# SELF-REPORTED QUESTIONNAIRE AND SALIVARY BIOMARKERS FOR PERIODONTITIS SCREENING IN MALAYSIAN ADULTS

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FACULTY OF DENTISTRY
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**SELF-REPORTED QUESTIONNAIRE AND SALIVARY BIOMARKERS FOR PERIODONTITIS SCREENING IN MALAYSIAN ADULTS**

## ABSTRACT

Salivary biomarkers and self-reported oral health questionnaire (SROH) could act as two convenient and non-invasive approaches for periodontitis screening. Their advantages are manifold, given that periodontal examination protocols such as full-mouth periodontal examination, Basic Periodontal Examination and Community Periodontal Index of Treatment Need require more manpower, equipment, time and cost. This study aimed to validate the SROH as a screening tool for periodontitis among the Malaysian population and to compare the salivary levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), matrix metalloproteinase-8 (MMP-8), matrix metalloproteinase-9 (MMP-9) and metallothionein (MT) between patients with periodontal health, gingivitis or periodontitis. First, content of the SROH was validated by experts followed by face validation by a pilot sample of subjects who were uninvolved in the main study. Next, a convenience sample of 77 systemically healthy adults was recruited consecutively and divided into groups of periodontal health, gingivitis and periodontitis. The participants were asked to answer the self-administered SROH, followed by collection of unstimulated saliva samples. Five millilitres of saliva were collected within 30 minutes. After centrifugation, the resultant supernatants were aliquoted and stored at -80°C until analysis. The SROH responses were used to determine the construct validity, concurrent validity, internal consistency and test-retest reliability of the questionnaire. Concurrently, quantification of salivary biomarker levels was determined using enzyme-linked immunosorbent assay. Differences in salivary biomarker levels between groups were compared with Kruskal Wallis H test with post-hoc Dunn test. Spearman correlation coefficient was used to study the linear relationship between salivary biomarker levels and clinical periodontal parameters. Multivariate

binary logistic regression for the presence of periodontitis was performed using demographic variables, salivary biomarker levels and responses to SROH as predictors. The diagnostic accuracy, sensitivity and specificity of the predictive models were determined by plotting a receiver operating characteristics curve. After some modifications, the final six-item SROH was considered to be valid and reliable, with scale-level content validity index of one, scale-level face validity index of 0.837, internal consistency/Cronbach alpha of 0.813, test-retest reliability of 0.975 and all items having factor loading score >0.5. Significant intergroup differences were observed in salivary levels of IL-1β, IL-6, MMP-8 and MMP-9. The levels of IL-1β and IL-6 were significantly higher in periodontal disease states relative to periodontal health. Meanwhile, the highest expression of MMP-8 and MMP-9 was found in the periodontitis group. Multivariate logistic regression analysis for the discrimination of periodontitis from non-periodontitis groups demonstrated good predictive ability of models combining social demographics, SROH responses and salivary biomarker levels, with diagnostic performance exceeding 90%. In conclusion, the development of prediction model that integrated patient characteristics, SROH responses and levels of selected salivary biomarkers offered a sufficiently accurate and non-invasive means of periodontitis screening that should be validated in future studies.

Keywords: gingivitis, periodontitis, questionnaire, salivary biomarkers

**SOAL SELIDIK YANG DILAPORKAN SENDIRI DAN BIOPENANDA AIR LIUR UNTUK SARINGAN PERIODONTITIS DALAM KALANGAN ORANG DEWASA MALAYSIA**

## ABSTRAK

Biopenanda (*biomarker*) air liur dan soal selidik kesihatan mulut yang dilaporkan sendiri (SROH) berpotensi untuk berfungsi sebagai dua kaedah saringan periodontitis yang mudah dan tidak invasif. Kelebihannya adalah pelbagai, memandangkan protokol pemeriksaan periodontal seperti pemeriksaan periodontal seluruh mulut, *Basic Periodontal Examination* dan *Community Periodontal Index of Treatment Needs* memerlukan tenaga kerja, peralatan tambahan, masa dan kos yang lebih tinggi. Kajian ini bertujuan untuk mengesahkan kesahihan SROH sebagai alat saringan periodontitis dalam kalangan populasi Malaysia serta membandingkan tahap air liur bagi *interleukin-1β* (IL-1β), *interleukin-6* (IL-6), *tumour necrosis factor-alpha* (TNF-α), *matrix metalloproteinase*-8 (MMP-8), *matrix metalloproteinase-9* (MMP-9) dan *metallothionein* (MT) antara kumpulan dengan kesihatan periodontal, gingivitis atau periodontitis. Soal selidik SROH telah dikemukakan kepada jawatankuasa pakar untuk pengesahan kandungan, diikuti oleh pengesahan dalam kalangan pesakit melalui sampel perintis subjek yang tidak terlibat dalam kajian utama. Kemudian, 77 orang dewasa yang sihat secara sistemik direkrut secara berturut-turut dan dibahagikan kepada kumpulan kesihatan periodontal, gingivitis, dan periodontitis. Peserta diminta menjawab soal selidik SROH yang ditadbir sendiri diikuti dengan pengumpulan sampel air liur tanpa rangsangan. Lima mililiter air liur dikumpulkan dalam tempoh 30 minit. Selepas proses sentrifugasi, supernatan yang diperoleh dibahagikan kepada *aliquot* dan disimpan dalam peti sejuk suhu -80°C sehingga proses analisis dijalankan. Respons SROH digunakan untuk menentukan kesahihan konstruk dan konkuren, serta konsistensi dalaman dan kebolehpercayaan uji ulang soal selidik tersebut. Pengukuran tahap biopenanda air liur

ditentukan melalui *enzyme-linked immunosorbent assay*. Perbezaan tahap biopenanda air liur antara kumpulan dianalisis menggunakan ujian *Kruskal-Wallis H* dengan ujian *post-hoc Dunn*. Korelasi *Spearman* digunakan untuk mengkaji hubungan linear antara tahap biopenanda air liur dengan parameter klinikal periodontal. Regresi logistik binari multivariat untuk status periodontitis dijalankan dengan menggunakan maklumat demografi sosial, tahap biopenanda air liur, dan respons SROH sebagai peramal. Ketepatan diagnostik, sensitiviti, dan spesifikiti model ramalan dinilai melalui plot *receiver operating characteristic*. Selepas beberapa pengubahsuaian, SROH versi akhir yang terdiri daripada enam soalan dinilai sebagai sah dan konsisten, dengan indeks kesahihan kandungan berskala satu, indeks kesahihan muka berskala 0.837, konsistensi dalaman (*Cronbach alpha*) sebanyak 0.813, kebolehpercayaan uji ulang sebanyak 0.975, dan semua item mencatatkan *factor loading score* melebihi 0.5 (kesahihan konstruk). Perbezaan signifikan antara kumpulan diperhatikan dalam tahap air liur IL-1β, IL-6, MMP-8, dan MMP-9. Tahap IL-1β dan IL-6 adalah lebih tinggi dalam keadaan penyakit periodontal berbanding kesihatan periodontal. Selain itu, ekspresi tertinggi MMP-8 dan MMP-9 ditemui dalam kumpulan periodontitis. Analisis regresi logistik multivariat untuk diskriminasi periodontitis daripada kumpulan bukan periodontitis menunjukkan keupayaan ramalan yang baik, dengan model yang menggabungkan demografi sosial, respons SROH, dan tahap biopenanda air liur mencatatkan prestasi diagnostik melebihi 90%. Kesimpulannya, model ramalan yang menggabungkan ciri-ciri pesakit, soal selidik SROH, dan tahap biopenanda air liur terpilih menawarkan kaedah saringan periodontitis yang tepat dan tidak invasif, yang wajar disahkan dalam kajian lanjutan pada masa hadapan.

Kata kunci: biopenanda air liur, gingivitis, periodontitis, soal selidik

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| | | |
|---|---|---|
| AAP | : | American Academy of Periodontology |
| aMMP-8 | : | Activated matrix metalloproteinase-8 |
| ANOVA | : | Analysis of variance |
| AUC | : | Area under the curve |
| AUROCC | : | Area under the receiver operating characteristic curve |
| BOP | : | Bleeding on probing |
| BPE | : | Basic Periodontal Examination |
| CAL | : | Clinical attachment level |
| CART | : | Classification and regression tree |
| CDC | : | Centers for Disease Control and Prevention |
| COSMIN | : | Consensus-based standards for the selection of health status measurement instruments |
| COVID-19 | : | Coronavirus disease 2019 |
| CPITN | : | Community Periodontal Index of Treatment Needs |
| CVI | : | Content validity index |
| DM | : | Diabetes mellitus |
| DNA | : | Deoxyribonucleic acid |
| ECM | : | Extracellular matrix |
| EFA | : | Exploratory factor analysis |
| ELISA | : | Enzyme-linked immunosorbent assay |
| GCF | : | Gingival crevicular fluid |

| | | |
|---|---|---|
| HbA1c | : | Glycated haemoglobin |
| HRP | : | Horseradish peroxidase |
| ICC | : | Intraclass correlation coefficient |
| I-CVI | : | Item-level content validity index |
| IFMA | : | Immunofluorometric assay |
| I-FVI | : | Item-level face validity index |
| IHC | : | Immunohistochemistry |
| IL-1β | : | Interleukin-1 beta |
| IL-6 | : | Interleukin-6 |
| IQR | : | Interquartile range |
| KMO | : | Kaiser-Meyer-Olkin |
| LFT | : | Lateral flow test |
| MDA | : | Malondialdehyde |
| MHC | : | Major histocompatibility complex |
| MMP-8 | : | Matrix metalloproteinase-8 |
| MMP-9 | : | Matrix metalloproteinase-9 |
| MPO | : | Myeloperoxidase |
| MT | : | Metallothionein |
| NETs | : | Neutrophil extracellular traps |
| NHANES | : | National Health and Nutrition Examination Survey |
| NSPT | : | Non-surgical periodontal therapy |
| OHRQoL | : | Oral health-related quality of life |

| | | |
|---|---|---|
| OPG | : | Osteoprotegerin |
| OR | : | Odds ratio |
| PCR | : | Polymerase chain reaction |
| *P. gingivalis* | : | *Porphyromonas gingivalis* |
| POCT | : | Point-of-care-testing |
| PPD | : | Probing pocket depth |
| PS | : | Plaque score |
| PSR | : | Periodontal Screening and Recording |
| qPCR | : | Quantitative real-time polymerase chain reaction |
| RANKL | : | Receptor activator of nuclear factor kappa-B ligand |
| RCF | : | Relative centrifugal force |
| REC | : | Gingival recession |
| ROC | : | Receiver Operating Characteristics |
| S-CVI | : | Scale-level content validity index |
| S-CVI/Ave | : | Scale-level content validity index, averaging calculation method |
| S-CVI/UA | : | Scale-level content validity index, universal agreement calculation method |
| SD | : | Standard deviation |
| SEPA | : | Spanish Society of Periodontics and Osseointegration |
| S-FVI | : | Scale-level face validity index |
| S-FVI/Ave | : | Scale-level face validity index, averaging calculation method |

| | | |
|---|---|---|
| SMD | : | Standardised mean difference |
| SPT | : | Supportive periodontal therapy |
| SROH | : | Self-reported oral health questionnaire |
| T2DM | : | Type II diabetes mellitus |
| *T. denticola* | : | *Treponema denticola* |
| *T. forsythia* | : | *Tannerella forsythia* |
| TIMP | : | Tissue inhibitor of matrix metalloproteinases |
| TLR | : | Toll-like receptor |
| TMB | : | Tetramethylbenzidine |
| TNF-α | : | Tumour necrosis factor-alpha |
| US | : | United States |

## LIST OF APPENDICES

# CHAPTER 1. INTRODUCTION

## 1.1 Background of the study

Periodontal diseases are complex, chronic inflammatory diseases of the periodontal supporting structures characterised by bacterial dysbiosis and dysregulated host immune-inflammatory response (Cekici et al., 2014). According to the Global Burden of Disease Study 2021, 1.07 billion people in the world had severe periodontitis, with an increase of 90.7% in global count of prevalent cases from 1990 – 2021. Moreover, South East Asia presented with the highest age-standardised prevalence of severe periodontitis, amounting to 15,900 per 100,000 population (GBD 2021 Oral Disorders Collaborators, 2025). In essence, severe periodontitis is commonplace, and its prevalence is in the ascendant (Chen et al., 2021; Kassebaum et al., 2014).

While periodontal diseases usually begin silently and insidiously, they can eventually lead to tooth loss, masticatory dysfunction, aesthetic impairment and an overall reduction in oral health-related quality of life (OHRQoL) (Buset et al., 2016). The impact extends beyond the oral cavity, with substantial evidence highlighting the close relationship between oral and systemic health. Periodontitis has been associated with multiple systemic diseases, such as diabetes mellitus (DM), cardiovascular diseases, adverse pregnancy outcomes, chronic kidney diseases and more, with the dissemination of oral microbiota and inflammatory mediators to distant organs being touted as the most probable mechanistic links (Beck et al., 2019; Bobetsis et al., 2020; Bobetsis et al., 2023; Chambrone et al., 2013; Daalderop et al., 2018; Sanz et al., 2018; Sanz et al., 2020; Schenkein et al., 2020; Tonetti & Van Dyke, 2013). In addition, periodontal therapy can improve the glycaemic control of patients with DM, as evident by a decrease in glycated haemoglobin (HbA1c) level that is equivalent to the addition of another oral hypoglycaemic drug (Chapple & Genco, 2013; Sanz et al., 2018; Simpson et al., 2022). The widespread prevalence of periodontal diseases and its detrimental impact on both

oral and systemic health testify to the imperative of periodontal diseases screening in the general population.

The hallmark biological mechanisms of periodontal disease pathogenesis revolve around dysregulated inflammation, connective tissue degradation by host-derived enzymes, bone resorption and increased oxidative stress (Meyle & Chapple, 2015; Page & Schroeder, 1976; Sczepanik et al., 2020). A multitude of molecules, cytokines or enzymes mediate these processes, which become detectable in the immediate milieu such as gingival tissues, saliva and gingival crevicular fluid (GCF).

Saliva, an oral fluid derived from salivary gland secretions, GCF, bronchial secretions, serum, blood cells, microorganisms (bacteria, virus, fungi and more) and their byproducts, oral squames and food debris can serve as a window into an individual's oral and systemic health. The coronavirus disease 2019 (COVID-19) pandemic has further pushed salivary diagnostics to the forefront, with salivary COVID-19 test kits gaining widespread acceptance. The development of biomarkers for early detection of periodontal diseases and the identification of disease progression is highly sought after to address the shortcomings of existing approaches. Interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNF-α) are well-established pro-inflammatory cytokines that regulate the local inflammatory processes in periodontal diseases, as well as being disseminated in the systemic circulation to affect distant organs (Cekici et al., 2014; Hajishengallis, 2014).

Connective tissue breakdown that entrains the loss of connective tissue attachment to the tooth root is characteristic of the advanced lesions of periodontitis (Lindhe et al., 1975; Page & Schroeder, 1976). Degradation of the connective tissue of the periodontium is modulated by host-derived proteases such as matrix metalloproteinase-8 (MMP-8) and matrix metalloproteinase-9 (MMP-9) (Luchian et al., 2022). These cytokines and host enzymes were associated with periodontal diseases in numerous human studies conducted

in different study populations (Arias-Bujanda et al., 2020; Blanco-Pintos et al., 2023; Kc et al., 2020). Metallothionein (MT) is a host protein involved in oxidative stress processes but is sparsely investigated in relation to periodontal diseases. Limited evidence supported a possible association between MT levels and periodontitis (Katsuragi et al., 1997; Yadav et al., 2021). Significant research efforts are currently underway to translate biomarker analysis into rapid, chairside point-of-care testing (POCT) implements such as biosensors and lab-on-a-chip microfluidics (Steigmann et al., 2020). These technological chairside molecular detection platforms allow rapid salivary sample analysis and immediate presentation of microbiological and immunological data that can aid the clinician's decision making while the patient is still in session (Bostanci & Belibasakis, 2023).

Furthermore, several self-reported tools have been developed for the purpose of periodontitis screening and surveillance (Abbood et al., 2016; Blicher et al., 2005; Eke & Genco, 2007; Renatus et al., 2016; Yamamoto et al., 2009). Eke and Genco (2007) designed a self-reported oral health questionnaire (SROH) containing eight questions, intended for the population-based surveillance of periodontal diseases. The SROH was validated against full-mouth periodontal examination among a nationally representative sample of 3743 US adults who participated in the 2009 – 2010 National Health and Nutrition Examination Survey (NHANES). The authors concluded that these self-reported models are viable alternative to clinical periodontal examination in population-based research and that the local adaptation of these self-report questions could enhance the global surveillance of periodontal diseases (Eke et al., 2013).

## 1.2 Problem statement

Despite significant improvement in the understanding of the aetiopathogenesis of periodontal diseases, the techniques used to screen and diagnose periodontal diseases remain unchanged. Comprehensive periodontal examination and radiographic assessment

are the mainstays of periodontal diagnostics. These clinical and radiographic parameters are required to arrive at the diagnosis and subsequent staging and grading of periodontitis, according to the latest classification of periodontal and peri-implant diseases and conditions (Caton et al., 2018; Chapple et al., 2018; Tonetti et al., 2018). These clinical procedures are time-consuming and labour intensive. The conduct of a full-mouth periodontal examination requires basic dental equipment, calibrated periodontal probes and trained dental personnel. In addition, they rely on detecting the loss of connective tissue and alveolar bone, which are irreversible and indicative of late stages of periodontal diseases. Additionally, these methods do not indicate current disease activity but rather reflect the cumulative effect of past periodontal tissue destruction. These shortcomings make them less than ideal for population-wide screening. Even the Basic Periodontal Examination (BPE), Community Periodontal Index of Treatment Needs (CPITN) or Periodontal Screening and Recording (PSR), designed for rapid screening of patients with periodontal diseases, require specialised probe, dental equipment and additional staff training. Consequently, dental researchers are shifting towards more convenient and simple methods to screen for periodontal diseases, with salivary biomarkers and self-reported questionnaire emerging as two promising options. At the time of conception of this study, the SROH has not been validated in the Malaysian population.

Recent systematic reviews showed a growing body of evidence supporting the diagnostic potential of several salivary biomarkers in periodontal diseases (Arias-Bujanda et al., 2020; Blanco-Pintos et al., 2023; de Lima et al., 2016). However, the range of concentration varied widely within and between studies. Therefore, it is uncertain if the thresholds established by these studies could be applied to our local population. Furthermore, there were much more studies looking into single biomarker as compared to biomarker combinations, although recent data suggested that the latter is better at detecting periodontal diseases (Blanco-Pintos et al., 2023; Ebersole et al., 2015; Wu et al.,

2018). In addition, there is a scarcity of evidence regarding the effect of both salivary biomarkers and SROH responses, and whether this combination offer improvement in the diagnostic accuracy of predictive models for periodontitis.

### 1.3 Rationale of the study

Screening for periodontitis at the population level relies on periodontal examination including full-mouth periodontal examination or simplified protocols such as BPE, CPITN or PSR. These methods share the limitation of requiring trained personnel, significant time and specialised equipment, making them cumbersome for large-scale screening and surveillance purposes. Such challenges emphasise the need for alternative means of periodontitis screening approaches that are accurate, simple, convenient and practical. Salivary biomarkers and SROH, being non-invasive and easy to collect, present promising alternatives. However, their validity for predicting periodontitis must first be verified before they can be adopted.

### 1.4 Significance of the study

If proven reliable and valid, the SROH and salivary biomarkers could offer alternative, less invasive methods for community-wide screening for periodontitis, reducing reliance on full-mouth periodontal examination or BPE protocols. These tools could be incorporated into future iterations of the National Oral Health Survey of Adults, enhancing periodontitis surveillance across the Malaysian population. The non-invasive and simplicity nature would enable more frequent and accessible screening, addressing current limitations in monitoring and improving early detection efforts.

## 1.5 Research questions

i. What are the validity and reliability of the SROH when used among Malaysian adults?

ii. How do the concentrations of salivary biomarkers (IL-1β, IL-6, TNF-α, MMP-8, MMP-9, and MT) differ among subjects with periodontal health, gingivitis, and periodontitis?

iii. What is the correlation between levels of salivary biomarkers and various clinical periodontal parameters, including probing pocket depth (PPD), clinical attachment level (CAL), plaque score (PS) and bleeding on probing (BOP)?

iv. What is the accuracy, sensitivity and specificity of multivariate models that combine patient characteristics, salivary biomarker levels, and SROH responses for discriminating between different periodontal health and disease states (periodontal health, gingivitis, and periodontitis)?

## 1.6 Research hypothesis

### 1.6.1 Null hypothesis

i. There are no significant differences in salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT between periodontal health, gingivitis and periodontitis groups.

ii. Salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT are not significantly correlated with clinical periodontal parameters.

iii. The performance of predictive models for periodontitis is no better than chance alone, with an area under the receiver operating characteristic curve (AUROCC) of 0.5.

### 1.6.2 Alternative hypothesis

i. Salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT are significantly different between periodontal health, gingivitis and periodontitis groups.

ii. Salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT are significantly correlated with clinical periodontal parameters.

iii. The performance of predictive models for periodontitis is greater than chance alone, with AUROCC >0.5.

## 1.7 Aims and Objectives

Therefore, the aim of this study was to validate the SROH as a screening tool for periodontitis among Malaysian adults and to investigate the relationship between salivary biomarkers and periodontal health status.

The specific objectives of this study were:

i. To assess the validity and reliability of the SROH among Malaysian adults.
ii. To compare the salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT among healthy adults with periodontal health, gingivitis and periodontitis.
iii. To correlate the salivary levels of IL-1β, IL-6, TNF-α, MMP-8, MMP-9 and MT with clinical periodontal parameters, including PPD, CAL, PS and BOP.
iv. To assess the accuracy, sensitivity and specificity of multivariate models combining patient characteristics, salivary biomarker levels, and responses to the SROH for the discrimination between periodontitis and non-periodontitis (periodontal health and gingivitis) groups.

## 1.8 Conceptual framework

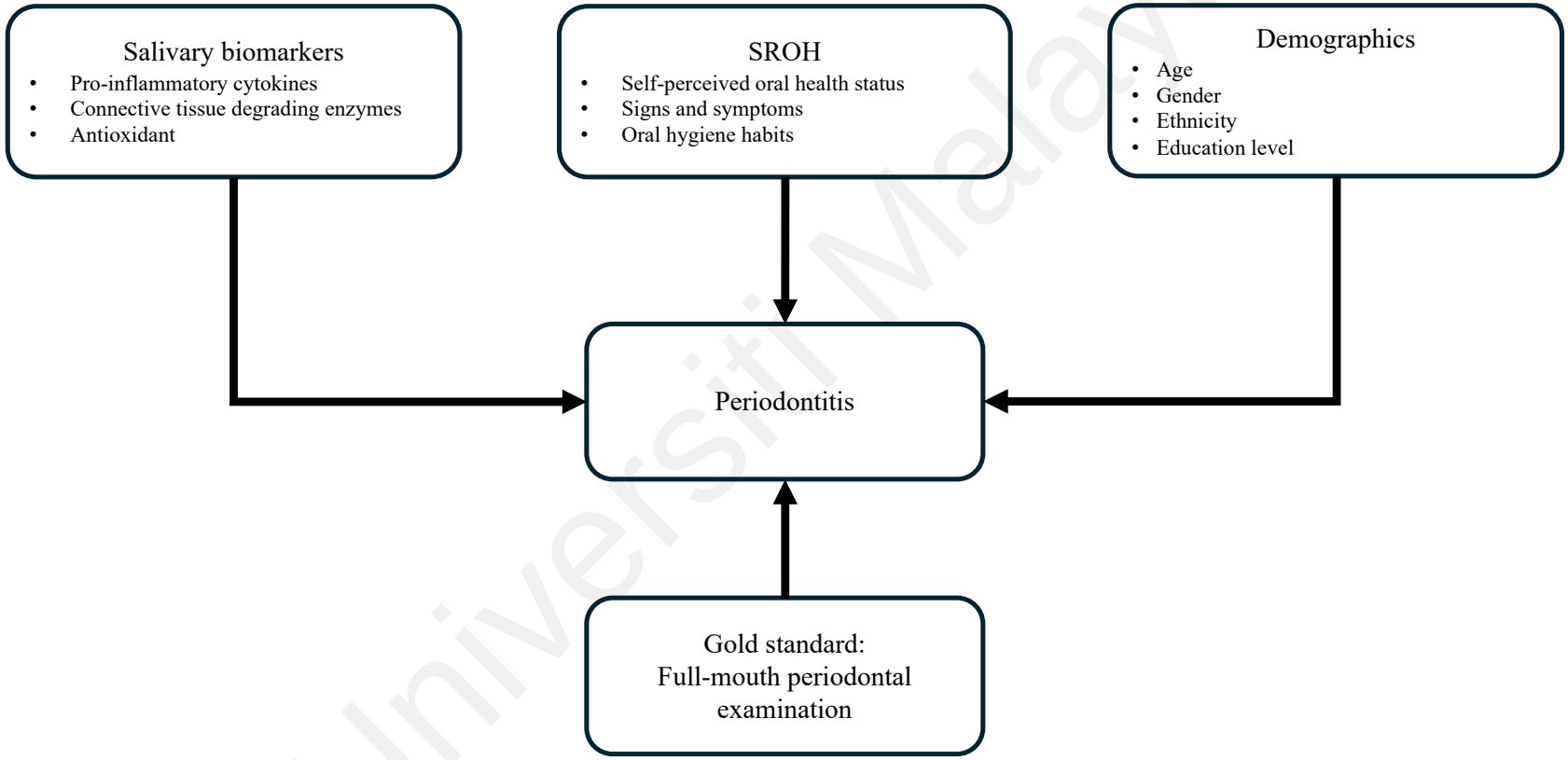



**Figure 1.1. Conceptual framework of this research study.**
**SROH, self-reported oral health questionnaire**

# CHAPTER 2. LITERATURE REVIEW

## 2.1 Periodontal health and diseases

Periodontal diseases are a group of chronic inflammatory diseases of the periodontium that if left untreated, can progress to irreversible loss of periodontal tissues and finally, tooth loss (Papapanou et al., 2022). The four components that make up the periodontium are the gingiva, periodontal ligament, root cementum and alveolar bone (Bosshardt et al., 2022).

### 2.1.1 Periodontal health

The World Health Organisation defined health as a state of complete physical, mental and social well-being and not merely the absence of disease and infirmity (World Health Organization, 2020). Such a holistic view of periodontal health should include the assessment of OHRQoL instead of merely the absence of disease. Nevertheless, from a more practical and clinical standpoint, periodontal health would denote a state free of inflammation that is associated with gingivitis or periodontitis. Histologically, an inflammatory infiltrate is always present subjacent to the junctional epithelium, even in clinically healthy gingival tissues (Brecx et al., 1987). This constitutes a form of physiological immune surveillance by the host.

Periodontal health has been defined as pristine clinical health or clinical periodontal health. The former comprises a set of stringent criteria including absence of attachment loss, BOP, pus discharge and clinical signs of inflammation such as erythema and oedema, as well as PPD ≤3 mm (Lang & Bartold, 2018). Pristine clinical health is rare, but achievable. Clinical periodontal health is a more reasonable and plausible state. It refers to tissues that are free of or having only a very low level of gingival inflammation that is compatible with health (Lang & Bartold, 2018). The term clinical periodontal health is hereinafter used to indicate periodontal health.

The definition of clinical periodontal health should also include the status of the periodontium. This pertains to whether the state of health is juxtaposed on an intact or reduced periodontium (Chapple et al., 2018; Lang & Bartold, 2018). Clinical periodontal health on an intact periodontium is a good preventive starting point. It is characterised by BOP<10%, physiological gingival sulcus depth and normal bone heights. For a periodontitis patient, the loss of connective tissue attachment and alveolar bone is irreversible sans periodontal regeneration. Therefore, clinical outcomes in a treated periodontitis patient can be attained at two levels. These are periodontal stability and periodontal disease remission/control. Periodontal stability connotes a successful treatment outcome whereby minimal BOP, optimal reduction in PPD and gain in CAL, concomitant with a lack of disease progression is attained. Meanwhile, periodontal disease remission/control is an intermediate state of low disease activity. This manifests in improvement in BOP, PPD reduction and CAL gain, but residual disease remains (Lang & Bartold, 2018).

When dental biofilm is allowed to accumulate adjacent to the tooth surface with clinical periodontal health, gingivitis will ensue (Löe et al., 1965).

### 2.1.2 Gingivitis

Gingivitis is a non-specific host inflammatory response to non-specific dental biofilm accumulation. The inflammatory infiltrate is confined to the gingival tissues. The reversibility of gingivitis differentiates it from periodontitis, which is accompanied by permanent loss of periodontal supporting structures (Lindhe et al., 1975; Löe et al., 1965). The inflammatory changes of a gingivitis lesion manifest in gingival bleeding, erythema, oedema and increase in GCF volume. Among these parameters, BOP is widely investigated and universally adopted for assessment of periodontal status. Gingival bleeding possesses multiple characteristics that bode well for its clinical applicability. As a clinical sign, BOP is easily accessible and can be recorded objectively. Moreover, BOP

is a good proxy for the inflammatory condition of the gingival tissues at the site level. Beyond that, BOP holds prognostic significance as repeated BOP at multiple examination visits during supportive periodontal therapy (SPT) increased the risk of further attachment loss (Lang et al., 1986). Conversely, absence of BOP during SPT is an excellent indicator of periodontal stability (Lang et al., 1990).

The 2017 World Workshop proposed the use of BOP score to define and classify a gingivitis case. Distinction should also be made on whether the gingivitis lesion is superimposed on an intact or reduced periodontium. For reduced periodontium, a further subdivision is made, differentiating between reduced periodontium in a non-periodontitis or a stable periodontitis patient. Regardless, the threshold and cut-off values for localised or generalised gingivitis for all three subclassifications are similar. Localised gingivitis is defined as a patient presenting with BOP score ≥10% and ≤30%. For a generalised gingivitis case, the BOP score should exceed 30% (Caton et al., 2018; Chapple et al., 2018; Trombelli et al., 2018).

### 2.1.3 Periodontitis

Classical studies from the natural history of periodontal diseases in man identified host susceptibility as a key determinant of periodontitis development and progression (Löe et al., 1986). This aspect was proven by longitudinal data collated from a group of tea plantation labourers in Sri Lanka. This study population was unique because the subjects did not perform routine oral hygiene practices and had no exposure to dental services. Despite the universal lack of access to dental care, gross accumulation of dental biofilm and presence of gingival inflammation across all participants, the amount of interproximal attachment loss and tooth mortality rates were not uniform. Three broad categories became evident. The bulk of the subjects (81%) showed moderate progression of periodontitis. Approximately 11% of the subjects presented with no progression of periodontal diseases beyond gingivitis. The remaining 8% comprised of subjects with

rapid progression of periodontal tissue loss. The mean annual rate of attachment loss in the rapid progression group varied between 0.1 to 1 mm. The corresponding ranges for the moderate progression and no progression groups were 0.05 to 0.5 mm and 0.01 to 0.09 mm, respectively (Löe et al., 1986). Hence, gingivitis does not lead invariably to periodontitis.

The principal characteristics of periodontitis are the apical migration of junctional epithelium, loss of connective tissue attachment and resorption of marginal alveolar bone (Page & Schroeder, 1976). However, variations in disease progression, age of onset, individual genetic susceptibility and the contribution by multiple risk factors culminate in a wide spectrum of clinical presentations of periodontitis. Whether these clinical phenotypes represent different disease entities, or a variation of a singular disease is often debated. This ambivalence is reflected by the changes made to the classification of periodontitis over the last few decades.

The 1999 International Workshop on the Classification of Periodontal Diseases was heavily influenced by the research that dominated that time period, namely the identification of specific bacteria species or bacterial complexes as aetiologic agents of periodontal diseases in general or certain periodontitis phenotypes specifically. Moreover, the notion of genetic polymorphism conferring individual susceptibility to periodontitis loomed large. This led to the recognition of four forms of periodontitis under the 1999 classification scheme. These are chronic periodontitis, aggressive periodontitis, necrotising periodontitis and periodontitis as a manifestation of systemic diseases (Armitage, 1999).

Chronic periodontitis and aggressive periodontitis are both biofilm-associated conditions, with apparently distinctive clinical phenotypes. However, subsequent studies failed to substantiate the notion that aggressive periodontitis and chronic periodontitis are fundamentally different. A series of review articles commissioned in 2010 further

suggested that these two conditions are not that dissimilar in terms of microbiology, immunology, histopathology, neutrophil function, clinical presentation, radiographic features or response to periodontal therapy (Armitage, 2010; Armitage & Cullinan, 2010; Deas & Mealey, 2010; Ford et al., 2010; Ryder, 2010; Smith et al., 2010; Stabholz et al., 2010). An exception could be argued for localised aggressive periodontitis, which has some unique features such as early age of onset, localisation of severe periodontal breakdown to incisors and first molar regions, rate of progression and its preponderance among certain ethnic populations (Fine et al., 2018). Nevertheless, existing evidence refuted the notion that these clinical phenotypes have different pathophysiology. They are all biofilm-associated inflammatory diseases. This paved the way for the revision of the classification of periodontal diseases in 2017, essentially combining aggressive periodontitis and chronic periodontitis into a single entity, periodontitis (Papapanou et al., 2018; Tonetti et al., 2018). Case definitions for a periodontitis case were proposed. A patient is considered a periodontitis case in the presence of interdental CAL at ≥2 non-adjacent teeth or buccal/oral CAL ≥3 mm with pocketing ≥3 mm at ≥2 teeth. In addition, the observed CAL cannot be attributed to non-periodontal causes such as trauma-induced gingival recession, cervical dental caries, pocketing distal to second molars associated with impacted third molars, a draining sinus tract originating from an endodontic lesion or vertical root fracture (Tonetti et al., 2018).

The current classification scheme and framework for periodontitis is designed to be amenable for future revision, as and when new knowledge arises. Interestingly, the research group behind the 2017 classification system acknowledged the potential role of biomarkers in improving the early detection of periodontitis, suggesting that validated biomarkers could possibly be included in the future revisions of the classification framework (Papapanou et al., 2022; Tonetti et al., 2018).

## 2.2 Periodontal screening and diagnostic modalities

Full mouth periodontal examination (FMPE) supplemented with dental radiographs when indicated remains the gold standard for diagnosis of periodontal diseases. However, periodontal disease screening through FMPE is highly laborious, necessitating significant investment in time, manpower and resources. Even tools devised specifically for periodontal screening such as BPE, PSR and CPITN are not completely free from the aforementioned shortcomings. In addition, many factors can affect the measurement of clinical periodontal parameters, leading to intra- and interindividual variation. Moreover, gingival margin that is positioned coronal to the cementoenamel junction (CEJ) also complicates the measurement of clinical attachment level, as it is difficult to distinguish the CEJ with tactile sensation alone.

In the light of these shortcomings, there is a genuine need for alternative periodontal screening tools that are simple, cost-effective and non-invasive. Salivary biomarkers and self-reported questionnaire emerge as two promising tools. If proven valid as compared to FMPE, they could potentially replace the need for periodontal probe for community-wide screening of periodontal diseases. But first, the biological rationale for using biomarkers for disease detection will be explored, starting with the aetiopathogenesis of periodontal disease in the following section.

## 2.3 Aetiopathogenesis of periodontal diseases

### 2.3.1 Bacterial aetiology of periodontal diseases

A seminal paper in 1965 had categorically proven that dental biofilm is the causative factor for the onset of gingivitis (Löe et al., 1965). The biofilm is defined as matrix-enclosed bacterial communities adherent to each other and to a shedding/non-shedding surface (Costerton, 1999; Hall-Stoodley et al., 2004). Biofilms are complex and dynamic structures that are functionally and spatially organised in patterns that favour the survival of its constituent species (Marsh & Zaura, 2017). In fact, microorganisms residing in biofilms may be 10 – 1000 times more resistant against antimicrobials than their planktonic forms (Costerton, 1999).

Infectious diseases such as syphilis and tuberculosis are caused by a specific and exogenous microorganism, the elimination of which will predictably lead to disease resolution. On the contrary, the bacterial infection underlying periodontal diseases is characterised by a polymicrobial and endogenous infection. A higher prevalence and proportion of a selected number of bacterial species are found in periodontitis patients, leading to their categorisation as periodontal pathogens. Most notably, a landmark publication employing the checkerboard deoxyribonucleic acid (DNA)-DNA hybridisation technique of plaque samples introduced the concept of microbial complexes, whereby the red complexes of *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*) and *Treponema denticola* (*T. denticola*.) were significantly associated with periodontitis (Socransky et al., 1998). Conversely, health-associated or host compatible species such as *Veillonella parvula*, *Actinomyces*, *Capnocytophaga* and *Streptococcus* were elevated in periodontal health and increased in proportions after periodontal therapy (Haffajee et al., 2006; Socransky et al., 1998; Socransky et al., 1988; Teles et al., 2013).

### 2.3.2 Host immune-inflammatory responses of periodontal diseases

In spite of dental biofilm's critical role in the initiation of periodontal diseases, most of the tissue damage accrued in periodontitis lesion can be attributed to the host immune-inflammatory responses, which paradoxically are called upon to protect the host from the microbial communities (Cekici et al., 2014; Meyle & Chapple, 2015; Seymour et al., 2022). In this sense, gingivitis and periodontitis are chronic inflammatory conditions orchestrated by complex interplay between pathogenic bacterial biofilms and the host immune response. This interaction is a dynamic process. In states of periodontal health, the bacterial communities and host response exist in homeostatic balance with one another. Continual bacterial accumulation at the gingival margin triggers a localised inflammatory response. As the biofilm matures, the symbiosis between biofilm and the host is perturbed, leading to dysbiosis. This microbial shift initiates an exaggerated immune response in susceptible hosts, predominantly characterised by the infiltration of neutrophils and macrophages into the periodontal tissues. Later on, lymphocytes are also recruited. The perpetuation of this dysregulated immune response underpins disease progression, as immune cells release cytokines and other molecular mediators that result in tissue destruction and bone loss (Cekici et al., 2014).

At the cellular level, innate immune cells like neutrophils and macrophages are the first responders. Chemotactic signals such as interleukin-8/chemokine (C-X-C motif) ligand 8 and monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 create a gradient that facilitate the entry of these phagocytes into the periodontal tissues (Hanazawa et al., 1993; Takashiba et al., 1992). These cells ingest bacteria and release an array of pro-inflammatory cytokines. However, the bacterial challenge, which is sheltered within the biofilm matrix is not cleared. As the inflammatory process becomes chronic, adaptive immune system is activated, further amplifying the inflammatory process (Cekici et al., 2014). Key molecular players in this immune cascade include cytokines

like IL-1β, IL-6, and TNF-α. These pro-inflammatory cytokines function as the driver of inflammation by promoting leukocyte recruitment, enhancing the production of matrix metalloproteinases and sustaining tissue degradation (Pan et al., 2019). Specifically, MMP-8 and MMP-9 are released to degrade extracellular matrix components, setting the stage for the breakdown of periodontal connective tissue. These proteases are produced by immune cells such as neutrophils as well as resident cells such as gingival fibroblasts. Additionally, IL-1β and TNF-α increase oxidative stress and sustain inflammation. Although the full extent of the cytokine network is not known, it is well-established that the balance of cytokines in inflamed periodontal tissues dictates if the lesion remains stable or progresses to tissue destruction (Seymour & Gemmell, 2001). Metallothionein also play a role in modulating oxidative damage and maintaining tissue homeostasis (Aziz et al., 2021).

The interaction between the upregulation of host immune-inflammatory cells and pro-inflammatory cytokines is partially mediated by the Triggering Receptor Expressed on Myeloid Cells-1 (TREM-1). This receptor belongs to the immunoglobulin superfamily and is expressed on the surfaces of various host cells such as neutrophils, monocytes, macrophages, dendritic cells, vascular smooth cells and some keratinocytes. It is upregulated in the presence of inflammation. When TREM-1 is engaged by bacterial antigens of the dental biofilm, IL-1β expression is upregulated (Willi et al., 2014). It was suggested that the elevated levels of IL-1β is mediated by TREM-1's effect on the polarization of pro-inflammatory M1 macrophages via the STAT3/HIF-1α signaling pathway (Wu et al., 2022). Moreover, TREM-1 can activate the NF-κβ pathway, leading to increased production of IL-1β and TNF-α (Rudick et al., 2017). These cytokines in turn stimulate fibroblasts to produce MMP-8 and MMP-9 that degrades the extracellular matrix of the periodontal connective tissue (Seymour et al., 2022).

Understanding the biological roles, molecular regulation and expression profiles of these cytokines and enzymes is critical for elucidating their respective roles in the pathogenesis of periodontitis. Moving on, these molecular mediators are described in greater detail individually, starting with an archetypal pro-inflammatory cytokine, IL-1β.

#### 2.3.2.1 Interleukin-1β

Interleukin-1 beta is a potent pro-inflammatory mediator derived from the IL-1 family of cytokines. The binding of IL-1β to its receptor activates a series of downstream signals that eventually stimulate the proliferation, differentiation and function of many innate and specific host immunocompetent cells such as neutrophils, T-cells and B-cells. It also possesses bone-resorptive properties via the activation of osteoclasts. Genetic polymorphisms in IL-1β are linked to susceptibility to chronic periodontitis (Lavu et al., 2015). Moreover, genetic polymorphism in the IL-1 gene, which resulted in elevated tissue and GCF levels of IL-1β, was associated with significantly greater risk of both periodontitis initiation and progression (Kornman & di Giovine, 1998).

A variety of host cells, including neutrophils, macrophages and B-cells secrete IL-1β. *P. gingivalis* is known to induce a higher percentage of peripheral blood B-cells from periodontitis patients to produce IL-1β compared to macrophages (Curtis et al., 2022; Seymour et al., 2022). B-cells, and not macrophages are the dominant cell populations in the advanced lesion (Page & Schroeder, 1976). Therefore, B-cells could be the major source of IL-1β in advanced lesions of periodontitis (Seymour et al., 2022). As IL-1β is known to regulate the balance between receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG), the increased secretion of IL-1β by B-cells could explain the contributory role of B-cells in alveolar bone destruction in periodontitis (Seymour et al., 2022).

A cross-sectional study was conducted among 36 periodontally healthy, 31 gingivitis and 60 periodontitis patients to elucidate the salivary concentrations of 10 candidate biomarkers using enzyme-linked immunosorbent assay (ELISA). All the participants were non-smokers. Statistically significant differences in salivary IL-1β concentrations were found for all comparison groups (healthy vs gingivitis, healthy vs periodontitis and gingivitis vs periodontitis). The periodontitis group presented with the highest level of IL-1β, with a fold-change of 3.34 and 2.17 relative to the healthy and gingivitis groups, respectively. In fact, classification and regression tree analysis identified IL-1β as one of the predictor variables for a statistically generated decision tree that discriminated healthy vs periodontitis groups (Bostanci et al., 2021). In a recent cross-sectional study of African population, significantly higher IL-1β levels were detected among periodontitis patients as diagnosed based on the 2017 World Workshop classification (Reddahi et al., 2022).

In another cross-sectional study that involved 493 Finnish patients, IL-1β concentration in saliva was associated with higher PPD, alveolar bone loss and BOP. However, the patients within this study were also diagnosed with coronary artery disease, which may be a confounding variable on the expression of inflammatory biomarkers (Salminen et al., 2014). Conversely, salivary IL-1β level was not associated with periodontal disease status in a cross-sectional study of 74 chronic periodontitis patients and 44 periodontally healthy patients (Teles et al., 2009). The laboratory test used for biomarker quantification was multiplexed bead immunoassay. The authors attributed the non-significant findings to the fact that GCF, which is the source of these biomarkers is extensively diluted in saliva. Moreover, the limited distribution of deep periodontal pockets in this study population (mean percentage of sites >4 mm and >6 mm of 14% and 2.7%, respectively) meant that the GCF derived from these deep pockets only made up a small portion of the whole saliva composition. Nevertheless, independent studies had

consistently demonstrated a reduction in salivary levels of IL-1β following periodontal treatment, in tandem with significant improvement in clinical periodontal parameters (Kaushik et al., 2011; Kinney et al., 2011; Sexton et al., 2011). The follow-up period for these interventional studies ranged from one to six months. Despite substantial evidence in favour of its association with periodontal disease states and clinical periodontal parameters, the range of salivary IL-1β levels varied widely across studies. In healthy states, the mean levels ranged from 7.24 ± 7.69 pg/ml to 633.91 ± 91pg/ml. For periodontitis patients, levels between 90.04 ± 85.22 pg/ml and 1312 ± 691.22 pg/ml had been reported (Jaedicke et al., 2016). These variations could be the results of different patient selection criteria or study population involved. As a whole, IL-1β was identified as a salivary biomarker with acceptable diagnostic accuracy for periodontal diseases in three separate systematic reviews (Blanco-Pintos et al., 2023; de Lima et al., 2016; Kc et al., 2020).

#### 2.3.2.2 Interleukin-6

As a pro-inflammatory marker, IL-6 is known to regulate the growth of T- and B-cells, direct the chemotaxis of leukocytes and induce the production of acute phase proteins during the course of periodontal diseases (Pan et al., 2019). Moreover, the expression of RANK is upregulated by IL-6, favouring osteoclastic differentiation and bone resorption (Wu et al., 2017). Cellular sources of IL-6 include macrophages, dendritic cells, $CD4^+$ T-cells, endothelial cells and fibroblasts (Rincon, 2012). Receptors for IL-6 exist in the transmembrane form in cells such as monocytes or lymphocytes, or as a soluble form following protease-mediated cleavage (Kang et al., 2019). The downstream signalling transduction of ligand-receptor interaction involving IL-6 is mediated via the transmembrane protein glycoprotein-130 (Kishimoto et al., 1992).

Studies looking into the salivary expression of IL-6 in periodontal diseases were conflicting and inconclusive. While elevated IL-6 levels were associated with

periodontitis in some studies, others presented with non-significant differences between periodontitis patients and healthy controls (Costa et al., 2010; Ebersole et al., 2013; Fine et al., 2009; Gursoy et al., 2009; Ng et al., 2007; Prakasam & Srinivasan, 2014; Ramseier et al., 2009; Rathnayake et al., 2013; Reddahi et al., 2022; Scannapieco et al., 2007; Teles et al., 2009). In the study by Prakasam and Srinivasan (2014), an 1.84-fold increase in salivary IL-6 level was observed in the chronic periodontitis group compared to healthy controls. However, scaling and root planing had no effect on IL-6 levels, six weeks post-therapy. Nonetheless, three separate systematic reviews agreed that IL-6 has diagnostic value as a salivary biomarker for periodontal diseases (Blanco-Pintos et al., 2023; de Lima et al., 2016; Kc et al., 2020).

#### 2.3.2.3 Tumour necrosis factor-alpha

As a pro-inflammatory cytokine, TNF-α is a prime candidate for periodontal disease biomarker owing to its multifarious functions in the pathogenesis of periodontal diseases. It is involved in the activation of macrophages, apoptosis of epithelial cells in the mucosa, regulation of major histocompatibility complex (MHC) class I and II protein expression, production of collagenase by gingival fibroblasts and induction of bone resorption indirectly via the regulation of the RANKL-OPG balance. It is released by several immune inflammatory cells such as macrophages, natural killer cells, neutrophils, B-cells, T cells and mast cells, as well as non-immune cells including endothelial cells and fibroblasts. The secretion of TNF-α by macrophages is mediated via the Toll-like receptor (TLR) signalling pathway (Sedger & McDermott, 2014). It is also involved in the development of the gingivitis lesion by promoting the expression of adhesion molecules by endothelial cells and the subsequent sticking and emigration of polymorphonuclear neutrophils from the gingival vasculature into the gingival tissues. TNF-α can also induce neutrophil extracellular traps (NETs), which are associated with severe tissue damage (Seymour et al., 2022). Its biological relevance in periodontal

disease pathogenesis has spurred numerous studies to investigate its differential expression in periodontal disease and health. In general, most studies reported minute to negligible levels of TNF-α in human saliva (Aurer et al., 2005; Ebersole et al., 2013; Gursoy et al., 2009; Kinney et al., 2011; Mirrielees et al., 2010; Ramseier et al., 2009; Rathnayake et al., 2013; Scannapieco et al., 2007). In a cross-sectional study, the salivary TNF-α levels of periodontitis subjects who exhibited ≥30% BOP, ≥20% sites with PPD ≥4 mm, ≥10% sites with interdental CAL ≥2 mm and evidence of alveolar bone loss at ≥20% sites based on posterior vertical bitewings were compared against healthy controls of similar age, gender and race. The mean concentration of 4.33 pg/ml of salivary TNF-α in the periodontitis group was significantly higher than healthy control (mean concentration of 2.03 pg/ml). Nonetheless, the actual concentration recorded was very low, with the maximum detectable level being 27.96 pg/ml found in the periodontitis group. When salivary TNF-α levels were at least two standard deviations above the mean concentration in the control group (≥5.75 pg/ml), significantly greater number of sites displayed BOP, PPD ≥4 mm and interdental CAL ≥2 mm (Frodge et al., 2008).

Contradictory findings were observed among a sample of Taiwanese adults, whereby salivary TNF-α level was higher in the non-periodontitis group relative to the periodontitis group (Wu et al., 2017). The reliability of the findings is uncertain, however, as the TNF-α levels were very low and the within-group fluctuations were high.

#### 2.3.2.4 Matrix metalloproteinase-8

As a potent host proteinase, MMP-8, otherwise known as collagenase-2 or neutrophil collagenase, is heavily involved in the pathogenesis of periodontitis (Luchian et al., 2022). It is well-established that the tissue destruction seen in periodontitis is primarily the corollary of the host's immune inflammatory response in response to the dental biofilm. Matrix metalloproteinases such as MMP-8 and MMP-9 belong to a family of proteinases whose primary function is the degradation of the extracellular matrix

(ECM). Collagen, which makes up the bulk of the ECM is cleaved into smaller fragments, before undergoing further denaturation or being phagocytosed by fibroblasts. In fact, these MMPs, together with the inflammatory cytokines might be sequentially expressed in a cascade of molecular events culminating in host-mediated tissue destruction. For example, IL-1, IL-6 and TNF-α stimulate fibroblasts to secrete MMP-8 and MMP-9 (Seymour et al., 2022). Initially produced in its inactive pro-form, MMPs are activated to degrade the ECM. The actions of MMP-8 and MMP-9 are counteracted by the naturally occurring inhibitors known as tissue inhibitor of metalloproteinases (TIMP).

Significant elevation in salivary levels of MMP-8 among periodontitis patients was consistently reported by numerous observational studies (Bostanci et al., 2021; Costa et al., 2010; Miller et al., 2021; Ramseier et al., 2009; Rathnayake et al., 2013; Reddahi et al., 2022). Similarly, the concentration of MMP-8 was positively correlated with BOP, PPD and CAL measurements (Reddahi et al., 2022). Even when no statistically significant differences were found, the levels of MMP-8 tended to be higher in the presence of periodontal destruction (Katsiki et al., 2021).

In a systematic review of clinical observational studies reporting on salivary MMP-8 levels between periodontitis patients and healthy controls, a total of ten studies with 485 periodontitis patients and 379 healthy subjects were included. Significantly higher levels of MMP-8 were found in eight studies, with a pooled SMD (standardised mean difference) of 1.195 (95% CI .0.720 – 1.670). The use of SMD allowed the mean differences to be compared across different studies where the SD (standard deviation) within groups was equivalent to one. However, the high $I^2$ value of 89.3% indicated high heterogeneity, which could be due to the different detection techniques used (ELISA, immunofluorometric assay and Luminex), variation in the criteria for periodontitis and controls, and different study populations. Moreover, the mean MMP-8 concentrations varied widely across studies, ranging from 2.95 ± 0.66 ng/ml to 888.6 ± 990.1 ng/ml for

periodontitis patients and 2.51 ± 0.81 ng/ml to 309.4 ± 183.4 ng/ml for healthy controls. In view of these limitations, the authors recommended further high quality studies with robust design and larger sample size to better characterise the expression of MMP-8 in the saliva of periodontitis patients (Zhang et al., 2018). Similarly, two systematic reviews agreed that MMP-8 in saliva possessed good capability to detect periodontitis (Arias-Bujanda et al., 2020; Kc et al., 2020).

The level of MMP-8 in saliva was significantly reduced six months after non-surgical periodontal therapy (NSPT). In addition, significantly lower MMP-8 levels were detected after treatment among responders compared to non-responders. In this case-control study, responders were defined as individuals exhibiting 20% improvement in percentages of sites with BOP, CAL≥2 mm, PPD>4 mm and PPD> 5 mm (Sexton et al., 2011). Similar outcomes were observed in another interventional study, although the reduction in MMP-8 levels was only significant three weeks after NSPT. The MMP-8 levels six weeks later were not significantly different from baseline values (Kim, 2022).

The ability of MMP-8 to discriminate periodontitis was apparent even in the presence of type II DM. Among 61 diabetic individuals, elevated levels of MMP-8 were predictive of periodontitis with an odds ratio (OR) of 5.09 (95% CI 1.24 – 20.92; p = 0.03) (Miller et al., 2021). This is instructive as DM has long been regarded as a confounder for the diagnostic capacity of saliva in periodontal disease and is usually part of the exclusion criteria for these studies.

These promising results have spurred efforts to conduct translational research that incorporates MMP-8 into chairside diagnostic test kits. A product of this research endeavour is the activated matrix metalloproteinase-8 (aMMP-8) PCOT, which demonstrated some value as a diagnostic tool for periodontal and peri-implant diseases, prognostic tool for disease progression and monitoring tool for treatment response (Räisänen et al., 2023). This chairside test had recently shown potential in the early

detection of incipient periodontitis among a cohort of Finnish adolescent, as all subjects with stage I periodontitis within this cohort tested positive for the aMMP-8 PCOT (>20 ng/ml) (Heikkinen et al., 2022). The practical applications of the aMMP-8 PCOT extend beyond systemically healthy individuals. It has been investigated in population groups with DM, COVID-19, reproductive health issues and those undergoing radiotherapy for head and neck cancer (Grigoriadis et al., 2019; Keskin et al., 2020; Sorsa et al., 2022; Sorsa et al., 2021).

In addition, a research team in the United Kingdom developed a novel prototype biosensor based on specific antibodies and surface acoustic wave technology to quantify the levels of aMMP-8 in patients with periodontal diseases (Taylor et al., 2019). The biosensor featured an assay time of 20 minutes and detection limit of 62.5 ng/ml. The measured salivary MMP-8 levels of the biosensor was compared against more conventional immunoassay techniques like the time-resolved immunofluorometric assay (IFMA) and ELISA, showing significant correlation with ELISA ($r = 0.681$, $P=0.001$) and IFMA ($r = 0.354$, $P<0.001$) (Umeizudike et al., 2022). The strength of correlation of the biosensor is considered to be moderate for ELISA and weak for IFMA (Schober et al., 2018). The lower correlation between biosensor and IFMA could be explained by their different affinities for the different forms of MMP-8. The specific antibodies of the biosensor detected both the active and latent forms of MMP-8, while the IFMA had higher affinity for aMMP-8. In other words, the IFMA was only detecting a fraction of the MMP-8 measured by the biosensors and ELISA. In addition, all three methods demonstrated the area under the curve (AUC) values approximating 0.8 for discriminating periodontitis and gingivitis from healthy controls. The actual AUC values for biosensor, IFMA and ELISA were 0.808, 0.782 and 0.857, respectively (Umeizudike et al., 2022). In Italy, a research group devised a surface plasmon resonance-plastic optic fiber-based biosensor to detect salivary MMP-8 with a lower detection limit of 9.9 ng/ml (Guida et al., 2023). A recent

systematic review, based on meta-analysis of six studies, demonstrated acceptable diagnostic performance of an aMMP-8 POCT, irrespective of the oral fluid types, diagnostic thresholds or POCT modalities. The thresholds for a positive test of the various POCT devices ranged from 10 ng/ml to 25 ng/ml. The pooled sensitivity and specificity rates were 63% and 84%, respectively (Wei et al., 2024).

The wealth of scientific evidence behind MMP-8 has led to a proposition for the inclusion of aMMP-8 as a key biomarker to supplement the existing classification of periodontitis and peri-implantitis. In this proposed modified classification, aMMP-8 levels in mouthrinse, GCF or peri-implant sulcular fluid of 0 – 19.9 ng/ml, >20 ng/ml and >30 ng/ml were designated as modifiers for grade A, B and C, respectively (Sorsa et al., 2022).

#### 2.3.2.5 Matrix metalloproteinase-9

The expression of latent form of MMP-9 had been demonstrated in gingival tissues of periodontitis patients, with its active form being detected in tissues associated with clinical disease. In addition, the amounts of the active form of MMP-9 were positively correlated with the number of CD22-positive B-cells, forming a plausible mechanism by which B-cells contribute to tissue destruction in periodontitis (Pan et al., 2019; Seymour et al., 2022).

Like MMP-8, MMP-9 was detected in higher concentration in the presence of periodontitis (Kim, Kim, et al., 2020; Mäkelä et al., 1994; Ramseier et al., 2009). Grant et al. (2022) conducted a study for biomarker discovery and validation in two stages. The first stage set out to identify differentially expressed proteins in saliva and GCF using mass spectrometry, a hypothesis-free proteomic technique. Subsequently, the levels of 15 candidate proteins were further measured using ELISA and compared between healthy and gingivitis, between healthy or gingivitis and periodontitis, and between stage I/II and

stage III/IV periodontitis. Across all comparisons, MMP-9, alongside other proteins presented significant discriminative ability between the paired periodontal status, with AUC ranging from 0.764 – 0.972 (Grant et al., 2022).

A lateral flow test (LFT) PCOT measuring MMP-9 levels was developed for the purpose of periodontitis screening. This novel MMP-9 LFT PCOT was tested among a sample population of 137 adults in a national dental hospital, showing good diagnostic accuracy for stage II – IV periodontitis with sensitivity and specificity values of 92% and 72%, respectively (Kim, Lee, et al., 2020).

#### 2.3.2.6 Metallothionein

The link between oxidative stress and periodontal diseases is supported by an increasing body of evidence, as indicated by a recent systematic review. In the review, significant decrease in total antioxidant capacity and a significant increase in malondialdehyde (MDA), nitric oxide, total oxidant status and 8-hydroxy-deoxyguanosine levels were observed in the saliva of chronic periodontitis patients (Chen et al., 2019). Moreover, there was a significant correlation between MDA and C-terminal telopeptide of type I collagen, a marker of alveolar bone loss (Miricescu et al., 2014).

Metallothionein is a cysteine-rich protein that serves a biological role in pathological processes associated with oxidative stress via scavenging of reactive oxygen species, heavy metal detoxification and regulation of zinc homeostasis (Aziz et al., 2021). Very little is known about the expression of MT in periodontitis patients. The review article did not identify any studies on MT in relation to periodontitis (Chen et al., 2019). One of the earliest documented evidence of such an association was found in a comparative study of MT levels and its distribution within gingival biopsies samples from smokers or non-smokers with advanced periodontitis. Tissues samples were harvested during periodontal flap surgery and subjected to mono-clonal antibody and

immunohistochemistry (IHC) staining. Smokers with periodontitis presented with higher MT levels and MT-positive cell ratio than non-smokers with comparable level of periodontal destruction (Katsuragi et al., 1997). As a control group of periodontally healthy subjects was not included, the effect of periodontal disease on MT levels cannot be ascertained.

More recently, a cross-sectional study compared the serum, saliva and GCF levels of MT among smokers and non-smokers with chronic periodontitis or periodontal health. The group of chronic periodontitis patients who were also smokers had significantly higher levels of MT compared to the other groups (Yadav et al., 2021). The same research group then performed an interventional study by providing NSPT for the periodontitis groups and measuring the levels of MT in biofluids three months later. In addition, the research group also measured the mRNA expression of MT in gingival tissue samples using quantitative real-time polymerase chain reaction (qPCR). A significant reduction in MT mRNA expression levels and levels of MT in biofluids was detected after NSPT (Yadav et al., 2024).

A summary of the biological roles and cellular sources of the various biomarkers included in the present study is outlined in Table 2.1. As discussed previously, the upregulation of these biomarkers could be detected in the saliva of periodontitis subjects, with more robust and consistent results being observed for IL-1β, IL-6, MMP-8 and MMP-9.

**Table 2.1. Biological functions and cellular sources of biomarkers investigated in the present study.**

| Biomarker | Function | Cellular source | Supporting literature |
|---|---|---|---|
| IL-1β | • Stimulate the differentiation and polarisation of myeloid and lymphoid cells.<br>• Induce the activation and expansion of Th1 and Th2 cells.<br>• Induce the expression of RANKL and stimulate osteoclastogenesis. | Macrophage<br>B-cell<br>Osteoclast precursor<br>Mature osteoclast | (Hofbauer et al., 1999) |
| IL-6 | • Activate B-cells.<br>• Regulate balance of $CD4^+$ T-cell populations.<br>• Influence myeloid cell differentiation. | Macrophage<br>T-cell<br>Endothelial cell<br>Fibroblast<br>Osteoclast precursor<br>Mature osteoclast | (Seymour et al., 2022) |
| TNF-α | • Stimulate production of chemokines.<br>• Induce release of neutrophil extracellular traps.<br>• Induce the expression of RANKL and stimulate osteoclastogenesis. | Macrophage<br>Fibroblast<br>Activated T-cell<br>Mast cell<br>Osteoclast precursor<br>Mature osteoclast | (Hofbauer et al., 1999; Remijsen et al., 2011) |
| MMP-8 | Degradation of extracellular matrix. | Neutrophil<br>Macrophage<br>Fibroblast<br>Epithelial cell | (Seymour et al., 2022) |
| MMP-9 | Degradation of extracellular matrix. | Fibroblast<br>Macrophage<br>Epithelial cell<br>Polymorphonuclear neutrophil | (Seymour et al., 2022) |
| MT | Free radicals scavenger.<br>Heavy metal detoxification.<br>Regulate zinc homeostasis. | No information | (Aziz et al., 2021) |

IL-1β, interleukin-1beta; IL-6, interleukin-6; TNF-α, tumour necrosis factor-alpha; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9, MT, metallothionein, Th1, T-helper 1; Th2, T-helper 2; RANKL, receptor activator of nuclear factor kappa-B ligand.

## 2.4 Panel of biomarkers

Findings from numerous clinical studies supported the notion that biomarker combinations exhibit better diagnostic performance in discriminating periodontal disease states than single biomarkers (Blanco-Pintos et al., 2023; Bostanci et al., 2021; Ebersole et al., 2015; Wu et al., 2017). In a clinical study of 57 Taiwanese adults, the combinations of IL-1β, interleukin-1 receptor antagonist and MMP-9 produced a high AUC of 0.853 with 73.3% sensitivity and 88.9% specificity (Wu et al., 2017).

A recent systematic review on the diagnostic accuracy of single molecular biomarkers for the detection of clinically diagnosed periodontitis in systemically healthy subjects identified five biomarkers that were eligible for meta-analysis using the Hierarchical Summary Receiver Operating Characteristic model. The sensitivity values for the diagnosis of periodontitis, in descending order belonged to IL-1β (78.7%), MMP-8 (72.5%), IL-6 (72%), haemoglobin (72%) and MMP-9 (70.3%) (Arias-Bujanda et al., 2020). Notably, the sensitivity estimates were below 80% for all of the biomarkers investigated.

Another systematic review was performed to determine the accuracy of biomarker combinations to diagnose clinically assessed periodontitis in systemically healthy subjects. Meta-analyses were performed for biomarker combinations evaluated in at least three contingency tables across two independent studies. Six out of 47 salivary biomarker combinations were eligible for meta-analyses using hierarchical summary receiver operating characteristic modelling (Blanco-Pintos et al., 2023). The analysis indicated that combining IL-1β, IL-6 and MMP-8 in pairs resulted in improved sensitivity (>82%) and specificity (>80%) estimates (Blanco-Pintos et al., 2023), which were higher than those reported for single biomarkers as reported by Arias-Bujanda et al. (2020).

Numerous studies highlighted tremendous diagnostic potential of selected biomarkers, either individually or in combination to detect periodontitis (Arias-Bujanda et al., 2020; Blanco-Pintos et al., 2023; Bostanci et al., 2021; de Lima et al., 2016; Kc et al., 2020). However, it is also evident that the trend of biomarker expression (upregulated or downregulated) did not correspond to similar concentrations in different populations. Other sources of heterogeneity aside, the wide range of salivary biomarker levels seen strongly suggested that different study populations harbour different biomarker expression profiles and hence, different thresholds when biomarkers are used to differentiate between different periodontal status. In other words, the thresholds determined in other clinical studies had low generalisability. In addition, preliminary evidence from a cross-investigation highlighted racial differences in the expression of IL-6 between Blacks and Whites, although no differences were seen for IL-10 and TNF-α (Paalani et al., 2011). Therefore, there is a genuine need to perform high quality clinical studies to validate the differential expression and diagnostic value of salivary biomarkers in the context of the local population.

The six candidate biomarkers in this cross-sectional study were selected by virtue of their known or purported biological roles in the pathogenesis of periodontal diseases. To recapitulate briefly, IL-1β, IL-6 and TNF-α are associated with host inflammation; MMP-8 and MMP-9 with tissue destruction; and MT with oxidative stress.

## 2.5 ELISA

Quantitative analytical tests that assess the presence and concentration of molecules in biological fluids by measuring the colour change induced by antigen-antibody reaction obtained through enzyme-linked conjugate and enzyme substrate are generally known as enzyme immunoassay or ELISA (Hornbeck, 2015). Out of the various techniques, ELISA is commonly used in salivary biomarker studies to quantify the concentration of selected biomarkers. Its high sensitivity to detect proteins present at low

abundance and high specificity due to strict antibody-antigen binding offer advantages over qualitative techniques such as immunohistochemistry.

The invention of ELISA can be traced back to the 1960s, when a radioactive label-based immunoassay was utilised to measure plasma insulin levels (Yalow & Berson, 1960). In 1971, two research groups independently developed the enzyme-based immunoassay to quantify the levels of immunoglobulin G in rabbit serum and human chorionic gonadotropin in urine samples (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971).

The concept of ELISA is based on the premise of using enzymes to detect and quantify the specific antigen-antibody interactions. Both antigens and antibodies are adsorbed to a solid phase that is usually manufactured in rigid polystyrene, polyvinyl or polypropylene tubes or microplates. Examples of enzymes used in ELISA include beta-galactosidase, glucose oxidase, peroxidase and alkaline phosphatase. Depending on the type of enzyme used, the substrate can either be alkaline phosphatase, P-nitro-phenyl phosphate, 5-amino salicylic acid or orthophenylene diamine. After allowing for 30 – 60 minutes for the enzyme substrate reaction to be completed, the reaction is stopped with sodium hydroxide, hydrochloric acid or sulfuric acid. The optical densities are then recorded using a spectrophotometer at a wavelength of 400 – 600 nm (Aydin, 2015).

Enzymatic immunoassay methods can be homogenous or heterogenous, the latter of which is by far more commonly used and involves washing steps to remove the free antigens from the bound antigen-antibody complexes adhering to the microplate walls (Aydin, 2015).

Different types of ELISA exist, namely direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA. For direct ELISA, the surface of the plate is directly coated with antigen or antibody for the protein of interest. The sequential steps of adding

enzyme-tagged antibody/antigen, incubation, washing and addition of substrate solution give rise to the colorimetric reaction that is measured to determine the concentration of antigen/antibody (Engvall, 2010; Hornbeck, 2015). The indirect ELISA differs in that an enzyme-tagged secondary antibody is used to isolate the target antigen by binding to the complexes formed from the interaction of the antigen and the primary antibody (Lindström & Wager, 1978). Meanwhile, the sandwich ELISA derives its name from the fact that the antigens present within the samples are stuck in between the antibody molecules coated onto the microplate wells and the enzyme-tagged antibody molecules. The sandwich ELISA is reported to be two to five times more sensitive than other ELISA types. Lastly, competitive ELISA is distinguished by the fact that samples and enzyme-tagged antibody/antigen are added to the well concurrently, which is coated with antigen-specific antibody or antibody-specific antigen. The tagged and untagged antigen/antibody molecules compete with one another to bind to the antibody/antigen coated onto the wells. As such, there is an inverse relationship between biomarker concentration and the intensity of colorimetric reaction. A higher absorbance value indicates a lower amount of target protein present in the sample (Aydin, 2015). In spite of inherent differences in the screening method and sensitivities of the different ELISA types, all share a common feature of being capable of detecting small quantities of substrates, antigens or antibodies in a rapid and reproducible manner.

## 2.6 Alternative protein measurement techniques

Aside from ELISA, other techniques commonly employed for protein detection include Western blot and mass spectrometry. Western blot analysis relies on the interaction between antibodies and specific antigens in the biological samples. Proteins are separated by electrophoresis and transferred to a membrane. Subsequently, primary and secondary antibodies are used to bind and identify target proteins. Protein extraction is a critical step in the Western blotting process, and the multiple extraction, fractionation

and purification methods have to be selected based on the location of target proteins (nuclear, mitochondrial or transmembrane) and the types of cells or tissues that contain the protein of interest (Pillai-Kastoori et al., 2020).

The protein analysis capabilities of mass spectrometry, on the other hand, are derived from its ability to provide highly accurate molecular weight information on peptide constituents, down to the attomole level (Trauger et al., 2002). These techniques are less equipped for measuring multiple different proteins in a single biological sample simultaneously (Cohen & Walt, 2019). Multiplexed protein detection assays are more suited for high throughput analyses due to the low cost and time involved. Examples of multiplex systems include protein microarrays and flow cytometry. However, multiplexing suffers from some drawbacks such as non-specific binding and cross reactivity between affinity reagents, secondary labels and other constituents in the biological sample (Cohen & Walt, 2019).

Nuclei acid-based detection methods are also used for protein detection, whereby the DNA of the target proteins can be amplified with polymerase chain reaction (PCR) (Fredriksson et al., 2002). However, nucleic acid-based approaches are more expensive to conduct and fraught with technical complexities. A cheap and rapid way of protein detection, that has generated significant clinical interest as POCT is the lateral flow assay. Pregnancy test and COVID-19 LFT are two examples of the lateral flow assay technology. However, LFT mostly offers qualitative or at best semiquantitative protein measurements (Posthuma-Trumpie et al., 2009).

In the techniques described hitherto, the proteins are removed from their native environment, which may impede the understanding of their role in health and disease states. In such instances, optimal imaging-based methods such as immunohistochemistry or immunocytochemistry are indispensable for the study of proteins in their native cellular

environment (Coons et al., 1941). This is not so pertinent for the present study, however, as saliva samples instead of tissue samples were used.

Among the numerous protein measurement options available, ELISA was chosen for this study as it is a widely used, sensitive and convenient technique. Western blot is not suitable as it measures proteins intracellularly, whereas the adoption of mass spectrometry is hampered by its high cost and technical complexity.

### 2.7 Self-reported oral health questionnaire

A collaboration between the Centers for Disease Control and Prevention (CDC) and the American Academy of Periodontology (AAP) in 2003 heralded the search for appropriate questions for a self-reporting questionnaire to predict the prevalence of periodontitis (Eke & Genco, 2007). A preliminary set of six questions was first field tested in the Australian population in conjunction with the Australian National Survey of Adult Oral Health. A multivariate model incorporating these self-reported measures and five risk indicators for periodontitis demonstrated modest predictive capacity for moderate/severe periodontitis, with a combined sensitivity and specificity value of 1.39 (Slade, 2007). The final version of the questionnaire contained eight questions, which was first field-tested in a pilot study involving a convenience sample of 456 volunteers (Eke & Dye, 2009), and thereafter in a nationally representative sample of 3743 individuals who participated in the 2009 – 2010 rendition of the NHANES (Eke et al., 2013).

Since its inception, the CDC/AAP eight-item self-reported measures were adapted and modified for populations in different parts of the world, including Portugal (Machado et al., 2022), Spain (Montero et al., 2020; Saka-Herrán et al., 2020), Japan (Iwasaki et al., 2021), New Zealand (Foster Page et al., 2016), Brazil (Cyrino et al., 2011), Hong Kong (Deng et al., 2021) and France (Carra et al., 2018). Iwasaki and co-workers validated a Japanese nine-item self-reported questionnaire against periodontitis diagnosis derived

from full-mouth clinical examination among 949 adults. They assessed the AUC, sensitivities and specificities of various predictive models incorporating the SROH, demographics and health-related variables. Parsimonious models were developed from multivariate logistic regression analysis, retaining the oral health questions on "gum disease", loose tooth", "lost bone" and "bleeding gums" as significant predictors in the prediction for both severe periodontitis and total periodontitis (Iwasaki et al., 2021).

The French version of SROH was a 12-item questionnaire that included, in addition to the original eight questions developed by CDC/AAP, four questions regarding gum bleeding, food impaction, longer teeth and root exposure. Multivariate logistic regression analysis of a model encompassing these 12 items yielded sensitivity of 71.8% and specificity of 70.9%. The sensitivity and specificity values increased to 77.2% and 76.7% when the questionnaire items were combined with demographic/clinical variables which included age, smoking, number of teeth and education level. Moreover, a simplified and reduced model using only predictors that were statistically significant or clinically relevant was used to generate a periodontal screening score. The seven predictors contained within this parsimonious model included age, smoking status and questions on self-appraisal of teeth and gum health, treatment for gum disease, loose teeth, lost bone and teeth appearance. The Periodontal Screening Score had an overall diagnostic accuracy of 82% in differentiating severe periodontitis group from the other subjects. The score ranged from 0 – 13, with the score ≥5 presenting with a good balance between sensitivity (78.9%) and specificity (74.8%) (Carra et al., 2018).

The diagnostic value of four self-reported periodontal questions was investigated among a New Zealand birth cohort at the age of 38. Out of the 1037 original members of the birth cohort, 895 individuals provided complete data on self-reported periodontal status, smoking status and periodontal examination for further analysis. The four questions were "gum disease", "lost bone", "loose teeth" and "had gum treatment before".

When the outcome of interest was one or more sites with CAL ≥4 mm, two self-reported questions (gum disease and loose teeth) were identified as significant predictors. These same two questions were retained in the predictive model for sites with CAL ≥5 mm and ≥6 mm, with the addition of a third question into the model relating to "history of gum treatment" and "lost bone", respectively. For the outcome CAL ≥5 mm, the question on "lost bone" was very close to statistical significance (P = 0.05) (Foster Page et al., 2016). The research team did not elaborate on the reasoning behind using only four out of the eight original SROH questions. While most studies combined different permutations of SROH questions under a single predictive model, a recent cross-sectional study of participants in the NHANES 2011 – 2014 documented varying levels of sensitivity and specificity for CDC/AAP's classification of moderate-to-severe periodontitis using individual SROH items (Bond et al., 2024).

Heterogeneity in the prevalence of periodontitis, case definitions for periodontitis, number and format of self-reported periodontal questions complicate direct comparison between these different studies. In addition, there were variations in the administration of the SROH. Some studies used self-administered questionnaire, whereas others delivered the questions via face-to-face interviews. Regardless, most studies agreed that SROH had tangible and measurable value in estimating the prevalence of periodontal disease as an alternative to full-mouth periodontal examination (Abbood et al., 2016; Carra et al., 2018; Cyrino et al., 2011; Eke et al., 2013; Foster Page et al., 2016; Iwasaki et al., 2021; Machado et al., 2022; Montero et al., 2020).

The CDC/AAP SROH is by no means the only self-reported questionnaire for periodontitis screening and surveillance. Other self-reported tools had been developed, but none were investigated as extensively or adopted as widely as the CDC/AAP SROH (Abbood et al., 2016; Blicher et al., 2005; Renatus et al., 2016; Taylor & Borgnakke, 2007; Yamamoto et al., 2009).

A systematic review of self-reported measures for periodontal diseases concluded that there was acceptable validity in using self-reported measures to screen for periodontitis. The sensitivities and specificities of the included studies ranged from 4% to 93% and 58% to 94%, respectively. The authors, however, stressed the need for more large, well-designed diagnostic studies assessing the validity of these self-reported questions (Abbood et al., 2016). To the best of our knowledge, self-reported measures for periodontitis have not been validated in the Malaysian population. An overview of studies published to date on the validity of the SROH in diverse demographic populations is presented in Table 2.2.

**Table 2.2. Overview of research studies reporting on the original or modified version of the CDC/AAP self-reported oral health questionnaire.**

| No. | Authors | Country | Study population | SROH items | Periodontal outcomes of interest | Diagnostic performance |
|---|---|---|---|---|---|---|
| 1 | Machado et al. (2022) | Portugal | 103 first-time patients to a university dental clinic. | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose teeth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Gum bleeding<br>9. Gum bleeding in last 3 months<br>10. Loose teeth lost<br>11. Gum pain<br>12. Gum recession<br>13. Visible roots | 1. 2017 World Workshop classification of periodontitis<br>2. CDC/AAP case definitions for mild and severe periodontitis | AUC: 0.49 – 0.86<br>Sensitivity: 79.5 – 100%<br>Specificity: 0 – 82.5% |
| 2 | Iwasaki et al. (2021) | Japan | 949 Japanese adults | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose tooth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Mouthwash<br>9. Bleeding gums | CDC/AAP case definitions for mild, moderate and severe periodontitis | AUC: 0.63 – 0.88<br>Sensitivity: 47.5 – 80.8%<br>Specificity: 44.3 – 82.6% |
| 3 | Foster Page et al. (2016) | New Zealand | 895 38-year-old adults from the Dunedin Multidisciplinary Health and Development Study | 1. Have gum disease<br>2. Lost bone<br>3. Had gum treatment<br>4. Loose tooth | Prevalence of patients with at least 1 site with attachment loss ≥4 mm, ≥5 mm or ≥6 mm | AUC: 0.69 – 0.84<br>Sensitivity: 25 – 52%<br>Specificity: 81 – 98% |

**Table 2.2, continued**

| No. | Authors | Country | Study population | SROH items | Periodontal outcomes of interest | Diagnostic performance |
|---|---|---|---|---|---|---|
| 4 | Carra et al. (2018) | France | A convenience sample of 232 adults who visited a Paris health centre. | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose tooth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Mouthwash use<br>9. Bleeding gums<br>10. Food impaction<br>11. Tooth getting longer<br>12. Exposure of tooth roots | CDC/AAP case definition for severe periodontitis | AUC: 0.778 – 0.845<br>Sensitivity: 71.8 – 77.2%<br>Specificity: 70.9 – 76.7% |
| 5 | Montero et al. (2020) | Spain | 231 Spanish adults from the Di@bet.es II study | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose tooth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Mouthwash use | 1. CDC/AAP case definition for severe periodontitis<br>2. ≥50% of teeth with CAL ≥5 mm<br>3. ≥25% of teeth with PPD ≥6 mm | AUC: 0.64 – 0.81<br>Sensitivity: 57.7 – 84.5%<br>Specificity: 40.1 – 82.8% |

**Table 2.2, continued**

| No. | Authors | Country | Study population | SROH items | Periodontal outcomes of interest | Diagnostic performance |
|---|---|---|---|---|---|---|
| 6 | Saka-Herrán et al. (2020) | Spain | 112 adults from two hospitals in Spain | 1. Periodontal disease<br>2. Gum disease<br>3. Periodontal disease diagnosis by dental professionals<br>4. Bone loss/deep pockets<br>5. Teeth movement or loosening<br>6. Long teeth/receding gums<br>7. Visible tooth roots<br>8. Gum pain<br>9. Floss use<br>10. Visit to periodontist<br>11. Scaling/root planing<br>12. Teeth loss due to mobility<br>13. Gum bleeding | 1. SEPA classification: PPD ≥4 mm in at least one sextant and/or grade II to III furcation defects.<br>2. 2017 World Workshop classification of stage I to IV periodontitis.<br>3. CDC/AAP case definitions for mild, moderate and severe periodontitis. | AUC: 0.69 – 0.89<br>Sensitivity: 66.7 – 92.2%<br>Specificity: 62.3 – 90.2% |
| 7 | Deng et al. (2021) | Hong Kong | A convenience sample of 408 adults who visited the Prince Philip Dental Hospital. | 1. Gum disease<br>2. Teeth/gum health<br>3. a) Coronal scaling<br>b) Scaling and root planing<br>4. Loose teeth<br>5. Bone loss<br>6. Tooth appearance<br>7. Floss use<br>8. Mouthwash | 2017 World Workshop classification<br>1. Periodontal disease (gingivitis and periodontitis)<br>2. Periodontitis<br>3. Stage I/II periodontitis<br>4. Stage III/IV periodontitis | AUC: 0.608 – 0.953<br>Sensitivity: 61.4 – 95.7%<br>Specificity: 35.3 – 91.1% |
| 8 | Eke et al. (2013) | United States | 3743 adults aged 30 years or older from the NHANES 2009 – 2010. | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose tooth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Mouthwash | 1. CDC/AAP case definitions for total periodontitis (mild, moderate and severe<br>2. CAL ≥3 mm<br>3. PPD ≥4 mm | AUC: 0.63 – 0.82<br>Sensitivity: 59.3 – 98.5%<br>Specificity: 0 – 58.4% |

**Table 2.2, continued**

| No. | Authors | Country | Study population | SROH items | Periodontal outcomes of interest | Diagnostic performance |
|---|---|---|---|---|---|---|
| 9 | Eke and Dye (2009) | United States | Convenience sample of 456 United States adults | 1. Have gum disease<br>2. Teeth/gum health<br>3. Had gum treatment<br>4. Loose tooth<br>5. Lost bone<br>6. Tooth does not look right<br>7. Floss use<br>8. Mouthwash | CDC/AAP case definitions for moderate and severe periodontitis | AUC: 0.7 – 0.94<br>Sensitivity: 48 – 63.6%<br>Specificity: 72 – 98.4% |
| 10 | Slade (2007) | Australia | 2999 adults from the Australian National Survey of Adults Oral Health | 1. Have gum disease<br>2. Lost bone<br>3. Had gum treatment<br>4. Loose tooth<br>5. Mouthwash<br>6. Floss use | CDC/AAP case definitions for moderate and severe periodontitis | AUC: 0.63 to 0.75 or higher<br>Sensitivity: 23 – 58%<br>Specificity: 69 – 96% |

AUC, Area under curve; CAL, clinical attachment loss; CDC/AAP, Centre for Disease Control/American Academy of Periodontology; NHANES, National Health and Nutrition Examination Survey; PPD, probing pocket depth; SEPA, Spanish Society of Periodontics and Osseointegration; SROH, self-reported oral health questionnaire.

## 2.8 Adaptation and validation process of a health measurement instrument

As a health measurement instrument, the SROH should undergo a rigorous and scientifically robust adaptation and validation process to ensure its accuracy, reliability, and cultural relevance in a new population (Guillemin et al., 1993). The Consensus-Based Standards for the Selection of Health Measurement Instruments (COSMIN) was developed as a foundational framework that can be applied for questionnaire validation, particularly in cross-cultural settings (Mokkink et al., 2010).

Content validity is a core aspect of the validation process when adapting an instrument for a new population (Shultz et al., 2020). It determines if the questionnaire's items adequately cover the constructs it is supposed to measure. Content validity is evaluated by expert panels who review the items for their relevance, clarity and comprehensiveness for the target population (Guillemin et al., 1993). With respect to oral health questionnaires, the expert panels would be comprised of dental professionals well-versed with Malaysian oral health issues and the socio-cultural factors that may influence oral health perceptions. A widely reported measure of content validity is the content validity index (CVI), which can be broadly divided into item-level content validity index (I-CVI) and scale-level content validity index (S-CVI). The definitions of the different types of CVI are shown in Table 2.3.

**Table 2.3. Definitions of content validity terms (Polit & Beck, 2006).**

| No. | Content validity terms | Definition |
|---|---|---|
| 1 | I-CVI | Validity of individual items: Proportion of content experts rating an item as 3 (quite relevant) or 4 (highly relevant) on a 4-point scale. |
| 2 | S-CVI/UA | Proportion of items on a scale that receive relevance rating of 3 or 4 by all content experts. |
| 3 | S-CVI/Ave | Average of the I-CVIs for all items on a scale. |

I-CVI, item-level content validity index; S-CVI/Ave, scale-level content validity index, averaging calculation method; S-CVI/UA, scale-level content validity index, universal agreement calculation method.

This is usually followed by a pilot study, involving a sample from the local population to further assess the face validity of the adapted instrument, ensuring that respondents comprehend the questions as intended. In addition, factor analysis is routinely employed to verify the dimensionality of the questionnaire, determining the number of underlying constructs and whether the original factors are retained in the new population. To ensure that the instrument consistently measures the same constructs, internal consistency is evaluated. To that end, Cronbach's alpha is the most commonly used statistical test, with a value of 0.70 or higher generally considered acceptable (Cronbach, 1951). Good internal consistency means that the items within the questionnaire are correlated, thus reliably capturing the target construct. Test-retest reliability is another critical metric, whereby the same participants complete the questionnaire after a time lapse, and their responses across time are compared to determine the stability of the instrument over time (Crocker & Algina, 2008). This is crucial in ensuring that the questionnaire elicits consistent responses when used in repeated assessments within the same population.

Criterion validity evaluates the performance of the instrument in comparison to a gold standard or benchmark test (Shultz et al., 2020). With respect to SROH, criterion validity would be demonstrated by correlating questionnaire responses with full-mouth periodontal examination. It is vital that self-reported data accurately reflect clinically assessed periodontal status. Studies in diverse populations have attested to the predictive validity of the SROH against periodontal examination (Carra et al., 2018; Eke et al., 2013; Foster Page et al., 2016; Iwasaki et al., 2021).

In summary, the validation of SROH for use in the Malaysian population requires a multi-step process rooted in well-established scientific principles. Content validity, construct validity, reliability, internal consistency and criterion validity should be evaluated and judged to be appropriate for application in clinical and research settings.

Now, we will explore if merging salivary biomarker levels and SROH into predictive models for periodontitis is a scientifically valid concept.

### 2.9 Predictive models for periodontal diseases combining self-reported oral health questions and salivary biomarker concentrations

A research group from Netherlands investigated the accuracy of predictive models incorporating a Dutch translation of the SROH, biomarkers from oral rinse samples and demographic characteristics to detect periodontitis. The biomarkers under investigation included albumin levels, chitinase activity, proteinase activity and MMP-8. It was demonstrated that the model combining all three groups of parameters had an AUROCC of 0.91 for total periodontitis, with sensitivity and specificity values of 80% and 88%, respectively. When salivary biomarkers were omitted from the model, the predictive performance dropped slightly to an AUROCC of 0.88, sensitivity of 78% and specificity of 84%. The authors concluded that predictive models for screening periodontitis based on SROH, demographic features with or without biomarkers were feasible and accurate (Verhulst et al., 2019). Two algorithms, one for total periodontitis and another for severe periodontitis based on age and SROH were devised and used to develop a screening tool.

This screening tool was later validated by the same research group among patients in an outpatient medical setting. Among this cohort, the algorithm for total periodontitis and severe periodontitis attained an AUROCC of 0.59 and 0.72, respectively (Nijland et al., 2021). The authors attributed the reduced accuracy of the prediction models to the fact that CPITN was used as the gold standard, which might have overestimated the prevalence of periodontitis cases by including all cases of CPITN code 3. It is plausible that a proportion of these CPITN code 3 cases were due to reduced resistance to probe penetration due to clinical inflammation instead of true attachment loss that is characteristic of periodontitis. The limited evidence to date justified the need to conduct

additional studies to assess the diagnostic accuracy of models combining salivary biomarkers and SROH responses in discriminating periodontitis patients.

# CHAPTER 3. METHODOLOGY

This cross-sectional study was conducted in three parts. Part I involved the adaptation and validation of the SROH. Psychometric properties of the SROH were analysed, including content validity, face validity, reliability (i.e., internal consistency and test-retest reliability), construct validity, and concurrent validity. A pilot study was conducted as part of the psychometric analysis, which included the clinical examination of patients with periodontal health, gingivitis, and periodontitis. Prior to the clinical examination, the SROH was administered, and the responses were compared to the findings from a full-mouth periodontal examination. Concurrently, saliva specimens were collected from participants for laboratory analysis. Part II focused on the measurement of selected biomarkers from saliva samples using ELISA. Finally, Part III centred on the development of multivariate predictive models for periodontitis. Various models were tested, including those incorporating all variables, social demographic characteristics and SROH, or SROH alone.

## 3.1 Ethical considerations

Ethical approval for the study was granted by the Faculty of Dentistry Medical Ethics Committee, Faculty of Dentistry, Universiti Malaya with the reference number DF RD2013/0064 (P).

## 3.2 Part 1: Questionnaire validation and reliability testing

Figure 3.1 illustrates the framework for the adaptation and validation of the SROH among the Malaysian population.

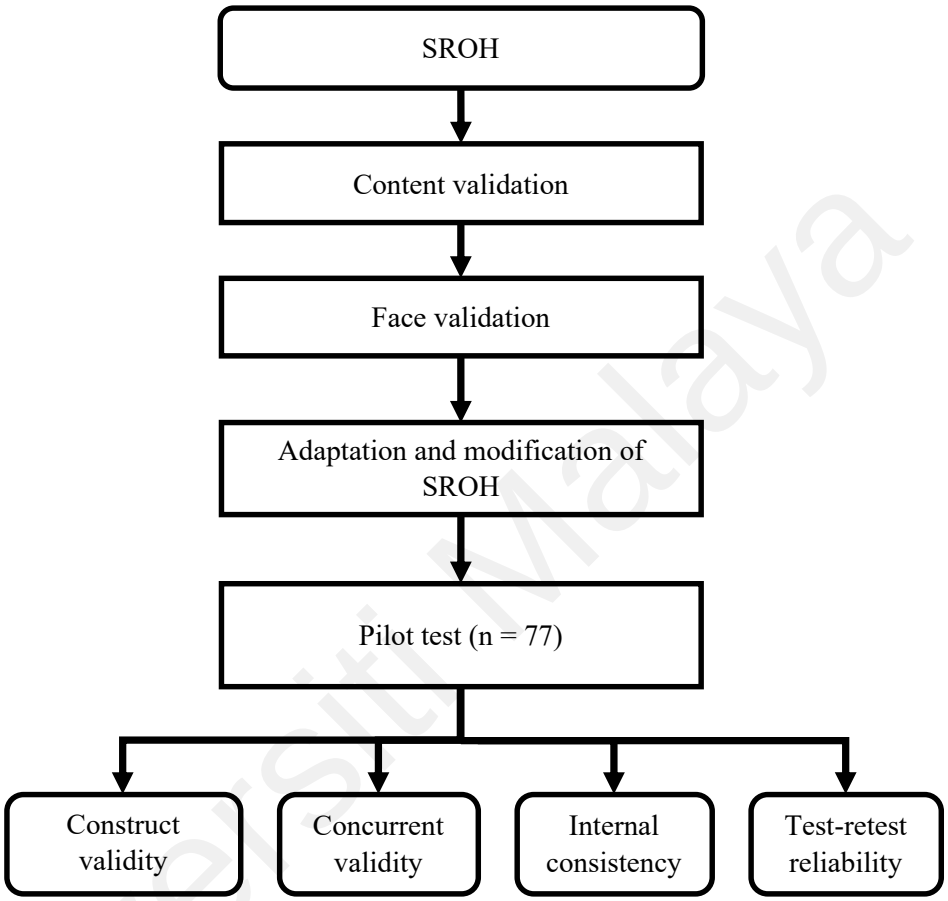



**Figure 3.1. The framework for the adaptation and validation of the self-reported oral health questionnaire among the Malaysian population.**
**SROH, self-reported oral health questionnaire.**

### 3.2.1 The self-reported oral health questionnaire

The original eight-item SROH by CDC/AAP was developed with the objective of predicting the prevalence of periodontitis in the US population (Eke & Genco, 2007). It has been validated in diverse populations around the world including the United States, Portugal, Spain, Japan, New Zealand, Japan, Brazil, and France (Carra et al., 2018; Cyrino et al., 2011; Eke et al., 2013; Foster Page et al., 2016; Iwasaki et al., 2021; Machado et

al., 2022; Montero et al., 2020; Saka-Herrán et al., 2020). The items in the SROH are presented in Table 3.1.

**Table 3.1. The original eight-item self-reported oral health questionnaire.**

| Question number | Question topic | Question | Response options |
|---|---|---|---|
| 1 | Gum disease | Do you think you might have gum disease? | Yes<br>No<br>Refused<br>Don't know |
| 2 | Teeth/gum health | Overall, how would you rate the health of your teeth and gums? | Excellent<br>Very good<br>Good<br>Fair<br>Poor<br>Refused<br>Don't know |
| 3 | Gum treatment | Have you ever had treatment for gum disease such as scaling and root planing, sometimes called "deep cleaning"? | Yes<br>No<br>Refused<br>Don't know |
| 4 | Loose teeth | Have you ever had any teeth become loose on their own, without an injury? | Yes<br>No<br>Refused<br>Don't know |
| 5 | Bone loss | Have you ever been told by a dental professional that you lost bone around your teeth? | Yes<br>No<br>Refused<br>Don't know |
| 6 | Teeth appearance | During the past 3 months, have you noticed a tooth that doesn't look right? | Yes<br>No<br>Refused<br>Don't know |
| 7 | Floss use | Aside from brushing your teeth with a toothbrush, in the last seven days, how many times did you use dental floss or any other device to clean between your teeth? | ____ : Number of days<br>77 = Refused |
| 8 | Mouthwash | Aside from brushing your teeth with a toothbrush, in the last seven days, how many times did you use mouthwash or other dental rinse product that you used to treat dental diseases or dental problems? | ____ : Number of days<br>77 = Refused |

The questions pertained to the respondent's self-perceived status of gum disease (question one), teeth/gum health (question two), previous history of gum treatment (question three) and common sequelae of gum diseases such as loose teeth (question four),

bone loss (question five) and abnormal teeth appearance (question six). In addition, self-reported frequencies of flossing (question seven) and mouthwash use (question eight) were inquired.

### 3.2.2 Content validation

The original eight-item SROH was submitted for content validation by a four-member expert committee comprising of three periodontists and a dental public health specialist. The expert committee members were asked to rate each question on a four-point Likert scale (1 = not relevant, 2 = somewhat relevant, 3 = quite relevant, 4 = highly relevant), indicating its relevance to the construct being measured within the local population. The experts were also invited to provide comments or feedback, if any.

The I-CVI and S-CVI were calculated (Lynn, 1986; Polit & Beck, 2006). The I-CVI was calculated by dividing the number of experts rating an item as 3 (quite relevant) or 4 (highly relevant) by the total number of experts. The cut-off value of acceptable I-CVI was one, as recommended by Lynn (1986) when there are five or fewer experts, in order to account for the possibility of agreement by chance alone. In other words, for each item to be deemed content valid, all experts had to rate it as either "quite relevant" or "highly relevant". The I-CVI for all items on the questionnaire was averaged to compute the scale-level content validity index, averaging calculation method (S-CVI/Ave). In addition, the proportion of items rated as "quite relevant" or highly relevant" by all content experts, termed as the scale-level content validity index, universal agreement calculation method (S-CVI/UA), was calculated. The cut-off values for S-CVI/Ave and S-CVI/UA for the scale to be considered valid were 0.9 and 0.8, respectively (Davis, 1992; Lynn, 1986; Waltz et al., 2016). The acceptable standard for S-CVI/Ave is lower than S-CVI/UA due to the former's more liberal requirements for congruence. While the S-CVI/Ave focuses on average agreement of the items, the S-CVI/UA is based on total agreement. The higher the number of experts, the lower the likelihood of achieving total

agreement. As our study employed a panel of four experts, it was decided that a S-CVI/UA of 0.8 as proposed by Davis (1992) is a reasonable threshold. If modifications to the questionnaire were recommended by the experts, the necessary changes were made, and the revised questionnaire was returned to the experts for review and feedback. The calculations of the content validity indices as described above were then repeated.

### 3.2.3 Face validation

Following the confirmation of content validity, the questionnaire was then administered to potential subjects, for this study, the dental patients (n = 20), to assess its face validity. Patients visiting the Dental Specialist and Research Tower, Faculty of Dentistry, Universiti Malaya, were approached to participate. They were provided with the list of the questions and asked to rate the comprehensibility of each question on a 5-point Likert scale: 1 = Very easy, 2 = Somewhat easy, 3 = Neutral, 4 = Somewhat difficult, 5 = Very difficult. Participants were also invited to provide any opinions or feedback regarding the questions. For face validity to be considered acceptable, each item in the questionnaire had to be rated as "very easy" or "somewhat easy" to understand by at least 80% of the participants. This corresponded to an item-level face validity index (I-FVI) of 0.8. Additionally, a scale-level face validity index, averaging calculation method (S-FVI)/Ave) value of ≥0.8 was considered acceptable (Yusoff, 2019).

### 3.2.4 Pilot study

Following the confirmation of both content and face validity, a pilot study was conducted to generate data for the assessment of construct validity, concurrent validity and reliability. Figure 3.2 illustrates the process and sequence of the pilot study.

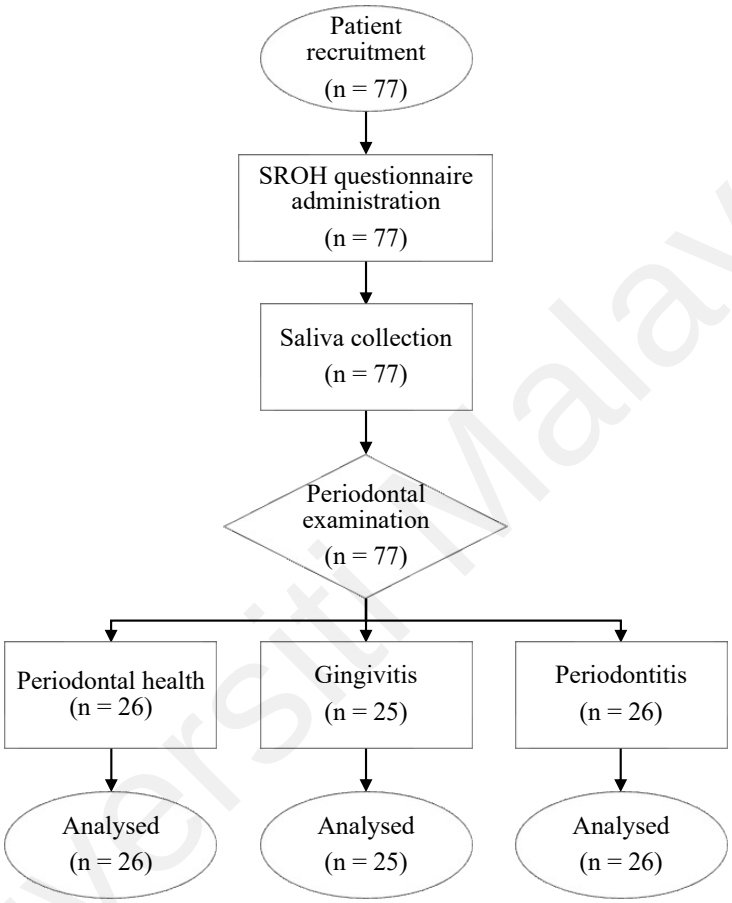



**Figure 3.2. Flowchart of clinical examination protocol. SROH, self-reported oral health questionnaire**

#### 3.2.4.1 Sample size calculation

The sample size required to validate the SROH was guided by the item-to-respondent ratio of 1:5 (Gorsuch, 2014; Tsang et al., 2017). Therefore, the eight-item questionnaire required a minimum sample of 40 respondents. Concurrently, the G* Power software was used to compute the sample size needed to detect a difference in the levels of salivary biomarkers (Faul et al., 2007). Based on a pooled standard deviation of 175.42 ng/ml for MT obtained from our pilot study, an effect size of 0.45 was derived from the

ANOVA, fixed effects, omnibus, one way test (Ho, 2022). The sample size required for this study to attain 95% confidence interval and 80% power was 51, divided equally into 17 subjects per group. Ultimately, 25 subjects per group was deemed adequate, with a total sample size of 75, which would satisfy the sample size requirements for both SROH validation and salivary biomarker comparisons.

#### 3.2.4.2 Calibration exercise

A single examiner (HJY) performed the clinical periodontal examination. To ensure the reliability of these measurements, the examiner was calibrated against a certified specialist in periodontology (NAB) for the clinical parameters PPD and CAL. Due to inherent difficulties in obtaining absolute conformity in probing measurements between different individuals or even within the same individual, variation in measurements up to 1 mm was deemed acceptable (Glavind & Löe, 1967). Inter-examiner reliability between HJY and NAB was assessed by examining two patients not involved in the primary study. Intra-examiner reliability for HJY was evaluated via measurement of the same clinical parameters on two patients, with repeated measurements 30 minutes apart. Intraclass correlation coefficient (ICC) was used for reliability analysis, using the two-way mixed effects, single measurement and absolute agreement model.

#### 3.2.4.3 Sampling and patient recruitment

A convenience sampling approach was employed to recruit 77 patients who visited the Postgraduate Periodontology Clinic, Faculty of Dentistry, Universiti Malaya between January 2023 to May 2024 for this study. Study participants were recruited based on the following inclusion criteria: adults aged 18 years and above; systemically healthy and having ≥20 permanent teeth. The exclusion criteria included: recent periodontal treatment in the last six months; past history of antibiotic, steroid or non-steroidal anti-inflammatory medication intake in the past three months; systemic diseases such as DM, coronary artery disease, osteoarthritis, rheumatoid arthritis, kidney diseases, liver diseases or

inflammatory bowel disease; pregnant or lactating mothers; history of cardiac conditions that necessitate antibiotic prophylaxis; and current smokers or former smokers who quit less than five years ago.

#### 3.2.4.4 Administration of the self-reported oral health questionnaire

Upon recruitment, each eligible participant was asked to answer an electronic version of the SROH through the web-based application Google Form. A quick response code leading to the Google Form was shown to the research participants, who then answered the SROH questionnaire on their personal electronic devices.

#### 3.2.4.5 Periodontal examination

Subsequent to completion of the questionnaire, each participant underwent a full-mouth periodontal examination. All teeth were examined except for third molars and retained roots. Probing pocket depth and CAL were measured with the UNC-15 periodontal probe which has 1 mm incremental markings up to 15 mm, at six sites per tooth. Bleeding on probing and the presence of plaque were assessed on a binary scale (presence or absence), also at six sites per tooth (Ainamo & Bay, 1975; O'Leary et al., 1972).

Based on these clinical parameters, they were grouped into periodontal health, gingivitis and periodontitis groups, in accordance with the case definitions proposed by the 2017 World Workshop classification (Table 3.2) (Caton et al., 2018; Chapple et al., 2018; Lang & Bartold, 2018; Papapanou et al., 2018; Tonetti et al., 2018; Trombelli et al., 2018).

**Table 3.2. Case definitions for periodontal health, gingivitis and periodontitis.**

| Group | Case definitions | Supporting literature |
|---|---|---|
| Periodontal health | • BOP <10%<br>• PPD ≤3 mm | Chapple et al. (2018); Lang and Bartold (2018) |
| Gingivitis | • BOP >30% (generalised gingivitis)<br>• PPD ≤3 mm | Chapple et al. (2018); Trombelli et al. (2018) |
| Periodontitis | • Interdental CAL is detectable at ≥2 non-adjacent teeth, OR<br>• Buccal or oral CAL ≥3 mm with pocketing >3 mm is detectable at ≥2 teeth but the observed CAL cannot be ascribed to non-periodontitis causes.<br>• PPD ≥5 mm | Papapanou et al. (2018); Tonetti et al. (2018) |

BOP, bleeding on probing; CAL, clinical attachment loss; PPD, probing pocket depth

### 3.2.4.6 Construct validity

Construct validity relates to how well the SROH measures the underlying construct of self-reported periodontal status. Exploratory factor analysis (EFA) was used to load the SROH items into a single latent construct. The decision to extract only one factor was based on the theoretical framework of the SROH, which was designed and used as a screening tool instead of a multi-dimensional psychometric scale. Principal component analysis with Varimax rotation was used for this purpose. The suitability of the dataset for factor analysis was determined by the Bartlett's test of sphericity and the Kaiser-Meyer-Olkin (KMO) measures of sampling adequacy. For the KMO test, a minimum value of 0.6 was required, indicating adequate dataset to perform EFA. Factor loadings denoted the strength of association between each individual item and the underlying construct. For this study, factor loadings of 0.3 to 0.4 were considered acceptable.

### 3.2.4.7 Concurrent validity

To assess the accuracy of the SROH for screening periodontitis, the responses were compared against full-mouth periodontal examination. The periodontal health and gingivitis groups were combined into a non-periodontitis group for this analysis. Binary

logistic regression analysis was used to determine the cut-off score that differentiates between periodontitis and non-periodontitis groups, using the SROH responses as categorical predictive variables. The resultant beta-coefficients (β-coefficient), which represented the weightage of each variable, were used to compute the screening score or the predicted probability for periodontitis. The cut-off value that distinguished between periodontitis and non-periodontitis groups was determined by the AUC test, using a threshold that provided the highest sum of sensitivity and specificity values.

#### 3.2.4.8 Internal consistency

Internal consistency of the SROH as a scale was evaluated using Cronbach's alpha test. A Cronbach's alpha value of at least 0.7 was considered acceptable (Lance et al., 2006; Nunnally, 1978). If the value was less than 0.7, the effect of removing each item on the Cronbach's alpha value was assessed. Items that led to a clear improvement in Cronbach's alpha when removed were discussed with the research team, on the feasibility of removing or retaining them.

#### 3.2.4.9 Test-retest reliability

To assess the stability of the SROH, test-retest reliability was assessed among 10 subjects two weeks after the initial administration of the questionnaire. The same questionnaire was administered with the questions arranged in different order. The responses between the two time points were compared using ICC test. The following ICC parameters were chosen: two-way mixed effects, single measurement and absolute agreement.

## 3.3 Part 2: Saliva collection and measurement of biomarker concentrations

### 3.3.1 Saliva collection

The same group of participants who answered the SROH for the concurrent validity test was asked to provide saliva samples for biomarker quantification. The sample

size calculation, calibration exercise, sampling technique, selection criteria and clinical examination protocol were as described from Chapter 3.2.4.1 to Chapter 3.2.4.3 and Chapter 3.2.4.5.

The saliva collection protocol was adapted from previous studies (Bostanci et al., 2021; Bostanci et al., 2018). Saliva collection was scheduled between 9 am to 11 am. Participants were asked to avoid drinking, eating or toothbrushing an hour before the procedure. The participants first rinsed their mouth with water for two minutes. Ten minutes later, five millilitres (mL) of unstimulated whole saliva were collected by passive drooling into sterile collection tubes.

The saliva samples were stored in ice and centrifuged at 4000 relative centrifugal force (RCF) for 20 mins at 4°C. The supernatants were then aliquoted into individual microcentrifuge tubes and stored in -80°C until further analysis, no longer than six months after sample collection.

### 3.3.2 Measurement of biomarker concentrations

Salivary biomarker levels were measured with commercially available ELISA kits in adherence to the manufacturer's instructions (Elabscience® for IL-1β, IL-6, TNF-α, MMP-8 and MMP-9; Cusabio® for MT). In general, saliva samples were allowed to thaw prior to analysis. Similarly, the ELISA kit reagents were brought out for equilibration to room temperature (18 - 25°C) 30 minutes before use. The optimal dilution factors for different biomarkers were determined beforehand based on literature review and a previous pilot test (Ho, 2022).

For standards preparation, seven 1.5 mL Eppendorf tubes were numbered and placed on a receptacle. An amount of reference standard and sample diluent stipulated by the manufacturer was added to each tube. Subsequently, an appropriate concentration of the stock standard solution, constituted according to manufacturer's instructions was

transferred to tube one and mixed using a calibrated pipette. An appropriate amount of standard from tube one was then transferred to tube number two and mixed thoroughly. This process of serial dilution was repeated until tube number six. No solution was transferred to the final tube, which served as the blank solution.

Using calibrated micropipettes, the requisite amount of standards and samples was added to the bottom of each well, paying attention to avoid touching the inside walls and causing foaming as much as possible. Each standard and sample was assayed in duplicates. The plate was sealed with a sticker (provided in the kit) and incubated at 37°C for a period of time (range between one hour thirty minutes to two hours with the kits used in the present experiment). The plate was then decanted and Biotinylated Antibody solution was immediately added to each well, sealed with a new sticker and incubated for a period of time. Next, the plate was decanted, and the wash process was performed using multichannel pipettes. With regards to the wash process, each well was filled with 200 – 350µl of wash buffer and allowed to soak for one minute, and then decanted and patted dry against clean absorbent paper. The wash process was repeated three times. Next, horseradish peroxidase (HRP) conjugate solution was loaded onto each well, followed by another incubation step. Subsequently, the wash process was repeated for five times, followed by addition of tetramethylbenzidine (TMB) substrate solution to each well. The plate was again placed inside the incubator for about 15 – 30 minutes, depending on the intensity of the colorimetric reaction. Acidic Stop solution was deposited in the same order as the substrate solution and the plate was immediately analysed with a microplate reader set at the 450 nm wavelength for all biomarkers except MT, which required a correction reading at 570 nm wavelength.

### 3.4 Part III: Statistical analysis and development of multivariate predictive models for periodontitis

Statistical tests were performed using IBM SPSS Statistics, version 26 (IBM Corp). Normality of data set was evaluated by the Shapiro-Wilk test. Continuous data was expressed as mean ± standard deviation (SD) or median ± interquartile range (IQR); whereas categorical data was presented in frequency distribution and percentages. Analysis of variance (ANOVA) with post-hoc Tukey test/Kruskal-Wallis H test with post-hoc Dunn test was used to compare the differences in continuous variables between groups. Salivary biomarker levels were related to clinical parameters using Pearson/Spearman correlation analysis. Association between categorical variables was analysed with the Chi-Square/Fisher Exact test.

Univariate binary logistic regression analysis was performed to establish candidate predictors that were associated with periodontitis, using a cut-off P-value of 0.2. Then, three predictive models were created and assessed:

- Model 1: All candidate predictors (demographics, SROH responses and salivary biomarkers).
- Model 2: Demographics and SROH responses only.
- Model 3: SROH responses only.

In these models, periodontitis status served as the dependent variable, while candidate predictors filtered from the univariate analysis acted as co-variates. Periodontal health and gingivitis groups were combined into a single non-periodontitis group to facilitate binary logistic regression. Stepwise backward elimination likelihood ratio method consecutively removed predictors with the highest P-value, until all the remaining co-variates retained statistical significance. The predicted probability values of the logistic regression models were saved as a separate variable.

The Receiver Operating Characteristics (ROC) curves with corresponding AUROCC were used to assess the discriminative abilities of the predictive models between periodontitis and non-periodontitis groups. Sensitivity and specificity were estimated based on the predicted probability cut-off value that maximized the sum of sensitivity and specificity across the ROC curve.

Individual sum scores were calculated for each subject with the formula below:

$$Y = B_1 \times X_1 + B_2 \times X_2 \dots \dots B_n \times X_n$$

In this formula, Y denotes the individual sum score, $B_n$ is the regression coefficient retrieved from the binary logistic regression, and X represents the predictors. For binary predictors such as SROH responses, a reference outcome was determined a priori by coding negative outcome as 1, and positive outcome as 0. No reference outcomes were necessary for biomarker predictors. Thus, the individual sum score was calculated by adding up all the predictors multiplied by their weightages (B). For all data analysis, a P-value of less than 0.05 was considered statistically significant.

# CHAPTER 4. RESULTS

## 4.1 Part I: Questionnaire validation and reliability testing

### 4.1.1 Content validation

The first round of content validation yielded a S-CVI/Ave of 0.9375 and S-CVI/UA of 0.75 (Table 4.1). All four content experts rated questions one (gum disease), three (gum treatment), four (loose teeth), five (bone loss), seven (floss use) and eight (mouthwash) as relevant or highly relevant. Questionnaire items number two (tooth/gum health) and six (teeth appearance) were rated by one expert to be "somewhat relevant", reducing their I-CVI to 0.75. For question two (tooth/gum health), an expert suggested to separate tooth/gum health into two separate questions on tooth health and gum health individually. Moreover, a concern raised was that question number six (teeth appearance) was too vague, which might confuse the respondents. Moreover, a remark was also made with regards to the inclusion of "Refused" as one of the answer choices.

**Table 4.1. Round one of content validation test among expert panel.**

| Item | Expert 1 | Expert 2 | Expert 3 | Expert 4 | Number in agreement | I-CVI |
|---|---|---|---|---|---|---|
| 1 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 2 | ✓ | - | ✓ | ✓ | 3 | 0.75 |
| 3 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 4 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 5 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 6 | ✓ | - | ✓ | ✓ | 3 | 0.75 |
| 7 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 8 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| Proportion Relevant: | 1 | 0.75 | 1 | 1 | | S-CVI/Ave: 0.9375 S-CVI/UA: 0.75 |

I-CVI, item-level content validity index; S-CVI/Ave, scale-level content validity index, averaging calculation method; S-CVI/UA, scale-level content validity index, universal agreement calculation method

The question on "teeth appearance" was restructured into "During the past 3 months, have you noticed a tooth that doesn't look right (e.g. shaky, tilted, drifted etc.)?" in order to enhance its clarity. The questionnaire was submitted to the expert committee for another round of content validation. Two members of the expert panel recommended to include a question on gingival bleeding into the questionnaire, citing the publication by Iwasaki et al. (2021) as a reference source. Therefore, the SROH was expanded to a nine-item questionnaire and resubmitted for content validation.

During the second round of content validation, all nine questions received I-CVI of 1, thereby attaining a S-CVI/UA score of 1 (Table 4.2).

**Table 4.2. Round two of content validation test among expert panel.**

| Item | Expert 1 | Expert 2 | Expert 3 | Expert 4 | Number in agreement | I-CVI |
|---|---|---|---|---|---|---|
| 1 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 2 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 3 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 4 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 5 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 6 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 7 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 8 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| 9 | ✓ | ✓ | ✓ | ✓ | 4 | 1 |
| Proportion Relevant: | 1 | 1 | 1 | 1 | | S-CVI/Ave: 1<br>S-CVI/UA: 1 |

I-CVI, item-level content validity index; S-CVI/Ave, scale-level content validity index, averaging calculation method; S-CVI/UA, scale-level content validity index, universal agreement calculation method

### 4.1.2 Face validation

Thirty-two patients participated in the face validity assessment, and their responses were tabulated in the percentage of participants who rated each question and its responses as either "somewhat easy" or "very easy" to understand (Table 4.3). Questions were considered as face valid when the combined positive responses ("somewhat easy" and "very easy") were ≥80%. In other words, I-FVI of each item should be 0.8 or higher.

**Table 4.3. Face validity test for the self-reported oral health questionnaire.**

| Question | Percentage of responses that were either somewhat easy" or "very easy" (%) | I-FVI |
|---|---|---|
| 1 | 87.5 | 0.875 |
| 2 | 81.3 | 0.813 |
| 3 | 87.5 | 0.875 |
| 4 | 81.3 | 0.813 |
| 5 | 87.5 | 0.875 |
| 6 | 68.8 | 0.688 |
| 7 | 84.4 | 0.844 |
| 8 | 84.4 | 0.844 |
| 9 | 84.4 | 0.844 |
| | | S-FVI/Ave = 0.83 |

I-FVI, item-level face validity index; I-FVI/Ave, item-level face validity index, averaging calculation method

Among the nine questions, only question number six (bone loss) failed to attain an I-FVI of 0.8. The question was graded as "difficult to understand" by 31.2% of the respondents. A recurring point of contention voiced by these respondents pertained to the incomprehensibility of the phrase "lost bone around your teeth". The S-FVI/Ave was 0.83.

Question number six was then reworded into "Have you been told by a dentist that the bone holding your teeth is lost?", in order to more clearly define the characteristic of the bone, which is holding the teeth in place. Moreover, the term "dental professional" was replaced by "dentist" because "dentist" is a more commonly used terminology among the laypeople in Malaysia. The updated question went through a second face validation process among 29 participants, but the I-FVI dropped slightly to 0.62. Similar comments were provided in the feedback section, alluding to a difficulty in relating the bone to the teeth, which is not visible in the mouth.

In light of the limited awareness of the presence of alveolar bone around natural teeth that became apparent during the face validity test, a decision was made to attach a picture depicting the radiographic appearance of moderate bone loss on the lower right posterior teeth. On the radiograph, the structures of bone and tooth root were labelled clearly (Figure 4.1).

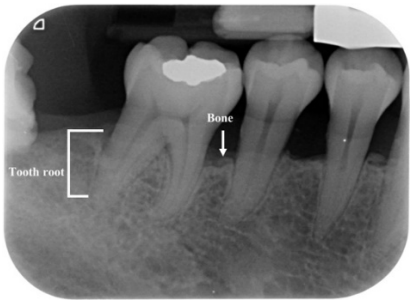
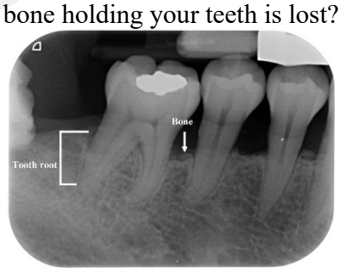



**Figure 4.1. Periapical radiograph depicting bone loss as a result of periodontitis to supplement question number six (bone loss).**

The question format, “Have you been told by a dentist that the bone holding your teeth is lost?” was retained. The revised question with its attendant illustration was submitted for a third face validity test. Thirty-two responses were collected, with 75.1% of them agreeing that the question was easy to understand, giving rise to I-FVI of 0.75. The S-FVI/Ave of the SROH after this final revision was 0.837. Although the I-FVI did not reach the stipulated criteria of 0.8, the question was retained as the improvement in patient comprehension after the two revision processes was considered adequate. Moreover, an ideal I-FVI has not been established in the scientific literature, and the threshold of 0.8 used in the present study was an arbitrary decision (Bolarinwa, 2015; Tsang et al., 2017).

The modified version of the nine-item SROH after content validation and face validation tests is illustrated in Table 4.4.

**Table 4.4. Modified version of self-reported oral health questionnaire after content and face validation tests.**

| Question number | Question topic | Question | Response options |
|---|---|---|---|
| 1 | Gum disease | Do you think you might have gum disease? | Yes<br>No<br>Refused<br>Don't know |
| 2 | Teeth/gum health | Overall, how would you rate the health of your teeth and gums? | Excellent<br>Very good<br>Good<br>Fair<br>Poor<br>Refused<br>Don't know |
| 3 | Gum bleeding | During the past three months, have you had bleeding gums? | Never<br>Hardly ever<br>Sometimes<br>Fairly often<br>Very often |
| 4 | Gum treatment | Have you ever had treatment for gum disease such as scaling and root planing, sometimes called "deep cleaning"? | Yes<br>No<br>Refused<br>Don't know |
| 5 | Loose teeth | Have you ever had any teeth become loose on their own, without an injury? | Yes<br>No<br>Refused<br>Don't know |
| 6 | Bone loss | Have you ever been told by a dentist that the bone holding your teeth is lost?<br>  | Yes<br>No<br>Refused<br>Don't know |
| 7 | Teeth appearance | During the past 3 months, have you noticed a tooth that doesn't look right (e.g., shaky, tilted, drifted etc.)? | Yes<br>No<br>Refused<br>Don't know |
| 8 | Floss use | Aside from brushing your teeth with a toothbrush, in the last seven days, how many days did you use dental floss or any other device to clean between your teeth? | ____ : Number of days<br>77 = Refused |
| 9 | Mouthwash | Aside from brushing your teeth with a toothbrush, in the last seven days, how many days did you use mouthwash or other dental rinse product that you used to treat dental diseases or dental problems? | ____ : Number of days<br>77 = Refused |

### 4.1.3 Pilot study

#### 4.1.3.1 Examiner calibration

The ICC values obtained for inter-rater reliability for PPD and CAL were 0.911 (95% CI 0.88 – 0.935) and 0.803 (95% CI 0.707 – 0.864), respectively. The corresponding ICC values for repeated measurements of the same examiner were 0.82 (95% CI 0.718 – 0.885) for PPD and 0.906 (95% CI 0.847 – 0.941) for CAL. Therefore, the intra- and interrater reliability of PPD and CAL measurements of the primary examiner (HJY) was considered to be moderate to excellent (Koo & Li, 2016).

#### 4.1.3.2 Demographic and clinical characteristics of study population

The baseline demographic and clinical characteristics of the study population are outlined in Table 4.5.

**Table 4.5. Demographic and clinical characteristics of the study population (n = 77).**

| | Periodontal health (n = 26) | Gingivitis (n = 25) | Periodontitis (n = 26) | P-value |
|---|---|---|---|---|
| Age (mean ± SD) | 32.23 ± 8.67[a] | 35.96 ± 9.33[a] | 44.54 ± 8.44[b] | <0.001† |
| Gender, n (%) | | | | 0.014‡ |
| Male | 3 (11.5) | 5 (20) | 12 (46.2) | |
| Female | 23 (88.5) | 20 (80) | 14 (53.8) | |
| Ethnicity n (%) | | | | 0.849‡ |
| Malay | 13 (50) | 17 (68) | 14 (53.8) | |
| Chinese | 11 (42.3) | 7 (28) | 9 (34.6) | |
| Indian | 1 (3.8) | 1 (4) | 2 (7.7) | |
| Others | 1 (3.8) | 0 (0) | 1 (3.8) | |
| Education level, n (%) | | | | 0.094‡ |
| Primary | 0 (0) | 0 (0) | 2 (7.7) | |
| Secondary | 6 (23.1) | 5 (20) | 8 (30.8) | |
| Diploma | 3 (11.5) | 8 (32) | 10 (38.5) | |
| Bachelor | 10 (38.5) | 7 (28) | 4 (15.4) | |
| Postgraduate | 7 (26.9) | 5 (20) | 2 (7.7) | |
| Clinical parameters (mean ± SD) | | | | |
| Number of teeth | 26.96 ± 2.29[a] | 26.88 ± 1.79[a] | 24.23 ± 4.62[b] | 0.001† |
| Probing pocket depth (mm) | 1.86 ± 0.24[a] | 2.19 ± 0.22[b] | 5.01 ± 0.57[c] | <0.001† |
| Clinical attachment level (mm) | 0.03 ± 0.06[a] | 0.07 ± 0.18[a] | 3.78 ± 1.41[b] | <0.001† |
| Bleeding on probing (%) | 4 ± 2.05[a] | 24.35 ± 12.49[b] | 48.15 ± 23.89[c] | <0.001† |
| Plaque score (%) | 13.74 ± 10.13[a] | 33.98 ± 15.32[b] | 45.29 ± 19.14[b] | <0.001† |

†Kruskal-Wallis test.
‡Fisher exact test.
Different alphabets denoted statistically significant differences between groups.

The mean age of the periodontitis group was significantly higher than the periodontal health and gingivitis groups. Moreover, a higher proportion of females made up the periodontal health and gingivitis groups, with an almost equal distribution in gender in the periodontitis group. Ethnicity and education level differences between the three groups were not statistically significant (P>0.05).

Intergroup differences for all clinical parameters achieved statistical significance (P<0.05), with the periodontitis group showing lesser number of teeth, deeper PPD, greater CAL and more bleeding sites than both periodontal health and gingivitis groups. With regards to plaque score, the periodontal health group presented with significantly less plaque accumulation than either the gingivitis or periodontitis groups (P<0.001).

#### 4.1.3.3 Self-reported oral health questionnaire response distribution

Table 4.6 illustrates the frequency distribution of the SROH responses. The number of missing/refused responses was low, recorded by only six and five respondents for the items on (Q8) “floss use” and (Q9) “mouthwash”, respectively. The highest tally of “don’t know” response was recorded by the item (Q1) “gum disease”, making up 15.6% of the total responses for this question. The percentage of “don’t know” responses for other SROH items was low, ranging from 2.6 – 6.5%.

**Table 4.6. Frequency distribution of responses to self-reported oral health questionnaire (n = 77).**

| Self-reported oral health questions | Responses | Overall (n = 77) | Periodontal health (n = 26) | Gingivitis (n = 25) | Periodontitis (n = 26) |
|---|---|---|---|---|---|
| Q1. Gum disease | Yes | 32 (41.6%) | 2 (7.7%) | 10 (40%) | 20 (76.9%) |
| | No | 33 (42.9%) | 22 (84.6%) | 8 (32%) | 3 (11.5%) |
| | Don't know | 12 (15.6%) | 2 (7.7%) | 7 (28%) | 3 (11.5%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q2. Teeth/gum health | Excellent | 1 (1.3%) | 1 (3.8%) | 0 (0%) | 0 (0%) |
| | Very good | 8 (10.4%) | 5 (19.2%) | 2 (8%) | 1 (3.8%) |
| | Good | 30 (39%) | 15 (57.7%) | 9 (36%) | 6 (23.1%) |
| | Fair | 21 (27.3%) | 3 (11.5%) | 10 (40%) | 8 (30.8%) |
| | Poor | 14 (18.2%) | 0 (0%) | 4 (16%) | 10 (38.5%) |
| | Don't know | 3 (3.9%) | 2 (7.7%) | 0 (0%) | 1 (3.8%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q3. Bleeding gums | Never | 22 (29.9%) | 12 (46.2%) | 8 (32%) | 3 (11.5%) |
| | Hardly ever | 13 (16.9%) | 7 (26.9%) | 3 (12%) | 3 (11.5%) |
| | Sometimes | 36 (46.8%) | 7 (26.9%) | 13 (52%) | 16 (61.5%) |
| | Fairly often | 3 (3.9%) | 0 (0%) | 0 (0%) | 3 (11.5%) |
| | Very often | 2 (2.6%) | 0 (0%) | 1 (4%) | 1 (3.8%) |
| Q4. Gum treatment | Yes | 10 (13%) | 4 (15.4%) | 3 (11.5%) | 3 (11.5%) |
| | No | 65 (84.4%) | 22 (84.6%) | 22 (84.6%) | 22 (84.6%) |
| | Don't know | 2 (2.6%) | 0 (0%) | 1 (3.8%) | 1 (3.8%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q5. Loose teeth | Yes | 15 (19.5%) | 0 (0%) | 1 (4%) | 14 (53.8%) |
| | No | 57 (74%) | 24 (92.3%) | 22 (88%) | 11 (42.3%) |
| | Don't know | 5 (6.5%) | 2 (7.7%) | 2 (8%) | 1 (3.8%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q6. Bone loss | Yes | 14 (18.2%) | 1 (3.8%) | 4 (16%) | 9 (34.6%) |
| | No | 61 (79.2%) | 25 (96.2%) | 21 (84%) | 15 (57.7%) |
| | Don't know | 2 (2.6%) | 0 (0%) | 0 (0%) | 2 (7.7%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q7. Teeth appearance | Yes | 16 (20.8%) | 0 (0%) | 2 (8%) | 14 (53.8%) |
| | No | 57 (74%) | 26 (100%) | 20 (80%) | 11 (42.3%) |
| | Don't know | 4 (5.2%) | 0 (0%) | 3 (12%) | 1 (3.8%) |
| | Refused | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q8. Floss use | Never | 28 (36.4%) | 8 (30.8%) | 8 (32%) | 12 (46.2%) |
| | 1 – 7 days | 43 (55.8%) | 17 (65.4%) | 13 (52%) | 13 (50%) |
| | Missing/Refused | 6 (7.8%) | 1 (3.8%) | 4 (16%) | 1 (3.8%) |
| Q9. Mouthwash | Never | 41 (53.2%) | 16 (61.5%) | 12 (48%) | 13 (50%) |
| | 1 – 7 days | 31 (40.3%) | 9 (34.6%) | 10 (40%) | 12 (46.2%) |
| | Missing/Refused | 5 (6.5%) | 1 (3.8%) | 3 (12%) | 1 (3.8%) |

#### 4.1.3.1 Internal consistency

The nine-item SROH questionnaire as a single scale yielded a Cronbach's alpha value of 0.673. In addition, the corrected item-total correlations and Cronbach's alpha values if an item was deleted were computed. It was observed that removal of questions number four (gum treatment), eight (floss use) or nine (mouthwash) increased the Cronbach's alpha value to above 0.7 (Table 4.7).

**Table 4.7. Internal consistency (Cronbach's alpha) of the nine-item self-reported oral health questionnaire.**

| | Corrected Item-Total Correlation | Cronbach's Alpha if Item Deleted |
|---|---|---|
| Q1 | .658 | .568 |
| Q2 | .604 | .582 |
| Q3 | .336 | .649 |
| Q4 | -.051 | **.707** |
| Q5 | .573 | .600 |
| Q6 | .480 | .622 |
| Q7 | .603 | .595 |
| Q8 | .091 | **.704** |
| Q9 | -.039 | **.730** |

#### 4.1.3.2 Test-retest reliability

The value of the ICC was 0.975 (95% CI 0.961 – 0.984), indicating excellent test-retest reliability of the SROH on repeated administration (Koo & Li, 2016).

#### 4.1.3.3 Construct validity

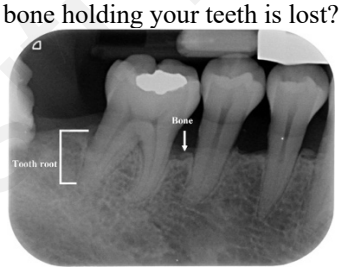
A KMO test of 0.7 and Bartlett's test of sphericity with a P-value of <0.001 indicated that the dataset was suitable for factor analysis using principal component analysis. The factor loading scores of each questionnaire item were arranged in descending order in Table 4.8.

**Table 4.8. Factor loadings of the self-reported oral health questionnaire items following principal component analysis.**

| Self-reported oral health questionnaire items | Factor loading score |
|---|---|
| Q1. Gum disease | 0.849 |
| Q5. Loose teeth | 0.779 |
| Q7. Teeth appearance | 0.772 |
| Q2. Teeth/gum health | 0.747 |
| Q6. Bone loss | 0.661 |
| Q3. Gum bleeding | 0.566 |
| Q9. Mouthwash | 0.111 |
| Q8. Floss use | <0.1 |
| Q4. Gum treatment | <0.1 |

Six out of nine items obtained factor loading values of greater than 0.5. The exceptions were question number four (gum treatment), eight (floss use) and nine (mouthwash). Their factor loadings of less than 0.3 were discussed among the research team. Combined with the three items' negative impact on internal consistency of the SROH as a scale, the research team arrived at a consensus to remove them from the questionnaire, essentially modifying it into a six-item SROH (Table 4.9).

**Table 4.9. Final version of the modified six-item self-reported oral health questionnaire.**

| Question number | Question topic | Question | Response options |
|---|---|---|---|
| 1 | Gum disease | Do you think you might have gum disease? | Yes<br>No<br>Refused<br>Don't know |
| 2 | Teeth/gum health | Overall, how would you rate the health of your teeth and gums? | Excellent<br>Very good<br>Good<br>Fair<br>Poor<br>Refused<br>Don't know |
| 3 | Gum bleeding | During the past three months, have you had bleeding gums? | Never<br>Hardly ever<br>Sometimes<br>Fairly often<br>Very often |
| 4 | Loose teeth | Have you ever had any teeth become loose on their own, without an injury? | Yes<br>No<br>Refused<br>Don't know |
| 5 | Bone loss | Have you ever been told by a dentist that the bone holding your teeth is lost?<br>  | Yes<br>No<br>Refused<br>Don't know |
| 6 | Teeth appearance | During the past 3 months, have you noticed a tooth that doesn't look right (e.g., shaky, tilted, drifted etc.)? | Yes<br>No<br>Refused<br>Don't know |

#### 4.1.3.4 Internal consistency and construct validity after removal of three SROH items

The internal consistency and construct validity of the six-item SROH were reassessed. Following the removal of three SROH items, the modified six-item SROH demonstrated improved internal consistency, with Cronbach-alpha value of 0.813. Further removal of any individual SROH items led to either negligible or deterioration in Cronbach's alpha value.

**Table 4.10. Internal consistency (Cronbach's alpha) of the six-item self-reported oral health questionnaire.**

| | Corrected Item-Total Correlation | Cronbach's Alpha if Item Deleted |
|---|---|---|
| Q1. Gum disease | .726 | .746 |
| Q2. Teeth/gum health | .632 | .771 |
| Q3. Gum bleeding | .444 | .816 |
| Q4. Loose teeth | .611 | .778 |
| Q5. Bone loss | .506 | .798 |
| Q6. Teeth appearance | .563 | .787 |

Principal component analysis confirmed the construct validity of the modified six-item SROH. All items loaded well to the construct of periodontitis, with factor loading scores ranging between 0.578 – 0.836 (Table 4.11), indicating good factor loading onto the construct of self-perceived periodontal status.

**Table 4.11. Factor loadings of the six-item self-reported oral health questionnaire using principal component analysis.**

| Self-reported oral health questionnaire items | Factor loading score |
|---|---|
| Q1. Gum disease | 0.836 |
| Q2. Teeth/gum health | 0.755 |
| Q3. Gum bleeding | 0.578 |
| Q4. Loose teeth | 0.769 |
| Q5. Bone loss | 0.654 |
| Q6. Teeth appearance | 0.732 |

#### 4.1.3.5 Concurrent validity

All six items of the modified SROH were entered simultaneously as predictive variables in binary logistic regression analysis using periodontitis as the outcome variable, to assess the concurrent validity of the SROH against periodontal status diagnosed by

full-mouth periodontal examination. The periodontitis screening score was derived from the logistic regression analysist based on the following formula:

Screening score = 1.488 (Q1 Gum disease) - 0.612 (Q2 Teeth/gum health) + 0.993 (Q3 Gum bleeding) + 2.377 (Q4 Loose teeth) - 0.37 (Q5 Bone loss) + 1.736 (Q6 Tooth appearance) - 2.421

The AUC of the six-item SROH in predicting periodontitis was 0.874, with a 95% CI of 0.783 – 0.965 (Figure 4.2). Therefore, the diagnostic accuracy was considered good to excellent. Using a threshold of 0.35 as a cut-off in weighted score between periodontitis and non-periodontitis groups, 77% sensitivity and 86% specificity were achieved. The classification of periodontitis vs non-periodontitis by the SROH as compared to full-mouth periodontal examination for each subject is presented in Table 4.12.

**Table 4.12. Weighted scores and classification of periodontal status by the self-reported oral health questionnaire compared to full-mouth periodontal examination for each study participant.**

| Subject | Weighted score | Periodontitis/non-periodontitis classification | |
|---|---|---|---|
| | | SROH | Full-mouth periodontal examination |
| 1 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 2 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 3 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 4 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 5 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 6 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 7 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 8 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 9 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 10 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 11 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 12 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 13 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 14 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 15 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 16 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 17 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 18 | 0.04595 | Non-periodontitis | Non-periodontitis |
| 19 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 20 | 0.17584 | Non-periodontitis | Non-periodontitis |
| 21 | 0.12846 | Non-periodontitis | Non-periodontitis |
| 22 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 23 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 24 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 25 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 26 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 27 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 28 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 29 | 0.81085 | Periodontitis | Non-periodontitis |
| 30 | 0.33513 | Non-periodontitis | Non-periodontitis |
| 31 | 0.04595 | Non-periodontitis | Non-periodontitis |
| 32 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 33 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 34 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 35 | 0.04595 | Non-periodontitis | Non-periodontitis |
| 36 | 0.11504 | Non-periodontitis | Non-periodontitis |
| 37 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 38 | 0.28461 | Non-periodontitis | Non-periodontitis |
| 39 | 0.36544 | Periodontitis | Non-periodontitis |
| 40 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 41 | 0.08241 | Non-periodontitis | Non-periodontitis |
| 42 | 0.36544 | Periodontitis | Non-periodontitis |
| 43 | 0.17584 | Non-periodontitis | Non-periodontitis |
| 44 | 0.36544 | Periodontitis | Non-periodontitis |
| 45 | 0.08155 | Non-periodontitis | Non-periodontitis |
| 46 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 47 | 0.19333 | Non-periodontitis | Non-periodontitis |
| 48 | 0.36544 | Periodontitis | Non-periodontitis |
| 49 | 0.28461 | Non-periodontitis | Non-periodontitis |
| 50 | 0.76575 | Periodontitis | Non-periodontitis |
| 51 | 0.36544 | Periodontitis | Non-periodontitis |
| 52 | 0.97239 | Periodontitis | Periodontitis |
| 53 | 0.19333 | Non-periodontitis | Periodontitis |
| 54 | 0.88828 | Periodontitis | Periodontitis |
| 55 | 0.51497 | Periodontitis | Periodontitis |

**Table 4.12, continued**

| Subject | Weighted score | Periodontitis/non-periodontitis classification | |
|---|---|---|---|
| | | SROH | Full-mouth periodontal examination |
| 56 | 0.08241 | Non-periodontitis | Periodontitis |
| 57 | 0.36544 | Periodontitis | Periodontitis |
| 58 | 0.97239 | Periodontitis | Periodontitis |
| 59 | 0.17584 | Non-periodontitis | Periodontitis |
| 60 | 0.36544 | Periodontitis | Periodontitis |
| 61 | 0.96053 | Periodontitis | Periodontitis |
| 62 | 0.08155 | Non-periodontitis | Periodontitis |
| 63 | 0.96053 | Periodontitis | Periodontitis |
| 64 | 0.96053 | Periodontitis | Periodontitis |
| 65 | 0.76575 | Periodontitis | Periodontitis |
| 66 | 0.96053 | Periodontitis | Periodontitis |
| 67 | 0.96053 | Periodontitis | Periodontitis |
| 68 | 0.86121 | Periodontitis | Periodontitis |
| 69 | 0.92883 | Periodontitis | Periodontitis |
| 70 | 0.94325 | Periodontitis | Periodontitis |
| 71 | 0.08155 | Non-periodontitis | Periodontitis |
| 72 | 0.19333 | Non-periodontitis | Periodontitis |
| 73 | 0.36544 | Periodontitis | Periodontitis |
| 74 | 0.97239 | Periodontitis | Periodontitis |
| 75 | 0.69310 | Periodontitis | Periodontitis |
| 76 | 0.88768 | Periodontitis | Periodontitis |
| 77 | 0.96009 | Periodontitis | Periodontitis |

The cut-off value of weighted score, above which indicated a periodontitis case based on the index test (self-reported oral health questionnaire) was 0.35.

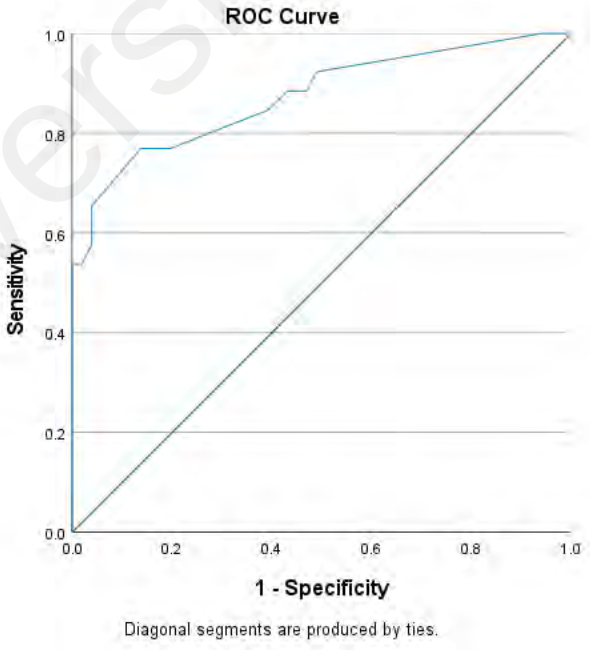



**Figure 4.2. Area under curve, sensitivity and 1-specificity of self-reported oral health questionnaire (all six items) in predicting periodontitis.**
**ROC, receiver operating characteristic**

## 4.2 Part II: Saliva collection and measurement of biomarker levels

Shapiro-Wilk test disclosed that the data for salivary biomarker concentrations were not normally distributed. The differences in biomarker concentrations between groups are shown in Table 4.13.

**Table 4.13. Concentrations of salivary biomarkers between periodontal health, gingivitis, and periodontitis groups.**

| | Periodontal health | Gingivitis | Periodontitis | P-value |
|---|---|---|---|---|
| IL-1β (pg/mL) | 0.22 ± 0.47[a] | 4.85 ± 6.62[b] | 5.98 ± 6.68[b] | <0.001 |
| IL-6 (pg/mL) | 2.09 ± 4.41[a] | 10.43 ± 19.21[b] | 8.35 ± 8.96[b] | <0.001 |
| TNF-α (pg/mL) | 15.13 ± 33.73 | 16.78 ± 40.52 | 30.46 ± 84.67 | 0.092 |
| MMP-8 (ng/mL) | 39.91 ± 53.7[a] | 91.6 ± 78.32[b] | 224.73 ± 160.51[c] | <0.001 |
| MMP-9 (ng/mL) | 75.91 ± 88.98[a] | 151.95 ± 113.26[b] | 332.8 ± 193.05[c] | <0.001 |
| MT (pg/mL) | 1327.56 ± 1045.18 | 1431.86 ± 1043.63 | 1789.23 ± 1206.31 | 0.458 |

Abbreviation: IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumour necrosis factor-alpha; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MT, metallothionein
Data was presented as mean ± standard deviation.
Intergroup differences were analysed with Kruskal Wallis test with post-hoc Dunn test.
Different alphabets between groups indicated statistically significant differences.

The levels of MMP-8 and MMP-9 were significantly higher in the saliva of periodontitis patients when compared to either gingivitis or periodontal health groups. The expression profile of IL-1β and IL-6 was similar, whereby significantly lower concentrations were observed in the periodontal health group ($P<0.001$), with no differences between gingivitis and periodontitis groups. Meanwhile, concentrations of TNF-α and MT were highest among the periodontitis group, but the differences were not statistically significant ($P>0.05$).

The correlations between salivary biomarker concentrations and clinical periodontal parameters are shown in Table 4.14. Levels of IL-1β, IL-6, MMP-8 and MMP-9 were significantly correlated with PPD, CAL and BOP. The strength of association was considered moderate, with correlation coefficients between 0.43 and 0.62. The relationship between the four biomarkers and plaque score was considered weak to moderate, as the correlation coefficient ranged from 0.32 to 0.49.

**Table 4.14. Correlations between clinical parameters and salivary biomarker concentrations.**

| | | IL-1β | IL-6 | TNF-α | MMP-8 | MMP-9 | MT |
|---|---|---|---|---|---|---|---|
| Probing pocket depth | Correlation coefficient | 0.585*** | 0.499*** | 0.161 | 0.602*** | 0.562*** | 0.168 |
| | P-value | <0.001 | <0.001 | 0.164 | <0.001 | <0.001 | 0.145 |
| Clinical attachment loss | Correlation coefficient | 0.564*** | 0.537*** | 0.134 | 0.572*** | 0.555*** | 0.26* |
| | P-value | <0.001 | <0.001 | 0.248 | <0.001 | <0.001 | 0.023 |
| Bleeding on probing | Correlation coefficient | 0.515*** | 0.433*** | 0.22 | 0.643*** | 0.604*** | 0.131 |
| | P-value | <0.001 | <0.001 | 0.056 | <0.001 | <0.001 | 0.257 |
| Plaque score | Correlation coefficient | 0.484*** | 0.323** | 0.089 | 0.491*** | 0.463*** | 0.064 |
| | P-value | <0.001 | 0.004 | 0.445 | <0.001 | <0.001 | 0.579 |

Abbreviation: IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumour necrosis factor-alpha; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MT, metallothionein
Spearman rank-order correlation
*P<0.05, **P <0.01 and ***P <0.001

Meanwhile, Table 4.15 describes the correlations between pairs of salivary biomarkers. There was a strong correlation between MMP-8 and MMP-9 levels, with a correlation coefficient of 0.894. The strength of association between pro-inflammatory cytokines IL-1β and IL-6 with the collagenases MMP-8 and MMP-9, as well as between IL-1β and IL-6 was considered as moderate, with Spearman coefficient ranging from 0.599 to 0.689.

**Table 4.15. Correlations between salivary biomarkers.**

| | | IL-1β | IL-6 | TNF-α | MMP-8 | MMP-9 |
|---|---|---|---|---|---|---|
| IL-1β | Correlation coefficient | | | | | |
| | P-value | | | | | |
| IL-6 | Correlation coefficient | 0.689*** | | | | |
| | P-value | <0.001 | | | | |
| TNF-α | Correlation coefficient | 0.066 | 0.049 | | | |
| | P-value | 0.569 | 0.673 | | | |
| MMP-8 | Correlation coefficient | 0.664*** | 0.648*** | 0.072 | | |
| | P-value | <0.001 | <0.001 | 0.536 | | |
| MMP-9 | Correlation coefficient | 0.599*** | 0.619*** | 0.095 | 0.894*** | |
| | P-value | <0.001 | <0.001 | 0.413 | <0.001 | |
| MT | Correlation coefficient | 0.297** | 0.353** | 0.24* | 0.34** | 0.361** |
| | P-value | 0.009 | 0.002 | 0.035 | 0.003 | 0.001 |

Abbreviation: IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumour necrosis factor-alpha; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MT, metallothionein
Spearman rank-order correlation.
*P<0.05, **P <0.01 and ***P <0.001

## 4.3 Part III: Development of multivariate predictive models for periodontitis

**Table 4.16. Univariate logistic regression analysis of demographic variables, self-reported oral health questionnaire items and salivary biomarker levels for periodontitis relative to non-periodontitis.**

| Variables | Univariate unadjusted odds ratio (95% CI) for periodontitis | P-value |
|---|---|---|
| Age | 1.13 (1.06 – 1.2) | <0.001* |
| Gender | | |
| Female | Reference | |
| Male | 4.61 (1.57 – 13.55) | 0.006* |
| Education level | | |
| Low education | 2.27 (0.81 – 6.39) | |
| Higher education | Reference | 0.048* |
| Ethnicity | | |
| Malay | Reference | |
| Chinese | 1.07 (0.39 – 2.98) | 0.895 |
| Indian | 2.14 (0.27 – 16.81) | 0.468 |
| Others | 2.14 (0.13 – 36.8) | 0.599 |
| SROH | | |
| Q1. Gum disease | 10.83 (3.54 – 33.15) | <0.001* |
| Q2. Teeth/gum health | 4.5 (1.63 – 12.43) | 0.004* |
| Q3. Bleeding gums | 4.76 (1.64 – 13.87) | 0.004* |
| Q4. Loose teeth | 58.33 (6.97 – 488.09) | <0.001* |
| Q5. Bone loss | 4.87 (1.43 – 16.61) | 0.011* |
| Q6. Teeth appearance | 26.54 (5.27 – 133.72) | <0.001* |
| Salivary biomarkers | | |
| IL-1β | 1.11 (1.01 – 1.21) | 0.024* |
| IL-6 | 1.01 (0.98 – 1.05) | 0.482 |
| TNF-α | 1 (0.99 – 1.01) | 0.33 |
| MMP-8 | 1.01 (1.01 – 1.02) | <0.001* |
| MMP-9 | 1.01 (1.005 – 1.014) | <0.001* |
| MT | 1 (1 – 1.001) | 0.127* |

Abbreviation: IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumour necrosis factor-alpha; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MT, metallothionein

* Candidate variables to be included into multivariate logistic regression as the criteria of P-value <0.2 was satisfied.

Based on univariate logistic regression analysis (Table 4.16), age, gender, education level, IL-1β, MMP-8, MMP-9, MT and all six SROH items were eligible for inclusion as predictors in the multivariate logistic regression analysis, by virtue of having P-value of <0.2. Using the backward elimination method, six statistically significant predictors were loaded into the model that incorporated all categories of variables, including social demographics, SROH responses, and salivary biomarker levels. For model two, which included only social demographics and SROH responses, five significant predictors were identified. The predictors were age, gender, education level,

question one on “gum disease” and question four on “loose teeth”. Model three, comprising only SROH responses, retained two variables: question one on “gum disease” and question four on “loose teeth” (Table 4.17).

**Table 4.17. Multivariate logistic regression models for predicting periodontitis.**

| | Model 1: All | | | | Model 2: Demographics and SROH | | | | Model 3: SROH only | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variables | Contributing to model | B | Adjusted OR (95% CI) | P-value | Contributing to model | B | Adjusted OR (95% CI) | P-value | Contributing to model | B | Adjusted OR (95% CI) | P-value |
| Age | | | | | + | 0.096 | 1.1 (1.02 – 1.19) | 0.014 | | | | |
| Gender | + | 2.613 | | | + | 1.874 | | | | | | |
| Female | | | Reference | | | | Reference | | | | | |
| Male | | | 13.64 (1.23 – 151.73) | 0.033 | | | 6.52 (1.07 – 39.54) | 0.042 | | | | |
| Education level | + | 2.546 | | | + | 1.752 | | | | | | |
| Low education | | | 12.75 (1.29 – 125.74) | 0.029 | | | 5.76 (0.96 – 34.58) | 0.055 | | | | |
| Higher education | | | Reference | | | | Reference | | | | | |
| Ethnicity | | | | | | | | | | | | |
| Q1. Gum disease | | | | | + | 2.439 | 11.46 (1.82 – 72.09) | 0.009 | + | 1.505 | 4.51 (1.25 – 16.23) | 0.021 |
| Q2. Teeth/gum health | + | 2.299 | 9.96 (1.16 – 85.28) | 0.036 | | | | | | | | |
| Q3. Bleeding gums | | | | | | | | | | | | |
| Q4. Loose teeth | | | | | + | 3.035 | 20.8 (1.87 – 231.04) | 0.013 | + | 3.321 | 27.69 (3.07 – 249.9) | 0.003 |
| Q5. Bone loss | | | | | | | | | | | | |
| Q6. Teeth appearance | + | 4.364 | 78.55 (4.32 – 1428.2) | 0.003 | | | | | | | | |
| IL-1β | + | -0.252 | 0.78 (0.64 – 0.94) | 0.01 | | | | | | | | |
| IL-6 | | | | | | | | | | | | |
| TNF-α | | | | | | | | | | | | |
| MMP-8 | + | 0.022 | 1.02 (1.01 – 1.04) | <0.001 | | | | | | | | |
| MMP-9 | | | | | | | | | | | | |
| MT | | | | | | | | | | | | |

Abbreviation: IL-1β, interleukin-1 beta; IL-6, interleukin-6; MMP-8, matrix metalloproteinase-8; MMP-9, matrix metalloproteinase-9; MT, metallothionein; OR, odds ratio; SROH, self-reported oral health questionnaire; TNF-α, tumour necrosis factor-alpha

Predictors marked with + were those that remained after stepwise backwards logistic regression modelling. B denoted the regression coefficient of the predictors, indicating its weightage.

The predicted probability cut-off values for each logistic regression model were saved as a separate variable and used to assess the diagnostic accuracies of the prediction models using AUROCC. The corresponding sensitivity and specificity values are shown in Table 4.18.

**Table 4.18. Parameters of multivariate logistic regression model for predicting periodontitis.**

| | Model 1 | Model 2 | Model 3 |
|---|---|---|---|
| AUC | 0.96 | 0.923 | 0.84 |
| 95% CI | 0.92 - 1 | 0.859 – 0.988 | 0.737 – 0.946 |
| P-value | <0.001 | <0.001 | <0.001 |
| Predicted probability cut-off | 0.4603 | 0.3525 | 0.248 |
| Sensitivity | 84.6% | 84.6% | 80.8% |
| Specificity | 94.1% | 88.2% | 76.5% |
| Positive predictive value | 84.6% | 77.8% | 63.6% |
| Negative predictive value | 92.2% | 90% | 88.6% |

In terms of diagnostic accuracy, model 1 exhibited the best performance with AUC of 0.96 (Figure 4.3), followed by model 2 (AUC 0.923) (Figure 4.4) and model 3 (AUC 0.84) (Figure 4.5). The sensitivity, specificity, positive predictive value and negative predictive value for model 1, based on the cut-off value of 0.4603, were 84.6% and 94.1%, 84.6% and 92.2%, respectively.

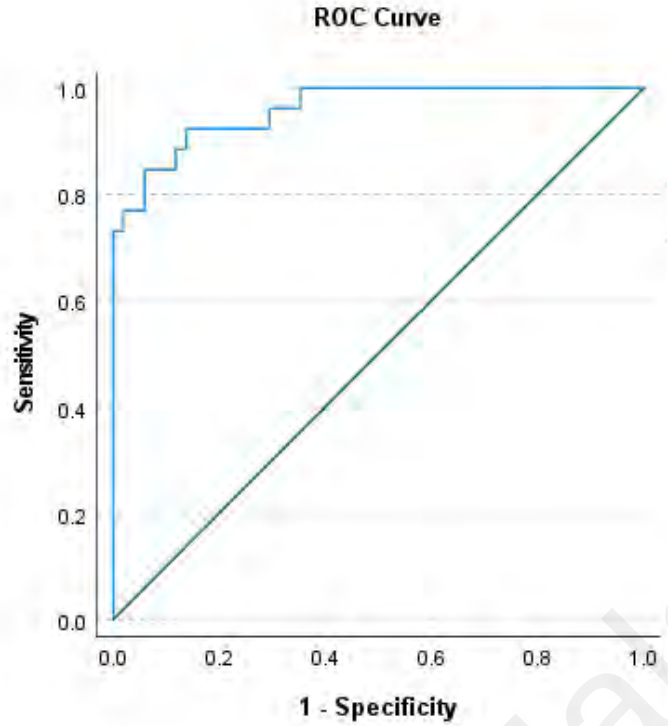



**Figure 4.3. Area under curve, sensitivity and 1-specificity of model 1 (all variables) for predicting periodontitis.**
**ROC, receiver operating characteristic**

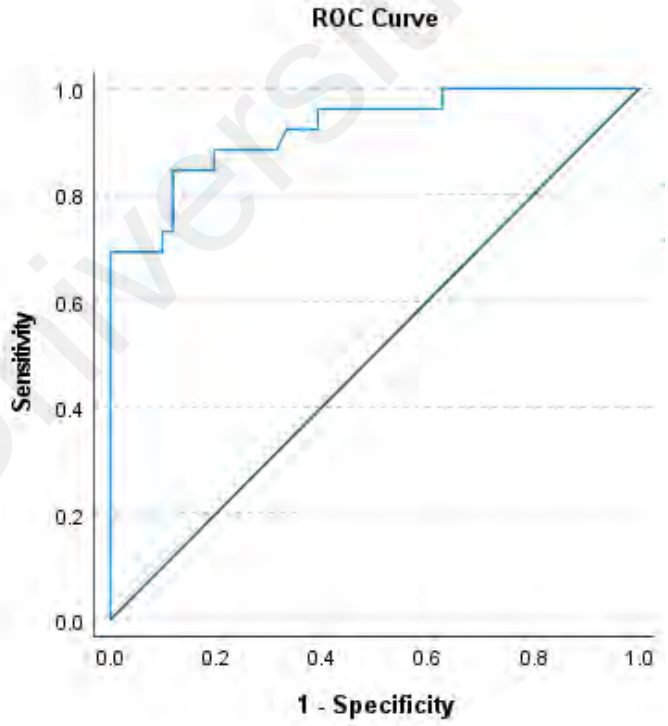



**Figure 4.4. Area under curve, sensitivity and 1-specificity for model 2 (social demographics and self-reported oral health questionnaire) for predicting periodontitis.**
**ROC, receiver operating characteristic**

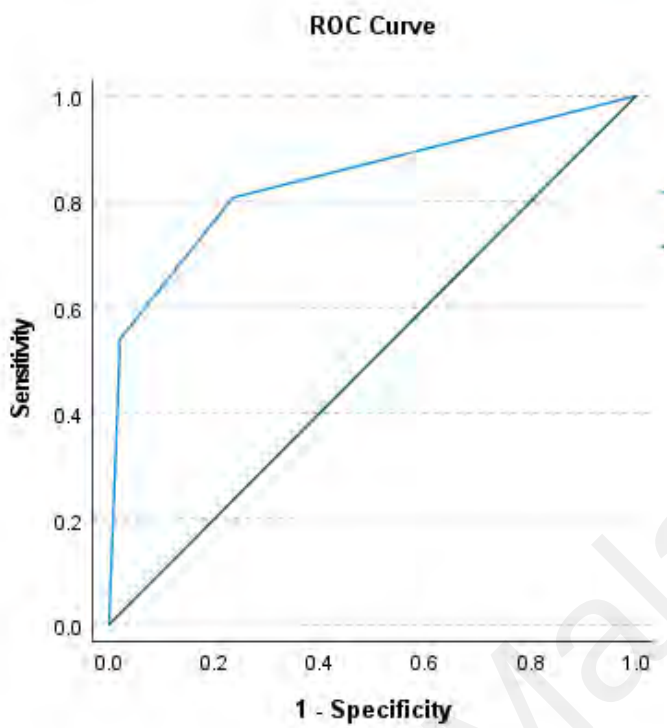



**Figure 4.5. Area under curve, sensitivity and 1-specificity for model 3 (self-reported oral health questionnaire only) for predicting periodontitis.**
**ROC, receiver operating characteristic**

# CHAPTER 5. DISCUSSION

## 5.1 Summary of research findings

After removal of three items, the six-item SROH proved to be valid and reliable for periodontitis screening in our study population. In addition, MMP-8 and MMP-9 levels were significantly higher in the periodontitis group, while IL-1β and IL-6 were overexpressed in both gingivitis and periodontitis groups. These observations were corroborated by positive correlations between IL-1β, IL-6, MMP-8 and MMP-9 with clinical parameters such as PPD, CAL, BOP and to a lesser degree, plaque score. Different predictors were incorporated into models to discriminate periodontitis from non-periodontitis subjects, with the best diagnostic performance being exhibited by the model containing social demographics, salivary biomarkers and SROH.

## 5.2 SROH

The acceptable threshold for I-CVI was fixed at 1.0 in accordance with the recommended threshold when there are five or fewer experts (Lynn, 1986). In the present study, four content experts contributed and participated in the validation exercise. After the first round of review, two items received I-CVI of 0.75, thus necessitating review or omission. The two revised items subsequently received I-CVI of 1.0 at the second validation exercise, thus satisfying the cut-off points of I-CVI, S-CVI/Ave and S-CVI/UA as advocated by Lynn (1986) and Waltz et al. (2016).

During the first round of content validation, a content expert raised concerns about the SROH item “Overall, how would you rate the health of your teeth and gums?”. She proposed for the question to be separated into two questions, addressing teeth and gum separately. During the development of the original eight-item SROH, this question only considered the respondents’ perception about their gum health. However, minor modification was made to expand the question to encompass teeth and gum health after

40 semi-structured cognitive interviews revealed that respondents routinely considered their teeth and gums together. Their responses towards this question were predicated on their existing condition, which went beyond having gingivitis, inflamed gums and bleeding on brushing to include conditions such as the need for tooth extraction (Miller et al., 2007). This line of reasoning and the context of the question on teeth/gum health was relayed to the content expert, and she concurred, and later indicated that the question was highly relevant during the second round of content validation.

Three SROH items, namely question four "gum treatment", question eight "floss use" and question nine "mouthwash" were removed due to poor factor loading scores with the construct of periodontitis and their negative impact on the internal consistency of the scale. For the question on "gum treatment", periodontitis prevalence could have been underestimated among respondents who are not regular dental attendees or lack awareness and knowledge about periodontal treatment. A systematic review of community-based investigations identified disease awareness, aetiology and periodontal-related risk factors as major knowledge deficits among the general public (Varela-Centelles et al., 2016). The majority of studies included were conducted in regions of high economic and human development, hence the findings from less affluent countries could be more dire. Since periodontal screening is not mandatory in the private general dental practice in Malaysia, it is plausible, but speculative that patients, including dental attendees, may receive little to no education about periodontal diseases by their general dentists. In fact, during the face validity test for the SROH, many participants were oblivious to the existence of bone holding the teeth in place, leading to difficulty in understanding the question inquiring about bone loss around their teeth. This is further compounded by the high cost associated with periodontal treatment, even within the heavily subsidised periodontal specialist clinics in the public sector. These costs include

the cost of treatment and biomaterials, transportation expenditure and the loss of income due to absence from work (Mohd-Dom et al., 2014).

The two questions on oral hygiene habits had poor construct validity and internal consistency with the scale. Although evidence existed to support the use of interdental brushes and chemical mouthrinse as an adjunct to toothbrushing alone in reducing plaque and gingival indices, it is less clear whether these oral hygiene habits can reduce the risk of developing periodontitis (Chapple et al., 2015; Escribano et al., 2016; Figuero et al., 2019; Figuero et al., 2020; Serrano et al., 2015). Moreover, flossing was shown in a majority of studies to be ineffective in plaque removal and reduction of gingival inflammation (Sälzer et al., 2015). It is also possible that flossing and the use of chemical mouthrinse are not routinely practiced by our study population, contributing to their poor performance in predicting periodontitis. A cross-sectional study of 787 Malaysian adults reported that the majority of respondents (75%) used additional oral hygiene aids alongside toothbrushing. Specifically, 20.2% of the respondents used mouthwash, while 18.9% used both dental floss and mouthwash (Mitha et al., 2018). However, the study was based on a convenience sample from two regions in Peninsular Malaysia (Kuala Lumpur and Johor Bahru), which are more affluent compared to other regions in Malaysia. The recruitment method may introduce social desirability bias, where respondents overreport behaviours like interdental cleaning or mouthwash use to align with perceived norms.

### 5.3 Selection of candidate biomarkers

Hypothesis free biomarker identification methods offer several advantages over conventional candidate biomarker approach, the latter of which was employed in the current study. Supporting evidence for this concept emerged from two clinical studies (Bostanci et al., 2018; Grant et al., 2022). One of them was a cross-sectional study conducted in England, involving 190 medically healthy adults. Clusters of protein

biomarkers capable of differentiating between different clinical phenotypes of periodontal diseases were determined using a two-stage approach. Phase I comprised of biomarker discovery via mass spectrometry, followed by the validation of these shortlisted proteins by ELISA. The mass spectrometry-based proteomic approach identified 95 proteins that were observed in both GCF and saliva. The protein profiles were then clustered to identify groups with the largest number of proteins present in discriminating between periodontal health and disease states. Finally, 15 candidate proteins were used for ELISA quantification. The best performing panel in discriminating between healthy or gingivitis from periodontitis, healthy from gingivitis and mild periodontitis from severe periodontitis consistently involved MMP-9, alpha-1-acid glycoprotein and pyruvate kinase (Grant et al., 2022). In another large-scale study involving 654 participants, targeted proteomics identified 14 proteins that improved the overall fit and discrimination of a predictive model for periodontal diseases when combined with well-established risk factors (Reckelkamm et al., 2023). The preliminary results of these proteomic analyses are promising and warrant further research (Hu & Leung, 2023).

### 5.4 IL-1β, IL-6 and TNF-α

In the present study, the salivary levels of IL-1β were significantly higher in both periodontitis (5.98 ± 6.68 pg/mL) and gingivitis groups (4.85 ± 6.62 pg/mL) relative to periodontal health (0.22 ± 0.47 pg/mL). Supporting evidence for overexpression of IL-1β in periodontitis state was provided by a case-control study. In that study, significantly higher mean concentration of IL-1β in the saliva samples of adults with moderate to severe periodontitis compared to age-, race- and ethnicity-matched controls was demonstrated. Moreover, significant decline in IL-1β levels was recorded one month after periodontal therapy but they were still higher than controls. In this study, the mean concentrations of IL-1β of the periodontitis and control groups were 1312.75 pg/ml and 161.51 pg/ml, respectively (Kaushik et al., 2011). In addition, Abdullameer and

Abdulkareem (2023) corroborated our study findings by demonstrating the ability of salivary IL-1β to differentiate between periodontal health from gingivitis and periodontitis. In that study, salivary levels of IL-1β were measured among 25 periodontally healthy patients, 25 gingivitis patients and 50 periodontitis patients. The proposed cut-off values for IL-1β to distinguish periodontal health from gingivitis and periodontitis were 103.8 pg/mL and 102 pg/mL, respectively. As in our study, distinction between gingivitis and periodontitis patients based on salivary IL-1β levels was not substantiated (Abdullameer & Abdulkareem, 2023). These concentration ranges for salivary IL-1β in the two preceding studies were much higher than our findings, which could be explained by the differences in the severity of periodontitis. In the study by Kaushik et al. (2011), the average proportion of sites presenting with CAL >2mm (76.5%), PPD ≥4 mm (68.4%) and BOP (86.4%) was very high, indicating a widespread distribution of periodontal inflammation and loss of supporting structures.

On the contrary, significant differences in IL-1β levels between gingivitis group and periodontitis group had been observed in a cross-sectional study of 80 subjects in China (Zhang et al., 2021). Although both our research group and the Chinese team used the 2017 World Workshop classification scheme to define gingivitis and periodontitis, the latter selected a higher threshold for periodontitis that was equivalent to stage III periodontitis. Subjects in the periodontitis group had interdental CAL ≥5 mm, PPD ≥6 mm and radiographic bone loss extending to two third of the root or beyond. The greater severity of periodontitis could explain why there were significantly higher IL-1β levels in periodontitis subjects relative to gingivitis subjects in their experiment.

The levels of IL-6 in the gingivitis group (10.43 ± 19.21 pg/mL) and periodontitis group (8.35 ± 8.96 pg/mL) in our study were significantly higher than the periodontal health group (2.09 ± 4.41 pg/mL), with no significant differences between gingivitis and periodontitis groups. On the contrary, Ebersole et al. (2015) reported significantly

elevated salivary IL-6 levels in the periodontitis group (22.8 ± 3.7 pg/mL) compared to both gingivitis (6.3 ± 2.7 pg/mL) and healthy subjects (3.7 ± 0.5 pg/mL). Moreover, the proportion of undiluted saliva samples that fell below the detection limit of the IL-6 assay was about ten times greater in the gingivitis and healthy groups when compared to periodontitis group. Using linear discriminant analysis, the research group reported improved diagnostic accuracy, sensitivity and specificity when biomarkers were combined. This was especially pronounced for the combination of IL-1β and IL-6 in differentiating between periodontitis from healthy subjects, with AUC of 0.79, sensitivity of 81% and specificity of 77% (Ebersole et al., 2015). Elevated IL-1β and IL-6 levels in periodontal disease states relative to healthy controls were consistent findings across different studies (Ebersole et al., 2013; Rathnayake et al., 2013), and this trend applied to our study population as well.

Chronic inflammation is a cardinal feature of both gingivitis and periodontitis, hence the upregulation of pro-inflammatory biomarkers in both disease groups (gingivitis and periodontitis) is biologically plausible. In an experimental gingivitis study, salivary levels of IL-1β and IL-6 increased in parallel with plaque accumulation, peaking at 14- and 21-days following cessation of oral hygiene practices, respectively (Zhou et al., 2012). Moreover, in that prospective study, the concentrations of IL-1β and IL-6 correlated positively with plaque index, gingival index and bleeding index, but not with PPD. The lack of an association between biomarkers levels and PPD was because all participants are periodontally healthy dental students, whereas the present study included periodontitis patients. The fact that both gingivitis and periodontitis are characterised by chronic inflammation could explain the lack of significant differences in IL-1β and IL-6 levels between periodontitis and gingivitis groups.

In this study, the level of TNF-α was higher in periodontitis group compared with gingivitis or periodontal health groups, but the differences were not statistically

significant. Conversely, another study of 57 Taiwanese adults showed significantly elevated levels of TNF-α among the non-periodontitis group (Wu et al., 2018). However, the authors noted that the detectable levels of TNF-α were very low with wide fluctuations within their study population. On the contrary, a lack of association between salivary TNF-α levels and periodontitis was reported by a higher number of clinical studies (Aurer et al., 2005; Ebersole et al., 2013; Gursoy et al., 2009; Ramseier et al., 2009; Scannapieco et al., 2007). Across these studies, very low to negligible levels of TNF-α in saliva specimens were consistently reported. Similarly, the majority of saliva specimens (70%) in our study presented with TNF-α levels that were below the lower end of the kit's detection range (7.81 pg/mL), which could account for the non-significant differences observed between groups. Altogether, the cumulative evidence suggested that TNF-α, in spite of its biological significance in the pathogenesis of periodontal diseases, is not a valid salivary biomarker for periodontitis.

Despite using undiluted samples, the levels of IL-1β, IL-6 and TNF-α for some specimens remained below the detection limit of the ELISA kits. A possible workaround is to employ standard addition-subtraction methods, whereby a known quantity of peptide is added to all biological samples. The actual concentration is then derived by subtracting the added concentration from the concentration obtained at the end of the experiment (Aydin, 2015).

### 5.5 MMP-8 and MMP-9

Similar to our results for MMP-8, significant elevation in this collagenase enzyme in periodontitis patients was a consistent finding across different studies (Bostanci et al., 2021; Ebersole et al., 2015; Gursoy et al., 2013; Miller et al., 2006; Rai et al., 2008; Rathnayake et al., 2013). This outcome is true whether the control group was patients with periodontal health, gingivitis or a combination of both conditions condensed into a non-periodontitis group. In the case-control study by Ebersole et al. (2015), the mean

MMP-8 level in the periodontitis group (314.1 ± 25.5 ng/mL) was significantly higher than both the gingivitis group (199 ± 29.1 ng/mL) and healthy group (130.7 ± 14.6 ng/mL). The average levels reported across this sample of US population were higher than the concentration range found in our study population (38.4 – 224.43 ng/mL). This could be attributed to variations in study population, inclusion criteria, case definitions for periodontal status and the type of biomarker assay technique used. For example, periodontitis in their study was defined as having BOP at >10% of sites, and ≥5% of sites with PPD ≥4 mm and CAL ≥2. Moreover, 28% of the periodontitis subjects were active tobacco users, which may promote further connective tissue destruction and contribute to increased expression of MMP-8 in the periodontitis group. Overall, our study findings on MMP-8 aligned with a recent systematic review of 10 studies with 485 periodontitis patients and 379 healthy controls. Meta-analysis calculated a SMD of 1.195 with significantly higher MMP-8 levels in periodontitis patients, albeit with high heterogeneity (Zhang et al., 2018).

Like MMP-8, salivary MMP-9 was significantly elevated in the presence of periodontitis in our study. This finding mirrored the observations by a research group in the United Kingdom, who found MMP-9 to be a regular member of a panel of biomarkers that discriminated between healthy and gingivitis, between healthy or gingivitis and periodontitis and between mild and advanced stages of periodontitis. In that study, the diagnostic potential of MMP-9 was demonstrated through a two-stage approach. Firstly, a discovery phase for differentially expressed proteins in saliva and GCF was carried out via mass spectrometry. Ninety-five proteins were detected in both saliva and GCF. Out of these, 15 candidate proteins were further validated by ELISA and tested for sensitivity and specificity as compared to clinical diagnostic criteria. In the UK study, two patient cohorts were recruited, one from Birmingham and the other from Newcastle. While the percentage BOP threshold for gingivitis cases in the Newcastle cohort was >10%, the

corresponding threshold for the Birmingham cohort was >30%. Moreover, gingivitis patients also satisfied an additional criterion, namely the presence of >30% sites with gingival index >2 or modified gingival index ≥3 for the Birmingham and Newcastle cohort, respectively (Grant et al., 2022). In other words, it can be surmised that gingivitis patients in the UK study presented with more widespread and severe gingival inflammation than our study population.

In another study, salivary MMP-9 levels were differentially expressed between periodontal health, gingivitis or periodontitis, with the highest mean concentration being reported in the periodontitis group followed by the gingivitis group. Moreover, the sum of MMP-8 and MMP-9 levels in the periodontitis group was 8.72 times higher than the healthy group. The interpretation of salivary biomarker combinations was further aided by classification and regression tree analysis (CART) (Bostanci et al., 2021). This analysis method processes all parameters based on an internal algorithm and develops a CART diagram, a classification tree of a set of binary if-then logical conditions that guide towards an accurate classification of the patient's periodontal status. Each split in the decision tree represents a sequential step to maximize the sensitivity and specificity of the classification. Using the CART analysis, MMP-9 levels served as a split in the decision tree for the differentiation between health vs gingivitis and health vs periodontitis groups. In fact, when MMP-9 levels were above 150.3 ng/mL, this parameter alone was able to classify gingivitis cases with an accuracy of 90.5% (Bostanci et al., 2021). This corresponded to our study findings, showing significantly higher salivary MMP-9 levels in the periodontitis and gingivitis groups relative to periodontal health. However, MMP-9 was not a significant predictor for periodontitis when combined with SROH response and demographic parameters. This discrepancy could be due to a multitude of factors. Firstly, the periodontitis group in Bostanci et al. (2021)'s study included both generalised aggressive periodontitis and generalised chronic periodontitis

patients. Moreover, the generalised chronic periodontitis patients were required to demonstrate ≥50% alveolar bone loss in at least two quadrants, which is equivalent to stage III/IV periodontitis patients. Furthermore, different statistical method was used. In our study, logistic regression analysis, which depended to a certain extent on user's choices in parameter selection was used. Conversely, CART analysis's selection of parameters and their order in the decision tree was based on its internal algorithm.

The significance of MMP-9 as a biomarker for periodontal disease was further highlighted in a proteomic study by Bostanci et al. (2018). Her research group first performed an open-ended label-free quantitative proteomics of saliva specimens, yielding 119 proteins with at least two-fold significant difference between health and disease states. The discriminative capacity of sixty-five proteins, the majority of which were derived from the label-free quantitative proteomic data were then validated in an independent cohort using selected-reaction monitoring-targeted proteomics. Aided by machine learning modelling, this two-step process pinpointed MMP-9 as part of a five-biomarker panel with high predictive value for periodontal disease. The maximum AUC of MMP-9 when paired with the protein deleted in malignant brain tumours-1 was 0.97 (Bostanci et al., 2018).

The MMPs tested in our study were significantly correlated with clinical periodontal parameters such as PPD, CAL and BOP. Similar outcomes were reported in other clinical studies (Rai et al., 2008). This aligns well with our understanding about the biological role of collagenases in periodontal disease pathogenesis, which mediate connective tissue destruction that occurs during active phases of both gingivitis and periodontitis (Page & Schroeder, 1976).

Levels of IL-1β and IL-6 were positively correlated with MMP-8 and MMP-9 in the present study. This was congruent with the prevailing understanding of the link between these two classes of molecules in the pathophysiology of periodontal diseases.

Fibroblasts, when stimulated by pro-inflammatory cytokines such as IL-1β and IL-6, secrete MMPs to degrade the extracellular matrix (Hajishengallis et al., 2020). This sequence of events facilitates the destruction of connective tissue, manifesting in loss of connective tissue attachment histologically and measurable attachment loss, clinically.

### 5.6 Metallothionein

Metallothionein had been detected in tissues afflicted with numerous chronic inflammatory conditions such as rheumatoid arthritis, inflammatory bowel disease, atherosclerosis and periodontitis (Brüwer et al., 2001; Göbel et al., 2000; Katsuragi et al., 1997; Sun et al., 2018; Winters et al., 1997). Nevertheless, its association with periodontitis is poorly understood.

In the present study, the highest level of MT was observed in the periodontitis group, followed by gingivitis and periodontal health. The mean MT levels ranged from 1342 to 1789 pg/mL. The differences, however, were not statistically significant. This contrasted the results by Yadav et al. (2021), who conducted a cross-sectional study to characterise the differential expression of MT in serum, saliva and GCF of patients with chronic periodontitis or periodontal health. Each group was further subdivided into smokers and non-smokers. Participants who were active smokers irrespective of the number of cigarettes consumed in a day were placed within the smoker group. Participants who had chronic periodontitis and were active smokers presented with the highest level of MT in serum, GCF and saliva samples. The differences were statistically significant when compared to periodontally healthy smokers, periodontally healthy non-smokers and non-smokers with periodontitis. In the absence of smoking, the median levels of MT were still significantly greater among the periodontitis group compared to those with healthy periodontium. Moreover, saliva MT levels were positively correlated with clinical periodontal parameters such as plaque index, gingival index, sulcus bleeding index, PPD and CAL, with correlation coefficients ranging from 0.336 to 0.646, which

were considered weak to moderate (Yadav et al., 2021). The disparity in study findings could be attributed to the inclusion of smokers, as cigarette smoking is a potent source of oxidative stress that could have stimulated the upregulation of MT irrespective of periodontal status.

### 5.7 Predictive modelling using self-reported questionnaire and salivary biomarkers

According to the threshold proposed by Akobeng (2007), the diagnostic accuracy as represented by the AUC of models 1 and 2 was high (>0.9). Meanwhile, the diagnostic performance of model 3 was rated as useful (0.71 to 0.9).

The present study showed that multidimensional model generally performed better than simpler models in predicting periodontitis. Model two, combining SROH responses and demographic parameters had higher AUC than model three, which was derived from SROH responses only. This concurred with findings from Carra et al. (2018), who found that the multivariate logistic regression model derived from their 12-item SROH and selected risk factors presented with better diagnostic performance, sensitivity and specificity metrics than SROH model or risk factor model alone. In that study, the curated risk factors for inclusion into predictive modelling included age, smoking, education level and number of teeth. The enhanced accuracy for periodontitis prediction when self-reported questionnaire was combined with demographics and lifestyle factors was further corroborated by a cross-sectional diagnostic study using a Cantonese version of the SROH. In this study, the best diagnostic performance by far was achieved when this combinatorial modelling was used to predict stage III/IV periodontitis. The AUC, sensitivity and specificity were 0.953, 95.7% and 89%, respectively (Deng et al., 2021).

Using stepwise backward elimination likelihood ratio test, the best reduced model of SROH items and demographic features that predicted periodontitis in our study

included two SROH items (questions on gum disease and loose teeth) and three demographic variables (age, gender and education level). This mirrored the findings in a Spanish population, whereby the best reduced model for predicting severe periodontitis based on the CDC/AAP case definition included only one SROH item on gum disease, and other factors such as age, gender, smoking status and tooth loss. This model yielded sensitivity of 72.2%, specificity of 60.6% and AUC of 0.75 (Montero et al., 2020). The notion that a reduced set of self-reported questions was sufficient for periodontitis discrimination was also corroborated by another study in Spain (Saka-Herrán et al., 2020). Out of the complete set of 12 self-reported questions, only three were significantly associated with periodontitis as defined by the Spanish Society of Periodontics and Osseointegration (SEPA), CDC/AAP and like our study, the 2017 World Workshop classification system. These questions were: "In the past year have you noticed that your teeth are longer or that you have receding gums?" (Q2.5), "have you lost teeth in recent years because of mobility?" (Q2.11) and "do your gums usually bleed either when brushing or chewing?" (Q2.12). This cluster of questions had a sensitivity of 90.2% and AUC of 0.87 after adjusting for age, gender, education status, monthly income and country of origin. Moreover, they were significantly associated with periodontitis with an odds ratio of 15.4 (Saka-Herrán et al., 2020).

In the present study, only two SROH questions were significantly predictive of periodontitis in each of the three models tested. This could be a corollary of combining every periodontitis patient under a single group, including stage I/II periodontitis patients. It appeared that segregating periodontitis cases into different levels of severity increased the number of significant predictors for the outcome of interest. In the Japanese validation study, four, three and two oral health questions were significantly predictive of severe periodontitis, combined moderate and severe periodontitis, and total periodontitis, respectively (Iwasaki et al., 2021). Similarly, lower accuracy and sensitivity of self-

reported periodontal questions were reported when all stages of severity of periodontal diseases were compressed into a single group (Lertpimonchai et al., 2023).

The study by Eke and Dye (2009) suggested that self-reported questions were more specific than sensitive. For example, models predicting the prevalence of total periodontitis had sensitivity that ranged from 48 – 60%, while the corresponding specificity values were between 72 and 88%. This disparity could be ascribed to the method employed by the research group to select the predicted probability threshold. In that study, the predicted probability for periodontitis was chosen to yield a proportion of predicted cases that was equivalent to the observed prevalence of clinically diagnosed periodontitis cases in the sample. Conversely, our study selected the predicted probability cut-off value based on the greatest sum of sensitivity and specificity values.

A source of the variation in diagnostic performances of self-reported questions is the adoption of different case definitions for periodontitis. By far the most used system was the CDC/AAP system, which was designed for population-based studies of periodontitis (Eke et al., 2012; Page & Eke, 2007). More recent studies applied the 2017 World Workshop classification, which were more directly comparable to our present study (Deng et al., 2021; Saka-Herrán et al., 2020).

A research group in Netherlands explored the additional value of adding salivary biomarkers to SROH (Verhulst et al., 2019). They performed a cross-sectional analysis of the predictive performance of the eight-item SROH, demographics and biomarker concentrations from oral rinse samples for the presence of periodontitis, among patients recruited from a general medical setting. Three predictive models were established, evaluating all possible predictors for model one (SROH, biomarkers and demographics), SROH and demographics only (model two) and SROH only (model three). An algorithm expressing the individual sum score for each patient was formulated and compared to the predicted probability cut-off value, to determine if the model classified the patient as

periodontitis patient or not. This methodology was comparable to our study, but differed due to difference in population setting, different biomarkers investigated as well as the inclusion of an additional question on gingival bleeding in the present study. Nevertheless, some similarities were found. The best diagnostic performance for predicting periodontitis was achieved by the model combining questionnaire, demographic data and biomarkers. The AUROCC of the study by Verhulst et al. (2019)was 0.89, with 95% CI of 0.85 – 0.95. This was comparable to but slightly lower than the AUROCC of the present study, which had a value of 0.96.

The logistic regression analytical method employed in the current study only permitted dichotomous classification of periodontal health/disease status, which is overly restrictive, considering that different levels of severity of periodontitis were masked . For example, periodontitis can be differentiated in terms of different stages (I, II, III and IV) and grades (A, B and C) according to the latest classification scheme (Caton et al., 2018; Papapanou et al., 2018; Tonetti et al., 2018). A recent cross-sectional study applied machine learning into multiclass classification of different periodontal health and disease states, reporting higher accuracy as compared to binary classification with logistic regression analyses. A six-class analysis between periodontal health, gingivitis, periodontitis stage I, II, III and IV yielded AUROCC between 0.94 – 0.97, with the best prediction for stage IV periodontitis. The four most important predictors in this model, in decreasing order were age, haemoglobin concentration, self-reported questions related to loose teeth and self-perceived teeth and gum health (Deng et al., 2023).

### 5.8 Inclusion and exclusion criteria

Current smokers and former smokers with less than five years of smoking cessation were excluded from the present study, due to the well-established impact of smoking on periodontal disease pathogenesis and the host's immune-inflammatory response (Apatzidou, 2022). With regards to the concentration of inflammatory mediators

in saliva, smoking can influence the measurements by altering the saliva and GCF flow rate, as well as interfering with the secretion of several cytokines and chemokines (Preshaw et al., 2024). Electronic cigarette users were not explicitly excluded from the present study, although none of the participants reported being active electronic cigarette users. At present, the available body of evidence does not support an association between electronic cigarette usage and worsened periodontal outcomes (Shabil et al., 2024). More research is needed to verify if electronic cigarette is a true risk factor for periodontal diseases.

A study was formulated to investigate the effect of pack years and time since cessation on the salivary levels of MMP-8, MMP-9, TIMP-1 and myeloperoxidase (MPO). The study population was derived from the PAROGENE cohort, compromising 508 patients who underwent coronary angiography and concomitantly, periodontal examination. Periodontitis in this patient cohort was defined as having any amount of alveolar bone loss (cervical third to root apex) and PPD ≥4 mm at ≥4 sites. Smoking cessation was divided into four distinct groups: never smokers, quit more than a year ago, quit less than a year ago and current smokers. Salivary MMP-9 levels were found to be significantly lower among current smokers (median 113 μg/ml) when compared to never smokers (median 242 μg/ml) (P=0.004). The odds ratios for the association between MMP-9, TIMP-1 and MPO with periodontitis were significantly greater for current smokers and those who quitted less than a year ago relative to never smokers. On the contrary, smoking cessation exceeding one year presented similar OR to never smokers. The diagnostic performance of MMP-8 was mainly affected by pack years of smoking, whereas MMP-9 was mostly influenced by the duration of smoking cessation (Lahdentausta et al., 2019).

## 5.9 Limitation

The absence of cognitive evaluation of the SROH questions among the current study population is a limitation of this study, as the respondents' understanding was not comprehensively evaluated. However, the high S-FVI/Ave score, as well as low frequency of missing values (answer response "refused") implied an adequate level of understanding of the SROH questions.

Another limitation is the cross-sectional nature of this present study. Although significant correlations were reported between salivary biomarker levels and clinical parameters of periodontal disease, only associations can be inferred from such an analysis.

Moreover, the study population comprised patients recruited from an academic dental centre, which is not representative of the general population. It is possible that this group of patients had a higher level of dental awareness or experienced severe periodontal problems that prompted the dental visit in the first place. The study should be conducted in different centres and among different populations in Malaysia to determine if the thresholds identified herein are applicable to the general Malaysian population. In addition, a small subset of participants lacked basic understanding of periodontal diseases, which made it difficult for them to answer the questionnaire. Although all participants were able to understand English, some subgroups, especially the Malay respondents voiced their preference for a Malay version of the questionnaire. To that end, a research project that ran parallel to this current study was devised to translate and validate the Malay version of the SROH. Preliminary findings from the pilot study had been reported (Lawrence, 2024). Moreover, the study findings are only applicable for systemically healthy individuals. Patients with underlying medical conditions such as DM could attenuate the discriminatory abilities of salivary biomarkers due to higher level of baseline inflammation. Nevertheless, a recent cross-sectional study affirmed the ability of salivary IL-1β and MMP-8 in distinguishing DM patients with periodontitis from systemically

healthy subjects without periodontitis and diabetic patients without periodontitis (Miller et al., 2021).

The cytokine network involved in periodontitis pathogenesis is complex, intricate and at the present time, not known in its entirety (Pan et al., 2019). It is not the intention of this study to oversimplify the complex cytokine network but rather, to try and identify cytokines that may have a more dominant and large effect on the periodontitis pathogenesis, with consequent enhanced diagnostic and prognostic value.

The participants of this study were presumed systemically healthy by means of self-reporting. However, it cannot be ruled out that some individuals might harbour undiagnosed diseases, due to recall bias or patient's own obliviousness. Subclinical/latent infectious/inflammatory processes that may modulate the protein expression levels could be present in all groups. Future studies could consider conducting medical examinations to verify the systemic conditions that are subjectively reported by participants. Moreover, other risk indicators associated with periodontal diseases such as chronic stress, nutritional status, obesity, undiagnosed DM and prediabetes were not accounted for and could act as confounders. Future studies could account for these factors by incorporating additional measurements such as salivary cortisol levels, body mass index and HbA1c levels.

The lack of blinding of the investigator who interpreted the full-mouth periodontal examination, salivary biomarkers and SROH data constituted another limitation of this study. Concerted effort was made to circumvent this source of bias by administering the SROH and collecting the saliva specimens prior to clinical examination. In addition, salivary biomarker quantification and clinical periodontal examination are both objective measures that are less susceptible to subjective interpretation.

Age differences between the diseased groups and the healthy group is a possible confounding factor on the protein expression levels. However, periodontitis being a chronic inflammatory condition is more prevalent among older adults, showing linear increase in mean CAL with age (Billings et al., 2018; Eke et al., 2018; van der Velden, 1991). This presented considerable difficulty in sampling patients that were matched for age across all three study groups. This limitation also existed for a large number of saliva studies in the literature. In a systematic review of clinical studies evaluating the diagnostic accuracy of biomarker combinations in saliva and GCF for periodontitis detection, the periodontitis groups of ≥70% of saliva studies were ≥40 years old; while control groups in 62% of saliva studies were <45 years old (Blanco-Pintos et al., 2023).

In addition, this study did not measure total protein content as a normalisation factor, which may have affected the biomarker levels due to variations in salivary flow rate, sample volume and participants' hydration status. A rigorous and standardised saliva collection protocol (early morning collection, unstimulated saliva and fasting prior to collection) was followed to minimise inter-individual variation. Future studies should include total protein as a normalisation factor to strengthen the accuracy and comparability of biomarker levels.

## 5.10 Strength

Conversely, the strengths of this study relate to the calibration of examiners for the measurement of clinical periodontal parameters and the adoption of the latest 2017 World Workshop classification scheme for periodontal diagnosis that can facilitate comparisons across different studies. Moreover, the statistical approaches used in this study were reviewed by a statistician and were chosen in accordance with the normality and interdependence of the dataset. A proper sample size calculation was also performed to ensure that the study was not underpowered. It should be noted that the lack of examiner calibration, inconsistent case definitions for periodontitis, inappropriate

statistical tests and underpowered studies were all common drawbacks of a majority of biomarker studies in periodontal disease based on a recent review article (Jaedicke et al., 2016). This study also attempted to characterise the association between salivary MT levels and periodontal disease alone by excluding smokers and former smokers who recently quitted. The validation of the SROH among the local population augurs well for its future integration into population-wide screening of periodontal diseases, such as the decennial National Oral Health Survey of Adults. Moreover, the notion of combining SROH responses and salivary biomarker levels into a predictive model for periodontitis is a relatively new concept that was explored and expanded upon with this present investigation.

# CHAPTER 6. CONCLUSION

Within the limitations of this study, the following conclusions were made.

1. The SROH is a valid and reliable instrument for predicting periodontitis in this sample of Malaysian population.
2. Selected salivary biomarkers such as IL-1β, IL-6, MMP-8 and MMP-9 were significantly overexpressed in periodontal disease states.
3. Selected salivary biomarkers such as IL-1β, IL-6, MMP-8 and MMP-9 correlated positively with clinical periodontal parameters.
4. Predictive model incorporating social demographics, SROH responses and salivary biomarker levels presented with excellent diagnostic accuracy for predicting periodontitis.

## 6.1 Recommendations

Longitudinal changes in salivary biomarker levels in patients who demonstrate periodontitis progression or following completion of periodontal treatment should be investigated in future studies to evaluate their prognostic abilities for periodontitis progression and response to therapy, respectively. Moreover, the SROH should be validated in large groups of people sampled from different population settings in order to verify its generalisability. Future studies should include cognitive evaluation to enable a more thorough analysis of the participants' level of understanding of the SROH items. This would reduce the potential biases from self-reported data. These study designs would further strengthen or refute the findings from this study.

Among the various protein quantification methods, ELISA was selected in the present study due to its high sensitivity in detecting low abundance proteins, such as the case with salivary proteins. The candidate biomarker approach adopted by this study is biased towards our limited understanding of periodontal disease pathogenesis. On the other hand, employment of unbiased, hypothesis-free approach such as label-free

quantitative proteomics in biomarker identification may uncover new protein markers with diagnostic or prognostic value.

Although saliva collection is perceived to be simple, straightforward and quick, our own experience suggested that some patients may encounter difficulty in expectorating the requisite amount of saliva. An alternative medium could be oral rinse samples, which can be collected in under a minute. A recent study suggested that oral rinse possessed better accuracy than saliva or GCF samples in segregating subjects into control or periodontitis group, based on a combination of MMP-8 and chitinase levels (Katsiki et al., 2021).

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