LOCALISATION OF CITRULLINATED AND CARBAMYLATED PROTEINS IN THE INFLAMED GINGIVAL TISSUES OF RHEUMATOID ARTHRITIS

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Localisation of citrullinated and carbamylated proteins in the inflamed gingival tissues of rheumatoid arthritis

ABSTRACT

Background: Antibodies to citrullinated proteins are widely used as diagnostic marker for rheumatoid arthritis (RA). Antibodies against carbamylated proteins were also suggested to be used as a potential biomarker for RA. It has been speculated that citrullination and carbamylation occur in inflamed periodontium which can be a source of autoantibodies to induce immune responses resulting in the development and progression of RA. **Aim**: To determine the presence and location of citrullinated and carbamylated proteins in the gingival tissues and compare their abundance in periodontitis (PD) participants with and without RA. **Materials and Methods**: Gingival tissue samples of healthy control (n = 5), PD with RA (n = 5) and PD without RA (n = 5) were collected. Specimens were formalin-fixed, paraffin embedded and sectioned at 4 μm. The tissue sections were analysed for the presence of citrullinated and carbamylated proteins by immunohistochemistry. Semi-quantitative analysis was performed to quantify and compare the protein abundance between groups. **Results**: Number of cells containing citrullinated and carbamylated proteins with higher intensity was markedly increased in gingival tissues from PD with or without RA in comparison with healthy controls. **Conclusion**: Inflamed gingival tissue is a potential extra-articular source of citrullinated and carbamylated proteins. The extent to which these proteins contribute to the pathogenesis of RA warrants further elucidation.

Pengesanan sitrulinasi dan karbamilasi protein dalam tisu gusi rheumatoid arthritis yang radang

ABSTRAK

Latar Belakang: Antibodi protein yang telah disitrulinasi digunakan secara meluas sebagai penanda diagnostik untuk rheumatoid arthritis (RA). Antibodi terhadap protein karbamilat juga disarankan penggunaannya sebagai biomarker RA yang berpotensi. Terdapat spekulasi mengatakan proses sitrulinasi dan karbamilasi berlaku di dalam tisu periodontium yang radang dan ianya boleh menjadi sumber autoantibodi pencetus tindak balas imun mengakibatkan pembentukan dan perkembangan RA. **Matlamat**: Menentukan kehadiran dan lokasi protein yang disitrulinasikan dan dikarbamilasikan dalam tisu gusi (gingiva) serta membandingkan kuantitinya di dalam peserta yang mengalami periodontitis (PD) dan RA dengan yang tanpa RA. **Bahan dan Kaedah**: Sampel tisu gusi (gingiva) sihat (n = 5), PD dengan RA (n = 5) dan PD tanpa RA (n = 5) dikumpulkan. Spesimen diawet formalin, dibenamkan dalam parafin dan dipotong setebal 4μm. Sampel tisu dianalisa untuk kehadiran protein sitrulinasi dan karbamilsai menggunakan kaedah immunohistochemistry. Analisasi semi kuantitatif dilakukan untuk mengukur dan membandingkan jumlah protein di antara kumpulan. **Keputusan**: Bilangan sel yang mengandungi protein sitrulinasi dan karbamilasi meningkat secara ketara di dalam tisu gusi (gingiva) PD dengan atau tanpa RA berbanding dengan kawalan sihat. **Kesimpulan**: Tisu gusi (gingiva) yang radang berpotensi sebagai sumber protein sitrulinasi dan karbamilsi di luar sinovial. Sejauh mana protein ini menyumbang kepada patogenesis RA memerlukan penyiasatan lanjut.

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LIST OF SYMBOLS AND ABBREVIATIONS

ACPA	:	anti-citrullinated protein antibodies
AKA	:	anti-keratine antibodies
Anti-Carp	:	anti-carbamylated protein antibodies
APF	:	anti-perinuclear factor
AR	:	antigen retrieval
CAL	:	clinical attachment level
CCP	:	anti-cyclic citrullinated peptide
CEJ	:	cemento-enamel junction
CIA	:	collagen-induced arthritis
CRP	:	C-reactive protein
DAB	:	3.3'-Diaminobenzidine
DAS28	:	disease activity score-28
DMARDs	:	disease-modifying anti-rheumatic drugs
ELISA	:	enzyme-linked immunosorbent assay
ESR	:	erythrocyte sedimentation rate
FFPE	:	formalin-fixed paraffin-embedded
FMBS	:	full mouth bleeding score
FMPS	:	full mouth plaque score
GR	:	gingival recession
H&E	:	haematoxylin & eosin
HLA	:	human leukocyte antigen
IHC	:	immunohistochemistry
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M

IL	:	interleukin
MHC	:	major histocompatibility complex
MMPs	:	matrix metalloproteinases
MPO	:	myeloperoxidase
NBF	:	neutral-buffered formalin
NETs	:	neutrophil extracellular traps
OPG	:	osteoprotegerin
PAD	:	peptidylarginine deiminase
PAR-2	:	proteinase-activated receptor 2
PD	:	periodontitis
PGE2	:	Prostaglandin E2
PPAD	:	*P. gingivalis* peptidylarginine deiminase
PPD	:	probing pocket depth
RA	:	rheumatoid arthritis
RANKL	:	receptor activator of nuclear factor kappa-B ligand
RF	:	rheumatoid factor
ROI	:	region of interest
SQA	:	semi-quantitative analysis
TNF-α	:	tumour necrosis factor-alpha
TRAIL	:	tumour necrosis factor-related apoptosis-inducing ligand
Tris	:	trisaminomethane
UMMC	:	University of Malaya Medical Centre
VAS	:	visual analog scale

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Chapter 1: Introduction

1.1 Background

Periodontitis (PD) is a chronic inflammatory disease of the periodontal tissues, often of insidious onset and triggered by oral bacteria. It is a common chronic inflammatory infection of the mouth, with a global prevalence of 30-35% (WHO, 2007). In Malaysia, up to 48.5% of the general population were affected by PD (MOH, 2013). If left untreated, PD can progress and destroy the soft and hard tissues, and eventually lead to tooth loss. In addition to local tissue destruction, studies have shown that PD is implicated in other systemic diseases, including cardiovascular disease, diabetes mellitus and osteoporosis (Beck et al., 1996; Taylor et al., 2013; Wactawski-Wende, 2001).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder with classic features of unremitting inflammation of the synovium and autoantibody production. It affects approximately 0.5-1% of the global population and has an obvious female gender predilection (Lee & Weinblatt, 2001). In Malaysia, RA affects about 5 in 1000 people (AFM, 2016). Similar to PD, RA can cause permanent damage to the joints and physical disability as the disease progresses.

The PD-RA link has been debated for decades owing to their common pathobiological features. Several observational studies revealed that the prevalence and severity of PD were higher in RA patients than non-RA counterparts (Dissick et al., 2010; Joseph et al., 2013). The same pattern was also observed even among the non-smoking RA subgroup (Potikuri et al., 2012). However, the exact mechanism underlying the relationship between these two diseases is still obscure despite the enormous amount of research on this topic. The current hypothesis suggests that

citrullination and carbamylation could be the mechanistic links between them (Pruijn, 2015).

1.2 Problem statement

There is a paucity of evidence on the presence of carbamylated and citrullinated proteins in the gingival tissues of RA patients. This study proposes to identify if carbamylated and citrullinated proteins are present in gingival tissues that could serve as possible triggers for the autoimmune response in RA.

1.3 Aim

Histological evidence that demonstrates the presence of carbamylated proteins in the gingival tissues is scarce given that anti-Carp autoantibody is a novel diagnostic biomarker for RA. Therefore, this study aims to locate and determine the level of carbamylated proteins as well as citrullinated proteins in the inflamed gingival tissues of rheumatoid arthritis.

1.4 Objectives

1. To locate the presence of carbamylated and citrullinated proteins in the gingival tissues of healthy control and PD with or without RA.
2. To determine the relative abundance of carbamylated and citrullinated proteins in the gingival tissues of healthy control and PD with or without RA.
3. To compare the level of carbamylated and citrullinated proteins in the gingival tissues between PD participants with RA and without RA.

Chapter 2: LITERATURE REVIEW

2.1 Background

There have been increasing evidences demonstrating links between oral and systemic health over the past two decades, marking a resurgence of the controversial focal infection theory. The relationship between PD and RA may not have been as extensively examined as other systemic conditions such as diabetes mellitus and cardiovascular disease. However, it has also garnered substantial attention among the dental and medical professionals in recent years. This chapter reviews current knowledge that relates periodontitis to rheumatoid arthritis, starting with a short description of these two diseases, followed by a detailed account of what has been learnt from previously published studies.

2.1.1 Periodontitis

Periodontitis (PD) is a chronic inflammatory disease of the periodontal tissues, often of insidious onset and triggered by dental biofilm. It is a prevalent chronic inflammatory infection of the oral cavity, affecting approximately 30-35% of the global population (WHO, 2007) and 48.5% of the Malaysian population (MOH, 2013). The severe form of periodontitis accounted for an overall worldwide prevalence of 11.2% and a global economic burden of 442 billion USD according to the recent Global Burden of Disease Study (GBD, 2016).

In general, PD is more common in adults but it can occur in any age group. The progression of PD is usually slow to moderate and during which bursts of exacerbation can occur over a short period of time followed by a long period of quiescence (Socransky et al., 1984). The diagnosis of PD is made through a combination of careful clinical and radiographic assessments of the periodontium based on the parameters

advocated by 1999 classification system (Armitage, 1999) such as bleeding on probing and clinical attachment loss (**Table 2.1**). If left untreated, PD can cause soft tissue destruction, alveolar bone resorption and ultimately tooth loss. In addition to local tissue destruction, considerable evidence has indicated that PD can go beyond the oral cavity. It has been associated with other systemic diseases, notably diabetes mellitus, cardiovascular disease and chronic respiratory disease possibly through systemic dissemination of both bacteria or bacterial products from the dental biofilm and inflammatory mediators originating from the inflamed periodontal lesions. The end result of these mechanisms is a heightened systemic inflammatory burden which contributes to the development of the aforementioned chronic diseases (Beck et al., 1996; Linden & Herzberg, 2013; Scannapieco & Ho, 2001; Taylor, 2001).

Table 2.1: Guidelines to determine the severity of periodontitis

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

Source: (Armitage, 1999; AAP, 2015)

2.1.2 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder, affecting primarily the synovial joints and it is characterised by auto-antibody production. The worldwide prevalence of RA is estimated at 0.5-1% (Smolen et al., 2016) with a regional variation, indicating that genetic factors may contribute to the disease susceptibility. The incidence is reported to be highest in the Pima Indians (5.3%) while lowest among Japanese and Chinese (0.2-0.3%) (Silman & Pearson, 2002). In Malaysia, RA affects about 5 in 1000 people according to statistics (AFM, 2016). Similar to other autoimmune diseases, RA has an obvious female gender predilection where the sex ratio is around 3:1 with an age of onset between 30 and 50 (Lee & Weinblatt, 2001; Wolfe et al., 1968). RA is initially presented as synovitis and as the disease progresses, persistent inflammation of the joints causes destruction of cartilage and bone which leads to functional disability as well as extra-articular manifestations. Therefore, it is not surprising that RA patients often present with other comorbidities such as cardiovascular disease and diabetes mellitus (Peters et al., 2009). Mortality rate is twice as high in RA patients as in the general population despite advancements in disease management (Gonzalez et al., 2007). The increased mortality in RA is not fully understood but it has been speculated that increasing age and the presence of comorbidities contribute to the overall mortality in RA. The diagnosis of RA requires thorough history taking, clinical assessment and biochemical tests which normally investigate the levels of acute phase reactants and antibodies. Using 2010 American College Rheumatology (ACR)/ European League Against Rheumatism (EULAR) classification criteria, a score of 6 and above is considered a definite RA (**Table 2.2**).

Table 2.2: The 2010 ACR/EULAR classification criteria for RA

Joint Distribution (0-5)	
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints (large joints not counted)	3
>10 joints (at least 1 small joint)	5
Serology (0-3)	
Negative RF AND negative ACPA	0
Low positive RF OR low positive ACPA	2
High positive RF OR high positive ACPA	3
Symptom Duration (0-1)	
< 6 weeks	0
≥ 6 weeks	1
Acute Phase Reactants (0-1)	
Normal CRP AND normal ESR	0
Abnormal CRP OR abnormal ESR	1

RF: rheumatoid factor; ACPA: anti-citrullinated peptide antibodies; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate

Source: (Kay & Upchurch, 2012)

2.2 Pathogenesis of periodontitis: Current understanding

The pathogenic concepts of periodontitis are evolving. From the linear model of disease progression where dental plaque was known as the principal culprit to initiate gingivitis and periodontitis prior to 1980s to the recognition of host inflammatory response in periodontal tissue destruction in 1980s. This was followed by the proposal of 1997 classical model (Page & Kornman, 1997) in which the clinical expression of

periodontitis is a consequence of the complex interplay between microbial challenge and host response in the presence of genetic and environmental modifying factors (Kornman, 2008). It is accepted that pathogenic biofilm is a prerequisite for the development of gingival inflammation but it is insufficient by itself to cause PD. In clinically healthy periodontium, microbial biofilm and host cells are constantly in a biological equilibrium that mutually benefits each other. Immune surveillance by the transmigrating neutrophils into the gingival sulcus in response to microbial challenge keeps the biofilm from advancing subgingivally. Other components of innate immune system including antimicrobial peptides found in the saliva, flushing action of gingival crevicular fluid and high turnover rate of junctional epithelium in eliminating bacteria-infected superficial cells, making them an efficient physical barrier to keep the equilibrium in check (Pöllänen, Salonen & Uitto, 2003). Biofilm benefits from the host-microbial interaction by securing nutrition, which is the by-product of inflammation, as well as restricting the colonization of pathogenic species. However, when this homeostasis is disrupted by a change in the environment which favours the proliferation of certain species for example *Porphyromonas gingivalis*, oral dysbiosis occurs. The environmental change can be a result of inadequate plaque control, giving rise to excessive microbial challenge which overwhelms the local defenses (Marsh, 1994). In susceptible individual, dysbiotic microbiota can trigger exaggerated host response that shifts the balance towards destructive sequelae (Hajishengallis & Lamont, 2012). Due to the impervious nature of biofilm, neutrophils are unable to phagocytose the bacteria when they reach the gingival sulcus and thus, performing 'abortive phagocytosis' by discharging their lysosomal contents into the gingival sulcus to kill the bacteria (Seymour, Berglundh & Trombelli, 2015). These lysosomal enzymes can revert into the tissues and cause a perivascular loss of collagen (White et al., 2016). Neutrophils can also kill bacteria by releasing neutrophil extracellular traps (NETs), which is probably

the last resort to control infection (Brinkmann et al., 2004). The process of pathogen-induced cell death is known as NETosis, which can be induced by lipopolysaccharide, streptococcal M protein, IL-8 and TNF-α found in the gingival sulcus (Remijsen et al., 2011; White et al., 2016).

As microbial challenge persists, the first line of defense fails to contain the infection so the adaptive immunity takes over. Collagenolytic activity increases as inflammation intensifies and this causes more degradation of connective tissue. Eventually, a periodontal pocket forms because of loss of connective tissue attachment and apical migration of the junctional epithelium along the root surface. Fibroblasts and epithelial cells can be activated by lipopolysaccharides to engage in tissue destruction process through the secretion of collagenases and MMPs (Takata & Donath, 1988). Meanwhile, increased ulceration of the pocket epithelium permits additional entry of the bacterial products. Subsequently, this allows persisting production of inflammatory cytokines (IL-1, TNF-α and PGE_2) which maintains the destructive inflammatory response (Reynolds & Meikle, 1997). The lesion takes on chronic traits, in favour of infection and non-resolving inflammation.

The robustness of innate immune response and the local cytokine production will determine whether or not the lesion progresses. It is generally accepted that a stable lesion is cell-mediated (T-cell lesion) whereas a progressive lesion is induced by humoral response (B-cell lesion) (Seymour, Powell & Davies, 1979). Bone resorption is the hallmark of PD (Taubman et al., 2005). Despite the formation of fibrous tissue band to wall off the progressing lesion, it is believed that tissue destruction takes place at such a rate that tissue repair fails to keep up. This in turn allows the extension of inflammation through the ulcerated pocket epithelium into the bone and ultimately elicits crestal bone resorption. Pro-inflammatory cytokines such as TNF-α and PGE_2, secreted by activated macrophages and T-cells can stimulate osteoclastogenesis. Hence,

bone resorption takes place. In PD, host inflammatory response acts as a double-edged sword. At tooth level, inflammation appears destructive because of the local tissue damage it results. However, at the host level, it is a protective mechanism employed to prevent the systemic spread of infection.

2.3 Pathogenesis of rheumatoid arthritis: An unsolved mystery

The exact cause of RA still remains unknown just like most other autoimmune diseases. Twin studies have revealed that genetic factors contribute to 50% of RA risk (MacGregor et al., 2000). Although the exact gene is yet to be defined, RA is most likely polygenic and the first genetic risk factor identified to be linked to RA susceptibility is human leukocyte antigen (HLA)-DRB1 cluster (Gregersen et al., 1987). More than 32 non-HLA risk loci have been identified such as PTPN22, PADI4 and STAT4 following the application of genome wide association studies (Chatzikyriakidou et al., 2013). Infection has long been speculated to be involved in the pathogenesis of RA but the evidence is still lacking. Interestingly, Fasano (2009) hypothesized that celiac disease and other autoimmune disorders including RA were a result of what he coined as a trio of causes: 1. an environmental trigger 2. genetic susceptibility and 3. unusually permeable intestines (Fasano, 2009). While the role of digestive system is speculative it gives a new insight into autoimmunity since neither genetic factors nor environmental factors can fully explain the development of RA. Nevertheless, the current understanding of RA pathophysiology encompasses three phases which are summarized in **Figure 2.1**.

The earliest event in RA is synovitis which is characterized by an influx of inflammatory leukocytes and angiogenesis. The activation of innate immune system may be due to a microbial insult or mechanical trauma to the synovium. Initially, tissue oedema and fibrin deposition predominate due to increased vascular permeability, followed by proliferation of synoviocytes. Subsequently, the synovial lining becomes

hyperplastic and forms surface projections known as villi. The synovial sub-lining also expands with the infiltration of mononuclear cells including macrophages, T and B cells and plasma cells. The synovial lining is made up of two types of synoviocytes known as Type A (macrophage-like) synoviocytes and Type B (fibroblast-like) synoviocytes. In RA fibroblast-like synoviocytes are phenotypically different and resistant to apoptosis (McInnes & Schett, 2011). It is believed that the altered fibroblast-like synoviocytes contribute to synovial inflammation and local tissue destruction through the expression of altered levels of cytokines, chemokines and proteolytic enzymes. Upon exposure to exogenous material or autologous antigens, the antigen-presenting cells such as dendritic cells present arthrogenic antigens to T cells, stimulating the production of antibodies, cytokines and autoantibodies to self-antigens (McInnes & Schett, 2011).

The common autoantibodies in RA are rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA) (Lee & Weinblatt, 2001). These autoantibodies bind to autoantigens and form immune complexes to further stimulate the secretion of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 which promote the recruitment of immune cells to inflamed synovium. Meanwhile, neutrophils and synoviocytes release MMPs into the synovial fluid which degrade the cartilage. Chondrocytes are also activated by the pro-inflammatory cytokines and cause direct cartilage degradation through the release of additional MMPs (Smolen et al., 2007). Another characteristic of RA is pannus formation at the interface with cartilage and bone which is a distinct portion of synovium rich in osteoclasts, mononuclear cells and fibroblasts (Shiozawa et al., 1983). Pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 secreted by activated fibroblasts, macrophages, T and B cells can stimulate the formation and maturation of osteoclasts, leading to bone erosion (Scott et al., 2010).

The relationship between autoimmunity development and synovial involvement is poorly understood. Studies have shown that the development of RF and ACPA can

precede the clinical articular involvement by up to 15 years (Isaacs, 2010). Therefore, it has been suggested that a 'second hit' is necessary to involve the synovium since autoantibody production precedes the clinical onset of joint symptoms in RA (van de Sande et al., 2011). Although not all RA patients are seropositive, the presence of autoantibodies in HLA-DRB1 positive individuals is associated with a more severe course of RA (van Gaalen et al., 2004). Currently, RF and ACPA are routinely utilised in the diagnosis of RA. Other autoantibodies such as anti-carbamylated and acetylated peptide antibodies have been identified in RA patients but are yet to be proven clinically useful (Shi et al., 2014). Many questions remain unanswered and majority of the studies focused on seropositive RA, leaving seronegative RA unexplored. It is unclear whether additional autoantibodies are waiting to be discovered for seronegative RA or it is simply a separate disease entity with different pathogenesis.

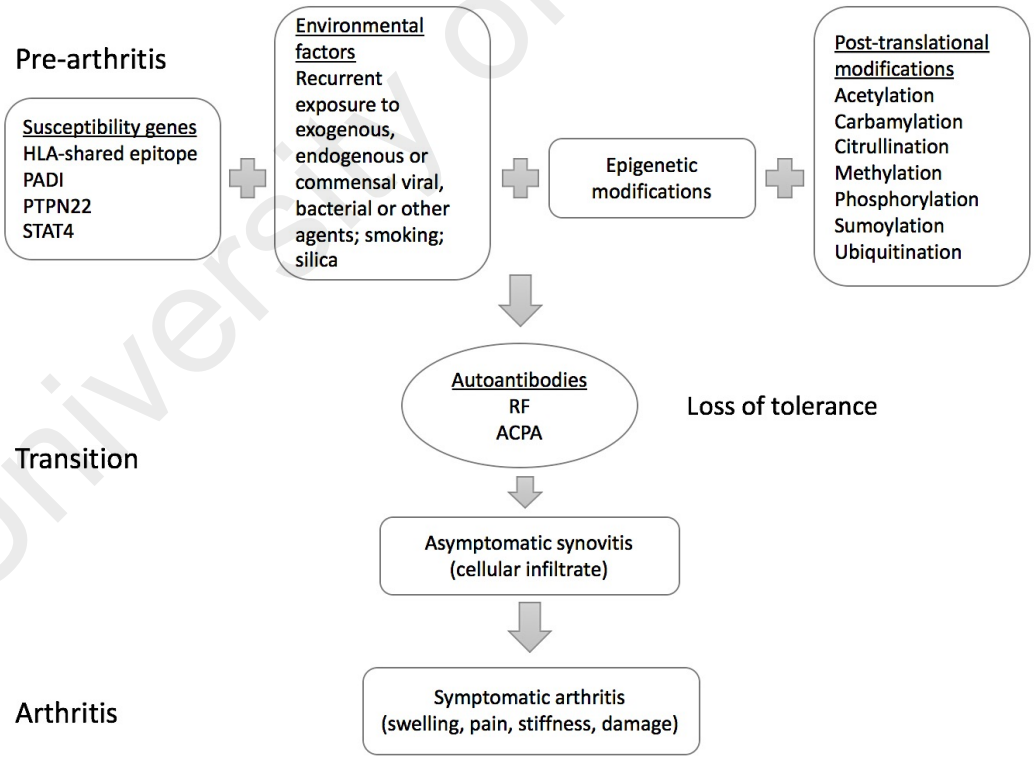

Figure 2.1: Current understanding of the aetiopathogenesis of RA.

Source: (Smolen et al, 2016)

2.4 Association between PD and RA: Old versus New hypotheses

The association between PD and RA has been discussed for decades owing to their common pathobiological features. Previously, RA was considered a risk factor for PD as the oral hygiene of participants with established RA tended to be poorer than non-RA counterparts due to their impaired manual dexterity. However, case control studies have reported an independent association between PD and RA which cannot be fully explained by oral hygiene and smoking (Kasser et al., 1997; Pischon et al., 2008). In response to the association studies, new hypotheses have been proposed to unravel the possible mechanisms underlying their association.

2.4.1 Hypothesis 1: Two-hit model

A hypothetical 'two-hit' model was proposed by Golub and colleagues (2006) to elucidate the connection between RA and PD. In this model, RA was suggested to be able to co-induce PD through exacerbation of systemic inflammation (Golub et al., 2006). The local inflammatory response within the synovium (first hit) elevates the levels of circulating pro-inflammatory mediators particularly TNF-α, IL-6 and CRP and increases systemic inflammation. In the presence of the second hit which is endotoxin produced by the microbial biofilm, the host inflammatory response is enhanced and subsequently results in periodontal tissue destruction (Golub et al., 2006). A similar two-hit phenomenon has been used to explain the pathogenesis of acute respiratory distress syndrome, in which pig models with both faecal peritonitis and ischaemic reperfusion injury developed lung injury compared to no sign of lung injury in pig models with faecal peritonitis only (Steinberg et al., 2005). One prospective cohort study investigating the mechanisms of generalised bone loss in early RA has shown that patients with RA have significantly higher plasma levels of IL-6 and CRP and lower bone mineral density than healthy controls, suggesting an influence of systemic inflammation on bone resorption. The authors concluded that increased levels of pro-

inflammatory cytokines in the circulation stimulated osteoclastic activation and caused bone loss in RA. Therefore, it is possible that alveolar bone resorption can occur through the same mechanism (Gough et al., 1998).

The concept that low-grade systemic inflammation can induce periodontal destruction is further supported by a recent longitudinal prospective population-based cohort study in Germany (Pink et al., 2015). The study followed up 1784 participants over a period of 11 years. Consistent associations were found between PD (measured in terms of probing depth and clinical attachment loss) and systemic low-grade inflammation (indicated by fibrinogen levels and white blood cell counts). To put it into perspective, a one unit increase in fibrinogen level is associated with 3% more sites with probing depth of ≥ 3 mm and 2.7% more sites with clinical attachment loss of ≥ 3 mm (Pink et al., 2015). However, the clinical significance of the findings was diluted by the consideration that systemic inflammation merely contributed to 12% of progression over 11 years. Clearly, systemic low-grade inflammation could be considered only one among other yet unknown pathways that leads to PD (Josey & Merchant, 2016).

2.4.2 Hypothesis 2: The humoral response to oral bacteria

Infection has long been suspected to be associated with the development of autoimmunity in genetically susceptible individuals but the scientific evidence is scanty (Shoenfeld et al., 2002). An assumption emerged that the infectious agent might not necessarily be a pathogen originating from the joint and this was supported by several studies that reported the detection of *P. gingivalis* DNA in the synovial fluids and tissues of participants with RA. In 2009, Martinez-Martinez and co-workers reported that 57.8% of *P. gingivalis* DNA was detected in the synovial fluid sample of RA patients and the migration of the bacterial DNA from the oral cavity to the joint could be in free DNA form (Martinez-Martinez et al., 2009). A more recent study with larger sample size also documented the presence of *P. gingivalis* DNA in the synovial tissue

and fluid of RA patients (Totaro et al., 2013). The study reported that the amount of *P. gingivalis* DNA detected was significantly higher in the synovial tissue than in the synovial fluid. Although no viable bacteria have been recovered from the synovial tissue, the presence of bacterial DNA implies that oral bacteria may be involved in the pathogenesis of RA. Rosenstein and colleagues hypothesized that the proteinases secreted by oral bacteria in the periodontal lesions were capable of converting fibrin into citrullinated antigens which could act as systemic immunogens (Rosenstein et al., 2004). The dissemination of locally produced citrullinated antigens triggers the production of IgG antibodies. The binding of IgG to citrullinated antigens allows them to become a more accessible antigenic target for RF. Immune complexes are formed following the binding of IgG and RF, activating the complement cascade and T-lymphocytes. What follows is a complex inflammatory cascade resulting in persisting release of pro-inflammatory mediators and destruction of soft and hard tissues observed in both PD and RA (Rosenstein et al., 2004).

2.4.3 Hypothesis 3: Common tissue destruction pathway

While the 'two-hit' model is a favourite concept used to explain the link between RA and PD, another hypothesis that has been proposed is that at least one common underlying dysregulation of the inflammatory pathway between RA and PD may lie within the RANKL/OPG/TRAIL axis (Bartold et al., 2005). A decrease in OPG expression leads to vascular damage whereas an increase in RANKL and TRAIL levels may result in the activation of osteoclasts and consequently bone resorption.

RANKL is a cell-surface protein and a member of the tumour necrosis factor (TNF) superfamily. It can be secreted by several cells including osteoblasts, stromal cells and dendritic cells as well as activated T and B cells. It binds to its receptor, known as RANK on osteoclast precursor cells and promotes the maturation of osteoclasts which in turn causes bone resorption. OPG, on the contrary, is a soluble

cytokine receptor that acts as a natural inhibitor of RANKL. Therefore, no bone destruction will occur when OPG binds to RANK (Simonet et al., 1997; Wang & El-Deiry, 2003). Arg-gingipain of *P. gingivalis* has also been shown to increase the RANKL/OPG ratio in gingival fibroblast and periodontal ligament cells and subsequently enhanced osteoclastogenesis (Belibasakis et al., 2007).

There has been growing literature pertaining to the role of TRAIL in tissue destruction pathway. There are 5 types of receptors for TRAIL, 2 death receptors with death domains and 3 decoy receptors without the death domains. Programmed cell death is activated when TRAIL binds to the death receptors whilst apoptosis is inhibited when the decoy receptors bind to TRAIL (Lucas et al., 2010). It is believed that in both RA and PD, either a reduced TRAIL death receptor expression or a raised expression of TRAIL decoy receptor may be the key to the chronicity of both diseases (Agnihotri & Gaur, 2014).

The current focus was on either the ratio of RANKL/OPG in periodontal lesions or the expression of TRAIL in RA tissues but none on the comparison of the RANKL/OPG/TRAIL axis in PD and RA. Perhaps, future studies could explore RANKL/OPG/TRAIL axis in both RA and PD for further clarification.

2.5 Epidemiologic perspective

The association between PD and RA has been reported by numerous observational studies on different populations across the world. In general, the prevalence and severity of PD are higher in RA patients than non RA counterparts. Similar pattern is also observed among the non-smoking RA subgroup. A pilot study in the United States evaluated the prevalence and severity of PD among the veterans with RA and their relationship to RA in terms of disease activity (Dissick et al., 2010). No association was found between the disease activity and PD status but moderate to severe PD was significantly more prevalent in RA patients (51%) than non-RA controls (26%).

The study was extended in 2014 to include a larger sample size from 4 rheumatology clinics. Significantly more sites with alveolar bone loss greater than 20% in ACPA-positive RA patients was found compared to controls (Gonzalez et al., 2015). One of the limitations of these two studies is the male-predominance in their study population.

A hospital based case control study in India compared PD status of 100 RA patients with 112 healthy control. RA patients were found to be 3 times more likely to have moderate to severe PD than non-RA controls (Joseph et al., 2013). The strength of this study is the exclusion of smokers and co-morbidities which are two known major confounders. However, the effect of RA medications and duration of RA on PD status were not examined so it remains unclear whether the immunosuppressive effects of their RA treatment could have influenced the results. This gap in the literature was addressed by another case control study in which non-smoking and disease-modifying anti-rheumatic drugs (DMARDs)-naïve RA patients were enrolled (Potikuri et al., 2012). RA patients were 4 times more likely to have PD than healthy controls, independent of oral hygiene status. As only patients with early RA and unaffected manual dexterity were recruited, the presumption that poor oral hygiene due to impaired motor function was ruled out. Likewise, patients with early RA in Germany had a greater number of missing teeth and increased clinical attachment loss than controls despite comparable oral hygiene (Wolff et al., 2014). A recent systematic review of 17 cross sectional, case-control studies revealed that RA patients had a 13% greater risk of PD, confirming an association between RA and PD (Fuggle et al., 2016).

Nevertheless, some studies have shown opposite findings. An Indonesian study reported no difference in both the prevalence and severity of PD between RA and non RA groups (Susanto et al., 2013). The same results were also revealed in a recent population-based study in Sweden (Eriksson et al., 2016). The conflicting findings may be ascribed to heterogeneity in study design, underpowered sample size and lack of

uniformity in RA and PD case definition. Therefore, the temporal relationship between these two diseases can only be ascertained through sufficiently powered, multicentre longitudinal studies.

2.6 Clinical perspective

Non-surgical periodontal treatment has been shown to reduce systemic inflammation and disease activity of RA in short term clinical trials. A pilot clinical trial in Ohio investigated the effect of non-surgical periodontal therapy on the severity of RA (Al-Katma et al., 2007). Twenty-nine patients were involved but only 17 received treatment consisting of scaling and root planing, weekly prophylaxis and oral hygiene reinforcement while the remaining 12 received no treatment. All the clinical and laboratory parameters including gingival index, plaque index, PPD, CAL, DAS28, ESR and VAS were recorded at baseline and 8 weeks after therapy. The disease severity as measured by DAS28, ESR and VAS had significant improvement in the treatment group compared to control group. Similarly, another single-centre intervention study in Turkey also reported a reduction in DAS28 and the level of IL-1ß in gingival crevicular fluid 6 months after non-surgical periodontal therapy (Biyikoglu et al., 2013). The authors suggested that periodontal treatment could act synergistically with systemic RA treatment to reduce the disease activity. However, the sample size in this study was small and there was no periodontally healthy RA group as a control.

As RA patients experience episodic exacerbation and remission due to the nature of the disease, frequent change of medications is not uncommon. Therefore, it is challenging to ascertain whether the improvements obtained come from periodontal treatment alone or the combination of medical and periodontal treatments. This issue has been addressed by an intervention study in University Hospitals of Cleveland in which 40 RA participants taking either DMARDs only or a combination of DMARDs and anti-TNF-α medication were subdivided into 4 groups: (A) DMARDs only, (B)

periodontal treatment and no anti-TNF-α drug, (C) periodontal treatment and anti-TNF-α drug and (D) anti-TNF-α drug only (Ortiz et al., 2009). RA disease severity was measured by ESR, VAS and DAS28 at baseline and 8 weeks after periodontal treatment. DAS28 and VAS in both treatment groups improved significantly compared to non-treatment groups, indicating that periodontal treatment had a beneficial effect on RA disease activity regardless of medications being taken.

A Japanese intervention study examined the effect of periodontal treatment on the serum antibodies to *P. gingivalis* and citrulline levels in relation to RA severity. The treatment group received oral hygiene instruction and supragingival scaling while the control group received no periodontal treatment at all. After 8 weeks, the treatment group showed significantly greater reduction in DAS28-CRP, serum levels of IgG to *P. gingivalis* and citrulline than the control group, suggesting a role of *P. gingivalis* in citrullination (Okada et al., 2013).

Two systematic reviews of 5 and 8 randomised clinical trials respectively supported the hypothesis that resolution of gingival inflammation following conventional periodontal treatment in some way has positive influence on systemic inflammation which explains the improvement of sign and symptoms of RA (Kaur et al., 2014; Silvestre et al., 2016). However, the level of evidence is low given all studies included suffered from small sample size and short trial duration.

2.7 Potential mechanisms underlying the association between PD and RA

2.7.1 Citrulline and citrullination

The human genetic code only directly encodes 20 amino acids (Massey et al., 1998). However, citrulline is not one of the common amino acids but a result of post-translational modification of protein-contained arginine residues. Arginine is a positively charged amino acid that forms hydrogen bonds with other amino acid side chains and the peptide backbone, making it a crucial amino acid in maintaining the three-dimensional structure and function of proteins (Mangat et al., 2010). The main difference between arginine and citrulline is the loss of one positive charge upon enzymatic conversion (citrullination) (**Figure 2.2**). This conversion is catalyzed by an enzyme known as peptidylarginine deiminase (PAD) in the presence of high concentrations of calcium. Five isotypes of PAD enzymes with different functions have been identified in human (PAD-1, PAD-2, PAD-3, PAD-4 and PAD-6) and they are distributed in various locations, ranging from epidermis, hair follicles, neuronal tissue, uterus to white blood cells (Wegner et al., 2010). The precise role of citrullination is still unclear but it is deemed to be involved in many physiologic processes in the body such as skin keratinization during the terminal differentiation of keratinocytes, gene regulation and innate immune functions (Baka et al., 2012). Its potential pathogenic role has also been implicated in RA and multiple sclerosis as citrullination causes changes in protein structure. These conformational changes may not only disrupt their physiologic function but may also trigger immunologic events through the formation of new epitopes which can become antigenic targets of autoantibodies in susceptible individuals. This hypothesis is supported by an animal study in which citrullination of arginine residues enhanced the peptide-MHC affinity and evoked an autoreactive T cell response to citrullinated peptides in HLA-DRB1*0401 transgenic mice (Hill et al., 2003).

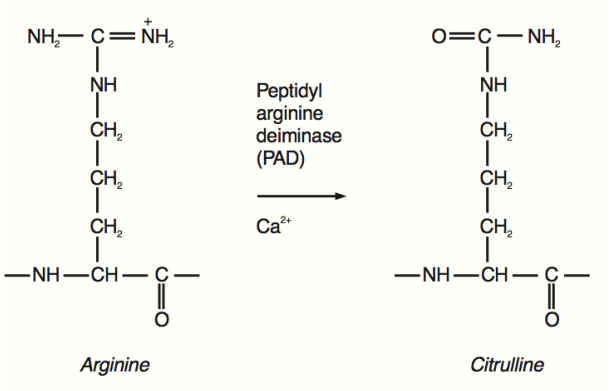

Figure 2.2: Conversion of arginine to citrulline

2.7.2 Anti-citrullinated protein antibodies (ACPA) and RA

In any autoimmune disease, the immune responses are directed against self-antigens. As a result, autoantibodies detection becomes a significant tool for aiding the diagnosis of RA. One of the routinely measured serum autoantibodies in RA patients is RF which recognizes the Fc portion of immunoglobulin G (IgG). The problem with RF is that it can be present in healthy individuals and other non-rheumatic conditions such as Sjorgren's syndrome and viral infections (Ingegnoli et al., 2013; Simard & Holmqvist, 2012). The low positive predictive value of RF renders it an unspecific test for RA diagnosis. Subsequently, a more specific autoantibody named anti-perinuclear factor (APF) that labeled the cytoplasmic granules in buccal mucosa cells was discovered and it was present in the sera of 50% of RA participants with specificity between 73 and 99% (Hoet & van Venrooij, 1992; Nienhuis & Mandema, 1964).

Meanwhile another class of autoantibodies called anti-keratine antibodies (AKA) that labeled the stratum corneum cells of epithelium was also found to be specifically present in the serum of RA patients (Hoet & van Venrooij, 1992). Despite the high specificity of APF and AKA, they are not routinely tested due to the inconvenience of the indirect immunofluorescence. Thus, RF was still used as the main diagnostic marker

for RA. In 1998, Schellekens and colleagues published an experimental study showing APF/AKA antibodies in the sera of RA participants were reactive with citrulline-containing peptides, with a sensitivity of 76% and specificity of 96% (Schellekens et al., 1998). Later on, they developed a cyclic citrullinated peptide and used it as an antigenic substrate in enzyme-linked immunosorbent assay (ELISA). Serum samples from early RA, established RA and non-RA participants including Crohn's disease, psoriasis, multiple sclerosis and healthy controls were taken at first visit and repeated at 1-year follow-up to determine the diagnostic usefulness of anti-cyclic citrullinated peptide (anti-CCP) in RA. It was found that anti-CCP was highly specific for RA (98%) and the combination of anti-CCP and IgM-RF ELISAs yielded 91% positive predictive value compared to IgM-RF ELISA alone (74%) (Schellekens et al., 2000). Presence of anti-CCP can be detected up to 10-15 years before the clinical manifestation of RA, making early disease detection possible and enabling new development of therapeutic strategies in RA (van de Stadt et al., 2011). The presence of ACPA at baseline is also associated with more severe radiologic damage over time, making it a potential prognostic marker for patients with early RA (Kroot et al., 2000). Therefore, both RF and ACPA have now become routinely tested serological markers as listed in 2010 ACR/EULAR classification criteria.

As mentioned previously, ACPA can be detected years before the disease onset but the epitopes recognised by ACPA are not well defined. Serum samples from RA participants were reactive with various citrullinated endogenous and exogenous peptides such as fibrin, vimentin, α-enolase, collagen and histones (Johansson et al., 2016) and such a broad reactivity expanded with disease development, a phenomenon known as epitope spreading (van der Woude et al., 2010). A longitudinal cohort study that investigated the sera collected from RA patients who donated blood before RA onset has shown that the number of recognised citrullinated epitopes increased throughout the

disease development, from before the onset of RA to established RA (van der Woude et al., 2010). Such expansion of immune response may be related to epitope spreading which is defined as the diversification of epitope specificity from the initial single epitope-specific immune response, directed against a self or foreign protein, to other epitopes on that protein (intramolecular spreading) or other proteins (intermolecular spreading) (Vanderlugt & Miller, 2002). An accumulation of ACPA reactivity over time was noted in the sera of RA participants who progressed from pre-clinical phase to development of clinical RA. Interestingly, the ACPA accumulation preceded the elevation of serum pro-inflammatory cytokines and chemokines, indicating a correlation between autoantibodies accumulation and clinical inflammation (Sokolove et al., 2012). However, what is unknown is whether the autoantibodies are generated intra-articularly or extra-articularly or both.

2.7.3 *P. gingivalis*: more than a periodontal pathogen

The concept of bacterial complexes introduced by Socransky and co-workers in their landmark study has highlighted that PD is not caused by a single bacterial species but a synergistic interaction between different bacterial species acting in concert as a complex (Socransky et al 1998). Red complex is the complex most strongly associated with PD and is composed of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*. Being one of the major periodontal pathogens, *P. gingivalis* has become the subject of particular interest in oral microbiology due to its capacity to colonise and evade the host defense mechanism as well as its ability to produce myriads of virulence factors that result in host tissue destruction. *P. gingivalis* is a non-motile, Gram-negative obligate anaerobe which relies on heme for its growth. It is frequently detected in PD with a prevalence of 85% (Yang et al., 2004) and its number increases with PPD (Kawada et al., 2004). A significant reduction in *P. gingivalis* numbers following periodontal therapy is related with an improvement in periodontal status

(Haffajee et al., 1997). As part of its survival strategies, it attaches to the early colonizers through its fimbriae (Lamont & Jenkinson, 1998), invades the gingival epithelial cells and disseminates from cell to cell without going through extracellular space to escape from the humoral immune response (Yilmaz, 2008). It is able to release proteolytic enzymes that degrade the host macromolecules such as collagen, fibrinogen and laminin to ensure its nutritional support. The strong association of *P. gingivalis* with PD is undoubtedly established but how it is related to RA is speculative.

The speculation that oral bacteria could initiate and perpetuate joint inflammation in RA is not without basis. Using checkerboard DNA-DNA-hybridization, Moen and colleagues detected the DNA of *P. gingivalis*, *Tanneralla forsynthia* and *Prevotella intermedia* in the synovial fluid samples of RA patients. The higher number of DNAs found in RA synovial fluid compared to RA serum implies that blood stream is not the only bacterial transmission route. The translocation of bacterial DNA from the mouth to joints could occur through phagocytosis of immune cells (Moen et al., 2006). Later on, another cross-sectional study also identified periodontal bacterial DNA in subgingival plaque, synovial fluid and serum obtained from participants with both RA and PD. It was reported that periodontal bacteria (57.8% of *P. gingivalis* and 73.6% of *Prevotella intermedia*) were frequently detected in the synovial fluid samples. In contradiction to (Moen et al., 2006), the authors hypothesized that the migration of the bacterial DNA from the periodontal sites to the joints could be in free DNA form due to the absence of viable bacterial cells in the synovial fluid samples as well as absence of bacterial DNA inside leucocytes isolated from the whole blood (Martinez-Martinez et al., 2009). Although the sample size of both studies was small, they gave new insights into the plausible aetiological link between periodontal pathogens and RA. Another study detected the presence of *P. gingivalis* DNA in both of the synovial tissue and fluid of RA patients. However, the amount of *P. gingivalis* DNA detected was significantly

higher in the synovial tissue than in the synovial fluid, suggesting a possible intracellular translocation of *P. gingivalis* (Totaro et al., 2013).

Apart from the detection of bacterial genetic material, some studies tested the immune response to *P. gingivalis* in RA samples. When genetically-modified mice were infected with 3 different bacterial species (*P. gingivalis*, *T. denticola* and *T. forsythia*), a significant elevated antibody level to periodontal pathogens was observed in infected mice than uninfected mice but only *P. gingivalis* DNA was detected in the inflamed joints of infected mice (Chukkapalli et al., 2016). This supports the hypothesis that *P. gingivalis* may be able to amplify autoimmune arthritis through systemic dissemination. A large epidemiologic assay of an immune response to *P. gingivalis* in patients with early onset RA shared a similar hypothesis (Kharlamova et al., 2016). Their data showed that 23.1% of RA participants had increased anti-*P. gingivalis* antibody levels compared to 9.6% of non-RA controls. It was the first observational study conducted using a large sample size and a more specific antibody as a serological marker to speak volumes about their results. The *P. gingivalis* link between PD and RA was further endorsed by a current systematic review that inferred RA patients tended to show a higher antibody response to *P. gingivalis* than systemically healthy individuals, irrespective of the presence of PD (Bender et al., 2017). Although it remains obscure how *P. gingivalis* DNA travels beyond the oral cavity, all the direct and indirect evidences mentioned previously have implied an association of *P. gingivalis* with RA. Here, we see *P. gingivalis* take on a role different from that of a classic periodontal pathogen, and act as a culprit in driving the production of autoantibodies against self-antigen expressed in the inflamed joints.

2.7.4 *P. gingivalis* peptidylarginine deiminase (PPAD) and citrullination

McGraw and colleagues were the first team to isolate and characterize one of the many virulent factors of P. gingivalis, PPAD which was an incidental discovery during the hemagglutinin preparation (McGraw et al., 1999). It was believed that PPAD was secreted to regulate the vascular flow in the sulcular area by converting arginine residues in peptides to citrulline residues. Through the control of vascular flow, *P. gingivalis* can sustain adequate nutritious supply and at the same time is not flushed away from the periodontal pocket by excessive exudation of plasma. The deiminase activity of PPAD also releases ammonia as a by-product, rendering the environment more alkaline and favourable for its growth (McGraw et al., 1999). The pathogenic impact of citrullination by PPAD in periodontal disease includes modified function of epidermal growth factor and reduced pro-inflammatory function of anaphylatoxin C5a which directly impairs healing of periodontium and diminishes chemotactic activity (Bielecka et al., 2014; Pyrc et al., 2013). Therefore, PPAD is important for both the viability and virulence of *P. gingivalis* in periodontal disease.

To date, *P. gingivalis* is the only prokaryote that is able to express PAD (Mangat et al., 2010). Unlike human PADs, PPAD is capable of citrullinating both C-terminal arginine residues and free arginine in the absence of calcium (Rodriguez et al., 2009). Intriguingly, gingipains of *P. gingivalis* has been shown to promote the release of pro-inflammatory cytokines by activating proteinase-activated receptor 2 (PAR-2), a G protein-coupled receptor found on the surface of neutrophils, epithelial cells and osteoblasts (Lourbakos et al., 1998). Increase in intracellular calcium concentration was observed as gingipains acted on PAR-2 which may activate human PADs citrullination in the oral environment.

Since human PADs are efficient at citrullinating arginine residues within polypeptide chains but not at their terminal, the complementary action of PPAD is

suggested to play a role in the pathogenesis of RA by generating a distant (extra-articular) source of epitopes for autoimmunity in RA progression (Maresz et al., 2013). This hypothesis is supported by (Quirke et al., 2014) who reported in their study that PPAD was capable of auto-citrullination and the antibodies produced against auto-citrullinated PPAD among PD patients with RA could heighten the immune response through epitope spreading and cross-reactivity with citrullinated human proteins. However, this finding was refuted by (Konig et al., 2015) as they did not detect auto-citrullinated PPAD in their study and the anti-PPAD levels were not correlated with ACPA levels or disease activity in RA, casting doubts over whether auto-citrullinated PPAD was the true antigenic target. Some clinical trials, on the other hand, showed a positive correlation between anti-PPAD and ACPA levels in RA patients and the level of anti-PPAD was significantly higher in RA patients than non-RA control (Kobayashi et al., 2016; Shimada et al., 2016). RA patients with low titers of anti-PPAD at baseline tend to respond better to biological disease-modifying anti-rheumatic drug (bDMARD) such as inhibitors of TNF and IL-6 receptor compared to those with high anti-PPAD, implying a negative effect of anti-PPAD on RA (Kobayashi et al., 2016).

2.7.5 Evidence on citrullination in the RA-PD link

Although citrullination has been proposed as one of the mechanisms underlying the link between RA and PD, there is a serious lack of direct evidence confirming this hypothesis. ACPA is often used as a surrogate marker in animal and human studies to depict the association between RA and PD with conflicting results. Using rats with experimentally-induced RA and PD, the levels of ACPA in serum and gingival tissues increased significantly in RA-PD group compared to RA alone, PD alone and healthy control (Correa et al., 2017). The paw swelling observed in RA group was worsened when PD was present, suggesting PD perpetuated the immune response in RA. Clinical studies, however, reported conflicting findings. (Bello-Gualtero et al., 2016) evaluated

the periodontal condition of both patients at risk of RA (pre-RA) and patients with early RA. A positive association was found between PD and pre-RA but not with early RA. In both groups, the immune response against *P. gingivalis* was associated with ACPA levels, suggesting that PD could be a risk factor for RA. Likewise, a Korean cross-sectional study revealed a significant correlation between periodontal parameters including bleeding on probing and ACPA levels. Disease activity and systemic inflammation, represented by ESR values were also correlated with periodontal involvement (Choi et al., 2016). A similar association was also reported by a large-scale prospective cohort study in Japan. Interestingly, the association between PD and ACPA remains relevant in non-smoking cohort, supporting the notion that PD may contribute to ACPA generation (Terao et al., 2015).

On the contrary, Reichert et al. (2015) failed to find any association between PD and ACPA when they assessed the plasma samples from non-RA patients with PD. Most of the periodontally compromised patients were tested negative for ACPA which contradicted the hypothesis that PD could lead to the development of ACPA. Clearly, these discrepancies are inevitable owing to methodological variations in study design, case definition and sample size. However, several immunohistochemical studies have repeatedly detected the presence of citrullinated proteins in inflamed gingival tissues obtained from patients with PD and the intensity of staining increased with the severity of PD (Engström et al., 2018; Harvey et al., 2013; Janssen et al., 2017). Janssen et al. (2017), in particular, found citrullinated histone H3 in periodontal tissue from PD patients and levels of anti-citrullinated histone H3 were higher in ACPA seropositive RA patients than ACPA seronegative RA patients. Nevertheless, the association between anti-citrullinated histone H3 levels and periodontal and smoking status of RA participants could not be established in this study, indicating that the development of anti-citrullinated histone H3 may have a genetic basis. ACPA level is always higher in

diseased tissues than serum which indicates a difference between local and systemic production of autoantibodies. The question whether local production of autoantigens targeted by ACPA in PD contributes to RA still remains equivocal. Additional studies are required to confirm this relationship.

2.8 Other potential mechanism

2.8.1 Carbamylation: another source of autoantigens in RA?

As opposed to citrullination, carbamylation is a non-enzymatic post-translational modification in which a cyanate group binds to the amine group of lysine residue, converting it to homocitrulline (**Figure 2.3**). Cyanate can be produced either internally through spontaneous dissociation of urea (isocyanic acid as the by-product) or from external source such as tobacco smoke, polluted air and foods containing thiocyanate which can be converted to cyanate once exposed to reactive oxygen species (Chung & Wood, 1970; Roberts et al., 2011). The blood cyanate concentration is always in equilibrium with urea in healthy individuals and thus, physiological carbamylation occurs at a rather slow rate over time. In conditions for example chronic kidney disease where the concentration of urea is elevated the intensity of carbamylation is expected to increase (Pietrement et al., 2013). Due to its time-dependent nature, proteins with long half-lives such as collagen and elastin are more commonly carbamylated which explains its role in aging. All proteins, however, are subject to carbamylation and such chemical reaction can cause structural changes to proteins and subsequently result in a decrease or loss of function. Besides, the conformational changes induced by carbamylation may generate new epitopes that are capable of breaching the tolerance of host immune system and trigger the production of autoantibodies in susceptible individuals (Shi et al., 2011).

The involvement of carbamylated proteins in the pathogenesis of RA has come into play following the discovery of anti-carbamylated protein (anti-Carp) in RA patients. It was initially thought that ACPA might not be able to differentiate homocitrulline from citrulline due to their great resemblance in terms of chemical structure (homocitrulline is merely one methylene group longer) so the potential for cross-reactivity of ACPA and anti-Carp was anticipated. However, inhibition studies showed that ACPA did not recognize homocitrulline residues, indicating a high specificity of ACPA for citrullinated peptides (Shi et al., 2011). Mice immunised with homocitrulline-containing peptides developed anti-Carp and intra-articular injection of homocitrulline-containing peptides stimulated the development of erosive arthritis. Arthritis was also induced in non-immunized mice when T and B cells were transferred from immunised mice to normal control whereas systemic injection of anti-Carp or intra-articular injection of homocitrulline did not elicit immune response in normal control, indicating that humoral response to carbamylated peptides is a potential mechanism for RA pathogenesis (Mydel et al., 2010). Anti-Carp was detected in about 16-30% of ACPA negative patients and similar to ACPA, they appeared years before the clinical onset of RA (Shi et al., 2011). The presence of anti-Carp was associated with RA progression, characterised by a more severe radiological joint damage over time (van der Linden et al., 2009). Different carbamylated peptides with different sensitivities in RA patients have been identified, ranging from 28-80% against carbamylated vimentin (Nakabo et al., 2017) to 31.4% against carbamylated albumin (Ospelt et al., 2017). Overall, a meta-analysis of 7 studies reported a relatively low sensitivity (42%) but a high specificity (96%) of anti-Carp for the diagnosis of RA (Li et al., 2016).

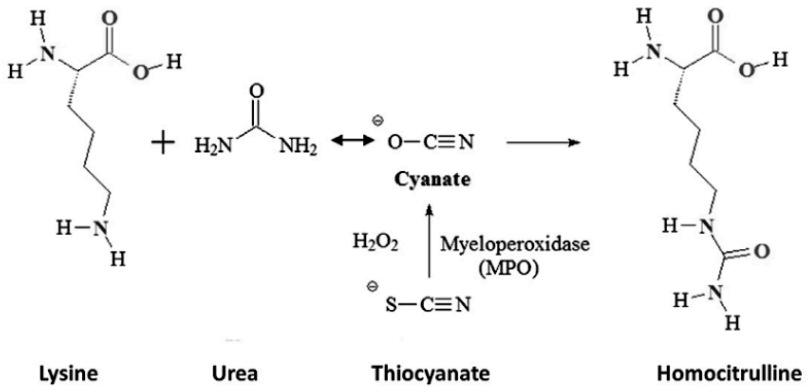

Figure 2.3: Conversion of lysine to homocitrulline

2.8.2 Carbamylation and inflammation-induced neutrophil extracellular traps (NETs)

Other than urea-dependent pathways, carbamylation can also occur during inflammation in the presence of myeloperoxidase (MPO). MPO is a heme-containing enzyme stored primarily in the azurophilic granules of neutrophils and released when neutrophils are recruited to sites of inflammation (Harrison & Schultz, 1976). The formation of reactive oxygen species which are important in antimicrobial defence is catalysed by MPO. Upon activation, neutrophils can kill invading microorganisms extracellularly by releasing NETs which contain MPO (Brinkmann et al., 2004). Therefore, high levels of MPO are released by NETs during inflammation which then catalyse the oxidation of thiocyanate to cyanate, resulting in carbamylation of proteins (Wang et al., 2007) (**Figure 2.3**). MPO-induced carbamylation has recently been implicated in the pathogenesis of numerous inflammatory conditions including renal failure, atherosclerosis and rheumatoid arthritis (Jaisson et al., 2011; Shi et al., 2011). Delporte et al. (2018) confirmed in animal study that MPO-induced carbamylation of proteins was the pathogenic mechanism causing accumulation of homocitrulline in the atheroma and it could take place via three pathways: i. by direct oxidation of cyanide into cyanate, ii. by reaction of hypochlorous acid with cyanide, iii. by reaction of

chloramines with thiocyanate. The release of NETs by neutrophils can be triggered by pathogens as a defense mechanism so infections have been linked to the generation of autoantigens through post-translational modification. In RA, NETs have been shown in *in vitro* research to stimulate pro-inflammatory response through upregulation of IL-6 and IL-8 mRNA levels by fibroblast-like synoviocytes (Khandpur et al., 2013). Therefore, one of the possible underlying mechanisms is that inflammation within the joint compartment enhances carbamylation of host proteins which could potentially become a source of immunogenic antigens in susceptible individuals and further perpetuate the destructive inflammatory response in RA.

2.8.3 Evidence on carbamylation in the RA-PD link

The role of carbamylation in the RA-PD connection has not been widely explored. The presence of carbamylated proteins was shown for the first time in inflamed gingival tissues in a review article, giving rise to a hypothesis that PD could be a site of inflammation-dependent carbamylation (Bright et al., 2015). There is a positive correlation between the intensity of immunostaining and the severity of periodontal disease (Bright et al., 2018). The serum levels of anti-Carp were significantly higher in PD patients with and without RA compared to healthy controls. In agreement with the histological findings by Bright et al. (2018), the serum levels of anti-Carp had a positive correlation with the severity of PD (Kaneko et al., 2018). In contrast with the Japanese study, a case-control study in the Netherlands found a negative association between PD severity and anti-Carp seropositivity (Janssen et al., 2015). The conflicting results could be attributed to differences in study design, case definition and study population. It is not known if ethnic differences have an impact on the levels of anti-Carp. However, if carbamylation is an inflammation-dependent process, it would be reasonable to expect a dose-related increase in the autoantibodies. There is still a lack of evidence supporting

carbamylation as one of the mechanistic links in the association between RA and PD and thus, further studies are warranted to clarify this.

Chapter 3: MATERIALS AND METHODS

3.1 Study design and population

This is an immunohistochemical study involving gingival tissue samples from participants with PD and RA. The study protocol has been approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DF RD1707/0029(L) (Appendix H) and the Medical Research Ethics Committee, University Malaya Medical Centre (MRECID NO: 2017510-5227) (Appendix I). This work was supported by Dental Postgraduate Research Grant (DPRG/27/17), University of Malaya and Frontier Research Grant, University of Malaya (FG040-17AFR).

3.2 Participant recruitment

The participants of the study were recruited from the Periodontology Clinic and Outpatient Clinic of Faculty of Dentistry, University of Malaya and the Rheumatology Clinic of UMMC. Initially a total of 87 participants from Rheumatology Clinic were screened based on the inclusion and exclusion criteria given below. Out of 87 participants, eight were found eligible. Eligible participants were invited to participate on a voluntary basis. The nature of the study was communicated verbally to all participants in addition to the patient information sheet (Appendix C & D). Written informed consent (Appendix F & G) was obtained prior to enrolment.

Inclusion criteria

1. Age 30 years and above
2. Dentate with at least 8 teeth, excluding third molars

a. PD with RA group

1. Confirmed diagnosis of RA for at least 1 year based on ACR/EULAR 2010 criteria
2. Diagnosed with moderate to severe chronic periodontitis (Armitage 1999)

b. PD without RA group

1. Medically healthy, with no family history of RA
2. Moderate to severe chronic periodontitis (Armitage 1999)

c. Healthy control group

1. Medically healthy, with no family history of RA
2. Periodontally healthy or gingivitis (Armitage 1999)
3. Absence of gingival inflammation at biopsy site.

Exclusion criteria

1. Pregnant or lactating females
2. Evidence of known systemic modifiers of periodontal disease (type 1 and 2 diabetes mellitus, other autoimmune diseases, infections, blood disorders, malignancies and medications known to affect periodontal tissues)
3. Known allergy to local anaesthetics
4. On systemic antibiotics in the past 3 months
5. Need prophylactic antibiotics before medical or dental procedures.
6. Smokers

3.3 Sample size calculation

The sample size was calculated on the basis that there was a true difference in the levels of citrullinated and carbamylated proteins between control and disease groups. Harvey et al (2012) reported the mean semi-quantitative analysis (SQA) scores for citrullinated proteins in PD samples were 1.8 (standard deviation of 0.24) while the mean SQA scores for non-periodontitis samples were 0.9 (standard deviation of 0.21).

Assuming a two-tailed 5% type I error rate and 80% power, the required sample size per group to detect a difference of 25% between the mean scores of two samples was 1 but a minimum 3 samples would be necessary for statistical analysis. Considering the risk of specimen damage during handling and processing, at least 5 gingival samples per group were targeted.

3.4 Data collection

3.4.1 Medical and Socio-demographic profile

Socio-demographic profile (age, gender and ethnicity), medical history and habits of all participants were recorded using a self-administered questionnaire (Appendix E) prior to periodontal examination.

3.4.2 Clinical parameters

Clinical parameters including plaque score, bleeding score, probing pocket depth, gingival recession and clinical attachment level were recorded for all teeth (excluding the 8's). UNC 15 colour coded periodontal probe (Hu-Friedy, Chicago, USA) was used in all the measurements. All data were recorded in the form shown in Appendix E.

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

   VPI was recorded at 4 sites of each tooth (mesio-buccal, mid-buccal, disto-buccal and palatal/lingual surface) using dichotomous scoring system. The presence or absence of visible plaque was scored (0 = no visible plaque, 1 = visible plaque) without the use of disclosing tablet.

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

   GBI was based on recordings from all 4 tooth surfaces of all teeth (mesio-buccal, mid-buccal, disto-buccal and palatal/lingual surface) using dichotomous scoring system.

   0 = Bleeding absent

   1 = Bleeding present

iii. Probing pocket depth (PPD)

PPD was measured from the free gingival margin to the base of the pocket at 6 sites.

iv. Gingival recession (GR)

GR was measured from the free gingival margin to cemento-enamel junction (CEJ) at 6 sites. A positive value was assigned if the gingival margin was below the CEJ while a negative value was assigned if the gingival margin was above the CEJ (Ramfjord, 1959).

v. Clinical attachment level (CAL)

CAL was calculated by summing up PPD and GR.

3.4.3 Examiner training

Training was done to ensure the reliability of the results obtained. Both intra-examiner and inter-examiner alignment and assessment for PPD and GR scores were performed on 2 neutral participants who volunteered for this calibration exercise in August 2017. The intra-examiner calibration was performed on different clinical sessions (morning/afternoon) while the inter-examiner calibration was done against a senior supervisor in the Periodontology discipline of University of Malaya. A Kappa score of more than 0.8 was obtained for both intra-examiner and inter-examiner reliability (**Table 3.1**)

Table 3.1: Kappa coefficients of intra- and inter-examiner reliability of PPD and CAL

Variables	**Intra-examiner reliability**			**Inter-examiner reliability**		
	Kappa value	Error	*P* value	Kappa value	Error	*P* value
PPD (N = 336)	0.983	0.017	< 0.001	0.935	0.032	< 0.001
CAL (N = 336)	0.983	0.016	< 0.001	0.887	0.041	< 0.001

CAL: clinical attachment level; PPD: probing pocket depth; N: total number of sites

3.4.4 Gingival biopsy collection

A total of 15 gingival samples (measuring 5 X 5 X 1 mm) were taken, five each from healthy control (HC), $PD^+ RA^-$, $PD^+ RA^+$ participants during surgical procedures such as minor oral surgery of third molars, crown lengthening and periodontal flap surgery at Postgraduate Periodontology Clinic, Faculty of Dentistry, University of Malaya (**Figure 3.1**). All participants diagnosed with PD were subjected to non-surgical periodontal therapy prior to periodontal surgery. Periodontal flap surgery was performed when there were persisting deep pockets exceeding 5 mm with bleeding on probing. Dental extraction was indicated for hopeless teeth.

Figure 3.1: Sources of gingival samples

Out of 8 participants who met all the criteria and consented to periodontal treatment, 5 participants managed to complete the initial cause-related therapy as planned while the other 3 participants are still at the initial phase of therapy due to belated participation. Therefore, only 5 gingival samples were available for immunohistochemistry (IHC) study (**Figure 3.2**). Another 10 gender-matched gingival samples were obtained from healthy participants with and without PD who were attended to in the Outpatient Clinic and Periodontology Clinic to allow comparison of the relative abundance of anti-citrullinated and anti-carbamylated proteins. Finally, a total of 15 gingival samples were biopsied and divided into 3 groups: healthy control (HC), PD without RA ($PD^+ RA^-$) and PD with RA ($PD^+ RA^+$).

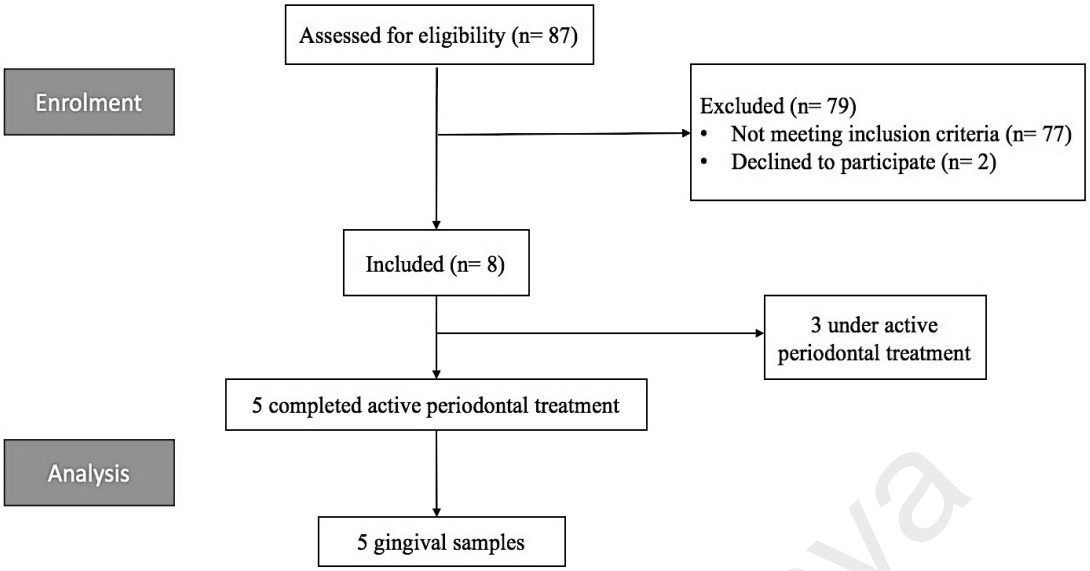

Figure 3.2: Flowchart for participant recruitment

3.5 Laboratory procedures

3.5.1 Fixation, processing, embedding and microtomy

Freshly cut gingival tissue was fixed in 10% neutral buffered formalin, pH 7.0 at room temperature for 6 hours. Following fixation, specimen was placed in a labelled cassette before being transferred to the automated tissue processor (PELORIS Rapid Tissue Processor, LEICA MICROSYSTEMS, USA) for dehydration, clearing and wax infiltration. When the specimen was thoroughly infiltrated with wax, it was embedded using a mould filled with molten wax. The cassette was placed on top of the mould and filled with more wax before putting on a cold plate to solidify. The mould was then detached from the cassette and the paraffin block was ready for microtomy. The paraffin block was cooled on the cold plate for 5 minutes prior to microtomy. After trimming the block at 10 μm to expose the tissue down to a level where a representative section could be obtained, a thickness of 4 μm section was cut using a slow, uniform cutting stroke. The section was placed onto the water surface (temperature 39℃) and left to flatten before mounting on the slides. Silane-coated slides (25 mm x 75 mm) were used for IHC staining while normal microscope plain slides (25 mm x 75 mm) were used for hematoxylin and eosin (H&E) staining. Slides were drained to remove excess water and dried in the oven (37℃) overnight.

3.5.2 Hematoxylin and eosin (H&E) staining

Histological staining with H&E was performed for the first section from each sample for histological assessment. Only samples with intact surface epithelium and sufficient underlying connective tissue were going to be used for IHC. Slides were incubated at 60℃ for 1 hour. Tissue sections were deparaffinised in 2 changes of xylene, 5 minutes each, followed by rehydration in absolute alcohol for 3 minutes, 95% alcohol for 3 minutes and 70% alcohol for 2 minutes. Slides were washed in running tap water for 3 minutes and stained in Harris hematoxylin for 5 minutes. Subsequently, slides

were immersed in 0.5% acid alcohol for 10 seconds to remove excess hematoxylin staining, followed by washing under running tap water for 3 minutes. Bluing was done by dipping the slides in 2% sodium acetate for 4 times, followed by washing under running tap water for 3 minutes. Slides were rinsed in 80% alcohol for 1 minute before being counterstained in eosin-phloxine solution for 2 minutes. After counterstaining, slides were dehydrated through 2 changes of 95% alcohol and absolute alcohol, 2 minutes each. Slides were then immersed in 3 changes of xylene for clearing, 3 minutes each. Mounting was done with xylene based mounting medium (Cytoseal 60, Thermo Scientific, US). Slides were ready for microscopic examination.

3.5.3 Immunohistochemical (IHC) staining

IHC staining procedure was performed according to the following protocol:

i. Slide preparation

The slides were incubated at 37℃ overnight. On the day of IHC procedure, the slides were transferred to a 60℃ oven and incubated for 1 hour prior to IHC staining.

ii. Rehydration

The slides were placed on hot plate for 15 minutes to melt the wax. The molten wax was dissolved in 2 changes of xylene, 5 minutes each. The slides were rehydrated in absolute alcohol, 95% alcohol and 70% alcohol for 3 minutes each. After rehydration, the slides were washed in running tap water for 3 minutes.

iii. Antigen retrieval

Rehydrated slides were placed into a microwave vessel filled with sufficient amount of antigen retrieval solution (citrate buffer pH 6.0) to cover the tissue sample. The slides were heated in the solution at the power setting of 40 P and temperature of 98℃ for 20 minutes in the microwave (LabPulse Staining Laboratory Microwave, EBSciences, LP2850P). Attention was given to the level of the solution from time to time to avoid drying of the slides. After the heating process, the microwave vessel was transferred to

a water bath. The slides were left to cool down in the retrieval solution for 20 minutes. As the slides were cooled, they were submerged in distilled water for 5 minutes to prepare for the blocking step.

iv. Blocking

The slides were removed from the distilled water and excess water was blotted off carefully to avoid contact with the tissue sample. The slides were placed on the staining tray and added with 2.5% normal goat serum (Vectorlabs, USA). Incubation of the slides was done at room temperature for 20 minutes. Slides were washed with freshly prepared Tris-buffered saline (1M, pH 7.6) twice, 1 minute each.

v. Primary antibody

Primary antibodies, rabbit polyclonal Citrulline antibody (ab6464 Abcam, UK) and rabbit anti-carbamyl-lysine polyclonal antibody (STA-078 Cell Biolabs, USA) were diluted according to the concentration predetermined through optimisation. The dilutions were 1:300 and 1:600 for rabbit polyclonal Citrulline antibody and rabbit anti-carbamyl-lysine polyclonal antibody respectively. Fifty µl primary antibody was pipetted to each sample and left to incubate for 1 hour at room temperature. For positive control, human breast carcinoma tissue and perineal skin tissue were used to optimise the antibody for citrullinated and carbamylated proteins respectively. For negative reagent control, primary antibody was omitted and substituted with antibody diluent (S2022, Dako REAL, Agilent, USA). After 1 hour of incubation, slides were submerged into Tris-buffered saline (1M, pH 7.6) twice, 1 minute each for washing.

vi. Endogenous blocking

One hundred µl of 3% hydrogen peroxide (Dako REAL ready-to-use, Agilent, CA, USA) was pipetted to each tissue sample and left for 15 minutes at room temperature to quench endogenous enzyme activity. This step was crucial to reduce background noise

in IHC. The slides were then submerged into Tris-buffered saline (1M, pH 7.6) twice, 1 minute each.

vii. Secondary antibody

Excess washing buffer was wiped off. The slides were returned to the staining tray. A drop of universal secondary antibody (ImmPRESS HRP anti-rabbit IgG (Peroxidase) polymer detection kit, made in goat, Vectorlabs, USA) was applied to each slide, enough to cover the entire tissue sample. The slides were left to incubate at room temperature for 30 minutes. After incubating with secondary antibody, the slides were washed in Tris-buffered saline (1M, pH 7.6) twice, 1 minute each.

viii. Antibody detection and counterstaining

DAB solution was prepared by mixing an equal volume of reagent 1 with reagent 2 (ImmPACT DAB EqV peroxidase substrate SK-4103, Vectorlabs, USA). Fifty µl DAB solution was pipetted to each tissue sample and left to incubate for 3 minutes or until optimum brown colour developed. The DAB reaction was stopped by submerging the slides into distilled water for 5 minutes. Subsequently, counterstaining was carried out by submerging the slides into Harris hematoxylin for 30 seconds, followed by washing in distilled water for 1 minute.

ix. Dehydration

During dehydration, the slides were immersed in 70% alcohol, 95% alcohol and absolute alcohol for 1 minute each. Immediately after that, the slides were cleared in 2 changes of xylene, 1 minute each. A drop of mounting medium (Cytoseal 60, Thermo Scientific, US) was applied on the dehydrated tissue sample. The cover slip was angled over the sample with one edge touching the slide and gently lowered to cover the sample. Care was taken to avoid air bubbles from being trapped under the cover slip. The slides were ready for viewing. Note: The protocols for buffer preparation: citrate buffer (pH 6.0) and Tris-buffered saline (1M, pH 7.6) are listed in Appendix A.

3.6 Immunohistochemical scoring

Semi-quantitative scoring evaluation was performed on all stained slides using Olympus dotSlide virtual slide system VS110 (Olympus, Tokyo, Japan) by two observers. Three regions of interest (ROI) of equal size were divided into coronal, middle and apical sections of the specimen and the number of all positively stained cells within oral epithelium and gingival connective tissues were counted manually. The number of positively stained cells from each ROI was summed up for individual observer to get a single value for each sample. The average of two single values from each observer was considered the mean total number of positively stained cells in each sample which was then expressed in percentage of positively stained cells per ROI.

Two observers were calibrated prior to the semi-quantitative scoring analysis. Intra- and inter-observer reliability measurements were done on 10 IHC stained slides on two different occasions (1 week apart). A kappa score of more than 0.8 was obtained for both intra-observer and inter-observer reliability measurements, indicating a high level of agreement (**Table 3.4**).

Table 3.2: Kappa coefficients for intra- and inter-observer reliability of SQA analysis

Variables	Intra-observer reliability			Inter-observer reliability		
	Kappa value	Error	*P* value	Kappa value	Error	*P* value
Number of cells (N = 10)	0.886	0.041	< 0.001	0.883	0.042	< 0.001
Intensity of staining (N = 10)	0.934	0.032	< 0.001	0.918	0.035	< 0.001

SQA: semi-quantitative analysis; N = total number of slides

3.7 Statistical analysis

Data were statistically analysed using SPSS for windows (IBM SPSS Statistics 25.0, Armonk, NY, USA: IBM Corp). Counts and percentages summarised the categorical data and Fisher's exact test was used to compare percentages in different groups. Descriptive statistics were used for mean and standard deviation of data. The distribution of numerical variables was assessed using Shapiro-Wilk test to determine if the data were normally distributed. Parametric test (One-way ANOVA) with a 95% confidence interval was used to compare the mean differences in age, full mouth plaque score, clinical attachment level and citrullinated protein semi-quantitative score of different groups and this was followed by a post hoc analysis (Tukey) when necessary. Kruskal-Wallis test was used to evaluate the mean differences in full mouth bleeding score, probing pocket depth and carbamylated protein semi-quantitative score of different groups, also followed by post-hoc comparison (Dunnett T3) when indicated. The level of statistical significance was set at $p \leq 0.05$.

Chapter 4: RESULTS

4.1 Demographic and clinical data of study participants

The study population consisted of 15 participants (5 healthy controls, 5 with periodontitis only and 5 with both periodontitis and rheumatoid arthritis). All participants were gender-matched and non-smokers. **Table 4.1** summarizes the characteristics of the study population. In this study, 60% of the sample population were women and 40% were men. Their ages ranged from 31 to 60 years old. There was no significant mean age difference and ethnicity distribution between groups. Clinically, the $PD^+ RA^+$ group had higher baseline full mouth plaque scores and bleeding scores than $PD^+ RA^-$ and HC groups. There was no significant difference between $PD^+ RA^-$ and HC groups in terms of mean plaque scores but $PD^+ RA^-$ group had higher bleeding scores than HC group. $PD^+ RA^+$ group and $PD^+ RA^-$ group had significantly higher PPD and CAL than HC group but these two parameters were not significantly different between $PD^+ RA^+$ and $PD^+ RA^-$ groups.

Table 4.1: Participant socio-demographic and clinical characteristics

Variables	HC n=5	$PD^+ RA^-$ n=5	$PD^+ RA^+$ n=5	*p*-value
Age (years)	43 (±12.5)	44.8 (±9.7)	52 (±7.2)	0.219[a]
Gender				
Female, n (%)	3 (60)	3 (60)	3 (60)	> 0.999[f]
Male, n (%)	2 (40)	2 (40)	2 (60)	
Ethnicity				
Malay, n (%)	1 (20)	0 (0)	1 (20)	0.434[f]
Chinese, n (%)	4 (80)	4 (80)	2 (40)	
Indian, n (%)	0 (0)	1 (20)	2 (40)	
FMPS (%)*	25.2	23.3	42.5	0.002[a]
FMBS (%) **	11.5	18.1	42.2	0.007[k]
PPD (mm)**	2.11 (±0.68)	3.48 (±1.64)	3.22 (±1.57)	0.007[k]
CAL (mm)*	0.16 (±0.71)	4.34 (±2.24)	3.70 (±1.94)	< 0.001[a]

HC: healthy controls; $PD^+ RA^-$: periodontitis only; $PD^+ RA^+$: periodontitis with rheumatoid arthritis; FMPS: full mouth plaque score; FMBS: full mouth bleeding score; PPD: probing pocket depth; CAL: clinical attachment level.

[a] Differences tested for significance using One-way Anova, $p < 0.05$

[k] Differences tested for significance using Kruskal-Wallis, $p < 0.05$

[f] Differences tested for significance using Fisher's exact test, $p < 0.05$

*Post-hoc analysis (Tukey's) for

FMPS showed significant difference between $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.002$) and between HC and $PD^+ RA^+$ ($p = 0.005$) but no significant difference between HC and $PD^+ RA^-$ ($p = 0.905$).

CAL showed significant difference between HC and $PD^+ RA^-$ ($p < 0.001$) and between HC and $PD^+ RA^+$ ($p < 0.001$) but no significant difference between $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.315$).

**Post-hoc analysis (Dunnett T3) for

FMBS showed significant differences between all groups, HC and $PD^+ RA^+$ ($p = 0.019$), $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.015$), HC and $PD^+ RA^-$ ($p = 0.048$).

PPD showed significant difference between HC and $PD^+ RA^-$ ($p = 0.006$) and between HC and $PD^+ RA^+$ ($p = 0.001$) but no significant difference between $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.525$).

4.2 Routine histological examination

All the gingival tissue samples (5 X 5 X 1 mm) were sectioned for initial histologic assessment by an oral pathologist who was masked to the status of the participants to determine the adequacy in terms of specimen size and cell morphology prior to immunohistochemical staining. Hematoxylin and eosin (H&E) sections showed histologic features consistent with the clinical diagnosis of periodontitis and non-periodontitis. A marked increase in inflammatory cell infiltration was demonstrated in gingival connective tissues from PD^+ RA^- compared to healthy control. Similarly, the inflammatory cell infiltration in the gingival connective tissues of PD^+ RA^+ was heavy as compared to the presence of scarce inflammatory cells in periodontally healthy gingival connective tissues. Representative H&E sections of gingival tissues from each group of participants are shown in **Figure 4.1 a-f**.

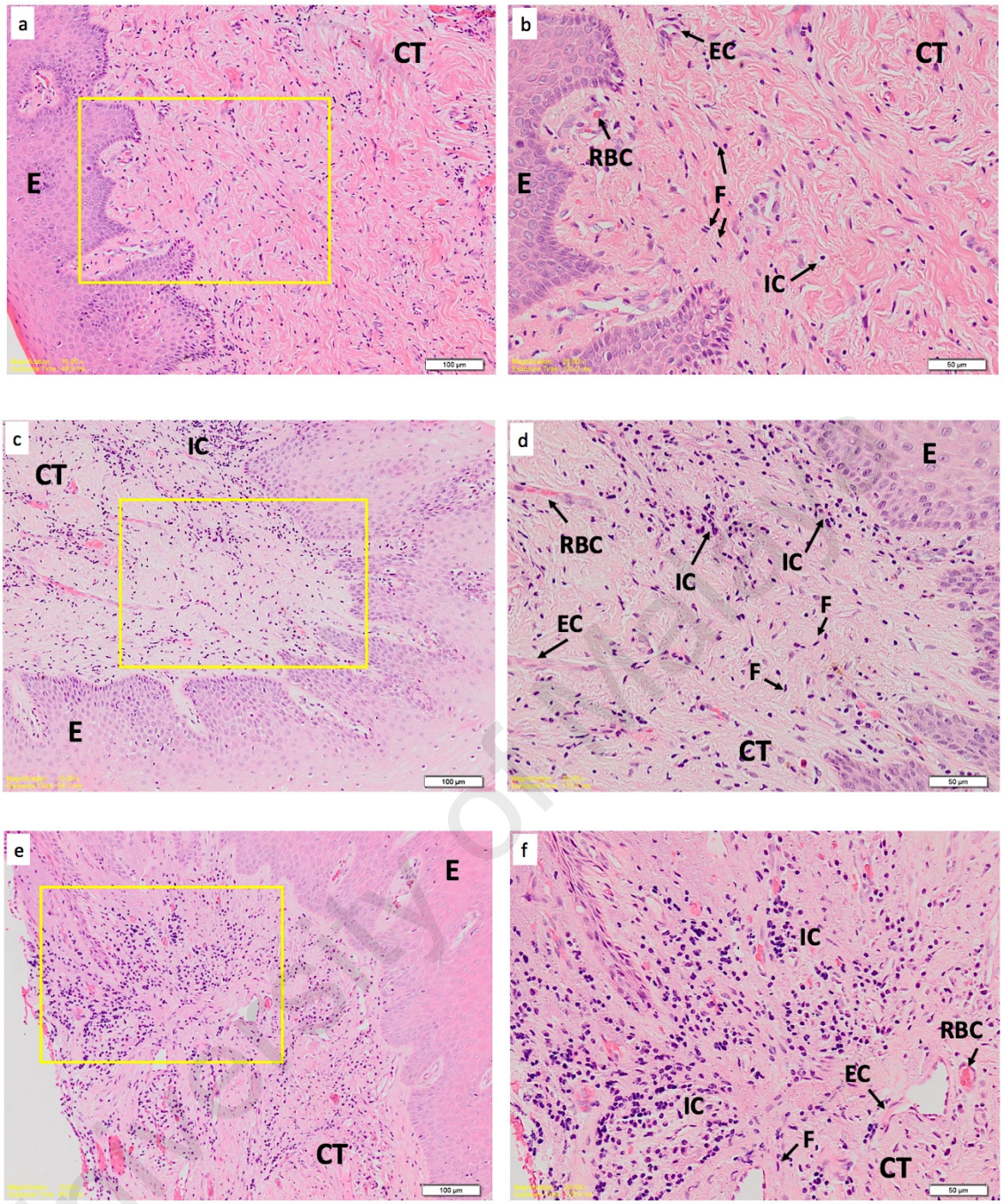

Figure 4.1

Photomicrograph showing H&E staining of gingival tissue sections (**a** & **b)** HC showing features of clinically healthy gingiva, characterised by collagen-dense connective tissue with little inflammatory cell infiltration. (**c** & **d)** PD^+ RA^- showing increased area of inflammatory cell infiltration and reduced density of collagen. (**e** & **f)** PD^+ RA^+ showing heavy inflammatory cell infiltration subjacent to oral epithelium.
(**a**, **c** & **e**. Magnification x 100; **b**, **d** &**f**. Magnification x 200). *E epithelium, CT connective tissue, F fibroblast, EC endothelial cell, IC inflammatory cell, RBC red blood cell.*

4.3 Presence of citrullinated proteins in gingival tissues

Brown staining of citrullinated proteins was observed in all gingival connective tissue and epithelium irrespective of disease status. However, staining increased markedly in tissues with periodontitis (both $PD^+ RA^-$ and $PD^+ RA^+$), along with the staining intensity. In healthy controls, citrullinated proteins were detected mainly in fibroblasts and epithelium (**Figure 4.2 a, b**). In $PD^+ RA^-$, citrullinated proteins were detected in inflammatory cells, endothelial cells, fibroblasts and epithelium (**Figure 4.2 c, d**). Similar pattern of staining was seen in $PD^+ RA^+$ (**Figure 4.2 e, f**). Human breast carcinoma tissue was used as positive and negative controls for citrullinated proteins (**Figure 4.3**).

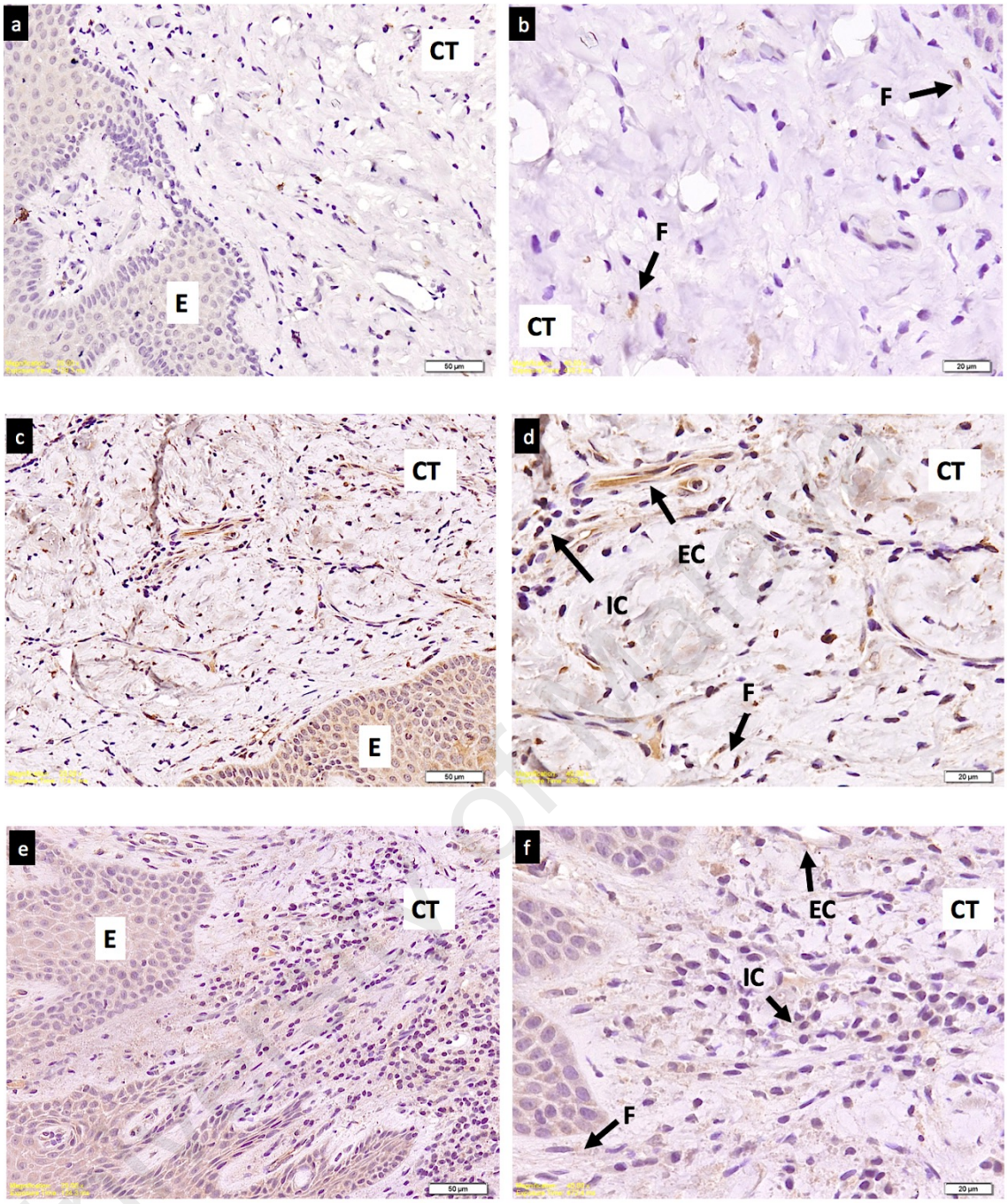

Figure 4.2

Immunohistochemical staining of gingival tissue from HC, $PD^+ RA^-$ & $PD^+ RA^+$ (**a** & **b)** HC, showing low expression of citrullinated proteins (arrows) within connective tissue and epithelium. (**c** & **d)** $PD^+ RA^-$, showing increased expression of citrullinated proteins within connective tissue and strong intensity in epithelium. (**e** & **f)** $PD^+ RA^+$, showing increased expression of citrullinated proteins within connective tissue compared to HC. Note the dense infiltration of inflammatory cells in connective tissue. (**a, c & e** Magnification x 200. **b, d & f** Magnification x 400). *E epithelium, CT connective tissue, F fibroblast, IC inflammatory cell, EC endothelial cell.*

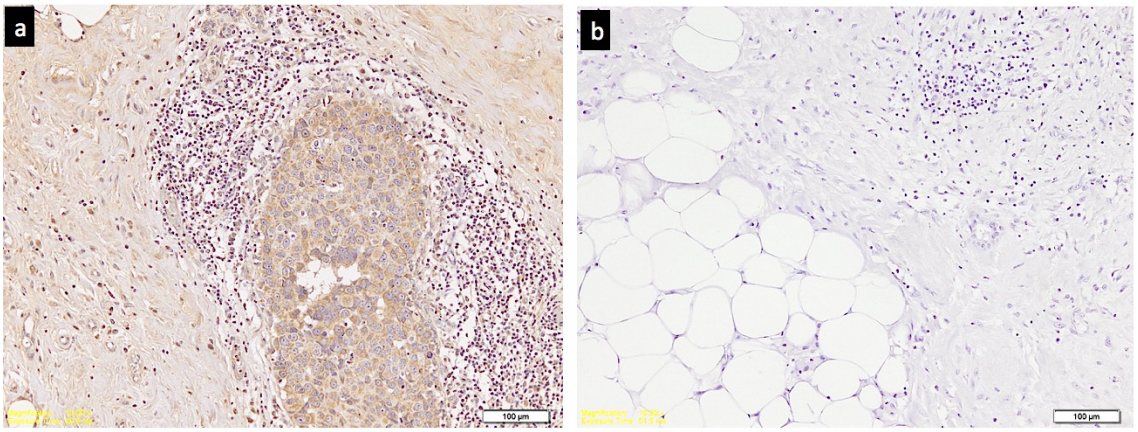

Figure 4.3

Immunohistochemical staining of breast carcinoma tissue used as positive and negative controls for citrullinated proteins. (**a)**. positive control (dilution 1: 300) (**b)**. negative control. Magnification x 100.

4.4 Presence of carbamylated proteins in gingival tissues

Brown staining of carbamylated proteins was prevalent and observed in all gingival connective tissue and epithelium irrespective of disease status. Carbamylated proteins were detected mainly in fibroblasts, inflammatory cells, endothelial cells and epithelium with increasing intensity from HC to PD^+ RA^- (**Figure 4.4**). However, there was no significant difference in staining intensity between PD^+ RA^- and PD^+ RA^+ groups, suggesting carbamylation in gingival tissue is inflammation dependent rather than RA-related. Human perineal skin was used as positive and negative controls for carbamylated proteins (**Figure 4.5**).

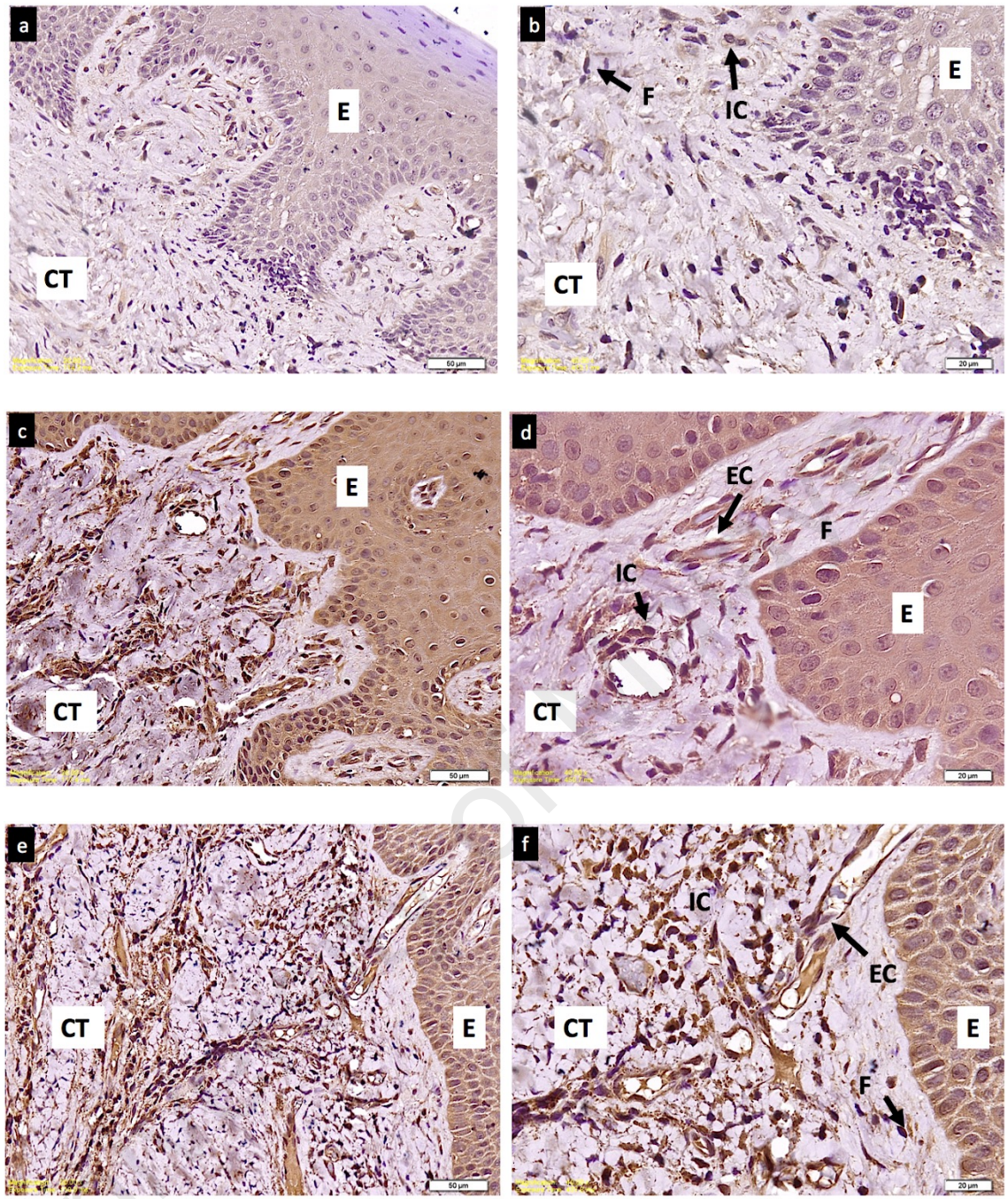

Figure 4.4

Immunohistochemical staining of gingival tissue obtained from HC, $PD^+ RA^-$ & $PD^+ RA^+$. **(a & b)** HC, showing the presence of carbamylated proteins within connective tissue and epithelium. **(c & d)** $PD^+ RA^-$, showing increased expression and intensity of carbamylated proteins in connective tissue and epithelium. **(e & f)** $PD^+ RA^+$, also showing increased expression and intensity of carbamylated proteins in connective tissue and epithelium with no marked difference from $PD^+ RA^-$. **(a, c & e** Magnification x 200. **b, d & f** Magnification x 400). *E epithelium, CT connective tissue, F fibroblast, IC inflammatory cell, EC endothelial cell.*

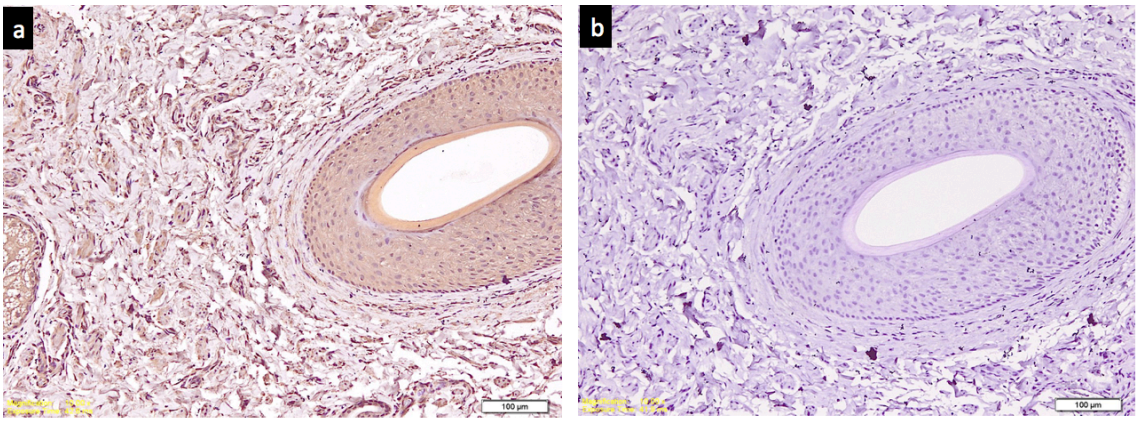

Figure 4.5

Immunohistochemical staining of perineal skin tissue used as positive and negative controls for carbamylated proteins. (**a**). positive control (dilution 1:600) (**b**). negative control. Magnification x 100.

4.5 Semi-quantitative analysis (SQA) for citrullinated and carbamylated proteins in gingival tissues

SQA was performed to determine the proportion of positively-stained cells within gingival connective tissues based on the total number of positively stained cells per region of interest (ROI). Mean values were obtained for each group and expressed in percentage. Overall, PD^+ RA^+ group scored the highest for citrullinated and carbamylated proteins, followed by PD^+ RA^- and HC (**Figure 4.6**). PD^+ RA^- and PD^+ RA^+ groups showed higher mean scores than healthy controls for both citrullinated and carbamylated proteins which were statistically significant. However, there was no statistically significant difference between PD^+ RA^- and PD^+ RA^+ groups for both protein markers. **Table 4.2** summarizes the mean scores for each group.

Table 4.2: Semi-quantitative analysis of immunostaining in gingival tissues for citrullinated and carbamylated proteins

Variables		HC	$PD^+ RA^-$	$PD^+ RA^+$	*p*-value
Citrullinated proteins	Positively stained cells (n/total)	82/2055	686/2845	860/2992	
	Mean Score (%)*	4.00 (±1.94)	24.10 (±2.56)	28.74 (±3.95)	$< 0.001^a$
Carbamylated proteins	Positively stained cells (n/total)	1198/1986	2380/2563	2734/2860	
	Mean Score (%)**	60.32 (±3.87)	92.90 (±4.77)	95.60 (±1.71)	$< 0.001^k$

[a] Differences tested for significance using One-way Anova.
[k] Differences tested for significance using Kruskal-Wallis.
*Post-hoc analysis (Tukey) showed significant difference between $PD^+ RA^-$ and HC ($p < 0.001$), $PD^+ RA^+$ and HC ($p < 0.001$) but no significant difference between $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.067$).
**Post-hoc analysis (Dunnett T3) showed significant difference between $PD^+ RA^-$ and HC ($p < 0.001$), $PD^+ RA^+$ and HC ($p < 0.001$) but no significant difference between $PD^+ RA^-$ and $PD^+ RA^+$ ($p = 0.591$).

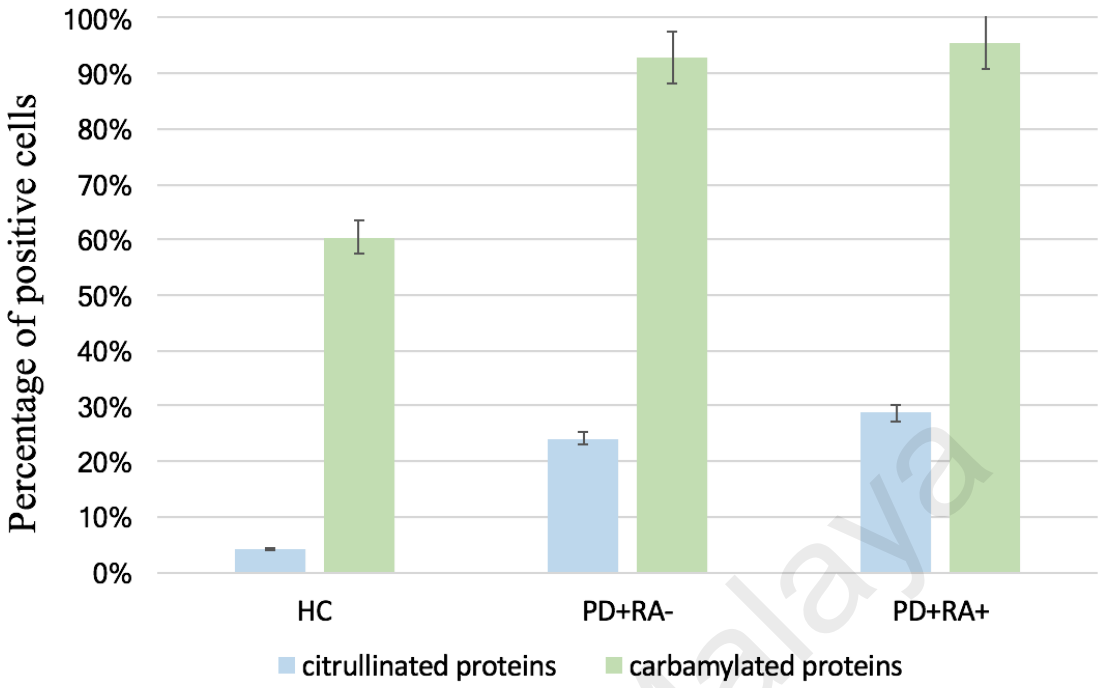

Figure 4.6

Semi-quantitative analysis (SQA) of positively-stained cells for citrullinated and carbamylated proteins in gingival tissue samples from HC, $PD^+ RA^-$ and $PD^+ RA^-$. Data are expressed as mean percentage ± standard deviation of the mean.

Chapter 5: DISCUSSION

The present study detected the presence of citrullinated and carbamylated proteins in the gingival tissues of periodontally healthy participants (HC) and PD participants with and without RA (PD^+ RA^- and PD^+ RA^+). To the best of our knowledge, this is the first study to attempt the identification of citrullinated and carbamylated proteins in the gingival tissues using IHC, and also the first study comparing the expression of these two protein markers in the presence of PD and RA.

5.1 Part 1: Discussion on materials and methods

5.1.1 Case definition

The importance of case definition in periodontal research cannot be emphasised more as it reflects a precise description of a disease under investigation. A standard definition of a case will ease data interpretation and comparison between studies, be it epidemiological or clinical research (Preshaw, 2009). However, this issue is yet to be resolved in periodontal research whereby various threshold values are employed to define a case across studies. The present study defined a periodontitis case based on the criteria proposed by Armitage (1999) mainly because it had been routinely used for clinical diagnosis in the department (Armitage, 1999). All participants with moderate to severe PD underwent a comprehensive periodontal assessment prior to their periodontal therapy and thus, the 1999 classification seemed more appropriate for diagnosis purpose.

All periodontitis cases included were moderate to severe form as they made up the majority of the patients seen in the Periodontology department. One of the challenges in measuring clinical attachment level (CAL) is to accurately identify the cemento-enamel junction (CEJ) when the gingival margin is more coronally positioned. Very often, the gingival margin is mistakenly charted as the CEJ level when it is not. As a result, CAL can be overestimated or underestimated depending on where the CEJ lies

and an error of 2.5 mm has been reported by the European taskforce (Tonetti & Claffey, 2005). Therefore, to avoid common errors resulting from misclassifying non-cases as mild periodontitis due to reduced periodontium and the difficulty in determining CEJ in the presence of pseudo pockets, CAL ≥ 3 mm is a more sensitive threshold to identify true cases. Based on the 1999 classification, CAL ≥ 3 mm is considered moderate and severe periodontitis (Armitage, 1999) or Stage 3 and 4 according to the new classification (Caton et al., 2018). In this study, non-periodontitis cases were restricted to those with no sign of clinical attachment loss and radiographic bone loss. Based on this definition, gingivitis at a patient level was not an exclusion criterion but gingivitis site was excluded from gingival biopsy, for clinically healthy periodontium was necessary to serve as a control. This was further confirmed with the histological examination.

5.1.2 Immunohistochemistry

Immunohistochemistry (IHC) is a method used to determine the expression of antigens in tissue sections using antibodies. This antigen-antibody interaction is highly specific and therefore, a valuable diagnostic, prognostic and research tool in histopathology. There are several methods available for tissue immunostaining but IHC labelling with DAB is one of the most commonly utilised IHC staining methods. It is also the most frequently used staining approach in the university laboratory for both diagnostic and research purposes. IHC with fluorescence, also known as immunofluorescence (IF) is gaining popularity in translational research due to its ability to enable multiple labelling or co-localisation of multiple antigens in one sample which can be difficult using the conventional IHC (Robertson et al., 2008). Although the idea of having double staining in one tissue section may seem attractive, extra reagents such as nonimmune serum and fluorescent dye will be needed for IF which implies added cost to the project. Given the objective of this study was to localise citrullinated and

carbamylated proteins in the gingiva tissues which could be achieved with the conventional method, it was not cost effective to apply a more expensive, albeit sophisticated method to obtain a similar outcome in terms of staining quality (Vega-Damián et al., 2016). The decision between IHC and IF was also based on the concern that one of the commercially developed antibodies (rabbit polyclonal anti-carbamyl-lysine) had not been tested then for IF and there is evidence showing the detection rate of IF can be affected by unsuitable primary antibody (Qi et al., 2017).

5.1.3 Fixation technique

Optimum fixation is critical to immobilise the proteins within the tissue in order to preserve their immunoreactivity. It is a good start for successful IHC. Perfusion and freezing methods are two commonly used fixation methods in IHC. Both methods have their own pros and cons. In this study, perfusion method with 10% neutral-buffered formalin was chosen for two reasons: 1. preservation of tissue histomorphology. 2. technical and storage ease. Formalin-fixed paraffin-embedded (FFPE) tissue is superior in retaining tissue morphology for histological examination (Paavilainen et al., 2010) while fresh frozen tissue is prone to artefacts due to thaw-and-refreeze cycles (Desciak & Maloney, 2000). The ease of handling FFPE samples is an important intraoperative consideration as the tissue can be fixed rapidly through immersion in the formalin without introducing any interruption to the surgery. On the contrary, time-out during surgery for snap freezing is inevitable if fresh frozen sample is desired. From clinical perspective, it is impractical and time-consuming.

5.1.4 Optimisation

In IHC, the quality of immunostaining depends on the application of an optimal protocol that has to be determined empirically, starting from tissue processing, antigen retrieval, antibody dilution, incubation time through counterstaining (Blaisdell, 2014). In the present study, tissue processing was done with an automated tissue processor while the remaining steps were manually performed. One of the essential steps in IHC of FFPE tissues is antigen retrieval (AR). Enzyme digestion was traditionally the primary method used to unmask the epitopes but the drawback was risk of tissue damage resulting from over-digestion (D'Amico et al., 2009; Shi et al., 2007). It was later on discovered that the cross-linkages formed between formalin and proteins could be disrupted at high temperature without compromising the tissue morphology (Shi et al., 1991). Therefore, heat-induced AR pre-treatment has now become a routine practice of IHC in the laboratory. The procedure was made easy in this study with the use of a laboratory grade microwave oven (LabPulse LP1850-120, EBSciences, USA) in which even heat distribution could be ensured compared to a domestic microwave. Apart from AR protocol, the optimisation of antibody dilution and incubation duration was the most tedious step in IHC. Different antibody dilutions ranged from 1: 100 to 1:1500 were tested at various durations (overnight, 1 hour and 30 minutes) in order to obtain an optimum staining quality. One of the setbacks that commonly occurred during the laboratory procedures was the detachment of tissue from the slides. This was probably caused by a stronger adherence force between the tissue and the paraffin than the bond strength between the tissue and the silane-coated glass slide that resulted in tissue being detached during clearing with xylene. This problem was then overcome by melting the wax on the hot plate for 15,, minutes prior to dewaxing and rehydration.

5.1.5 Semi-quantitative analysis

Absolute quantification is a challenge in IHC due to the variability in the staining quality and subjectivity in interpreting the severity and extent of the lesion as well as the intensity of the staining (Renshaw & Gould, 2007). As a result, semi-quantitative analysis (SQA) is still widely used in histopathologic research based on an ordinal scoring system. Ideally, a standard, validated scoring system should be implemented to allow for meaningful comparisons between studies. However, there is still a lack of uniformity in the scoring system in IHC as it is difficult to establish a universal approach that is able to answer all the scientific questions in pre-clinical studies (Fedchenko & Reifenrath, 2014). Consequently, various scoring systems have been developed or customised to studies' requirements. The present study adopted the scoring system used in previous studies which also investigated the presence of citrullinated proteins in the gingival tissues (Janssen et al., 2017; Nesse at al., 2012). There is limited literature in this area and most of the studies did not describe their scoring systems. To minimise subjectivity in scoring, two calibrated observers performed the scoring and only the mean scores for each sample were used for statistical analysis (Obuchowski, 2004).

Another issue arises from histopathologic analysis is that using a two-dimensional section to measure a three-dimensional structure may influence the accuracy of the interpretation. This can still happen even if the calculation is done with a digital image analysis despite the fact that computer-aided analysis has higher reproducibility than manual scoring (Riber-Hansen et al., 2012). A more sophisticated method such as stereological analysis may resolve this shortcoming but it was not available in the laboratory. Due to the small sample size in this study, it was less laborious and less expensive to do the scoring manually.

5.2 Part 2: Discussion on results

5.2.1 Sample characteristics

Clinically, the periodontal status of both PD^+ RA^- and PD^+ RA^+ groups was reflected in their parameters with significantly higher full mouth bleeding tendency, PPD and CAL compared to healthy control. However, the full mouth bleeding scores and plaque scores of PD^+ RA^- participants were significantly lower than PD^+ RA^+ participants mainly because the PD^+ RA^- participants were the existing patients in Periodontology Department who had received oral hygiene instructions and undergone non-surgical periodontal therapy prior to the enrolment. Hence, the clinical parameters measured during enrolment were used as the baseline measurements to represent the disease status of the participants at that time. PD^+ RA^+ participants, on the other hand, had not received periodontal treatment previously during recruitment and thus, demonstrated the highest plaque scores and bleeding scores among all groups. This discrepancy would not affect the results as gingival biopsies were only done at the diseased tissues which provided site-specific characteristic. Besides, it was not the aim of this study to investigate the effect of periodontal treatment on protein markers in the gingival tissues.

5.2.2 Histopathological features

The microscopic examination of the haematoxylin and eosin (H&E) sections demonstrated two distinctive histopathological features between gingival tissues from healthy control and gingival tissues with periodontitis, irrespective of the systemic status. The gingival biopsies from the healthy periodontium composed of oral epithelium and connective tissue. Other microscopic features observed included rete pegs projecting from oral epithelium into the connective tissue, dense collagen fibres with the spindle-shaped fibroblasts residing in between and sub-epithelial vessels within the connective tissue which are typical of normal gingiva (Lindhe et al., 2008). Type I

collagen is the main composition of the gingival connective tissue (Chavrier et al., 1984). Healthy gingival tissue was harvested from the coronal portion of the dento-alveolar gingival unit during surgical removal of third molar or crown lengthening surgery. Despite the absence of clinical sign of inflammation such as bleeding and erythema, inflammatory cells were observed histologically. This is in agreement with the histopathologic findings by Page & Schroeder (1976) and Kinane (2001), whereby inflammatory infiltrate is a common histological feature in clinically healthy gingiva. The inflammatory cells are predominantly polymorphonuclear leukocytes (PMNs) or neutrophils and lymphocytes. Under physiologic condition, about 1% to 2% of all neutrophils exit from the vessels of the gingival plexus through the junctional epithelium, into the gingival sulcus (Ryder, 1980). This trans-endothelial emigration of neutrophils forms the first line of the defence to maintain gingival health through phagocytosis of invading microorganisms and thus, preventing the extension of subgingival biofilm (Tonetti et al., 1998).

The gingival biopsies from periodontitis lesions of both $PD^+ RA^-$ and $PD^+ RA^+$ participants were characterised by an increased infiltration of inflammatory cells in the connective tissue. The vascular changes were prominent with the proliferation of capillaries compared to the healthy gingiva. Almost one third of the connective tissue was occupied by the inflammatory infiltrate in most of the histological sections. The content of collagen was markedly reduced due to inflammation-induced collagenolytic activity. Two mechanisms are involved in the collagen loss. One is driven by PMNs during the bactericidal activity. The lysosomal enzymes secreted by PMNs can revert back to into the tissues and cause local tissue destruction (Scott & Krauss, 2012). Another mechanism of collagen loss is induced by resident fibroblasts. The function of fibroblasts is altered during inflammation. When stimulated by inflammatory cytokines, fibroblasts secrete collagenases and MMPs that cleave collagen fibrils into small

peptides which are then phagocytosed by the surrounding fibroblasts (Bhide et al., 2005). The histopathological features in the gingival tissues were closely similar in both $PD^+ RA^-$ and $PD^+ RA^+$ groups, which resembled the established and advanced lesions depicted in Page & Schroeder (1976). This observation confirms that the histopathology of periodontitis is not modified by the presence of RA. Similar histopathologic findings have also been reported in chronic and aggressive periodontitis despite different clinical presentation in extent and severity of the diseases (Johnson et al., 1980; Smith et al., 2010). Likewise, RA patients have been shown to have worse periodontal health in terms of gingival inflammation (Gingival Index 0.51 ± 0.43 vs. 0.16 ± 0.19, $p < 0.001$) and clinical attachment loss (3.25 ± 0.73 mm vs. 2.90 ± 0.51 mm, $p < 0.001$) than non-RA counterparts (Choi et al., 2016) but this variation was not reflected histologically in the present study. What can be hypothesised from this finding is that inflammation is the common feature shared by PD and RA.

5.2.3 Citrullinated proteins

The current study showed that citrullinated proteins were present in all gingival tissues with different patterns of distribution and staining intensities. In HC, a relatively weak positive staining was observed in oral epithelium and fibroblasts within gingival connective tissue but the proportion of positively-stained fibroblasts was very low (mean SQA score 4% ± 1.94, CI 95%), constituting less than 10% of the total cells per region of interest (ROI). However, the observation that all healthy control specimens were stained positively suggested a physiologic role for citrullination in normal gingiva. The presence of citrullinated proteins may not be entirely pathogenic as citrullination is commonly involved in many physiological processes in the body. Citrullinated keratin forms a protective barrier in the skin while in the immune cells especially neutrophils, citrullination is necessary for the formation of NETs (Baka et al., 2012; Wang et al., 2009). The physiological citrullination may be facilitated by neutrophils. Peptidyl-

arginine deiminase-2 (PAD2) and -4 (PAD4) can be expressed extracellularly by inactive neutrophils under normal conditions, which may promote extracellular protein citrullination (Zhou et al., 2017). In human oral epithelia, expression of keratin has been detected in the basal and suprabasal layers, in both health and disease (Mackenzie & Gao, 1993). Therefore, citrullination may play a role in epithelial differentiation. This explains the presence of citrullinated proteins at the epithelial layer in all gingival tissues regardless of the disease status. However, the intensity of the staining was relatively stronger in the epithelia of $PD^+ RA^-$ and $PD^+ RA^+$ gingiva tissues than of HC. This is probably due to the presence of inflammation as *in vitro* study has shown the staining of keratin increased in inflamed gingival tissue in contrast to normal gingiva but the underlying mechanism is poorly understood (Pritlove-Carson et al., 1997). Similar results were reported in other IHC studies that investigated the presence of citrullinated proteins in inflamed and non-inflamed gingival tissues (Engström et al., 2018; Nesse et al., 2012). Nesse and colleagues also found that while epithelium always stained positive for citrullinated polyclonal antibody, it was not the case for monoclonal antibody. It appeared that only periodontitis-affected epithelium was positively stained for F95, a specific peptidyl-citrulline which was also detected in the synovial tissue of RA patients (Nesse et al., 2012). It remains to be determined whether only a specific antigen is citrullinated during inflammation.

In both $PD^+ RA^-$ and $PD^+ RA^+$, citrullinated proteins were detected in fibroblasts, endothelial cells and inflammatory cells in gingival connective tissues. There was no statistically significant difference in the mean SQA scores between $PD^+ RA^-$ (24.1%±2.56, CI 95%) and $PD^+ RA^+$ (28.7%±3.95, CI 95%), indicating on average one third of the total cells per ROI were positively stained. The lack of clear distinction in the abundance of citrullinated proteins between these two groups coincided with the histological findings in which both groups of gingival tissues exhibited similar degree

of inflammation. A reasonable explanation for such findings is that increased expression of citrullinated proteins within the gingival tissues is inflammation-dependent rather than RA-related. This is in agreement with results of other studies that looked at the association between inflammation and expression of citrullinated proteins in the periodontal tissues (Harvey et al., 2013; Janssen et al., 2017; Nesse et al., 2012). Citrullinated histone H3, a component of NETs that is released by neutrophils as a defence mechanism against infection (Neeli et al., 2009) was detected in the inflamed periodontal tissues but not in any of the healthy tissues. This finding was matched with higher counts of neutrophils in inflamed tissues, implying that neutrophil-related cell death (NETosis) may be responsible for increasing the amount of citrullinated proteins in periodontitis (Janssen et al., 2017). As mentioned earlier, citrullination of histone H3 is essential for NETs formation and this process is catalysed by PAD4 which is expressed at high levels by neutrophils under inflammatory conditions (Darrah et al., 2012). Upon stimulation by bacterial lipopolysaccharide (LPS), neutrophils degranulate and release citrullinated histone H3 into the surrounding area. It has been speculated that citrullinated histone H3 can be released extracellularly into the circulation based on the finding that serum citrullinated histone H3 was detectable within 3 hours following LPS injection (Li et al., 2011). However, evidence from human trial showed that no association was found between serum citrullinated histone H3 level and periodontal status of RA patients (Janssen et al., 2017).

Neutrophils have garnered increasing attention as key players in the pathogenesis of RA due to their ability to generate autoantigens through citrullination (Darrah & Andrade, 2018). This is in contrast to the hypothesis that periodontal pathogen, specifically *P. gingivalis* and its unique enzyme, PPAD are responsible for citrullination in gingiva (Rosenstein et al., 2004). The speculation that citrullination is driven mainly by human PAD rather than PPAD, is favoured due to the consistent

findings that the expression of PAD2 and PAD4 is increased together with citrullinated proteins in inflamed gingiva (Engström et al., 2018; Harvey et al., 2013). Moreover, these studies failed to show any correlation between citrulline level and the presence of *P. gingivalis* and *A. actinomycetemcomitans*, suggesting that these two pathogens may not be necessary to trigger citrullination. A recent study in Japan did not find any association between anti-PPAD IgG levels and the severity of periodontitis, indicating *P. gingivalis* might not be present in all periodontitis cases (Kobayashi et al., 2016). This argument is plausible as periodontitis is polymicrobial in nature and *P. gingivalis* is considered a low-abundance biofilm species in active periodontitis (Kumar et al., 2006). It remains unclear to what extent a low number of *P. gingivalis* contributes to protein citrullination as compared to the highly abundant neutrophils present in inflamed gingiva. The local citrullinated protein production shown in the present study was likely influenced by the inflammatory status of the gingiva rather than the microbial composition or RA status. To validate this hypothesis, it may be worth investigating the presence of *P. gingivalis* in the plaque sample and relating the level of PPAD to citrullinated proteins within the gingiva in future studies.

Finding the right candidate autoantigen in RA has been a challenge as the autoantibodies of RA patients show reactivity for various citrullinated peptides, including citrullinated fibrinogen, type II collagen, vimentin and α-enolase (Goules et al., 2013). Although anti-citrullinated protein antibodies (ACPA) are highly specific for RA (98% specificity, 65% sensitivity), the presence of ACPA in healthy individuals is not uncommon (Schellekens et al., 2000). A large population-based study in the Netherlands reported a prevalence of 1% ACPA positive in the general population while only 22.4% of the seropositive participants had RA (van Zanten et al., 2017). It is not known if the remaining 77.6% are at risk of RA since ACPA can be present more than 10 years before the clinical onset of RA (Nielen et al., 2004). Or, it could simply mean

that the mere presence of ACPA in the circulation might not be pathogenic except for susceptible individuals. Autoantibodies are not all destructive. For instance, autoantibody IgG4 has important physiological role in maintaining immune homeostasis through phagocytosis of dead cells and debris during tissue degradation in periodontal disease (Nair et al., 2014). The antibody used in this study was polyclonal ACPA which bound to all citrulline residues so it was not possible to distinguish which type of citrullinated protein was detected.

5.2.4 Carbamylated proteins

Comparable to citrullinated proteins, carbamylated proteins were prevalent in the gingival tissues in all groups, confirming the results from an *in vivo* study that carbamylation is a naturally occurring mode of post-translational modification (PTM) (Pietrement et al., 2013). In healthy control, carbamylated proteins could be observed in oral epithelium and connective tissues. As opposed to citrullination, carbamylation is an enzyme-independent PTM that takes place during spontaneous urea dissociation, in which lysine is converted into homocitrulline (Shi et al., 2014). This happens at a slow rate under physiological conditions and carbamylated proteins have been shown to accumulate in the tissues until degradation (Balion et al., 1998). However, the turnover rate depends on the half-life of the protein being carbamylated. Type I collagen, being a long-lived and the most abundant extracellular matrix protein in the connective tissue and skin, is therefore more prone to carbamylation than other short-lived proteins (Jaisson et al., 2007; Pietrement et al., 2013). The persistence and accumulation of carbamylated collagen were reflected by profuse weak staining (50% - 75% of total cells per ROI) in healthy gingiva tissues in this study. The presence of carbamylated proteins *per se* is not pathogenic but an increase in the amount could be problematic (Carracedo et al., 2018; Pietrement et al., 2013). The pathological role of excessive carbamylated proteins has been reported in autoimmune and chronic inflammatory

diseases such as systemic lupus erythematosus, chronic kidney disease and atherosclerosis (Massaro et al., 2018; Speer et al., 2014; Wang et al., 2007; Wynckel et al., 2000). It has been postulated that chronic exposure to carbamylated proteins can trigger autoimmunity through T cells activation, followed by autoantibody generation by B cells (Mydel et al., 2010).

Interestingly, carbamylation is involved in aging and hence, the expression of carbamylated proteins is expected to be higher in mature skin than in young skin (Gorisse et al., 2016). More importantly, increased carbamylation is a sign of age-related damage due to oxidation stress rather than an indication of chronological age (Martinez de Toda et al., 2016). However, there was no significant age difference between groups so the increased expression and intensity of carbamylated proteins seen in $PD^+ RA^-$ and $PD^+ RA^+$ gingiva tissues was not age-related. Similar to citrullinated proteins, there was no significant difference in staining expression and intensity between $PD^+ RA^-$ and $PD^+ RA^+$ gingival tissues. Both tissues were heavily infiltrated by inflammatory cells, suggesting that carbamylated proteins were upregulated by inflammation. Other than spontaneous urea dissociation, carbamylation can also be mediated by myeloperoxidase (MPO) which is most abundantly expressed by neutrophils during inflammation (Wang et al., 2007). In periodontal disease, the migration of neutrophils through the vascular plexus increases following the upregulation of IL-8 by resident cells (Kinane, 2001). The heightened MPO activity of neutrophils increases the availability of isocyanic acid in the tissue and subsequently, the rate of carbamylation locally. The magnitude of carbamylation is determined by the severity of inflammation. An increase in staining intensity for carbamylated and citrullinated proteins from mild to moderate periodontitis was observed in the gingival tissues, implying that both citrullination and carbamylation are inflammation-dependent events (Bright et al., 2018). In the current study, more than 90% of the total cells per

ROI were positively stained in periodontitis lesions with no marked differences between $PD^+ RA^-$ and $PD^+ RA^+$ groups. The reason being both $PD^+ RA^-$ and $PD^+ RA^+$ participants had moderate to severe PD and gingival biopsy was performed at the site with the deepest pocket. Clearly, there was no difference in terms of severity of the disease and the presence of RA did not seem to affect the results.

It is unclear how the local production of autoantibodies in the target organs such as gingival tissue and synovial tissue, contributes to systemic autoimmunity. It has been hypothesised that B cells mediate autoimmune diseases due to their multiple roles in humoral immune response – presentation of self-antigen (citrullinated and carbamylated proteins) to T cells and with the help of activated T cells, B cells can produce autoantibodies (Mydel et al., 2010) or B cells can become autoreactive and generate autoantibodies without the presence of autoreactive T cells (Dekkers et al., 2017). In severe PD, the distribution of B cell subset was altered compared to mild and moderate periodontitis. Significantly higher proportion of switched memory B cells than normal regulatory B cells were found in the periphery blood of severe PD patients, suggesting a systemic effect of severe PD on serum antibody level (Demoersman et al., 2018). However, the role of these memory B cells and their antigenic target, whether bacterial products or PTM proteins, is yet to be determined. Besides, the commercial carbamylated antibody used in the present study was polyclonal and had a wide cross-reactivity with various carbamylated proteins. It was not possible to determine the antigen specificity of this antibody in the gingival tissues. To verify the link between PD and RA, it will be useful to identify the autoantibody in the synovial tissue and compare with that in the gingival tissue, in order to find out if the same autoantibody can be generated outside the synovial compartment.

Taken together, it is interesting to realise how citrullination and carbamylation are possibly brought together by neutrophils through PAD enzyme-mediated pathway

and MPO-mediated pathway (Darrah & Andrade, 2015). Future studies should evaluate the function of neutrophils in the development of autoimmunity.

5.2.5 Strengths of the study

To the best of our knowledge, this is the first Malaysian study that investigated the presence of citrullinated and carbamylated proteins in inflamed gingival tissues of RA participants and compared with those of PD participants and healthy controls. Besides, the stringent selection criteria including age and gender matching cases, exclusion of smokers and other comorbidities enabled us to minimise the potential confounders.

5.2.6 Limitations of the study

There are several limitations in this study. First, there was no synovial tissue from RA participant available for IHC as the Rheumatology Clinic in UMMC adopted a conservative treatment approach and none of their RA patients were indicated for surgical management during the study period. Second, the samples were not spread out according to the ratio of ethnicity in Malaysia which might reflect the lack of representativeness of the population. Third, the commercial antibodies used in this study lacked specificity and thus, no inferences could be made on the type of protein detected. Forth, the smoking status was self-reported and the type, dosage and duration of RA medications taken varied from individual to individual which could potentially confound the results. Finally, the main drawback of semi-quantitative analysis is variability due to its inherent subjectivity. Although it can be minimised through increasing the number of observers or computer-aided analysis, it is at best a semi-quantitative but not an absolute quantitative measurement.

5.2.7 Clinical relevance

Anti-Carp is a potential biomarker for RA. ACPA, despite its high specificity, is only present up to two third of RA patients, rendering the remaining one third ACPA-negative undiagnosed (Darrah & Andrade, 2018). The discovery that anti-Carp is present in 16-30% of ACPA-negative patients and it does not cross react with citrulline provide a novel insight into the pathogenesis of ACPA-negative RA (Shi et al., 2011). The present study has demonstrated the ability of periodontitis in promoting citrullination and carbamylation locally, which could have pathogenic implication in susceptible individuals. If periodontal disease is a potential source of PTM proteins, there is a possibility that chronic exposure of dysregulated PTM proteins may induce local and systemic autoimmune response or perhaps, exacerbate the pre-existing autoimmune disease. Hence, untreated PD should not be taken lightly in the context of RA. Given the inflammatory link between many chronic inflammatory diseases, PD can be regarded as a risk indicator for RA. On that account, addressing gingival inflammation should be viewed as an important preventive measure in the general population not only for the progression of periodontal disease but also reducing the risk of developing extra-oral comorbidities.

5.2.8 Future recommendations

1. Citrullination and carbamylation are not the only two PTM of proteins involved in the pathogenic mechanisms of RA and evidence is emerging for possibility of other PTMs involvement notably glycosylation, acetylation and oxidation in RA (de Brito Rocha et al., 2019). Considering the knowledge gap in this area, it may be worth exploring the role of other PTM proteins and other autoantibody systems in the association between PD and RA.

2. The use of mass spectrometry will allow a more accurate quantification of proteins and identification of other unknown PTM proteins that may be involved in the pathogenesis of RA.

3. The hypothesis that citrullination and carbamylation are inflammation-dependent phenomena can be validated by directing the focus on the host response especially the role of neutrophils in regulating autoimmunity.

Chapter 6: CONCLUSION

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

1. In the present study, citrullinated and carbamylated proteins were observed in both oral epithelia and gingival connective tissues. Within the connective tissues, some endothelial cells, fibroblasts and inflammatory cells were positively-stained, indicating the presence of these two proteins.
2. The presence of citrullinated and carbamylated proteins was prevalent in the gingival tissues but the expression of these proteins appeared to increase with inflammation. Higher abundance of citrullinated and carbamylated proteins was observed in $PD^+ RA^-$ and $PD^+ RA^+$ than HC.
3. The $PD^+ RA^-$ and $PD^+ RA^+$ had similar level of carbamylated and citrullinated proteins in the gingival tissues.

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