DETECTION OF SUBGINGIVAL PERIODONTO-PATHOGENS AND INFLAMMATORY CYTOKINES IN MALAYSIAN ADULTS WITH CHRONIC PERIODONTITIS

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE MASTER OF DENTAL SCIENCE

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ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Nursolehah Binti Shahruddin

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Title of Dissertation ("this Work"): Detection of subgingival periodonto-pathogens and inflammatory cytokines in Malaysian adults with chronic periodontitis.

Field of Study: Periodontology

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ABSTRACT

Detection of subgingival periodonto-pathogens and inflammatory cytokines in Malaysian adults with chronic periodontitis

Objectives: In periodontitis, the interaction between lipopolysaccharide from gram-negative bacteria with host cells initiates the secretion of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α). The aim of this study was to assess the frequency of detection of subgingival microbiota *Porphyromonas gingivalis* (*P. gingivalis), Tannerella forsythia (T. forsythia), Prevotella intermedia (P. intermedia)* and *Aggregatibacter actinomycetemcomitans (A. actinomycetemcomitans)* and the levels of IL-6, IL-17, resistin and TNF-α among selected population of Malaysian chronic periodontitis (CP) subjects and to assess their association with periodontal parameters.

Methods: A total of 167 CP and 134 healthy subjects were recruited. Visible plaque index (VPI), gingival bleeding index (GBI), probing pocket depths (PPD) and clinical attachment levels (CAL) were recorded. Subgingival plaque and blood were sampled from all subjects. Quantification of resistin, IL-17, IL-6 and TNF-α was performed by ELISA. Real time PCR (qPCR) was used to frequency of detection and quantitate of *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans.*

Results: Prevalence and mean counts of *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* were higher in CP subjects [(94.50%, 24.66×10^5 cells/µl), (92.10%, 7.70×10^5 cells/µl), (72.60%, 6.61×10^5 cells/µl), (7.30%, 0.14×10^5 cells/µl) respectively] (p <0.05) compared to healthy controls [(73.70%, 8.32×10^5 cells/µl), (62.00%, 4.75×10^5 cells/µl), (58.40%, 5.17×10^5 cells/µl), (not determined (ND)) respectively]. Similarly level of resistin, IL-17, IL-6 and TNF-α levels were higher in CP subjects [(100%, 274620 pg/ml),

(100%, 727.13pg/ml), (100%, 26.84pg/ml), (100%, 54.91pg/ml) respectively] (p <0.001) compared to healthy controls ((94.90%, 22.12pg/ml), (45.30%, 0.19pg/ml), (30.70%, 0.006pg/ml), (ND) respectively). CP was found as the only confounder that influenced all clinical parameters, periodontal pathogens and inflammatory mediators. Additional confounders were CAL for *P. gingivalis*, PPD for *T. forsythia* and, VPI and CAL for IL-17. In the CP group, there was a positive but weak correlation between *T. forsythia* and IL-17 (r=0.189, p=0.016) and *A. actinomycetemcomitans* with TNF-α (r=0.166, p<0.033). Healthy group showed no significant correlation between periodontal pathogens and all inflammatory mediators.

Conclusion: Adults with CP were found to have significantly higher prevalence and mean counts of *P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans* and inflammatory mediators (resistin, IL-17, IL-6 and TNF-α) as compared to healthy controls. *P. gingivalis* was found to be positively associated with mean CAL while IL-17 was negatively associated with mean CAL. *T. forsythia* was positively associated with mean PPD. There was a weak but significant correlation between *T. forsythia* with IL-17 and *A. actinomycetemcomitans* with TNF-α.

ABSTRAK

Pengesanan patogen periodontal subgingiva dan sitokin radang pada rakyat Malaysia dewasa dengan periodontitis kronik

Objektif: Dalam penyakit periodontitis, interaksi antara lipopolisakarida dari bakteria gram negatif dengan sel-sel perumah dimulakan dengan rembesan sitokin seperti *interleukin*-1, (IL-1), *interleukin*-6 (IL-6) dan faktor nekrosis tumor-α (TNF-α). Tujuan kajian ini adalah untuk menilai tahap kekerapan mikrobiota subgingiva *Porphyromonas gingivalis* (*P. gingivalis*), *Tannerella forsythia* (*T. forsythia*), *Prevotella intermedia* (*P. intermedia*) dan *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) dan tahap IL-6, IL-17, resistin dan TNF-α dalam populasi terpilih periodontitis kronik di Malaysia dan menilai hubungan mereka dengan parameter periodontal.

Kaedah: Sebanyak 167 subjek CP dan 134 subjek yang sihat telah direkrut. Indeks plak yang kelihatan (VPI), indeks pendarahan gingiva (GBI), kedalaman poket periodontal (PPD) dan paras atakmen klinikal (CAL) telah direkodkan. Plak subgingiva dan sampel darah diambil dari semua subjek. Analisis paras mediator keradangan resistin, IL-17, IL-6 dan TNF-α di lakukan menggunakan teknik ELISA. *Real Time*-PCR (qPCR) digunakan untuk menentukan kekerapan dan kuantiti *P. gingivalis*, *T. forsythia*, *P. intermedia* dan *A. actinomycetemcomitans*.

Keputusan: Tahap kekerapan dan purata kuantiti *P. gingivalis, T. forsythia, P. intermedia* dan *A. actinomycetemcomitans* lebih tinggi dalam subjek CP [(94.50%, 24.66×10^5sel/µl), (92.10%, 7.70×10^5sel/µl), (72.60%, 6.61×10^5sel/µl), (7.30%, 0.14×10^5sel/µl)] (p <0.05) berbanding dengan subjek sihat [(73.70%, 8.32×10^5 cells/µl), (62.00%, 4.75×10^5 cells/µl), (58.40%, 5.17×10^5 cells/µl), (tidak di kesan (ND) masing-masing]. Begitu juga paras

mediator keradangan lebih tinggi dalam subjek CP (100%, 274620pg/ml), (100%, 727.13pg/ml), (100%, 26.84pg/ml), (100%, 54.91pg/ml) berbanding subjek sihat [(94.90%, 22.12pg/ml), (45.30%, 0.19pg/ml), (30.70%, 0.006pg/ml), (ND) masing-masing]. CP didapati sebagai satu-satunya faktor pengasas yang mempengaruhi semua parameter klinikal dan mediator keradangan. Tambahan faktor pengasas untuk *P. gingivalis* ialah CAL, untuk *T. forsythia* adalah PPD, untuk IL-17 adalah VPI dan CAL. Dalam kumpulan CP, terdapat hubungan positif yang lemah antara *T. forsythia* dengan IL-17 (r=0.189, *p*=0.016) dan *A. actinomycetemcomitans* dengan TNF-α (r=0.166, *p*=0.033). Kumpulan yang sihat tidak menunjukkan korelasi yang signifikan antara patogen periodontal dengan semua mediator keradangan.

Kesimpulan: Individu dewasa dengan CP secara signifikan menunjukkan kekerapan dan jumlah min bagi *P. gingivalis, T. forsythia, P. intermedia* dan *A. actinomycetemcomitans* serta mediator keradangan (resistin, IL-17, IL-6 dan TNF-α) yang lebih tinggi berbanding individu sihat. *P. gingivalis* mempunyai kaitan positif dengan min CAL sementara IL-17 mempunyai kaitan negatif dengan min CAL. *T. forsythia* mempunyai kaitan positif dengan min PPD. Terdapat korelasi yang lemah tetapi signifikan di antara *T. forsythia* dengan IL-17 dan *A. actinomycetemcomitans* dengan TNF-α.

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LIST OF SYMBOLS AND ABBREVIATIONS

AAP	:	The American Academy of Periodontology
ATCC	:	American Type Culture Collection
BF	:	Bunch of fimbriae
CAL	:	Clinical attachment loss
CDC	:	Centre for Disease Control
CP	:	Chronic periodontitis
DEF1	:	First definition
DEF2	:	Second definition
DEF3	:	Third definition
DNA	:	Deoxyribonucleic acid
ELISA	:	Enzyme-linked immunosorbent assay
GBI	:	Gingival bleeding index
GCF	:	Gingival crevicular fluid
GF	:	Gingival fibroblasts
GI	:	Gingival index
HGF	:	Human growth factor
IL	:	Interleukin
LPS	:	Lipopolysaccharide
LtxA	:	Leukotoxin A
MMPs	:	Matrix metalloproteinases
MPDBS	:	Malaysian Periodontal Database and Biobank System
NOHSA	:	National Oral Health Survey of Adults

OLP	:	Oral lichen planus
PBS	:	Phosphate Buffer Solution
PCR	:	Polymerase chain reaction technique
PDLFs	:	Periodontal ligament fibroblasts
PMN	:	Polymorpho-nuclear leukocytes
PPD	:	Periodontal pocket depth
PrtH	:	Trypsin-like protease
qPCR	:	Real time PCR
PSD	:	Polymicrobial synergy and dysbiosis
RELMs	:	Resistin-like molecules
RIA	:	Radioimmunoassay
RNA	:	Ribonucleic acid
SD	:	Standard deviation
Th17	:	T-helper 17
TIMPs	:	Tissue inhibitors of metalloproteinases
TNF-α	:	Tumour necrosis factor-α
UiTM	:	Universiti Teknologi Mara
USA	:	United State of America
VPI	:	Visible plaque index
WHO	:	World Health Organisation

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of study

Chronic gingivitis and periodontitis are the two main forms of periodontal diseases (Taylor & Borgnakke, 2008). It is the outcome of inflammatory reactions caused by bacteria residing in the subgingival biofilm (Haffajee et al., 2006). Although it is known that plaque is the main aetiological agent for periodontal disease, however it is the host immune response initiated by this infection which has been regarded as the main mechanism for periodontal ligament and alveolar bone destruction (Bartold & Van Dyke, 2013; Gemmell et al., 1997; Page & Kornman, 1997). Factors such as smoking, diabetes mellitus, stress and obesity are modifying risk factors which determine the progression of the disease (Grossi et al., 1994; McKaig et al., 2000; Ng & Keung Leung, 2006). Thus, periodontal disease is also known as a multifactorial inflammatory disease.

It is well understood that periodontitis is initiated by the imbalance in the composition of normal flora (indigenous bacteria) of the mouth. Various bacterial hypotheses have been proposed for initiating and sustaining the progression of periodontal disease. These range from the specific to non-specific plaque theories (Loe et al., 1965; Theilade et al., 1966), ecological plaque hypothesis (Marsh, 2003) and more recently, 'polymicrobial synergy and dysbiosis (PSD)' model (Hajishengallis & Lamont, 2012b) theory. Most theories adopted plaque as the main role for disease initiation. As a result, a number of periodontal pathogens have been identified and termed putative periodontal pathogens. These pathogens include *Porphyromonas gingivalis* (*P. gingivalis*), *Tanarella forsythia* (*T. forsythia), Prevotella intermedia* (*P. intermedia*), *Treponema denticola* (*T. denticola*) and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*).

The microbiological profile of subgingival plaque of chronic periodontitis patients has been the subject of interest in many studies. Low levels of bacteremia and endotoxins derived from Gram-negative microorganisms and other bacterial components provides a stimulus for the systemic inflammatory response (Williams & Offenbacher, 2000). Periodontal breakdown occurs when bacterial load within a periodontal pocket overwhelms the local and systemic host defence mechanisms (Quirynen et al., 2005).

In periodontitis, the interaction of lipopolysaccharide (LPS) from Gram-negative bacteria with host cells initiates the secretion of cytokines such as Tumour necrosis factor-α (TNF-α), Interleukin-1, (IL-1) and Interleukin-6 (IL-6) and these cytokines were found to be systemically elevated during the inflammatory process in the periodontium (Page & Kornman, 1997). The expression of cytokines in systemic inflammation favours the cell adhesion molecules in gingival tissues, leading to loss of alveolar bone and loss connective tissues supporting the teeth. The altered inflammatory and immune responses at systemic and/or periodontal levels could be the possible trigger for chronic periodontitis progression. Likewise, chronic periodontitis may also unfavourably affect the systemic levels of cytokines in favour of pro-inflammation (Zimmerman, Peters, Altaras, & Berg, 2013).

1.2 Significance of study

The presence and levels of specific periodontal pathogens play an important role in disease initiation which produces inflammatory cytokines which causes disease progression if untreated. This results in an increase in the subgingival periodonto-pathogenic microbiota due to the changes in the environment of the periodontal pocket. Many studies have been conducted to reveal such a relationship. Studies comparing the prevalence as well as frequency of detection of pathogenic species between healthy and non-healthy sites and their

association with inflammatory cytokines have been conducted with varying results. Recently, Interleukin-17 (IL-17), a proinflammatory cytokine and resistin which induces expression of proinflammatory cytokines have also been implicated in this association. Thus, this study was initiated to investigate the relationship between the frequency of detection of subgingival microbiota *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* and the production of cytokines such as IL-6, IL-17, Resistin and TNF-α in a selected population of chronic periodontitis subjects in Malaysia.

1.3 Aim of the study

To detect the levels of periodonto-pathogenic species namely *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* and the serum levels of IL-6, IL-17, resistin and TNF-α in adults with moderate to severe chronic periodontitis and to assess the correlation between quantity of periodonto-pathogens and serum levels of cytokines in these subjects.

1.4 Objectives of the study

1. To determine and compare the prevalence and level of periodonto-pathogenic species (*P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans*) between adults Malaysian population with moderate to severe chronic periodontitis and healthy periodontium.
2. To determine and compare the serum levels of IL-6, IL-17, resistin and TNF-α between adults Malaysian population with moderate to severe chronic periodontitis and healthy periodontium.
3. To investigate the association between serum levels of inflammatory mediators and periodonto-pathogens with periodontal parameters (visible plaque index (VPI), gingival bleeding index (GBI), periodontal pocket depth (PPD), clinical attachment loss (CAL)).
4. To investigate the correlation between level of periodonto-pathogens and serum levels of cytokines in the adult Malaysian population with moderate to severe chronic periodontitis.

1.5 Hypothesis

1.5.1 Null Hypothesis

1. There is no difference in the presence and level of periodonto-pathogenic species (*P. gingivalis, T. forsythia, P. intermedia* and *A. actinomycetemcomitans*) between adults Malaysian population with moderate to severe chronic periodontitis and healthy periodontium.

2. There is no difference in the serum levels of IL-6, IL-17, resistin and TNF-α between adults Malaysian population with moderate to severe chronic periodontitis and healthy periodontium.
3. There is no association between the level of periodonto-pathogenic species and the serum levels of inflammatory mediators with clinical periodontal parameters.
4. There is no correlation between level of periodonto-pathogens and serum levels of cytokines in the adult Malaysian population with moderate to severe chronic periodontitis.

CHAPTER 2: LITERATURE REVIEW

2.1 Periodontal Disease

Periodontal disease is a chronic inflammatory disease affecting the tooth supporting structures which comprises the gingiva, cementum, alveolar bone and periodontal ligament. Two major forms of the disease are chronic gingivitis and periodontitis. Chronic gingivitis occurs when the disease is confined to the gingiva. Periodontitis results from the apical extension of gingival inflammation to involve the supporting tissues resulting in loss of alveolar bone, gingival swelling, periodontal pocket formation and movement of teeth (Vanarsdall, Blasi, & Secchi, 1994). Destruction of the periodontal supporting tissues results in periodontal pocket formation.

2.1.1 Classification of Periodontal Disease

Periodontal disease is thought to comprise a family of related but distinct diseases that differ in precise aetiology, natural history and response to therapy (Tylor & Borgnakke, 2008). The clinicians realize the importance of a systematic classification of periodontal disease as it allows them to provide effective treatment.

Over the years, classifications of periodontal disease were made according to clinical characteristics and etiology of the disease. The American Academy of Periodontology (AAP) classification in 1999 (Armitage, 1999) proposed the new classification based on principles of basic pathology (Wiebe & Putnins, 2000).

Overall, this classification has been well accepted. The 1999 classification was proposed due to weaknesses in the previous systems as the diagnosis criteria remained unclear, disease categories overlapped as well as an emphasis on the age of disease onset and rate of progression. The 1999 classification is summarized in Table 2.1.

Table 2.1: Abbreviated version of the 1999 classification of periodontal diseases and conditions (Armitage, 1999)

I. Gingival Diseases
A. Dental plaque-induced gingival diseases
B. Non-plaque-induced gingival lesions
II. Chronic Periodontitis
A. Localized
B. Generalized
III. Aggressive Periodontitis
A. Localized
B. Generalized
IV. Periodontitis as a Manifestation of Systemic Diseases
V. Necrotizing Periodontal Diseases
VI. Abscesses of the Periodontium
VII. Periodontitis Associated With Endodontic Lesions
VIII. Developmental or Acquired Deformities and Conditions

AAP had announced modification and updating of the 1999 classification. Discussions on this commenced in 2017 (American Academy of Periodontics Dentistry, 2014) and was published in 2018. Concerns for the update of the 1999 Classification focused on three specific areas (i) attachment level, (ii) chronic versus aggressive periodontitis and (iii)

localized and generalized periodontitis. The new classification by American Academy of Periodontics Dentistry, 2014, has reclassified periodontitis as follows:

1. *necrotizing periodontitis*,
2. *periodontitis as a manifestation of systemic disease*,
3. "chronic" or "aggressive", now grouped under a single category, "periodontitis".

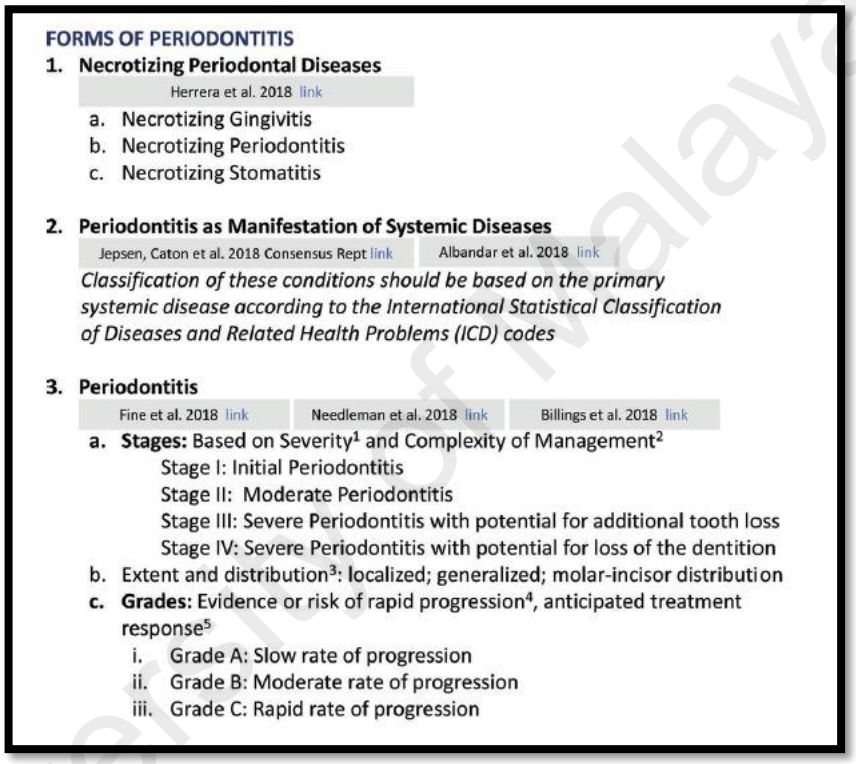

Figure 2.1: The new classification by American Academy of Periodontics Dentistry, 2014, by Caton et al., 2018.

2.1.2 Case Definition

A case definition is a set of criteria in order to determine conditions present in a population for the purposes of analysis based on precise and accurate counts. Moreover, it may be convenient to frame a set of rules that would be able to distinguish both doubtful and confirmed cases by including the incidence of cases, distribution of frequency of the number of cases and so on.

In most studies, the case definitions were intended to be used as standardization of diagnostic criteria with a view to clinical intervention, to ensure the case definitions had accuracy, sensitivity and specificity (Whittington et al., 2017). For example, Ioannidou et al. (2010), defined chronic periodontitis using three different definitions:

1. First definition (DEF1) - Chronic periodontitis was defined as two or more interproximal sites with CAL≥4mm (not on the same tooth) or two or more interproximal sites with PD≥5mm (not on the same tooth).
2. Second definition (DEF2) - PD≥5 or CAL ≥ 4 in at least 6 proximal sites.
3. Third definition (DEF3) - PD≥5 or CAL ≥ 4 in at least 2 proximal sites in each quadrant.

Both DEF2 and DEF3 were arbitrary definitions with clinical cut off thresholds representing an escalating extent of periodontal destruction compared to DEF1 (Centre for Disease Control (CDC)/AAP definition).

In this context, the standard case definition of periodontal disease is necessary for use in epidemiology and clinical based studies. The different case definitions used will gives impact on the published data of periodontal disease across worldwide populations (Costa & Ceser, 2009). Hence, Page and Eke (2007) proposed a CDC/AAP case definition for moderate and severe chronic periodontitis based on clinical diagnosis of periodontitis for population based surveillance. In 2012, this case definition was updated with the addition of mild chronic periodontitis. The details of the case definition are shown in Table 2.2.

Table 2.2: Case definition of periodontitis (Page and Eke, 2007**;** Eke, 2012)

Case Definition	Descriptions
Severe periodontitis	• 2 or more interproximal sites with ≥ 6 mm attachment loss (not on same tooth) And • 1 or more interproximal site(s) with ≥ 5 mm pocket depth
Moderate periodontitis	• 2 or more interproximal sites ≥ with 4 mm attachment loss (not on same tooth) Or • 2 or more interproximal sites with ≥ 5 mm pocket depth (not on same tooth)
Mild periodontitis	• 2 or more interproximal sites with ≥ 3 mm attachment loss And • 2 or more interproximal sites with ≥ 4 mm pocket depth (not on same tooth) Or • 1 site with ≥ 5 mm

2.1.3 Epidemiology of Periodontal Disease

Periodontal disease, which comprises both gingivitis and periodontitis is one of the most common disease to affect oral health as up to 90% of the global adult population has been reported to have some form of the disease (Taylor & Borgnakke, 2008). Periodontitis resulting in loss of alveolar bone, gingival swelling and movement of teeth was reported to affect up to 20-50% of the world population (Nazir, 2017).

According to Eke et al., (2012), over 47% of adult population in United State of America (USA) had periodontitis. Based on severity of disease, the numbers were distributed as 8.7%, 30.0%, and 8.5% with mild, moderate, and severe periodontitis, respectively. It was also reported that periodontitis was highest amongst men, Mexican-American ethnicity group, low education level and current smokers. This condition provides direct evidence for a high burden of periodontitis on health and wellbeing in the American population.

Countries such as China and Europe have reported that periodontitis affected more than half of the adult population (Patel et al., 2012; Eke & Dye, 2009; Hu, Hong, & Li, 2011; Holtfreter, Kocher, Hoffmann, Desvarieux, & Micheelis, 2010).

The prevalence of periodontitis is even higher in the elderly population. Reports have shown that 70–90% of Europeans aged between 60 and 74 years suffer from periodontal disease (Holtfreter et al., 2010; Mattila, Niskanen, Nordblad, & Knuutile, 2010).

In a study done by Corbet, (2006), it was shown that Asians were more prone to suffer from periodontitis as compared to Caucasians. In Malaysia, several oral health surveys in adults were carried out from 1990s till present. The National Oral Health Survey for Adults (NOHSA) (2010) has reported an upward trend in the periodontal status amongst Malaysians from 1990 till 2010. Prevalence of periodontal disease in 1990 was 92.8% and has risen to 94% in the 2010 survey. Meanwhile, prevalence of severe chronic periodontitis has also shown an increment from 6.0% in 1990 to 18.2% in 2010.

A study investigating the oral health of elderly in East Malaysia estimated approximately 98.4% subjects were affected with chronic periodontitis (Tuti Ningseh et al., 2013). The highest proportion of chronic periodontitis was amongst male gender (94.2%), aged 50-59 years (96.9%) and 36.8% subjects with moderate pocket depths (4-5 mm).

2.1.4 Aetiology

The search for aetiological agents of periodontal diseases has been conducted by many investigators as early as 1880-1920 during the early golden era of medical bacteriology (Feres, Cortelli, Figueiredo, Haffajee, & Socransky, 2004). However, following the experimental gingivitis study done by Loe et al. (1965) it has been accepted that dental plaque is the main aetiology of the disease. The accumulation of dental plaque causes gingivitis and reinstitution of oral hygiene can reverse gingivitis into healthy periodontal condition. Subsequently, it was also found that the degree of tissue damage is dependent on the interaction between plaque bacteria and host defence mechanisms which is determined by disease susceptibility (Loesche, 1996). Local environmental factors may also favour the accumulation of bacterial plaque.

Various bacterial hypotheses have been proposed for the initiation and progression of periodontal disease.

2.1.4.1 Specific plaque hypothesis

The specific plaque hypothesis proposed by Loesche (1976) stated that periodontal disease was caused by only a few species of the total microflora. It was the preferred opinion at that time. Basically, the hypothesis focused at the plaque establishment in periodontitis by taking into consideration the relationship between periodonto-pathogens and its virulence, environmental aspects, structure of plaque biofilm and the host response. The basic argument

was that there were some specific pathogenic bacteria in the biofilm which were responsible for disease even though the oral environment was healthy (Loesche, 1976).

Hence, this hypothesis also proposed that antibiotics would be best to treat specific bacterial species and prevent periodontitis (Loesche & Nafe, 1973 ; Loesche, 1976 ; Loesche, Bradbury, & Woolfolk, 1977). Later findings from clinical studies, were not very promising as they did not support this hypothesis. For example, a study by Banas (2009), found the failure in the function of kanamycin to penetrate certain bacterial wall allowing accumulation of more virulent periodonto-pathogen species. It also stated that antibiotics was able to reduced but not eliminate the periodonto-pathogens and in certain cases, their numbers increased immediately after treatment was stopped (Loesche & Nafe, 1973; Loesche, Bradbury, & Woolfolk, 1977).

2.1.4.2 Non-specific plaque hypothesis

The non-specific plaque hypothesis proposed by Theilade (1986) stated that inflammation which was caused by accumulation of dental biofilm at or below the gingival margin would then lead to periodontal tissue destruction. The destruction process could be triggered by sufficiently large numbers of bacteria present without discriminating the level of virulence of bacteria (Lindhe et al., 1992). This hypothesis was proposed after they realised that even in the absence of specific bacteria, disease still occurred.

The host would have a threshold capacity to fight this disease (Theilade, 1986). If the host was no longer able to detoxify the bacterial products present in the pockets, then virulence factors from periodonto-pathogen would no longer be neutralized. However, this hypothesis was also disputed as they found that certain periodonto-pathogens such as *A. actinomycetemcomitans* were specific and predominant in aggressive periodontitis (Theilade, 1986).

2.1.4.3 Ecological plaque hypothesis

Ecological plaque hypothesis was developed in 1994 by combining the earlier two hypotheses (Marsh, 1994). It is known that inflammation in periodontitis causes swelling, bleeding gums and increased pocket depths. In the ecological plaque hypothesis, it was suggested that changes in ecological factors caused by increasing pocket depths would cause an increase in the presence of essential cofactors and nutrients. This would then result in an imbalance in the microflora due to the ecological stress (Marsh 1994, Rosier et al., 2014). Ultimately, this would lead to progression of the disease.

2.1.4.4 Polymicrobial synergy and dysbiosis (PSD) model

Most recently, the polymicrobial synergy and dysbiosis (PSD) model was proposed in 2012 (Hajishengallis et al.,, 2012). Polymicrobial communities ensure opportunities for competitive and cooperative interspecies interactions. This hypothesis states that certain low-abundance microbial pathogens known as keystone-pathogens can initiate inflammatory disease. An important keystone pathogen, *P. gingivalis*, through its virulence factor gingipain has been clearly shown to cause periodontitis through dysbiosis of the symbiotic microbial community. However, the hypothesis believed that the inflammatory disease could be interfered by host response modifying factors (genetic/environmental) and remodelling of the microbiota by protective members of the microbiota (Hajishengallis et al.,, (2012); Hajishengallis & Moutsopoulos, (2016)).

2.1.5 Oral Microbiome

There are approximately 700 species of the oral microbiome found in the subgingival plaque (Aas et al., 2005). Although the subgingival plaque in periodontitis can act as a harbour for hundreds of bacterial species, however only a small number such as gram

negative spirochetes and anaerobic rods have been associated with the progression of periodontal disease (Özçaka et al., 2011b).

P. gingivalis, *P. intermedia* (Ioannou et al., 2009) and *A. actinomycetemcomitans* (Campus et al., 2005; Wang et al., 2010) have been found to be associated with pathogenesis of chronic periodontitis. Table 2.3 shows the common isolates, frequently constituting more than 2-3% and sometimes more than 50% of the cultivable subgingival flora, and possessing potent virulence factors.

Table 2.3: Common isolates from subgingival plaque

Bacteria	**Characteristics**		
Aggregatibacter actinomycetemcomitans	Gram –ve	Non-motile	Capnophilic rod
Eikenella corodens	Gram –ve	Non-motile	Capnophilic rod
Eubacterium species	Gram +ve	Non-motile	Anaerobic rod
Tannerella forsythia	Gram –ve	Non-motile	Anaerobic rod
Fusobacterium nucleatum	Gram –ve	Non-motile	Anaerobic rod
Porphyromonas gingivalis	Gram –ve	Non-motile	Anaerobic rod
Prevotella intermedia	Gram –ve	Non-motile	Anaerobic rod
Treponema sp.	Gram –ve	Motile	Anaerobic spirochaete
Peptostreptococcus micros	Gram +ve	Non-motile	Anaerobic coccus
Campylobacter rectus	Gram –ve	Motile	Anaerobic curved rod

The ability to evade host defences and survive in low-oxygen environment is the main reason for subgingival down-growth of periodontitis–associated bacteria in subgingival plaque (Loesche, 1996). Haffajee et al., 1998 have described the subgingival flora to comprise a loosely adherent component as well as a layer of tooth-attached plaque in direct

association with the pocket epithelium. The tooth-attached plaque comprises mainly of gram-positive cocci and rods while the loosely attached plaque consists predominantly of gram-negative organisms including motile forms. The relatively stagnant environment of the pocket will encourage the colonization of these bacteria to adhere readily to the tooth surface (Haffajee et al., 1998).

Motile organisms, are found in higher proportions within loosely adherent subgingival plaque than anywhere else in the mouth. A subgingival flora, once established, is protected both from the natural cleansing action of tongue, lips, cheeks and saliva, and from tooth brushing (Haffajee et al., 1998). Subgingival bacteria can also penetrate into the tissues where they control their own environment and can, to some extent, evade the inflammatory and immune defence mechanisms (Karpiński & Szkaradkiewicz, 2013; Kebschull & Papapanou, 2011).

Bacterial colonization of the subgingival plaque by certain species of bacteria can trigger the development of periodontitis (Haffajee & Socransky, 1994; Socransky & Haffajee, 1994). Each colonization species does not cause periodontal disease on its own, however it has been demonstrated that periodontal disease is more likely to occur as the result of an imbalanced interaction between the pathogenic and beneficial species in which the pathogenic species dominate (Roberts & Darveau, 2002; Socransky et al., 1988b).

2.1.5.1Microbial complexes

Socransky et al. (1998) performed a study to look at the association among bacterial species within the dental plaque. Over 13 000 subgingival plaque samples from 185 adults' subjects were examined to identify these specific microbial groups. The examination used community ordination techniques and cluster analysis to determine these groups and six closely associated groups of bacterial species were reported. These periodonto-pathogenic

bacterial groups were categorised by colour coding as shown in Figure 2.2. Their characterisation was based on their role in the development of plaque and the level of pathogenicity. These designed complexes were strongly related to the pocket depth and bleeding on probing (Dwyer & Socransky, 1968).

The most virulent complex; the red complex consists of three tightly related species: *T. forsythia, P. gingivalis* and *T. denticola.*

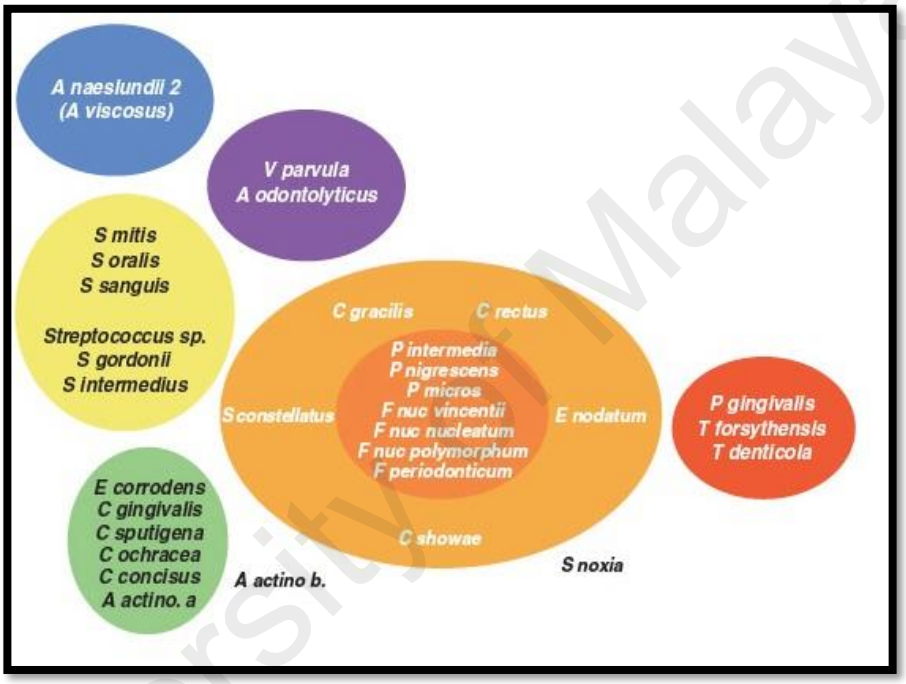

Figure 2.2 : Microbial complexes (adapted from Socransky et al., 1998)

The orange complex which includes species like *F. nucleatum, P. intermedia* and *C. rectus* were understood to precede the colonization by the red complex species. The yellow complex consists mainly of streptococcus species whereas the green complex exhibits species from *Capnocytophaga* sp. and *A. actinomycetemcomitans* serotype a. The last complex; the purple complex has contributions from *V. parvula and A. odontolyticus*. *A. actinomycetemcomitans* serotype b and *S. noxia* do not fall into any of these complexes as they are related more to cases of aggressive periodontitis. While species from the red and

orange complexes show significant association with each other, the species from yellow, green and purple complex also have demonstrated strong associations among themselves (Socransky, 1998). These associations may indicate possible pattern or sequence of colonization in the dental plaque (Bogren et al., 2004).

2.1.5.2*Porphyromonas gingivalis*

More than 40% of patients with periodontitis have been found to have *P. gingivalis* and it is a clinically important species in progression of the disease (Cutler et al., 1999). *P.gingivalis* has fimbriae with numerous adhesions that ensure adherence of the bacteria to the periodontal tissues. This also allows co-aggregation with other species and induces pro-inflammatory cytokine response. Cysteine proteinases (gingipains) are considered the marked virulence factor of the pathogen. These proteinases are highly responsible for the high proteolytic activity of the bacterium. Gingipains are divided into lysine-specific (known as lys-gingipain) and arginine-specific (called arg-gingipains) (RgpA and RgpB), according to substrate specificity (Curtis & Korach, 1999; Imamura et al., 2003; Lamont & Jenkinson, 1998; Potempa & Pike, 2009). Gingipains play a role in regulating the activity of cytokines in the immune system (Holt et al., 1999; Stathopoulou et al., 2009). Thus, *P. gingivalis* actively induces the production of pro-inflammatory cytokines TNF-α, IL-1, IL-6, and IL-8 by monocytes, neutrophils and macrophages. Previous studies have demonstrated that arg-gingipain destroys complement protein C3 related with opsonization, causing *P. gingivalis* to be resistant to phagocytosis by neutrophils. In turn, lys-gingipain degrades C5, releasing the C5a component and therefore stimulates inflammation (Holt & Ebersole, 2005; Holt et al., 1999; Rot & von Andrian, 2004; Stathopoulou et al., 2009). *P. gingivalis* also undergoes fermentation which produces end-products such as propionic acid, acetic acid and butyric acid, and volatile sulfur compounds. These end products are produced in large quantities

which affects the cytotoxicity of the host cells. Capsular polysaccharides are also produced by *P. gingivalis* strains. This results in reduced binding of *P. gingivalis* to polymorpho-nuclear leukocytes (PMN) and subsequently inhibits phagocytosis. In addition, lipopolysaccharide (LPS) present in the cell wall plays the role of the antigen and activates cytokines (Al-Shibani & Windsor, 2008; Jain & Stylianopoulos, 2010).

2.1.5.3 *Aggregatibacter actinomycetemcomitans*

A. actinomycetemcomitans has been found to be associated with periodontal disease (Li et al., 2010). Strains of this species are anaerobic and capnophilic with a microcapsule and bunch of fimbriae (BF) with the ability to adhere to the periodontal tissues and cause aggregation (Table 2.3). Strains of *A. actinomycetemcomitans* produce leukotoxin-A (LtxA). LtxA is cytotoxic to monocytes/macrophages and neutrophils which are able to damage cell membranes and by apoptosis leads to lysis of PMN, monocytes and T cells (Henderson et al., 2010; Jamieson et al., 2012; Kachlany, 2010). The second major toxin of *A. actinomycetemcomitans* is cytolethal distending toxin (CDT toxin) which induces apoptosis in cells by blocking the cell cycle in G2 phase in T cells. The development of periodontal diseases worsens when cells infected by *A. actinomycetemcomitans* undergo apoptosis (Saiki et al., 2004). Lipopolysaccharide (LPS) of *A. actinomycetemcomitans* can induce tolerant response in macrophages which secrete TNF-α, IL-1β and methylproteinase-9 enzyme which able to degrades tissues. This process is responsible in the modulation of the host inflammatory response and progression of periodontitis (Fives-Taylor et al., 1999).

2.1.5.4 *Tannerella forsythia*

Tannerella forsythia (*T. forsythia*) has an additional protective structure over the outer membrane surface layer (S-layer) formed by regularly arranged two protein subunits. The S-layer of *T. forsythia* strains may provide their adherence to host cells, and add to

invasiveness, as has been shown in experimental studies. Trypsin-like protease (PrtH) enzyme, and glycosidases which is an important factor for *T. forsythia* strains pathogenicity are also produced at the same time. The end products of the fermentation of *T. forsythia*, such as propionic acid, acetic acid and butyric acid can have a cytotoxic effect on host cells (Boutaga et al., 2006; Tanner & Izard, 2006).

2.1.5.5 *Prevotella intermedia*

Prevotella intermedia (*P. intermedia*) is characterized by hemolytic activity. This is due to proteases produced that are capable of destroying a number of proteins including collagen and fibronectin. It has been demonstrated that proteases possess trypsin-like properties characteristic for cysteine proteinases. These properties are capable of damaging antibodies, in particular IgG and fibrinogen which may reduce the effectiveness of the host immune and inflammatory defense (Mallorquí-Fernández et al., 2008; Takahashi, 2015). According to Mallorquí-Fernández et al. (2008), LPS produced by *Prevotella* sp. may participate in the periodontal destruction and alveolar bone loss through osteoclastogenesis stimulation, as well as reduction in bone formation.

2.1.6 Pathogenesis of Chronic Periodontitis

Pathogenesis of chronic periodontitis involves complex interactions between microbial factors and the susceptible host (Aguenda et al., 2008; Kazmierczak et al., 2005). Generally, both bacteria and host tissue will release their products which are responsible in bone resorption and destruction of connective tissue during inflammation and immune reaction in chronic periodontitis.

There are two immune mechanisms (i) innate immune mechanism (ii) adaptive immune mechanism involved in the pathogenesis of periodontal disease.

2.1.6.1 Innate Immune Mechanism

Innate immunity or also known as native immunity provides the beginning line of defence mechanism against microbes. This consists of cellular and biochemical defence mechanisms. These are in place even before an infection and are composed to respond rapidly to infections. They are specific for structures which are common to groups of related microbes and may not distinguish fine differences between microbes.

Plaque accumulation on a completely clean tooth stimulates the early inflammatory response in the gingival tissues after 0 to 4 days. Once it is fully developed, the subgingival environment alters to favour the growth of Gram-negatives bacteria. Gingival swelling and a slight deepening of the sulcus creates an area for Gram negative bacteria to be localized. The inflammatory changes cause the permeability of the junctional epithelium to increase. Thus, bacteria products may easily penetrate tissue resulting in further inflammation (Casanova, 2014). Hence, there is a relationship between subgingival biofilm and the host inflammatory response together with the development of chronic cycles of inflammation.

The bacterial components such as LPS activate the macrophages to produce cytokines such as TNF- α and IL-1 (Vogt et al., 2011). The activation of macrophages and fibroblasts by these cytokines in the periodontal tissues then produce matrix metalloproteinases (MMPs)which are plasminogen activators. The plasminogen is able to activate plasmin. Plasmin, in turn, then activates some other type of latent MMPs, while tissue inhibitors of metalloproteinases (TIMPs) can inactivate the active MMPs (Baskaradoss, Geevarghese, & Kutty, 2011). Among susceptible individuals, the prolonged and excessive bacterial promotions of the MMPs induces the primary component of the periodontal matrix by enhanced degradation of collagen. The polymorphonuclear leukocytes (PMN) releases MMP-8 and -9 and which is responsible for a substantial part of the destruction caused by

the host response. MMP-13 also facilitates bone resorption by degrading the collagenous matrix of the bone after bone is demineralized by osteoclasts (Collins et al., 1994). The increased plasma levels of MMP-8 and MMP-9 in chronic periodontitis patients emphasizes the importance of periodontal treatment to avoid elevated MMP-8 and -9 levels which are associated with many systemic diseases, particularly cardiovascular disorders (Collins et al., 1994).

2.1.6.2 Adaptive Immune Mechanism

Specific immunity or adaptive immune mechanism is where the host recognizes and reacts to a large number of groups of microbial or non-microbial substances known as antigens. This mechanism, known as specificity, has the ability to distinguish different substances (Casanova, 2014). Additionally, it also has the ability to respond more vigorously to repeated exposures to the same microbe. Lymphocytes and their antibodies are the unique components of adaptive immunity. The antigens induce specific immune responses or are recognized by lymphocytes or antibodies (Casanova, 2014).

As the inflammation persists, an acute state changes to a chronic state. At this stage additional cellular players become involved, notably B-cells, T-cells macrophages, mast cells, plasma cells, and eosinophils (Nagata, 1997). When the microbial accumulation is controlled or damaged, inflammation will subside, and tissue starts being repaired. Inflammation resolution requires leukocytes in the area of inflammation to allow cell death process (apoptosis), which will then be cleared from the area (without additional leukocytes being drawn to the region during the resolution process) (Van Dyke & Serhan, 2003). When there is no resolution, inflammation persists, and disease such as periodontitis occurs. However, it is well recognized that not all individuals are equally susceptible to develop

periodontal disease, or if they do it may be at different levels of severity (Van Dyke & Serhan, 2003).

2.2 Risk factors for periodontal Disease

According to World Health Organisation (WHO), risk factors are any characteristics, attribute or exposure of an individual that increases the likelihood of developing a disease or injury (WHO). In periodontitis, several risk factors have played a role in modifying the host response to the disease.

2.2.1 Tobacco smoking

Higher levels of periodontal disease have been reported among smokers (Graves & Welsh, 2004). Tobacco smoking causes a substantial destructive effect on the periodontal tissues which increases the progression rate of periodontal disease (Nishimura, Soga, Iwamoto, Kudo, & Murayama, 2005). This is due to the fact that tobacco smoking modifies the host response to the challenge of bacteria in microbial dental plaque (Stegeman, 2005). Nicotine causes local vasoconstriction, edema, reducing blood flow, and clinical signs of inflammation (Grossi & Genco, 1998; Pucher & Stewart, 2004). The disease progresses silently as gingival bleeding and clinical inflammation in smokers with periodontal disease was shown to be much less compared to non-smokers (Thorstensson & Hugoson, 1993). It has also been reported that nicotine acetylcholine receptor plays an important role in the development of nicotine related periodontitis (Fiorini et al., 2014).

2.2.2 Diabetes Mellitus

Patients with poorly controlled or undiagnosed type 1 or type 2 diabetes are at higher risk for periodontal disease (Buzinin et al., 2014). Studies have shown that there is an association between diabetes and an increased susceptibility to oral infections which includes

periodontal disease (Al-Mubarak et al., 2002; Janket, Wightman, Baird, Van Dyke, & Jones, 2005). This is due to impaired neutrophil function and poor healing in uncontrolled diabetics and leads to an increase in the progressesion of periodontitis (Perrino, 2007). Periodontal treatment however, has been shown to significantly improve glycemic control of diabetes patients (Taiyeb-Ali, Raman, & Vaithilingam, 2011).

2.2.3 Obesity

Obesity is a condition associated with storage of excess fat tissues and driven largely by other co-morbidities. Obesity is known to be associated with production of cytokines by adipose tissue which leads to a hyper–inflamed state (Devanoorkar, Dwarakanath, Gundanavar, Kathariya, & Patil, 2012). Obesity is involved in many metabolic and physiological pathways such as inflammation, vascular homeostasis and insulin sensitivity (Moreno-Aliaga, Santos, Marti, & Martínez, 2005). In dentistry, obesity has been associated with periodontal disease (Mathur, Shankarapillai, Manohar, & Pandya, 2011). It is hypothesized that obesity could contribute to the overall inflammatory burden, and subsequently alters the course of periodontal disease (Saito et al., 2005).

2.2.4 Stress

Stress is associated with poor oral hygiene, increased glucocorticoid secretions that depress the immune function, increased insulin resistance, and potentially increased risk of periodontitis (Baxter et al., 1987). Patients under stress are at higher risk to get severe periodontal disease (Chaffee & Weston, 2010; Pischon et al., 2007; Shimazaki et al., 2010; Suvan, D'Aiuto, Moles, Petrie, & Donos, 2011). Studies have demonstrated that some periodontal disease indicators such as gingival bleeding and tooth loss are associated with work stress and financial strains (Jeffcoat, 2005).

2.3 Inflammatory Molecules in Periodontal Tissue

Cytokines play an important role in the pathology associated with chronic inflammatory diseases. In periodontitis, patients with gingival inflammation are found associated with a high levels of inflammatory burden due to the large numbers of pro-inflammatory cytokines released (Cochran, 2008). These pro-inflammatory cytokines include interleukin-1a (IL-1a), IL-1b, tumour necrosis factor-a (TNF-a) and IL-6 (Cochran, 2008).

2.3.1 Interleukin-6

IL-6 is secreted by T cells and macrophages. Secretion of IL-6 is to stimulate immune response to trauma, especially burns or other tissue damage which leads to inflammation. IL-6 is a protein which is encoded by the "IL6" gene in humans and plays a key role in systemic inflammation (Fonseca et al., 2009). IL-6 is able to cause periodontal tissue destruction by stimulating bone resorption. It is produced when neutrophils enter the periodontal environment (Trevani et al., 2003).

IL-6, IL-1 and TNF-α are cytokines that have a wide variety of biological functions (Wei et al., 2011). They are potent inducers of the acute phase response. They act as a pro-inflammatory and also anti-inflammatory cytokines in periodontitis (Wei., 2011). The main cells of periodontal soft connective tissue are gingival fibroblast (GF) and periodontal ligament fibroblasts (PDLFs). They respond through the secretion of cytokines and degradation molecules. TNF-α and IL-6 produced by GFs are important regulators of bone metabolism and inflammatory process in periodontitis (Ekhlassi et al., 2008 ; Taguchi et al., 2015).

2.3.2 Interleukin-17

The IL-17 family consists of IL-17A until IL-17F (Gu et al., 2013). IL-17 commonly known as a pro-inflammatory cytokine and the prototype cytokine for a subset of T-helper (Th17) cells. However, recent study has demonstrated IL-17 subfamily have pro and anti-inflammatory properties for example IL-17A and IL-17E (Awang et al., 2014). IL-17 is responsible for stimulating a variety of cell types to produce inflammatory mediators such as TNF-α, IL-1, IL-6, metalloproteinases, and chemokines (Beklen et al., 2007). Cytokines of the Th17 lineage are thought to contribute to the pathology noted in a variety of inflammatory and autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, inflammatory bowel disease, asthma, ulcerative colitis and allergic diseases. Tesmer, Lundy, Sarkar, and Fox (2008) have reported that Th17 inflammatory responses are thought to provide protection against a variety of microbial infections.

There are many examples of the role of Th17 cytokines in protection against periodontal infections. For example, Yu et al. (2008) reported that IL-17 is protective against *Porphyromonas gingivalis*. *P. gingivalis* induced bone loss *via* mobilization of neutrophils in a murine model. However, clinical studies reported increased IL-17 levels associated with chronic periodontitis (Vernal et al., 2005; Ohyama et al., 2009; Takahashi, 2015). In addition, peripheral blood mononuclear cells in patients with gingivitis and periodontitis' stimulates the outer membrane protein of *P. gingivalis* which then express IL-17 (Oda et al., 2003). IL-17 mRNA was shown to be expressed in gingival tissue, with higher levels reported in diseased tissues (Cardoso et al., 2009; Honda et al., 2008).

In vitro, IL-17 stimulates human growth factor (HGF) to produce IL-6, IL-8, and MMPs (Beklen et al., 2007; Mahononda et al., 2008). Moreover, isolated CD4+ T-cell clones from gingival tissues of periodontitis patients have been found to have significant expression

of IL-17 (Ito et al., 2005). Murine models of periodontitis and other chronic diseases have demonstrated both protective aspects of Th17-induced immune responses due to neutrophil mobilization and destructive aspects due to lesion chronicity.

2.3.3 Resistin

Resistin is a member of a secretory protein family, known as resistin-like molecules (RELMs) (Holcomb et al., 2000). The family is characterized by a highly conserved, cysteine-rich C terminus (Holcomb et al., 2000). Resistin, RELMα, RELMβ and RELMg are the four members in the mouse RELMs family and only resistin and RELMβ are the two counterparts found in human (Patel et al., 2014).

Initially, resistin was suggested to contribute to insulin resistance. However, recent evidence indicated that it may also be involved in the inflammatory process (Akram et al., 2017). Resistin levels have been reported to be upregulated by pro-inflammatory agents (Kaser et al., 2003). Expression of resistin will only be stimulated by secretion of IL-1b, IL-6 and TNF-α (Kaser et al., 2003 ; Zhang et al., 2010). Plasma resistin levels were found associated with many inflammatory markers in some pathophysiological conditions (Stejskal et al., 2003) Saito et al., (2005) reported increased serum resistin levels in middle-aged Japanese women with periodontitis when compared with the healthy controls. Hiroshima et al. (2012) have reported the presence of resistin in gingival crevicular fluid (GCF) which was correlated with gingival index (GI) scores.

There are many studies conducted demonstrating the link between resistin to insulin resistance (Stejskal et al., 2003). In a study conducted by Piestrzeniewicz et al. (2008) blood resistin concentration was positively correlated to obesity in patients with acute myocardial infarction. Recently, studies have indicated the ability of resistin to promote the initiation or perpetuation of the atherosclerotic state by activating vascular endothelial cells.

2.3.4 Tumour Necrosis Factor-alpha

Tumor necrosis factor alpha (TNF-α) is also known as cachexin, or cachectin. It is a cell signalling protein (Yarilina et al., 2011). TNF-α is an adipokine that is produced by activated macrophage and involved in systemic inflammation (Yarilina et al., 2011). It is also one of the cytokines that makes up the acute phase reaction. However, it can also be produced by many other cell types such as CD4+ lymphocytes, neutrophils, NK cells, mast cells, neurons and eosinophils (Verma et al., 2003). The primary role of TNF-α is in the regulation of immune cells. TNF-α, being an endogenous pyrogen, is able to induce apoptotic cell death, cachexia, fever, inflammation and to inhibit tumorigenesis and viral replication and respond to sepsis via IL-1 & IL-6 producing cells (Locksley et al., 2001).

IL-1 and TNF-α are key regulators of the host responses to microbial infection (Graves & Cochran, 2003). These cytokines stimulate a number of events which occur during periodontal disease. Events include the induction of adhesion molecules and other mediators that facilitate and amplify the inflammatory response, bone resorption and the stimulation of matrix metalloproteinase (Graves & Cochran, 2003). Critical events of periodontitis namely, loss of attachment and bone resorption are connected with the activity of these cytokines (Yarilina et al., 2011).

2.4 Identification Techniques

2.4.1 Microbiological Identification Techniques

In the past, study of microbiological profile has traditionally been based on bacterial cultivation methods (Boutaga et al., 2003) which is the gold standard technique for microbial identification. This technique requires specific growth condition in terms of types of anaerobic environment, media used, optimum temperature and period of growth. The cultivation method is also widely used to determine the *in-vitro* antimicrobial susceptibility

of oral pathogens. Additional series of biochemical tests need to be performed in order to confirm the types of microorganism. However, these microbiological and biochemical techniques provide the results in a qualitative form. Thus, culture methods are more time consuming, laborious and limit the number of samples that can be evaluated (Boutaga et al., 2003).

It has been found that there is significant difference in microbiological profile of periodontitis and healthy adults by using culture technique (Renvert et al., 1990) and deoxyribonucleic acid (DNA)-DNA hybridization methods (Haffajee et al., 1997). Studies however showed that a significant portion of oral microflora may not grow under some experimental conditions (Weiger et al., 1992). This study also supports that culture method has low sensitivity when compared to other molecular biology methods.

Alternatively, polymerase chain reaction (PCR) technique has become one of the most frequently used techniques as it can detect cultivable and non -cultivable bacteria (Saez-Llorens & McCracken Jr., 2003). This technique also permits rapid assessment of the predominant bacterial species in complex samples such as saliva and plaque (Saez-Llorens & McCracken Jr., 2003). Another great advantage of this technique is that it does not require viable bacterial cells for detection (Li et al., 2010). However, PCR method does not allow quantification of the bacteria present (Nonnenmacher et al., 2004).

Real time PCR or quantitative PCR (qPCR) is one of the most popular techniques used to identify and quantify anaerobic bacteria with high sensitivity and specificity (Boutaga et al., 2006). It is better than conventional PCR based on the fact that it allows quantification of bacteria during the amplification cycle by detection of fluorescence produced by reporter molecules which increases as the reaction precede in real time (Thornton & Basu, 2011). In a comparison study done by (Soleimani et al., 2013), it was shown that qPCR had a tenfold increase in sensitivity of detection for *T. forsythia* and *A. actinomycetemcomitans* when

compared with PCR method. A comparison study was carried out by Boutaga et al. (2003) to identify the presence of *P. gingivalis* by using culture and qPCR methods. Results of positive sample detection of *P. gingivalis* using qPCR turned out to be negative detection when using culture method due to unculturable bacteria. Moreover, few samples which were initially undetected using culture method subsequently showed positive detection results after attempting the prolonged culture. This study showed the limitations of using culture method and the advantages of qPCR in terms of time efficiency and sensitivity.

Recently, Al-hebshi et al. (2014) have detected and quantified putative periodontal pathogens in Yemenis with chronic periodontitis by using TaqMan real-time PCR technology. They found the qPCR showed excellent linearity and sensitivity when all the tested bacteria were detected in 100% of the samples except for *P. gingivalis* (97.5%) and *A. actinomycetemcomitans* (67.5%). Another study carried out by Ismail et al., (2015) have demonstrated the similar method was used for identification of microbial analysis for 9 periodontal pathogens namely *A. actinomycetemcomitans*, *P. gingivalis*, *T. denticola*, *T. forsythia*, *P. intermedia*, *Peptostreptococcus micros* (*P. micros*), *Fusobacterium nucleatum* (*F. nucleatum*), *Eubacterium nodatum* (*E. nodatum*) and *Capnocytophaga gingivalis* (*C. gingivalis*) which show the capability of this method for bacterial identification.

2.4.2 Immunological Techniques

Various immunological techniques for detection of inflammatory mediators which are available and recently used in biochemistry are radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) and Luminex assay. RIA is a technique for detecting antigen and antibody while ELISA is a technique used to quantify the specific antibody and cytokines in serum. ELISA has also been known as a classic method to analyse cytokine expression

pattern. Luminex, which is a new improved technology to evaluate multiple immune mediators simultaneously has recently been introduced.

RIA and ELISA methods offer highly sensitive and specific analyte detection. However, compared with the RIA method, ELISA does not require radioisotopes (radioactive substance) and a radiation counting counter. However, the analysis using ELISA technique requires a long time to analyse multiple analytes separately. Alternatively, Luminex comes with several advantages such as requiring smaller sample volume, lower cost for the analysis with higher throughput and simultaneous analysis of multiple analytes (Dupont et al., 2005). However, this instrument is expensive to procure. Studies performed on validation and comparison of detection and quantification of IL-1β, IL-4, IL-5, IL-6, IL-10 and TNF-α using Luminex and ELISA showed excellent correlation (Dupont et al., 2005).

In the search for possible biomarkers for chronic periodontitis, previous studies have used Enzyme-linked immunosorbent assay (ELISA) in the measurement of the inflammatory mediators such as IL-6, IL-17, resistin and TNF-α levels in chronic periodontitis patients (Cui et al., 2014; Duarte et al., 2010; Honda et al., 2008; Kim et al., 2013; Özçaka et al., 2011b).

CHAPTER 3: METHODOLOGY

3.1 Study Design

This was a comparative cross-sectional study conducted at the Faculty of Dentistry, University Malaya. Clinical sessions and sample collection were done by previous calibrated clinicians. All the data and sample was managed under the Malaysian Periodontal Database and Biobank System (MPDBS) (Vaithilingam et al., 2015). Ethical approval for this study was obtained from the Medical Ethics Committee Faculty of Dentistry, University of Malaya (DF PE1103/0037(L)) (Appendix A).

3.2 Sample Size Calculation

The sample size calculation was performed by considering the detectable mean difference of at least 1mm of CAL between groups (Feres et al., 2009) in the deepest site (PPD $\geq$ 5 mm), the ideal sample size to achieve 80% power of study with an α of 0.05 is at least 150 subjects per group (Dupont & Plummer, 1990).

3.3 Sample Selection

Based on the inclusion and exclusion criteria, the samples were grouped into periodontally diseased (test) and healthy (control) groups. Those who fulfilled the inclusion criteria were invited to participate in this study. Written informed consent was obtained from each participant.

The inclusion and exclusion criteria for the samples were as follows:

(a) Inclusion Criteria

1. Patients who were Malaysians

2. Patients who were 35 years old and above

3. Patients who were diagnosed with moderate to advanced chronic periodontitis (CP group) had 5 or more pockets with PPD ≥ 5mm depth or CAL of ≥ 4 mm in at least 2 different quadrants which bled on probing. The healthy group comprised subjects with PPD < 4 mm.

4. Patients who had at least 12 teeth present excluding third molars.

(b) Exclusion Criteria

1. Patients who had periodontal treatment within the past 6 months

2. Patients who had taken antibiotics within the past 4 months

3. Patients who were pregnant or lactating mothers

3.4 Clinical Parameters

The data for the clinical parameters were obtained from the MPDBS database. The clinical parameter measurements for the database were performed by trained and calibrated clinicians. Intra- and inter-examiner calibration was achieved amongst all examiners with Kappa score of more than 80% (Vaithilingam et al., 2015). A full mouth periodontal examination was carried out on all teeth except third molars.

The periodontal clinical measurements included the following:

(a) Visible Plaque Index (VPI)

Assessment of VPI was using the dichotomous scoring system based on the presence of visible plaque on tooth surface or while scrapping with the probe. The visible detection of

plaque was marked as present (1) or absent (0) (Ainamo & Bay, 1975). Assessment was performed at 4 sites per tooth (Mesio-buccal, mid-buccal, disto-buccal and mid-palatal).

(b) Gingival Bleeding Index (GBI)

Assessment of GBI was using the dichotomous scoring system based on the presence of bleeding within 10 seconds after probing of the gingiva. The presence of bleeding was marked as present (1) or absent (0) (Ainamo & Bay, 1975). Assessment was performed at 4 sites per tooth (Mesio-buccal, mid-buccal, disto-buccal and mid-palatal).

(c) Periodontal Pocket Depth (PPD)

PPD is the measure of the distance from the gingival margin to the base of the pocket. The measurement was carried out using a William's probe (Hu-Friedy, Chicago USA) by placing the probe parallel to the long axis of the tooth. The PPD was assessed at 6 sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-palatal, mid-palatal and disto-palatal).

(d) Clinical Attachment Loss (CAL)

CAL was measured as the distance from the cemento-enamel junction to the base of the pocket. The CAL was assessed using PPD and recession measurements and was assessed at 6 sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-palatal, mid-palatal and disto-palatal).

3.5 Sample Collection

3.5.1 Blood Collection

Blood samples were collected for serum inflammatory cytokines analysis. A total of 10 ml of venous blood sample was collected from each participant using the butterfly syringe

and transferred into plain blood tubes (red lid) and kept at temperature <10°C.(either in an ice box or refrigerator). Within 24 hours after collection, the blood samples were centrifuged for 15 minutes at a speed of 15000 x g to aliquot the serum from other blood contents. Then, 1 ml serum was transferred into 1.5 ml Eppendorf tubes and stored at temperature of - 80^0C till serum inflammatory mediator analysis was performed.

3.5.2 Plaque Collection

Subgingival plaque samples were collected for microbiological profiling. Sample sites were isolated with cotton rolls, supragingival plaque was removed and finally subgingival plaque samples were collected using sterile Hu-Friedy curettes (Hu-Friedy Mfg LLC, Chicago). The sampling sites were pockets from the most diseased sites (PPD ≥ 5 mm) for the test group while for the control group it was collected from interdental sites.

The plaque samples were then pooled in sterile DNase-free and RNase-free polyethelene tube (Li et al., 2006) containing 1.5 ml of phosphate buffer solution (PBS) and stored at - 80^0C till microbiological analysis.

3.6 Identification of Serum Interleukin using Sandwich ELISA Technique

Serum interleukins (resistin, IL-17, IL-6 and TNF-α) were analysed using Sandwich ELISA test. The Sandwich ELISA protocol was followed as recommendations from the manufacturer. Each ELISA kit was provided with reagents and plates. The plates contain 96 wells which 8 wells for standard and the remaining 88 wells for serum samples and its duplicates.

3.6.1 Principle of Sandwich ELISA

Enzyme-linked Immunosorbent Assays (ELISA) is a technique for detecting and quantifying such as peptides, proteins, antibodies and hormones in blood that use plate based

assay. In this study the technique of sandwich ELISA was used. Briefly, sandwich ELISA technique involves an enzyme conjugated with an antibody and reacts with a colourless substrate called chromogenic substrate to produce a coloured product. In detail, the antibodies or antigen present in serum is captured by corresponding antigen or antibodies coated on the solid surface of the 96 well plate. After incubation period, the plate is washed with series of wash buffer in order to remove the serum and unbound antibodies or antigens. Then the plate continues with second incubation period with secondary antibodies that are attached to enzyme (i.e. peroxidase or alkaline phosphatase). The plate is washed off to remove the unbound secondary antibodies. The chromogenic substrate is then added and allowed to react with the enzyme to produce colour. The quantity of antigen or antibody present can be measured through the colour produced at optical density 450 nm. The intensity of colour measured may indicate the amount of antigen or antibody present.

3.6.2 Serum Dilution and Reagent Preparation

Prior to analysis, all serum samples and reagents were allowed to thaw to room temperature before use. All reagents were gently mixed prior to the experiments. All sera samples were diluted 1:25 to avoid the samples producing signals greater than the highest standard concentration. A 20 ml of Wash Buffer Concentrate was added to deionized or distilled water to prepare 500 ml of Wash Buffer. Number of 8-well strips needed for the assay was determined. Extra strips were re-packed and stored in the refrigerator for future use. One well was reserved for chromogen blank.

3.6.3 Determination of Resistin

(a) Human Resistin Standard Dilution

Human Resistin Standard was reconstituted with 1.0 ml of deionized or distilled water. This reconstitution produced a stock solution of 100 ng/ml. Then the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilution.

A 900 µl of *Calibrator Diluent RD5K* was pippeted into Tube 1 and 500 µl was pippeted into the remaining tubes (Tube 2 till Tube 7).

Human Resistin Standard stock solution had undergone serial dilution (Figure 3.1). A 100 µL from *Human Resistin Standard* stock solution was transferred to the Tube 1 with 900 µL diluent, producing total of 1000 µL solution. Then, 500 µL of this solution in Tube 1 was transferred into the Tube 2. Process of extracting 500 µL continued from the Tube 3 until the final serial dilution completed in Tube 7.

Each tube was mixed thoroughly before the next transfer. The 10 ng/ml standard served as the high standard while the *Calibrator Diluent* served as the zero standards (0 ng/ml).

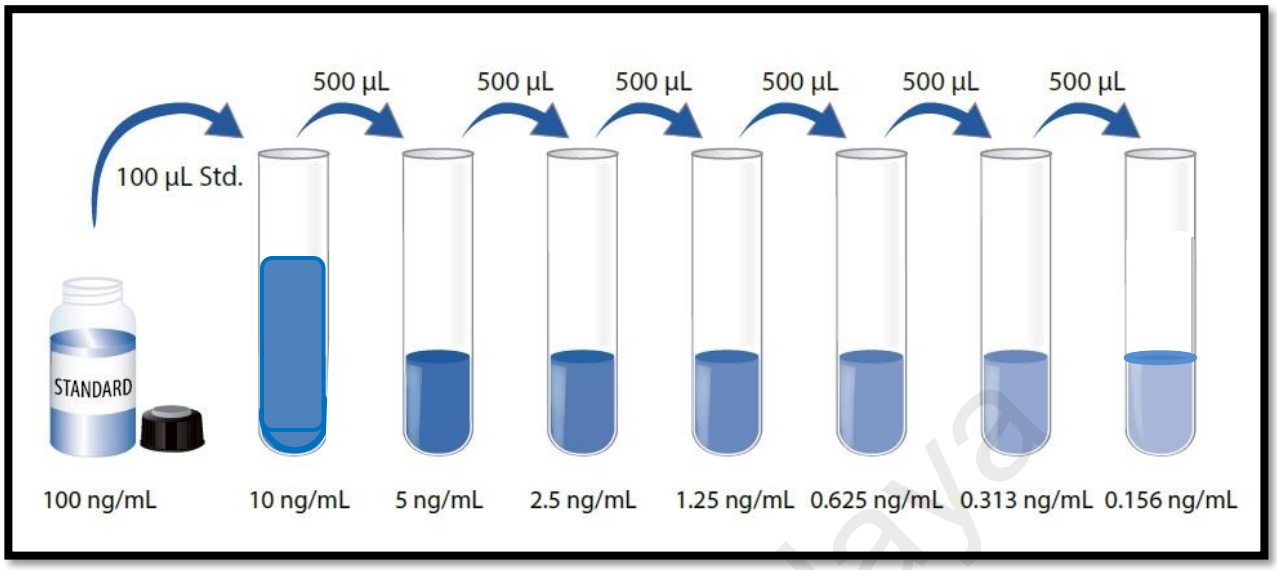

Figure 3.1: Serial Dilution of Human Resistin Standard in calibrator diluent RD5K (Concentration for each tube after serial dilution was labelled below the tubes)

(b) Determination of Resistin using ELISA

A 100 µl of *Assay Diluent RD1-19* solution was pipetted into each well of plate. A 100µL of standard and samples (serum) were added to the appropriate microtiter wells. Then the plate was tapped gently at the side to allow mixing. Next, the plate was covered with adhesive strip and incubated for 2 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *Resistin Conjugate* was added to each well. Again, the plate was covered with a new adhesive strip and was incubated for another 2 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining of *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *Substrate Solution* was added to each well. Then the plate was incubated for 30 minutes at room temperature in the dark condition. A 50 µl of *Stop Solution* were added to each well. Then the plate was tapped gently at the side of the plate to mix. The liquid in the wells were turn into blue. Determination of optical density of each well was done within 30 minutes, using a microplate reader set to 450 nm. The absorbance of the standards against the standard concentration was plotted on graph paper.

3.6.4 Determination of Interleukin-17

(a) Human IL-17 Standard Dilution

Human IL-17 Standard was reconstituted with 1.0 ml of deionized or distilled water. This reconstitution produces a stock solution of 20,000 pg/ml. Then the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

A 900 ml of the *Calibrator Diluent RD6-21* was pipetted into the into Tube 1 and 500 µl was pippeted into the remaining tubes (Tube 2 till Tube 7).

The *Human IL-17 Standard* stock solution was used to produce a serial dilution (Figure 3.2). A 100 µL from *Human IL-17 Standard* stock solution was transferred to the Tube 1 with 900 µL diluent, producing total of 1000 µL solution. 500 µL of this solution in Tube 1 was transferred into the Tube 2. Process of extracting 500 µL continued from the Tube 3 until the final serial dilution completed in Tube 7. Each tube was mixed thoroughly before the next transfer. The 2000 pg/ml standard served as the high standard, while the appropriate *Calibrator Diluent* served as the zero standard (0 pg/ml).

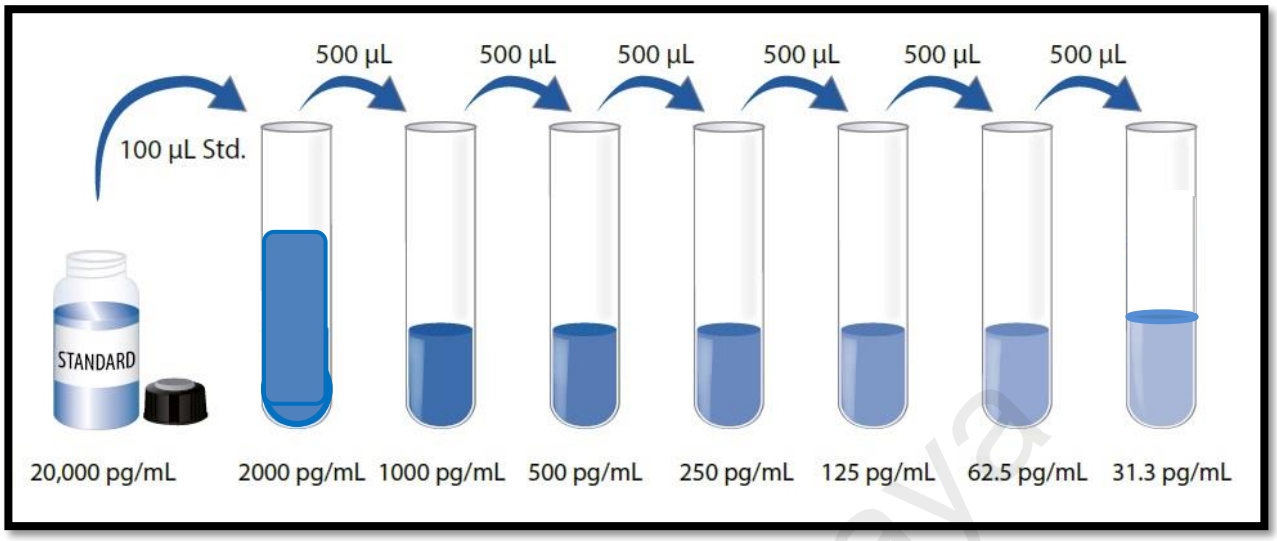

Figure 3.2: Serial Dilution of Human IL-17 Standard in calibrator diluent RD6-21 (Concentration for each tube after serial dilution was labelled below the tubes)

(b) Determination of IL-17 using ELISA

A 100 µl of *Assay Diluent RD1-36* solution was pipetted into each well. Then, 100 µl of standards, samples (serum) and controls were added to the appropriate microtiter wells. All reagent addition was ensured uninterrupted and completed within 15 minutes. Then the plate was tapped gently at the side of the plate to mix. Next, the plate was covered with adhesive strip and incubated for 3 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *Human IL-17 Conjugate* was added to each well. Again, the plate was covered with a new adhesive strip and was incubated for another 1 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells

were washed 4 times. After the last wash, any remaining *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

Following that, 200 µl of *Substrate Solution* was added to each well. Then the plate was incubated for 30 minutes at room temperature in the dark condition. Then, 50 µl of *Stop Solution* were added to each well. The plate was tapped gently at the side of the plate to allow mixing. Later, the liquid in the wells were turn into blue. Determination of optical density of each well was done within 30 minutes, using a microplate reader set to 450 nm. The absorbance of the standards against the standard concentration was plotted on graph paper.

3.6.5 Determination of Interleukin-6

(a) Human IL-6 Standard Dilution

Human IL-6 Standard was reconstituted in 5.0 ml of *Calibrator Diluent RD6F* (for serum samples). This reconstitution produces a stock solution of 300 pg/ml. Then the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

A 667 µl of the *Calibrator Diluent* RD6F was pipetted into the Tube 1 and 500 µl was pippeted into the remaining tubes (Tube 2 till Tube 6).

The *Human IL-6 Standard* stock solution was used to produce a serial dilution (Figure 3.3). A 333 µL from the human standard mixture was added to the Tube 1 with 667 µL diluent, producing total of 1000 µL solution. A total of 500 µL of this solution in Tube 1 was aliquoted and added into the Tube 2. Process of extracting 500 µL continued from the Tube 3 until the final dilution in Tube 6. Each tube was mixed thoroughly before the next transfer. The undiluted standard was served as the high standard (100 pg/ml). The *Calibrator Diluent* RD6F was served as the zero standards (0 pg/ml).

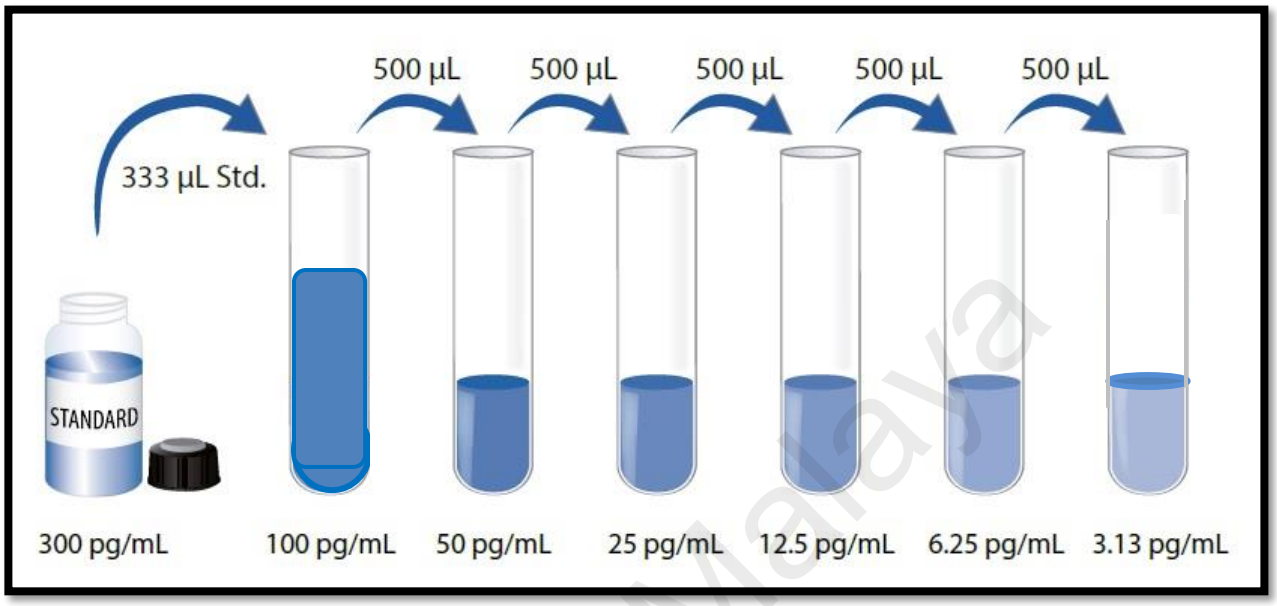

Figure 3.3: Serial Dilution of Human IL-6 Standard in calibrator diluent RD6F (Concentration for each tube after serial dilution was labelled below the tubes)

(b) Determination of IL-6 in serum using ELISA

Assay Diluent RD1W solution of 100 µl was pipetted into each well. Meanwhile, 100 µl of standards, samples (serum) and controls were added to the appropriate microtiter wells. Then the plate was tapped gently at the side of the plate to mix. Next, the plate was covered with adhesive strip and incubated for 2 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining of *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *Human IL-6 Conjugate* was added to each well. The same process was repeated again.

In the next step, a 200 µl of *Substrate Solution* was added to each well. Then the plate was incubated for 20 minutes at room temperature in the dark condition. Finally, 50 µl of *Stop Solution* were added to each well. Then the plate was tapped gently at the side to ensure a thorough mix and the liquid in the wells will changed its color to blue. Determination of optical density of each well was done within 30 minutes, using a microplate reader set to 450 nm. The absorbance of the standards against the standard concentration was plotted on graph paper.

3.6.6 Determination of Tumour Necrosis Factor-alpha (TNF-α)

(a) Human TNF-α Standard Dilution

TNF-α Standard was reconstituted with 1.0 ml of deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/ml. Then the standard was allowed to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

A 900 µl of *Calibrator Diluent RD6-35* was pippeted into the Tube 1 and 500 µl was pippeted into the remaining tubes (Tube 2 till Tube 7).

The *Human TNF-α Standard* stock solution was used to produce a serial dilution (Figure 3.4). A 100 µL from the human standard mixture was added to the Tube 1 with 900 µL diluent, producing total of 1000 µL solution. A total of 500 µL of this solution in Tube 1 was aliquoted and added into the Tube 2. Process of extracting 500 µL continued from the Tube 3 until the final dilution in Tube 7. Each tube was mixed thoroughly before the next transfer. The 1000 pg/ml standard served as the high standard. The *Calibrator Diluent RD6-35* served as the zero standard (0 pg/ml).

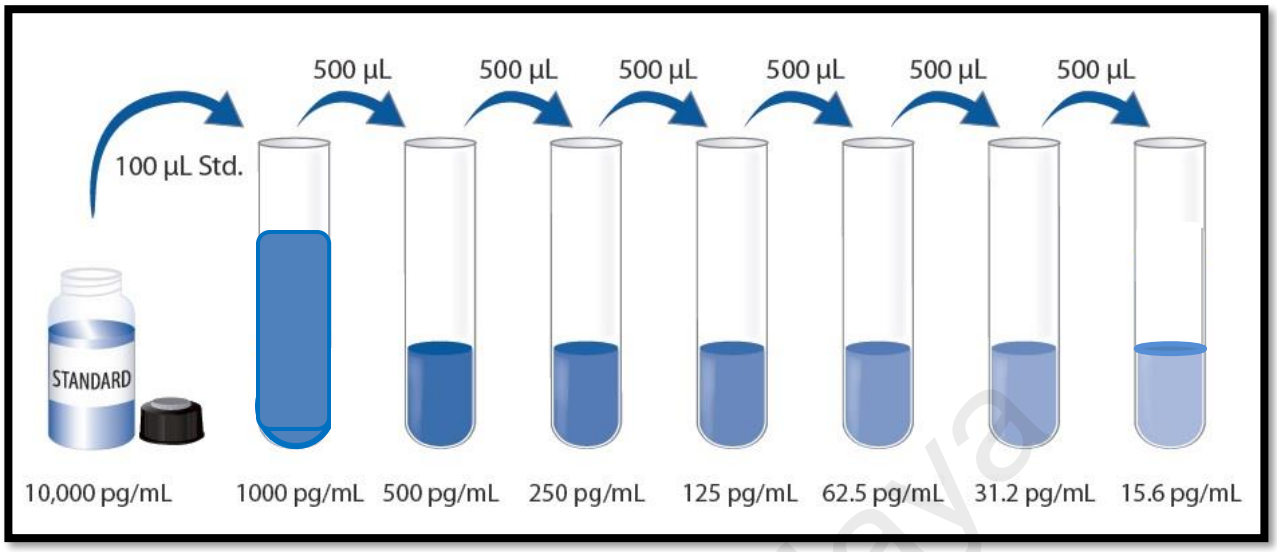

Figure 3.4: Serial Dilution of Human TNF-α Standard in calibrator diluent RD6-35 (Concentration for each tube after serial dilution was labelled on the tubes)

(b) Determination of TNF-α in serum using ELISA

A 50 µl of *Assay Diluent RD1F* solution was pipetted into each well. 200 µl of standards, samples (serum) and controls were added to the appropriate microtiter wells. Then the plate was tapped gently at the side of the plate to mix the solution. Next, the plate was covered with adhesive strip and was incubated for 2 hours at room temperature. After incubation, all solution from wells were thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining *Wash Buffer* was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *TNF-α Conjugate* was added to each well. Similar process was repeated again, the plate was covered with a new adhesive strip and was incubated for another 2 hours at room temperature. After incubation all solution from wells was thoroughly aspirated and discarded. The wells were washed 4 times. After the last wash, any remaining *Wash Buffer*

was removed by aspirating or decanting. The plate was inverted and blotted against clean paper towels.

A 200 µl of *Substrate Solution* was added to each well. Then the plate was incubated for 20 minutes at room temperature in the dark condition. 50 µl of *Stop Solution* were added to each well. Then the plate was tapped gently at the side of the plate to allow mixing. Until the liquid in the wells were turn into blue. Determination of optical density of each well was done within 30 minutes, using a microplate reader set to 450 nm. The absorbance of the standards against the standard concentration was plotted on graph paper.

3.7 Identification of Subgingival Microbiota using qPCR Technique

The detection of periodontal pathogens was done using Real Time Polymerase Chain Reaction (qPCR). Four microorganisms responsible for periodontal disease *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* were identified and quantified using qPCR method.

To determine the sensitivity of the qPCR technique, serial samples containing known concentrations of individual microorganism were processed for qPCR analysis. The lowest concentration that resulted in positive PCR product was regarded as the sensitivity of the assay (Yuan et al., 2001).

3.7.1 DNA Extraction

The protocol of DNA extraction was followed as manufacturer recommendation. Tubes containing the sampled plaque were centrifuged at a speed of 5,000 rpm for 10 minutes on a tabletop centrifuge to pellet the cells. Supernatant were discarded. Then the bacterial pellet was placed on the QIAcube-shaker for DNA extraction. DNA extraction was done automatically using automated QIAcube extractor (Qiagen, Netherlands).

(a) Principle of DNA extraction

DNA extraction involved four steps (i) lysis, (ii) precipitation, (iii) wash and (iv) resuspension in order to get the pure DNA. Briefly, nucleus is surrounded with cell membrane and cell wall which need to be removed from other to get the purified DNA. The lysis steps were referred to process of breaking the cell wall and cellular membrane that achieved by incubation of the sample material in Proteinase K supplemented lysis buffer. The precipitated DNA is washed with ethanol solution to remove soluble impurities. Finally, the pure DNA is resuspended in a buffer to ensure the stability and for long term storage. The purity of extracted DNA was measured using Nano Drop 2000 Spectrophotometer. Ratio absorbance between ~1.8 to 2.0 was considered as acceptance range for DNA purity. A 100 µl of the eluted DNA was stored in -80◦C for bacterial identification using qPCR.

3.7.2 qPCR

All DNA extraction sample will undergo qPCR using ABI 7500 Fast Real-Time PCR (Applied Biosystems) and One-Step Plus (Applied Biosystems) real-time PCR cycler in 96 well plates in order to identify and quantify the microbes. The qPCR reaction was prepared in 20 µl volumes containing the TaqMan mastermix, TaqMan assay, nuclease-free water and DNA samples. Each custom TaqMan Assays contain sequence of primer and probes representing different bacteria. The sequence of primer and probes are shown on Table 3.1.

Table 3.1: Sequence of primers and probes for microbes

Microbes	Sequence (5'→ 3')	Reference
P. gingivalis		
Forward	GCG CTC AAC GTT CAG CC	Boutaga et al., 2003
Reverse	CAC GAA TTC CGC CTG C	
Probe	CAC TGA ACT CAA GCC CGG CAG TTT CAA	
T. forsythia		
Forward	GGG TGA GTA ACG CGT ATG TAA CCT	Boutaga et al., 2006
Reverse	ACC CAT CCG CAA CCA ATA AA	
Probe	CCC GCA ACA GAG GGA TAA CCC GG	
P. intermedia		
Forward	CGG TCT GTT AAG CGT GTT GTG	Boutaga et al., 2006
Reverse	CAC CAT GAA TTC CGC ATA CG	
Probe	TGG CGG ACT TGA GTG CAC GC	
A. actinomycetemcomitans		
Forward	GAA CCT TAC CTA CTC TTG ACA TCC GAA	Boutaga et al., 2006
Reverse	TGC AGC ACC TGT CTC AAA GC	
Probe	AGA ACT CAG AGA TGG GTT TGT GCC TTAGGG	

For negative control, ribonucleic acid (RNA)se free water was used to replace bacterial DNA in each qPCR procedures while DNA extract of the above microorganisms were purchased from American Type Culture Collection (ATCC) to act as the positive control (Yuan et al., 2001).

This mixture will then be subjected to an initial amplification cycle of 50ºC for 2 minutes and 95ºC for 10 minutes, followed by 45 cycles at 95ºC for 15s and 60ºC for 1 minute (Boutaga et al., 2006).

3.8 Statistical analysis

Data entry and statistical analysis was carried out using Statistical Package for the Social Sciences (SPSS), version 23.0 (SPSS Inc. Chicago, IL, USA). The data was tested for normality of distribution using Shapiro–Wilk test before the test of hypothesis analysis. *P*–value of less than 0.05 was considered statistically significant. Mann-Whitney U test was used to analyze the significant difference in age distribution while chi-square test was used to analyze significant difference of the distribution of subject characteristics.

The distributions of clinical parameters, serum interleukins and microbes were measured using either Independent Sample T–test or Mann Whitney U test depending on the distribution of the data.

The relationship between clinical parameters, serum interleukins and microbes were measured using either Pearson's coefficient correlation or Spearman coefficient correlation.

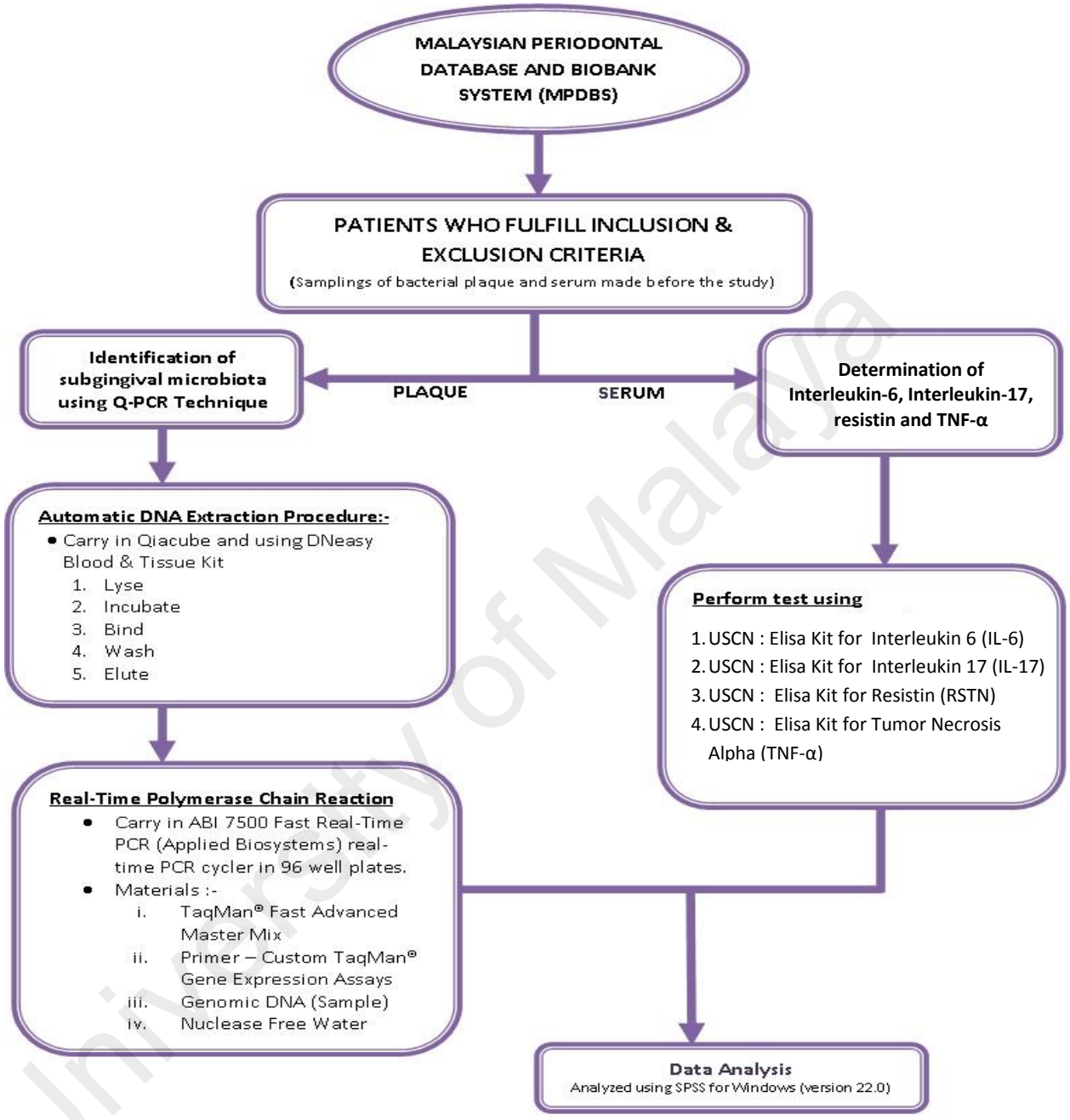

Figure 3.5: Flowchart of the study

CHAPTER 4: RESULTS

4.1 Demographic characteristics of study population

Demographic characteristics of study population is summarized in Table 4.1. There was a total of 164 subjects in the test group (CP group) and 137 for the control group (non-CP group). The mean age and standard deviation were 47.32 ± 7.56 for the test group and 47.47 ± 10.28 for the control group, with no significant difference ($p = 0.378$) between both groups.

There were about twice the number of males in the CP group as compared to the non-CP group ($p = 0.004$). In addition, Malays were the dominant ethnic group in both CP and non-CP groups. Within each ethnic group, there were significantly more Chinese and Indians in the CP group whereas the Malays were higher in number in the non-CP group ($p < 0.01$).

Chi-Square analysis showed no significant difference in terms of education level between CP and non-CP groups ($p > 0.05$). More than 80% of the participants in each group had at least secondary education level. There was no significant difference between smokers and non-smokers in both groups. However, there were significantly more diabetic subjects in the non-CP group ($p < 0.01$).

Table 4.1: Demographic characteristics and habits of the study population according to the groups CP (test) and non-CP (control) groups

Characteristics	Groups (N = 301)		*p* – value
	CP (N = 164)	Non – CP (N = 137)	
	Mean (±SD)	Mean (±SD)	
Age (years)	47.32 (7.56)	47.47 (10.28)	0.378 [a]
	N (%)	**N (%)**	
Gender			
Male	81 (49.40)	45 (32.80)	0.004 [b*]
Female	83 (50.60)	92 (67.20)	
Ethnicity			
Malay	80 (48.80)	92 (67.20)	0.009 [b*]
Chinese	61 (37.20)	28 (20.40)	
Indian	22 (13.40)	16 (11.70)	
Others	1 (0.60)	1 (0.70)	
Education level			
Tertiary	53 (32.30)	51 (37.20)	0.180 [b]
Secondary	86 (52.40)	70 (51.10)	
Primary	11 (6.70)	12 (8.80)	
Others	14 (8.50)	4 (2.90)	
Smoking habit			
Smoking	27 (16.50)	25 (18.20)	0.683 [b]
Non–smoker	137 (83.50)	112 (81.80)	
Diabetic status			
Diabetic	2 (1.20)	12 (8.80)	0.002 [b*]
Non-diabetic	162 (98.80)	125 (91.20)	

CP: Chronic periodontitis
[a] Mann-Whitney U test
[b] Chi-Square test
[*] *p*-value < 0.05

4.2 Comparison of mean clinical parameters between groups

Statistical analysis to compare mean clinical parameters between groups is shown in Table 4.2. The mean percentage of both VPI and GBI were significantly higher in the CP group [53.56% ± 29.98 and 48.64% ± 27.61 respectively] compared to the non-CP group [19.52% (± 19.11) and 9.78% (± 16.06) respectively] with p=0.001.

Similar observations were noted for mean PPD and CAL. In the CP group, the mean PPD and CAL 3.07 ± 0.88 mm and 3.83 ± 1.37 mm respectively were significantly higher than the mean PPD and CAL in the non-CP group 1.26 ± 0.44 mm and 1.45 ± 0.43 mm respectively ($p < 0.001$).

Mean PPD and CAL were further analysed based on the severity of the disease (Table 4.2). In both parameters, severity was indicated by the pocket depth (PPD < 4 mm, PPD 4–6 mm and PPD > 6 mm) and attachment loss (CAL < 4 mm, CAL 4–6 mm and CAL > 6 mm). Overall, subjects in the non-CP group showed that all of them had PPD and CAL less than 4 mm (100% each).

When comparing within the CP groups, majority of the subjects had % of sites PPD < 4 mm (70%) and CAL < 4 mm (54.08%) followed by PPD 4-6 mm (24.5%) and CAL 4-6 mm (32.92%) and finally PPD > 6 mm (5.5%) and CAL > 6 mm (13%).

Table 4.2: Comparison of Visible Plaque Index (VPI), Gingival Bleeding Index (GBI), Probing Pocket Depth (PPD) and Clinical Attachment Loss (CAL) in CP and non – CP subjects

Clinical parameters	Groups (N=301)		*p* – value
	CP (N=164)	Non – CP (N=137)	
	Mean (SD)	Mean (SD)	
Mean VPI (%)	53.56 (29.98)	19.52 (19.11)	< 0.001[a*]
Mean GBI (%)	48.64 (27.61)	9.78 (16.06)	< 0.001[a*]
Mean PPD (mm)	3.07 (0.88)	1.26 (0.44)	< 0.001[a*]
% sites with PPD > 6 mm	5.50 (7.86)	0.00 (0.00)	< 0.001[a*]
% sites with PPD 4-6 mm	24.50 (14.71)	0.00 (0.00)	< 0.001[a*]
% sites with PPD < 4mm	70.00 (19.14)	100.0 (0.00)	< 0.001[a*]
Mean CAL (mm)	3.83 (1.37)	1.45 (0.43)	< 0.001[b*]
% sites with CAL > 6 mm	13.00 (16.05)	0.00 (0.00)	< 0.001[a*]
% sites with CAL 4-6 mm	32.92 (16.22)	0.00 (0.00)	< 0.001[b*]
% sites with CAL < 4 mm	54.08 (25.12)	100.00 (0.00)	< 0.001[a*]

CP: Chronic periodontitis, SD: Standard deviation
VPI: Visible Plaque Index, **GBI**: Gingival Bleeding Index, **PPD**: Probing Pocket Depth, **CAL**: Clinical Attachment Loss
[a] Mann-Whitney U test;
[b] Independent Samples T Test;
[*] $p < 0.05$

4.3 Multiple regression analysis for clinical parameters with subject characteristics.

Linear regression analysis was performed to find out the possible confounders for all clinical parameters (VPI, GBI, PPD, CAL, PPD < 4 mm, PPD 4–6 mm, PPD > 6 mm, CAL < 4 mm, CAL 4–6 mm and CAL > 6 mm).

Generally, every additional individual from CP group in this study may increase the mean VPI and GBI by 34% and 38% respectively. Likewise, every additional individual in CP group also may increase the mean PPD and CAL by 1.81 mm and 2.38 mm respectively.

Furthermore, additional number of individuals in CP group may decrease the percentage of sites with PPD < 4 mm by 29% but increase the percentage sites with PPD 4–6 mm and PPD > 6 mm by 24% and 5%. Similarly, the percentage of sites with CAL < 4 mm may decrease by 45% for every additional individual in CP group. However, it may increase percentage sites with CAL 4–6 mm by 32% and CAL 6 mm by 13%.

After controlling subjects' characteristics such as group, gender, ethnicity and diabetic status, CP group was found as the only significant predictor for all clinical periodontal parameters (Table 4.3).

Table 4.3: Multiple regression analysis for clinical parameters with subjects' characteristics

Variables	Model	B	t	*p* – value
Mean clinical parameter				
VPI (%)	CP	34.041	11.445	< 0.001*
GBI (%)	CP	38.856	14.503	< 0.001*
PPD (mm)	CP	1.812	21.799	< 0.001*
CAL (mm)	CP	2.384	19.586	< 0.001*
% sites with PPD < 4mm	CP	- 29.998	- 18.343	< 0.001*
% sites with PPD 4-6 mm	CP	24.499	19.491	< 0.001*
% sites with PPD > 6 mm	CP	5.502	8.188	< 0.001*
% sites with CAL < 4mm	CP	- 45.923	- 21.389	< 0.001*
% sites with CAL 4-6 mm	CP	32.926	23.749	< 0.001*
% sites with CAL > 6 mm	CP	13.001	9.481	< 0.001*

Adjusted group, gender, ethnicity and diabetic status to predict the mean clinical parameters.

CP: Chronic periodontitis; * $p < 0.05$

4.4 Prevalence of periodontal pathogens in study population

Table 4.4 presents the prevalence of periodontal pathogens in the study population. The CP subjects showed significantly higher presence of *P. gingivalis* (94.50%), *T. forsythia* (92.10%), *P. intermedia* (72.60%) and *A. actinomycetemcomitans* (7.30%) as compared to the non-CP subjects (73.7%, 62.00%, 58.4% and undeterminable respectively) (p <0.05). *A. actinomycetemcomitans* which was present 7.3% in CP subjects was however not present in the non-CP subjects.

The CP group presented with higher mean counts of periodontal pathogens as compared to non-CP group. The CP group showed higher detection level of *P. gingivalis* (24.66×10^5 cell/µl), *T. forsythia* (7.70×10^5 cell/µl), *P. intermedia* (6.61×10^5 cell/µl) and *A. actinomycetemcomitans* (0.14×10^5 cell/µl) as compared to non-CP group (8.32×10^5, 4.75×10^5, 5.17×10^5 µl and 0.00 respectively) ($p < 0.05$).

Table 4.4: Prevalence of periodontal pathogens in study population

Periodontal pathogens	Frequency, N (%)		*p*–value	Mean count ± SE ($\times 10^5$ cells/µl)		*p*–value
	CP (N = 164)	Non – CP (N = 137)		CP (N = 164)	Non – CP (N = 137)	
P. gingivalis	155 (94.50)	101 (73.70)	< 0.001[a*]	24.66 (1.05)	8.32 (0.62)	< 0.001[b*]
T. forsythia	151 (92.10)	85 (62.00)	< 0.001[a*]	7.70 (0.34)	4.75 (0.38)	< 0.001[b*]
P. intermedia	119 (72.60)	80 (58.40)	0.010[a*]	6.61 (0.69)	5.17 (0.46)	0.047[b*]
A. actinomycetemcomitans	12 (7.30)	0 (0.00)	0.001[a*]	0.14 (0.07)	0.00 (0.00)	0.005[b*]

CP: Chronic periodontitis, SD: Standard deviation
[a] Chi-Square test;
[b] Mann-Whitney U test;
[*] $p < 0.05$

4.5 Multiple regression analysis of periodontal pathogens level with subject characteristics

A linear regression analysis was run to find out the possible confounders for mean level of periodontal pathogens. After controlling the groups (CP and non-CP), subject characteristics (gender, ethnicity, and diabetic status) and all clinical parameters, the confounder for mean level of periodontal pathogens are shown in Table 4.5.

The confounding factor for *P. gingivalis* was CP group and mean CAL. It can be explained that the mean level of *P. gingivalis* may increase by additional individual in CP group (9.836×10^5 cell/µl) and mean CAL (2.638×10^5 cell/µl). The mean level of *T. forsythia* was influenced by mean PPD (1.452×10^5 cell/µl).

Overall, only *P. gingivalis* was found to be a significant predictor of CP among the study population. Gender, ethnicity and diabetic status were not confounders for all periodontal pathogens.

Table 4.5: Multiple regression mean of periodontal pathogen level with subject characteristics.

Variables	Model	B	t	*p* - value
Mean level detection				
***P. gingivalis* (×10^5)**	CP	9.836	5.212	< 0.001*
	CAL (mm)	2.638	4.432	< 0.001*
***T. forsythia* (×10^5)**	PPD (mm)	1.452	6.870	< 0.001*

Adjusted gender, ethnicity, diabetic status and all clinical parameters to predict the mean detection level of periodontal pathogens.

CP: Chronic periodontitis; * $p < 0.05$

4.6 Inflammatory mediators level in study population

The CP group showed significantly higher presence of all inflammatory mediators (100.00%) as compared to the level in non-CP group where presence of resistin was 94.90%, IL-17 was 45.30%, IL-6 was 30.70% and TNF-α was undeterminable ($p < 0.001$).

The level of resistin (274620pg/ml), IL-17 (727.13pg/ml), IL-6 (26.84pg/ml) and TNF-α (54.91pg/ml) were found to be significantly higher in the CP group as compared to the non-CP group, (22.12pg/ml, 0.19pg/ml, 0.006pg/ml and 0.00pg/ml respectively) ($p < 0.001$).

Table 4.6: Inflammatory mediators level in study population

Inflammatory mediators	Frequency, N (%)		*p*–value	Mean count ± SE (pg/ml)		*p*–value
	CP (N = 164)	Non – CP (N = 137)		CP (N = 164)	Non – CP (N = 137)	
Resistin	164 (100.00)	130 (94.90)	< 0.001[a*]	274620 (19.000)	22.120 (1.820)	<0.001[b*]
IL-17	164 (100.00)	62 (45.30)	< 0.001[a*]	727.13 (0.048)	0.190 (0.011)	< 0.001[b*]
IL-6	164 (100.00)	42 (30.70)	< 0.001[a*]	26.84 (0.002)	0.006 (0.001)	< 0.001[b*]
TNF-α	164 (100.00)	0 (0.00)	< 0.001[a*]	54.91 (0.002)	0.000 (0.000)	< 0.001[b*]

CP: Chronic periodontitis, SD: Standard deviation

[a] Chi-Square test;

[b] Mann-Whitney U test;

[*] $p < 0.05$

Minimum detection limits resistin, 2.6pg/ml; IL-17, <15pg/ml; IL-6, 0.07pg/ml and TNF- α, 1.6pg/ml.

4.7 Multiple regression analysis of inflammatory mediators level with subject characteristics

The possible confounders for mean level of inflammatory mediators was determined by using linear regression analysis. After controlling the groups (CP and non-CP), subject characteristics (gender, ethnicity, and diabetic status) and all clinical parameters, the confounders for mean detection level of inflammatory mediators are shown in Table 4.7.

The only confounder for mean level of resistin, IL-6 and TNF-α was CP status. An addition of a CP individual may increase the level of resistin by 226 pg/ml, IL-6 by 20.7pg/ml and TNF-α by 49.2pg/ml. Addition of a CP individual would increase the mean level of IL-17 by 569.5 pg/ml and increase of mean % VPI would increase the mean level of IL-17 by 3.4pg/ml. However, mean level of IL-17 may decrease by 134pg/ml for every addition of mean CAL (mm).

Overall, all inflammatory mediators were found to be significant predictors of CP in this study population. In addition, IL-17 was found to be the only significant predictor for % VPI and CAL (mm). Gender, ethnicity and diabetic status were not confounders for all inflammatory mediators.

Table 4.7: Multiple regression analysis of inflammatory mediators level with subject characteristics.

Variables	Model	B	t	*p* - value
Mean level detection				
Resistin	CP	226.451	7.375	<0.001*
IL-17	CP	569.522	6.037	< 0.001*
	VPI (%)	3.381	2.939	0.004*
	CAL (mm)	-134.369	-4.240	< 0.001*
IL-6	CP	20.720	8.522	< 0.001*
TNF-α	CP	49.211	13.807	< 0.001*

Adjusted group, gender, ethnicity diabetic status and all clinical parameters to predict the mean detection level of serum interleukins.

CP: Chronic periodontitis; * $p < 0.05$

4.8 Correlation between the level of periodontal pathogens and inflammatory mediators for both groups.

Spearman correlation coefficient was used to determine the strengths of the correlation coefficient (r) values between level of periodontal pathogens and inflammatory mediators for both groups as shown in Table 4.8. The correlation between variables were based on the normal distribution of the data.

The CP group showed two significant positive correlations with weak strengths which were between *T. forsythia* and IL-17 ($r = 0.189$; $p = 0.016$) and between *A. actinomycetemcomitans* with TNF-α ($r = 0.166$; $p = 0.033$). Overall, non-CP group showed no significant correlation between periodontal pathogens with all inflammatory mediators.

Table 4.8: Correlations between the levels (mean count) of periodontal pathogens and inflammatory mediators in the study population

Parameters / Pathogen	*P. g*		*T. f*		*P. i*		*A. a*	
	CP	Non-CP	CP	Non-CP	CP	Non-CP	CP	Non-CP
	r (p-value)	r (p value)	r(p value)	r (p-value)	r (p-value)	r (p-value)	r (p-value)	r (p-value)
Resistin	- 0.025 (0.753)	0.016 (0.857)	0.145 (0.063)	0.053 (0.536)	- 0.055 (0.485)	0.012 (0.885)	0.084 (0.248)	-
IL-17	0.093 (0.237)	- 0.120 (0.161)	0.189 (0.016)*	0.071 (0.413)	- 0.053 (0.504)	0.030 (0.727)	- 0.003 (0.974)	-
IL-6	0.028 (0.721)	0.072 (0.401)	- 0.051 (0.516)	0.068 (0.430)	0.050 (0.524)	- 0.082 (0.340)	0.011 (0.886)	-
TNF-α	0.068 (0.387)	-	0.027 (0.731)	-	0.005 (0.945)	-	0.166 (0.033)*	-

P.g: P. gingivalis, T.f: T. forsythia, P.i: P. intermedia, A.a: A. actinomycetemcomitans; IL-17: Interleukin 17, IL-6: Interleukin 6, TNF-α: Tumour necrosis factor alpha; * $p < 0.05$

CHAPTER 5: DISCUSSIONS

5.1 Experimental Design

This study was conducted as a case-control comparative study and was part of a large multi-centre study looking at genetic associations with periodontal disease. Sample collection for the main study was initiated in November 2011 and continued to March 2015. Sample collection and clinical examination for all subjects was done by calibrated clinicians from the University of Malaya, Universiti Sains Islam Malaysia, Universiti Teknologi Mara (UiTM) and Ministry of Health Malaysia. All data and samples were managed and banked under the Malaysian Periodontal Database and Biobank System (MPDBS) (Vaithilingam et al., 2015) which is based in the Faculty of Dentistry, University Malaya. For the current study, all laboratory procedures were conducted at the Faculty of Dentistry, University of Malaya. Ethical approval (DF PE1103/0037(L)) had been obtained for the current study from the ethical committees of Faculty of Dentistry, University of Malaya and also the various collaborating institutions.

A number of previous studies have been carried out to determine the clinical relationships between periodontal pathogens and inflammatory mediators (Cheng et al., 2017). It is known that periodontitis is initiated by dental plaque (complex and diverse microbial biofilms) on the teeth. The activation of host defence cells starts with substances released from the biofilm such as lipopolysaccharides, antigens and other virulence factors (Yucel-Lindberg & Bage, 2013). These bacterial products provide access to the gingival tissue and subsequently initiates inflammatory and immune response and results in periodontal tissue destruction and bone resorption.

To the best of our knowledge, no previous study in Malaysia has looked at the presence of all four inflammatory mediators namely resistin, IL-17, IL-6 and TNF-α and its

association with periodontal pathogens in the Malaysian population. Hence this case-control study was designed to compare clinical parameters, inflammatory mediators and periodontal pathogens profile of Malaysian patients diagnosed with moderate to chronic periodontitis versus healthy individuals. Furthermore, this study also looked at the predictors for chronic periodontitis from periodontopathogens (*P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans)* and inflammatory markers (resistin, IL-17, IL-6 and TNF-α).

5.2 Sample population

In the current study, the test group comprised Malaysian subjects who were 35 years old and above and were diagnosed with moderate to severe chronic periodontitis. The case definition used for this study was that provided by Page and Eke (2007) as they had proposed case definitions for moderate and severe chronic periodontitis based on the clinical diagnosis of periodontitis for population-based surveillance. This case definition was further updated in 2012 with the addition of a case definition for mild chronic periodontitis. This is in contrast to the 1999 classification (Armitage, 1999) which does not specify very distinctive case definitions for varying severity of disease. The Page and Eke (2007) case definition has also been used in other case-control studies (Haffajee et al., 1998) as well as in interventional clinical trials (Buzinin et al., 2014).

The control group in this study also comprised Malaysians who were 35 years old and above with a healthy periodontium. The age of patients in both test and control groups was 35 years to 85 years old. It has been established that the most frequently occurring form of periodontitis which is CP is commonly detected in adults (Highfield, 2009). Highfield (2009) also stated, the severity and prevalence of chronic periodontitis also increases with age.

Selection of subjects more than 35 years old was to avoid including patients with aggressive periodontitis who were generally under the age of 30 (Highfield, 2009).

In this study, subjects were not age- and gender- matched. When designing a study, it is important to have both test and control groups to be similar in terms of socio-demographic parameters. When comparisons for demographic data and systemic factors were then made between test and control groups, the subjects were found to be equally distributed between test and control groups except for ethnicity and gender. Therefore, these factors were potential confounding factors for this study population and subsequent multiple regression was performed to control for these factors.

There is a lot of evidence in the literature about systemic diseases such as Type 2 diabetes being a risk factor for periodontitis (Nazir, 2017). The progression of periodontitis is affected by uncontrolled diabetes as there will be an increase in the levels of TNF-α and IL-6 in these subjects (Furugen et al., 2008). Thus, including diabetic patients in this study may have an effect on the results for inflammatory mediators. However, due to the strict subject inclusion criteria and the need for sufficient numbers of sample size, our study included 14 subjects with Type 2 diabetes (2 in the test group and 12 in the control group). It is noted that the diabetes status was self-reported by the subjects and there was no information gathered about their diabetic control (ie glycated haemoglobin levels or random glucose levels) during the time of sampling. When comparisons were done between the test and control groups, it was found that there were significantly higher number of diabetics in the control group ($p<0.005$). Therefore, multiple regression analysis was performed to control for this confounding factor in all subsequent analysis.

In this study, smokers were also included. This was due to the difficulty in getting sufficient subjects for the sample size. Take into consideration from research done by Bergstrom, 2014, which stated smoking can be one of the important risk factors for

periodontitis, and drop in smoking rate could reduce prevalence of periodontal disease. So, comparisons were made between test and control group and found that there was no significant difference in the number of smokers in both groups. Thus, there was no bias included in the samples in both groups. Comparison also made due to there is evidence that oral microbial flora will increase (Bergstrom, 2014) and also affects host response (Fiorini et al., 2014).

5.3 Sample Size Determination

A sample is a set of participants selected from a population, which is less in number (size) but adequately represents the population so that true inferences about the population can be made from the results obtained (Noordzij et al., 2010). Sample size calculation for case control study depends to a large extent on the study population size, nature of the case being studied whether it is a commonly seen case or a rare case (Lui, 1991). In this study, we included samples that were diagnosed with moderate to severe CP. According to National Oral Health Survey of Adults (NOHSA), Oral Health Division, Ministry of Health Malaysia the prevalence of this category of disease in 2010 is 18.2% of the Malaysian population (NOHSA, 2010).

To calculate the sample size, it is important to have an idea of the results expected for the current study. Basically, the larger the variability in the outcome variable, the greater the sample size needed in assessing if an observed effect is a true effect or not (Jeffcoat, 1992). In this study, the calculation was done after determination of the standard deviation for CAL difference of 1.0 mm at deep sites based on a previous study (Carvalho et al., 2004). By considering the ideal sample size to achieve 80% power of study with an α of 0.05, at least 150 subjects per group were required (Dupont & Plummer, 1990). However, due to time

constraints and difficulty in getting suitable patients that fulfilled the inclusion criteria, only 137 subjects for control group and 164 subjects for test group were recruited in the study. The data for these subjects was obtained from the MPDBS data-base and was used for the statistical analyses of all parameters.

5.4 Clinical Measurements

All clinical measurements were done by previously calibrated clinicians (Vaithilingam et al., 2015). Kappa score obtained for intra- and inter-examiner reproducibility of PPD and CAL was more than 80%. Clinical periodontal measurements for CAL has become a standard for determining periodontal status of an individual. (Armitage et al., 1977; Pihlstrom, 1992). Measurements of CAL continue to be dependent on the accuracy of PPD measurements. As stated in studies by Armitage et al.,(1977) and Listgarten (1980), PPD values were highly affected by the degree of inflammation, location of probe tip and probing force. For instance, measurement of PPD in an inflamed human periodontal tissue was proven histologically to be overestimated by approximately 0.3mm-0.5mm apical to the junctional epithelium (Listgarten, 1980). On the other hand, for subjects with healthy gingiva, this value may become underestimated when probing is introduced into healthy gingiva. This is due to relatively tighter junctional epithelium attachment in healthy gingiva (Armitage et al, 1977, Listgarten, 1980). To control for this problem, all examiners who participated in providing data and samples to the MPDBS had been calibrated (Vaithilingam et al., 2015).

Upon analyzing the difference between the 2 groups, the results demonstrated that VPI and GBI scores were significantly higher in the CP group as compared to non-CP group ($p < 0.001$). Similar findings have been reported in other studies (Haffajee et al., 1998).

These results reinforce the findings from the classic study done by Loe, 1965 which demonstrated that plaque is the main aetiology of the disease. Deep periodontal pockets offer difficulties to remove plaque deposits. Many studies have shown that the formation of subgingival plaque and to a lesser extent subgingival microflora was influenced by supragingival plaque which was measured by VPI (Guo et al., 2017). Moreover, bleeding index has been regarded as the most reliable indicator for periodontal health. It has been shown that bleeding on probing has 30% predictive value for periodontal breakdown (Lang et al., 1986 ; Guo et al., 2017) and its absence indicates a 98% prediction of periodontal health (Lang et al., 1990). In this study GBI in the non-CP group was 9.78% as compared with 48.66% in the CP group. Thus, the relative absence in the non-CP group indicated the healthy condition of the periodontium.

The results also demonstrated that PPD and CAL scores were significantly higher in the CP group as compared to non-CP group ($p < 0.001$). Similar findings have been reported in other studies (Haffajee et al., 1998 ; Stingu et al., 2012). When PPD and CAL were further assessed at site level, it was found that all PPD and CAL 4-6mm and >6mm were significantly higher in the CP group ($p<0.001$). The significant difference in both groups were influenced by increase in the sites of PPD by 4–6 mm and PPD > 6 mm and similarly to the sites with CAL < 4 mm in CP group. It is also found that CAL and PPD <4mm were significantly low in CP group. After controlling for all confounding factors, it was found that CP group was the only confounder that influenced all clinical parameters ($p <0.001$). These findings are expected as studies have shown that subjects with CP have greater PPD and CAL compared to healthy periodontium (Haffajee et al., 1998; Matuliene et al., 2008; Chen et al., 2015).

The results of this study also indicate that the extent and severity of disease in the subject was localized severe chronic periodontitis as less than 30% sites were found to have PPD and CAL of more than 6mm (Armitage, 2008). However, when considering subjects

with moderate and severe CP, a total of 30% subjects had PPD ≥ 4mm and 45.9% had CAL ≥ 4mm and thus they can be termed as generalized moderate to severe CP subjects. Benefit of having sample subject from on generalized CP subjects was it involves more than 30% of sites on teeth as compared to localized CP subjects (Armitage, 2008). Furthermore, comparing localized only involves first molar or incisor interproximal attachment loss, whereas generalized involves the interproximal attachment loss affecting at least three permanent teeth other than incisors and first molar (Armitage, 1999).

5.5 Sample Preparation

In this study, pooled plaque samples were taken from most diseased site (PPD ≥ 5 mm) for the test group while for the control group it was collected from interdental sites of each subject to detect the frequency of all four periodonto-pathogen (at subject level) as well as to determine its detection level (counts) in the sample. Samples were kept in sterile DNase-free and RNase-free polyethylene tube (Li et al., 2006) containing 1.5 ml of Phosphate Buffer Solution (PBS) and stored at -80°C till microbiological analysis.

Assessment of serum level of inflammatory mediators of all four was done from the serum sample. The inflammatory markers found in the serum is representative of systemic inflammatory changes (Buduneli et al., 2012). Other methods of measuring inflammatory markers will be from gingival cervical fluid (GCF) or saliva which is more representative of the inflammatory changes at the site level (Buduneli et al., 2012). Using saliva and GCF samples are also non-invasive and preferred by subjects (Buduneli et al., 2012). It is suggested that a future study will be done using either GCF or saliva samples to assess more localized inflammatory markers for these subjects.

Throughout the study, appropriate storage time and conditions of all collected

samples were maintained to avoid degradation of proteins if kept too long or not in optimum temperature (Jurowski et al., 2017). It has been recommended that biological samples should ideally be extracted immediately after removal from their origin. This will directly minimize changes which occur to the protein components (Ennis et al., 2010). Thus, in this study, fresh plaque samples and blood samples taken from patients were stored immediately at 4-6°C before being transferred and stored at -80°C. Plaque and serum samples can be kept up to 6-12 months until analyses. This was to allow maximizing the availability of obtaining adequate samples for conducting our study (Zain et al., 2013).

5.6 Real time PCR

It has been reported that studies using culture methods had low chances to detect non-viable bacteria (Sakamoto et al., 2005, Li et al., 2006) and some bacteria only can be detected using PCR methods compared to cultivation (Stingu et al., 2012). Therefore, DNA-based techniques such as DNA probes and PCR are regarded as extremely sensitive bacterial detection tools (D'Ercole et al., 2008) and has been advocated as the best method for periodontal pathogen determination. As the subgingival microbiota comprises mainly of obligate anaerobic bacteria which are difficult to be cultured (Sakamoto et al., 2005), this study has made use of current technological advancements in microbiological diagnostics by using Real Time PCR (qPCR). Moreover, this method could maximize the chances of detecting and quantifying the composition of the subgingival plaque anaerobic bacteria (Doungudomdacha et al., 2001). Besides, qPCR has also been shown to have good sensitivity and specificity for detection of periodontal pathogens (Boutaga et al., 2003; Loesche, 1992). Hence, the utilization of qPCR for this study was justified in order to produce meaningful and reliable results.

The detection limit was determined using a similar study protocol mentioned in a previous study that assessed the presence for all four periodonto-pathogens (K. Boutaga et al., 2003). The detection limits for each bacterium was as follows; *P. gingivalis* was 3.897 x 10^5 cells/µl, *T. forsythia* was 2.679 x 10^5 cells/µl, *P. intermedia* was 3.38 x 10^5 cells/µl and *A. actinomycetemcomitans* was 4.336 x 10^5cells/µl. This detection limit for *P. gingivalis*, *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* was used since it was considered to be the cut-off point beyond which the early signs of clinical changes manifested.

5.7 Microbiological Data

The qPCR result for CP subjects showed significantly higher prevalence and mean counts of all tested periodontal pathogens namely *P. gingivalis, T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* as compared to the non-CP subjects (p <0.05). The prevalence for *P. gingivalis* and *T. forsythia* was more than 90% in the CP group while *P. intermedia* was more than 70% in the CP group. Similar findings have been reported by Conrads et al. (1996) who also found *P. gingivalis, T. forsythia* and *P. intermedia* significantly higher in diseased subjects than periodontally healthy subjects. Furthermore, research done by Rajakaruna et al. (2018) also found using PCR, 75% of CP patients had high counts of *P. gingivalis* and *T. forsythia*.

A study done by Stingu et al. (2012) based on conventional cultivation method has reported the most frequently isolated species in CP subjects was *P. intermedia* which was present in 79% of diseased subjects compared with 30% healthy subjects. This study has also carried out bacteria identification using qPCR and failed to detect *P. intermedia.* Nevertheless *P. gingivalis, T. forsythia* and *A. actinomycetemcomitans* were detected, with the highest percentage of detection in CP subject was *T. forsythia* > *P. gingivalis,* > *A.*

actinomycetemcomitans. Factors that could have caused this may be due to the fact that their test group samples included both aggressive periodontitis and chronic periodontitis subjects.

In the current study, the prevalence for *A. actinomycetemcomitans* in the CP group was lowest which was less than 10% of the CP subjects. Similar findings were reported in the study by Umeda et al. (2004) where out of 6 periodontal pathogens being tested, *A. actinomycetemcomitans* was found to have the lowest percentage among diseased patients which was only 1.8% (Umeda et al., 2004). Furthermore, none of the healthy subjects reported the presence of *A. actinomycetemcomitans* in this study. Stingu et al. (2012) have also reported only 10% detection in CP subjects and there was no significant difference compared to the healthy group. The low detection of *A. actinomycetemcomitans* in this study could be explained by the fact that *A. actinomycetemcomitans* does not fall into any of microbial complexes for CP as explained by Socransky et al. (1998) and they are more related to cases of aggressive periodontitis (Socransky et al., 1998).

The different identification methods used in each reported study; the different case definition used for the disease groups as well as the age range of subjects have made comparisons of the microbiologic results of the present study with other previous study more complicated. Future studies should use standardized case definitions to allow for better comparisons.

P. gingivalis was the only significant predictor associated with CP after controlling for the confounding factors. This is similar to the findings by Haffajee et al. in 1998 which provided evidence of an association between *P. gingivalis* with chronic periodontitis. Similar to our study, Haffajee et al., (1998) also found that *P. gingivalis* was not only high in numbers in CP but also in healthy subjects. This may be due to the fact that in this current study healthy subjects were found to have VPI (19.53%) and GBI (9.78%). Even though the quantity is quite low this may contribute to the reason why *P. gingivalis* was also found in healthy

subjects (Lee et al., 2005). Hajishengallis et al., (2012) have stated that in polymicrobial communities, there are interspecies interaction, and this ensures opportunities for competitive and cooperative interspecies interactions. For example, certain low-abundance microbial pathogens, such as *P. gingivalis*, known as keystone-pathogens, it able to initiate inflammatory disease and also may provide growth factors or attachment opportunities for others (Stingu et al., 2012).

Similar to our study, Haffajee et al (1998) did not find any significant association between *T. forsythia*, *P. intermedia* and *A. actinomycetemcomitans* with CP. A later study by Stingu et al. (2012) however reported that *T. forsythia* which is a member of the red complex and *P. intermedia* which is from the orange complex (figure 2.2) were significantly associated with periodontitis patients. However, their study did not provide a clear case definition for their periodontitis classification and this may have caused overlaps in the groups.

When, multiple regression analysis was performed, it was found that the presence of *P. gingivalis* was influenced by CAL (mm) while the presence of *T. forsythia* was influenced by PPD (mm) ($p < 0.001$). Similar findings were reported by Stingu et al. (2012) where it was found that *T. forsythia* was significantly associated with PPD ($p < 0.001$). Our results strengthen the findings by (Socransky et al., 1998) and (Dwyer & Socransky, 1968) who reported that periodontal pathogens in the red complex such as *P. gingivalis* and *T. forsythia* were strongly related to pocket depths and attachment loss. The inability to remove the plaque debris causes the retention of periodontal pathogenic bacteria in deep periodontal pockets (Rabbani et al., 1981). The higher the PPD, the higher the CAL which then leads to increase in number of periodontal pathogens in the site (Gemmell et al., 2007).

5.8 Enzyme-linked Immunosorbent Assay (Elisa)

Many studies on protein determination, either to assess inflammatory mediators for periodontitis or other studies have been conducted using ELISA (Cui et al., 2014; Duarte et al., 2010; Honda et al., 2008; Kim et al., 2013; Özçaka et al., 2011b). ELISA was found to be the method that offers highly sensitive and specific analyte detection (Lequin, 2005). Unlike qPCR, the detection limit for protein quantification is stated on individual kits. Minimum detection limit for resistin was 0.026ng/ml, IL-17 was <0.015ng/ml, IL-6 was 0.00007ng/ml and TNF- α was 0.0016ng/ml. The very minimum detection limit of all four inflammatory mediators was due to the minimum concentration of a particular substance that measured and reported with 99% confidence (Lequin, 2005).

5.9 Inflammatory Mediators Data

The protein determination data from ELISA kits showed significantly higher presence of all inflammatory mediators in CP group as compared to non-CP group ($p < 0.001$). A multiple regression analysis was subsequently performed to control for confounding factors and to find out the possible significant predictors of chronic periodontitis among Malaysian adults. In this study, all inflammatory mediators were associated with CP group. However, only IL-17 showed significant association with clinical parameters CAL and VPI.

Similar findings have also been reported for resistin in a systematic review done by Akram et al., (2017) which found resistin was significantly higher in CP as compared to healthy subjects. They reported that resistin acts as an inflammatory modulator in chronic periodontitis. Contrary findings were however reported by Devanoorkar, Dwarakanath, Gundanavar, Kathariya, & Patil, (2012) which found no significant difference in the presence of resistin in non-CP and CP groups. Similar to our findings, they also reported that resistin

had no significant correlation with clinical parameters. This may indicate that resistin levels were more associated with obesity levels rather than periodontal destruction (Furugen et al, 2008) However, in our study we did not assess obesity levels and therefore are not able to discuss the effect of obesity on resistin levels of our patients.

Similar to our findings, IL-17 was also found to be significantly higher in CP subjects compared to healthy subjects by Awang et al. (2014). In another study comparing CP subjects with and without oral lichen planus (OLP) (Wang et al., 2013), it was also demonstrated that levels of serum IL-17 in OLP-CP group was higher compared with OLP group ($p < 0.05$) and healthy group ($p < 0.01$). None of the previous studies reported contrasting findings of IL-17 in CP group. None of the previous studies found that IL-17 was a predictor for CP status however previous studies found that IL-17 had a strong correlation with cytokines such as IL-4 and IL-10 (Chen et al., 2015) and IL-6 and IL-1β (Cardoso et al., 2009; Mitani et al., 2015) in periodontitis as compared to healthy group. In our study we did not test this correlation with other cytokines.

Awang et al. (2014) explained that there are subfamilies in IL-17 that have different functions. Both IL-17A and IL-17E have opposing roles in the pathogenesis of periodontitis. They have proposed that IL-17A is pro-inflammatory while 1L-17E is anti-inflammatory. IL-17E can negatively regulate periodontal pathogens and provide a protective role against pathogens while IL-17A induces other cytokines to enhance bone loss (Awang et al., 2014). Currently the role of the other IL-17 subfamilies IL-17B, IL-17C and IL-17D is still unknown (Awang et al, 2014). IL-17F has also been detected in periodontal tissues (Konermann et al., 2013) and derived from the same subset with IL-17A from TH17 (Kolls & Khader, 2010). In this study the IL-17 levels assessed was a general IL-17 levels and not the sub-families of IL-17. Among all inflammatory mediators assessed, only IL-17 showed association with other clinical parameters which were CAL and VPI. VPI had a positive correlation with IL-

17 whereby concentration of IL-17 increased by 3.4 pg/ml for every addition of mean % VPI. With the increase in plaque accumulation, there was an increase in the inflammatory process and this may explains the increase in IL-17 levels.

In this study however, CAL was negatively correlated with IL-17 whereby IL-17 levels decreased by 134 pg/ml for every addition of mean CAL. This is in contrast to the findings reported by Awang et al, (2014) where it was reported that IL-17 had a significant positive correlation with CAL, PPD and GBI. The reason for the opposite findings may be due to the fact that our subjects may have secreted higher levels of IL-17 E that has an anti-inflammatory effect as reported by Awang et al, (2014).

As for IL-6, studies by Becerik et al. (2012) and Tymkiw et al. (2011) found similar findings as the current study whereby CP groups had significantly higher IL-6 levels compared with the healthy group ($p < 0.05$). A contrary finding for IL-6 was reported by Chen et al. (2015), where they found no significant difference between CP and healthy group. However, in a recent study by Batool et al. (2018) it was reported that as the disease severity gradually increased from mild to moderate and severe, the levels of IL-6 and IL-17 will also increase. In the current study, the diseased group had localized severe CP and this could explain the reason for the significant levels for IL-6.

The current study demonstrated that TNF-α was detected in all CP subjects but none of the healthy subjects. Study done by Tymkiw et al. (2011) also reported that TNF-α was significantly higher in CP subjects compared to non-CP group. In contrast, Chen et al. (2015), demonstrated that there was no difference in TNF-α between CP and healthy groups. In another study it has also been reported that TNF-α was depressed in smokers with periodontitis as compared with non-smokers with periodontitis (Tymkiw et al., 2011, Cesar-Neto et al., 2007). In the current study, none of the healthy subjects had any TNF-α present.

5.10 Correlation between periodontal pathogens and inflammatory mediators

In the present study, the CP group only showed significant but weak correlation between *T. forsythia* and IL-17 ($r = 0.189$; $p = 0.016$) and between *A. actinomycetemcomitans* and TNF-α ($r = 0.166$; $p = 0.033$).

Similar findings were reported by Kesavalu et al. (2002), which stated that *A. actinomycetemcomitans* had a significant correlation with expression of TNF-α. However, in their study they also reported that *P. gingivalis* was more potent than *A. actinomycetemcomitans* in inducing cytokine expression which was not shown in the current study. Yamaguchi et al. (2004) have explained more on the relationship between *A. actinomycetemcomitans* and TNF-α. *A. actinomycetemcomitans* produces a leukotoxin (Ltx) that is able to destroy specific target cells via an apoptotic effect (Lally et al., 1989; Mombelli et al., 1999). Byrne & Reen (2002) and Zychlinsky & Sansonetti (1997) have also shown that the lipopolysaccharide of *A. actinomycetemcomitans* was able to regulate the apoptosis of neutrophils and monocytes/macrophages. The regulation was through the effect of endogenous cytokines (Zychlinsky & Sansonetti, 1997). This may explain the correlation between *A. actinomycetemcomitans* and TNF-α. However Kesavalu et al. (2002) have reported that non-pathogens are also able to induce TNF-α and therefore more research needs to be done to look into this link.

To date, no studies have examined any correlation between *T. forsythia* and IL-17. *T. forsythia* which falls in the 'red complex group' is involved in periodontal disease initiation and progression as well as periodontal tissue destruction (Yu et al., 2007, Li et al., 2012). The accumulation of bacteria from the 'red complex' then causes release of IL-17 (Li et al., 2012) which either has pro or anti-inflammatory effects on the periodontium of patients (Awang et al., 2014).

The results show that there was no significant correlation between *P. gingivalis* and *P. intermedia* with any inflammatory mediators. However, these findings are in contrast to studies by Tamura et al. (1992); Yu et al. (2008); Guan et al. (2009); Nelwan et al. (2019). The reason why there was no significant correlation between *P. gingivalis* and *P. intermedia* with any inflammatory mediators being detected in our study might be due to the fact that our samples were pooled and not site-specific (Stingu et al., 2012). Also, the inflammatory mediators used were taken from serum samples which reflects systemic inflammatory response as compared to samples taken from GCF or saliva which represent local inflammatory response at the site of inflammation.

5.11 Limitation of the study:

The following were limitations to the current study:

1) Inflammatory markers were assessed only from serum samples. This will give results which were more reflective of systemic host response. Samples should also have been procured from GCF or saliva so as to reflect or more localised inflammatory response.
2) Obesity levels such as BMI levels should have been assessed to see the relationship of resistin with obesity markers.
3) For the control group, the sample size was slightly smaller than the numbers required by sample size calculation.
4) In the methodology, we have ensured that the samples used were stored less than 12 months before being processed in the laboratory. However, this duration of samples kept in the freezer may have had an effect on the DNA levels of the plaque samples.

5.12 Suggestion for future studies

The following are possible suggestions which may be undertaken when conducting a future study:

1. To obtain saliva and GCF samples to better reflect localised host response in the oral cavity.
2. To increase the number of samples to better reflect the profiling based on sample size calculation.
3. To look further into the link between *P. gingivalis*, *T. forsythia* and IL-17 and its role on chronic periodontitis.
4. To study further on association of chronic periodontitis and the sub-families of IL-17 and their role in periodontitis.

CHAPTER 6: CONCLUSIONS

Within the limitations of the current study, the following can be concluded:

- Adults with chronic periodontitis were found to have significantly higher mean counts of *P. gingivalis* and also higher levels of resistin, IL-17, IL-6 and TNF-α as compared to healthy controls.
- *P. gingivalis* was positively associated with mean CAL while IL-17 was negatively associated with mean CAL.
- *T. forsythia* was positively associated with mean PPD.
- *T. forsythia* was found to have a weak but significant positive correlation with IL-17 in adults with CP.
- *A. actinomycetemcomitans* was found to have a weak but significant positive correlation with TNF-α in adults with CP.

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