EVALUATING PRESENCE OF ANTIBODIES AGAINST CITRULLINATED PROTEINS IN RELATION TO RHEUMATOID ARTHRITIS AND PERIODONTITIS

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2019

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF CLINICAL DENTISTRY (PERIODONTOLOGY)

FACULTY OF DENTISTRY
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

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Matric No: DGL160003

Name of Degree: MASTER OF CLINICAL DENTISTRY (PERIODONTOLOGY)

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

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EVALUATING PRESENCE OF ANTIBODIES AGAINST CITRULLINATED PROTEINS IN RELATION TO RHEUMATOID ARTHRITIS AND PERIODONTITIS

ABSTRACT

Background and objective(s): Presence of antibodies against citrullinated proteins (anti-CitP) are highly specific for Rheumatoid Arthritis (RA). Recent studies have revealed the occurrence of citrullination in periodontitis (PD) which can induce anti-CitP production, allowing for a potential link between PD and development of RA. However, conflicting findings have been reported to associate RA and PD in relation to the expression of anti-CitP. This study aimed to compare the presence of serum anti-CitP with clinical periodontal and RA parameters in patients with RA and/or PD.

Materials and Methods: A total of 80 participants were recruited, each of 20 from those diagnosed with both RA and PD (RAPD), only RA, only PD, or no RA or PD (control). Full mouth periodontal examination was conducted, and 10 mL of serum sample was collected from each participant for quantification of anti-CitP using enzyme-linked immunosorbent assay (ELISA). ESR levels and RA disease duration were also recorded.

Results: Highest mean (±SD) levels of serum anti-CitP were found in RAPD group (228.82±219.09 IU/mL) followed by RA group (204.01±202.41 IU/mL), PD group (102.62±75.46 IU/mL) and lowest in healthy group (68.73±52.49 IU/mL). Anti-CitP levels were statistically significant higher in RAPD and RA groups compared to that of healthy group ($p < 0.05$). Multiple regression analysis to control for age and gender confirmed the statistical significance. Anti-CitP levels were not correlated with any of the clinical periodontal parameters between all four groups. For RAPD and RA groups, no statistical significant correlations were observed between serum anti-CitP levels with ESR and RA disease duration.

Conclusion: There is a trend for an increase in anti-CitP levels from healthy to PD to RA and RAPD groups. The underlying biological mechanisms for the relationship between PD and RA needs to be further investigated in future studies

Keywords: Periodontitis, Rheumatoid arthritis, anti-citrullinated protein antibodies (anti-CitP)

MENGKAJI KEHADIRAN ANTIBODI TERHADAP PROTEIN SITRULINASI BERKAITAN DENGAN RHEUMATOID ARTHRITIS DAN PERIODONTITIS

ABSTRAK

Latar belakang dan objektif kajian: Kehadiran antibodi terhadap protein sitrulinasi (anti-CitP) adalah sangat khusus untuk penyakit Rheumatoid Arthritis (RA). Kajian terbaru menunjukkan kewujudan proses sitrulinasi dalam penyakit periodontitis (PD) boleh menyebabkan penghasilan anti-CitP, yang berpontensi untuk menghubungkait RA dengan PD. Walau bagaimanapun, penemuan dan hasil kajian mengenai anti-CitP yang menghubungkait kedua-dua penyakit adalah terhad dan bercanggah. Kajian ini bertujuan untuk membandingkan kehadiran serum anti-CitP dengan parameter klinikal PD dan RA di kalangan pesakit yang mengalami RA dan/atau PD.

Bahan dan kaedah: Seramai 80 peserta telah menyertai kajian ini, 20 peserta dalam setiap kumpulan. Sebanyak 4 kumpulan yang terlibat: kumpulan yang menghidapi kedua-dua penyakit RA dan PD (RAPD), kumpulan yang hanya menghidapi RA (RA), kumpulan yang hanya menghidapi PD, dan kumpulan yang bukan RA dan PD (sihat). Pemeriksaan gusi dijalankan dan 10 ml sampel darah telah diambil dari setiap peserta untuk penilaian paras anti-CitP menggunakan ujian makmal "ELISA". Tahap ESR dan tempoh penyakit RA juga direkodkan.

Keputusan: Paras tertinggi (purata ± SD) serum anti-CitP dilaporkan di kalangan kumpulan RAPD (228.82 ± 219.09 IU/mL) diikuti oleh kumpulan RA (204.01 ± 202.41 IU/mL), kumpulan PD (102.62 ± 75.46 IU/ml) dan paras terendah adalah di kalangan kumpulan sihat (68.73 ± 52.49 IU/mL). Paras anti-CitP adalah jauh lebih tinggi dan ketara secara statistik dalam kumpulan RAPD dan RA ($p < 0.05$) berbanding dengan kumpulan yang sihat. Walau bagaimanapun, paras serum anti-CitP tidak berkorelasi dengan mana-mana parameter periodontal klinikal di antara semua kumpulan. Bagi kumpulan RAPD dan RA, tiada korelasi dilaporkan di antara paras serum anti-CitP dengan paras ESR dan tempoh penyakit RA.

Kesimpulan: Trend peningkatan paras anti-CitP telah dilaporkan dari kumpulan sihat diikuti dengan kumpulan PD, kumpulan RA dan akhirnya kumpulan RAPD. Walaubagaimanapun, mekanisme biologi yang menghubungkaitkan PD dan RA perlu diselidiki dengan lebih lanjut dalam kajian masa depan.

Kata kunci: Periodontitis, Rheumatoid Arthritis, antibodi terhadap protein yang tersitrulinasi (anti-CitP)

ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my supervisor, Associate Prof. Dr. Rathna Devi Vaithilingam who supervised me dedicatedly throughout my research project. I am truly blessed to have such a wonderful supervisor who has been guiding me and correcting my research report with great attention and care. She has been giving me continuous encouragement and support from initial stage until completion of my research report.

Secondly, I would like to express my deepest gratitude to my co-supervisors, Prof. Dr. Mohammad Tariqur Rahman and Dr. Syarida Hasnur Safii for their continuous support, motivation, insightful comments throughout my research period. Prof Dr. Tariq has always been there to listen and give me advice in conducting my laboratory procedures correctly while Dr. Syarida has been kind and giving me positive strength all the time throughout the whole research project.

Next, I am heartily thankful to Porf. Dr. Noor Lide Abu Kassim and Dr Zabri for their guidance in statistical input and solution throughout my statistical analyses and sample size calculation. I am also indebted to Prof Sargunan and members of Rheumatology clinic in University Malaya Medical Centre and the research assistants (Mr. Jazli and Miss Azkiyah) and all researchers involved in the whole recruitment process and project.

I would also like to extend my sincere thanks to my beloved family members and friends who have been patiently offering all sorts of help and support throughout my study especially my two other colleagues (Yin Hui and Philip) who are also involved in this same large-scale project. Finally, I appreciate the financial support from Dental Postgraduate Research Grant (DPRG) University of Malaya and Frontier Research Grant (FRG) University of Malaya that had funded this research project. Without these grants, this research would not be able to be conducted.

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LIST OF ABBREVIATIONS

AAP	:	American Academy of Periodontology
ACR	:	American College of Rheumatology
AFM	:	Arthritis Foundation Malaysia
Anti-CitP	:	Anti-citrullinated protein antibody
Anti-CarP	:	Anti-carbamylated protein antibody
BOP	:	Bleeding on probing
BPE	:	Basic periodontal examination
CAL	:	Clinical attachment level
CDC	:	Centre of Disease Control and Prevention
CEJ	:	Cemento-enamel junction
CRP	:	C-reactive protein
EFP	:	European Federation of Periodontology
ELISA	:	Enzyme-linked immunosorbent assay
ESR	:	Erythrocyte sedimentation rate
EULAR	:	European League Against Rheumatism
FLS	:	Fibroblast like synoviocytes
GBI	:	Gingival bleeding index
GCF	:	Gingival crevicular fluid
GR	:	Gingival recession
Ig	:	Immunoglobulin
IL	:	Interleukin
LPS	:	Lipopolysaccharides
MMPs	:	Matrix metalloproteinases
MREC	:	Medical Research Ethic Committee

NHANES	:	National Health and Nutrition Examination Survey
NOHSA	:	National Oral Health Survey of Adults
PAD	:	Peptidyl arginine deiminase enzyme
PD	:	Periodontitis
PGE-2	:	Prostaglandin E-2
PIS	:	Patient information sheet
PPD	:	Probing pocket depth
RANKL	:	Receptor activator of nuclear factor kβ ligand
RA	:	Rheumatoid Arthritis
ROS	:	Reactive oxygen species
SD	:	Standard deviation
TNF-α	:	Tumour necrosis factor-alpha
UMMC	:	University of Malaya Medical Centre
VPI	:	Visible plaque index
WHO	:	World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Background of study

Periodontitis (PD) is a chronic inflammatory disease associated with a dysbiotic dental biofilm and is characterised by both soft and hard tissue destruction around the teeth (Mercado et al., 2000; Papapanou et al., 2018). It is a major oral health problem affecting 48.5% of the Malaysian population (Oral Health Division, 2012) as it will lead to irreversible attachment loss, bone destruction and tooth loss if left untreated. The aetiology of periodontitis is the dental biofilm while the host immune-inflammatory response causes the resulting tissue damage.

Rheumatoid Arthritis (RA) is a chronic inflammatory autoimmune disease which is characterised by inflamed synovial as well as destruction of cartilage and bone in the joints. It has a global prevalence of 1% and may lead to severe disabilities and early premature mortality as it progresses with age (Scott, Wolfe, & Huizinga, 2010). To date, the aetiology of RA is still unclear.

Currently, many studies have reported a considerable association between PD and RA whereby PD is more common and severe in patients with established RA and vice versa (Berthelot & Le Goff, 2010; Demmer, Molitor, Jacobs, & Michalowicz, 2011; Detert, Pischon, Burmester, & Buttgereit, 2010; Pischon et al., 2008; Rutger Persson, 2012). Although both PD and RA have similar host mediated pathogenesis, however the actual mechanism through which RA and PD are interrelated is still unclear. Bright and colleagues (2015) have proposed the dual hit hypothesis for pathogenesis of RA being driven by protein citrullination or carbamylation, or both, in inflamed periodontal tissues that are indirectly linked with the pathogenesis of RA (Bright, Proudman, Rosenstein, & Bartold, 2015).

Citrullination is a post translational modification of amino acid arginine to citrulline mediated by peptidyl arginine deiminase enzymes (PADs). This results in citrullinated proteins that will induce the production of autoantibody, anti-citrullinated protein antibody (anti-CitP) in RA (Ioan-Facsinay et al., 2010). Anti-CitP is known to be present in patients' sera prior to the onset of the clinical manifestations of RA (Demoruelle, Deane, & Holers, 2014). Due to its high specificity and predictive value for RA, it has been used as an important serological marker for RA (Mangat, Wegner, Venables, & Potempa, 2010).

Citrullinated proteins have also been reported to be present in inflamed periodontal tissues (Nesse et al., 2012; Wegner et al., 2010). These findings suggest that citrullination can occur within the inflamed periodontal tissues, priming the susceptible individuals to a heightened anti-CitP response which may contribute to the association of RA and PD. In addition, some researchers have postulated that periodontal pathogens especially *Porphryomonas gingivalis (P.gingivalis)* could play a role in the propagation of RA. It could be due to the capability of *P. gingivalis* to citrullinate proteins using its peptidyl arginine deiminase (PPAD) enzymes (Mcgraw, Potempa, Farley, & Travis, 1999), contributing to RA-autoantibody (anti-CitP) production through post-translational modification of proteins. Beside generation of auto-antibodies, those oral bacteria that invade into blood may also contribute to chronic inflammatory responses.

Over the past few years, important insights have been gained into the development and aetiopathology of RA by the discovery of RA-specific autoantibodies directed against citrullinated proteins (anti-CitP). The production of Petidyl Arginine Deiminase enzyme (PPAD) by *P.gingivalis* can citrullinate proteins and trigger the formation of autoantibodies allowing for a link between periodontitis and development of RA. Despite all the evidences supporting a link between RA and PD, the underlying molecular mechanisms have yet to be completely identified as those studies associating both

diseases are still inadequate especially in the Malaysian setting. Therefore, we plan to carry out this study to assess the serum levels of anti-CitP in four groups of participants (RAPD, RA, PD and healthy control groups). We will then identify the possible association between RA and PD by correlating anti-citrullinated protein antibodies expression to both clinical RA and PD parameters.

1.2 Aim of the study

The aim of this study is to evaluate the expression of anti-citrullinated protein antibodies (anti-CitP) in the serum of RAPD, RA, PD and healthy control groups as well as to investigate its association with RA and PD.

1.3 Objectives of the study

- To identify and compare the presence of serum anti-CitP in RAPD, RA, PD and healthy control groups.
- To correlate the presence of anti-CitP with clinical periodontal parameters and RA parameters.

CHAPTER 2: LITERATURE REVIEW

2.1 Periodontitis (PD)

2.1.1 Introduction

Periodontitis is a chronic multi-factorial inflammatory disease initiated by dental biofilm (Loe, Theilade, & Jensen, 1965). It is the most prevalent form of destructive periodontal disease in adults. Together with caries, it makes up the two major oral diseases with a high prevalence rate that affects populations worldwide (Papapanou, 1999). It is modulated by the host immune system which results in the soft and hard tissue destruction around the teeth (Mercado et al., 2000; Papapanou et al., 2018).

The clinical features of PD include signs and symptoms such as alterations of the colour, volume and texture of the gingiva, bleeding upon probing, increased periodontal pocket depth due to reduction of resistance to probing of soft marginal gingival tissues, clinical attachment loss (CAL), gingival recession, alveolar bone loss, root furcation exposure, increased tooth mobility and drifting. This will eventually lead to tooth loss if it is left untreated (Lang & Lindhe, 2015).

In addition, the progression and severity of the periodontal destruction is highly dependent on both the amount and virulence of the microorganisms, the host immune-resistance (Mysak et al., 2014) and environmental factors (Page & Schroeder, 1976). The role of host response in the development of inflammatory response is governed by the individual's heritability factor which will then determine the individual susceptibility to periodontal inflammation (Kinane & Hart, 2003).

2.1.2 Prevalence of periodontitis

Periodontitis is the 6th most prevalent oral condition worldwide that represents a major contributor to the global burden of disease (Marcenes et al., 2013). Current epidemiological evidence suggests that periodontitis is moderately prevalent globally

while its severe subtype displays lower prevalence (Kassebaum et al., 2014; Sheiham & Netuveli, 2002). Global prevalence of the severe form of periodontitis in adult population is reported to be approximately 11% (Kassebaum et al., 2014).

It is understandable that these prevalence rates vary from region to region across the globe due to the different indices and methodology used for different population surveys. In the United States of America (USA), the National Health and Nutrition Examination Survey (NHANES 2009-2012) estimated that 46% of adults aged 30 and older suffer from chronic periodontitis with 8.9% of them having severe periodontitis (Eke et al., 2015). In the United Kingdom, periodontitis affecting more than 30% of adults aged 65 years or above (Morris, Steele, & White, 2001).

In addition, the prevalence of chronic periodontitis within Asia was between 15 to 20% which is consistent with the global prevalence (Corbet, Zee, & Lo, 2002). Malaysia's National Oral Health Survey of Adults (NOHSA) 2010, involved 8332 dentate subjects reported 48.5% of Malaysian adults had periodontitis and 18.2% suffered from severe periodontitis (NOHSA, 2010). Middle-aged urban population and educated men were commonly affected. Males had higher proportion of deep pockets (20.3%) in comparison to females (16.5%).

2.1.3 Classification of periodontitis

According to the 1999 International Classification of Periodontal Disease and Condition, chronic periodontitis and aggressive periodontitis are the two major forms of periodontitis (Armitage, 1999). Chronic periodontitis is most prevalent in adults and usually affects an individual at older stage of life (more than 35 years old). However, it can also be found in children and adolescents (Armitage, 2004; Baer, 1971).

Under this 1999 classification, chronic periodontitis can be further classified based on extent and severity. Based on the extent of disease, it is classified as localised

chronic periodontitis when there is less than 30% of sites are affected. If there is more than 30% of sites affected, it is known as generalised chronic periodontitis (Armitage, 1999). On the other hand, severity can be characterised based on the clinical attachment loss (CAL) as following: mild periodontitis if the CAL measured is 1–2mm, moderate periodontitis if the CAL is 3–4mm and severe periodontitis if the CAL is >5mm (Armitage, 1999).

Recently, American Academy of Periodontology (AAP) and European Federation of Periodontology (EFP) have proposed a new classification of Periodontal and Peri-implant Diseases and Conditions. According to this 2017 classification, the two forms of periodontitis which are previously known as chronic and aggressive periodontitis are now under the same category, known as "periodontitis". Under this revised classification, periodontitis can be further categorised and characterised based on staging and grading system (Papapanou et al., 2018).

Classification of periodontitis based on stages are defined by the disease's severity and complexity of disease management as well as its extent and distribution. There are four stages of periodontitis ranging from stage I to IV**,** which represent an increased severity and complexity. On the other hand, grading system is used as an indicator of disease progression. In addition, for each stage, the extent and distribution of periodontitis can be classified based on the number of diseased sites involved. It is considered as localised periodontitis if it affects < 30% of teeth and generalised if >30% of teeth are involved.

2.1.4 Aetiology of periodontitis

The dental biofilm has long been established as the aetiological factor of gingival inflammation and subsequent periodontal tissue destruction (Loe et al., 1965). Over the past decades, a few hypotheses describing the aetiology of periodontal disease have been

proposed. During the 1970s, periodontal disease has been considered to be caused solely by specific bacteria or group of bacteria residing in the dental biofilm as advocated in "specific plaque hypothesis" (Loesche, 1979). Since then, several disease related micro-organisms have been identified as periodontal pathogens such as *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Treponema denticola* and others.

However, it was argued that those specific periodontal pathogens were also indigenous microflora that was present in healthy individuals as well. Consequently, the notion of "non-specific aetiology" proposed by Theilade in 1986 came to the forefront. At that time point, it was believed that periodontal disease was a non-specific inflammatory response to a complex indigenous microbiota and their virulence factors caused the subsequent periodontal tissue destruction (Theilade, 1986). Later, "ecological plaque hypothesis" was proposed after combining the key concepts of the earlier hypotheses. According to this hypothesis, environmental conditions determine the composition of microbial communities. For instance, ecological stress can result in breakdown of homeostasis, leading to a shift in the balance of the microflora in favour to disease related pathogens, therefore predisposing to disease (Marsh, 1994).

Recently, it was proposed that periodontal disease is attributed to polymicrobial synergy and dysbiosis, leading to host mediated disruption of microbial homeostasis, particularly subgingival bacterial communities (Hajishengallis, Darveau, & Curtis, 2012). Presence of certain microbial pathogens at low abundance can lead to a change in normal microbiota (in terms of quantity and composition) as described in "the keystone pathogen hypothesis" (Hajishengallis et al., 2012). The changes in subgingival microbial communities will then induce gingival inflammation, together with immune cell infiltration, leading to subsequent periodontal tissue destruction (Cekici, Kantarci, Hasturk, & Van Dyke, 2014; Darveau, 2010). Furthermore, this model also highlighted that the host immune response is initially subverted by *P.gingivalis* as a "key-stone

pathogen" with the aid of accessory pathogens (Hajishengallis, 2015), leading to breakdown of homeostasis with destructive inflammation in those susceptible individuals.

2.1.5 Pathogenesis of periodontitis

Plaque bacteria releases various virulence factors such as lipopolysaccharides (LPS) and microbial peptides that can induce inflammation and cause direct or indirect tissue damage mediated by host immune mechanisms. Direct effects occur when the bacteria or bacterial substances directly stimulate host cells such as monocytes, lymphocytes and fibroblasts to release inflammatory cytokines and result in direct tissue damage. However, dental biofilm appears to elicit most periodontal tissue injury through indirect mechanisms by activating a number of host-mediated destructive processes (Cekici et al., 2014).

Pathogenic bacteria that colonises the subgingival biofilm will release chemotactic peptides such as lipopolysaccharides (LPS) and microbial peptides. Subsequently, the circulating neutrophils will be recruited to the periodontal pocket or the gingival crevice as the first line of defense. Once the LPS gains access into the underlying gingival connective tissues, both epithelial cells and connective tissues will be stimulated to release a cascade of cytokines and inflammatory mediators, allowing inflammatory response to occur within the tissues. The neutrophils not only release enzymes that are capable of degrading pathogens, but also destroy the pathogens by phagocytosis. If the neutrophil becomes overloaded with bacteria, it will degranulate and release reactive oxygen species (ROS) that can lead to further gingival connective tissue destruction.

Apart from neutrophils, macrophages also being recruited to the site of infection after several days to aid in elimination of pathogens via phagocytosis. Activation of humoral activity (neutrophils and macrophages) leads to an accumulation of plasma cells

and the productions of immunoglobulin which comprises of immunoglobulin G (IgG) and immunoglobulin M (IgM). These antibodies will then activate the complement cascade which in turn facilitates phagocytosis and complement mediated lysis. Host defense activities (innate and adaptive immune responses) may contain the microbial challenge, resulting in resolution of inflammation, or allow the condition to worsen if the microbial challenge eventually overcomes the host defense. If containment does not occur, the inflammation will worsen, and disease will then progress to a more advanced stage.

In the advanced lesion stage, the degree of destruction is more apparent clinically. There will be more ingress of bacterial products into the connective tissues thus stimulating more of an immune-inflammatory response with more recruitment of neutrophils and macrophages, IgG, IgM as well as activation of T lymphocytes. Activated T lymphocytes will produce lymphokines which will modulate macrophage activity leading to improved phagocytosis and intracellular killing. Activated macrophages also release abundant amounts of cytokines including Interleukin-1 (IL-1), Tumor necrosis factor-alpha (TNF-α), Prostaglandin E2 (PGE2) and Matrix- metalloproteinases (MMPs). IL-1 induces the release of collagenase from a variety of connective tissue cells including fibroblasts and osteoblasts whereas MMPs, are a group of collagenases that are responsible for breakdown of collagen fibres.

These actions will lead to gingival and periodontal connective tissue degradation. Prostaglandin E2, MMPs, IL1 and TNF-α are factors that causes stimulation of functional osteoclasts from precursor cells and aids in preparing bone surfaces for osteoclastic resorption. Thus functional (mature) osteoclasts are activated and bone resorption occurs. Differentiation and maturation of osteoclasts are also stimulated by receptor activator of nuclear factor κB ligand (RANKL). All the above events causes junctional epithelium to regrow more apically thus forming the periodontal pocket in PD (Cekici et al., 2014).

2.2 Rheumatoid Arthritis (RA)

2.2.1 Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterised by persistent synovial inflammation, destruction of cartilage and bone in the joints as well as presence of serum autoantibodies specific to RA (particularly rheumatoid factors and anti-citrullinated protein antibody). RA mainly attacks the joints and may affect multiple joints at once which is known as polyarthritis. It usually affects joints in the hands, wrists, feet and knees, causing joint tissue destruction and may lead to severe disabilities and early premature mortality (Scott et al., 2010). The mortality rates in patients with RA are increased at least two folds as compared to the general population and this mortality gap seems to be widening in recent years (Gonzalez et al., 2007; Wolfe et al., 1994).

RA is characterised by periods of disease exacerbation (flares) and remission. The sign and symptoms may flare up at some point of time and undergo remission later. The signs and symptoms include pain and stiffness in one or more joints, tenderness and swelling in one or more joints, fever, weakness, fatigue, joint dysfunction and weight loss. These signs and symptoms usually affect both sides of the body in a symmetrical presentation. Furthermore, the corresponding systemic inflammation may result in a number of extra-articular manifestations and comorbidities such as disorders of multiple organ systems, which leads to increased mortality (Gibofsky, 2012; McInnes & Schett, 2011).

Prior to the clinical manifestation of RA, a preclinical immunological phase shown by the identification of serum autoantibodies is seen years before the onset of RA (Demoruelle et al., 2014). This suggests that systemic inflammation and autoimmunity in RA begins long before the onset of clinically evident joint inflammation. These autoantibodies also serve as an important serological marker for RA.

Diagnosis of RA is primarily based on the clinical history, blood results and physical examination findings. Clinically, RA is diagnosed according to the 2010 American College of Rheumatology and European League Against Rheumatism (ACR-EULAR) classification criteria (Aletaha et al., 2010). For instance, patients with a score of at least 6 out of 10 according to the classification would be indicated as having RA and hence treatment would be considered for this patient.

2.2.2 Prevalence of Rheumatoid Arthritis

Rheumatoid arthritis has an estimated 0.5%-1% prevalence globally and its prevalence increases with age (Scott et al., 2010). It is three times more likely to affect females compared to males (Arnett et al., 1988). RA affects people of all races equally and has a highest prevalence in women aged 65 and above. Basically, the prevalence of RA varies from region to region across the globe (Biver et al., 2009; Costenbader, Chang, Laden, Puett, & Karlson, 2008). These variations may be attributed to regional variation in genetic factors, RA diagnosis and environmental exposures (Costenbader et al., 2008).

An epidemiological study shows that RA is more common in industrialized countries such as Northern Europe and North America which was estimated to be 0.5% to 1 % of prevalence (Kalla & Tikly, 2003). On the other hand, the overall prevalence of RA in United States was estimated as 0.8% (Helmick et al., 2008) in which the highest prevalence was observed among the North America Indians, ranging from 1.4% to 7.1%. (Ferucci, Templin, & Lanier, 2005).

Currently, the epidemiological evidences for other parts of the world are still scanty including Malaysia (Symmons, Mathers, & Pfleger, 2000). According to Arthritis Foundation Malaysia (AFM), the prevalence of RA in Malaysia is estimated to be 0.5% of the population, affecting about 135,000 Malaysians. Females are more commonly affected as compared to males (Arthritis Foundation Malaysia, 2017; Malaysia).

2.2.3 Classification of Rheumatoid Arthritis

The first classification criteria set to define RA that has been widely used is the 1987 American College of Rheumatology (ACR) classification. This classification criteria is particularly useful in identifying patients with more established RA disease from those with combination of other rheumatological diagnoses (Arnett et al., 1988). However, these criteria has been criticised as lacking in sensitivity due to its failure to identify patients with early RA and therefore not helpful in achieving early diagnosis and early effective intervention.

Subsequently, a joint working group of ACR and European League Against Rheumatism (EULAR) was created to develop a new classification criterion with the aim of identifying early RA cases to improve treatment outcome and prognosis of RA by early detection. Similar to the 1987's classification, the 2010 American College of Rheumatology and European League Against Rheumatism (ACR-EULAR) classification criteria is meant for identifying patients for epidemiological reasons, clinical trials as well as to discriminate patients with synovitis and those with highest risk for progressing to more erosive forms of RA. Furthermore, this 2010 ACR/EULAR classification criteria can also be adopted as a scheme to identify patients with definite RA (Aletaha et al., 2010).

It is also important to note that this classification criteria is only applicable for those eligible patients with the evidence of "currently active clinical synovitis in at least one joint in the body with the exception of the distal interphalangeal (DIP) joints, first metatarsophalangeal (MTP) joint and first carpometacarpal (CMC) joint as diagnosed by an expert assessor". Subsequently, four additional criteria can be applied to identify those with "definite RA" which are shown in Table 2.1.

To arrive to the classification of definite RA, a thorough history of symptoms, duration and careful evaluation of total joint involvement are mandatory. Besides that, at least one serological test, either Rheumatoid Factor (RF) or Anti-Citrullinated Protein Antibodies (ACPA) and one acute phase response measure (erythrocyte sedimentation rate, ESR or C-reactive protein, CRP) must be performed. Consideration of these criteria provides a score of 0-10 for the patient based on the scoring system. The classification criteria is shown in Table 2.1. For instance, patient with a score of at least 6 out of 10 according to the classification would be indicative of having definite RA and hence treatment would be considered (Aletaha et al., 2010).

Table 2.1: ACR-EULAR classification criteria for RA (Aletaha et al., 2010)

Classification criteria	Score
A. Joint involvement	
1 large joint	0
2-10 large joints	1
1-3 small joints (with or without involvement of large joints)	2
4-10 small joints (with or without involvement of large joints)	3
>10 joints (at least small joint)	5
B. Serology (at least 1 test result is needed for classification)	
Negative RF and negative ACPA	0
Low-positive RF or low-positive ACPA	2
High-positive RF or high-positive ACPA	3
C. Acute-phase reactants (at least 1 test result is needed for classification)	
Normal CRP and normal ESR	0
Abnormal CRP or abnormal ESR	1
D. Duration of symptoms	
<6 weeks	0
≥6 weeks	1

2.2.4 Aetiology and pathogenesis of Rheumatoid Arthritis

To date, the exact aetiology of RA is still unknown. Although the pathogenesis of RA has not been completely elucidated yet, what is known from the literature is that the aetiopathogenesis of RA is rather complex. Current evidence suggests that a combination of genetic and environmental risk factors with loss of immunological tolerance to self-antigens underpin the pathogenesis of RA. The interaction between environmental factors and genetic factors will result in a cascade of immune-inflammatory reactions, leading to production of autoantibodies and pro-inflammatory cytokines in the joint synovium. Ultimately, this will result in manifestation of synovitis, cartilage and joint destruction as well as structural bone damage in RA (Gibofsky, 2012; Horta-Baas et al., 2017).

Although the exact cause of disease is not clear, but the individual risk is thought to be increased by combination of both environmental and genetic factors. Certain environmental triggers such as viral infections (Epstein-Barr virus, Cytomegalovirus), stress, cigarette smoking and infectious agents can precipitate post-translational modification of proteins in genetically susceptible individuals, leading to the production of autoantibodies directed against their own cellular structures (Gibofsky, 2012; Horta-Baas et al., 2017).

The process is important as it contributes to the production of anti-citrullinated protein (anti-CitP) and anti-carbamylated protein (anti-CarP), autoantibodies from post-translational modification of proteins (particularly citrullination and carbamylation) which may play a key role in the pathogenesis of RA (Bright et al., 2015). These pre-clinical phase autoantibodies in RA (RF, anti-CitP, anti-CarP) are thought to first develop outside the synovial joints, possibly in the lungs, gut and oral cavity. The presence of increased autoantibodies and inflammatory cytokines as well as chemokines can be detected via blood tests during the early phase.

Environment factors such as smoking can modify a self-protein, making them to be targeted by the immune system as 'self-antigen'. When the host immune system recognises the altered self-protein, autoreactive T cells and B cell will be produced as a result of break of self-tolerance, which is the hall mark of autoimmune disease. The actual relationship between loss of self-tolerance and synovial involvement is still unclear currently. Only in certain patients, these autoimmune responses will lead to synovial joint inflammation which is possibly induced by an increased permeability in the synovial joint that permits the entry for autoantibodies (Choy, 2012; McInnes & Schett, 2011).

After entering the joints, the autoantibodies will bind to the autoantigens in the cartilage, bone and lining of the synovial joint. As a result, the classical complement pathway is activated, and leukocyte recruitment is stimulated. Once the leukocytes infiltrate the synovium, synovitis occurs. This triggers an inflammatory reaction and activates the immune-inflammatory cells in the joints. Together with other cells in the joints, activated monocytes will then differentiate to yield more macrophages, leading to a cascade of soluble inflammatory cytokines such as TNF, IL-6, IL-17. Damage caused by the inflammatory environment can expose new self-antigens to the immune system, continuing the cycle. At this stage the first clinical symptoms are joint pain and swelling. Some patients may recover but most commonly patients will continue with inflammatory joint disorders.

As the disease progresses, dendritic cells display newly exposed self-antigens and activate the T cells in the joint itself and in the local lymph nodes. In addition, B cells infiltrate the joints and proliferate to produce antibodies and a series of pro-inflammatory cytokines and chemokines, which further amplifies the autoimmune response. Cells in the joint lining called fibroblast-like synoviocytes (FLS) also proliferate and grow into the joint space, spreading across to the cartilage surface. The pro-inflammatory cytokines generated by immune cells alter the metabolism of FLS. Instead of synthesizing

extracellular matrix components, FLS secrete matrix-metalloproteinase (MMPS) and cytokines that degrade the cartilage tissues and induce osteoclastogenesis (Choy, 2012; McInnes & Schett, 2011; Shiozawa, Tsumiyama, Yoshida, & Hashiramoto, 2011).

Later, the inflammatory cytokines released will further enhance the recruitment of leukocytes and thus, creating a positive feedback loop mediated by complex interplay shown among leukocytes, synovial fibroblasts, chondrocytes, and osteoclasts. The resultant event is the persistence of chronic inflammatory response within the joint which eventually leads to irreversible soft and hard tissue destruction in RA (Choy, 2012; McInnes & Schett, 2011).

2.2.5 Citrullination and Rheumatoid Arthritis

2.2.5.1 Citrullination

Citrullination is a conversion process of positively charge arginine (Arg) residue to neutral citrulline (Cit) residue by a family of enzymes called peptidyl arginine deiminase (PAD) (Figure 2.1). This process takes place in the presence of calcium as part of the normal physiological process and normal functioning of immune system (Wegner et al., 2010). Five isoforms of PAD have been identified in humans, which include PAD-1, PAD-2, PAD-3, PAD-4/5, and PAD-6 which are distributed at different locations with different functions (Valesini et al., 2015). For instance, PAD-1 induced citrullination is essential in skin keratinization whereas PAD-2 is actively involved in myelin sheath stability in brain (Valesini et al., 2015).

Apart from the involvement in physiological processes, citrullination also takes part in pathological inflammatory conditions as part of the innate response to bacterial infection and cell death mechanism. For example, PAD-4 induced citrullination is known to play an important role in altering chemokines' functions and have been actively participating in neutrophils extracellular traps (NETs) formation as part of the

antibacterial mechanism. It is often being linked to chronic inflammatory disorders such as Multiple Sclerosis, Alzheimer's disease, psoriasis and RA (van Gaalen, Visser, & Huizinga, 2005; Wegner et al., 2010). So far, only PAD-2 and PAD-4 have been expressed in joints and identified to be associated to RA (Foulquier et al., 2007; Wegner et al., 2010).

The enzymatic deimination of arginine residues to citrulline by PAD (post-translational modification) alters the tertiary structure, antigenicity and function of proteins (Schellekens, de Jong, van den Hoogen, van de Putte, & van Venrooij, 1998; Schellekens et al., 2000). Subsequently, this may expose the previously hidden immune epitopes and induce an auto-immune response as citrulline is not a standard amino acid of proteins (Gyorgy, Toth, Tarcsa, Falus, & Buzas, 2006). In a susceptible patient, these citrullinated peptide will act as an antigenic determinant that could break the immunological tolerance and evoke an autoimmune response by binding onto the antigen presenting cells. As a result, pathogenic T and B cells will be activated, leading to the RA-specific anti-citrullinated protein autoantibodies (anti-CitP) formation (Schellekens et al., 1998). These anti-CitP will then form immune complexes with citrullinated peptides, resulting in the production of inflammatory mediators and ultimately causing joint destruction in RA (Koziel, Mydel, & Potempa, 2014; Liao et al., 2009).

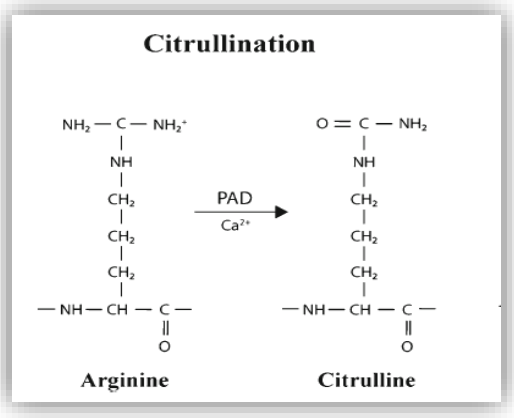

Figure 2.1: Schematic illustration of citrullination, taken from (Bax, Huizinga, & Toes, 2014)

2.2.5.2 Anti-citrullinated proteins (anti-CitP) and Rheumatoid Arthritis development

Anti-citrullinated protein antibodies (anti-CitP) and anti-cyclic citrullinated peptide antibodies (anti-CCP) are the known autoantibodies that act against these citrullinated proteins in RA (Schellekens et al., 2000). It has been shown that anti-CCP antibodies are actually a collection of anti-CitP that recognised multiple citrullinated proteins, which play a crucial role in the pathogenesis of RA (Ioan-Facsinay et al., 2010). It can recognise citrullinated autoantigens comprised of various extracellular proteins such as fibrinogen, vimentin, enolase, type II collagen, filaggrin and histones. The binding between anti-CitP and citrullinated autoantigens will then evoke autoimmunity via activation of autoreactive B and T cells, eventually leading to RA (Valesini et al., 2015).

Anti-CitP can be detected in about 70%-80% of RA patients. In a meta-analysis, anti-CitP positivity has been reported to be more specific (ranging from 88%-95%) than rheumatoid factor (RF) positivity in terms of diagnosing RA especially in those early RA cases (Nishimura et al., 2007; van Venrooij, Hazes, & Visser, 2002). It also shares a comparable sensitivity to RF (about 68%) (Schellekens et al., 2000). Furthermore, patients with anti-CitP positivity are often associated with more severe and worsening

clinical outcomes in RA and therefore it is a better predictor of erosive disease (Bright et al., 2015; Nishimura et al., 2007). Therefore, Anti-CitP has emerged as a specific serological marker for RA due to its high specificity and predictive value for RA (Mangat et al., 2010). Although the serum anti-CitP positivity has been frequently related to more severe disease, study have also reported that the serum concentration of anti-CitP do not correlate well with RA disease severity and their disease activity (Papadopoulos, Tsiaousis, Pavlitou-Tsiontsi, Giannakou, & Galanopoulou, 2008).

The presence of anti-CitP in RA sera years before the clinical disease becomes apparent have implicated the pathogenic role of anti-CitP in RA development. This can be substantiated by several studies using experimental animal models. By using collagen induced arthritis mice immunized with bovine type II collagen, Kuhn and co-workers demonstrated the presence of antibodies against both collagen type II and cyclic citrullinated peptide before joint swelling was evident. Moreover, they also observed a reduction in disease severity and incidence following the induction of tolerance towards the cyclic citrullinated peptides in the mice which led to a significant reduction in collagen induced arthritis susceptibility (Kuhn et al., 2006).

In another study, Hill et al reported that all genetic susceptible transgenic mice immunized with human citrullinated fibrinogen developed arthritis but none from those administered with unmodified fibrinogen. Citrullinated fibrinogen is a known antigen that is frequently targeted by anti-CitP in RA. Thus, they concluded that citrullinated fibrinogen can drive an autoimmune response that will lead to development of arthritis in genetically susceptible transgenic mice (Hill et al., 2008). Both these studies confirmed the important role of selective citrullinated proteins in inducing autoimmunity associated with development of RA.

As for human studies, most cases were triggered by the autoimmune response to citrullinated proteins which were generated under physiological conditions. However, loss of tolerance in genetically susceptible individuals may initiate the production of anti-CitP in the synovium and subsequently lead to a cascade of events, leading to the induction of RA (van Gaalen et al., 2005; Wegner et al., 2010). Citrullinated proteins and anti-CitP are reported to be found abundantly in inflamed joints, again suggesting that they might play a pathological role in the development of RA (van Gaalen et al., 2005). Production of citrullinated antigens locally in the inflamed synovium has further implicated the fact that these resulting immune complexes are directly involved in the pathogenesis of chronic inflammation in the RA joints (Kinloch et al., 2008).

2.3 Periodontitis-Rheumatoid Arthritis association

2.3.1 Association between periodontitis and Rheumatoid Arthritis

The historical evidence of a link between RA and PD has long been recognised since the last few decades. The common inflammatory mechanisms between RA and PD were first highlighted by Snyderman and McCarty in year 1982. Since then, there has been increasing evidence linking periodontal disease to other systemic diseases including rheumatoid arthritis (Scannapieco, 1998). Currently, there are emerging evidences from numerous clinical and epidemiological studies suggesting a positive association between RA and PD (Berthelot & Le Goff, 2010; Demmer et al., 2011; Detert et al., 2010; Rutger Persson, 2012).

Existence of RA may promote emergence and progression of PD (Detert et al., 2010) and on the other hand, PD is found to be more common and severe in patients with established RA (Bingham & Moni, 2013). Despite the differences in their initiating aetiological mechanisms, both chronic inflammatory conditions actually share a similar host mediated pathogenesis characterised by similar set of pro-inflammatory cytokines, connective tissue destruction as well as bone erosion as their pathological hallmark of

disease (Potempa, Mydel, & Koziel, 2017; Rosenstein, Greenwald, Kushner, & Weissmann, 2004). They also share numerous characteristic and pathogenic similarities with regards to risk factors (such as smoking, obesity and ageing), immunogenetics, disease progression, and tissue destruction pathways to justify the hypothesis that there is a plausible link between them. However, the actual mechanisms through which RA and PD are interrelated is still unclear.

Over the years, several hypotheses have been proposed on how PD interact or link with RA. One of the earliest hypotheses was proposed by Rosenstein et al (2004) suggesting that PD preceded the development of RA and RA was initiated by humoral response to periodontal pathogens. Central to this hypothesis is the production of PAD enzymes by *P.gingivalis* (*Prophyromonas gingivalis* Peptidyl Arginine Deiminase Ezyme, PPAD) that can induce autoantibodies via citrullination, allowing for a link between PD and development of RA (Rosenstein et al., 2004). The fact that the autoimmune response in RA in the form of anti-CitP often preceded the clinical onset of RA by several years may also suggest that RA may have originated from mucosal sites distant to the joints, potentially from the gums and lungs (Rantapaa-Dahlqvist et al., 2003).

Subsequently, another hypothesis was proposed by Golub et al (2006) which was known as the "two-hit" model. This model suggested that a systemic disease like RA can potentially exacerbate or initiate periodontitis and vice versa (Golub, Payne, Reinhardt, & Nieman, 2006). It was proposed that the first hit of chronic inflammation via chronic periodontitis is at the periodontal microbial biofilm, followed by second hit to induce systemic inflammation in RA, leading to exacerbated inflammatory response (Golub et al., 2006).

Recently, Bright and colleagues (2015) have proposed a dual hit hypothesis for pathogenesis of RA being driven by citrullination or carbamylation, or both, in inflamed periodontal tissues of a genetically susceptible individual (Figure 2.2). In this model, chronic inflammation seen in periodontitis (first hit) will result in the extra-articular formation of antibodies directed against the citrullinated and carbamylated peptides, leading to the initial break of immune tolerance. These autoimmune responses will then be translated into inflammatory joint disease in the susceptible individuals (second hit). This hypothesis has suggested a plausible mechanistic link associating PD in the pathogenesis of RA via citrullination and carbamylation (Bright et al., 2015).

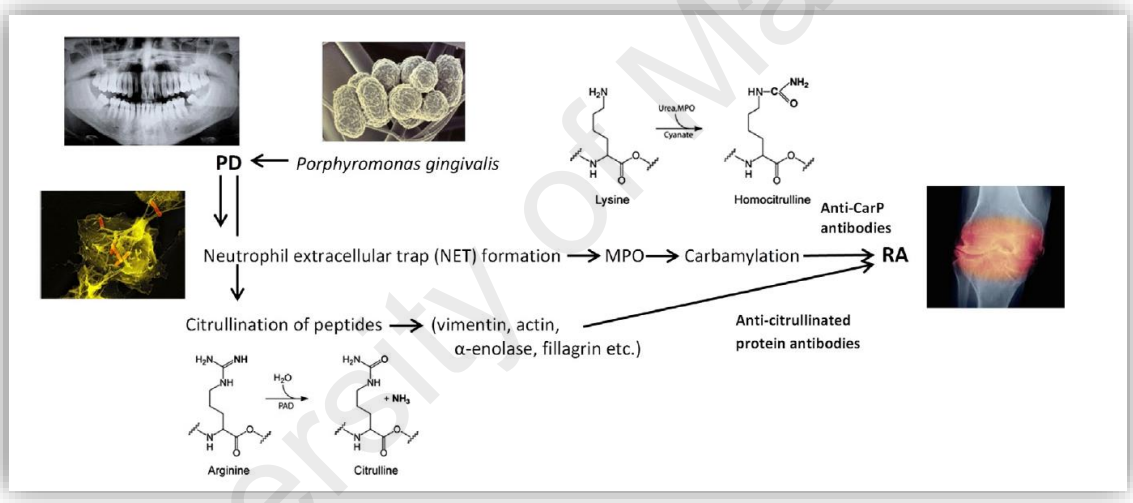

Figure 2.2: Dual hit hypothesis model for pathogenesis of RA, taken from (Bright et al., 2015)

2.3.2 Citrullination, anti-citrullinated protein antibodies (anti-CitP) and periodontitis (PD)

Citrullination is an inflammatory-dependent process that plays a central role in autoimmune diseases (Valesini et al., 2015). Apart from inflamed synovial joints in RA, citrullinated proteins have also been detected in inflamed periodontal tissues of patients with periodontitis. The presence of citrullinated proteins in periodontal tissues was first revealed by Nesse et al (Nesse et al., 2012) . The citrullinated proteins, PAD-2 and PAD-4 were also found in inflamed periodontal tissues in PD patients without any signs of RA

(Harvey et al., 2012). Based on these findings, it was postulated that PD could provide a conducive environment for citrullination and initiation of anti-CitP targeting citrullinated peptides in joint.

Subsequently, a few studies had been carried out to identify the presence of anti-CitP in the serum of PD patients. Two case–control studies demonstrated higher percentage of anti-CitP positivity in patients with aggressive periodontitis (7.4% and 8 % respectively) when compared to the control groups (0%) (Havemose-Poulsen et al., 2006; Hendler et al., 2010). However, none was detected in patients with gingivitis and chronic periodontitis. Although no strong conclusion could be drawn from these findings, the authors proposed that autoimmunity might play a role in the pathogenesis of aggressive periodontitis, supporting the possible link between RA and PD via citrullination.

On the other hand, one study has reported a significant correlation between chronic periodontitis and serum anti-CitP expression. RA patients with anti-CitP seropositivity were more likely to suffer from moderate to severe periodontitis than those seronegative patients (Dissick et al., 2010). Some other studies also demonstrated that patients with chronic periodontitis have a slightly increased presence of titres of anti-CitP in their sera compared to controls (P. de Pablo, Dietrich, & McAlindon, 2008; Harvey et al., 2012; Lappin et al., 2013). However, these findings were not conclusive as these studies had small sample sizes and the differences between patients and controls were not statistically significant. Nevertheless, these results had shown a considerable potential that citrullination can occur within the inflamed periodontal tissues, priming the susceptible individuals to a heightened anti-CitP response during the development of RA later.

Furthermore, a significant association between anti-CitP seropositivity and oral mucosal inflammation had also been reported in a case control study, suggesting that anti-

CitP formation could be induced at inflamed oral mucosa of a non-RA subjects (Janssen et al., 2015). Recently, a large-scale prospective cohort study was conducted to assess the association between PD status and anti-CitP production in 9554 healthy Japanese population. The number of missing teeth, Community Periodontal Index (CPI) and clinical attachment loss were evaluated as PD parameters in this study. As a result, a significant association was reported between all these PD parameters and levels of anti-CitP positivity, suggesting the fundamental involvement of PD in anti-CitP production (Terao et al., 2015). These association patterns were also observed in the non-smokers when sub-analysis was carried out focusing on 6206 non-smokers subjects from the same cohort.

Therefore, all these findings have further strengthened the possible involvement of PD in citrullination process, leading to systemic anti-CitP production that was specific to the onset of RA in a genetically susceptible patient.

2.3.3 Periodontal pathogens and Rheumatoid Arthritis

The presence of periodontal pathogens in the subgingival biofilm has been known to be one of the essential factors in the initiation and progression of periodontitis. The most virulent pathogens related to chronic periodontitis are from the red complex (based on (Socransky, Haffajee, Cugini, Smith, & Kent, 1998) bacterial grouping) which consists of *Porphyromonas gingivalis (P. gingivalis), Tannarella forsythia (T. forsythia) and Treponema denticola (T. denticola)*. The red complex has been strongly associated with advanced periodontal destruction and lesion (Mysak et al., 2014).

Numerous infectious agents, including periodontal pathogens have been implicated as contributory factors in the aetio-pathogenesis of RA (Moen et al., 2006; Ogrendik, 2009). This assumption arises following the detection of bacterial DNA of *P. gingivalis* within the synovial fluid of inflamed joints in RA subjects (Moen et al., 2006).

Apart from anaerobic bacterial DNA, high levels of oral anaerobic bacterial antibodies have also been detected in both synovial fluid and serum from RA patients (Ogrendik, 2009). Martinez-Martinez et al discussed and further confirmed the possibility of transportation of oral genetic material to the joint compartment in free form DNA as they found bacterial DNA of *P. gingivalis* more frequently in the synovial fluid than in serum (Martinez-Martinez et al., 2009).

The aforementioned assumption that genetic material was carried from teeth to joints in free form of DNA via bloodstream was further confirmed by more recent studies whereby *P. gingivalis* DNA was detected in the synovial tissue of RA patients (Reichert et al., 2013; Totaro et al., 2013). This further reinforces the fact that *P. gingivalis* may play a central role in inducing or perpetuating RA. Although the sample size of these above-mentioned studies was relatively small, they have given new insights into the plausible aetiological link between periodontopathogens and RA.

Recently, another specific periodontal pathogen, *Aggregatibacter actinomycetemcomitans (A.actinomycetemcomitans)* has been proposed by Konig and colleagues as an autoimmunity trigger in rheumatoid arthritis. In their study, *A.actinomycetemcomitans* was shown to be able to dysregulate peptidyl arginine deiminases (PAD) in neutrophils, leading to the extracellular release of autoantigens following neutrophil apoptosis via *A.actinomycetemcomitans*-induced hypercitrullination. This process is mediated by its major virulence factor, leukotoxin A (LtxA) which forms pores on the cell membrane of neutrophils, allowing PAD activation and citrullination of a broad range of peptides. (Konig et al., 2016). Thus, their study provides new mechanistic insights into a biologically plausible link between *A.actinomycetemcomitans* in periodontitis and the promotion of autoimmunity directed against citrullinated proteins. However, direct demonstration on how *A.actinomycetemcomitans* can induce an anti-CitP response *in vivo* is still needed.

2.3.3.1 *Porphyromonas gingivalis* , citrullination and Rheumatoid Arthritis

Over the years, *P.gingivalis* has always been the focused periodontal pathogen in the research of the putative link between periodontitis and RA. This gram negative anaerobic bacteria has been the subject of particular interest among researchers in the last decade owing to its unique properties and virulence factors that allow it to evade host defence mechanisms and manipulate the complement pathway to its own benefit (Rosenstein et al., 2004). *P. gingivalis* is rarely present in periodontally healthy subjects but its colonization is mandatory for the progression of the periodontal lesion (Bostanci & Belibasakis, 2012; Detert et al., 2010; Farquharson, Butcher, & Culshaw, 2012). As an opportunistic pathogen, *P. gingivalis* possesses a number of virulence factors such as lipopolysaccharide (LPS), capsule, fimbriae and cysteine proteases (gingipains) that are detrimental to host cells (Bostanci & Belibasakis, 2012).

To date, *P.gingivalis* is the only prokaryote that expresses peptidyl arginine deiminase (PAD) which is known as *Porphyromonas gingivalis* peptidyl arginine deiminase (PPAD). It is an enzyme that catalyses citrullination of arginine in both host and bacterial proteins (Mcgraw et al., 1999). This citrullinated peptide antigen will then be present in PD patient's periodontium and further activate the adaptive immune response that is selective to RA (Mikuls et al., 2012). Since *P. gingivalis* is the only known microorganism with the ability to produce PPAD, therefore it may play an active role in peptide citrullination which might be involved in RA development (Liao et al., 2009; Potempa et al., 2017).

Unlike the human PAD, PPAD is able to drive the irreversible, post-translational conversion of both free arginine and peptidylarginine to citrulline without requiring calcium (Rosenstein et al., 2004; Schellekens et al., 2000). PPAD also citrullinates C-terminal arginine residues and free arginine at a slightly alkaline pH, while PADs cannot do this (Koziel et al., 2014). Besides that, the ability of *P. gingivalis* in increasing

intracellular calcium concentration through induction of defensive enzymatic activity may have an indirect role in promoting human PAD activity (Koziel et al., 2014; Loyola-Rodriguez, Martinez-Martinez, Abud-Mendoza, Patino-Marin, & Seymour, 2010; Mangat et al., 2010).

The ability of PPAD to citrullinate host proteins and lead to early anti-CitP response has also implicated *P. gingivalis* in the development and progression of RA (Maresz et al., 2013). In their animal study, it was demonstrated that collagen-induced arthritis was aggravated in mice infected with live *P. gingivalis* compared to no difference in disease progression noted in mice injected with heat-killed *P. gingivalis*, implying that exacerbation of RA was dependent on PPAD expression by *P. gingivalis*. In another study, mice inoculated with genetically modified PPAD deficient strains of *P. gingivalis* were associated with reduced amount of periodontal bone loss, less severe experimental arthritis and lower levels of anti-CitP as compared to mice inoculated with wild type *P.gingivalis* (Gully et al., 2014). This adds further evidence to the contributory role of *P. gingivalis* and its secreted PPAD enzyme in the pathogenesis of PD and RA.

Furthermore, higher levels of antibodies against *P. gingivalis* were found to be related to elevated levels of autoantibody, anti-CitP in RA patients compared to healthy controls. These findings further implicate *P. gingivalis* in the role that it might play in disease risk and progression in RA (Mikuls et al., 2009). Similarly, Hitchon et al demonstrated that anti-*P. gingivalis* levels were higher in anti-CitP positive patients with RA than anti-CitP negative patients with RA (Hitchon et al., 2010). In a recent Swedish population-based case–control study, Epidemiological Investigation of Rheumatoid Arthritis (EIRA), anti-*P. gingivalis* levels were found to be significantly elevated in anti-CitP positive RA patients compared to anti-CitP negative RA patients (Kharlamova et al., 2016). Based on these studies, it was suggested that *P. gingivalis* could be a credible candidate that may trigger and drive autoimmunity in RA patients.

2.3.4 Anti-citrullinated protein antibodies (anti-CitP) in Rheumatoid Arthritis patients with periodontitis

In view of the positive association between RA and PD via citrullination, various studies have been carried out to assess and compare the anti-CitP levels in RA patients with and without PD. In a case-control study conducted among a cohort of non-smoker RA patients, anti-CitP titres were reported to be significantly higher in RA patients with PD than those without PD. The PD status was also found to be more severe in these RA patients. These strong association of PD and high anti-CitP titres could serve as a potential environmental trigger in RA development (Potikuri et al., 2012). Therefore, these findings have further strengthened the association between PD and RA via citrullination.

Subsequently, a multicentre case– control study reported a significant association between serum anti-CitP and alveolar bone loss >20 % in 287 RA patients in comparison to patients with osteoarthritis. Among the RA subjects, those who presented with greater alveolar bone loss was significantly associated with higher serum anti-CitP concentration regardless of smoking habit. These could provide a novel insight into the important association between RA and PD as alveolar bone loss is an important hall mark of PD (Gonzalez. et al., 2015).

However, in two other studies conducted to investigate the levels of anti-CitP antibodies in RA patients with and without PD, none of these studies reported significantly higher levels of anti-CitP antibodies in RA-PD patients as compared to those RA non-PD patients (Dissick et al., 2010; Pischon et al., 2008). Thus, these results do not provide good evidence for the association between increased anti-CitP antibodies' level and the presence of PD and RA. Furthermore, in a recent study, serum anti-CitP Ig G was only found to be higher in RA patients, but no differences were observed between RA-non-PD and RA-PD patients (Laugisch et al., 2016), suggesting PD might not be contributing to the anti-CitP positivity in RA patients.

As a summary, the findings from the above-mentioned studies were found to be contradicting and inconclusive. Such discrepancy between the findings may be due to differences in patient cohorts and ELISA methodology. Thus, further investigations need to be performed to assess the actual contribution of PD in anti-CitP production which is specified to RA development.

2.4 Methods for serum antibodies quantification

2.4.1 Enzyme linked immunosorbent assay (ELISA)

The ELISA is widely utilized in clinical laboratories and biomedical research to detect and quantify a specific protein (such as cytokine, antibody, and antigen) in a biological sample. It is available for biological samples such as plasma, serum, GCF and tissue homogenous (Mire-Sluis, Gaines-Das, & Thorpe, 1995). ELISA enables the analysis of the protein samples using specific antibodies directed against the target protein to be measured. This method involves immobilization of the target protein onto a solid surface (usually a 96 wells' microplate) either by direct adsorption to the surface or via another antibody which is also specific to the same antigen (in "sandwich" ELISA) (Engvall & Perlmann, 1971).

After the immobilisation and plate blocking process, the target protein will be chemically linked to the detection antibodies and biological enzymes to produce a measurable signal with substrate solutions. The signal observed is proportional to the quantity of protein of interest in the sample. The washing steps in between procedures are to ensure that only specific "high-affinity" binding events are retained to produce signal at the final step for quantification (Avrameas, 1969; Clark, Lister, & Bar-Joseph, 1986).

ELISA is a validated immunoassay to detect the presence of antigen or antibody in liquid sample. Therefore, it is useful to determine the concentration of serum antibody. Currently, there are various types of ELISA available. For instance, direct ELISA,

sandwich ELISA and competitive ELISA are among the most commonly used kits. Among all, sandwich ELISA has been recognised as one of the most accurate tests due to its high specificity and sensitivity (Engvall & Perlmann, 1971).

The advantages of ELISA include high sensitivity and specificity because it can detect target proteins at the picogram level in a very specific manner using a specific antibody. It is also a high throughput, easy to perform test as commercial ELISA kits are easily available in a 96 wells format and suitable for various sample types such as plasma, serum, saliva and tissue extracts. Having said that, the handling procedures of ELISA can be technique sensitive and its performance might varies depending on the protein quality and operator's experiences (Aziz, Nishanian, Mitsuyasu, Detels, & Fahey, 1999). Furthermore, ELISA can only measure one specify protein in liquid sample at one time and not able to distinguish the biological activity of the target protein (Leng et al., 2008; Malone et al., 2001).

2.4.2 Multiplex assay

Multiplex assays have been developed and promoted from traditional ELISA due to its superiority over ELISAs. Multiplex assay can be used to measure multiple cytokines and antibodies in the same sample in a single analysis simultaneously. They are commercially available in different formats based on the usage of chemiluminescence, electrochemiluminescence as well as flow cytometry. They can perform equivalent to or better than currently available ELISA assays in terms of sensitivity, specificity, simplicity, and reliability (Kingsmore, 2006; Leng et al., 2008).

Multiplex assays have several advantages over ELISA including faster hybridisation kinetic enabling high throughput analysis, greater accessibility to antibody, higher efficiency as it requires less time for data acquisition and thus reducing assay time from several hours to less than an hour. Furthermore, the ability to measure multiple target

proteins in one liquid sample can reduce the amount of sample required and thus reducing potential errors arise from sample splitting. In addition to saving samples volume, multiplex assay also requires a shorter duration to complete. Simultaneous analysis can be carried for multiple cytokines within the same biological sample also facilitates investigation of the roles of different cytokine combinations with regards to disease progression which can be useful for clinical diagnosis of a disease (Leng et al., 2008).

However, multiplex assay is more expensive than ELISA due to its costlier instruments and consumables. Nevertheless, if we need to measure more target proteins, multiplex may be a cost-effective option rather than using separate ELISA assays. Despite its superiority over traditional ELISA, implementation of multiplex technology in the clinical setting and diagnostic laboratories require a more extensive validation to overcome its technical complexity and developmental challenges (Ellington, Kullo, Bailey, & Klee, 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

This was a cross-sectional comparative study conducted at Faculty of Dentistry, University of Malaya. Ethical approval was obtained from the Medical Research Ethics Committee (MREC), University Malaya Medical Centre (UMMC) [MRECID NO. 2017510-5227] (Appendix A) and Medical Ethics Committee, University of Malaya's Faculty of Dentistry [DF-RD1707/0029(L)] (Appendix B) prior to the study.

3.2 Study participants recruitment

This was part of a large study investigating the relationship between periodontitis and RA. RA participants were recruited from the Rheumatology Clinic of University Malaya Medical Centre (UMMC). Other participants were recruited among patients who came for dental treatment at the Outpatient Clinic of Faculty of Dentistry, University of Malaya. A total of 80 participants fulfilled the inclusion criteria and were invited to participate in this study. The recruitment was conducted between November 2017 and December 2018. Informed consent was obtained from all participants (Appendix C and D).

3.2.1 Inclusion Criteria

1. Participants who were Malaysian.
2. Participants who were 30 years old and above.
3. Participants who had at least eight remaining teeth excluding third molars.

A) Participants with RA

Those who diagnosed with Rheumatoid Arthritis (RA) based on 2010 American College of Rheumatology/ European League Against Rheumatism (ACR/EULAR) classification criteria for RA (Aletaha et al., 2010) with >1 year of diagnosis.

B) Participants with PD

Those who diagnosed with moderate to severe chronic periodontitis according to the 1999 AAP Classification of Periodontal Diseases and Conditions (Armitage, 1999).

C) Participants without PD

Those who were periodontally healthy or had gingivitis; PPD ⩽3mm and had ≤15% horizontal bone loss.

3.2.2 Exclusion Criteria

1. Non-Malaysian.
2. Participants who were on antibiotic usage within the past 4 months before study.
3. Participants who had received periodontal therapy within the past 4 months before the study.
4. Participants who had any concurrent systemic or debilitating conditions such as diabetes mellitus and other autoimmune diseases or malignancies.
5. Participants who were pregnant and lactating mothers.

All eligible participants were allocated into the four groups either RAPD group, RA group, PD group, or healthy group (control).

3.2.3 Sample size calculation

The sample size was calculated using PS (Power and Sample Size Calculation) Software version 3.0.43. Estimation of the sample size was based on the mean levels and standard deviations (SD) of serum anti-CitP in RA group (86.0±73.0 U/mL) and non-RA group (7.5±7.4 U/mL) (Karkucak, 2011). Each group must be represented by 20

participants in order to provide 80% power at a significance level of 5%. Thus, the total sample size for this study was 80 (N=80).

3.3 Questionnaire

Prior to the clinical examination, the participants completed a questionnaire (Appendix E) consisted of socio-demographic information (age, gender, and ethnicity), medical and dental histories, including lifestyle practices or habits (smoking status and oral hygiene habits).

3.4 Clinical measurements

3.4.1 Rheumatoid Arthritis

Participants diagnosed with RA were recruited from the Rheumatology Clinic of University Malaya Medical Centre (UMMC). RA parameters recorded were erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and disease duration of RA. These details were obtained from the medical records.

Diagnosis of RA:

All RA participants were diagnosed by a rheumatologist in accordance with the classification criteria of the 2010 American College of Rheumatology (ACR) / European League against Rheumatism (EULAR) (Aletaha et al., 2010). Those who fulfilled at least six of the ACR/ EULAR criteria for RA were recruited.

3.4.2 Periodontal disease

All participants were screened for PD by three trained examiners using a WHO probe (Hu-Friedy, Chicago America). Periodontal screening was performed using the Basic Periodontal Examination (BPE) by the British Society of Periodontology. Those who fulfilled the inclusion criteria were recruited. Full-mouth periodontal examination was performed. The number of teeth present was recorded. Third molars were excluded.

3.4.3 Clinical periodontal parameters:

The UNC 15 colour coded periodontal probe (Hu-Friedy, Chicago USA) was used to measure the periodontal clinical outcomes. Data were recorded in a charting form as shown in Appendix F and G. All teeth except wisdom teeth were examined.

i. Visible Plaque Index (VPI) (Ainamo & Bay, 1975)

VPI was carried out at four sites in each tooth (mesio-buccal, mid-buccal, disto-buccal and palatal/lingual surface) using a dichotomous scoring system. The visible detection of plaque was recorded as presence (1) or absence (0) without the use of disclosing tablet.

0 = No visible plaque

1 = Visible plaque

ii. Gingival Bleeding Index (GBI) (Ainamo & Bay, 1975)

GBI was assessed at four sites of each tooth using a dichotomous scoring system. If there was bleeding within 10 seconds of probing, it was recorded as score "1" (presence) and score "0" was given if there was no bleeding within 10 seconds upon probing.

0= No visible bleeding

1= Visible bleeding

iii. Probing Pocket Depth (PPD)

Probing pocket depth was measured from the gingival margin to the base of the pocket or gingival sulcus. It was measured at six points per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) using periodontal probe with calibrated markings by placing the probe parallel to the long axis of the tooth. PPD measurement was recorded to the nearest millimeter.

iv. Recession (GR)

Recession was measured from cementoenamel junction (CEJ) to gingival margin at six sites per tooth (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual) using periodontal probe with calibrated markings. The probe was placed parallel to the long axis of the tooth and measurement was recorded to the nearest millimeter.

v. Clinical Attachment Level (CAL)

Clinical attachment level was measured from the CEJ to the base of the pocket. CAL was estimated as the sum of PPD and GR.

3.4.4 Standardisation of examiners

This study was part of an on-going large-scale RA-PD research project. Therefore, three trained examiners were involved in the patient examination and data collection. The standardisation exercise for the measurements of clinical parameters were carried out among the three examiners (LPH, LYH, and PHSH) to ensure the reliability of the results obtained.

The intra-examiner reproducibility was performed for PPD and CAL measurements on two volunteers. A total of 56 teeth and 336 sites were examined. The procedure was performed by an examiner (LPH) in two sessions (morning and afternoon) on two different days. The intra-examiner agreement (Kappa value) for PPD and CAL was calculated using Cohen's kappa coefficient (κ). The κ value for intra-examiner reproducibility was 0.934 for PPD and 0.918 for CAL. These values showed excellent intra-examiner reproducibility.

As for the inter-examiner agreement and reproducibility, all three examiners were trained by an experienced periodontist before the commencement of the study. Each examiner measured PPD and CAL on two volunteers with total of 336 sites on 56 teeth.

The level of agreement against the gold standard examiner was κ value of 0.883 for PPD and 0.832 for CAL which indicated excellent intra-examiner reproducibility. The intra- and inter-examiner reproducibility agreements are reported in Table 3.1.

Table 3.1: Intra-examiner and inter-examiner reproducibility for PPD and CAL

Index	Number of sites	Intra-examiner reproducibility		Inter-examiner reproducibility	
		Kappa value	Asymptotic standard error	Kappa value	Asymptotic standard error
PPD	336	0. 934	0.032	0.883	0.042
CAL	336	0. 918	0.035	0.832	0050

PPD, periodontal pocket depth; CAL, clinical attachment level

3.4.5 Blood sampling procedures

Peripheral blood (10 mL) was obtained from venous cubital fossa using a butterfly needle and transferred into BD vacutainer blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, USA). The tubes were immediately placed in an ice box before being transported to the lab. After coagulation, the blood sample was centrifuged for 15 minutes at 1000× g. The serum was aliquoted into labelled microcentrifuge tubes (1.5 μL) and stored at -80°C until analysis.

3.5 IMMUNOASSAY ANALYSIS

3.5.1 Enzyme Linked Immunosorbent Assay (ELISA)

Levels of anti-CitP in serum samples were determined using Human CCP-Ab (Anti-cyclic citrullinated peptide) ELISA Kits (Elabscience, USA). Standard "Sandwich" ELISA was performed according to the manufacturer's instructions (Appendix J). A 96-well microtiter plate that had been pre-coated with antigens specific to Human CCP-antibody was used. Prior to the experiment, assay optimization was conducted to determine the dilution factor for the autoantibody.

3.5.2 Serum samples dilution and preparation

The serum samples were thawed to room temperature and centrifuged at 1000× g for 15 minutes. The samples were diluted with sample diluents provided in the kit according to dilution factor of four which was previously determined from the assay optimization procedure.

3.5.3 Standard dilution and standard working solution preparation

The standard was centrifuged at 10,000× g for one minute. The reference standard (1 mL) was added to sample diluent and allowed to stand for 10 minutes before being inverted 5 times to make up 400 IU/mL working solution. Two-fold serial dilutions (400, 200, 100, 50, 25, 12.5, 6.25, 0 IU/mL) were prepared according to the manufacturer's protocol by adding sample diluent to the reference standard. Dilution series of the standard solution is illustrated in Figure 3.1.

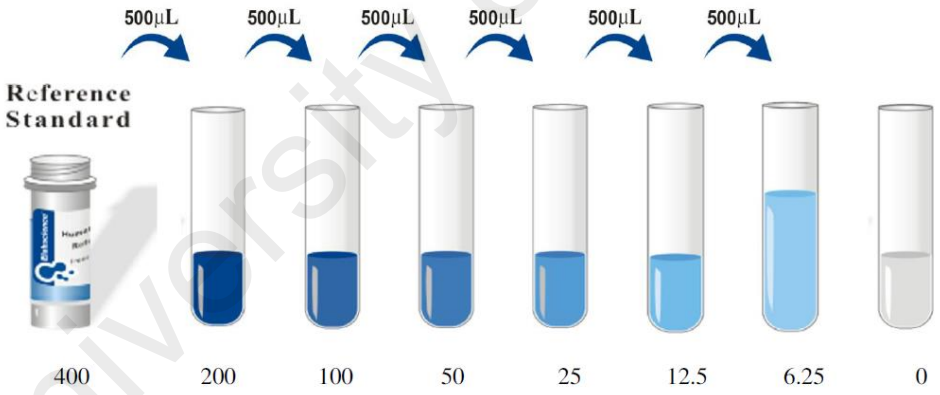

Figure 3.1: Dilution series of standard (Human CCP-Ab, Elabscience USA)

3.5.4 Reagent preparation

All reagents were brought to warm to room temperature for 30 minutes before use. Wash buffer was prepared by mixing 30 mL of Wash Buffer Concentrate with 720 mL distilled water. Biotinylated Detection Ag working solution was prepared by diluting the 100× Concentrated Biotinylated Detection Ag to 1× working solution with Biotinylated Detection Ag Diluent. For Concentrated HRP Conjugate working solution, it was

prepared by diluting the 100× Concentrated HRP conjugate to 1× working solution with Concentrated HRP Conjugate Diluent.

3.5.5 Human CCP-Ab ELISA procedures

All reagents and samples were prepared as mentioned in previous sections (3.5.2, 3.5.3. and 3.5.4). For assay procedures, 100 μL of the diluted standard working solution was added to the first two columns of the microplates except one well was left blank as the zero standard. Diluted samples (100 μL) were then added to each of the remaining wells. The plates were covered with adhesive strip and then incubated at 37°C for 90 minutes. After incubation, the wells were aspirated and 100 μL of Biotinylated Detection Ag working solution was added to each well immediately. The microplates were then covered with plate sealer and incubated at 37°C for an hour. After incubation, all the wells were aspirated and washed for three times using the wash buffer (350 μL). After the last wash, the remaining wash buffer were removed by aspiration and the plate was inverted and blotted against the clean paper towels to ensure complete removal of liquid. One hundred microliter (100 μL) of HRP-conjugate working solution was added to each well except the blank well and incubated for 30 minutes at 37°C. After incubation, the washing process was repeated five times. Following this, 90 μL of substrate reagent was added to each well and incubated for 15 minutes at 37°C, protecting from light. Lastly, 50 μL of stop solution was added to each well and mixed thoroughly by gentle tapping. The assay was performed in triplicates. The optical density (absorbance) of each well was determined within 10 minutes by using a microplate reader set at 450nm wavelength. Standard curve was generated using standard optical density and the concentration of anti-CitP in serum samples were determined based on the standard curve and multiplied by the dilution factor of four.

3.6 Data collection and study flow chart

All participants who fulfilled the inclusion and exclusion criteria from Rheumatology Clinic in UMMC and the dental outpatient clinic in the University of Malaya's Faculty of Dentistry were invited to participate in the study. Patient information sheet (PIS) which are available in both the English and Malay languages (Appendix H and I) was presented to the participant. A booklet with an identification number was assigned to each participant who volunteered for this study. The booklet contained the study's consent form, questionnaire and recording forms for the clinical examination component.

Prior to the examination, informed consent was obtained from each participant. All participants were required to complete the questionnaires and Basic Periodontal Examination (BPE) was carried out to screen for periodontal disease. Comprehensive full mouth periodontal examination was performed to measure VPI, GBI, PPD, GR and CAL from all included participants.

Blood samples were collected from all the consented participants. After that, the blood sample was delivered to laboratory for processing and storage. All the four groups of serum samples (RAPD, RA, PD, control group) were assayed using ELISA to quantify the serum levels of anti-CitP following the manufacturer's protocol. All data collected was then analysed using SPSS statistical analysis. The summary of the study design was demonstrated in the flow chart in Figure 3.2.

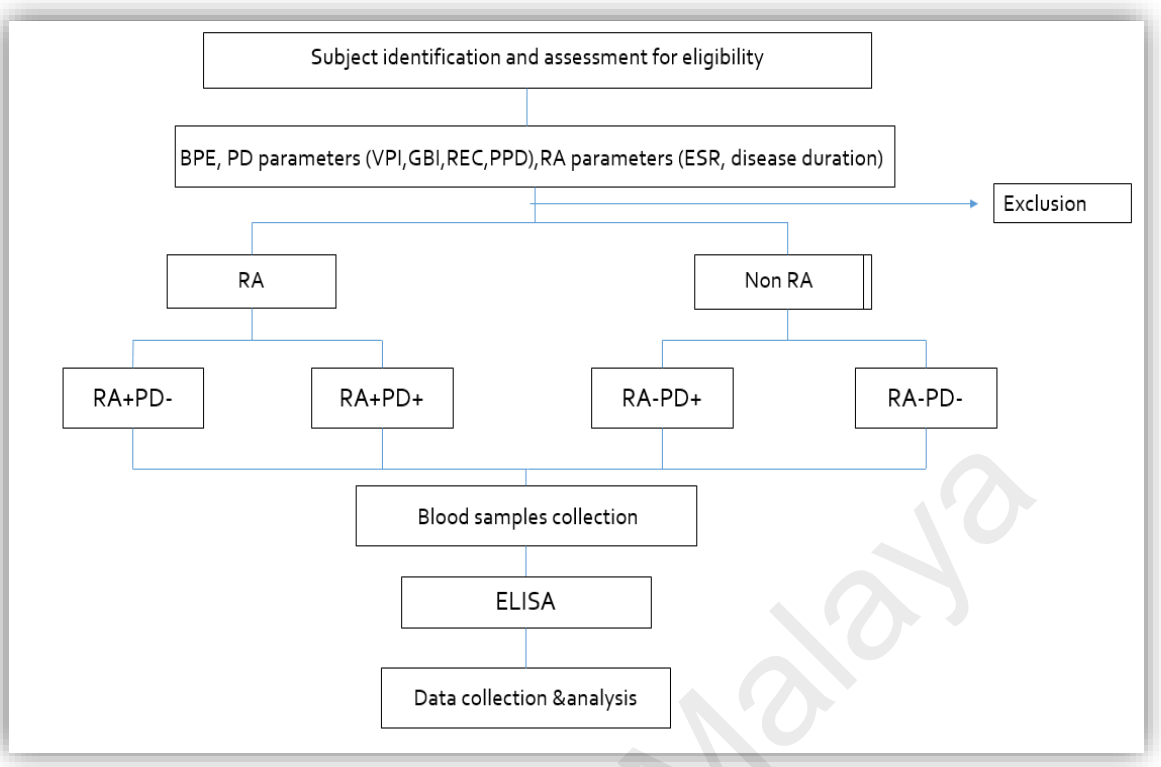

Figure 3.2: Flow chart of the study design

3.7 Statistical analysis

Statistical analysis was performed using SPSS 25.0 statistical software (IBM, Chicago, IL, USA). The statistical significant level was set at $p < 0.05$ at 95% confidence interval (CI).

First, all the metric demographic and clinical data were checked for normal distribution using the Kolmogorov–Smirnov test. The Kolmogorov- Smirnov test showed most of the data were normally distributed with $p > 0.05$ except for CAL and anti-CitP that showed non-normally distributed data with $p < 0.05$. Therefore, parametric test (one-way ANOVA) and non-parametric test (Kruskal-Wallis) were used to compare the periodontal parameters (VPI, GBI, PPD and CAL), RA parameters (ESR, RA disease duration) as well as the laboratory biomarker (serum anti-CitP levels) between the four groups of participants in this study.

The differences in the distributions of categorical outcomes were analysed using the Pearson chi-square test while the differences in continuous outcome between the four groups were determined by either one-way ANOVA or Kruskal-Wallis test depending on the normality of data. The correlation between the serum level of anti-CitP and clinical periodontal and RA parameters were evaluated using Pearson correlation test.

CHAPTER 4: RESULTS

4.1 Characteristics of study population

Table 4.1 summarises characteristics of the four groups of participants included in this study (RAPD, RA, PD and control groups). There were 20 participants in each group (n=20). For the RAPD group, the mean age was 54.25±7.47 years, whereas in RA group mean age was 52.70±9.49 years. The PD and control group participants were generally younger than RA group with the mean age of 44.0±11.56 and 39.60±11.55 years respectively. For all the four groups, most of the participants were females.

Ethnicity distribution in RAPD group comprised of 55% Chinese, 20% Malay and 25% Indian. There was almost equal distribution of three ethnic groups. Similarly, there were equal numbers of Malay and Chinese participants in PD group (45%), while only 10% of the participants were Indian. However, most of the control participants were Malay (65%), followed by Chinese and Indian (25% and 10% respectively). For the RA, PD and control groups, majority of the participants had tertiary education level (65%, 65% and 90% respectively). The rest of the participants in these groups completed secondary education and only one participant in RA group completed primary education. On the contrary, those in RAPD group mostly completed secondary education and only 30% of them finished tertiary education.

With regards to smoking habits, all participants from the RA group and most of the participants from the other three groups were non-smokers. Only two participants from the RAPD group , one from the PD and control groups were smokers. About 40% of the RAPD group had been diagnosed with RA less than 5 years ago at the time of recruitment, 30% of them had suffered from RA for 5 to 10 years and 30 % of them had suffered from RA for more than 10 years duration. Almost half of the participants (45%) in the RA group had RA for 5 to 10 years. Seven participants (35%) were diagnosed with

RA less than 5 years and four of the RA participants had suffered from RA for more than 10 years.

Most of the participants in the RAPD and PD groups were diagnosed with localised moderate to severe PD. There were more participants diagnosed with generalised moderate to severe PD (30%) in the PD group than the RAPD group (15%). However, the difference was not statistically significant. There were statistical significant differences in age and gender between the four groups ($p < 0.05$), however, no statistical significant differences were observed for the education level, ethnicity, smoking status and duration of RA.

Table 4.1: Sample characteristics of study population

Variables/ Characteristics	RAPD (n=20)	RA (n=20)	PD (n=20)	Control (n=20)	*p*-value
Age (Means±SD)	54.25± 7.47	52.70± 9.49	44.0± 11.56	39.60± 11.5	**<0.00[a]
	n (%)	**n (%)**	**n (%)**	**n (%)**	
Gender					
• **Female**	14 (70)	19(95)	11(55)	16(80)	**0.028[b]
• **Male**	6 (30)	1(5)	9(45)	4(20)	
Ethnicity					
• **Malay**	4 (20)	7(35)	9(45)	13(65)	0.086[b]
• **Chinese**	11(55)	7(35)	9(45)	5(25)	
• **Indian**	5(25)	6(30)	2(10)	2(10)	
Educational level					
• **Tertiary**	6(30)	13(65)	13(65)	18(90)	0.050[b]
• **Secondary**	14(70)	6(30)	7(35)	2(10)	
• **Primary**	0(0)	1(5)	0(0)	0(0)	
Smoking status					
• **Smoker**	2(10)	0(0)	3(15)	3(15)	0.457[b]
• **Former-smoker**	2(10)	0(0)	1(5)	1(5)	
• **Non-smoker**	16(80)	20(100)	16(80)	16(80)	
RA disease duration (years)					0.587[b]
• **< 5**	8(40)	7(35)	NA	NA	
• **5 – 10**	6(30)	9(45)	NA	NA	
• **>10**	6(30)	4(20)	NA	NA	
PD status					
• **Localised**	17(85)	NA	14(70)	NA	0.110[b]
• **Generalised**	3(15)	NA	6(30)	NA	

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; PD: Participants without RA but has PD; Control: Participants without both RA and PD; [a] One-way ANOVA ,**significant set at $p<0.05$; [b] Pearson Chi-square test, ** significant set at $p<0.05$.

4.2 Mean ESR levels for RA and RAPD groups

The mean values (±SD) of ESR for both RAPD and RA groups, which is the clinical parameter for RA were demonstrated in Figure 4.1. The mean ESR for RAPD group was 32.75±21.38 mm/hr, which was slightly higher than the RA group, 27.90±11.56 mm/hr. However, there was no statistical significant difference for mean ESR between these 2 groups ($p > 0.05$).

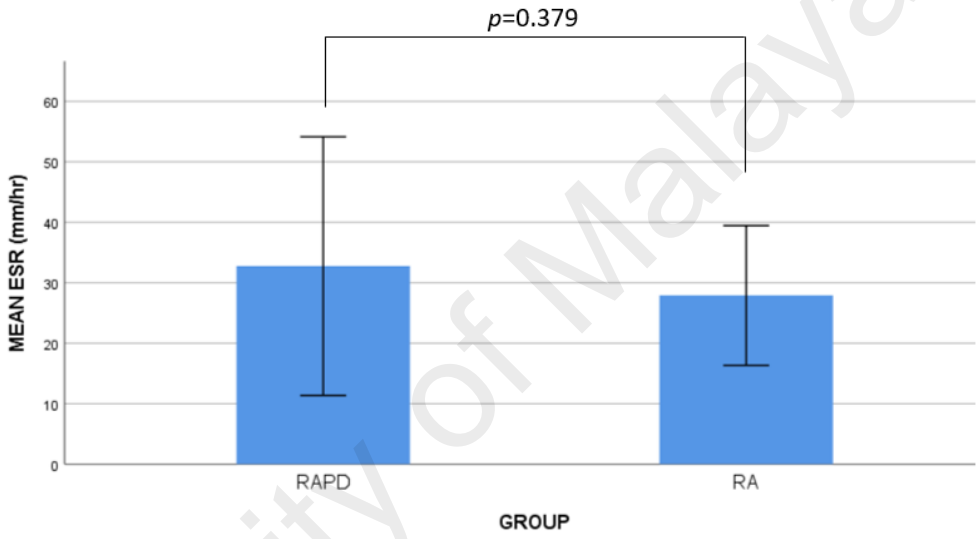

Figure 4.1: Mean (±SD) ESR between RAPD and RA groups. Comparison was analysed using Independent sample T-test, significant level was set at $p<0.05$.

4.3 Comparison of periodontal parameters (means VPI, GBI, PPD and CAL) between groups

Table 4.2 shows the mean values (±SD) of the clinical periodontal parameters (VPI, GBI, PPD and CAL) for the four groups. The mean (±SD) VPI was found to be the highest in the RAPD group (55.24±25.13%) followed by PD group and RA group. The control group showed the lowest VPI among all groups with mean VPI of 28.47±20.78%. Meanwhile for GBI, PD group was found to have the highest mean GBI among all four groups which was about 40.02±24.45%. In contrast, the RA group was found to have the lowest mean GBI among the 4 groups with the mean values of 7.29±6.54%.

For PPD and CAL, PD group was found to have the highest mean PPD and CAL with the mean values of 3.62±0.92mm and 4.33±1.78mm respectively, followed by RAPD group with the mean PPD of 3.07±0.72mm and mean CAL of 3.77±1.12mm. On the other hand, both the RA and control group were found to have similar mean values of PPD and CAL, which were much lower than the periodontitis group as expected. For the RA group, the mean ± (SD) value was 1.92±0.28mm for the PPD and 0.61±0.10mm for the CAL. Likewise, the mean PPD and CAL for the control group were 1.95±0.32mm and 0.63±0.19mm respectively.

Statistical significant difference ($p < 0.05$) were observed for all the clinical periodontal parameters measured (VPI, GBI, PPD and CAL) between the four groups of participants (RAPD, RA, PD and control groups). The post hoc comparison between groups is shown in Table 4.2 and Figures 4.2-4.5.

Table 4.2: Comparison of the mean ± (SD) VPI, GBI, PPD and CAL between groups

Clinical parameters	RAPD (n=20)	RA (n=20)	PD (n=20)	Control (n=20)	*p*-value
	Means±SD	Means±SD	Means±SD	Means±SD	
VPI (%)	55.24±25.13[f]	36.04±23.17	55.18±26.94[g]	28.47±20.78[f,g]	**0.001[d]
GBI (%)	33.52±23.41[f]	7.29±6.54	40.02±24.45[g]	12.92±15.39[f,g]	**<0.001[d]
PPD (mm)	3.07±0.72[j,f]	1.92±0.28[j,k]	3.62±0.92[k,g]	1.95±0.32[f,g]	**<0.001[e]
CAL (mm)	3.77±1.12[j,f]	0.61±0.10[j,k]	4.33±1.78[k,g]	0.63±0.19[f,g]	**<0.001[e]

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; PD: Participants without RA but has PD; Control: Participants without both RA and PD; VPI: visible plaque index; GBI: gingival bleeding index; PPD, probing pocket depth; CAL: clinical attachment level; [d] One-way ANOVA; [e] Kruskal- Wallis Test **significant at $p<0.05$; [f] Significant different between RAPD and control; [g] Significant different between PD and control; [j] Significant different between RAPD and RA; [k] Significant different between RA and PD

Post-hoc comparison between groups:

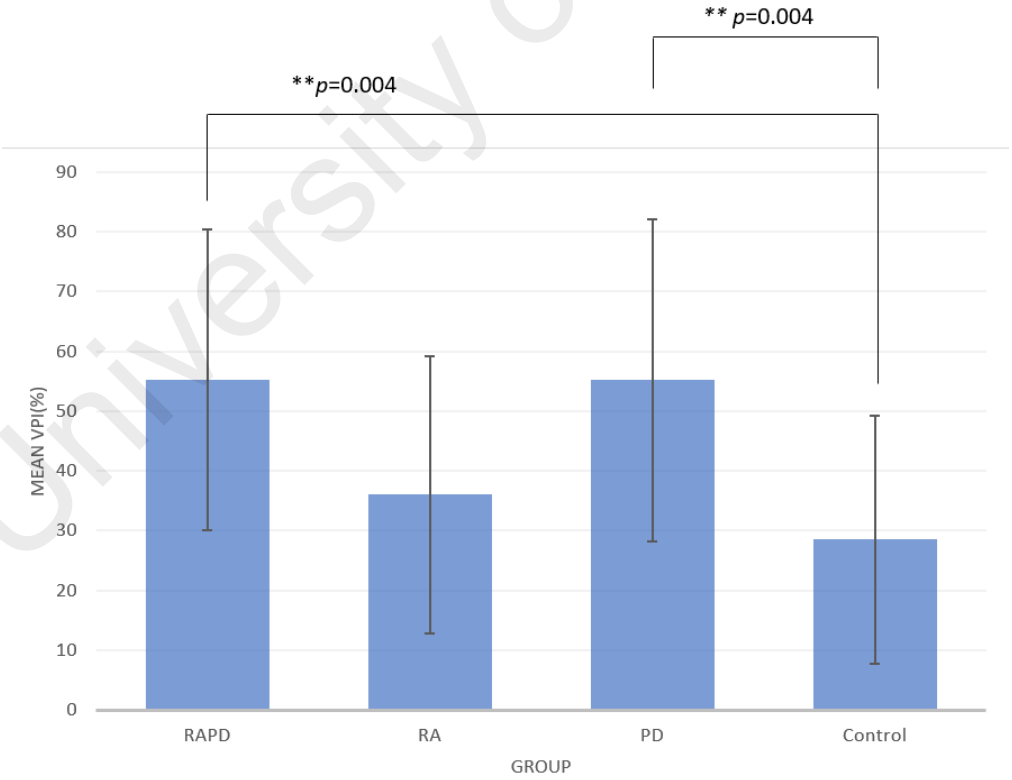

Figure 4.2: Mean VPI (%) of the four groups of participants. ** indicates significant difference inter-group at $p<0.05$.

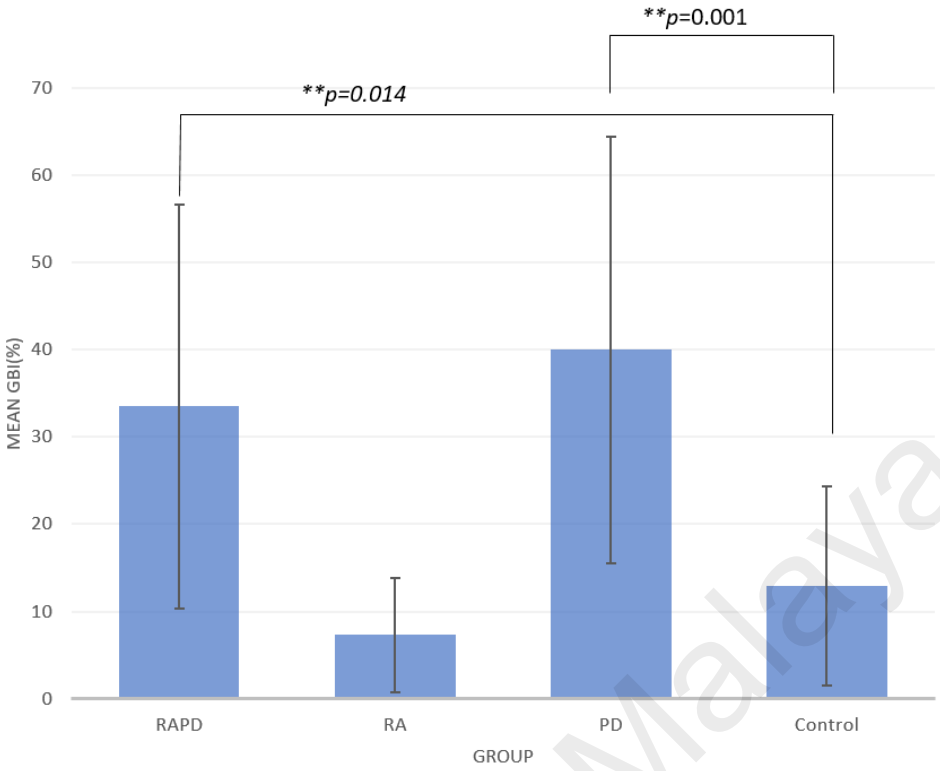

Figure 4.3: Mean GBI (%) of the four groups of participants. ** indicates significant difference inter-group at $p<0.05$.

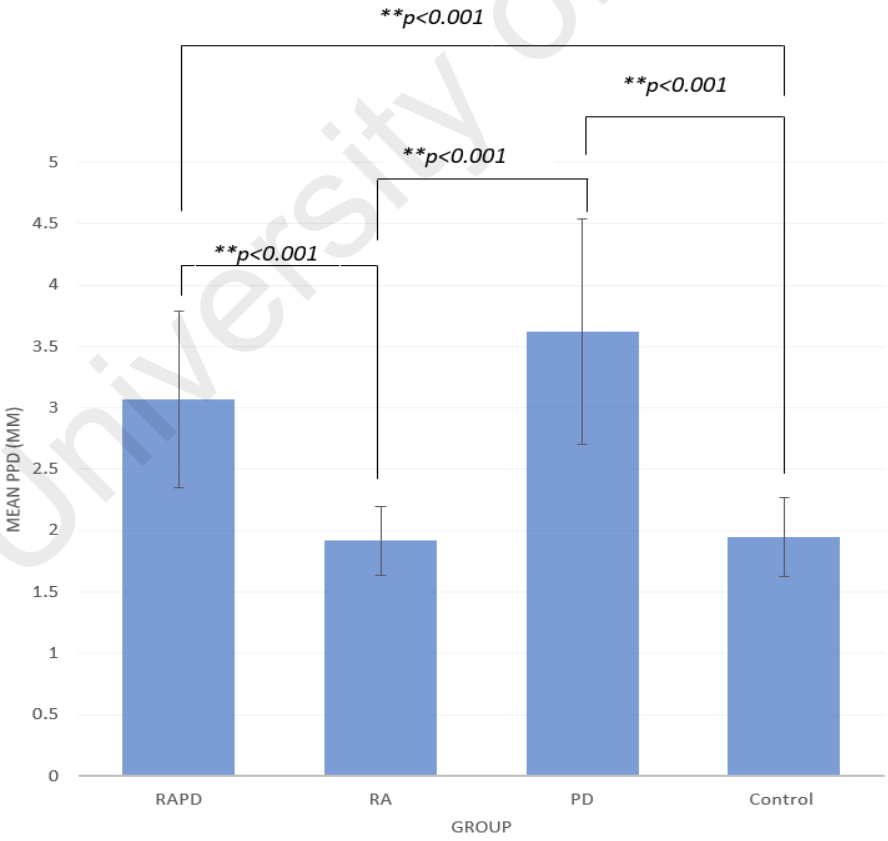

Figure 4.4: Mean PPD (mm) of the four groups of participants. ** indicates significant difference inter-group at $p<0.05$

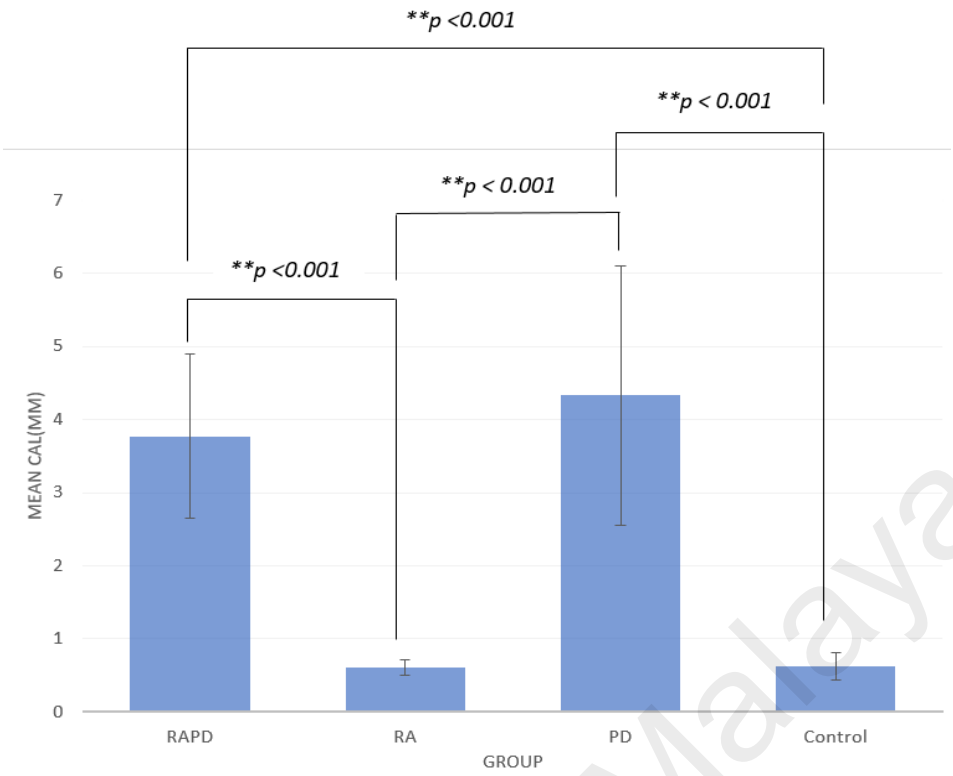

Figure 4.5: Mean CAL (mm) of the four groups of participants. ** indicates significant difference inter-group at $p<0.05$.

4.4 Comparison of mean serum anti-citrullinated protein antibody levels between groups

Overall results show the highest mean (± SD) levels of serum anti-CitP in RAPD group (228.82±219.09 IU/mL) as compared to the other three groups. The mean (±SD) levels of anti-CitP in the RA group and PD group were 204.01±202.41 IU/mL and 102.62±75.46 IU/mL respectively while the subjects in control group showed the lowest concentration of anti-CitP in their serum (68.73±52.49 IU/mL).

Furthermore, there were statistical significant differences ($p < 0.05$) observed for mean serum level of anti-CitP between these four groups of participants (RAPD, RA, PD and control groups). Within groups, there were statistical significant difference observed for anti-CitP levels between RAPD and control group as well as between RA and the control group.

Table 4.3: Comparison of the mean (±SD) serum anti-citrullinated protein antibody level between groups

Serum antibodies level	**RAPD (n=20)**	**RA (n=20)**	**PD (n=20)**	**Control (n=20)**	***p*-value**
	Means±SD	**Means±SD**	**Means±SD**	**Means±SD**	
anti-CitP level (IU/mL)	228.82 ±219.09[r]	204.01 ±202.41[s]	102.62 ±75.46	68.73 ±52.49[r,s]	**0.005[k]

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; PD: Participants without RA but has PD; Control: Participants without both RA and PD; Anti-CitP: anti-citrullinated protein antibodies; [k]Kruskal- Wallis test, **significant at $p<0.05$; [r] Significant different between RAPD and control group ($p=0.026$); [s] Significant different between RA and control group ($p=0.048$)

4.5 Distribution of serum anti-citrullinated proteins for all participants

The distribution of the serum anti-CitP levels for all the four groups of participants are shown in ascending order in Figure 4.6. There is a trend of an increase in anti-CitP levels from control, PD group, RA group to RAPD group. All the participants in the control group exhibited less than 200 IU/mL of anti-CitP while participants in PD group demonstrated anti-CitP levels of less than 300 IU/mL. The mean anti-CitP in the control group (68.73±52.49 IU/mL), indicated by dotted line in Figure 4.6, was used as the cut-off value for comparisons between groups using mean of the control as relative comparison.

Table 4.4 shows comparisons of anti-CitP levels between groups relative to the cut-off value. Participants with anti-CitP levels higher than 68.73±52.49 IU/mL were considered positive for anti-CitP. The highest number/percentage of positive participants were in the RAPD group followed by RA, PD and control group as demonstrated in Table 4.4.

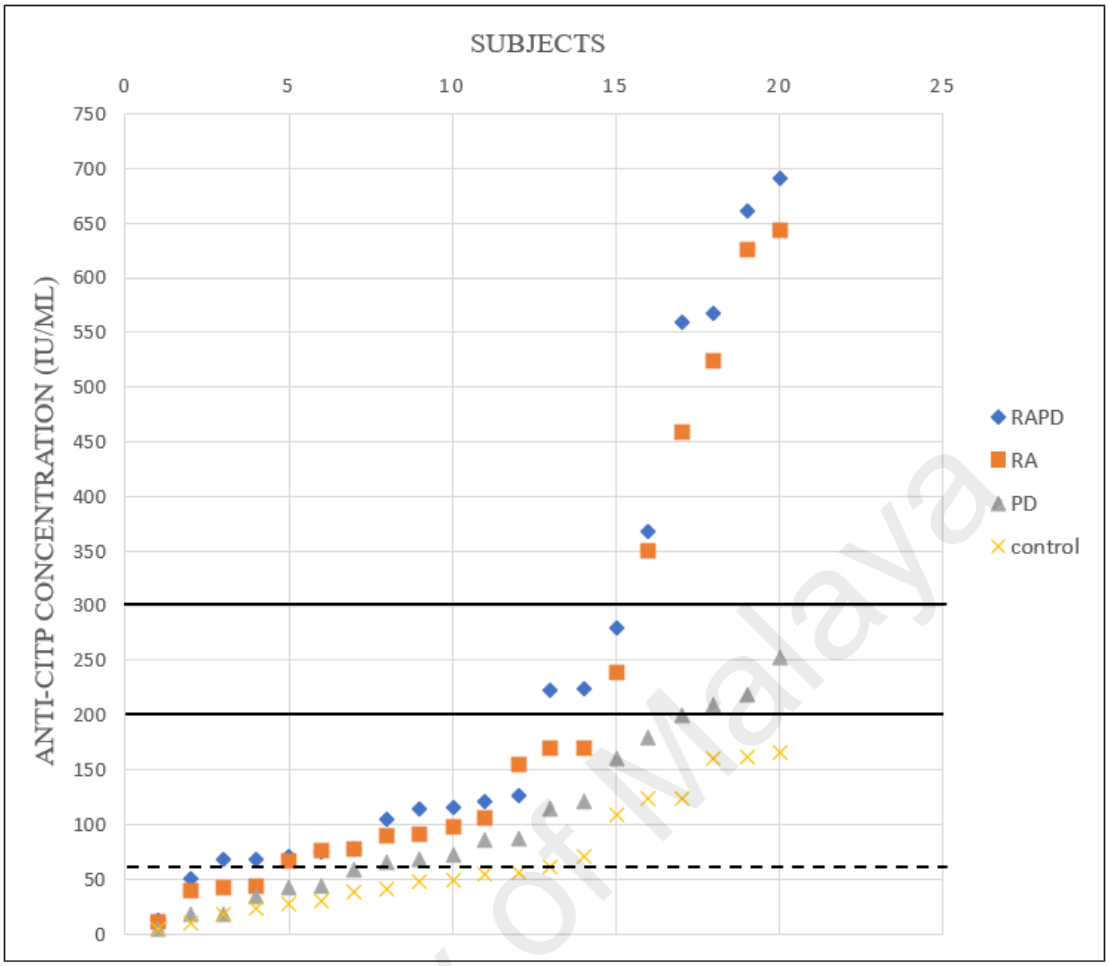

Figure 4.6: Distribution of serum anti-CitP concentration in four groups of participants (in ascending order)

Table 4.4: Comparison of subject distribution between groups using mean serum anti-citrullinated protein antibody in control group as cut off point

Serum anti-CitP	**RAPD (n=20)**	**RA (n=20)**	**PD (n=20)**	**Control (n=20)**
	n (%)	n (%)	n (%)	n (%)
Positive	16(80)	15(75)	11(55)	7(35)
Negative	4(20)	5(25)	9(45)	13(65)

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; PD: Participants without RA but has PD; Control: Participants without both RA and PD; Anti-CitP, anti-citrullinated protein antibodies; Positive: participants with anti-CitP level > 68.73±52.49 IU/mL; Negative: participants with anti-CitP level < 68.73±52.49 IU/mL

4.6 Standard multiple regression analysis for serum anti-citrullinated protein levels

The combination of age, gender and smoking status accounted for 1.5% of the variability in serum anti-CitP levels, with the $R^2 = 0.015$, F (3, 76) = 0.609, p= 0.611. The results for each predictor (age, gender and smoking status) were reported in Table 4.5, showing age, gender and smoking status did not contribute significantly to the model.

Table 4.5; Relationship between serum anti-citrullinated protein antibody levels and potential predictors (age, gender, smoking status)

Variable	*B* [95% CI]	β	sr^2	*p*-value
Age	1.71[-1.49,4.91]	0.12	0.014	0.290
Gender	-28.18[-95.55,77.47]	0.023	0.001	0.836
Smoking Status	19.28[-27.67,66.22]	0.105	0.093	0.416

Multiple linear regression analysis, **significant at p<0.05; B = Unstandardised regression coefficients; β= Standardised regression coefficients; CI= Confidence interval; Sr^2= Squared Semi-Partial Correlations

4.7 Bivariate correlation analysis of serum anti-citrullinated protein levels with RA parameters

Bivariate correlation analyses were performed to assess the relationship of serum anti-citrullinated proteins levels with clinical parameters of RA in participants with RA (comprised of RAPD and RA groups). The findings are presented in Table 4.6. Pearson correlation test was used to assess the correlation. If the "r" value (referring to Pearson correlation coefficient) is between 0.50-1.0, 0.30-0.50 and below 0.30, the correlation will be considered as strong, moderate and weak to no correlation respectively.

In both RAPD and RA groups, there were no correlations between the serum anti-CitP levels with ESR and RA disease duration ($r < 0.30$; $p > 0.05$).

Table 4.6: Bivariate correlation analyses of serum anti-citrullinated protein with clinical RA parameters

RAPD (n=20)			**RA (n=20)**		
anti- CitP	**r**	***p*-value**	**anti-CitP**	**r**	***p*-value**
ESR	0.242	0.141	**ESR**	-0.133	0.577
Disease duration	0.262	0.264	**Disease duration**	-0.012	0.961

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; Anti-CitP: anti-citrullinated protein antibodies; ESR: Erythrocyte sedimentation rate; Pearson correlation test; r-value: 0.5 to 1.0 (strong correlation); -0.3 to 0.5 (moderate correlation); below 0.3 (weak to no correlation), **significant at $p<0.05$

4.8 Bivariate correlation analysis of serum anti-citrullinated protein levels with PD parameters

Bivariate correlation analyses were performed to assess the relationship of serum anti-citrullinated proteins levels with clinical periodontal parameters in all four groups of participants as presented in Table 4.7. Pearson correlation test was used to assess the correlation. If the "r" value (referring to Pearson correlation coefficient) is between 0.50-1.0, 0.30-0.50 and below 0.30, the correlation will be considered as strong, moderate and weak to no correlation respectively.

In all the four groups, none were statistically significant between the serum anti-CitP levels with all the clinical periodontal parameters ($r < 0.30$; $p > 0.05$).

Table 4.7: Bivariate correlation analyses of serum anti-citrullinated protein antibodies with clinical periodontal parameters

	RAPD (n=20)		**RA (n=20)**		**PD (n=20)**		**Control (n=20)**	
anti-CitP	**r**	***p*-value**	**R**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
VPI	0.228	0.334	0.254	0.28	0.119	0.618	0.085	0.723
GBI	0.052	0.828	-0.030	0.90	0.045	0.850	-0.058	0.808
PPD	-0.03	0.899	-0.057	0.811	0.051	0.830	-0.182	0.441
CAL	0.099	0.678	0.275	0.240	0.107	0.654	-0.201	0.196

RA: Rheumatoid Arthritis; PD: Periodontitis; RAPD: Participants with RA and PD; RA: Participants with RA but without PD; PD: Participants without RA but has PD; Control: Participants without both RA and PD; Anti-CitP: anti-citrullinated protein antibodies; VPI: visible plaque index; GBI: gingival bleeding index; PPD: probing pocket depth; CAL: clinical attachment level; Pearson correlation test; r-value : 0.5 to 1.0 (strong correlation); -0.3 to 0.5 (moderate correlation); below 0.3 (weak to no correlation)),**significant at $p<0.05$

CHAPTER 5: DISCUSSION

5.1 DISCUSSION OF MATERIALS AND METHODS

5.1.1 Study design and sample population

This study was a cross sectional comparative study, which was conducted to provide a comparative evaluation of the serum levels of anti-CitP together with clinical RA and PD parameters in relation to PD and RA. Healthy participants without both RA and PD were also recruited as the control group for the present study. Over the years, different study designs have been used in studies that investigate serum anti-CitP in RA and/or PD patients ranging from case control, cross sectional to prospective cohort studies. For example, a large scale cohort study was carried out in Japan to assess the association between anti-CitP and PD status among the healthy population (Terao et al., 2015). However, for this study, a cross sectional study design was adopted as it could be conducted in a shorter time frame and was inexpensive as compared to a prospective study design. Furthermore, it is a type of observational study that allowed data analysis from the defined population of RA, PD and healthy groups, at a specific time point.

RA participants were recruited from those who were under RA follow-up at Rheumatology Clinic of University Malaya Medical Centre (UMMC) while the non-RA groups (PD group and control group) were recruited from the Outpatient Clinic of Faculty of Dentistry, University of Malaya. This strategy was a convenience sampling technique dictated by access to patients attending the clinic. Whilst a probability sampling technique that was based on the principle of randomisation would have been more ideal and preferred, convenience sampling was used as this technique allowed the desired sample size to be achieved in a relatively fast and cost-effective way. Furthermore, this study only included those participants who fulfilled the inclusion and exclusion criteria in order to ensure the homogeneity of the sample population as well as to reduce potential confounding factors.

This present study chose to exclude participants who had other autoimmune diseases and diabetes mellitus as well as those who had been on antibiotics or received periodontal therapy within the past four months before the study. All these were potential confounders that might affect our study outcomes. Diabetes mellitus has long been identified as the true risk factor that can contribute to an increase in the prevalence or severity of PD (Papapanou, 1996) while usage of systemic antibiotics were shown to cause fluctuation of serum antibody levels (Zhang, Minardi, Kuenstner, Zekan, & Kruzelock, 2018). Furthermore, periodontal treatment had also been shown to reduce serum anti-CitP levels in participants with RA and PD (Lappin et al., 2013; Okada et al., 2013).

However, we did not exclude smokers during subject recruitment. We acknowledge that smoking is a true risk factor for PD that contributes to an increase in prevalence and severity of periodontitis (Papapanou, 1996; Susin, Oppermann, Haugejorden, & Albandar, 2004). In RA, cigarette smoking has also been identified as an important environmental risk factor as it may lead to increased formation of citrullinated proteins and anti-CitP (Baka, Buzás, & Nagy, 2009; Belakova, Manka, Racay, & Zanova, 2017). However, due to the high prevalence of smokers reported in Malaysia whereby 22.8% (five million) of Malaysian adults were current tobacco smokers (National Healthy and Morbidity Survey, NHMS 2015), it was difficult to exclude smokers from the study. There was also a concern that the exclusion of smokers may hinder the desired sample size within the sampling time frame. Thus, it was decided not to exclude smokers during recruitment and to use statistics to control for this confounder. For instance, we applied multiple linear regression model, adjusting for smoking habit for anti-CitP level as the outcome variable.

5.1.2 Case definition

In this study, the 1999 AAP Classification of Periodontal Diseases and Conditions was used to define cases of periodontitis (Armitage 1999). It is the most widely used classification in daily clinical practice. This classification categorised the disease severity based on the amount of clinical attachment loss (CAL) as follows: mild= 1 to 2 mm CAL, moderate = 3 to 4 mm CAL, and severe = ≥5 mm CAL. With regards to the extent of disease, PD can be further classified as localised or generalised depending on whether the patient had less than 30% or more than 30% of sites involved. Most previous studies investigating serum anti-CitP in RA and PD had used this 1999 classification to assign and define disease severity for their PD participants and thus allowing less heterogeneity during comparisons (Harvey et al., 2012; Mohamad et al., 2018; Reichert et al., 2015)

Different case definitions have been proposed and used to define PD cases in previous studies. The suggested threshold values used for PPD and/or CAL used in the case definition was also inconsistent to determine the true evidence of periodontal destruction. These variations had rendered difficulties in comparing the prevalence across different population in different studies (Papapanou, 1999). For instance, the case definition proposed by Centre for Disease Control and Prevention and the American Academy of Periodontology (CDC/AAP) was based on PPD and CAL (Eke, Page, Wei, Thornton-Evans, & Genco, 2012). However, this case definition was more widely used in epidemiological studies and had limited usage in clinical setting.

Hence, in this cross-sectional study, we chose to use the 1999 classification rather than other case definitions because this classification had a more stringent threshold for PD and hence was more sensitive to capture specific PD cases according to their severity. Besides that, it also required full mouth periodontal charting that can reflect the actual periodontal condition, allowing for more accurate diagnosis and analysis.

For this study, we only included PD participants with moderate and severe disease severities (CAL≥ 3mm). Epidemiological studies have also reported that patients with RA were more likely to have moderate to severe periodontitis (Detert et al., 2010; Dissick et al., 2010; Mercado et al., 2000). A full mouth periodontal charting was thus required to diagnose these patients. Furthermore, heterogeneity in PD severity definitions have been identified as a major confounding factor that could lead to diversity in research findings as some studies have allocated all PD cases in one entity despite different severities (Mawardi, Elbadawi, & Sonis, 2015). Therefore, we chose to only include moderate and severe PD subjects to prevent the dilution of our study outcomes.

On the other hand, for RA case definition, both American College of Rheumatology (ACR) 1987 and American College of Rheumatology /European League Against Rheumatism (ACR/EULAR) 2010 classifications have been widely used to define RA cases in previous studies. However, the ACR/EULAR 2010 was shown to perform better than ACR 1987 due to its high sensitivity and specificity in predicting and diagnosing RA (Cornec et al., 2012). Therefore, it is now commonly used to diagnose RA cases in the clinical setting. Therefore, we chose to define our RA cases with ACR/EULAR 2010 which was also in accordance to the diagnostic criteria used by the rheumatologist in the medical centre (UMMC) where we recruited our RA participants.

5.1.3 Clinical assessment

A comprehensive full-mouth periodontal examination (FMPE) protocol that involved measurement of six sites on all teeth (excluding third molars) was utilized in this study. It is also known as the gold standard for individual periodontal assessment prior to clinical diagnosis (Preshaw, 2015).

Alternatively, partial mouth periodontal examination that involves only measurements from representative sets of teeth or probing sites can also be used in a

research study. Although the partial-mouth examination procedure appears to be faster, it may not adequately reflect the actual periodontal status of the entire dentition and may significantly underestimate or overestimate the prevalence and severity of PD (Susin, Kingman, & Albandar, 2005). Therefore, full-mouth periodontal examination was chosen over partial mouth periodontal examination in this present study to avoid bias and under/ over-estimation of PD prevalence and severity.

The present study used a manual probe, UNC 15 colour coded periodontal probe (Hu-Friedy, Chicago USA) to measure all periodontal clinical parameters due to its availability in our clinic. The probing force used during the clinical examination has been shown to influence the recorded measurements (Preshaw, 2015; Van Der Velden, 1979). To overcome this, the use of computerised pressure-controlled probe such as Florida probe as an alternative may have an advantage in improving the reproducibility of measurements by providing a constant probing force. However, this instrument is expensive and currently unavailable in our clinic.

Apart from probing force, the accuracy of probing depths can also be affected by a multitude of factors such as probe type and design, location and angulation of the probe as well as gingival inflammation (Buduneli, Aksoy, Köse, & Atilla, 2004; Bulthuis, Barendregt, F. Timmerman, Loos, & Velden, 1998). To overcome these problems, a standardisation exercise was carried out prior to recruitment to achieve accuracy and reproducibility of measurements as well as to minimise both intra- and inter-examiner errors during periodontal examination between and within all three examiners involved in the study. Furthermore, correct placement of the probe was confirmed by inserting the probe tip parallel to the long axis of the tooth at six sites per tooth. At interproximal sites, the probe should be inserted as far interproximal as possible, contacting the contact point in order to avoid over-angulation of the probe.

5.1.4 Serum sample

For the laboratory assessment in this study, serum samples from all the study participants were used to determine the levels of anti-CitP. Serum sample had been used for analysis in most of the previous studies that assessed levels of anti-CitP in RA and non-RA subjects (Janssen et al., 2015; Laugisch et al., 2016; Mohamad, Jia, Ghazali, & Taib, 2018). Basically, serum sample was chosen because it is still considered as the gold standard and required sample for most of the assays available (Leng et al., 2008). After adequate coagulation is completed, serum is the undiluted liquid part of blood which is typically free of clotting proteins, other cellular components and platelets. Thus, it appears to be a cleaner sample with least contamination as compared to other biological samples such as plasma and saliva (Guder, 2002). Furthermore, the serum sample is more stable once separated as it does not contain cellular debris or blood cells which are still metabolically active and capable of altering the analysis (Oddoze, Lombard, & Portugal, 2012). Therefore, serum sample was used in this study to detect anti-CitP by using commercial ELISA kits.

Proper sample handling and storage are critical for reliable measurement of circulating anti-CitP. In this study, blood samples were transported almost immediately in an ice box to the laboratory for sample processing and storage at -80°C until they were assayed. The samples were stored at -80°C rather than -20°C in order to prevent protein degradation during storage (Aziz et al., 1999). All serum samples were allowed to achieve fully retracted clot (standardised 30 minutes) before centrifugation in the laboratory to prevent latent fibrin formation above the serum which may affect the subsequent analysis.

Alternatively, other biological samples like gingival crevicular fluid (GCF) can also be used to determine anti-CitP levels. GCF is a physiological fluid comprised of a complex mixture of substances or biomarkers derived from both local and systemic source. The local expression of numerous inflammatory biomarkers or autoantibodies in

PD cases have been closely associated with systemic expression in serum (Ebersole, 2003). Given the promising results by Harvey et al (2012) that showed that anti-CitP could be detected in 9 out of 11 PD patients, it would be more valuable if we could analyse GCF and compare them to our serum samples. Unfortunately, GCF samples were not obtained in this study. It is also important to note that there are number of distinct technical challenges involved when collecting GCF samples. GCF samples are easily contaminated with blood, saliva and plaque. Prolonged sampling time is usually required. Nevertheless, research using GCF samples will serve as a promising avenue for future research as quantitative analysis of anti-CitP in GCF with comparisons to serum levels might shed some light on the possibility of local production of anti-CitP in PD.

5.1.5 ELISA method

ELISA was utilized in this study to evaluate levels of anti-CitP in serum samples. There are four different types of ELISA techniques that can be applied in a research study such as direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA. Sandwich ELISA was chosen over the other types of ELISA as it is the most widespread technique used to identify and quantify a specific protein in a complex mixture (Mire-Sluis et al., 1995). It has high sensitivity and specificity since two antibodies are used in the assay.

To date, ELISA remains as the best validated method for quantifying a single protein and most of the published studies used the ELISA technique to measure serum levels of anti-CitP in RA and PD. The ELISA method was used in this study rather than multiplex assay as we only measured a single protein (anti-CitP) in each sample and ELISA reader was readily available in our laboratory. In contrast, multiplex assay requires more specialised equipment that may incur a higher cost if compared to traditional ELISA (Leng et al., 2008). Nevertheless, multiplex assay is an emerging

advanced technique that offers both cost and sample savings over ELISA. It can be used in future studies in quantifying multiple proteins at one time.

5.2 Discussion of results

5.2.1 Characteristics of study population

In this study, a total of 80 RA and non-Ra participants were recruited and allocated into four groups based on the inclusion and exclusion criteria. Based on our knowledge, this is the first study in Malaysia that assessed and compared anti-CitP levels in four groups of participants, including healthy controls. Previous studies mainly focused on either RA cohorts or only among PD cohorts. The mean age of the RA cohorts involved in this study was 54.25 for RAPD group and 52.7 for RA group. These results are almost similar to the 2 local studies conducted in University Kebangsaan Malaysia Medical Centre (UKMMC) and Hospital University Science of Malaysia (HUSM) that reported mean age of 51.0 years and 51.9 years respectively for RA patients (Abdul Wahab, Mohammad, Rahman, & Mohamed Said, 2013; Mohamad et al., 2018). The mean age of RA cohorts in this study was also consistent with previous reports which showed the onset of RA is most evident during fourth and fifth decades of life (Arnett et al., 1988; Hootman, Helmick, Barbour, Theis, & Boring, 2016).

Majority of our study participants were females, indicating that RA more commonly affects middle aged women. This female predominance in RA was also reported in previous local studies in Malaysia (Gomez et al., 2011; Sockalingam, Khuan, & Sthaneshwar, 2009). The sample characteristics and demographic distribution of the study participants were equally distributed between the groups except for age and gender. The non-RA groups (PD and control groups) were significantly younger than the RA groups. This could be due to volunteer bias that occurred during our recruitment and consent for blood sampling. Our RA cohorts were mostly from the middle age group and routinely attended hospital for their follow-up appointments and thus were more willing

to consent for blood samples. On the other hand, our non-RA cohorts who were willing to participate in this study and consent for blood sample were mostly from the younger age group as the older population appeared to be more reluctant to participate in this study when they knew that blood sample would be taken.

Besides that, females have been reported to have a more positive attitude and better oral health awareness and behaviours than males (Furuta et al., 2011), resulting in more female volunteers in all four groups of participants. To control for the bias in the distribution of age and gender among participants in the four groups, multiple linear regression analysis was carried out. With the absence of significant influence of both age and gender in the regression model, we assume that both age and gender differences between these four groups did not influence the serum anti-CitP levels.

5.2.2 Periodontal parameters

Our results showed that majority of our participants in both RAPD and PD groups were diagnosed with localised forms of moderate to severe PD. There were significantly higher means for VPI, GBI, PPD and CAL observed in both RAPD and PD groups as compared to the healthy control group. This was an expected result as we only included patients with moderate to severe PD in the RAPD and PD groups. However, there was no statistical significant difference between RAPD and PD groups in terms of their periodontal status measured by the above-mentioned PD parameters. This findings were in agreement with a recent study that have reported no statistical significant difference for periodontal status and severity between RAPD and PD groups, suggesting that presence of RA in PD participants does not seem to have an effect on the status and severity of PD (Zhao et al., 2018).

However, there have been contradictory results that showed presence of PD was more common in patients with RA. Epidemiological studies have reported that the

existence of RA may promote emergence and progression of PD (Detert et al., 2010). On the other hand, PD was also reported to be more common and severe in patients with established RA (Bingham & Moni, 2013). However, these findings were not demonstrated in our study as there was no difference in PPD and CAL levels in both RAPD and PD groups in our study samples. This could be due to methodological limitations as our study was a cross-sectional study which only included participants based on our inclusion and exclusion criteria. The sample recruitment was solely based on voluntary consent basis which could lead to a volunteer bias as the data gathered cannot be generalised to represent the whole population, but merely of those that chose to volunteer. Those RA cohorts who volunteered themselves in this study were mostly highly motivated participants with better oral health awareness. It has been shown that volunteers tend to be more health conscious than the general population thus resulting in reduced incidence of a true disease (Jordan et al., 2013). Majority of the participants in both groups were also diagnosed as localised moderate to severe PD. Therefore, the similarity in periodontal disease severity in both RAPD and PD groups might be due to our inability to capture those RA participants with generalised severe periodontal inflammation.

On the other hand, there was no statistical significant difference observed between RA and control groups for all periodontal parameters. This was consistent with several studies that revealed no evidence of an increased prevalence and severity of periodontitis in patients with RA when compared with healthy controls, suggesting that not all RA patients were susceptible to the initiation and progression of PD (Eriksson et al., 2016; Susanto et al., 2013; Zhao et al., 2018). A previous study has also reported no difference in plaque and bleeding indices between RA and healthy controls, supporting the notion that limited manual dexterity in RA patients will not contribute to greater plaque accumulation as well as periodontal inflammation in these cohorts (Mercado et al., 2000).

5.2.2 RA parameters

Both RA and PD are chronic inflammatory diseases that are known to elevate the systemic inflammatory marker, ESR. In this study, there was no statistical significant difference in ESR levels between RAPD and RA groups. This finding is similar to that reported by Joseph et al (2013). In their study, they reported no significant difference for the ESR levels between RAPD and RA groups, suggesting that PD severity did not significantly affect the serum ESR levels in RA patients (Joseph, Rajappan, Nath, & Paul, 2013). This could be also due to the similar high RA disease activity in both RAPD and RA groups that could have masked the possible effects of PD since ESR levels were more closely related to RA disease activity.

However, our findings were in contrast with the findings from Zhao et al (2018) and Mercado et al (2000). Both studies reported statistical significantly higher serum levels of ESR in RAPD group as compared to RA group, suggesting that existence of PD might contribute to the disease activity and RA development (Mercado et al., 2000; Zhao et al., 2018). The discrepancy in the findings could be possibly caused by the PD disease severity among the cohort of participants recruited as most of our RAPD group participants were diagnosed with only localised PD that might not be severe enough to contribute to significant increased ESR in RA.

In this current study, although there was no statistical significant difference between the 2 values, there was higher disease activity indicated by higher mean ESR noted in RAPD group as compared to RA group. The higher disease activity in RAPD group could be due to the combined effect of underlying dysregulation of the inflammatory mechanism in RA and PD. Presence of periodontal inflammation can contribute to additional systemic inflammatory burden in RA patients. It has been proposed that PD contributes to the systemic inflammation by generating citrullinated proteins in inflamed periodontal tissues, subsequently leading to the formation of anti-

CitP. PD was proposed as the potential environmental risk factor that can trigger the development of RA as well as maintenance of systemic inflammation in RA (Detert et al., 2010). The lack of significant difference between the two groups may be due to the small sample size. Further large-scale prospective studies are needed to assess this potential association.

5.2.3 Mean serum anti-citrullinated protein antibody levels

The first objective of this study was to identify and compare the presence of serum anti-CitP in RA-PD, RA, PD and healthy controls. Results showed the highest mean levels of serum anti-CitP in RAPD group followed by RA group and PD group while the subjects in healthy group showed the lowest concentration of anti-CitP in their serum. The mean levels of serum anti-CitP were found to be statistical significantly higher in the RA cohorts (both RAPD and RA groups) as compared to the healthy control group. This finding was in line with a study by (Karkucak, 2011) who compared the serum anti-CitP in RA subjects and healthy control group and found that RA cohorts had significantly higher anti-CitP levels than the controls. Zendman et al (2006) in their multicentre study also demonstrated that anti-CitP antibodies were detected in 80% of RA patients but rarely present in healthy controls. It is an established fact that anti-CitP represents the specific humoral immune response that is highly specific to RA (Schellekens et al., 2000).

Biologically, anti-CitP had been shown to be involved in the inflammatory processes that occur in RA. Some studies have suggested that anti-CitP can interact directly with citrullinated antigens presence in the joints or immune cells in serum, subsequently leading to a cascade of pro-inflammatory reactions and high concentration of serum anti-CitP (Kerkman et al., 2016). Therefore, the serum anti-CitP levels in established RA patients is remarkably high which is in agreement with our study. This finding also reflects the important role of anti-CitP as a specific serological marker for disease activity in RA (Mangat et al., 2010).

It was reported that anti-CitP could be detectable at low levels in about 1 to 3 % of healthy subjects without any joint symptoms (Terao et al., 2015; van Zanten et al., 2017). However, in our study we could not differentiate which subjects were truly anti-CitP seropositive as there was no diagnostic cut off point provided in our quantitative ELISA kit. We could only quantify the serum anti-CitP levels and compare them between the groups. Nevertheless, we did observe a trend in the serum anti-CitP concentration in which the RAPD group had the highest mean concentration followed by RA group, PD group and healthy group (RAPD>RA>PD>control). This trend was also observed when we made relative comparisons of the anti-CitP levels for all participants to the mean anti-CitP of our control group. This might suggest the possible role of PD as a source of auto-antigens for anti-CitP production. However, the actual biological mechanism still needs to be further investigated in future studies.

In this study, it was also demonstrated that there was no statistical significant difference in the serum anti-CitP levels between the RAPD and RA groups. Similar findings were also revealed by previous studies (Dissick et al., 2010; Pischon et al., 2008) that compared the levels of anti-CitP antibodies in RA patients with and without PD. None of these studies reported statistical significantly higher levels of anti-CitP antibodies in RAPD patients as compared to the RA patients. Furthermore, in a more recent study, no significant difference was observed in serum anti-CitP expression between RAPD and RA groups (Laugisch et al., 2016). Thus, together with our findings, it can be postulated that PD might not be contributing to the anti-CitP positivity in RA patients. Nevertheless, these were all cross-sectional studies thus prospective study should be carried out to obtain more conclusive findings.

The results of the present study were contradictory to other studies that reported statistical significantly higher titres of anti-CitP in RAPD group as compared to RA group (Mikuls et al., 2014; Potikuri et al., 2012). This could be attributed to the differences in

the ELISA kits used in experiments with different diagnostic cut off or due to the differences in the PD disease severity among the cohort of participants recruited. In our study, most of our RAPD group participants were diagnosed with localised moderate stage of PD while the afore-mentioned studies (Mikuls et al., 2014; Potikuri et al., 2012) involved those with more generalised and severe stage of PD. Furthermore, our present study had a smaller sample size compared to the other studies which had sample sizes of more than 180 participants (Mikuls et al., 2014; Potikuri et al., 2012). This may have also contributed to our inability to distinguish smaller differences in anti-CitP expression between RAPD and RA groups. Thus, we suggest that studies with larger sample size should be carried out to obtain more conclusive findings.

On the other hand, our study also found no statistical significant difference in anti-CitP levels between PD group and controls although there was an increased titre of anti-CitP in patients with PD than the healthy controls. Similar findings were also reported in other studies that compared serum anti-CitP in patients with PD to the healthy controls. No significant differences were reported between the anti-CitP level of PD group as compared to control group (Reichert et al., 2013; Zhao et al., 2018). Thus, it can be suggested the role of anti-CitP in PD is unlikely and thus doesn't contribute to significant results in our PD group. The increased anti-CitP via citrullination is more likely to be caused by increased inflammation rather than being associated with the specific disease process (Makrygiannakis et al., 2006).

In contrast, some studies showed that patients with PD had a significantly higher anti-CitP level in their sera and GCF compared to controls, suggesting the potential role of PD as an inducer of anti-CitP formation and possible involvement in RA development later (de Pablo et al., 2014; Harvey et al., 2012; Lappin et al., 2013). The differences between the findings revealed in our study in comparison to these previous studies can be explained as follows: Firstly, anti-CitP is known to be highly specific for RA and thus

rarely present in non-RA groups. Although the results in these studies were significantly different, the published differences for the anti-CitP levels between the PD patients and controls were rather low. For instance, in the study by Lappin et al (2013) , only 3 out of 39 PD patients and none from the 36 controls were anti-CitP positive. Additionally, there were differences in patient cohorts and ELISA methodology. There was higher prevalence of smokers among the PD subjects than the control group in both studies by Lappin et al (2013) and de Pablo et al (2014) which could contribute to the significant higher anti-CitP level in PD patients. Furthermore, these studies also had a larger sample size than our present study.

The present study reported a large variation in the mean serum level of anti-CitP. This may be attributed to methodological limitations and other possible confounders. The wide variation of results could be due to the differences in periodontal inflammation and RA disease activity at the time of collection of serum samples. For RA participants (both RAPD and RA groups), this variation could be due to the distinct difference in the serum anti-CitP levels between sero-positive and sero-negative RA population. In addition, fluctuations of serum antibody levels could happen depending on the underlying systemic condition of the participants and any drugs taken before or at the time of serum sample collection. Although we had set our inclusion or exclusion criteria to control all possible confounders, these were all patient reported outcomes which might affect our results.

5.2.4 Correlation between serum anti-citrullinated protein antibody level with clinical periodontal parameters

The second objective of this study was to correlate the serum anti-CitP with periodontal parameters. Over the years, many studies have been carried out to find possible associations between RA and PD by correlating anti-CitP (RA marker) with clinical periodontal parameters. However, different clinical periodontal parameters have been measured in these studies. Thus, comparisons were made based on the available

studies correlating serum anti-CitP level to clinical periodontal parameters among patients with RA and PD.

This present study demonstrated no statistical significant correlations between serum levels of anti-CitP with VPI, GBI, PPD and CAL in all the four groups of subjects. These findings agreed with a recent study conducted locally on a cohort of RA subjects whereby no significant association was reported between level of anti-CitP and periodontal status based on CAL (Mohamad et al., 2018). Similarly, anti-CitP also showed no correlation with clinical parameters of disease activity of PD among PD patients based on PPD, CAL and BOP (Reichert et al., 2015). This can be interpreted as neither PD disease activity nor severity seems to influence the serum anti-CitP levels.

In contrast, a statistical significant correlation was reported between PD parameters and anti-CitP in a case control study conducted on RA and control patients with or without PD (Potikuri et al., 2012). It was suggested that PD was strongly associated with the presence of anti-CitP and could be a potential environmental risk factor in the pathogenesis and in the maintenance of systemic inflammation in RA. Furthermore, a significant association between PD parameters (CAL, number of missing teeth) and serum anti-CitP levels was demonstrated, supporting the notion of fundamental involvement of PD with anti-CitP production (Terao et al., 2015).

The contradictory results in the present study may suggest that PD per se does not contribute to increased serum anti-CitP that is specific to RA development. Indeed, RA is a complex disease that can only be triggered in genetically susceptible individuals by multiple environmental risk factors. The presence of anti-CitP alone is not sufficient to induce RA, hence, an additional trigger such as formation of immune complexes or microvascular insults is needed to initiate synovitis in RA (Smolen et al., 2018). There is a possibility that non-specific oral inflammation by PD is important for anti-CitP

production. Therefore, it is logical to widen our consideration of mechanisms beyond that of citrullination. This warrants more future studies that should include more inflammatory markers.

5.2.5 Correlation between serum anti-citrullinated protein antibody level with RA parameters

Different parameters have been used to assess and monitor RA disease activity and severity including Disease Activity Score with 28 joint counts (DAS-28), C reactive protein (CRP) and erythrocyte sedimentation rate (ESR). In our study, we used ESR for assessing RA disease severity and activity as other parameters could not be sufficiently retrieved from the medical records.

In this study, we did not demonstrate any statistical significant correlation between serum levels of anti-CitP and the RA parameter measured in this study (ESR) for both RAPD and RA groups. Our findings were consistent with some studies that reported no correlation between serum levels of anti-CitP with disease severity and activity (Gupta et al., 2014; Papadopoulos et al., 2008). This finding supports the notion that the quality of the anti-CitP response defined by molecular characteristics or functional features of anti-CitP expressing autoreactive B cells are much more important and relevant than its quantity (serum level of anti-CitP) in determining the outcome of established RA. It has been shown that the fluctuations in serum anti-CitP levels do not reflect RA disease activity and are not clinically useful in predicting the progression and flare-up of the disease (Landmann et al., 2010). Therefore, measurements of serum anti-CitP was not useful in monitoring RA disease activity. This warrants more research to develop more inflammatory markers for RA.

In contrary, the presence of serum anti-CitP in RA patients has been linked to higher disease activity and severity in RA. Anti-CitP has long been used as a promising

diagnostic marker for RA due to its high specificity, sensitivity and early detection in disease development (Schellekens et al., 2000; Zendman, van Venrooij, & Pruijn, 2006). Previous studies have shown that anti-CitP positivity had been correlated with RA disease activity as well its severity (Bongi et al., 2004; van der Helm-van Mil, Verpoort, Breedveld, Toes, & Huizinga, 2005). However, this was not demonstrated in our study population. This difference in findings could be due to the different RA parameters used in our study. Previous studies reported RA disease activity based on the combination of DAS-28 and ESR or CRP. However, our study only recorded the ESR of RA patients as DAS-28 and CRP could not be sufficiently retrieved.

It has been suggested that ESR is a good inflammatory marker that can reflect RA disease activity over the preceding few weeks. However, it could be affected by other confounding factors such as age, gender, fibrinogen levels and rheumatoid factor (RF) (Wolfe, 1997). Meanwhile, CRP is shown to be more sensitive to short term evaluation of disease activity. Therefore, it was suggested that combination of DAS-28 with CRP or ESR or both would be a better indicator for evaluating RA disease activity accurately. Furthermore, different types of ELISA kits used in these studies could also be one of the reasons that caused these result discrepancies.

5.3 Strengths and limitations of study

The main strength of this present study is the study design comparing the expression of anti-CitP in 4 groups of subjects in relation to RA and PD. Previous studies were focused on comparisons among the RA cohort (Dissick et al., 2010; Laugisch et al., 2016; Mercado et al., 2000). In our study, we included periodontally healthy subjects without RA and PD as the negative control group which is an advantage to compare or verify whether results were contributed by RA alone, PD alone or both as we were investigating both diseases at a single time point.

Besides that, although convenience sampling was used in this study, our selection of subjects was based on strict inclusion and exclusion criteria to ensure the homogeneity of the sample population. This can also aid in reducing potential confounding factors that might affect the study outcomes. We also did sample size calculation prior to our recruitment to make sure that our study was not underpowered. Furthermore, for PD case definition, 1999 AAP classification was used in this study as this was the most widely used case definition in the other clinical studies that previously investigated on anti-CitP in RA and PD patients. This will provide an advantage of allowing better comparisons with other studies since a similar case definition had been used. Furthermore, we only included moderate to severe PD patients since it was shown in epidemiological studies that RA patients were more commonly affected and associated with moderate to severe PD. By doing this, dilution of study outcomes was avoided.

However, there were several limitations in this study that needs to be taken into consideration when interpreting the present results. Firstly, this study was a cross sectional study which only shows association while no causal relationship can be determined. Thus, it limits the value in identifying a temporal relationship as all the assessments/measurements related to RA and PD were assessed at single time points concurrently.

Secondly, although adjustments for age and gender as well as strict selection of study subjects have been done to reduce the confounders and bias in this study, the observed results could be affected by some other unidentified factors such as the use of antibiotics or drugs, underlying systemic diseases as well as smoking status. All this information was self-reported by the subjects. For instance, some patients might not be aware of their underlying systemic condition. In addition, our recruitment of subjects was based on voluntary basis. This might lead to an underlying volunteer bias as these volunteered participants might be different in some ways from the general population.

The true PD status among all the RA subjects might not be accurately revealed in this study. Thus, these results cannot be generalised to the whole population.

Another limitation of this study would be the ELISA kits used in this study did not provide a cut off value to aid in distinguishing the sero-positivity of subject. This precluded our ability to identify the true sero-positive anti-CitP subjects as no physiological anti-CitP cut off value was provided by the kit. Lastly, insufficient data on the inflammatory markers such as DAS-28 and CRP may limit our data interpretation in relation to RA disease activity.

CHAPTER 6: CONCLUSIONS AND FUTURE RECOMMENDATIONS

6.1 Conclusions

Within the limitations of the study, the following conclusions can be drawn based on the findings:

1. Anti-CitP levels were significantly higher in RAPD and RA groups as compared to the healthy control group.
2. There was an increasing trend seen in anti-CitP levels from healthy, PD, RA to RAPD groups suggesting an influence of inflammation in the increase in anti-CitP levels.
3. There was no correlation between anti-CitP expression with the clinical parameters of PD and RA.

6.2 Future recommendations

In view of the limitations of this study, this cross-sectional study did not provide a good evidence of temporal relationship between RA and PD due to the methodological limitations. Therefore, future prospective cohort study with larger sample size is required to validate the possible causal-relationship between these two diseases via citrullination. Furthermore, quantitative ELISA kits with physiological cut off value for anti-CitP should be used in future studies.

Although the present study did not provide good evidence to support the hypothesis of periodontitis-induced anti-CitP which could predispose individuals for the development of RA, the underlying biological mechanisms for the relationship between periodontitis and RA cannot be denied. This needs to be investigated in further studies by using other biomarkers as anti-CitP alone might not be enough to show potential association between these two complex diseases. Apart from serum, future studies examining the presence of antibodies or biomarkers in other biological samples such as

GCF and saliva for both RA and PD patients may provide more insight into the relevance of their presence in RA and PD.

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