treatment, were higher than those observed in this study after SNI stimulation. In the same way, intracellular vacuoles observed in the seromucous cells after Cch treatment were only rarely observed after SNI stimulation. These morphological changes confirm the massive action of Cch and the milder activity of SNI on salivary parenchyma.

Under physiological conditions, it is well known that the stimulation of muscarinic receptors on salivary glands induces fluid secretion. Intracellular $Ca^{2+}$ concentration and aquaporin (AQP)-5 expression play an important role in this type of secretion. Fluid secretion is caused by calcium-evoked intracellular and transcellular Cl transport that drives $Na^{+}$ and $K^{+}$ secretion into saliva and the consequent movement of water in it (Melvin et al., 2005; Kondo et al., 2015). Water can reach saliva through two routes: the transcellular route, through aquaporins, and the paracellular route due to the loosing of tight junctions (Murakami et al., 2001; Narita et al., 2019). SNI increases AQP-5 occurrence in the apical membrane in rat parotid acinar cells and induces the release of AQP-5, lipid rafts, amylase, and mucin into saliva (Ishikawa et al., 2000; Inoue et al., 2003; Takakura et al., 2007; Pan et al., 2009). Moreover, SNI increases the intracellular $Ca^{2+}$ concentration of rat parotid gland acinar and duct cells in a dose-dependent manner (Ono et al., 2009). Suzuki and colleagues (2013) observed that SNI increases the human salivary volume and releases Substance P into saliva demonstrating a close connection between this neuromodulator and the secretion evoked by SNI. Cong and colleagues (2015) showed that Cch and SNI also increased paracellular permeability in cultured epithelial cells from rat.

An increase in secretion due to SNI stimulation can be useful to enhance saliva production in physiological conditions and diseases such as SS (Ono et al., 2004; Beroukas et al., 2002; Iwabuchi and Masuhara, 1994; Takagi et al., 2004), not only in major salivary glands but also in MSGs. A recent study demonstrated that in diabetic patients, the protein secretion of labial glands is heavily damaged (Isola et al., 2011). Considering that pharmacological treatments, chemo- and radio-therapies can affect the function of the labial glands and that pretreatment with

SNI could prevent radiation-induced xerostomia in mice (Takakura et al., 2007), the secretagogue effect of SNI might ensure a better quality of life in SS or cancer patients, ensuring adequate mouth lubrication during functions such as eating, perceptions of taste, and speaking. Furthermore, being milder than the pan-muscarinic agents on the parenchyma of the labial glands, SNI ensures better physiological activity with less potential damage after long-term treatment.

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## AUTHORS' CONTRIBUTION

F. Loy, R. Isola, and M. Isola contributed to the experimental design, acquisition of data, and data interpretation. C. Masala and R. Isola performed the statistical analyses and drew the graphs. F. Loy, R. Isola, and M. Isola wrote the manuscript.

## REFERENCES

Beroukas D, Goodfellow R, Hiscock J, Jonsson R, Gordon TP, Waterman SA. 2002. Up-regulation of M3-muscarinic receptors in labial salivary gland acini in primary Sjögren's syndrome. Lab Invest 82:203–210.

Braga MA, Tarzia O, Bergamaschi CC, Santos FA, Andrade ED, Groppo FC. 2009. Comparison of the effects of pilocarpine and cevimeline on salivary flow. Int J Dent Hyg 7:126–130.

Brimhall J, Jhaveri MA, Yepes JF. 2013. Efficacy of cevimeline vs. pilocarpine in the secretion of saliva: a pilot study. Spec Care Dent 33:123–127.

Cong X, Zhang Y, Li J, Mei M, Ding C, Xiang RL, Zhang LW, Wang Y, Wu LL, Yu GY. 2015. Claudin-4 is required for modulation of paracellular permeability by muscarinic acetylcholine receptor in epithelial cells. J Cell Sci 128:2271–2286.

Crawford JM, Taubman MA, Smith DJ. 1975. Minor salivary glands as a major source of secretory immunoglobin A in the human oral cavity. Science 190:1206–1209.

Ekström J. 1989. Autonomic control of salivary secretion. Proc Finn Dent Soc 85:323–331.

Eliasson L, Carlén A. 2010. An update on minor salivary gland secretions. Eur J Oral Sci 118:435–442.

Farkaš R. 2015. Apocrine secretion: New insights into an old phenomenon. Biochim Biophys Acta 1850:1740–1750.

Ferguson DB. 1999. The flow rate and composition of human labial gland saliva. Arch Oral Biol 44:S11–S14.

Fife RS, Chase WF, Dore RK, Wiesenhutter CW, Lockhart PB, Tindall E, Suen JY. 2002. Cevimeline for the treatment of xerostomia in patients with Sjögren syndrome: a randomized trial. Arch Intern Med 162:1293–1300.

Fisher BA, Jonsson R, Daniels T, Bombardieri M, Brown RM, Morgan P, Bombardieri S, Ng WF, Tzioufas AG, Vitali C, Shirlaw P, Haacke E, Costa S, Bootsma H, Devauchelle-Pensec V, Radstake TR, Mariette X, Richards A, Stack R, Bowman SJ, Barone F. 2017. Standardisation of labial salivary gland histopathology in clinical trials in primary Sjögren's syndrome. Ann Rheum Dis 76:1161–1168.

Fox PC, Brennan M, Di Sun P. 1999. Cytochine expression in human labial minor salivary glands in health and disease. Arch Oral Biol 44:S49–S52.

Fox RI, Konttinen Y, Fisher A. 2001. Use of muscarinic agonists in the treatment of Sjögren's syndrome. Clin Immunol 101:249–263.

Fullwood NJ, Lawlor AJ, Martin-Hirsch PL, Matanhelia SS, Martin FL. 2019. An analysis of benign human prostate offers insights into the mechanism of apocrine secretion and the origin of prostasomes. Sci Rep 9:4582.

Gesase AP. 2007. Apocrine secretory processes in the goblet cells of rat colon following stimulation with carbamylcholine. Ital J Anat Embryol 112:117–129.

Gesase AP, Satoh Y. 2003. Apocrine secretory mechanism: recent findings and unresolved problems. Histol Histopathol 18:597–608.

Gobeljić SM, Milić V, Pejnović N, Damjanov N. 2020. Chemosensory dysfunction, oral disorders and oral health-related quality of life in patients with primary Sjögren's syndrome: comparative cross-sectional study. BMC Oral Health 20:187.

Guellec D, Cornec D, Jousse-Joulin S, Marhadour T, Marcorelles P, Pers JO. 2013. Diagnostic value of labial minor salivary gland biopsy for Sjögren's syndrome: a systematic review. Autoimmun Rev 12:416–420.

Humphrey SP, Williamson RT. 2001. A review of saliva: normal composition, flow, and function. J Prosthet Dent 85:162–169.

Kondo Y, Nakamoto T, Jaramillo Y, Choi S, Catalan MA, Melvin JE. 2015. Functional differences in the acinar cells of the murine major salivary glands. J Dent Res 94:715–721.

Inoue N, Iida H, Yuan Z, Ishikawa Y, Ishida H. 2003. Age-related decreases in the response of aquaporin-5 to acetylcholine in rat parotid glands. J Dent Res 82:476–480.

Ishikawa Y, Skowronski MT, Ishida H. 2000. Persistent increase in the amount of aquaporin-5 in the apical plasma membrane of rat parotid acinar cells induced by a muscarinic agonist SNI-2011. FEBS Lett 477:253–257.

Isola M, Cossu M, Massa D, Casti A, Solinas P, Lantini MS. 2010. Electron microscopic immunogold localization of statherin in human minor salivary glands. J Anat 216:572–576.

Isola M, Ekström J, Diana M, Solinas P, Cossu M, Lilliu MA, Loy F, Isola R. 2013. Subcellular distribution of melatonin receptors in human parotid glands. J Anat 223(5):519–524.

Isola M, Ekström J, Isola R, Loy F. 2019. Melatonin release by exocytosis in the rat parotid gland. J Anat 234:338–345.

Isola M, Lantini MS, Solinas P, Diana M, Isola R, Loy F, Cossu M. 2011. Diabetes affects statherin expression in human labial glands. Oral Dis 17:685–689.

Isola R, Solinas P, Concettoni C, Atzeni F, Loy F, Diana M, Isola M, Giacchetti G, Boscaro M, Riva A, Mariotti S. 2013. Cortical adrenal mitochondrial morphology changes in functional state: new insights. Cell Tissue Res 351:409–417.

Isola R, Solinas P, Loy F, Mariotti S, Riva A. 2010. 3-D structure of mitochondrial cristae in rat adrenal cortex varies after acute stimulation with ACTH and CRH. Mitochondrion 10:472–478.

Iwabuchi Y, Masuhara T. 1994. Sialogogic activities of SNI-2011 compared with those of pilocarpine and McN-A-343 in rat salivary glands: identification of a potential therapeutic agent for treatment of Sjögren's syndrome. Gen Pharmacol 25:123–129.

Lilliu MA, Loy F, Cossu M, Solinas P, Isola R, Isola M. 2015. Morphometric study of diabetes related alterations in human parotid gland and comparison with submandibular gland. The Anat Rec (Hoboken) 298:1911–1918.

Lovelace TL, Fox NF, Sood AJ, Nguyen SA, Day TA. 2014. Management of radiotherapy-induced salivary hypofunction and consequent xerostomia in patients with oral or head and neck cancer: meta-analysis and literature review. Oral Surg Oral Med Oral Pathol Oral Radiol 117:595–607.

Loy F, Diana M, Isola R, Solinas P, Isola M, Conti G, Lantini MS, Cossu M, Riva A, Ekström J. 2012. Morphological evidence that pentagastrin regulates secretion in the human parotid gland. J Anat 220:447–453.

Loy F, Isola M, Isola R, Lilliu MA, Solinas P, Conti G, Godoy T, Riva A, Ekström J. 2014. The antipsychotic amisulpride: ultrastructural evidence of its secretory activity in salivary glands. Oral Dis 20:796–802.

Loy F, Isola M, Isola R, Solinas P, Lilliu MA, Puxeddu R, Ekstrom J. 2015. Ultrastructural evidence of a secretory role for melatonin in the human parotid gland. J Physiol Pharmacol 66:847–853.

Loy F, Serra MP, Boi M, Isola R, Ekström J, Quartu M. 2020. Tyrosine-hydroxylase, dopamine β-hydroxylase and choline acetyltransferase-like immunoreactive fibres in the human major sublingual gland. Arch Oral Biol 109:104571.

Mei L, Roeske WR, Izutsu KT, Yamamura HI. 1990. Characterization of muscarinic acetylcholine receptors in human labial salivary glands. Eur J Pharmacol 176: 367–370.

Melvin JE, Yule D, Shuttleworth T, Begenisich T. 2005. Regulation of fluid and electrolyte secretion in salivary gland acinar cells. Ann Rev Physiol 67:445–469.

Murakami M, Shachar-Hill B, Steward MC, Hill AE. 2001. The paracellular component of water flow in the rat submandibular salivary gland. J Physiol 537:899–906.

Narita T, Qi B, Murakami M, Sugiya H. 2019. Pilocarpine induces the residual secretion of salivary fluid in perfused submandibular glands of rats. PLoS One 14:e0221832

Ono K, Inagaki T, Iida T, Hosokawa R, Inenaga K. 2009. Effects of pilocarpine and cevimeline on $Ca^{2+}$ mobilization in rat parotid acini and ducts. J Med Investig 56:375.

Ono M, Takamura E, Shinozaki K, Tsumura T, Hamano T, Yagi Y, Tsubota K. 2004. Therapeutic effect of cevimeline on dry eye in patients with Sjogren's syndrome: a randomized, double-blind clinical study. Am J Ophthalmol 138:6–17.

Pan Y, Iwata F, Wang D, Muraguchi M, Ooga K, Ohmoto Y, Takai M, Cho G, Kang J, Shono M, Li XJ, Okamura K, Mori T, Ishikawa Y. 2009. Identification of aquaporin-5 and lipid rafts in human resting saliva and their release into cevimeline-stimulated saliva. Biochim Biophys Acta 1790:49–56.

Petrone D, Condemi JJ, Fife R, Gluck O, Cohen S, Dalgin P. 2002. A double-blind, randomized, placebo-controlled study of cevimeline in Sjögren's syndrome patients with xerostomia and keratoconjunctivitis sicca. Arthritis Rheumatol 46:748–754.

Riva A, Congiu T, Faa G. 1993. The application of the $OsO_4$ maceration method to the study of human bioptic material. A procedure avoiding freeze-fracture. Microsc Res Techn 26:526–527.

Riva A, Lantini MS, Testa Riva F. 1990. Normal human salivary cells. In: Riva A., Motta PM. Editors. Ultrastructure of extraparietal glands of the digestive tract. Boston, Dordrecht, London: Kluwer Academic Publishers. p. 53–74.

Riva A, Loy F, Isola R, Isola M, Conti G, Perra A, Solinas P, Testa Riva F. 2007. New findings on 3-D microanatomy of cellular structures in human tissues and organs. An HRSEM study. Eur J Histochem 51:53–58.
Riva A, Puxeddu R, Loy F, Isola M, Cabras T, Testa Riva F. 2003. Serous and mucous cells of human submandibular salivary gland stimulated in vitro by isoproterenol, carbachol and clozapine: An LM, TEM, and HRSEM study. Eur J Morphol 41:83–87.

Riva A, Puxeddu R, Loy F, Testa Riva F. 2002. Morphofunctional studies on human labial salivary glands. Eur J Morphol 40:227–233.

Riva A, Puxeddu R, Uras L, Loy F, Serreli S, Testa Riva F. 2000. A high resolution SEM study of human minor salivary glands. Eur J Morphol 38:219–226.

Riva A, Tandler B, Ushiki T, Usai P, Isola R, Conti G, Loy F, Hoppel, CL. 2011. Mitochondria of human Leydig cells as seen by high resolution scanning electron microscopy. Arch Histol Cytol 73:37–44.

Riva A, Testa Riva F. 1998. Historical note: Giuseppe Oronzo Giannuzzi and the discovery of the serous demilunes and of the intercellular canaliculi. Eur J Morphol 36:15–18.

Ryberg AT, Warfvinge G, Axelsson L, Soukup O, Götrick B, Tobin G. 2008. Expression of muscarinic receptor subtypes in salivary glands of rats, sheep and man. Arch Oral Biol 53:66–74.

Scott JBDS, Path MRC. 1986. Structure and function in aging human salivary glands. Gerodontology 5:149–158.

Specian RD, Neutra MR. 1980. Mechanism of rapid mucus secretion in goblet cells stimulated by acetylcholine. J Cell Biol 85:626–640.

Suzuki Y, Itoh H, Amada K, Yamamura R, Sato Y, Takeyama M. 2013. Significant increase in salivary Substance P level after a single oral dose of Cevimeline. Int J Pept 2013:284765

Takagi Y, Kimura Y, Nakamura T. 2004. Cevimeline gargle for the treatment of xerostomia in patients with Sjögren’s syndrome. Ann Rheum Dis 63:749.

Takakura K, Takaki S, Takeda I, Hanaue N, Kizu Y, Tonogi M, Yamane G. 2007 Effect of cevimeline on radiation-induced salivary gland dysfunction and AQP5 in submandibular gland in mice. Bull Tokyo Dent Coll 48:47–56.

Tandler B, Denning CR, Mandel ID, Kutscher AH. 1969. Ultrastructure of human labial salivary glands. I. Acinar secretory cells. J Morphol 127:383–407.

Tavoni AG, Baldini C, Bencivelli W, Cavazzini L, Covelli M, De Vita S, Caporali R, Gerli R, Giansanti M, Govoni M, Donati V, Montecucco CM, Morbini P, Pilato FP, Resta L, Scott CA, Mosca M, Vitali C, Bombardieri S. 2012. Minor salivary gland biopsy and Sjögren's syndrome:

comparative analysis of biopsies among different Italian rheumatologic centers. Clin Exp Rheumatol 30:929–933.

Testa Riva F, Puxeddu R, Loy F, Conti G, Riva A. 2006. Cytomorphological study on human submandibular gland following treatment with secretagogue drugs. Cell Tissue Res 324:347–352.

Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE. 2002. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 61:554–558.

Wang Z, Shen MM, Liu XJ, Si Y, Yu GY. 2015. Characteristics of the saliva flow rates of minor salivary glands in healthy people. Arch Oral Biol 60:385–392.

Xu F, Laguna L, Sarkar A. 2018. Aging-related changes in quantity and quality of saliva: Where do we stand in our understanding? J Texture Stud 50:27–35.

Yamada H, Nakagawa Y, Wakamatsu E, Sumida T, Yamachika S, Nomura Y, Mishima K, Saito I. 2007. Efficacy prediction of cevimeline in patients with Sjögren's syndrome. Clin Rheum 26:1320–1327.

Yuan Z, Iida H, Inoue N, Ishikawa Y, Ishida, H. 2003. Effect of SNI-2011 on amylase secretion from parotid tissue in rats and in neuronal nitric oxide synthase knockout mice. Eur J Pharmacol 464:197–206.

## FIGURE LEGENDS

FIGURE 1: Untreated labial salivary gland as seen using LM (A-D) and TEM (E-F). A: Several mucous tubules, both in longitudinal and cross sections, show cells filled with mucous droplets. B-D: In the seromucous cells, the secretory granules are in the central and apical portions of the cells. E: TEM cross section of a mucous tubule. The mucous cells are filled with large clear rounded mucous droplets. F: A seromucous acinus as seen using TEM. In the center of the micrograph is a seromucous cell, with serous granules (which appear smaller, less clear, and sometimes with a small dark core). In the bottom, a mucous cell with some droplets. Arrows (in A-D) or L: lumina; S: seromucous cells; M: mucous tubules; N: nucleus; G: Golgi apparatus; RER: rough endoplasmic reticulum; I: intercellular canaliculus. Arrowheads (in B and E): myoepithelial cells. Bars: A-E, 10 µm; F, 1 µm.

FIGURE 2: Untreated labial salivary gland as seen using HRSEM. A: Labial gland mucous tubule obtained *via* the NaOH maceration method. Myoepithelial cells (M), were wrapped around the tubule, bordering the secretory cells, as seen from the interstitial space. B: Cross-sectioned mucous tubule obtained *via* the osmium maceration method. The cells are full of mucous droplets. C: Osmium maceration of seromucous cells. The nuclei and rough endoplasmic reticulum are clearly located in the basal part of the cells, whereas on the apical part some granules are visible. D: Intercellular canaliculus, as seen from the cytoplasmic side of the seromucous cell after discarding the cytoplasm and organelles from the cells. Holes (arrowhead)

represent the bases of the microvilli deprived of the cytoskeleton and the microbuds (arrows) represent membrane recycling processes or microexocytosis events. L: lumen. N: nuclei; G: Golgi apparatus; RER: rough endoplasmic reticulum. Bars: A-B, 10 µm; C, 5 µm; D, 1 µm.

FIGURE 3: Labial gland treated with cevimeline as seen using LM (A-B) and TEM (C-F). A: The mucous tubules show dilated lumina (L) where the mucus flux is evident. Fragments of the cells can be observed inside the flux (asterisks). Note that the mucous cells are also reduced in size. B: The seromucous acini show dilated lumina (L) and intercellular canaliculi (arrowheads). C-D: The mucous tubules show an enlarged lumen with some cellular debris (asterisks) that were part of the mucous flux; observed using TEM. In the cells, the mucous droplets are mostly fused. C Inset: High magnification of a mucous cell. E: High magnification of the lumen of a mucous tubule. #: Adjacent fused mucous droplets and mucous granule discharging its content into the lumen. F: Seromucous acinus. Note granule exocytosis (arrow) into the lumen (L), and coated pits (arrows) on the apical membrane (Inset). L: lumina. G: Golgi apparatus. N: nucleus. I: intercellular canaliculi. M: myoepithelial cell. Arrowheads (in E): membranaceous profiles of mucous droplets released into the lumen. Bars: A-B, 10 µm; C-F and C inset, 2 µm; F inset: 250 nm.

FIGURE 4: Labial gland treated with cevimeline as seen using HRSEM (A-C). A: After cevimeline administration, the mucous cells display numerous droplets fused (arrowheads). B: High magnification of the figure A. Note the flux of mucus (O) into the lumen (L) with fragments of cells inside (asterisk). C: Seromucous acini. N: nuclei. RER: rough endoplasmic reticulum. Asterisk: hole due to nucleus removal. Bar: A-C, 2 µm.

FIGURE 5: Labial gland subjected to atropine pretreatment before cevimeline administration. The morphology was similar to that of the untreated samples. A: TEM. High magnification of mucous cells; inset: LM, low magnification image of mucous tubules and seromucous acini. Arrows: lumina. B: HRSEM cross section of a mucous tubule. L: lumen. Bar: A-B, 2 µm; Inset, 10 µm.

FIGURE 6: Pattern of measurements and graphs of morphometric data collected upon the secretion of the mucous tubules.
A: LM image of a cross section of the mucous tubule. Dashed line: lumen (L) area. Solid line: area of mucous droplets (G). Dotted line: area of the whole tubule (A). Bar: 10 µm.
B: TEM image of a mucous cell. Solid line: area occupied by fused droplets (F). Dotted line: area of mucous droplets. G: single mucous droplet. L: lumen. Bar: 5 µm.
Graphs: Area of the lumina of the tubule, DS ratio (ratio between the area of tubular mucous droplets and the cytoplasm free from the droplets), LE ratio (ratio between the area of tubules and the related lumen), FD ratio (ratio between the area of the total droplets and that of fused droplets in the same cell) in the control, cevimeline (SNI)-, carbachol (Cch)-stimulated, and atropine pretreatment before cevimeline (A+SNI) samples. *, **, ***: statistically significant, $p<0.05$, $p<0.001$, $p<0.0001$, respectively.