“Expression of Bone Resorption Markers in Gingival Crevicular Fluid of Rheumatoid Arthritis Patients with and without Periodontitis. A Pilot Study”

A report submitted to the University of Adelaide in partial fulfilment of the requirements of the Degree of Doctor of Clinical Dentistry (Periodontology)

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Finally, I would like to dedicate this to my grandparents Matt and Jean Kobayashi and Noel Nicholls who saw me begin this journey but unfortunately were not able to see me finish.

Chapter 1. "A Review of Bone Resorption Markers in Rheumatoid Arthritis and Periodontitis and the Current Literature Linking the Diseases"

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1.1 Introduction

Periodontitis is a complex disease in which disease expression involves intricate interactions between the dental biofilm, environmental, genetic factors and the host immunoinflammatory response with subsequent alterations in bone and connective tissue homeostasis (Kornman, 2008).

Its primary aetiology is the subgingival biofilm that naturally forms on the teeth, which in a diseased state is comprised of a series of gram negative anaerobic bacteria, such as *Porphyromonas gingivalis* and *Tannerella forsythia* in the case of chronic periodontitis, and *Aggregatibacter actiniomycetemcomitans* in the case of localised aggressive periodontitis (Offenbacher, 1996).

While the aetiology of periodontitis is bacterial, it is becoming clear that the pathogenesis of disease is mediated by the host response. Although the presence of bacteria is essential, it not sufficient for disease to occur and the disease severity is dependant upon a dynamic equilibrium of host-bacterium interactions, which are significantly influenced by various genetic, epigenetic and environmental factors in a susceptible host (Page and Beck, 1997, Kornman, 2008, Gomez *et al*., 2009, Nunn, 2003).

Periodontitis has been proposed as having an etiologic or modulating role in cardiovascular and cerebrovascular disease, diabetes, respiratory disease and adverse pregnancy outcome. Several mechanisms have been proposed to explain or support these relationships. In recent years, epidemiological and pathological relationships between periodontal diseases and rheumatic diseases, especially rheumatoid arthritis (RA), have been presented.

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease primarily affecting the joints. Progressive inflammation may lead to joint destruction and, subsequently, physical and quality-of-life impairments.

The evidence for a possible association between RA and periodontitis stems from both animal and clinical studies. However, despite the growing support relating the two diseases, there is currently no clear explanation of how periodontal and rheumatic diseases are related.

1.2 Biology of the Alveolar Bone

The alveolar process is that bony portion of the maxilla and mandible where the teeth are embedded (sockets) and by which tooth roots are supported. The alveolar socket is the cavity within the alveolar process in which the root of the tooth is held by the periodontal ligament. It consists of outer cortical plates of compact bone, a central spongiosa and alveolar bone, and the bone lining the alveolus. The bone lining the socket is specifically referred to as bundle bone because it provides attachment for PDL fibre bundles. It is perforated by many foramina, which transmit nerves and vessels; thus it is sometimes referred to as the cribiform plate (Nanci and Bosshardt, 2006).

1.2.1 Bone Cells

In bone, separate cells are responsible for the formation, resorption and maintenance of osteoachitecture. These cells are osteoblasts; which form bone, osteocytes; which, together with inactive osteoblasts, maintain bone, and osteoclasts; which resorb bone.

Osteoblasts are mononucleate bone-forming cells which descend from osteoprogenitor cells (mesenchymal cells). They are responsible for the production of the bone matrix constituents. They are located on the surface of osteoid seams and make a protein mixture known as osteoid, which mineralises to become bone. Osteoid is primarily composed of Type I collagen.

Osteocytes originate from osteoblasts which have migrated into and become trapped and surrounded by bone matrix, which they themselves produce. These cells still produce matrix proteins. The spaces which they occupy are known as lacunae. Osteocytes have many processes which reach out to meet osteoblasts and other osteocytes, probably for the purposes of communication. Their functions include to varying degrees: formation of bone, matrix maintenance and calcium homeostasis. They have also been shown to act as mechano-sensory receptors, regulating the bone's response to stress and mechanical load. They are mature bone cells. Despite the complete organisation of the osteoclastic network, the exact function of these cells remains obscure. It has been proposed that they respond to tissue strain and enhance bone remodelling activity by recruiting osteoclasts to sites where bone remodelling is required (Nanci and Bosshardt, 2006).

Osteoclasts are cells responsible for bone resorption (remodelling of bone to reduce its volume). Osteoclasts are large, multinucleated cells located on bone surfaces in what are called Howship's lacunae or resorption pits (that they themselves created). These lacunae, or resorption pits, are left behind after the breakdown of the bone surface. They have deep folds on the plasma membrane that faces the bone matrix (called ruffled border) and the surrounding area (sealing zone) and copious transport vesicles that contain lysozymal enzymes.

Because the osteoclasts are derived from a monocyte stem-cell lineage, they are equipped with phagocytic like mechanisms similar to circulating macrophages. Osteoclasts mature and/or migrate to discrete bone surfaces. Upon arrival, active enzymes, such as tartrate resistant acid phosphatase, and cathepsin K are secreted via the ruffled border against the mineral substrate (Sodek and McKee, 2000).

1.2.1 Bone Formation

Bone formation occurs in three successive phases: the production and the maturation of the osteoid, followed by the mineralisation.

Initially, osteoblasts produce osteoid by rapidly depositing collagen. This is followed by an increase in the mineralisation rate (by alkaline phosphatise) equal to that of collagen synthesis. In the final stage the rate of collagen synthesis decreases and mineralisation continues until the osteoid becomes fully mineralised.

Osteoblasts produce a range of growth factors under a variety of stimuli including insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF) β, and bone morphogenetic proteins (BMP); which play a critical role in the differentiation of undifferentiated mesenchymal cells into osteoblasts (Hughes *et al*., 2006).

Osteoblast activity is regulated in an autocrine and paracrine manner. Receptors for parathyroid hormone, thyroid hormone, growth hormone, insulin, progesterone and prolactin are located on osteoblasts as well.

1.2.2 Bone Resorption

The process of the osteoclast attachment to the bone surface involves binding of integrins to the surface of the bone matrix. After adhesion, cytoskeletal reorganisation occurs within the osteoclast. Attachment usually occurs via dynamic structures called podosomes. Through their continual assembly and disassembly they allow osteoclast movement across the bone surface during which bone resorption proceeds (Sodek and McKee, 2000).

Osteoclasts resorb bone by acidification and proteolysis of the bone matrix and the hydroxyapatite crystals encapsulated within the sealing zone. The first process during resorption is mobilisation of the hydroxyapatite crystals by digestion of their link to collagen. Then the residual collagen fibres are digested by either cathepsins or activated collagenases and the residues from this digestion are either internalised or transported across the cell and released. Osteoclast function is regulated both by locally acting cytokines and by systemic hormones (calcitonin, androgens, thyroid hormone, insulin, PTH, IGF-1, interleukin (IL) 1, and PDGF) (Saffar *et al*., 1997).

1.3 Bone Remodelling

In bone, there is a constant process of modelling and remodelling. In this process a constant resorption of the bone occurs on a particular bony surface, followed by a phase of bone formation. In normal adults, there is a balance between the amount of bone resorbed by osteoclasts and the amount of bone formed by osteoblasts (Parfitt, 1982).

The current concept of bone remodelling is based on the hypothesis that oseoclastic precursors become activated, migrate and differentiate into osteoclasts, which begin the process of bone resorption (Charles *et al*., 1987).

This phase is followed by a bone-formation phase. The number of sites entering the bone formation phase, together with the individual rates of the two processes, determines the rate of tissue turnover. After the completion of osteoclastic resorption, there is a reversal phase when mononuclear cells appear on the bone surface. These cells prepare the surface for new osteoblasts to begin bone formation and provide signals for osteoblast differentiation and migration.

The formation phase follows with osteoblasts laying down bone until the resorbed bone is completely replaced by new. When this phase is complete, the surface is covered with flattened lining cells and a prolonged resting period begins until a new remodelling cycle is initiated.

The stages of the remodelling cycle have different lengths. Resorption continues for about 2 weeks, the reversal phase may last up to 4 or 5 weeks, while formation can continue for 4 months until the new bone structural unit is completely created (Hill, 1998).

Molecular communication between osteoblasts and osteoclasts and between bone cells and other bone marrow cells is a fundamental mechanism that regulates bone formation and resorption with remarkable precision. Interestingly, the exact molecular mechanism that describes this interaction has only recently been identified (Suda *et al*., 1999).

Regulation of the bone remodelling process is complex and involves hormonal and local factors acting in an autocrine or paracrine manner on the generation and activity of differentiated bone cells (Galli *et al*., 2010).The main switch for initiation of osteoclastic bone resorption is the receptor activator for NF-κB-ligand (RANKL), a cytokine that is released by activated osteoblasts. Its action on the RANK receptor is regulated by osteoprotegerin (OPG), a decoy receptor, which is also derived from osteoblasts (Kong *et al*., 1999b).

1.3.1 Osteoimmunology – The Expression of RANKL/RANK/OPG

RANKL is a member of the tumour necrosis factor (TNF) superfamily of proteins and exists in two forms: one which is presented on cell surfaces with a molecular weight of 35.5 kDa (RANKL) and one soluble form with a molecular weight of 27.7 kDa soluble RANKL (sRANKL). It also possesses various names (TRANCE: TNF-related activation-induced cytokine; ODF: osteoclast differentiating factor; OPGL: osteoprotegerin ligand; TNFSF11: TNF superfamily member 11).

RANKL mRNA is expressed by osteoblastic lineage cells as well as on activated T-cells and this expression is increased in the presence of osteoclastic agents (such as vitamin D3, IL-1) (Lacey *et al*., 1998, Takayanagi *et al*., 2000, Quinn *et al*., 2000). RANKL is expressed at its highest levels in osteoblasts and stromal cells (Yasuda *et al*., 1998b). It is

also expressed by fibroblasts (Takayanagi *et al*., 2000), activated T-cells (Teng *et al*., 2000) and activated B cells (Yun *et al*., 1998)

RANKL stimulates osteoclastic activity and differentiation and inhibits osteoclast apoptosis. It binds to osteoclast progenitor cells inducing changes in their gene expression that modulate the formation of bone resorbing osteoclasts (Hsu *et al*., 1999). Excessive bone resorption associated with the presence of T-cells (Teng *et al*., 2000) is promoted directly by RANKL expression and indirectly by cytokine production that induce RANKL expression in osteoblasts (Hofbauer and Heufelder, 2001). Its receptor RANK is present on pre-osteoclasts. In bone, (in the presence of macrophage colony-stimulating factor (M-CSF)) when RANKL binds to RANK it allows the maturation, differentiation and activation of osteoclasts.

On the other hand, osteoblasts also secrete a factor that exerts a protective effect on bone, soluble decoy receptor OPG (Simonet *et al*., 1997). OPG is a member of the TNF receptor superfamily and is mainly secreted by cells of osteoblastic origin and other lineages, i.e. human periodontal ligament cells, gingival fibroblasts and epithelial cells (Sakata *et al*., 1999), and human microvascular endothelial cells (Kanzaki *et al*., 2002). It has a very important role in the skeletal system, acting as a decoy receptor for RANK–RANKL binding (Yasuda *et al*., 1998a, Yun *et al*., 1998). The effects of RANKL are counteracted by OPG, which prevents the binding of RANKL to its receptor RANK on osteoclasts (Akatsu *et al*., 1998).

OPG binds to RANKL with high specificity, preventing RANKL binding to RANK. Thereby OPG prevents osteoclast differentiation and activation and promotes osteoclast apoptosis. Stimulators of the osteoclastogenesis, such as IL-1 β, IL-6, IL-11, IL-17, and TNF-α, increase the expression of RANKL and decrease OPG expression in osteoblasts/stromal cells. Cytokines inhibiting the osteoclastogenesis, such as IL-13, INF-γ, and TGF-β1, suppress the expression of RANKL and stimulate OPG expression (Saidenberg-Kermanac'h *et al*., 2004). Therefore it is the balance between RANKL and OPG that determines bone resorption.

When RANKL expression is enhanced relative to OPG, RANKL is available to bind RANK on osteoclast precursors, tipping the balance to favour activation of osteoclast formation and bone resorption (Boyle *et al*., 2003). When OPG concentrations are high

relative to RANKL expression, OPG binds RANKL, inhibiting it from binding to RANK. Preventing the binding of RANKL to RANK leads to reduced formation of osteoclasts and apoptosis of pre-existing osteoclasts (Boyle *et al*., 2003).

Signalling through RANK–RANKL is the key factor for osteoclast proliferation and differentiation. However, RANK by itself does not have intrinsic enzymatic activity and needs to recruit adaptor proteins, such as the TNF-receptor-associated factor (TRAF) family of proteins (Takayanagi, 2007), especially TRAF6. The cytoplasmic tail of RANK contains three TRAF6-binding domains and the binding of this protein to RANK induces trimerisation of TRAF6 leading to the activation of nuclear factor kappa-B (NF-κB) and mitogen-activated kinases (MAPK), including Jun N-terminal kinase (JNK) and p38 (Asagiri and Takayanagi, 2007).
Therefore, TRAF6 acts downstream of RANK inducing, in pre-osteoclasts, the expression of the target genes activator protein-1 (AP1) and nuclear factor of activated T-cells, and cytoplasmic calcineurin dependent 1 (NFATc1), leading to pre-osteoclasts fusion and to osteoclasts differentiation (Takayanagi, 2007, Kobayashi *et al*., 2001). TRAF6 also plays an important role in osteoclastic bone resorption by inducing membrane ruffling and actin ring formation through the activation of c-Src signalling cascade (Wu and Arron, 2003).

Although RANK–RANKL signalling plays a central role, osteoclast proliferation also depends on the presence of macrophage colony-stimulating factor, produced by several cell types including osteoblasts, that upon binding to its receptor, c-fms, at the surface of pre-osteoclast cells, activates an intracellular cascade that ultimately leads to proliferation and survival of osteoclasts (Takayanagi, 2007). In this way, osteoblasts produce the key factors RANKL and M-CSF that promote osteoclasts proliferation and differentiation. This is illustrated in Figure 1.

Figure 1. Roles of RANKL and OPG on osteoblasts and osteoclasts (Boyle *et al*., 2003). Schematic representation of the mechanism of action of **a**, pro-resorptive and calcitropic factors; and **b**, anabolic and anti-osteoclastic factors. RANKL expression is induced in osteoblasts, activated T cells, synovial fibroblasts and bone marrow stromal cells, and subsequently binds to its specific membrane-bound receptor RANK, thereby triggering a cascade that promote osteoclast differentiation, activation and survival. Conversely, OPG expression is induced by factors that block bone catabolism and promote anabolic effects. OPG binds and neutralizes RANKL, leading to a block in osteoclastogenesis and decreased survival of pre-existing osteoclasts.

1.4 How Does The Expression Of RANKL/RANK/OPG Relate To Periodontitis?

OPG and RANKL are believed to be the key regulators regulating bone metabolism and alveolar bone destruction in periodontitis and are both modulated by the inflammatory cytokines present in periodontitis (Nakashima *et al*., 2000, Brandstrom *et al*., 1998, Hofbauer *et al*., 1998).

In animal models of periodontitis, it has been shown that transplanted human T-cells are able to produce RANKL, leading to activation of osteoclasts and consequent bone loss (Teng *et al*., 2000).

Further clinical studies investigated the concentrations of RANKL and OPG in gingival tissues and crevicular fluid extracted from individuals with periodontitis to determine the RANKL/OPG ratio. In human gingival tissues, periodontitis patients exhibit higher expression of RANKL (Crotti *et al*., 2003, Liu *et al*., 2003, Garlet *et al*., 2004, Vernal *et al*., 2006, Bostanci *et al*., 2007b), and lower expression of OPG (Crotti *et al*., 2003, Liu *et al*., 2003) compared with healthy controls. Similar results were also reported in gingival crevicular fluid (GCF) (Mogi *et al*., 2004, Vernal *et al*., 2004, Lu *et al*., 2006, Bostanci *et al*., 2007a). The RANKL/OPG ratio has also been found to be increased in patients with periodontitis compared to healthy controls (Liu *et al*., 2003, Mogi *et al*., 2004, Bostanci *et al*., 2007a, Bostanci *et al*., 2007b).

Tissue studies found an increase in soluble RANKL concentrations without a corresponding change in OPG levels in individuals with chronic periodontitis compared to healthy controls (Kawai *et al*., 2006, Wara-aswapati *et al*., 2007). However, a reciprocal relationship was also found, in which RANKL protein expression was higher and OPG levels were lower in diseased gingival tissues compared to healthy controls (Crotti *et al*., 2003).

When examining the concentrations of RANKL and OPG expression, the results varied from study to study, although the trend was generally the same; the RANKL/OPG ratio was higher in individuals with periodontitis than in healthy controls (Kawai *et al*., 2006, Wara-aswapati *et al*., 2007, Lu *et al*., 2006). These trends toward a net increase in the RANKL/OPG ratio in periodontal disease have been observed in gingival tissue as well as in GCF.

A summary of human studies investigating RANKL and OPG in periodontal disease is shown in Table 1 (Cochran, 2008).

Table 1. A summary of human studies looking at RANKL and OPG in PD (Cochrane, 2008)

1.4.1 RANKL and OPG Levels in Different Forms of Periodontal Disease

To discover if the expression of OPG and RANKL varied in different forms of periodontal disease, a study by Bostanci *et al* (2007b) obtained gingival tissues from periodontally healthy subjects, patients with gingivitis, chronic periodontitis, aggressive periodontitis and chronic periodontitis who were receiving immunosuppressant therapy.

They found that that levels of RANKL and OPG in tissues were reciprocally regulated in periodontitis; i.e., an elevation in RANKL protein and a decrease in OPG were observed in

the GCF of individuals with periodontitis compared to healthy controls, hence a higher RANKL to OPG ratio compared with healthy controls. However, there was no difference in RANKL/OPG ratio between the two forms of periodontitis.

The same research group (Bostanci *et al*., 2007a), using a similar study design, then examined this ratio in GCF. They found that there was no difference in the GCF concentrations of RANKL and OPG between both groups, and that again; the RANKL/OPG ratio was much higher in chronic and aggressive periodontitis.

In contrast, OPG mRNA expression is higher in chronic periodontitis patients compared to aggressive periodontitis (Garlet *et al*., 2004). These results suggest that higher levels of OPG may offer some protective role and that lower levels, as occurring in aggressive periodontitis, could account for the early onset and more rapid progression of this clinical form of the disease.

One explanation for these inconsistencies between the tissue expression and GCF levels of these cytokines, is that there could be a lag between production of these molecules in the tissue and their release into the periodontal pocket. Varying levels may also reflect different stages of disease chronicity, or even the time elapsed between the conservative treatment and surgical treatment may reflect a site undergoing a healing process. It also further demonstrates the complexity of the different cytokine interactions and that more research is required to fully understand the mechanisms that are occurring in this pathway.

1.4.2 Does the Expression of RANKL and OPG Correlate to Periodontal Disease Severity and Disease Progression?

Although periodontal disease is associated with an increased RANKL/OPG ratio compared to healthy controls, the ratio may not necessarily distinguish between mild, moderate, and severe forms. This was shown in studies of GCF and tissue samples where there was an overall increase in the ratio in patients with periodontal disease compared to healthy controls; however, there was no difference in the ratio between patients with mild, moderate, or severe periodontitis (Garlet *et al*., 2004, Lu *et al*., 2006, Mogi *et al*., 2004). However, Bostanci *et al*. (2007a, 2007b) found that the levels of RANKL and RANKL/OPG ratio in gingival tissues and GCF were significantly correlated with the clinical parameters such as attachment level, probing depth and bleeding on probing.

Similar results were reported by Vernal *et al* (2004) who studied the GCF levels of RANKL in relation to disease progression in a group of untreated periodontitis patients. Higher total amounts of RANKL were found to be expressed in active sites (≥2 mm attachment loss) compared to inactive counterparts.

These data seem to indicate that gingival tissues and GCF RANKL/OPG ratios are regulated in a similar manner in periodontal diseases, and suggest that an increase in the ratio may indicate the occurrence of periodontitis.

Considering periodontitis is characterised by bone loss and attachment loss, markers specific to bone matrix such as RANKL and OPG may be a reliable indicator of disease activity. The involvement of RANKL/OPG axis in periodontitis opens up a whole new area of periodontal research.

1.4.3 The Use of RANKL/OPG as a Treatment Possibility for Periodontitis

With a net increase in the ratio of RANKL/OPG in gingival and crevicular fluids associated with bone loss and maybe with the increasing severity of periodontitis (Bostanci *et al*., 2007b), the possibility that interference with the RANK/RANKL/OPG axis may lead to novel treatments is promising.

The desired outcome would be an increase in OPG or a decrease in RANKL that brings the RANKL/OPG ratio to a balance where bone formation is equal to bone resorption.

The RANKL/RANK/OPG axis has been targeted directly for the treatment of periodontal disease even though RANKL-mediated osteoclastogenesis may not be exclusively responsible for induction of bone loss in periodontal or other experimental systems (Kobayashi *et al*., 2000).

Most strategies developed to date have aimed to block RANKL. The development of small molecules that mimic OPG action by targeting the RANKL/RANK signalling pathway provided proof-of-principle experiments that these agents may indeed prevent osteoclast-mediated bone loss (Cheng *et al*., 2004, Onyia *et al*., 2004). OPG was thus an obvious first choice to combat inflammation-induced bone resorption. The anti-erosive effect of OPG

had been demonstrated in rats especially when started early in disease (Campagnuolo *et al*., 2002).

The use of OPG as an inhibitor of alveolar bone loss in periodontitis was investigated in mice orally infected with *A. actinomycetemcomitans* (Teng *et al*., 2000, Mahamed *et al*., 2005). Inhibition of RANKL function with OPG treatment significantly reduced the number of osteoclasts and the alveolar bone loss in both studies. In another rat model of periodontitis using T-cell adoptive transfer, systemic administration of OPG significantly reduced periodontal bone loss compared to the control (Taubman *et al*., 2005).

Similarly in murine models, systemic or local injection of OPG was also found to inhibit B-cell mediated periodontal bone resorption (Han *et al*., 2006). Jin *et al* (2007) also reported that OPG has a strong preventive effect on alveolar bone loss in ligature-induced experimental periodontitis for treatment of periodontal disease (Jin *et al*., 2007, Mahamed *et al*., 2005).

A trial of OPG in postmenopausal women reported that a single injection of OPG-Fc resulted in sustained suppression of bone resorption, as measured by levels of urinary excretion of deoxypyridinoline (Bekker *et al*., 2004).

However, there were some potential concerns for using OPG. One was the risk for generation of anti-OPG antibodies, especially with chronic use, leading to cross-reactivity with endogenous OPG, thereby neutralising the activity of OPG. Another concern was the possibility of OPG binding to TNF-related apoptosis-inducing ligand (TRAIL), a survival factor for tumour cells, resulting in an interference with the natural defence mechanism against tumorigenesis (Fouque-Aubert and Chapurlat, 2008).

The development of anti-RANKL monoclonal antibody, known as denosumab or AMG 162, does not appear to have this drawback, and data suggests that it is effective in the treatment of osteoporosis (Bekker *et al*., 2004, McClung *et al*., 2006).

However, it should be noted that in normal function RANKL enhances the survival and activity of dendritic cells and macrophages and promotes their antigen-presenting function (Park *et al*., 2005). Therefore blockade of the RANK/RANKL interaction may not only affect bone metabolism but may also interfere with the normal function of the immune

system. Moreover, a limitation of RANKL antagonistic approaches in rheumatoid arthritis is that they do not treat synovitis (Romas and Gillespie, 2006).

In short, RANKL inhibition in periodontitis may inhibit bone resorption but will probably not treat inflammation or control the infection.

1.4.4 Periodontopathogens and Their Influence on RANKL and OPG

It has been shown that several stimuli can influence the expression of RANKL and OPG in PDL cells. For instance, stimulation of these cells with bacterial supernatants of periodontal pathogens induces RANKL expression, while OPG expression was down-regulated or remained the same (Yamamoto *et al*., 2006, Belibasakis *et al*., 2005b, Belibasakis *et al*., 2005a, Kiji *et al*., 2007, Tiranathanagul *et al*., 2004, Belibasakis *et al*., 2007).

However, other virulence factors have also been demonstrated to cause activation of RANKL expression in PDL cells *in vitro*. For example cytolethal distending toxin (Cdt) and Arg- proteinases (Belibasakis *et al*., 2007, Belibasakis *et al*., 2005a).

Furthermore, lipopolysaccharide (LPS) treatment on osteoblasts increases RANKL gene expression through activation of Toll-like receptors (Kikuchi *et al*., 2001). In endothelial cells, *P. gingivalis* upregulates the expression of OPG but not RANKL (Kobayashi-Sakamoto *et al*., 2004).

Interestingly, Kawai *et al* (2007) demonstrated RANKL-dependent bone loss in mice as a result of the cross-reactivity between *A. actinomycetemcomitans and* oral commensal bacteria in the induction of an adaptive immune response (Kawai *et al*., 2007). More recently Krajewski *et al* (2009) demonstrated that stimulation of PDL cells with *P. gingivalis* LPS leads to an increased release of sRANKL, rather than increased RANKL expression. They hypothesised that through this action, *P. gingivalis* LPS may exert its biological effect on osteoclast formation and bone resorption. In addition a study by Wara-aswapati *et al* (2007), demonstrated an association between upregulated RANKL levels and the number of *P. gingivalis* in clinically obtained periodontal tissues. Taking all of these studies into consideration there seems little doubt that periodontal pathogens are able to modulate the expression of OPG and RANKL in cells found in periodontal tissues.

1.5 Gingival Crevicular Fluid

GCF is an inflammatory exudate that is composed of serum and locally generated materials such as tissue breakdown products, inflammatory mediators, and antibodies directed against dental plaque bacteria. For these reasons it has been suggested that analysis of GCF might provide a means to quantitatively evaluate the inflammatory status of gingival and periodontal tissues, and subsequently there has been intense interest in the diagnostic potential of GCF (Armitage, 2004).

Traditionally several different techniques have been employed for the collection of GCF each technique has its own advantages and disadvantages. The techniques can be divided into three basic strategies; gingival washing methods, capillary tubing or micropipettes and, more commonly, the use of absorbent filter paper strips. The advantages in using this technique are that it is quick and easy to use, and can be applied to individual sites and, possibly, is the least traumatic when correctly used.

As previously discussed, GCF picks up several factors along its way into the periodontal pocket. The components are derived from plasma, locally produced host factors and microbial sources and thus these molecules may be of interested as they may represent factors of disease and health in the tissues (PM Bartold and Narayanan, 1998). More than 65 GCF constituents have been evaluated as a potential diagnostic marker of periodontal disease progression (Armitage, 2004). These markers can be divided into three groups: host derived enzymes and their inhibitors, inflammatory mediators and host response modifiers and by products of tissue breakdown (Lamster and Ahlo, 2007). In addition, collagen breakdown products are found in GCF, and may serve as direct measures of connective tissue catabolism for both soft and hard tissues (Champagne *et al*., 2003).

Several investigators have examined GCF in periodontal disease for cellular immune-response indicators prostaglandin E (Offenbacher *et al*., 1986), IL-1 and IL-6 (Reinhardt *et al*., 1993), IL1β, IL-6 and TGFα (Mogi *et al*., 1999). It was Mogi *et al* that first showed that the ratio of the concentration of RANKL to that of OPG in GCF was significantly higher for periodontal disease patients than for healthy subjects (Mogi *et al*., 2004). This has subsequently been confirmed (Vernal *et al*., 2004, Bostanci *et al*., 2007b).

Controversy still exists as to whether GCF is present at sites designated as clinically healthy. This dispute probably arises, at least in part, as a result of the different techniques used to sample the GCF. The initial experiments in healthy crevices used the intracrevicular sampling technique (inserting strips until resistance is felt) and yielded detectable levels of GCF (Brill and Krasse, 1958).

In contrast, if filter paper strips were placed just at the entrance to the gingival crevice GCF was seldom detected (Oliver *et al.*, 1969). The proponents of the latter technique concluded that the 'deep' intracrevicular technique induced trauma, producing an artefactual flow of fluid into the healthy crevice. Although these arguments were derived from the filter paper strip method of collection, similar arguments of the effect of trauma during sampling apply equally to the other sampling techniques, such as those using capillary tubes (Griffiths, 2003).

Further discrepancies can be caused by (1) contamination with blood, saliva and plaque (2) sampling times; which can influence the volumes obtained (3) evaporation, and (4) Recovery from strips. Thus when studying potential markers in GCF it is important to remember that the method of collecting GCF may have a significant effect upon the nature of the sample collected and will therefore prejudice results assessing diagnostic markers.

For many years researchers have been searching for a marker for periodontitis in GCF. The goal is to develop a simple chairside test which can determine whether a patient suffers from periodontitis or whether further therapy is required for maintenance patients. GCF has been the subject of intense research in periodontology and readily lends itself to comparative studies of various conditions. However, the reliability of data is sometimes problematic because of the difficulty in measuring and assaying the small volumes obtained in many cases, and the variations in collection protocols (different collection strips, times and numbers of samples) and processing methodology.

1.5.1 Gingival Crevicular Fluid Related to Systemic Conditions

GCF analysis can be used to study how systemic diseases and conditions may influence the progression of periodontal disease, and may eventually be used to assess how periodontal disease influences the progression of certain systemic diseases. In addition, GCF can be analysed to determine whether specific markers of systemic disease can be identified in the oral cavity.

1.5.2 Alterations in GCF Associated with Systemic Disorders

Studies have been conducted to examine mediators in GCF from patients with diabetes mellitus. These mediators were higher in the insulin dependent diabetes mellitus group (regardless of the severity of periodontal disease) versus the systemically healthy group (Salvi *et al*., 1997). A study by Engebretson *et al* (2004) found that there was a correlation between poor glycemic control and increased levels of IL-1 levels in GCF, emphasising a link between poorly controlled diabetes and periodontal disease severity.

1.6 Periodontitis and Systemic Disease

The possible links between oral and systemic health have been increasingly recognised over the past two decades, with a large number of epidemiological studies now linking poor oral health with cardiovascular diseases, poor glycaemic control in diabetics, adverse pregnancy outcomes, obesity, respiratory diseases and rheumatoid arthritis (Cullinan *et al*., 2009). Whilst many of these studies have shown some association between oral and systemic health, the strength of this association has varied between reports. As a result, a number of meta-analyses have been conducted and confirmed the associations but at the same time cautioned that further studies are required, particularly with regard to the effect of periodontal treatment in reducing risk. As research in this area continues, numerous possible mechanisms have been put forward to explain the associations and whilst evidence continues to grow, significant “cause and effect” relationships have not yet been established (Williams *et al*., 2008, Cullinan *et al*., 2009)

1.7 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a complex genetic disease, involving multiple genes and environmental factors. Twin studies have estimated that the relative contribution of genetic factors to be about 50% (MacGregor *et al*., 2000). In one twin study the genetic element

was controlled while one environmental factor, smoking, was studied. In a series of monozygotic twin pairs discordant for RA and smoking, the smoker was the one with the disease in 12 of 13 pairs (Silman *et al*., 1996).

The natural history of RA is poorly defined; its clinical course fluctuates and the prognosis is unpredictable. RA affects up to 1-3% of the population, with a 3:1 female preponderance disappearing in older age (Spector, 1990). RA is characterised by progressive and irreversible damage to the synovial-lined joints causing loss of joint space, bone and function, as well as deformity. Extracellular matrix degradation is a hallmark of RA which is responsible for the typical destruction of cartilage, ligaments, tendons, and bone.

Extra-articular signs can involve pulmonary, cardiovascular, nervous, and reticuloendothelial systems. The clinical presentation of RA varies, but an insidious onset of pain with symmetric swelling of the small joints is the most frequent finding. RA onset is acute or subacute in about 25% of patients, but its patterns of presentation also include palindromic onset, monoarticular presentation (both slow and acute forms), extra-articular synovitis (tenosynovitis, bursitis), polymyalgic-like onset, and general symptoms (malaise, fatigue, weight loss, fever). The palindromic onset is characterised by recurrent episodes of oligoarthritis with no residual radiologic damage, while the polymyalgic-like onset may be clinically indistinguishable from polymyalgia rheumatica in elderly subjects (Anderson, 2005).

Early RA is characterised by symmetric polyarthritis involving the small joints of the hands and feet with no radiologic changes. RA most frequently affects the metacarpophalangeal, proximal interphalangeal and wrist joints. Although any joint, including the cricoarytenoid joint, can be affected, the distal interphalangeal, the sacroiliac and the lumbar spine joints are rarely involved, which is peculiar because these are some of the most typical targets of seronegative spondylarthropathies, such as psoriatic arthritis and ankylosing spondylitis. Simultaneous involvement of the same joint areas on both sides of the body should always be investigated even when it is not apparent. The clinical manifestations of RA vary depending on the involved joints and the disease stage (Anderson, 2005).

1.7.1 The Course of Rheumatoid Arthritis

The clinical course of RA is mostly progressive, with remission and worsening but continuing disease activity. Slow progression of joint symptoms is typical in RA, with destruction beginning within a few weeks of symptom onset. Several clinical, laboratory, and imaging findings can indicate the disease process and may play a relevant role in predicting good or poor patient outcome and patient risk stratification. The erythrocyte sedimentation rate and acute phase reactants, i.e. C-reactive protein, best reflect disease activity fluctuations. The main prognostic signs that may help identify the patients with more severe disease include several swollen joints, high serum levels of acute phase reactants or IgM rheumatoid factor, and early radiographic and functional abnormalities (Kazis *et al*., 1990). The radiologic progression is unpredictable and varies among patients. Long-term disability is associated with hand function loss and failure of the larger weight-bearing joints.

1.7.2 Diagnosis of Rheumatoid Arthritis

The diagnosis of RA is primarily clinical; the typical presentation is polyarticular, with pain, stiffness, and swelling of multiple joints in a bilateral, symmetric pattern. Weakness is out of proportion to tenderness. Only a minority of patients present with oligoarticular asymmetric involvement (Grassi *et al*., 1998).

In most patients, symptoms emerge over weeks to months, starting with one joint and often accompanied by prodromal symptoms of anorexia, weakness, or fatigue. In approximately 15% of patients, onset occurs more rapidly over days to weeks. In 8-15% of patients, symptoms begin within a few days of a specific inciting event, such as an infectious illness (Walton *et al*., 1985).

In clinical trials, RA is diagnosed formally using seven American Rheumatism Association (ARA) criteria (Arnett *et al*., 1988). A summary of ARA classification criteria for RA is presented in Table 2.

Table 2. Classification criteria for rheumatoid arthritis

Morning stiffness (lasting > 1 hour)†
Arthritis of 3 or more joint areas (areas are right or left proximal interphalangeal, metacarpophalangeal, wrist, elbow, knee, ankle, and metatarsophalangeal joints)†
Arthritis of hand joints (proximal interphalangeal or metacarpophalangeal joints)†
Symmetric arthritis, by area†
Subcutaneous rheumatoid nodules
Positive rheumatoid factor
Radiographic changes (hand and wrist, showing erosion of joints or unequivocal demineralization around joints)

(Majithia and Geraci, 2007)

Initial laboratory tests should include a complete blood cell count, rheumatoid factor (present in about 80% of cases of RA), and erythrocyte sedimentation rate or C-reactive protein (CRP) (Block, 2002). Further testing of anticyclic citrullinated peptide antibody (ACPA) carries high specificity and positive predictive value but is present in fewer than 60% of rheumatoid arthritis patients (van Gaalen *et al*., 2004). Sensitivity of this test is increased when used in combination with rheumatoid factor. Table 3 lists the more common tests used for diagnosis. Unfortunately, no single test definitively confirms the diagnosis of rheumatoid arthritis. However, multiple tests can provide objective data that increase diagnostic certainty and allow disease progression to be followed. One such test for RA activity is the disease activity score (DAS28). The score is calculated by a complex mathematical formula, which includes the number of tender and swollen joints (out of a total of 28), the erythrocyte sedimentation rate (ESR, a blood marker of inflammation), and the patient's 'global assessment of global health'. A DAS28 score greater than 5.1 implies active disease, less than 3.2 well controlled disease, and less than 2.6 remission.

Table 3. Summary of the test findings associated with RA (Rindfleisch and Muller, 2005).

NOTE:
This table is included on page 22 of the print copy of the thesis held in the University of Adelaide Library.

1.7.3 Rheumatoid Arthritis Therapy

It was previously believed that anti-rheumatic therapy made little, if any, difference to the long term outcome of the disease. Part of this reasoning was that it was thought to be difficult to see patients early enough in the disease's progression, so that an accurate diagnosis was not possible, and that therapies were ineffective.

Now, newer techniques have been developed that make earlier diagnosis easier and more accurate. Examples of this are genetic testing methods (HLA class II), immunological tests (rheumatoid factor and citrullinated peptides) and the development of magnetic resonance imaging that can pick up changes in over 80% of cases (compared to conventional radiographs of rheumatoid patients showing normal radiographs in 80% of cases), and high resolution ultrasonography that can detect synovitis and classic erosions in seven times more patients than can radiography (Wakefield *et al*., 2000).

Strategies for treatment of RA have evolved greatly over the past decade (Emery, 2006). Three ideas have driven this alteration. First, early and consistent reduction of

inflammation is the key; i.e., if there is no inflammation, there is little joint damage. Second, specific molecular mechanisms implicated in pathogenesis of the disorder should be targeted. Third, RA is a diverse and dynamic disease, for which different treatments are effective on individual patients and at various time points.

Findings of several studies during the past decade have provided definite proof that early and aggressive treatment with conventional disease modifying anti-rheumatic drugs (DMARDs) can be highly beneficial in controlling inflammatory activity, increasing remission rate and stopping the development of erosions in many patients (Svensson *et al*., 2005). It is therefore imperative to diagnose the disease and initiate treatment as soon as possible (Emery *et al*., 2002).

1.7.4 Pharmacotherapeutic Management of Rheumatoid Arthritis

Non-steroidal anti-inflammatory drugs (NSAIDS) have long been the initial treatment of patients with RA. They act mainly by inhibiting cyclooxygenase and thus reduce the production of prostaglandins. However, although these drugs improve signs and symptoms, they do little to alter the progressive structural damage and long term disability associated with RA.

In the past, further therapy using DMARDs was only prescribed when there was radiographic evidence of bony erosions. This has now changed, as DMARDs are now considered appropriate for all patients with RA (Emery, 2006).

The most commonly used DMARDs are gold, penicillamine, glucocorticoids, and sulfasalazine, all of which produced a slow response and a high level of toxicity. Consequently, few patients take these drugs long term. The addition of methotrexate produced an improvement, but still less than 50% of patients remain on DMARD treatment long term (Emery, 2006).

Active treatment can lead to a striking change in the long term course of RA (Grassi *et al*., 1998). Because of such treatments, the clinical face of the disorder is changing, in that previously feared extra-articular manifestations and subcutaneous nodules are diminishing in frequency (Watts *et al*., 2004).

Several new drugs with novel mechanisms of action have emerged in recent years. This latest generation of antirheumatic drugs have been called "biologics". These have novel molecular mechanisms that target cytokines, signalling molecules and cells involved in inflammation and joint destruction. These include the TNF antagonists: adalimumab, etanercept, and infliximab (first line agents); the IL-1 antagonist, anakinra; the anti-B cell antibody, rituximab; and the down-regulator of T-cell co-stimulation, abatacept. Unfortunately, all biologics are associated with an increased risk of infection (bacterial, viral, and fungal) and tuberculosis reactivation.

1.7.5 Rheumatoid Arthritis: Pathogenesis

Although the early events in RA are not well understood, it is now known that the onset of clinically detectable disease in the joints is preceded by a variable period of "benign autoimmunity," a period in which RA autoantibodies, such as rheumatoid factor and anti-citrullinated protein antibodies, are detectable in the serum (Rantapaa-Dahlqvist *et al*., 2003, Nielen *et al*., 2004).

At some point, undefined environmental factors trigger the synovial membrane of one or more joints to become inflamed. It is unknown whether RA autoantibodies preceding joint disease actually play a role in triggering the synovial inflammation. The reasons the immune/inflammatory response specifically targets the synovial membranes are also unclear. Over time, particularly if initiation of effective therapy is delayed, the inflammatory process becomes chronic and leads to the activation and proliferation of the synovial tissue in a process called pannus formation. Similar to the chronic inflammation of periodontitis, TNF-α, IL-1β, and other key proinflammatory cytokines play a key role in the activation, proliferation, and destructiveness of the synovial pannus (Firestein, 2003). The proliferative pannus tissue attaches to the adjacent cartilage and bone and the subsequent degradation of cartilage is mediated by matrix-degrading proteases, such as matrix metalloproteinases (MMPs). Osteoclastogenesis then occurs and ultimately, if the synovitis is not effectively controlled, this process completely destroys the articular cartilage and periarticular bone, causing severe deformity and functional loss.

Unlike periodontitis, in which a bacterial aetiology is well defined, a single specific aetiology for RA has not been determined, despite extensive investigation during the past

half century. The accepted model involves one or more environmental factors acting on a predisposing genetic background.

The major genetic risk for RA is now well known to reside in the human leukocyte antigen (HLA) locus, which is a cluster of genes that serves to shape adaptive immune responses mediated by T-cells. In RA, the strongest association is with the major histocompatibility complex, class II, DRβ1 (HLA-DRβ1) gene. Several variants of HLA-DRβ1 have been shown to be associated with RA (Marotte *et al*., 2006, Mattey *et al*., 2002). Intriguingly, the same variants also are associated with periodontitis (Ogrendik *et al*., 2005, Rosenstein *et al*., 2004).

This positively charged motif appears to facilitate the presentation of peptides containing the amino acid citrulline to T-cells (Rosenstein *et al*., 2004, Nibali *et al*., 2007). The amino acid citrulline is generated by an enzymatic modification of the amino acid arginine by a family of enzymes called peptidylarginine deiminase (PADs). Citrullination plays a physiologic role in the regulation of protein folding and degradation and is prominently involved in processes such as cornification of the skin. Interestingly the development of antibody responses to citrullinated peptides, is specific to RA (Hirsch *et al*., 1998, Templin *et al*., 1994)

Thus the generation of citrullinated peptides is certainly not unique to RA, but the development of antibody responses to citrullinated peptides is quite specific to RA. This discovery of the generation of anticyclic citrullinated peptide antibodies has been a major step forward in the understanding of RA pathogenesis and the genetic basis for this disease (Ogrendik *et al*., 2005, Rosenstein *et al*., 2004).

1.8 Rheumatoid Arthritis and Periodontitis

RA and chronic periodontitis are arguably the most prevalent chronic inflammatory diseases in humans and associated with significant morbidities. Periodontitis and RA are remarkably parallel disease processes that share not only some clinical features, but pathophysiologic, epidemiological, and therapeutic features as well (Bartold *et al*., 2005).

Whether RA is associated with the progression of existing inflammatory conditions, such as periodontitis, has historically been a controversial topic. A 1972 review of the literature

by Helminen-Pakkala indicated that periodontal disease does not seem to be a prominent feature in persons suffering from RA (Helminen-Pakkala, 1972). The author reported similar numbers of edentulous RA patients and control group subjects, and the number of missing teeth was similar in both groups.

1.8.1 Clinical Evidence for an Association between Rheumatoid Arthritis and Periodontal Disease

More recent clinical and animal studies have shown an association between RA and periodontal disease. The largest of these studies examined data from the Third National Health and Nutrition Examination Survey (NHANES III). This is a nationally representative cross-sectional survey of the non institutionalised civilian US population, which included home interviews and medical and dental examinations performed by physicians and dentists. Investigators found that those with RA had a higher rate of periodontitis (odds ratio [OR] = 4.13) than people without RA, independent of age, race/ethnicity, sex, and smoking (de Pablo *et al*., 2008).

In a pilot study, it was reported that subjects with aggressive periodontitis had a four-fold increased incidence of RA, although it must be noted that this study used a self-reported diagnosis of RA (Mercado *et al*., 2000). In a subsequent study, this same group performed careful dental exams on 65 subjects with validated RA and matched controls. They reported a significantly high prevalence of moderate to severe periodontitis in individuals with RA. The converse is also true: periodontitis patients have a higher prevalence of RA compared to the general population (Mercado *et al*., 2001).

Several other studies have suggested a relationship between RA and periodontitis; RA may have a negative impact on periodontal condition and vice versa (Kasser *et al*., 1997, Bozkurt *et al*., 2006, Biyikoglu *et al*., 2006, Pischon *et al*., 2008,).

One recent study examined the prevalence and severity of periodontitis in United States veterans with RA (Dissick *et al*., 2010). They found that moderate to severe periodontitis was more common and had greater severity in patients with RA (51%) compared with controls (26%). A comprehensive comparison of RA and periodontitis has also been published which concluded that “a general and underlying dysregulation of the host inflammatory response is present in both conditions seems very likely” (Mercado *et al*., 2003).

1.8.2 Pathogenesis Theories linking Rheumatoid Arthritis and Periodontitis

1.8.2.1 Genetics factors in RA and Periodontitis

Genetic factors are thought to contribute considerably (~50%) to the etiology of both RA and periodontitis (Michalowicz *et al*., 1991, MacGregor *et al*., 2000). In humans, many of the genes that regulate monocytic cytokine responses have been mapped to the HLA-DR region of chromosome 5 in the area of the TNF-β genes (Bendtzen *et al*., 1988). Both RA and progressive periodontitis are associated with this HLA complex (Ollier and Thomson, 1992), which suggests a genetic basis for the observed monocyte trait, linking RA, progressive periodontitis and other systemic diseases.

In both diseases, antigenic challenge (e.g. LPS) to the monocytic/lymphocytic axis would result in the secretion of catabolic cytokines and inflammatory mediators, of which prostaglandin E_2 (PGE_2), IL-1, TNF-α and IL-6 would appear to dominate (Mercado *et al*., 2003).

1.8.2.2 Inflammation in Rheumatoid Arthritis and Periodontitis

Both periodontitis and RA present an imbalance between pro-inflammatory and anti-inflammatory cytokines, which is deemed responsible for the tissue damage and, in this sense, both cellular and humoral immune reactions have been shown to contribute to the pathogenesis of the two diseases. Activation of monocytes by stimulated T-lymphocytes is a major initiator of the production of large amounts of TNF-α and IL-1β (Graves and Cochran, 2003, Page *et al*., 1997). These cytokines further stimulate the expression of adhesion molecules and other inflammatory mediators that amplify the local inflammatory reaction, leading to the generation of proinflammatory mediators such as cytokines, eicosanoids, proteolytic enzymes, activated oxygen, and nitrogen species and increased MMP production (Page *et al*., 1997, Cochran, 2008). Immune complexes and complement are the final common mediators of inflammation in both conditions, with the ultimate result being destruction of adjacent bone (Weissmann, 2004). Figure 2 illustrates a model of the complex relationships between RA and periodontitis.

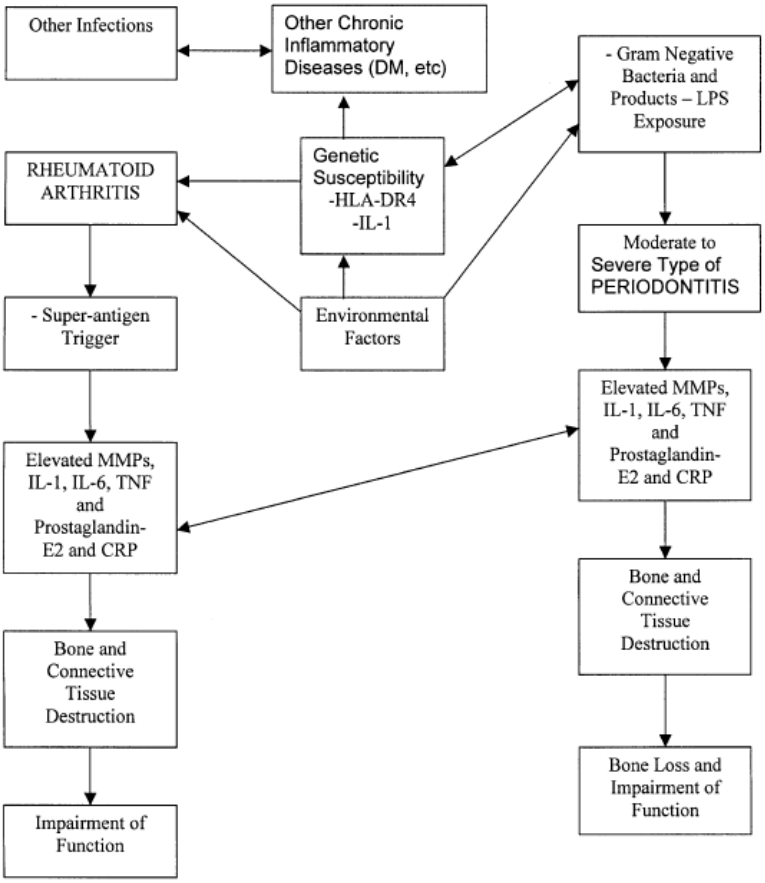

Figure 2. Common model of relationship between RA and periodontitis, other inflammatory diseases with genetic, environmental and microbial exposures as the common link (Mercado *et al*., 2003)

1.8.2.3 Autoimmunity in Rheumatoid Arthritis and Periodontitis

As previously stated, the amino acid citrulline is generated by an enzymatic modification of the amino acid arginine by a family of enzymes called PADs. This citrullination process is not unique to RA, however anticyclic citrullinated peptide antibodies are highly specific for RA (Hirsch *et al*., 1998, Templin *et al*., 1994)

It has been hypothesised that in a genetically susceptible host, environmental factors can potentially play a role in breaking tolerance to citrullinated autoantigens. One example is *P. gingivalis*, which is the only prokaryote known to express the PAD enzyme and is capable of citrullinating terminal arginine residues on peptides (Rosenstein *et al*., 2004). Thus, it is possible that the presence of *P. gingivalis*, in chronically inflamed periodontal tissue may lead to the local generation of citrullinated peptides. This results in an altered three-dimensional structure and function of that protein.

In such a chronic inflammatory oral lesion, presentation of citrullinated antigens to T-cells by local antigen presenting cells would be facilitated by a microenvironment rich in proinflammatory cytokines, such as TNF-α and IL-1β, which serve to stimulate and accelerate this process. In turn, through a process of molecular mimicry, the immune response would be directed toward other homologous citrullinated human autoantigens and become progressively amplified and evolved. One such autoantigen is the citrullinated enzyme α-enolase; a highly conserved, multifunctional protein that is substantially similar between human and prokaryotic cells. PAD provides a mechanism by which antibacterial antibodies cross-react with endogenous citrullinated proteins and initiate loss of tolerance (Kinloch *et al*., 2005).

Moreover, the same group of researchers have shown the human enzyme PAD and the bacterial enzyme PAD have striking homology in the immunodominant epitopes recognised by the immune system. These studies provide a conceptual framework around which chronic periodontitis, associated with *P. gingivalis*, could be involved in the initiation of the autoimmune processes that precede the onset of RA. This is illustrated in Figure 3.

Further evidence supporting the role of bacteria in RA pathogenesis has been found by Moen *et al* (2006), who detected oral bacterial DNAs in serum and synovial fluid of patients with RA. Patients with RA also have a significantly higher level of immunoglobulin G antibody against *P. gingivalis*, *P. intermedia*, and *T. forsythia* (Ramamurthy *et al*., 2005).

Figure 3. Schematic illustration of the etiological hypothesis for *P. gingivalis* and citrullinated α-enolase involvement in RA. A) Infection by *P. gingivalis* leads to citrullination in the gingiva of bacterial and/or human proteins (by either PPAD, human PAD, or both). In the presence of danger signals, such as LPS and DNA, pathogenic T-cells are activated by APCs (citrullinated antigen in the context of HLA-DRB1 SE). T-cell mediated activation of pathogenic B cells results in the production of antibodies specific for citrullinated proteins. B) A second inflammatory event occurs in the joint, leading to citrullination of joint proteins and the formation of immune complexes in the joint (through epitode spreading, cross-reactivity with citrullinated joint proteins, or both). The resulting perpetuation of the inflammatory process eventually causes chronic RA. (Lundberg *et al*., 2010)

Abbreviations: ACPA, anti-citrullinated protein/peptide antibody; APC,. antigen presenting cell; LPS, lipopolysaccharide; *P. gingivalis, Porphyromonas gingivalis*; PAD, peptidylargine deiminases; PPAD, *P. gingivalis* PAD; RA, rheumatoid arthritis; SE, shared epitope.

1.8.3 Rheumatoid Arthritis and Periodontitis Animal models: Periodontitis Primes Rheumatoid Arthritis and Vice Versa

Periodontal disease and RA share many common pathophysiologic features, but a clinical relationship between the two conditions remains controversial, in part because of the confounding effects of anti-inflammatory drug therapy universally used to treat RA. Animal models provide a method to overcome this. There are currently several animal models used in examining the link between RA and periodontitis.

Adjuvant arthritis (AA) is a well established model of RA in rats. One study found that induction of experimental arthritis in rats resulted in periodontal destruction and increased cytokines and MMPs in the periodontal tissues (Ramamurthy *et al*., 2005).

Park *et al* (2010) examined the cellular/molecular mechanisms of periodontal breakdown in a collagen-induced arthritis (CIA) model in mice to enhance the understanding of RA-associated alveolar bone loss in humans. Their results show that osteoclasts were induced in CIA and noted an increased apoptosis of osteoblasts leading to diminished bone formation. It is suggested that these mechanisms could account for the same outcome in human RA.

Most recently Bartold *et al* (2010) and Cantley *et al* (2011) developed a mouse model to assess the association between pre-existing periodontitis and experimental arthritis. They found mice with pre-existing periodontitis developed more severe arthritis, which developed at a faster rate. They discovered that severe arthritis developed more rapidly in animals with a pre-existing *P. gingivalis* induced inflammatory lesion, and that mice with experimental arthritis alone showed evidence of bone loss. They concluded that pre-existing periodontitis exacerbated experimental arthritis in a mouse model. Thus providing further evidence for a relationship between the presence of periodontal pathogen-associated inflammation and the development of RA (Bartold *et al*., 2010, Cantley *et al*., 2011).

One hypothesis that links RA and periodontitis is the published "two-hit" model that attempts to link experimental evidence from animal models and is supported by evidence from human clinical studies (Golub *et al*., 2006).

In this theory, the first “hit” involves the periodontopathic subgingival biofilm and its microbial products, such as endotoxin. The second “hit” involves a medical systemic disease, such as rheumatoid arthritis, which increases biomarkers of systemic inflammation in the circulation, including C -reactive protein (CRP), cytokines (e.g., IL-6), prostanoids (e.g., PGE_2), and matrix metalloproteinases (e.g., MMP-9), and TNF-α. These cytokines are thought to stimulate resident cells in the synovium and the periodontium to produce MMPs mediating connective tissue destruction, and to induce the differentiation and activity of osteoclasts to destroy bone (McGee *et al*., 1998). (See figure 4 below from Golub *et al* (2006)).

In particular, TNF-α, also promotes bone resorption: (i) by up-regulating inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO); and (ii) by modulating the RANKL in osteoblasts, and its antagonist OPG, thus altering the RANKL/OPG ratio, which enhances osteoclast activity, and, finally, lead to periodontal breakdown (Haynes, 2004) (See Figure 4).

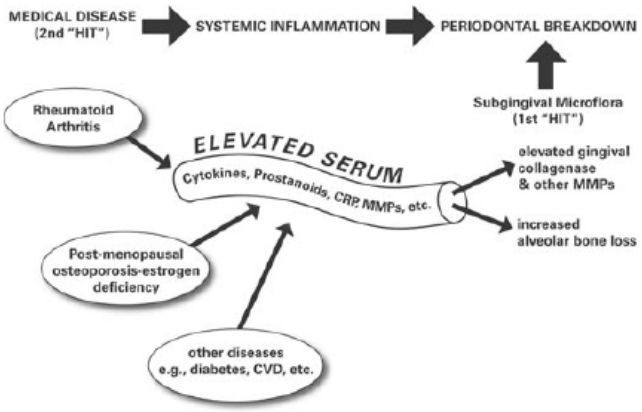

Figure 4. A hypothetical "two-hit" model of induction of chronic destructive periodontitis. The first "hit" involves the periodontopathic subgingival biofilm and its microbial products, such as endotoxin. The second "hit" involves a medical systemic disease, such as (but not limited to) rheumatic arthritis and post-menopausal osteoporosis, which increases biomarkers of systemic inflammation in the circulation, including C-reactive protein (CRP), cytokines (e.g. IL-6), prostanoids (e.g. PGE_2), and matrix metalloproteinases (e.g. MMP-9) (Golub *et al*., 2006)

1.8.4 Rheumatoid Arthritis and Bone Resorption Markers

RA synovial fluid has been shown to contain a wide range of effector molecules including pro-inflammatory cytokines (IL-1β, IL-6, TNF-α and IL-18), chemokines (such as IL-8, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), and regulated upon activation normal T-cell (RANTES), MMPs (MMP-1, -3, -9 and -13) and metabolic proteins (Cox-1, Cox-2 and iNOS). As previously described, this complex interaction is thought to cause a cycle of pro-inflammatory signals resulting in chronic and persistent inflammation (Muller-Ladner *et al*., 2005).

TNF-α is a prime inflammatory mediator. Importantly, the genes encoding TNF-α and many of the other factors mentioned above are now known to be under the control of NF-κB transcription factors, suggesting that RANK/RANKL pathway could be one of the master regulators of inflammatory cytokine production in RA (May and Ghosh, 1998). Studies demonstrate that synovial T-cells express RANKL mRNA *in vitro*, whereas tissue from normal patients did not express RANKL provided the insights into the role of RANKL in the pathogenesis of focal bone erosions, in patients with RA (Gravallese *et al*., 2000, Horwood *et al*., 1999). Other studies have provided compelling evidence that activated T-cells from the RA synovium and cultured synovial fibroblasts produce RANKL and RANKL mRNA (Takayanagi *et al*., 2000, Kong *et al*., 1999a). Further research has found that RA synovial tissues exhibit an increased ratio of RANKL/OPG mRNA expression, indicating that pro-osteoclastogenic conditions dominate within the microenvironment of the RA joint (Haynes *et al*., 2001).

The presence of activated RANK/RANKL transcription factors has also been demonstrated in cultured synovial fibroblasts (Yamasaki *et al*., 2001), human arthritic joints (Benito *et al*., 2004) and the joints of animals with experimentally induced RA (Han *et al*., 1998). In addition, ELISA analysis has shown increased levels of RANKL and OPG in the joints and in the sera of patients with RA (Ziolkowska *et al*., 2002, Vanderborght *et al*., 2004, Romas *et al*., 2002, Geusens *et al*., 2006).

Taken together, these studies indicate that excess production of RANKL by activated T-cells, which play a central role in the pathogenesis of RA, may contribute to focal bone erosions and periarticular bone loss in RA.

OPG, the decoy receptor for RANKL, acts as a key negative regulator of osteoclastogenesis by binding soluble RANKL and preventing its binding to its receptor RANK, thereby inhibiting osteoclast differentiation. In a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation accompanied by bone and cartilage destruction, administration of OPG prevented bone and cartilage destruction but not inflammation, indicating the ability of OPG to block RANK signalling and focal bone erosion (Kong *et al*., 1999a, Schett *et al*., 2003).

In RA, the balance between RANKL and OPG expression levels is a fundamental regulator of osteoclast differentiation and function (Skoumal *et al*., 2005, Geusens *et al*., 2006). Thus, targeting the RANKL/RANK/OPG pathway to inhibit osteoclast differentiation represents an important potential therapeutic strategy to prevent bone erosion in inflammatory arthritis.

1.8.5 Gingival Crevicular Fluid Studies and Rheumatoid Arthritis

Numerous studies have used GCF and gingival tissues to examine the levels of various Th1 and Th2 cytokines in patients with RA and chronic periodontitis (Masada *et al*., 1990, Rossomando *et al*., 1990, Bozkurt *et al*., 2000).

IL-6 is a proinflammatory cytokine, which has long been assumed to be involved in the pathology of periodontal disease. It was reported that IL-6 levels in inflamed gingival tissues were higher than those in healthy control tissues (Bartold and Haynes, 1991). Similarly the amounts of IL-6 in gingival crevicular fluid samples are significantly correlated with probing depth and bleeding indices, and furthermore with sites which showed further attachment loss during periodontal therapy compared to remaining sites (Geivelis *et al*., 1993). IL-6 is also assumed to be involved in the pathogenesis of rheumatic arthritis (Park and Pillinger, 2007).

In 2000, Bozkurt *et al* were the first to investigate the relationship between IL-6 levels in GCF of RA patients (Bozkurt *et al*., 2000). They found there was no significant difference between IL-6 levels and periodontal status when compared to patients with chronic perodontitis. In a later study, the same group examined the levels of the Th2 cytokines IL-4 and IL-10 cytokines in periodontitis and RA patients. Preliminary data suggested that there

was a lack or inappropriate response of these anti-inflammatory cytokines in these patient groups (Bozkurt *et al*., 2006, Biyikoglu *et al*., 2006, Biyikoglu *et al*., 2009).

Biyikoglu *et al* (2006) found no differences in the GCF levels of PGE_2 and IL-1β between periodontitis patients and RA individuals with a long history of usage of corticosteroids and methotrexate. The same authors, several years later, also examined MMP-8 and -13 and tissue inhibitor of metalloproteinases-1 (TIMP-1) levels in GCF in the same two groups and discovered that the coexistence of RA and periodontitis did not significantly affect levels (Biyikoglu *et al*., 2009).

When considering the current literature it can be concluded that these studies show that GCF sampling in RA patients is an acceptable method for measuring cytokine levels and that to date RANKL and OPG have not yet been tested in GCF of patients with RA.

1.9 Periodontal Treatment Affects Rheumatoid Arthritis

To determine if eliminating periodontal infection and gingival inflammation affects the severity of active RA in patients with chronic inflammatory periodontal disease, one study examined patients with both diseases and completed scaling/root planing and plaque control, and then compared erythrocyte sedimentation rate, periodontal indices and before and after Disease Activity Scores (DAS). They concluded that periodontal treatment may reduce the severity of RA. There was also a reported subjective improvement in treated patients (Al-Katma *et al*., 2007).

A second clinical study also suggested that the treatment of periodontal disease might have a significant impact on RA severity (Ribeiro *et al*., 2005). In a small cohort of patients with RA with periodontitis, full-mouth scaling and root planing resulted in significantly reduced periodontitis severity in addition to significant reductions in ESR and DAS28 scores compared to those receiving oral hygiene instruction only (Ribeiro *et al*., 2005).

Similarly, subjects with RA have significantly increased periodontal attachment loss compared to controls, and oral hygiene may only partially account for this association (Pischon *et al*., 2008). Therefore, it is possible that the prevention and appropriate treatment of periodontitis could improve RA disease activity.

Recently another study confirmed that control of periodontal infection and inflammation by means of scaling and root planing and oral hygiene in subjects with moderate to severe periodontal disease might contribute to a reduction in the signs and symptoms of active RA as well as to a reduction in the serum levels of TNF-α (Ortiz *et al*., 2009).

However, these studies are equivocal and potentially confounded by competing compliance between the required regular follow-up for patients with RA and dental care. Two studies have reported the relative lack of dental care and resulting poor oral hygiene as explanations for the higher frequency of periodontitis occurring in patients with RA compared to controls (de Pablo *et al*., 2008, Pischon *et al*., 2008).

1.10 Effect of Rheumatoid Arthritis Treatment on Bone Remodelling and Periodontitis

The traditional drugs used to treat RA include NSAIDs, corticosteroids, and DMARDs such as methotrexate. This intensive, systemic anti-inflammatory therapy, which is aimed at reducing RA disease activity, retarding joint erosions, and improving the patient's quality of life, has the potential to also interfere with destructive processes in the periodontium (Kasser *et al*., 1997).

One study validating this link found that methotrexate, sulfasalazine (SSZ), infliximab, and IL-4 inhibited human osteoclastogenesis by modulating the interaction of RANKL, RANK, and OPG, primarily by decreasing expression of RANKL and increasing expression of OPG (Lee *et al*., 2004).

Another study examined the effect of glucocorticoids and showed that their anti-inflammatory efficacy is accompanied by deleterious effects on bone. These effects include up-regulation of the RANKL/OPG ratio to promote bone-resorbing osteoclasts and include inhibition of bone-forming osteoblasts (Rauch *et al*., 2011).

Biyikoglu *et al* (2006) found no differences in the GCF levels of PGE_2 and IL-1β between periodontitis patients and RA individuals with a long history of usage of corticosteroids and methotrexate.

In an effort to evaluate the effect of rheumatologic treatment on periodontal inflammation, Miranda *et al* (2007) examined GCF levels of IL-1β, IL-18 and elastase activity in patients

with RA and compared them to healthy controls. They discovered that the anti-inflammatory treatment taken by RA patients might influence the periodontal inflammation status represented by IL-1β and elastase in the GCF and that these findings probably are a consequence of the intensive anti-inflammatory treatment (NSAIDs, prednisone, and methotrexate) received by RA individuals (Miranda *et al*., 2007).

In the past decade, the introduction of targeted biologic therapy has resulted in significantly improved clinical and structural outcomes for patients with RA. These therapeutic agents have specific mechanisms of action, including inhibiting the action of individual cytokines, blocking cell-cell interactions, and depleting certain cell types.

As described previously, the therapeutic potential of blocking the biologic actions of RANKL with therapeutics such as OPG-Fc and the RANKL antibody Denosumab, suggest the promise of osteoclast inhibition as a therapeutic modality to protect against focal and systemic bone loss in RA, osteoporosis, periodontitis and other diseases in which bone is destroyed (Body *et al*., 2003).

TNF-α is one of the key cytokines in rheumatoid inflammation and is present in serum and inflammatory tissues; its levels correlate with disease activity and the degree of tissue damage by upregulating osteoclastogenesis and down regulating osteoblastogenesis (Nanes, 2003).

Because of its role in inflammation, TNFα is now a target for various biologic agents that are now used to treat RA, and the use of these TNFα antagonists in patients with active early RA has been shown to significantly reduce clinical signs of disease activity and effect clinical remission in some patients (Bathon *et al*., 2000, Breedveld *et al*., 2006).

Studies in animals (Di Paola *et al*., 2007) and in humans (Mercado *et al*., 2001, Pers *et al*., 2008) suggested that anti-TNF-α therapy may also reduce the severity of periodontitis.

Recently a study (Mayer *et al*., 2009) evaluated the influence of anti-TNF-α therapy on the clinical and immunologic parameters of the periodontium. In this study, patients with RA receiving anti-TNF-α medication had lower periodontal indices and GCF TNF-α levels. This supports the hypothesis that suppression of proinflammatory cytokines might prove beneficial in suppressing periodontal diseases.

1.11 Conclusion

Bone resorption is a key factor of both RA and periodontitis. A balance between bone resorption by osteoclasts and bone formation by osteoblasts determines the level of bone mass. The cellular and molecular mechanisms remain complex (Petropoulos *et al*., 2004) but RANKL and OPG, which are both modulated by inflammatory cytokines (Nakashima *et al*., 2000), are believed to be the key regulators in bone metabolism and alveolar bone destruction in periodontitis and RA (Crotti *et al*., 2003, Kearns *et al*., 2008).

Increased RANKL expression and/or a decreased OPG production, and, hence a high ratio of RANKL-to-OPG expression, has been reported in periodontal disease and in RA (Kearns *et al*., 2008, Cochran, 2008).

Investigating the ratio of OPG and RANKL in human GCF in patients with rheumatoid arthritis and periodontitis and comparing these levels to either patients with periodontitis or rheumatoid arthritis alone may help in explain how these two diseases are linked. Further to this it may assist in developing a test capable of detecting subclinical levels of RA in patients with periodontitis, may be useful in determining disease activity and provide further evidence that supports a "two hit" theory linking the two diseases.

1.12 Aims of the Study

The aim of this study was to evaluate influence of rheumatoid arthritis on the expression of RANKL and OPG in GCF of patients with and without chronic periodontitis.

1.13 Hypothesis

Our hypothesis was that at sites of periodontal destruction, levels of RANKL in GCF will be increased in patients with both rheumatoid arthritis and periodontitis compared to either periodontitis or rheumatoid arthritis alone.

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Chapter 2. "Expression of Bone Resorption Markers in Gingival Crevicular Fluid of Rheumatoid Arthritis Patients with and without Periodontitis. A Pilot Study"

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2.1 Introduction

Periodontitis is a chronic inflammatory disease of the supporting structures of teeth that is present in a severe form in 15% to 20% of the general population (Brown and Loe, 1993) and is characterised by progressive destruction of the tissues supporting the teeth (Listgarten, 1986). Whilst the aetiology of periodontitis is bacterial, relating to the accumulation of subgingival plaque, it is becoming clear that the pathogenesis of the disease represents a disequilibrium between the plaque biofilm and the host immune response, resulting in the overexpression of proinflammatory cytokines, with subsequent destruction of periodontal connective tissue and alveolar bone (Kornman, 2008). The severity of periodontitis is dependant upon a dynamic equilibrium of host-bacterium interactions, which are significantly influenced by various genetic, epigenetic and environmental factors in a susceptible host (Page and Beck, 1997, Kornman, 2008, Gomez *et al*., 2009).

Rheumatoid arthritis (RA) is also a chronic destructive inflammatory disorder. It is characterised by inflammation and tissue damage in the synovial membrane, leading to synovitis, pannus formation and subsequent joint destruction. A single specific aetiology for RA has not been determined. It is thought to be a complex disease in which multiple genetic and environmental factors are involved. RA affects approximately 1% of the population, with a female to male ratio of 3:1 (Spector, 1990). Over time it can lead to joint damage, loss of function and disability.

The effects of periodontal disease not just confined to the periodontium, it is thought that inflammatory mediators of periodontal origin, as well as periodontal bacteria and their products, may have widespread systemic effects. In particular, periodontitis has been implicated as a potential risk factor for various systemic conditions including cardiovascular disease, cerebrovascular disease (Janket *et al*., 2003), diabetes (Taylor *et al*., 1996), respiratory disease (Scannapieco and Mylotte, 1996), adverse pregnancy outcomes (Scannapieco *et al*., 2003, Offenbacher *et al*., 1996) and cancer (Hujoel *et al*., 2003).

There has also been a growing interest in the relationship between periodontitis and RA. They are reported to share numerous pathogenic characteristics (Mercado *et al*., 2003) and a number of studies have found a potential association between RA and periodontitis, with

reports that the prevalence of RA is greater in periodontitis patients, and that RA patients have more severe periodontitis (Mercado *et al*., 2000, Mercado *et al*., 2001, de Pablo *et al*., 2008, Pischon *et al*., 2008). Most recently Bartold *et al* (2010) and Cantley *et al* (2011) developed a mouse model to assess the association between pre-existing periodontitis and experimental arthritis. They found mice with pre-existing periodontitis developed more severe arthritis, which developed at a faster rate. Thus, providing further evidence for a relationship between the presence of periodontal pathogen-associated inflammation and the development of rheumatoid arthritis (Bartold *et al*., 2010, Cantley *et al*., 2011).

It also appears that periodontal treatment may reduce the severity of RA. Al Katma *et al* (2007) examined patients with both diseases, completed scaling and plaque control and then compared erythrocyte sedimentation rate, periodontal indices and RA disease activity scores (DAS28) before and after treatment. They concluded that periodontal treatment may reduce the severity of RA. There was also a reported subjective improvement in treated patients (Al-Katma *et al*., 2007). Recently, another study confirmed that control of periodontal infection and inflammation by means of scaling and root planing and oral hygiene in subjects with moderate to severe periodontal disease might contribute to a reduction in the signs and symptoms of active RA as well as lead to a reduction in the serum levels of tumor necrosis factor (TNF)-α (Ortiz *et al*., 2009). Furthermore, both diseases are characterised by the presence of similar pro-inflammatory mediators, including prostaglandins, cytokines, matrix metalloproteinases, as well as inflammatory cell types, which, among other functions, serve as regulators of bone turnover (Bozkurt *et al*., 2000, Mercado *et al*., 2003, Petropoulos *et al*., 2004).

Bone is a dynamic tissue that is continuously remodelled by bone-resorbing osteoclasts and bone-forming osteoblasts (Zaidi, 2007). In both periodontitis and RA, alveolar bone or joint destruction is due to an inflammatory induced perturbation of this process, which is regulated at both the systemic and local levels by several hormones, growth factors and cytokines (Hofbauer and Heufelder, 2001, Cheung *et al*., 2003). The key molecular factors involved in the control of bone remodelling include receptor activator of nuclear factor-kappa B (RANK), its ligand (RANKL) and osteoprotegerin (OPG); the decoy receptor for RANKL (Gallagher, 2008). RANKL, a membrane-bound or soluble protein (sRANKL) belonging to the tumor necrosis factor (TNF) superfamily, is primarily produced in cells of osteoblastic lineages and activated T-cells. Binding of RANKL to its cellular receptor RANK, which is expressed on the surfaces of osteoclasts and their precursors, promotes

osteoclast differentiation and activation, and inhibits osteoclast apoptosis (Yasuda *et al*., 1998b). The effects of RANKL are counteracted by OPG which is secreted by various tissues and acts as an endogenous soluble receptor antagonist (Simonet *et al*., 1997, Yasuda *et al*., 1998a). When OPG is secreted in sufficient quantities, RANKL-RANK binding is diminished and bone resorption is suppressed. In both periodontitis and RA, abnormalities of the RANKL-OPG axis have been implicated in disease progression. In RA patients, a high serum RANKL/OPG ratio is associated with increased bone resorption (Pilichou *et al*., 2008). In adjuvant arthritis animal models increased serum RANKL levels have been associated with local bone erosion and systemic bone loss (Stolina *et al*., 2005). In addition, serum RANKL levels in RA patients positively correlate with disease progression (Oelzner *et al*., 2007). In patients with periodontitis, it has been demonstrated that the gingival tissues exhibit higher expression of RANKL (Crotti *et al*., 2003, Vernal *et al*., 2004, Bostanci *et al*., 2007b) and lower expression of OPG (Liu *et al*., 2003) compared with healthy controls. Similar results have been reported for levels in gingival crevicular fluid (GCF) (Mogi *et al*. 2004, Bostanci *et al*. 2007a). As a consequence, RANKL/OPG ratios are found to be increased in periodontitis patients compared with healthy controls (Mogi *et al*., 2004, Liu *et al*., 2003, Bostanci *et al*., 2007a, Bostanci *et al*., 2007b). In a group of untreated periodontitis patients, Vernal *et al* (2004) showed that higher levels of RANKL in GCF were associated with active sites compared with inactive sites. Thus, the RANKL/OPG ratio regulates the equilibrium between bone formation and bone resorption, and can be used as a marker of disease activity in RA and periodontitis (Catrina *et al*., 2006, Wara-aswapati *et al*., 2007, Bostanci *et al*., 2007a).

Although several studies have established a relationship between RA and periodontitis, the exact mechanisms underlying the relationship are currently unknown. The coexistence of periodontal disease and RA in patients offers a unique opportunity to study the possible influence of RA on the periodontal inflammatory process and vice versa. Therefore, the objective of this study was to evaluate the influence of rheumatoid arthritis on the expression of RANKL in GCF of patients with and without chronic periodontitis. We hypothesised that at sites of periodontal destruction, levels of RANKL in GCF would be increased in patients with both rheumatoid arthritis and periodontitis compared to either periodontitis or rheumatoid arthritis alone.

2.2 Methods and Materials

2.2.1 Patient Selection

2.2.1.1 Study Population and Clinical Examination

Subjects were selected from patients and staff members in the Department of Periodontology at the University of Adelaide, or from patients attending the Royal Adelaide Hospital Rheumatology Clinic. Written and informed consent was obtained from each subject before enrolment in the study. Complete medical histories were recorded for all subjects. Subjects were excluded if they were current smokers, had a history of cardiovascular or respiratory disease, diabetes mellitus, hepatitis or HIV infection, immunosuppressive chemotherapy, current pregnancy or lactation, or use of antibiotics at least 3 months prior to the study. The study was approved by the Human Ethics Research Committee of the University of Adelaide.

All subjects underwent a periodontal examination; including the measurement of probing pocket depth (PPD) and clinical attachment level (CAL) at six sites around each tooth, using a periodontal probe with William's markings. The periodontal condition was classified according to the clinical and radiographic criteria proposed by the 1999 International World Workshop for a Classification of Periodontal Disease and Conditions (Armitage, 1999). This system grades the severity of periodontitis by the amount of clinical attachment loss, and also categorises periodontitis as either localised (if ≤30% of the teeth are affected by periodontitis) or generalised (if >30% of sites are affected). No patients had received any periodontal therapy in the preceding 6 months.

All patients with RA had been diagnosed according to the criteria of the American College of Rheumatology (Arnett *et al*., 1988) and were receiving anti-rheumatic drug therapy which included various combinations of non-steroidal anti-inflammatory drugs (NSAIDS), disease modifying anti-rheumatic drugs (DMARDS) or corticosteroids.

A total of 63 subjects were recruited, and were classified into four categories: patients who were periodontally and systemically healthy; patients with chronic periodontitis only, patients diagnosed with RA but without signs of periodontal disease; and patients with both chronic periodontitis and RA.

2.2.2 Site Selection and GCF Sampling

2.2.2.1 GCF Sampling

GCF samples were taken from the two periodontal sites with the deepest pockets using a modification of previously published methods (Offenbacher *et al*. 1986). Briefly, the area was isolated, all detectable supragingival plaque was carefully removed, and the site was gently air-dried to prevent salivary contamination. GCF was obtained using sterile filter paper strips (Periopaper™; Oraflow, New York, NY, USA) that were carefully inserted into the gingival crevice until mild resistance was felt, and left in place for 30 seconds. Strips that were contaminated with blood or debris were discarded.

The volume of GCF collected was measured by immediate transfer to a Periotron 8000 (Harco, Tustin, CA, USA). These readings were then converted to an actual volume (microlitres) by reference to a standard curve generated during monthly calibration (as per manufacturer's instructions).

The paper strips from the individual sites were wrapped in foil, placed in microcentrifuge tubes and stored at -20°C until processed. GCF was eluted from the paper strips with phosphate-buffered saline, pH 7.2 as described previously (Megson *et al*., 2010). Briefly, each strip was individually eluted in 225 µl phosphate buffered saline in microtitre plates by incubating at room temperature on a shaking platform for 30 minutes. Eluted samples were then transferred to microcentrifuge tubes containing a further 225 µL (total volume 450 µL) and stored at -20°C until further analysis.

2.2.3 sRANKL Analysis in GCF

2.2.3.1 Enzyme Immunoassay

The amount of total soluble RANKL (sRANKL) in GCF was determined using commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits (Biomedica, Vienna, Austria) in accordance with the manufacturers' instructions. Briefly, samples and standards were added in duplicate to 96-well plates precoated with appropriate antibodies. Detection antibody was then added and incubated at 4°C overnight (18 to 24 hours). After washing, conjugated antibody was added and incubated for 60 minutes at room temperature. The plates were washed again, and the substrate was added and incubated for 30 minutes at room temperature in the dark allowing for optimum optical density to

develop. The stop solution was then added and the optical density was read at 450 nm with 650 nm as a reference wavelength using a BioTek Powerwave microplate reader (BioTek Instruments, Winooski, VT, USA). Standard curves were then generated using KC4 software (BioTek Instruments) and used to determine the sRANKL concentration in each eluted sample. The concentration of RANKL in GCF was determined by dividing the total amount by the volume of GCF collected. The total amount of sRANKL per strip was calculated by dividing the concentration by the total elution volume (0.450 mL). The lower detection threshold for the sRANKL ELISA was 1.6 pg/ml. Data were reported as the amount of sRANKL (pg) or concentration (pg/μL).

2.2.4 Statistical Analysis

The distribution of age, recession, PPD, and GCF volume were compared between groups using the non-parametric Kruskal-Wallis test. To further explore differences between groups for age, recession and pocket depth, post-hoc Mann Whitney tests were performed. Descriptive statistics and frequency distribution curves were generated for sRANKL. Since the data were not normally distributed (right skewed) they were log-transformed prior to analysis. To compare log RANKL values between the groups (healthy, periodontitis only, RA only, RA and periodontitis), a linear generalised estimating equation was fitted to the data. This was chosen so as to account for the dependence in repeated measurements (2 sites) from the same subject. An independent working correlation matrix was assumed in the model. Wald test analysis was then used to compare the mean log total RANKL amount and concentrations between the four groups (with the null hypothesis assuming the means were the same in the 4 groups). All calculations were performed using SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA).

2.3 Results

As described, 63 patients were recruited and separated into four groups; healthy, periodontitis only, RA only, and both periodontitis and RA. None of the patients were current smokers. The clinical periodontal measurements at the GCF sampling sites are presented in Table 1.

Subsequent analysis of age, gingival PPD and recession demonstrated statistically significant different distributions between the four groups ($p < 0.05$). As expected, the periodontitis only and RA and periodontitis group had significantly higher mean PPD and

recession scores at sampling sites than the healthy and RA only groups ($p < 0.05$). No significant differences in recession were detected between healthy and RA only groups, periodontitis only and RA only groups, and periodontitis only and RA and periodontitis group (>0.05). Furthermore, there was no difference in GCF volume between the four groups ($p = 0.48$).

Table 1. Demographic and clinical data of subject groups

	Healthy	Periodontitis	RA	RA and Periodontitis
Number of subjects	15	15	17	16
Number of female:male	10:05	10:05	14:03	11:05
Age (years) *	35.3 ± 10	47.9 ± 13.8 §	56.9 ± 18.8 § †	57.8 ± 13.9 § †
Age Range (years)	19 - 55	39 - 82	22 - 90	20- 84
PPD of sites sampled (mm) *	1.6 ± 0.49	5.6 ± 0.97 §	2.6 ± 0.49 § †	4.8 ± 0.78 § † ‡
Recession (mm) *	0.59 ± 0.57	0.97 ± 0.61 §	0.74 ± 0.71	1.22 ± 0.66 § ‡
GCF Volume (µl)	0.47 ± 0.35	0.55 ± 0.49	0.41 ± 0.22	0.36 ± 0.26

* Values represent mean ± SD

§ Statistically different from healthy group ($p < 0.05$ Mann Whitney test)

† Statistically different from periodontitis groups. ($p < 0.05$ Mann Whitney test)

‡ Statistically different from RA group ($p < 0.05$ Mann Whitney tests)

2.3.1 Comparison of RANKL Concentrations Between Groups

sRANKL was detected in 27 out of 30 sites (90%) in both the healthy group and the periodontitis group samples. In the RA group RANKL was detected in 33 from 34 sites (97%) of the samples. All samples from the periodontitis and RA group contained sRANKL.

Healthy, periodontitis only, RA only and periodontitis and RA groups demonstrated median sRANKL levels of 15.06 pg/µl of GCF, 33.03 pg/µl, 35.67 pg/µl, and 37.28 pg/µl respectively (Figure 1). Although the average medians appear similar, there was a trend of increasing concentration of sRANKL with disease states, with the highest in the RA and periodontitis group. Due to the right skewed nature to the data it was analysed using logarithmic adjusted values.

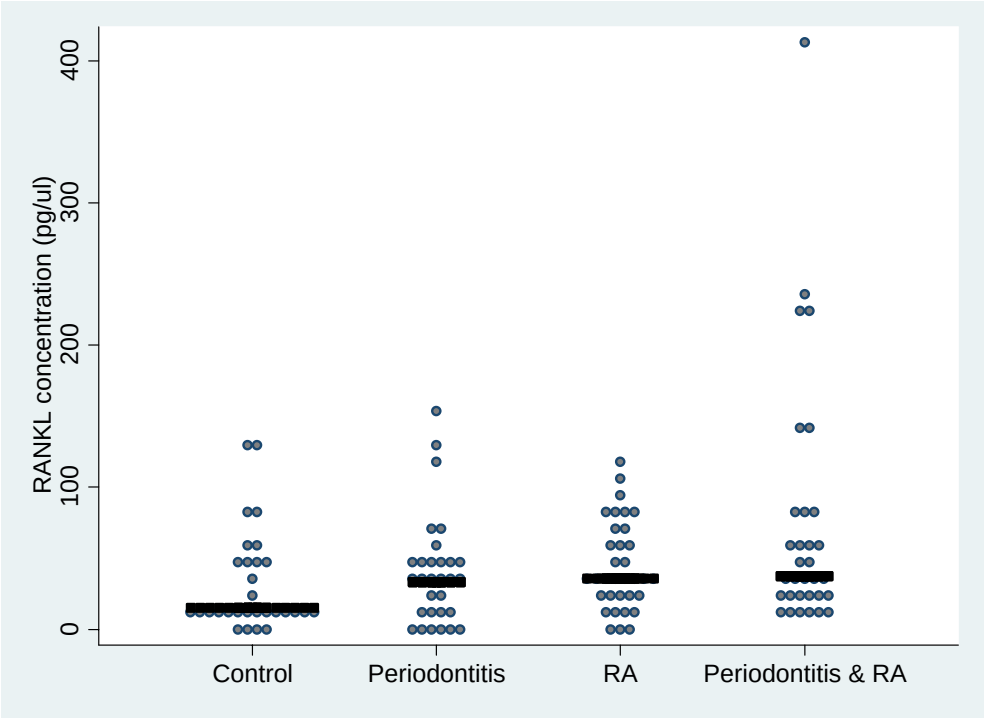

Figure 1. Distribution of RANKL levels (pg/µl) in GCF from healthy, periodontitis, RA, RA and periodontitis groups. Bars represent median RANKL concentration (pg/µl of GCF).

Table 2 shows the log adjusted mean concentration of sRANKL for all groups. Results showed that the difference in mean log RANKL between the four groups approached significance (p = 0.07) but is not considered statistically significant.

Table 2. RANKL concentration (pg/µl) across different groups.

	Log–transformed Adjusted Means	
	Mean log RANKL	**Standard Error**
Healthy	2.9981	0.2517
Periodontitis	3.2469	0.2715
RA	3.5305	0.2062
RA and periodontitis	3.8315	0.2138

2.3.2 Comparison of Total RANKL Values Between Groups

The total amount (pg) of RANKL in GCF sample per sampling time were analysed using the same statistical approach as used for concentrations. Variations in the total amount (pg) of sRANKL in all groups are plotted in Figure 2. Similar to the results for the concentration, the total amount of sRANKL shows an increasing trend in the amount of sRANKL, with the highest levels found in the periodontitis and RA group. Due to the right skewed nature of the data logarithmic adjusted analysis was performed.

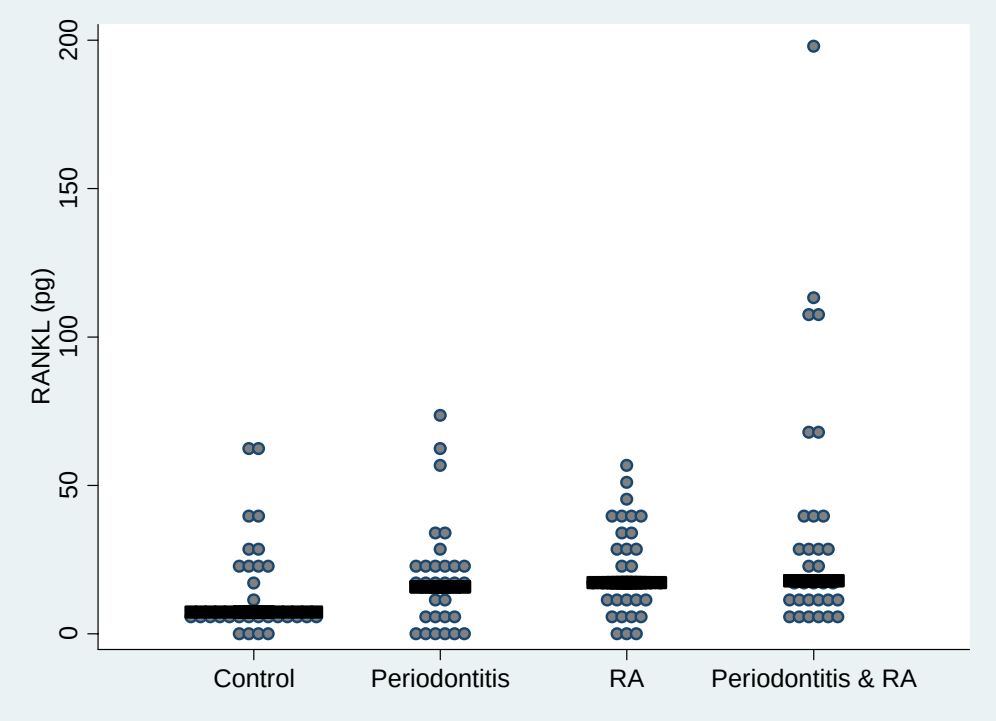

Figure 2. Distribution of amount of RANKL (pg) in GCF from healthy, periodontitis, RA, RA and periodontitis groups. Bars represent median total amount of RANKL (pg).

Table 3 shows the log adjusted mean total amount RANKL of all groups. The log transformed sRANKL amount is higher in the RA and periodontitis group but there was no statistical significance found between any of the groups. The differences between the four groups approached significance ($p = 0.08$), but are not considered statistically significant.

Table 3. RANKL amount (pg) across different groups.

	Log–transformed Adjusted Means	
	Mean log RANKL	**Standard Error**
Healthy	2.4330	0.2162
Periodontitis	2.6621	0.2286
RA	2.8935	0.1805
RA and periodontitis	3.1626	0.2039

2.4 Discussion

In the present study, we analysed levels of sRANKL in the GCF of RA patients with and without chronic periodontitis. Since RA, a chronic inflammatory disorder, has a positive association with periodontitis, (Mercado *et al.*, 2000, 2001, Pischon *et al.*, 2008, Cantley *et al*., 2011) we hypothesized that the presence of RANKL in areas of periodontal inflammation would be influenced by increased systemic levels of RANKL associated with RA. Although sRANKL was detected in nearly all samples obtained, and there was a trend of increasing amounts (pg) and concentrations (pg/μl) of sRANKL, with the lowest in the healthy, followed by periodontitis, RA, and RA and periodontitis groups, there was no statistically significant difference between sRANKL in any of the groups.

RANKL was studied because elevated levels of this mediator occurs in the serum of RA patients, (Simmonds and Foxwell, 2008) and in the GCF of patients with periodontitis (Bostanci *et al*., 2007a, Lu *et al*., 2006, Vernal *et al*., 2004). To our knowledge, this is the first study to (1) examine levels of sRANKL in the GCF of periodontally healthy patients with RA and compare to patients with chronic periodontitis, and, (2) analyse the possible effect of comorbidity of periodontitis and RA on sRANKL levels in the GCF.

Clinical recording and analysis of our groups revealed that GCF volumes were similar for each group, a finding that is contrary to what is often observed, as GCF flow is usually increased at periodontally involved sites (Griffiths, 2003). RA is a chronic disease and is more frequently identified later in life; it is not surprising then that the ages of RA only group and the RA and periodontitis group were not statistically different. Furthermore, recession between the healthy group and the RA group and between the periodontitis only group and the periodontitis and RA group was not significantly different. It is also worth

noting that the recession between the periodontitis only group and RA only group were not significantly different ($p = 0.2$). It would be convenient to surmise that this may be because systemic inflammation in RA patients may contribute to recession, but unfortunately low patient numbers and the difference of mean (unadjusted) recession scores (0.23 mm) in the current study make it unfeasible to draw such conclusions.

This study demonstrated that GCF sRANKL concentrations and detection rates were similar among our groups. A study by Lu *et al* (2006) had similar results to ours, where RANKL was detected in 93% of healthy and 100% of periodontitis patients.

However these results are inconsistent with other previously published studies examining periodontally healthy and diseased patients. Bostanci *et al* (2007a) found that RANKL was detected in only 33% of healthy patients compared to 100% of chronic periodontitis patients. They found significant differences in the concentrations between healthy and diseased groups (Bostanci *et al*., 2007a). A study by Vernal *et al* (2004) found that RANKL was detected in only 46% healthy patients, but in 85% of sites from chronic periodontitis patients. However, they reported that that, the concentrations (pg/µl) of RANKL were higher in the healthy group than the periodontitis group, but when examining the amount present (pg/site) the diseased groups had significantly higher levels compared to the healthy subjects (Vernal *et al*., 2004).

There are several reasons which may explain these differences between our study and previous studies. There are numerous commercially available ELISA kits to test for RANKL, each with varying limits of detection, and a number of manufacturer's ELISA kits have previously been used. The final Biomedica kit used in this study was based on the sensitivity of the assay and consistent detection rate in GCF samples. It should also be noted that some previous researchers tested GCF for total RANKL as opposed to sRANKL which may account for the discrepancies in amounts reported. The data analysis in the current study did not combine the two site values for each patient, unlike a number of other studies. Instead we used all the data collected and accounted for these repeated measures in the statistical analysis, thus providing site specific data and a wider overview of the presence of sRANKL in the GCF of these subject groups.

Our observations of a lack of differences in markers of inflammation between patients with RA and periodontitis is consistent with two studies by Biyikoglu *et al* (2006, 2009). They

found that the co-existence of RA and periodontitis did not significantly affect levels of matrix metalloproteinases (MMP) -8, MMP-13 (collagenases) tissue inhibitors of MMPs -1 (TIMP-1), IL-1β and PGE_2 and it was noted that all RA patients in those studies were taking anti-inflammatory drugs. Similarly, Miranda *et al* (2007) found that GCF levels of Interleukin (IL) 1β and elastase were significantly lower in patients taking DMARDS, and concluded that the anti-inflammatory treatment taken for RA may have been a contributing factor to this finding. This literature suggests that the intensive, systemic anti-inflammatory therapy which is aimed at reducing RA disease activity, retarding joint erosions, and improving the patient's quality of life, may also interfere with destructive processes in the periodontium.

Traditionally the drugs used to treat RA include non-steroidal anti-inflammatory (NSAIDS), corticosteroids, and DMARDS, such as methotrexate (Majithia and Geraci, 2007). The mechanisms of action of these drugs are only partially understood; however, it is known that they modulate the cytokine network (Barrera *et al*., 1996) and inhibit human osteoclastogenesis by modulating the interaction of RANKL, RANK, and OPG (Lee *et al.*, 2004, Lien *et al*., 2010). This is further supported by a previous study which demonstrated that GCF levels of RANKL and OPG were significantly decreased in a group of chronic periodontitis patients that were immunosuppressed (cyclosporine-A) compared to chronic periodontitis patients (Bostanci *et al*., 2007a).

Although in our study it is difficult to evaluate the contribution that the individual immunomodulatory drugs or DMARDS have on the regulation of RANKL expression, it is possible that they had a significant impact on the results. Given the above mentioned studies, it is plausible that DMARDS could have affected the GCF concentrations of sRANKL. It may be speculated that if these patients had not been receiving NSAIDs, corticosteroids and DMARDs, the sRANKL levels in our RA patient cohort would have been higher.

Our results can be interpreted in several ways; firstly, it is possible that the RANKL/OPG axis is not part of the underlying mechanism that links RA and periodontitis. Secondly, it is also possible that (locally produced) GCF levels of RANKL are not influenced by the high systemic/serum levels of RANKL normally associated with RA. Thirdly, the long term use of antirheumatic drugs may suppress sRANKL levels, thereby resulting in a lack of difference in RANKL levels in patients with RA. Fourthly, if anti-RA therapy influences

the underlying mechanisms of periodontitis then it is possible that the deep PPD found are from a period prior to RA treatment, and, therefore 'inactive' periodontal pockets would have been sampled, thus resulting in lower RANKL levels. Lastly, it is also possible that since all of the RA patients had "inactive" rheumatoid disease as measured by DAS28, the systemic influence of RA would be negligible. Any of these scenarios may explain the lack of differences between our groups.

This study has a number of limitations that could be addressed in future studies, namely the cross sectional design of the study, relatively low subject numbers and lack of matched groups with respect to age and sex. It is probable that the RANKL/OPG ratio is a better indicator of the occurrence and activity of periodontitis, as well as osteoclast activity, than RANKL alone (Buduneli and Kinane, 2011, Taubman *et al*., 2007). It is feasible that the proposed disruption to the RANKL/OPG balance in these subjects is influenced more heavily by OPG than first expected, rather than RANKL. The small differences that were observed may be better analysed together with OPG. In addition, the combination of examining both mediators concurrently may provide a better understanding of the relationship of bone regulation between periodontitis and RA. Furthermore, the fact that the majority of RA subjects all took at least one DMARD may have also confounded results by modulating the expression of RANKL. It should also be recognised that RANKL does exist in several different isoforms, and may co-exist as a complex with OPG. The clinical and pathological significance of this is unknown, and current detection assays are not able to detect all of these isoforms (Belibasakis and Bostanci, 2012).

Future work in this area should be aimed at examining a larger cohort of patients and examining the ratio of RANKL/OPG. More importantly, it would be beneficial to examine RA patients prior to DMARD therapy, as it may be speculated that in these patients RANKL levels would be higher. It may also be interesting to examine mediators of bone regulation in GCF and serum in RA patients.

In conclusion, our present findings show that despite being a marker for bone resorption in both RA and periodontitis, the co-existence of RA and periodontitis did not significantly influence sRANKL levels in the population studied.

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