EVALUATION OF SALIVARY CORTISOL, RANKL AND OPG LEVELS IN CHRONIC PERIODONTITIS PATIENTS ATTENDING HOSPITAL UNIVERSITI SAINS MALAYSIA

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LIST OF ABBREVIATIONS

%	Percentage
A.a	Aggregatibacter actinomycetemcomitans
ACTH	Adrenocorticotrophic hormone
AVP	Arginine vasopressin
B-cells	B lymphocyte cells
CAL	Clinical attachment loss
CBG	Corticosteroid- binding globulin
CEJ	Cemento enamel junction
CI	Confidence interval
CLSI	Clinical and Laboratory Standard Institute
CP	Chronic periodontitis
CRF	Corticotrophin releasing factor
CXC	Chemokines
DM	Diabetes mellitus
ELISA	Enzyme-linked immunosorbent assay
ECLISA	Enzymechemical immunosorbent assay
GBI	Gingival bleeding index
GCF	Gingival crevicular fluid
HPAA	Hypothalamus pituitary adrenal axis activity
HUSM	Hospital Universiti Sains Malaysia
ICC	Intraclass correlation coefficient
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8

IL-10	Interleukin 10
IL-11	Interleukin 11
IL-17	Interleukin 17
IL-21	Interleukin 21
IL-23	Interleukin 23
JEPeM	Jawatankuasa Etika Penyelidikan Manusia
LPS	Lipopolysachharide
mg/L	Milligram/litre
mL	Milliliter
mm	Millimeter
mmol/L	Millimoles per liter
mRNA	Messenger ribonucleotide acid
MMP's	Matrix metalloproteanases
NFκB	Nuclear factor κB
NHANES	National Health and Nutrition Examination Survey
NOHSA	National Health Survey
NO	Nitrous oxide
Ng/mL	Nanogram per mililters
NSPT	Non-surgical periodontal therapy
OCPs	Osteoclast precursor cells
ODF	Osteoclast differentiation factor
OPG	Osteoprotegerin
OPGL	Osteoprotegerin ligand
PDL	Periodontal ligament
PGE2	Prostaglandin E2 cells
PMN	Polymorphonuclear cells
PPD	Periodontal pocket depth
PPSG	Pusat Pengajian Sains Pergigian
PS	Plaque score

PVN	Paraventricular nucleus
PTH	Parathyroid hormone
RANKL	Receptor Activator of Nuclear Factor kappa-B Ligand
ROS	Reactive oxygen species
SD	Standard deviation.
SE	Standard Error
T-cells	T lymphocyte cells
Th-cells	T helper cells
TGF-b	Tumor growth factor beta
TIMP	Tissue inhibitor of matrix metalloprotein
TNF-α	Tumor necrosis factor alpha
TNFSF11	Tumor necrosis factor super family member 11 gene
TRL	Toll-like receptors
TRAIL	TNF-related apoptosis-inducing ligand
TRANCE	TNF related activation induced cytokine receptor
USM	Universiti Sains Malaysia
USA	United States of America
UTR	Untranslated region
WHO	World Health Organization

PENILAIAN PARAS KORTISOL, RANKL DAN OPG AIR LIUR DALAM PESAKIT PERIODONTITIS KRONIK DI HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRAK

Periodontitis kronik (CP) adalah jangkitan inflamasi gred rendah yang berlaku pada struktur periodontal yang disebabkan oleh kehadiran flora patogenik dan plak biofilem yang berinteraksi dengan sistem imun dalam tempoh tertentu. Tabiat merokok, pengambilan alkohol, tekanan, kegemukan, sindrom metabolik, diabetes melitus, keadaan-keadaan serta penyakit-penyakit sistemik yang lain telah dikenal pasti sebagai faktor berisiko terhadap penyakit ini. Tujuan kajian ini adalah untuk mengkaji dan menilai perkaitan di antara paras kortisol serta biopenanda protein RANKL dan OPG dalam air liur pesakit CP. Dalam kajian keratan rentas ini, data sosio-demografik, parameter periodontal klinikal [kedalaman poket periodontal (PPD), aras atakmen klinikal (CAL), skor plak (PS) dan indeks pendarahan gingiva (GBI)] telah diambil dan skor DASS-21 telah dinilai berdasarkan borang soal selidik. Kepekatan kortisol serta protein RANKL dan OPG dalam air liur telah dinilai dalam kumpulan pesakit yang terdiri daripada subjek yang sihat iaitu kumpulan 1, pesakit CP tahap ringan iaitu kumpulan 2 serta pesakit CP tahap sederhana hingga teruk iaitu kumpulan 3 di Klinik Periodontik, Pusat Pengajian Sains Pergigian, Hospital Universiti Sains Malaysia. Min kortisol, protein RANKL dan OPG dalam air liur adalah tinggi dalam kumpulan 3 (pesakit CP tahap sederhana hingga teruk) iaitu sebanyak 7.46 ng/ml, 0.23 ng/ml, dan 1.78 ng/ml, mengikut turutan. Keputusan soal selidik DASS-21 menunjukkan kumpulan

3 mempunyai skor yang lebih tinggi terhadap kemurungan, kebimbangan dan tekanan. Skor DASS-21 juga menunjukkan perkaitan secara signifikan di antara paras kortisol air liur dengan kedua-dua kumpulan pesakit CP iaitu tahap ringan (kumpulan 2) serta tahap sederhana hingga teruk (kumpulan 3). Kesimpulannya, tekanan yang tinggi berpotensi menjadi faktor berisiko yang berkait dengan peningkatan kelesapan tulang bagi pesakit CP di Kelantan. Penurunan paras protein OPG dan peningkatan paras protein RANKL menyumbang kepada peningkatan kelesapan tulang dalam pesakit CP tahap awal.

EVALUATION OF SALIVARY CORTISOL, RANKL AND OPG LEVELS IN CHRONIC PERIODONTITIS PATIENTS ATTENDING HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRACT

Chronic periodontitis is a low grade inflammatory infection of supporting periodontal structure ocurring in the presence of pathogenic flora and plaque biofilm interaction with immune system over a period of time. Multiple risk factors such as smoking, alcohol intake, stress, obesity and metabolic syndrome, diabetes mellitus, other systemic diseases or conditions have been identified as a potential risk factors for this disease. The aim for this study is to assess and evaluate the association between levels of salivary cortisol, RANKL and OPG protein biomarkers in chronic periodontitis (CP) patients. In this cross sectional study, clinical periodontal parameters [periodontal pocket depth (PPD), clinical attachment level (CAL), plaque score (PS) and gingival bleeding index (GBI)] were performed and DASS-21 scores were assessed from the survey form. Protein concentrations for salivary cortisol, RANKL and OPG were assessed in study groups consisting of mild chronic periodontitis patients in group 2, moderate to severe chronic periodontitis patients in group 3 and healthy subjects in group 1 at Periodontic Clinics of School of Dental Sciences, Hospital Universiti Sains Malaysia. Mean levels for salivary cortisol, RANKL and OPG protein were higher for group 3 (moderate to severe chronic periodontitis) with 7.46 ng/ml, 0.23 ng/ml and 1.78 ng/ml. Higher depression, anxiety and stress score were also noted for group 3 (moderate to severe chronic periodontitis) from DASS-21 questionairre. DASS scores were significantly

associated with salivary cortisol levels in both mild and moderate to severe chronic periodontitis groups. In conclusion, high stress is considered as a potential risk factor associated with increase bone loss in chronic periodontitis in Kelantanese population. Low OPG and high RANKL protein levels are responsible for higher bone loss in mild chronic periodontitis patients.

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CHAPTER 1.

INTRODUCTION

1.1 Background of the study

The oral cavity is a unique diverse environment which harbors an immense number of microscopic organisms of various species within the soft tissues like oral mucosa and hard tissues of the teeth. Oral environment consists of aerobic and anaerobic microorganisms that favours the temperate and gentle environment of oral cavity forming a multiplex structure and a stable biological community (Marsh and Martin, 1992). However, often these microbes can cause periodontal diseases like chronic periodontitis and gingivitis, which are seen to be the most commonly detected microbial diseases affecting the humans across the globe (Albandar 2002).

The bacterial species especially the red-complex are actively linked with periodontal disease, which include the bacteria *Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola.* Other than the red complex, *Aggregatibacter actinomycetemcomitans* and *Eubacterium nodatum* are notably associated with periodontal disease (Darveau, 2010). The gram-negative microorganisms were considered as the etiologic determinants of the periodontal diseases and affected the clinical parameters which led to periodontitis (Darveau 2010; Yang et al., 2016). Initiation and advancement of periodontitis occur as a combination of genetic and ecological variables including negligence of oral hygiene, stress/anxiety/depression, smoking and systemic diseases (Dibart, 2009).

Many studies have noted that if proper cleaning and brushing of teeth were neglected, the oral bacteria form a microbial film on the teeth surfaces. This microbial film includes many enzymes, proteins and cellular debris, which is present in the saliva, in addition to the food particles that enter the mouth. All these factors are responsible for the formation of a plaque biofilm on the surface of the teeth. This plaque film leads to a further bacterial invasion in the deeper periodontal structures, like the collagen layers. This triggers the breakdown of the periodontal structure due to the microbial attack and the corresponding host responses (Hajishengallis, 2014). The different external or internal stimuli activate the host immune-inflammatory response, which is the main mechanism that maintains the oral periodontal health. The inflammatory response triggers many macrophages, neutrophils, cytokines, and activates the T- and B-lymphocytes and finally, the complement system (Abbas *et al.*, 2014). Cytokines are produced and secreted by immune and non-immune cells in our body. Cytokines are able to initiate and control a channel of events that cause the up or down regulation of specific genes. Hence, these cytokines are responsible for modifying pro-inflammatory and anti-inflammatory response occuring within the body (Garlet, 2010; Graves, 2008).

A persistent and chronic inflammatory reaction activates some inflammatory molecules like matrixmetalloproteinases (MMPs) and the receptor activator of nuclear factor-κB (RANKL) ligand (Lacey *et al.*, 1998; Sorsa *et al.*, 2004). Two pre-inflammatory cytokine molecules like the Tumour Necrosis Factor-alpha (TNF-α) and Intereukin-1 beta (IL-1β) increase the RANKL secretion via the T and B cells (Van Dyke 2007; Chen et al., 2014). However, OPG was a soluble antagonist receptor that inhibited the osteoclast differentiation as it competed with the RANKL for the receptor

(Simonet et al., 1997). The ratio of the RANKL and OPG molecules is a good indicator of the bone tissue degradation during the progression and severity of the periodontal diseases (Belibasakis and Bostanci 2012).

Stress is the protective response of the body against any internal or external factor that can harm the body, i.e., stressors. McEwen (2000) stated that any event or scenario which is regarded as scary or frightening to the person can trigger the behavioural and physiological responses in the person. During this process, the central nervous system gets activated via the Hypothalamus Pituitary Adrenal Axis (HPAA) activity, which triggers the hypothalamus to activate and secrete Corticotropin-Releasing Hormone (CRH). The adrenal cortex is also triggered to release glucocorticoids like cortisol (Chrousos 1995). These molecules increase the stress levels in the individual.

Glucocorticoids downregulate the immunological expression by activating the leukocyte molecules and inhibiting the growth of cytokine molecules like the Intereukin 1-beta (IL-1b), IL-6 and other inflammatory mediators (Chrousos 1995). The physiological events which are further activated due to the HPAA mechanism also decrease the immunity of the body since they suppress the activities of the IgA, IgG and neutrophils. All these factors increase the probability of a severe infection, which is responsible for causing destructive periodontitis (Wein 2000; Khayat 2007). Psychosocial stress triggers many autonomic nervous system-based reactions, which cause a catecholamine secretion (i.e., epinephrine and norepinephrine) that affects the prostaglandins and increases the activity of the proteases. This complete chain of events significantly aggravates the periodontal breakdown and damage (Dimsdale and Moss 1980). The health of the periodontal tissue is affected by the daily stresses, which affect

the lifestyle of the person in many ways. Some of the other factors which affect periodontal health include alcohol consumption, smoking, neglecting oral hygiene and improper dental care (Jaiswal *et al.*, 2016).

Many researchers have considered the salivary cortisol levels as an important stress biomarker in their studies. The cortisol hormone is a glucocorticoid, which is synthesised and released by the adrenal cortex. It is present and detected in all the body fluids, like the gingival crevicular fluid (GCF), serum, urine, saliva, and blood (Genco et al., 1998; Mengel et al., 2002). This hormone shows many immunosuppressive and anti-inflammatory functions and properties, which prevent the lymphocyte formation and induces lymphatic tissue hyperplasia (Snell 1976). This further decreases the humoral immune defence of the body. Cortisol is a common stress biomarker, and the salivary cortisol levels are measured for assessing many systemic and psychological diseases (Hjortskov et al., 2004). Cortisol is seen to inhibit the proliferation of the fibroblasts in the inflammatory granulation tissue, which affects the homeostasis of the immune system (Hagan *et al.*, 1992).

Saliva is a very important body fluid which displays many advantages. It possesses many properties that assist in diagnosing the different oral conditions and diseases like oral cancer, periodontitis, Alzheimer's disease, etc. (Nagler 2009). Furthermore, it shows many advantages, like it is easily available, inexpensive, can be collected and stored without any hassle (Dolan et al., 2004). Also, a saliva sample can be collected at any time of the day, without any specific clinical setting. Unlike the other body fluids like the serum, blood, CSF, etc., saliva collection does not require an invasive or difficult process (Pfaffe et al., 2011). Saliva contains many proteins and

biomarkers that can offer an important link to the different diseases and conditions affecting the human body. These biomarkers are still under investigation (Al Kawas *et al.*, 2012).

In this study, the researchers have investigated the salivary cortisol levels as the stress biomarker. Additionally, they circulated a Malay-translated version of the DASS-21 questionnaire for determining the stress levels of the people included in the study groups. This Malay-translated, DASS 21 questionnaire was a shorter version of the original DASS 42 questionnaire that was developed by Lovibond (1995). It shows internal consistency and reliability and can be used for determining the stress-related symptoms. However, it was not used for the clinical diagnosis of psychological disorders (Musa et al., 2007; Tran et al., 2013; Wang et al., 2016).

There are very few studies which have determined the stress levels and its subsequent effect on oral health in the Malaysian population. This is the first study which has analysed the mean concentration of the salivary cortisol hormone, OPG, RANKL, along with anxiety, stress and depression levels in the respondents who suffered from mild or severe periodontitis. These levels were correlated to the periodontal parameters noted for every group.

1.2 Problem statement & justification of the study

In view of periodontal disease initiation and progression, the role of protein biomarkers such as RANKL and OPG in inflammatory process could be correlated with the stress condition among chronic periodontitis patients. The studies investigating such relationship are scarcely reported. Thus, present study will be looking on the salivary

cortisol and protein biomarkers RANKL and OPG levels. Stress scores using DASS-21 questionairre between healthy subjects, mild and moderate to severe chronic periodontitis patients will be assessed in this study. Association among all these variables with periodontal clinical parameters will be assessed. Additionally, correlation among all these variables with periodontitis disease severity and progression was also evaluated in this study.

The earlier studies noted a positive correlation between the serum and salivary cortisol levels and the initiation and progression of the periodontal disease. However, these studies did not indicate a clear cause and effect relationship between the stressors, salivary cortisol levels and the periodontitis disease. Hence, the actual mechanism which involves the salivary cortisol levels and the periodontal disease is still unknown (Rai et al., 2011). Stress was seen to be a potential risk factor which led to the onset of the periodontal disease as it could affect the local oral hygiene of the individual. This affected the release of the cortisol hormone from the HPPA centre into the blood or other body fluids.

1.3 Objectives of the study

1.3.1 General objective

To evaluate the association among levels of salivary cortisol, RANKL and OPG protein biomarkers in chronic periodontitis (CP) patients.

1.3.2 Specific objectives

i. To assess and compare the levels of salivary cortisol, protein biomarkers RANKL and OPG and periodontal parameters among healthy subjects with mild and moderate to severe chronic periodontitis patients

ii. To assess and compare the stress (DASS-21 scores) among healthy subjects with mild and moderate to severe chronic periodontitis patients.

iii. To assess the association of the salivary cortisol level, protein biomarkers (RANKL and OPG), RANKL/OPG ratio and stress (DASS-21) with periodontal parameters in chronic periodontitis patients.

1.3.3 Research questions

1. Is there any difference between the levels of salivary cortisol, protein biomarkers RANKL and OPG among healthy group, mild and moderate to severe chronic periodontitis patient groups?
2. Is there any difference between the scores of DASS-21 among healthy group, mild and moderate to severe chronic periodontitis patient groups?

3. Are there any association in the levels of salivary cortisol, protein biomarkers RANKL and OPG, RANKL/OPG ratio to the periodontal clinical parameters and stress in chronic periodontitis patients?

1.3.4 Research hypothesis

i) There is a significant difference in the levels of salivary cortisol, protein biomarkers (RANK and OPG) levels with increase periodontal parameters between the healthy subjects, mild periodontitis and moderate to severe chronic periodontitis patients.

ii) There is a significant association of salivary cortisol levels, protein biomarkers (OPG and RANKL) and stress levels in healthy subjects, mild periodontitis and moderate to severe chronic periodontitis patients.

iii) There is a significant difference in the DASS-21 score between healthy subjects, mild periodontitis and moderate to severe chronic periodontitis patients.

CHAPTER 2.

LITERATURE REVIEW

2.1 Periodontium

Periodontium is the fundamental part of the tooth which consists of four major components named gingiva, cementum, periodontal ligament and alveolar bone (Reddy, 2017) as shown in the Figure 2.1 adapted from Xu and coworkers (Xu *et al.*, 2018).

Healthy periodontium is superficially covered with the resilient layer of gingiva, forming a barrier against mechanical and microbial damages. Histologically, gingiva comprises of gingival epithelium and the gingival connective tissue (Bashir *et al.*, 2018). A shallow crevice between gingival epithelial lining and tooth is known as gingival sulcus. Clinically this space is measured using a periodontal probe (a metalic instrument). In clinically healthy gingiva, this probing depth is up to 3 mm. Probing is one of the most significant clinical parameters used to assess the periodontal status (Lang and Bartold, 2018).

Beneath the gingiva, teeth are anatomically positioned within the alveolar bone (Listgarten *et al.*, 1991). The periodontal ligament fibers (PDL) are the connective tissues produced by the fibroblasts, they stretch across the space between the alveolar bone and root surface (de Jong *et al.*, 2017). Cementum is the hard tissue covering the root surface. It aids in anchoring the tooth through insertion of Sharpey's fiber and providing support to periodontal ligament fibers. It also helps in protecting the pulp (Menicanin *et al.*, 2015).

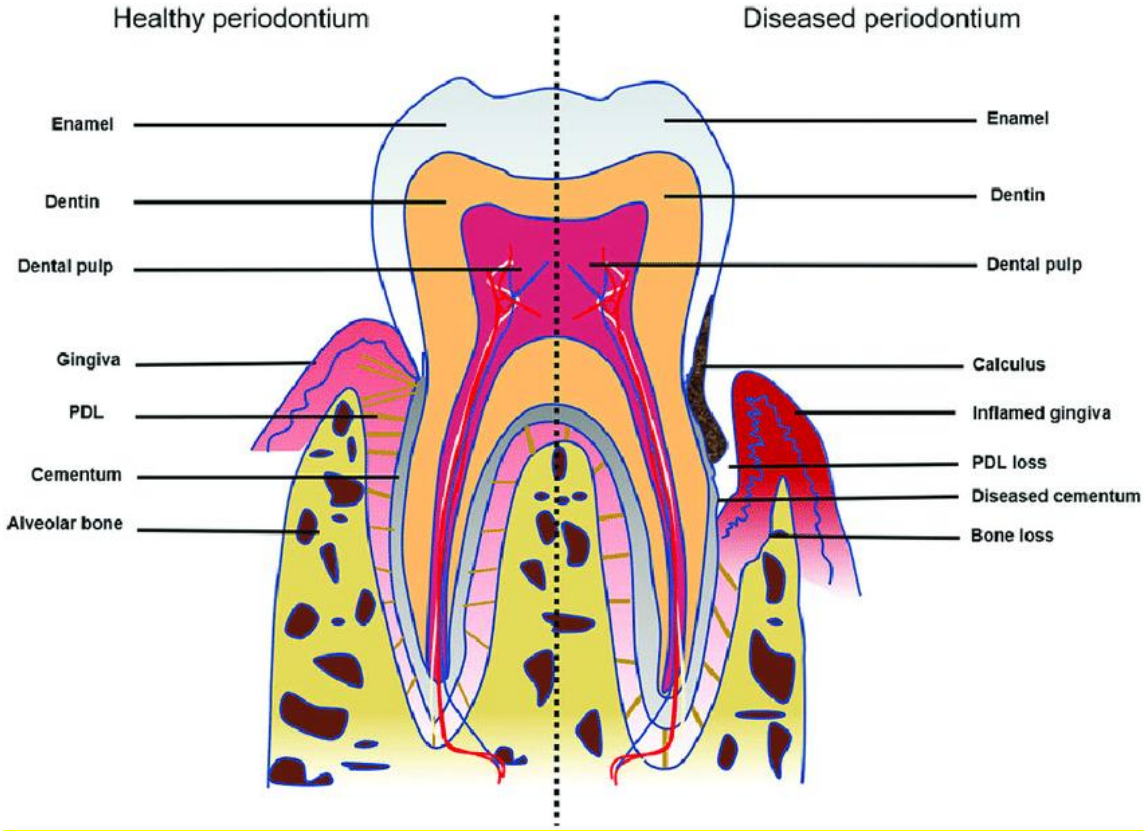

Figure 2.1 Schematic drawing of healthy and diseased periodontium (Figure adapted from Xu et al.,2018).

2.2 Periodontal disease

Periodontal diseases are microorganism induced inflammatory chronic condition that affect the teeth supporting structure. This polymicrobial infectious disease starts with establishment and growth of a small group of gram-negative anaerobic bacteria mainly consisting of *P. gingivalis, Aggregatibacter actinomycetemcomitans, P. intermedia*, spirochetes and others (Mira et al., 2017). These bacteria along with thousands of other bacterial species within the plaque biofilms, made their way apically along the teeth's root surface to initiate the formation of periodontal pockets, they

slowly cause the degradation of the alveolar bone and collagenous fibers of the periodontal ligament (Page et al., 1997).

Bacterial biofilm forms on the teeth surface shelter and provides a chronic microbial stimulus which initiates a local inflammatory response within the gingival mesh (Pihlstrom et al., 2005). Although bacteria are mandatory for periodontal disease to initiate but they also require various factors or determinants such as multiple host and environmental aspects for example genetic predisposition, lifestyle, oral hygiene measures, systemic conditions and diseases and psychological stress contribute towards onset and progression of periodontitis (Genco and Borgnakke, 2013).

Periodontal disease stability is defined as a state in which the periodontitis has been successfully treated through control of local and systemic factors, resulting in minimal bleeding on probing (BoP), optimal improvements in periodontal pocket depth (PPD) and clinical attachment levels (CAL), and a lack of progressive destruction. Clinical signs of the disease do not appear to worsen in extent or severity despite the presence of a reduced periodontium (Lang and Bartold, 2018; McKay, 2019).

Periodontal disease remission/control is defined as a period during disease when symptoms become less severe, but the diseases is not in its active state or condition. This may be a reasonable treatment outcome for individuals with uncontrollable modifying factors. Indeed, for many chronic inflammatory medical conditions such as diabetes mellitus, cardiovascular disease, hyperlipidemia, and rheumatoid arthritis, the goal of disease remission is important and is based on the emerging concept of treat to target (Lang and Bartold, 2018).

The non-surgical periodontal therapy is to halt the progression and control the factors contributing in the periodontal inflammation by the mechanical and chemical debridement of subgingival bacterial deposits and the subsequent control of plaque levels by patients (Greenstein, 1992). Non-surgical therapy consists of several basic clinical procedures including scaling, root planing, oral hygiene instruction, proper counselling as well as other therapies such as caries control, replacement of defective restorations, occlusal therapy, orthodontic movement, and smoking cessation (Pihlstrom et al., 1981).

2.3 Periodontitis

Periodontitis is the inflammatory condition of the periodontium induced by bacteria. As the disease progresses, it causes degenerative changes in the periodontium, resulting in the loss of periodontal ligament and alveolar bone. Subsequently, the gingival sulcus deepens and forms periodontal pocket (Kurgan and Kantarci, 2018).

A patient is defined as periodontitis case as reported in the concensus report 2017 (Papapanou *et al.*, 2018).

1. Interdental CAL is detectable at ≥2 nonadjacent teeth, or

2. Buccal CAL ≥3 mm with pocketing ≥3 mm is detectable at ≥2 teeth but the observed CAL cannot be ascribed to non-periodontitis related causes such as: gingival recession of traumatic origin; dental caries extending in the cervical area of the tooth; the presence of CAL on the distal aspect of a second molar and associated with malposition or

extraction of a third molar, an endodontic lesion draining through the marginal periodontium; and the occurrence of a vertical root fracture.

It is proposed that there are 4 levels of periodontal health, depending on the state of the periodontium (structurally and clinically sound or reduced) and the relative treatment outcomes:

(1) pristine periodontal health, with a structurally sound and uninflamed periodontium;

(2) well- maintained clinical periodontal health, with a structurally and clinically sound (intact) periodontium;

(3) periodontal disease stability, with a reduced periodontium, and

(4) periodontal disease remission/control, with a reduced periodontium.

2.3.1 Classification of periodontitis

Classification of any disease is of great significance, when it comes to diagnose down the disease along with provision of better treatment regimen. Multiple classification schemes had been proposed, adopted and acknowledged in the past, of which the most renowned was from the Armitage (Armitage, 1999) classification for periodontal disease and conditions in 1999. However, recently in 2017, a world workshop was organized in which consensus was made after reviewing, debating and mutually agreeing on the overall conclusions made by the committee members and prominent reviewers from the specialty of periodontology (Papapanou *et al.*, 2018).

1. In this new periodontitis classification scheme forms of the disease previously recognized as “chronic” or “aggressive” are now grouped under a single category “periodontitis” and are further characterized based on a multi- dimensional staging and grading system.

2. Staging is largely dependent upon the severity of disease at presentation as well as on the complexity of disease management, while grading provides supplemental information about biological features of the disease including a history- based analysis of the rate of periodontitis progression; assessment of the risk for further progression; analysis of possible poor outcomes of treatment; and assessment of the risk that the disease or its treatment may negatively affect the general health of the patient.

2.3.2 Prevalence of periodontitis

According to the reports 13-35% of adult population residing in industrialized countries are suffering from periodontitis disease and approximately 5-8% of them suffer from aggressive periodontitis disease (Johannsen, 2006; Milosavljevic, 2018). The prevalence of severe chronic periodontitis (CP) varies worldwide from 10% to 15% in adult population according to World Health Organisation (WHO) (Petersen and Ogawa, 2012). The latest epidemiological data in the USA has corroborated the high prevalence of periodontitis disease in more than 47% of adult population (Eke *et al.*, 2012b).

In the National Oral Health Survey of Adults undertaken by the Oral Health Division, Ministry of Health, Malaysia, the prevalance rate was found high.

Approximately, 90% of the population aged between 45 and 70 apparently had periodontal problems (Chan *et al.*, 2012). Extreme cases of periodontal disease severity with more pocket depths and cinical attachment loss were reported in Songkhla, which is a province of Southern Thailand. It was predicted that these individuals showed more severity of periodontal diseases when compared to the rest of Asian populations (Corbet *et al.*, 2002). A higher rate of periodontal disease was also found out in Taiwan, according to one of the community-based studies in Keelung (Lai *et al.*, 2007).

Another cross-sectional periodontal study was carried out in Vietnam, targeting middle aged group people from two different provinces in which periodontal disease status was measured clinically showed a significant periodontal attachment loss (Do *et al.*, 2003). In Japan a national level study was conducted and demonstrated that 42% of the tooth loss was due to periodontal diseases and conditions (Aida *et al.*, 2006).

2.3.3 Aetiology of periodontitis

The aetiology and pathogenesis for gingivitis and periodontitis disease has been studied vastly in the past. With the emerging modern technology more advance genetic and molecular studies have been carried out on this disease which revealed the complex microcellular pathways through which periodontal disease initiates and progresses (Bostanci and Bao, 2017; Dentino *et al.*, 2013). The comprehension of periodontal disease in early 1950's was that when left untreated disease pursued an advancement from gingivitis to periodontitis leading ultimately to tooth loss (Arnold *et al.*, 1966; Lang *et al.*, 2009; Moosani, 2012; Schätzle *et al.*, 2009; Teles *et al.*, 2013). Later, in the mid 1960's and early 1970s, studies contemplated the key role of microorganisms in the

etiology of the periodontal disease (Jordan and Keyes, 1964; Kahnberg *et al.*, 1976; Socransky and Haffajee, 1992). This concept was elaborated and practiced by some of the longitudinal studies carried out during 80's to 90's decades. These longitudinal studies proposed that it is not only the bacterial plaque microorganism interaction which initiates the periodontal disease but the underlying host immune reaction within the body also contributes towards disease initiation and progression (Dentino *et al.*, 2013; Kulkarni and Kinane, 2014).

Although this dental plaque or biofilm is considered as a primary requisite to promote the local infection or disease. It also depends upon the host's inflammatory-immune responses to occur in the presence of the microbial overload which in turn causes the initiation, progression and destruction of the periodontium (Darveau, 2010; Li *et al.*, 2017). A local inflammatory reaction activating the innate immune system of the body takes place in the very first stage in response to the underlying bacterial infection (Graves and Cochran, 2003). Recent studies concluded that periodontitis pathogenesis occurs as a result of overload and shifting of multiple bacterial species from healthy to diseased state under the influence of immune-inflammatory reactions which causes the bone loss and degradation of periodontal structures (Abusleme *et al.*, 2013; Hajishengallis *et al.*, 2012).

2.3.4 Pathogenesis of periodontitis

Periodontitis is seen as a long-term plaque persistence at the dento-gingival interface resulting in an advancement and maturation of the dental biofilm, along with low-grade ongoing inflammation in which over the time there occurs an irreversible loss

of the supporting tooth structures (Lalla and Papapanou, 2011). Subgingival habitat under the normal conditions contains a balanced homeostatic relation between innate and immune inflammatory mediators and microbial niche (Hajishengallis, 2015; Meyle and Chapple, 2015).

A pathogenic biofilm is considered as a primary prerequisite for the periodontal inflammation to start and initiate in the first place (Johansson and Dahlén, 2018) however, this dental biofilm on its own cannot initiate any inflammatory reaction or process because the whole procession requires an interaction of host response, inflammatory immune responses within the body (Kurgan and Kantarci, 2018).

Initial host immune inflammatory reaction mediates neutrophils, which fails to maintain this homeostatic balanced environment, paving path for the penetration and intrusion of pathogenic microorganisms within the connective tissues and there they interact with various other types of immune cells like macrophages, dendritic cells, T cell types and a subset of innate like cells. These cells further produce proinflammatory mediators like bone degrading cytokines like interleukin 1-beta (IL-1b), interleukin 17 (IL-17), tumour necrosis factor (TNF) subclass enhancing destructive process. Moreover, Th helper (Th) cells production and regulation during this process is under the control of these pro inflammatory mediators, which are highly responsible for escalating inflammatory response (Hajishengallis, 2014).

More recently a number of independent multi genomic and programmatic oral microbial studies revealed a polymicrobial synergy and dysbiosis (PSD) model. This model provides a concept which explains a synergistic and dysbiotic association of oral

microflora. Numerous species of microorganisms take part in the pathogenesis of periodontitis, which are considered to play an important role during disease course (Hajishengallis, 2014; Hajishengallis and Lamont, 2012; Lasserre *et al.*, 2018). According to this PSD model, *Porphyromonas gingivalis* is the main pathogen responsible for disruption of periodontal homeostasis as well as it compels and obligates the surrounding flora, pathogens called as pathobionts consisting of various microbial colonies to stampede and results in the inflammatory tissue disruption (Zenobia and Hajishengallis, 2015).

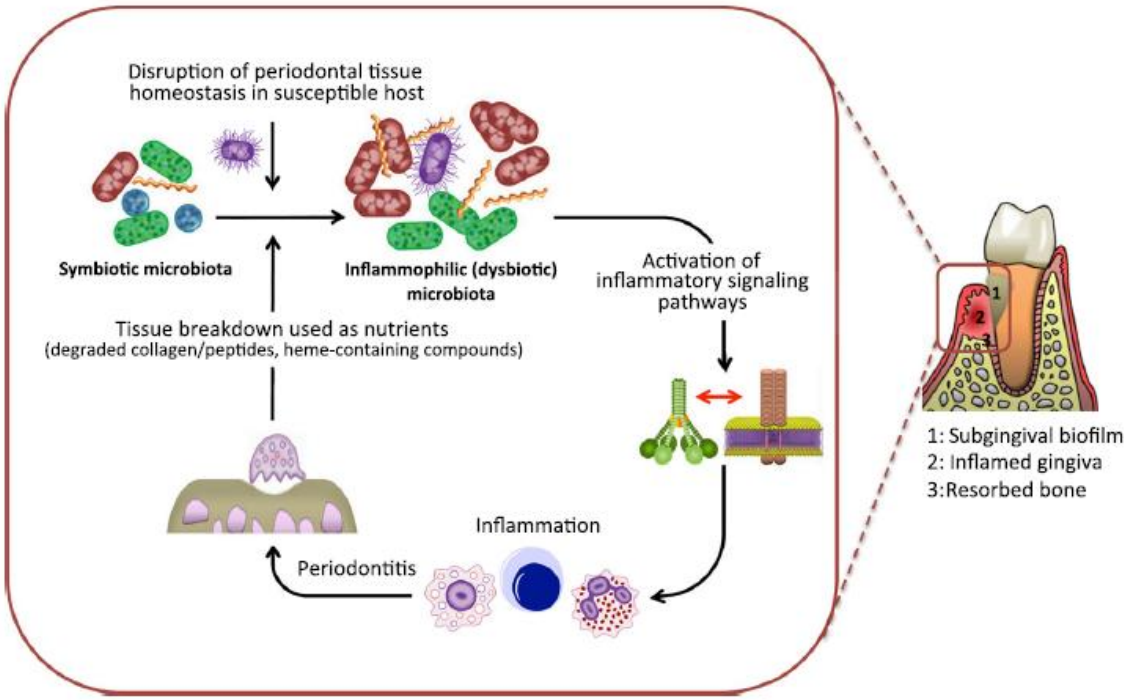

Figure 2.2 A schematic diagram showing keystone pathogens influencing the periodontal inflammation. (Figure adapted from Hajishengallis and Lamont 2014)

2.3.5 Bone Metabolism

Bone is the specialized connective tissue comprising of various cells, collagenous and inorganic matrix (Green and Kleeman, 1991). Alveolar bone resorption is the result of excessive osteoclastic activity that leads to an imbalance in bone remodeling (Mundy, 1993). The level of bone mass which is essential to execute various functions is maintained by a balanced act of bone formation and bone resorption (Hadjidakis and Androulakis, 2006). This process is highly coordinated and regulated by two specialized cells, osteoblasts the bone forming cells and osteoclasts the bone

resorbing cells (Rodan, 1992). These cells are controlled by various hormones, inflammatory mediators, cytokines and growth factors (Lu *et al.*, 2006; Lumachi *et al.*, 2009). Remodeling of alveolar bone occurs in different environments. Physiologically, it occurs during tooth eruption as well as during normal occlusal forces (Saffar *et al.*, 1997). It also occurs as a result of clinical interventions such as orthodontic tooth movements. Pathological conditions that cause bone resorption include periodontal disease, periapical pathologies, granulomas and tumors (Lerner, 2004).

2.3.6 Osteoclastogenesis occurring at cellular level in periodontitis

Osteoclasts are large multinucleated cells characterized by their ability to resorb bone (Feng and Teitelbaum, 2013). They are derived from hematopoietic stem cells within the bone marrow and their mononuclear precursors are typically found circulating in peripheral blood (Väänänen, 2005). Osteoclasts are differentiated through two regulatory cells including RANKL and macrophage colony stimulating factor (M-CSF) (Boyle *et al.*, 2003).

The great amount and interactions of bacterial end products with various inflammatory stimuli primarily lipopolysaccharide with the periodontal and immune cells stimulates the differentiation of osteoclasts and thus activates pathologic bone loss. These factors influence the state and progression of the periodontal disease (Garlet, 2010). Several biomolecules are produced during periodontal inflammation, these biomolecules are also known as biomarkers (Ebersole *et al.*, 2015). These biomarkers lead towards connective tissue invasion and breakdown of alveolar bone. Levels of these biomarkers are readily and easily detectable within the saliva and GCF of periodontitis

patients (Frodge *et al.*, 2008). RANKL and OPG are two important biomarkers that plays a significant role in periodontal bone loss process.

Various inflammatory cytokines including IL-1, IL-6, IL-17, TNF-a, PGE-2, MMP's are involved in the pathogenesis of periodontitis. However, the key cytokine system that regulates bone remodeling process is dependent on the receptor activator of nuclear factor-kappa B ligand (RANKL) and Osteoprotegerin (OPG) balance (Hofbauer *et al.*, 2001). RANKL causes the maturation of osteoclast precursors (OCPs) leading to bone resorption in periodontitis (Hajishengallis, 2014). This mechanism is shown in the figure 2.3.

Infiltrated leukocytes produce IL-1 & prostaglandins E2 (PGE2) through osteoblasts, periodontal fibroblast & gingival fibroblasts (Lu et al., 2006). PGE2 is produced by immune cells, fibroblasts and other resident gingival cells and has a wide range of biological effects on the cells of the diseased gingiva (Zhang *et al.*, 2010). PGE2 include the stimulation of MMPs and causes osteoclast formation via receptor activator of nuclear factor-κB ligand (RANKL) (Bage *et al.*, 2011; Hienz *et al.*, 2015b). Interleukin-17 (IL-17) is also thought to contribute in the bone resorption process in periodontitis. IL-17 mediates the production of matrix metalloproteinases (MMPs), chemokines along with other mediators of bone metabolism (TGF-β, PTH, 1,25-dihydroxyvitamin D3, glucocorticoids and estrogen) and tissue-destructive molecules such as reactive oxygen species (ROS), and RANKL production enhances destruction of periodontal tissues.

TNFα and IL-1 are synthesised by many cell types in the periodontal tissue such as monocytes/ macrophages, PMN cells, fibroblasts, epithelial cells, endothelial cells and osteoblasts (Cekici *et al.*, 2014; Kayal, 2013). TNFα is released from mast cells in response to bacterial challenge and triggers the bone loss process in periodontitis (Yucel-Lindberg and Bage, 2013). In clinical context, TNFα and IL-1β have been found in increased concentrations in GCF and gingival tissue of periodontitis sites (Hernandez *et al.*, 2011). At the cellular level, these two cytokines can induce and trigger several other mediators including IL-6, IL-8, matrix metalloproteinases (MMPs) and PGE2 (Yucel-Lindberg and Bage, 2013).

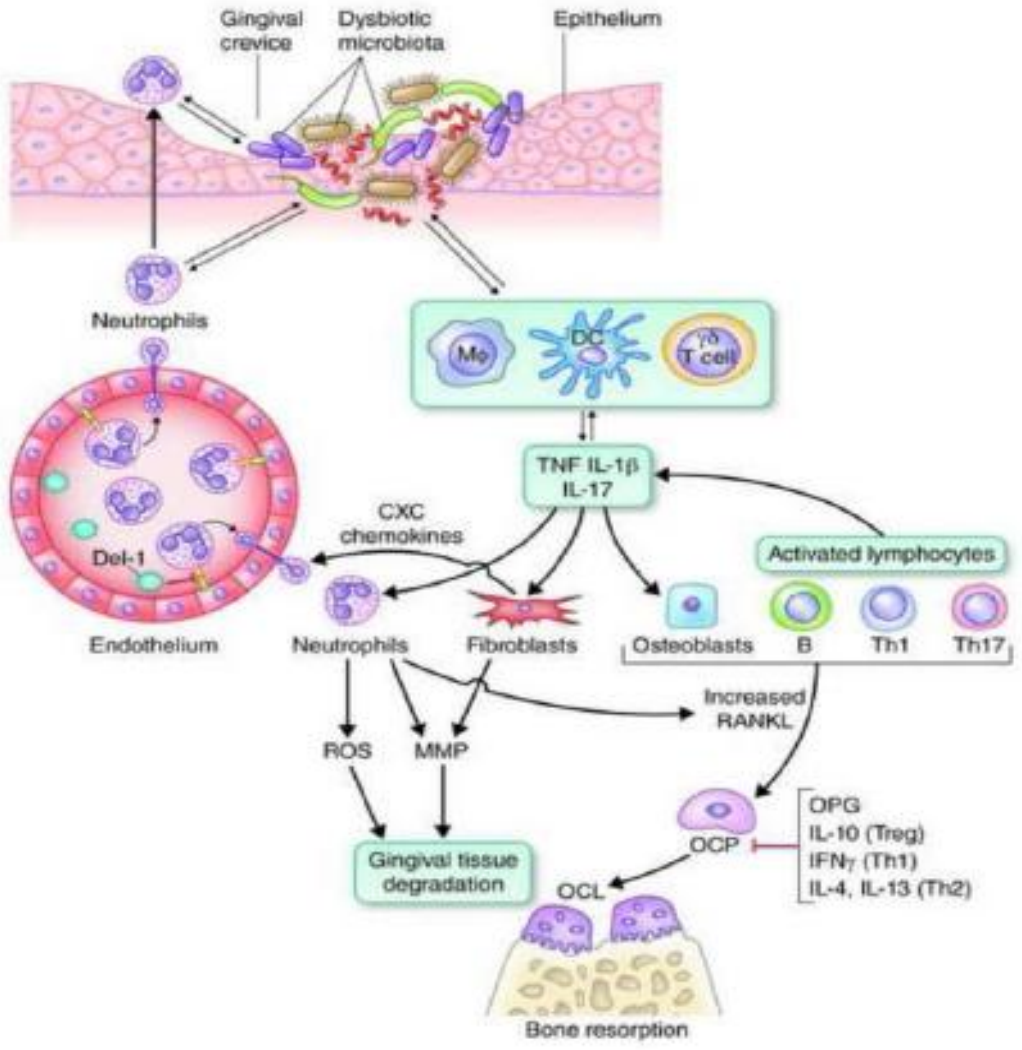

Figure 2.3 A schematic diagram showing the inflammatory mechanisms leading to bone loss in periodontitis (Figure adapted from Hajishengallis, 2014).

Thus, if the molecular mechanism of alveolar bone were elucidated in detail, prognosis of the periodontal lesion will be more accurately diagnosed & appropriate treatment modalities will be proposed. Since the RANKL-OPG system is crucial for

controlling bone resorption, imbalances in its expression may cause a switch from a physiological to a pathological bone loss in different sites.

2.3.7 Physiological role of RANK, RANKL and OPG system

RANKL acts by connecting to RANK on the surface of preosteoclast/ osteoblast cells, resulting in the enhancement of osteoclasts activity (Cochran, 2008; Martin and Ng, 1994). Through this activation, it triggers the fusion and differentiation of pro osteoclasts into mature osteoclasts, thus activating bone resorption (Kostenuik, 2001; Quax *et al.*, 2018; Theoleyre *et al.*, 2004; Xiao *et al.*, 2016). At times this action is blocked by the homologous decoy receptor known as osteoprotegerin (OPG), which is also a member from TNF superfamily (Locksley *et al.*, 2001; Sojod *et al.*, 2017). OPG attaches itself onto the surface of RANK, causing deregulation of molecular and cellular events taking place in osteoclastic process (Cavalla *et al.*, 2018; Theoleyre *et al.*, 2004). Below mentioned figure 2.4 is showing the schematic illustration and representation of RANKL-RANK-OPG system (Khosla, 2001). In this figure RANK-RANKL and OPG system and its brief process has been summarized. RANKL expressed on the surface of preosteoblastic/stromal cells, binds to RANK on the osteoclastic precursor cells. Macrophage-colony stimulating factor (M-CSF), which binds to its receptor, colony stimulating factor gene encoded CSF1R (c-Fms), on to the preosteoclastic cells, appears to be necessary for osteoclast development because it is the primary determinant of the pool of these precursor cells (Udagawa *et al.*, 1990). A number of proresorptive cytokines, such as TNF-α and IL-1, modulate this system primarily by stimulating M-