# COMPARISON OF SALIVARY AND SERUM LEVELS OF SOLUBLE TOLL-LIKE RECEPTOR 4 (TLR4) IN PATIENTS WITH SJÖGREN'S SYNDROME (SS), A CASE:CONTROL STUDY

A Thesis

by

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## ABSTRACT

Sjögren's syndrome (SS) is an auto-immune (AI) mediated destruction of the major and minor salivary glands, and is the second most common AI disease in the United States. Typical treatment palliative in nature and no treatment is available that will reverse or halt the progression of the disease process.

SS poses many dental problems for affected patients due to a loss of the protective effects of the saliva, resulting in increased caries incidence. However, whether SS patients are more at risk for periodontal disease is controversial. Periodontal disease is a gram-negative bacterial infection that may or may not elicit an immune response resulting in the destruction of the attachment apparatus of the teeth.

Toll-like receptors (TLRs) are highly conserved pattern recognition receptors found throughout the body. TLR4 recognizes lipopolysaccharide (LPS) on gram negative bacteria, and is involved in mucosal immunity. Many *in vitro* studies have reported an increased expression of TLR4 in both SS and periodontal disease. Whether or not TLR4 is a contributor to the induction and/or progression of SS and/or periodontal disease is unknown.

This cross-sectional study examined potential differences in concentration of TLR4 in serum and citric acid-stimulated saliva from patients with SS and healthy age-, sex-, and periodontal disease matched control patients.

Salivary function was stimulated with 5.0 mLs of 2% citric acid, and whole saliva samples were obtained. Full-mouth clinical periodontal measurements were

recorded from 21 SS patients and 22 control patients. Salivary and plasma TLR4 concentrations were determined by enzyme-linked immunoassays in 20 patients per study group. Collected data was statistically analyzed for its respective distribution.

No statistical differences in age, body mass index (BMI), salivary TLR4 concentrations, or clinical periodontal measurements were evident between either group. Salivary flow rates were significantly lower ($P<0.001$) whereas plaque % ($p=0.048$) and serum TLR4 concentrations ($p=0.011$) were significantly higher in SS patients.

The present findings support a hypothesis that a hyper-inflammatory systemic state, as seen in SS patients, can independently raise serum TLR4 concentration in the absence of periodontal disease and may be important in the induction and / or progression of SS-mediated pathophysiology in affected patients.

## DEDICATION

This paper is dedicated to my family, mentors, and friends who drove me and supported me in completing this endeavor, as well as my Creator, who gave me the ability to derive and complete this project.

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# CHAPTER I

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 Sjögren's syndrome overview

Sjögren's syndrome (SS) is the second most common autoimmune disorder, behind rheumatoid arthritis. In the United States, the prevalence is estimated at 2-4 million people. Like many other autoimmune diseases, SS affects women more than men with reported ratios above 9:1, with peri- and post-menopausal women most often affected. SS is an autoimmune exocrinopathy and classically affects the lacrimal and salivary glands, resulting in dryness of the eyes and oral cavity; however, it is a systemic disorder and can affect any of the secretory tissues throughout the body. SS is categorized as either primary or secondary, where primary SS occurs without the presence of another connective tissue or autoimmune disease and secondary SS occurs in concert with another connective tissue disease such as systemic scleroderma, rheumatoid arthritis, or systemic lupus erythematosus (SLE). The reports of primary and secondary SS demonstrate an approximately even distribution of each type of SS in affected patients.(1)

In SS, there are consistent dental findings related to autoimmune-mediated salivary dysfunction and share similar findings with other conditions characterized by salivary hypofunction. Clinically, SS patients often present with a high caries rate, mucosal dryness, increased fungal infections, altered salivary flow rate, and often a painless enlargement of the salivary glands. Following loss of normal salivary function, difficulties are almost always encountered with chewing, swallowing, and speaking.

Alterations in taste and subsequent compromises in nutrition coupled with the above mentioned findings lead to a decreased quality of life.(2) Biopsies of minor salivary glands reveal changes consisting of an inflammatory cell infiltrate around periductal tissues with a resulting loss of acinar cells.(3) Histologic analysis of the infiltrate reveals it to be composed mainly of lymphocytes, with T-lymphocytes predominating. The pathologic outcome is a loss of functional acinar tissues resulting in a loss of secretory output and symptoms of oral dryness.(4)

Non-oral manifestations of SS include dry eyes, other xeroses including dryness of the nasopharyngeal, vaginal, and respiratory alveolar membranes. Also, renal disorders and muscle and joint inflammation are common findings. Serum changes in SS patients, both primary and secondary, include a notable hypergammaglobulinenemia, elevated total protein and sedimentation rate, decreased white blood cell counts, persistent rheumatoid factors, and the autoantibodies anti-sicca-syndrome-A (anti-SS-A/Ro), anti-sicca syndrome-B (anti-SS-B/La). These changes are nonspecific, and occur in the majority of affected SS patients, but also are found to be elevated in other autoimmune conditions. SS, especially primary SS, predisposes patients to lymphomas, with a 2000-4000% increased incidence of the neoplasm(s) compared to unaffected patients.(5) In almost all cases, these tumors are mucosally associated lymphoid tissue (MALT) tumors and commonly involve the salivary glands themselves.

The skin may be affected and display palpable or non-palpable purpura associated with cryoglobulianemia and/or hyperglobulinaemia.(6) Also, some patients may display medium vessel pathology and present with urticarial vasculitis, necrosing

vasculitis, or thrombotic lesions of the more superficial veins and arteries. Other epidermal associated findings include vitiligo, anetoderma, alopecia, cutaneous lymphomas, and xerosis.(7)

Arthalgia and arthriris are in general similar to rheumatoid arthritis and SLE and are usually symmetrical in the affected joint type and location.(8) Furthermore, some patients will also display an arthritic pattern consistent with erosive osteoarthritis.(9) Asymmetrical joint edema may suggest an additional process such as crystalline or infectious arthropathy. Muscle pain (myalgia) and muscle weakness are frequent findings in SS patients.(10) Polymyositis is often associated with sicca symptoms. Other conditions, such as polymyalgia rheumatic, myopathy, and myostitis due to medications should also be considered and treated appropriately. Neurologic problems including vasculitis, thrombitic and/or paraneoplastic processes can also cause symptoms of weakness and generalized fatigue. Analysis of acute-phase reactants, muscle enzymes, electromyography, or biopsy may be necessary if symptoms progress or are debilitating. Also, fibromyalgia is a common finding associated with SS and may cause myalgia and weakness independently.(11)

The most common presentation of pulmonary involvement in SS are interstitial pneumonitis and tracheobronchial sicca.(12) Furthermore, SS patients can develop MALTs of the lung.(13) Other common pulmonary findings include hypersensitivity of the lung and toxic effect of drugs (alkylating agents, methotrexate, etc) as well as bacterial and/or fungal pneumonia from opportunistic infections in immunosuppressed patients.(14) Pneumonitis in patients receiving monoclonal antibody therapy

(infliximab, rituximab) have been reported and signs/symptoms of pneumonitis must be evaluated in patients on this medication regimen.(15, 16)

Pulmonary hypertension and pericarditis are common findings in SS patients. Alterations in cardiophysiologic function may also manifest in clinical examinations as abnormal findings in physiologic compensation for the Valsalva maneuver, heart-rate response to deep breathing, and heart-rate and blood-pressure response to standing are abnormal in some patients with SS.(17) Also, the incidence of congenital heart block is higher in infants born to anti-SS positive mothers(18), however other auto-antibodies have been implicated in addition to anti-SS.(19, 20)

The most common renal finding in SS patients is interstitial nephritis,(21) although hypokalaemic paralysis,(22) osteomalacia, and renal calculi may be found in addition.(23) Loss of function of the kidneys should primarily be attributed to medications, such as chronic non-steroidal anti-inflammatory drugs (NSAIDs).(24) Patients may develop glomerulonephritis associated with hyperglobulinaemia. Specifically, antibodies to double-stranded DNA may require testing for amyloidosis, immune-complex disorder, and/or undiagnosed systemic lupus erythematosus.(25) Other common findings of the renal system are interstitial cystitis that may be antagonized by large fluid intake in SS patients to treat chronic dry mouth.(26)

The most common G/I complaint in SS patients is dysphagia due to a combination of xerostomia and esophageal dysfunction.(27) Also, gastritis is common and patients complaining of this symptom should be tested and treated (if positive) for

Helicobacter pylori infection, as this bacterium has been positively associated with MALT lymphomas in SS patients.(28) Primary biliary cirrhosis and celiac sprue may also be found in SS patients, and identification and treatment of these conditions in SS patients is important.(29)

Hypothyroidism and hypofunction of the pituitary and adrenal gland axis are common endocrine findings in patients with SS. 10% of patients with autoimmune thyroiditis also have similar findings, and the differential diagnosis must be confirmed and treated appropriately.(30),(31) Alterations in thyroid function may manifest as lethargy, loss of cognitive function, and generalized fatigue.

The risk of developing lymphoma is about 40 times that in the general population in SS patiets, and consequently lymphoproliferative disease is a particular concern.(32) Most lymphomas in SS patients are marginal-zone B-cell neoplasms that arose in extranodal and nodal sites, and were not associated with concomitant viral infection.(33) However, case reports of lymphomas in SS patients associated with H. pylori(34)[100], human herpes virus 6(35), human t-lymphotropic virus type I,(36) and Epstein-Barr virus.(37) Usually, lymphoma is initially recognized as progressively enlarged parotid glands, regional or general lymphadenopathy, pulmonary infiltrates, hypergammaglobulinemia, hepatosplenomegaly, and vasculitis. All of these are non-specific findings, but should be treated with suspicion and serologic testing and/or biopsy should be performed to make a diagnosis and treated appropriately.

Central and peripheral neurologic problems are reported in approximately 20% of SS patients.(38),(39) Sensory neuropathies are typically the most common, and

epineural inflammatory changes are often evident on nerve biopsy samples.(40) Asymmetrical motor and/or sensory neuropathy can signal vasculitis of blood vessels.(6) The frequency of central demyelinating disease in SS patients appears to be similar to that in systemic lupus erythematosus,(41) and SS patients may display abnormal oligoclonal bands in the cerebrospinal fluid and abnormal brain MRI.

The most common psychiatric disorder in SS patients are anxiety and depression.(42) These symptoms commonly are a prelude to a formal diagnosis of SS.(43) Subtle changes to cognitive function and memory may be evident in SS patients, and may coincide with abnormal positron emission tomography scans largely in the frontal lobe(44),(45). Another extremely common finding in SS patients is generalized fatigue that does not improve with increased sleep. As many as 30% of patients with SS are affected in this manner. Much effort has been made to identifity a serologic marker that coincides with fatigue, however to date hemoglobin, erythrocyte sedimentation rate, and C-reactive protein levels did not predict fatigue.(46, 47)

### 1.2 Treatment strategies

Conventional treatment of SS has been aimed at improving flow from the affected glands. Recommendations include staying well hydrated plus preventative therapy to reduce damage to the teeth and periodontium and include supplemental fluoride application, use of xylitol-containing gums and rinses, a diet low in acidic foods and refined carbohydrates, and rigorous oral hygiene. FDA-approved prescription treatment of the condition includes cevimeline or pilocarpine to increase salivary production and hydroxychloroquine to improve overall symptoms of affected patients.

While these therapies are effective, they are often not long-term solutions and slow, not stop, glandular deterioration. Thus the search for more efficacious medications to treat this condition continues.(48)

### 1.3 Diagnosis

The establishment of a definitive clinical diagnosis for SS has remained an elusive goal, in light of its non-specific clinical symptoms and a lack of both specific and sensitive lab findings. The American–European Consensus Classification Criteria has improved the diagnosis dilemma in clinical trials,(49) but a definitive diagnostic and disease severity marker is still needed. Recent investigations into serum and salivary biomarkers have been undertaken to address this issue, from analysis of macromolecular structures such as whole intra and/or extracellular receptors themselves to micromolecular structures such as genomic analysis. The findings from these studies may help elucidate etiological mechanisms and provide subsequent novel strategies to treat this disease.(50) Notably, alterations in the type 1 interferon pathway have been demonstrated in SS, a feature that it shares with only systemic lupus erythematosus, dermatomylsitis, and psoriasis. This pathway has been demonstrated to be upregulated in pSS patients and blockade of this pathway may be an important future therapy for treatment of SS.(51)

### 1.4 Immunopathegensis of sjögren's syndrome

In healthy patients, normal immune function requires a coordination of both the innate and acquired arms of the immune system. In many disease states a deficiency or hyperactivity of one pathway adversely affects the function of the other and leads to

development of clinical disease in the patient, as seen in SS. SS is characterized by B-lymphocyte hyperactivity and is evident on laboratory examination of serum by elevated amounts of gamma-globulins which includes a variety of autoantibodies. The lymphocytic infiltrate around the exocrine glands consists of mainly CD4+ T-lymphocytes (70%), with smaller amounts of B-, CD8+ T-, and CD17+ T-lymphocytes.(52) Recent investigations of inflammatory mediators and immune cells themselves have been undertaken to explore what contribution, if any, the given molecule or cell has on B-cell expression and their effects on SS induction, progression, and resolution.

Levels of TNF-alpha in the tears and circulation of SS patients are elevated, indicating a possible role in pathogensis of the disease.(53) Besides its known pro-inflammatory roles, TNF-alpha is known to be a direct inducer of cell-death, and in SS the cytokine has been shown to be localized around salivary epithelial cells.(54) However, clinical trials of two different TNF-alpha blocking medications (Infliximab and Enteracept) have shown mixed results in treating SS in vivo. Infliximab is a monoclonal antibody that binds to TNF-alpha directly and impairs the molecules ability to agonize its respective receptor. Enteracept is a fusion protein consisting of a human TNF-alpha receptor linked to an Fc IgG1 molecular tail. TNF-alpha binds to the TNF-alpha receptor end of the drug and once bound is cleared from the body by phagocytes after recognition of the Fc region, and subsequent opsonization and clearance of endogenous TNF-alpha. Small uncontrolled trials with Infliximab on SS patients resulted in decreased symptoms after one year of treatment along with restoration of

normal distribution of aquaporin-5 to the cell membrane of salivary acinar cells. Aquaporin-5 was previously demonstrated to be down-regulated in SS, and is necessary for proper fluid transport into the salivary ducts.(55) Larger-scale randomized controlled clinical trials demonstrated no beneficial outcomes when compared to placebo.(56) Likewise, pilot studies on Enteracept were shown to be ineffective in improving symptoms in a small scale study of SS patients.(57),(58)

Following case reports of development of SS following treatment for hepatitis-C, investigators explored the potential role of endogenous anti-viral molecules, mainly interferon-alpha, in SS patients.(59) Their investigations found that increases in mRNA expression for IFN-alpha corresponded with increases in B-cell activating factor (BAFF) in salivary gland cells taken from SS patients compared to controls. When these same cell populations were exposed to other cytokines, an increase in BAFF expression did not occur, suggesting a key role for the signaling of interferon-alpha in the disease process.(60) However, clinical treatment of primary SS patients with interferon-alpha supplementation has shown improvement in salivary flow (61) along with and increased levels of aquaporin-5 on the cell membrane of parotid gland cells.(62) Clearly interferon-alpha levels are altered in SS, but to what extent it determines disease presence or severity is unknown and the cell-culture and clinical studies have contradictory findings.

Treatment involving B-cell depletion with Rituximab has shown promise. Rituximab is a monoclonal antibody that recognizes CD20, an extracellular molecule found only on B-cells. Small pilot studies revealed reductions of lymphocytic infiltrates

and lymphoepithelial lesions. T-lymphocyte populations were decreased as well. Also, acinar structures returned to normal appearance in some patients.(63, 64) In clinical trials, the efficacy of B-cell depletion directly corresponded to the remaining residual glandular function present when the therapy was started, meaning that the therapy was more effective in patients who had less pre-existing salivary gland damage from the disease. Symptoms of decreased fatigue and other comorbid conditions along with increases in salivary flow were demonstrated in all patients with remaining salivary gland function at the start of the study. More investigation of this drug's effect on affected patients may be of great value in future treatment of the disease.(64)

It has previously been shown that B-cell activating factor (BAFF) is upregulated in SS and correlates with titers of anti-SS-A and anti-SS-B antibodies.(65) BAFF is also overexpressed in salivary glands of patients with SS.(66) After previous studies with B-cell depletion with rituximab, it was realized that there was increased BAFF expression on blood monocytes. This led to the discovery of a negative feedback system that B-cells exert on BAFF, and with a loss of this signal from B-cell depletion therapy, levels of BAFF increased.(66) The concentration of BAFF seems to determine the rate of B-cell repopulation as well as the rate of reappearance of memory B-cells in the circulation and peripheral tissues.(67)

Monoclonal antibodies targeting co-stimulatory bonds between antigen presenting cells and CD8+ T-cells, are under investigation. Salivary gland epithelial cells in SS have been demonstrated to express HLA class II and co-stimulatory molecules and may function as antigen-presenting cells in pSS, besides dendritic cells

and B cells. Interfering in co-stimulation in pSS could, theoretically, inhibit both systemic and local autoimmune responses in pSS. Abatacept, a fusion molecule of IgG–Fc and cytotoxic T-lymphocyte antigen 4, modulates CD28-mediated T-cell co-stimulation and a controlled trial with abatacept in pSS has been undertaken but the results are not published to date.(68) More studies looking at various putative biomarkers are being reported using a proteonomics approach, but much more investigation is needed to fully characterize the molecules involved in the initiation, progression, and treatment of SS.(69)

The initiation and underlying etiology of SS is complex and multifactorial, with suspected contributions from any one or a combination of the following events: viral infection, aberrant activation of B- and T-lymphocytes, cytokine profiles favoring survival and multiplication of defective cell lines. Recent studies on monoclonal antibody therapies have revealed much about the etiology of SS by characterizing how patients respond when contributions from a given cytokine or cell is blocked. TNF-alpha has been demonstrated to be of questionable significance in pathology of SS, with mixed results obtained in small pilot studies and larger randomized controlled studies. Interferon-alpha has had similar mixed results but is likely to be involved in BAFF signaling and as such merits further investigation. B-cells are the major player in the disease progression of SS and BAFF signaling is suspected to be involved in repopulation of auto-reactive B-cells. From a treatment efficacy standpoint, the disease duration is most important in predicting therapeutic benefit. The disease duration dictates the amount of gland destruction and subsequent loss of salivary flow, and

treatment aimed at increasing or restoring lost flow by reversing the source of glandular destruction will not be beneficial if there is little or no residual function of the affected gland(s).(64)

**1.5 Periodontal disease**

Periodontal disease has been demonstrated in numerous studies to be bacterial in origin, with development and maintenance of a complex bacterial biofilm extending subgingivally on the roots of the teeth. In the supragingival areas, gram positive, aerobic bacteria predominate, with the bacterial population(s) becoming more anaerobic and gram (-) in nature as you progress apically down the root surface. Gram (-) bacteria possess lipopolysaccharide (LPS), which is highly recognized and bound by innate immune cells.(70),(71) Binding of LPS causes activation of the innate immune system, specifically polymorphonuclear cells (PMNs) and macrophages. Activated PMNs and macrophages release a variety of lytic enzymes and cytokines. These cytokines amplify the inflammatory response by priming localized gingival cells (fibroblasts, gingival epithelial cells, etc) to release matrix metalloproteinases (MMPs) and target the acquired immune system to the site of infection. IL-1 beta, IL-6, and TNF-alpha have been associated with recruitment of pro-inflammatory cells, and are upregulated in the saliva of individuals with periodontitis.(72) Six bacterial clusters were described by Socransky et al. in subgingival biofilms and related to structural characteristics of the biofilm extending away from the tooth surface and consist of the yellow, purple, green, orange, red, and blue. The yellow cluster is composed of the Streptococcus species including S. sanguis and S. oralis and the purple cluster consists of Actinomyces odontolyticus and

Veillonella parvula. The yellow and purple clusters are thought to be early colonizers and express ligands that allow for attachment to surfaces found in the host's subgingival environment (cementum, gingival epithelial cells, enamel pellicle) and facilitate attachment to the subgingival microenvironment of other species. The green cluster consists of Capnocyopha spp, Campylobacter concisus, Eikenella corrodens, and Actinomyces actinomycemcomitans. The orange cluster consists of Fusobacterium spp, Prevotella spp, Micromonas micros (aka Peptostreptococcus micros), Campylocabeter spp, Eubacterium spp, and Streptococcus constellatus. The red cluster consists of Porphyromonas gingivalis, Tanerella forsythia, and Tanerella denitcola and the blue cluster consists of Actinomyces species. The orange and green clusters are thought to be a bridge between the early colonizers and the more virulent members of the red cluster, since they can bind to the already present members of the yellow and purple cluster bacteria and members of the red cluster.(73) Specific gram negative bacteria have been implicated in the etiology of periodontal destruction, and will be further reviewed below.

Actinobacter actinomycetem (AA) is a small, nonmotile, gram-negative, saccharolytic, capnophilic, round-ended rod. It was first suspected as a major contributor to periodontal destruction by its frequent detection in subgingival samples from patients with localized aggressive periodontitis (previously juvenile periodontitis) (74-77) and the fact that most subjects with localized aggressive periodontitis have an exaggerated serum antibody response to this pathogen when challenged. ((78-80). Furthermore, successful treatment of these patients was associated with elimination and/or reduction in the AA levels recovered in the saliva, whereas failures were

associated with inability to lower counts of AA. ((81-83) AA produces a number of damaging by-products, including a leukotoxin(84), is able to induce periodontal disease in animal experiments.(85), and can invade human gingival epithelial cells and escape host-immune detection.(86-88) Also, increased concentration of AA were recovered from sites undergoing progressive attachment loss (up to 2mm in 3 months) in human subjects. The collective evidence suggests that AA is a particularly virulent organism to the attachment apparatus around teeth, and may be a main etiologic contributor in localized aggressive periodontal disease.(89, 90)

Porphyrmonas gingivalis (Pg) is a gram-negative, anaerobic, nonmotile, asaccharolytic rod. It is a member of the black-pigmented bacteroides group and member of the red complex family. Pg has long been associated with chronic periodontal disease and (91) produces a wide array of virulence factors, including collagenase, multiple proteases, and endotoxin. Early investigations indicated that Pg was rarely recovered from samples of periodontally healthy subjects, but was a frequent finding in destructive periodontal disease. Like AA, in successfully treated sites, an elimination and/or reduction of Pg is often found in recurrent sites after treatment. ((92-94) Antibody studies have reliably demonstrated that most subjects undergoing attachment loss exhibit elevated antibodies to antigens of Pg. Animal studies in dogs and mokeys have revealed that ligature induced periodontitis occurs with a coincident increase in Pg.(95) Pg also has the ability to invade human gingival epithelial cells in vitro (96, 97) and has been recovered in epithelial cells recovered from the periodontal pocket.(98)

Like Pg, Prevotella intermedia (Pi) is gram-negative, anaerobic, nonmotile, asaccharolytic rod and is also a member of the black-pigmented bacteroides. Pi has been demonstrated to be elevated in number in acute necrotizing ulcerative gingivitis (ANUG) (99) and chronic periodontitis.(100). This species appears to have a number of similar virulence factors like Pg, including fimbriae specific for gingival epithelial cell-surface targets, allowing for internalization of the bacteria.

Tannerella forsythia (Tf) is a gram-negative, anaerobic, spindle-shaped, highly pleomorphic rod and is a member of the red complex family. It is extremely slow growing in-vitro, but it's growth may be enhanced by co-culturing with Fusobacterium nucleatum.(101) and the addition of N-acetylmuramic acid (102). Tf is often recovered from patients with chronic periodontal disease and from sites actively undergoing attachment loss.(103, 104) Increasing numbers of Tf have been shown to be strongly correlated with increasing pocket depth(103, 105). Also, refractory sites after treatment display a higher level of Tf and that it is the most commonly observed species in or on epithelial cells recovered from periodontal pockets.(105) Serum antibody to Tf is elevated in patients with chronic periodontal disease, and may be extremely elevated in refractory periodontitis.(106)

**1.6 Sjögren's syndrome and periodontal disease**

The periodontal status of SS patients has been studied previously, with mixed findings. In a studies by Kuru et al. (evaluated primary and secondary SS patients) and Jorkjend et al. evaluated secondary SS patients only), the periodontal status of SS patients in terms of clinical attachment level, probing depths, bleeding on probing,

plaque index, and bacteriological profiles were no different from age and sex-matched controls. They found that secondary SS patients had significantly more missing teeth, but the periodontal status of the teeth they had was no different from controls. (107, 108) Shiodt et al. again found no differences in all periodontal parameters in their study of only primary SS patients compared to controls.(109) Boutsi et al. found no increased risk for developing periodontal disease in their study of primary and secondary SS patients, although they did find that SS patients had better oral hygiene and more frequent dental visits.(110) In contrast, Antoniazzi et al. studied the periodontal status of primary and secondary SS patients compared to healthy age and sex-matched controls, and also measured the amount of interleukin 1-beta in their gingival crevicular fluid (GCF). They found that patients with SS had increases in all measured periodontal parameters including bleeding on probing, probing depth, and clinical attachment level compared to healthy controls. Also, they demonstrated that secondary SS patients had higher levels of periodontal disease than primary SS patients. Primary SS patients had significantly lower levels of IL1-beta in their GCF than patients with secondary SS or control patients.(111) Seck-Diallo et al. also found significant increases in inflammation (as measured by the gingival index of Sillness and Loe) and had a calculated odds ratio of 5.5 for developing periodontal disease after their study of 103 patients with SS compared to healthy controls.(112) Another study by Najera et al. demonstrated an odds ratio of 2.2 for developing the disease in SS patients.(113) The mechanism for increased susceptibility to periodontal disease in SS patients, if any indeed exists, is under investigation currently. Pers et al. investigated the salivary levels of BAFF and

correlated them to periodontal findings in SS patients compared to patients with clinical signs of xerostomia but did not have SS. They found that SS patients have increased levels of salivary BAFF compared to other dry mouth patients without SS, and that the levels of salivary BAFF correlated with increased probing depths in SS patients.(67) Scardina et al., using microangioscopy, determined that the microvasculature in the gingiva of SS patients was altered compared to healthy controls, and postulated that a decrease in local microcirculation would predispose this patient group to periodontal disease.(114) It has been shown previously from studies in the periodontal literature that progressive periodontal disease is mediated by B-cells. After clinical gingivitis develops, a T-cell mediated condition, the progression to periodontal disease occurs when the inflammatory infiltrate switches from T-cell dominated to B-cell dominated lesion.(115) In SS, increased levels of salivary BAFF coupled with decreased vascularity of the periodontal complex would favor development of clinical periodontal disease due to alterations in normal immune function (hyperactive B-cell dominated lesions) and blood supply. In summary, previous investigations have yielded contradictory findings about the prevalence of periodontal disease in both primary and secondary SS patients and the risk for developing periodontal disease once diagnosed with SS. Plausible molecular explanations exist if SS does in fact favor clinical periodontitis, but the issue is unclear and needs to be investigated further.

**1.7 Toll-like receptors**

TLRs are highly conserved pattern recognition receptors found in humans. They recognize pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan,

double stranded RNA, and bacterial DNA and allow for first-line activation of the innate immune system in response to infection.(116) To date, there have been 10 TLRs identified in humans.

In the gingiva, TLRs have been identified on gingival epithelial cells, gingival fibroblasts, osteoclasts, endothelium, osteoblasts, cementoblasts, and periodontal ligament fibroblasts. TLR4 is the only TLR that is present on each of the above cell types in the gingival tissues, and binds LPS present on the cell membrane of gram(-) bacteria, and is responsible for bacterial mucosal immunity. TLR4 is a localized to the cell membranes of these gingival cells as well as PMNs, macrophages, and natural killer cells. Once TLR4 binds LPS, it activates the innate immune system effector cells directly (in the case of TLR4 on leukocytes) or indirectly (in the case of TLR4 bound to gingival epithelial cells or fibroblasts) by promoting proinflammatory cytokine release and causing migration of the innate immune effector cells through chemotactic stimuli.(117) The presence of LPS in the periodontium induces an influx of immune cells to clear the infection, and in the process of clearing the infection, bone and the supporting structures of the teeth are destroyed from the host of proteolytic enzymes and free radicals released from the inflammatory and local area cells. A previous study had found that TLR4 was elevated in the saliva of adults diagnosed with chronic periodontitis.(118) Also, it has been shown that there are soluble TLR4 receptors in saliva that mediate macrophage activity and TNF-alpha secretion.(119) Overexpression of TLR4 could cause an exaggerated pro-inflammatory response and lead to increased periodontal destruction; conversely, defective TLR4 function and/or localized blockade

of the TLR4 could lead to decreased destruction compared to healthy individuals. The activation of TLR4 in the gingival epithelial cells lining the gingival sulcus also leads to stimulation and maturation of area dendritic cells, which process area bacterial antigens and present them to both the innate and acquired immune systems, causing a further influx of inflammatory cells and periodontal destruction.(117)

TLRs have been identified in SS patients. Ittah et al. demonstrated the presence of TLR3 and TLR7 in salivary gland epithelial cell cultures of SS patients. Both TLR3 and TLR7 are responsible for recognizing viral components. TLR3 is an extracellular receptor that recognizes double stranded RNA and TLR7 is an intracellular endosomal receptor that recognizes single stranded RNA. When the cell cultures were stimulated with double single and double stranded RNA and interferon-alpha (another anti-viral mediator), high levels of BAFF were excreted by these cells regardless of stimulatory source. This finding that stimulation of anti-viral pathways resulted in high levels of BAFF secretion. These findings provide more evidence that SS may be initiated by viral infection (not clear) and that malfunctions in viral immune mechanisms may inadvertently trigger BAFF expression and cause subsequent B-cell proliferation.(120) Furthermore, recent evidence indicates that a synergism exists between TLRs and B-cell receptors (BCRs) in activation of the B-cell itself. When TLRs and BCRs are crosslinked by a common antigen, such as LPS (the ligand for TLR4), the B-cell may become activated without additional co-stimulation from area immune cells or cytokines.(121) Other cell culture studies from labial minor salivary glands from SS patients and controls demonstrated increased expression of TLRs 2, 3, and 4, and when

stimulated with their respective ligands, increases in IL-6 production was produced from the cell cultures.(122) Also, in a study that measured mRNA expression for TLRs 1, 2, 3, and 4 by salivary gland epithelial cells from SS patients, an increase in expression was found for mRNA for the genes encoding TLRs 1, 2, and 4 compared to control data.(123) In summary, recent studies have found that TLR levels are elevated in cell culture studies of salivary gland epithelium from SS patients, specifically TLRs 1, 2, 3, 4, 7, and 9. TLR4 is a good candidate to study any molecular correlation between SS and periodontal disease because TLR4 is secreted in a soluble form in the saliva(119) and is upregulated in patient groups that have either SS(122) or chronic periodontal disease.(118) Analyzing salivary and serum levels of TLR 4 in SS patients may correlate with an increased risk of developing periodontal disease in this patient population and warrants further investigation.

**1.8 Toll-like receptors and other autoimmune diseases**

TLRs are the first line defense in humans for protection against microbial challenge, they have also been implicated in the development of many inflammatory and immune diseases, which are not thought to be directly related to bacterial or viral infection.(124) This property of TLRs is believed to be from their ability to recognize host-derived mediators released during the course of diseases. Through their ability to activate antigen presenting cells, TLRs provide a bridge between the innate and adaptive immune response.(125). The ubiquitous distribution of TLRs and changes in their signaling both with each other and the host cells have been implicated in altered susceptibility to infection as well as autoimmune disease.(126)

Rheumatoid arthritis (RA) is an autoimmune disease primarily associated with inflammation of the synovial membranes and subsequent destruction of adjacent cartilage and bone. TLR ligands have been demonstrated to be significantly elevated in RA synovial fluid and serum.(127) Specifically, expression of TLR2 and TLR4 is increased in RA-fibroblast-like synoviocytes (FLS). TLR2 and TLR4 ligands can induce receptor activator of nuclear factor-kappa beta ligand (RANKL) in RA-FLS and promote osteoclastic differentiation in the synovium of RA positive patients.(128)]. Furthermore, TLR2 activation in RA-FLS by TLR2 ligands causes an induction of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8) causing inflammation directly by activation of local inflammatory mechanisms and indirectly by aberrant angiogenesis.(129) In adjuvant-induced, zymosan-induced, and streptococcal cell wall-induced arthritis in animal models, TLR2 can induce and/or exacerbate pathogenesis.(130). Activation of TLR2 with peptidoglycan or bacterial lipopeptide causes nuclear factor kappa beta (NF-kb) translocation and increases expression of pro-inflammatory cytokines, adhesins, matrix metalloproteinases, and chemokines.(131). Heat shock protein bacteria 8 (HSPB8), a proposed TLR4 agonist, has been found to be highly expressed in the synovial tissue of RA patients and likely to be released during subsequent synovial inflammation. The release of HSPB8 into the synovial environment could lead to continuous activation of area inflammatory cells, amplifying synovial inflammation and providing a positive feedback mechanism to exacerbate the disease. Ex-vivo cultures of human synovial cultures from human patients with RA was shown to have TLR2 and TLR4 present in the cultured media and responsive to exogenous

ligands.(132) More importantly, signaling mediated by the pan-TLR adaptor Myeloid differentiation factor 88 (MyD88) and MyD88 adapter-like toll-interleukin 1 receptor domain containing adaptor protein (Mal/TIRAP), which is utilized by both TLR2 and TLR4, is involved in spontaneous production of cytokines and MMPs in RA synovial membranes. Additionally, human RA synovial membrane cultures release factors that stimulate macrophages in a MyD88- and Mal-dependent mechanism. In conclusion, TLR2 and TLR4 can both protect the host against infection, but also may promote the inflammatory cascade in RA by their interactions with exogenous and endogenous ligands present in situ.

Experimental autoimmune encephalitis (EAE) is the animal equivalent of multiple sclerosis in humans, a common inflammatory demyelinating disease of the central nervous system (CNS). Within the CNS, resident glial cell activation causes an inflammatory cascade that can lead to tissue destruction, neurologic deficits, and demyelination of neuronal cell membranes. Recently, TLR agonists of TLR2, TLR4, TLR9 have demonstrated adjuvant properties in EAE,.(133, 134) providing a mechanism of limiting autoimmune responses in the CNS by negative regulation of TLR pathways. In a rodent experiment, rats immunized with a combination of myelin basic protein peptide (MBP), CpG-Oligodeoxynucleotide (ODN) in incomplete freund's adjuvant (IFA) or LPS in IFA did not develop EAE. However, rats immunized with a combination of MBP, CpG-ODN AND LPS developed clinical EAE. In the same experiment, it was observed that spleen cells proliferated and secreted interferon-gamma in response to MBP, and caused secretion of IL-12p40 and IL-6 in response to CpG-

ODN and LPS. However, rats immunized with MBP, CpG-ODN, and Poly(I:C), a TLR3 ligand, did not develop EAE. Collectively, this study demonstrated that different combinations of TLR agonists can induce or protect the host from EAE.(135) In a follow-up study, wild type and TLR2 double knockout mice developed EAE in a similar time course of disease onset and severity after inoculation with myelin oligodendrocyte glycoprotein (MOG) peptide emulsified in complete freund's adjuvant (CFA). In contrast, under the same exposures, mice that are MyD88 double knockout are completely EAE resistant and TLR9 double knockout mice develop EAE but both the onset is slower and the severity is reduced. Thus, both TLR9 and MyD88 are required for normal EAE induction and progression. Stimulation of TLR3 by Poly(I:C) suppresses demyelination in EAE and suppression is associated with an induction and increase of endogenous interferon beta (IFN-B) and chemokine (C-C motif) ligand 2 (CCL2)-monocyte chemoattractant protein 1 (MCP-1). That study demonstrated that preferential activation of the MyD88-dependent, type 1 interferon-inducing TLR pathway may block the progression of tissue specific autoimmune disease.(136) Also, activation of TLR3 and TLR4 by their respective ligands, dsRNA and LPS, consistently induces secretion of C-X-C motif chemokine 10 (CXCL10) by human microglial cells. CXCL10 has been implicated as a major chemoattractant of T-lymphocytes in the MS lesion.(137, 138) The above studies have provided a plausible mechanism for T-cell infiltration in EAE via a TLR-dependent signaling cascade, but further study is needed before definite conclusions may be drawn.

Myocarditis is a major cause of sudden death in adolescents and young adults from sudden heart failure. A large portion of these cases are believed to be caused by cardiac myosin acting as an auto-antigen. In mice, dendritic cells primed with a cardiac-specific self peptide induce CD4+ T-cell mediated myocarditis. After the acute myocarditis resolved, these mice developed heart failure, and further stimulation of TLRs resulted in relapse of inflammatory cell infiltration in the heart. Also, injection of damaged syngeneic cardiomyocytes induced myocarditis in mice if TLRs were activated in vivo. (46). Protein kinase C-e (PKC-e)-deficient mice failed to succumb to experimental autoimmune myocarditis (EAM), but administration of the TLR9 ligand CpG restored EAM in PKC-e-deficient mice. It is believed the mechanism was from administered CpG directly restoring cell proliferation signals in TLR9-expressing T-lymphoctyes. Therefore, TLR mediated signaling in mice is adequate to overcome the normal regulatory signals of PKC and cause proliferation of a T-cells and cause the development of autoimmunity.(139) In another murine model, coxsackie virus infection was found increase expression of TLR4 on mast-cells and macrophages immediately following infection. TLR4 signaling can also increase acute myocaridits and elevate the production of inflammatory cytokines in cardiac tissue.(140) Coxsackie B virus (CBV) infection is believed to be a main culprit in the development of myocarditis associated with inflammatory heart disease death in young adults. Although these viruses are cytopathic, their RNA can persist in myocardium and contribute to a chronic inflammatory cardiomyopathy that is believed to be mediated through TLR8 and TLR7, but these claims require further investigation.(141) In conclusion, myocarditis is usually

an acute condition with fatal and/or debilitating outcomes. TLR-dependent pathways have been demonstrated to be a plausible mechanism for a healthy immune system going rogue and attacking the myocardial cells, with often times fatal outcomes.

Hepatitis is defined as inflammation of the liver and may be caused by liver injurty by toxins, autoimmune attack from the host, and microbial and/or viral infection. There are several different forms of hepatitis, but the main forms are caused by three viruses, hepatitis A, B, and C viruses respectively. Hepatitis C virus (HCV) infection usually causes a persistent inflammatory state of the liver via the host's immune system and usually ends with sclerosis and/or cancer of the organ. Recently, a correlation between the expression of TLR2, 3, and 6 in peripheral blood mononuclear cells (PBMC) and the severity of inflammation in the liver has been discovered in human chronic hepatitis C subjects.(142) Also, it has been shown that RNA levels for TLRs 2, 4, 5,6, 7, 8, 9, and 10 were increased in HCV-infected human monocytes and T-cells. TLR4 was only increased in T-lymphocytes, while TLR5 was selectively increased in monocytes.(143) TLR3 recognizes double stranded RNA and induces the production of type 1 interferon to help combat the perceived viral infection. In vitro, when the expression of TLR3 is suppressed in hepatocytes transfected with HCV, a continuous HCV infection is evident presumably due to decreased ability to combat viral infection in these cell lines.(144) Furthermore, in HCV infection, dendritic cell expression of TLR2 is reduced and TLR2-stimulated dendritic cells are less competent in stimulating T-cell proliferation in vitro and combating HCV, thus providing evidence that TLR2 may also be an important mechanism to protect the host from HCV-mediated

pathogenesis.(145) In murine models, macrophages the expression of HCV nonstructural proteins NS3, NS3/4A, NS4B, or NS5A was found to impair activation and signaling of TLR2, TLR4, TLR7, and TLR9 pathways in macrophage cell lines. NS5A and MyD88 interact via the interferon sensitivity-determining region (ISDR) and inhibits cytokine production from macrophages. Under normal conditions, TLR2-mediated pathways are turned on by HCV core and NS3 proteins, and requires homo- or heterodimerization with TLR1 or TLR6 in humans.(146-148) In chronic HCV infection in humans, there is a diminished number and function of HCV-reactive T-cells and antigen presenting cells and both cell types react with reduced function when stimulated with TLR ligands compared to wild-type cells from healthy hosts.(149) Immunotherapy is the main treatment modality for chronic HCV infection, which is causes problematic side effects and is only efficacious in roughly 50% of patients. Recently, it has been demonstrated that systemic administration of the TLR7 agonist isatoribine has a significant anti-viral effect and improves serum biomarkers with fewer and less severe systemic side effects.(150) Furthermore, in another murine model affected with Hepatitis B virus (HBV), ligands for TLR3, TLR4, TLR5, TLR7, and TLR9 cause reduced HBV replication in hepatocytes within 24 hours of their systemic administration in an interferon-dependent manner. The ability of TLR ligands to upregulate antiviral molecules at the site of infection may represent a novel, powerful therapy treat chronic hepatits infections.(151)

TLRs present on renal cells help coordinate and amplify host immune responses to kidney infection by recognition of endogenous molecules released from injured cells

and may contribute to acute injury to tubular cells and provide and adjuvant effect on renal inflammation(152) In a murine model, cultured renal tubular epithelial cells express TLRs 1-4, and TLR6 constituitively. When stimulated with LPS, TLRs 2-4 were upregulated and inflammatory cytokines were secreted in a dose-dependent manner by these cells.(153) TLR2 was detected in renal tubule, vascular, glomeruli, and medullary renal cells in human and murine renal cells. Mice deficient in TLR2 were more protected from ischemic renal injury than mice deficient in MyD88, demonstrating a plausible role for a TLR2-dependent/MyD88-independent pathway(s) that regulate renal injury during ischemia.(154) Further evidence came from a murine study in which a TLR2 ligand was given at time of immunization and caused profoundly increased nephrotoxic nephritis by promoting hyperactive adaptive immune responses against renal cells.(155) Other TLRs, such as TLR7 and TLR9 have been implicated in antigen-induced immune complex glomerulonephritis and lupus nephritis.(156, 157) Mice deficient in TLR9 display increased production of autoantibodies and acceleration of lupus nephritis compared to wild-type mice.(158) Furthermore, cultured proximal tubular cells display increased TLR9 when stimulated with immune complexes from sera from human patients with systemic lupus erythematosus. Other studies with MyD88 knockout mice displayed that MyD88 contibutes to the pathogenesis of TLR-mediated injury to renal cells. Also, TLRs have been demonstrated to be involved in transplant tolerance and rejection of grafted organs. In conclusion, TLR-mediated pathology in renal cells has been demonstrated in human and animal studies, and may provide an

interesting target for therapies in the future, however these pathways are in their infancy of medical understanding.(159, 160)

Systemic lupus erythematosus (SLE) is characterized by production of autoantibodies against a relatively narrow range of nuclear antigens by auto-reactive plasma cells. These autoantibodies form immune complexes that deposit in tissues of the skin, kidneys, and other organs and induce inflammation. A growing role for TLR-mediated contributions to the pathology of this disease are being elucidated, however under different conditions, they may be either a protective or damaging partner to the host with respect to SLE-associated pathology. Specifically, TLR5 and TLR7 signalling have been shown to enhance the inflammatory damage from SLE.(161, 162) A stop codon polymorphism in the gene endcoding TLR5 has been recently identified in humans that protects humans from development of SLE. Subjects with this polymorphism produce significantly lower levels of inflammatory cytokines compared to wild-type individuals.(161) In murine models, duplication of TLR7 enhances autoimmune disease in SLE-prone mice. Mice that are deficient in TLR7 but have had experimental SLE induced, display decreased lymphocyte activation and serum IgG responses, thereby improving the symptoms of SLE.(163) Other TLRs, such as TLR9 have a controversial role in SLE induction.(163, 164) In murine studies, TLR9 deficient mice display enhanced SLE-pathology compared to wild-type mice. Without TLR9, activated lymphocytes and plasmocytoid dendritic cells increased in number and displayed elevated levels of serum IgG and interferon alpha.(163) It has been hypothesized that TLR9's protective effect may be due to the ability of certain anti-

dsDNA antibodies to clear endogenous cellular debris thereby reducing the available substrate for anti-nuclear auto-antibodies.(165) TLR9 can be stimulated by endogenous hypomethylated DNA in addition to it's normal bacterial substrate. Like other studies, MyD88-dependent and independent pathways in SLE models have been shown to co-regulate TLR-mediated pathways. TLR9/MyD88 signaling is required for a switch to production of pathologic auto-antibodies in murine models of SLE,(166) and in TLR9 deficient mice, the generation of anti-dsDNA and anti-chromatin autoantibodies in inhibited. In contrast to TLR9, mice deficient in TLR3 does not inhibit formation of auto-antibodies to either DNA- or RNA-antigens.(164) Thus, TLR signaling can induce and/or reduce autoimmunity in SLE models and elucidation of more TLR-mediated pathways is a promising goal of future therapies to treat this debilitating disease.(167)

In conclusion, the complex interplay of TLRs with endogenous and exogenous ligands has the capacity to bring about a protective or inductive reaction and trigger and/or exacerbate many different inflammatory states. In addition to those mentioned above, diabetes mellitus types 1 (168) and 2 (169), autoimmune uveitis (170, 171), and the process of aging (172, 173), have all been investigated and confirmed inflammatory changes involving a collage of TLR-modulated signaling to both accelerate and/or ameliorate the conditions. SS, being an inflammatory condition as well, is highly likely to have multiple TLR-mediated mechanisms for the induction, progression, and arresting of the condition and warrants further investigation.

### 1.9 Salivary analysis in disease states

Previous studies have successfully analyzed saliva for determining disease states (HIV, hepatitis, bacteremias), hormonal levels, cancer status, and drug usage.(174) Salivary analysis of periodontal infections has been useful in determining bacterial profiles, indirect determinations of cellular damage by examining enzymatic levels associated with apoptosis/necrosis (aspartate and alanine aminotransferases, lactate dehydrogenase, creatine kinase, alkaline and acidic phosphatases, and g-glutamyl transferase), and degree and type of immune response by analysis of immunoglobulin type and numbers.(175) Serum components such as drug, drug metabolites, immunoglobulins, complement proteins, and hormones also reach the saliva, usually through gingival crevicular fluid outflow. Recently, studies analyzing the salivary and plasma levels of TLR2 and TLR4 have been performed on patients with chronic periodontitis. It was found that TLR4 was significantly elevated in both plasma and serum in periodontal patients, but TLR2 was elevated only in the plasma of periodontal patients compared to healthy controls. Therefore, TLR4 is present in detectable quantities in control patients, but is increased in patients with chronic periodontal disease and may be a useful diagnostic biomarker of treatment efficacy and/or disease status in future studies.(118)

### 1.10 Serologic analysis in disease states

Blood analysis has long been useful for diagnosing a variety of disorders, from diabetes mellitus to malignancies. Almost all of the secretions including, lacrimal and salivary, are derived from the bloodstream. When blood is analyzed and allowed to clot,

the clear supernatant that forms is the serum component, and contains no blood cells or clotting factors. Serum does, however, contain various other proteins that do not participate in the clotting process, various electrolytes, hormones, drugs and drug metabolites, antidodies, antigens, and can carry numerous types of microbial and/or viral intruders and their metabolites if present in the patient's blood. In the context of periodontal disease, serologic analysis been useful in determining immunologic, bacteriologic, and inflammatory mediator profiles, and how these profiles change in the face of different treatment modalities.(176, 177)

# CHAPTER II

# COMPARISON OF SOLUBLE TOLL-LIKE RECEPTOR 4 IN PATIENTS WITH SJÖGREN'S SYNDROME, A CASE-CONTROL STUDY

## 2.1 Synopsis

This cross-sectional study examined potential differences in concentration of Toll-like receptor 4 (TLR4) in serum and citric acid-stimulated saliva from patients with Sjögren's Syndrome (SS) and healthy age-, sex-, and periodontal disease matched control patients.

Salivary function was stimulated with 5.0 mLs of 2% citric acid, and whole saliva samples were obtained. Full-mouth clinical periodontal measurements were recorded from 21 SS patients and 22 unaffected control patients. Salivary and plasma TLR4 concentrations were determined by enzyme-linked immunoassays in 20 patients per study group. Data were tested statistically as appropriate for the data distribution.

No statistical difference in age, body mass index (BMI), salivary TLR4 concentrations, or clinical periodontal measurements were evident between either group. Salivary flow rates were significantly lower ($P<0.001$) whereas plaque % ($p=0.048$) and serum TLR4 concentrations ($p=0.011$) were significantly higher in SS patients.

The present findings support a hypothesis that a hyperinflammatory systemic state, as seen in SS patients, can independently raise serum TLR4 concentration in the absence of periodontal disease and may be important in the induction and / or progression of SS-mediated pathophysiology in affected patients.

## 2.2 Introduction

SS is an autoimmune exocrinopathy and classically affects the salivary and lacrimal glands. Approximately 2-4 million people in the United States are affected by the condition, with women diagnosed nine times more often than men. SS is classified as either primary or secondary, depending on the absence or simultaneous presence of another autoimmune disease (AI).(1) A definitive biomarker or serum finding signifying positive disease status has remained elusive, and most diagnoses are made later in the disease process and are usually classified by the American-European Consensus Classification Criteria from 2002. This classification system assesses six categories and includes ocular and oral symptoms, ocular signs, histopathology of minor salivary gland biopsies, salivary gland involvement, and autoantibodies upon serologic analysis. A definitive diagnosis is made if four of six items are positive as long as the histopathology and serologic findings are positive. Alternatively, if three out of the four objective items (clinical tests vs. symptoms) are positive a diagnosis may be made.(2) A revised definition was made by the American Academy of Rheumatology in 2012. This definition requires two out of three objective features be met for a positive diagnosis and assesses serologic findings, minor gland histopathology, and ocular signs.(3) Consistent oral manifestations include increased caries rate, dryness, increased fungal infections, painless enlargement of salivary glands, altered salivary flow, difficulty in swallowing, speaking, chewing, and alterations in taste. Biopsies of minor salivary glands are considered the "gold standard" in SS diagnosis. SS is diagnosed by the presence of pathologic changes consisting of a T-lymphocyte predominated inflammatory cell

infiltrate around periductal tissues with a resulting loss of acinar cells and secretory function.(4) Nonspecific serum changes in SS patients may include hypergammaglobulinemia, elevated total protein and erythrocyte sedimentation rate (ESR), decreased white blood cell count, persistent elevated rheumatoid factors, and autoantibodies anti-sicca-syndrome-A (Anti-SS-A, also known as Ro) and Anti-SS-B (also known as La). SS also predisposes patients to an increased risk of developing lymphoma(s) with a reported 4000% higher incidence than the general population..(5) Treatment is palliative in nature and often includes sialagogues to increase salivary flow; however, these medications have many potential side-effects and do not slow the destruction of the salivary glands. New therapies, such as TNF-alpha blocking agents or B-cell depletion therapy show promise but need more study before routine clinical application.(6)

Chronic periodontal disease is among the most common oral diseases in humans. It is believed to be initiated by a complex interplay between bacterial biofilm colonization in the periodontal pocket and the host immune response and is marked by inflammatory changes in the supporting structures of the teeth. Initially, reversible gingival inflammation, gingivitis, is characterized by a largely T-lymphocyte infiltration of the soft tissues causing edema and erythema with an increase in gingival bleeding. If allowed to progress, gingivitis may remain stagnant or may progress to periodontitis,where the infiltrate switches to a plasma cell dominated lesion. The immune system is activated and an inflammatory destruction of the attachment

apparatus and bone around the teeth ensues(7). However, the exact mechanism of the inflammatory signaling process is unknown.

TLRs are highly conserved pattern recognition receptors found in humans. They recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycans, and various bacterial and/or viral genetic materials such as double stranded RNA. This allows for first-line activation of the innate immune system in response to infection.(8) To date, there have been 11 TLRs identified in humans. TLR4 is membrane bound and secreted in a soluble form, recognizes LPS, and is responsible for mucosal surveillance of gram(-) bacterial infection. In the oral cavity, it is found on epithelial cells, gingival fibroblasts, osteoclasts, endothelial cells, osteoblasts, cementoblasts, and periodontal ligament fibroblasts it activates local polymorphonuclear neutrophils (PMNs) and macrophages in the face of gram(-) infection. Previous in vitro cell-culture studies have indicated that SS patients secrete more inflammatory cytokines and have increased mRNA levels of TLR4 when stimulated with LPS.(9), (10)

In the gingiva, TLRs have been identified on gingival epithelial cells, gingival fibroblasts, osteoclasts, endothelium, osteoblasts, cementoblasts, and periodontal ligament fibroblasts. TLR4 is the only TLR that is present on each of the above cell types in the gingival tissues. It binds LPS present on the cell membrane of gram(-) bacteria and is responsible for bacterial mucosal immunity. TLR4 is also localized to the cell membranes of PMNs, macrophages, and natural killer cells. Once TLR4 binds LPS, it activates the innate immune system effector cells directly (in the case of TLR4 on

leukocytes) or indirectly (in the case of TLR4 bound to gingival epithelial cells or fibroblasts) causing proinflammatory cytokine release and causing migration of the innate immune effector cells through chemotactic stimuli.(11) Immune cells infiltrate the affected areas to clear the infection, and in the process bone and the supporting structures of the teeth are destroyed from the host of proteolytic enzymes and free radicals released from the inflammatory and local area cells. A previous study had found that TLR4 was elevated in the saliva of adults diagnosed with chronic periodontitis.(12) Also, it has been shown that there are soluble TLR4 receptors in saliva that mediate macrophage activity and TNF-alpha secretion.(13) Overexpression of TLR4 could cause an exaggerated pro-inflammatory response and lead to increased periodontal destruction; conversely, defective TLR4 function and/or localized blockade of the TLR4 could lead to decreased destruction compared to healthy individuals. The activation of TLR4 in the gingival epithelial cells lining the gingival sulcus also leads to stimulation and maturation of area dendritic cells, which process area bacterial antigens and present them to both the innate and acquired immune systems, causing a further influx of inflammatory cells and periodontal destruction.(11)

TLRs have been identified in SS patients. TLR3 and TLR7 have been identified in salivary gland epithelial cell cultures of SS patients, both of which are responsible for viral recognition. Stimulation of these cells with the respective ligands of TLR3 and TLR7 caused an elevated release of B-cell activating factor (BAFF), causing B-cell proliferation. These findings provide evidence that SS may be initiated by viral infection and that malfunctions in viral immune mechanisms may inadvertently trigger

BAFF expression and cause subsequent B-cell proliferation.(14) Furthermore, recent evidence indicates that a synergism exists between TLRs and B-cell receptors (BCRs) in activation of the B-cell itself. When TLRs and BCRs are cross-linked by a common antigen, such as LPS (the ligand for TLR4), the B-cell may become activated without additional co-stimulation from area immune cells or cytokines.(15) Other cell culture studies from labial minor salivary glands from SS patients and controls demonstrated increased expression of TLRs 2, 3, and 4, and when stimulated with their respective ligands, increases in IL-6 production was produced from the cell cultures.(9) Also, in a study that measured mRNA expression for TLRs 1, 2, 3, and 4 by salivary gland epithelial cells from SS patients, an increase in expression was found for mRNA for the genes encoding TLRs 1, 2, and 4 compared to control data.(10) In summary, recent studies have found that TLR levels are elevated in cell culture studies of salivary gland epithelium from SS patients, specifically TLRs 1, 2, 3, 4, 7, and 9. TLR4 is a good candidate to study any molecular correlation between SS and periodontal disease because TLR4 is secreted in a soluble form in the saliva(13) and is upregulated in patient groups that have either SS(9) or chronic periodontal disease.(12) Analyzing salivary and serum levels of TLR 4 in SS patients may correlate with an increased risk of developing periodontal disease in this patient population and warrants further investigation.

Saliva is a useful diagnostic fluid and has been used to determine disease states and assay cellular damage, cancer biomarkers, hormonal levels, and drug use.(16) Saliva is readily available and conveniently collected, and has previously been shown to contain inflammatory mediators collected from periodontal patients including

measurable amounts of TLR4 (12) Whole saliva comes from exocrine glands of the oral cavity and contains inflammatory mediators of pooled sites from the oral cavity and may provide a useful assessment of general inflammation in contrast to gingivocrevicular fluid which is site-specific.(17)

Blood analysis has long been the gold standard for diagnosing a variety of disorders. Almost all secretions of the lacrimal and salivary glands are derived from the blood. When blood is collected and is allowed to clot, the clear supernatant that forms is the serum component, and contains no cells or clotting factors but does contain electrolytes, hormones, drugs and drug metabolites, antibodies, antigens, bacteria, viruses, and bacterial and viral metabolites. Serum analysis has been used to assess immunologic, bacteriologic, and inflammatory mediator profiles and how these values change with treatment with respect to periodontal disease.(18, 19)

To the best of our knowledge, neither salivary nor circulating levels of TLR4 have been analyzed in patients with SS. Our hypothesis is that salivary and serum levels of TLR4 are increased in SS patients due to upregulation by stimuli generated from receptor crosstalk involved in the signaling cascades of induction and progression of SS compared to age-, sex-, and periodontal disease status-matched control patients.

## 2.3 Materials and methods

### 2.3.1 Study population

A total of 43 subjects were included in the study. The study protocol was approved by the Institutional Review Board of Texas A&M Health Science Center (TAMUHSC), and conducted in full compliance with ethical principles, including the

Helsinki Declaration of 1975, as revised in 2000. Twenty-one non-smoking patients with diagnosed SS and twenty-two, non-smoking, age and sex-matched control patients were recruited from the staff and faculty of Texas A&M University-Baylor College of Dentistry (TAMUBCD). Patients were recruited from October 2012 to August 2013 from the Stomatology and Salivary Dysfunction Clinic. The American-European Consensus Classification Criteria was used to make a positive diagnosis (49), and was used because many of these patients were diagnosed prior to the 2012 diagnostic revision by the American Academy of Rheumatology.(178) The study protocol was explained and written informed consent was obtained from each individual before salivary and venous blood collection, and periodontal measurements were made. Medical and dental histories were obtained. All patients had 16 teeth present with at least two molars. Patients with diabetes mellitus, hepatitis, malignancies, oral and/or mucosal ulcerations, clinically detectable oral/pharyngeal candidiasis, pregnant and/or lactating females, those under the age of 18, visible oral/mucosal ulcerations, clinically detectable untreated odontogenic infections, or who had periodontal therapy six months previously were excluded from the study (Table 1). Periodontal disease was assessed by the clinical case definitions proposed by the Centers for Disease Control working group for use in population based surveillance of periodontitis.(179) An absence of periodontitis was confirmed if patients did not have ≥ 2 interproximal sites with a clinical attachment loss (CAL) of ≥3 mm or ≥ 2 sites with interproximal probing depths (PD) of ≥ 4mm (excluding mild, moderate, and severe periodontitis).(180) After data collection from SS patients, age and sex-matched control patients were recruited and

were excluded if any of the above conditions existed or if the periodontal status differed from the SS-matched patient in an attempt to exclude the effect periodontal disease may have on serum and salivary levels of TLR4.

### 2.3.2 Salivary collection

Stimulated salivary collection was completed as described by Navazesh et al.(181) Briefly, patients were instructed to brush and floss their teeth, rinse, and fast for two hours immediately before salivary collection. At the time of collection, patients rinsed with 10.0 mL's of sterile deionized water for one minute. Salivary flow was stimulated with a five second swish of 5.0 mLs of 2% citric acid. After one minute, the patients expectorated any accumulated secretions into a separate container that was discarded. Then, expectorated whole saliva was collected on ice into pre-weighed, pre-chilled sterile 50.0 mL polypropylene tubes until a total volume of 5.0 mL's was reached or twenty minutes had elapsed. Salivary collection was timed and weighed in order to calculate a salivary flow rate. Saliva was immediately processed by centrifugation (800g) for 10 minutes at 4ºC and aliquoted into 500 uL amounts with sterile water. The samples were immediately frozen and stored at -80ºC until the sample collection period ended and thawed immediately before assays.

### 2.3.3 Serum collection

Ten mLs of venous blood was taken from the antecubital fossa by standard venipuncture with Vacutainers®.[k] The Vacutainer was inverted five times immediately after collection and allowed to sit undisturbed for one hour per manufacturer's recommendations. Serum was separated from blood by centrifugation at 12,000 rpm for

ten minutes and stored at -80ºC until the sample collection period ended and thawed immediately before assays.

### 2.3.4 Periodontal measurements

After saliva and serum collection, clinical periodontal measurements including PD, CAL, presence of bleeding upon probing (BOP), and plaque from six sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) of all teeth were recorded with a UNC15 periodontal probe.[‡] Furcations (Glickman classification) and mobility (Miller classification) were assessed with a Nabors probe and bi-digital manipulation. CAL was assessed from the cementoenamel junction (CEJ) to the base of the probable pocket and BOP was positive if it occurred within 30 seconds after probing. Finally, a disclosing agent[††] was applied to the teeth per manufacturer's recommendations, and plaque was recorded dichotomously as present or absent at six sites per tooth. Measurements were recorded by two calibrated examiners (JB, HP). Examiners were calibrated before the study began and again three months into the study by comparing full mouth probing exam values. Demographic and periodontal measurements were reported for all patients in the study (n=21 SS, n=22 control).

### 2.3.5 Measurement of toll-like receptor 4 in serum and salivary samples

One test sample and two control sample were randomly selected and used for calibration purposes and were not used for ELISA analysis. The remaining 20 test samples and 20 control samples were selected for analysis with TLR4 enzyme-linked immunosorbent assay (ELISA) kits[§] following the manufacturer's protocol. Samples were not diluted. The concentrations of TLR4 in the saliva and serum samples were

determined by comparing the average sample optical density (OD) reading to concentrations from the assay standard curve. For samples that had an optical density above the maximum detectable range, a linear equation was derived from the standard curve and measured OD values were used to derive the concentration of TLR4. The lower detection thresholds for the TLR4 assay was 0.016 ng/mL, the upper detection limit was 10.0 ng/mL.

### 2.3.6 Statistical analysis

A power analysis was performed from a previous study, and with a power of 80% and alpha at 0.05, the minimum number of subjects to detect a difference was twenty per group. Shapiro-Wilk normality tests were performed on all numerical variables with the null hypothesis that the variable in question was normal versus the alterative that the variable was not normal. For variables that were normal, a t-test was performed between the control group and the SS group. For variables that were not normal, Mann-Whitney was used, and to settle disputes caused by ties in Mann-Whitney, jittering was used and repeated (Monte-Carlo) with 10,000 permutations. The p-value was averaged over the 10,000 permutations, creating a simulated p-value, and reported. Correlation coefficients were found using Pearson's correlation coefficient between variables, and correlation coefficients greater than 0.6 were considered to be noteworthy. Finally, for the binary data if the patient visited the dentist in the past 6 months (1 = Yes and 0 = No), the Fisher's Exact Test was used because this data was structured as a 2x2 contingency table.

The number of patients taking an individual medication was counted and summed according to the pharmacological category of that particular drug (e.g. antihypertensives, psychologics, biologics, etc.). A percent difference was calculated as

$$percent\ difference = \frac{(\#of\ patients\ with\ SS\ taking\ Drug - \#of\ patients\ as\ Control\ taking\ Drug)}{\#of\ patients\ with\ SS\ taking\ Drug}$$

The distribution of the number of drugs taken by patients was tested for normality using the Shapiro-Wilk statistic, which indicated that the distribution was in fact not normal. Therefore, the total number of drugs used by the SS group and control group was analyzed using a Mann-Whitney test to see if there was a significant difference.

Serum and saliva were analyzed for TLR4 using Mann-Whitney U test. The first two consecutive patients that completed the study protocol were excluded for calibration and training purposes. One more patient was randomly eliminated three months into the study and used for re-calibration purposes. Thus, twenty patients per group remained. All statistical tests were performed at alpha = 0.05. All statistical tests were completed with SPSS (IBM).

## 2.4 Results

### 2.4.1 Clinical analyses

Inter-examiner differences were not statistically different for measured clinical variables (Table 2). Clinical variables and mean/median values of clinical measurements are outlined in Table 3. The healthy control group exhibited no significant differences with regard to age, BMI, PD, CAL, furcation involvements, and mobility as the SS group, but had significantly less plaque ($p<0.041$) than the SS group.

The male/female ratio was 3:18 for the SS group and 2:20 for the control group, with no significant difference in sex distribution between either group. Salivary collection mass and salivary flow rates were significantly different, with the SS group having significantly lower salivary flow ($p<0.001$) than the control group. No significant difference was observed in frequency of dental visits, with both groups reporting they received dental care more than twice annually. Prescription drug information is displayed by drug class in Table 4. Some patients were on multiple classes of drugs to treat the same condition (e.g. hypertension), therefore the total number of drugs was tabulated per class per patient group instead of the number of patients taking a drug in each group. As expected, SS patients were on significantly more prescription medication (6.61 medications/patient vs. 2.36 medications/patient in the control group). SS patients took significantly more anti-malarial medication (hydroxychloroquine) and medication for analgesia, gastro-esophageal reflux disease (GERD), psychiatric conditions, dry mouth, insomnia, thyroid dysfunction, and hormone replacement therapy (HRT). No significantly different amounts of medication were taken to treat allergies, hypertension, or hyperlipidemia. Significantly fewer SS patients took no medication compared to control patients (2 SS patients, 8 control patients). In addition to the tables listed above, correlations between variables were calculated. Significant correlations were determined to be correlations with $r > .6$. Notable correlations were Flow with Saliva Mass (r=.863), Flow with Collection Time (r=-.759), Average PD with Average CAL (r=.604), PD > 5 mm with Collection Time (r=.655), and number of Grade 1 Furcation with Grade 2 Furcation (r=.617). Salivary volume (lower SS group, $p < .001$),

Salivary Flow (lower SS group, p < .001), and plaque index (higher SS group, p = .049) were all statistically significantly different. Finally, a z-proportion test was performed to see if the 3:18 ratio of men-women in the SS group of the study reflected the population proportion of 1:9 accurately. The result of the test was $p = .735$, indicating there was no significant difference between the study and the true population sex ratio.

### 2.4.2 Salivary and serum toll-like receptor 4 concentration

There was no statistically significant difference in salivary TLR4 levels between patient groups (p = 0.312), however; serum TLR4 was significantly elevated in SS patients vs. controls (p =0.011) as seen in Table 5.

## 2.5 Discussion

The present study compared salivary and serum values of TLR4 in patients with SS and with age, sex, BMI, and periodontal disease-matched control patients. As expected, significant differences in salivary flow rates were found between groups. The decreased salivary function in SS patients may account for this and consequently these patients were taking more sialagogue medications than control patients (14/21 SS patients, 0/22 control patients). The loss of protective salivary functions, such as decreased clearance from diminished quantities of IgA, IgM, mucins, and salivary washing action possibly explains the higher plaque percentage scores observed in the SS patients in the present study.

While no direct statement may be made on the prevalence of periodontal disease in SS patients from our study, it provides supporting evidence that SS is not a risk factor for periodontal disease because of our ability to recruit 22 patients that were

periodontally healthy with SS. This is in agreement with Kuru et al. and Jorjend et al. who found no higher prevalence of periodontal disease in SS patients.(182) Also, similar to a study by Boutsi et al., we found that SS patients visit their dentist more than recommended (>2 times/year) and tend have meticulous oral hygiene. Even though supragingival plaque was statistically higher than control patients, bleeding on probing was less (although the difference was not statistically different) and frequent trips to their dental professionals for prophylaxis and reduction in the intra-oral bioburden may be protective in nature from developing periodontal disease in SS patients in the absence of normal salivary flow. Also, the role of anti-inflammatory medication cannot be ignored as more SS patients were taking immunomodulating medications which may have masked clinical oral/gingival inflammation.

We found no differences in salivary concentrations of TLR4 between SS and control patients. This was somewhat expected since we excluded patients with periodontal disease. Periodontal disease, being a predominantly gram (-), anaerobic infection, provides antigens for both membrane-bound and soluble TLR4 and causes activation of the innate and consequently acquired immune responses.(116, 117) Without a suitable number of antigens in the absence of periodontal disease, it follows that TLR4 would not be expressed more than at a basal level for necessary surveillance of the oral cavity. Also, the present study found salivary TLR4 concentrations of 2.650 ng/mL and 2.364 ng/mL for the SS and control patients, respectively, in contrast to Buduneli et al.'s study who found 14.44 ng/mL average salivary concentration in healthy control patients. This difference may be partially explained by the different methods of

salivary collection. Both studies collected whole saliva, however, the present study utilized stimulated saliva with 2% citric acid and Buduneli's study utilized unstimulated saliva by expectoration only. Stimulated saliva differs in composition by its source, with approximately 50% coming from the parotid gland while unstimulated saliva is composed of ~20% parotid, 65% submandibular, 7-8% sublingual, and <10% from minor glands).(184) The contribution of each major and collective minor salivary glands has on TLR4 concentration requires further study in disease and health in multiple age groups before definitive conclusions may be drawn. Also, citric acid may chelate proteins, thus decreasing the amount of soluble TLR4 available for capture; however, the method of collection should have greatly reduced and/or eliminated this interference. Citric acid was used in the present study, despite its limitations, because it is one of the most potent stimulators of salivary flow, and was necessary to collect an adequate salivary volume in SS patients. Furthermore, geographic differences in dietary composition, genetic factors, influence of medication, and age-related differences also partially explain the differences between our findings and those of Buduneli et al. Finally, 'normal' values of TLR4 in saliva are unknown. To our knowledge, this is the only study that has described the concentration of TLR4 in stimulated human saliva in healthy and SS affected patients.

Differences in serum TLR4 values may be explained by increased molecular crosstalk from autoimmune complexes potentially creating a hyperactive systemic immune state. These findings are in agreement with in the vitro findings of Spachidou et al.(123), Kamachi et al. (122), and Berglova (121) that demonstrated increased

expression of TLR4 mRNA in salivary gland epithelial cells, increased expression of membrane-bound TLR4 complexes, and hyperactive B-lymphocyte function when challenged with LPS respectively. Serum TLR4 concentrations of the control patients in the present study were lower than those reported in Budeneli et al. (0.840 ng/mL in the present study vs. 0.97 ng/mL in Budeneli et al.) Like the salivary values, differences in geography and demographics, medication regimen, and patient age may potentially be the cause of a different result. Again, 'normal' TLR4 serum concentration expression in health and disease in different age groups requires more investigation to define what a true normal/abnormal value is in health and disease.

SS patients were on significantly more prescription medication than control patients, and took more anti-malarial medication (hydroxychloroquine), analgesics, HRTs, and medication to treat GERD, psychiatric conditions, dry mouth, insomnia, and thyroid dysfunction. No differences were observed with frequency of patients taking medication to treat allergies, hypertension, or hyperlipidemia. Also, only two SS patients were not taking any prescription medication whereas eight control patients did not take any. Patients were not instructed to discontinue the use of any prescribed medication they currently were taking, as doing so would be unethical. However, the local and systemic effects cannot be ignored and may have skewed our data. For example, SS patients may in fact have more periodontal disease and inflammation than unaffected patients, as reported by Najera et al. (113), Seck-Diallo et al.(112), and Antoniazzi et al. (111), but the clinical presentation of inflammation as bleeding on probing was masked by immunomodulatory medication. Also, stimulated salivary

secretion volume, secretion rate, and TLR4 concentration would probably be different in the absence of sialagogue medication in SS patients. Interestingly, 9/21 SS patients were being treated with proton-pump inhibitors (PPIs) for GERD. A recent in vitro study by Ubagai et al. reported that PPI's decreased PMN function with respect to release of inflammatory mediators (various cytokines, TNF-alpha, TLR4) when challenged with LPS and found a down-regulation of all gene transcripts analyzed when the duration of treatment exceeded 3 hours.(184) The effects of PPIs on TLR4 expression in the serum and saliva in vivo requires further study but the potential of PPI therapy to influence our observed results exists and may have affected our findings. Finally, the anti-malarial drug hydroxychloroquine has long been used for treatment of various rheumatic diseases and has potent immunomodulatory effects. In vitro human cell-culture studies and murine models have demonstrated that hydroxychloroquine causes a down-regulation of TLR4 when challenged with LPS (185, 186). The potential influence of this unique drug also could further contribute to the observed findings. The systemic effect of medication regimens cannot be ignored in the present study and therefore the current findings should be interpreted with caution until there is further study on the independent and interactive effects of different medications on salivary and serum TLR4 concentrations.

# CHAPTER III

# CONCLUSIONS

## 3.1 Summary of findings

The present study demonstrated that stimulated salivary flow rates are statistically significantly lower and plaque percentage is statistically significantly higher in SS patients than controls. Also, SS patients on average visited their dental professional >2 times per year and enhanced dental care may have promoted good periodontal health in the absence of normal salivary function. Furthermore, stimulated salivary concentrations of TLR4 were not statistically significantly different than control patients but serum concentrations of TLR4 were statistically significantly elevated compared to control patients.

## 3.2 Conclusions

These finding may represent an enhanced systemic inflammatory state and prove to be a useful marker to gauge the efficacy of a given therapeutic regimen. The study could not control for the systemic and local effects of medications, and further investigation is needed to elucidate the "normal and abnormal" values of TLR4 in disease and health.

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## APPENDIX A

## TABLES

**Table 1. American-European consensus classification of Sjögren's syndrome (2)**

| | |
|---|---|
| I. Ocular symptoms: a positive response to at least one of the following questions: | 1. Have you had daily, persistent, troublesome dry eyes for more than 3 months?<br><br>2. Do you have a recurrent sensation of sand or gravel in the eyes?<br><br>3. Do you use tear substitutes more than 3 times a day? |
| II. Oral symptoms: a positive response to at least one of the following questions: | 1. Have you had a daily feeling of dry mouth for more than 3 months?<br><br>2. Have you had recurrently or persistently swollen salivary glands as an adult?<br><br>3. Do you frequently drink liquids to aid in swallowing dry food? |
| III. Ocular signs—that is, objective evidence of ocular involvement defined as a positive result for at least one of the following two tests: | 1. Schirmer's I test, performed without anaesthesia (< 5 mm in 5 minutes)<br><br>2. Rose bengal score or other ocular dye score (>4 according to van Bijsterveld's scoring system) |
| IV. Histopathology: In minor salivary glands | (Obtained through normal-appearing mucosa) focal lymphocytic sialoadenitis, evaluated by an expert histopathologist, with a focus score >1, defined as a number of lymphocytic foci (which are adjacent to normal-appearing mucous acini and contain more than 50 lymphocytes) per 4 mm2 of glandular tissue |
| V. Salivary gland involvement: objective evidence of salivary gland involvement defined by a positive result for at least one of the following diagnostic tests: | 1. Unstimulated whole salivary flow ( < 1.5 ml in 15 minutes)<br><br>2. Parotid sialography showing the presence of diffuse sialectasias (punctate, |

**Table 1. Continued**

| | |
|---|---|
| | cavitary or destructive pattern), without evidence of obstruction in the major ducts<br><br>3. Salivary scintigraphy showing delayed uptake, reduced concentration and/or delayed excretion of tracer |
| VI. Autoantibodies: presence in the serum of the following autoantibodies: | 1. Antibodies to Ro(SSA) or La(SSB) antigens, or both |

**Table 1: American-european consensus classification of sjogren's syndrome.(2)** For primary SS, a positive diagnosis if four out of the six items are positive as long as either item IV or VI is positive. Also, if three of the four objective criteria are positive (items III, IV, V, and VI), a positive diagnosis is made. For secondary SS, the presence of item I or item II plus any 2 from among items III, IV, and V may be considered as indicative of secondary SS.

**Table 2. Exclusion and inclusion criteria**

| Exclusion | Inclusion |
|---|---|
| < 18 years of age | ≥18 years of age |
| <16 teeth or < 2 molars | ≥16 teeth, ≥2 molars |
| Pregnant and/or lactating females | Documented history of SS (for SS patients) |
| Past or present history of malignancies, HIV/AIDS, hepatitis, diabetes mellitus | Absence of periodontal disease (< 2 sites with interproximal CAL of ≥3mm and/or < 2 interproximal sites with PD ≥4mm |
| Periodontal therapy in the past six months | Periodontal therapy > 6 months previously |
| Oral/mucosal ulcerations | |
| Clinically detectable untreated odontogenic infection(s) | |
| Clinically detectable oropharyngeal candidiasis | |

**Table 2: Exclusion and inclusion criteria.** Subjects were excluded or included based upon the above parameters.

**Table 3. Inter-examiner calibration**

| | Buccal | | Palatal | | Total | |
|---|---|---|---|---|---|---|
| **Percent Agreement (%)** | 65.7 | | 54.3 | | 60.0 | |
| **T-test p-value** | .459 | | .122 | | .496 | |
| **ICC** | .628 | | .491 | | .558 | |
| **T-test p-value (Bias)** | .229 | (0.047) | .519 | (-0.114) | .750 | (-0.033) |

**Table 3: Inter-examiner calibration.** Calibration statistics for buccal, palatal, and combined buccal and palatal (total). From the table above, it is evident that both raters were similar. The total percent agreement was above 60%, and any differences were 1 mm or less. The total t-test for the raw difference between both raters was not significant (p-value = .496). Additionally, the t-test for bias was insignificant (p-value = .75).

**Table 4. Patient demographics and periodontal parameters**

| | SS Patients n = 21 Median (IQR)* | Control Patients n = 22 Median (IQR)* | p-value |
|---|---|---|---|
| **Age (years)*** | 59.3 (12.07) | 52.6 (12.99) | 0.085 |
| **Saliva Mass (g)*** | 3.31 (1.48) | 4.99 (1.37) | <0.001 |
| **Salivary Flow (g/min)** | 0.30 (0.19) | 0.89 (0.57) | <0.001 |
| **Body Mass Index*** | 28.6 (7.24) | 29.9 (7.53) | 0.549 |
| **Average Probing Depth (mm)*** | 2.27 (0.42) | 2.33 (0.25) | 0.552 |
| **Average Clinical Attachment Loss (CAL) (mm)*** | 2.58 (.58) | 2.50 (0.41) | 0.569 |
| **Bleeding on Probing** | 1.00 (8.00) | 0.50 (2.00) | 0.225 |
| **Plaque %** | 25.6 (35.90) | 19.5 (10.00) | 0.049 |
| **Class 1 Mobility (# teeth)** | 0.00 (0.00) | 0.00 (0.00) | 0.547 |
| **Class 2 and 3 Mobility (# teeth)** | N/A | N/A | N/A |
| **Probing depth ≥4mm (# teeth)** | 0 (4.00) | 0 (1.00) | 0.568 |
| **Probing depth ≥5mm (#** | 0 (0.00) | 0 (0.00) | 0.423 |

**Table 4. Continued**

| | | | |
|---|---|---|---|
| **Grade 1 Furcations (# teeth)** | 1.00 (2.00) | 0.00 (1.75) | 0.397 |
| **Grade 2 Furcations (# teeth)** | 0.00 (0.00) | 0.00 (0.00) | 0.408 |
| **Grade 3 Furcations (# teeth)** | N/A | N/A | N/A |
| **Dental visits in last >2 times per year (% of group who did)**** | 67 | 54 | 0.132 |

**Table 4: Patient demographics and periodontal parameters.** The single star (*) indicates a variable that was determined to be normal by Shapiro-Wilk statistic. For each normal variable the mean and standard deviations were reported. Additionally, a t-test was performed, and its associated p-value was reported. For those not marked with a single star (*) or a double star (**), the median and interquartile range (IQR) was reported, as well as the p-value from the Mann-Whitney test, the final row (designated as "**") represents the percent of patients who did visit the dentist within 6 months and the p-value from Fisher's exact test was reported**.**

**Table 5. Medication regimens per group**

| Medication | # Medications in SS patients | | Percent Difference (%) |
|---|---|---|---|
| **ANTIHYPERTENSIVES** | **8** | **15** | |
| • Beta-blockers | 2 | 4 | |
| • Diuretics | 1 | 6 | **-87.5** |
| • Ca++ blockers | 4 | 2 | |
| • Angiotensin 2 inhibitors | 1 | 3 | |
| **ANTI-PLATELET** | **3** | **5** | |
| • Salicylic Acid | 3 | 3 | **-66.7** |
| • Clopidrigel | 0 | 2 | |
| **ANTI-HYPERLIPIDEMICS** | **6** | **4** | **33.3** |
| **GASTROESOPHAGEAL REFLUX** | **11** | **5** | |
| • Proton Pump Inhibitors | 9 | 5 | **54.5** |
| • Antacids | 2 | 0 | |
| **PYSCHOLOGIC** | **10** | **5** | |
| • Selective serotonin reuptake | 5 | 2 | |
| • Norepinephrine reuptake inhibitors | 1 | 1 | **50.0** |
| • Lithium | 2 | 1 | |
| • Tricyclic anti-depressants | 1 | 1 | |
| • Atypical anti-psychotic | 1 | 0 | |
| **ANALGESICS** | **28** | **11** | |
| • Nsaids | 8 | 4 | |
| • Barbituates | 1 | 0 | |
| • Opiods | 5 | 1 | |
| • Systemic Steroids | 1 | 0 | |
| • Muscle Relaxers | 6 | 3 | **60.7** |
| • Anti-migraine | 1 | 1 | |
| • Gabapentin | 3 | 1 | |
| • Cox-2 Inhibitors | 0 | 1 | |
| • Pregabalin | 4 | 0 | |
| • Anti-gout | 0 | 1 | |
| **ALLERGY** | **5** | 7 | |
| • Leukotriene receptor antagonist | 1 | 1 | |
| • Histamine receptor antagonist | 0 | 2 | **-40.0** |
| • Nasal corticosteroid | 3 | 2 | |
| • Inhaled corticosteroid | 1 | 1 | |
| • Ophthalmic antihistamine | 0 | 1 | |
| **HORMONE REPLACEMENT** | **10** | **1** | |
| • Estrogen receptor agonist | 9 | 1 | **90.0** |
| • Progesterone receptor agonist | 1 | 0 | |

**Table 5. Continued**

| | | | |
|---|---|---|---|
| • Estrogen receptor agonist | 9 | 1 | |
| • Progesterone receptor agonist | 1 | 0 | |
| **HYPOTHYROID** | 7 | **2** | **71.4** |
| **SIALOGOGUES** | **18** | **0** | **100.0** |
| **INSOMNIA** | **10** | **0** | |
| • Benzodiazepines | 6 | 0 | **100.0** |
| • Zolpiderm | 4 | 0 | |
| **ANTI-MALARIALS** | **9** | **0** | **100.0** |
| **MISCELLANEOUS** | **6** | **2** | |
| • Ophthalmic drops for dry eye | 4 | 1 | **66.7** |
| • Bladder-selective Alpha-1 | 0 | 1 | |
| • Bisphosphonates | 2 | 0 | |
| **BIOLOGICS** | **1** | **0** | **100.0** |
| ***Total # medications*** | ***117*** | ***57*** | ***51.3*** |
| ***Average # medications/pt*** | ***6.61*** | ***2.36*** | ***64.3*** |

**Table 5: Medication regimens per group.** This table shows the aggregate number patients taking drugs and types of drugs. The bolded numbers along with the bolded and capitalized category shows the total number of patients taking the drugs listed per the specific test group status.

**Table 6. Salivary and serum toll-like receptor 4 concentrations**

| | SS Patients<br>n = 20<br>Median (IQR) | Control Patients<br>n = 20<br>Median (IQR) | p-value |
|---|---|---|---|
| **Salivary TLR4 (ng/mL)** | 2.650 (1.80) | 2.364 (1.54) | 0.312 |
| **Serum TLR4 (ng/mL)** | 0.987 (0.144) | 0.840 (0.159) | **0.011** |

**Table 6: Salivary and serum toll-like receptor 4 concentrations.** Mann-Whitney U test found statistically significantly higher serum TLR4 concentration between SS and control patients (p=0.011).