SALIVARY MUCINS IN PATIENTS WITH BURNING MOUTH SYNDROME

Sumeia G. Werfalli

A dissertation submitted in fulfillment of partial requirements for the degree of

Doctor of Philosophy

University of Washington

2019

Reading Committee:

Dr. Linda LeResche, Chair

Dr. Jill Johnsen

Dr. Richard Presland

Program authorized of offer degree:

School of Dentistry

Oral Health Sciences

University of Washington

Abstract

Salivary Mucins in Patients with Burning Mouth Syndrome

Sumeia Werfalli

Chair of the Supervisory Committee:

Linda LeResche

Department of Oral Medicine, Adjunct, Department of Oral Health Sciences

Abstract

Burning Mouth Syndrome (BMS) is a chronic pain condition that most commonly affects post-menopausal women. Pain is constant or intermittent, ranging from mild to severe. Treatment of BMS remains unsatisfactory, and there is no definitive cure. Mechanisms underlying BMS remain unclear. Evidence of changes in oral mucosal epithelial cells has been found in BMS patients. However, the status of the protective salivary barrier in BMS patients has not been investigated.

Salivary mucins represent the first line of defense for oral epithelial cells, and the principal lubricating constituents of saliva. Mucins participate in the formation of the salivary pellicle that covers and protects the oral epithelium. This research is a case-control study that aims to determine whether BMS cases have impaired mucosal barrier function compared to controls without BMS, focusing on the role of mucins as the first line of defense for oral epithelial cells. Our primary aims are to assess: 1) the quantity of MUC1 in unstimulated whole saliva (USWS) in cases and controls; 2) the levels of

glycosylation in USWS in cases and controls; and 3) the associations between mucin levels and mucin glycosylation and the severity of oral burning in cases.

A total of 50 women (22 BMS cases and 28 healthy controls) age 50 years or older were included in the analysis of this study. Participants attended a clinical visit for salivary sample collection, oral examination, and swab for candida detection and DNA collection. They also completed a pain and health history questionnaire. Total protein concentration was measured using BCA. MUC1 protein was detected and quantified using ELISA. Mucin glycosylation was assessed using dot blots stained with three different lectins (MAL-II, WGA, and UEA). Secretor status was determined by performing targeted candidate gene DNA sequencing of the FUT2 gene.

Compared to controls, cases had significantly lower USWS flow rates (p-value <0.001) and had a higher prevalence of xerostomia (p-value=0.001), GI disease (p-value <0.001), and vaginal dryness (p-value=0.01). Cases also consumed a higher number of medications (p-value=0.01).

The average salivary protein concentration was 1.30 mg/ml in cases vs. 0.94 mg/ml in controls (p-value= 0.15). Mann Whitney U analysis showed that the levels of MUC1 were lower in cases (n=20, sum of ranks=392.50) compared to controls (n=24, sum of ranks=597.50), p-value=0.17. Although not statistically significant, levels of UEA, MALII, and WGA lectins were lower in cases compared to controls. The percentage of non-secretors was 23% among cases vs. 38% among controls (p-value=0.28).

In conclusion, BMS patients had a statistically significant reduction in USWS, higher prevalence of xerostomia, vaginal dryness and GI disease. Underlying pathophysiological mechanisms related to minor salivary gland dysfunction and GI disease should be further investigated.

ABBREVIATIONS

BMS: Burning Mouth Syndrome

ELISA: Enzyme linked immunosorbent assay

FUT2: Fructosyltransferase 2

GalNAc: N-acetylgalactosamine

GlcNAc: N-acetylglucosamine

MALII: Maackia amurensis lectin II

MUC5B: Mucin 5B

MUC7: Mucin 7

MUC1: Mucin 1

NGF: Nerve growth factor

OHIP-14: Oral health impact profile-14

PCR: Polymerase chain reaction

PHQ-8: Patient health questionnaire

RAS: Recurrent aphthous stomatitis

RMS: Residual mucosal saliva

SS: Sjögren Syndrome

SWS: Stimulated Whole Saliva

TBS: Tri-buffered saline

SXI-D: Short version of xerostomia inventory

UEA-I: Ulex europeaeus agglutinin-I

USWS: Unstimulated Whole Saliva

WGA: Wheat Germ Agglutinin

Table of Contents

ACKNOWLEDGEMENTS

This research was funded by grants from the University of Washington Dr. Douglass L. Morell Dentistry Research Fund and the Department of Oral Medicine, as well as a Pain Innovative Research Grant from the Scan Design Foundation.

I would like to express my deepest appreciation to my committee chair, Dr. Linda LeResche, for providing valuable guidance, supervision, mentoring as well as encouragement throughout the development of this dissertation. Dr. LeResche's support and advice have been invaluable over the years and I feel incredibly privileged to have her as my supervisor.

The completion of my dissertation would not have been possible without the support and nurturing of Dr. Jill Johnsen who welcomed me generously into her lab and provided continuous advice and support with the research experiments.

I'd also like to extend my gratitude to my committee members: Dr. Richard Presland, Dr. Susanne Jeffrey, and Dr. Mark Drangsholt for providing continuous help and scientific advice throughout the study period.

I gratefully acknowledge the invaluable assistance of Kerry Lannert with all the laboratory experiments and Sumia Dakhil for her great help with the study data collection sessions.

I have been extremely blessed with having a supportive husband, wonderful children and my loving mother who provided continuous inspiration and support throughout this journey. I would also like to thank the Libyan Ministry of Higher Education for supporting me financially throughout my postgraduate study period.

Chapter I: Introduction

Burning Mouth Syndrome (BMS) is a chronic pain condition characterized by intraoral burning or abnormal sensation that recurs daily for more than 2 hours per day over more than 3 months, without clinically evident causative lesions. It most commonly affects post-menopausal women. Pain is constant or intermittent, ranging from mild to severe. Mechanisms underlying BMS are not fully understood. Treatment remains unsatisfactory, and there is no definitive cure. Dry mouth is common among BMS patients, and some studies have found evidence of epithelial changes in BMS patients, although the specific deficit in the epithelial barrier has not been identified.

Our global hypothesis is that the symptoms of BMS are due to changes in the oral mucosal barrier, specifically in salivary mucins. Salivary mucins are the first line of defense for the oral epithelial barrier. They represent the principal lubricating constituents of saliva and participate in the formation of the salivary pellicle that covers and protects the oral epithelium. The main mucins found in the oral cavity include: mucin 5B (MUC5B), mucin 7 (MUC7), and mucin 1 (MUC1). MUC5B and MUC7 are secreted into the saliva mainly by the submandibular and sublingual salivary glands, whereas MUC1 is a membrane-associated mucin, which has an extracellular part protruding into the oral cavity that can be cleaved into a secretory MUC1 found in saliva. Changes in mucins due to inflammatory, microbial, genetic or environmental causes are thought to cause the loss of integrity of the mucosal barrier. Such changes have been implicated in a number of diseases outside the oral cavity.

We conducted a case-control study to examine whether female BMS cases have impaired mucosal barrier function compared to age- and gender-matched controls without BMS, focusing on the role of mucins as the first line of defense for oral epithelial cells. We assessed salivary flow rates, mucin

concentrations and mucin glycosylation in female BMS cases and controls. Self-report data, salivary samples and other clinical exam data were collected at a single session.

Specific Aims

Primary Aims:

Aim 1: To quantify MUC1 in unstimulated whole saliva (USWS) in cases and controls

Hypothesis: There will be no statistically significant difference in levels of MUC1 between cases and controls.

a. Collect unstimulated saliva samples from BMS cases and controls.
b. Measure the quantity of MUC1 glycoproteins in each sample initially using enzyme-linked immunosorbent assays (ELISAs) (Lopez-Jornet, Camacho-Alonso, & Molino-Pagan, 2013).

Aim 2: To assess the levels of glycosylation in USWS in cases and controls

Hypothesis: The levels of glycosylation will be significantly lower in cases compared to controls.

a. Use dot blots stained by three lectins: MAL-II, UEA, and WGA which are able to bind to different sugars.
b. Use PCR and sequencing of FUT2 for assessment of secretor status.

Aim 3: To assess the association between levels of glycosylation and the severity of oral burning in cases

Hypothesis: There will be an inverse relationship between levels of glycosylation and severity of oral burning in cases.

a. Correlate levels of glycosylation with pain intensity rating levels reported by BMS patients using Spearman's correlation coefficient

Secondary Aims:

In addition to the primary aims, this study aimed to assess:

- The prevalence of dry mouth complaint in cases and controls
- The prevalence of hyposalivation in cases and controls
- The level of mucosal hydration in cases and controls
- The association of mucin levels/glycosylation and the severity of oral dryness sensation in cases and controls who report a dry mouth complaint.
- Secretor status in cases and controls

Background and Significance

Burning Mouth Syndrome (BMS) -- also referred to in the literature as Burning Mouth Disorder, stomatopyrosis, orodynia, glossitis or glossodynia -- is a chronic oral condition (Minor & Epstein, 2011), (Jaaskelainen, 2012). The International Headache Society classification ICHD-III classifies BMS under the category "Painful cranial neuropathies and other facial pains" and describes it as, "An intraoral burning or dysaesthetic sensation, recurring daily for more than 2 hours per day over more than 3 months, without clinically evident causative lesions" (Headache Classification Committee of the International Headache, 2013).

The prevalence of BMS in the general population is reported to vary from 0.7% - 15% depending on the diagnostic criteria used. BMS is more common in women than men with a reported ratio of approximately 1:5 to 1:7 men to women, depending on the study population. BMS is most

commonly reported in post-menopausal women aged 60-69 years (Jaaskelainen, 2012). Prevalence appears to increase with age in both men and women (Klasser, Grushka, & Su, 2016). Dry mouth complaint (xerostomia) is reported by about 54% to 75% of patients with BMS. Up to 69% of BMS patients complain of taste disturbances (Jaaskelainen, 2012; Thoppay, De Rossi, & Ciarrocca, 2013; Woda, Dao, & Gremeau-Richard, 2009).

Several classification systems have been proposed for BMS. It is sometimes classified as primary or secondary, based on whether there is an identifiable cause. It has also been classified based on pathophysiologic mechanisms, but, to date, none of these mechanisms has been validated (Bender, 2018).

Despite the existing data gained from recent research, BMS remains an unclear, misunderstood, and under-recognized painful condition. Patients have often sought help from dentists, general practitioners, gastroenterologists and neurologists prior to diagnosis (Gao, Chen, Zhou, & Peng, 2009). Because of such diagnostic challenge, diagnosis is often delayed, with previous studies reporting an average delay of between 34 and 41 months from initial presentation to diagnosis (Klasser et al., 2016; Mignogna, Fedele, Lo Russo, Leuci, & Lo Muzio, 2005). A recent study found that the average time from onset of symptoms to diagnosis was 13 months (Ni Riordain, O'Dwyer, & McCreary, 2019). The diagnostic delay may result in increased anxiety in these patients. Gao and his colleagues reported that BMS patients had statistically significant higher depression and anxiety scores in comparison to age and sex-matched healthy controls (Gao et al., 2009). Both BMS and dry mouth have a negative impact on the oral health-related quality of life (OHRQoL) (Locker, 2003; Lopez-Jornet, Camacho-Alonso, & Lucero-Berdugo, 2008).

Clinical Characteristics of Burning Mouth Syndrome

Clinically, the classic symptom experienced by BMS patients is burning pain of the oral mucosa (Braud, Toure, Agbo-Godeau, Descroix, & Boucher, 2013; Scala, Checchi, Montevecchi, Marini, & Giamberardino, 2003). Patients with BMS usually use the following words to describe their symptoms: painful, burning, tender, tingling, hot, scalding, and numbness. Sometimes the sensation is only described as discomfort, raw, and annoying. Symptoms usually affect multiple areas in the mouth, most commonly involving the anterior two thirds of the tongue, hard palate and lips (Minor & Epstein, 2011), (de Moraes et al., 2012; Sun et al., 2013). The onset of pain may be gradual or sudden. Usually, there are no identifiable precipitating factors. However, some cases relate pain onset to a certain event, such as a dental procedure, trauma, medication, illness, or a stressful life event (Bender, 2018).

BMS pain may be constant or intermittent, varying in intensity from mild to severe. Pain is usually bilateral and symmetrical, not restricted to the anatomical distribution of peripheral sensory nerves. The majority of BMS patients experience minimal symptoms on awakening, and symptoms tend to build up over the day, reaching maximum severity in the evening, but the pain only rarely disturbs nighttime sleep (Bergdahl & Bergdahl, 1999; Forssell et al., 2012; Grushka, 1987). In some cases, eating temporarily relieves the symptoms. Most patients, however, avoid hot, spicy, or acidic food, or alcoholic beverages, as they tend to worsen their symptoms. Some patients also report the pain is more intense when they feel more stressed or fatigued (Bender, 2018).

In terms of clinical management, BMS remains a poorly-understood disorder. It is widely accepted that BMS is likely more than one disease process with a multifactorial cause, including oral, systemic and psychosocial factors, thereby making it a diagnosis of exclusion (Klasser et al., 2016). The diversity of symptoms associated with BMS provides a challenge for practitioners

managing these patients (Klasser et al., 2016). Initial management aims at elimination of secondary causes of oral burning such as ill-fitting dentures, candidiasis, nutritional deficiencies and allergy. If symptoms persist, pharmacotherapy might be considered. Treatment of BMS remains unsatisfactory, and there is no definitive cure. To date, the best available evidence for pharmacotherapy is for alpha lipoic acid, capsaicin and clonazepam with equal efficacy for local or systemic forms when these alternatives are available (Kisely, Forbes, Sawyer, Black, & Lalloo, 2016).

Proposed Mechanisms Underlying BMS

Although the pathophysiology remains mostly unknown, the ICHD-III beta suggests BMS is a neuropathy (Bender, 2018). Several peripheral and/ or central mechanisms have been proposed to explain development and maintenance of BMS. Recently, it has been suggested that BMS is a result of nerve damage or dysfunction along the trigeminal neuraxis. Trigeminal small fiber sensory neuropathy has been the most accepted theory based on significant evidence found in BMS patients (Balasubramaniam, Klasser, & Delcanho, 2009; Lauria et al., 2005), including abnormal quantitative sensory testing findings and reduced epithelial nerve fiber density in oral epithelium of BMS patients (Beneng et al., 2010) as well as increased levels of markers for nerve damage such as Nerve Growth Factor (NGF) and substance P (Borelli et al., 2010). Findings from a recent systematic review suggest that BMS depends on specific local mechanisms, possibly at the trigeminal level, as it revealed that there is no evidence for an association between BMS and other pain symptoms, and BMS patients do not exhibit clear somatosensory patterns (Moisset, Calbacho, Torres, Gremeau-Richard, & Dallel, 2016). Despite the substantial evidence for the role of nerve damage in BMS, there are no systematic studies assessing the potential causative factors of the small fiber neuropathy in this condition.

The Potential Role of Mucosal Barrier Dysfunction in BMS

Defects in mucosal barrier function have been implicated in the development of different chronic pain syndromes such as interstitial cystitis (painful bladder syndrome), irritable bowel syndrome, and ulcerative colitis (Merga, Campbell, & Rhodes, 2014). The loss of integrity of the mucosal barrier is thought to be due to changes in mucin expression and structure, which may be related to immunological, microbial, and genetic or environmental causes (Boltin, Perets, Vilkin, & Niv, 2013).

We hypothesize that a similar salivary mucin-related defect in oral mucosal barrier function occurs in BMS cases. In the oral cavity, the oral mucosal epithelium together with the saliva-derived pellicle play many vital roles in the protection of oral soft tissues. Oral mucosal surfaces are covered by mucus, the first line of the local innate defense system against mechanical and chemical damage as well as the passage of microorganisms and toxic materials (Kullaa, Asikainen, Herrala, Ukkonen, & Mikkonen, 2014). Studies examining the oral mucosa of BMS patients are very limited. These studies have found abnormalities such as epithelial atrophy, markers of epithelial injury, and disrupted epithelial maturation among individuals with BMS (Hershkovich & Nagler, 2004).

Overall, it has been suggested that impaired epithelial barrier due to parafunction-induced microtrauma to the oral mucosa, or decreased lubricating quality of saliva could lead to subclinical inflammation and nerve damage in BMS patients (Boras, Brailo, Lukac, Kordic, & Blazic-Potocki, 2006; Kho, Lee, Lee, & Lee, 2010). The tip or lateral borders of the tongue that are frequently exposed to such traumatic irritation are in fact the most commonly involved areas in BMS patients (Ko, Park, Park, & Kho, 2011). This hypothesis is supported by the effectiveness of the use of tongue protectors, and oral lubricants in the treatment of BMS symptoms (Kho et al., 2010; Lopez-

Jornet et al., 2013). However, the role of the oral mucosal barrier status in the development of BMS remains unclear.

Salivary Mucins as the First Line of Defense for the Oral Mucosa

Mucins are glycoproteins normally produced by mucosal surfaces that line cavities exposed to the external environment such as the respiratory, digestive, and reproductive tracts, and the ocular surface. These glycoproteins have important roles in maintaining surface hydration and forming a protective physical barrier against pathogens. Excessive mucin production and/or abnormal mucus properties can lead to duct obstruction, bacterial colonization and chronic inflammation, potentially causing irreversible tissue damage, similar to changes seen in cystic fibrosis, chronic otitis media and cervicovaginal infection (Ramsey, Rushton, & Ehre, 2016).

Disruption of this protective role has been hypothesized in several oral disease models including HIV/AIDS, oral candidiasis, and dental caries (Visvanathan & Nix, 2010). The high viscosity of mucins and their ability to strongly stick together and to other salivary proteins helps in forming the basis of the mucosal pellicle layer which coats epithelial surfaces, functioning both as a lubricant and a selectively permeable barrier, protecting against dryness, and mechanical and chemical damage (Kullaa et al., 2014). In addition, mucins can function to modulate the oral microbial flora providing selective attachment of certain microorganisms and/or by facilitating the clearance of others.

Three types of mucins are predominantly expressed in the oral cavity: membrane-associated mucin, MUC1(Forssell, Jaaskelainen, Tenovuo, & Hinkka, 2002), and the soluble secreted mucins, MUC7 and MUC5B. MUC7 (formerly known as MG2) is a low-molecular weight, monomeric mucin with molecular mass of approximately 130–180 kDa. MUC5B (formerly known as MG1)

is a high-molecular weight, oligomeric mucin with molecular mass of 20000–40000 kDa (Takehara, Yanagishita, Podyma-Inoue, & Kawaguchi, 2013) and the membrane-bound MUC1 is approximately 120-300 kDa in size (Hattrup & Gendler, 2008).

Structurally, membrane-associated mucins can be divided into three main regions: 1) the cytoplasmic tail, 2) membrane spanning domain, and 3) extracellular domain. The cytoplasmic tail is mainly an anchoring region of the membrane-associated mucin that is incorporated through the cell membrane of the epithelial cells. It is currently believed that the cytoplasmic tail may also have a role in cellular signaling particularly in MUC1. The intermediate region is hydrophobic and spans the cellular membrane. The extracellular domain extrudes from the plasma membrane, promoting direct contact with salivary mucins. The cross-links created between the extracellular domain of the membrane-bound MUC1 and both MUC7 and MUC5B, as well as other salivary proteins, participate in mucus gel formation in the mucosal pellicle creating the first line of mucosal defense (Almstahl, Wikstrom, & Groenink, 2001; Kho, Chang, Kim, & Kim, 2013; Kullaa et al., 2014). Studies on MUC1-null mice have shown defective barrier function resulting in chronic inflammation and opportunistic bacterial or fungal infections of the lower reproductive tract, eyes, and oral cavity (DeSouza et al., 1999; Kardon et al., 1999; McAuley et al., 2007). However, the function of the shed soluble form of MUC1 in the oral cavity has not yet been reported.

From a biological standpoint, mucins are high-molecular-weight O-linked glycoproteins. Membrane-bound mucins and secreted mucins share several common features. They both consist of a protein backbone that makes up nearly 20% of the molecular mass and is arranged into two different regions: 1) a central glycosylated region, which includes a large number of tandem repeats that are rich in serine, threonine and proline (STP repeats); and 2) regions at the amino and

carboxy terminals with an amino acid structure more typical of globular proteins, relatively few O-glycosylation and very few N-glycosylation sites (Bell et al., 2003). These second regions are rich in cysteine and contain domains that have sequence resemblance to von Willebrand factor (vWF) C and D domains, and C-terminal cystine knot domains, and are involved in dimerization through disulfide bond formation, and subsequent polymerization of the dimers to form multimers (Bell, Xu, & Forstner, 2001; Perez-Vilar & Hill, 1999; Turner, Bhaskar, Hadzopoulou-Cladaras, & LaMont, 1999). Both membrane-bound and secreted mucins are heavily glycosylated, consisting of 80% carbohydrates -- mainly N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), fucose, galactose, and sialic acid (N-acetylneuraminic acid) -- and small amounts of mannose and sulfate. The oligosaccharide chains are arranged in a ‘‘bottle brush’’ configuration around the protein core. The chains consist of carbohydrate monomers which are attached to the protein core by O-glycosidic bonds to the hydroxyl side chains of serine and threonine amino acids (O type glycosylation). These carbohydrate monomers differ in composition, branching, length and modification by sulfation and acetylation, thus contributing to the heterogeneity of mucins. O-linked carbohydrate chains are synthesized by specific glycosyltransferases. During this synthesis, GalNAc is attached to a serine or threonine, and can be further elongated by addition of galactose (Gal) or GlcNAc. The carbohydrate chains can be terminally glycosylated by addition of different sugars such as fucose, sialic acid, or sulfate, as well as by blood-group determinants. The presence of sialic acid and sulfates on Gal or GlcNAc moieties imparts negative charges to mucins, whereas fucose imparts hydrophobicity. Thus, these terminal sugars are believed to determine the physical and biological properties of mucins, and changes in terminal glycosylation of mucins, which can arise in disease conditions, may alter a variety of properties of mucins (Rose & Voynow, 2006).

Salivary Factors in BMS: The Potential Role of Salivary MUC1

Salivary factors have a direct effect on oral mucosal barrier function. Several studies have examined the saliva of BMS patients. While dry mouth is a common complaint among BMS patients (Kolkka-Palomaa et al., 2015), salivary flow rates in BMS patients differ between studies. Some studies have found a significantly lower unstimulated salivary flow rate among BMS cases compared to healthy controls (Poon, Su, Ching, Darling, & Grushka, 2014), whereas others have found no significant differences in unstimulated salivary flow rates between BMS patients and controls (Boras et al., 2010; Nagler & Hershkovich, 2004). The sensation of oral dryness in BMS patients has been generally attributed to neurosensory changes, rather than an actual hyposalivation problem (Poon et al., 2014). While little correlation has been found between symptoms of oral dryness and salivary flow rates (Castro et al., 2013), unstimulated whole saliva flow rate has been found to relate more closely with symptoms of xerostomia than stimulated salivary flow rate (Wang et al., 1998). Salivary compositional changes have been found in patients with dry mouth, as well as those with BMS (Kolkka-Palomaa et al., 2015). Of specific interest are salivary mucins, which are mainly excreted from minor salivary glands into the unstimulated saliva. Because mucins represent the principal lubricating constituents of saliva, it has been suggested that alterations in mucins might play a role in the sensation of oral dryness (Tabak, 1995).

A few studies have examined the relationship between salivary mucins and dry mouth. Patients reporting oral dryness have been found to have similar or higher mucin protein concentrations compared with controls (Chaudhury, Shirlaw, Pramanik, Carpenter, & Proctor, 2015; Randall et al., 2013). However, recently, an assessment of the quality of mucins in patients with Sjögren syndrome (SS – a disease characterized by oral and ocular dryness) revealed a statistically

significant reduction in mucin glycosylation, specifically in sialic acid, and in sulfation among patients compared to healthy controls. Such loss of negatively-charged glycan residues is a proposed mechanism for oral dryness through the diminished water retention ability of mucins, causing a reduction in mucosal hydration (Chaudhury et al., 2015). Recently, a study found higher levels of MUC5B and MUC1 in saliva of adolescents with high number of carious lesions compared with those with smaller number of lesions. The authors proposed that MUC1 acts as a scaffold for MUC5B, and when MUC1 is shed into saliva then MUC5B will follow, reducing the amount of MUC5B in the enamel pellicle, thereby making the enamel more susceptible to adherence of *S. mutans*, which can cause enamel demineralization (Lynge Pedersen & Belstrom, 2019). Salivary mucins have not been evaluated in BMS patients, except for two studies. One study found an increased expression of oral epithelial MUC1 mRNA among BMS patients compared to controls (Kho et al., 2013), while another study found no association between genotypic polymorphism of MUC7 with the development of BMS (Kim, Kim, Chang, Kim, & Kho, 2017).

Mucin Glycosylation

Glycosylation is one of the most important posttranslational modifications of proteins and is of great biological significance. The two major types of protein glycosylation, N-linked and O-linked, are both involved in the maintenance of protein structure and activity, and in protein intracellular trafficking and secretion, as well as in protein protection from proteolytic degradation (Oliveira-Ferrer, Legler, & Milde-Langosch, 2017). The carbohydrate constituents of glycoproteins like mucins have been shown to provide the molecules with anti-proteolytic properties. Although not fully understood, it is believed that the removal of carbohydrate chains could make mucins more susceptible to proteolytic degradation (Takehara et al., 2013). Altered mucin glycosylation has

significant implications in several diseases, such as cystic fibrosis, inflammatory bowel disease and several cancers (Corfield et al., 1992; Flowers, Ali, Lane, Olin, & Karlsson, 2013). In the oral cavity, the carbohydrate components of salivary mucins are able to bind to and interact with different microorganisms such as bacteria and *candida albicans*, preventing their attachment to the oral mucosal surface (Everest-Dass et al., 2012). For example, oral commensal Mitis group streptococci recognize the terminal sialic acid (a nine-carbon monosaccharide) on salivary mucins (Kozak et al., 2016), particularly MUC7 (Takamatsu, Bensing, Prakobphol, Fisher, & Sullam, 2006). In addition, sialylated mucins have the ability to retain large quantities of water and thus contribute to hydration and lubrication of the oral cavity (Castro et al., 2013). Carbohydrate chains attached to salivary MUC7 showed changes in patients with recurrent aphthous stomatitis (RAS). Patients with RAS have less complex carbohydrate chains, likely caused by bacterial oligosaccharide-degrading enzymes that reduce glycan complexity (Kozak et al., 2016; Zad, Flowers, Bankvall, Jontell, & Karlsson, 2015).

There is extensive normal variation in the glycosylation of MUC5B oligosaccharides, which may have significant effects on the microenvironment in the oral cavity of different individuals. Such variation is, in part, genetically controlled through blood group and secretor status (Thomsson, Schulz, Packer, & Karlsson, 2005). Salivary glycoproteins carry the carbohydrate epitopes that include the ABO, Lewis (Le) and other blood group determinants. Secretor status (as defined by alleles of the fucosyltransferase 2 gene – FUT2) additionally modifies glycosylation patterns, as the FUT2 enzyme is responsible for providing the glycan scaffold for Leb/y and blood-type motifs. FUT2 regulates the expression of the H antigen, a precursor of the blood group A and B antigens, on the gastrointestinal mucosa. Approximately 20% of Caucasians are non-secretors who do not express ABO antigens in saliva, as they are homozygous for FUT2 null alleles, having an inactive

FUT2. Consequently, individuals who have a deficiency in this enzyme would have less fucosylated glycoforms than secretors who have an active FUT2 (Henry, Oriol, & Samuelsson, 1995). The association between secretor status (which would provide indirect information about glycosylation patterns) and BMS has not been previously studied. Examination of this relationship could provide important information about the potential role of a genetic factor in the development of BMS.

Chapter II: Materials and Methods

Overview

To assess the possible role of mucosal barrier dysfunction in BMS, we conducted a case-control study comparing salivary flow rates, mucin concentrations and mucin glycosylation in female BMS cases and controls. Self-report data, salivary samples and other clinical exam data were collected at a single session. Samples were analyzed using ELISA, dot plots stained with different lectins, and PCR/sequencing. University of Washington Institutional Review Board (IRB) approval was obtained for this study.

Participants

A total of 57 female participants (28 cases and 29 controls) were enrolled in this study.

Definition of cases: Female patients, age 50 years or older recruited from the University of Washington Oral Medicine Clinical Services with the following complaint, "intraoral burning or dysesthetic sensation, recurring daily for more than 2 hours per day for more than 3 months" (ICD-10-CM diagnosis code K14.6).

Definition of controls: Women volunteers, age 50 years or older who responded to the study advertisement posted in the University of Washington Health Sciences Building. Controls were eligible to enter the study if they met both of the following criteria:

1. They had not been previously diagnosed with BMS, or any other medical condition known to cause oral burning except hyposalivation.

2. They answered "no" to the following question: In the past 3 months, did you have a

burning pain, altered sensation, or disturbed taste inside your mouth, including on your lips or tongue?

Exclusion criteria for both cases and controls:

1. Current smokers or a history of smoking habit within the last 10 years.

2. A history of head and neck radiation.

3. Have a clinically obvious abnormality of the oral mucosa such as lichen planus or oral ulceration

4. Have a history of trigeminal nerve injury.

Recruitment of cases:

A chart review of all the patients who attended the Oral Medicine clinic from January 1, 2017 through December 20, 2018 identified 102 patients who had a diagnosis of BMS (ICD-10 K14.6). Fifty-five of these patients didn't meet eligibility criteria for this study (16 were males; 6 had an oral epithelial abnormality including oral ulcerations, lichen planus or leukoplakia; 3 had a history of trigeminal nerve injury; 11 were younger than 50 years old; and 5 reported that their oral burning had resolved). Eligible patients were approached either by phone or in person at the end of their regular follow-up visit to the oral medicine clinic and invited to participate in this study. Nine refused, and we were not able to contact 12 patients. Twenty-eight cases were enrolled and attended the study visit.

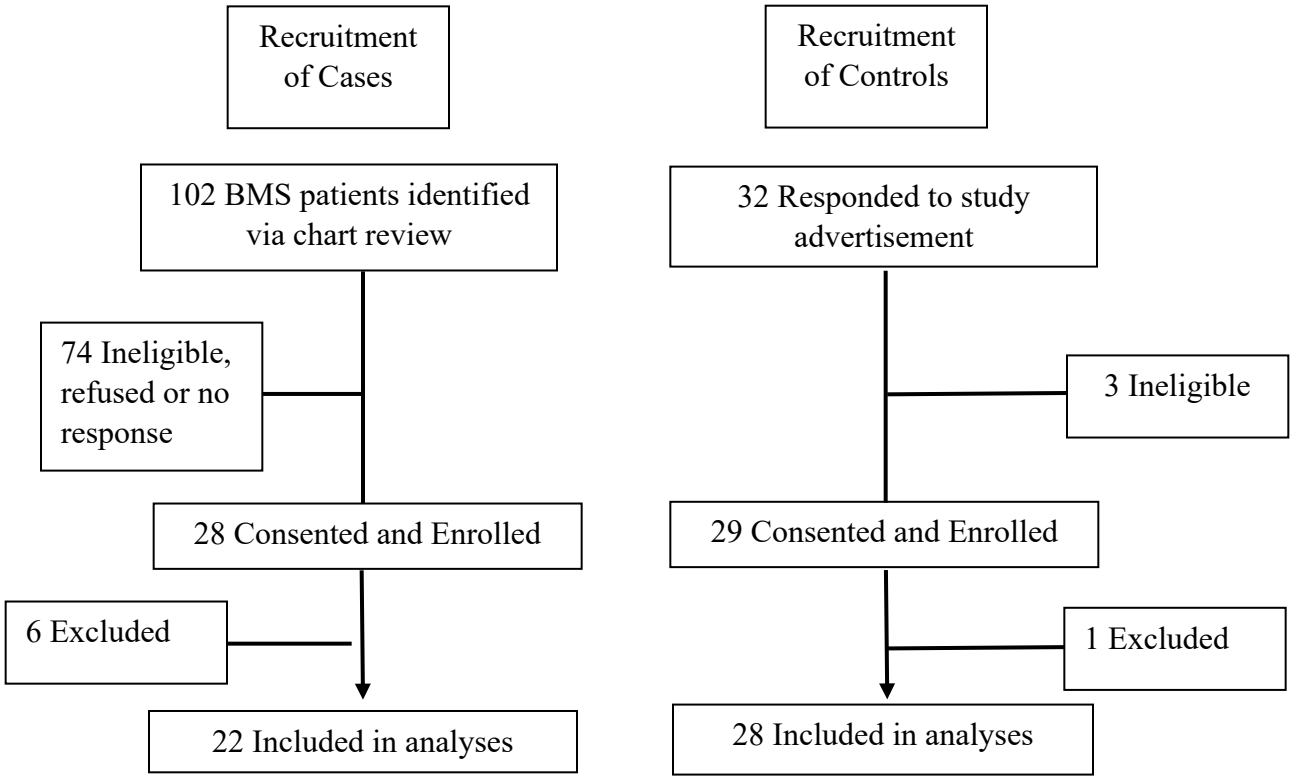
Recruitment of controls:

Thirty-two people responded to the study advertisement. Three were ineligible (one was a male, two were younger than 50 years old). Twenty-nine controls were enrolled and attended the study visit.

Analytic Sample

After the study visit, data for six cases were excluded from further analysis, due to one or more of the following reasons: two were below 50 years old, one had oral lichenoid lesions, one geographic tongue lesions, two had oral pain that did not meet criteria for BMS. Data from one control were excluded because of the presence of depapillated lesions on her tongue, leaving a sample of 22 cases and 28 controls included in the analyses presented here. Figure 1 shows the study flow.

Figure 1. Study flow diagram



Data Collection

Potential participants were invited for a single one-hour visit for collection of salivary samples and other data. To avoid diurnal variations in salivary flow rate and composition, all sessions were held at the same time of day (9:00 am to 12:00 pm) for all participants. Participants were instructed to avoid eating, drinking, chewing gum, or brushing their teeth in the 3 hours before sample collection. Also, they were instructed not to consume alcohol or caffeinated beverages in the 12 hours prior to participation. At the beginning of the study session, study procedures were reviewed, and written consent was obtained from each participant. All study procedures were conducted by the candidate who is a dentist specializing in Oral Medicine.

Participants were seated in a dental chair and instructed to rinse their mouths about 10 minutes prior to saliva collection. The salivary collection protocol was as follows:

- Collection of unstimulated (resting) whole saliva (USWS):

  Unstimulated whole saliva was collected under resting conditions by asking the participant to tilt her head forward, allow saliva to collect in the mouth, and to let the saliva passively drool into a pre-weighed, dry, sterilized and chilled plastic tube. The participant was instructed not to make any chewing movements, and not to swallow any saliva. The collection was timed for 10 minutes. The tube was reweighed after collection.

- Collection of residual mucosal saliva (RMS):

  RMS was collected 5 minutes after the completion of USWS collection. Collection was completed using 2cm x 0.5cm sized filter paper strips. The pre-weighed filter paper strip was placed on the mucosa immediately after swallowing. Four mucosal sites were

measured; anterior hard palate, buccal mucosa, anterior tongue, and lower lip. After 10 seconds of collection time the paper strip was placed in a microcentrifuge tube and weighed.

- Collection of stimulated whole saliva (SWS)

  Stimulated whole saliva was collected while having the subjects chew 1 g of gum base at her habitual pace. After discarding the saliva collected during the first minute, SWS samples were collected for 5 minutes into pre-weighed, dry, sterilized and chilled tubes.

  Saliva sample weight was calculated by subtracting the empty tube weight from the weight of the one filled with saliva. UWS and SWS flow rates were calculated and expressed in (ml/min), taking 1 g saliva = 1 ml. USWS and SWS samples were kept on ice at all times during the study visit. At the end of the visit, 2mL of each of the USWS and SWS saliva for each patient was mixed with an equal volume of protease inhibitor/Tri-buffered saline (TBS). 250µl aliquots of the diluted samples were prepared. The remaining collected saliva was divided in aliquots of 1ml in prelabeled tubes. All tubes were transferred to the freezer for storage at -80 °C (Takehara et al., 2013; Zad et al., 2015).

- DNA collection

  A salivary collection device called the DNA•SAL™ device (Oasis Diagnostics Vancouver, WA) was used to abrade cells and saliva in the mouth. This sample collection involved scraping the inner surface of the cheek using a plastic brush and swishing with a solution for 15 seconds then spitting into a bottle. Saliva in the collection device was stored at 4°C.

- Intraoral examination

  An intraoral examination was conducted to rule out any mucosal abnormalities. An oral smear was taken from all participants to assess for candida infection. Candida swabs were sent to the lab within 24 hours of collection.

- Completion of questionnaires:

  Participants completed the questionnaires shown in Table 1. These variables were used to describe the sample and assess for differences between cases and controls.

Table 1. Questionnaires completed by study participants:

Questionnaire	**Data provided**
Pain and Health History questionnaire	Demographic variables, medical conditions, medications, menopausal status in cases and controls. Pain characteristics* in cases only.
Patient Health Questionnaire eight-item depression scale (PHQ-8)	Measures the prevalence and severity of depression in cases and controls (Kroenke et al., 2009)
Short-form version (SXI-D) of the Xerostomia Inventory (XI),	Assesses the prevalence of xerostomia in cases and controls (Thomson, 2015)
Oral health impact profile-14 (OHIP-14)	Measures social impact of oral burning in cases (Slade & Spencer, 1994).

*What was related to onset of symptoms?
How many years/months did burning pain begin?
When is the pain worse?
What do you use to alleviate symptoms?
Questions about disability days and disability scores
Pain averseness using VAS (Visual Analog Scale)
Pain intensity rating using VAS
What does your pain feel like?
How has your pain changed since onset?
What is the recurrence pattern of your pain?
What are treatments you have received for burning mouth?

- Periodontal probing depth was assessed using the Periodontal Disease Index (Ramfjord, 1967) for all study subjects. The index used measurements for six teeth which are:

  -Tooth #3 (maxillary right first molar)

  -Tooth #9 (maxillary left central incisor)

  -Tooth #12 (maxillary left first bicuspid)

  -Tooth #19 (mandibular left first molar)

  -Tooth #25 (mandibular right central incisor)

  -Tooth #28 (mandibular right first bicuspid).

Analysis of Sample Composition

Assessment of Secretor Status:

Total DNA was extracted from saliva using Qiagen DNeasy Blood & Tissue Kit using a modified version of manufacturer's protocol (v.170927).

FUT2 Genotyping:

The *FUT2* (secretor) genotype was determined using sequence-specific primers and PCR. Four primers were used: two forward and two reverse (Table 2). Sanger sequencing was performed for only the coding region of the FUT2 gene to determine carriage of FUT2 variants. Sequence analysis and alignments were performed using the DNAstar Lasergene 13 v.190201.

Table 2. Primers used for genotyping

Primer name	Sequence	Note
PCR_Fwd	GCCTGTGCACATAGGCAAGTAT	Used for PCR
PCR_Rev	CAGGCCACTGTTCACTGAGA	Used for PCR
Fut2Seq_Fwd	CCCCCATCTTCAGAATCACCC	Used for PCR and sequencing
Fut2Seq_Rev	CCACCACCCCCTTCCACACT	Used for PCR and sequencing

Saliva clarification:

Samples were thawed at 37°C and spun at 3000 x G for 10 minutes. The supernatant was removed and transferred to a sterile vial. Clarified samples and pellets were stored separately at -80°C.

Quantification of protein in USWS:

Total protein in USWS was quantified to provide a standard for mucin glycosylation assessment. Protein concentrations of the USWS samples from 28 controls and 21 cases (one case produced no USWS and therefore was not included in this analysis) were quantified using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific) as per the manufacturer's protocol. Samples were run in duplicate.

MUC1 ELISA:

The MUC1 ELISA was performed using the MUCIN 1/MUC1 Human ELISA Kit - Invitrogen (Thermo Fisher Scientific) according to the manufacturer's protocol. The unknowns were initially diluted 1:5 and serially diluted three times at 1:2, for a total of four dilutions.

Lectin Dot Blots:

Dot blots were prepared using an initial protein concentration of 500 ng/µl for the standard and a concentration of 200 ng/µl for the study samples. All samples were serially diluted in duplicates for seven additional times for a total of eight concentrations. 5µl of each sample were spotted on a nitrocellulose paper which was then left to dry overnight. The blots were then blocked using ELISA SynBlock (from Bio-Rad Laboratories, Inc.) We used biotinylated lectins (from Vector Laboratories, Inc., Burlingame, CA, U.S.A). Optimal concentrations and sugar specificity of the lectins used are listed in Table 3. Peroxidase-conjugated streptavidin was applied, and they were developed with Millipore Chemiluminescence and imaged using GE ImageQuant 350 imaging system to estimate concentrations of the different lectins in saliva samples.

Table 3. Binding specificity of the lectins used in the present study

Lectin	Abbreviation	Concentration (µl/ml)	Binding Specificity
Wheat Germ Agglutinin	WGA	1:1000	GlcNAc
Ulex europeaeus agglutinin-I	UEA-I	1:500	α-Fuc
Maackia amurensis lectin II	MAL-II	1:500	NeuAc

Fuc: fucose; GlcNAc: N-acetylglucosamine; N-acetylneuraminic acid.

Statistical Analysis

A total of 22 cases and 28 controls were included in the statistical analysis. Statistical analyses of all data were performed using the SPSS 19 for Windows program (SPSS Company, Chicago, IL, USA). Descriptive statistics were calculated for cases and controls. Percentages are presented for categorical variables. Means and standard deviations are presented for continuous variables. Chi-square test was used for comparison of qualitative data and the independent sample T-test for comparison of saliva flow rates. For comparison of MUC1 and lectins levels we used the Mann Whitney test as these variables were not normally distributed. Spearman correlation tests were used to evaluate the association between continuous variables. Significance was assessed at $p < 0.05$ level.

Our power analysis was based on the work of Kang and his collegues, who found a difference between MUC1 RNA expression levels in BMS patients vs. controls (Kang, Kim, Chang, & Kho, 2017). Our power calculations indicate that our sample size yields > 80% power to detect a similar difference in mucin levels between cases and controls (effect size of 3⁄4 standard deviation). Power to detect differences in glycosylation should be similar. For Aim 3, this sample size provides 80% power to detect a correlation of 0.49 between levels of mucin glycosylation and the severity of oral burning in cases.

Chapter III: Results/Demographic and Clinical Data

Demographic and descriptive data:

A total of 50 women (22 cases and 28 controls) were included in the data analysis of this study. Tables 4-11 describe the characteristics of the cases and controls. As shown in Table 4, mean age was 62 years for cases and 56 years for controls (p=0.05). Compared to controls, alcohol consumption was higher among cases (p = 0.01). The majority of each group were postmenopausal. Two cases and two controls had a hysterectomy and were not assigned a menopausal stage score. Overall, systemic disease was more common among cases (p = 0.01). Specifically, gastrointestinal disease was more common among cases (p= <0.001). No other category of disease showed a statistically significant difference between cases and controls. On average, cases used about two medications, vs. a mean of one medication in controls (p = 0.01).

Table 4. Demographic and descriptive data: *n* (%) is presented for categorical variables. Mean (*SD*) is presented for continuous variables.

Variables	**BMS (n=22)**	**Controls (n=28)**	**p-value**
Age, mean (SD)	62 (6.5)	56 (12.6)	0.05
Race (number/percent white)	15 (68%)	22 (78%)	0.40
Education			0.94
High school	3 (13.6%)	3 (10.7%)	
College	14 (63.6%)	18 (64.2%)	
Postgraduate	5 (22.7%)	7 (25.0%)	
Alcohol (drinks/week)			0.01
0	5 (22.7%)	17 (60.7%)	
1-2	12 (54.5%)	6 (21.4%)	
3 or more	5 (22.7%)	5 (17.8%)	
Physical activity			0.54
Vigorously active	12 (54.5%)	11 (39.2%)	
Moderately active	8 (36.3%)	11 (39.2%)	
Seldom active	2 (9.0%)	5 (17.8%)	
Menopausal stage (postmenopausal)	17/20 (85.0%)	22/26 (84.6%)	0.63
Systemic disease present *	12 (54.5%)	6 (21.4%)	0.01
GI disease present	8 (36.3%)	0	<0.001
Total number of drugs, mean (SD)	2.1 (2.0)	0.9 (1.2)	0.01
Use xerostomic medication(s)	5 (22%)	6 (21.4%)	0.91

*Rheumatiod arthritis, Lupus Erythematosis, autoimmune disease, gastrointestinal problems, heart disease, anemia, respiratory disease, hypertension, diabetes, thyroid problems, hepatitis, osteoarthritis, reflux, bladder disease, osteoporosis, kidney disease, cancer.

Compared to controls, more cases reported the following symptoms: dry mouth (p-value= 0.001), and vaginal dryness (p-value=0.01). The mean PHQ-8 score was higher among cases than controls, although this difference was not statistically significance (3.90 vs. 2.25, p-value=0.14) (Table 5).

Table 5. Self-report of dry mouth, dry eyes, vaginal dryness, and PHQ-8 scores in cases and controls.

	BMS (n=22)	Controls (n=28)	p-value
Dry Mouth	11 (50.0%)	2 (7.1%)	0.001
Dry Eyes	12 (54.5%)	11 (39.2%)	0.61
Vaginal Dryness	11 (50.0%)	5 (17.8%)	0.01
PHQ-8, mean (SD)	3.9 (4.9)	2.2 (3.0)	0.14

Saliva Flow Rates and Clinical Exam Data:

Salivary flow rates and clinical data are presented in Table 6. Overall, compared to controls, cases had lower USWS flow rates (p value <0.001), and lower SWS flow rates, although the latter finding did not reach statistical significance (p-value=0.11). Significantly more cases had lower than normal saliva flow rates (defined by USWS flow rate of ≤0.1 ml/min, SWS flow rate of ≤0.7 ml/min). Amounts of lingual, labial, and buccal residual saliva were lower among cases vs. controls, however the differences were not statistically significant. There was no statistically significant difference in the percentage of participants having a positive candida culture or in periodontal index scores between cases and controls.

Table 6. Biological and clinical data: *n* (%) is presented for categorical variables. Mean (*SD*) is presented for continuous variables.

Variable	BMS (n=22)	Controls (n=28)	*p*-value
USWS (ml/min)	0.1 (0.1)	0.3 (0.2)	<0.001
UWS ≤0.1 (ml/min)	17 (77.2%)	10 (35.7%)	<0.001
SWS (ml/min)	0.9 (0.6)	1.2 (0.5)	0.11
SWS ≤0.7(ml/min)	12 (54.5%)	7 (25.0%)	0.03
Lingual residual saliva (mg)	9.0 (9.0)	11.0 (6.0)	0.52
Palatal residual saliva (mg)	6.0 (6.0)	5.0 (8.0)	0.64
Labial residual saliva (mg)	5.0 (6.0)	7.0 (8.0)	0.32
Buccal residual saliva (mg)	5.0 (6.0)	6.0 (5.0)	0.44
Positive candida culture	3 (13.6 %)	3 (10.7%)	0.75
Periodontal index	4.1 (0.2)	4.1 (0.2)	0.57

Thirty-six percent of cases and fourteen percent of controls reported having current pain in the head and neck region (other than burning mouth pain) (p-value=0.14). The most common pain was TMJ pain (62%) in cases and neck pain (75%) in controls. Average duration of this head and neck pain was lower among cases 4.5 years compared to 13.5 years among controls (p-value=0.28). (Table 7)

Table 7. Frequency, duration of head and neck pain complaints among cases and controls, and cases' feelings about association of head and neck pain with BMS pain.

	BMS (n= 22)	Controls (n= 28)	p-value
Any head/ neck pain, n (%)	8 (36.3%)	4 (14.2%)	0.14
Neck pain	4 (18.2%)	3 (10.7%)	
Shoulder pain	3 (13.6%)	2 (7.1%)	
Ear pain	1 (4.5%)	1 (3.6%)	
Eye pain	1 (4.5%)	0	
TMJ pain	5 (22.7%)	0	
Forehead pain	0	1 (3.6%)	
Temple pain	1 (4.5%)	1 (3.6%)	
Sinus pain	2 (9.1%)	0	
Upper jaw pain	1 (4.5%)	0	
Lower jaw pain	2 (9.1%)	0	
Average duration (years), mean (SD)	5 (2.2)	13.5 (19.3)	0.28
Do you feel it is associated with your BMS problem? n (%)			
Yes	4 (50%)		
No	1 (12%)		
Unsure	3 (37%)		

Oral Burning Pain Characteristics among Cases:

Self-report data obtained from study questionnaires was used to assess characteristics of oral pain among cases. All cases described their oral pain as "burning." Additional pain descriptors commonly used by cases to describe their pain included: tender, aching, and exhausting (see Table 8 for full list of pain descriptors).

Table 8. Terms used by cases to describe their burning mouth pain:

Descriptor	N (%) of cases
Burning	22 (100%)
Tender	7 (32%)
Tiring	6 (27%)
Throbbing	5 (23%)
Exhausting	5 (23%)
Gnawing	4 (18%)
Hot	4 (18%)
Aching	4 (18%)
Sharp	2 (9%)
Unbearable	2 (9%)
Splitting	2 (9%)
Sickening	2 (9%)
Stabbing	1 (4%)
Fearful	1 (4%)
Cruel	1 (4%)

Cases reported using several methods to alleviate their oral burning. Most commonly reported methods were: having frequent sips of water or liquid (40%), followed by candies or mints and chewing gum (31%) (See Table 9).

Table 9. Oral Burning alleviating methods:

Alleviating factor	N (%) of cases
Frequent Sips of Water or Liquid	9 (40%)
Candies or Mint	7 (31%)
Chewing Gum	5 (22%)
Get Up at Night to Drink Water	4 (18%)
Other	3 (13%)
Salivary Substitute	2 (9%)
Hot or Cold Packs	1 (4%)

Regarding BMS treatments that have been used by cases, the most common treatment was topical clonazepam (54%), followed by mouth stents (36%). Topical clonazepam was reported by cases to be the most effective treatment with all those who used it reporting it to be a successful treatment.

Table 10. Treatments for oral burning used by cases

Type of treatment	Using treatment	No success	Some success	Good success
Topical clonazepam	12	0	4	8
Appliance	8	1	3	4
Pilocarpine	2	1	0	1
Lyrica	1	0	1	0

Regarding the characteristics of oral burning pain among cases (summarized in Table 11), most cases (41%) reported an overall improvement of their pain since onset. They most commonly reported that pain is worse during the day (73%), and the most common recurrence pattern was "daily" (64%).

Table 11. Oral burning pain characteristics among cases. *N (%)* is presented for categorical variables. Mean (SD) is presented for continuous variables.

Time Since Onset (years)	**Pain Aversive-ness Score**	**Pain Intensity Score**	**Change Since Onset**	**Time pain is Worst**	**Recurrence**	**OHIP-14**	**Taste Disturb-ance**
6.3 (5.75)	6.4 (2.66)	5.7 (3.25)	Improved (41%) Worse (27%) Unchanged (32%)	At night 11 (50%) Upon awakening 2 (9%) During day 16 (73%) When eating 2 (9%) Specific foods 6 (27%) During stress 9 (41%) At work 4 (18%)	Recurs daily 14 (64%) Recurs several times a day 3 (14%) Recurs weekly 1 (5%) Constant 4 (18%)	13.4 (9.8)	7 (31%)

Chapter IV: Results/Saliva Analyses Data

Secretor Status Analysis:

Secretor status analysis was conducted in order to detect variants in the FUT2 gene that could potentially alter glycosylation. Samples were examined for the presence of common variants known to partially or completely inactivate FUT2 gene resulting in a non-secretor phenotype. A total of 21 cases and 26 controls were included in the analysis of secretor status. The mean DNA concentration extracted from saliva samples was 36.08 ng/µl. A total of 26 homozygous variants were found in the total sample, five were synonymous, eighteen were missense variants, and three were loss of function variants (Table 12). Two variants were common variants for Fut2 (non-secretors) (428G>A, nonsense variant and 385 A>T, missense variant). Participants who carried any of the two common variants or a nonsense variant were considered "non-secretors". All other participants were considered "secretors". The percentage of non-secretors was 23% among cases vs. 38% among controls (p-value=0.28).

Table 12. Types and frequencies of variants found in FUT2 gene among cases and controls:

Variant	Frequency (controls n=26)	Frequency (cases n=21)	Type	Note
95 T>A	1	0	missense	
171 A>G	8	2	synonymous	
192 G>T	1	0	missense	
216 C>T	8	3	missense	
311 A>G	1	0	missense	
357 C>T	6	3	synonymous	
358 G>T	1	0	missense	
365 T>A	5	4	missense	
369 G>C	1	0	missense	
371 A>G	2	0	missense	
376 T>G	1	0	missense	
385 A>T	1	1	missense	Variant associated with non-secretor
428 G>A	9	3	nonsense	Variant associated with non-secretor
463 C>T	0	1	nonsense	
529 A>T	0	1	missense	
532 G>T	0	2	missense	
533 G>A	1	1	missense	
534 G>A	1	0	synonymous	
539 G>A	1	0	missense	
651 C>T	0	1	synonymous	
696 T>G	1	0	missense	
697 A>G	1	0	missense	
698 A>G	1	0	missense	
739 G>A	6	4	missense	
960 A>G	5	5	synonymous	

Quantification of protein in USWS:

Mean protein concentration was obtained in order to normalize saliva samples to protein content when assessing saliva glycosylation differences (Aim 2). The mean protein concentration was 0.94 mg/ml in controls (SD, 1.17), vs. 1.30 mg/ml (SD, 1.19) in cases (P value= 0.15). Pearson's

correlation analysis showed that salivary protein content was negatively correlated with the USWS flow rate ($r = –0.296$, $p < 0.05$) (Figure 1).

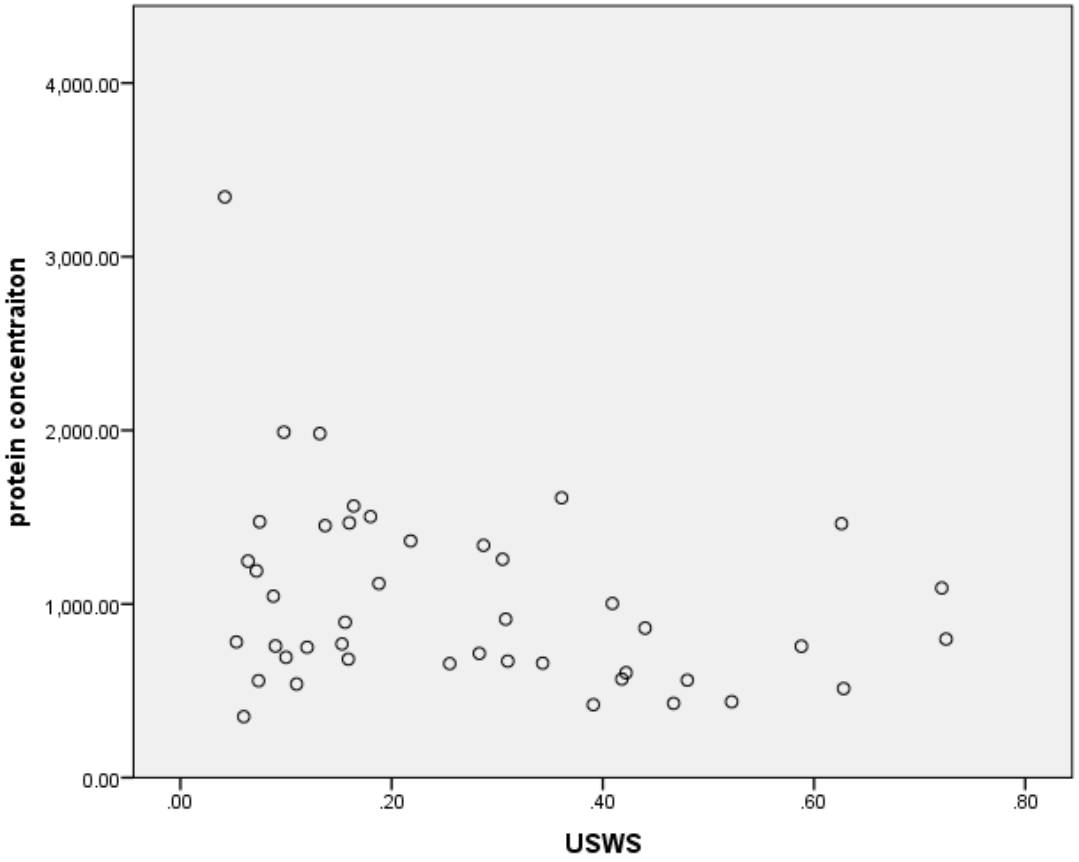


Figure 1. Correlation of USWS protein concentration with USWS flow rates in total sample ($r = –0.296$, $p < 0.05$).

MUC1 ELISA:

Mann Whitney U test showed that the levels of MUC1 were lower in cases (n=20, sum of ranks=392.50) compared to controls (n=24, sum of ranks=597.50), p-value=0.17 (Figure 2,3). In the total sample, Spearman correlation analysis showed a negative correlation between MUC1 levels and RMS levels on the hard palate (r=-0.36, p=0.01).

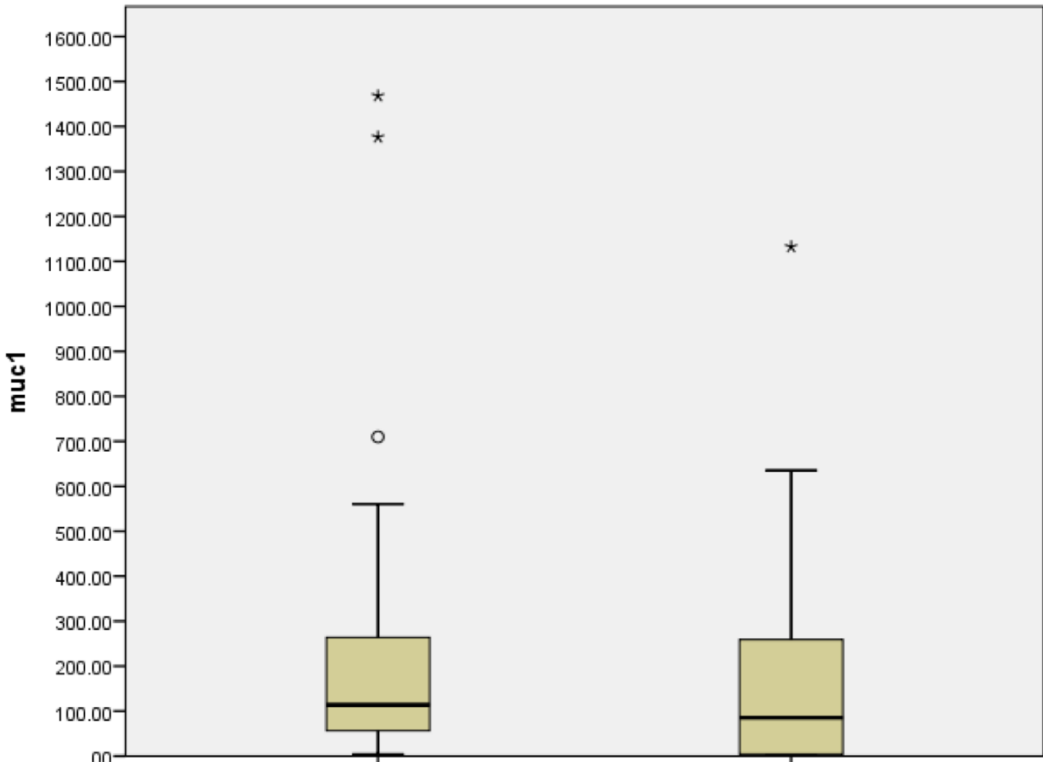


Figure 2. Mann Whitney U test results displayed in box plots showing differences in MUC1 levels between cases and controls (p-value=0.17).

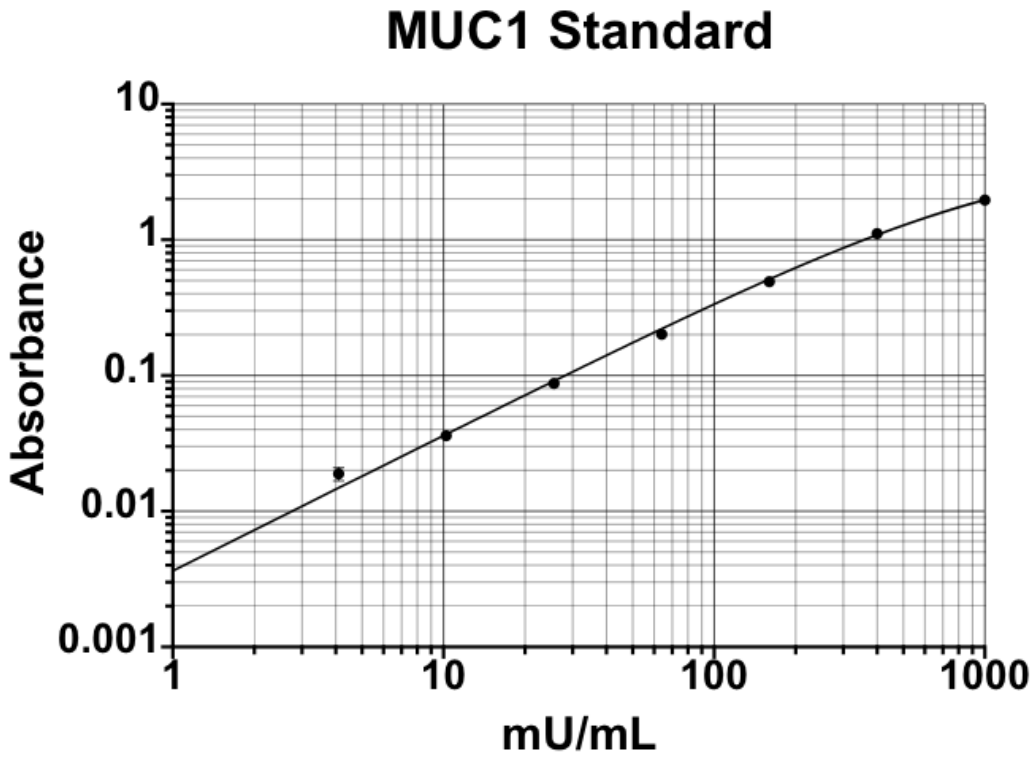


Figure 3. Standard curve for MUC1 protein levels.

Lectin Dot Blots:

Results of lectin dot blots (samples shown in Figures 4-9) showed that, although there was a great deal of individual variation and findings did not achieve statistical significance, levels of UEA, MALII, and WGA lectins were lower in cases compared to controls (Table 13). Secretors had significantly more UEA levels compared to non-secretors (p-value<0.001).

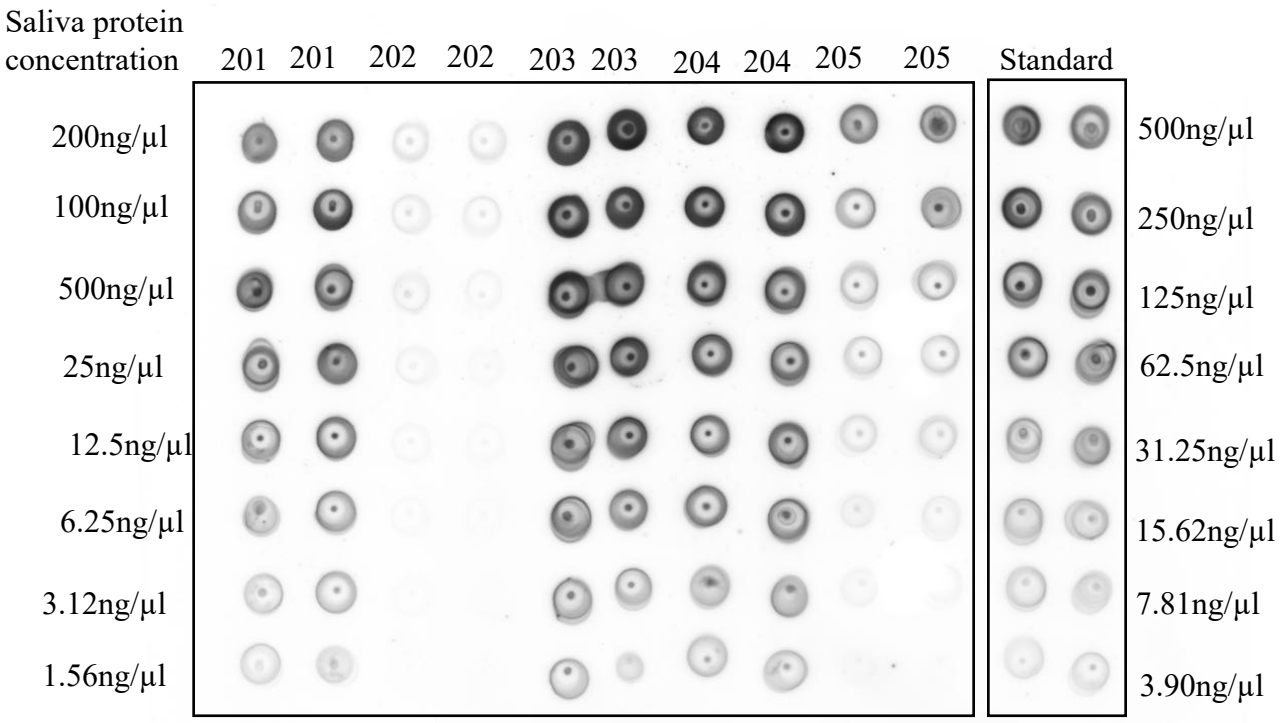


Figure 4. Dot blot stained with UEA lectin showing subject #202 a non-secretor.

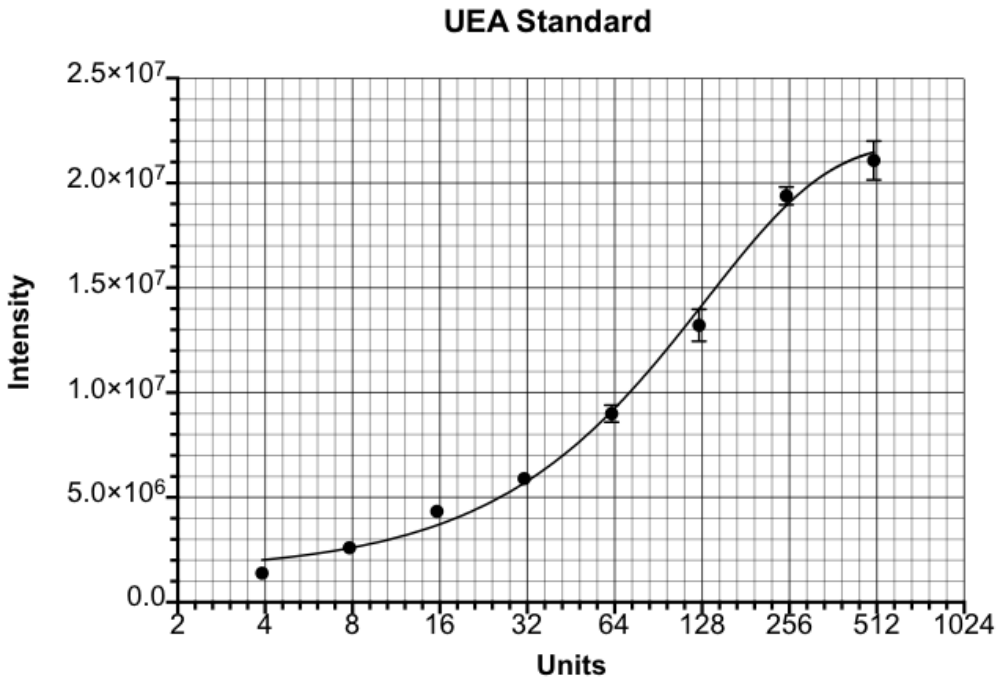


Figure 5. Standard curve for UEA levels.

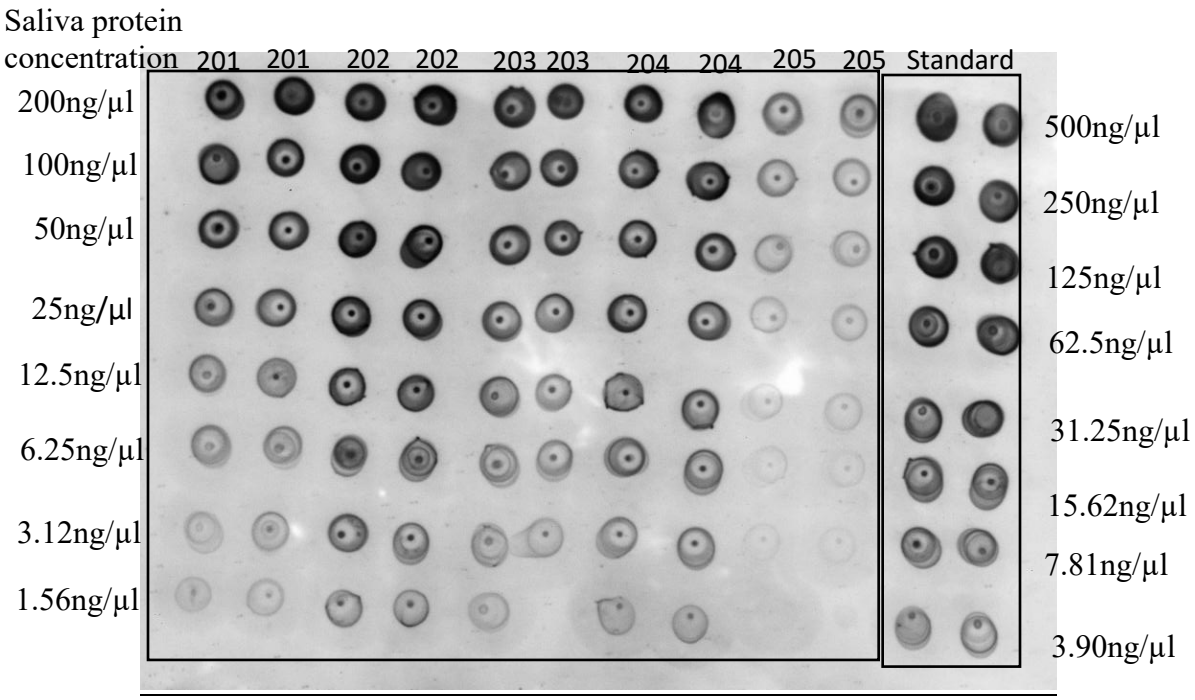


Figure 6. Dot blot stained with WGA lectin.

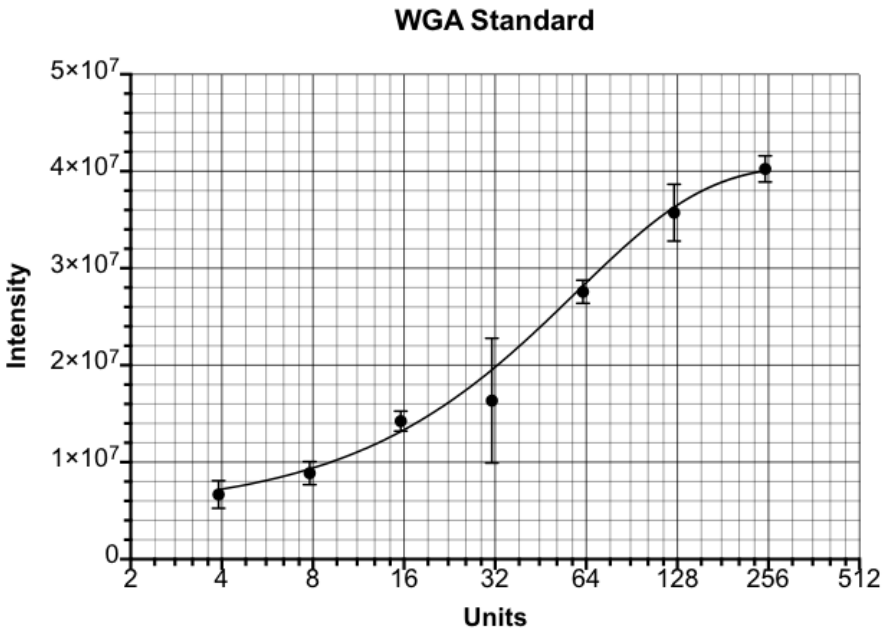


Figure 7. standard curve for WGA levels.

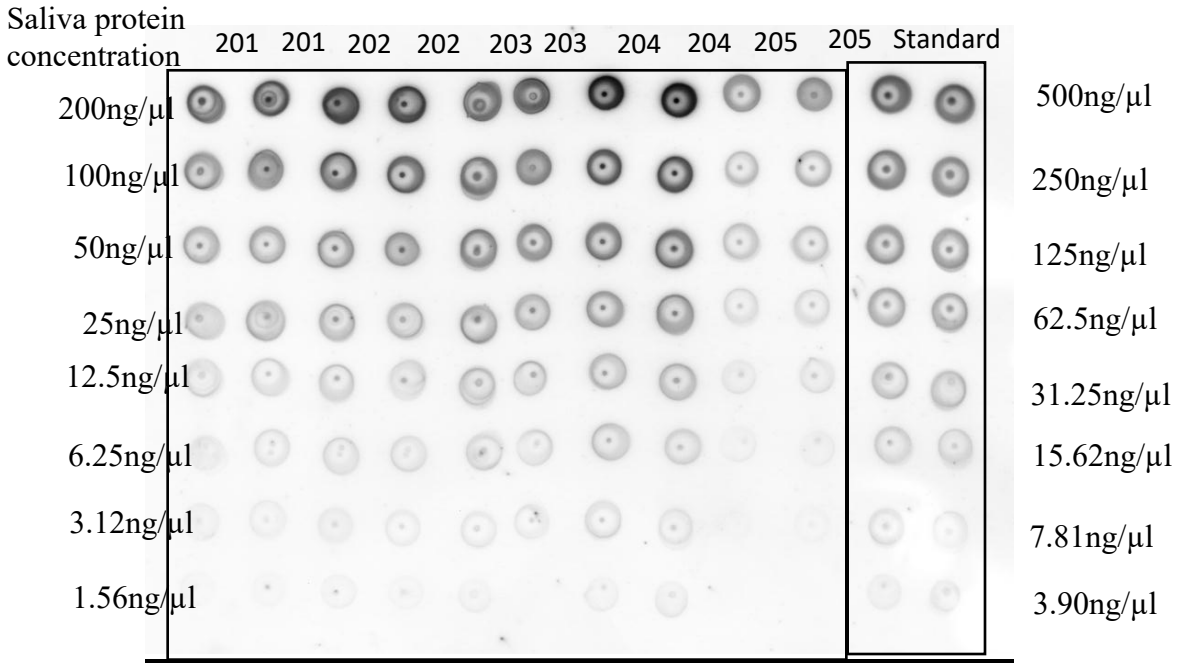


Figure 8. Dot blot stained with MAL-II lectin.

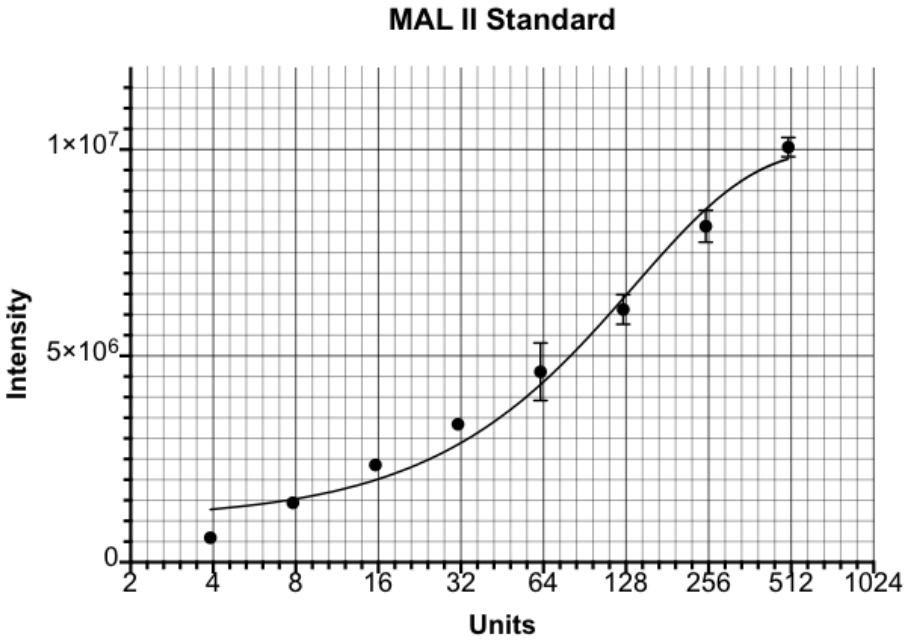


Figure 9. Standard curve for MALII levels.

Table 13. Mann Whitney U test results displayed in table showing differences in UEA, MALII, and WGA lectin levels between cases and controls.

Lectin	Cases (n=21)	Controls (n=27)	p-value
UEA	497.50	678.50	0.72
MALII	488.50	687.50	0.58
WGA	506.50	718.50	0.70

Spearman correlation analysis showed no significant correlation between lectin levels and the severity of oral burning (Aim 3) (Table 14). Similarly, no correlation was found between lectin levels and the severity of oral dryness (Table 15).

Table 14. Spearman correlation analysis between lectin levels and the severity of oral burning

Lectin	Correlation coefficient	p-value
UEA	-0.009	0.96
MALII	0.24	0.28
WGA	-.022	0.32

Table 15. Spearman correlation analysis between lectin levels and the severity of oral dryness

Lectin	Correlation coefficient	p-value
UEA	0.21	0.13
MALII	0.14	0.31
WGA	-0.17	0.23

Chapter V: Discussion

This is a case-control study of women age 50 years or older with and without BMS. The main findings of this study were that cases had lower USWS flow rates and a higher prevalence of xerostomia, vaginal dryness and GI disease compared to controls. No statistically significant difference in MUC1 levels or glycosylation levels were observed between cases and controls.

In this study, systemic disease, specifically GI disease, was more commonly reported among cases. Similar findings have been reported previously (Lamey & Lamb, 1988; Netto et al., 2011), where BMS patients were found to be over three times more likely to have GI problems compared to healthy individuals. Gastritis and gastroesophageal reflux (GERD) are the most commonly reported GI problems (Brailo et al., 2006). Also, dry mouth and oral burning were found to be the most common symptoms associated with GERD (Campisi et al., 2008; Di Fede et al., 2008). The nature of the association between GI disease and BMS remains unclear. In our study, more cases reported having GERD, however, the difference was not statistically significant (p-value=0.12). An association of H. pylori and BMS has been suggested (Gall-Troselj, Mravak-Stipetic, Jurak, Ragland, & Pavelic, 2001). One study showed that about 12.7% of patients with BMS had a *Helicobacter pylori* infection, however, more studies are needed to understand the role of this infection in the pathogenesis of BMS (Brailo et al., 2006).

A previous study reported no significant difference in USWS flow rate between BMS and controls (Nagler & Hershkovich, 2004) and another study reported a non-statistically significant decrease in USWS flow rate in BMS cases compared with controls (Zhao, Chen, & Lin, 2001). In this study,

the mean USWS flow rate in cases was three times lower than controls, and this was highly significant. In addition, similar to findings from a previous study (Hershkovich & Nagler, 2004), our BMS cases had higher total protein concentrations in USWS compared to controls (difference is not statistically significant). In our sample, there was a negative correlation between total protein concentrations and USWS flow rates.

Clinically, in addition to the burning of the oral mucosa, BMS is often accompanied by complaints of xerostomia. Since examination of the oral cavity often shows no evident pathological change that would explain the discomfort, it has been suggested that the sensation of oral dryness in BMS is a sensory change, rather than a result of a reduction in salivation (Bergdahl & Bergdahl, 1999; Grushka, 1987; Scully, 2003; Shetty, Bhowmick, Castelino, & Babu, 2012). However, in our study, all BMS cases who reported oral dryness had a reduced USWS flow rate. The majority of BMS patients are over the age of 50 years and an association of age with oral dryness has been found in the older population, likely as a result of increased medication usage, especially medications with a xerogenic effect (i.e., medications causing dry mouth). In our study, however, we found no significant difference in xerogenic medication intake in BMS cases compared to controls. Similarly, Poon et al. found no significant differences between BMS who did vs. did not take xerogenic medications, suggesting that oral dryness experienced by BMS patients is not significantly affected by medications (Poon et al., 2014). However, in our study and in the study by Poon et al. a statistically significant difference in the number of medications was found between BMS cases and controls, suggesting that medication usage is possibly a contributing factor in those patients using more than one medication. It has been suggested that the reduction in USWS flow rates found in BMS patients may be due to the loss of parasympathetic secretomotor innervation

carried by the chorda tympani, which is further reduced as a result of medication usage (Poon et al., 2014).

Vaginal dryness, as well as oral dryness, was significantly more common among cases (p-value<0.001). This finding is interesting because the mechanisms associated with vaginal and oral dryness could be similar. Menopause-related anatomic and physiologic changes in the vagina are directly related to reduced circulating estrogen levels and aging. Such changes include thinning of the epithelium, reduced vaginal blood flow, and diminished lubrication. These changes potentially lead to vaginal epithelial damage, causing vaginal pain, burning, and discomfort. Epithelial thinning with decreased glycogenated superficial cells alters the vaginal flora and causes loss of lactobacilli, increased pH, and a subsequent change in the microbiome. Such changes in vaginal flora and their role in menopausal-related symptoms are being examined in different vaginal microbiome research studies (Brotman et al., 2014; Hummelen et al., 2011). Because the oral mucosa contains estrogen receptors, variations in hormone levels associated with menopause have direct effects on the oral cavity. A significant number of menopausal women suffer from oral discomfort. The mechanisms of hormone-related oral symptoms and signs are not fully understood. Given that there is a great similarity in the histology of oral and vaginal mucosa it has been suggested that their symptoms might share a common cause. However, symptoms such as xerostomia and burning mouth are not generally linked with such hormonal changes and, there is no strong evidence showing that hormone replacement therapy prevents or helps women with oral symptoms (Suri & Suri, 2014).

Kho and his collogues found a significantly higher MUC1 RNA expression in BMS patients (Kho et al., 2013) Our study did not find a significant difference in MUC1 protein levels between BMS

cases and controls. MUC1 is detected in the residual mucosal saliva that coats the oral mucosa (J. Y. Lee, Chung, Kim, Chung, & Kho, 2007; S. K. Lee, Lee, Chung, Kim, & Kho, 2002). MUC1 is thought to play a role in the formation of the oral mucus gel that protects and hydrates the oral mucosa. MUC1 expression is decreased in the elderly population (Chang, Chang, Kim, Lee, & Kho, 2011). Kho et al., 2013 suggested that the increased expression of MUC1 in BMS may be an adaptive mechanism of the oral mucosal epithelial cells to chronic local irritation One explanation of our findings regarding MUC1 levels, is that the increase in MUC1 expression is accompanied by an increase in the rate of MUC1 degradation. It is also possible that the difference in MUC1 protein levels was too small to be detected by ELISA. Another explanation is that MUC1 protein levels are highly variable in our sample population and a larger sample size is needed to detect any differences.

This is the first study to assess glycosylation in saliva of BMS patients. We did not observe a significant difference in any of the lectins between cases and controls. One study of dry mouth patients found a reduction in mucin glycosylation in dry mouth patients, specifically in the amount of sialic acid which alters the ability of mucins to retain moisture causing less hydration of the oral mucosa (Chaudhury et al., 2015). We found a significant individual variation in lectin dot blot results and a larger sample size is likely needed to observe any real differences in lectin levels. As expected, UEA (which recognizes Fucα1-2Gal-R present in saliva of secretors) levels were significantly higher in secretors compared to non-secretors.

This study has several strengths and a few limitations. Study strengths include the following: well-characterized cases and controls, our sample size is larger than some other studies of BMS, and this study is the first study to measure MUC1 protein, glycosylation, and secretor status in BMS patients. In terms of study weaknesses, the sample size was relatively small and possibly

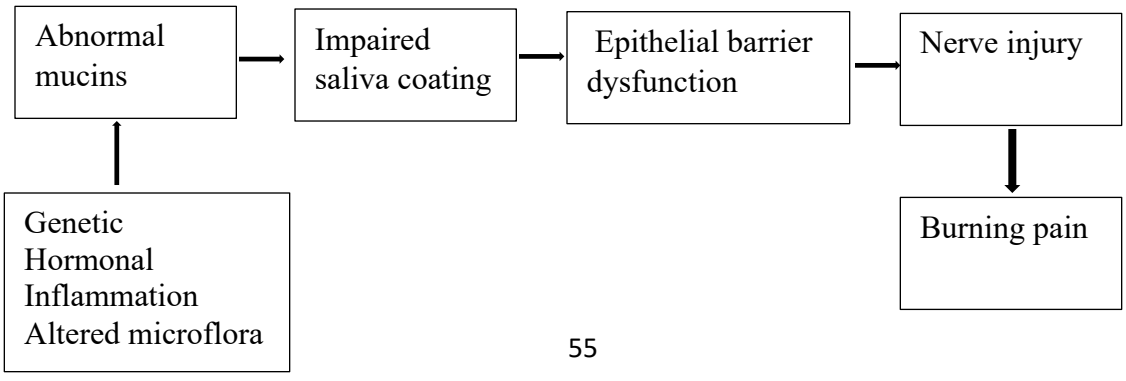
heterogeneous with potential unidentified BMS subgroups despite efforts to keep it as uniform as possible. Our controls were slightly younger than the cases. Cases and controls also differed in their alcohol consumption. We also had some technical issues with the MUC1 ELISA assay as some samples were below the detection threshold of the test. We used strict inclusion criteria for BMS cases which limited the number of eligible BMS cases and excluded a number of participants. In addition to restrictions based on BMS diagnostic criteria, we used age 50 years as a cutoff to achieve a homogenous sample and enrich for postmenopausal phase in an attempt to control for hormonal factors. Another exclusion criterion for both cases and controls was having an oral mucosal abnormality detected by clinical examination. This was based on our hypothesis that BMS is caused by an epithelial barrier dysfunction related to alterations in salivary mucins. Overall, we excluded six cases and one control with no predominant reason for exclusion of our study participants. In terms of recruitment, we attempted to recruit controls who are similar in many ways to the cases. Female patients attending the University of Washington Oral Medicine Clinic who have a diagnosis other than BMS, would have been potentially a very similar control group for our cases, but we were unable to include this group since a large number of these patients have an oral mucosal abnormality that can be identified clinically.

It is widely accepted that BMS is a neuropathic condition caused by deafferentation of thin nerve fibers. The challenge remains to explain the trigger that lead to such a deafferentation especially in the absence of macro-trauma to the nerve. It has been proposed that reduced synthesis of ovarian steroids after menopause causes a deficiency in adrenal steroids, which reduces the neuroprotective effects of steroids on neural tissues (Imamura et al., 2019; Woda et al., 2009). There are no animal models that clearly demonstrate the effect of reduced gonadal steroids on somatosensory and gustatory fibers in the tongue. Ovariectomized animals are often used to mimic some major

symptoms of BMS. Ovariectomy induces thinning of tongue epithelium. There is also a decrease in the number of vaginal nerve fibers after ovariectomy (Imamura et al., 2019). Besides direct effects on epithelium and nerve fibers, it is hypothesized that the lack of neuroprotective steroids leads to hypofunction of minor salivary glands that causes oral dryness and preclinical inflammation of oral mucosa and such changes may be involved in triggering burning mouth symptoms (Shinozaki et al., 2016).

In terms of our global hypothesis, we proposed that BMS is caused by an oral mucosal epithelial barrier dysfunction, which leads to nerve damage and subsequent neuropathic pain (Figure 6). In this study we hypothesized that subtle changes in mucin glycosylation are the main cause of the epithelial defect. MUC1 was the salivary mucin we chose to examine. This was based on results from previous literature assessing MUC1 expression in BMS (Kho et al., 2013). To assess glycosylation, we chose to use three lectins: UEA, MALII, and WGA which have the affinity to bind to terminal fucose, sialic acid, and *N*-acetylglucosamine respectively. These sugars play a key role in the hydration and bacterial colonization of the healthy GI epithelium (Sicard et al., 2018). Although our quantification of saliva glycosylation revealed no significant differences between cases and controls, all results were in the hypothesized direction. Other aspects of the oral epithelial barrier, including the quantity of other mucins and other lectins associated with glycosylation remain to be examined.

Figure 6. Proposed model for factors leading to BMS



References:

Almstahl, A., Wikstrom, M., & Groenink, J. (2001). Lactoferrin, amylase and mucin MUC5B and their relation to the oral microflora in hyposalivation of different origins. *Oral Microbiol Immunol, 16*(6), 345-352.

Balasubramaniam, R., Klasser, G. D., & Delcanho, R. (2009). Separating oral burning from burning mouth syndrome: unravelling a diagnostic enigma. *Aust Dent J, 54*(4), 293-299.

Bell, S. L., Xu, G., & Forstner, J. F. (2001). Role of the cystine-knot motif at the C-terminus of rat mucin protein Muc2 in dimer formation and secretion. *Biochem J, 357*(Pt 1), 203-209.

Bell, S. L., Xu, G., Khatri, I. A., Wang, R., Rahman, S., & Forstner, J. F. (2003). N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2. *Biochem J, 373*(Pt 3), 893-900.

Bender, S. D. (2018). Burning Mouth Syndrome. *Dent Clin North Am, 62*(4), 585-596.

Beneng, K., Yilmaz, Z., Yiangou, Y., McParland, H., Anand, P., & Renton, T. (2010). Sensory purinergic receptor P2X3 is elevated in burning mouth syndrome. *Int J Oral Maxillofac Surg, 39*(8), 815-819.

Bergdahl, M., & Bergdahl, J. (1999). Burning mouth syndrome: prevalence and associated factors. *J Oral Pathol Med, 28*(8), 350-354.

Boltin, D., Perets, T. T., Vilkin, A., & Niv, Y. (2013). Mucin function in inflammatory bowel disease: an update. *J Clin Gastroenterol, 47*(2), 106-111.

Boras, V. V., Brailo, V., Lukac, J., Kordic, D., & Blazic-Potocki, Z. (2006). Salivary interleukin-6 and tumor necrosis factor-alpha in patients with burning mouth syndrome. *Oral Dis, 12*(3), 353-355.

Boras, V. V., Savage, N. W., Brailo, V., Lukac, J., Lukac, M., & Alajbeg, I. Z. (2010). Salivary and serum levels of substance P, neurokinin A and calcitonin gene related peptide in burning mouth syndrome. *Med Oral Patol Oral Cir Bucal, 15*(3), e427-431.

Borelli, V., Marchioli, A., Di Taranto, R., Romano, M., Chiandussi, S., Di Lenarda, R., . . . Zabucchi, G. (2010). Neuropeptides in saliva of subjects with burning mouth syndrome: a pilot study. *Oral Dis, 16*(4), 365-374.

Brailo, V., Vueiaeeviae-Boras, V., Alajbeg, I. Z., Alajbeg, I., Lukenda, J., & Aeurkoviae, M. (2006). Oral burning symptoms and burning mouth syndrome-significance of different variables in 150 patients. *Med Oral Patol Oral Cir Bucal, 11*(3), E252-255.

Braud, A., Toure, B., Agbo-Godeau, S., Descroix, V., & Boucher, Y. (2013). Characteristics of pain assessed with visual analog scale and questionnaire in burning mouth syndrome patients: a pilot study. *J Orofac Pain, 27*(3), 235-242.

Brotman, R. M., Shardell, M. D., Gajer, P., Fadrosh, D., Chang, K., Silver, M. I., . . . Gravitt, P. E. (2014). Association between the vaginal microbiota, menopause status, and signs of vulvovaginal atrophy. *Menopause, 21*(5), 450-458.

Campisi, G., Lo Russo, L., Di Liberto, C., Di Nicola, F., Butera, D., Vigneri, S., . . . Di Fede, O. (2008). Saliva variations in gastro-oesophageal reflux disease. *J Dent, 36*(4), 268-271.

Castro, I., Sepulveda, D., Cortes, J., Quest, A. F., Barrera, M. J., Bahamondes, V., . . . Gonzalez, M. J. (2013). Oral dryness in Sjogren's syndrome patients. Not just a question of water. *Autoimmun Rev, 12*(5), 567-574.

Chang, W. I., Chang, J. Y., Kim, Y. Y., Lee, G., & Kho, H. S. (2011). MUC1 expression in the oral mucosal epithelial cells of the elderly. *Arch Oral Biol, 56*(9), 885-890.

Chaudhury, N. M., Shirlaw, P., Pramanik, R., Carpenter, G. H., & Proctor, G. B. (2015). Changes in Saliva Rheological Properties and Mucin Glycosylation in Dry Mouth. *J Dent Res, 94*(12), 1660-1667.

Corfield, A. P., do Amaral Corfield, C., Wagner, S. A., Warren, B. F., Mountford, R. A., Bartolo, D. C., & Clamp, J. R. (1992). Loss of sulphate in human colonic mucins during ulcerative colitis. *Biochem Soc Trans, 20*(2), 95S.

de Moraes, M., do Amaral Bezerra, B. A., da Rocha Neto, P. C., de Oliveira Soares, A. C., Pinto, L. P., & de Lisboa Lopes Costa, A. (2012). Randomized trials for the treatment of burning mouth syndrome: an evidence-based review of the literature. *J Oral Pathol Med, 41*(4), 281-287.

DeSouza, M. M., Surveyor, G. A., Price, R. E., Julian, J., Kardon, R., Zhou, X., . . . Carson, D. D. (1999). MUC1/episialin: a critical barrier in the female reproductive tract. *J Reprod Immunol, 45*(2), 127-158.

Di Fede, O., Di Liberto, C., Occhipinti, G., Vigneri, S., Lo Russo, L., Fedele, S., . . . Campisi, G. (2008). Oral manifestations in patients with gastro-oesophageal reflux disease: a single-center case-control study. *J Oral Pathol Med, 37*(6), 336-340.

Everest-Dass, A. V., Jin, D., Thaysen-Andersen, M., Nevalainen, H., Kolarich, D., & Packer, N. H. (2012). Comparative structural analysis of the glycosylation of salivary and buccal cell proteins: innate protection against infection by Candida albicans. *Glycobiology, 22*(11), 1465-1479.

Flowers, S. A., Ali, L., Lane, C. S., Olin, M., & Karlsson, N. G. (2013). Selected reaction monitoring to differentiate and relatively quantitate isomers of sulfated and unsulfated core 1 O-glycans from salivary MUC7 protein in rheumatoid arthritis. *Mol Cell Proteomics, 12*(4), 921-931.

Forssell, H., Jaaskelainen, S., Tenovuo, O., & Hinkka, S. (2002). Sensory dysfunction in burning mouth syndrome. *Pain, 99*(1-2), 41-47.

Forssell, H., Teerijoki-Oksa, T., Kotiranta, U., Kantola, R., Back, M., Vuorjoki-Ranta, T. R., . . . Estlander, A. M. (2012). Pain and pain behavior in burning mouth syndrome: a pain diary study. *J Orofac Pain, 26*(2), 117-125.

Gall-Troselj, K., Mravak-Stipetic, M., Jurak, I., Ragland, W. L., & Pavelic, J. (2001). Helicobacter pylori colonization of tongue mucosa--increased incidence in atrophic glossitis and burning mouth syndrome (BMS). *J Oral Pathol Med, 30*(9), 560-563.

Gao, J., Chen, L., Zhou, J., & Peng, J. (2009). A case-control study on etiological factors involved in patients with burning mouth syndrome. *J Oral Pathol Med, 38*(1), 24-28.

Grushka, M. (1987). Clinical features of burning mouth syndrome. *Oral Surg Oral Med Oral Pathol, 63*(1), 30-36.

Hattrup, C. L., & Gendler, S. J. (2008). Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol, 70*, 431-457.

Headache Classification Committee of the International Headache, S. (2013). The International Classification of Headache Disorders, 3rd edition (beta version). *Cephalalgia, 33*(9), 629-808.

Henry, S., Oriol, R., & Samuelsson, B. (1995). Lewis histo-blood group system and associated secretory phenotypes. *Vox Sang, 69*(3), 166-182.

Hershkovich, O., & Nagler, R. M. (2004). Biochemical analysis of saliva and taste acuity evaluation in patients with burning mouth syndrome, xerostomia and/or gustatory disturbances. *Arch Oral Biol, 49*(7), 515-522.

Hummelen, R., Macklaim, J. M., Bisanz, J. E., Hammond, J. A., McMillan, A., Vongsa, R., . . . Reid, G. (2011). Vaginal microbiome and epithelial gene array in post-menopausal women with moderate to severe dryness. *PLoS One, 6*(11), e26602.

Imamura, Y., Shinozaki, T., Okada-Ogawa, A., Noma, N., Shinoda, M., Iwata, K., . . . Svensson, P. (2019). An updated review on pathophysiology and management of burning mouth syndrome with endocrinological, psychological and neuropathic perspectives. *J Oral Rehabil, 46*(6), 574-587.

Jaaskelainen, S. K. (2012). Pathophysiology of primary burning mouth syndrome. *Clin Neurophysiol, 123*(1), 71-77.

Kang, J. H., Kim, Y. Y., Chang, J. Y., & Kho, H. S. (2017). Relationships between oral MUC1 expression and salivary hormones in burning mouth syndrome. *Arch Oral Biol, 78*, 58-64.

Kardon, R., Price, R. E., Julian, J., Lagow, E., Tseng, S. C., Gendler, S. J., & Carson, D. D. (1999). Bacterial conjunctivitis in Muc1 null mice. *Invest Ophthalmol Vis Sci, 40*(7), 1328-1335.

Kho, H. S., Chang, J. Y., Kim, Y. Y., & Kim, Y. (2013). MUC1 and Toll-like receptor-2 expression in burning mouth syndrome and oral lichen planus. *Arch Oral Biol, 58*(7), 837-842.

Kho, H. S., Lee, J. S., Lee, E. J., & Lee, J. Y. (2010). The effects of parafunctional habit control and topical lubricant on discomforts associated with burning mouth syndrome (BMS). *Arch Gerontol Geriatr, 51*(1), 95-99.

Kim, M. J., Kim, J., Chang, J. Y., Kim, Y. Y., & Kho, H. S. (2017). Polymorphisms of interleukin-1beta and MUC7 genes in burning mouth syndrome. *Clin Oral Investig, 21*(3), 949-955.

Kisely, S., Forbes, M., Sawyer, E., Black, E., & Lalloo, R. (2016). A systematic review of randomized trials for the treatment of burning mouth syndrome. *J Psychosom Res, 86*, 39-46.

Klasser, G. D., Grushka, M., & Su, N. (2016). Burning Mouth Syndrome. *Oral Maxillofac Surg Clin North Am, 28*(3), 381-396.

Ko, J. Y., Park, I. H., Park, H. K., & Kho, H. S. (2011). Outcome predictors of initial treatment with topical lubricant and parafunctional habit control in burning mouth syndrome (BMS). *Arch Gerontol Geriatr, 53*(3), 263-269.

Kolkka-Palomaa, M., Jaaskelainen, S. K., Laine, M. A., Teerijoki-Oksa, T., Sandell, M., & Forssell, H. (2015). Pathophysiology of primary burning mouth syndrome with special focus on taste dysfunction: a review. *Oral Dis, 21*(8), 937-948.

Kozak, R. P., Urbanowicz, P. A., Punyadeera, C., Reiding, K. R., Jansen, B. C., Royle, L., . . . Wuhrer, M. (2016). Variation of Human Salivary O-Glycome. *PLoS One, 11*(9), e0162824.

Kroenke, K., Strine, T. W., Spitzer, R. L., Williams, J. B., Berry, J. T., & Mokdad, A. H. (2009). The PHQ-8 as a measure of current depression in the general population. *J Affect Disord, 114*(1-3), 163-173.

Kullaa, A. M., Asikainen, P., Herrala, M., Ukkonen, H., & Mikkonen, J. J. (2014). Microstructure of oral epithelial cells as an underlying basis for salivary mucosal pellicle. *Ultrastruct Pathol, 38*(6), 382-386.

Lamey, P. J., & Lamb, A. B. (1988). Prospective study of aetiological factors in burning mouth syndrome. *Br Med J (Clin Res Ed), 296*(6631), 1243-1246.

Lauria, G., Majorana, A., Borgna, M., Lombardi, R., Penza, P., Padovani, A., & Sapelli, P. (2005). Trigeminal small-fiber sensory neuropathy causes burning mouth syndrome. *Pain, 115*(3), 332-337.

Lee, J. Y., Chung, J. W., Kim, Y. K., Chung, S. C., & Kho, H. S. (2007). Comparison of the composition of oral mucosal residual saliva with whole saliva. *Oral Dis, 13*(6), 550-554.

Lee, S. K., Lee, S. W., Chung, S. C., Kim, Y. K., & Kho, H. S. (2002). Analysis of residual saliva and minor salivary gland secretions in patients with dry mouth. *Arch Oral Biol, 47*(9), 637-641.

Locker, D. (2003). Dental status, xerostomia and the oral health-related quality of life of an elderly institutionalized population. *Spec Care Dentist, 23*(3), 86-93.

Lopez-Jornet, P., Camacho-Alonso, F., & Lucero-Berdugo, M. (2008). Quality of life in patients with burning mouth syndrome. *J Oral Pathol Med, 37*(7), 389-394.

Lopez-Jornet, P., Camacho-Alonso, F., & Molino-Pagan, D. (2013). Prospective, randomized, double-blind, clinical evaluation of Aloe vera Barbadensis, applied in combination with a tongue protector to treat burning mouth syndrome. *J Oral Pathol Med, 42*(4), 295-301.

Lynge Pedersen, A. M., & Belstrom, D. (2019). The role of natural salivary defences in maintaining a healthy oral microbiota. *J Dent, 80 Suppl 1*, S3-S12.

McAuley, J. L., Linden, S. K., Png, C. W., King, R. M., Pennington, H. L., Gendler, S. J., . . . McGuckin, M. A. (2007). MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest, 117*(8), 2313-2324.

Merga, Y., Campbell, B. J., & Rhodes, J. M. (2014). Mucosal barrier, bacteria and inflammatory bowel disease: possibilities for therapy. *Dig Dis, 32*(4), 475-483.

Mignogna, M. D., Fedele, S., Lo Russo, L., Leuci, S., & Lo Muzio, L. (2005). The diagnosis of burning mouth syndrome represents a challenge for clinicians. *J Orofac Pain, 19*(2), 168-173.

Minor, J. S., & Epstein, J. B. (2011). Burning mouth syndrome and secondary oral burning. *Otolaryngol Clin North Am, 44*(1), 205-219, vii.

Moisset, X., Calbacho, V., Torres, P., Gremeau-Richard, C., & Dallel, R. (2016). Co-occurrence of Pain Symptoms and Somatosensory Sensitivity in Burning Mouth Syndrome: A Systematic Review. *PLoS One, 11*(9), e0163449.

Nagler, R. M., & Hershkovich, O. (2004). Sialochemical and gustatory analysis in patients with oral sensory complaints. *J Pain, 5*(1), 56-63.

Netto, F. O., Diniz, I. M., Grossmann, S. M., de Abreu, M. H., do Carmo, M. A., & Aguiar, M. C. (2011). Risk factors in burning mouth syndrome: a case-control study based on patient records. *Clin Oral Investig, 15*(4), 571-575.

Ni Riordain, R., O'Dwyer, S., & McCreary, C. (2019). Burning mouth syndrome-a diagnostic dilemma. *Ir J Med Sci*.

Oliveira-Ferrer, L., Legler, K., & Milde-Langosch, K. (2017). Role of protein glycosylation in cancer metastasis. *Semin Cancer Biol, 44*, 141-152.

Perez-Vilar, J., & Hill, R. L. (1999). The structure and assembly of secreted mucins. *J Biol Chem, 274*(45), 31751-31754.

Poon, R., Su, N., Ching, V., Darling, M., & Grushka, M. (2014). Reduction in unstimulated salivary flow rate in burning mouth syndrome. *Br Dent J, 217*(7), E14.

Ramfjord, S. P. (1967). The Periodontal Disease Index (PDI). *J Periodontol, 38*(6), Suppl:602-610.

Ramsey, K. A., Rushton, Z. L., & Ehre, C. (2016). Mucin Agarose Gel Electrophoresis: Western Blotting for High-molecular-weight Glycoproteins. *J Vis Exp*(112).

Randall, K., Stevens, J., Yepes, J. F., Randall, M. E., Kudrimoti, M., Feddock, J., . . . Miller, C. S. (2013). Analysis of factors influencing the development of xerostomia during intensity-modulated radiotherapy. *Oral Surg Oral Med Oral Pathol Oral Radiol, 115*(6), 772-779.

Rose, M. C., & Voynow, J. A. (2006). Respiratory tract mucin genes and mucin glycoproteins in health and disease. *Physiol Rev, 86*(1), 245-278.

Scala, A., Checchi, L., Montevecchi, M., Marini, I., & Giamberardino, M. A. (2003). Update on burning mouth syndrome: overview and patient management. *Crit Rev Oral Biol Med, 14*(4), 275-291.

Scully, C. (2003). Drug effects on salivary glands: dry mouth. *Oral Dis, 9*(4), 165-176.

Shetty, S. R., Bhowmick, S., Castelino, R., & Babu, S. (2012). Drug induced xerostomia in elderly individuals: An institutional study. *Contemp Clin Dent, 3*(2), 173-175.

Shinozaki, T., Imamura, Y., Kohashi, R., Dezawa, K., Nakaya, Y., Sato, Y., . . . Taira, M. (2016). Spatial and Temporal Brain Responses to Noxious Heat Thermal Stimuli in Burning Mouth Syndrome. *J Dent Res, 95*(10), 1138-1146.

Sicard, J. F., Vogeleer, P., Le Bihan, G., Rodriguez Olivera, Y., Beaudry, F., Jacques, M., & Harel, J. (2018). N-Acetyl-glucosamine influences the biofilm formation of Escherichia coli. *Gut Pathog, 10*, 26.

Slade, G. D., & Spencer, A. J. (1994). Development and evaluation of the Oral Health Impact Profile. *Community Dent Health, 11*(1), 3-11.

Sun, A., Wu, K. M., Wang, Y. P., Lin, H. P., Chen, H. M., & Chiang, C. P. (2013). Burning mouth syndrome: a review and update. *J Oral Pathol Med, 42*(9), 649-655.

Suri, V., & Suri, V. (2014). Menopause and oral health. *J Midlife Health, 5*(3), 115-120.

Tabak, L. A. (1995). In defense of the oral cavity: structure, biosynthesis, and function of salivary mucins. *Annu Rev Physiol, 57*, 547-564.

Takamatsu, D., Bensing, B. A., Prakobphol, A., Fisher, S. J., & Sullam, P. M. (2006). Binding of the streptococcal surface glycoproteins GspB and Hsa to human salivary proteins. *Infect Immun, 74*(3), 1933-1940.

Takehara, S., Yanagishita, M., Podyma-Inoue, K. A., & Kawaguchi, Y. (2013). Degradation of MUC7 and MUC5B in human saliva. *PLoS One, 8*(7), e69059.

Thomson, W. M. (2015). Dry mouth and older people. *Aust Dent J, 60 Suppl 1*, 54-63.

Thomsson, K. A., Schulz, B. L., Packer, N. H., & Karlsson, N. G. (2005). MUC5B glycosylation in human saliva reflects blood group and secretor status. *Glycobiology, 15*(8), 791-804.

Thoppay, J. R., De Rossi, S. S., & Ciarrocca, K. N. (2013). Burning mouth syndrome. *Dent Clin North Am, 57*(3), 497-512.

Turner, B. S., Bhaskar, K. R., Hadzopoulou-Cladaras, M., & LaMont, J. T. (1999). Cysteine-rich regions of pig gastric mucin contain von willebrand factor and cystine knot domains at the carboxyl terminal(1). *Biochim Biophys Acta, 1447*(1), 77-92.

Visvanathan, V., & Nix, P. (2010). Managing the patient presenting with xerostomia: a review. *Int J Clin Pract, 64*(3), 404-407.

Wang, S. L., Zhao, Z. T., Li, J., Zhu, X. Z., Dong, H., & Zhang, Y. G. (1998). Investigation of the clinical value of total saliva flow rates. *Arch Oral Biol, 43*(1), 39-43.

Woda, A., Dao, T., & Gremeau-Richard, C. (2009). Steroid dysregulation and stomatodynia (burning mouth syndrome). *J Orofac Pain, 23*(3), 202-210.

Zad, M., Flowers, S. A., Bankvall, M., Jontell, M., & Karlsson, N. G. (2015). Salivary mucin MUC7 oligosaccharides in patients with recurrent aphthous stomatitis. *Clin Oral Investig, 19*(8), 2147-2152.

Zhao, M., Chen, Q., & Lin, M. (2001). [Relationship between the symptom of xerostomia and non-stimulated salivary flow rates in patients with burning mouth syndrome]. *Hua Xi Kou Qiang Yi Xue Za Zhi, 19*(3), 169-170, 177.