IMMUNOHISTOCHEMICAL EVALUATION OF LABIAL SALIVARY GLANDS
IN XEROSTOMIC PATIENTS

By

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This thesis is submitted by Yat-Ho Ma and has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Georgia Health Sciences university.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore in partial fulfillment of the requirements for the degree of Master of Science.

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I. Introduction

A. Statement of the Problem

Xerostomia is the subjective sensation of dry mouth. In many cases, it is caused by true hyposalivation that can be diagnosed objectively (Nederfors 2000). Xerostomia affects about 25% of the older adults in North America (Quandt et al. 2011). The prevalence increases up to 44% in the general world population for those who are 65 years and older (Orellana et al. 2006). Xerostomia can have a significant detrimental effect on a person’s quality of life. Not only does it affect chewing and swallowing, but xerostomia can lead to a clinically important increase in caries, mucosal soreness, and candidiasis (Guggenheimer and Moore 2003). There are a number of factors that cause dry mouth, including medications, systemic diseases such as diabetes, and radiation treatment to the head and neck region. Another significant cause of xerostomia is certain autoimmune diseases, such as Sjögren’s Syndrome (SS) (Sreebny and Schwartz 1997; Porter et al. 2004). Currently, treatment of dry mouth is limited to alleviating the symptoms using salivary substitutes, oral lubricating agents, sugar-free gum, and specific medications like Pilocarpine and Cevimeline, however these approaches do not address the underlying pathology (Guggenheimer and Moore 2003; Mavragani et al. 2006; Dickinson et al. 2010).
B. Significance

The mechanism(s) underlying the presentation of xerostomia in autoimmune diseases such as SS are poorly understood, although reactive oxygen species (ROS) are thought to play a role in damaging glandular epithelial cells. By examining the glandular expression of proteins involved in cellular damage, further insight into the relationship between SS, hyposalivation, xerostomia, and cellular damage might be gained. The development of immunohistochemical strategies to highlight differences between SS subjects, xerostomic non-SS, and non-xerostomic subjects following labial salivary gland (LSG) biopsies could provide new and objective diagnostic tools. Further, recent research in SS has shown that EGCG (epigallocatechin-3-gallate), a component of green tea extract, has the potential to delay the onset of the disease in a mouse model for SS (Gillespie et al. 2008). Examination of the effect of EGCG on immunohistochemically identifiable markers for xerostomia and/or SS could provide further insight into the etiology of these disorders, and may provide support for the use of EGCG as a promising alternative treatment for patients suffering from xerostomia.

C. Review of the Literature

Function of Saliva

Saliva is a multifunction secretion essential to oral health. Salivary protection of the oral tissues and maintenance of health is mediated by the many electrolytes and proteins within the fluid. The water content cleans the oral cavity, and acts as a solvent for food. The thick, ropy, viscous mucin acts as a lubricant to protect the hard and soft
oral tissues and allow free movement of the tongue and lip, facilitating speech as well as denture retention (Tandler and Phillips 1993). Another well known salivary function involves the \[ \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+ \] buffering system and carbonic anhydrase VI, the enzyme that catalyses the reversible reaction of this system (Kivelä et al. 1999). This buffering system neutralizes the acidiogenic bacterial by-products from dental plaque, acidic food, or food rich in carbohydrates, and decreases the demineralization of calcium hydroxyapatite in teeth (Geddes 1994; Lenander-Lumikari and Loimaranta 2000). Furthermore, the mineral content within saliva also assists in tooth remineralization.

Currently, 309 proteins have been identified in saliva (Dawes 2008). Enzymatic proteins such as amylase, lipase, and proteases initiate the digestion process. Saliva also has antimicrobial properties provided by the complement, lysozyme, lactoferrin, histatin, and secretory IgA content. Aggregation of IgA activates the complement system and eliminates bacteria and reduces the infectivity of viruses (Boackle 1991). Lysozyme, an antimicrobial enzyme, cleaves chemical bonds in the bacterial cell wall by hydrolyzing glycosidic linkages of peptidoglycan. Lactoferrin inhibits microbial growth by sequestering iron from the environment. Histatins neutralize lipopolysaccharide, inhibit enzymes involved in periodontal destruction, inhibit release of histamine from mast cells (Giannobile et al. 2009), and serve as bactericidal and fungicidal agents.

The protein products and other contents within saliva also possess diagnostic value and can serve as markers for screening for certain diseases. Defensins were found to have a high positive correlation in patients diagnosed with squamous cell carcinoma. Elevated tumor markers such as cancer antigens were detected in saliva and may provide an early breast and ovarian cancer detection tool. As a route of disease transmission,
saliva may be used as an alternative for detection and diagnosis of viral hepatitis or herpes viruses (Kaufman and Lamster 2002). *Heliobacter pylori* has also been detected in the oral cavity and may serve as a re-infection route for gastric ulcers (Madinier et al. 1997). Saliva has also been shown to have inflammation and connective tissue breakdown products such as PGE2, IL-6, TNF-α, Matrix Metalloproteinases (MMP)-8, gelatinase (MMP-9), and collagenase (MMP-13) (Giannobile 2008). Currently, the Integrated Microfluidic Platform for Oral Diagnostics (IMPOD) is being developed and could revolutionize the diagnosis of periodontitis using saliva (Gioannobile et al. 2009).

**Salivary production**

Saliva production consists of two stages. The first stage involves the secretion from the acinar cells of the salivary epithelium of an isotonic fluid that also contains >85% of the protein in saliva. This fluid, rich in NaCl, is then modified by ductal cells. Ions are resorbed and proteins secreted as the saliva travels through a series of intercalated, striated, and excretory ducts. An intracellular Ca²⁺ increase is the primary fluid secretion signal while cAMP-signaling regulates protein-containing granule discharge from the acinar cells (Turner and Sugiya 2002; Melvin et al. 2005).

Salivary fluid secretion by acinar cells is regulated by two ion-secretion dependent mechanisms: Cl⁻ and HCO₃⁻ (Figure 1). In the Cl⁻ dependent mechanism, the intracellular Cl⁻ concentration is increased by a combination of a Na⁺ pump, an Na⁺/K⁺/2Cl⁻ cotransporter (primary uptake), and the Cl⁻/HCO₃⁻ and Na⁺/H⁺ exchangers at the basolateral membrane. The high (5x) intracellular concentration of Cl⁻ is then allowed to exit the cell into the acinar lumen via a regulated Ca²⁺ gated Cl⁻ channel. This
creates a negative electrical potential that drives Na\(^+\) ions into the lumen through the interstitial spaces between cells, and the resulting osmotic gradient drives the water movement to the lumen (Turner and Sugiya 2002; Melvin et al. 2005).

The HCO\(_3^-\) dependent pathway plays a lesser role in salivary secretion. The Cl\(^-\)/HCO\(_3^-\) and Na\(^+\)/H\(^+\) exchangers and intracellular carbonic anhydrase generate intracellular HCO\(_3^-\). As in the Cl\(^-\) efflux, the HCO\(_3^-\) also pass through an apical channel and drives fluid secretion (Melvin et al. 2005).

**Figure 1.** Stage one: Salivary secretion through Cl\(^-\) (A) and HCO\(_3^-\) (B) dependent pathway (Melvin et al. 2005). (A) Cl\(^-\) dependent pathway: Intracellular Cl\(^-\) concentration is increased by a Na\(^+\) pump, an Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter, and the Cl\(^-\)/HCO\(_3^-\) and Na\(^+\)/H\(^+\) exchangers at the basolateral membrane. Cl\(^-\) exits the cell into the acinar lumen via a regulated Ca\(^{2+}\) gated Cl\(^-\) channel. (B) HCO\(_3^-\) dependent pathway: The Cl\(^-\)/HCO\(_3^-\) and Na\(^+\)/H\(^+\) exchangers and intracellular carbonic anhydrase generate intracellular HCO\(_3^-\). HCO\(_3^-\) exits into the acinar lumen through an apical channel.

As the isotonic fluid flows through the ductal lumen, NaCl is reabsorbed resulting in a hypotonic secretion. In contrast to the acinar cells, the apical membrane Cl\(^-\) channels of the ductal cell facilitate the influx of Cl\(^-\), and Na\(^+\) channels at the apical membrane
allow the influx of Na\(^+\) ions. The low intracellular concentration of Cl\(^-\) and the Cl\(^-\)/HCO\(_3^-\) and Na\(^+\)/H\(^+\) exchangers at the apical membrane drives the Cl\(^-\) movement into the cells (Figure 2). At the basolateral membrane, the Na\(^+\)/H\(^+\) exchanger, Na\(^+\) pump, and Cl\(^-\) channel transfer intracellular NaCl to the interstitium, further lowering the intracellular NaCl concentration for NaCl reabsorption. Unlike the acinar cells, ductal cells are impermeable to water; therefore the modified salivary secretion becomes hypotonic (Melvin 1999).

![Diagram of salivary gland structure](image)

**Figure 2.** Stage two: Ductal modification of secretory fluids (Melvin 1999). Na\(^+\) and Cl\(^-\) ions are reabsorbed into the ductal cell from the apical membrane and into the interstitium. Ductal cells are impermeable to water and salivary secretion becomes hypotonic.

**Salivary stimulation**

Salivary secretion is control synergistically by both parasympathetic (PS) and sympathetic (Sym) innervation. The two primary neurotransmitters used in the salivary autonomic secretion system are acetylcholine (Ach) and norepinephrine (Norepi). All postganglionic PS neurons use Ach as a neurotransmitter and the corresponding receptors are known as muscarinic receptors (mAchRs). Postganglionic Sym neurons use NorEpi
and the receptors are considered adrenergic. There are several classes of the adrenergic receptors. The α1-adrenergic receptor response is similar to muscarinic activation in which salivary fluid secretion is increased, the α2-adrenergic receptor activation inhibits salivary secretion response, while the β-receptor stimulates protein secretion (Proctor and Carpenter 2007).

Neurotransmitter interaction with mAchRs or adrenergic receptors activate their coupled G (guanine nucleotide-binding) proteins. In mAchRs, the coupled heterotrimeric G protein activation leads to the production of inositol trisphosphate (IP$_3$) and diacylglycerol (DAG) through phospholipase C activation. IP$_3$ binds to the IP$_3$ receptor at the endoplasmic reticulum to release intracellular Ca$^{2+}$ (Turner and Sugiya 2002; Dawson et al. 2006). This leads to the Ca$^{2+}$ gated Cl$^-$ channel opening at the apical membrane and Cl$^-$ and HCO$_3^-$ exiting the cell. Thus, PS stimulation through binding of Ach to mAchRs promotes the secretion of watery saliva (Melvin 1999). In contrast, β-adrenergic receptor stimulation leads to an increase in the intracellular level of cAMP through its coupled monomeric G proteins. This results in the activation of protein kinase A and exocytosis of secretory granules (Turner and Sugiya 2002).

The specific sequence for the exocytosis mechanism is currently unknown. It is believed that cAMP promote the docking of the secretory vesicles to the cell membrane and the increase in Ca$^{2+}$ finalized the fusion and secretion process. SNARE (Soluble N-ethylmaleimide-sensitive-factor attachment protein receptors), a membrane-fusion-related protein complex, has been hypothesized to play a major role in exocytosis of salivary secretory granules (Turner and Sugiya 2002). VAMP2 (vesicle-associated membrane
protein 2/synaptobrevin) in neuronal vesicles is associated with the release of neurotransmitter. Recently, VAMP8 (Endobrevin), was suggested to perform a similar role in parotid acinar cells. VAMP8 null mice were found to have defective salivary gland exocytosis resulting in the accumulation of mucin, amylase, and carbonate anhydrase VI in secretory granules (Wang et al. 2007).

**Structures of the salivary gland**

The serous, mucous, and the seromucous cells make up the secretory endpieces of the salivary gland. According to Tandler, cells with granules that appear blue-black in toluidine blue staining under the light microscope and appear to be electron-dense under the electron microscope are considered serous. In contrast, seromucous cells are hardly stained by toludine blue and have low electron density under the electronic microscope. Serous cells have a rich network of rough endoplasmic reticulum (RER) and Golgi apparatus. They secrete an abundant amounts of protein and carbohydrate. The serous granules differ in structure among the different salivary glands but contain amylase and other non-enzymatic proteins such as Proline-rich proteins. Serous cells may contain two nuclei and have a prominent nucleolus (Tandler and Phillips 1993a).

Mucous cells produce mucin, which consists of 50-80% carbohydrates. They discharge mucin by exocytosis. Mucous cells have fewer RER, lysosomes and mitochondria; they are often associated with demilune serous secretory cells. Optimal fixation for light microscopy is difficult however, under electronic microscopy, mucous droplets can be seen filling the cytoplasm’s centrally located nucleus (Tandler 1993b).
The salivary secretion from the salivary parenchymal cells flows through a series of ducts, i.e. the intercalated duct to the striated duct to the excretory duct, and in the major salivary glands, to the main excretory duct. As mentioned before, saliva is modified in the ductal pathways through the interplay of ATPase, Na\(^+\)K\(^+\) ATPase, and carbonic anhydrase isozyme VI found in the ductal cells. Structurally, intercalated duct cells are simple cuboidal in shape and have a centrally placed nucleus, prominent RER, and an abundance of lysosomes. Secretory granules are found in intercalated ductal cells. These granules contain epidermal growth factor, agglutinin, Lewis blood group antigens, and acid mucin. Myoepithelial cells are connected through desmosomes to the intercalated ductal cells. These cells change the morphology of the intercalated ducts and lower the salivary flow resistance (Tandler et al. 1998).

Striated duct cells vary from simple columnar to pseudostratified in shape. They have an abundance of mitochondria and glycogen particles. Unlike those in the intercalated duct, cells of the striated duct perform most of the ion resorption as well as protein secretion and absorption during the second stage of salivary secretion (Tandler 1993c).

Excretory ducts consist of simple cuboidal and pseudostratified epithelium. The excretory ductal cells are filled with mitochondria and lysosomes. They also produce dehydrogenases, oxidases, acid phosphatase, and acid hydrolases that contribute to the salivary content. The main excretory duct is formed from the union of many excretory ducts and as it reaches the oral mucosa the ductal epithelium resembles squamous epithelium. The main excretory duct serves as a reservoir for saliva and the content is released when the surrounding muscles contract (Tandler 1993c; Tandler et al. 2006).
The parotid gland is situated superficial to the masseter muscle bilaterally. It secretes a predominately serous secretion via Stensen’s duct that opens near the vestibule of the second maxillary molar. The sublingual gland lies beneath the tongue and above the mylohyoid muscle. It secretes mucin-rich saliva into the oral cavity through the sublingual duct known as Bartholin’s duct. The submandibular gland resides posterior to the sublingual gland and beneath the mylohyoid muscle. It secretes a mixture of serous and mucin components. This mixture is secreted into the mouth via Wharton’s duct (Ross et al. 1995). The minor salivary glands produce predominantly mucin-rich saliva and are scattered throughout the oral mucosa (Hand 1999).

Xerostomia definition and prevalence

Xerostomia is the subjective sensation of dry mouth, and is therefore self-identified (Nederfors 2000). Studies on older adult populations (≥55 years old) conducted in North Carolina (Quandt et al. 2011) and Korea (So et al. 2010) show a prevalence of about 25%. In general, prevalence is higher in females and the elderly. In the United States, 20% of females and 13% of males between the ages of 65-84 have xerostomia. Globally, the prevalence increases up to 44% of the population for those who are 65 years and older (Orellana et al. 2006).

Since it is a subjective complaint, a survey instrument has been routinely used as the primary diagnostic tool for xerostomia. Although many survey methodologies have been developed over the years (Thomson 2005), questionnaires in these different surveys consisted of variations of the same questions relating to the subjectively rated difficulty in speaking, eating, and swallowing resulting from xerostomia. Thomson developed a more detailed survey, an 11-question Xerostomia Inventory, however, only two questions
in the survey ("My mouth feels dry when eating a meal" and "My mouth feels dry") correlate xerostomia to the amount of salivary volume (van der Putten et al. 2011). Unfortunately, a measurable objective method for xerostomia is currently unavailable at this time.

As a subjective condition, xerostomia differs from true hyposalivation, which is an objective measurement of reduced salivary flow that can in turn produce the sensation of dry mouth (Nederfors 2000). According to Dawes (Dawes 2004), if loss of saliva film thickness on the mucosa (notably the palate) by evaporation from mouth-breathing and absorption of fluid through the oral mucosa exceeds the restoration by salivary flow rate, then mucosal dryness in localized areas may cause the subjective complaint. Dawes suggested that an unstimulated salivary flow rate > 0.1 ml/min may avoid the dry sensation (Dawes 2004). An unstimulated salivary flow rate <0.1 ml/min and a stimulated flow rate of <0.7 ml/min are considered abnormal (Navazesh 2003). A normal unstimulated salivary flow rate averages 0.3 ml/min (Dawes 2008).

Though in principle a definitive flow rate (in ml/min) could delineate hyposalivation and normal flow rate, there are problems with this description. Measurement of salivary flow rate can have wide ranges, both between individuals and in the same individual at different times of the day. While normal salivary flow rates in some studies were reported between 0.1-2.0 ml/min (Fenoll-Palomares et al. 2004), healthy individuals without xerostomia may have an unstimulated flow rate between 0.008-1.85 ml/min (Dawes 2004). There are also variations of normal salvary flow rate and hyposalivation between individuals and within the same individual (Ghezzi et al. 2000). While little saliva is produced during the sleeping hours (Humphrey and
Williamson 2001), it is normal for the flow rate to gradually increase from the morning to noon. Thus the time of salivary collection in the evaluation of flow rate can be important (Flink et al. 2005). In addition, age may also play a factor in salivary flow but there are conflicting studies. While one study showed the healthy elderly produced less saliva than young individuals (Zussman et al. 2007), another study found no association between age and salivary flow rate (Sevón et al., 2008). Collectively, subjects who truly have hyposalivation may not have xerostomia and subjects who have xerostomia may not have hyposalivation (Wiener et al. 2010). As a result, it is not possible to assign a "normal" or "abnormal" flow rate that could be used to provide a statistically reliable assessment of an individual for hyposalivation related to xerostomia, unless it is extreme.

**Etiology of xerostomia**

The most common reversible cause of xerostomia is medications. There are over 400 commonly prescribed medications that induce dry mouth. These drugs may block either the cholinergic or adrenergic receptors, thereby decreasing salivary stimulation. Hypertension patients taking diuretics may also be affected by the dehydration effect. With greater than 52% of the population taking at least one medication and between 11-24% taking greater than four drugs per day, it is likely that an adverse xerostomic reaction from medication will occur. Fortunately, drug-induced xerostomia is not irreversible and normal salivary flow returns once the offending medication is either removed or the dosage reduced (Sreebny and Schwartz 1997). However, this may not be possible due to the need to continue treatment of an underlying condition.

Head and neck radiation cancer treatment may also predispose patients to xerostomia through acinar cell atrophy, fibrosis, and chronic inflammation of the salivary
glands. Most cancer patients received a total dose of 50-70 Gy that is given 5 days a week for 5-7 weeks with a daily dose of about 2 Gy (Guchelaar et al. 1997). A 50-60% reduction of salivary flow had been shown after just one week of radiation therapy. Permanent salivary hypofunction can occur in some receiving < 25 Gy of radiation (Grundmann et al. 2009). In a recent head and neck cancer treatment study, 36 patients were exposed to 70 Gy over 6 weeks. Overall, the stimulated and unstimulated saliva flow decreased to 29% and 33%, respectively, of the pretreatment level 12 months after therapy (Lal et al. 2010). In rats, healing after radiation therapy results in serous acinar cell fibrosis. Some studies show a down-regulation of the aquaporin-5 water channel at the apical membrane of the acinar cells, while others indicate a dose-dependent apoptosis of the salivary gland (Grundmann et al. 2009).

**Sjögren’s Syndrome**

Sjögren’s syndrome (SS) is a chronic autoimmune disorder characterized by lymphocytic infiltration and malfunction of the salivary and lacrimal glands (Fox 2005). The disease affects 3 million people in the US (Helmick et al. 2008) and is the second most common autoimmune connective tissue disorder.

The diagnosis of SS is made by meeting a selective number of subjective and objective criteria according to the Revised International Classification Criteria for Sjögren’s Syndrome (RICCSS) (Vitali et al., 2002). Subjective criteria include ocular (I) and oral dryness (II) complaint. Objectively, ocular criteria (III) includes positive Schirmer’s test (<5mm in 5min) or ≥4 ocular dye score, while oral criteria (V) includes low unstimulated whole salivary flow (≤1.5ml/15mins), parotid sialography indicating diffuse sialectasias without major ducts obstruction, or salivary scintigraphy with delayed
uptake and excretion. In addition, labial salivary gland (LSG) biopsy with \( \geq 1 \) focus score (IV) (area with \( \geq 50 \) lymphocytes per 4mm\(^2\)) and presence of serum autoantibodies (VI) to either or both SSA (Ro) and SSB (La) antigens are also indicative of a positive criteria. A patient can be classified as either primary SS (pSS) or secondary SS (sSS). They are classified as pSS when no associated diseases are present and they meet the following criteria: any 4 of the 6 criteria with either positive histology/serology or the presence of any 3 of the 4 objective criteria (objective ocular, objective oral, histology, serology). When patients are diagnosed with other autoimmune diseases, sSS may be considered in the presence of any subjective criteria plus any 2 of the following: objective ocular, objective oral, or positive histology (note that positive serology is not necessary for sSS diagnosis).

Early studies suggested that apoptosis of acinar epithelial cells in salivary glands was responsible for the xerostomic symptoms of SS patients (Maganelli and Fietta 2003); sufficient cell death would lead to a functionally significant decline in saliva production. One possible trigger may be the autoantibodies such as anti-Ro and anti-La that are expressed in autoimmune disease of SS. Recently, anti-Ro and anti-La were shown to penetrate LSG cells inducing apoptosis (Sisto et al. 2007). This mechanism consists of the activation of the cysteine proteases, the caspases, by the interaction of transmembrane Fas antigen and Fas Ligand. Through a series of pathways, caspases-3 was activated and induced nuclear DNA fragmentation (Maganelli and Fietta 2003). Recently, caspases 3 and FAS/FAS Ligand were shown to be expressed in the LSG of SS subjects (Herrera-Esparza 2008), consistent with apoptosis as a mechanism involved in SS.
However, questions have been raised that challenge the apoptosis model for loss of salivary function through cell death (Dawson et al. 2006). As suggested by Ohlsson et al. 2001, apoptosis is rare in SS. In addition, the proportional loss of saliva flow appears to greatly exceed the loss of tissue. The management of xerostomia by application of cholinergic agonists such as pilocarpine and cevimeline or mechanical stimulation from chewing gum (Porter et al. 2004) strongly suggests that the acinar cells of SS salivary glands are hypofunctional. The glandular dysfunction may be caused by a non-apoptotic mechanism in which there is a chronic immune-mediated inhibition of the acinar secretory cells (Dawson et al. 2006). Such a mechanism(s) may either involve the inhibition of muscarinic acetylcholine receptors (subtype M3R) by antimuscarinic autoantibodies (Nikolov and Illei 2009; Tsuboi et al. 2010), the impaired release of acetylcholine (Zoukhri and Kublin 2001), or the rapid degradation of neurotransmitter by acetylcholinesterase (Dawson et al. 2005). This model does not preclude a role for apoptosis in the generation of autoantibodies.

Overexpression of pro-inflammatory cytokines had also been implicated in both the systemic as well as exocrine manifestations of SS. Current autoimmune research indicates that an over-expression of IFNα (interferon α), BAFF (B cell activating factor), IL-6 (interleukin-6), IL-12/IL-23, and IL-17, is present in the salivary glands or serum of SS patients (Roescher et al. 2010). An elevated level of IFNα, produced by plasmacytoid dendritic cells from toll-like receptor activation, were detected in LSG biopsies of pSS (Gottenberg et al. 2006). IFNα induces BAFF, a cytokine that promotes B cell survival and is involved in germinal center formation. The ability of IFNα to induce BAFF production and the elevated BAFF in SS supported the treatment potential of anti-IFNα...
and anti-BAFF antibodies, however, cytokine-based SS treatment studies have reported
only limited efficacy (Mavragani and Crow 2010; Roescher et al. 2010).

**Xerostomia treatment options**

Treatment of patients with xerostomia currently revolves around mechanical
stimulation and topical and systemic agents. Unlike radiation-induced xerostomia,
(which is caused by salivary glandular damage and atrophy), or SS, (where the
mechanism underlying the pathology is still unknown), xerostomia induced by
medications may be controlled by modification of dosage or a medication change by the
primary physician. However, the underlying medical conditions may prevent such an
alternative. Until the salivary damage can be reversed, delayed, or diminished, or the
underlying mechanism(s) of xerostomia be elucidated, treatment modalities that focus
primarily on relieving the symptom —as opposed to curing the underlying pathology—
will remain the best that can be offered.

To prevent dental caries, proper oral hygiene, sealants, fluoridated mouthwash,
varnish, and topical fluoride treatment have been recommended (Guggenheimer and
Moore 2003). Mechanical stimulation from sugar-free gum, candy, or lozenges, along
with localized agents, such as salivary substitutes, water, lubricating gels, and oral
moisturizers are often used to help relieve xerostomic symptoms. However, both types of
treatment provide only short duration of comfort, are inconvenient (Porter et al. 2004),
and increased water consumption often disrupting daily activities due to frequent and
nocturnal urination.
Systemic agents such as Pilocarpine (Salagen) and Cevimeline (Evoxac) are two of most widely available pharmacological treatments for dry mouth. Pilocarpine, a cholinergic agonist, enhances salivary secretion by activating the M3R receptor in salivary glands cells. One study showed that a 5mg tablet taken 4 times a day (at mealtimes and bedtime) significantly relieved the subjective complaint of xerostomia and increased the objective salivary flow (Vivino et al. 1999). Cevimeline, an acetylcholine analogue with 40 times greater affinity for M3R than to the cardiac muscarinic receptor is another popular oral systemic agent. One earlier study showed that taking 30mg 3 times a day can significantly relieved subjective xerostomic symptoms and increase salivary flow (Fife et al. 2002).

Although these systemic agents were effective, there were dosage-dependent adverse effects due to the unwanted extraoral cholinergic receptor stimulation. Greater than 10% of subjects taking Pilocarpine complained of excessive sweating, headache, nausea, flu syndromes, diarrhea, dizziness, and dyspepsia (indigestion) (Vivino et al. 1999). Similar adverse effects also occured with subjects who took Cevimeline. Greater than 10% of subjects had excessive sweating, nausea, vomiting, dyspepsia, and frequent urination (Fife et al. 2002). Due to these unwanted conditions, some subjects prefer the alternative and discontinued their systemic medication.

**Recent research**

The mechanisms of xerostomia associated with pSS and sSS arising from autoimmune disease are still unclear. Although treatments are available, they provide only temporary relief and come with multiple adverse effects that lead to cessation of use. Thus, understanding the differences in cell metabolism and protein expression between
asymptomatic and xerostomia salivary glands associated with different causes may provide a clue to the pathology of SS.

One theory to the possible mechanism of xerostomia included apoptosis of the ductal and acinar epithelial cells of salivary glands (Manganelli and Fietta 2003). Even though this mechanism for xerostomia is contested, there is a lack of an animal model to set a foundation for a study (because it is impossible to obtain a subjective complaint from an animal). Therefore, the NOD (non-obese diabetic) mice model has been used. The NOD strain of mice spontaneously develops autoimmune diabetes, and also shows characteristics of human SS with lymphocytic infiltration and salivary hyposcretion by 16 weeks of age (Cha et al. 2002); however, the diabetic onset and the loss of secretory function are independent of each other (Humphreys-Beher et al. 1998), thus it was used for diabetic and SS research.

When compared to non-NOD mice, a statistically significant increase in apoptosis in the NOD (non-obese diabetic) mice was detected using the TUNEL (terminal deoxynucleotidyl-transferase dUTP nick end labeling) method (Gillespie et al. 2008), in contrast to the rare event from human study (Ohlsson et al. 2001). Interestingly, protein markers for the detection of proliferation, Ki-67 and PCNA (proliferating cell nuclear antigen), were also over-expressed (although only roughly 3% of cells were positive using Ki-67 compared to 15% of PCNA) in the salivary epithelial cells in the NOD mouse model. The TUNEL assay is a method used to detect DNA fragmentation by labeling the terminal end of nucleic acids, thus it detects DNA strand breakage, which occurs during apoptosis (but can also result from DNA damage). PCNA and Ki-67 are nuclear antigens expressed during the cell cycle, and signify DNA replication and cell
proliferation. In addition PCNA is also expressed during DNA damage repair. Thus, the TUNEL assay was used for detection of apoptosis while PCNA and Ki-67 were used to detect cell proliferation and/or DNA repair.

The mechanism of xerostomia in human SS had been linked previously to increased apoptotic activity, antimuscarinic autoantibodies, M3R inhibition and pro-inflammation cytokines overexpression. The histology of NOD mice submandibular glands shows that SS-like pathology not only includes increased apoptotic activity, but it also has a proliferation component that has not been investigated. Furthermore, when EGCG (epigallocatechin-3-gallate, a green tea polyphenol) was added to the NOD mouse drinking water, not only was the onset of diabetes delayed, but also the apoptosis and proliferative signals of salivary epithelial cells were significantly reduced (Gillespie et al. 2008). EGCG has been shown to have chemoprevention, antioxidant, and anti-inflammation properties (Nagle et al. 2006). EGCG was also shown to reduce the level of reactive oxidative species (ROS) in normal epithelial cells (Yamamoto et al. 2003). Whether the elevation of cell proliferation markers in the NOD model is also reflected in human salivary glands, or whether there is a relationship between antioxidation and xerostomia (as suggested by EGCG application) remained unknown. The ability of green tea polyphenol to delay symptom onset and its effect on apoptosis and proliferation in NOD mice model may in the near future translate into a new therapeutic approach for xerostomia.
D. Purpose

The purpose of this study was to determine if elevated protein levels of markers for cell proliferation and apoptosis are associated with xerostomia in humans using immunohistochemistry; and to establish proliferation and apoptosis indexes in labial salivary gland biopsies from xerostomia patients.

E. Hypotheses

Elevated proliferation cell nuclear antigen (PCNA) expression, independent from cell proliferation, serves as a marker for abnormality in human salivary glands associated with xerostomia.

F. Specific Aims

1. To determine protein expression of PCNA and Ki-67 by immunohistochemistry, and establish apoptosis index by TUNEL in archived human labial salivary gland specimens.

2. To perform a quantitative analysis of immunohistochemistry data and correlate with a subject’s xerostomia survey, autoantibody serology, and histological diagnosis.
II. Materials and Methods

Overview of Methods

Archived labial salivary gland (LSG) biopsies from 16 xerostomic subjects and 4 normal non-xerostomic subjects were retrieved from the archives of the Georgia Health Sciences University (GHSU) Clinical Center of Oral Medicine (CCOM) (Figure 3). Subjects' personal data, xerostomic condition, serological data, and pathological diagnosis were performed previously by GHSU-CCOM. Hematoxylin (H) and eosin (E) staining and terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) assay of all biopsies were performed by the GHSU core lab. In addition, immunohistochemistry using Ki-67 and proliferating cell nuclear antigen (PCNA) were performed in Dr. Kalu Ogbureke's lab.

Focus scores (FS, the number of isolated inflammatory cell aggregates containing 50 or more lymphocytes in each 4-mm² area (equivalent to 100X magnification)), were obtained from the H&E histology (Figure 5). The proportion of cells positive for TUNEL, Ki-67 and PCNA was determined by counting, and the value converted into apoptotic and proliferation indices (expressed as a percentage of total counted cells). The biopsies were categorized into six subject groups: normal (non-xerostomic), Sjögren's Syndrome (SS), xerostomia from medications (XMeds), xerostomia with autoimmune disease (XDz), xerostomia with negative serology (XNeg), and xerostomia without serology (Xw/oS). The mean and standard deviation of the apoptotic and proliferative
indices of each group were calculated. One-way analysis of variance (ANOVA) was performed to compare the means of each group on apoptotic and proliferation indices. Due to the small sample size of each subject group and the comparison of multiple data, statistically significant differences between subject groups were determined with P < 0.01.

**Figure 3.** Experimental design. This retrospective study consists of 20 subjects. Xerostomic Questionnaire, serologic data, and LSG biopsies were taken. Biopsy specimens were stained with H&E. Immunohistochemistry staining using the TUNEL method, Ki-67, and PCNA were performed.

**Patient Data and Biopsies:**

Sixteen subjects were previously referred from various medical specialties to Dr. DeRossi at the GHSU-CCOM to examine for Sjögren’s Syndrome (SS) while 4 subjects were referred for labial salivary gland (LSG) biopsy for pathology other than SS. CCOM collected serological and pathological data, and obtained subjects’ age, sex and
disease history. Subjective information was obtained using the Xerostomia questionnaire (Figure 4). Inclusive of the questionnaire was a visual analog scale (VAS) (Figure 4) that allowed the subjects to rate the severity of their dry mouth condition subjectively by marking a vertical line on a horizontal scale with “Dry as a desert” and “Not dry at all” corresponding to a VAS score of zero and 100, respectively. In addition, Dr. De Rossi also performed a LSG biopsy that was subsequently stained with H&E by the GHSU Core lab. Using the collective information, pathologic diagnosis was made for each subject by Dr. Kalu Ogbureke, a board-certified oral pathologist.

<table>
<thead>
<tr>
<th>Question</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Does the amount of saliva in your mouth seem to be too little, too much, or you do not notice it? (circle one)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have any difficulties swallowing?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Does your mouth feel dry when eating a meal?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you sip liquids to aid in swallowing dry food?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Please rate your present condition of dry mouth by marking a vertical line on the horizontal line</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry as a desert (0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not dry at all (100)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Figure 4. Xerostomia questionnaire. Subjects answer these questions and were asked to rate the severity of their dry mouth condition by marking on the horizontal line.*

**Histopathological Analysis**

The archived H&E specimens from the 20 subjects were retrieved from the
GHSU Core lab. The inflammatory cell infiltrate was determined by a standardized scoring system called a focus score (FS). As depicted on the H&E slide below (Figure 5), each circled area indicates a focus, and a FS of two was recorded for this biopsy. All LSG H&E specimens were examined and all FS were recorded.

![H&E staining of the LSG biopsy. Each blue circle indicates a focus. In the current biopsy, a focus score (FS) of 2 was recorded.](image)

**Figure 5.** H&E staining of the LSG biopsy. Each blue circle indicates a focus. In the current biopsy, a focus score (FS) of 2 was recorded.

**Sjögren’s Syndrome (SS) criteria:**

The diagnosis of SS is made upon meeting a selective number of subjective and objective criteria. A patient is classified as pSS (primary Sjogren Syndrome) when no associated diseases are present and they meet any 4 of the 6 criteria with either positive histology/serology or the presence of any 3 of the 4 objective criteria. When patients are diagnosed with autoimmune diseases, the presence of any subjective criteria and any 2 of objective ocular, objective oral, and positive histology, may be considered as sSS (secondary Sjogren’s syndrome). Subjective criteria include an ocular (I) and oral
dryness (II) complaint. Objective criteria include positive Schirmer's test or ≥4 ocular dye score (III), low unstimulated whole salivary flow (≤1.5ml/15mins) (IV), LSG biopsy with ≥1 focus score (IV), and presence of serum autoantibodies (VI) to either or both SSA(Ro) and SSB(La) antigens (Vitali et al. 2002).

In the present retrospective study, objective ocular and oral criteria were not recorded from the subjects. Therefore, a true SS diagnosis based on the RICCSS is not possible for certain subjects. However, a SS diagnosis could still be made if the objective ocular and oral criteria were omitted. According to Fox and Saito (Ryo et al. 2006), patients are diagnosed with SS when 3 of the following criteria are met: (1) the presence of keratoconjunctivitis sicca, where the tear volume was measured by the Schirmer I test (< 5 mm/5 min), (2) dry mouth, (3) extensive lymphocytic infiltrate on minor salivary gland biopsy, and (4) laboratory evidence of a systemic autoimmune disease, which includes positive rheumatoid factor (titer 1: 160), positive antinuclear antibodies (titer 1: 160), or positive SS-A or SS-B antibodies.

**Immunohistochemical Analysis**

Immunohistochemical staining is a process in which proteins in tissues are specifically recognized by binding of primary antibodies, followed by colorimetric reaction using a secondary antibody, enzyme coupling and chromogenic substrates to detect the location of the primary antibody (and hence, the target antigen). This process identifies whether and how much a specific protein is expressed in the tissue samples, and if so, where. In the present study, immunohistochemical staining using TUNEL, Ki-67 and PCNA was performed as described in detail below.
**Apoptosis: TUNEL Assay**

The archived LSG specimens were sectioned and sent to the GHSU Core lab for TUNEL processing. Cells with nuclear DNA fragmentation was visualized by the TUNEL assay using the ApopTag Plus Peroxidase in situ apoptosis detection kit (Chemicon International, CA, U.S.A.) according to the manufacturer’s directions (Figure 6). Briefly, after deparaffinization, protein digestion enzyme was applied to the specimen for 15 minutes at room temperature. Endogenous peroxidase activity was quenched using 3% hydrogen peroxide in PBS (phosphate buffered saline) for 5 minutes at room temperature. An equilibration buffer was used to incubate the specimen for 10 seconds at room temperature. Then the sections were incubated with 55 uL/5 cm² concentration of terminal deoxynucleotidyl-transferase (TdT) enzymes incorporated with digoxigenin-conjugated nucleotides in a moist chamber for 60 minutes at 37°C. Incubation with 65 uL/5 cm² concentration antidigoxigenin antibody conjugated with peroxidase for 30 minutes at room temperature was then conducted, followed by color development with DAB (3,3'-DiAminoBenzidine), a peroxidase substrate. Hematoxylin was used for counterstaining.

Using 200X magnification light microscopy, each archived LSG specimen returned from the GHSU Core lab were photographed and quantitatively analyzed. Positive cells were identified by the brownish DAB chromogen against a blue hematoxylin-stained background. TUNEL Indices (TI) were recorded. TI was defined as the number of TUNEL-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage. As depicted in the diagram below, after 1000 cells
counted, a TI of 0.2% was recorded. All archived LSG biopsy specimens were examined and the TI recorded.

\[
TUNEL \text{ index (\%)} = \frac{\text{Number of TUNEL positive cells}}{1000} \times 100
\]

\[
67\% = \frac{670}{1000} \times 100
\]

**Figure 6. TUNEL. Apoptosis assay. Brown chromogenic staining indicates apoptosis.**

**Cell Proliferation: Ki-67**

Cell proliferation was detected using anti-Ki-67 polyclonal antibody (Abcam Inc, MA, U.S.A.) (Figure 7). Briefly, the archived LSG deparaffinized sections were immersed in methanol containing 3% hydrogen peroxide for 20 minutes. Antigen retrieval was performed with a 10% solution of Diva Decloaker© at a 125° Celsius for 30 seconds and cool at 90° Celsius for 10 seconds. The sections were incubated with anti-Ki-67 polyclonal antibody diluted to 1:1000 and incubated for 1 hour at room temperature. A biotinylated secondary antibody conjugate with horse radish peroxidase
(HRP) was incubated for another 10 minutes. Peroxidase staining was performed for 3 to 7 min using a solution of AEC (3-Amino-9-EthylCarbazole) chromogen. Hematoxylin was used for counterstaining.

Using 200X magnification light microscopy, each Ki-67 positive cell was identified by the brownish AEC chromogen on a blue hematoxylin stained background. Ki-67 Indices (KI) were recorded. KI was defined as the number of Ki-67-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage. As depicted on the diagram below, if the slide shows the same proportion of positive and negative cells, a KI of 2.8% will be recorded. All archived LSG biopsies specimens were immunohistochemically stained, examined, and KI recorded.

\[
Ki-67 \text{ index (\%)} = \frac{\text{Number of Ki-67 positive cells}}{1000} \times 100
\]

\[
2.8\% = \frac{7}{250} \times 100
\]

Figure 7. Ki-67. Proliferation assay. Brown chromogenic cells indicate proliferation.

Cell Proliferation and Repair: PCNA
Cell proliferation and repair were detected using anti-PCNA polyclonal antibody (FL-261; Santa Cruz Biotechnology, CA, U.S.A.) (Figure 8). Briefly, the archived LSG deparaffinized sections were immersed in methanol containing 3% hydrogen peroxide for 20 minutes. After antigen retrieval using Diva Decloaker©, the sections were incubated with anti-PCNA polyclonal antibody diluted to 1:100 and incubated overnight at 4°C. A biotinylated secondary antibody conjugate with HRP was incubated for another 10 minutes. Peroxidase staining was performed for 3 to 7 min using a solution of AEC chromogen. Hematoxylin was used for counterstaining.

Using 200X magnification light microscopy, PCNA positive cells were identified by the brownish AEC chromogen in a blue hematoxylin stained background. PCNA Indices (PI) were recorded. PI was defined as the number of PCNA-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage. As depicted on the diagram below, if the slide shows the same proportion of positive and negative cells, a PI of 67% will be recorded. All archived LSG biopsies specimens were immunohistochemically stained, examined, and PI recorded.

\[
PCNA \text{ index } (\%) = \frac{\text{Number of PCNA positive cells}}{1000} \times 100
\]

\[
67\% = \frac{670}{1000} \times 100
\]
Figure 8. PCNA. Proliferation and DNA repair assay. Brown chromogenic cells indicate proliferation and/or repair.

Statistical analysis:

After the focus score and various indices of apoptosis, proliferation, and DNA repair were recorded, subject data (such as serology, pathologic diagnosis, subjects age, sex, and disease history) collected by Dr. DeRossi from GHSU-CCOM corresponding to each biopsy specimen were released. According to the common etiology of xerostomia, subjects were separated into six groups (Table 1, 3). Group 1—Sjögren’s Syndrome (SS). These were subjects who met a set of selective criteria made by the RICCSS or according to Fox and Saito. Group 2—Xerostomia from medications (XMeds). These were subjects who are taking xerostomia-inducing medications. Group 3—Xerostomia with autoimmune disease (XDz). These were subjects who were diagnosed with autoimmune disease by their physician and have xerostomia that was not caused by medications, radiation therapy, or autoimmune diseases other than SS. Group 4—
Xerostomia with negative serology (XNeg). These were subjects in which the etiology of their xerostomia was unknown. When the serology test was performed, these subjects show no SSA, SSB, AntiNuclearAntibodies (ANA), or Rhematoid Factor (RF). Group 5—Xerostomia without serology test (Xw/oS). These subjects also have unknown etiology for xerostomia however, serology exam was not provided. Group 6—Normal. These subjects were not diagnosed with Sjögren’s Syndrome, were not xerostomic, and serology test were not performed.

The apoptotic and proliferation indices of each subject for SS, XMeds, XDz, XNeg, and Xw/oS were compared to normal subjects. The mean, standard error, and standard deviations were calculated from each group. One-way analysis of variance (ANOVA) was then performed using these results to compare the means of each group on apoptotic and proliferation indices. Due to the small sample size of each subject group and the comparison of multiple data, statistically significant differences between subject groups were determined with P <0.01.
III. Results

In this retrospective study, information from a total of 20 subjects was evaluated. As defined, 4 subjects met the criteria for SS, one subject had xerostomia from medication (XMeds), 6 subjects had xerostomia with autoimmune disease (XDz), 2 subjects had xerostomia with unknown causes showing negative serology (XNeg), 3 subjects had xerostomia without serology performed (Xw/oS) (Table 1,3), while 4 subjects were non xerostomic normal. Thirteen out of 16 xerostomic subjects took the Xerostomia questionnaire presented to them by Dr. DeRossi. The referring physicians had provided serologic information for 13 subjects. Normal subjects did not participate in the Xerostomia questionnaire or present with serology report. Dr. DeRossi also performed LSG biopsy from all subjects to be evaluated using H&E, TUNEL, Ki-67, and PCNA immunohistochemistry.

Xerostomia questionnaire

A total of 13 subjects took the Xerostomia questionnaire (Figure 4, Table 2). Two of the 3 SS subjects who took the questionnaire stated there was too little saliva in their mouth, while the remaining 10 subjects who took the questionnaire from the XMeds, XDz, XNeg, Xw/oS groups indicated too little saliva. Twelve of 13 subjects indicated that there seemed to be too little saliva in their mouths. Seven subjects stated that they had difficulty in swallowing. Seven subjects also complained of their mouth feeling dry.
Table 1: Group categorization. Objective ocular and oral criteria were not recorded from any patients, while subjective ocular symptoms were only provided by some, therefore a true SS diagnosis based on the Revised International Classification Criteria for Sjögren’s Syndrome (RICCSS) is not possible for certain subjects, therefore Fox and Saito criteria were substituted. Normal subjects did not provide Serology. (Note: ANA/RF are not RICCSS criteria, ANA=AntiNuclearAntibody, RF=Rheumatoid Factor, NP=not provided)

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sjögren’s Syndrome (SS)</th>
<th>Xerostomia from Medications (XMeds)</th>
<th>Xerostomia with Autoimmune Disease (XDz)</th>
<th>Xerostomia with Negative Serology (XNeg)</th>
<th>Xerostomia without Serology (Xw/oS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>08-839</td>
<td>09-417</td>
<td>09-898</td>
<td>08-1171</td>
<td></td>
</tr>
<tr>
<td>Subjective Ocular Symptoms</td>
<td>NP</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Subjective Oral Symptoms</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Objective Ocular Symptoms</td>
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<td></td>
<td></td>
<td></td>
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<td>Histology</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Objective Oral Symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autoantibodies (SSA and/or SSB)</td>
<td>SSA/SSB</td>
<td>SSA</td>
<td>NEG</td>
<td>SSA</td>
<td>NEG</td>
</tr>
<tr>
<td>ANA/RF</td>
<td>NEG</td>
<td>ANA</td>
<td>ANA</td>
<td>RF</td>
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</tr>
</tbody>
</table>
when eating while 9 required the aid of liquid to swallow dry food. Only 4 subjects that noted difficulties in swallowing, eating a meal, and needing liquid for dry food indicated too little saliva.

Upon examining the visual analog scale (VAS) score, the xerostomic subjects had a VAS score ranging from 0-98 (n=13, mean=38, SD=25). The VAS score range of SS was 25-98 (n=3, mean=59, SD=37), XMeds was 37 (n=1), XDz was 18-52 (n=5, mean=33, SD=13), XNeg was 15-54 (n=2, mean=35, SD=28), while Xw/oS was 0-3 (n=2, mean=1.5, SD=2). When one-way ANOVA was performed, the VAS mean scores between groups was not significantly different (p>0.20) (Figure 9).

The questionnaire results indicate that each subject with dry mouth, even those with SS, have various xerostomic experiences when encountering different activities, such as swallowing, eating a meal, or swallowing dry food. The subjective report of xerostomia using the VAS score provides an indication that the severity of xerostomia is not significantly different between SS, XMeds, XDz, XNeg, Xw/oS groups.
Table 2: Summary of the Xerostomia questionnaire categorized by group. Thirteen out of 16 xerostomic subjects participated in the questionnaire. Twelve stated too little saliva, while 7 indicated difficulty swallowing and mouth feels dry when eating. Nine subjects stated that they need to sip liquid to aid in swallowing dry food. Normal subjects did not participate in the Xerostomia questionnaire. (Note: NP=Not provided)

<table>
<thead>
<tr>
<th>Questionnaire</th>
<th>Sjögren’s Syndrome (SS)</th>
<th>Xerostomia from Medications (XM)</th>
<th>Xerostomia with Autoimmune Disease (XDz)</th>
<th>Xerostomia with Negative Serology (XNeg)</th>
<th>Xerostomia without Serology (Xw/oS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Saliva</td>
<td>NP</td>
<td>Not noticed</td>
<td>Too little</td>
<td>Too little</td>
<td>Too little</td>
</tr>
<tr>
<td>Difficulty swallowing</td>
<td>NP</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Feel dry when eating</td>
<td>NP</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Need liquid to swallow dry food</td>
<td>NP</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>VAS (mm)</td>
<td>NP</td>
<td>98</td>
<td>25</td>
<td>55</td>
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</table>

(Note: NP=Not provided)
**Figure 9:** Visual analog scale from xerostomic subjects. Although the VAS score ranges from 0-98 between all groups, the mean VAS score between each groups were not significantly different (p>0.20). Normal subjects did not participate in the Xerostomia questionnaire.

**Serology**

Serological titers for AntiNuclearAntibodies (ANA), Rhematoid Factor (RF), Anti-Sjögren’s Syndrome A (SSA), and Anti-Sjögren’s Syndrome B (SSB) obtained from subjects with xerostomia were provided by Dr. DeRossi (Table 1). A total of 13 xerostomia subjects presented with ANA, RF, SSA, or SSB serology information, while serologic information from 3 Xerostomic subjects (Xw/oS) were not provided. Serology was recorded as either positive or negative for each autoantibody category. Ten out of 13 subjects were positive for at least one autoantibody. Out of the 13 subjects with serology,
ANA titer was positive in 6 subjects and RF was present in 3 subjects. SSA and SSB were positive for 4 and 1 subjects, respectively. Two subjects were positive for both SSA and RF, 1 subject was positive for both SSA and SSB, and 1 subject was positive for both ANA and SSA.

Subjects with SS showed mixed serology presentations. One SS subject did not have either SSA or SSB. The 3 other SS subjects were all positive for SSA while one subject was also SSB positive. Within this subject group in the present study, there was no SS subject with only SSB serology. Two SS subjects also showed ANA positivity (Table 1).

Except for one subject with both SSA and RF positivity, there were no SSA or SSB positive results within subjects who provided serology information in the XDz group. ANA positive serology was found in 4 subjects while 2 were RF positive (Table 1).

Labial Salivary Gland Biopsies

Histology using H&E

In total, 20 LSG biopsies from 16 xerostomic subjects and 4 normal subjects were examined in this study (Table 3). Upon examining the LSG biopsy using H&E (Figure 10), normal subjects did not show clusters of lymphocytes around the LSG ducts. Clusters of >50 lymphocytes surrounding the LSG ducts were clearly identifiable in all LSG biopsies of the SS and the XMeds groups. In the majority of the LSG biopsies of the XDz, XNeg, and Xw/oS groups, lymphocytic clusters were not consistently seen.
Pathologic diagnosis of all 20 biopsies (xerostomia and normal) were performed by Dr. Ogbureke. The pathology reports confirmed 4 subjects consistent with SS. Seven subjects were not SS (4 subjects of which were normal), while 9 subjects’ pathologic results could not confirm or rule out SS. Upon examination with H & E, all 4 confirmed SS subjects showed a focus score > 1. Of the 9 patients whose diagnosis could not confirmed or disapproved, 4 had focus scores of 1 while the other 5 had focus score =0. Subjects who were not SS all have focus score of 0 (Table 3).
Table 3: Histopathology SS diagnosis and immunohistochemistry analysis of Apoptosis (TUNEL) and Proliferation (Ki-67 and PCNA) categorized by group. All 4 SS subjects show a focus score > 1. Out of the 9 patients who cannot be ruled out as SS, 4 have foci score of 1 while the others 5 have foci score =0. Subjects who are not SS all have Focus score of 0. Apoptosis as measured using the TUNEL Index has a range of 0-1.9 (Mean=0.6, SD=0.42). Proliferation as measured using the Ki-67 Index and Proliferation with DNA repair measured using the PCNA Index range from 0.4-3.0 (Mean=1.6, SD=0.66) and 12-91 (Mean=63, SD=23), respectively. (Note: CR/O=Cannot rule out Sjögren’s Syndrome)

<table>
<thead>
<tr>
<th>Pathology Diagnosis</th>
<th>Normal</th>
<th>Sjögren’s Syndrome (SS)</th>
<th>Xerostomia from Medications (XMedS)</th>
<th>Xerostomia with Autoimmune Disease (XDz)</th>
<th>Xerostomia with Negative Serology (XNeg)</th>
<th>Xerostomia without Serology (Xw/oS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focus Score (H&amp;E)</td>
<td>0.19</td>
<td>0.45</td>
<td>0.46</td>
<td>0.30</td>
<td>0.10</td>
<td>0.00</td>
</tr>
<tr>
<td>TUNEL Index (Apoptosis)</td>
<td>0.31</td>
<td>0.30</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Ki-67 Index (Proliferation)</td>
<td>1.13</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
<td>1.11</td>
</tr>
<tr>
<td>PCNA Index (Proliferation/DNA repair)</td>
<td>12</td>
<td>22</td>
<td>30</td>
<td>16</td>
<td>73</td>
<td>61</td>
</tr>
</tbody>
</table>
Figure 10. H&E histological staining representative of each subject group. Hematoxylin stains the nucleus of cells and lymphocytes blue while eosin stains the cytoplasm and connective tissue red. 10A. Normal (09-456) 10B. SS (09-498) 10C. XMeds (08-1171) 10D. XDz (09-611) 10E. XNeg (09-1000) 10F. Xw/oS (09-1272)
Immunohistochemistry

*TUNEL—Apoptosis Assay*

Upon examining the LSG biopsy using the TUNEL method (Figure 11), apoptotic positive brown chromogenic cells were rarely found in all 6 subject groups. Overall, as recorded in Table 3, apoptosis as noted utilizing the TUNEL method demonstrated TUNEL Indices ranging from 0%-1.9% (n=20, mean=0.3, SD=0.42). Normal subjects had TI ranging from 0.1%-1.9% (n=4, mean=0.62, SD=0.85). SS subjects had TI ranging from 0.1%-0.2% (n=4, mean=0.12, SD=0.05). The one XMeds subject had TI of 0.2%; XDz subjects had TI ranging from 0%-0.4% (n=6, mean=0.2, SD=0.15); the 2 XNeg subjects had TI of 0.1% and 0.3%; and the Xw/oS subjects had TI ranging from 0%-0.7% (n=3, mean 0.4, SD=0.38). Statistical analysis using the One Way ANOVA showed no significant differences in apoptosis (p>0.61) between each of our observation groups (Figure 12). Thus, this data suggested that apoptosis (as determined by the proportion of TUNEL-positive cells) may not play a prominent role in xerostomia within this subject sample, at least in terms of gross tissue damage.
Figure 11. TUNEL, Apoptotic expression representative from each subject group. Nuclei of cells were stained blue (Hematoxylin). Apoptotic cells were stained brown (DAB). 11A. Normal (subject 09-456) 11B. SS (subject 09-498) 11C. XMeds (subject 08-1171) 11D. XDz (subject 09-611) 11E. XNeg (subject 09-1000) 11F. Xw/oS (09-1272)
Ki-67—Proliferation Assay

Brown chromogenic cells were counted as positive in this Ki-67 assay and a KI was determined (Figure 13). Below is a representative immunohistochemistry result. Overall, proliferation of epithelial cells observed utilizing Ki-67 had a Ki-67 Index (Table 3) ranging from 0.4%-3.0% (n=20, mean=1.6%, SD=0.42%). Normal subjects had KI ranging from 1.1% to 2.3% (n=4, mean=1.7%, SD=0.55%); SS subjects had a KI ranging from 1.0%-1.9% (n=4, mean 1.5%, SD=0.45%); the one XMeds Subject had KI of 1.7%; XDz subjects had KI ranging from 0.4% to 1.9% (n=6, mean=1.2%, SD=0.6%); the 2 XNeg subjects had KI of 1.2 and 2.1; and the Xw/oS subjects had KI ranging from 0.8%-3% (n=3, mean=2.1%, SD=1.2%). Statistical analysis using One Way ANOVA showed no statistical differences (p>0.53) between any of our observation groups. Therefore, this data suggested that proliferation of epithelial cells (as determined by the
proportion of Ki67-positive cells) may not play a role in xerostomia in this subject sample (Figure 14).

**Figure 13.** Ki-67, Proliferation assay representative of each subject group. Nuclei of cells were stained blue (Hematoxylin). Ki-67 positive cells were stained brown (AEC). 13A. Normal (subject 09-456) 13B. SS (subject 09-498) 13C. XMed (subject 08-1171) 13D. XDz (subject 09-611) 13E. XNeg (subject 09-1000) 13F. Xw/oS (09-1272)
Brown chromogenic cells were counted as positive in this PCNA assay and a PI was determined (Figure 15). Below is a representative immunohistochemistry result.

Overall, proliferation and DNA repair of epithelial cells observed utilizing PCNA had a PCNA Index (Table 3) ranging from 12%-91% (n=20, mean 63%, SD=23%). Normal subjects had PI ranging from 12%-30% (n=4, mean=20, SD=7.8); SS subjects had a PI ranging from 61%-80% (n=4, mean=72, SD=7.9); the one XMed subject had PI of 77%; XDz subjects had PI ranging from 67%-81% (n=6, mean=74, SD=5.8); the 2 XNeg subjects had PI of 78% and 91%; and the Xw/oS subjects had PI ranging from 62%-74% (n=3, mean=66, SD=6.7%). When the mean PI of the normal group was compared to each of the xerostomic groups, there was a 3 to 4 fold increase in PCNA expression in the
xerostomic subjects (Table 4). Statistical analysis using One Way ANOVA showed a statistically significant differences (p<0.001) between the normal group to every other observation groups. This suggested that proliferation and DNA repair of epithelial cells (as determined by the proportion of PI-positive cells) may play a role in xerostomia in the current subject samples (Figure 16).
Figure 15. PCNA, Proliferation and DNA repair assay representative of each subject group. Red arrows indicate PCNA positive chromogenic cells. Black arrows indicate PCNA negative cells. 15A. Normal (subject 09-456) 15B. SS (subject 09-498) 15C. XMeds (subject 08-1171) 15D. XDz (subject 09-611) 15E. XNeg (subject 09-1000) 15F. Xw/oS (09-1272)
Ki-67 is a proliferation assay whereas PCNA is both a proliferation as well as a DNA repair assay. To estimate the isolated DNA repair activity, data from Ki-67 and PCNA of the normal and all xerostomic subjects were examined. As shown in table 4, the mean Ki was 1.7% in normal and 1.5% in xerostomic subjects while the mean PI was 20% in normal and 74% in xerostomic subjects. The estimated ratio of proliferation to DNA repair indexes in the normal subjects was 1:11 while in the xerostomic subjects it was 1:49. This is consistent with a 5-fold increase in DNA repair-positive cells in the xerostomia groups compared to normal subjects. When the normal subjects were compared to each individual xerostomia group, there appeared to be 3 to 6 times more DNA repair in each xerostomia group when compared to normal.
Table 4. Proliferation and DNA repair comparison using mean KI and mean PI. (Note: Xerostomia consists of the SS, XMeds, XDz, XNeg, and Xw/oS groups)

<table>
<thead>
<tr>
<th></th>
<th>Mean KI</th>
<th>Mean PI</th>
<th>Proliferation: DNA repair ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.7%</td>
<td>20%</td>
<td>1:11</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>1.5%</td>
<td>74%</td>
<td>1:49</td>
</tr>
<tr>
<td>SS</td>
<td>1.5%</td>
<td>72%</td>
<td>1:48</td>
</tr>
<tr>
<td>XMeds</td>
<td>1.7%</td>
<td>77%</td>
<td>1:45</td>
</tr>
<tr>
<td>XDz</td>
<td>1.2%</td>
<td>74%</td>
<td>1:62</td>
</tr>
<tr>
<td>XNeg</td>
<td>1.7%</td>
<td>85%</td>
<td>1:50</td>
</tr>
<tr>
<td>Xw/oS</td>
<td>2.1%</td>
<td>66%</td>
<td>1:31</td>
</tr>
</tbody>
</table>
IV. Discussion

Sjögren’s Syndrome (SS), is a leading cause of xerostomia due to salivary gland hypofunction. This study shows for the first time that labial salivary glands (LSG) of xerostomic subjects (with or without a confirmed diagnosis of SS) have a consistently high level of proliferating cell nuclear antigen (PCNA) expression, but not Ki-67 expression, in comparison to normal non-xerostomic subjects. The PCNA expression in xerostomia subjects was independently elevated with respect to the focus score or serology, standard criteria for diagnosis of SS. Apoptosis, as determined by immunohistochemical analysis using the TUNEL method, was not significantly different (Figure 12, p>0.61) between the xerostomic and non-xerostomic control groups.

Although multiple reports in the literature have implicated apoptosis as the source of salivary dysfunction, (Patel et al. 2000; Maganelli and Fietta 2003; Ping et al. 2005; Herrera-Esparza et al. 2008), the apoptotic theory for the pathogenesis of autoimmune disease-related xerostomia has been put into questions by many researchers (Esch 2001; Dawson et al. 2006; Nikolov and Illei 2009). Today, apoptosis remains an active research topic; however, alternative theories of xerostomia pathogenesis have focused on cytokine dysregulation (Mavragani and Crow 2010; Roescher et al. 2010), free radical damage from ROS and RNS stress (Dickinson et al. 2010), and autoantibodies interaction with M3R cholinergic receptors (Dawson et al. 2006; Nikolov and Illei 2009).
It is important to note (1) the specificity of the TUNEL assay was 87% while the sensitivity ranged from 61 to 90% (Kelly et al. 2003), (2) the TUNEL method labels DNA breakage at free 3’-OH ends regardless of how those ends are generated; this can be by apoptosis, but also by necrosis, or DNA damage and repair processes (Baima and Sticherling 2002), and (3) since apoptosis is a rapid process, histology may only provide us a glimpse of the apoptotic activity at a specific moment in time rather than an overall activity of the tissue in question (Hall 1999). Other apoptotic labeling methods focus on the antigenic presentation of Fas/FasL receptor proteins (Ping et al. 2005). Antigen may degrade and affect immunoreactivity or fixation may alter dimensional structure (Yaziji and Barry 2006) leading to contrasting results. Data obtained in this study do not support a role for apoptosis in gross loss of glandular tissue as a precursor to xerostomia. Immunohistologically, no significant apoptosis were seen from the TUNEL assay between xerostomic and normal groups. Histologically, LSG structure remained intact with no gross fibrosis observed even in confirmed SS subjects.

While Ki-67 expression was similar (P>0.53) between all subjects, PCNA expression in the normal subject group was significantly different (P<0.001) than xerostomic subject groups regardless of a positive SS diagnosis. Both Ki-67 and PCNA are markers for cell proliferation (Gerdes et al. 1984; Celis et al., 1987). The lack of elevation of Ki-67 in xerostomic LSGs (with or without SS) indicates that cell proliferation does not increase in the human LSG. This is in contrast to the modest increase in Ki-67 expression seen in the ductal epithelium of submandibular glands in the non-obese diabetic (NOD) mouse (Gillespie et al. 2008). The NOD mouse is a widely used animal SS model that is genetically predisposed to phenotypically express
autoimmune diabetes and hyposalivation (Humphreys-Beher et al. 1998). Whereas Ki-67 expression was localized to ductal epithelial cells in the NOD mouse, low numbers of Ki-67 positive cells were randomly distributed between the acinar and ductal epithelium in human. In addition, Ki-67 positive cells in 22-weeks non-EGCG feed NOD mice increased nearly 20-fold compared to the non-NOD mice. In the present human study however, Ki-67 expression was not significantly different (Figure 14, p>0.53) between xerostomic groups with or without SS compared to non-xerostomic controls. While the Ki-67 expression (3.7%) in the 22-weeks NOD mice was significant (p<0.0001) (Gillespie et al. 2008), the human xerostomic subjects showed a non-significant average of 1.5% Ki-67 positivity compared to normal individuals (Table 4).

The differences between the mouse and human study of xerostomia may be due to the fact that: 1) the mouse model inherently possesses a genetic disorder (Cha et al. 2002) that may skew the Ki-67 expression; 2) although the mouse model demonstrates hyposalivation, the subjective complaint of xerostomia cannot be validated with murine histology; 3) the inflammatory changes in the NOD mouse salivary gland do not correlate with the severity of secretory symptoms (Jonsson et al. 2006); 4) the phenotype of the mouse seromucous submandibular salivary glands are different than the labial minor salivary glands in human (Tandler and Phillips 1993); and 5) the biopsy in the mouse model consists of a large portion of the submandibular gland while only a small sample of 5-7 minor glands (Greenspan et al. 1974) were examined in the human LSG biopsy.

Unlike Ki-67, PCNA expression shows considerable similarities between the mouse submandibular glands and the human LSG biopsies. Firstly, PCNA expression
was elevated dramatically in both xerostomic human LSG and non-EGCG feed NOD mice. Whereas a 13-fold increase was seen in the PCNA expression in the 22-weeks non-EGCG fed NOD mice, as compared to non-NOD mice (Gillespie et al. 2008), there was a significant increase (p<0.001) of 3 to 6 fold in PCNA expression in the LSG of xerostomic subjects compare to non-xerostomic subjects (Table 4). Secondly, ductal epithelium showed a similar PCNA response between the salivary glands of non-EGCG feed NOD mice and xerostomic humans. In the NOD mice, a high level of PCNA expression was located primarily in the ductal epithelium, and the ductal epithelial cells of the human LSG always showed positive PCNA expression. Although there was only 14.6% of PCNA positive cells in the submandibular glands of mice (Gillespie et al. 2008), human xerostomic subjects showed an average of 74% PCNA positivity in the LSG (Table 4). Thirdly, when the proportion of Ki-67 and PCNA positive cells at 22 weeks of age in the water-fed mice group were examined, (3.7% vs 14.6%) a 4-fold increased in DNA repair was seen (Gillespie et al. 2008), similar to the 5-fold increase in DNA repair in the human xerostomia groups compare to normal subjects in the current study (Table 4).

The similar PCNA expressivity between NOD mice submandibular glands and human LSG may point toward the critical role PCNA plays in DNA repair (Maga et al. 2003; Ulrich 2004; Kelman 1997). PCNA is a ring shaped homotrimer (a protein composed of 3 identical polypeptide units) of 29-kDa subunits that belongs to the DNA sliding clamp family (Figure 17). The central channel that accommodates DNA is 34Å wide. Its inner surface is positively charged, allowing it to associate and encircle the
negatively charge phosphodiester backbone of DNA (Kelman 1997). The outer surface consists of a conserved motif termed the PIP (PCNA-interacting proteins) box that allows PCNA-protein interaction. The homotrimeric structure allows multiple different PIP interactions with other proteins simultaneously (Ulrich 2004).

Figure 17: PCNA homotrimer structure (Maga et al. 2003). PCNA protein consists of three identical polypeptides. Its inner surface is positively charged, allowing it to associate and encircle the negatively charge phosphodiester backbone of DNA. The outer surface consists of a conserved motif termed the PIP (PCNA-interacting proteins) box that allows PCNA-protein interaction.

The 4961 base pair PCNA gene is located on chromosome 20 and consists of 6 exons and 5 introns (Travali et al. 1989, Ottavio et al. 1990). Transcription of the PCNA gene is very low in quiescent cells but once stimulated, the rate of transcription is similar between Go and S phase. The signal for transcription is growth regulated, as seen when the PCNA mRNA levels are increased when cells are experimentally stimulated with platelet derived growth factor (PDGF) or epidermal growth factor (EGF) (Chang et al. 1990). PCNA also undergoes post-translational modification that may be essential for DNA replication. PCNA affinity to DNA polymerases increases when PCNA is
acetylated and decreases when deacetylated (Naryzhny and Lee 2004). Degradation of PCNA occurs at a low level during S and G2 phases and is proteosome-dependent (Naryzhny and Lee 2003).

PCNA is a multifunctional protein. It is found at high levels in the nucleus during S phase of the cell cycle (Kelman 1997). However, it is also present in the cytoplasm and has been found to interact with the enzymes involved in glycolysis as well as enzymes of the mitochondria that regulate energy generation. (Naryzhny and Lee 2010). Though upregulation of PCNA expression has yet to be linked directly to disease causality, PCNA expression had been found to be correlated to tumor stages in adrenal carcinoma (Martins et al. 2005), recurrence in primary basal cell carcinoma (Toth et al. 1996), advanced prostate cancer (Zhao et al. 2011) and as a prognostic factor for oral cancer patients undergoing radiation therapy (Mallick et al. 2010).

Structurally, PCNA contains 3 types of binding sites: an interdomain connecting loop, N-terminus, and C-terminus. Proteins that bind to PCNA are involved in chromatin metabolism, DNA replication, and DNA repair. (Maga et al. 2003; Kelman 1997) In Chromatin metabolism, one function of PCNA is involved in the degradation of cdt1. The PCNA-cdt1 interaction promotes the ubiquitylation of cdt1, leading to cessation of DNA replication. Without this interaction, cdt1 will not be degraded and DNA replication will continue, leading to aneuploidy. (Moldovan et al. 2007)

In DNA replication, PCNA is first loaded onto DNA by clamp loader RF-C. (Maga et al. 2003; Kelman 1997). DNA polymerase is then bound to the PIP box of PCNA as it encircles and slides along the DNA. The PCNA sliding is bidirectional following the helix of the DNA (Kochaniak et al. 2009). Subsequently, polymerase δ
will perform both continuous and discontinuous DNA strand synthesis. At the completion of the discontinuous synthesis, RF-C unloads the PCNA from the Okazaki fragment. Ligation of the Okazaki fragments is then performed by PCNA-binding protein Fen1 and Lig1. In the absence of PCNA, abnormal leading and lagging strand replication occurs (Maga et al. 2003; Kelman 1997).

When DNA damage occurs, DNA replication can either stall, continue from the undamaged sister chromatid, or continue from the damaged strands (Figure 18). In damaged strand replication, low fidelity DNA polymerases, such as Polymerase η, which insert adenine to an opposing thymine dimer, are activated. This translesion synthesis (TLS) is error-prone and often highly mutagenic. The RAD6 pathway, which encodes ubiquitin-conjugated enzyme (E2), is important in controlling the error-prone TLS. However, RAD6 also play a role in the error-free DNA repair (Ulrich 2004).

**Figure 18:** PCNA and DNA repair. Error-prone or error-free DNA repair (Andersen 2008) may occur subsequent to DNA damages using the sister chromatid DNA strand. DNA repair may also performed by homologous recombination.
Ubiquitin (Ub) is a highly conserved 76 amino acid protein. When Ub is covalently linked to a protein by Ub-activating enzyme, the activity of the protein changes. PCNA is a protein whose function is modified when ubiquinated. When PCNA is monoubiquitinated, an error-prone (mutagenic) TLS is promoted. (Ulrich 2004, 2005; Andersen et al. 2008) PCNA monoubiquitylation requires ubiquitin-activating enzyme E1, E2 (Rad6 protein), and E3 (Rad 18). When PCNA is polyubiquitinated, it is believed an error-free pathway utilizing the sister chromatid as a template for repair occurs. Polyubiquitination requires E2, E3, and Rad5 Ub enzymes (Moldovan et al. 2007).

PCNA can also be modified by small ubiquitin-like modifier (SUMO). PCNA Sumoylation occurs during S phase in the absence of DNA damage. This process prevents DNA repair by homologous recombination (Ulrich 2004, 2005; Moldovan et al. 2007; Andersen et al. 2008) which otherwise increases the incidence of deletion/duplication and chromosomal alteration, or leads to apoptosis (Maga et al. 2003). Thus, various mechanisms involving PCNA protein binding interactions are in place to manage a replication fork stalled due to DNA damage.

Immunohistochemical measurement of Ki-67 is a proliferation assay, whereas measurement of PCNA is both a proliferation as well as a DNA repair assay. By calculating the ratio between Ki-67 and PCNA in nonxerostomic and xerostomic subjects, the xerostomic subjects show an estimated 4X higher underlying DNA repair than nonxerostomic subjects (Table 4). The simplest explanation for the difference in PCNA and Ki-67 indices is that in xerostomic subject LSGs, there is an increase in DNA damage within the glandular epithelium that induces PCNA-dependent DNA repair.
mechanisms. Oxidative DNA damage was previously reported in SS and has been thought to be involved in the pathogenesis of the disease (Koufuchi et al. 2006; Kurimoto et al. 2007). However, oxidative DNA damage of salivary glands in xerostomic subjects had not been confirmed. This study therefore provides a potentially important extension of these data. If the elevation of PCNA expression indeed reflects an increase oxidative DNA damage, then the data presented here suggest that glandular epithelial cell oxidative DNA damage may be a product of hyposalivation, regardless of cause, rather than (or at least, as well as) a cause of glandular dysfunction. Further work would be required to clarify this possibility.

Increased expression of DNA polymerase, DNA ligases, and PCNA and their recruitment to DNA sites with ROS (reactive oxygen species) related damage has been shown (van Loon and Hubscher 2009). DNA damage from excess free radicals is induced by the formation of ROS (Wells et al. 2009). ROS damages DNA by forming apurinic and apyrimidinic sites, DNA strand breakage, and oxidized bases (Burkovics et al. 2009). Apyrimidinic exonucleases, such as Ape2, are DNA repair enzymes that can remove the apyrimidinic sites. Ape2 has a PIP motif. In the presence of DNA-loaded-PCNA, Ape2 co localizes with PCNA and enhances the exonuclease activity in the repair and prevents the synthesis of ROS-damaged DNA (Burkovics et al. 2009).

ROS also have been a source of DNA damage that leads to teratogenesis. Studies of pregnant animals given either thalidomide or phenytoin, two well known teratogenic medications, show that newborn abnormality can be prevented if treated with free radical traps or antioxidants. Therefore, it is possible that ROS can promote mutation that can lead to cellular damage and oncogenesis (Wells et al. 2009). Such a relationship had
been explored by Pan et al., in their proposed Ras oncogenesis pathway mediated by ROS (Pan 2009). Recently, oxidative stress, specifically from reactive nitrogen species (RNS) had been implicated in the dysregulation of salivary gland function (Cal et al. 2006). One such RNS is nitric oxide (NO) that is synthesized from L-arginine by NO synthase (NOS). NOS has three isoforms, endothelial NOS (eNOS or NOS1), inducible (iNOS or NOS2), and neuronal NOS (nNOS or NOS3). nNOS and eNOS are constitutively expressed while iNOS expression is induced (Dröge 2002).

The role of NO in signal transduction and salivary secretion is derived from its ability to activate guanylyl cyclases, leading to the synthesis of cyclic GMP (cGMP) and an increase in intracellular Ca\(^{2+}\) (Looms et al. 2002). The elevated intracellular Ca\(^{2+}\) activates a Ca\(^{2+}\) gated Cl\(^{-}\) channel, resulting in both granule discharge and fluid secretion from salivary acinar cells (Melvin et al. 2005). Thus, NO appears to stimulate salivary secretion (Rettori et al. 2000), as was shown by the increase in salivary secretion by elevated expression of iNOS in LPS-induced inflamed salivary glands (Correia et al. 2010), and a decreased secretion with reduced NOS activities (Cal et al. 2006).

However, chronic exposure to NO was also shown to have an inverse effect on Ach stimulated salivary secretion (Caulfield et al. 2009). The excess NO may initiate the closing of the Ca\(^{2+}\) channel through positive feedback, resulting in salivary hypofunction (Dawson 2006). It was found that at a low concentration, NO has benign and regulatory effect. At high levels however, NO can initiate inflammation and stimulate tumor growth and metastasis (Hussain and Harris 2007; Paradise et al. 2010). Increased NO interaction with ROS such as superoxide can also increase peroxynitrite formation (Hanaue et al. 2007) which does not participate in guanylyl cyclase activation but lead to modification.
of proteins, lipids, and DNA structure. These modified proteins may alter enzymatic reactions (Hill et al. 2010) whereas interaction with DNA may lead to DNA strand breaks and mutations in cancer-related genes (Hussain and Harris 2007).

pSS is a chronic autoimmune disease that affects 0.4 – 3.1 million Americans (Helmick et al. 2008). The triad symptoms of SS consists of: 1) dry mouth—xerostomia, 2) dry eyes—xerophthalmia, and 3) lymphocytic infiltration of salivary glands (Fox et al. 2000). Patients suffering from SS have significant oral complications such as severe dental caries, candidiasis, and oral mucosal soreness that are detrimental to their quality of life (Guggenheimer and Moore 2003). In addition, extraglandular manifestation from possible hypergammaglobulinemia or inflammatory infiltrates can lead to other systemic conditions such as nephropathy (Pessler et al. 2006), arthralgia, Raynaud phenomenon, cutaneous vasculitis, autoimmune thyroiditis, and interstitial lung disease (Launay et al. 2007). A recent meta-analysis also showed that non-Hodgkin lymphoma (NHL) is more common in patients with autoimmune diseases, especially patients with primary SS, who have an incidence rate of 18.8. This suggests that for every NHL incidence occurred in the general population, an estimate of 18.8 cases of NHL from pSS patients are anticipated (Zintzaras et al. 2005). Therefore, the diagnosis of SS as a cause of xerostomia has a profound effect on the treatment approach and prognosis.

According to the revised international classification criteria for SS, meeting any 4 out of 6 criteria leads to a diagnosis of SS. The 6 criteria are ocular symptoms, ocular objective signs, oral symptoms, oral objective evidence, histopathology, and autoantibodies in serology (Vitali et al. 2002). In this current retrospective study, a Xerostomia questionnaire was adapted to evaluate the subjects' risk of developing
salivary gland hypofunction and its complications (Navazesh 2003). Note that xerostomia is a subjective sensation, whereas hyposalivation denotes signs of decreased saliva flow rate (Nederfors 2000).

Thirteen out of 16 xerostomic subjects who took the Xerostomia questionnaire indicated some salivary abnormality had VAS ranging from 0-98 (Table 2). The questionnaire result indicates that each subject with dry mouth, even those with SS, have various xerostomic experiences when encountering different activities, such as swallowing or eating. The subjective report of xerostomia using the VAS score provides an indication that the severity of xerostomia may not be different (Figure 1, p>0.20) between SS, XM, XDz, XNeg, and Xw/oS groups. When subjects with autoantibodies from serology were examined, the VAS ranged from these individuals ranged from 15-98, and 1 subject did not report any dry mouth complaint. When subjects with LSG biopsies with lymphocytic infiltrations (≥1 FS) were examined, the VAS ranged remained from 15-98, while the same subject who did not have any dry mouth complaint with a positive antiautoantibody serology, also show lymphocytic infiltration. These subjective data from both the questionnaire and VAS show inconsistency in the way the subjects perceived their xerostomia. These subjects perceived abnormality in salivary flow, serology indicated some form of autoantibodies, LSG biopsy demonstrated lymphocytic infiltration, yet each subject rated their xerostomia VAS differently, yet virtually all complained of dry mouth. Thus, each subject perceived their xerostomic condition differently and their perception may not correspond with the objective finding from histology and serology.
Lip biopsy as a diagnostic tool is invasive since it requires the removal of tissue. Complications such as temporary lost of sensation, hematoma, and local swelling may occur (Colella et al. 2010). Therefore the risks and benefits should be examined before a biopsy is conducted. Bamba et al. have suggested that patients presenting with typical SS symptoms and SS serology should not be subjected to this procedure (Bamba et al. 2009) because it does not add to the diagnostic value. Studies have shown that neither xerostomic symptoms nor autoantibody serology can predict a positive lip biopsy (Langerman et al. 2007). Thus, the use of lip biopsy should only be used as a last resort to diagnose patients with equivocal or negative serology and questionable symptoms (Bamba et al. 2009; Colella et al. 2010).

LSG biopsies in the current study were obtained to confirm the SS diagnosis. The lack of positive serology and questionable sicca symptoms had prompted the referring physicians to request a LSG biopsy. In total, 20 LSG biopsies from 16 xerostomic subjects and 4 normal subjects were examined in this study (Figure 3, Table 3). Four subjects, as defined by Morbini et al., showed lymphocytic infiltration with focus score > 1 that had histopathologically consistent with SS (Morbini et al. 2005). One subject from each of the categories, XMeds, XDz, XNeg, and Xw/oS, had a focus score equal to 1 and thus, histologically could not confirmed or ruled out SS. The 4 normal subjects did not have any lymphocytic infiltration, therefore, absence of xerostomia was predicted. However, 8 subjects from the XDz, XNeg, and Xw/oS groups who showed no lymphocytic infiltration were xerostomic. Though lymphocytic infiltration is a criterion in diagnosing patients with SS with xerostomia, the absence of lymphocytic infiltration does not indicate the subject is non-xerostomic. In fact, the
sensitivity and specificity of LSG biopsy with ≥1 focus score for SS diagnosis was found to be 82.4% and 86.2%, respectively (Vitali et al. 1994). This suggests that xerostomia can occur regardless of the presence or absence of lymphocytic infiltration of the LSG as seen in the 8/16 of xerostomic subjects. This also suggests that histologically, lymphocytic infiltration may not provide a strong diagnostic value for patients suffering from xerostomia.

In patients suffering from SS, autoantibodies have been used as a criterion for diagnosis (Vitali et al. 2002). Antinuclear antibodies (ANA), rheumatoid factors (RF), anti-SSA/Ro (SSA), and anti-SSB/La (SSB) were frequently found in serology of patients with SS. Whereas ANA binds to nucleus content, RF binds to the Fc portion of the IgG antibodies (Routsias and Tzioufas 2007). The function of the 60-kd and 52-kd Ro protein is unknown, however, the 48-kd La serves as a factor for RNA polymerase III (Scofield et al. 1999). Other autoantibodies such as anti-α-Fodrin and anti-β-Fodrin have also been associated with SS (Routsias and Tzioufas 2007). Although multiple autoantibodies were found previously to be associated with SS, only SSA and SSB were used for the SS diagnostic criteria (Vitali et al. 2002). In addition, these autoantibodies are not exclusive to SS and may be expressed in other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (Routsias and Tzioufas 2007).

The percentage of autoantibodies in the sera of subjects with SS differs between reports. According to Routsias and Tzioufas (2007), the percentage of patients with SS who are positive for ANA is about 90%. Subjects with pSS show 40-50% RF positivity. For anti-α-Fodrin, 95% and 63% of pSS and sSS patients were positive respectively. Subjects with pSS and sSS have 51% and 84% anti-β-Fodrin positivity respectively. For
patients with pSS, SSA is positive in 60-90% while SSB is between 30-60%. Patients with sSS however, show SSA and SSB positivity of 5-38% depending on the associated autoimmune disease (Routsias and Tzioufas 2007, Table 5).

<table>
<thead>
<tr>
<th>Autoantibodies</th>
<th>% in pSS</th>
<th>% in sSS</th>
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<tbody>
<tr>
<td>ANA</td>
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<tr>
<td>RF</td>
<td>40-50</td>
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<tr>
<td>α-Fodrin</td>
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<td>B-Fodrin</td>
<td>51</td>
<td>84</td>
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<tr>
<td>SSB</td>
<td>30-60</td>
<td>5-38</td>
</tr>
</tbody>
</table>

Table 5: Percentage of autoantibodies in sera of subjects with SS (Routsias and Tzioufas 2007).

There is currently no set autoantibody profile specific for patients suffering with xerostomia. Therefore, ANA, RF, SSA, and SSB were considered in this study with respect to the xerostomia from SS. Out of the 13 serology reports, 11 subjects were positive for at least one autoantibody; Seven subjects were ANA+, 3 subjects were RF+, 4 subjects were SSA+, while 1 subject was SSB+ (Table 1). Interestingly, all 4 SSA+ subjects were also present with positive serology for either ANA, RF, or SSB. There were 2 xerostomic subjects who were negative for all of these autoantibodies. When the subjective VAS was used to compare the serology, subjects with positive autoantibodies had a VAS ranged from 18-98, while the 2 subjects who were negative had VAS of 15 and 54. Xerostomic subjects with or without positive serology were not significantly different (p>0.76). Thus, the autoantibody serologic exam did not differentiate between
subjects with or without xerostomia. This suggests that xerostomia can occur regardless of the presence or absence of ANA, RF, SSA, or SSB autoantibody. This also suggests that serological examination may not provide a strong diagnostic value for patients suffering from xerostomia.

In the current study, subjective complaints of xerostomia in regard to activities such as swallowing, eating a meal, or swallowing dry food, were not consistent between subjects from all xerostomic groups regardless of whether they were SS, XMeds, XDz, XNeg, or Xw/oS. When the VAS were compared, the groups were not statistically different from one another (p>0.20). Apoptosis detection using the TUNEL (p>0.61) method and Proliferation assay using Ki-67 (p>0.53) did not show any significant differences between the xerostomic groups. When lymphocytic infiltration were examined using H&E, there was no correlation between xerostomia and focus score. Similar findings were seen when the xerostomia were related to autoantibodies such as ANA, RF, SSA, or SSB. In fact, subjects who have zero focus score or negative autoantibody serology also presented with dry mouth. However, when proliferation and DNA repair were examined using the PCNA assay, a statistically significant difference (p<0.001) was found between subjects with and without xerostomia, regardless of the underlying conditions (SS, XMeds, XDz, XNeg, Xw/oS). Therefore, PCNA overexpression, as identified by immunohistochemistry markers, may provide an objective criterion that may be used to diagnose a subjective condition.

As reviewed by Dickinson et al., it is possible that the salivary dysfunction in xerostomic subjects may be mediated, at least in part, by ROS (Dickinson 2010). As proposed by Dawson et al., subsequent salivary dysfunction may be the result of NO’s
positive feedback on the Ca\textsuperscript{2+} channel (Dawson et al. 2006). DNA damaged cells may also up-regulate their DNA repair mechanism(s) as a sequela to ROS stress. Salivary epithelial cells have demonstrated a response to autoimmune diseases such as SS by upregulating p53, a tumor suppressor gene involved in DNA repair (Tapinos et al. 1999). Oncogenesis had been seen in SS patients with xerostomia. The oxidative stress may increase the burden on activated lymphocytes such that DNA repair becomes inadequate. A recent systematic review has reported an increase in lymphoma development in patients suffering from autoimmune diseases, such as SS, SLE, or RA, who are also predisposed to xerostomia (Zintzaras et al. 2005). As such, DNA damage from oxidative stress may promote repair that requires gene activation, detected here as an upregulation of PCNA in the current xerostomic subjects.

However, the DNA repair mechanism itself may be another reason for the decreased salivary function. Current data here indicate that PCNA is over-expressed in xerostomic specimens compare to non-xerostomic subjects (Figure 16). It is possible that the altered signaling process from the increased ROS leads to the over-expression of PCNA which may disrupt and prevent key salivary secretion signaling components from DNA transcription, or post-translational protein modification from RNS may leads to the disruption of secretory processes.

While sparse PCNA expression was seen throughout the acinar epithelial cells of LSG biopsy in the nonxerostomic group, nearly all ductal cells of the LSG biopsy in the current study showed positive PCNA expression. Increased ductal PCNA expression was seen in both the NOD mouse model (Gillespie et al. 2008) and human (Herrera-Esparza et al. 2008) SS model. Another study also showed that SSA and SSB were expressed by
the ductal cells of SS patients as well (Barcellos et al. 2007). In addition, significant production of an oxidative stress marker was found at the salivary ductal cells of SS subjects (Kurimoto et al. 2007). Furthermore, Thioredoxin, an antioxidant protein, was co-expressed with the oxidative stress marker in the ductal cells in SS subjects (Kurimoto et al. 2007). Since the ductal cells are responsible for re-absorption of NaCl and modification of the salivary secretion (Melvin 1999), it is possible that DNA damage of the ductal cells—as indicated by the increased PCNA expression found in the current study—may play a larger role in hyposalivation and xerostomia that has been thought previously.

If oxidative stress resulting in DNA repair is involved in the etiology of salivary dysfunction, ROS and RNS such as NO, should be consistently found in the LSG epithelial cells of xerostomic patients. Application of antioxidant enzymes such as catalase or the application of antioxidants should also reverse or delay the ROS induced damage. Antioxidants such as superoxide dismutase, have been shown to protect salivary gland from secretory dysfunction due to ROS induced radiation (Tai et al. 2009).

Another antioxidant, EGCG (epigallocatechin-3-gallate), has also been examined for an effect on oxidatively stressed epithelial tumor cells. EGCG is a green tea polyphenol extracted from the leaf of the plant *Camellia sinensis*. EGCG has been shown to have chemoprevention, antioxidant, and anti-inflammation properties (Nagle et al. 2006). EGCG was also shown to reduce the level of ROS in normal epithelial cells while promoting apoptosis in epithelial tumor cells (Yamamoto et al. 2003). In addition, EGCG decreased the expression of ANA, SSB, and α-fodrin autoantigens (Hsu et al. 2005), which are found in the serum of patients suffers from autoimmune disorders.
Recently, EGCG had been shown to delay the onset of disease and lymphocytic infiltration in submandibular glands of Non-Diabetic mice (Gillespie et al. 2008). This mouse SS model study showed that PCNA (which the current study indicates may be an objective marker for xerostomia), was reduced after EGCG treatment. Since EGCG has the potential to reduced oxidants and autoantigens, the decrease in PCNA expression may indicate a state of decrease oxidative stress and requisite DNA repair. If similar result occurs with xerostomic patients taking EGCG, then EGCG might be a new treatment alternative for the multiple autoimmune diseases that result in xerostomia.
V. Summary

For the first time, the labial salivary glands (LSG) of xerostomic subjects (including, but not limited to, definitive SS patients) have been shown to have a consistently high level of proliferating cell nuclear antigen (PCNA) expression, in comparison to LSGs of normal non-xerostomic subjects, independent from cell proliferation measured by Ki-67 expression, PCNA is known to play a crucial role in DNA replication in both error-free and error-prone DNA damage repair mechanisms (Kelman 1997; Andersen et al. 2008). Its expression has been found to correlate with tumor stages of various carcinomas (Toth et al. 1996; Martins et al. 2005; Zhao et al. 2011). PCNA has been shown to be a prognostic factor for an oral cancer patients undergoing radiation therapy (Mallick et al. 2010). In addition, PCNA has been shown to be involved in the repair of oxidative DNA damage from ROS (Burkovics et al. 2009).

The high expression of PCNA (but normal Ki-67 expression) observed in LSG from xerostomic subjects suggests that there is a significant increase in cells which are actively undergoing DNA repair relative to normal subjects. That is, DNA damage could be an objective marker for xerostomia, a subjective condition. Further, since many of the patients likely had true salivary hypofunction, this suggests that reduced saliva flow per se, regardless of cause, could result in DNA damage from oxidative stress, and that elevation of PCNA expression reflects an associated salivary gland dysfunction in xerostomic patients. ROS have been shown to be a possible mechanism in salivary
dysfunction by activation of guanylyl cyclases (Looms 2002; Caulfield et al. 2009) and has also been linked to DNA damage that leads to oncogenesis (Wells et al. 2009). The high PCNA expression, likely reflecting DNA damage and repair, may be correlated to the increase lymphoma rates in xerostomic patients diagnosed with SS (Zintzaras et al. 2005). Since ROS also serve as signaling molecules, an increase in RNS, specifically NO, may alter intracellular signals and be involved in salivary dysfunction. It is possible that the altered signaling process from the increase in ROS and RNS may lead to post-translational protein modification that disrupt salivary secretory processes and the oxidative stress may upregulate DNA damage repair as seen by the over-expression of PCNA. Importantly, the correlation of elevated PCNA with xerostomia, regardless of underlying cause, may provide an objective test for a subjective condition.
VI. Acronyms and definitions

Ach—Acetylcholine—neurotransmitter for the Parasympathetic and preganglionic sympathetic system. It binds to M3R to stimulate salivary secretion.

Acinar cells—cells responsible for the production of salivary secretion.

Adrenergic receptors—NorEpinephrine or Epinephrine sensitive receptors found at the postganglionic sympathetic system. The β-receptor stimulates protein secretion. The α1-receptor stimulates salivary fluid secretion, while α2-receptor activation inhibits salivary secretion (Proctor 2007).

ANA—Antinuclear Antibodies

ANOVA—analysis of variance: used to compare the means of each group using the standard deviation.

Ape2—Apyrimidinic exonucleases: DNA repair enzymes that can remove the apyrimidinic sites. When Ape co-localizes with PCNA, repair and prevention of the synthesis of ROS-damaged DNA occurs (Burkovics 2009).

Apoptosis—program cell death that do not cause an inflammation response.

Aquaporin-5—water channel at apical membrane of the acinar cells that allows water passage (Grundmann 2009).

BAFF—B cell activating factor: Pro-inflammatory cytokine associated with SS that induced by INFα and promote B cell survival and involved in germinal center formation.

Bartholin’s duct—drains the sublingual salivary glands

Ca2+—Calcium, a signal messenger with many functions. It regulates Cl− ions and serves as the primary salivary secretion signal

cAMP—Cyclic adenosine monophosphate: derived from ATP, is a signal messenger which regulates granules discargues from the acinar cells.

Carbonic anhydrase IV—an enzyme in saliva that catalyses the reversible reaction of the buffer system (Kivela 1999)
Caspases—cysteine pro tease that is activates by Fas/Fas Ligand interaction and causes DNA fragmentation through the process apoptosis (manganelli and Fietta 2003).

Cevimeline: Trade name Evoxac. An acetylcholine analogue. Rx: 30mg TID.

EGCG--epigallocatechin-3-gallate: A green tea polyphenol extracted from the leave of the plant Camellia sinensis. EGCG had been linked to have chemoprevention, antioxidant, and anti-inflammation properties (Nagle et al. 2006). EGCG was also shown to reduce the level of ROS in normal epithelial cells while promoting apoptosis in epithelial tumor cells (Yamamoto 2003).

Excretory ducts—the ductal portion that is next to the oral cavity. Excretory ductal cells also produce dehydrogenases, oxidases, acid phosphatase, acid hydrolases that can contribute to the salivary content. In the major salivary glands, the main excretory duct also serves as a salivary reservoir (Tandler 1993c, 2006).

Fas/Fas Ligand—Fas Ligand is a transmembrane protein that once binds to Fas antigen, apoptosis is initiated (manganelli and Fietta 2003).

FS—Focus score: A focus is an area of salivary gland in which ≥50 lymphocytes per 4mm² is seen. Focus score is the number of focus.

G proteins—Guanine nucleotide-binding proteins: Transmembrane protein that convey signals between outside and inside the cells. In mAchRs, G protein activation leads to the production of inositol trisphosphate (IP₃) and diacylglycerol (DAG) through phospholipase C. In contrast, β-adrenergic receptor activation coupled G proteins that increases the intracellular level of cAMP. This results in the activation of protein kinase A and exocytosis of secretory granules (Turner 2002).

H&E—Hematoxylin and Eosin.

Hyposalivation—Objective tool to measure dry mouth. An unstimulated salivary flow rate <0.1 ml/min and a stimulated flow rate of <0.7 ml/min are considered hyposalivation (Navazesh 2003). Normal unstimulated salivary flow rate averages 0.3 ml/min (Dawes 2008).

Ig—Immunoglobulin—secretory antibodies secreted by Plasma cells to facilitate microbial clearance. It consists of IgA, IgD, IgG, and IgM.

Immunohistochemical staining— is a process in which proteins in tissues are specifically recognized by the primary antibodies followed by colorimetric reaction using a secondary antibody. This process identifies whether and how much a specific protein is expressed in the tissue samples, and if so, where.

being developed that will detect salivary biomarker for disease diagnosis (Gioannobile 2008).

INF\(\alpha\)—interferon \(\alpha\): Pro-inflammatory cytokine associated with SS that induces BAFF, a cytokine that promotes B cell survival and involved in germinal center formation.

Intercalated ducts—most proximal to the secretory cells. Cell membrane is associated with ATPase, \(\text{Na}^+\text{K}^+\text{ATPase}\), and carbonic anhydrase isozyme VI promote salivary secretion modification. They are connected to Myoepithelial cells which facilitate fluid flow (Tandler 1998).

\(\text{IP}_3\)—inositol trisphosphate: Secondary messenger that binds to \(\text{IP}_3\text{R}\).

\(\text{IP}_3\text{R}\)—Receptor for \(\text{IP}_3\): Activation by \(\text{IP}_3\) results in the release of intracellular \(\text{Ca}^{2+}\) from the endoplasmic reticulum (Dawson 2006).

KI—Ki-67 Index: the number of Ki-67-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage.

Ki-67—Proliferation marker used to detect Ki-67 nuclear antigens.

LSG—Labial salivary glands or minor salivary glands. Produce predominantly mucin-rich saliva and are scattered throughout the oral mucosa (Hand 1999). For LSG biopsy, 5-7 glands are normally needed for diagnosis (Greenspan et al. 1974).

M3R—Type 3 Muscarinic acetylcholine receptor found at the salivary gland.

mAchRs—Muscarinic acetylcholine receptors—acetylcholine receptors which is more sensitive to muscarine than to nicotine. Found in the parasympathetic innervation system. Muscarinic activation will increases salivary fluid secretion.

Mucous cells—produces mucin, which consists of 50-80% carbohydrates (Tandler 1993b).

NOD—Non-Obese Diabetic mice: A strain of mice used for diabetic and SS research because it spontaneously develops diabetes and shows characteristic of human SS with lymphocytic infiltration and salivary hyposecretion by 16 weeks of age (Cha et al. 2002).

NO—Nitric oxide: synthesized from L-arginine by NO synthase. It is a signal molecule that can cause an increase in intracellular \(\text{Ca}^{2+}\) (Looms 2002), leading to salivary secretion (Rettori et al. 2000). However, chronic exposure to NO was also shown to have an inverse effect on Ach stimulated salivary secretion (Caulfield 2009). It was found that at low concentration, NO have a benign and regulatory effects. At high level however, NO can initiates inflammation and stimulate tumor growth and metastasis.
NOS—Nitric Oxide Synthase. Consists of 3 isoforms. Endothelial NOS (eNOS or NOS1), Inducible (iNOS or NOS2), and Neuronal NOS (nNOS or NOS3). nNOS and eNOS are constitutively expressed while iNOS expression is induced (Dröge 2002).

Parotid glands—produces serous saliva

PCNA—Proliferative Cell Nuclear Antigen or Cyclin: Proliferation and DNA repair marker that used to detect PCNA nuclear antigens. It is expressed at high levels in the nucleus during S phase of the cell cycle (Nelman 1997). However, it is also expressed in the cytoplasm and had been found to interact with the enzymes involved in glycolysis as well as enzymes of the mitochondria that regulate energy generation. (Naryzhny and Lee 2010).

Pilocarpine: Trade name Salagen. A cholinergic agonist, enhances salivary secretion by activating the M3R receptor of the cells of salivary glands. Rx: 5mg QID.

PI—PCNA Indices: (PI) were recorded. the number of PCNA-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage.

PIP—PCNA-interacting proteins: conserved motif at the outer surface of PCNA that allows PCNA-protein interaction (Maga 2003).

pSS—primary Sjogren's Syndrome—See SS.

RF—Rheumatoid Factor

RICSS—Revised International Classification Criteria for Sjögren's Syndrome: Set criteria for primary SS (pSS) and secondary SS (sSS) (Vitali 2002).

RNS—Reactive Nitrogen species: Similar to ROS except that instead of oxygen, nitrogen is the free radical bearing element. Nitric oxide is an RNS.

ROS—Reactive oxygen species. “Molecules or ions formed by the incomplete one-electron reduction of oxygen. They contribute to the microbicidal activity of phagocytes, regulation of signal transduction and gene expression, and induce oxidative damage to nucleic acids, proteins, and lipids.” (Pan et al. 2009) ROS also have been a source of DNA damage and can promote mutation that can lead to cellular damage and oncogenesis (Wells 2009).

Seromucous cells—According to Tandlers, cells that hardly stained by toluidine blue and have low electron density under the electronic microscope (Tandler 1993a).

Serous cells—According to Tandler, cells with granules that appear blue-black in toluidine blue staining under the light microscope and appear to be electron-dense under
electron microscope are considered serous. Serous cells produce protein rich secretion (Tandler 1993a).

SNARE-- Soluble N-ethylmaleimide-sensitive-factor attachment protein receptors. A membrane-fusion-related protein complex had been hypothesized to play a major role in exocytosis of salivary secretory granules (Turner 2002).

SS-- Sjögren's Syndrome. An autoimmune disease characterized by dry eye, dry mouth, and lymphocytic infiltration of the labial salivary glands (Fox 2005). According to the RICCSS, patient is classified as pSS when no associated diseases are presence and met the following criteria: any 4 of the 6 criteria with either positive histology/serology or the presence of any 3 of the 4 objective criteria (objective ocular, objective oral, histology, serology). When patients are diagnosed with autoimmune diseases, the presence of any subjective criteria and any 2 of objective ocular, objective oral, and positive histology, may be considered as sSS. Subjective criteria include ocular (I) and oral dryness (II) complaint. Objectively, ocular criteria (III) includes positive Schirmer's test or ≥4 ocular dye score, while oral criteria (V) includes low unstimulated whole salivary flow (≤1.5ml/15mins), parotid sialography indicating diffuse sialectasias without major ducts obstruction, or salivary scintigraphy with delayed uptake and excretion.

SSA/Ro—Anti-Sjögren's Syndrome A/Ro autoantibodies.

SSB/La—Anti-Sjögren's Syndrome B/La autoantibodies.

sSS—secondary Sjogren's Syndrome—See SS

Stensen's duct—drains the parotid salivary glands

Striated ducts—the second most proximal ducts to the secretory acinar cells. The striated duct perform most of the ion resorption, protein secretion and absorption during the second stage of salivary secretion (Tandler 1993c).

Sublingual glands—produces mucin-rich saliva

Submandibular—produces a mixture of serous and mucin salivary secretion.

SUMO-- Small Ubiquitin-like Modifier: When PCNA is modified by SUMO, it prevents DNA repair by homologous recombination (Ulrich, 2004, 2005, Moldovan 2007, Andersen 2008) which otherwise increases the incidence of deletion/duplication and chromosomal alteration, or leads to apoptosis (Maga 2003).

TI—TUNEL Index: the number of TUNEL-positive LSG epithelial cells (both acinar and ductal) in 1000 count and expressed as a percentage.

TLS--Translesion Synthesis: Error-prone DNA repair that is often mutagenic.
TUNEL—Terminal deoxynucleotidyl-transferase dUTP Nick End Labeling: An immunohistochemistry assay that detect DNA strand breakage.

Ubiquitylation—When Ub is covalently linked to a protein by Ub-activating enzyme. When PCNA is monoubiquitinated, an error-prone (mutagenic) TLS is promoted. (Ulrich, 2004, 2005, Andersen 2008) PCNA monoubiquitylation requires ubiquitin-activating enzyme E1, E2 (Rad6 protein), and E3 (Rad 18). When PCNA is polyubiquitinated, it is believed an error-free pathway utilizing the sister chromatid as a template for repair occurs. Polyubiquitination requires E2, E3, and Rad5 Ub enzymes (Moldovan 2007).

Ub—Ubiquitin: A highly conserved 76 amino acid protein. When Ub is covalently linked to a protein by Ub-activating enzyme, the activity of the protein changes.


VAS—Visual analog scale: A tool that allows patients to rate their dry mouth condition subjectively by marking a vertical line on a horizontal scale with "Dry as a desert" and "Not dry at all" corresponding to a VAS score of zero and 100, respectively.

Wharton’s duct—drains the submandibular salivary glands.

XDz—Xerostomia with autoimmune disease: Subjects with xerostomia who don’t meet the criteria for SS and have autoimmune disease diagnosed by physician and autoantibody serology.

Xerostomia—Subjective complaint of dry mouth.

XMeds—Xerostomia from medications: Subject with xerostomia related to medication usage.

XNeg—Xerostomia with negative serology: Subjects with xerostomia who don’t meet the criteria for SS, don’t have autoimmune diseases, and did not have autoantibody serology.

Xw/oS—Xerostomia without serology: Subjects with xerostomia who don’t meet the criteria for SS, don’t have autoimmune diseases, and did not take get a blood drawn (therefore autoantibody profile is unknown).
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VIII. Appendix

A. H&E stained slides

1. Sjögren’s Syndrome subjects
2. Xerostomia from Medication subjects
3. Xerostomia with Autoimmune Disease subjects

08-195

09-577

09-611
4. Xerostomia with Negative Serology subjects

09-1000

09-1124
5. Xerostomia without Serology subjects
6. Normal subjects
B. TUNEL (Apoptosis assay)

1. Sjögren’s Syndrome subjects

08-889

09-417
2. Xerostomia from Medication subjects

08-1171
3. Xerostomia with Autoimmune Disease subjects

08-195

09-577

09-611
4. Xerostomia with Negative Serology subjects

09-1000

09-1124
5. Xerostomia without Serology subjects
6. Normal subjects
C. Ki-67 (Proliferation Assay)

1. Sjögren’s Syndrome subjects

![Image 08-889]

![Image 09-417]
2. Xerostomia from Medication subjects
3. Xerostomia with Autoimmune Disease subjects

08-195

09-577

09-611
4. Xerostomia with Negative Serology subjects

09-1000

09-1124
5. Xerostomia without Serology subjects
6. Normal subjects

09-456

09-460

09-497
D. PCNA (Proliferation and DNA Repair assay)

1. Sjögren’s Syndrome subjects

![Images of tissue samples labeled 08-889 and 09-417]
2. Xerostomia from Medication subjects
3. Xerostomia with Autoimmune Disease subjects

08-195

09-577

09-611
4. Xerostomia with Negative Serology subjects

09-1000

09-1124
5. Xerostomia without Serology subjects
6. Normal subjects

09-456

09-460

09-497
E. Clinical information of each subject.

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**QUESTIONNAIRE**

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F. Summary of Subjects personal information, serology report, pathology diagnosis, Focus score, apoptosis and proliferative indices.

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