AQUAPORINS AS SALIVARY PROTEIN BIOMARKERS FOR XEROSTOMIA AND THE EFFECTS OF XEROSTOMIA ON ORAL HEALTH-RELATED QUALITY OF LIFE IN PATIENTS WITH PERIODONTITIS ATTENDING COMBINED MILITARY HOSPITAL, LAHORE

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF APPENDICES

LIST OF SYMBOLS

χ	Chi
r	Correlation
°	Degree
μ	Mean
σ	Standard deviation

LIST OF ABBREVIATIONS

ALM	Apicolateral membrane
AM	Apical membrane
AQP	Aquaporin
BAFF	B-cell activating factor
BLM	Basolateral membrane
BM	Basal membrane
BMI	Body mass index
C	Celsius
CI	Confidence interval
CMH	Combined Military Hospital
CP	Cytoplasm
CPI	Community Periodontal Index
CPITN	Community Periodontal Index of Treatment Needs
CV	Coefficient of variance
df	Degree of freedom
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPV	Events per variable
ERC	Ethical Review Committee
ESSPRI	European League Against Rheumatism Sjögren's Syndrome Patient Reported Index
FDI	Federation Dentaire Internationale
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOHAI	General Oral Health Assessment Index
HRP	Horseradish peroxidase

ICC	Intraclass correlation coefficient
ID	Identification
IL	Interleukin
IOD	Institute of Dentistry
IQR	Interquartile range
JEPeM-USM	Jawatankuasa Etika Penyelidikan Manusia Universiti Sains Malaysia
mRNA	Messenger ribonucleic acid
MUC5B	Mucin protein 5B
OD	Optical density
OHIP	Oral Health Impact Profile
OHRQOL	Oral health-related quality of life
OPD	Outpatient department
pH	Power of hydrogen
PTFE	Polytetrafluoroethylene
RT-PCR	Reverse transcription-polymerase chain reaction
SES	Socioeconomic status
SF-36	Short Form Health Survey
SKU	Stock keeping unit
S-OHIP	Shortened Oral Health Impact Profile
SPSS	Statistical Package for Social Sciences
Sr No.	Serial number
SXI	Shortened Xerostomia Inventory
Th	Helper T
TMJ	Temporomandibular joint
USA	United States of America
VIP	Vasoactive intestinal peptide

WHO	World Health Organization
XI	Xerostomia Inventory
XQ	Xerostomia Questionnaire
2D	2-dimensional

AKUAPORIN SEBAGAI BIOPENANDA PROTIN AIR LIUR BAGI XEROSTOMIA DAN KESAN XEROSTOMIA TERHADAP KUALITI HIDUP YANG BERKAITAN DENGAN KESIHATAN MULUT DALAM PESAKIT PERIODONTITIS YANG HADIR KE COMBINED MILITARY HOSPITAL, LAHORE

ABSTRAK

Xerostomia adalah keadaan mulut kering yang boleh menyebabkan banyak masalah kesihatan mulut yang mempengaruhi kualiti hidup berkaitan dengan kesihatan mulut (OHRQOL). Objektif umum kajian ini adalah untuk mengenal pasti potensi penggunaan protin air liur akuaporin (AQP)-3, AQP-4 dan AQP-5 sebagai biopenanda bagi xerostomia dalam pesakit dengan periodontitis dan untuk mengkaji kesan xerostomia terhadap OHRQOL. Dalam kajian deskriptif ini, 140 peserta yang sihat, berumur 20 – 55 tahun, dengan skor Community Periodontal Index (CPI) ≥ 2 dilibatkan melalui persampelan rawak secara sistematik. Shortened Xerostomia Inventory (SXI) digunakan bagi penilaian subjektif berkenaan xerostomia, sementara rembesan air liur yang tidak dirangsang dikumpulkan bagi penilaian secara objektif. Pesakit dengan skor SXI > 5 dianggap mengalami xerostomia; skor 6 – 8 sebagai gred 1 dan skor 9 – 15 sebagai xerostomia gred 2. Soal selidik Shortened Oral Health Impact Profile (S-OHIP) digunakan bagi menilai OHRQOL. Pengukuran kuantiti AQP-3, AQP-4 dan AQP-5 dilakukan dengan ujian imunosorben berkait enzim (ELISA). Setelah membuang data ekstrem, data daripada 132 peserta dianalisis. Nilai kebarangkalian ditetapkan pada 5%. Ujian bukan parametrik digunakan dalam analisis data. Xerostomia dilaporkan dalam 78% pesakit dengan periodontitis. Xerostomia gred 1 dilaporkan pada 74.8% dan gred 2 pada 25.2% dalam kalangan pesakit

periodontitis dengan xerostomia. OHRQOL adalah lebih teruk secara signifikan dalam pesakit dengan xerostomia berbanding dengan pesakit tanpa xerostomia, $p = .001$, dan dalam gred 2 berbanding dengan gred 1 xerostomia, $p = .001$. Kadar aliran air liur yang tidak dirangsang adalah jauh lebih rendah dalam pesakit periodontitis dengan xerostomia gred 1 berbanding gred 2, $p = .013$. Kepekatan AQP-3 jauh lebih tinggi dalam pesakit dengan xerostomia berbanding dengan pesakit tanpa xerostomia, $p < .001$. Tambahan lagi, kepekatan AQP-5 berkurangan dengan ketara dalam gred 2 berbanding dengan gred 1 xerostomia, $p = .002$. Dalam pesakit dengan periodontitis, terdapat hubungan yang signifikan di antara SXI dan S-OHIP ($p <.001$), SXI dan AQP-3 ($p = .004$), AQP-3 dan AQP-4 ($p = .001$), kadar aliran air liur dan AQP-4 ($p = .035$), dan kadar aliran air liur dan AQP-5 ($p = .007$). SXI adalah peramal yang sesuai bagi kemerosotan OHRQOL. Lebih-lebih lagi, skor S-OHIP dan AQP-3; dan skor S-OHIP dan AQP-5 masing-masing merupakan peramal xerostomia dan xerostomia gred 2. Kesimpulannya, AQP air liur merupakan biopenanda yang sesuai bagi xerostomia dalam pesakit dengan periodontitis. Xerostomia perlu dirawat segera sebelum kesihatan mulut terkesan dengan pengenalpastian masalah ini melalui biopenanda tersebut, kerana xerostomia agak sering berlaku dan menyebabkan kemerosotan OHRQOL.

AQUAPORINS AS SALIVARY PROTEIN BIOMARKERS FOR XEROSTOMIA AND THE EFFECTS OF XEROSTOMIA ON ORAL HEALTH-RELATED QUALITY OF LIFE IN PATIENTS WITH PERIODONTITIS ATTENDING COMBINED MILITARY HOSPITAL, LAHORE

ABSTRACT

Xerostomia is a debilitating condition of oral dryness which may lead to numerous oral health conditions affecting oral health-related quality of life (OHRQOL). The general objectives of this study were to identify the potential of using salivary aquaporin (AQP)-3, AQP-4, and AQP-5 proteins as biomarkers for xerostomia in patients with periodontitis and to investigate the effects of xerostomia on OHRQOL. In this descriptive study, 140 healthy participants, 20 – 55 years old, with Community Periodontal Index (CPI) score ≥ 2 were included through systematic random sampling. Shortened Xerostomia Inventory (SXI) was used for subjective assessment of xerostomia, while unstimulated saliva was collected for its objective measurement. Patient with SXI score > 5 was considered xerostomic; score 6 – 8 as grade 1, and score 9 – 15 as grade 2 xerostomia. Shortened Oral Health Impact Profile (S-OHIP) questionnaire was utilised to assess OHRQOL. Quantification of AQP-3, AQP-4, and AQP-5 was done by enzyme-linked immunosorbent assay (ELISA). After removing extreme outliers, data of 132 participants were analysed. Probability value was set at 5%. Non-parametric tests were used for data analyses. Xerostomia was reported in 78% of the patients with periodontitis. Grade 1 xerostomia was reported in 74.8% whereas, grade 2 xerostomia in 25.2% of the periodontitis patients with xerostomia. OHRQOL was significantly poor in xerostomics compared to non-

xerostomics, p = .001, and in grade 2 compared to grade 1 xerostomia, p = .001. Unstimulated salivary flow rate was significantly lower in periodontitis patients with grade 1 compared to grade 2 xerostomia, p = .013. Concentration of AQP-3 was significantly higher in xerostomics compared to non-xerostomics, $p < .001$. Moreover, concentration of AQP-5 was significantly reduced in grade 2 compared to grade 1 xerostomia, p = .002. In the patients with periodontitis, there were significant correlations between SXI and S-OHIP ($p < .001$), SXI and AQP-3 (p = .004), AQP-3 and AQP-4 (p = .001), unstimulated salivary flow rate and AQP-4 (p = .035), and unstimulated salivary flow rate and AQP-5 (p = .007). SXI was a suitable predictor for poor OHRQOL. Moreover, S-OHIP score and AQP-3; and S-OHIP score and AQP-5 were suitable predictors of xerostomia and grade 2 xerostomia, respectively. In conclusion, salivary AQPs are suitable protein biomarkers for xerostomia in patients with periodontitis. It is important to manage xerostomia before it affects oral health by early detection using these biomarkers, as xerostomia is considerably prevalent and causes a deterioration of OHRQOL.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Xerostomia is defined as a subjective sensation of oral dryness (Thomson *et al.*, 2006b). It is a complex phenomenon; a person may experience xerostomia with or without reduced salivary secretions, or vice versa (Navazesh, 2002; Navazesh and Kumar, 2008; Thomson *et al.*, 2019). It can occur as a result of aging, side effects of medications (such as anticoagulants, antidepressants, antihypertensives, antiretrovirals, hypoglycaemics, levothyroxine, non-steroidal anti-inflammatory drugs, steroid inhalers, multivitamins, and iron supplements), diseases (such as diabetes mellitus and Sjögren's syndrome), anxiety, stress, depression, and radiation exposure to the oro-facial region (Guggenheimer and Moore, 2003; Villa *et al.*, 2015). The overall global prevalence of xerostomia has been projected at 23% with high heterogeneity among different studies (Agostini *et al.*, 2018). In Pakistan, the prevalence is 55% in 21 – 65 years old (Shah *et al.*, 2017). Prolonged xerostomia secondary to hyposalivation may lead to increased incidence of periodontal disease, dental caries, halitosis, candida infections, mucositis, tongue fissuring, tongue depapillation, burning mouth syndrome, and dental prostheses instability (Bossola, 2019), which negatively impact oral health-related quality of life (OHRQOL) (Chamani *et al.*, 2017; Kakoei *et al.*, 2012). These oral problems may cause difficulty in chewing and swallowing, altered gustatory and olfactory functions, and restriction of type and amount of food taken, which may lead to malnutrition and subsequent weight loss (Nihtilä *et al.*, 2019; Rusthen *et al.*, 2017).

Xerostomia due to hyposalivation may lead to reduced clearance of microbial and food debris from the oral cavity, which may cause periodontal disease. Based on pathophysiology, there are three distinct forms of periodontitis: (a) Necrotising periodontitis, (b) periodontitis as a manifestation of systemic diseases, and (c) periodontitis (Caton *et al.*, 2018; Papapanou *et al.*, 2018). Periodontitis is an inflammatory disease of multifactorial aetiology associated with plaque biofilms causing progressive destruction of the periodontium (Nesse *et al.*, 2008). It is characterised by activation of the host-immune inflammatory cascade as a result of microbial deposits and their associated endotoxins. This results in infiltration of periodontal tissues by inflammatory cells such as polymorphonuclear leukocytes, macrophages, and monocytes. The cytokines produced by these cells can exert local and systemic effects (Page and Kornman, 1997). The assessment of periodontitis is based on clinical attachment loss, periodontal pocket depth, and gingival inflammation (Genco and Williams, 2010). The disease usually advances slowly, over a period of many years, and varies significantly among different individuals owing to their inherent susceptibility to the disease, host-defence mechanisms, and interaction of risk factors with the host defences and the bacterial plaque (Tatakis and Kumar, 2005).

Periodontitis affects an estimated 47% of the USA population, with 38% having moderate to severe periodontitis (Eke *et al.*, 2012). In developing and under-developed countries, the number of the affected population is higher (Albandar and Tinoco, 2002; Kassebaum *et al.*, 2017). According to a cross-sectional study conducted on Pakistani army soldiers, the prevalence of periodontitis was reported as 32% (Chaudhry *et al.*, 2008), while in the Pakistani general population, it was 83% (Anwar *et al.*, 2015). It is the second most common cause of tooth extraction in Pakistan (Haseeb *et al.*, 2012) and, therefore, a major health concern in this country.

An autoimmune disease, Sjögren's syndrome, affects salivary and lacrimal glands and results in dry mouth and dry eyes. Variation in periodontal microcirculation and vascular reactivity has been linked to the pathogenesis of periodontitis in patients with Sjögren's syndrome (Scardina *et al.*, 2010). Salivary B-cell activating factor (BAFF) protein was associated with probing pocket depth in patients with periodontal disease and in patients with symptoms of xerostomia, with or without autoimmune disorders (Pers *et al.*, 2005), which shows that certain biomarkers in saliva may be associated with xerostomia in patients with periodontitis. Xerostomia had been indirectly linked to gingival disease and plaque accumulation in healthy young adults (Mizutani *et al.*, 2014). Moreover, chronic mouth-breathers may suffer from xerostomia, eventually predispose these patients to periodontal disease (Dawes, 2008; Wagaiyu and Ashley, 1991).

Aquaporins (AQPs) are water-permeable transmembrane proteins responsible for transcellular transport of water and some other solutes in most of the internal organs including the exocrine glands such as salivary, lacrimal, and sweat glands (Day *et al.*, 2014; Delporte, 2014). Conditions resulting in salivary gland hypofunction and xerostomia such as Sjögren's syndrome, diabetes, aging, salivary gland tumours, and radiation therapy have been associated with altered expression of AQPs (D'Agostino *et al.*, 2020). Currently, seven out of 13 AQPs have been identified in human salivary glands (Delporte, 2014; Wang *et al.*, 2003). The first AQP to be quantified in whole human saliva was AQP-5 in 2009 (Pan *et al.*, 2009).

In patients who are immunocompromised and have underlying medical conditions, xerostomia can be a debilitating factor. For this reason, identifying protein biomarkers such as AQPs for xerostomia may have a positive impact on these patients

for its early and timely management. Furthermore, complications of xerostomia, such as caries, periodontitis, mucosal infections, recurrent ulcers, tongue fissures, and difficulty in speech, mastication, and swallowing, can be prevented. Hence, patients will have a better quality of life when their xerostomia-related complications are prevented or treated early. Identifying AQPs as protein biomarkers in saliva, which are linked to a specific condition, may help us to achieve this goal.

1.2 Justification of the study

Periodontitis is one of the most prevalent conditions of the oral cavity in Southeast Asian countries (Petersen and Yamamoto, 2005). There is a growing need to identify the molecular biomarker of xerostomia in patients with periodontitis and to investigate how it may affect the quality of life of these patients. This study aimed to explore the potential of salivary AQP proteins as biomarkers of xerostomia in patients with periodontitis. It may help us to identify the possibility of using these proteins as predictive or diagnostic biomarkers for xerostomia. Early detection and diagnosis of xerostomia in patients with periodontitis are critical to avoid complications which may have a negative impact on the quality of life of the patients. Identification of potential biomarkers will be helpful for screening and risk assessment and can be used for disease monitoring and for selecting appropriate therapy. Identification of molecular salivary biomarkers such as AQPs may prove to be an important step towards the development of a point-of-care type of diagnostic kit for xerostomia. To date, robust molecular salivary biomarkers for xerostomia in humans have not been identified.

1.3 Problem statement and hypotheses

The current methods to diagnose xerostomia are semi-quantitative by using a questionnaire and by collecting saliva samples. The subjectivity of questionnaires cannot be ruled out, and their reliability is questionable, especially with individuals of limited cognitive ability. Moreover, saliva sampling is affected by many factors even within the same person such as time of the day, state of hydration, level of stress, and habits such as smoking, caffeine intake, and mouth breathing. Therefore, other objective methods need to be identified for early screening of the condition to prevent associated complications. As the current method of assessment of xerostomia is subjective, identification of molecular salivary biomarkers such as AQPs may prove to be helpful for early screening of this condition. AQPs as potential salivary biomarkers for xerostomia in patients with periodontitis have not been established. We hypothesised the following:

1. Patients with xerostomia will have decreased unstimulated salivary flow rate.
2. With increase in severity of xerostomia as assessed by Shortened Xerostomia Inventory (SXI), unstimulated salivary flow rate will decrease.
3. Patients with xerostomia will have lower OHRQOL as measured by Shortened Oral Health Impact Profile (S-OHIP) questionnaire compared to patients without xerostomia.
4. Patients with xerostomia will have higher Community Periodontal Index (CPI) score.
5. Salivary AQP-3, AQP-4, and AQP-5 proteins will be potential biomarkers of xerostomia in patients with periodontitis.

6. SXI, OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins will be correlated with each other in patients with periodontitis.
7. In xerostomic patients, SXI, OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins will be correlated with each other.
8. SXI, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins will be suitable predictors of poor OHRQOL.
9. OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins will be suitable predictors of presence of xerostomia.
10. OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins will be suitable predictors of grade 2 xerostomia.

1.4 Objectives of the study

1.4.1 General objectives

The general objectives of this study were to identify the potential of using salivary AQP-3, AQP-4, and AQP-5 proteins as biomarkers for xerostomia in patients with periodontitis and to investigate the effects of xerostomia on OHRQOL.

1.4.2 Specific objectives

The specific objectives for this study were:

1. To determine prevalence of xerostomia (xerostomia status/presence or absence of xerostomia) in patients with periodontitis using SXI
2. To determine severity of xerostomia (grade 1 and grade 2) in periodontitis patients with xerostomia using SXI
3. To determine OHRQOL in patients with periodontitis using S-OHIP questionnaire
4. To determine, compare, and associate OHRQOL in periodontitis patients with xerostomia and periodontitis patients without xerostomia using S-OHIP questionnaire
5. To determine, compare, and associate OHRQOL in periodontitis patients with grade 1 and grade 2 xerostomia using S-OHIP questionnaire
6. To determine unstimulated salivary flow rate in patients with periodontitis
7. To determine, compare, and associate unstimulated salivary flow rate in periodontitis patients with xerostomia and periodontitis patients without xerostomia
8. To determine, compare, and associate unstimulated salivary flow rate in periodontitis patients with grade 1 and grade 2 xerostomia
9. To determine periodontal status by utilising CPI in patients with periodontitis
10. To determine, compare, and associate CPI score in periodontitis patients with xerostomia and periodontitis patients without xerostomia
11. To determine, compare, and associate CPI score in periodontitis patients with grade 1 and grade 2 xerostomia

12. To measure concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins in patients with periodontitis using enzyme-linked immunosorbent assay (ELISA)
13. To measure, compare, and associate concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins in periodontitis patients with xerostomia and periodontitis patients without xerostomia using ELISA
14. To measure, compare, and associate concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins in periodontitis patients with grade 1 and grade 2 xerostomia using ELISA
15. To investigate the relationships between SXI, OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins in patients with periodontitis
16. To investigate the relationships between SXI, OHRQOL, unstimulated salivary flow rate, CPI score, and concentration of salivary AQP-3, AQP-4, and AQP-5 proteins in periodontitis patients with xerostomia
17. To investigate the relationships between SXI, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins with OHRQOL in patients with periodontitis
18. To investigate the relationships between OHRQOL, unstimulated salivary flow rate, CPI score, and concentration of salivary AQP-3, AQP-4, and AQP-5 proteins with xerostomia status in patients with periodontitis
19. To investigate the relationships between OHRQOL, unstimulated salivary flow rate, CPI score, and concentration of salivary AQP-3, AQP-4, and AQP-5 proteins with severity of xerostomia in xerostomic patients with periodontitis

1.5 Research questions

The research questions for this study were:

1. Is xerostomia prevalent in patients with periodontitis based on SXI score?
2. Is there any association between xerostomia and OHRQOL in patients with periodontitis?
3. Is there any association between severity of xerostomia and OHRQOL in patients with periodontitis?
4. Is there any association between unstimulated salivary flow rate and xerostomia in patients with periodontitis?
5. Is there any association between unstimulated salivary flow rate and severity of xerostomia in patients with periodontitis?
6. Is there any association between CPI score and xerostomia?
7. Is there any association between concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins and xerostomia in patients with periodontitis?
8. Is there any association between concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins and severity of xerostomia in patients with periodontitis?
9. Is there any correlation between SXI, OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins in patients with periodontitis?
10. Can SXI, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins predict poor OHRQOL in patients with periodontitis?
11. Can OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins predict xerostomia in patients with periodontitis?

12. Can OHRQOL, unstimulated salivary flow rate, CPI score, and concentrations of salivary AQP-3, AQP-4, and AQP-5 proteins predict grade 2 xerostomia in xerostomic patients with periodontitis?

CHAPTER 2

LITERATURE REVIEW

2.1 Saliva

Saliva is a lubricating fluid which coats the hard and soft tissues of the oral cavity. It performs numerous functions which include food digestion, barrier against oral microbes, buffering action, protection against dental caries, wound healing, phonation, and smell and taste recognition (Zhang *et al.*, 2016). These functions contribute to the overall well-being and health of the oral cavity. Whole saliva consists of water, electrolytes, cells, microorganism, microparticles, exosomes, immunoglobulins, proteins and peptides, lipids, small signalling molecules, enzymes, and nitrogenous products (Dawes *et al.*, 2015). Saliva has important diagnostic implications as it contains several biomarkers that can be helpful in the detection and monitoring of oral and systemic diseases. Saliva is secreted by major and minor salivary glands.

2.1.1 Anatomy, histology, and physiology of salivary glands

In humans, saliva is secreted by three pairs of major salivary glands and numerous minor salivary glands. The major glands are the parotid, submandibular, and sublingual. Parotid is the largest salivary gland located external to the ramus of mandible. Submandibular gland consists of a superficial and a deep lobe attached around the posterior part of the mylohyoid muscle, medial to the angle of mandible. The smallest of the three major glands is the sublingual gland, which is located in the submucosa of the floor of mouth. The minor salivary glands are present in the submucosa of all parts of the oral cavity except the gingiva and the anterior part of hard

palate. Stensen's duct is the excretory duct of the parotid gland arising from its anterior part, running across the masseter, and piercing the buccinator muscle to open in the buccal mucosa of the oral cavity at the level of the second maxillary molar. Wharton's duct is the excretory duct of the submandibular gland arising from the deep lobe, running beneath the mucosa of the floor of mouth, and opening lateral to the lingual frenum (Carpenter, 2013). Saliva from sublingual gland is excreted by a series of short ducts called ducts of Rivinus, which open directly into the floor of mouth or through the Wharton's duct (Ellis, 2012). Ducts in major salivary glands are comparatively long and branched with variable lumen size compared to ducts in minor salivary glands (Pedersen *et al.*, 2018).

Histologically, salivary glands consist of two components: Parenchyma and stroma. The parenchyma is composed of acinar cells and a complex ductal system. The acinar cells are saliva-producing epithelial cells arranged in the form of grape-like acini; they are also known as secretory endpieces. Acinar cells synthesise, store, and secrete different proteins. Based on the histology and secretions, these acini can be either serous, mucous, or seromucous. The ductal system comprises intercalated, striated, and excretory ducts. The acinar cells are arranged around a central lumen forming the acini. The base of each cell is resting on the basement membrane separating the cell from the surrounding stroma, and the apex of the cell is towards the lumen or centre of the acini. The lumen of the acini is continuous with the lumen of the intercalated, striated, and excretory ducts, in that order, in all salivary glands except sublingual and minor glands which lack the striated ducts (Pedersen *et al.*, 2018). The stroma consists of connective tissue, cells, vessels, and nerves that surround the acini and the ductal system. The connective tissue capsule surrounding the major salivary glands forms septae dividing the gland into lobes and lobules. Cells that surround the acini include myoepithelial,

immune, endothelial, and stromal cells (Holmberg and Hoffman, 2014). A diagram showing the histology of the salivary gland is shown in Figure 2.1.

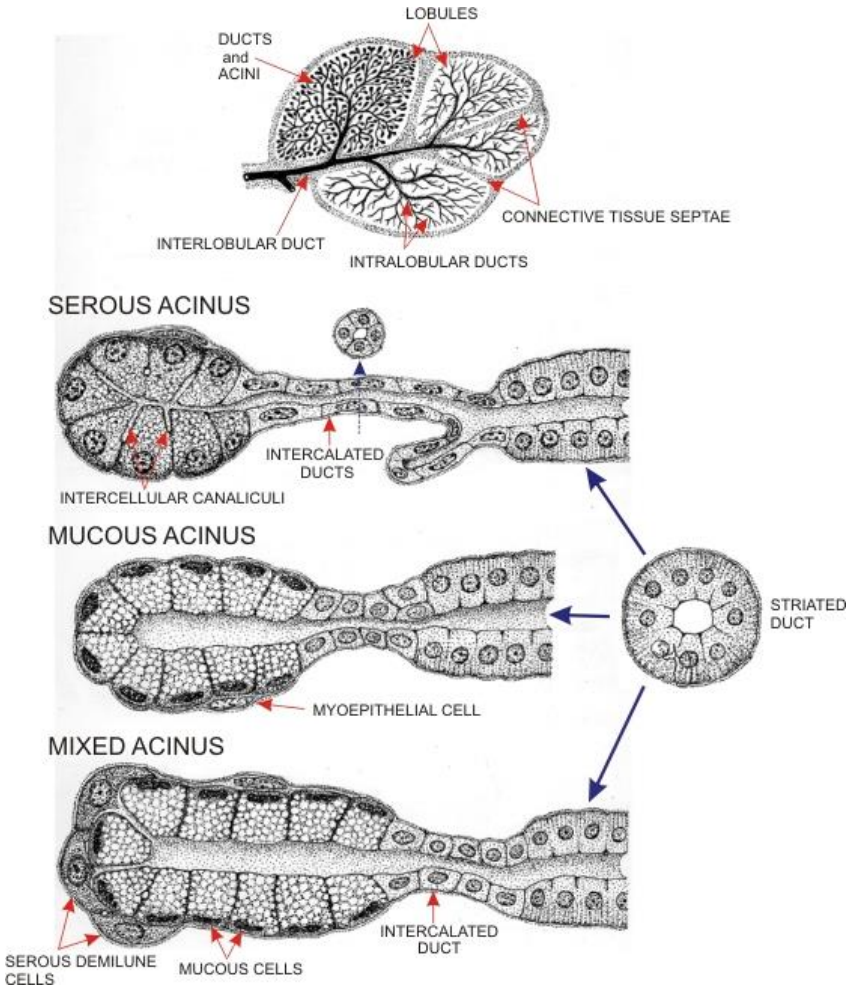

Figure 2.1: Histology of salivary glands showing the three different types of acini and ductal system (Clermont *et al.*, 2013).

Saliva is classified as serous, mucous, or mixed. Saliva produced from parotid gland is predominantly serous. The minor salivary glands produce predominantly mucous saliva. The submandibular and sublingual glands produce mixed saliva (Carpenter, 2013). Saliva is pertinent for maintenance of oral health, including the protection of oral mucosa from infections, modulation of microbial flora, facilitation of wound healing, prevention of demineralisation of teeth, maintenance of the taste

receptors, and ability to communicate through speech by means of the unique composition of saliva (Carpenter, 2013; Dawes *et al.*, 2015).

The secretion of saliva, including water transport, involves a two-stage secretory process: (i) The initial formation stage and (ii) ductal modification stage. In the first stage, upon receiving a neural signal from the brain, the acinar cells secrete primary saliva, which is similar in composition to plasma. During this stage, active secretion of sodium and chloride ions by the acinar cells into the lumen of the salivary acini occurs, involving certain ionic transporters. The luminal ion accumulation generates a transepithelial osmotic gradient. Subsequently, transepithelial water transport occurs through AQP channels and paracellular pathways as a result of which, isotonic saliva is formed in the acinar lumen. In the second stage, when the primary isotonic saliva flows through the lumen of the salivary gland ductal system, the ionic composition of saliva is modified. The duct cells reabsorb part of the sodium chloride and secrete bicarbonate and potassium ions into the saliva. These ductal cells are relatively impermeable to water. Therefore, the secondary or final saliva, secreted through the salivary gland excretory ducts into the oral cavity, is hypotonic. Transcellular water permeability in the acini is ensured by the AQP water channels (Carpenter, 2013; D'Agostino *et al.*, 2020).

2.1.2 Nervous control of salivary secretion

Salivary glands are innervated with sympathetic and parasympathetic nerves of the autonomic nervous system. Sympathetic stimulation is responsible for the activation of adrenergic receptors involving norepinephrine as a neurotransmitter and results in vasoconstriction, hence, there is a low flow of saliva, but with high protein secretion

(Edwards, 1998; Holmberg and Hoffman, 2014). Parasympathetic nerve stimulation results in the activation of muscarinic cholinergic receptors involving acetylcholine as a neurotransmitter and leads to vasodilation and increases the blood flow to salivary glands, thereby resulting in watery and high-flow saliva secretion enriched with ions but having low protein content (Edwards, 1998; Holmberg and Hoffman, 2014). In salivary glands, parasympathetic and sympathetic nervous systems work together rather than antagonistically, unlike the rest of the body (Carpenter, 2013). Parasympathetic stimulation has a dominant role in the regulation of salivary secretions. Functional parasympathetic innervation is important for the maintenance of neuronal-epithelial interaction and repair and regeneration of saliva-producing acinar cells in salivary glands (Ferreira and Hoffman, 2013). Subjective xerostomia has not been significantly associated with acinar atrophy and fibrosis in labial minor salivary glands (Sørensen *et al.*, 2014). In contrast, a negative correlation was found between the total nerve length and xerostomia, which suggested that reduced parasympathetic innervation impaired the distribution efficiency of cholinergic stimuli to mucin-producing acinar cells, whereas, reduced functional acinar cells did not have an effect on xerostomia (Sørensen *et al.*, 2014). This may also affect protein levels in saliva as denervation studies on rats revealed that autonomic nerve resection led to altered salivary protein levels (Proctor and Asking, 1989; Proctor *et al.*, 1990). In comparison to sympathectomy, parasympathectomy significantly decreased AQP-5 protein expression levels in the salivary gland without affecting its mRNA expression through a post-transcriptional mechanism, involving protein degradation in autophagosomes and/or lysosomes (Azlina *et al.*, 2010; Hosoi *et al.*, 2020; Li *et al.*, 2008).

Besides acetylcholine and norepinephrine, other neurotransmitters such as calcitonin gene-related peptide, encephalin, neuronal nitric oxide synthase, neurokinin

A, neuropeptide Y, pituitary adenylate cyclase-activating peptide, substance P, and vasoactive intestinal peptide (VIP) are also associated with salivary gland function (Ekström *et al.*, 2017). Upregulated or downregulated neuropeptides expressed in saliva can give useful information about the functioning of the salivary glands. These expressed peptides may guide us to which neural pathway is affected in conditions of hyposalivation and xerostomia. VIP and neuropeptide Y outputs and VIP concentrations were higher in stimulated saliva of Sjögren's syndrome patients compared to healthy controls, indicating their increased leakage and reduced watery salivary flow (Santavirta *et al.*, 1997). Treatment with VIP improved submandibular gland function in Sjögren's syndrome mouse model (Li *et al.*, 2017). Stress was also associated with higher salivary VIP concentrations in patients with Sjögren's syndrome and healthy controls in stimulated saliva (Santavirta *et al.*, 1997). However, the study comprised a small sample of 22 female participants. Moreover, 2 out of 10 healthy participants were smokers, which may have affected the results. Sjögren's syndrome has a female preponderance (Maciel *et al.*, 2017) and gender-based differences are reported in the proteomic profile of saliva (Xiao *et al.*, 2017). Hence, the results of this study may not apply to both genders.

2.1.3 Saliva as a diagnostic tool

Whole saliva is a remarkable oral fluid and has been studied extensively over the past few years as a potential diagnostic medium which can be non-invasively and easily obtained from the patients (Carpenter, 2013). Besides the secretions from salivary glands, whole saliva also consists of gingival crevicular fluid, desquamated epithelial cells, nasal and bronchial secretions, microorganisms and their products, inflammatory cells, serum components, and food debris. Saliva has approximately 99% water and a

variety of electrolytes, proteins, and other small organic products such as glucose, urea, uric acids, and lipids (Humphrey and Williamson, 2001; Ilea *et al.*, 2019). Salivary proteins include enzymes, immunoglobulins, mucosal glycoproteins, and polypeptides (Carpenter, 2013; Lamy *et al.*, 2018). Biomarkers are these biological molecules, which can be measured and analysed objectively as indicators of normal biological or pathological processes or to monitor pharmacological response to therapeutic modality (Hartenbach *et al.*, 2020; Zia *et al.*, 2011). These molecules play an important role in differentiating presence or absence of diseases. Saliva has an abundance of protein biomarkers which can reflect the physiological state of the individual. Various salivary biomarkers have been studied for diagnosis and prognosis of periodontal disease such as inflammatory mediators, enzymes, keratins, immunoglobulins, ions, and hormones (Hartenbach *et al.*, 2020; Kaufman and Lamster, 2000). However, no published study has reported AQP proteins as biomarkers of xerostomia in individuals who have periodontitis.

2.1.4 Saliva collection

Saliva has many purposes; it can aid in disease detection, helpful in diagnosis, useful in monitoring treatment effects, and helps in understanding disease progression. Collection of saliva is an easy, cost-effective, non-invasive, and rapid method which does not require specialised skills or armamentarium (Zhang *et al.*, 2010). Detection through oral fluids is preferred in areas where large-scale screening is required and spares the time, effort, and cost of blood collection, serum separation, and storage of samples (Abraham *et al.*, 2012). Saliva sampling is done through collection of stimulated or unstimulated saliva. Stimulated saliva sample is collected by providing a stimulant such as gum base, paraffin, or citric acid to the patient. This type of saliva has

major contribution from parotid gland followed by submandibular and sublingual glands, and even minor contribution from minor salivary glands. Unstimulated or resting saliva is collected by drooling, spitting, swab, or suction methods. Unstimulated saliva represents basal flow rate which coats and protects oral cavity for about 14 hr a day (Sreebny, 2000). Hence, it is more important for maintenance of oral health (Mese and Matsuo, 2007). Major components of this unstimulated saliva are contributed by submandibular and sublingual glands with minor contributions from parotid and minor salivary glands. The contributions from individual salivary glands alter following stimulation; parotid serous secretion increases from 7 – 8% in unstimulated saliva to more than 50% in stimulated saliva (Edgar, 1990). Unstimulated saliva is, therefore, more suitable for biomarker studies because of its higher protein levels per ml of saliva which can be quantitatively analysed, whereas stimulated saliva has more serous secretions (Lamy *et al.*, 2018; Mohamed *et al.*, 2012; Principe *et al.*, 2013). Moreover, studies have shown that xerostomia was more significantly associated with unstimulated salivary flow rate than stimulated salivary flow rate (Flink *et al.*, 2008; Iwasaki *et al.*, 2016). Decreased unstimulated salivary flow rate was also found to be associated with severe subjective xerostomia symptoms (Márton *et al.*, 2008). Moreover, besides effecting salivary flow rate, xerostomia also caused changes in the composition of saliva (Billings *et al.*, 2016; Islas-Granillo *et al.*, 2017).

Saliva can also be collected from the individual salivary gland but requires an intra-oral device to be inserted in the ductal orifice which requires special skills. The ductal orifices of the submandibular and sublingual glands are in proximity; hence, difficult to correctly isolate. Moreover, these glands may share a common duct. The rationale behind unstimulated whole saliva collection relates to application of this work to develop a point-of-care type of kit to diagnose and monitor xerostomia; this type of

saliva is, therefore, more convenient to collect and comfortable for the patient than glandular saliva (Wong, 2006).

Different types of saliva such as glandular or whole and stimulated or unstimulated saliva, offer apparent variation in the snapshot of the salivary proteome (Jasim *et al.*, 2016). Proteins present in pure glandular saliva are secreted solely by saliva-producing cells called acini of salivary glands. In contrast, proteins in whole saliva have less contribution from salivary acini (only one-tenth) and more input from gingival crevicular fluid, exfoliating epithelial cells, and oral microorganisms (Ekström *et al.*, 2017), which become particularly important in patients with compromised oral health. Certain laboratory procedures require a sufficient amount of saliva and/or proteins in saliva for successful protein identification (Jasim *et al.*, 2016). Unstimulated parotid saliva displayed a very slow and variable flow rate, yielding insufficient saliva for bottom-up protein identification through 2-dimensional (2D) gel electrophoresis. Moreover, 2D gels from participants with enough glandular saliva had fewer protein spots than gels from whole saliva (Jasim *et al.*, 2016). Therefore, whole saliva is more suitable for quantitative and qualitative assessment of proteins in saliva.

Salivary flow rate is another variable that must be taken into account during saliva collection. Patients with very low salivary flow rates may need more than one sitting to collect an adequate amount of saliva for protein analysis.

2.1.5 Salivary flow rate

Normal whole salivary flow rate in health varies from 1 to 1.5 litre per day, with maximum contribution (65%) from submandibular glands followed by 20% from parotid, less than 10% from minor salivary glands, and approximately 7 – 8% from

sublingual glands (Humphrey and Williamson, 2001). This variation in salivary flow rate may be affected, reversibly or irreversibly, by various physiological and pathological factors (Tanasiewicz *et al.*, 2016). Measurement of salivary flow rate has been the most commonly applied objective measure of evaluating salivary gland function. Reduced saliva production by the gland is a hallmark of salivary gland dysfunction or hypofunction, which is separate from xerostomia. The best way to diagnose hyposalivation is to measure salivary flow rate (Wiener *et al.*, 2010). Salivary flow rate of stimulated saliva varies between 1.6 – 2 ml/min. Unstimulated salivary flow rate is 0.29 – 0.41 ml/min, and the rate below 0.1 ml/min indicates salivary gland hypofunction (Sreebny, 2000). There is also variation in the cut-off values of salivary flow rate in studies on xerostomia; some authors have considered unstimulated salivary flow rate > 0.25 ml/min as normal and values ≤ 0.25 ml/min were considered as objective xerostomia (Nascimento *et al.*, 2019).

Reduced unstimulated salivary flow rate negatively affects oral and dental health. This condition also affects the amount of protective calcium, phosphate, bicarbonate ions, and proteins in saliva (Bardow *et al.*, 2001). Some exogenous substances such as glucose, sucrose, or acids from food and drinks consumed are dissolved in saliva, and the longer these molecules are retained in the oral cavity, the greater the damage to the tooth surfaces. The reduction in the concentration of these potentially harmful elements depends on the flow of freshly secreted saliva and the swallowing reflex—the phenomenon called salivary clearance. According to the Dawes model of oral clearance, unstimulated salivary flow rate of 0.3 ml/min generates a clearance half-time of 2.2 min, suggesting that it takes 2.2 min to reduce the concentration of any unnecessary and potentially harmful constituent of saliva to half (Dawes, 2012). Individuals with a lower unstimulated salivary flow rate will have a low

oral clearance of sugars and food debris. Hence, these individuals will have a higher caries risk (Bardow *et al.*, 2001). Studies focusing on the association between reduced salivary flow rate and periodontal disease are inconclusive. Authors have reported that reduced salivary flow rate affected periodontal health (Crow and Ship, 1995; Márton *et al.*, 2008; Samnieng *et al.*, 2012). Individuals with a stimulated salivary flow rate of ≤ 1.4 ml/min had a higher odds of having periodontal disease compared to those with flow rate > 3.5 ml/min (Shimazaki *et al.*, 2017). On the contrary, others have reported that there was no association between periodontal disease and reduced salivary flow rate, especially in elderly patients (Hirotomi *et al.*, 2006; Hirotomi *et al.*, 2008; Koss *et al.*, 2009). Syrjälä *et al.* (2011) reported that elderly individuals of 75 years and older with unstimulated salivary flow rate of < 0.1 ml/min and stimulated salivary flow rate of < 0.7 ml/min had a decreased likelihood of having teeth with deep periodontal pockets. The authors proposed that it could be due to less calculus deposits owing to a low salivary output of different ions and a change in the composition and pH of the dental biofilm. Considering the influence of circadian rhythm on salivary flow (Flink *et al.*, 2005) and possibly on the composition of saliva, it is important to collect saliva samples of all participants at the same time of the day, which could affect the outcome of the study on salivary biomarkers (Proctor and Shaalan, 2021). Syrjälä *et al.* did not collect saliva samples of all participants at the same time of the day, which may have affected the findings of their study. Salivary flow rate alone is not the defining factor for periodontal disease (Hirotomi *et al.*, 2006) and elderly individuals with viscous saliva are also susceptible to periodontal disease progression (Hirotomi *et al.*, 2008). Hence, quantity and quality of saliva are equally important for the surface-associated function of saliva such as lubrication (Christersson *et al.*, 2000), for which hydration plays a vital role (Bongaerts *et al.*, 2007). Mucin proteins are abundant in saliva and impart the

properties of lubrication and adhesion by binding with water and forming a protective coating over the oral mucosa. Saliva stringiness (spinnbarkeit) and mucosal hydration (wetness) were significantly lower in xerostomic patients compared to healthy subjects because the structure of mucin was altered due to glycosylation despite its normal concentration in saliva (Chaudhury *et al.*, 2015). Spinnbarkeit is a property of viscoelastic fluids such as saliva that measures the capacity of a fluid to be drawn into an unbroken strand and indirectly measures its adhesive property (Lai *et al.*, 2009). Hirotomi *et al.* (2006) reported that 76 years old elderly participants with a combination of reduced stimulated salivary flow rate of < 0.7 ml/min and salivary spinnbarkeit > 2 mm had significantly higher mean periodontal pocket depth than those participants who only had reduced salivary flow rate or high spinnbarkeit. They divided the study participants into four groups based on combination of increased or decreased salivary flow rate and high or low spinnbarkeit. The study findings suggested that a combination of reduced salivary flow rate and high salivary spinnbarkeit could be potential risk factors for periodontal disease, whereas, reduced salivary flow rate alone is not related to periodontal conditions. The authors did not disclose the time of the day for saliva collection. In older individuals, xerostomia is caused by alterations in salivary glands' function, dehydration, and changes in saliva composition (Islas-Granillo *et al.*, 2017). Moreover, level of hydration also influences the composition of saliva (Proctor and Shaalan, 2021).

In summary, unstimulated whole saliva is an ideal fluid for biomarker research. It may give useful information about the diseases affecting the oral cavity such as xerostomia in patients with periodontitis. Special skills are not required for saliva collection. The collection method is easy to understand and to follow by the patient at home for a point-of-care type of kit. It has the benefits of being rich in proteins and

peptides, which can be identified by bottom-up or top-down proteomic techniques. These proteins may be unique to xerostomia and may help the patients and caregivers to monitor and manage the condition effectively. There is considerable variation in salivary flow rate and most likely in the proteomic profiles within the same individual at different times of the day; therefore, following a standard protocol for all research participants is important.

2.2 Xerostomia

Xerostomia is a subjective feeling of oral dryness which may or may not be associated with an actual reduction in salivary flow. Oral dryness is a complex phenomenon. A person may experience xerostomia with or without hyposalivation, or vice versa (Thomson *et al.*, 1999). Xerostomia, due to salivary gland hypofunction, is dryness of the oral cavity resulting from insufficient secretion of saliva or a complete absence of saliva (Wiener *et al.*, 2010). When xerostomia correlates with clinical signs of salivary gland hypofunction, the salivary flow rate has decreased by 40 – 50% (Dawes, 1987; Ship *et al.*, 1991). Xerostomia is therefore classified as true xerostomia or symptomatic/pseudo xerostomia. True xerostomia, also known as "xerostomia vera" or "xerostomia primaria" results from malfunction of the salivary glands. It is also referred to as objective xerostomia. In symptomatic/pseudo xerostomia, also known as "xerostomia spuria" or "xerostomia symptomatica", patient has a subjective perception of oral dryness despite normal function of the salivary glands (Tanasiewicz *et al.*, 2016). It is generally referred to as subjective xerostomia. Psychological factors, such as depression, anxiety, and stress, are associated with xerostomia (Bergdahl and Bergdahl, 2000). Increased plaque and gingival inflammation were reported in patients with subjective xerostomia (Mizutani *et al.*, 2014). In mouth-breathers, the amount of water

lost in exhaled air is 42% higher than in nose-breathers (Svensson *et al.*, 2006) and may result in oral dryness (Närhi, 1994). Adjunctive use of salivary substitutes in mouth-breathers resulted in significant improvement in periodontal inflammation after scaling and root planing (Kaur *et al.*, 2018). If the salivary flow rate exceeds the rate of oral fluid loss by evaporation and mucosal absorption of saliva from the oral cavity, xerostomia may be avoided (Dawes, 2004).

2.2.1 Prevalence of xerostomia

The prevalence of xerostomia varies from 0.9 to 64.8% (Orellana *et al.*, 2006). The overall prevalence is estimated at 23%, according to a systematic review and meta-analysis (Agostini *et al.*, 2018). In a prospective study on 20 – 59 years old, xerostomia prevalence was 11% (da Silva *et al.*, 2017). The prevalence has been differently expressed in different studies. It shows a wide variation, which could be due to differences in geographical location, ethnicity, inclusion and exclusion criteria, various assessment tools, different age groups, health condition of participants, medications in use, and psychological factors (Agostini *et al.*, 2018; Billings *et al.*, 2016).

Xerostomia is more frequently reported in females compared to males (Abdullah, 2015; Benn *et al.*, 2015; da Silva *et al.*, 2017; Mao *et al.*, 2019; Niklander *et al.*, 2017). Nevertheless, some authors have not reported gender-based differences in the prevalence of xerostomia (Barbe *et al.*, 2017; Johanson *et al.*, 2015; Matear *et al.*, 2006; Mizutani *et al.*, 2014; Thomson *et al.*, 2006b).

In a questionnaire-based cross-sectional study on 21 – 65 years old individuals accompanying patients at the dental outpatient department (OPD) in Mirpurkhas Pakistan, subjective xerostomia prevalence assessed through the Fox questionnaire was