Assessment of Salivary Flow Patterns in Sjögren's Disease (SjD)

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ABSTRACT

Aim and Hypothesis: Salivary flow follows a circadian pattern and is influenced by clock genes, which also regulate Aquaporins (AQPs), water channels responsible for salivary flow regulation in salivary glands. A deficiency in AQP5 in mouse salivary glands disrupted this pattern. We hypothesized that patients with Sjögren's Disease (SjD) do not exhibit the same circadian salivary flow pattern as healthy controls. The study compared salivary flow patterns in healthy individuals and SjD patients. To do this, we assessed stimulated whole saliva (SWS) and unstimulated whole saliva (UWS) patterns in SjD subjects compared with healthy controls.

Methods: Thirteen subjects per group were required to achieve 80% power. SjD subjects met the ACR/EULAR2016 criteria. During the first visit, participants provided consent. They received instructions for saliva collection at home at two time points (5 AM and 10 AM) for unstimulated whole saliva (UWS, 5 minutes) and stimulated whole saliva (SWS, 2 minutes), with a third sample collected on-site at 3 PM. Friedman's test compared salivary flow within each group across time points, while the Mann-Whitney U test compared salivary flow between groups. Generalized linear mixed models assessed group-time point interactions. Significance was set at α=0.05.

Results: The study analyzed 13 healthy controls (10 female, 3 male, mean age 48.9 ± 10.7 years) and 13 SjD subjects (all female, mean age 65.3 ± 8.4 years). In SjD subjects, SWS 0.84 ± 0.83 mL/min, 1.08 ± 1.13 mL/min, and 1.00 ± 1.12 mL/min ($p = 0.037$) respectively. UWS ranged from 0.17 ± 0.21 mL/min at 5 AM to 0.24 ± 0.22 mL/min at 3 PM ($p=0.46$). No significant differences in control SWS; $p = 0.527$, with respective means of 2.80 ± 1.41 mL/min, 3.25 ± 1.81 mL/min, and 2.70 ± 1.42 mL/min. similar results were found in UWS: 1.03 ± 1.24 mL/min at 5 AM, 1.16 ± 1.34 mL/min at 10 AM, and 1.07 ± 1.29 mL/min at 3 PM ($p = 0.527$). SjD subjects

had significantly lower UWS and SWS rates than healthy controls ($p<0.001$). No significant group interactions were observed ($p>0.05$).

Conclusion: The data do not support the hypothesis that SjD subjects have an altered salivary flow pattern compared to healthy controls. Neither SWS nor UWS exhibited a clear circadian pattern in healthy individuals, suggesting a need for further research into circadian influences on salivary flow in the healthy population.

DEDICATION

To my parents, Abdulshaheed and Ramlah – Thank you for always holding my hands since the beginning, your prayers, and believing in me.

To my husband, Mohammed – Thank you for all the sacrifices you have made for our little family to make my dream come true

To my daughter, Danah for inspiring me to complete the journey

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TABLE OF CONTENTS

Thesis Committee……Page ii

Abstract……Page iii

Table of Contents……Page vii

List of Figures……Page viii

List of Tables……Page ix

List of Abbreviations……Page x

Introduction……Page 2

Materials and Methods……Page 18

Study Design ……Page 20

Inclusion Criteria……Page 19

Exclusion Criteria……Page 19

Sample Size and Power……Page 18

Statistical Analysis ……Page 18

Results……Page 24

Discussion……Page 28

Conclusion……Page 35

Appendix A……Page 36

Appendix B……Page 40

Bibliography ……Page 42

LIST OF FIGURES

Figure 1: Mean and Standard Deviation of Whole Salivary flow (mL/min) by group and time point

Figure 2: Stimulated Whole Salivary Flow (mL/min) – Healthy Controls

Figure 3: Unstimulated Whole Salivary Flow (mL/min) – Healthy Controls

Figure 4: Stimulated Whole Salivary Flow (mL/min) – Sjögren's Disease Subjects

Figure 5: Unstimulated Whole Salivary Flow (mL/min) – Sjögren's Disease Subjects

Figure 6: Mean Stimulated Whole Salivary Flow Pattern

LIST OF TABLES

Table 1: Whole Stimulated Salivary Flow in Healthy Controls (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Table 2: Whole Unstimulated Salivary Flow in Healthy Controls (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Table 3: Whole Stimulated Salivary Flow in Sjögren Disease Subjects (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Table 4: Whole Unstimulated Salivary Flow in Sjögren Disease Subjects (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Table 5: Fulfillment of ACR/EULAR 2016 criteria for Sjögren's Disease diagnosis among Sjögren's Disease group

LIST OF ABBREVIATIONS

ACTH: Adrenocorticotropic Hormone

AEP: Acquired Enamel Pellicle

AQP: Aquaporin

AQP5: Aquaporin 5

DM: Diabetes Mellitus

EGF: Epidermal Growth Factor

SGECs: Salivary Glands Epithelial Cells

SjD: Sjögren Disease

SWS: Stimulated Whole Saliva

TLRs: Toll-Like Receptors

UWS: Unstimulated Whole Saliva

Assessment of Salivary Flow Patterns in Sjögren's Disease (SjD)

Saliva is a medically necessary fluid that is produced in the mouth. It is composed of 99.5% water and the remaining components have several roles including transporting digestive enzymes (amylase and lipase) and nutrients, providing lubrication and barrier against microbial invasion (salivary mucins), helping with speech and swallowing, antimicrobial capacity (lysozyme, lactoferrin, and histatins), healing of oral mucosa after injury (salivary epidermal growth factors) and aiding in tooth remineralization. Three paired major glands (parotid, submandibular, sublingual) are responsible for secreting 90% of saliva, and the minor salivary glands produce the remaining 10%. In healthy subjects, the normal unstimulated whole saliva (UWS) flow rate ranges between 0.3-0.4 ml/min, and the normal stimulated whole saliva (SWS) flow is 1.2-2.0 ml/min.[1] The average daily saliva production ranges between 0.5-1.5 liters.[2] UWS of < 0.1 mL/min is an essential classification for diagnosing SjD. Patients with reductions in stimulated whole saliva (SWS) are classified as having mild, moderate, and severe dysfunction, respectively, as follows: > 0.7 ml/min, 0.7–0.1 ml/min, and < 0.1 ml/min[3] by the European Alliance of Associations for Rheumatology (EULAR) which guides management.

Mucosal lubrication and moisture provided by saliva facilitate the swallowing process, remove epithelial cell desquamation, leucocytes, and food debris, and lower susceptibility to mucosal abrasion.[4] Mucins provided by the submandibular, sublingual, and minor salivary glands are essential for providing a viscous coat. There are two main heavily glycosylated glycoproteins mucins in the saliva (MUC5B and MUC7) that have also been reported to be present in the acquired enamel pellicle and mucosal pellicle with a mean thickness of 0.07-0.10 mm. This pellicle helps to separate the shedding and non-shedding surfaces in the mouth.[5-7]

Saliva plays a crucial role in taste and smell. Several neurological and chemosensory mechanisms control taste and smell. Taste buds are present around the oral cavity (tongue, soft

palate, epiglottis, nasopharynx, esophagus) and in extraoral sites, such as the cilia of airway epithelial cells and the digestive tract. Normal salivary flow is needed to deliver the food particles (tastants) to the taste buds and aid in dissolving, leading to taste perception.[1,8] Doyennette et al. have found that the smell of food is proportionally linked to the time of chewing, which is directly proportioned to the amount of saliva secretions.[9]

The digestion of food begins in the oral cavity, where food is subjected primarily to the mechanical actions of chewing and grinding, which breaks down large-sized food pieces into smaller ones. In addition to this mechanical processing, food is mixed with a digestive enzyme called amylase, produced by the salivary glands secreted in the mouth. Amylase is secreted at a very high concentration from the parotid glands, at about one-quarter of this concentration from the submandibular and sublingual salivary glands, and in trace amounts from the minor salivary glands. The primary function of amylase is to facilitate the dissolution of starch-containing food debris that remains in the oral cavity after having a meal. Lipase is also present in the oral cavity. However, it does not have a significant role in digestion compared to gastric lipase in fat digestion.[10-12]

In addition to this physical barrier, the content of EGF in saliva promotes tissue regeneration, thus accelerating and enhancing the repair of the mucosal lining following injury. If saliva production is insufficient or the concentration of EGF is lower than average, the mucosal lining is more susceptible to injury and less likely to heal. This can lead to localized disorders such as esophagitis, oral ulcers, or other types of traumatic injury to the mucosa. Thus, the protection given by saliva, coupled with its ability to stimulate the proliferation of mucosal cells via EGF, is essential in preventing injury and facilitating the healing of the lining in the oral cavity and esophagus.[13]

Lastly, saliva aids in tooth protection through several methods. The main method is through the acquired enamel pellicle (AEP). It has been reported that AEP contains about 130 proteins, which aid in coating the enamel and, therefore, protect it from acidic erosions, mechanical wear demineralization, and bacterial adhesion. 14.4% of these proteins originate from saliva, with others from cells and plasma.[1]

Hyposalivation and Xerostomia

Hyposalivation is the objective finding of a decreased UWS rate of ≤0.3 mL/min or SWS rate of ≤1.2 mL/min.[1,14] When UWS or SWS flow is reduced by half, subjects may experience subjective sensations of oral dryness or xerostomia.[15] Xerostomia is a subjective sensation of oral dryness and can occur with or without objective evidence of decreased salivary flow.[15] There is often confusion between the two terms, making interpreting the findings in scientific literature difficult. The prevalence of xerostomia ranges between 5.5% to 46%, is more prevalent in women than men, is frequently associated with systemic diseases or as a side effect of hundreds of medications, and is, therefore, more common in the elderly population.[16,17] Xerostomia is a sign of multiple conditions rather than being a separate disease entity. One of the most common pathological causes of xerostomia is SjD, an autoimmune disease that will be discussed in detail. Over 85% of primary SjD patients report xerostomia.[18,19] Other conditions causing xerostomia include diabetes Mellitus (DM), even though a clear cause-and-effect relationship between diabetes and xerostomia cannot be determined. It has been suggested that the state of diuresis secondary to diabetes is the cause; others have hypothesized that it is secondary to diabetic neuropathy affecting the nerves supplying the salivary glands.[20,21] The cross-sectional study by Cindel Balbinot Fornari et al. studied xerostomia in the elderly population in detail. Key findings of this study are the following: a significant association of xerostomia was found with diabetes,

hypertension, and rheumatoid arthritis. They also studied the relationship with medications, with the most important relationship with anticholinergic medicines. They have suggested that clinicians should be aware of this and offer alternative medicines if needed. They also explored the relationship between gender and xerostomia. No significant difference in this study was found between genders in relation to xerostomia.[22] Patients with hyposalivation present with multiple signs that include but are not limited to dry, chapped lips, increased risk of candidiasis and dental caries, and gingivitis. Symptoms reported by patients include dry mouth, nose, and throat, difficulty in swallowing, bad taste or breath, oral soreness, oral burning sensation, frequent need to sip water, and frequent night awakening with dry mouth.[2]

Sjögren's Disease

Sjögren's Disease (SjD) was named after the Swedish ophthalmologist Henrik Sjogren. It is a systemic autoimmune disease affecting exocrine glands. It usually presents as oral and ocular dryness due to functional impairment of salivary and lacrimal glands and multisystem manifestations.[23,24] The multisystem manifestations of SD include constitutional symptoms, renal, articular-like arthralgia and/or synovitis, cutaneous lesions like vasculitis, central nervous system like cerebral vasculitis, palpable salivary and lacrimal glands, peripheral neuropathy, myositis, pulmonary disorders, lymph adenopathy, and, more seriously a 5-10% chance of MALT lymphomas.[25,26] SjD is more prevalent in females in their fifth to sixth decade, with a female-to-male ratio of 9:1 with an overall prevalence of 60.82 cases per 100,000 inhabitants. The incidence rate is 6.92 per 100,000 people/year.[23,27] Although the exact etiology of SD is not well established, it is explained that autoimmune epithelitis could play a role in its pathogenesis. In SjD, salivary glands epithelial cells (SGECs) express immune competent molecules like toll-like receptors (TLRs). When the TLR signaling pathway gets activated in the salivary glands

epithelium, cytokines and chemokines are released; with the help of the B-cell activating factor (BAFF), B-cells produce autoantibodies targeting the epithelial cells, causing cell apoptosis. Another pathway is when the dendritic cells produce IL-7, which activates CD4+ T cells to produce IL-6,12,21,22,23, leading to the germinal center formation and activation of more B cells or producing IL-17 and IFN-y, causing direct tissue damage. These two mechanisms eventually lead to gland hypofunction, manifesting as mucosal changes and dry surfaces.[25] The diagnosis of SD is a collaborative process between rheumatologists, oral medicine specialists, and ophthalmologists. The diagnosis of SjD has gone through several iterations, with salivary flow rates being established as one of the diagnostic criterion in 1967, followed by the addition of labial salivary glands biopsy a year after, and detection of anti-Ro/SSA and anti-La/SSB antibodies in SD patients in the early 80s.[25] In 2016, the American College of Rheumatology/European League Against Rheumatism (ACR-EULAR) proposed the most recent diagnostic criteria because of international collaboration. It consists of the following five Items: labial salivary gland with focal lymphocytic sialadenitis and focus score ³ 1 foci/4mm^2, anti-SSA/anti-Ro positive, an ocular staining score ³ 5 (or van Bijsterveld score equal or more than 4) in at least one eye, Schirmer's test 5 mm/5 min in at least one eye, and UWS flow rate less than or equal to 0.1 mL/min.[2] Any individual with a score four or more who does not have a history of head and neck radiation treatment, active hepatitis C infection (confirmed by PCR), AIDS, sarcoidosis, amyloidosis, graft-vs.-host disease, and IgG4-related disease meet the criteria.[28]

Despite the multiple systemic treatments for SjD and the need for referral to rheumatology and ophthalmology to manage the systemic manifestations, oral medicine specialists play an essential role in managing SjD patients' salivary hypofunction, as well as periodically following them up to assess lymphoma risk. In addition to preventing dryness, proper hydration with 64 oz. of water

daily is also necessary, as well as avoidance of mouth breathing and use of humidifiers. Mechanical and gustatory stimulation of salivary flow can provide symptomatic relief to SD patients. Salivary gland stimulation can be done with over-the-counter sugar-free gums, topical oral inserts, and OTC oral lubrication. However, since the salivary gland function is compromised in this population, systemic cholinergic agents are advised, such as pilocarpine 5 mg four times a day, a nd cevimeline 30 mg tablets three times daily.[29-31]

Circadian Rhythm

Salivary flow from the submandibular glands primarily occurs at rest, while stimulation like chewing or tasting activates the parotid glands. Autonomic nervous system signals or a stimulus, such as expecting food, control both processes. Salivary flow rates can fluctuate with things such as dehydration, side effects of medications, and natural circadian rhythms. The National Institute of General Medical Sciences defines circadian rhythm as the physical, mental, and behavioral changes an organism experiences over a 24-hour cycle. Circadian rhythm is associated with multiple normal physiological functions of the human body, such as sleep and secretions of substances such as human milk, cortisol, and saliva. [32]

Positive and negative molecular feedback loops control the circadian rhythm in the human body. Different clock genes linked to the circadian rhythm in humans have been identified. BMAL1/BMAL2, CLOCK, CRY1/CRY2, and PER1/PER2/PER3 clock genes regulate and control the transcription and translation process related to circadian rhythm. As a result of multiple signaling pathways on a cellular level, the expression of single or numerous clock genes allows the cells to identify and differentiate the time of the day and ultimately perform/execute

the appropriate function accordingly. However, one cannot ignore that external factors, such as light, also contribute to regulating circadian rhythms.[32]

Development of circadian rhythm starts postnatally as the fetus is not subjected to external stimuli while inside the womb. An immature circadian system develops over the first four months of the newborn life. Exposure to multiple rapid physiological changes leads the newborn to adapt to the environment and identify the 24-hour cycle. Body temperature and perception of day and night are the first circadian-related physiological activities to develop. Melatonin and cortisol secretion follow and aid in regulating the newborn circadian rhythm.[33]

Circadian Pattern of Salivary Flow

Dawes was the first to assess salivary flow patterns in healthy adults and concluded that unstimulated whole saliva followed a circadian pattern of secretion. He also measured changes in salivary composition. He found that salvary secretions were lowest at 4 am(1.4 ml/min), gradually increased throughout the day reaching its peak ~at 3:26 pm (3.00 ml/min) then gradually decreased again to 1.8 ml/min at midnight. In Dawes' study, saliva samples were collected from 8 subjects. One subject participated for four consecutive days, three for 11 days, three for two days, and one for three days. Five collections were obtained throughout the day (07:00, 11:00, 14:00, 17:00, and 22:00) over 4-26 days. While stimulated, left parotid saliva was collected over two minutes; this sample was used to analyze the protein concentration throughout the day. Temperature and electrolytes were also recorded. Temperature showed circadian pattern, peaking at 4:41. Electrolytes did not show circadian pattern, At midnight, the average UWS rate was about; it gradually decreased until 4AM.[34]

It was found by the research of J.R. Martinez on 18 dogs that antidiuretic hormone decreases salivary flow volume in a dose-related manner; this effect occurs 45 minutes after injection, while there was no change within the groups that received the saline injection. [35] Dawes has assumed that this circadian pattern in salivary flow rate was related to increased secretion of antidiuretic hormones at night. His finding supported the importance of oral hygiene practices before bed, as salivary flow decreases significantly during sleep, and therefore reducing the protective means of saliva during sleep. Although Dawes did not justify his hypothesis of a relationship between the antidiuretic hormone and the circadian pattern of salivary flow, it could be related to the effect of antidiuretic hormone on water reabsorption from the striated ducts. However, no literature supports a direct relationship between antidiuretic hormone levels and salivary flow patterns.[34]

Ferguson et al. studied the saliva composition and flow rate in a convenience sample of 16 healthy young individuals (nine males and seven females) without further details related to subject characteristics. Ferguson tried to determine if the circadian pattern differs between individual gland secretions and whole saliva secretion. In this study, 5 ml samples were collected from each parotid gland and both submandibular glands after stimulation with ascorbic acid. After which, whole saliva was collected. Afterward, the time required to collect this amount was calculated to measure salivary flow per minute. The minimum interval between sample collection was eight hours, with patients instructed not to have a meal for two hours before collection (without mention of refraining from drinking), with no more than two samples collected on the same day. The total sampling period was sixteen days for each type of collection (parotid, submandibular, and whole), making the entire sampling period 48 days. In addition to salivary flow, electrolytes, protein, and nitrogen were analyzed. Ferguson et al. concluded that

the peak whole saliva flow occurs at 4:30 pm similar to Dawes.[6] The parotid gland peak salivary flow was at 3:00 pm, and the submandibular peak salivary flow was at 5:00 pm. UWS had a statistically significant increase at 11:00 a.m. compared to 7:30 a.m. Ferguson has concluded that the circadian pattern of salivary flow is similar among all kinds of secretions, regardless of the gland in healthy subjects.[22]

While studies have been conducted to determine salivary flow rates in conditions such as diabetes, where peak flow rates were detected between noon and evening, and schizophrenic subjects as a side effect of clozapine, no studies have been done to investigate salivary flow patterns regarding the circadian pattern in subjects with SjD.[36,37]

Salivary flow patterns can vary related to underlying diseases. S Ionesacu studied salivary flow patterns in 17 insulin-dependent diabetes (IDDM) (ages ranging from 7 to 61 years old) and 14 non-insulin-dependent diabetes (NIDDM) (ages ranging from 38 to 77 years old), compared with sixteen healthy controls (age ranging from 29 – 82 years old). His findings were published as a note without extensive detailed methodology or results. UWS was collected on a single day during two periods (7:00-8:00 AM) before breakfast and (5:30-6:00 PM) before dinner, without details if the subjects refrained from food intake before collections other than having their meals. Salivary flow rate, pH, and lactate dehydrogenase (LDH) were compared at the two periods. In all groups of the study population, the minimal salivary flow was found in the morning in comparison to the afternoon period, with mean UWS of 0.425 mL/min for healthy controls at 7:30-8:00 and 0.5 mL/min at 17:30-18:00; however, this difference was not statistically significant[7]. There was a non-statistically significant difference in the unstimulated salivary flow at 7:30-8:00 and 17:30-18:00 in the IDDM (0.225 mL/min and 2.75 mL/min, respectively). The only group with statistically significant differences in the unstimulated salivary flow values

between the two-time points was the NIDDM group, with 0.25 mL/min at 7:30-8:00 and 0.41 mL/min at 17:30 – 18:00. pH levels in the morning were low compared to the afternoon period, and lactate dehydrogenase (LDH) did not show any significant change in the level between the compared periods.[36]

The results of this study cannot be assumed reliable due to the lack of detailed methodology, results, and conclusions.

Another study by G. Palmai 1967 investigated the diurnal variation of salivary flow in depressive patients during treatment and compared those with and without diurnal variation in their mood[45]. The study included 20 female subjects in each group compared with ten depressive non-treated controls. Each subject refrained from eating or drinking for an hour before each collection. UWS was collected every two hours over 24 hours. The control group's mean age was 37.9 years, and the data was obtained from the nursing staff with whom the study was conducted. The saliva collection method in this study consisted of placing pre- and post-weighted dental cotton rolls measuring 4 x 2 cm and left to absorb the saliva. Even though it is not practical to compare these results with ours given the difference in the study population and different collection methods, data is to be presented. All groups in this study followed a diurnal pattern regardless of treatment of their depression or having diurnal mood variation. However, the diurnal pattern differed between groups. The untreated depressive controls had high salivary flow during the early morning when reduced until reaching lowest values at 6:00 PM, which increased again until the end of the day. Both treated groups had lower values of their salivary flow in comparison to the non-treated group. Those without diurnal variation in their mood had higher salivary flow values at 6:00 AM, 10:00 AM, and 2:00 AM. Subjects with diurnal mood variation had their salivary flow peak at 8:00 PM.[38]

Circadian Rhythm, Central Clock Genes, and Aquaporins

Historically, the mammalian circadian rhythm was thought to be only regulated by external factors, most importantly light. However, this has been disputed as research has found that circadian rhythm continues despite the absence of external cues. However, external factors are still believed to be essential in synchronizing the rhythms. It is hypothesized that the suprachiasmatic nucleus of the hypothalamus (SCN) is the central pacemaker. The current understanding is that each organ throughout the body has different receptors and transcription genes leading to its circadian rhythm, with the first clock gene discovered named "Period" in 1971, formulating a basis for further research on "clock genes"[26]. The human circadian clock is regulated by a daily expression of 20 different transcription factors named clock genes, with a central clock in the brain linked to several peripheral clocks.

Aquaporins (AQPs) are a family of membrane water channels that transport water across plasma membranes, with 13 members identified in mammals.[24,39-42] AQP1, AQP4, AQP5, and AQP8 were demonstrated in the salivary glands, with aqp5 being the primary influencer on salivary gland secretions. AQP5 has been linked to the central clock genes and is directly affected by them. As a result, the expression of AQP5 mean number in the salivary glands shows a circadian pattern based on PCR gene expression. [24,39-42] We will discuss AQPs in detail in the next section.

To elaborate, the human body's biological rhythm is regulated by multiple mechanisms, the simplest and most known being environmental cyclic events like dark and light. However, the literature has illustrated the presence of biological clocks in the mammalian body.[43] According to Weaver DR, the mammalian central clock genes are located in suprachiasmatic nucleus in the hypothalamus, and other clocks are spread throughout the body called peripheral clocks.[44] The

central clock remains connected and greatly influenced by environmental factors, most notably the light and dark cycle. Whereas the peripheral clocks are not affected by environmental factors, they stay connected to the central clock and are hence indirectly affected by the dark/light cycle. They can also be independent of the central clock in the sense of being controlled by other physiological factors like feeding. Despite the clear connection between central and peripheral clocks, their interaction mechanism remains unclear.[43,45,46]

Circadian rhythm generation requires multiple signaling pathways that involve transcription-translation feedback loops (TTFLs). Two negative feedback loops control the human body's circadian rhythm; the first one involves PER and CRY proteins that form a hetero-multimeric complex with casein kinase 1-δ that translocate into the nucleus and suppress PER and CRY proteins by BMAL1/CLOCK inhibition. The second pathway is controlled by Rev-Erb genes that control and inhibit the activation of BMAL1 and CLOCK.[43] The clock genes have been linked to regulating the formation of craniofacial tissues. It was thought that the incremental development of the three vital tissues comprising teeth—enamel, dentin, and cementum—was controlled by biological clocks. The growth lines produced in tooth structures by such rhythmic deposition of mineralized tissue are periodic. For example, enamel forms in daily increments to produce "cross-striations," whereas longer cycles—called Retzius lines—occur in approximate weekly cycles of 6-12 days. Likewise, incrementing growth patterns have also been observed in other tissues such as dentin and cementum of teeth; therefore, the development of the latter is similarly thought to be coupled with circadian and other physiological rhythms of the body.[47] Increased clock genes expression have also been detected in craniofacial epithelial tissues, like the epithelial rests of Malassez, and basal cells of oral epithelium, including the palatal and junctional epithelium. The circadian clocks have also been suggested to affect saliva and its

different flow patterns based on the findings of Dawes, making saliva peak at 3:26 PM, decrease at night, and reach its lowest value around 4 AM.[34,43]

The expression of Aquaporin 5 (AQP5), serving as a water-soluble protein, was also investigated to see if it shows a circadian expression in the mice like the rhythm followed by the clock genes BMAL1, CLOCK, PER1, and PER2. The AQP5 pattern was assessed using quantitative PCR gene expression. Aquaporin 5 showed a significant circadian pattern when studied both in light/dark and dark/dark conditions. It has also been shown that when the Bmal1 gene is overexpressed, AQP5 RNA is up-regulated.[43]

Animal studies in mice have shown that salivary glands have peripheral clock proteins that bind to salivary gland genes for AQP5 and carbonic anhydrase (Car2), and therefore, associated with differences in mRNA expression. In addition, AQP5 expression levels supported the circadian pattern in both dark and light conditions, indicating that it is a direct target for clock genes.[43]

AQP5 functions as a water channel in secretory glands and have been found to be downregulated in labial and submandibular glands in subjects with SjD, and associated with hyposalivation.[39,41,48] Based on the link between clock genes and AQP5, this could mean that subjects with SjD may not present with standard circadian salivary flow patterns.

Since AQP5 is deficient in SjD patients, it could be hypothesized that subjects with SjD might not follow the typical circadian pattern in saliva secretion compared with healthy individuals, as stated by Dawes and Ferguson. To our knowledge, no studies have been conducted to investigate the circadian pattern of salivary flow in various disease populations, particularly in patients with SjD. Therefore, the need to explore this is raised.

Sialometry is an integral part of the diagnosis/classification of SjD, as shown in the ACR-EULAR criteria, and it guides clinicians in independently selecting suitable treatment modalities for each case. Since the timing of saliva collection due to diurnal variation may significantly influence salivary flow rates, this could lead to a difference between someone meeting diagnostic/classification criteria for SjD or not meeting the criteria.[42] AQP5 is found in salivary acinar cells and controls salivary secretion. It plays a profoundly important part in the transport of water into the lumen of the gland, contributing to normal salivary flow. In diseases like SjD, AQP5 expression is radically lowered. The decrease in AQP5 levels may be causally linked to chronic inflammation and immune-mediated destruction in the salivary glands, disrupting a water transport pathway necessary for saliva production. Impaired function and reduced expression of AQP5 in SjD account for the critical reduction of the salivary flow, contributing to xerostomia or dry mouth. Yoshimura S et al. (2016) demonstrated that AQP5 is either deficient or improperly localized in labial and submandibular gland biopsies from mouse models of SjD. Uchida H et al. (2018) expanded on this by showing that AQP5 expression is regulated by central clock genes, which control the circadian pattern of various physiological functions in the human body.

Several diseases have been suggested to be linked to dysregulation of deficient clock genes. It has been hypothesized that perturbations in the expression of clock genes that regulate cell growth and maturation could predispose the individual to downstream in the immunological functions in several autoimmune conditions like Rheumatoid arthritis. Key modulators of the circadian clock, such as Cry1 and Cry2, control rheumatoid arthritis by means of controlling proinflammatory cytokines like TNF-α. Similarly, Cry1-/-Cry2-/- mice exhibit worse RA, characterized by higher production of TNF-α and increased joint inflammation. This study

illustrates that Cryptochrome directly represses the TNF-α gene and that treatment with anti-TNF-α dramatically improves symptoms in Cry-deficient mice. These findings associate the circadian clock with RA and indicate yet another direction circadian-based therapies may take in inflammatory diseases. It has also been shown that collagen-induced arthritis was more severe in mice lacking the core clock genes Cry1 and Cry2 without light stimuli. mRNA and protein levels of clock genes have been found to be expressed differently in the serous acini and duct cells of all major salivary glands of SjD.[43,49]

L. Zheng (2012) studied the relationship between clock genes and circadian patterns in salivary glands. This was hypothesized based on the similarities in the acinar cells, which are responsible for protein and water secretion, and the physiological mechanism on which kidneys secrete fluid with clock genes in the kidneys. As mentioned previously, the circadian pattern is controlled by central and peripheral clock genes, with about 20 genes involved. The central clock contains about 20,000 neurons that send output signals driving the circadian pattern of central and peripheral tissue's clock gene expression and regulation. The study obtained mice submandibular, sublingual, parotid, and human submandibular tissue. Circadian clock genes were found to be strongly expressed in human submandibular tissue.[43]

Furthermore, the four clock genes in mouse model of SjD (BMAL1, CLOCK, PER1, PER2) were analyzed using real-time PCR to explore whether proteins show circadian expression in the salivary glands. Expressions were assessed during two consecutive days and tested every four hours, starting at 6 AM. To assess the effect of light, the same mice were tested for expression in two different circumstances at the same specified time points (light/dark cycle and dark/dark cycle). All clock genes showed circadian expression of gene proteins in both light/dark and dark/dark patterns, with different patterns in each condition. For each clock gene, the pattern was

flipped between light/dark and dark/dark, making the peaks in a particular condition the lowest in the opposite one; this indicates that the expression of clock genes in salivary glands is highly affected by the light given the total alteration of expression mode according to light/dark environment vs. dark/dark.[43]

The hypothesis was that SjD subjects do not follow the same circadian pattern of salivary flow seen in healthy controls. This study aimed to determine if salivary flow patterns differ between healthy and SjD subjects. To do this, we assessed stimulated whole saliva (SWS) and unstimulated whole saliva (UWS) patterns in SjD subjects compared with healthy controls.

Sample size calculation:

A sample size calculation was performed using nQuery Advisor v. 9.1.1.0 (Statistical Solutions Ltd., Cork, Ireland) to determine the sample size needed for the study's primary aim (comparison of SWS rates across time points for the Sjögren's group). Based on the results of Alvarino et al. that suggest SWS is more reliable in the diagnosis of salivary hypofunction in SjD.[50] Sjögren's group was assumed to have a mean salivary flow rate of 0.35 ml/min for the 5:00 AM time point, along with a standard deviation of 0.34 ml/min. Assuming that the pattern of change across time varies proportional to the results of Dawes[34], and conservatively assuming a within-subject correlation of $\rho=0$ between time points, a sample size of n=13 was adequate to obtain 80% power for the comparison of time points in conjunction with a Type I error rate of $\alpha=.05$. Therefore, a final sample size of n=13 was taken for each group. 20% attrition was anticipated and compensated for by increasing the initial sample size to 34 (17 per group); however, the study was finished upon completing 13 subjects per group.

Statistical analysis:

Descriptive statistics (means, medians, standard deviations, and interquartile ranges for continuous variables; counts and percentages for categorical variables) were calculated. Due to the non-normality of the data (based on the Shapiro-Wilk test), comparisons of salivary flow across the time points (for each group separately) were conducted using Friedman's test. The comparison of salivary flow between the groups (SjD subjects and healthy subjects) was done for each time point using the Mann-Whitney U test. Generalized linear mixed models were used to assess a potential interaction effect between the group and the time point. The latter analysis was performed in two ways: using the original data (and therefore examining interactions in terms of

an “absolute difference”) and using log-transformed data (and therefore examining interactions in terms of a “proportional difference”). The significance level was set at α=.05. SPSS 28 (IBM Corp., Armonk, NY, USA) was used in the analysis.

Study Protocol:

The study was conducted after obtaining ethical approval from Tufts University Health Sciences Institutional Review Board (IRB) number #4223.

Inclusion criteria:

1. All subjects older than 18-years-old (26 subjects)
2. SjD subjects (13 subjects):
    a. Diagnosed according to 2016 ACR-EULAR14 criteria (having 4 points confirming the diagnosis):
    b. Basal whole UWS rate ≥ 0.01 mL/min at screening
    c. Subjects willing to refrain from taking sialagogues on the day of the study visit
3. Control group (13 subjects):
    a. Healthy subjects with no complaints of dry mouth
    b. Not taking any medications associated with dry mouth.
    c. SWS of ≥ 1.5mL/min on day of the first visit
    d. UWS rate of ≥0.3mL/min on day of the first visit
4. Subjects willing to refrain from eating or drinking 90 minutes before each saliva sample (26 subjects)

Exclusion criteria:

1. SjD subjects:
    a. Exclusion according to the 2016 ACR-EULAR14 criteria that includes subjects with the following systemic diseases:
        i. Sarcoidosis
        ii. Amyloidosis
        iii. IgG4-related disease
        iv. AIDS
        v. History of head and neck radiation.
    b. SjD subjects with the inability to produce any unstimulated saliva (basal flow rate < 0.01 ml/min)
    c. Taking sialagogues on day of study
2. Healthy controls:
    a. Drug-induced xerostomia subjects
    b. SWS of ≤ 1.5mL/min at day of first visit
    c. UWS rate of ≤ 0.3mL/min at day of first visit
3. Those who self-report as pregnant
4. Those who display any evidence of ulcerations or oral cancer during the clinical exam after consent.

Recruitment:

The source of patients was from the population of the Tufts University School of Dental Medicine (TUSDM) Oral Medicine Clinic for SjD subjects. Healthy controls were recruited through flyers posted at the TUSDM community, and they were not targeted; they voluntarily chose to enroll in the study since flyers were posted around the building. Within TUSDM Oral

Medicine practice, the study team reviewed AxiUm records and clinic schedules to identify potential subjects. Permission to access this schedule was obtained. All recruitment and consent conversations took place in a private room or bay to ensure the subject's privacy. If they contacted the study team by phone, recruitment was conducted over the telephone, assuring the patient feels their environment is conducive to the discussion; if not, arrangements were made to call back at another time. During phone calls, names and phone numbers were collected, and three attempts were made to make phone calls before leaving a voice message. Patients were informed about the study title and procedures. The purpose of the study was also shared with the participants. The initial criteria for withdrawing subjects were as follows, nevertheless; no subjects were withdrawn:

- Unwillingness to participate in the research visit.

- The PI may withdraw a subject if stopping their sialagogues medications impairs their medical interest.

-for SjD subjects, salivary rates were confirmed after consent, and subjects were withdrawn if they were unable to produce enough saliva during the demonstration

Once healthy controls and SjD subjects arrived for the first visit; general informed consent was obtained and kept in Tufts' secure location as per Tufts standards and in coordination with HIPAA rules. Before consent, general screening took place via AxiUm chart reviews of patients who may qualify and verbal confirmation of any medical conditions or lack thereof. After the subject was consented, medical history, demographics (age, sex), and SjD diagnostic criteria were confirmed, and inclusion/exclusion criteria were confirmed after consent was obtained. Saliva flow criteria were confirmed only after instructions on unstimulated and stimulated saliva

collection were demonstrated and conducted. In other words, the saliva collected during the demonstration was used to verify inclusion criteria. It was planned that if the subject was unable to produce the minimum saliva requirements for their category (healthy control or diseased), they would be considered a screen failure and would be withdrawn from the study.

Methods:

Saliva Collection:

Subjects were instructed not to eat or drink 90 minutes before each saliva collection and to delay their sialagogue medication for research purposes on the days of sample collection. For unstimulated saliva, the patient was asked to sit in a quiet room and drool saliva in a sample dilution vial (25) for 5 minutes. For stimulated saliva, subjects were asked to chew on a 2 cm wax piece and spit saliva that accumulated in their mouth into the sample vial for 2 minutes. After weighing, all saliva samples were measured and discarded in a specified biohazard dispensary. After confirming subjects were able to produce a minimum of 0.01 ml/min of saliva, a clinical exam included a brief exam of the soft tissue to ensure there were no abnormalities, such as ulcerations or oral cancer. Subjects who did not qualify after this exam were planned to be withdrawn from the study.

If subjects met all inclusion/exclusion criteria, the second visit was scheduled within a month from the first visit, aiding in maintaining the same medical history the patient presented initially with. The subject was given four labeled (time and date of collection of each sample, unique ID, and stimulated or unstimulated) sample vials to take home.

<u>VISIT 2:</u>

On the day of Visit 2, subjects were to use the labeled vials and begin with the 5:00 AM unstimulated one, collect saliva by drooling into the container for 5 minutes. After that, they will close the vial, open the 5:00 AM stimulated saliva vial, and collect the stimulated saliva over 2 minutes. This was repeated at 10:00 am with the 10:00 AM unstimulated collection vial for 5 minutes, followed by the 10:00 AM stimulated collection for 2 minutes. The subject brought all four vials into the clinic on Visit 2, where the samples were to be collected, weighed, and discarded as described above, and the third saliva sample was collected at 3:00 PM.

At this point, the study-related procedures were considered complete, and subjects were provided with a parking voucher and compensation. All activities during all visits were solely for research purposes.

List of variables that were collected:

Demographics: age and sex

Medical history:

1. Diagnosed medical conditions in relation to SjD inclusion/exclusion criteria
2. List of medications.

Research variables:

1. SjD diagnosis of healthy
2. SjD diagnosis method (minor salivary glands biopsy vs. serology for SSA)
3. Stimulated saliva and non-stimulated saliva amount in each time points

The healthy control group consisted of 13 subjects, of whom 10 were female (76.9%) and three identified as male (23.1%). The mean ± SD age for healthy controls was 48.9 ± 10.7 years. Five members of this group identified as White (38.5%), while three identified as Asian (23.1%), two identified as Black/African American (15.4%), and three did not report race (23.1%). The SjD group also consisted of 13 subjects, all 13 identifying as female. The mean ± SD age for the SjD group was 65.3 ± 8.4 years. In this group, 12 subjects identified as White (92.3%), while one identified as Black/African American (7.7%). Among the SjD group, the primary method of diagnosis was minor salivary gland biopsy with a focus score ≥ 1 for nine subjects (69.2%). The primary method of diagnosis for the remaining four subjects (30.8%) was positive SSA serology. Table 5 shows the fulfillment of the ACR/EULAR2016 criteria of Sjögren's Disease subjects.

Controls:

The mean ± SD SWS for healthy controls at 5 am was 2.80 ± 1.41 mL/min, 3.25 ± 1.81 mL/min at 10 am and 2.70 ± 1.42 mL/min at 3 pm (Figure 2). Friedman's test comparing the time points SWS was insignificant ($p = 0.527$). Means, medians, standard deviations, and interquartile ranges are presented in Table 1.

Among the 13 healthy controls, two subjects had their lowest SWS values at 5 am, an increase at 10 am, and a peak at 3 pm. A single subject had a peak at 5 am, a decrease at 10 am, and a lowest value at 3 pm. Seven subjects had their lowest SWS values at 5 am, a peak at 10 am, and a subsequent decrease at 3 pm. Two subjects followed this pattern and had a drop in their while-stimulated saliva values between 11 am and 3 pm. Two subjects had SWS values peaking at both 5 am and 3 pm, with lower values at 10 am. A single subject had their highest SWS at 5 am, a decrease at 10 am, and an increase (but not a peak) at 3 pm (Figure 2).

The mean ± SD UWS for healthy controls at 5 am was 1.03 ± 1.24 mL/min, 1.16 ± 1.34 mL/min at 10 am, and 1.07 ± 1.29 mL/min at 3 pm (Figure 3). Friedman's test comparing the time points UWS was not statistically significant for this group ($p = 0.527$). Means, medians, standard deviations, and interquartile ranges are presented in Table 2.

Among the 13 healthy subjects, only 4 followed the expected pattern with their lowest UWS at 5 am, an increase at 10 am, and a peak at 3 pm. Two subjects had a reverse circadian pattern with their peak whole UWS at 5 am, a slight decrease at 10 am, and the lowest value at 3 pm. Two subjects had their lowest UWS at 5 am, peaked at 10 am, and decreased at 3 pm. 5 subjects had high UWS at 5 am and at 3 pm, with the lowest values of whole UWS at 10 am. (Figure 3).

Sjögren's Disease's:

The mean ± SD whole SWS for SjD patients was 0.84 ± 0.83 mL/min at 5 am, 1.08 ± 1.13 mL/min at 10 am, and 1.00 ± 1.12 mL/min at 3 pm (Figure 4). Means, medians, standard deviations, and interquartile ranges are presented in Table 3. Friedman's test comparing the three-time points SWS was statistically significant ($p = 0.037$). However, all post-hoc tests using the Wilcoxon signed-rank test and Bonferroni correction were insignificant (all $p > 0.05 / 3 \approx 0.0167$).

Among the 13 SjD patients, four SjD subjects had their lowest whole SWS at 5 am, an increase at 10 am, and a peak at 3 pm. A single subject had a reversed pattern with a peak at 5 am, a decrease at 10 am, and the lowest value at 3 pm. Six subjects had low whole SWS at 5 am, a peak at 10 am, and a decrease at 3 pm. One of these six subjects had only a 0.001 mL/min difference in the SWS between 10 am and 3 am. Two SjD patients had their whole SWS peak at 5 am, decreased at 10 am, and reached their lowest values at 3 pm. Lastly, a single SjD patient

had their peak whole SWS at 5 am, a decrease at 10 am, and an increase at 3 pm to a level 0.04 mL/min lower than the peak at 5 am (Figure 4).

The mean ± SD whole UWS for SjD patients was 0.17 ± 0.21 mL/min at 5 am, 0.20 ± 0.21 mL/min at 10 am, and 0.24 ± 0.22 mL/min at 3 pm (Figure 5). Friedman's test comparing the three-time points was insignificant ($p = 0.463$). Means, medians, standard deviations, and interquartile ranges are presented in Table 4.

Among the 13 SjD subjects, four SjD subjects had the lowest whole UWS at 5 am, an increase at 10 am, and a peak at 3 pm. Additionally, two SjD subjects had their whole UWS peak at 5 am, decrease at 10 am, and reach the lowest value at 3 pm. Five had a low whole UWS at 5 am, a peak at 10 am, and a decrease at 3 pm. Lastly, two SjD subjects had high salivary flow at both 5 am and 3 pm, with their lowest whole UWS at 10 am; one of these two subjects had the whole UWS rate peak at 3 pm, while the other had the peak whole UWS at 5 am (Figure 5).

Comparison between groups:

SjD patients had lower mean unstimulated whole salivary flow values than healthy controls at each time point. SjD patients also had lower mean stimulated whole salivary flow values at each time point than healthy controls (Figure 6). Every between-groups comparison (i.e., the comparison of SjD patients and healthy controls for every combination of parameter [stimulated or unstimulated whole salivary flow] and time point [5 am, 10 am, or 3 pm]) was statistically significant (all $p < 0.001$).

Testing for interactions:

When investigating a potential interaction between group and time point using generalized linear mixed models, no results were statistically significant ($p = 0.147$ for stimulated whole salivary

flow and an absolute difference, $p = 0.934$ for unstimulated whole salivary flow and an absolute difference, $p = 0.634$ for stimulated whole salivary flow and a proportional difference, and $p = 0.573$ for unstimulated whole salivary flow and a proportional difference).

The overarching aim of our study was to see if salivary circadian patterns were altered in SjD patients, given the deficiency of AQP5 water channels in the acini of salivary glands of mouse models with SjD and the control of these channels by central clock genes.[43,49] We measured SWS and UWS at (5 AM, 10 AM, and 3 PM) in SjD patients and healthy controls. Our results show no circadian pattern in the SWS or UWS salivary flow in SjD patients. Surprisingly, we also found no circadian pattern in SWS or UWS in the healthy controls.

When comparing the UWS and SWS flow rates at each time point in the healthy controls vs. SjD , the SjD group showed statistically significant lower salivary flow as was expected..

Using generalized linear models, we further explored the interaction of salivary flow values between the groups (SjD population and healthy controls) and time points. All results (absolute and proportional differences) were not statistically significant.

Given our unexpected findings that controls did not follow an apparent circadian pattern of salivary flow, we took a closer look at previous studies that established claims that saliva flow followed a circadian pattern. We found that these studies had several flaws; they were of very small sample size, not all subjects followed a circadian pattern, there were variations in saliva collection methods, there was a lack of clear explanation of the subject's characteristics, and essential factors affecting salivary flow, such as medication history and age.

Dawes (1972) has previously studied unstimulated salivary flow patterns in only eight healthy controls collected at 7:00, 11:00, 14:00, 17:00, and 22:00. This was a pilot study, and no sample size determination or power calculations were performed. Stimulated left parotid saliva was collected to assess salivary metabolite patterns, not the flow rates, so we will not discuss stimulated parotid findings. Each subject had variability in the number of saliva collections obtained, given the total number of days over which samples were obtained. There was no

standardized time span for which subjects collected saliva, ranging between 4-26 days. Subjects were appropriately instructed to refrain from eating for 90 minutes before each saliva collection however it was not clear if they were also asked to avoid drinking fluids. If subjects were allowed to drink fluids, this might could affect salivary flow rates by temporarily increasing hydration levels. Dawes indicated that the whole UWS peaked at 3:26 PM. A closer look at the results reveals that this only occurred in six out of eight subjects. He elucidated in one of the two subjects that did not follow a circadian pattern. He stated that this single subject had samples obtained over the course of only four days and that the short collection cycle showed "least diurnal variation" in salivary flow compared to those who collected saliva for a longer time span. Dawes did not further explain the characteristics of the other subject, who did not have a circadian pattern of saliva secretion.

Another potential weakness was that only 8 subjects' data were entered into a Monte Carlo simulation to create the sin wave. It is advised statistically to have at least 30 subjects to assume a normal sin wave.

In our study, only 4 of 13 healthy subjects followed the expected pattern reported by Dawes. Our results do not replicate Dawes methodology regarding time points for saliva collection and the number of samples obtained (5 versus 3), which may be a reason for not detecting a similar pattern as found in the Dawes study[5]. We initially intended to replicate Dawes's methodology of having more frequent salivary collections in a day and to have saliva collected over several days. However, our study design was limited by logistical barriers such as funding, compensation for subjects, and the timeframe allowed to complete the study. Another difference was we only collected saliva over the course of a single day for each subject. While having several collections over many days might better indicate circadian patterns, confounders such as hydration levels,

humidity, caffeine intake, etc., would cause more significant subject variation. Our study eliminated this by measuring saliva in a single day. If multiple-day collections are considered a better method for assessing circadian patterns, an ideal way would be standardizing the total days on which saliva would be collected and to account for confounders in the statistical analysis. We believe that Dawes' study's results were overstated due to the previously mentioned flaws in his paper.

D. B. Ferguson and C. A. Botchway (1980) assessed for differences in circadian pattern between SWS and individual gland saliva (parotid and submandibular). Ferguson and Botchway based the assumption of the presence of circadian pattern on Dawes's results. They hypothesized that there might be a difference in the pattern between each gland's secretion due to the different salivary composition of each.[22] Similar to our findings, Ferguson found that only a few participants (4 out of 16) followed the expected pattern. Participants were young and healthy, but no specific demographic data were mentioned. This study had a sound saliva collection method (aside from any mention of whether they were instructed to avoid drinking fluids) for both UWS and SWS. Saliva sampling was obtained at two-hour intervals during the day over 16 days in each subject. Since collection occurred over 16 days, a short number of days for saliva collection does not appear to be an essential factor in whether flow rates followed a circadian pattern or not. Six subjects failed to wake up during the night for the night-time collections, so there were some missing data points. Unstimulated salivary flow reported means and modal peaks where peak mean SWS occurred at 4:30 PM, parotid salivary flow at 3:00 PM, and submandibular salivary flow at 5:00 PM. The times of the lowest values were not reported.

The variability in Ferguson's paper resembles the individual variability we saw in our results. Four subjects out of sixteen agreed on the peak salivary flow rates in all collections within ± 2-

hour period[6]. Regarding stimulated salivary flow patterns, these findings reflect the pattern followed by two out of thirteen of our healthy subjects who had their lowest flow at 5 AM, increased at 10 AM, and peaked at 3 PM. Correlating the findings of our study with Ferguson's might be more reliable due to the similar sample size. In both studies, means were reported, in contrast to Dawes' paper, which had only 8 subjects, and salivary flow values over the time points were reported based on the sine wave rather than actual saliva collection values.[22,34] As with Dawes, the lack of power calculation makes it challenging to generalize the findings and clearly state that SWS follows a circadian pattern. Also, because only 4 of 16 subjects in the Ferguson paper and 6 of 8 of the Dawes subjects followed the expected pattern, it is questionable if these results could be considered solid evidence for a circadian pattern of salivary flow in both unstimulated and stimulated conditions.

Another possible explanation for our subjects' inability to detect a circadian pattern of salivary flow rate is that we looked at patients with SjD. To our knowledge, flow rates have been investigated in only two other diseased populations: diabetes and depression. In diabetic patients, the flow rates indicated a diurnal variation of salivary flow with lower flow rates in the morning compared with the afternoon for all groups (IDDM, NIDDM, and controls). No statistically significant difference was seen between 16 IDDM and healthy controls. The NIDDM group (n =16) had statistically significant lower flow rates of ~0.25 mL/min in the morning compared to ~0.41 mL/min in the afternoon. pH levels were lower in the morning than in the afternoon, and lactate dehydrogenase was not significantly different among all the measurements compared to the two time points. The detailed methodology for collecting saliva was not mentioned, thus making direct comparison difficult; nevertheless, the results did indicate a pattern of UWS in a diurnal manner for all the groups.

Palmai et al. showed that having a diagnosis of depression can cause variation in the diurnal pattern of whole salivary flow when people with diurnal mood variations were compared with those without diurnal mood variations. Another difference was seen in subjects who were treated for their depression and those who were not treated. The healthy controls of our study excluded any subjects on medications known to be xerogenic; however, one subject reported an occasional episode of depression without the need for treatment. Mood changes may have influenced the results. This subject showed a salivary flow pattern opposite to the pattern reported by Dawes and had peak UWS and SWS at 5 AM, decreased at 10 AM, and reached its lowest at 3 PM, which agrees with the results of G. Palmai's mean salivary flow values in the untreated depressive population.[38] However, as saliva was collected on a single day, we do not expect that this could have affected the results in comparison to if saliva was collected on multiple days, which subjects the study participant to more variation in their mood throughout the study period, and, hence affecting their salivary flow. Of note, we did not ask about mood on the day of the study.

H. Ben-Aryeh et al. (1996) measured salivary flow rates and subjective xerostomia levels in schizophrenic subjects taking clozapine. Seventeen schizophrenic subjects medicated with clozapine (mean age 39.65 years, mean of fifteen years with schizophrenia diagnosis, and mean 12.8 months on 338.2 mg of clozapine) were matched with healthy unmedicated controls. Xerostomia was assessed through a survey on follow-up visits (number and time of follow-up not specified). UWS was measured, SWS was also collected, both at a single time point, so a diurnal pattern was not studied. Twenty schizophrenic subjects reported hypersalivation, with five of them reporting hypersalivation at nighttime. There was no significant difference in the salivary flow between schizophrenic and healthy subjects. No significant difference in salivary

flow was detected between subjects who reported hypersalivation and those who did not. Schizophrenic subjects were further divided into subjects on clozapine only (group A) and (group B) subjects on clozapine in addition to other medications (anticholinergics, benzodiazepines, antidepressants). The flow rate was 0.34 ± 0.12 mL/min in group A and 0.31 ± 0.61 mL/min in group B. The stimulated flow rate was 0.70 ± 0.19 mL/min in group A and 0.76 ± 0.36 mL/min in group B. No circadian pattern was assessed in this study.[37]

We initially suspected that SjD subjects would have a different diurnal pattern in their salivary flow in comparison to healthy controls in both stimulated and unstimulated whole salivary flow patterns based on the results of Yoshimura S et al 2016 of deficiency and relocation of the water channels (AQP5) in SjD subjects and their link to the central clock genes in the human body which could ultimately affect the circadian pattern of salivary flow in this population.[39,41,48] Considering that this finding was solely based on salivary gland biopsies obtained from mouse models with SjD is essential. To our knowledge, this finding was not studied and established in humans with SjD. Although we did not look at levels in SjD subjects, the link between Aquaporins and central clock genes in humans has already been illustrated, so this may be contributing to the lack of traditional circadian patterns in our study. We suggest that to better evaluate the link between aquaporins and salivary flow patterns in the SjD population, future studies should be designed to investigate the level and location of aquaporins in the SjD population. If deficiency or relocation is indeed present in SjD subjects' salivary glands biopsies, the next step is to design a study that correlates individualized levels of Aquaporins and their effect on different patterns of salivary flow.

Another factor that could have affected the salivary flow pattern is that our inclusion and exclusion criteria did not consider the effect of medications that SjD subjects were taking for

their other systemic medical conditions. Given the age group for SjD and the likelihood of this population having at least one other medical comorbidity necessitating being on medication that has xerostomia as a known side effect, it would have been impractical to exclude subjects with comorbidities from our study[45]. We did not instruct our subjects to withhold systemic medications for comorbidities. If they took these medications after the 5 AM collection, salivary flow rates might decrease after the 5 AM collection. Our study did not consider this; subjects were only instructed to skip their sialagogues medication during the study visits. Ideally, we recommend that further studies consider this when developing the study procedure and correlating the saliva collection time points with the time the subjects take their xerogenic medications.

As we ended with a non-statistically significant difference in the flow pattern, we suggest that future studies might consider a larger sample size and a wider range of time points for the saliva to be collected, as well as a longer time span, to be able to explore changes over several days. Considering that more complex inclusion and exclusion criteria would be appropriate to eliminate or account for confounders like mood and medications. We also suggest that further studies classify their results based on the number, pharmacological group, or potency of xerogenic medications the SjD subjects might be taking.

Our results demonstrated no significant evidence of a circadian salivary flow pattern in healthy control and SjD patients. We found significant flaws with previous literature concluding that saliva does follow a circadian pattern and believe that the results were overstated. There is a need to design further studies to examine salivary flow patterns.

Our study also shows that despite the link between aquaporins and central clock genes, there is no significant evidence of a difference in the salivary flow pattern in Sjögren's Disease population. To truly investigate this relationship, studies that look into levels of Aquaporin 5 in humans with Sjögren's Disease rather than basing conclusions on mouse studies remain needed.

APPENDIX A

Figures:

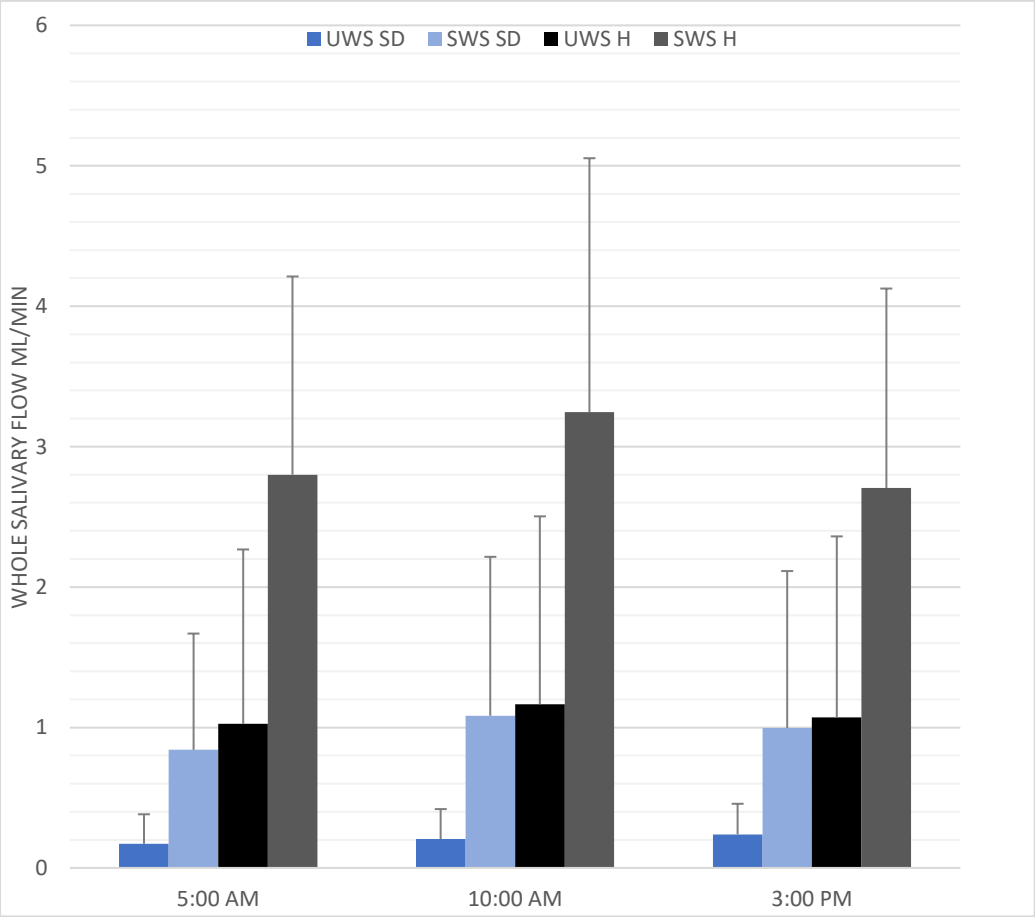

Figure 1: Mean and Standard Deviation of Whole Salivary flow (mL/min) by group and time point

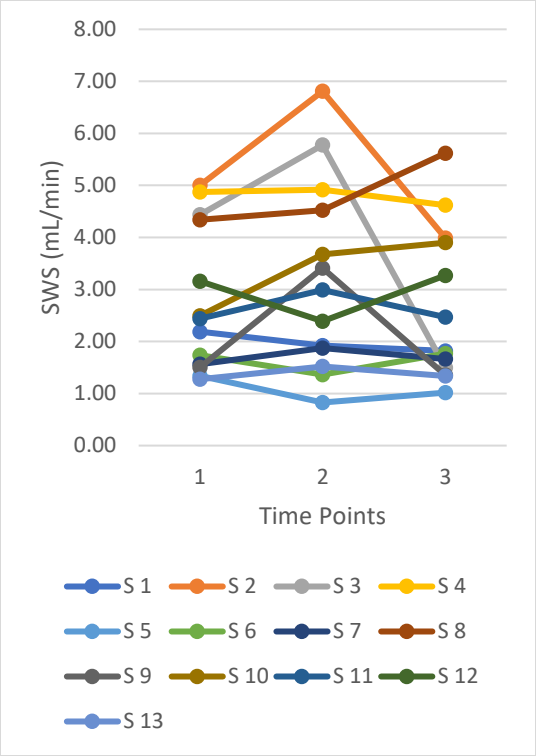

Figure 2: Stimulated Whole Salivary Flow (mL/min) – Healthy Controls

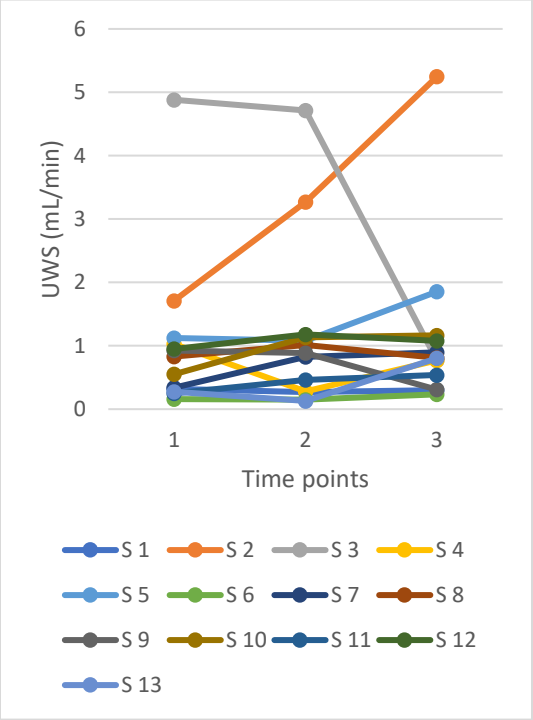

Figure 3: Unstimulated Whole Salivary Flow (mL/min) – Healthy Controls

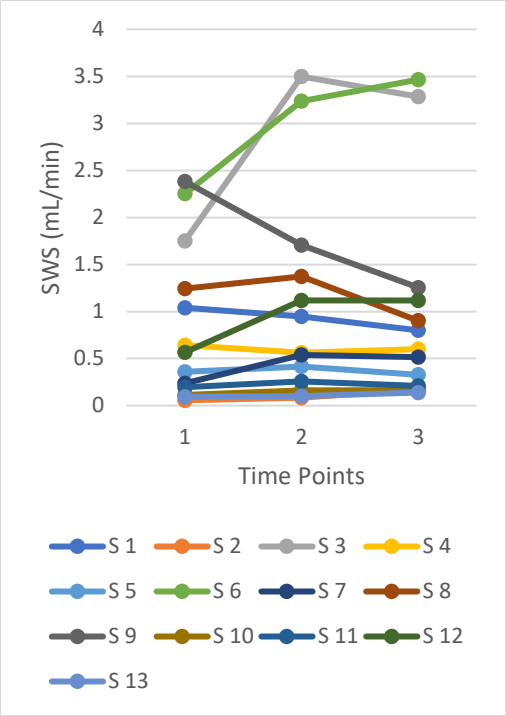

Figure 4: Stimulated Whole Salivary Flow (mL/min) – Sjögren Disease Subjects

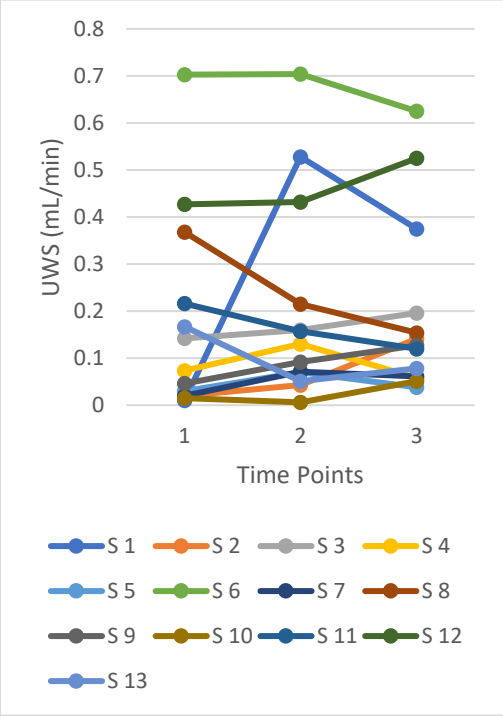

Figure 5: Unstimulated Whole Salivary Flow (mL/min) – Sjögren Disease Subjects

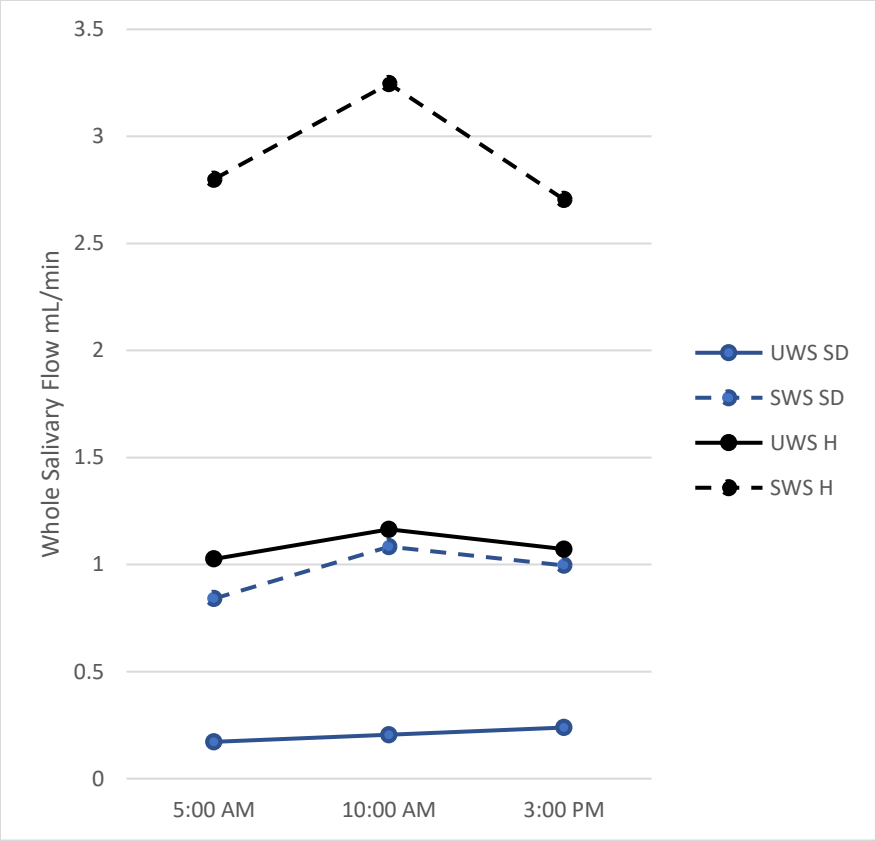

Figure 6: Mean Stimulated Whole Salivary Flow Pattern

APPENDIX B

Time Point	Mean (SD)	Median (IQR)
5:00 AM	1.03 (1.24)	0.83 (0.30-1.08)
10:00 AM	1.16 (1.34)	0.83 (0.28-1.16)
3:00 PM	1.07 (1.29)	0.80 (0.42-1.05)

Table 1: Whole UWS in Healthy Controls (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Time Point	Mean (SD)	Median (IQR)
5:00 AM	2.8 (1.41)	2.44 (1.53-4.39)
10:00 AM	3.25 (1.81)	2.99 (1.70-4.70)
3:00 PM	2.70 (1.42)	1.86 (1.58-3.95)

Table 2: Whole SWS in Healthy Controls (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Time Point	Mean (SD)	Median (IQR)
5:00 AM	0.17 (0.21)	0.07 (0.20-0.29)
10:00 AM	0.20 (0.21)	0.13 (0.06-0.32)
3:00 PM	0.24 (0.22)	0.14 (0.07-0.45)

Table 3: Whole UWS in SjD Subjects (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Time Point	Mean (SD)	Median (IQR)
5:00 AM	0.84 (0.83)	0.57 (0.15-1.50)
10:00 AM	1.08 (1.13)	0.65 (0.21-1.54)
3:00 PM	0.99 (1.12)	0.6 (0.19-1.19)

Table 4: Whole SWS in SjD Subjects (mL/min). SD: Standard Deviation; IQR: Interquartile Range

Subject	Labial salivary gland with focal lymphocytic sialadenitis and focus score ≥ 1.	Anti-SSA (Ro)	Ocular staining score ≥ 5 (or van Bijsterfeld score ≥ 4) on at least one eye	Schirmer ≤ 5 mm/5min on at least one eye	Unstimulated whole saliva flow rate ≤ 0.1 ml/min
1	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
2	**Not performed**	**Yes**	**Not recorded**	**Not recorded**	**Yes**
3	**Not performed**	**Yes**	**Not recorded**	**Yes**	**Yes**
4	**Yes**	**No**	**Not recorded**	**Yes**	**Yes**
5	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
6	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**

7	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
8	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
9	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
10	**Not performed**	**Yes**	**Not recorded**	**Yes**	**Yes**
11	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
12	**Yes**	**No**	**Not recorded**	**Not recorded**	**Yes**
13	**Not performed**	**Yes**	**Not recorded**	**Yes**	**Yes**

Table 5: Fulfillment of ACR/EULAR 2016 criteria for Sjögren's Disease diagnosis among Sjögren's Disease group

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