RELIABILITY OF ORAL BIOMARKERS IN THE PREDICTION AND DIAGNOSIS OF PERIODONTAL DISEASE

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ABSTRACT

Introduction: Research has been intense in investigating the possibility of using the ideal oral fluids in the prediction and diagnosis of periodontal disease. In the last few decades, there has been a growing trend to develop better methods to monitor periodontal disease. **Objectives:** The aim of this study was to compare and correlate between the microbiological findings and levels of oral biomarkers at different periodontal disease stages among the three ethnic groups of the Malaysian population. This study is aimed to detect the most accurate biomarkers that can be used to predict and diagnose periodontal disease in these populations. **Methods:** Gingival crevicular fluid (GCF) and subgingival plaque samples from 28 healthy subjects (H), 30 patients with generalized gingivitis (G) and 30 patients with moderate to severe chronic periodontitis (CP) were collected. The levels of biomarkers (LL-37, MMP-8, MMP-9, IL-6, IL-1β, TNF-α, OPG, OC and PGE_2) were quantified by enzyme-linked immunosorbent assay (ELISA) while the subgingival periodontal pathogens (*Tannerella forsythia, Treponema denticola, Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*) were identified using Real Time-polymerase chain reaction (RT-PCR). Cumulative Risk Score (CRS), a new statistical approach, was used to evaluate the accuracy of applying oral biomarkers in the diagnosis of periodontal disease based on three selected biomarkers. **Results:** The results of this study indicated that the ethnic backgrounds of the subjects had no noticeable effect on the periodontal pathogens count and the expression of a majority of oral biomarkers during the progression of periodontal disease. However, only LL-37 levels among the selected biomarkers showed significant difference between the ethnic groups ($p<0.05$). Each biomarker was associated with periodontal disease in the different stages of disease progression. The results of this study showed that MMP-8, IL-1β, PGE2 and IL-6 in GCF are associated with increased count of periodontal pathogens and different clinical

periodontal parameters when compared to the other biomarkers. This association increased significantly by using CRS, which had 2 to 3 times higher odds ratios than the use of any selected biomarkers alone. The area under the curve (AUC) values also increased from 0.653–0.704 for the selected biomarkers separately up to 0.749 for the three selected biomarkers cumulatively represented by CRS. **Conclusions:** In conclusion, this study showed that the levels of biomarkers in the GCF mainly MMP-8, IL-1β, PGE2 and IL-6, if used separately, could be useful in the prediction and diagnosis of periodontal disease to a certain degree of accuracy. The study also showed that the combination of three GCF biomarkers in a single biomarker package to establish the CRS index is more precise in the prediction and diagnosis of periodontal disease than the use of other biomarkers.

ABSTRAK

Pengenalan: Penyelidikan sedang menyiasat kemungkinan untuk menggunakan cecair oral yang ideal dalam ramalan dan diagnosis penyakit periodontal. Aliran semakin berkembang dalam beberapa dekad yang lalu untuk membangunkan kaedah yang betul dalam pemantauan penyakit periodontal. **Objektif:** Tujuan kajian ini adalah untuk membandingkan dan menghubungkait antara penemuan mikrobiologi dan tahap penanda biologi oral bagi peringkat penyakit periodontal yang berbeza antara tiga kumpulan etnik dalam kalangan penduduk Malaysia. Kajian ini bertujuan untuk mengesan penanda biologi yang paling tepat yang boleh digunakan untuk meramal dan mendiagnosis penyakit periodontal dalam kumpulan penduduk tersebut. **Kaedah:** Cecair gingiva krevis (GCF) dan sampel plak subgingival diambil daripada 28 subjek yang sihat (H), 30 pesakit dengan gingivitis umum (G) dan 30 pesakit dengan tahap periodontitis kronik (CP) daripada peringkat sederhana sehingga peringkat teruk. Peringkat penanda biologi (LL-37, MMP-8, MMP-9, IL-6, IL-1β, TNF-α, OPG, OC dan PGE2) diukur oleh asai enzim berkaitan imunoserapan (ELISA) manakala patogen periodontal subgingiva (*T. forsythia, T. denticola, gingivalis P.* dan *Aa*) dikenal pasti menggunakan tindak balas reaksi masa nyata rantai polimerase (PCR). Nilai Risiko Kumulatif (CRS) yang merupakan pendekatan statistik baharu yang digunakan untuk menilai ketepatan menggunakan penanda biologi oral dalam diagnosis penyakit periodontal telah digunakan ke atas tiga penanda biologi terpilih. **Keputusan:** Keputusan kajian menunjukkan bahawa latar belakang etnik subjek tidak memberi kesan yang ketara pada patogen periodontal dan majoriti ekspresi penanda biologi oral pada tahap perkembangan penyakit periodontal yang berbeza. Walau bagaimanapun, hanya peringkat LL-37 menjadi penanda biologi terpilih yang menunjukkan perbezaan yang ketara antara kumpulan etnik ($p < 0.05$). Setiap penanda biologi dikaitkan dengan penyakit periodontal pada tahap perkembangan yang berbeza bagi penyakit tersebut.

Keputusan kajian tersebut menunjukkan bahawa MMP-8, IL-1β, PGE2 dan IL-6 dalam GCF dikaitkan dengan peningkatan bilangan patogen periodontal dan parameter periodontal klinikal yang berbeza jika dibandingkan dengan penanda biologi lain. Hubungan tersebut meningkat dengan ketara dengan penggunaan CRS, yang memiliki nisbah mungkin 2 hingga 3 kali lebih tinggi daripada penggunaan penanda biologi terpilih sahaja. Kawasan di bawah nilai lengkung (AUC) juga meningkat daripada 0.653-0.704 untuk penanda biologi terpilih secara berasingan sehingga 0.749 secara kumulatif untuk tiga penanda biologi terpilih yang dihasilkan oleh CRS. **Kesimpulan:** Sebagai kesimpulan, kajian ini menunjukkan bahawa tahap penanda biologi dalam GCF terutamanya MMP-8, IL-1β, PGE2 dan IL-6, jika digunakan secara berasingan boleh menjadi berguna dalam ramalan dan diagnosis penyakit periodontal sehingga suatu tahap ketepatan tertentu. Kajian ini juga menunjukkan bahawa gabungan tiga penanda biologi GCF dalam pakej penanda biologi tunggal untuk mewujudkan indeks CRS adalah lebih tepat dalam ramalan dan diagnosis penyakit periodontal berbanding penggunaan penanda biologi lain.

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LIST OF SYMBOLS AND ABBREVIATIONS

A.a	: Aggregatibacter actinomycetemcomitans
P.gingivalis	: *Porphyromonas gingivalis*
T.forsythia	: *Tannerella forsythia*
T.denticola	: *Treponema denticola*
PI	: Plaque Index
BOP	: Bleeding On Probing
PD	: Pocket Depth
CAL	: Clinical Attachment Loss
GCF	: Gingival Crevicular Fluid
IL	: Interleukin
TNF-α	: Tumor necrosis factor-alpha
OC	: Osteocalcin
PGE2	: Prostaglandin E2
OPG	: Osteoprotegerin
RANKL	: Receptor Activator of NF-β Ligand
MMP	: Matrix Metalloproteinase
AMPs	: Antimicrobial Peptides
LL-37	: Human Cathelicidine LL-37
ELISA	: Enzyme-Linked Immunosorbent Assay
RT-PCR	: Real-time Polymerase Chain Reaction
FGM	: Free Gingival Margin
CEJ	: Cement Enamel Junction
PPD	: Probing Pocket Depth
CBCT	: Cone Beam Computed Tomography
AgP	: Aggressive Periodontitis

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CHAPTER 1: INTRODUCTION

1.1 Inflammatory periodontal disease

Periodontal disease is a common public health concern that poses a significant challenge globally. Progressive periodontal disease, if not managed efficiently, results in tooth loss eventually (Kassebaum et al., 2014; Marcenes et al., 2013). Furthermore, studies focusing on chronic periodontal disease have gained increased attention as they have been found to potentially play a significant role in pathogenesis and/or progression of various systemic ailments including Alzheimer‟s disease, cardiovascular diseases, diabetes, pre-term births and rheumatoid arthritis (Cullinan & Seymour, 2013; Klukowska et al., 2015).

1.1.1 Diagnosis of periodontal disease

Clinical evaluation and radiographic assessment are primarily carried out to diagnose a periodontal condition. These methods of evaluation provide a cross-sectional measure of pathological condition and cannot solely provide an early diagnosis (Frodge et al., 2008). An ideal aim of any periodontal diagnostic measure is to provide particular information about periodontal status, site, type and severity which facilitates the management of pathology of the disease. It should also provide longitudinal information of the maintenance and monitoring phases of periodontal therapy (Khiste et al., 2011).

1.2 Oral biomarkers

Serum and plasma are among the primary blood components and serve as a source of oral biomarkers. Such as plasma C - reactive protein which is a non-specific marker of inflammation and is known to be elevated in person with periodontal disease (Kanaparthy et al., 2012) and also in normal pregnancy (Larsson et al., 2011).

As blood sampling is an invasive procedure, saliva and gingival crevicular fluid (GCF) have also been considered as alternative sources of biomarkers (Khashu et al., 2012).

GCF comprises of substances derived from oral tissues as well as microflora inhabiting supragingival and subgingival dental biofilm (Lamster & Ahlo, 2007). Biomarkers in GCF can be identified to make an early diagnosis periodontal disease (Shimada et al., 2013).

The pathological breakdown of periodontal tissues is regulated by the host response. Immune responses of host cells to periodontal pathogens and their virulent by-products result in local release of inflammatory mediators (Van Dyke & Serhan, 2003). Several biomarkers have been found in GCF as a result of cellular responses around periodontally healthy and diseased tissues. These include; (1) Proinflammatory markers like interferon-γ (IFN-γ), interleukin (IL)-17, IL-23 and tumour necrosis factor-α (TNF-α), (2) anti-inflammatory markers like IL-4, and (3) osteoclastogenesis-related factors like receptor activator of NF-β (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG). Hence, identification of these biomarkers from GCF could serve as valid means of studying the role of systemic disorders in the pathogenesis and physiology of periodontal disease and the cellular host response to periodontal therapies (Santos et al., 2012) .

1.3 Aims of the study

1- To evaluate the accuracy of a new diagnostic method in early prediction and diagnosis of periodontal disease.

2- To compare between the microbiological findings, oral biomarkers and antimicrobial peptide levels in different ethnic groups within the Malaysian population.

3- To compare between the microbiological findings, oral biomarkers and antimicrobial peptide in subjects with different periodontal diseases.

1.4 Research objectives

1-Establishing criteria for the diagnosis and prediction of periodontal disease by the following:

A. Microbial criterion: by determining the average count of the red complex and *A.a* species in the subgingival biofilm among healthy, gingivitis and chronic periodontitis subjects.

B. Oral biomarkers criterion: by determining the average concentration of the oral biomarkers in the GCF among healthy, gingivitis and chronic periodontitis subjects.

C. Antimicrobial peptide criterion: by determining the average concentration of the antimicrobial peptide in the GCF among healthy, gingivitis and chronic periodontitis subjects.

2- Evaluate the reliability of the established criteria in the detection and diagnosis of periodontal disease.

1.5 Hypothesis

We hypothesized that this diagnostic method will be efficient in the prediction of periodontal disease and can give us a clearer picture for disease prognosis.

1.6 Null hypothesis

This diagnostic method will not be able to predict the status and progression of periodontal disease.

1.7 Significance of the research

The diagnosis of periodontal disease depending on the measurement of many clinical criteria such as bleeding on probing, clinical attachment loss and probing depth can be lengthy and considerably difficult. However, it is the most accurate and dependable method in the monitoring and diagnosis of periodontal disease.

Recently, researchers have been investigating oral biomarkers which include cytokines, chemokines and enzymes that are derived from different biofluids in an attempt to discover a method that can assist in the prediction and diagnosis of periodontal disease.

Although many oral biomarkers that are derived from GCF which has been proven to be associated with the different stages of periodontal disease and could be used as indicator for disease progression. There was no attempt to use these biomarkers in the prediction and/or diagnosis of periodontal disease until now. In this study, the CRS which is a new statistical approach was used for determining the diagnosis of

periodontal disease. This new method proved to have a good potential in the diagnosis of periodontal disease, especially in a large population.

CHAPTER 2: LITERATURE REVIEW

2.1 Periodontal disease

2.1.1 Introduction

One of the main causes of tooth loss is periodontal disease. It is amongst the serious public health problems affecting individuals socially and economically (Makiura et al., 2008).

In periodontally healthy patients, periodontal tissues enclose each tooth circumferentially and a shallow space exists between the crest of the gingiva and the adjacent tooth surface. This V- shaped space is termed the gingival sulcus. Microorganisms present within the gingival sulcus initiate an inflammatory process that leads to disease progression. Gingival sulcus gradually deepens and gradually results in the formation of a pathological periodontal pocket. Inflammation of periodontal tissues begins with gingivitis, followed by apical migration of clinical attachment and eventual loss of periodontal tissue and bone (Lockhart et al., 2012).

Prevalence of severe generalized disease is comparable in different populations, however a higher prevalence has been found in developing countries (Quijano et al., 2010). Prevalence rates ranging from 24.5% to 79.6% have been reported globally, with respect to their country developmental status. Variations in the prevalence rates can be related to various behavioural, socioeconomic and demographic factors. These factors include oral health habits, smoking habits, level of income, age, gender and ethnicity (Pei et al., 2015), socioeconomic status in developing countries playing the major role in the prevalence of periodontal disease.

2.1.2 Classification of periodontal disease

In order to study the etiopathogenesis and treatment of any disease, prior classification provides a general framework. It also assists clinicians to organize health care needs of the patients (Armitage, 1999). For decades inherent differences in periodontal disease have been identified by clinicians and attempts have been made to classify these diseases accordingly. With the integration of classification systems, clinicians can easily identify and characterize the disease based on classification. These classifications also give specific information regarding possible aetiology and suggest evidence based therapies to clinicians. Widely accepted classifications facilitate global communication for remote health care provision (Highfield, 2009). Periodontium is mainly affected by two common diseases namely gingivitis and chronic periodontitis.

2.1.2.1 Gingivitis

Gingivitis can be defined as gingival inflammation without any loss of connective tissue attachment. Only the soft tissue compartment including the connective tissue and gingival epithelium is involved. Clinical presentation of gingivitis is dependent upon the severity of the disease itself. It can range from mild redness or swelling of gingiva to bleeding on probing up to spontaneous gingival bleeding (Newman et al., 2011). Bleeding on probing on a long term basis has peculiar importance in terms of disease severity and is considered a sign of progression to periodontitis (Klukowska et al., 2015).

2.1.2.2 Chronic periodontitis

Chronic periodontitis occurs due to the complex nature of the interactions between periodontal pathogens and cellular immune response. Dental biofilms have been considered necessary for the initiation of the disease, however, its presence alone can only result in varying expression of disease progression (i.e., 20–30%) rather than causing the disease itself (Grossi et al., 1994). Microbial dental biofilm has been considered ultimately responsible for long standing destruction of periodontium in patients who have an exaggerated inflammatory response (Kirkwood et al., 2007). The National Health and Nutrition Examination Survey suggested that over 47% prevalence of chronic periodontitis was detected among adult Americans, which represented 64.7 million adult individuals (Eke et al., 2012).

The oral cavity harbours countless bacteria out of which many hundreds types have been isolated (Paster et al., 2001). Periodontitis appears to differ from other diseases since a group of different bacteria have been held responsible instead of a single culprit bacterium. Chronic gingivitis and periodontitis present almost similar clinical symptoms in different populations (Cekici et al., 2014).

Table 2.1: Guidelines for Determining Severity of Periodontitis (Periodontitis, 2015)

	Mild	Moderate	Severe (Advanced)
Bleeding on probing	Yes	Yes	Yes
Probing depths	>3 & <5 mm	≥5 & <7 mm	≥7 mm
Clinical attachment loss	1 to 2 mm	3 to 4 mm	≥5 mm
Radiographic bone loss	Up to 15% of root length or ≥2 mm&≤3 mm	16% to 30% or >3 mm & ≤5 mm	>30% or >5 mm

2.1.3 Diagnosis of periodontal disease

Diagnosis can be defined as the identification of a pathology from its symptoms and signs (Van Der Velden, 2005). Accurate diagnosis is only ascertained by evaluating the collected information thoroughly. The information includes: l) detailed patient history, 2) medical consultation, 3) periodontal clinical assessment, 4) radiographic evaluation and 5) laboratory investigations as required (Pihlstrom, 2001).

Recently, elaborate information has been added to the diagnosis and classification of periodontal disease. Numerous classifications have been proposed based on clinical signs of periodontal disease (Armitage, 1999; Picolos et al., 2005; Van Der Velden, 2005). Nevertheless, diagnosis and differentiation solely based upon clinical signs is not always accurate.

Though recent classification to diagnose periodontal disease was based on microbial infection/cellular response paradigm, information regarding etiological diagnosis is still scarce (Van Der Velden, 2005). Subsequently, the diagnostic potential of microbiological characterization (Listgarten & Loomer, 2003), assessment of host

cellular response (Loos & Tjoa, 2005) and genetic evaluation (Schäfer et al., 2011) have been explored.

Clinical measures of periodontal disease such as clinical attachment level (CAL), bleeding on probing (BOP) and pocket depth (PD) provides limited real-time information to the clinician about the disease status. In addition, these measures serve as poor predictors for future disease progression (Lindhe et al., 1983). An ideal diagnostic tool should therefore account for the present status and severity of disease and also predict the clinical course of the periodontal disease (Mcculloch, 1994). However, prediction of clinical course of disease progression is still uncertain despite the advances in research methodologies and laboratory assays (Kinney et al., 2014).

2.1.3.1 Plaque Index (PI)

A significant role of plaque biofilm deposits and retention in the development of disease has been established. Plaque Index (PI) is based following the same principle as the Gingival Index that clearly distinguishes between the severity and site of plaque aggregation. This system was introduced to homogenize the data with the Gingival Index (Löe, 1967). The Plaque Index criteria (Silness & Löe, 1964) can be described as:

0 = Absence of plaque biofilm from the gingival region.

1 = Presence of thin films of plaque attached to the free gingival margin (FGM) and surrounding tooth surfaces. A probe is run across the surfaces of the tooth to recognize plaque.

2 = Presence of moderate aggregates of soft deposits within or above the gingival margin and/or involving the surrounding tooth surfaces that can be clinically identified.

3 = Presence of abundant soft deposits within and/or above the gingival margin and the surrounding adjacent tooth surfaces.

2.1.3.2 Gingival Index (GI)

The primary objective of devising the Gingival Index system was to provide a measure of the gingival condition which is able to distinguish between different severities of lesion (quality) and the site (quantity) as related to the four (mesial, buccal, lingual, distal) locations which make up the entire circumference of the gingival margin.

The Gingival Index is entirely based upon the qualitative facets of gingival tissues (Löe, 1967). It does not take into account the pocket depth, degree of connective tissue loss or any type of quantitative change. The Gingival Index criteria can be described as:

0 = Healthy gingiva

1 = Mild inflammation: Exhibiting a slight change in colour with slight oedema and absence of bleeding on probing

2 = Moderate inflammation: Exhibiting obvious redness, glazing and oedema and bleeding upon probing

3 = severe inflammation: Exhibiting marked redness, ulceration, oedema and spontaneous bleeding tendency

2.1.3.3 Bleeding on probing (BOP)

Bleeding on probing (BOP) is a clinical sign of periodontal stability and disease progression. Absence or presence of BOP has been used as a clinical indication to determine the existence of periodontal disease and its progression (Joss et al., 1994).

BOP documents two different risks of disease progress: (1) Number of locations (percentage) which bleed on probing in a set of dentition may predict the risk of disease progression in an individual (2) The assessment of whether an individual tooth bleeds on probing or not may predict the local risk of peculiar tooth sites (Lang et al., 1996).

BOP is conventionally evaluated by using a periodontal probe. The probe is inserted in the bottom of the gingival pocket or sulcus. If no bleeding occurs a score of zero is given, whereas if bleeding ensues within 10-15 seconds, one score is given (Ainamo et al., 1975).

2.1.3.4 Probing pocket depth

Probing pocket depth (PPD) gives a measure of distance to the location where the probe meets physical resistance by the connective tissue attachment. Clinically, the linear distance between the crest of gingival margin and the point where the probe first meets resistance has been frequently measured. These measures have been used to guide management plan, but longitudinal comparison by providing treatment utilizing these measures has been considered difficult. Difficulty was faced because unstable reference locations which could move with oedema or recession of tissue or may remain stationary with respect to progression of disease. To address this issue, clinical attachment loss (CAL) have been commonly measured to evaluate the extent of soft tissue attachment (Reddy, 1997).

2.1.3.5 Clinical attachment loss

Linear distance from the cement enamel junction (CEJ) to the base of the periodontal pocket is defined as CAL. CAL is calculated by performing two measurements of each tooth of particular concern. These measurements are: (1) Gingival recession (REC) [defined as the linear distance between FGM and CEJ] and (2) PPD [defined as the distance between FGM and the bottom of the sulcus or pocket]. A negative score is given for REC when FGM is apically positioned with respect to the CEJ and if coronally positioned a positive score is given. Six sites per tooth are scored which are, mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual for entire dentition. A periodontal probe is parallelly positioned along the long axis of individual tooth at each of the six sites and scores are rounded off to the closest millimetre (Eke et al., 2015).

2.1.3.6 Radiographs

Accurate evaluation of the condition of the bone is of prime importance for the diagnosis, management and prediction of prognosis of disease (Langen et al., 1995). Information from CAL measurement can be used in conjunction with radiographic diagnostic imaging to assess alveolar bone height and for detection of vertical bone defects (Reddy, 1992). Radiographs can detect the hard tissue changes brought about by the inflammatory response to microorganisms and can distinguish between different patterns of bone resorption (Mol, 2004). Among two-dimensional (2D) radiographic imaging techniques, periapical and bitewing radiographs are considered more suitable as they can be acquired easily, economically and have suitable quality of images. However, these techniques have inherent limitations such as difficulty in standardization, overlapping of anatomical structures and underestimation of the size

and occurrence of bone defects. Numerous studies have been conducted to document the accuracy of these techniques, however precise diagnosis of bone loss still remains a challenge (De Faria Vasconcelos et al., 2014), even with the advent of orthopantomograph (OPG) and more recently the cone beam computed tomography (CBCT).

2.1.4 Microbiological assessment for periodontal diagnosis

It has been established that the human microbiome plays an essential role in the well-being of the host. Countless efforts have been made to isolate and characterize the diverse microbial communities that colonize in the human host. Among these sites oral microbiota have been commonly studied, and the microbiota colonizing in oral habitats have been extensively characterized (Consortium, 2012; Duran-Pinedo & Frias-Lopez, 2015).

Numerous researches have focused on the identification of disease specific species such as caries, gingivitis and periodontitis (Paster et al., 2006). More than 250 different species have been isolated, characterized and classified with advanced culturing techniques and the virulence of numerous microbes such as *Streptococcus mutans, Actinobacillus actinomycetemcomitans, Tannerella forsythia* and *Porphyromonas gingivalis* have been extensively documented (Holt & Ebersole, 2005; Takahashi & Nyvad, 2011). Socransky et al. (1998) proposed that particular focus on the interactions between different microorganisms can lead to better understanding of oral diseases rather than studying individual pathogens. This concept led to the introduction of „holistic" or „systematic thinking" into oral microbiological research. Following this idea, various studies accepted the polymicrobial nature of complex oral diseases like caries, gingivitis and periodontitis (Darveau, 2010; Kuramitsu et al., 2007). Therefore,

to develop comprehensive understanding of healthy microbial physiology, it is essential to elucidate the microbial composition and diversity in healthy individuals. This knowledge could facilitate development of novel diagnostic tools, corrective and preventive therapies for these polymicrobial diseases (Xu et al., 2015).

Comprehensive characterization of microbes responsible for different types of periodontal disease is still not described (Armitage & Cullinan, 2010). The diversity of microbiota, as well as varying inter- and intra-species may be responsible for such difficulties (Heller et al., 2011; Teles et al., 2006). Evidence suggests that the distribution of microbiota differs among different populations (Rylev & Kilian , 2008). Most of the microbial species are also members of health microbiota which further complicates the identification (Socransky & Haffajee, 2002). Hence, low levels of periodontitis associated microbes are commonly found in healthy individuals (Colombo et al., 2002). These particular complications emphasize the need of development of a definitive microbial diagnostic tool which may assist the clinical diagnosis and facilitate a better management plan (Shaddox & Walker, 2009).

2.2 Dental plaque biofilm

Dental plaque can be defined as a matrix of bacterial and salivary polymers harbouring microbial community on tooth surfaces in the form of biofilm (Marsh & Martin, 2009). Formation of an species-rich plaque matrix structure and function occurs as result of an ordered sequence of events (Kolenbrander et al., 2005; Marsh et al., 2011). Dental biofilm naturally forms on tooth surface and benefits tooth structure by preventing exogenous species from colonization. Despite of the minor environmental perturbations microbial composition of biofilm remains stable which is known as microbial homeostasis. This homeostasis occurs as a result of dynamic balance between

both antagonistic and synergistic microbial interactions. However, microbial shifts can occur which can lead to predisposition at the site of shift. An example of a shift is frequent episodes of low pH environment which leads to the selective inhibition of acid-sensitive microbes and favours the growth of aciduric microbes such as mutans streptococci and lactobacilli (Marsh, 1994).

2.2.1 Development of dental plaque biofilm

Dental plaque harbours structurally and functionally organized polymicrobial species as a result of ordered sequence of events (Marsh, 2004). Plaque formation takes place in distinct stages that include:

(1) Formation of acquired pellicle
(2) Long-range weak physico-chemical interactions between the pellicle and the cell surface
(3) Stronger attachment mediated by adhesin-receptor
(4) Attachment of secondary colonizers by co-adhesion to already attached cells (Kolenbrander et al., 2000)
(5) Microbial multiplication
(6) Synthesis of exopolysaccharides leading to biofilm formation
(7) Occasional detachment

An increase in understanding of the mechanisms of microbial attachment and intermicrobial adhesions can lead to restrict or modify pattern of biofilm formation. Analogs can be developed to hinder attachment of adhesin-receptor or co-adhesion of various microbes and chemical modification of colonizing surfaces could also hinder microbial colonization. However, microbes have been found to express different types of adhesins (Zhang et al., 2005), hence even successful blockade of

one type of adhesin could still lead to unaffected attachment mechanisms. Although colonization in biofilm matrix is essential for microbes, growth of microbes is dependent upon survival capability of a microbe in an environment (Marsh, 2006).

2.3 Periodontal pathogens

The microbes which increase the risk of periodontitis and alter immune responses of host individuals are known as periodontopathogens. They are also stimulate the secretion of proinflammatory cytokines by inflammatory cells that are essential for destruction of periodontal tissue (Kato et al., 2014).

A cascade of events occurs in the subgingival environment, which includes changes in the pH, increase in colonization of anaerobic bacteria and nutritional disturbances. These events eventually lead to the assimilation of periodontopathogens in the subgingival biofilm, which results in the initiation of periodontitis (Marquis, 1995; Mettraux et al., 1984; Takahashi, 2003). The initiation and progression of periodontal disease has been closely associated with the quantitative and qualitative changes in subgingival biofilms (Marsh, 1994; Takahashi, 2005).

Five microbial complexes have been frequently isolated together in the subgingival biofilms of healthy and diseased subjects (Socransky et al., 1998). *P. gingivalis*, *Treponema denticola*, *and Tannerella forsythia* together form the red complex, which is found in the later stages of biofilm development. The bacteria comprising the red complex have been previously considered as the most pathogenic microbial complex (Holt & Ebersole, 2005; Socransky et al., 1998). These bacteria have been investigated due to their detected association with gingival BOP, which has been considered an important clinical sign and measure of periodontal disease (Sundqvist & Figdor, 2003).

Much information needs to be added to the dynamic interactions and complexity of the periodontal ecosystem. Quorum sensing focuses on studying the oral ecology and it has been found essential to understand the physicochemical interactions of microbial and human cells and their subsequent effects on the disease process. Such information can ultimately lead clinicians to the development of novel, advanced and/or more adequate therapeutic and preventive approaches This information could also assist in designing diagnostic applications in periodontics (Da Silva-Boghossian et al., 2011).

It has been suspected that the presence of microbes of this complex can influence the capacity for colonization of other microbes. This can lead to the selective increase in the number of certain microbial virulent strains, hence affecting the virulence of the entire biofilm structure (Thurnheer et al., 2014).

Moreover, microorganisms such as *Campylobacter rectus*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Peptostreptococcus micros and Prevotella* species have been isolated in deep periodontal pockets in increased amounts, thus suspected as possible periodontopathogens (Colombo et al., 2009; Paster et al., 2006). Single microbial type is seldom isolated, rather a synergistic microbial role in tissue destruction has been suggested (Darveau et al., 1997).

Previously most of the clinical studies determining the association between various microbes effecting periodontal disease have been retrospective or cross-sectional instead of prospective longitudinal studies. These studies used semi-quantitative methods for microbial enumeration. Associations between microbial species and clinical tools to measure the periodontal disease cross-sectionally or retrospectively have failed to address arguably the most essential question in the determination of a microbial aetiology. It has been frequently asked whether the identified microbe or a complex of polymicrobes were present before the tissue destruction occurred or they accumulated

as a consequence of environmental and nutritional disturbances during and preceding the tissue destruction. Associations between periodontopathogens and periodontal disease have been reported at an individual level rather than at specific site level in the dentition. Although specific information about individuals at higher risks is substantially important to the clinician, site-specific information has been considered ideal for prompt diagnosis (Byrne et al., 2009). Routine monitoring of the levels of red complex microbes in the subgingival biofilm may aid in identification of locations, which have an increased risk of tissue breakdown and this information could assist target specific treatment (Byrne et al., 2009).

2.3.1 *Porphyromonas gingivalis (P. gingivalis)*

Porphyromonas gingivalis is a gram-negative anaerobe that has been identified as one of major periodontopathogens. It is involved in the initiation and progression of periodontal disease (Hajishengallis, 2015; Socransky & Haffajee, 2002). It has been frequently isolated from dental biofilm and a considerable involvement in the immunopathogenesis of periodontal disease has been documented (Ramos-Junior et al., 2015). Its presence in the subgingival plaque biofilm enhances its ability to tolerate the environmental and nutritional insults/signals external to the cytoplasmic membrane (Onozawa et al., 2015).

P. gingivalis is a predominant periodontopathogen that expresses multitude of potential virulence factors contributing to the pathogenesis. These pathogens attack epithelial cells of gingiva and the cells inhibit the deeper invasion in to the tissues. Two peculiar fimbriae of *P. gingivalis* have been identified: Mfa1 and FimA (Nagano et al., 2015). Fimbria is essential for the microbial-host interaction, as they facilitate the invasion of target sites and microbial adhesion. These fimbriae have the potential of

binding to the salivary components, matrix proteins and intermicrobial complexes. Fimbriae strongly adhere to the cellular α5β1- integrin. Following these, *P. gingivalis* is engulfed by cellular pseudopodia that ensues invagination process through an actin-mediated pathway. The event of invasion requires actin fibers, microtubules, host cellular dynamin and lipid rafts. After passage through the gingival epithelial barrier, these pathogens impair cellular function. Six genotypes of fimbriae from type I to V and Ib have been classified based upon the genotypic diversity. FimA genes are responsible for encoding each fimbria subunit. At the intracellular level, *P. gingivalis* having type II fimbriae significantly degrade paxillin, integrin-related signalling molecules and focal adhesion kinase that in turn lead to the down regulation of migration and proliferation of epithelial cells. These physico-chemical events describe the potential microbial strategy for persistence in epithelial cells (Amano, 2007). In addition, *P. gingivalis* induces peripheral CD4+ T helper cells to release increased levels of pro-inflammatory cytokines (IL-1β and IL-6) which leads to the development of aggressive periodontitis (AgP) (Gonzales et al., 2014).

2.3.2 *Treponema denticola (T. denticola)*

Treponema denticola is an anaerobic spirochete which is found in limited concentration in healthy subgingival area whereas it occurs in high concentrations in diseased periodontal pockets (Miller et al., 2014). It is also among the major periodontopathogens that have been associated with periodontal disease (Shin & Choi, 2012). It has flagella and it preferentially colonizes in the deepest area of pockets (Noiri et al., 2001; Omar et al., 1990).

The microbial ecology of subgingival crevice is greatly influenced by the chemotaxis of *T.denticola* (Ruby et al., 2008). The dmcA and dmcB genes have been

characterized to be responsible for chemotaxis in *T. denticola* (Li et al., 1999). Lux et al. (2001) suggested that this ability of *T. denticola* helps transportation of non-motile bacteria by inter-bacterial adhesion known as piggybacking.

The outer membrane of *T. denticola* is composed of dentilisin (lipoprotein–protease complex) and polypeptide products [monocistronic prcB-prcAprtP operon (Bian et al., 2005; Godovikova et al., 2010)]. This operon is identified and limited various oral *Treponema* microbes (Correia et al., 2003). The dentilisin plays its role in tissue destruction by decomposing extracellular matrix and serum components (Mcdowell et al., 2009), disturbing intercellular junctions (Chi et al., 2003) and by causing an imbalance in tissue homeostasis (Miao et al., 2011).

2.3.3 *Tannerella forsythia (T. forsythia)*

Tannerella forsythia was formerly known as *Bacteroides forsythus* as it was initially isolated at The Forsyth Institute in the mid-1970s. The subjects from which this microbe was isolated had progressing advanced periodontitis. Difficulties were faced in understanding the taxonomy of this microbe as it behaved differently from other gram-negative anaerobes. Primarily its slow and fastidious proliferation needs and cell morphology was alien to the existing knowledge (Tanner et al., 1979). It is a fusiform type of rod-like bacteria. *T. forsythia* is among the major etiopathological microbes that have been associated with periodontal disease, including aggressive and chronic periodontitis. It is involved in the initiation and progression of periodontal disease (Tanner & Izard, 2006).

The polymicrobial flora that are responsible for epithelial invasion include *T. forsythia* (Rudney et al., 2005). This capability of invasion into the epithelial cells makes these periodontopathogens more virulent and facilitates the evasion and/or

inhibition of the host defence responses. It also helps in reservoir formation which is essential for recurrent infections. Multiple virulence factors have been identified in different strains of *T. forsythia* that include BspA, sialidase, hemagglutinin, trypsin-like protease and bacterial S-layer components. BspA is a protein secretion that has associated with cell surface and its significant role in alveolar bone loss was detected in an animal study (Sharma et al., 2005).

2.3.4 *Aggregatibacter actinomycetemcomitans (A. a)*

Aggregatibacter actinomycetemcomitans (A. a) also termed as *Actinobacillus actinomycetemcomitans* is a nonmotile, gram-negative and facultative anaerobe. It frequently colonizes in the oral cavity of humans and it belongs to the Pasteurellaceae family. Significant associations with periodontal and other systemic ailments, like brain abscess and thyroid, have been determined (Rahamat-Langendoen et al., 2011) . *A.a* is considered to have a primary role in early onset periodontal disease and its refractory forms, with a particular focus on localized aggressive periodontitis (Asikainen et al., 1991; Elamin et al., 2011; Zambon, 1985).

A.a possesses the ability to ferment different forms of sugars, including fructose, gluctose and maltose. Numerous virulence factors have been identified that contribute in the onset of periodontal disease. These factors include leukotoxins, collagenases, lipopolysaccharides (LPS), cytolethal distending toxins (CDT) and few other membrane proteins (Åberg et al., 2013; Kachlany, 2010; Rogers et al., 2007; Schreiner et al., 2003). In addition, it has been increasingly documented that *A.a* induces cell apoptosis in gingival epithelium (Kang et al., 2012; Li et al., 2011). The exact molecular interaction of its microbial colonization in various human organs including the oral cavity is still unclear. This lack of information could be due to the scarcity of genetic

and molecular assessment tools available for it and other members of Pasteurellaceae family (Torres-Escobar et al., 2014).

Six serotypes (a to f) of *A.a* have been identified. This identification has been based upon the differential antibody response due to the presence of specific capsular proteins. (Kaplan et al., 2002). The identified genotypic variations have a significant association with differential virulence as serotype "a" and "c" have been associated with healthy periodontal tissues whereas serotype "b" has been associated with periodontal disease (Asikainen et al., 1995; Haffajee & Socransky, 1994; Zambon et al., 1983).

The distribution of different serotypes of *A. a* has geographical heterogeneity and the association between various serotypes and periodontal status of individuals may vary based on ethnicity and/or geographical location of populations (Dahlen, 2002; Fine et al., 2007). While studying a large population sample of Philadelphia, Yang et al. (2004) observed that serotype "b" was commonly isolated from samples having aggressive periodontitis rather than the chronic form. Information on whether the colonization ensues through unique serotype or multiple serotypes is still in debate (Asikainen et al., 1991; Chung et al., 1989; Mombelli et al., 1999).

2.4 Real-time polymerase chain reaction (PCR)

To develop a deeper understanding of the periodontal disease process, it is essential to acquire information about its aetiology. Culture techniques have been conventionally used to isolate and characterize microbes in a sample, however these approaches evidently underestimate the presence of certain microbial strains due to lack of specificity. Polymerase chain reaction is a molecular based method which can be used to rapidly identify specific microorganisms with superior sensitivity (Boutaga et al., 2006; Nonnenmacher et al., 2004).

Reliable and quick methods of microbial identification are desirable in microbiology. These requirements directly affect the clinical microbial diagnosis and also influence basic microbiological research. Although conventional culture methods have been used for more than a century, the molecular methods have gained increasing attention since their introduction in 1990‟s. These methods, in addition to the quantification of previously culture sensitive microbes also allow analysis and screening of complex samples rapidly. Nevertheless, even molecular methods have certain limitations and practical implications (Ammann et al., 2013).

Previous culturing methods were unable to quantify any specific microbes and also lacked the ability to allow proliferation of certain culture sensitive bacteria. It has been hypothesized that increased levels of certain periodontopathogens in specific location could result in commensal or rapid destruction of periodontal tissue. Real-time PCR might overcome certain limitations and fulfil shortcomings of previous methods. Real-time PCR evaluation not only identifies the periodontopathogens, in addition it can also give an account of the total bacterial load in a relative proportion (Boutaga et al., 2006). The identification of these microbes is essential for accurate diagnosis and for adjunctive therapeutic management (Loomer, 2004).

The disadvantage of conventional PCR is its lack of accurate quantification, which results in lack of information regarding important diagnostic aspects. The designing of fluorogenic probes and the development of continuous fluorescence monitoring tools has allowed real-time PCR detection. These assays allow automated detection and quantification of specific amplified bacterial products. The TaqMan system with the help of fluorogenic probe allows real-time laser scanning of the PCR products accumulating in a 96-well plate. Quicker assays are possible with this technique as no post sample handling is required. The log phase of the reaction allows quantification of a large dynamic range of the amplified target molecules (Nonnenmacher et al., 2004).

Previous studies have reported reliable quantification of numerous viral and bacterial microbes using PCR methods (Asai et al., 2002; Corless et al., 2001; Ke et al., 2000; Lyons et al., 2000; Mcavin et al., 2001).

The identification and quantification of oral microbes for clinical diagnosis using nucleic acid base PCR techniques has been extensively reported (Aas et al., 2008; Boutaga et al., 2006; Park et al., 2011; Preza et al., 2008; Sanz et al., 2004; Suzuki et al., 2004; Teles et al., 2012) , however information regarding assessment of antimicrobial efficacy is scarce. These techniques have also been complemented for their superior sensitivity, specificity and rapid provision of results (Sanchez et al., 2014).

2.5 Gingival crevicular fluid (GCF)

A mixture of different substances derived from host inflammatory cells, serum, microbes and structural cells of the periodontium is collectively known as gingival crevicular fluid (GCF). GCF is derived from adjacent vessels of gingival plexus and travels through the junctional epithelium and the external basement membrane to appear in the gingival sulcus. It can be extracted in trace amounts from healthy gingival sulcus. GCF isolated from healthy gingival sulcus results as a consequence of shift in osmotic gradient (Alfano, 1974). GCF contains substances released due to the inflammatory response. Information regarding these substances can be beneficial in assessing disease status and/or therapeutic outcomes (Toker et al., 2006).

Biomarkers in GCF can be identified to diagnose and monitor periodontal disease (Loos & Tjoa, 2005). The easy and non-invasive collection of GCF and the diverse array of biochemical and cellular molecules isolated from GCF explain its diagnostic potential. In healthy gingival sulcus, GCF exists as transudate, whereas in case of periodontal disease, it exists as an inflammatory exudate, which contains substances

derived from the serum, periodontal tissues and colonizing periodontopathogens (Delima et al., 2003). GCF collected from specific sites can be used to assess the microbial relationships with the host mediators (Shimada et al., 2013).

GCF has been previously used as a rich source of biomolecules which represent periodontal disease status (Bakri et al., 2013; Luo et al., 2011). More than 65 oral biomarkers representing possible disease progression have been identified from GCF samples. These biomarkers include host-response modifiers, inflammatory mediators, tissue breakdown products, host-derived enzymes and their inhibitors (Khongkhunthian et al., 2014).

The proximity of GCF to the diseased periodontal tissues increases its ability to provide more relevant information as compared to saliva. As saliva originates from salivary glands it is suspected that it could better serve as a predictor of salivary gland diseases rather than periodontal disease (Ozmeric, 2004).

2.5.1 Methods of GCF collection:

2.5.1.1 Intracrevicular washing technique

One of the sampling techniques used to collect GCF employ two injection needles, in which one needle is placed inside another needle. During sampling the 18 gauge “collection needle” is placed at the gingival margin whereas a 27 gauge “ejection needle" is positioned at the deepest point in the gingival sulcus. An incessant suction is used to collect the expelled solution from the crevice (Salonen & paunio, 1991).

2.5.1.2 Microcapillary technique

Different types of microcapillary pipettes are used to collect the GCF. These include non-calibrated pipette of known volume and calibrated volumetric pipette (Figure 2.1). To collect the sample without causing an irritation to gingival tissues, supragingival plaque is carefully removed by cotton pellets or suitable instruments. Then cotton rolls or swabs are used to achieve isolation and then the oral cavity is gently dried with air. Following the extracrevicular approach, volumetric micro-capillary pipette is used to collect one, two, or three µl of GCF as required. GCF samples that had an inadequate volume and/or were suspected to be contaminated are excluded.

Figure 2.1: Microcapillary pipettes (Drummond 2µL)

2.5.1.3 Absorption technique

Paper strips (Figure 2.2) or paper points (Figure 2.3) have been mainly used to perform this technique. Briefly, cotton rolls or swabs are used to achieve isolation and

then the oral cavity is gently air dried. Then, paper points or strips are positioned in the deepest point in the sulcus or pocket until minimum resistance for 30 seconds. Contaminated paper points or strips are ideally discarded.

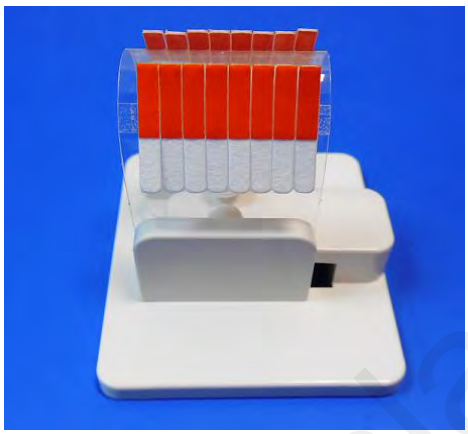

Figure 2.2: Periopaper, Oraflow

Figure 2.3: Absorbent paper points

2.6 Assessment of biomarkers in periodontal disease

Periodontal disease is an inflammatory condition that induced by periodontopathogens (Merchant & Pitiphat, 2007; Pihlstrom et al., 2005). These responses to pathogenic microbes lead to the progressive development of various periodontal disease (Bascones-Martínez et al., 2009; Ohlrich et al., 2009). Diagnosis of

periodontal disease is primarily based on subjective clinical parameters (Andrea Mombelli, 2005). The GCF components can be utilized to objectively analyse immune and inflammatory mediators responsible for inflammation and destruction of periodontal tissues (Lamster & Ahlo, 2007). There are three categories of biomarkers (Alrowis et al., 2014):

1. Indicators which exhibit activity of current disease
2. Predictors of disease initiation at healthy sites
3. Predictors of disease progression.

Three general categories of potential biomarkers in the GCF include:

• Inflammatory mediators and their by- products

• Host-derived enzymes

• Tissue-breakdown products

Table 2.2: Possible gingival crevicular fluid biomarkers for diagnosis of periodontal diseases (Ozmeric, 2004)

Enzymes	Proteins	Immunoglobulins	Cytokine	Others
Lysozyme	Lactoferrin	IgA	VEGF	PAF
MMP-2	Neopterin	IgM	TNF-α	thromboxane B2
MMP-8 (collagenase 2)	cystatins C, S	IgG	IL-1β	leukotriene B4
MMP-9	β-NAH	IgE	IL-2	hydroxyproline
MMP-13 (collagenase-3)	TIMP		INF-α	lipoxin A (4)
Neutral protease	osteopontin		IL-10	keratin
Alkaline phosphatase	hyaluronic acid		IL-8	PGE2
Dipeptidylpeptidase	calprotectin		RANTES	substance P
Aspartate aminotransferase	chondroitin sulphate		IL-1ra	glucose
Plasminogen	α-1-antitrypsin		EGF	volatile sulphur compounds
Gingipain	osteocalcin		HGF	glutathione
Cathepsin G, D, B	α-2-macroglobulin		TGF-β	phylloquinone
h-Glucuronidase	C-reactive protein		IL-6	butyric acid
Elastase	Transferrin		IL-4	propionic acid
Lactate dehydrogenase	Thrombomodulin			lactic acid
Creatine kinase	proteoglycan			methylglyoxal
Myeloperoxidase	endothelin			ICAM-1

	osteonectin			hydroxylysylpyridinoline
	hyaluronan			
	ICTP			
	fibronectin			
	α-1-EPI			
	NTx			
	E-selectin			
	neurokinin-A			
	MRP-8			
	Calcitonin			
	Albumin			

MMP: matrix metalloproteinase, ICTP: pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen, β-NAH: β-N-acetyl hexosaminidase, α-1-EPI: elastase-α-1-proteinase inhibitor complex, TIMP: tissue inhibitor of matrix metalloproteinase, NTx: cross-linked N telopeptide of type I collagen, E-selectin: endothelial leucocyte adhesion molecule, IL-: interleukin, IL-1ra: interleukin 1 receptor antagonist, TGF-β: transforming growth factor β, IFN-α: interferon α, TNF-α: tumor necrosis factor α, VEGF: vascular endothelial growth factor, HGF: hepatocyte growth factor, EGF: epithelial growth factor, $_{PGE2}$: prostaglandin E2, ICAM-1: intercellular adhesion molecule-1, PAF: platelet activating factor.

2.7 Biomarkers

2.7.1 Interleukin-1beta (IL-1β)

One of the important mediators that are involved in periodontitis is interleukin-1β (IL-1β) (Sánchez et al., 2013). IL-1β is a key mediator of the inflammatory response and significant involvement in differentiation and apoptosis, cell proliferation and in the pathophysiology of periodontal disease (Faizuddin et al., 2003). Several studies have linked increased levels of IL-1β in GCF with the inflammatory status of periodontal

disease (Al-Shammari et al., 2001; Faizuddin et al., 2003). Oh et al. (2015) demonstrated that the levels of IL-1β and GCF are closely associated with severity of disease and that these variables proved more reliable than subjective clinical measures like PPD and BOP.

IL-1β synergistically acts with various pro-inflammatory cytokines to induce osteoclastic activity in periodontitis (Graves & Cochran, 2003). IL-1β plays a key role in controlling the synthesis of dendritic cells (antigen presenting), regulating adaptive immunity and it is also suspected to influence the functioning and differentiation of T cell subsets (Taylor, 2010). In addition, recently two significant functions in T cell mediation were reported.

1. Enhancement of antigen-mediated stimulation of T cells
2. Stimulation of IL-6 secretion by macrophages (Ben-Sasson et al., 2009).

2.7.2 Interleukin-6 (IL-6)

Interleukin-6 is a pro- and anti-inflammatory cytokine that plays a vital role in acute-phase immune response, haemopoiesis and inflammation. Following secretion of TNF-α and interleukin-1 (IL-1), IL-6 is produced during inflammation and thereby subsequently inhibits secretion of TNF-α and IL-1 (Schindler et al., 1990). It is secreted by immune cells, adipose tissue and muscles (Ridker et al., 2000). It has also been held responsible for osteoclastic differentiation and osseous resorption, which is a typical presentation of chronic periodontitis (Hughes et al., 2006). Attachment loss of periodontal tissues had been associated with the intensity of expression of IL-6 (Moreira et al., 2007). It is also closely associated with continuous tissue destruction in samples having chronic periodontitis (Mccauley & Nohutcu, 2002). It is abundantly found in

inflammatory periodontal lesions and fibroblast activation in the presence of soluble IL-6 receptor has been observed (Takashiba et al., 2003).

2.7.3 Tumor necrosis factor-alpha (TNF-α)

Two functionally and structurally related proteins belong to this family, "Tumor necrosis factor-alpha" (TNF-α) and "Tumor necrosis factor-beta" (TNF- β). TNF-α or cachectin is mainly a by-product of macrophages and/or monocytes and TNF-β or lymphotoxin is a by-product of lymphoid cells (Vilcek & Lee, 1991).

TNF‑α is considered an important soluble pro‑inflammatory mediator that has been known to cause periodontal tissue destruction (Varghese et al., 2015a). It has a pivotal role in initiation and coordination of cascade of cellular events in the immune response to the microbial infection. It is primarily produced by macrophages, as well as mastocysts, lymphoid cells, myocytes, endothelial cells, adipocytes, neural tissue and fibroblasts. Copious amounts of TNF-α are secreted in the presence of interleukin-1, lipopolysaccharides and other microbial products (Brekalo Prńo et al., 2006; Safavi & Rossomando, 1991). It can be regarded as an "early" mediator that amplifies innate host responses. It increases the attachment of monocytes and polymorphonuclear leucocytes by affecting their endothelial cells and thus modulates their recruitment into inflammatory sites. It also enhances induction of matrix-metalloproteinases, osteoclast activity and synthesis, and stimulation and expression of PGE2. All of these events enhance bone and connective tissue destruction. It induces apoptosis of fibroblasts, causing stunted repair of the periodontal tissues. TNF-α antagonists have been used to reduce of periodontal inflammation, which indicates a strong relation between TNF-α and periodontal infection (Assuma et al., 1998; Delima et al., 2001; Graves et al., 1998).

2.7.4 Osteocalcin (OC)

Osteocalcin (OC) is primarily secreted during bone formation by osteoblasts. It is the most frequently found, non-collagenous, calcium-binding protein of the mineralized tissue (Kâ et al., 2014; Lian & Gundberg, 1988). OC exists in two distinct forms (Lee et al., 1999): (1) carboxylated and (2) undercarboxylated (ucOC). The former has a marked affinity for the hydroxyapatite minerals of bone and latter commonly exists in the blood stream. ucOC is considered the active form and involvement in the regulation of fat and glucose metabolism has been found (Lee et al., 2007; Wolf, 2008).

Previous studies investigated the association between osteocalcin levels in the GCF and periodontal disease (Golub et al., 1997; Nakashima et al., 1996; Nakashima et al., 1994). In a cross-sectional study by Kunimatsu et al. (1993) a significant correlation existed between N-terminal OC peptide levels in the GCF and the clinical measures of individuals with periodontitis and gingivitis.The authors also reported that osteocalcin could not be detected in patients with gingivitis. In addition, Nakashima et al. (1994) also reported a strong correlation between osteocalcin levels of the GCF and individuals suffering from gingivitis and periodontitis.

2.7.5 Prostaglandin E2 (PGE2)

Arachidonic acid metabolism commonly give rise to prostaglandin levels, which are commonly increased at inflammation sites (Kuehl & Egan, 1980). Prostaglandin E2 (PGE2) are chemically potent molecules that have been associated with changes in fibroblast metabolism, tissue destruction and bone resorption (Offenbacher et al., 1993). Increased levels of PGE2 in GCF have been strongly associated with inflammation and impending periodontal tissue destruction (Nakashima et al., 1994). PGE2 has diverse immunomodulatory and proinflammatory effects. In addition, greater elevation in PGE2

levels in the GCF has been noted in individuals with juvenile periodontitis compared to individuals with gingivitis and chronic periodontitis (Salvi & Lang, 2005). Based on theoretical knowledge, increased levels of PGE2 in the GCF can be held solely responsible for most of the inflammatory and tissue destructive changes such as edema, collagen degradation erythema and bone loss. The ability of PGE2 to induce vasodilatation and to increase the capillary permeability attributes to the erythema and oedema of gingival tissue. Inflammatory mediators such as histamine and bradykinin synergistically enhance the vasoactive effects of PGE2 (Salvi & Lang, 2005). It can cause an increase in the number of osteoclasts and induce bone resorption, modulate osteoclast and osteoblast levels of adenosine 3', 5'-monophosphate (Dziak, 1993). It regulates bone resorption by stimulation of osteoclasts (Chambers & Dunn, 1983). This is strong proof of a strong correlation of PGE2 levels in the GCF to the periodontal disease (Offenbacher et al., 1993).

ELISA has been considered an effective method to assess the levels of PGE2 in the GCF which can be used to assess disease status. Kumar et al. (2013) demonstrated that the levels and concentration of PGE2 in GCF correlated with severity of periodontal tissue inflammation and their clinical parameters. Measurement of PGE2 in GCF by ELISA may be an effective method for assessing periodontal inflammation(Kumar et al., 2013).

2.7.6 Osteoprotegerin (OPG)

Osteoprotegerin (OPG) is a bone-regulating protein. It is a soluble receptor decoy which can inhibit or block the action of RANKL (Teitelbaum, 2000). OPG, also termed “osteoclastogenesis inhibition factor”, inhibits osteoclastic activity (Boyce & Xing,

2007) and it plays a vital role in the immune and vascular systemS (Vieira Ribeiro et al., 2011).

Bone resorption is modulated by the interplay of OPG and RANKL, which belong to the tumor necrosis receptor and ligand families, respectively (Teitelbaum & Ross, 2003). RANKL is expressed as a secreted or membrane-bound ligand (Liu et al., 2010). The binding of the expressed ligand to its receptor activates the fusion and morphodifferentiation process. The osteoclast precursor cells from the monocyte/macrophage lineage differentiate into multi-nucleated osteoclasts, which in turn cause bone resorption (Lacey et al., 1998). Contrary to this process if OPG competes with RANKL to bind with the targeted receptor and when the former succeeds, successful inhibition of precursor differentiation and bone resorption takes place (Simonet et al., 1997). OPG synthesis is regulated by several local and systemic stimuli. These stimuli include inflammatory mediators, hormones and bacterial products (Lerner, 2006).

RANKL/OPG have been found particularly significant, primarily for bone regulation and resorption (Mogi et al., 2004). With recent advances in identification of peridontopathogenesis, the RANK/RANKL/OPG system is characterized as a dominant mediator of osteoclastogenesis (Lu et al., 2006; Vernal et al., 2004). The influence of RANK/RANKL/OPG system in precursor activated synthesis of osteoclasts and contemporaneous inhibition of synthesis of osteoclasts, has been extensively studied (Khosla, 2001) and these ligand-receptor based interactions have been extensively reviewed (Belibasakis et al., 2012; Kajiya et al., 2010). An imbalance in this system, can lead to increased concentration of one type of cytokine resulting in disease progression. Periodontal disease has been detected when concentration of RANKL in GCF exceeded over OPG levels. This evidence proves RANKL cytokine mediated osteoclastic activity in periodontal disease and thus we can infer that OPG, acts as a

decoy receptor thereby protecting bone against osteoclastic activity (Crotti et al., 2003; Liu et al., 2003). In conclusion, strategies to modulate the RANK/RANKL/OPG system hold promising and novel treatment strategies for the inhibition of bone destruction in periodontal diseases (Cutando et al., 2014).

Differences in the concentrations of RANKL/OPG have also been linked to give an account of the severity of periodontal disease (Costa et al., 2010; Duarte et al., 2007; Lappin et al., 2009). Previous studies have suggested that increased levels of RANKL and reduced levels of OPG may indicate presence of periodontal disease (Duarte et al., 2012; Lappin et al., 2007).

2.7.7 Matrix metalloproteinase (MMPs)

Matrix metalloproteinases (MMPs) are primarily responsible for extracellular matrix (ECM) and collagen degradation of periodontal tissue. MMPs are host-derived proteinases that mediate different processes associated with gingival inflammation and periodontal disease (Alfant et al., 2008). 28 members of the MMP family are categorized into subgroups, which include:

1. Gelatinases (MMP-2 and -9)
2. Stromelysins (MMP-3)
3. Collagenases (MMP-1, -8, and -13)
4. Membrane-type MMPs
5. Matrilysins
6. Others (Gonçalves et al., 2013)

Numerous physiological and pathological conditions are modulated by MMPs. MMPs are physiologically balanced by endogenous inhibitors, like tissue inhibitors of metalloproteinase (TIMP). Imbalance in any of these levels can predispose to

periodontal disease progression. Assessment of MMP levels in GCF, saliva and periodontal tissues may aid in prompt diagnosis of periodontal disease and also give an account of prognosis. Target specific therapies focussing on inhibition of MMPs may provide an adjunctive treatment approach (Sapna et al., 2014).

MMP-8 (collagenase-2) has been commonly detected in increased concentrations within GCF or periodontally compromised locations (Ingman et al., 1996). Polymorphonuclear neutrophils (PMNs) are primarily responsible for producing MMP-8. However, endothelial cells, gingival fibroblasts, plasma cells, sulcular epithelial cells and odontoblasts have also been known to synthesize this particular enzyme (Wahlgren et al., 2001). MMP-8 is considered a primary enzyme responsible for connective tissue degradation (Mancini et al., 1999; Sorsa et al., 2006; Sorsa et al., 2004; Sorsa et al., 1988) and its diagnostic potential has also been previously demonstrated (Mäntylä et al., 2006).

Various cell lines, including fibroblasts, eosinophils, keratinocytes, macrophages, osteoclasts and neutrophils express MMP-9 (gelatinase B). It is primarily responsible for degradation of various components of ECM during physiological as well as pathological conditions (Rai et al., 2008). Levels of this enzyme in salivary fluid reflect the severity of periodontal disease (Sorsa et al., 2006).

Several studies have suggested ECM metalloproteinases, especially MMP-8 and -9, have an important role in periodontal tissue destruction (Kumar et al., 2006). Leppilahti et al. (2014a) found that GCF of periodontally diseased cases had increased levels of MMP-8 and -9 as compared to healthy controls. Levels were especially high in cases of chronic and aggressive periodontitis (Gonçalves et al., 2013; Marcaccini et al., 2010).

Increased concentrations of MMP-8 in GCF were detected in subjects who had chronic periodontitis (Kinane et al., 2003; Mäntylä et al., 2003; Sorsa et al., 1999).

Hence, MMP-8 can be used as a potential biomarker for identifying or assessing periodontal tissue destruction.

2.8 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs) are components of defensive host response, which play an important physiological role (Wang et al., 2014). More than 45 AMPs extracted from the oral cavity have been identified (Gorr & Abdolhosseini, 2011; Tonetti & Chapple, 2011). Numerous AMPs exhibit strong activity against certain types of microorganism, even microbes that show resistance to traditional antibiotics. Since the action of AMPs is mostly targeted specific types of bacteria, their antimicrobial effect will be decreased on other beneficial bacterial species (Upton et al., 2012).

They are abundantly found in saliva and some are present in the GCF. Eleven of these AMPs are down-regulated in pathological conditions whereas 13 are up-regulated. Defensins and LL-37 are major components of the host antimicrobial defensive response. These AMPs are synthesized by gingival epithelial cells in response to bacterial stimulation, whereas peripheral blood neutrophils induced only LL-37 expression (Hosokawa et al., 2006). The role of AMP expression is unclear, it could be due to immune response to the microbial insult or it can occur as a part of general stimulation of target cells. It has been documented that stimulated levels of various AMPs were at the minimal inhibitory levels for microbes (Gorr et al., 2011). Therefore, certain biological interactions of the AMPs might modulate gingival inflammation.

AMPs have been classified into different functional families which include (Gorr, 2009):

1. Cationic peptides

2. Bacterial agglutination and adhesion molecules
3. Metal ion chelators
4. Peroxidases
5. Protease inhibitors
6. AMPs with activity against bacterial cell walls

These proteins are considered responsible in functioning of host innate immune response against various microbes entering the oral environment. The decreased ability of bacterial AMP resistance has been attributed to the capability of AMPs to perform multiple biological functions. Interestingly, absence of even a single AMP has been associated with predisposition to periodontal disease. Presence of periodontal disease and severe neutropenia are characteristic features of Morbus-Kostman disease. Particularly, a lack of LL-37 has been strongly associated with periodontal disease (Carlsson et al., 2006).

2.8.1 Human Cathelicidine LL-37

Cathelicidine is considered an exceptional group of antimicrobial peptides that have pleiotropic effects. They exhibit immunostimulatory/-modulatory and chemotactic abilities. In addition, it is capable of inducing angiogenesis, wound healing and modulating apoptosis (Vandamme et al., 2012). While analysing site specific samples high levels of cathelicidine are found, particularly at sites of inflammation. They function as a primary microbial and pathogenic defence system. Cathelicidine peptides exhibit antibacterial (Dean et al., 2011), antifungal (Wong et al., 2011) and antiviral (Barlow et al., 2011; Rolka, 2013) properties. Interestingly, chemokinetic function of these peptides has been considered responsible to stimulate and/or modulate host immune system. These peptides bridge the interaction between innate and adaptive

immune systems. They play an important role in stimulating angiogenesis and re-epithelialization thereby promoting wound healing (Pfosser et al., 2010).

The detected levels of LL-37 in GCF and saliva are lower than the minimal inhibitory levels for microbes, which suggested that increased concentration levels are required to utilize its antimicrobial action. The primary role of these AMPs is considered to be of an alarm system which acts as an endogenous mediator involved in recruitment and activation of antigen-presenting cells that enhances the adaptive and innate immune responses (Yang & Oppenheim , 2009).

2.9 The enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay is a well-known method to detect antibodies or antigens in a given sample. It has multiple applications in disease, pregnancy testing and drug screening. Different techniques are dependent on attachment to a solid surface, however there are multiple variations, such as indirect, sandwich, competitive and reverse techniques (Beaux et al., 2016). ELISA uses colour changes and different antibodies to identify a substance, like biomarkers. It is a popular “wet lab” type assay. It uses a solid-phase enzyme immunoassay (EIA) to identify the presence of a substance, typically an antigen. Dr. Dennis E Bidwell and Alister Voller introduced the technique of ELISA and it was intended to detect various kinds of diseases. Various uses of ELISA include medicinal diagnostic tool, antibody concentration determinant, monoclonal antibody screening, pregnancy testing, viral testing (Ebola, human immunodeficiency virus, etc.), plant pathological analysis and quality-control check in various industries. ELISA combines the sensitivity of simple enzyme assays and the specificity of antibodies, by using antigens or antibodies which is coupled to an assayed enzyme. ELISA tests are sensitive, accurate, specific and do

not require radioisotopes for functioning. Target antigens from the collected samples are attached to a solid surface. Then, a rather specific antibody is allowed to attach over the solid surface, to allow binding to the antigen. The attached antibody is then linked to an enzyme. In the last step, an enzyme‟s substrate containing substance is added. Preceding reaction emits a detectable signal, which is observed commonly as a colour change in the substrate (Greenberger & Patterson, 1982).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

1. Periodontal instrumentation.
    a. Examination Kit consisting of a mouth mirror, tweezer and probe.
    b. Gracey curettes.
    c. Williams periodontal probe.
2. Real time PCR equipment.
    a. DNA extraction kit (DNeasy® Blood and Tissue Kit)
    b. PrimerDesign™ Ltd genesig kit (Appendix G).
    c. Primer Design Ltd Oasig™ 2X lyophilised Mastermix
3. Eppendorf Thermomixer® comfort (Figure 3.1).
4. Refrigerated Eppendorf centrifuge 5415R (Figure 3.3).
5. Eppendorf centrifuge 5418.
6. Vortexer (VELP®) (Figure 3.2).
7. Autoclave (TOMY SX-500).
8. Microcentrifuge.
9. NANODROP 2000 spectrophotometer (Figure 3.4).
10. ELISA kit.
11. TECAN plate reader, infinite M200 PRO (Figure 3.5).

Figure 3.1: Eppendorf Thermomixer®

Figure 3.2: Vortexer (VELP®)

Figure 3.3: Refrigerated Eppendorf centrifuge

Figure 3.4: NANODROP 2000

Figure 3.5: TECAN plate reader, infinite M200 PRO

3.2 Methods

3.2.1 Study design

This was a cross sectional study. It was conducted in the Faculty of Dentistry, University of Malaya. Subjects were diagnosed as healthy, gingivitis, or chronic periodontitis (Figure 3.6), and those who agreed to participate in this study were considered for inclusion into the study. The ethical approval for this study was given by the Faculty of Dentistry, University of Malaya‟s Ethical Committee (reference number: DF RD1514/0047(P)) for the use of human individuals in this clinical study (Appendix A and B).

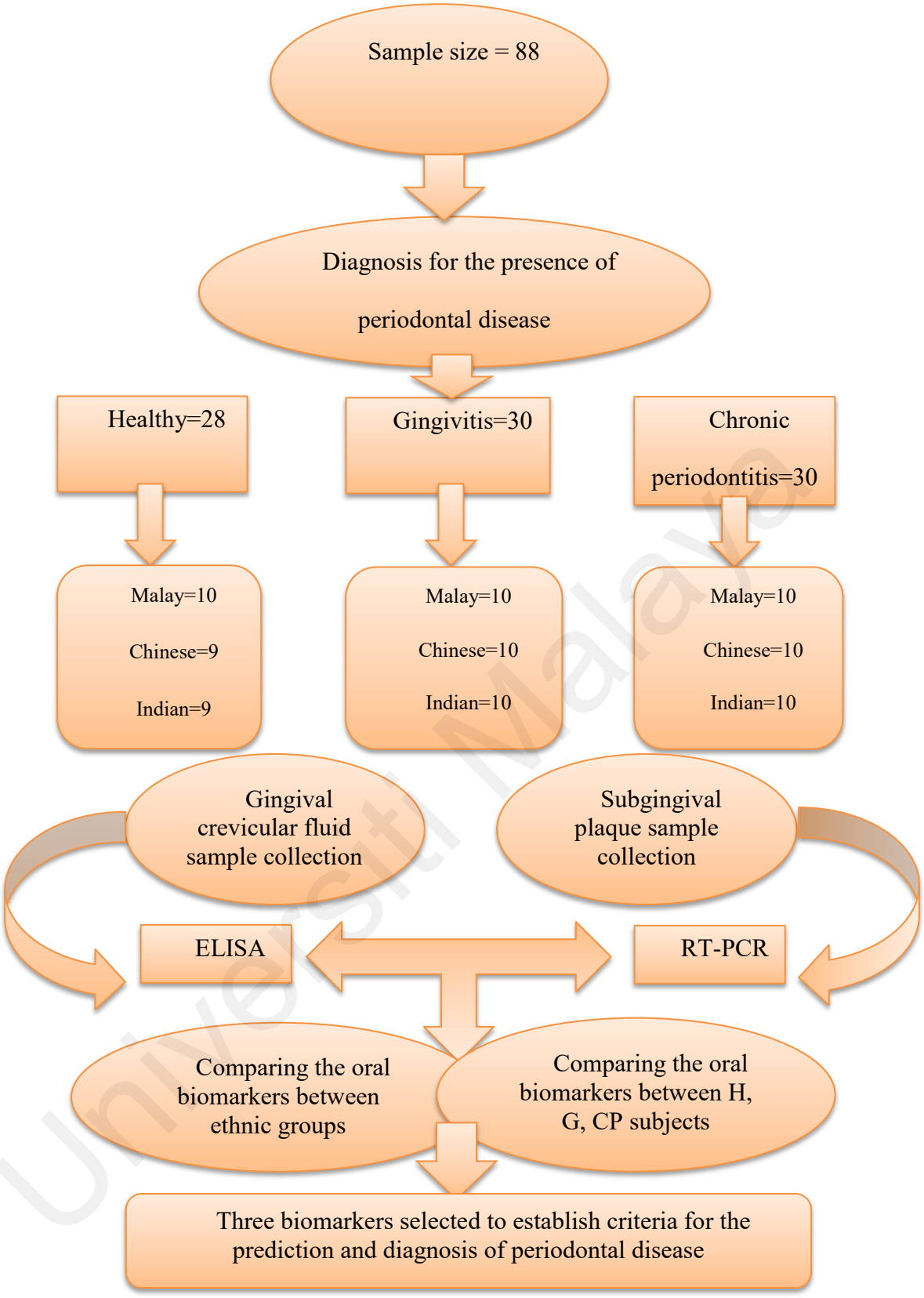

Figure 3.6: Flow chart

3.2.1.1 Subject selection

Case reports of patients with gingivitis (G) and chronic periodontitis (CP) were obtained from the Registration and Records Office of the Faculty of Dentistry, University of Malaya. Basic Periodontal Examination (BPE) was used in the initial screening for periodontal disease. Healthy subjects were identified and recruited from the staff and students of the Faculty of Dentistry.

Sample size was determined from previous studies (Kinney et al., 2014; Salminen et al., 2014) and by using the following statistical formula:

$$n = (z_\alpha + z_\beta)\,(\sigma_1^2 + \sigma_2^2) / \delta^2$$

σ: standard deviation

δ: mean1 – mean2

z_α: 2.58

z_β: 0.84

The identified subjects were contacted and appointments made to visit the Faculty of Dentistry. All subjects were acquainted about the nature of the study, the patient information sheet (Appendix C and D) was read and a consent form (Appendix E and F) was signed by the subjects who agreed to take part in the study. The research protocol was approved by the Review Committee/Restorative Department/Faculty of Dentistry. The subjects were categorized into:

1- Periodontally Healthy (28 subjects), which were subdivided into:

a- 10 subjects of Malay origin.

b- 9 subjects of Indian origin.

c- 9 subjects of Chinese origin.

2- Patients with chronic gingivitis (30 patients), which were subdivided into:

a- 10 patients of Malay origin.

b- 10 patients of Indian origin.

c- 10 patients of Chinese origin.

3- Patients with chronic periodontitis (30 patients), which were subdivided into:

a- 10 patients of Malay origin.

b- 10 patients of Indian origin.

c- 10 patients of Chinese origin.

3.2.1.2 Selection criteria of healthy subjects (H)

The inclusion criteria were absence of any periodontal disease, absence of systemic disease, no history of medication and no periodontal treatment in the previous 6 months, and age above 18 years. Smokers and women who were pregnant or receiving hormone treatment were excluded.

3.2.1.3 Selection criteria for patients with gingivitis (G)

The patients should be above 18 years old with a minimum of 20 teeth present in the dentition; diagnosed with mild to moderate gingivitis depending on the clinical periodontal parameters, patients with bleeding on probing present and patients who had not received any periodontal therapy for the past 6 months. Smokers were excluded.

3.2.1.4 Selection criteria for patients with chronic periodontitis (CP)

The selection criteria for this group were as follows: at least 18 teeth had to be present, excluding third molars, of which at least 12 had to be posterior teeth, presence of moderate to severe chronic periodontitis, absence of systemic disease and no history of medication in the previous 6 months and no previous periodontal treatment. Pregnant women and smokers were excluded.

The clinical diagnosis of periodontal status was established for all subjects based on the following criteria: periodontally healthy (H), ≤10% of sites with BOP, no PD or CAL>3 mm, although PD or CAL = 4 mm in up to 5% of the sites without BOP were allowed; chronic gingivitis (G), >10% of sites with BOP, no PD or CAL >3 mm, although PD or CAL = 4 mm in up to 5% of the sites without BOP was allowed; chronic periodontitis (CP), >10% of teeth with PD and/or CAL ≥5 mm and BOP (Da Silva-Boghossian et al., 2011). If the PD ≥ 5 mm and CAL ≥ 3-4, patients were considered to have moderate chronic periodontitis. When the PD ≥ 7 mm and CAL > 5 mm, patients were considered to have severe chronic periodontitis (Periodontitis, 2015). However, in this study all the above periodontal status of the patients was collectively considered as chronic periodontitis patients. Furthermore, all the CP patients underwent OPG for standardization.

3.2.2 Intraexaminar reliability

One non-blinded examiner was trained to measure the different periodontal parameters. Repeated measurements were done on three randomly selected subjects prior to the initiation in the course of the study in order to identify the level of intraexaminar reliability. The Intra Class Correlation Coefficient (ICCC) test was used to identify the degree of agreement.

Table 3.1 shows that the ICCC test results for plaque score (PS), bleeding on probing (BOP), pocket depth (PD) and clinical attachment loss (CAL) were 0.90, 0.92, 0.86 and 0.85 respectively. These results indicated perfect agreement as it was above 0.8.

Table 3.1: Results of Intra Class Correlation Coefficient test

Indices	No. of examined sites	Degree of agreement
Plaque score	504	0.90
Bleeding on probing	504	0.92
Probing depth	504	0.86
Clinical attachment loss	504	0.85

3.2.3 Periodontal examination

Periodontal examination was performed to enable categorization of the selected subjects into three groups (healthy, gingivitis and chronic periodontitis). Six sites per tooth were scored using a Williams‟s periodontal probe (Hu-Friedy, Chicago, IL), which were: mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual for the entire dentition; and the third molar was excluded. This was performed by measuring the following periodontal parameters:

1- Visible Plaque Index (VPI) or Plaque Score (PS)

The method of O'leary et al., 1972 was used to perform the PS. Disclosing solution was used to paint the expose the tooth surface. When the patient rinses his mouth, the researcher (ZN) checked the stained surfaces for plaque accumulations. The subjects were given a score zero (0) in the case of no plaque, and a score one (1) if plaque is present.

2- Bleeding On Probing (BOP)

BOP was measured using Ainamo & Bay‟s (1975) index. Williams‟s periodontal probe was inserted in the bottom of the gingival pocket or sulcus. If there was no bleeding, a score of zero was given; whereas if bleeding ensue within 10-15 seconds, a score of one was given.

3- Probing Depth (PD)

This index was performed by measuring the linear distance between the crest of the gingival margin and the point where the probe first meets resistance. PD was recorded in millimetres.

4- Clinical Attachment Loss (CAL)

CAL was measured by determining the linear distance from the cementoenamel junction to the base of the periodontal pocket.

3.2.4 Subgingival plaque collection

Subgingival plaque samples were collected from four sites in each healthy, gingivitis and chronic periodontitis subjects. A total of 352 subgingival plaque samples were collected from all of the subjects. Briefly, the upper posterior teeth, excluding the third molar, were selected initially for the subgingival plaque samples collection, which were taken from four sites in each healthy, gingivitis and chronic periodontitis subject. If those teeth did not show PD ≥ 5 mm and CAL ≥ 3-4 mm in chronic periodontitis patients, it was replaced by the adjacent teeth.

After gentle removal of supra gingival plaque by cotton pellets, the test site was dried gently by air and kept dry using cotton rolls. Sterile paper points (Elamin et al., 2011; Tsuzukibashi et al., 2008) (size #30) were inserted into the selected area (periodontal pockets or gingival sulcus) for 30 seconds to obtain subgingival plaque samples (Figure 3.7). The paper point from each sampling site was immediately placed into a microcentrifuge tube containing 1ml phosphate buffer saline. Samples for the RT-PCR analysis were stored at −80°C.

Figure 3.7: Subgingival plaque collection

3.2.5 Gingival crevicular fluid collection

Gingival crevicular fluid (GCF) samples were collected according to the method of (Tezel et al., 2005). GCF samples were collected from the same teeth that were chosen to collect the subgingival plaque samples. Following site selection (interproximal crevicular sites / in the upper posterior region excluding third molar), the sites were isolated with cotton rolls and the tooth surface were air dried to avoid contamination with saliva. The GCF samples were collected using absorbent paper strip (Periopaper®, ProFlow Inc., Amityville, NY, USA), which were placed into the sulcus / pocket until mild resistance was felt and then held in place for 30 seconds (Figure 3.8). Strips contaminated with saliva or blood were excluded. Six paper strips were obtained from each subject. A total of 528 GCF samples were collected from all of the subjects.

All strips with GCF were immediately placed into a sterile polypropylene tube and kept at −80°C until further analysis. Then, 700 µl of phosphate buffer saline was added to each tube and the tubes were shaken gently for 1 hour at room temperature. Next, the strips were removed and the fluids were stored at -–80°C until assayed by ELISA.

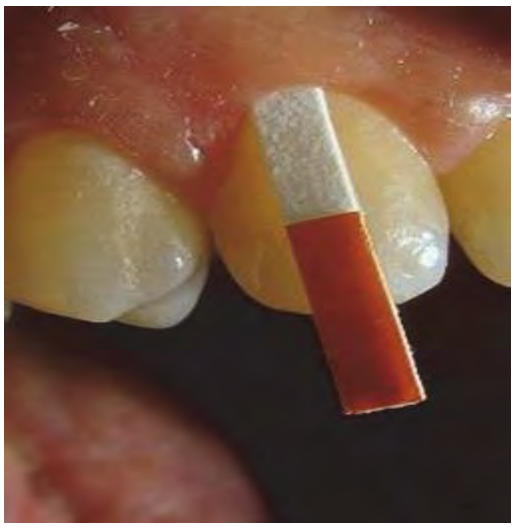

Figure 3.8: Gingival crevicular fluid collection

3.2.6 DNA extraction

DNA extraction kit [DNeasy® Blood and Tissue Kit (50), Cat. No. 69504] was used for bacterial DNA extraction. Samples were thawed for 20-30 minutes and centrifuged in microcentrifuge for 10 seconds. After the paper points were removed from the tubes, samples were pelleted using refrigerated centrifuge at 13, 2000 rpm for 30 minutes at 4°C and then the supernatant was discarded. After that, 180 µl of tissue lysis buffer (ATL) and 20 µl of proteinase K was added to the pelleted cells followed by vortexing for 10 seconds and centrifugation for 10 seconds. Then, the samples were placed in a thermo mixer set at 56°C for 3 hours of incubation. After the samples were removed from the thermo mixer, they were centrifuged for 10 seconds. A 200 µl of buffer (AL) was added to the tubes followed by vortexing for 10 seconds, centrifugation for 10 seconds and then incubated in the thermo mixer at 65°C for 10 minutes. The samples were centrifuged for 10 seconds after being removed from the thermo mixer. A 200 µl of cold absolute ethanol was added to each sample and the mixture transferred to an DNeasy mini spin column and centrifuged at 8,000 rpm for 1 minute and then the flow was discarded. A 500 µl of washing buffer 1 (AW1) was added and centrifugation at 8,000 rpm for 1 minute and the flow was also discarded. A 500 µl of washing buffer 2 (AW2) was added and centrifuged at 8,000 rpm for 1 minute and the flow was discarded. Then, a new collection tube was placed and centrifuged at 13, 2000 rpm for 3 minutes for drying. The total bacterial DNA was then transferred to a new microcentrifuge tube and eluted with 35 µl of elution buffer (AE)., Then, the DNA concentration and quality was measured using a NANODROP 2000 spectrophotometer device and the total product was stored at –20°C until real time polymerase chain reaction analysis.

3.2.7 Real Time PCR procedures

3.2.7.1 PCR primers

Table 3.2: PCR primers and TaqMan probes for detecting bacteria

Target gene	Oligonucleotides
**Aggregatibacter actinomycetemcomitans*	Forward: 5′-CGGTTACCGTTATGACCGTGTGA-3′ Reverse: 5′-GCCCGGAATGCTTTGCTATATTTC-3′ Probe: FAM-5′-AGGCAAGACGGGAAGCTAACGCAAA-TAMRA-3′
**Porphyromonas gingivalis*	Forward: 5′-TGGGACTTGCTGCTCTTGCTATG-3′ Reverse: 5′-GATGGCTTCCTGCTGTTCTCCA-3′ Probe: FAM-5′-CAAAGACAACGAGGCAGAACCCGTTA-TAMRA-3′
**Tannerella forsythia*	Forward: 5′-GCGTATGTAACCTGCCCGCA-3′ Reverse: 5′-CCGTTACCTCACCAACTACCTAATG-3′ Probe: FAM-5′-AGGGATAACCCGGCGAAAGTCGGA-TAMRA-3′
***Treponema denticola*	Forward: 5′-GGGCGGCTTGAAATAATRATG Reverse: 5′-CTCCCTTACCGTTCGACTTG Probe: FAM-5′-CAGCGTTCGTTCTGAGCCAGGATCA-BHQ

*(Saygun et al., 2008),**(Al-Hebshi et al., 2010)

3.2.7.2 Oasig^{TM} lyophilised 2X qPCR Mastermix preparation

The Primer Design Ltd Oasig^{TM} 2X lyophilised Mastermix is optimised for use in qPCR. The kit contains the lyophilised Mastermix, a tube of ROX dye which can be added as required and re-suspension buffer. The manufacture protocol was followed to prepare Oasig^{TM} lyophilised 2X qPCR mastermix. Briefly, the lyophilised mastermix was re-suspended in 525 µl of re-suspension buffer in order to be ready for use as a 2X qPCR mastermix. Since the biosystems 7500 platform was used, the ROX dye was added with 700 µl re-suspension buffer to be used as a passive reference guide according to the manufacturer‟s instructions. Finally, 10 µl of the mixed ROX dye was added into the mixed 2X qPCR mastermix.

3.2.7.3 Preparation of the *P. gingivalis, T. forsythia, T. denticola, and A. actinomycetemcomitans* standard curve:

The manufacturer‟s protocol (PrimerDesign ^{TM} Ltd genesig) was followed to prepare the standard curve for 4 types of bacteria (*P. gingivalis, T. forsythia, T. denticola*, *and A. actinomycetemcomitans*) in a 6 series dilution from 2 x 10^5 to 2x10^0 (Table 3.3). The lyophilised positive control was added with 500 µl of RNAse/DNAse free water. Then, the following steps were followed:

900 µl of RNAse/DNAse free water was pipetted into 5 tubes each (1.5 ml Eppendorf tubes) and labelled from 2 – 6. Then, 100 µl of positive control template which contains a high copy number template was pipetted into tube No.2 and vortexed thoroughly. After that, 100 µl was pipetted from tube 2 into tube 3 and also vortexed thoroughly. These steps were repeated with tubes 4, 5 and 6 to complete the series of dilution.

Due to the large sample size (88 samples), for each bacterial species (*P. gingivalis, T. forsythia, T. denticola, and A. actinomycetemcomitans*) the PCR test was performed twice because the maximum samples that could be examined per test is 80 samples. Therefore, two standard curves for each bacterial species was generated.

Table 3.3: Series of dilution

Standard curve	Copy number
Tube 1 positive control	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

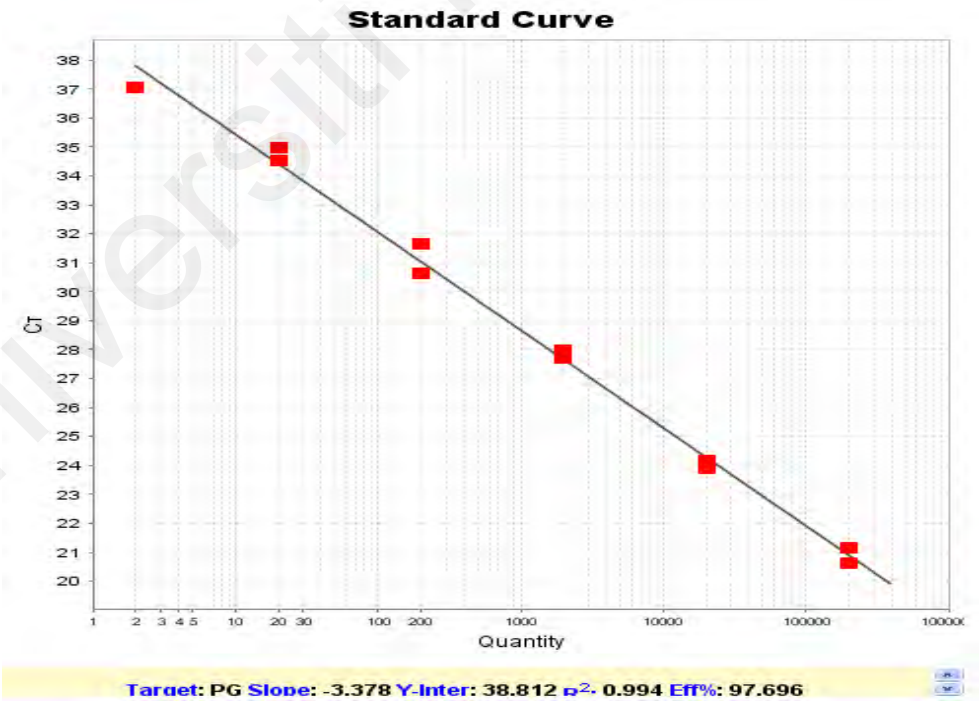

Figure 3.9: Standard curves of *P. gingivalis*

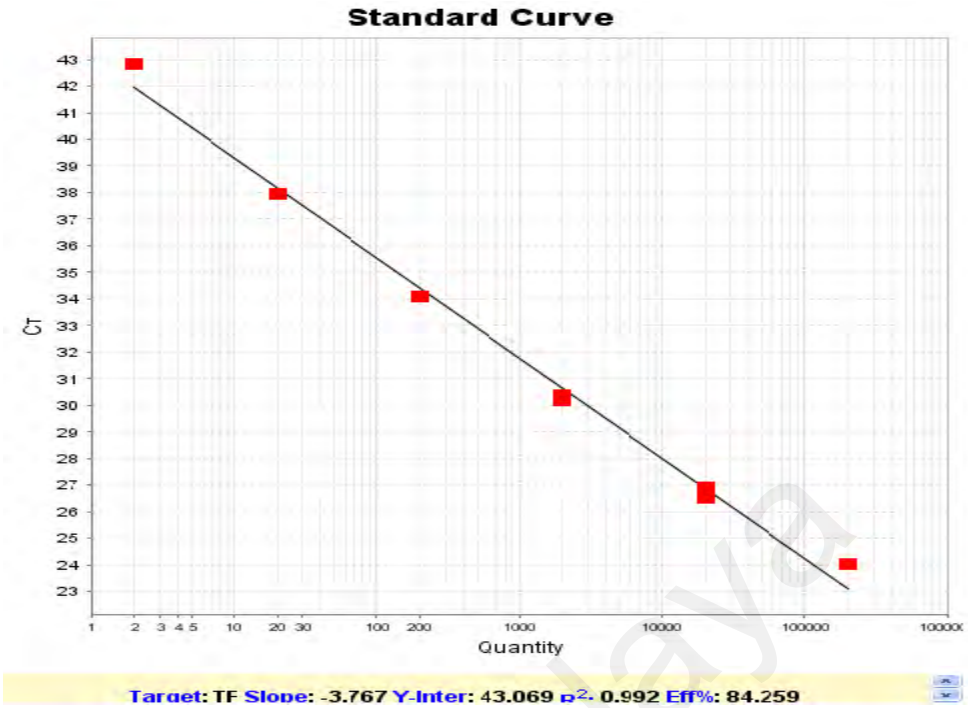

Figure 3.10: Standard curves of *T. forsythia*

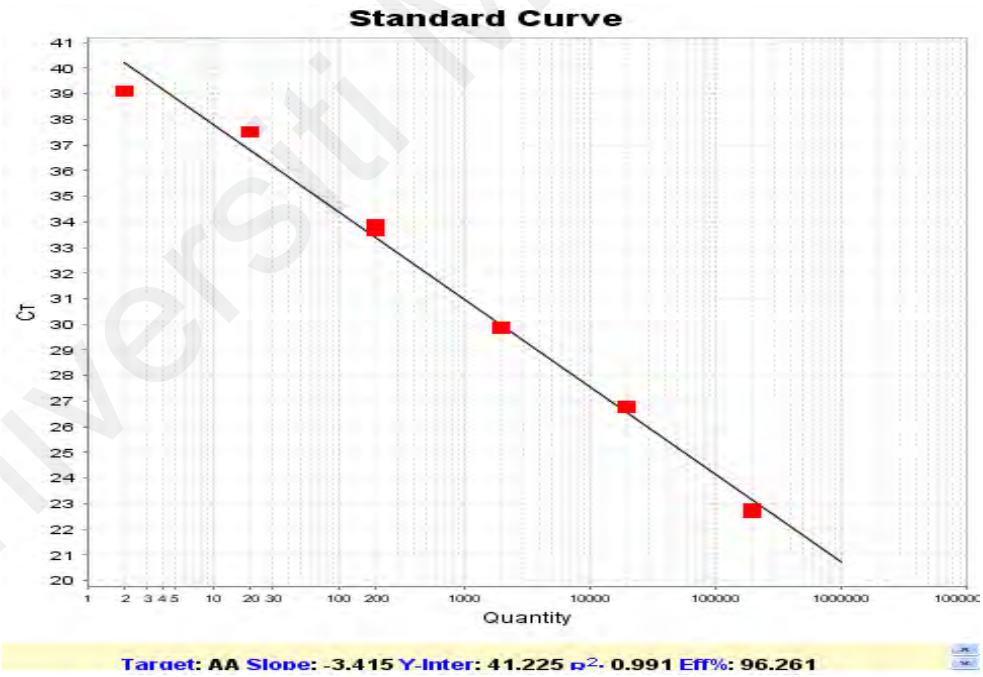

Figure 3.11: Standard curves of *A.a*

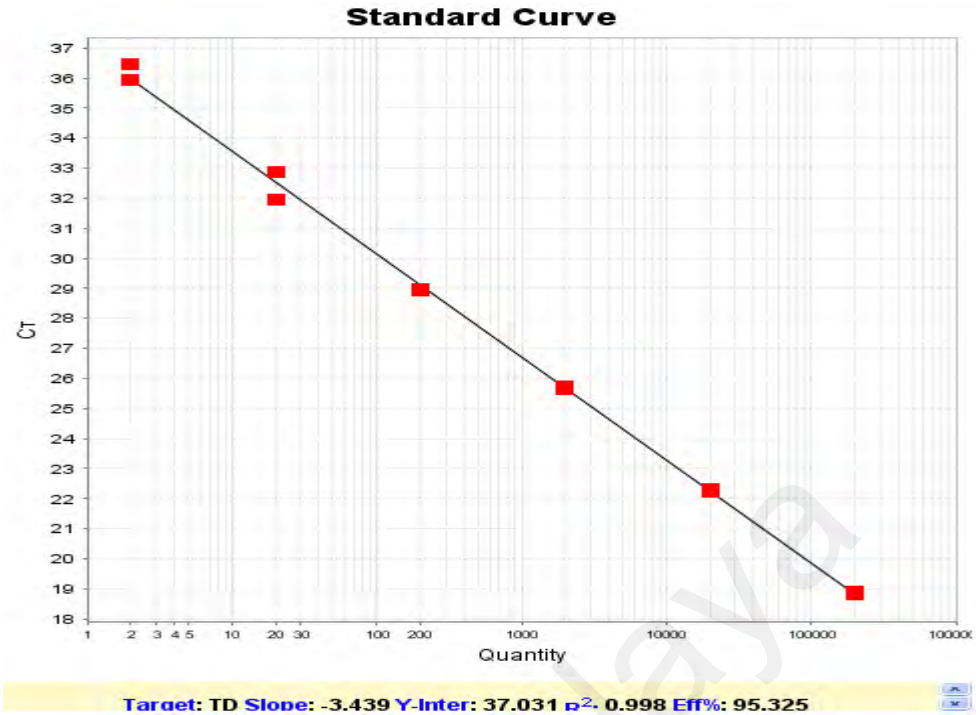

Figure 3.12: Standard curves of *T.denticola*

3.2.7.4 Bacterial quantification by real-time PCR

PrimerDesign ™ Ltd genesig kits were used for the quantification of *P. gingivalis* (Serial No.JN133243-41024, Catalogue No.Path-P.gingivalis-standard), *T. forsythia* (Serial No.JN133242-41024, Catalogue No.Path-T.forsythia-standard), *T. denticola* (Serial No.JN133246-41024, Catalogue No.Path-P.denticola-standard) and *A. actinomycetemcomitans* (Serial No.JN133244-41024, Catalogue No.Path-A.actino-standard). The head shock protein 60 gene was used to detect and quantify *P. gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans* genomes. Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (Figure 3.13) was used for quantification of bacterial genomic DNA according to the manufacturer‟s protocol. The reaction mix was prepared, including sufficient reactions for the negative control and the standard curve wells. Then, 15 µl of this mix was pipetted into each well according to real-time PCR plate set up. PCR was performed in a total volume of 20 µl: consisting

of 5 µl of genomic DNA, 10 µl of Primer Design Ltd Oasig™ 2X lyophilised mastermix, 4 µl of RNAse/DNAse free water and 1 µl of each bacterial species primer/probe mix. 5 µl RNAse/DNAse free water was added to the negative control well, making the final volume 20 µl in each well. Finally, the quantitative PCR was done on the Fast Real-Time PCR System, using the manufacturer"s PCR conditions as follows: initial denaturation for enzyme activation for 15 minutes at 95°C, followed by 50 cycles for denaturation (10 seconds at 95°C) and annealing (60 seconds at 60°C).

The quantity of *P. gingivalis, T. forsythia, T. denticola, and A. actinomycetemcomitans* DNA was generated by Fast Real-Time PCR System.

Figure 3.13: Fast Real-Time PCR System

3.2.8 Enzyme-linked immunosorbent assay (ELISA) procedures

3.2.8.1 Human cathelicidine LL-37

The ELISA kit (Hycult® biotech Human LL-37, Cat. No. HK321-01) was used for quantitative determination of the human gingival crevicular fluids LL-37. Samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and centrifuged in microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of the samples, which can be illustrated as follows:

All reagents were left at room temperature 20 – 25°C before use. 50 µl of standards and samples were transferred into appropriate wells without touching the side or bottom of the wells. The tray was covered and tapped to eliminate any air bubbles, and then the plate was incubated for 1 hour at room temperature. After incubation, the plates were washed 4 times with 200 µl wash/dilution buffer each time and then a 100 µl of diluted tracer was added into each well using the same pipetting order and also without touching the side or bottom of the wells. The plate was covered and tapped, and incubated for 1 hour. Similarly, after incubation the plate was washed 4 times and then 100 µl of diluted streptavidin-peroxidase was added into each well. The tray was covered and incubated for 1 hour. The washing procedure was also repeated 4 times, after that 100 µl of tetramethylbenzidine (TMB) substrate was added into each well using the same pipetting order and the tray was incubated for ½ hour at room temperature. Finally, 100 µl of the stop solution was added into each well with the same sequence, and then the plate was read within 30 minutes after addition of stop solution at 450 nm using a plate reader, following the instructions provided by the instrument‟s manufacturer.

Finally, a standard curve was drawn in order to calculate the LL-37 concentrations (Figure 3.14).

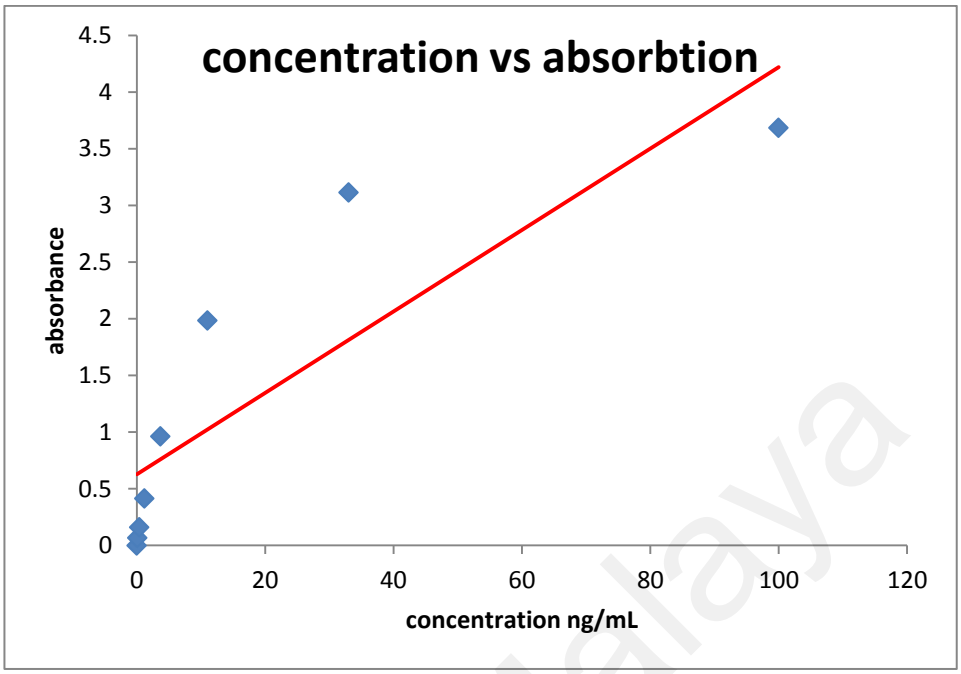

Figure 3.14: Human cathelicidine LL-37 ELISA standard curve

3.2.8.2 Human matrix metalloproteinase-9 (MMP-9)

R & D SYSTEMS™ Human MMP-9 Immunoassay Quantikine® ELISA kit (Cat. No. DMP900) was used to determine the MMP-9 concentration. Samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and centrifuged in a microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of the samples, which can be illustrated as follows:

100 µL of the assay diluent RD1-34 was added into each well, and then 50 µL of sample was added per well. The plate was covered with the adhesive strip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (450 rpm).

Each well was aspirated and washed, where the process of washing was repeated another three times for a total of four washes. The washing was done by filling each

well with wash buffer (400 µL). The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels.

200 µL of human MMP-9 conjugate was added into each well. The plate was covered with adhesive strip and incubated for 1 hour at room temperature on the shaker.

Washing was also repeated 4 times, and then 200 µL of substrate solution was added into each well and incubated for 30 minutes at room temperature and protected from light. 50 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. Finally, a standard curve was drawn in order to calculate the MMP-9 levels (Figure 3.15).

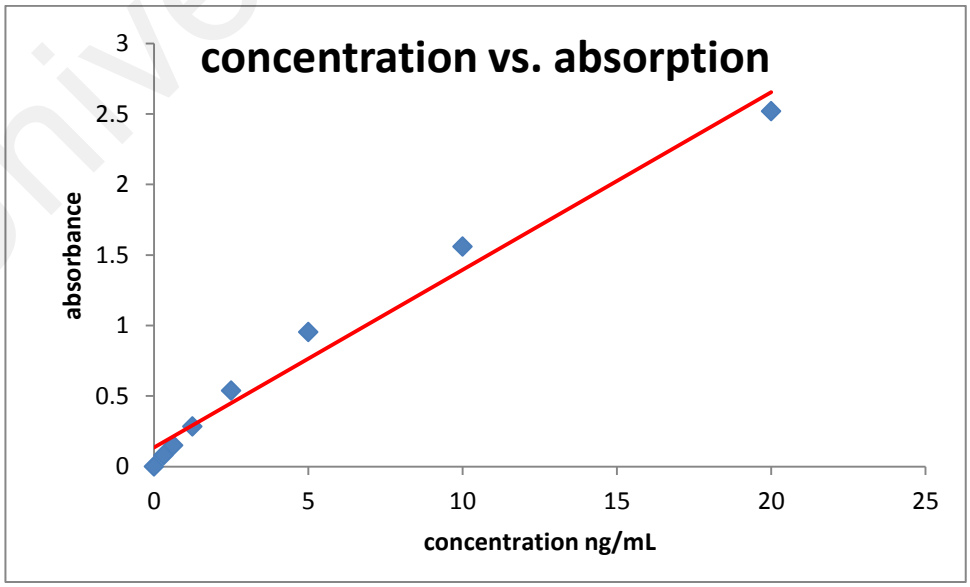

Figure 3.15: Human MMP-9 ELISA standard curve

3.2.8.3 Human interleukin-6 (IL-6)

IL-6 concentration was determined by using R & D SYSTEMS™ Human IL-6 Immunoassay Quantikine® ELISA kit (Cat. No. D6050). Samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of samples, which can be illustrated as follows:

100 µL of assay diluent RD1W was added to each well, then 50 µL of samples were added per well. The plate was covered with the adhesive strip provided and incubated for 2 hours at room temperature. After the aspiration and washing was done, the process of washing was repeated another three times for a total of four washes. Washing was done by filling each well with 400 µL wash buffer. The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels. Then, 200 µL of Human IL-6 Conjugate was added into each well; the plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. Washing was also repeated 4 times. Then, 200 µL substrate solutions were added into each well. The plate was incubated for 20 minutes at room temperature and away from light. Finally, 50 µL of stop solution was added to each well. The colour in the wells changed from blue to yellow.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. A standard curve was drawn in order to calculate the IL-6 concentrations (Figure 3.16).

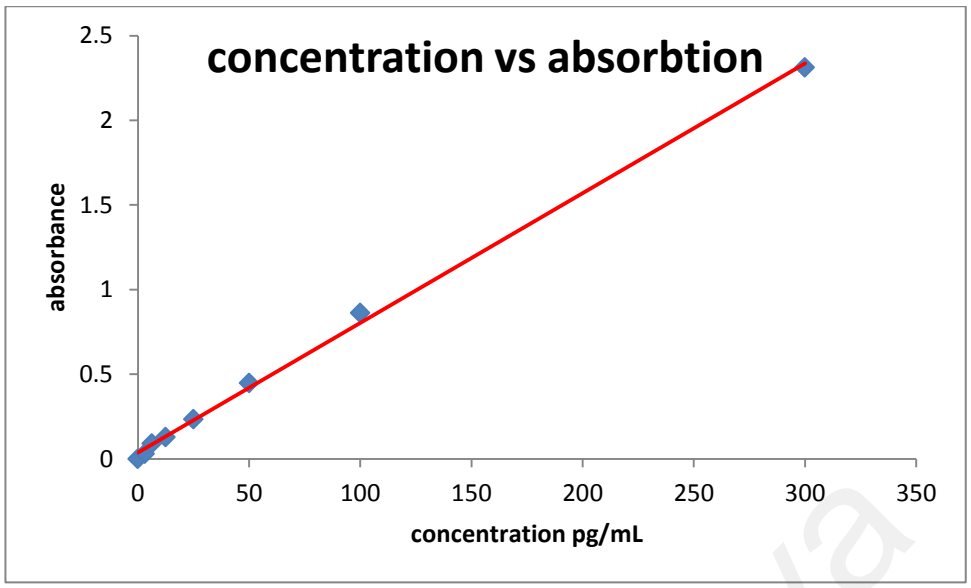

Figure 3.16: Human IL-6 standard curve

3.2.8.4 Human interleukin-1β (IL-1β)

R & D SYSTEMS™ Human IL-1β Immunoassay Quantikine® ELISA kit (Cat. No. DLB50) was used to quantify IL-1β. The samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and centrifuged in the microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of samples, which can be illustrated as follows:

100 µL of samples were added per well. The plate was covered with the adhesive strip and incubated for 2 hours at room temperature. After that, aspiration and washing was done; the process of washing was repeated another two times for a total of three washes. Washing was done by filling each well with 400 µL wash buffer. The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels. After that, 200 µL of Human IL-6 conjugate was added into each well, the plate covered with a new adhesive strip and incubated for 1 hour at room temperature. The washing was also repeated three times. Then, 200 µL of substrate

solution was added into each well; and incubated for 20 minutes at room temperature and away from light. Finally, 50 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. Finally, a standard curve was drawn in order to calculate the IL-1β concentrations (Figure 3.17).

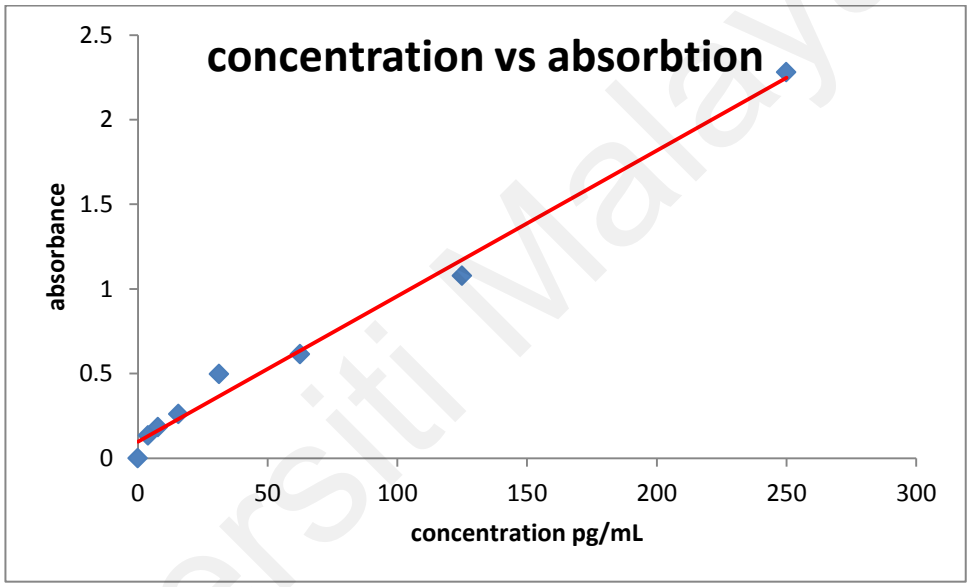

Figure 3.17: Human IL-1β standard curve

3.2.8.5 Human prostaglandin E_2 (PGE_2)

PGE_2 concentration was determined quantitatively using R & D SYSTEMS™ Human PGE2 Immunoassay Quantikine® ELISA kit (Cat. No. KGE004B). Samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. The manufacturer"s protocol was followed to calculate the mean absorbance of samples, as follows:

200 µL of calibrator diluent RD5-56 was added to the non-specific binding (NSB) wells. 150 µL of calibrator diluent RD5-56 was added to the zero standard (B_0) wells and 150 µL of standard and samples were added to the remaining wells. Then, 50 µL of the primary antibody solution was added into each well (excluding the NSB wells). The plate was covered with a plate sealer and incubated for 1 hour at room temperature on a horizontal orbital microplate shaker set at 500 rpm. After the incubation, 50 µL of PGE2 conjugate was added into each well. The plate was covered with the sealer and incubated for 2 hours at room temperature on the shaker. After the aspiration and washing was done, the process of washing was repeated another three times for a total of four washes. Washing was done by filling each well with 400 µL wash buffer. The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels. 200 µL of substrate solution was added into each well. Then, the plate was incubated for 30 minutes at room temperature and away from light. Lastly, 100 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. A standard curve was drawn in order to calculate the PGE_2 concentrations (Figure 3.18).

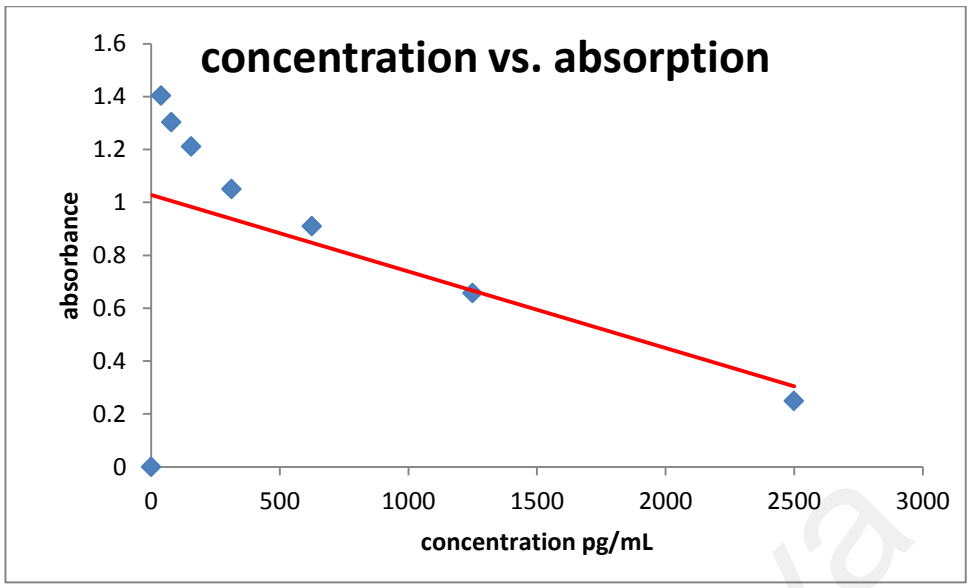

Figure 3.18: Human prostaglandin E_2 standard curve

3.2.8.6 Human osteoprotegerin (OPG)

Human Osteoprotegerin TNFRSF11b ELISA Kit (Product Number: RAB0484, Lot Number 0630C0178) was used for the quantitative determination of osteoprotegerin concentration. Samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. Prior to use, all samples and kit reagents were kept at room temperature (18 – 25°C).

50 µl of sample was added into the wells. The plate was covered and incubated for 2.5 hours at room temperature with gentle shaking. The solution was discarded and washed four times with 1x wash solution. Washing was done by filling each well with 300 µl wash buffer using a multichannel pipette. The complete removal of liquid at each step was done to ensure good performance. After the last wash, any remaining wash buffer was removed by aspirating. The plate was inverted and blotted against clean paper towels. 100 µl of biotinylated detection antibody was added into each well and incubated for 1 hour at room temperature with gentle shaking. After the incubation, the solution was discarded and the washing process was repeated. Then, 100 µl of HRP-

Streptavidin solution was added into each well and incubated for 45 minutes at room temperature with gentle shaking. After the incubation, the solution was discarded and the washing process was repeated. 100 µl of colorimetric TMB reagent was added into each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. Finally, 50 µl of stop solution was added into each well and the optical density of each well was determined immediately, using a microplate reader set to 450 nm. A standard curve was drawn in order to calculate the OPG concentrations (Figure 3.19).

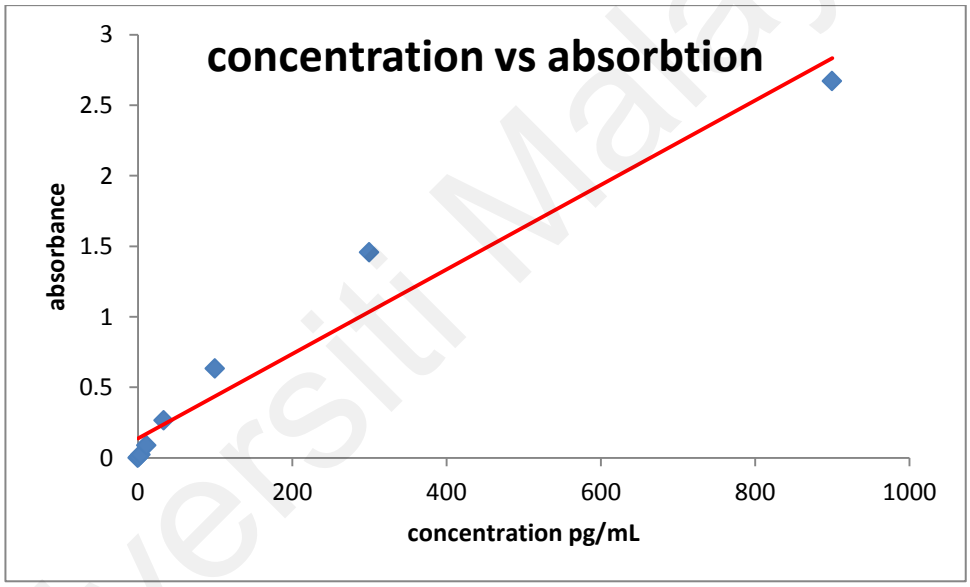

Figure 3.19: Human Osteoprotegerin standard curve

3.2.8.7 Human tumour necrosis factor-alpha (TNF-α)

R & D SYSTEMS™ Human TNF-α Immunoassay Quantikine® ELISA kit (Cat. No. DTA00C) was used to quantify TNF-α. The samples were thawed for 20 – 30) minutes, centrifuged for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of samples, as follows:

50 µL of assay diluent RD1F was added into each well. Then, 100 µL of each sample was added per well. The plate was covered with the adhesive strip provided and incubated for 2 hours at room temperature. After the aspiration and washing was done, the process of washing was repeated another three times for a total of four washes. Washing was done by filling each well with 400 µL wash buffer. The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels. After washing, 200 µL of TNF-α conjugate was added into each well. The plate was covered with a new adhesive strip and incubated for one hour. At the end of incubation, the washing was also repeated four times. Then, 200 µL of substrate solution was added into each well. The plate was incubated for 20 minutes at room temperature and away from light. Finally, 50 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. A standard curve was drawn in order to calculate the TNF-α level (Figure 3.20).

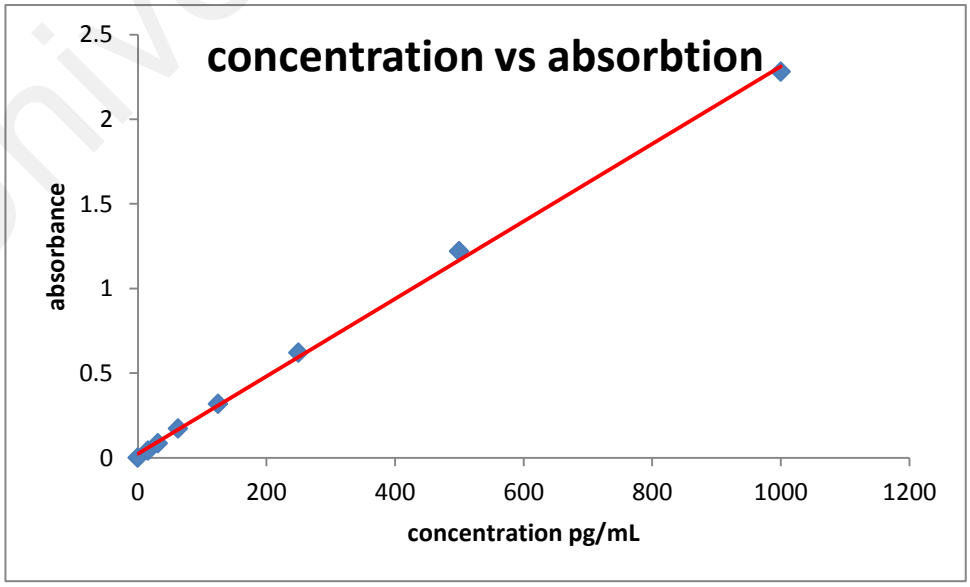

Figure 3.20: Human TNF-α standard curve

3.2.8.8 Human Osteocalcin (OC)

The osteocalcin concentration was determined using R & D SYSTEMS™ Human OC Immunoassay Quantikine® ELISA kit (Cat. No. DSTCN0). The samples were thawed for 20 – 30 minutes, centrifuged for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of samples, which can be illustrated as follows:

100 µL of assay diluent RD1-117 was added into each well, and then 25 µL of samples were added per well. The plate was covered with the adhesive strip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 450 rpm. Each well was aspirated and washed. The process of washing was repeated another three times for a total of four washes. Washing was done by filling each well with wash buffer (400 µL). The complete removal of liquid at each step was done to achieve good performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels. 200 µL of human osteocalcin conjugate was added into each well. The plate was covered with adhesive strip and incubated for 2 hours at room temperature on the shaker. Washing was also repeated 4 times. Then, 200 µL of substrate solution was added into each well. The plate was incubated for 30 minutes at room temperature and protected from light. 50 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow. The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. Finally, a standard curve was drawn in order to calculate the OC concentrations (Figure 3.21).

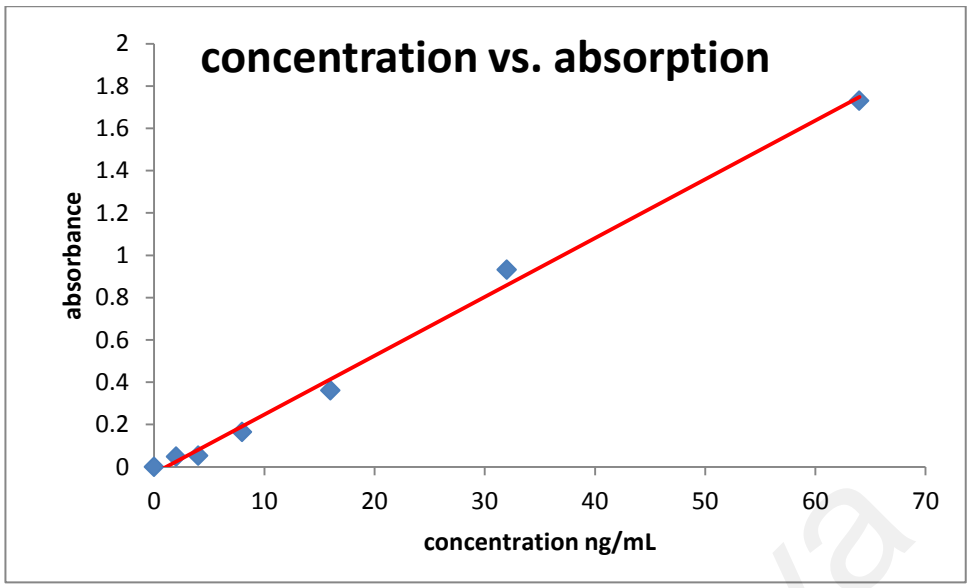

Figure 3.21: Human Osteocalcin standard curve

3.2.8.9 Human matrix metalloproteinase-8 (MMP-8)

R & D SYSTEMS™ Human MMP-8 Immunoassay Quantikine® ELISA kit (Cat. No. DMP800) was used to determine the MMP-8 concentration. The samples were thawed for 20 – 30 minutes, vortexed for 10 seconds and then centrifuged in the microcentrifuge for 10 seconds. The ELISA protocol supplied with the kit was followed to calculate the mean absorbance of samples, which can be illustrated as follows:

150 µL of assay diluent RD1-52 was added into each well and then 25 µL of the samples were added per well. The plate was covered with the adhesive strip provided and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker set at 450 rpm.

Each well was aspirated and washed. The process of washing was repeated another three times for a total of four washes. Washing was done by filling each well with 400 µL wash buffer. The complete removal of liquid at each step was done to achieve good

performance. After the last wash, any remaining wash buffers were removed by aspirating and the plate was blotted against clean paper towels.

200 µL of human MMP-8 conjugate was added into each well. The plate was covered with adhesive strip and incubated for 2 hours at room temperature on the shaker.

Washing was also repeated 4 times. Then, 200 µL of substrate solution was added into each well. The plate was incubated for 30 minutes at room temperature and protected from light. 50 µL of stop solution was added into each well. The colour in the wells changed from blue to yellow.

The optical density of each well was determined within 30 minutes, using a microplate reader set to 450 nm and the wavelength corrections were set to 540 nm. A standard curve was drawn in order to calculate the MMP-8 concentrations (Figure 3.22).

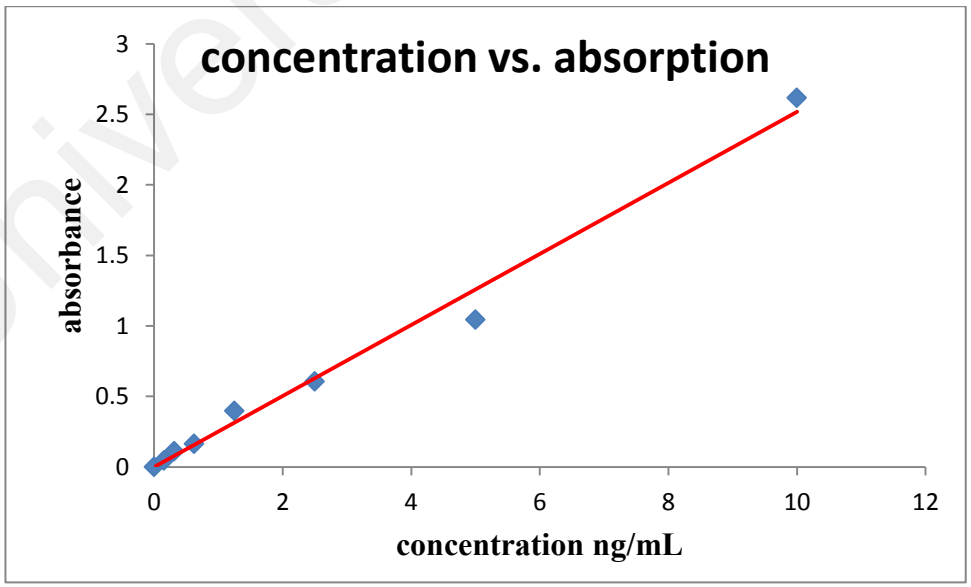

Figure 3.22: Human MMP-8 ELISA standard curve

3.3 Calculation of Cumulative Risk Scores (CRS)

In this study, we collected and utilized clinical, microbiological and oral biomarker data to propose a statistical approach for the diagnosis of periodontitis (Gursoy et al., 2011; Salminen et al., 2014). In this approach, the GCF concentrations of the selected oral biomarkers have been combined to obtain a cumulative risk score (CRS).

The GCF concentrations of each selected oral biomarker were divided into three tertiles 1 – 3. The cumulative sub-scores for each subject were calculated by the multiplication of the corresponding tertile values. According to this calculation, for example; if we chose three biomarkers, then the subject"s cumulative sub-score can be 1, 2, 3, 4, 6, 8, 9, 12, 18, or 27. Moreover, based on these cumulative scores, three CRS groups were formed as follows:

CRS I: Low risk (the cumulative scores of 1, 2, 3)

CRS II: Moderate risk (the cumulative scores of 4, 6, 8, 9)

CRS III: High risk (the cumulative scores of 12, 18, 27)

3.4 Statistical Analysis

Statistical analyses were performed using parametric and non-parametric methods. GCF biomarkers were analysed parametrically or non-parametrically depending on the type of data distribution (normally or abnormally) and on the homogeneity of variances, with one-way ANOVA test or Kruskal-Wallis test. Differences among the three groups were analysed with a post hoc Bonferroni test. The clinical periodontal parameters (PS, BOP, PD, and CAL) and subgingival plaque pathogens were analysed non-parametrically with a Kruskal-Wallis test and the differences among the three groups were analysed with a post hoc Dunn test. A two-way ANOVA test was used to analyse

the ethnic subgroups data. The correlations between the variables were achieved using Spearman rho rank test. The logistic regression model and receiver operating characteristics (ROC) curves were generated to study the association of oral biomarkers and CRS as tertiles with periodontal disease. The data analyses were done using IBMSPSS version 20 software package.

CHAPTER 4: RESULTS

4.1 Socio-demographic data of study sample

4.1.1 Age

The average age of the subjects who participated in this study is demonstrated in Table 4.1. The mean age of subjects in the healthy, gingivitis and chronic periodontitis groups was 25.35, 32.70 and 51.86 years, respectively.

Table 4.1: Demographic: Age

Group	Number of subjects	Mean Age	Standard deviation	Minimum	Maximum
H	28	25.35	4.97	21	37
G	30	32.70	16.25	18	67
CP	30	51.86	10.77	28	71

H: healthy, G: gingivitis, CP: chronic periodontitis

4.1.2 Gender

Table 4.2 shows the gender distribution among the groups. 21.4% of the subjects in healthy group were male, while 78.6% of the subjects were female. In the gingivitis group 46.7% of the subjects were male and 53.3% were female. The chronic periodontitis group showed equal distribution 50% for male and female among the participants.

Table 4.2: Demographic: Gender

Group	Number of male	% of male	Number of female	% of female
H	6	21.4	22	78.6
G	14	46.7	16	53.3
CP	15	50	15	50

4.1.3 Race

All the groups showed a similar distribution of the races among their participants, in which 10 subjects from each race (Malay, Chinese and Indian) involved in each test group, and for the healthy group in which 9 Indian and Chinese subjects were participated.

Table 4.3: Demographic: Race

Group	Malay	Chinese	Indian
H	10	9	9
G	10	10	10
CP	10	10	10

4.2 Clinical periodontal parameters of the study population

Intra-oral assessment involved the measurement of the clinical parameters that includes the plaque score (PS), bleeding on probing (BOP), pocket depth (PD) and clinical attachment lose (CAL) was done. PS, BOP, PD and CAL had been assessed from six sites for each tooth. The data of the clinical parameters were not normally distributed when checked by the Shapiro-Wilk test, so nonparametric tests were used in the comparison between the groups.

From the data analysis using Kruskal-Wallis Test, Table 4.4 showed that there was a significant difference ($p<0.05$) between the H, G and CP groups in all clinical parameters (PS, BOP, PD and CAL).

The PS and BOP scores were significantly higher ($p<0.05$) in the G and CP groups compared with the H group, in which there was no statistical significant difference between G and CP groups. The CP group had significantly higher PD and CAL scores compared with the G and H groups ($P <0.005$).

Table 4.4: Clinical periodontal parameters

Clinical parameters	H (n=28)	G (n=30)	CP (n=30)	P-value between and within groups
PS (%)	10.57 ± 3.31	49.43 ± 13.93	43.53 ± 14.95	0.000*
BOP (%)	5.10 ± 1.40	38.46 ± 9.78	36.03 ± 10.07	0.000*
PD (mm)	1.68 ± 0.35	2.00 ± 0.29	3.66 ± 0.80	0.000*
CAL (mm)	00 ± 00	00 ± 00	3.63 ± 0.45	0.000*

* P<0.05: significant difference. Kruskal-Wallis Test.

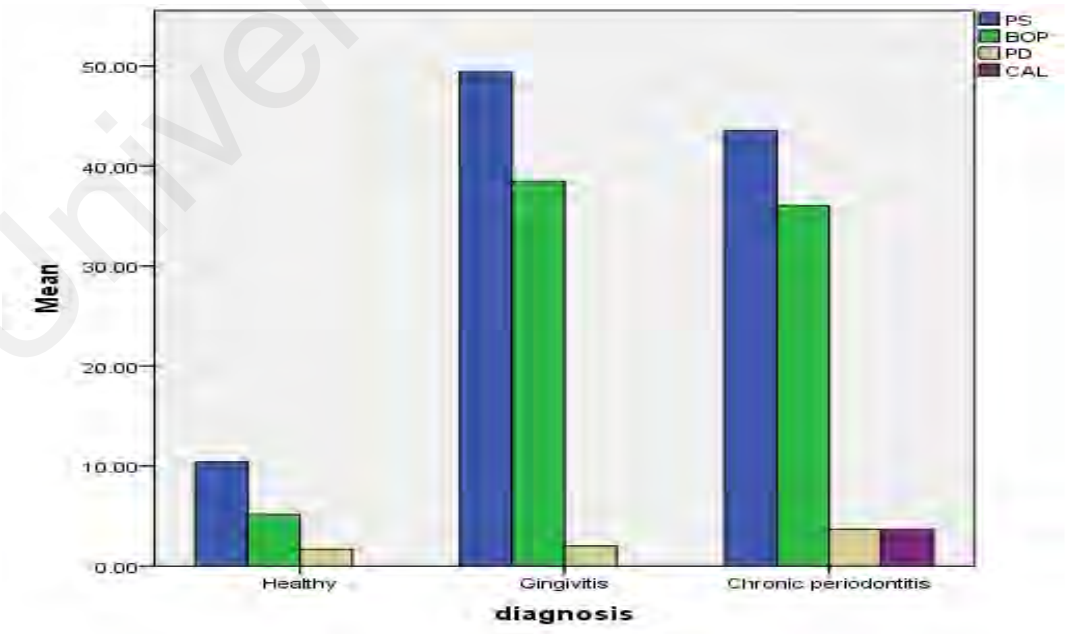

Figure 4.1: Clinical parameters in healthy and diseased groups

4.3 Subgingival plaque pathogens

4.3.1 *Porphyromonas gingivalis*

4.3.1.1 Comparison of *P. gingivalis* count between different ethnic groups

Two-Way ANONA test was used to determine the differences in the count of *P.gingivalis* between three ethnic subgroups in the healthy, gingivitis and chronic periodontitis groups. There was no significant difference between the ethnic groups as shown in Table 4.5.

Table 4.5: *P. gingivalis* count between different ethnic groups

	P. gingivalis mean and SD			
	Malay	Chinese	Indian	P value between groups
H	9828.98±21896.60	396.84±660.56	335.334±438.13	0.198
G	68064.67±149535.13	13494.00±25624.88	98970.67±117714.59	
CP	519478.97±605198.18	155416.76±268687.71	209052.18±511651.05	

P<0.05: significant difference. Two-Way ANONA

4.3.1.2 Comparison of *P. gingivalis* count between healthy, gingivitis and chronic periodontitis groups

The *P. gingivalis* population data in different groups appeared not normally distributed when checked by Shapiro-Wilk test in which it was highly significant (0.000). Non parametric test was used to determine the differences in *P. gingivalis* populations between the H, G and CP groups as shown in Table 4.6.

There was high statistical significant difference (p = 0.001) between the three groups. This difference was mainly between the H and CP groups (p = 0.002) which

determined by using pairwise comparisons, also between G and CP groups (p = 0.014), while between the H and G groups the difference was not significant.

Table 4.6: *P. gingivalis* count differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	P value between groups
H	5093.75	15740.89	2.79	62694	0.001*
G	54847.33	102620.81	0.36	335538.1	
CP	292414.49	481941.09	26.57	1545425	

*P<0.05: significant difference. Kruskal-Wallis Test

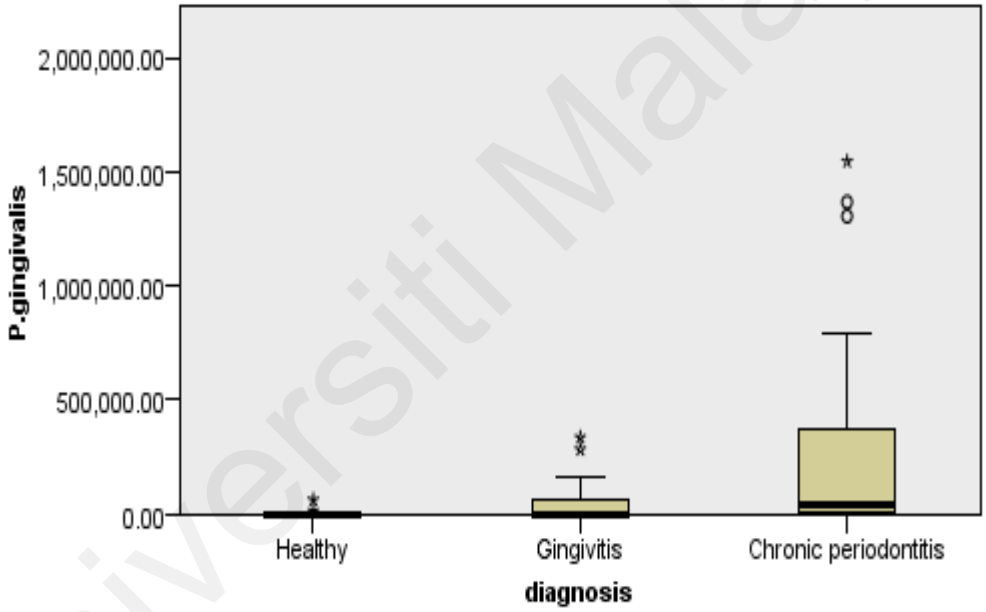

Figure 4.2: Population of *P. gingivalis* in healthy, gingivitis and chronic periodontitis groups

4.3.2 *Tannerella forsythia*

4.3.2.1 Comparison of *T. forsythia* count between different ethnic groups

There was no statistical difference (p>0.05) in the population of *T. forsythia* between the three ethnic subgroups in the healthy, gingivitis and chronic periodontitis groups as shown in Table 4.7.

Table 4.7: *T. forsythia* count between different ethnic groups

	T. forsythia mean and SD			
	Malay	Chinese	Indian	P value between groups
H	1637.04±3195.48	455.44±1086.45	690.13±1432.97	0.366
G	20751.03±57196.26	9677.61±15893.19	33074.56±57023.47	
CP	156190 ±139919.61	154736.25±197852.59	308266.01±459433.02	

P<0.05: significant difference. Two-Way ANONA

4.3.2.2 Comparison of *T. forsythia* count between healthy, gingivitis and chronic periodontitis groups

Table 4.8 showed that there was a highly significant difference (p<0.05) between the H, G and CP groups. Pairwise comparisons showed a high significant difference (p = 0.000) between the H and CP groups and between the G and CP groups, also there was significant difference between H and G groups (p = 0.049).

Table 4.8: *T. forsythia* count differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	P value between groups
H	1027.56	2287.84	1.66	10266.56	0.000*
G	22358.42	47997.48	1.38	198427.5	
CP	204552.47	294406.85	71.47	1150220	

*P<0.05: significant difference. Kruskal-Wallis Test

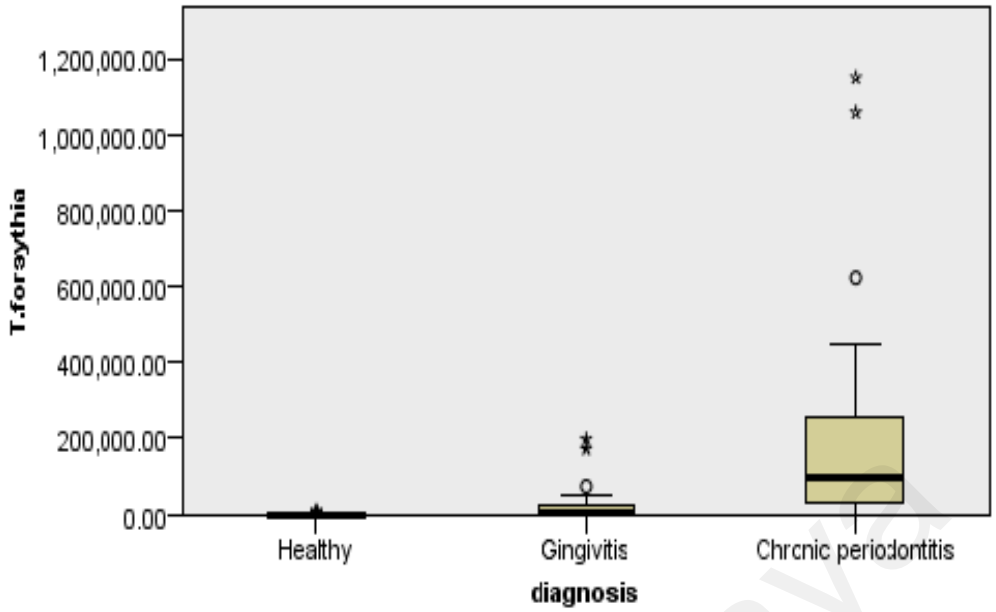

Figure 4.3: Population of *T. forsythia* in healthy, gingivitis and chronic periodontitis groups

4.3.3 *Treponema denticola*

4.3.3.1 Comparison of *T. denticola* count between different ethnic groups

No statistical difference ($p>0.05$) was found between the Malay, Chinese and Indian ethnic subgroups in the count of *T.denticola* as seen in Table 4.9.

Table 4.9: *T.denticola* count between different ethnic groups

	T. denticola mean and SD			
	Malay	Chinese	Indian	P value between groups
H	868.93±1360.92	15312.00±30459.99	172.24±269.34	0.854
G	4350.33±12254.90	527.63±1364.50	9547.86±23579.58	
CP	10621.37±9507.91	10995.94±17918.15	8379.65±13546.66	

P<0.05: significant difference. Two-Way ANONA

4.3.3.2 Comparison of *T. denticola* count between healthy, gingivitis and chronic periodontitis groups

There was high statistical significant difference (p = 0.000) between the three groups as shown in Table 4.10. This difference was mainly between the H and CP groups (p = 0.000) which was determined by using pairwise comparisons. This difference was also similar between G and CP groups (p = 0.002), while between the H and G groups, the statistical difference was not significant.

Table 4.10: *T. denticola* count differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	P value between groups
H	4394.36	15988.65	00	77100.71	0.000*
G	4681.18	15144.87	4.56	76158.35	
CP	9921.19	14023.74	19.36	56714	

*P<0.05: significant difference. Kruskal-Wallis Test

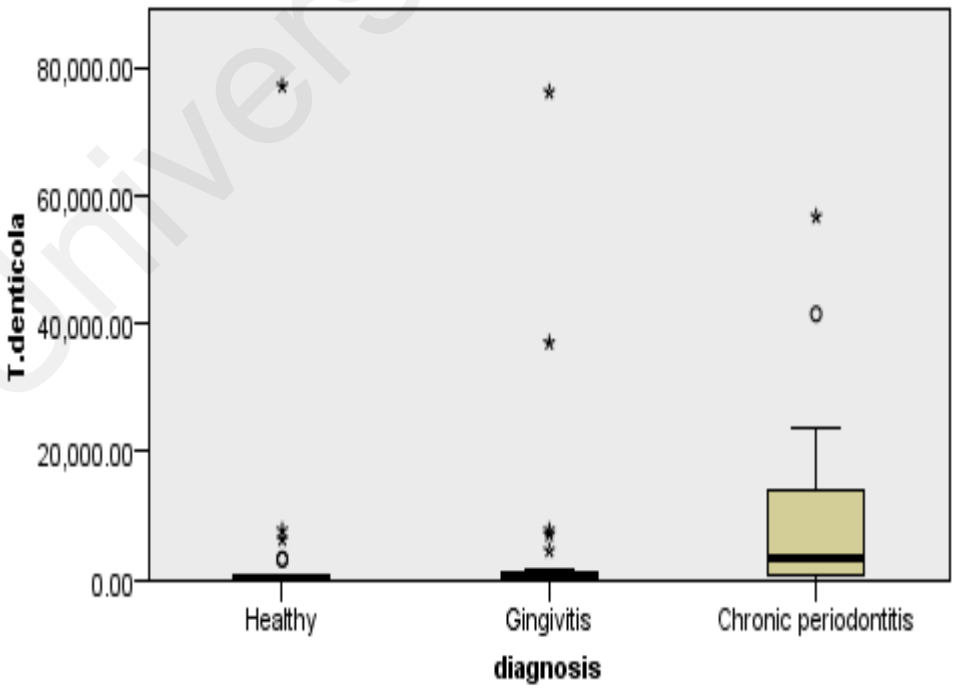

Figure 4.4: Population of *T. denticola* in healthy, gingivitis and chronic periodontitis groups

4.3.4 Aggregatibacter actinomycetemcomitans

4.3.4.1 Comparison of *A. a* count between different ethnic groups

Table 4.11 shows no statistical difference (p>0.05) between the three ethnic subgroups regarding the *A.a* count in H, G and CP groups.

Table 4.11: *A. a* count between different ethnic groups

	A.a mean and SD			
	Malay	Chinese	Indian	P value between groups
H	21907±38655.09	6114.99±10830.27	75.42±78.82	0.681
G	3612.10±4026.87	2802.94±5666.05	35282±68674.29	
CP	22674.30	126200.93±332197.78	115374.69±195455.28	

P<0.05: significant difference. Two-Way ANONA

4.3.4.2 Comparison of *A. a* count between healthy, gingivitis and chronic periodontitis groups

There was statistical significant difference (p = 0.037) between the three groups as shown in Table 4.12. The pairwise comparisons showed that this statistical difference between G and CP groups only (p = 0.020).

Table 4.12: *A. a* count differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	P value between groups
H	15230.05	31662.08	8.40	97928.09	0.037*
G	17135.37	46975.49	6.55	220584.5	
CP	94521.68	235243.54	6.11	1010484	

*P<0.05: significant difference. Kruskal-Wallis Test

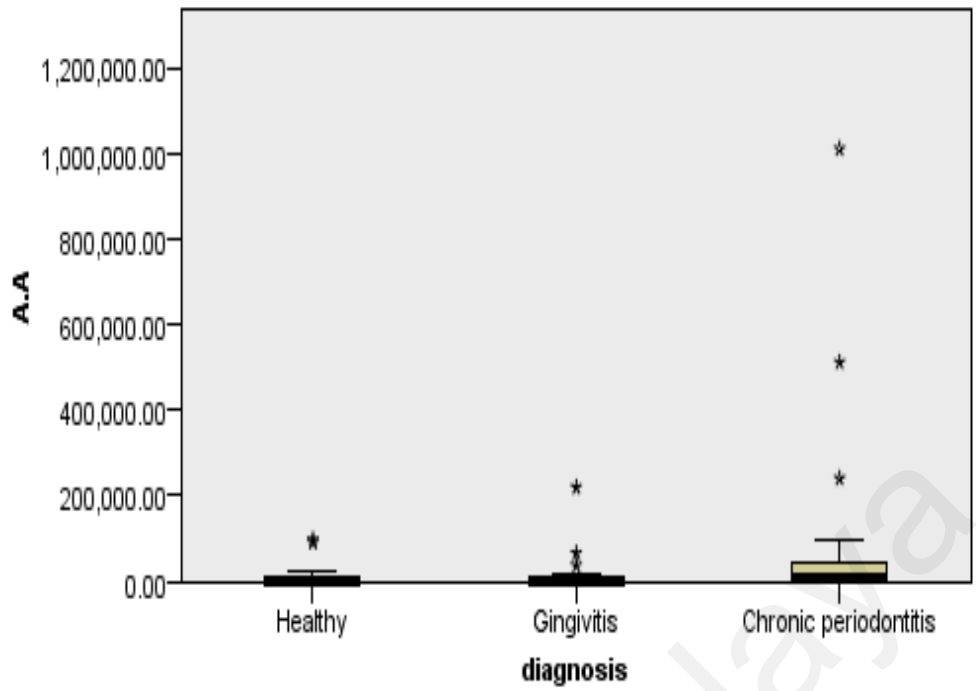

Figure 4.5: Population of *A. a* in healthy, gingivitis and chronic periodontitis groups

4.3.5 Correlation of *P. gingivalis, T. forsythia, T. denticola* and *A. actinomycetemcomitans* with the clinical periodontal parameters

The correlation between *P. gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans* population with the clinical periodontal parameters (PS, BOP, PD and CAL) in the healthy, gingivitis and chronic periodontitis groups were analyzed using the Spearman rho rank test.

Table 4.13 shows the analyzed data of these correlation tests. The results for *P.gingivalis* and its correlation to clinical periodontal parameters showed that there was no correlation between *P. gingivalis* count with PS and BOP, but there was a significant positive correlation between *P.gingivalis* count with PD and CAL (r_s = 0.442, r_s = 0.494, respectively). *T. forsythia* count was found to be positively correlated to all the clinical periodontal parameters (PS, BOP, PD and CAL) in which (r_s) values were as

follows 0.347, 0.415, 0.619 and 0.660, respectively. While *T. denticola* count was not correlated to the PS and BOP, but it was positively correlated with PD and CAL (r_s = 0.443 and r_s = 0.5, respectively). Finally the *A.a* count was not correlated to PS and BOP but positively correlated to PD and CAL (r_s = 0.345, r_s = 0.327).

Table 4.13: Correlation of periodontal pathogens with clinical parameters

	P. gingivalis		*T. forsythia*		*T. denticola*		*A. a*	
	r	p-value	R	p-value	r	p-value	r	p-value
PS	0.097	0.467	0.347*	0.001	0.197	0.086	0.055	0.675
BOP	0.223	0.089	0.415*	0.000	0.177	0.124	-0.037	0.774
PD	0.442*	0.000	0.619*	0.000	0.443*	0.000	0.345*	0.006
CAL	0.494*	0.000	0.660*	0.000	0.5*	0.000	0.327*	0.010

* Correlation significant at the 0.01 level (two-tailed).

4.4 Antimicrobial peptide (Human cathelicidine LL-37)

4.4.1 Comparison of LL-37 levels between different ethnic groups

The differences in LL-37 levels between the three ethnic subgroups in the healthy, gingivitis and chronic periodontitis groups were determined by using the Two-way ANOVA. There was slight significant difference (p = 0.029) between the ethnic groups as shown in Table 4.14.

Furthermore, the data were analyzed using the Post Hoc Bonferroni test to obtain multiple comparisons between the ethnic subgroups. The multiple comparison results showed that there was slight difference between the Malay and Chinese ethnic groups (p= 0.041) only, while no statistical difference between the Malay and Indian or the Chinese and Indian groups was detected.

Table 4.14: LL-37 concentrations (ng/mL) between different races

	LL-37 mean and SD			
	Malay	Chinese	Indian	P value between groups
H	1.31 ± 1.16	1.26 ± 1.11	1.84 ± 1.17	0.029*
G	2.06 ± 1.24	1.42 ± 1.53	2.05 ± 1.17	
CP	4.28 ±1.65	2.00 ± 1.37	2.79 ± 1.96	

*P<0.05: significant difference. Two-way ANONA

4.4.2 Comparison of LL-37 levels between healthy, gingivitis and chronic periodontitis groups

Table 4.15 shows a highly significant difference in LL-37 levels between the H, G and CP groups. The multiple comparisons between groups by using the Post Hoc test showed that this difference was mainly between H and CP groups (p = 0.000) and between G and CP groups (p = 0.014), and there was no statistical difference (p>0.05) between H and G groups (Fig.4.7).

The detection rates of LL-37 in the H, G and CP groups were as follows: 78.5%, 93.3% and 100%, respectively.

Table 4.15: LL-37 levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	1.46	1.14	00	3.52	22/28 (78.5%)	0.000*
G	1.85	1.27	00	4.44	28/30 (93.3%)	
CP	2.99	1.87	0.33	6.97	30/30 (100%)	

*P<0.05: significant difference. One-way ANONA

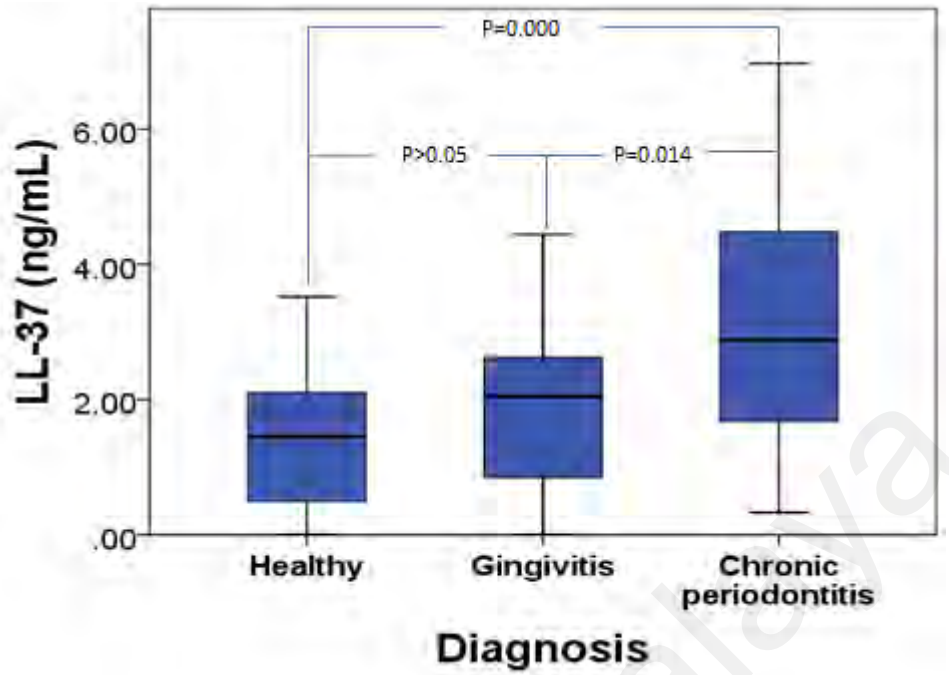

Figure 4.6: LL-37 levels in healthy, gingivitis and chronic periodontitis groups

4.4.3 Correlations of LL-37 levels with the clinical periodontal parameters and periodontal pathogens

Spearman rho rank test was used to determine if there were any correlations between the LL-37 levels with the clinical periodontal parameters (PS, BOP, PD, CAL) and the periodontal pathogens populations (*P.gingivalis, T. forsythia, T. denticola and A. actinomycetemcomitans*) as shown in Table 4.16.

With regards to clinical periodontal parameters it was found that there were no correlations between the LL-37 levels with the PS and BOP, but there were positive correlations between the LL-37 levels with the PD and CAL.

Furthermore, LL-37 levels were positively correlated to the *P.gingivalis, T. forsythia, T. denticola* and *A. actinomycetemcomitans* population ($r_s = 0.338$, $r_s = 0.365$, $r_s = 0.371$ and $r_s = 0.298$) respectively.

Table 4.16: Correlations of LL-37 levels with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	LL-37	
	Correlation (r_s)	p-value
PS	0.123	0.255
BOP	0.201	0.063
PD	0.307**	0.004
CAL	0.368**	0.000
P.gingivalis	0.337*	0.013
T.forsythia	0.377**	0.001
T.denticola	0.363**	0.001
A.a	0.296*	0.025

** Correlation significant at the 0.01 level (two-tailed).

* Correlation significant at the 0.05 level (two-tailed).

4.5 Cytokines

4.5.1 Interleukin-6 (IL-6)

4.5.1.1 Comparison of IL-6 levels between different ethnic groups

Two-way ANOVA test was used to determine the statistical differences of IL-6 levels between the three ethnic groups as shown in Table 4.17. There were no statistical differences ($p>0.05$) between the ethnic groups.

Table 4.17: IL-6 concentrations (pg/ml) between different races

	IL-6 mean and SD			
	Malay	Chinese	Indian	P value between groups
H	1.66 ± 1.86	1.46 ± 0.73	0.97 ± 0.80	0.72
G	1.88 ± 0.70	1.87 ± 1.15	1.29 ± 0.62	
CP	12.17 ±16.25	3.76 ± 4.36	3.40 ± 2.01	

*P<0.05: significant difference. Two-way ANONA

4.5.1.2 Comparison of IL-6 levels between healthy, gingivitis and chronic periodontitis groups

Non-parametric test was used to analyze the IL-6 data, in which Kruskal-Wallis test was used to show the statistical differences in the IL-6 levels between the study groups (H, G and CP). There was high statistical difference between the three groups (p=0.00) as shown in Table 4.18. The pairwise comparisons test (Figure 4.7) showed that there was high statistical difference between the H and CP groups (p=0.00) also there was statistical difference between the G and CP groups (p=0.035), while there was no statistical difference between the H and G groups (p>0.05).

The IL-6 detection rates in the H, G and CP groups were 96.4%, 100% and 100%, respectively.

Table 4.18: IL-6 levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	1.37	1.26	00	4.98	27/28 (96.4%)	0.00*
G	1.67	0.88	0.40	3.48	30/30 (100%)	
CP	6.35	10.12	0.49	49.22	30/30 (100%)	

*P<0.05: significant difference. Kruskal-Wallis Test

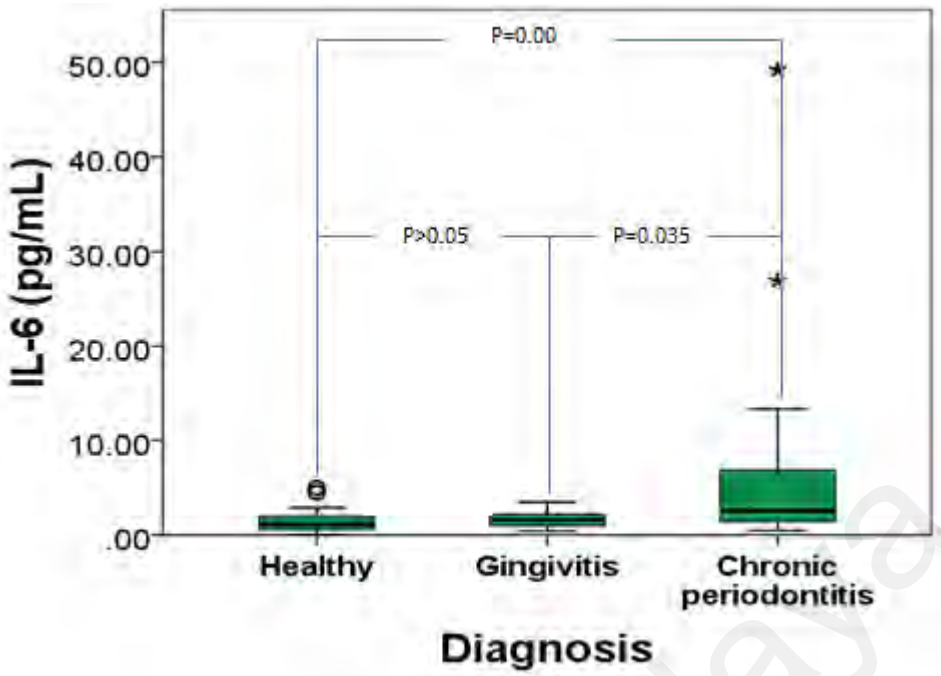

Figure 4.7: IL-6 levels in healthy, gingivitis and chronic periodontitis groups

4.5.1.3 Correlation of IL-6 levels with the clinical periodontal parameters and the periodontal pathogens

There were no correlations between the levels of IL-6 and the PS and BOP (P>0.05), but positive correlations were found between the IL-6 levels and PD and CAL (r=0.298, r=0.387) respectively.

Furthermore, no correlations were detected between the IL-6 levels with the count of *P.gingivalis, T. forsythia, T. denticola* and *A. actinomycetemcomitans* pathogens (P>0.05) as shown in Table 4.19.

Table 4.19: Correlations of IL-6 levels with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	IL-6	
	Correlation (r_s)	p-value
PS	0.187	0.083
BOP	0.095	0.381
PD	0.298*	0.005
CAL	0.387*	0.000
P.gingivalis	0.177	0.200
T.forsythia	0.203	0.071
T.denticola	0.065	0.582
A.a	0.137	0.308

* Correlation significant at the 0.01 level (two-tailed).

4.5.2 Interleukin-1β (IL-1β)

4.5.2.1 Comparison of IL-1β levels between different ethnic groups

The statistical differences in the levels of IL-1β between the three ethnic groups were determined by the Two-way ANOVA test as shown in Table 4.20. The results showed no statistical difference in IL-1β levels ($p>0.05$) between the ethnic groups.

Table 4.20: IL-1β concentrations (pg/mL) between different races

	IL-1β mean and SD			
	Malay	Chinese	Indian	P value between groups
H	84.5 ± 33.4	85.2 ± 37.8	97.4 ± 47.5	0.278
G	76.4 ± 32.3	93.4 ± 34.2	107.3 ± 50.4	
CP	145.7 ±82.3	135.4 ± 67.6	166.6 ± 85.7	

*P<0.05: significant difference Two-way ANONA

4.5.2.2 Comparison of IL-1β levels between healthy, gingivitis and chronic periodontitis groups

Parametric test was used to analyze the IL-1β data, in which One-Way ANOVA test was used to show the statistical differences in the IL-1β levels between the study groups (H, G and CP). There was high statistical difference between the three groups (p=0.00) as shown in Table 4.21. The Post Hoc test (Figure 4.8) showed that there was high statistical difference between the H and CP groups (p=0.00) and also there was statistical difference between the G and CP groups (p=0.001). However there was no statistical difference between the H and G groups (p>0.05).

IL-1β was detected in all study samples among the three study groups (H, G and CP).

Table 4.21: IL-1β levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	88.90	38.93	23.03	173.49	28/28 (100%)	0.00*
G	93.53	40.84	19.84	177.40	30/30 (100%)	
CP	148.80	76.76	29.63	301.65	30/30 (100%)	

*P<0.05: significant difference. One-way ANONA Test

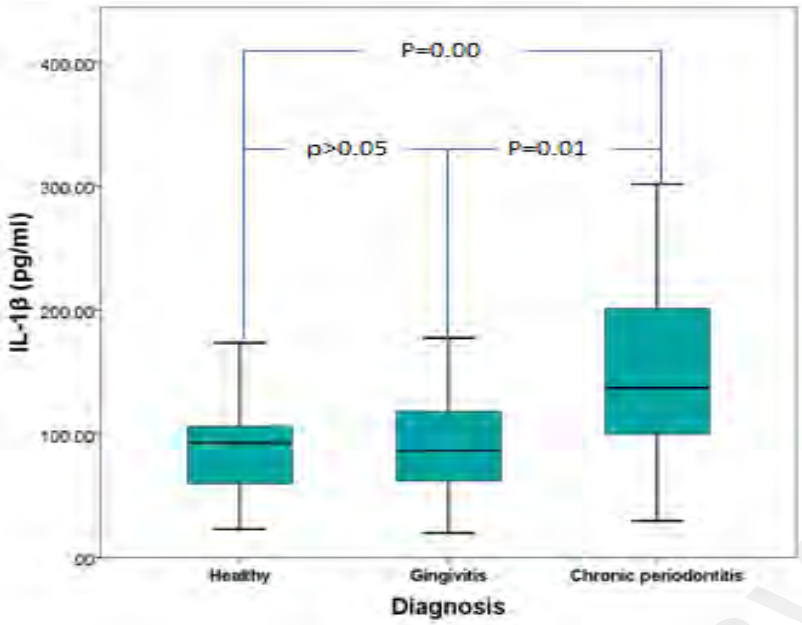

Figure 4.8: IL-1β levels in healthy, gingivitis and chronic periodontitis groups

4.5.2.3 Correlation of IL-1β levels with the clinical periodontal parameters and periodontal pathogens

Table 4.22 showed that the levels of IL-1β were no correlated to the PS and BOP ($p>0.05$), but positive correlations were detected between the IL-1β levels with the PD and CAL ($r_s=0.301$, $r_s=0.416$) respectively.

Furthermore, IL-1β levels were positively correlated to the *P.gingivalis, T. forsythia, T. denticola* and *A. actinomycetemcomitans* population ($r_s = 0.349$, $r_s = 0.484$, $r_s = 0.467$ and $r_s = 0.477$) respectively.

Table 4.22: Correlations of IL-1β levels with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	IL-1β	
	Correlation (r_s)	p-value
PS	0.100	0.355
BOP	0.173	0.108
PD	0.301*	0.005
CAL	0.416*	0.000
P.gingivalis	0.349*	0.010
T.forsythia	0.484*	0.000
T.denticola	0.467*	0.000
A.a	0.477*	0.000

* Correlation significant at the 0.01 level (two-tailed).

4.5.3 Tumor necrosis factor-alpha (TNF-α)

4.5.3.1 Comparison of TNF-α level between different ethnic groups

Two-way ANOVA statistical analysis was used to determine the differences in the levels of TNF-α between the three ethnic groups as shown in Table 4.23. The results showed no statistical difference in TNF-α levels ($p>0.05$) between the ethnic groups.

Table 4.23: TNF-α concentration (pg/mL) between different races

	TNF-α mean and SD			
	Malay	Chinese	Indian	P value between groups
H	4.31 ± 1.99	4.3 ± 1.65	8.27 ± 4.3	0.668
G	5.5 ± 3.7	5.38 ± 3.42	3.72 ± 2.76	
CP	8.73 ±5.28	6.05 ± 4.94	5.87 ± 3.52	

*P<0.05: significant difference Two-way ANONA

4.5.3.2 Comparison of TNF-α levels between healthy, gingivitis and chronic periodontitis groups

The non-parametric test was used to analyze the TNF-α level, in which Kruskal-Wallis test was used to show the statistical differences in the TNF-α level between the study groups (H, G and CP). There was no statistical difference between the three groups ($p>0.05$) as shown in Table 4.24.

TNF-α was detected in all study samples from the three study groups (H, G and CP).

Table 4.24: TNF-α levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	5.59	3.35	1.77	15.34	28/28 (100%)	1.76
G	4.82	3.27	1.20	12.15	30/30 (100%)	
CP	6.86	4.67	1.52	18.21	30/30 (100%)	

*P<0.05: significant difference. Kruskal-Wallis Test

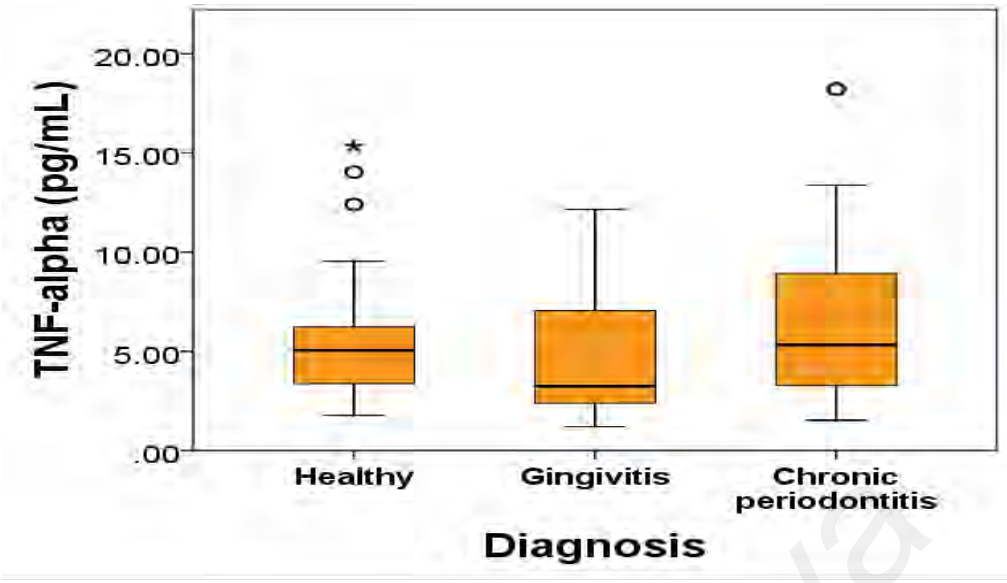

Figure 4.9: TNF-α levels in healthy, gingivitis and chronic periodontitis groups

4.5.3.3 Correlation of TNF-α level with the clinical periodontal parameters and periodontal pathogens

No correlations were detected by Spearman rho rank test between TNF-α level and the clinical periodontal parameters.

Furthermore, the statistical analysis revealed no correlations between the levels of TNF-α with periodontal pathogens count (Table 4.25).

Table 4.25: Correlations of TNF-α level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	TNF-α	
	Correlation (r_s)	p-value
PS	-0.032	0.768
BOP	0.040	0.714
PD	0.076	0.489
CAL	0.160	0.138
P.gingivalis	0.033	0.812
T.forsythia	-0.022	0.845
T.denticola	-0.138	0.242
A.a	0.033	0.807

* Correlation significant at the 0.01 level (two-tailed).

4.5.4 Prostaglandin E_2 (PGE_2)

4.5.4.1 Comparison of PGE_2 levels between the different ethnic groups

There was no statistical difference ($p>0.05$) in the PGE_2 levels between the Malay, Chinese and Indian subgroups as shown in Table 4.26.

Table 4.26: PGE_2 concentration (pg/mL) between different races

	PGE_2 mean and SD			
	Malay	Chinese	Indian	P value between groups
H	400.6 ± 77.3	381.6 ± 76	375.9 ± 60.1	0.446
G	443.7 ± 49.8	430.8 ± 66.1	395 ± 48.5	
CP	486 ± 46.9	479.7 ± 55.3	500 ± 52.6	

*P<0.05: significant difference Two-way ANONA

4.5.4.2 Comparison of PGE_2 levels between healthy, gingivitis and chronic periodontitis groups

The results of the statistical test that was used to detect the difference in the PGE_2 levels between the study groups showed highly significant difference, in which $p=0.00$ as shown in Table 4.27. This statistical difference was mainly between the H and CP groups ($p=0.00$) and between G and CP groups ($p=0.00$) as shown in Figure 10.The difference in PGE_2 levels between the H and G groups was not significant ($p>0.05$).

As shown in Table 4.27, PGE_2 was detected in all samples among the study groups.

Table 4.27: PGE_2 levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	386.59	70.23	212.3	481.9	28/28 (100%)	0.00
G	421.76	57.65	321.9	510.9	30/30 (100%)	
CP	488.34	50.68	426.6	589.4	30/30 (100%)	

*P<0.05: significant difference. One-way ANOVA Test

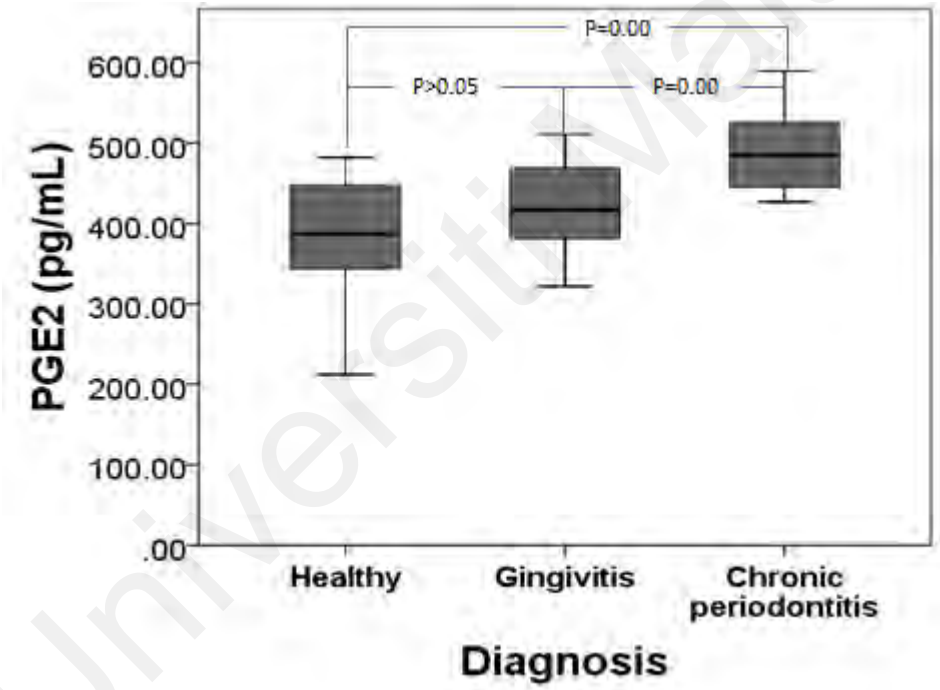

Figure 4.10: PGE_2 levels in healthy, gingivitis and chronic periodontitis groups

4.5.4.3 Correlation of PGE_2 levels with the clinical periodontal parameters and periodontal pathogens

Table 4.28 showed that the levels of PGE_2 were positively correlated to PS, BOP, PD and CAL (r_s=0.383, r_s=0.306, r_s=0.507, r_s=0.498) respectively. Moreover, PGE_2 levels were positively correlated to *T. forsythia* count (r_s=0.400). No statistical correlation was detected between the PGE_2 levels and the *P.gingivalis, T. denticola and A. actinomycetemcomitans* populations (p>0.05).

Table 4.28: Correlations of PGE_2 level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	PGE_2	
	Correlation (r_s)	p-value
PS	0.383*	0.000
BOP	0.306*	0.04
PD	0.507*	0.000
CAL	0.498*	0.000
P.gingivalis	0.157	0.258
T.forsythia	0.400*	0.000
T.denticola	0.173	0.141
A.a	0.105	0.438

* Correlation significant at the 0.01 level (two-tailed).

4.5.5 Matrix Metalloproteinase-8 (MMP-8)

4.5.5.1 Comparison of MMP-8 levels between the different ethnic groups

Comparison between the mean of MMP-8 levels in the three ethnic groups was statistically performed. No statistical difference (p>0.05) was detected between the Malay, Chinese and Indian groups as shown in Table 4.29.

Table 4.29: MMP-8 concentration (ng/mL) between different races

	MMP-8 mean and SD			
	Malay	Chinese	Indian	P value between groups
H	9.2 ± 7.6	6.9 ± 6.1	5.6 ± 5.2	0.817
G	4.5 ± 1.6	9.9 ± 8.1	12.8 ± 8.5	
CP	15.9 ± 8.8	17.2 ± 11.2	16 ± 11.1	

*P<0.05: significant difference Two-way ANONA

4.5.5.2 Comparison of MMP-8 levels between healthy, gingivitis and chronic periodontitis groups

The non-parametric test was used to analyze the differences in MMP-8 levels between the study groups (H, G and CP). There was high statistical difference between the three groups (p=0.001) as shown in Table 4.30. The pairwise comparison (Fig 4.11) showed that there was high statistical difference between the H and CP groups (p=0.000), and there was statistical difference between the G and CP groups (p=0.033), while there was no statistical difference between the H and G groups (p>0.05).

The MMP-8 was detected in all study samples among the three study groups (H, G and CP).

Table 4.30: MMP-8 levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	7.3	6.4	0.45	19.20	28/28 (100%)	0.001*
G	9.4	7.6	0.47	24.7	30/30 (100%)	
CP	16.4	10.1	3.90	30.18	30/30 (100%)	

*P<0.05: significant difference. Kruskal-Wallis Test

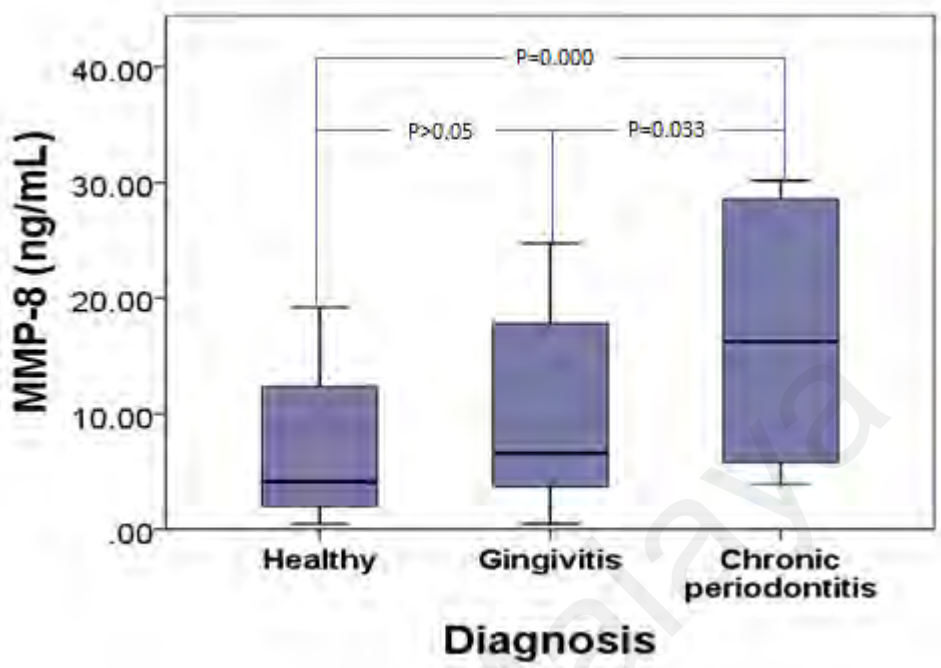

Figure 4.11: MMP-8 levels in healthy, gingivitis and chronic periodontitis groups

4.5.5.3 Correlation of MMP-8 levels with the clinical periodontal parameters and periodontal pathogens

The statistical correlations test between the MMP-8 levels and clinical periodontal parameters showed that there was no correlation between the MMP-8 levels and PS, while there was a positive correlation between MMP-8 levels and BOP, PD and CAL (r_s=0.222, r_s=0.404 and r_s=0.387) respectively.

Positive correlation between the MMP-8 levels and *P.gingivalis, T.forsythia* and *T.denticola* populations (r_s=0.363, r_s=0.349 and r_s=0.358 respectively) was detected, and there was no correlation between MMP-8 levels and *A.a* count.

Table 4.31: Correlation of MMP-8 level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	MMP-8	
	Correlation (r_s)	p-value
PS	0.211	0.05
BOP	0.222*	0.039
PD	0.404**	0.000
CAL	0.387**	0.000
P.gingivalis	0.363**	0.007
T.forsythia	0.349**	0.002
T.denticola	0.358**	0.002
A.a	0.113	0.404

** Correlation significant at the 0.01 level (two-tailed).

* Correlation significant at the 0.05 level (two-tailed).

4.5.6 Matrix Metalloproteinase-9 (MMP-9)

4.5.6.1 Comparison of MMP-9 levels between different ethnic groups

The two-way ANOVA statistical test did not show significant difference in the mean value of the MMP-9 levels between the Malay, Chinese and Indian subgroups in which the p value was > 0.05 as shown in Table 4.32.

Table 4.32: MMP-9 concentration (ng/mL) between different races

	MMP-9 mean and SD			
	Malay	Chinese	Indian	P value between groups
H	23.5 ± 14.8	13.4 ± 8.3	20.1 ± 13.7	0.533
G	18.3 ± 8.2	20.1 ± 11.9	23.8 ± 7.6	
CP	20.9 ± 9.5	22.8 ± 14.7	22.4 ± 13.2	

*P<0.05: significant difference Two-way ANONA

4.5.6.2 Comparison of MMP-9 levels between healthy, gingivitis and chronic periodontitis groups

The parametric test was used to analyze the MMP-9 levels, in which One-Way ANOVA test was used to show the statistical differences in the MMP-9 levels between the study groups (H, G and CP). There was no statistical difference between the three groups ($p>0.05$) as shown in Table 4.33.

MMP-9 was detected in all study samples among the three study groups (H, G and CP).

Table 4.33: MMP-9 levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	19.1	13	6.95	49.8	28/28 (100%)	0.627
G	20.9	9.5	7.06	39.2	30/30 (100%)	
CP	22.1	12.4	10.23	54.4	30/30 (100%)	

*P<0.05: significant difference. One-Way ANOVA Test

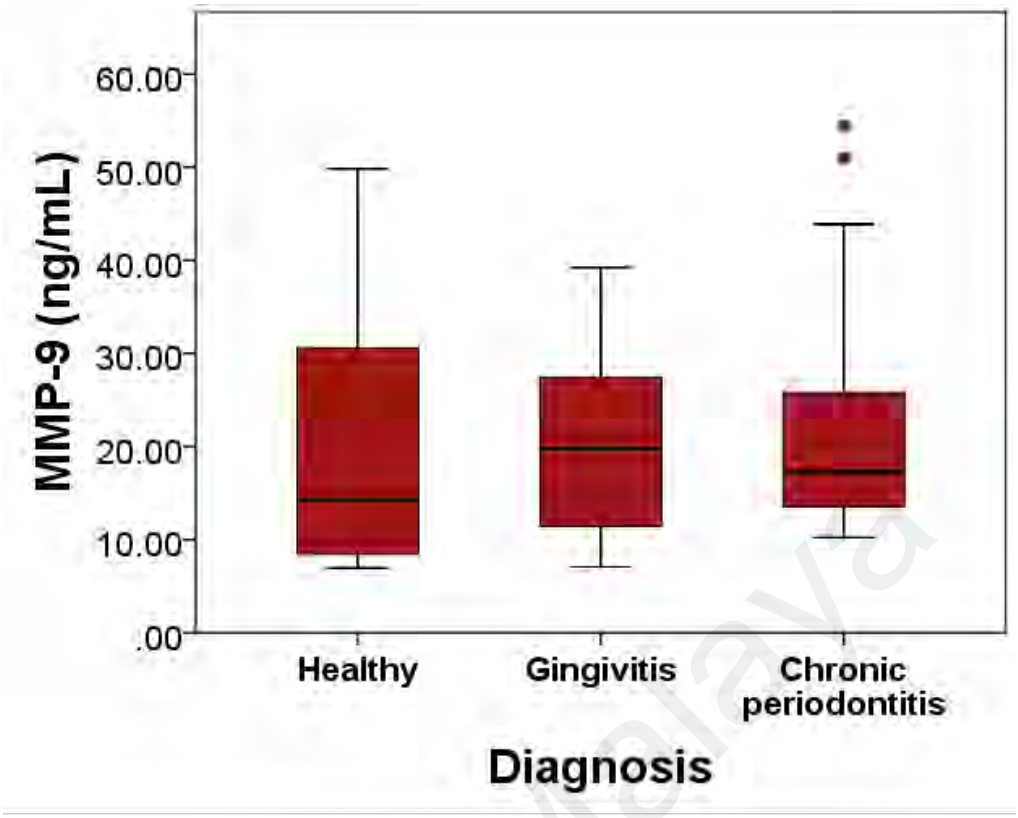

Figure 4.12: MMP-9 levels in healthy, gingivitis and chronic periodontitis groups

4.5.6.3 Correlations of MMP-9 levels with the clinical periodontal parameters and periodontal pathogens

No statistical correlations were detected by Spearman rho rank test between MMP-9 levels and the clinical periodontal parameters as shown in Table 4.34.

Furthermore, the non-parametric correlation statistical analysis revealed no correlations also between the levels of MMP-9 with periodontal pathogens populations.

Table 4.34: Correlations of MMP-9 level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	MMP-9	
	Correlation (r_s)	p-value
PS	0.128	0.239
BOP	0.151	0.167
PD	0.148	0.176
CAL	0.101	0.355
P.gingivalis	0.132	0.345
T.forsythia	0.193	0.088
T.denticola	0.145	0.220
A.a	-0.072	0.600

** Correlation significant at the 0.01 level (two-tailed).

4.5.7 Osteocalcin (OC)

4.5.7.1 Comparison of OC levels between the different ethnic groups

There was no statistical difference (p>0.05) in the levels of OC between the three ethnic subgroups as shown in Table 4.35.

Table 4.35: OC concentration (ng/mL) between different races

	OC mean and SD			
	Malay	Chinese	Indian	P value between groups
H	2.1 ± 2	1.5 ± 0.8	1.3 ± 0.1	0.587
G	1.4 ± 0.2	1.5 ± 0.7	2.4 ± 3	
CP	1.6 ± 0.4	3.3 ± 3.1	3.4 ± 3.7	

*P<0.05: significant difference Two-way ANONA

4.5.7.2 Comparison of OC levels between healthy, gingivitis and chronic periodontitis groups

Non-Parametric test was used to analyze the OC levels; Kruskal-Wallis Test was used to show the statistical differences in the OC levels between the study groups (H, G and CP). There was no statistical difference between the three groups ($p>0.05$) as shown in Table 4.36.

OC was detected in all study samples among the three study groups (H, G and CP).

Table 4.36: OC levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	1.7	1.3	0.99	6.39	28/28 (100%)	0.914
G	1.8	1.8	1.09	10.95	30/30 (100%)	
CP	2.8	2.8	1.26	11.35	30/30 (100%)	

*P<0.05: significant difference. Kruskal-Wallis Test

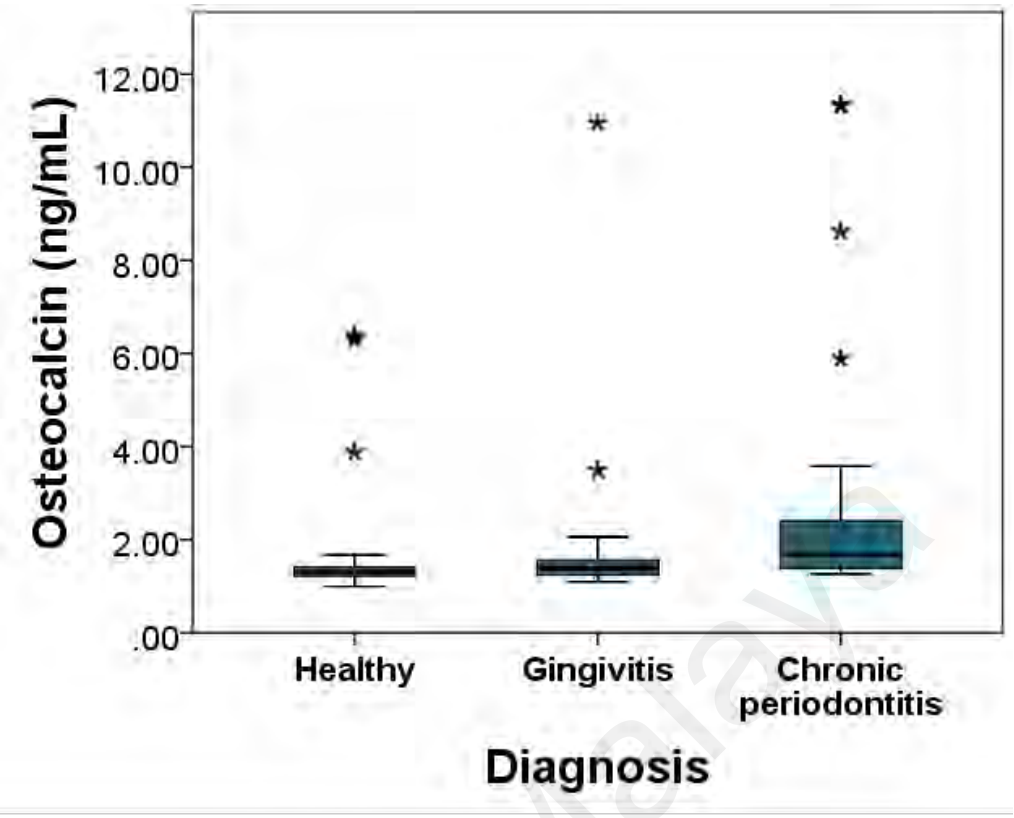

Figure 4.13: OC levels in healthy, gingivitis and chronic periodontitis groups

4.5.7.3 Correlations of OC levels with the clinical periodontal parameters and periodontal pathogens

No statistical correlations were found between OC levels and the clinical periodontal parameters as shown in Table 4.37.

Moreover, the statistical analysis revealed no correlations also between the levels of OC and the periodontal pathogens populations.

Table 4.37: Correlations of OC level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	OC	
	Correlation (r_s)	p-value
PS	0.119	0.274
BOP	0.239*	0.026
PD	0.244*	0.024
CAL	0.455**	0.000
P.gingivalis	0.165	0.233
T.forsythia	0.291**	0.009
T.denticola	0.198	0.091
A.a	0.057	0.672

** Correlation significant at the 0.01 level (two-tailed).

* Correlation significant at the 0.05 level (two-tailed).

4.5.8 Osteoprotegerin (OPG)

4.5.8.1 Comparison of OPG levels between different ethnic groups

There was no statistical difference (p>0.05) in the levels of OPG between the three ethnic subgroups as shown in Table 4.38.

Table 4.38: OPG concentration (pg/mL) between different races

	OPG mean and SD			
	Malay	Chinese	Indian	P value between groups
H	23.7 ± 16.7	11.2 ± 8.5	19.9 ± 13.2	0.237
G	24.5 ± 14.6	14.3 ± 11.1	9.9 ± 14.2	
CP	9.4 ± 8.02	15.3 ± 9.8	16 ± 9.1	

*P<0.05: significant difference Two-way ANONA

4.5.8.2 Comparison of OPG levels between healthy, gingivitis and chronic periodontitis groups

Parametric test was used to analyze the OPG levels. In spite of the mean value of OPG were higher in the H groups in comparison to other study groups (G and CP). The One-Way ANOVA Test that used to demonstrate the statistical differences in the OPG levels between the study groups (H, G and CP), showed no statistical difference between the three groups ($p>0.05$) as shown in Table 4.39.

The OPG was detected in all study samples among the three study groups (H, G and CP).

Table 4.39: OPG levels differences in healthy, gingivitis and chronic periodontitis groups

	Mean	Standard deviation	Minimum	Maximum	Detection rate	P value between groups
H	18.4	14	1.18	69.9	28/28 (100%)	0.353
G	15.6	14.1	1.66	48.52	30/30 (100%)	
CP	13.7	9.2	1.30	34.52	30/30 (100%)	

*P<0.05: significant difference. One-Way ANOVA Test

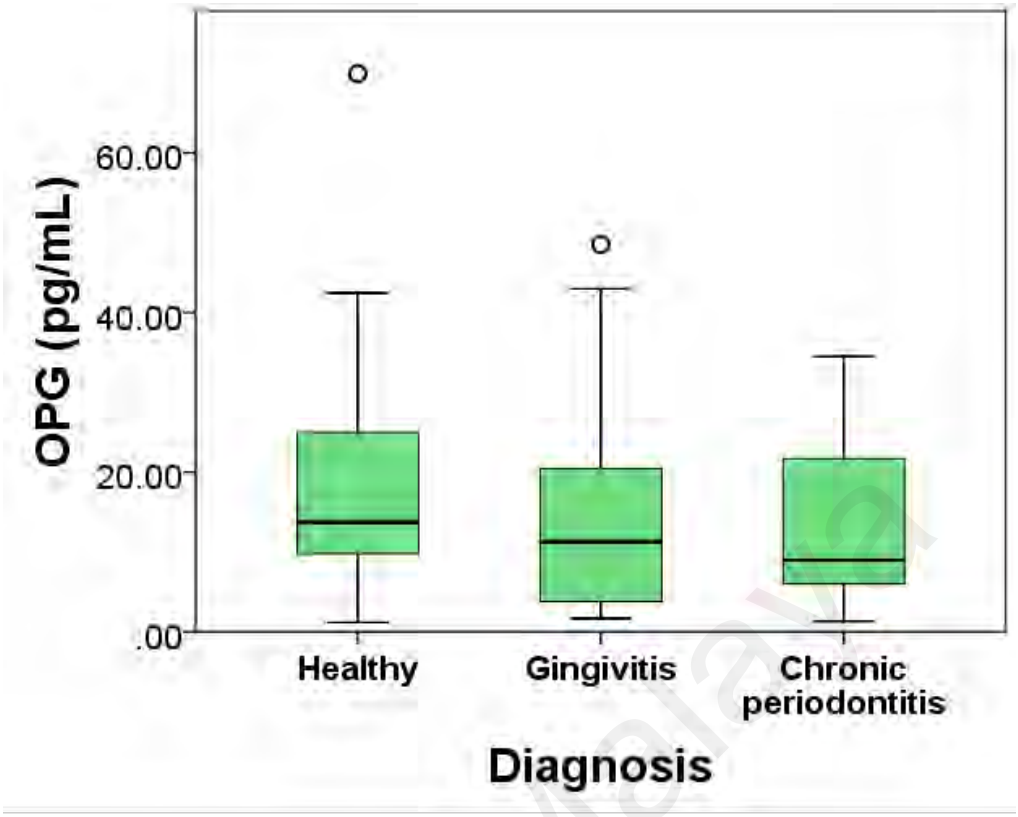

Figure 4.14: OPG levels in healthy, gingivitis and chronic periodontitis groups

4.5.8.3 Correlations of OPG levels with clinical periodontal parameters and periodontal pathogens

No statistical correlations were found between OPG levels and the clinical periodontal parameters as shown in Table 4.40.

Furthermore, the statistical analysis revealed no correlations between the levels of OPG and *P. gingivalis* and *A.a* count, while negative correlations was found between OPG levels and count of *T. forsythia* and *T. denticola* (r_s=-270, r_s=-299) respectively.

Table 4.40: Correlations of OPG level with the clinical periodontal parameters and periodontal pathogens

Clinical parameters and periodontal pathogens	OPG	
	Correlation (r_s)	p-value
PS	-0.173	0.109
BOP	-0.075	0.489
PD	-0.122	0.303
CAL	-0.150	0.165
P. gingivalis	-0.230	0.095
T. forsythia	-0.270*	0.016
T. denticola	-0.299*	0.010
A. a	-0.084	0.532

* Correlation significant at the 0.05 level (two-tailed).

4.6 Statistical correlations between the oral biomarkers

Spearman rho rank and Pearson correlation tests were used to determine the statistical correlation between the different oral biomarkers that were examined in this study. The aim of this study was to discover biomarkers that statistically correlated to each other in order to be used collectively in the diagnosis of periodontal disease.

Positive and negative statistical correlations were found between different oral biomarkers as shown in Table 4.41. The oral biomarkers that showed positive correlations between each other were MMP-8, IL-6 and IL-1β, in which the correlation of MMP-8 to IL-6 and IL-1β was 0.411 and 0.441 respectively, while the correlation between IL-6 and IL-1β was 0.304.

Furthermore, positive correlations were recorded between other oral biomarkers such as: MMP-9 and MMP-8 (0.537), PGE_2 and IL-6 (0.381), IL-1β and LL-37 (0.390).

Table 4.41: Correlations between the oral biomarkers

	LL-37 CC P-value	IL-6 CC P-value	MMP-9 CC P-value	IL-1β CC P-value	PGE_2 CC P-value	TNF-α CC P-value	OPG CC P-value	OC CC P-value	MMP-8 CC P-value
LL-37	1	0.126 0.242	0.187 0.083	0.390* 0.000	0.201 0.060	0.015 0.890	-0.176 0.102	0.044 0.684	.242* .024
IL-6	0.126 0.242	1	0.153 0.157	0.304** 0.004	0.381** 0.000	0.028 0.795	-0.160 0.139	0.282** 0.008	0.411** 0.000
MMP-9	0.187 0.083	0.153 0.157	1	0.116 0.285	0.048 0.660	-0.214* 0.047	-0.084 0.440	-0.084 0.441	0.537** 0.000
IL-1β	0.390* 0.000	0.304** 0.004	0.116 0.285	1	0.174 0.106	-0.042 0.694	- 0.333** 0.002	0.169 0.116	0.441** 0.000
PGE_2	0.201 0.060	0.381** 0.000	0.048 0.660	0.174 0.106	1	0.117 0.279	0.103 0.340	0.300** 0.004	0.223* 0.038
TNF-α	0.015 0.890	0.028 0.795	-0.214* 0.047	-0.042 0.694	0.117 0.279	1	0.094 0.385	0.062 0.569	-0.222* 0.039
OPG	-0.176 0.102	-0.160 0.139	-0.084 0.440	-0.333** 0.002	0.103 0.340	0.094 0.385	1	-0.133 0.219	-0.212* 0.048
OC	0.044 0.684	0.282** 0.008	-0.084 0.441	0.169 0.116	0.300** 0.004	0.062 0.569	-0.133 0.219	1	0.022 0.842
MMP-8	242* 0.024	0.411** 0.000	0.537** 0.000	0.441** 0.000	0.223* 0.038	-0.222* 0.039	-0.212* 0.048	0.022 0.842	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

CC = Correlation Coefficient

4.7 Calculation of cumulative risk scores (CRS)

4.7.1 Distribution of tertiles among the selected biomarkers

In order to calculate the CRS, the GCF concentrations of each selected oral biomarker (IL-6, IL-1 β and MMP-8) were divided into three tertiles 1–3 (Table 4.42). The tertiles values (T1, T2 and T3) for IL-6 were as follows: (≤ 1.150, 1.151-2.220 and >2.220) respectively, while the tertiles values (T1, T2 and T3) for the IL-1β was

(≤77.950, 77.951-124.040 and 124.040) respectively and for the MMP-8 was as follows: (≤4.200, 4.201-14.590 and >14.590).

Table 4.42: Distribution of tertiles

	Interluekin-6			Interluekin-1β			MMP-8		
Tertiles	Distribution of tertiles	Min	Max	Distribution of tertiles	Min	Max	Distribution of tertiles	Min	Max
T1	≤ 1.150	.00	1.15	≤77.950	19.84	77.95	≤4.200	.45	4.20
T2	1.151-2.220	1.18	2.22	77.951-124.040	78.43	124.04	4.201-14.590	4.39	14.59
T 3	>2.220	2.28	49.22	124.040	126.63	301.65	>14.590	14.76	30.18

For example, a subject with GCF concentrations: 2.2 pg/ml for IL-6, 95 pg/ml for IL-1β, and 3.5 ng/ml for MMP-8 will have tertile values of 2, 2, and 1, respectively. When these tertile values are multiplied (2 × 2 × 1), a cumulative score will be 4, and the subject will be categorized as being at moderate risk of having periodontitis (CRS II).

4.7.2 Distribution of subjects in the CRS groups

Following the determination of tertiles values of the selected oral biomarkers, the percentage of study subjects that belong to each CRS groups was determined. As shown in Table 4.43, 28.4% of the subjects were at low risk for having periodontitis (CRS I) and 39.7% of subjects being at moderate risk for having periodontitis (CRS II), while 31.8% of subjects had high risk to develop periodontitis (CRS III).

Table 4.43: Distribution of subjects on the CRS groups

CRS groups	Frequency	Percentage %
CRS I = 1-3	25	28.41
CRS II = 4-9	35	39.77
CRS III > 9	28	31.81
Total	88	100.0

Furthermore, the percentage of subjects that belong to each tertile (T1, T2 and T3) in the control (H and G) and CP groups for each selected biomarker was determined (Table 4.44). There was significant difference between the number of subjects that belong to each tertile for the three selected biomarkers (IL-6=0.004, IL-1β=0.002 and MMP-8=0.047), and there was a significant difference between the number of subjects that belongs to the CRS groups (0.003).

Table 4.44: Distribution of subjects among the tertiles for each selected biomarker

		Diagnosis				
		Healthy and Gingivitis (control)		Chronic periodontitis		P-value
		n	%	n	%	
IL-6	T1 ≤ 1.150	25	83.3	5	16.7	0.004*
	T2= 1.151-2.220	21	75.0	7	25.0	
	T 3 >2.220	13	43.3	17	56.6	
IL-1β	T1 ≤77.950	25	83.3	5	16.7	0.002*
	T2=77.951-124.040	22	75.9	7	24.1	
	T3 > 124.040	12	41.3	17	58.6	
MMP-8	T1 ≤4.200	26	81.2	6	18.8	0.047*
	T2= 4.201-14.590	18	69.2	8	30.8	
	T3 >14.590	15	50	15	50	
CRS	Low 1-3	23	92.0	2	8.0	0.003*
	Medium 4-9	23	65.7	12	34.3	
	High > 9	13	46.4	15	53.5	

*The Chi-square statistic is significant at the 0.05 level.

4.7.3 Association between the selected biomarkers and the presence of periodontitis

The Odds ratios (OR) for each selected biomarker and for the CRS were determined to detect the subjects that have higher risk to develop periodontitis (Table 4.45). The OR for the three biomarkers was significantly higher in T3 than in T1 and T2, also it was significantly higher in CRS III than the other CRS groups.

Furthermore, the area under curve (AUC) values were measured for the three biomarkers and also for the CRS in order to determine the sensitivity for each biomarker and for the CRS to detect periodontitis.

The results showed that the AUC value for CRS was higher than the AUC value for each biomarker separately, in which the AUC value for CRS was 0.749 and for the IL-6, IL-1β and MMP-8 were as follows: 0.697, 0.704 and 0.653 respectively.

Table 4.45: Odds ratios (OR) and area under curve (AUC) values (95% confidence interval) for the three GCF biomarkers divided into tertiles and for the CRS

Marker	P-value	OR (95% CI)	OR (95% CI)		AUC (95% CI)	AUC (95% CI)	
			Lower	Upper		Lower	Upper
IL-6T1		1			0.697	.576	.817
IL-6T2	.436	1.667	.461	6.030			
IL-6T3	.003	6.154	1.840	20.581			
IL-1βT1		1			0.704	.583	.824
IL-1βT 2	.478	1.591	.441	5.737			
IL-1βT 3	.002	6.667	1.973	22.524			
MMP-8T1		1			0.653	.530	.777
MMP-8T2	.291	1.926	.570	6.505			
MMP-8T3	.017	4.044	1.283	12.751			
CSR I		1			0.749	.645	.853
CSR II	.029	6.000	1.206	29.857			
CSR III	.002	12.385	2.426	63.224			

CHAPTER 5: DISCUSSION

5.1 Introduction

This cross sectional comparative study was conducted on subjects at different stages of periodontal disease (gingivitis and chronic periodontitis) and from different ethnic origins in the Malaysian population. The majority of previous studies on oral biomarkers have been conducted on subjects with gingivitis or chronic periodontitis who have the same ethnicity. However, the reliability of using GCF derived oral biomarkers in the prediction and diagnosis of periodontal disease in different ethnic groups has not been thoroughly researched.

5.2 Study Design

This clinical study was designed to measure and compare the levels of oral biomarkers in GCF and the microbiological profile in the subgingival plaque among subjects with different severity of periodontal disease.

GCF and subgingival plaque samples were collected, in addition to the measurement of clinical periodontal parameters (PS, BOP, PD and CAL) by the researcher (ZN) from all the subjects that participated in this study.

All the subjects in this study received oral hygiene instructions, full mouth scaling and / or root planing, if needed, following the collection of samples. This was done by the undergraduate or postgraduate students of the faculty of Dentistry, University of Malaya (not the researcher).

ELISA was used to measure the biomarkers in GCF, while RT-PCR was used to analyse the red complex species and *A. actinomycetemcomitans* (*A.a*) in the collected subgingival plaque samples.

5.3 Sample

A total of 96 Malaysian subjects aged between 21 – 71 were screened for enrolment into this study. Among these, 88 subjects were suitable according to the inclusion and exclusion criteria. Since the subjects were needed for one visit only, there were no cases of drop out from the study.

5.4 Clinical Periodontal Examination

A detailed periodontal examination is a procedure that includes the collection of data which is important to reach a correct diagnosis and subsequently plan the necessary treatment. Though the understanding of the pathogenesis of periodontal disease has developed significantly in the last 40 years, the traditional methods of periodontal disease diagnosis have remained almost unchanged (Wolf & Lamster, 2011).

The standard diagnosis of periodontal disease is currently achieved by measuring the clinical periodontal parameters such as PI, BOP, PD and CAL, and then confirmed by the radiographic findings for the assessment of the alveolar bone levels (Armitage, 2004; Laudenbach & Simon, 2014; Wolf & Lamster, 2011). Clinicians perform their diagnosis by referring to the classification system developed at the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions. A complete periodontal examination needs oral health professionals and can be relatively difficult and lengthy, particularly when performing measurements on large sample size in oral

health surveys (Gursoy et al., 2011). This issue can be attributed to the time taken to perform these periodontal examinations.

5.5 Subgingival plaque bacteria

One of the aims of this study was to measure the bacterial count in the subgingival plaque samples in H, G and CP subjects among the different ethnic groups in the Malaysian population. To the best of our knowledge, this is the first study that compared the subgingival bacterial count in three ethnic groups within the Malaysian population.

The composition, complexity and strain variation of oral bacteria has been studied in depth and extensively in recent years. These advances in information were mostly a consequence of development in molecular biotechnology, which allowed a wide analysis of the bacteria and / or permitted the discovery of uncultivable microorganisms (Abusleme et al., 2013; Oliveira et al., 2016; Park et al., 2015). However, more detailed information is needed on the red complex and on the relationship between the bacterial species and clinical periodontal parameters (Da Silva-Boghossian et al., 2011; Lanza et al., 2016).

Many studies have demonstrated that the composition of subgingival pathogens amongst different populations in the world is important in order to provide information on the bacterial changes during the different stages of periodontal disease progression that occurs in individuals from different geographical regions (Ximenez-Fyvie et al., 2006).

5.5.1 Red complex species and *Aggregatibacter actinomycetemcomitans* (*A. a*)

Subgingival plaque bacteria are the main causative factor for periodontal disease. However, a few species, such as *A. actinomycetemcomitans* and *P. gingivalis* , have been considered as the common periodontal pathogens for the progression of periodontal disease (Slots, 1999). Nevertheless, the red complex is presently considered as the most pathogenic bacterial component (Holt & Ebersole, 2005; Lanza et al., 2016).

This study observed that the red complex species strongly colonised the subgingival microbiota in chronic periodontitis patients compared to healthy subjects or patients with gingivitis. This finding is consistent with other studies in which periodontopathic bacteria were less prevalent in healthy subjects (Dogan et al., 2003; Tomita et al., 2013; Wara-Aswapati et al., 2009) and patients with gingivitis (Ashimoto et al., 1996; Tanner et al., 1998). The pathological deepening of the periodontal pocket and the increased amount of dental plaque biofilm encourages further increase of periodontopathic bacteria (Botero et al., 2007). The findings of this study also showed that the count of *A.a* in the Malaysian population were not correlated with the periodontal disease status; in which there were no significant differences in the count between the H and CP groups and only a slight difference between the G and CP groups. This finding is in accordance with a study done on the Thai population that also showed no correlation between *A.a* and periodontal disease status. That study reported that the prevalence of *A.a* in moderate to severe CP patients (35%) was less than the prevalence of *A.a* in the mild CP group (45%) in the Thai population (Wara-Aswapati et al., 2009). Furthermore, a study done on Japanese population was unable to confirm any significant difference bacterial count between the various types of periodontal disease. The study showed that there was a low prevalence of *A.a* in localised aggressive

periodontitis, generalised aggressive periodontitis and CP in the study groups that were investigated (Takeuchi et al., 2003).

The data discrepancy regarding the quantification and prevalence of the subgingival periodontal pathogens in patients with different stages of periodontal disease between different populations may be due to numerous reasons. Among those reasons, the differences in classification of periodontal disease, data analysis methods, techniques used for sample analysis (Dogan et al., 2003), demographic features of the study population (Braga et al., 2010), ethnicity, cultural factors (Botero et al., 2007), socioeconomic status and geographic locations may have contributed to these data differences (Wara-Aswapati et al., 2009).

Few studies have been able to investigate the differences between ethnic groups within the same geographical location (Ellwood et al., 1997; Sirinian et al., 2002) or more than one population under the same conditions of investigation. This could have been due to the difficulties in performing such studies (Haffajee et al., 2004), as it is difficult to standardize the methods and sites of sample collection and, in addition, difficulties in standardizing the laboratory analysis.

One of the main goals of the present study was to explore whether the genetic diversity between different ethnic groups has an effect on subgingival pathogens that are distributed in different stages of periodontal disease. The results of our study showed that there was no marked effect of the genetic diversity on the count of subgingival pathogens. There were also no statistical differences in the *T. forsythia, T. denticola, P. gingivalis* and *A.a* count between the Malay, Chinese and Indian populations in the H, G and CP groups. This result is in accordance with a study performed by Sirinian et al. (2002) who investigated the presence of *T. forsythia, T. denticola, P. gingivalis* and *A. a* in the saliva samples taken from young subjects in the Caucasian, Hispanic and Asian-

American ethnic groups residing in Los Angeles. The authors found that periodontopathic bacteria were not correlated to the ethnicity but related to the education level of the mother, gender and environmental factors (Sirinian et al., 2002). In a different study, Haffajee et al. (2004) explored the composition of the subgingival plaque bacteria in CP patients from four countries (USA=115 subjects; Sweden=101 subjects; Chile=26 subjects; Brazil=58 subjects) using almost standardised methods in the sample collections and analysis. A marked difference in the mean proportions of many types of subgingival plaque bacteria was reported. This mainly contributed to the differences in probing depths of the sampled sites between the groups and some groups receiving periodontal treatment or systemic antibiotics, in addition to diet, culture, genetic background and differences in socio-economic status (Haffajee et al., 2004).

5.6 GCF as a source of oral biomarkers

In the current study, GCF biomarkers were used rather than other biomarkers in biofluids (saliva or serum). Many studies have suggested that GCF is a source of biomolecular sampling to investigate the condition of the periodontal tissues (Bakri et al., 2013; Luo et al., 2011). GCF is composed of many components that have been described as markers for periodontal disease development. These comprise host-derived enzymes, host-response modifiers and tissue breakdown products (Khongkhunthian et al., 2014) .

It is known that biomarkers are objective and measurable characteristics of biological processes (Colburn et al., 2001). These biomarkers can support clinical evaluation if we fully understand the normal physiology of the biological processes of periodontal disease diagnosis and progression (Strimbu & Tavel, 2010). Many biomarkers that can be derived from different biofluids such as blood, serum, saliva and

GCF, and from different sources such as microbial dental plaque biofilm, connective tissue breakdown products, inflammatory mediators and host derivatives. For example, MMPs that exist in GCF, saliva, mouth-rinses and peri-implant sulcular fluid (PISF) can be used to discover a novel chair-side and point-of-care analytical test, which is a non-traumatic method for the diagnosis of periodontal disease (Sorsa et al., 2016; Sorsa et al., 2006).

In this study, the focus was on GCF biomarkers because of their close proximity to periodontal tissue. This minimises the possibility of detecting a response on other inflammatory processes in the body as may occur with other biofluids and the fact that the development of periodontitis is site-specific (Fujita et al., 2012).

5.7 Oral Biomarkers

Increased biomarker levels in GCF and saliva of patients with periodontal disease can be a good marker of the initiation and maintenance of periodontal disease (Gomes et al., 2016). Therefore, these biomarkers should be related to periodontal disease, in which the changes in their quantity might have a diagnostic significance (Rosa et al., 2014).

In the present study the concentrations of nine oral biomarkers (LL-37, MMP-8, MMP-9, IL-6, IL-1β, TNF-α, OPG, OC and PGE_2) derived from GCF of subjects with different periodontal disease were measured among three ethnic groups in Malaysia.

The correlations of the levels of oral biomarkers to clinical periodontal parameters and subgingival periodontal pathogens were also statistically analysed in order to determine the most accurate oral biomarkers for used in the detection and diagnosis of periodontal disease.

5.7.1 Antimicrobial peptides (Human cathelicidine LL-37)

In humans, LL-37 appears to play a major role in protecting the periodontal tissue against dental plaque microbes (Puklo et al., 2008); although the concentration of LL-37 is insufficient to prevent the initiation and progression of periodontal disease. This is especially present in genetic disorders such as the morbus Kostmann syndrome, a severe congenital disease that causes a deficiency of LL-37 (Pütsep et al., 2002), in addition, another congenital disease namely Papillon–Lefe`vre syndrome leads to lack of LL-37 (De Haar et al., 2006) which plays a key role in the development of severe periodontal destruction in patients with these syndromes.

The stimulation of periodontal pathogens during inflammation leads to the expression of LL-37 from the gingival epithelial cells (Hosokawa et al., 2006), salivary glands (Tao et al., 2005), monocytes (Scott et al., 2002) and mainly from neutrophils (Puklo et al., 2008). In this study, the cathelicidine LL-37 levels from the GCF samples were significantly elevated in CP patients compared to H subjects. This finding is consistent with other studies conducted (Makeudom et al., 2014; Puklo et al., 2008; Türkoglu et al., 2009). The significant differences in LL-37 levels were also found between the G patients and other study groups whereby the same findings were demonstrated by Türkoglu et al. (2009) in which the CP patients were reported to have higher LL-37 levels than in G patients who in turn had elevated LL-37 levels compared to the H subjects. Additionally, in this study, the LL-37 levels were found to be positively correlated with the probing depths and clinical attachment loss which are considered the most accurate clinical parameters together with radiographic interpretations in determining the severity of periodontal disease. These findings are consistent with a study by Hosokawa et al. (2006) measuring the LL-37 levels in

gingival tissue from different gingival sulcus and periodontal pocket depths. They reported a positive correlation between the LL-37 and periodontal pocket depths. Türkoglu et al. (2009) also demonstrated a positive correlation between the LL-37 and pocket depths. They attributed this relationship to the stimulation of an inflammatory response during the periodontal disease progression caused by dental plaque biofilm. This stimulation leads to migration of neutrophils into the periodontal pocket which causes LL-37 to be released from the stimulated neutrophils.

These findings of cathelicidine LL-37 in the GCF relative to the periodontal disease severity may assist in the prediction and diagnosis of different periodontal diseases; as LL-37 plays an important role in the innate immune response, thus making LL-37 a useful biomarker for the progression of periodontal diseases. This is especially because cathelicidine LL-37 in the GCF was also detected in healthy subjects, G and CP groups (78.5%, 93.3% and 100%, respectively) in different concentrations. This could be due to neutrophils stimulation by many periodontal pathogens leading to LL-37 secretions through the junctional epithelium in the gingival sulcus. The neutrophil‟s sensitivity to a minor bacterial stimulus in healthy subjects could be useful in the early prediction and monitoring of periodontal disease through the monitoring of LL-37 levels in the GCF. The occurrence of LL-37 when neutrophils are present only in the periodontally healthy subjects suggested that neutrophils are the essential factor of the LL-37 release in the healthy periodontium (Puklo et al., 2008).

Furthermore, the present study demonstrated a positive correlation between the LL-37 levels and population of *T. forsythia, T. denticola, P. gingivalis* and *A. a* in the subgingival plaque. These microbes are considered to be the most pathogenic for the initiation and progression of periodontal disease. The same correlation was discovered by Puklo et al. (2008) who demonstrated that the unprocessed cathelicidine and mature

LL-37 levels in the GCF were positively correlated to *T. forsythia, T. denticola* and *P. gingivalis*. This positive correlation between LL-37 levels and the red complex species, and *A.a* explained the elevation of LL-37 levels in accordance with the severity of periodontal disease which supported our study of using LL-37 as a biomarker for periodontal disease.

The variations in the expressions of GCF LL-37 levels in three ethnic groups within the Malaysian population were investigated in this study in an attempt to explore the effects of any genetic discrepancies on the cathelicidine LL-37 levels. A statistical difference (p=0.41) was detected between the Malay and Chinese groups and there was no statistical difference between the other study groups. This difference between the Malay and Chinese groups may be due to the small sample size (n=10) of the ethnic subgroups enrolled in each of the study groups (H, G and CP). Since the study samples were collected from the same geographical region and had similar nutritional and environmental conditions, the authors were of the opinion that demographic and socioeconomic variations between the study groups may have played a role in this difference between the Malay and Chinese ethnic groups although the difference was minimal.

5.7.2 Cytokines levels in the different study groups

5.7.2.1 Interleukin-6 (IL-6)

IL-6 is considered a multifunctional cytokine that is synthesised as a result of stimulation of many cells by trauma and infection (Kishimoto et al., 1995) such as neutrophils, macrophages, keratinocytes, endothelial cells and fibroblasts (Matsuki et al., 1992).

In the present study, the levels of IL-6 in GCF were significantly higher in chronic periodontitis patients compared to healthy subjects or patients with gingivitis. There was no statistical difference in the IL-6 levels between healthy and gingivitis groups in spite of the IL-6 levels in gingivitis patients being higher than in the healthy subjects. Furthermore, the IL-6 levels in GCF were positively correlated to CAL and PD but not correlated to PI and BOP. These results may be due to the variations in the secretion of IL-6 by their producing cells according to the type and / or severity of the periodontal disease (Takahashi et al., 1994). Furthermore, it could be due to the effect of bone resorption and / or remodelling process at the inflammation sites (Baylink et al., 1993).

These results are consistent with previous studies (Becerik et al., 2012; Mogi et al., 1999). Becerik et al. (2012) compared the GCF levels of different cytokines, among them the IL-6, between H, G, CP and GAgP groups. The results of these studies showed that the CP and GAgP patients had higher GCF IL-6 levels when compared to the H subjects. The GCF IL-6 total amounts of the GAgP group were not significantly different from the CP group. The G patients had higher levels of IL-6 than the H subjects, but the difference was not significant. In addition, they also documented a positive correlation between the IL-6 levels, CAL and PD. This is in contrast to the results in this study as they recorded a positive correlation between the levels of IL-6, the PI and papilla bleeding index (PBI).

Moreover, our results of the IL-6 GCF levels partially agreed with a study that was conducted by Ramseir et al. (2009) in which they measured the levels of IL-6 in saliva among H, G, mild CP and moderate to severe CP groups. They also recorded increased levels of IL-6 in CP groups compared to H and G groups. However, those differences were not statistically significant. This may be due to periodontal diseases being site

specific and GCF reflected the immune response better compared to other biofluids due to its proximity to the infection sites.

5.7.2.2 Interleukin-1 beta (IL-1β)

Many studies have demonstrated a correlation between the severity of periodontal disease and IL-1β levels in GCF and gingival tissue (Al-Shammari et al., 2001; Faizuddin et al., 2003; Ishihara et al., 1997). It has been reported that the GCF IL-1β levels are increased in samples from patients with periodontitis, regardless of the severity of the disease at the sampled site. This suggests that the levels of IL-1β in GCF are indicator for specific patients (Figueredo et al., 1999).

The results of this study showed significantly higher levels of the GCF IL-1β in the chronic periodontitis group compared to healthy and gingivitis groups. The mean value of the IL-1β levels was higher in gingivitis patients than in healthy subjects although no statistical difference was recorded. Moreover, there was a positive correlation between the IL-1β levels, the PD and CAL. IL-1β is known to be one of the main cytokines that play an important role in the alveolar bone destruction and IL-1β induce the migration of neutrophils to the periodontal tissue. Which will lead to the elimination of pathogens. The stimulation of primary mediators such as IL-1β, leads to the secretion of secondary mediators (cyclooxygenase). This will induce chemokines to perform like chemotactic cytokines. This situation keeps the inflammatory response in two ways: bone resorption by osteoclastic activity and tissue destruction via enzymes secretion such as MMPs (Graves et al., 2003).

The significantly higher levels of GCF IL-1β in the chronic periodontitis group than in healthy groups that was recorded in this study is consistent with previous studies

(Becerik et al., 2012; Chaudhari et al., 2011; Ertugrul et al., 2013; Konopka et al., 2012; Teles et al., 2010). Moreover, we agreed with other studies (Becerik et al., 2012; Ertugrul et al., 2013; Ülker et al., 2008) in which they also recorded non-significant difference between healthy and gingivitis groups. Conversely, these studies results that indicated non-significant differences between H and G groups were not inconsistent with a study performed by Ertugrul et al. (2013) which showed that the GCF IL-1β levels in the G group were significantly higher than the H group.

Becerik et al. (2012) conducted a study to investigate the plasma and GCF cytokine levels in four groups (H, G, CP and GAgP). They reported significantly higher levels of GCF IL-1β in the CP and GAgP groups compared to the H group, and there was no significant difference between the H and G groups, while the plasma levels of IL-1β were not significantly different among the study groups. A positive correlation was also recorded between the IL-1β levels and clinical periodontal parameters except for the plaque index. Almost similar findings were also produced by Ertugrul et al. (2013) in which they also investigated GCF IL-1β levels in H, G, CP and GAgP groups. Their results showed that the total IL-1β levels were significantly lower in H subjects than in the other groups. The total IL-1β levels in the GAgP group was significantly higher than in the CP and G groups. Meanwhile, the total IL-1β levels in the G group were also significantly higher than in the H group.

5.7.2.3 Human prostaglandin E2 (PGE2)

PGE_2 is an inflammatory mediator that mediates between bacterial infection and damage of periodontal tissue (Offenbaceer et al., 1986; Taxman et al., 2012). PGE2 has many functions, such as vasodilatation and enhancement of vascular permeability, although regarding the pathology of periodontitis, the initiation of osteoclastogenesis is considered the most important function (Brechter & Lerner, 2007; Gawron et al., 2014).

In this study, we found that the levels of PGE_2 in GCF were significantly higher in the chronic periodontitis group compared to the healthy and gingivitis groups. Although the mean value of the PGE_2 levels was higher in gingivitis patients than in healthy subjects, no statistical difference was recorded. Furthermore, positive statistical correlations were found between the PGE_2 levels and the clinical periodontal parameters (PS, BOP, PD and CAL).

Any stimulus which irritates or harms the cell wall will trigger the arachidonic acid pathway, prompting the creation of prostaglandins. These stimuli incorporate a bunch of inflammatory responses, for example, endotoxin secretion, lysis of cells, phagocytosis and so on. In this way, PGE2 levels may essentially reflect arachidonic acid enrolment and therefore indicate the inflammatory cell activation and cell death (Offenbaceer et al., 1986).

The results of this study are consistent with previous studies (Alpagot et al., 2007; Kumar et al., 2013). In 2013, Kumar et al. conducted a study to estimate PGE_2 levels in H and CP subjects. Their results showed that the PGE_2 levels in CP groups were significantly higher than the H group. They also documented positive correlations between the PGE_2 levels in GCF and the clinical periodontal parameters. Similar results were obtained by Alpagot et al. (2007). However, they conducted their study on patients

with HIV and they also found that the production of PGE_2 is up-modulated in an HIV infection.

5.7.2.4 Tumour necrosis factor-alpha (TNF-α)

TNF-α was found to initiate the resorption of the bone and up-controls the PGE2 secretion. They are created by numerous cells such as neutrophils, macrophages, keratinocytes, fibroblasts and T and B cells in the periodontal tissue (Gomes et al., 2016).

The results of this study showed that the mean values of TNF-α in the CP group is higher than the mean value of TNF-α in the H and G groups; but there was no significant difference in TNF-α levels between the three groups (H, G and CP). Besides that, no statistical correlation was detected between the TNF-α levels and clinical periodontal parameters. Actually, our results have contributed to the inhibition of TNF-α by other cytokines that are present in GCF, also to the extreme dilution of the GCF (Varghese et al., 2015b).

Our results indicated non-significant difference in the TNF-α levels between healthy and diseased groups and conformed to other previous studies (Oliveira et al., 2012; Teles et al., 2010; Ülker et al., 2008).

In 2005, Kurtis et al. determined the GCF levels of TNF-α in H, CP and AgP groups. They found that TNF-α levels in CP and AgP groups were significantly higher than the H group and there was no significant difference between the CP and AgP groups. Moreover, a positive correlation was detected between the TNF-α level and the clinical periodontal parameters (Kurtis et al., 2005). Almost similar findings were

reached by Gokul (2012) in which they conducted their study on H, G and CP groups. Their results showed that the TNF-α level in CP and G groups were significantly higher than the H group; and also, there was no difference in TNF-α level between the G and CP groups.

5.7.2.5 Matrix metalloproteinase-8 (MMP-8)

Gupta et al. (2016) reported the importance of MMP-8 (collagenase-2) as a key biomarker in periodontal disease. It has a specific capability to precisely measure the prevalence of active periodontal disease (Janska et al., 2016; Xu et al., 2008).

Our results showed that the GCF levels of MMP-8 increased with the severity of periodontal disease. The MMP-8 levels were significantly higher in the CP group compared to the H and G groups. Simultaneously, a non-significant difference was reported between the H and G groups although the mean values of the MMP-8 in the G group were higher than the H group. Moreover, there was a positive correlation between the GCF MMP-8 levels and the clinical periodontal parameters (PS, BOP, PD and CAL).

The significantly lower concentrations of MMP-8 in healthy subjects compared to chronic periodontitis patients observed in this study are consistent with other studies (Konopka et al., 2012; Rai et al., 2010; Teles et al., 2010). These studies only included H and CP groups in their study samples. Mantyla et al. (2003) investigated the MMP-8 levels in GCF among H, G and CP groups using the MMP-8 chair side test and they showed that the MMP-8 levels were significantly higher in CP groups compared to H and G groups.

On the other hand, Teles et al. (2010) found that the MMP-8 levels were positively correlated to the recession, BOP and gingival redness; and such correlation was absent between the MMP-8 levels, PD and PI.

Our study's results did not conform to a study by Yakob et al. (2013), in which they recorded a non-significant difference in the MMP-8 levels between healthy and diseased groups. This may be attributed to the difference in the MMP-8 analysis method. They used time-resolved immunofluorometric assay to measure MMP-8 levels while the ELISA technique was used in our study.

5.7.2.6 Matrix metalloproteinase-9 (MMP-9)

MMP-9 (gelatinase B) is the main gelatinase in oral biofluids (Westerlund et al., 1996). The major source of MMP-9 is neutrophils. Nonetheless, it is also secreted by other cells such as epithelial cells, fibroblasts and monocyte. The levels of MMP-9 in the oral biofluids together with clinical periodontal parameters reflect the severity of periodontal disease (Sorsa et al., 2006).

The results of this study showed that the levels of MMP-9 in GCF were not significantly different between the H, G and CP groups. In addition, MMP-9 levels were not statistically correlated to any of the clinical periodontal parameters (PS, BOP, PD, and CAL). These results reinforce a previous study conducted by (Yakob et al., 2013), in which they did not find significant difference in the MMP-9 levels between H and CP groups.

Inversely, Rai et al. (2010) showed in their study that there was significant difference between the MMP-9 levels in the study groups; in which it was significantly higher in the CP group compared to H group. This may be contributed to the small

sample size that was included in their study; only 10 subjects were enrolled in each study group.

5.7.2.7 Osteocalcin (OC)

The origin of OC in GCF is unclear. It may be produced locally, either through the resorption of the alveolar bone or synthesised by local active osteoblast (Bullon et al., 2005). The presence of OC in the periodontium such as cementum, gingiva, periodontal ligament and alveolar bone has been proven. Meanwhile, OC may play a major role in osteoblast differentiation, cementogenesis and mineralization of bone (Ivanovski et al., 2001).

The mean value of the level of OC in GCF in this study was found to be higher in the CP group than the other study groups. However, no statistical difference was recorded between the three groups. Furthermore, a positive correlation was found between the OC levels and the clinical periodontal parameters (BOP, PD and CAL).

The findings of this study are consistent with these of (Nakashima et al., 1996). They measured the OC levels in diseased and non-diseased sites and their results showed no significant difference in the OC levels. This study‟s results are also in agreement with Bullon et al.(2005), in which a positive correlation between the OC levels and clinical periodontal parameters was recorded.

In other studies (Ahmed et al., 2016; Özçaka et al., 2011), the OC levels were found to be significantly higher in the periodontally diseased group than the healthy group. However, it is important to clarify that Ozcaka et al. (2011) measured the OC levels in saliva instead of GCF and the sample size was small (15 subjects) in the study of Ahmed et al. (2016).

In view of all previous results, Becerik et al. (2011) measured the levels of OC in four groups (H, G, CP and GAgP). Their results showed that the total amount of OC in CP and GAgP was less than in the other groups (H and G). They also indicated a negative correlation between the OC levels and the clinical periodontal parameters. They attributed this results to the abnormality of bone turnover that takes place during periodontal disease progression (Becerik et al., 2011).

5.7.2.8 Osteoprotegerin (OPG)

Although alveolar bone loss is one of the important signs of periodontal tissue breakdown (Cochran, 2008), the mechanisms that are responsible for bone resorption in periodontal disease are not fully understood. OPG has physiological function, in addition to increasing evidence on the essential role of OPG in human periodontal disease (Bostanci et al., 2011), that could be used for diagnostic or treatment purposes (Bartold et al., 2010).

In the present study, the mean value of the OPG levels in GCF was higher in the H group compared to the other study groups. However, the statistical analysis showed no significant difference in the OPG levels between the three groups (H, G and CP). Furthermore, no statistical correlation was found between the clinical periodontal parameters and OPG levels. The absence of significant differences in GCF levels of OPG between the three groups proposes that there is no significant alteration in OPG levels in periodontally diseased patients compared to periodontally healthy subjects (Baltacıoğlu et al., 2014).

The results from many studies (Baltacıoğlu et al., 2014; Bandari et al., 2012; Mogi et al., 2004; Tobón-Arroyave et al., 2012) are consistent with the present study findings.

Recently, Baltacioglu et al. (2014) measured the OPG levels in serum and GCF among three groups (H, CP and GAgP). GCF and serum OPG levels were lower in CP and GAgP than in controls. However, a negative correlation between the levels of OPG and clinical parameters was reported. Almost similar findings were reported by Bandari et al. (2012); that conducted their study of four groups (H, G, slight CP and moderate to severe CP) in order to detect the OPG levels in GCF. The highest mean value of OPG levels in GCF was obtained for the H group and the least was in the moderate to severe CP group, suggesting a negative correlation between CAL and OPG levels.

5.8 Correlation between Periodontal Pathogens and Oral Biomarkers

The role of oral pathogens in the etiology of periodontal disease is well-established and there is evidence for specificity amongst some bacterial species and the different forms of periodontal disease (Socransky & Haffajee, 2005). Although the diversity and complexity of the periodontal pathogens has been established by several studies (Park et al., 2015; Socransky & Haffajee, 2005; Vengerfeldt et al., 2014; You et al., 2013), currently only three bacterial types have been documented as real periodontal pathogens namely *A. actinomycetemcomitans, P. gingivalis* and *T. forsythia* (Oliveira et al., 2016).

Interestingly, there was a correlation between the subgingival plaque bacterial species and the oral biomarkers in the GCF, which revealed that the composition of the subgingival pathogens is obviously related to GCF biomarkers expression.

Since this present study was cross sectional, it is still too early to draw inferences on cause–effect relationships. However, this study‟s data are in consensus with the concept of „reciprocal interaction‟, which was described by Socransky & Haffajee (2005). Conferring to their theory, gingival inflammation occurs as a result of the early

colonising bacteria during microbial progression. In turn, the local environment will change when inflammation increases. This leads to increase in the colonising species and foster the development of additional pathogenic bacteria, causing tissue destruction and more inflammation.

Many cytokines are secreted in the periodontal connective tissue during the beginning of the inflammatory response such as IL-6, TNF-α and IL-1β, from the junctional epithelia cells, macrophages, neutrophils and connective tissue fibroblasts. Furthermore, many enzymes, such as calprotectin, MMP-9 and MMP-8, are formed by polymorph nuclear and osteoclasts, causing the degradation of alveolar bone and connective tissue collagen. During inflammation of the connective tissue and the resorption of bone, bone resorptive proteins and cytokines move into the gingival sulcus or periodontal pocket and then into GCF (Ramseier et al., 2009).

5.9 Cumulative Risk Scores (CRS)

One of the main aims of this study was to establish diagnostic criteria based on the clinical, microbiological and oral biomarkers findings; and then to evaluate the accuracy of this new diagnostic method in early prediction and diagnosis of periodontal disease. In order to achieve this, a new statistical method was used (Gursoy et al., 2011; Salminen et al., 2014). In this method, the GCF concentrations of the three selected oral biomarkers (MMP-8, IL-1β and IL-6) have been combined to obtain a cumulative risk score (CRS).

To the best of our knowledge, this is the first study that has used CRS to diagnose and predict periodontal disease depending on three oral biomarkers (MMP-8, IL-1β and IL-6) that were derived from GCF. In previous studies (Gursoy et al., 2011; Salminen et

al., 2014), they used the salivary MMP-8, IL-1β and *P. gingivalis* as biomarkers to diagnose periodontitis.

Several studies have suggested that IL-1β levels can be used as a good biomarker to differentiate between healthy and chronic periodontitis sites (Becerik et al., 2012; Chaudhari et al., 2011; Ertugrul et al., 2013; Fujita et al., 2012; Teles et al., 2010). They can also be used to differentiate between healthy subjects and patients with AgP (Becerik et al., 2012; Ertugrul et al., 2013; Oliveira et al., 2012; Teles et al., 2010). Owing to the slight differences in IL-1β levels between healthy and gingivitis sites (Becerik et al., 2012; Ertugrul et al., 2013; Ülker et al., 2008), it is difficult to use them as indicators or predictors for disease initiation from healthy status to gingivitis.

Much greater levels of MMP-8 in GCF have been observed in periodontitis patients than in healthy subjects (Figueredo et al., 2004; Kinane et al., 2003; Konopka et al., 2012; Mäntylä et al., 2003; Rai et al., 2010; Teles et al., 2010). This variation in MMP-8 levels can serve as an indicator for periodontal disease development. Furthermore, Leppilahti et al. (2014b) found that the levels of MMP-8 in GCF at baseline can predict the behaviour of MMP-8 levels during the phase of maintenance.

For CRS calculation, the concentrations of MMP-8, IL-1β and IL-6 in GCF were selected instead of the other biomarkers due to: a) their high prevalence in the study population, b) their levels in GCF showing a significant difference between the CP group and the other study groups, c) these three biomarkers showed statistical positive correlation to CAL and PD, d) they are positively correlated to each other. The reasons that led to the exclusion of the other biomarkers were: a) there was a difference in the biomarker levels between the different ethnic groups such as LL-37, b) the levels of the biomarker did not show statistical difference between the CP groups and the other study

groups such as TNF-α, MMP-9, OC and OPG, c) the biomarker levels did not statistically correlate with the other biomarkers such as PGE_2.

However, the periodontal pathogens in this study were also proven to be excellent biomarkers for periodontal disease, especially the red complex species which they showed a high prevalence in the study population. In addition to that there was a highly significant difference in the count of *P. gingivalis, T. denticola and T. forsythia* in the CP groups compared to the H and G groups. Furthermore, these bacterial species were statistically correlated with CAL and PD.

The present study aimed to establish easy and convenient diagnostic criteria that could predict periodontal disease and serve as an adjunctive to traditional diagnostic procedures. This study only depended on the GCF biomarkers without the involvement of subgingival plaque microbial markers that will lead to the subgingival plaque collection and microbiological analysis, which were relatively considered complicated procedures.

The findings of this study showed that the levels of MMP-8, IL-1β and IL-6 in GCF are associated with increased periodontal pathogens count and different clinical periodontal parameters. The combination of the three GCF biomarkers in a single biomarkers package to establish the CRS index is more precise in the prediction and diagnosis of periodontal disease than the use of other biomarkers alone.

The odds ratios to detect the subjects that have a higher risk of developing periodontitis and the area under curve (AUC) values to determine the sensitivity for the three biomarkers separately and cumulatively as CRS to detect periodontal disease were measured. Each biomarker was associated with periodontitis at an altered level. This association increased significantly using CRS, which had 2 to 3 times higher odds ratios

than any single biomarkers. The increase in the diagnostic sensitivity was also confirmed in the receiver operating characteristics (ROC) analyses, in which the AUC value increased from 0.653 – 0.704 to 0.749. These findings are consistent with previous studies that were conducted (Gursoy et al., 2011; Salminen et al., 2014). The main difference between this study and the two previously mentioned studies are the selection of the biofluids from which the biomarkers were derived. The previous studies used salivary biomarkers instead of the GCF biomarkers used in this study. Furthermore, the former also included a microbiological biomarker (*P. gingivalis*) due to the possibility of measuring it together with other biomarkers in the same biofluids (saliva).

CHAPTER 6: CONCLUSION

6.1 Conclusions

1) CRS indicates that the combination of three GCF biomarkers in a single biomarker package rather than a single biomarker is more precise in the prediction and diagnosis of periodontal disease.

2) CRS provides a non-traumatic model for the differentiation of subjects with risk of developing periodontal disease and to evaluate their periodontal status, which is especially useful in large-scale population studies or investigations where traditional periodontal examination cannot be easily performed.

3) Red complex bacterial species count within the different Malaysian ethnic groups can be useful in the differentiation between the different stages of periodontal disease progression.

4) In this study the ethnic backgrounds of the subjects did not have clear and noticeable effect on periodontal pathogens and oral biomarkers expression in periodontal disease.

5) Increase levels of GCF biomarkers such as MMP-8, IL-1β, PGE2 and IL-6 are more associated with increased count of periodontal pathogens and different clinical periodontal parameters when compared to the other biomarkers.

6.2 Limitations of the study

1) Although the numbers of sites from which GCF and subgingival plaque samples were collected can be considered statistically sufficient to perform such kind of study, the number of subjects in each ethnic subgroup could have been higher to be more descriptive for each of the ethnic group.

2) In this clinical study, only four subgingival plaque bacterial species and nine GCF oral biomarkers were investigated to check their reliability in supporting the clinical diagnosis. This was mainly due to financial limitations and difficulties in obtaining the samples especially from healthy subjects.

6.3 Recommendations

1) This study should be performed as an epidemiological study on a larger scale of the Malaysian population, despite the financial constrains which will be involved.

2) Additional oral biomarkers could be investigated in order to calculate their reliability in the diagnosis of periodontal disease.

3) Different laboratory analytical techniques could be used to determine the most accurate technique for determining the levels of oral pathogens and biomarkers for standardization purposes.

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