Effect of Smoking on Concentrations of RANKL and OPG in Human Gingival Crevicular Fluid

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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Declaration

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// Acknowledgements

My special thanks must go to my supervisor, Professor Mark Bartold, for the original idea for this research project. His inspiration, help and encouragement for the last three years has had a significant impact on my personal development and completion of this research project.

I am very grateful to Dr Tracy Fitzsimmons for her incredible patience, helpfulness and expertise, especially during those long hours in the laboratory. Thanks must also go to Mr Victor Marino for the technical support with the Periotron 8000. The invaluable advice and assistance with the statistics from Professor Gary Slade is also gratefully acknowledged.

Lastly but not least, a big thank-you to my colleagues, Ms Yen Tu, Mrs Kerry Page, Dr Raymond Chan, Dr Christopher Bates, Dr Nupur Kataria, Dr Andre Bendyk and the staff of Clinic 1.4 for their assistance in the GCF collection. Lastly, but not least, I would like to thank Ms Catherine Offler for the final editing of this manuscript.

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Chapter 1

A REVIEW OF SMOKING EFFECTS ON THE PERIODONTIUM AND ITS POSSIBLE IMPLICATIONS ON EXPRESSION OF RANKL AND OPG

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1.1 Smoking and periodontal disease

Smoking is recognised as one of the major risk factors for chronic periodontitis. It is associated with degradation of the periodontal tissues leading to attachment loss, bone loss and eventually tooth loss (Johnson and Hill 2004).

In 1947, Pindborg reported an association between acute necrotizing ulcerative gingivitis and smoking (Pindborg 1949). However, it was not for another 34 years that the relationship between smoking and periodontitis was systematically evaluated. In 1983 a report was published regarding the periodontal status of 124 pairs of twins with different smoking status (Bergstrom and Floderus-Myrhed 1983). This study found that heavily smoking twins had more bone loss and tooth loss. Later, Bergstrom and Eliasson (1987) reported that the increased alveolar bone loss seen in smokers was independent of plaque levels and age. These findings suggested that smoking could be an independent risk factor for periodontitis.

Since the early 1980's, numerous cross-sectional population studies have reported a higher frequency of smoking among periodontitis patients compared to the general population (Bergstrom 1989, Preber and Bergstrom 1986a). In addition, more severe periodontitis has been reported among smokers compared to non-smokers (Bergstrom 1989). Similar findings were also reported in a more defined periodontal practice setting (Haber and Kent 1992).

In an attempt to identify risk indicators for attachment loss and bone loss, Grossi and co-workers performed cross-sectional studies on more than 1000 metropolitan dwellers living in Erie County, New York (Grossi *et al* 1995, Grossi *et al* 1994). They found that smoking was one of the major risk factors for periodontitis. In addition, they found that the deleterious effect of smoking was dose-dependent. Numerous other studies have supported these findings (Bergstrom *et al* 2000a, Bergstrom *et al* 2000b, Norderyd and Hugoson 1998, Persson *et al* 1998).

In general, smokers have gingival tissues of fibrotic appearance and fewer sites that bleed on probing (Bergstrom and Bostrom 2001, Bergstrom and Floderus-Myrhed 1983), greater attachment loss (Grossi *et al* 1994), greater alveolar bone loss (Grossi *et al* 1995), greater

gingival recession (Calsina *et al* 2002, Martinez-Canut *et al* 1995) and greater tooth loss (Holm 1994, Jette *et al* 1993).

All the studies mentioned above are of varying populations, clinical parameters, study design and length of study, but the results are generally consistent. Thus, the evidence is clear that smoking is closely associated with periodontitis.

To study the causal relationships between smoking and periodontitis, longitudinal studies are required. To date, there are only a few published longitudinal studies and cohort studies available. These longitudinal studies have emanated mainly from the US and Sweden.

For the US studies, 6-year data from the Veterans Administration Dental Longitudinal Study indicated greater alveolar bone loss in cigarette smokers compared to non-smokers (Feldman *et al* 1983). In another larger scale epidemiological survey over 28 years, smoking was identified as a risk marker of periodontal attachment loss (Ismail *et al* 1990).

For the Swedish studies, a 10-year study involving 349 individuals showed that smokers lost up to 6% of the root length of bone compared to 4.4% in those who gave up smoking during the study and 3.6% in non-smokers (Bolin *et al* 1993). In another 10-year study involving individuals matched for oral hygiene, smokers were found to have more periodontal disease sites concomitant with periodontal bone loss while the periodontal health of non-smokers and former smokers remained stable (Bergstrom *et al* 2000a). Similar findings were also reported in a 20-year prospective study in Sweden involving 507 dentate individuals (Jansson and Lavstedt 2002). From these studies, it is clear that, in addition to non-smoking, smoking cessation may be favourable for periodontal health.

In terms of disease progression, smoking has also been found to be a good prognostic factor for further periodontal breakdown (Machtei *et al* 1997, Norderyd *et al* 1999). In the study by Machtei *et al* (1997), the patients' cotinine level, a major metabolite of nicotine, was directly correlated with outcomes of periodontal breakdown.

1.1.1 Periodontitis and risk

The concept of risk was introduced into periodontology about 20 years ago (Preber and Bergstrom 1986a) and this has been applied in several studies to estimate the risk

associated with smoking. However, the estimates of the relative risk vary between studies due to the different study designs, populations chosen, clinical parameters, definition of disease and definition of smoking exposure.

In a survey of smoking habits among 369 adult periodontitis patients, Preber and Bergstrom (1986) found that the odds ratio for a smoker to be designated a periodontitis patient was doubled. In another study of 615 American adults controlled for confounding factors such as age, gender, plaque, and calculus, it was found that smokers had an odds ratio of 5.3 for having a mean probing depth of ≥3.5 mm as compared to non-smokers (Stoltenberg *et al* 1993). In addition, the odds ratio for periodontal disease among 82 young regular dental attendees and smokers was found to be 14.1 (Linden and Mullally 1994). Overall, the majority of studies suggest that smoking increases the risk of periodontal disease between 2 and 6 times. The relative risk can vary between 5 and 20 depending on the definition of disease and prevalence (Bergstrom 2003, Bergstrom 2004b). In addition, there is a relationship between the relative risk and the etiologic fraction (Bergstrom 2004b). A relative risk of 5 to 6, as normally found in smokers, relates to an etiologic fraction of about 80% and a relative risk of 10 to 15, as found in heavy smokers, relates to an etiologic fraction of approximately 90%. Following these estimates, Bergstrom (2004b) estimated that some 80% of total periodontal disease among smokers could be attributable to smoking. This corresponds fairly well with the figures from an US epidemiologic study based on the Third National Health and Nutrition Examination Survey (1988-1994) which showed that 74.8% of periodontal disease in current smokers was attributable to smoking (Tomar and Asma 2000).

There are also interactions between smoking and systemic factors such as diabetes and Interleukin-1 (IL-1) genetic polymorphism which further modulates the pathogenesis of periodontitis (Meisel *et al* 2004, Syrjala *et al* 2003).

In summary, the evidence to support smoking as a risk factor for periodontitis is strong.

1.1.2 Dose response

Over the years, researchers have attempted to find out whether there is a dose response relationship between smoking and periodontal status.

Grossi *et al* (1994) demonstrated that for periodontitis light smokers attracted a relative risk of 2.05 while heavy smokers attracted a relative risk of 4.75. In a comparable study on risk factors associated with alveolar bone loss, Grossi *et al* (1995) showed that light smokers had an odds ratio of 3.25 and heavy smokers had an odds ratio of 7.28 for more severe bone loss as compared to non-smokers.

Exposure of smoking in terms of packyears (packs of cigarettes smoked per day multiplied by the number of years the subject has smoked) was found to be significantly correlated with probing depth (Alpagot *et al* 1996).

In terms of number of cigarettes smoked per day, it was found that moderate to heavy smoking (greater than or equal to 10 cigarettes per day) was associated with severe periodontitis, but light smoking was not (fewer than 10 cigarettes per day) (Norderyd and Hugoson 1998). The presence of a minimum threshold of tobacco usage before it increases the risk of disease severity has also been shown by Martinez-Canut *et al* (1995).

From these studies, it is clear that smoking can be considered a true risk factor for development of periodontitis. In general, smokers attract a relative risk of 5 to 6 for having periodontitis and it can be higher among younger populations. In addition, there is a clear dose-dependent relationship between periodontitis and smoking. Bergstrom (2004) concluded that a smoker who has smoked 20 cigarettes a day for over 20 years runs a 20-fold increased risk. He also noted that the high level of risk is comparable to the smoking-associated risk for lung cancer.

1.1.3 Tobacco exposure

It is always difficult to measure a subject's exposure to tobacco products over many years. Therefore, it remains challenging to determine the strength of smoking as a risk factor. Most of the published studies have relied on questionnaires to assess smoking history and these may be subject to recall bias. Additionally, current levels of smoking may not reflect past exposure. Another useful measurement of smoking status is the serum level of cotinine, the principal metabolite of nicotine. In one of the few studies that have measured serum cotinine, Gonzalez *et al* (1996) showed that the clinical attachment level or crestal bone height was significantly correlated with serum cotinine levels. Patients' cotinine

levels have also been shown to correlate directly with periodontal disease progression (Machtei *et al* 1997).

In the gingival crevicular fluid (GCF) of smokers, nicotine concentrations are about 300 times those found in plasma (Benowitz and Jacob 1984, Ryder *et al* 1998).

1.1.4 Smoking and outcomes of periodontal treatment

The deleterious effects of smoking on the outcomes of periodontal treatment have been widely documented. Smokers have less periodontal pocket reduction and less gingival bleeding reduction after non-surgical periodontal treatment (Preber and Bergstrom 1986b, Preber and Bergstrom 1986c). In longer term studies, smokers have been shown to have less probing depth reduction and less attachment gain following a variety of different treatment modalities (Ah *et al* 1994, Bostrom *et al* 1998b, Kaldahl *et al* 1996). In terms of chronic and aggressive periodontitis, Darby *et al* (2005) showed that smokers had less probing depth reduction in both types of periodontitis patients after scaling and root planing.

A less favourable response in smokers compared to non-smokers has also been reported for almost all periodontal procedures. These include surgical management of furcation defects (Trombelli *et al* 2003), GTR procedures in furcation defects (Rosenberg and Cutler 1994), GTR procedures and GTR with demineralised freeze-dried bone allograft (DFDBA) in the treatment of intrabony defects (Rosen *et al* 1996, Tonetti *et al* 1995), root coverage procedures with various procedures (Miller 1987, Silva *et al* 2006, Trombelli and Scabbia 1997), and implant therapy (Haas *et al* 1996, Jones and Triplett 1992). In addition, the use of local adjunctive antimicrobials has also been reported to lead to poorer response among smokers compared to non-smokers (Kinane and Radvar 1997).

Heavy smoking has also been related to more tooth loss in patients under periodontal maintenance. McGuire and Nunn (1999) reported that heavy smoking increased the risk of tooth loss by 2.9 times while the combination of IL-1 genotype and smoking increased the risk of tooth loss by 7.7 times for patients maintained for 14 years.

1.1.5 Refractory periodontitis

Several studies have shown that the vast majority of refractory periodontitis patients are smokers. MacFarlane *et al* (1992) performed a retrospective study to look for environmental variables in 31 refractory periodontitis patients and found that 90% of them were smokers. Unfortunately, the literature on the association of smoking and refractory periodontitis is rather limited. No studies have been published yet evaluating the effect of smoking cessation in the management of refractory periodontitis.

1.2 Smoking and bone loss

1.2.1 Smoking and general bone loss

Smoking has an adverse effect on bone turnover. It has been reported that smoking lowers bone mineral density in postmenopausal women due to increased bone resorption and decreased calcium absorption (Rapuri *et al* 2000). Not only is smoking a risk factor for hip and spine fractures (White *et al* 2006), it also impairs osseous healing and affects bone mineral content in both genders (Benson and Shulman 2005, Hollinger *et al* 1999). In addition, nicotine was found to inhibit early revascularization of cancellous bone grafts (Daftari *et al* 1994).

1.2.1 Smoking and alveolar bone loss

In terms of bone loss as a result of periodontitis, numerous studies have consistently shown the deleterious effects of smoking.

In 1991, Bergstrom and co-workers studied the relationship between periodontal bone height and smoking in 210 Swedish dental hygienists with good oral hygiene (Bergstrom *et al* 1991). They found that loss of periodontal bone height increased with greater smoking exposure. They concluded that smoking related bone loss is not correlated with plaque infection. In addition, smokers are two to four times more likely to have teeth with furcation involvement (Mullally and Linden 1996).

The most convincing results have been reported from longitudinal studies. In a 1-year study, Meinberg *et al* (2001) reported significantly more interproximal bone loss and a higher percentage of moderate and severe periodontal pockets in smokers compared with

non-smokers. The effect of smoking on alveolar bone loss was also observed in a group of postmenopausal women observed for 2 years (Payne *et al* 2000). As shown in Figure 1.1, the progression of bone loss was seven-fold higher in smokers compared to non-smokers in a 10-year study and smoking cessation was shown to be beneficial for periodontal health (Bergstrom *et al* 2000a). Similar findings were also reported in a 20-year prospective study (Jansson and Lavstedt 2002).

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Figure 1.1 Ten-year change in periodontal bone height according to smoking status. Reproduced from Bergstrom et al (2000a).

Increased exposure of smoking also results in greater severity of alveolar bone loss as can be seen in Figure 1.2 (Grossi *et al* 1995). Similar findings were also reported in terms of vertical bone loss (Baljoon *et al* 2004).

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Figure 1.2 Cigarette smoking by level of alveolar bone loss. Reproduced from Grossi et al (1995).

As shown in Figure 1.3, Bergstrom (2004a) showed that the mean 10-year bone height reduction was 0.74 mm for smokers, 0.26 mm for former smokers and 0.27 mm for non-smokers. Based on that observation, the authors hypothesized that smoking was related to accelerated rate of bone loss and smoking cessation would result in a return towards non-smokers' rate. This is in general agreement with earlier longitudinal studies (Bergstrom *et al* 2000a, Bolin *et al* 1993, Feldman *et al* 1983, Krall *et al* 1999, Machtei *et al* 1997, Payne *et al* 2000).

Bergstrom (2004a) also calculated that approximately 80% of the premolar supporting bone will remain intact until old age in non-smokers as compared to approximately 40% in average chronic smokers. This further suggests that smokers are at increased risk of tooth loss due to accelerated alveolar bone loss. The increased risk of tooth loss in smokers is supported by several studies (Feldman *et al* 1983, Holm 1994, Hugoson and Laurell 2000, Krall *et al* 1999).

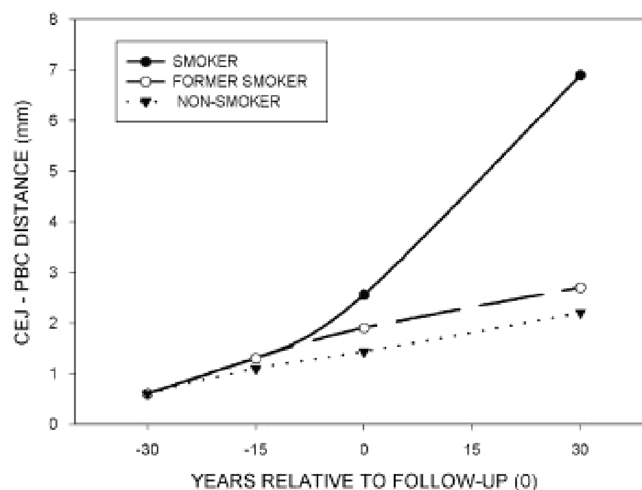

Figure 1.3 Smoking induced acceleration of periodontal bone height reduction rate. Hypothetical model based on present observations and the assumption of a constant reduction rate in non-smokers. 0 = time point of present 10-year follow-up, -15 years = time point of smoking cessation in former smokers, -30 years = time point for starting to smoke in current and former smokers, 30 years = prediction found by extrapolation. Reproduced from Bergstrom (2004a).

Apart from alveolar bone loss as a result of periodontitis, smoking is also related to higher incidence of localised alveolitis (Sweet and Butler 1979). It has been shown in a rat study that nicotine delayed alveolar healing in extraction sockets (Pinto *et al* 2002). In a prospective human study, it has been reported that smoking leads to greater dimensional

reduction of the residual alveolar ridge and delays socket healing (Saldanha *et al* 2006). In addition, rats receiving cigarette smoke inhalation were also found to have reduced healing in created fenestration defects (Benatti *et al* 2005).

Overall, the evidence is clear that smoking is associated with general bone loss and periodontal bone loss and this is supported by data showing that smoking cessation reduces the risk of further progression of alveolar bone loss.

1.3 How does smoking contribute to alveolar bone loss?

Although the evidence is convincing that smoking is one of the major risk factors for periodontitis, its role in the mechanisms of the pathogenesis are still poorly understood. Considering that periodontitis is a complex disease, with genetic and environmental factors involved, there is a possibility that smoking can act at many levels leading to a disturbance of overall tissue homeostasis.

1.3.1 Effects of smoking on subgingival microflora

Numerous studies have reported a difference in subgingival microflora composition between smokers and non-smokers. Zambon *et al* (1996) found a higher prevalence of *A. actinomycetemcomitans*, *T. forsythensis* and *P. gingivalis* in current or former smokers compared to non-smokers. In another study, current smokers were found to have an increased risk of harbouring *T. denticola* in periodontal pockets (Umeda *et al* 1998). Using checkerboard DNA–DNA hybridization technology, Haffajee and Socransky (2001) found that the prevalence of 8 species of subgingival microflora was higher in current smokers, especially in shallow pockets in comparison to non-smokers. This suggests that smoking favours the colonisation by pathogens of shallow sites leading to initiation of disease at new sites and in early smokers. In addition, there are also reports showing specific clusters of periodontal pathogens in moderate to deep probing depths in smokers (Kamma *et al* 1999, van Winkelhoff *et al* 2001).

Several studies have shown no difference in the prevalence of major periodontal pathogens between smokers and non-smokers in periodontitis patients, even with checkerboard DNA–DNA hybridization technology (Bostrom *et al* 2001, Preber *et al* 1992) and polymerase chain reaction (PCR) (Darby *et al* 2000).

In terms of microbiological changes following periodontal treatment, Grossi *et al* (1996) reported a less pronounced reduction of periodontal pathogens among smokers 3 months after treatment and significantly fewer smokers became negative for *P. gingivalis* and *B. forsythus* as a result of therapy. In addition, Darby *et al* (2005) reported that smokers had less reduction in *T. forsythensis* in chronic periodontitis sites compared with non-smokers following scaling and root planning. In aggressive periodontitis sites, smokers had less reduction in *P. intermedia* compared with non-smokers. The authors believed that the findings were related to deeper pockets in smokers and inadequate clearance by smokers due to poorer immune response. However, Preber *et al* (1995) revealed no difference in the microflora between smokers and non-smokers following therapy even though smokers had less pocket depth reduction. This is in agreement with the findings from a later study in which PCR technique was used to detect putative periodontal pathogens (Apatzidou *et al* 2005).

Studying the microflora in periodontitis has always been taxing. The number of different bacterial species that have been isolated from both supragingival and subgingival plaque is well over 400 (Socransky *et al* 1998). Yet, only less than 20 pathogens have been associated with progression of periodontitis. In addition, no single species or strain has been demonstrated to be exclusive to an individual periodontal disease state, whether the data are site or patient based (Newman 1990). Above all this, there are also issues related to culturing certain species and sampling. Therefore, more studies with adequate number of subjects and latest technologies should be carried out in the future to further define the specific roles of various periodontal pathogens in the pathogenesis of periodontitis.

1.3.2 Effect of smoking on gingival vasculature or microcirculation

Cigarette smoke contains over 6000 chemicals (Huber *et al* 1991). All these chemicals, including nicotine, have the potential to harm the periodontal tissues via different mechanisms.

Considering periodontal tissues are well vascularised, the effect of nicotine on gingival vasculature or microcirculation has been studied. In an animal model, Clarke *et al* (1981) found that epinephrine and nicotine caused a severe reduction in gingival flow rate. This is consistent with the findings that smokers have less gingival bleeding and GCF volume

compared to non-smokers (Bergstrom and Bostrom 2001, Bergstrom and Preber 1986, Preber and Bergstrom 1985). Gingival blood flow has been reported to increase following cessation of tobacco use (Morozumi *et al* 2004, Nair *et al* 2003).

In contrast to the above, the vasoconstrictive effect of nicotine has been questioned by Baab and Oberg (1987) who found that the acute effect of smoking caused a temporary increase in gingival blood flow. In addition, Meekin *et al* (2000) found no statistically significant changes in the relative gingival flow rates between light smokers, heavy smokers and non-smokers.

In terms of the effects of smoking on blood vessels, Bergstrom *et al* (1988) found that smokers had approximately half the number of visible vessels compared with the non-smokers in an experimental gingivitis study. In addition, Rezavandi *et al* (2002) observed a significantly larger number of vessels in inflamed tissues of non-smokers compared to smokers. In another study, smokers were found to have a higher percentage of smaller blood vessels and a lower percentage of larger vessels than non-smokers (Mirbod *et al* 2001). In contrast, one study has reported that vascular surface density and gingival microvessel number between the two groups appears to be similar (Sonmez *et al* 2003).

These conflicting results may be related to different study designs and different doses of nicotine. In addition, the effect of smoking on gingival vasculature is not just mediated by nicotine, but all of the chemicals in the smoke. Moreover, the acute and chronic effects of smoking must be differentiated. Smoking seems to have a long term chronic effect on the vasculature of the periodontal tissues. The impact on vasculature certainly has relevance to the healing capability of the periodontal tissues.

1.3.3 Effects of smoking on neutrophil number and function

The role of hyperresponsive neutrophils in the degradation of periodontal tissues has been increasingly recognised (Van Dyke and Serhan 2003). It has been shown that neutrophils from periodontitis patients exhibit hyperactivity with or without exogenous stimulation leading to increased release of cytokines and oxygen radicals (Gustafsson *et al* 2006, Matthews *et al* 2007a). Not surprisingly, studies have focused on the effects of smoking on the number and function of neutrophils.

Tobacco smoke exposure increases the number of circulating neutrophils (Sorensen *et al* 2004). However, neutrophil numbers in smokers have been found to be lower in saliva and similar in GCF (Pauletto *et al* 2000, Petropoulos *et al* 2004). There is evidence suggesting that PMNs viability and function are affected by smoking (Guntsch *et al* 2006).

In an in vitro study, Ryder *et al* (1998) showed an increased release of superoxide and hydrogen peroxide in unstimulated neutrophils which were exposed to tobacco smoke. In another study, the priming effect of TNF-α on neutrophils was found to be higher in smokers compared to non-smokers in subjects with or without periodontitis. In the group with periodontitis, smoking caused an even greater increase in generation of oxygen radicals (Gustafsson *et al* 2000). The increased levels of reactive oxygen radicals in the periodontal tissues of smokers lead to disruption of cellular proteins and matrix components (Ward *et al* 1988). In addition, reactive oxygen species have been found to activate neutrophil collagenases, thus mediating further tissue degradation (Sorsa *et al* 1989). Even passive smoking may be associated with increased leucocyte counts, chemotaxis and increased release of reactive oxidants from stimulated neutrophils (Anderson *et al* 1991).

There is evidence that neutrophil elastase is involved in the initial destruction of human periodontal ligament due to its role in degrading non-collagenous protein-covered collagen fibrils (Ujiie *et al* 2007). Furthermore, lower concentrations of α2-macroglobulin and total amounts of α1-anti-trypsin in severe lesions have been found in smokers compared to non-smokers (Persson *et al* 2001). In addition, the levels of α1-anti-trypsin and α2-macroglobulin appear to be unchanged in smokers after periodontal surgery while non-smokers had decreased levels of those inhibitors (Persson *et al* 2003). Considering both of those proteins are the main inhibitors of elastase, this may result in higher levels of functional elastase in smokers (Soder *et al* 2002).

Neutrophils express functional receptors for several components and metabolites of tobacco smoke, such as nicotine, cotinine (Benhammou *et al* 2000) and aryl hydrocarbons (Ackermann *et al* 1989). The numbers of nicotinic receptors expressed by human neutrophils are increased in smokers and decline on cessation (Lebargy *et al* 1996). In addition, neutrophils also express receptors for ICAM-1 (Scott *et al* 2000, Selby *et al* 1992) which are increased in smokers and decreased on cessation (Palmer *et al* 2002). The

binding of neutrophils to ICAM-1 stimulates elastase and matrix metalloproteinase (MMP) release leading to neutrophil-mediated tissue injury (Scott *et al*, 2000).

In summary, the involvement of neutrophils in periodontal tissue degradation is clear (Chapple and Matthews 2007, Matthews *et al* 2007b, Van Dyke and Serhan 2003). Therefore, the increased activation of neutrophils by smoking may lead to increased tissue degradation.

1.3.4 Effects of smoking on the humoral immune response

Reduced serum immunoglobulin G (IgG) (Graswinckel *et al* 2004, Moszczynski *et al* 1989, Quinn *et al* 1998) has been noted in smokers, and of relevance to periodontitis has been the observation of reduced IgG to *A. actinomycetemcomitans* in smokers compared to non-smokers (Apatzidou *et al* 2005).

With regards to the many subclasses of IgG, it has been shown that smoking is associated with lower IgG2 levels (Graswinckel *et al* 2004). It has also been shown in the US that Caucasian periodontitis patients who smoke have depressed levels of IgG2 in contrast to smoking black patients, who had comparable levels to control subjects without disease (Gunsolley *et al* 1997, Quinn *et al* 1998, Quinn *et al* 1996). In addition, smoking is also associated with lower IgG2 against *A. aggregatibacter* (Tangada *et al* 1997).

The questions remain as to whether all antibodies are protective in periodontitis and whether systemic immunoglobulins correlate with local immunoglobulins. However, it is clear that smoking has an effect on antibody production.

1.3.5 Effects of smoking on cytokines

It has been shown that current and former smokers with untreated moderate to severe periodontitis have significantly higher GCF levels of tumour necrosis factor-alpha (TNF-α) compared to non-smokers (Bostrom *et al* 1998a, Bostrom *et al* 1999). In addition, GCF concentrations of IL-1α and IL-1β are significantly lower in smokers compared to non-smokers, among periodontitis patients (Rawlinson *et al* 2003, Petropoulos *et al* 2004). However, some studies showed that GCF levels of IL-6, IL-1β and IL-1ra were not affected by smoking (Bostrom *et al* 1999, Bostrom *et al* 2000). In terms of IL-4 and IL-8,

Giannopoulou *et al* (2003) showed that smokers had lower amounts of IL-4 and higher amounts of IL-8 in GCF compared to non-smokers throughout a 10-day experimental gingivitis study.

In gingival tissues, smokers express higher levels of interferon-γ (IFN-γ) as compared with non-smokers with a comparable type of periodontitis (Cesar-Neto *et al* 2006). In a separate study, smoking was found to lower the gene expression of IL-1α, IL-8, IL-10, TNF-α, MMP-8 and OPG, and increased the levels of IL-6 and IL-1ra in sites with a comparable type of periodontitis (Cesar-Neto *et al* 2007).

Overall, the above studies show that smoking is able to modulate the expression of various cytokines in GCF and gingival tissues in periodontitis and periodontally healthy patients. However, the evidence is conflicting at times and more studies are required to determine the effects of smoking on the expression of cytokines in periodontitis patients. It is possible that smoking affects the balance of Th-1 and Th-2 cytokines, leading to tissue degradation (Alayan *et al* 2007).

1.3.6 Summary

Findings from studies related to multiple possible mechanisms of how smoking affects the periodontium are conflicting. However, these observations indicate that smoking may interfere with several reparative and destructive factors in the periodontal tissues of smokers.

1.4 Biology of alveolar bone

Alveolar bone loss is a striking feature and outcome of periodontitis in smokers. To understand the mechanisms of alveolar bone loss, a thorough understanding of the biology of alveolar bone is required.

The alveolar process is the specialised part of the jaw bones that contains sockets for the teeth. It consists of outer cortical plates of compact bone, a central spongiosa and alveolar bone. The compact bone is confined by the periosteum on the outside and endosteum on the inside. The inner cortical plate, the endosteal surface exhibits pronounced osteoclastic and osteoblastic activity. The periosteum is important during growth, regeneration of

periodontal defects and healing around implants. The alveolar bone is composed of bundle bone which contains several layers of bone deposited in an orientation parallel to the tooth socket wall which has Sharpey's fibers emanating from it at right angles (Schroeder 1992). It is also formed in layers in a parallel orientation to the coronal-apical direction of the tooth.

Alveolar bone contains collagens, bone-specific and non-specific noncollagenous proteins (Bronckers *et al* 1994, McKee and Nanci 1996) and proteoglycans (Bartold 1990, Waddington and Embery 1991).

1.4.1 Bone remodelling

The alveolar bone is subjected to continual and rapid remodelling associated with tooth eruption and functional demands of mastication (Saffar *et al* 1997). Bone remodelling involves the coordination of activities of two main cell types, osteoblasts and osteoclasts.

Regulation of bone remodelling is a complex process involving hormone and local factors acting in autocrine or paracrine manner on the generation and activity of differentiated bone cells.

1.4.2 Bone cells

The cells involved in the homeostasis of bone are osteoblasts, osteocytes and osteoclasts.

Osteoblasts are of mesenchymal origin. They secrete bone matrix actively when fully differentiated. They contribute to the organic extracellular matrix that consists of mainly type I collagen and various other noncollagenous born proteins and plasma proteins (Nanci and Bosshardt 2006).

Once the osteoblast is surrounded by a mineralized matrix, it becomes an osteocyte. Due to the abundance of osteocytes in the bone matrix, they appear to respond rapidly to calciotropic hormones, moving calcium and phosphate into and out of the bone mineral.

Cells of the osteoblast lineage are involved in the production of cytokines and growth factors, which affect bone resorption as well as bone formation. Osteoblasts and osteocytes

also produce proteases that are involved in degrading and remodelling the extracellular matrix, resulting in its maturation and mineralisation.

Osteoclasts are large, multinucleated cells that have the ability to resorb bone. Resorption of bone occurs in an acidified extracellular matrix compartment as a result of the combined actions of several enzymes associated with their ruffled membrane.

1.4.3 Coupling mechanisms

It is well-established that, in a healthy young adult, bone resorption is followed by an equivalent amount of bone formation, which is referred to as coupling (Parfitt 1982). The coupling of bone resorption with bone formation allows the bone to be remodelled throughout life (Hayden *et al* 1995). The individual rate of bone resorption and bone formation determines the rate of tissue turnover (Eriksen 1986). Bone resorption which occurs in diseases such as periodontitis or rheumatoid arthritis is a result of uncoupling of the normally coupled processes of bone formation and bone resorption.

1.4.4 Bone formation

Bone formation is always coupled with bone resorption in order to maintain bone mass (Parfitt 1982). This involves proliferation and differentiation of stromal stem cells to osteoblasts. This occurs under a combination of genetic programming and gene regulation by various hormones, cytokines and growth factors (Hughes *et al* 2006). In addition, there are progenitor cells in the periodontal ligament that can differentiate into osteoblasts for physiological maintenance and repair (Isaka *et al* 2001).

1.4.5 Bone resorption

Resorption of bone requires the recruitment of osteoclasts, which is assisted by the monocyte/macrophage lineage of hematopoietic cells (Baron *et al* 1986). Osteoclasts are stimulated to proliferate and differentiate under the influence of monocyte-macrophage colony-stimulating factor. A variety of biological molecules such as growth factors, systemic hormones and cells in the marrow, especially osteoblasts, play a critical role in regulating osteoclast formation. Cell-to-cell interactions are also important in the formation and activity of osteoclasts.

1.4.6 Formation and activity of osteoclasts

Formation of osteoclasts involves the fusion of monocytic precursors at the site of resorption. On the bone surface, osteoclasts become polarized and form a ruffled border beneath which bone resorption takes place (Blair 1998). Demineralisation of the bone matrix occurs through the acidification of a protected environment beneath the ruffled border. Lysosomal enzymes released by the osteoclasts then degrade the matrix macromolecules. Matrix metalloproteinases (MMPs) have also been observed in resorption lacunae (Sodek and Overall 1992). Osteoclasts require both MMP activity and Cathepsin K to resorb bone. Following resorption, osteoclasts may undergo apoptosis, thus limiting resorptive activity (Hughes *et al* 1995). Factors such as transforming growth factor-ß, estrogen and bisphosphonates promote apoptosis while factors such as parathyroid hormones and IL-1 suppress apoptosis. Therefore, the formation, activity and survival of osteoclasts are potential targets for regulation of osteoclast-mediated bone resorptive activity.

1.5 Interplay between Receptor Activator Nuclear Factor Kappa B Ligand (RANKL), Receptor Activator Nuclear Factor Kappa B (RANK) and Osteoprotegerin (OPG)

Bone remodelling and resorption is the result of coordinated molecular interplay between RANKL, its cellular receptor, RANK and the decoy receptor, OPG (Figure 1.4). This has been identified as the key regulation system for bone remodelling and bone destruction (Kong *et al* 1999), and has been demonstrated in disease models such as rheumatoid arthritis and periodontitis (Firestein 2003, Taubman *et al* 2005).

RANKL and OPG are expressed by multiple cells in periodontal tissues (Takayanagi *et al* 2000, Sakata *et al* 1999). In addition, RANKL is also expressed by T cells and B cells especially in periodontitis lesions (Horwood *et al* 1999, Teng *et al* 2000, Han *et al* 2006). This supports the role of immune cells in alveolar bone loss as a result of periodontitis and a new paradigm of periodontal pathogenesis is now emerging towards the "osteoimmunology" interactions (Teng 2003).

Apart from being involved in bone homeostasis, the OPG/RANKL/RANKL axis has been increasingly implicated in atherosclerosis and cardiovascular diseases (Bennett *et al* 2006, Bucay *et al* 1998, Ueland *et al* 2005).

NOTE:
This figure is included on page 19 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4 Molecular interplay between RANKL, RANK and OPG. Adapted from Yasuda et al (1998).

1.5.1 RANKL

RANKL is a member of the tumour necrosis factors (TNF) family of ligands. It is expressed at its highest levels in osteoblasts and stromal cells (Kong *et al* 1999, Lacey *et al* 1998, Udagawa *et al* 1999). It is also expressed in lesser amounts by human periodontal ligament cells, fibroblasts (Quinn *et al* 2000, Takayanagi *et al* 2000), activated T cells and B cells (Horwood *et al* 1999, Teng *et al* 2000, Han *et al* 2006). In addition, RANKL is also produced in a soluble form, but the majority of RANKL is cell bound. In the presence of macrophage colony-stimulating factor, RANKL is required for osteoclast differentiation. RANKL also has the capacity to inhibit or induce osteoclast apoptosis (Wu *et al* 2005).

RANKL binds directly to RANK on the surface of pre-osteoclasts and osteoclasts, stimulating both the differentiation of osteoclast progenitors and activity of mature osteoclasts (Burgess *et al* 1999, Hsu *et al* 1999, Lacey *et al* 1998, Matsuzaki *et al* 1998). RANKL knockout mice show severe osteopetrosis due to absence of osteoclasts (Kong *et al* 1999). Inflammatory cytokines such as IL-1 and TNF-α are reported to stimulate production of RANKL mRNA in human periodontal ligament, human microvascular endothelial cells, human osteoblasts and human mesenchymal stem cells (Collin-Osdoby *et al* 2001, Nukaga *et al* 2004, Wei *et al* 2005). The effects of RANKL are counteracted by OPG, which prevents the binding of RANKL to its receptor RANK on osteoclasts (Akatsu *et al* 1998).

It has been reported that RANKL is able to promote the interaction between endothelial cells and leukocytes and this is correlated with increased expression of the adhesion molecules in endothelial cells (Min *et al* 2005). In fact, upregulation of RANKL and an increased RANKL to OPG ratio may be involved in causing endothelial dysfunction and atherosclerosis (Collin-Osdoby 2004, Kaden *et al* 2004).

RANKL is able to stimulate differentiated osteoclasts to produce hydrogen peroxide which is prevented by the use of antioxidants (Ha *et al* 2004). In addition, osteoclasts are able to be activated by hydrogen peroxide (Fraser *et al* 1996). Apart from that, reactive oxygen species (ROS) are also found to induce RANKL expression in osteoblasts (Bai *et al* 2005). Therefore, lowering ROS levels may be beneficial to preventing bone loss by reducing RANKL-induced activities.

1.5.2 RANK

RANK is expressed on osteoclast precursors, mature osteoclasts and dendritic cells. RANK can bind several cytosolic TNF receptor-associated factors (TRAFs), which then trigger multiple downstream signalling pathways (Wong *et al* 1999).

1.5.3 OPG

OPG, a soluble TNF receptor-like molecule, is the naturally occurring inhibitor of osteoclast differentiation. OPG binds to RANKL with high affinity and blocks RANKL from interacting with RANK (Lacey *et al* 1998, Yasuda *et al* 1998). OPG knockout mice

show severe osteoporosis due to excessive osteoclast differentiation and activity (Bucay *et al* 1998). OPG is produced by human periodontal ligament cells, gingival fibroblasts, epithelial cells and human microvascular endothelial cells (Kanzaki *et al* 2002, Sakata *et al* 1999). RANKL and OPG are both modulated by inflammatory cytokines present in periodontitis (Brandstrom *et al* 1998, Hofbauer *et al* 1998, Nakashima *et al* 2000). Crotti *et al* (2003) demonstrated the presence of RANKL and OPG protein in human periodontal diseased tissues and are believed to the key regulators regulating bone metabolism and alveolar bone destruction in periodontitis.

The use of OPG as an inhibitor of alveolar bone loss in periodontitis was investigated in mice orally infected with *A. actinomycetemcomitans* (Mahamed *et al* 2005, Teng *et al* 2000). Inhibition of RANKL function with OPG treatment significantly reduced the number of osteoclasts and the alveolar bone loss in both studies. In a 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, local injection of OPG was also found to inhibit B-cell mediated periodontal bone resorption (Han *et al* 2006). Recently, Jin *et al* (2007) also reported that OPG has a strong preventive effect on alveolar bone loss in ligature-induced experimental periodontitis.

1.6 How does the expression of RANKL/RANK/OPG relate to periodontitis?

There are several studies showing that various cytokines such as IL-1, TNF-α, IL-6 and prostaglandin-E2 are involved in periodontal bone loss, but the exact mechanism is poorly understood (Graves and Cochran 2003, Offenbacher *et al* 1986).

In animal periodontitis models, transplanted human T cells are able to produce RANKL which leads to activation of osteoclasts and consequently bone loss (Teng *et al* 2000). Human studies were then carried out in GCF and gingival tissues to study the expression of RANKL and OPG in periodontitis. In gingival tissues, periodontitis patients exhibit higher expression of RANKL (Bostanci *et al* 2007b, Cesar-Neto *et al* 2007, Crotti *et al* 2003, Garlet *et al* 2004, Liu *et al* 2003, Vernal *et al* 2006), and lower expression of OPG (Crotti *et al* 2003, Liu *et al* 2003) compared with healthy controls. Similar results were also reported in GCF (Bostanci *et al* 2007a, Lu *et al* 2006, Mogi *et al* 2004, Vernal *et al* 2004). As a consequence, the RANKL/OPG ratio was also found to be increased in periodontitis

patients compared with healthy controls (Bostanci *et al* 2007a, Bostanci *et al* 2007b, Liu *et al* 2003, Mogi *et al* 2004).

In order to understand the expression of OPG and RANKL in different forms of periodontal disease, 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 all subjects with periodontitis had higher RANKL expression, lower OPG expression and hence higher RANKL to OPG ratio compared with healthy controls. OPG mRNA expression was weaker in chronic periodontitis while RANKL expression was stronger in aggressive periodontitis. However, there was no difference in RANKL/OPG ratio between the two forms of periodontitis. In a GCF study with similar study design, there was no difference in the GCF concentrations of RANKL, OPG or the RANKL/OPG ratio between chronic and aggressive periodontitis (Bostanci *et al* 2007a). In contrast, Garlet *et al* (2004) found similar RANKL mRNA expression between aggressive and chronic periodontitis but OPG mRNA expression was higher in chronic periodontitis patients. In addition, chronic periodontitis subjects receiving immunosuppressant therapy have been found to have stronger OPG expression compared to chronic periodontitis subjects (Bostanci *et al* 2007b). These inconsistencies between the tissue expression and GCF levels of these mediators highlight the complexity of clinical data interpretation and may relate to factors such as variations in disease progression. In addition, the authors suggested that there could be a lag between production of these molecules in the tissue and their release into the periodontal pocket (Bostanci *et al* 2007b).

1.7 Does the expression of RANKL and OPG correlate to disease severity and disease progression?

Several studies have found no correlation of RANKL and OPG to the clinical measurement of disease severity (Garlet *et al* 2004, Lu *et al* 2006, Mogi *et al* 2004). However, Bostanci *et al* (2007b) found that the levels of RANKL and RANKL/OPG ratio in gingival tissues were significantly correlated with clinical parameters such as attachment level, probing depth and bleeding on probing. In addition, in a GCF study, Bostanci *et al* (2007a) also found the positive correlation between RANKL/OPG ratio and periodontal probing depth and clinical attachment level. Recently, the positive correlation between salivary OPG concentration and probing depth, clinical attachment level and bleeding on probing were also reported in a group of untreated periodontitis patients (Buduneli *et al* 2008).

The study of GCF has allowed understanding of pathophysiology of periodontitis and identification of a diagnostic marker for active periodontitis. There have been methological concerns with GCF collection and analysis in the development of GCF-based diagnostic tests. Therefore, the use of who salva as a means of evaluating GCF constituents and some other exogenous components has received more attention in recent years.

Vernal *et al* (2004) studied the GCF levels of RANKL in relation to disease progression in a group of untreated periodontitis patients. The higher total amount of RANKL was found to be expressed in active sites (≥2 mm attachment loss) compared to inactive counterparts and may suggest the association of RANKL with the progression of periodontal destruction. Recently, Silva *et al* (2008) also reported a higher RANKL total amount in GCF of active sites compared to non-active sites. This may suggest that local levels of RANKL are indicative of alveolar bone loss as seen in periodontal disease progression. There is evidence in a study by Buduneli *et al* (2008) that untreated periodontitis patients had significantly greater salivary concentrations of RANKL than patients on maintenance in a group of non-smokers.

In an animal study, it was demonstrated that periodontal disease activity was related to the RANKL/OPG balance (Garlet *et al* 2006). In addition, the expression of RANKL was positively correlated to Th1-type cytokines such as IL-1β, TNF-α and IFN-γ. Conversely, the levels of Th2-type cytokines such as IL-4 and IL-10 were correlated positively with OPG expression (Garlet *et al* 2006). The authors suggested that the regulation of RANKL/OPG system was determined by the balance of T helper responses and could control the disease severity.

Considering periodontitis is characterised by bone loss and attachment loss, markers specific to bone matrix such as RANKL and OPG may be reliable indicators of disease activity. So far, a multi-centre study has shown that RANKL/OPG ratio predicts 5-year radiographic progression of joint damage in rheumatoid arthritis patients (Geusens *et al* 2006). It may be interesting to find out if RANKL/OPG ratio has some prognostic values in periodontal disease progression.

All the studies mentioned above did not exclude smokers and the proportions of smokers was not reported. So far, there are only three studies that have looked at the effect of

smoking on the expression of RANKL and OPG. Lappin *et al* (2007) studied the serum concentrations of RANKL and OPG in a group of treated periodontitis patients under supportive periodontal therapy. They reported lower serum concentration of OPG in smokers and it was negatively correlated with tobacco consumptions in terms of pack-years. However, there was no correlation between tobacco exposure and RANKL or RANKL/OPG. Cesar-Neto *et al* (2007) took gingival biopsies from periodontally healthy patients, smokers with severe chronic periodontitis and never smokers with chronic severe periodontitis. They confirmed the previous findings that periodontitis patients express higher levels of RANKL mRNA and lower levels of OPG mRNA compared with healthy controls. In addition, RANKL mRNA expression was found to be similar between smokers and never-smokers group. However, OPG mRNA expression was found to be lower in the smokers group. Thus, the RANKL to OPG ratio is increased in the smokers group. Most recently, Buduneli *et al* (2008) measured the saliva concentrations of RANKL and OPG in smokers and non-smokers with periodontitis. They reported that the salivary OPG concentration was significantly lower and the sRANKL:OPG ratio was higher in smokers than non-smokers. Furthermore, OPG concentration also correlated negatively with pack-years.

From the above, it can be concluded that periodontitis patients express higher levels of RANKL and lower levels of OPG compared with healthy controls. This results in a higher RANKL to OPG ratio in the periodontitis group. The involvement of the RANKL/OPG axis in periodontitis opens up a whole new area of periodontal research.

1.8 Involvement of periodontopathogens

In vitro studies have shown that periodontopathogens such as *P. gingivalis* and *A. actinomycetemcomitans* are able to affect RANKL and OPG mRNA expression by human periodontal ligament cells and gingival fibroblasts (Belibasakis *et al* 2005a, Belibasakis *et al* 2005b, Kiji *et al* 2007, Yamamoto *et al* 2006).

It was also reported that the LPS of *A. actinomycetemcomitans* was able to induce the expression of RANKL in human periodontal ligament cells (Tiranathanagul *et al* 2004). Levels of *P. gingivalis* have also been found to correlate with the upregulated levels of RANKL in gingival tissues from periodontitis patients (Wara-aswapati *et al* 2007). In addition, *P. gingivalis* induces RANKL and reduces OPG mRNA expression in human

periodontal ligament cells and gingival fibroblasts, resulting in an increased RANKL/OPG ratio (Belibasakis *et al* 2007). As well as affecting fibroblasts, *P. gingivalis* is also able to induce more RANKL expression and less OPG expression in osteoblasts via the mediation of PGE2 (Choi *et al* 2005, Okahashi *et al* 2004). Furthermore, expression of RANKL by cementoblasts can also be induced upon stimulation by *P. gingivalis* (Nemoto *et al* 2006). The lipopolysaccharide effect on osteoblasts via Toll-like receptors also increases RANKL gene expression (Kikuchi *et al* 2001). In endothelial cells, *P. gingivalis* upregulates the expression of OPG but not RANKL (Kobayashi-Sakamoto *et al* 2004). Interestingly, Belibasakis *et al* (2007) demonstrated that periodontal ligament cells challenged by *P. gingivalis* express less RANKL and more OPG over time, suggesting an initial more pathogenic process and longer term protective function. Furthermore, gingival fibroblasts challenged by the same pathogen expressed more RANKL and less OPG over time steadily, which may relate to a greater pathogenic potential of gingival fibroblasts (Belibasakis *et al* 2007). On the contrary, Kiji *et al* (2007) demonstrated that lipopolysaccharide from *P. gingivalis* and *A. actinomycetemcomitans* augmented the production of OPG by gingival fibroblasts possibly via PGE2. The authors suggested that this could be a self-defence mechanism of gingival fibroblasts in inhibiting alveolar bone loss. The stimulation of OPG expression by gingival fibroblasts after lipopolysaccharide treatment has also been reported by Nagasawa *et al* (2002).

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.

Periodontal pathogens are able to modulate the expression of OPG and RANKL in cells found in periodontal tissues. Considering smoking possibly affects the subgingival microflora, the modulating effect of smoking on RANKL and OPG expression could be mediated via the altered subgingival microflora.

1.9 Influence of gene polymorphisms

Periodontitis is a multifactorial disease which involves the complex interactions between host response and plaque biofilm. This is further modified by genetics and environmental factors (Page and Kornman 1997, Kornman 2008). It has been demonstrated that approximately half of the variability in periodontitis is attributed to genetics (Michalowicz

et al 2000). Kornman *et al* (1997) reported that an IL-1 polymorphism was associated with severity of chronic periodontitis in non-smokers. This study sparked interest in studying gene polymorphisms in periodontitis. Some of the gene polymorphisms that have been studied so far include IL-1 (Kornman *et al* 1997), TNF-α (Soga *et al* 2003) and FcγR (Loos *et al* 2003). Loos *et al* (2005) published a review article on genetic risk factors associated with periodontitis. Numerous gene polymorphisms were included in the review but they concluded that most available studies were under-powered and did not consider some of the relevant risk factors for periodontitis.

Eight OPG gene polymorphisms have been identified (Park *et al* 2008). Out of those, two have been associated more closely with periodontitis patients than control subjects in Korean populations and they are T950 and G1181 (Park *et al* 2008). Therefore, future studies in larger populations with different ethnic backgrounds are required to determine whether these alleles contribute to periodontitis susceptibility. The possibility remains that smoking may modulate the expression of specific OPG gene alleles, in turn contributing to smoking-related periodontitis.

1.10 Possible modulating effects of smoking on expression of RANKL and OPG

Considering RANKL and OPG are expressed by several types of cells, understanding the effects of smoking on those selected cell types may yield some information on the modulated expression of RANKL and OPG by smoking.

1.10.1 Osteoblasts

Studies have shown that nicotine has negative effects on the function of human osteoblasts (Walker *et al* 2001, Yuhara *et al* 1999). Tanaka *et al* (2006) demonstrated that nicotine and lipopolysaccharide could lead to reduced OPG production by osteoblasts in later stages of culture in a dose-dependent manner. It led to the stimulation of osteoclast precursors via an increase in PGE2 and macrophage colony stimulating factor. In the same study, it was also reported that proliferation of osteoblastic cells as well as alkaline phosphatase activity was also decreased by the addition of nicotine and lipopolysaccharide. Alkaline phosphatase activity plays a role in bone calcification. These findings suggest that bone formation is decreased and bone resorption is increased in smokers compared with non-smokers. The

effect is even greater when nicotine stimulation is combined with lipopolysaccharide compared with nicotine stimulation alone.

1.10.2 Osteoclasts

An in vitro study has shown that nicotine is able to stimulate osteoclast differentiation and resorption of calcium phosphate in a dose-dependent manner (Henemyre *et al* 2003). This may explain the greater periodontal bone loss among smokers.

1.10.3 T Cells

The expression of RANKL has been demonstrated on activated T cells and B cells, which implicates the involvement of lymphocytes in bone resorption (Brunetti *et al* 2005, Teng *et al* 2000, Kawai *et al* 2006). The complex interactions between activated lymphocytes, osteoblasts and macrophages in modulating RANKL expression and subsequent osteoclast activation have been shown in Figure 4. T cells may promote bone resorption directly via RANKL expression and indirectly via the production of cytokines such as TNFα, IL-11 and IL-17 that would induce RANKL expression by osteoblasts and bone marrow stromal cells (Theill *et al* 2002). Chronically activated T cells also affect the function of osteoclasts via the production of other factors such as IFN-γ, IL-4 and IL-10 (Theill *et al* 2002).

IFN-γ from T cells and lipopolysaccharide from subgingival bacteria can induce the production of IL-1, TNF-α or IL-6 by macrophages, which in turn increase RANKL expression in osteoblasts and bone marrow stromal cells. IFN-γ can also promote osteoclast differentiation by direct binding on the osteoclasts (Theill *et al* 2002). In a mouse periodontitis model, Baker *et al* (1999) demonstrated that IFN-γ positive T helper1 cells were associated with enhanced alveolar bone loss in periodontitis. Kotake *et al* (2005) showed that IFN-γ positive human T cells induced osteoclast formation through the expression of RANKL. The positive role of IFN-γ in modulating RANKL positive T helper (Th) cell-mediated bone loss was also demonstrated after microbial challenge leading to inflammatory conditions (Teng *et al* 2005). So far, it has been shown that smokers express higher mRNA and protein levels of IFN-γ than non-smokers with comparable degrees of periodontitis (Cesar-Neto *et al* 2006).

In terms of T cells, Loos *et al* (2004) reported higher total systemic leukocyte counts in smokers compared to non-smokers irrespective of periodontal disease status. In addition, smokers with periodontitis also have increased T cell proliferation and higher counts of CD3+, CD4+ and CD8+ T cell subsets in peripheral blood compared with non-smokers. The number of CD4+ cells have been shown to return to normal values on cessation of smoking (Loos *et al* 2004). Nevertheless, factors such as age, race and gender were found to be associated with significant differences in peripheral blood mononuclear-cell subsets (Tollerud *et al* 1989, Tollerud *et al* 1991).

Conflicting results have also been reported from animal studies regarding the effects of smoking on the immune response. This is probably due to different methods of exposure used in those studies such as nicotine pumps and tobacco glycoprotein isolated from cured tobacco leaves (Francus *et al* 1988, Geng *et al* 1995, Geng *et al* 1996). The effect of smoking on T cells is still poorly understood, and more studies are required to clarify the relationship.

1.10.4 B Cells

Activated B-cells produce RANKL and other cytokines, but they do not produce OPG (Yun *et al* 1998). Depending on whether they are stimulated in the presence of Th1- or Th2-like cytokines, B cells can affect osteoclastogenesis in a positive or a negative fashion (Choi *et al* 2001). It has been reported that a subset of the B-lymphoid lineage, B220+, can serve as an osteoclast precursor cell (Manabe *et al* 2001). In addition, it has recently been found recently that B lymphocytes are able to contribute to increased bone resorption in the absence of T lymphocytes through up-regulation of RANKL (Han *et al* 2006).

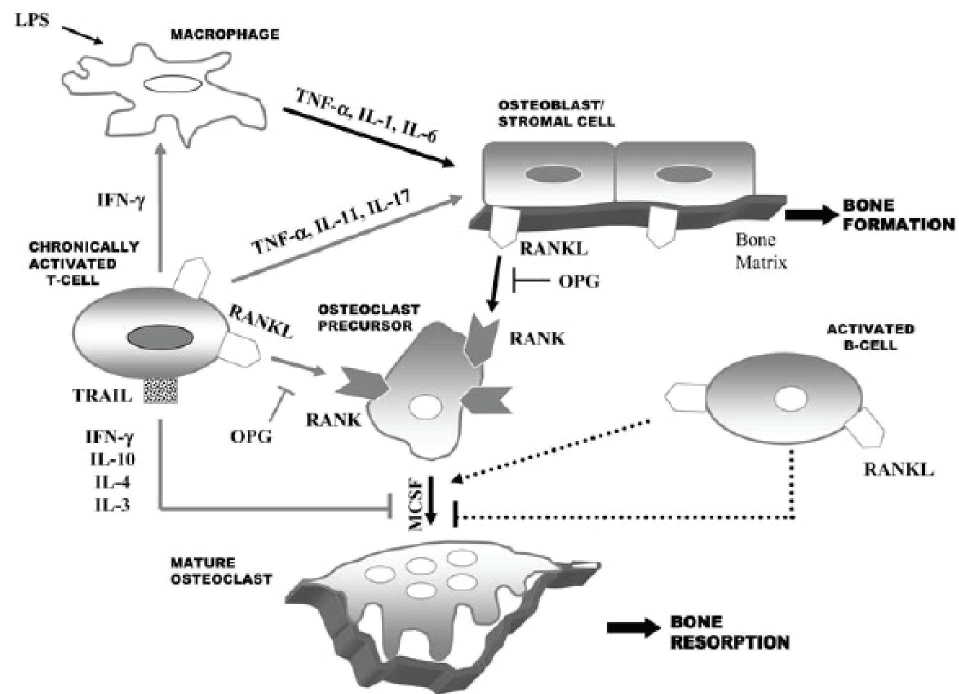

Figure 1.5 Involvement of activated lymphocytes and macrophages in bone remodelling. Adapted from Valverde et al (2005).

Interestingly, the percentage of RANKL positive B cells (>90%) was substantially higher than the percentage of RANKL positive T cells (50-60%) in periodontitis (Han *et al* 2006). It was also shown in a rat periodontitis model that activated antigen specific B cells can induce RANKL-dependent periodontal bone loss (Kawai *et al* 2006). In the same study, it was also shown that stimulation of peripheral T and B cells did not produce detectable levels of OPG. This indicated that the OPG in gingival tissues may be produced by fibroblasts in periodontal tissues.

Smoking has been found to have no effect on the proportions of B cells in lymphoid tissues in rats (Sopori *et al* 1989) or in human serum (Loos *et al* 2004). However, Mili *et al* (1991) showed that the average absolute B count of heavy smokers was 37% higher than that of non-smokers and it returned to normal on cessation of smoking. There is also evidence that B cells are functionally compromised under the influence of smoking leading to reduced proliferative responses to oral pathogens and subsequently reduced immunoglobulins production (Mooney *et al* 2001).

Smoking has been associated with reduced serum levels of IgG2 in white subjects with healthy periodontal status, generalised early-onset periodontitis and adult periodontitis, but serum IgG2 levels in black subjects were not depressed (Graswinckel *et al* 2004,

Gunsolley *et al* 1997, Quinn *et al* 1996, Quinn *et al* 1998). This may indicate a possible lower antibody response in smokers as supported by animal studies (Geng *et al* 1996, Sopori *et al* 1998).

The expression of RANKL on B cells mediated by smoking is most likely to be insignificant.

1.10.5 Fibroblasts

Fibroblasts are one of the most abundant cells in the periodontium. They are essential for the maintenance of gingival and periodontal ligament extracellular matrix as well as for wound healing. It is possible that smoking suppresses the normal functions of fibroblasts leading to reduced healing capacity of the periodontium. This may be consistent with the findings of a statistical model by Faddy *et al* (2000) that showed cumulative effect of impaired repair processes due to smoking. Periodontal ligament fibroblasts stimulated with *A. actinycetemcomitans* lipopolysaccharide were found to produce RANKL which was mediated by PGE2 (Tiranathanagul *et al* 2004). Gingival fibroblasts produce more OPG and less RANKL compared to periodontal ligament fibroblasts (Hormdee *et al* 2005). This may mean that gingival fibroblasts might prevent alveolar bone loss through the production of OPG, but osteoblasts and periodontal ligament fibroblasts might augment alveolar bone loss if inflammatory mediators penetrate these cells. The effects of nicotine and some other components of tobacco smoke have been tested in in vitro studies on gingival and periodontal ligament fibroblast cell lines.

1.10.5.1 Human gingival fibroblasts

The cytotoxic effects of smoking on fibroblasts certainly impact on the healing capability of the periodontium. Not only is nicotine harmful to fibroblasts, nicotine has also been found on root surfaces of periodontally affected teeth extracted from smokers (Cuff *et al* 1989). Nevertheless, the levels of nicotine may be reduced by thorough root planing (Cuff *et al* 1989). It has been found that nicotine can bind to gingival fibroblasts but they did not demonstrate any specific receptor binding for nicotine (Hanes *et al* 1991). In terms of attachment to glass and non-diseased root specimens, they found that the varying concentrations of nicotine (25-100 ng/ml) affected the orientation of fibroblasts, with cells overlapping and displaying vacuolization (Tanur *et al* 2000). The growth of fibroblasts and

the production of fibronectin and type I collagen were also inhibited and collagenase activity was significantly increased (Tipton and Dabbous 1995). However, the cytotoxic effects of nicotine seem to be reduced in human gingival fibroblasts from smokers and older donors (Checchi *et al* 1999).

Collagen production by gingival fibroblasts was inhibited when epithelial cells were treated by different concentrations of nicotine (Giannopoulou *et al* 2001). However, the production of non-collagenous proteins reduced by 50% only when epithelial cells were treated by the highest concentration of nicotine (500 µg/ml). The authors suggested that the mechanical barrier of epithelial cells can reduce but not eliminate the deleterious effects of nicotine on gingival fibroblasts.

When human gingival fibroblasts were treated with two volatile components of cigarette smoke, acrolein and acetaldehyde, their proliferation and attachment were inhibited in a dose-dependent manner (Cattaneo *et al* 2000). Not only that, their cytoskeleton was also disturbed leading to alteration in cell shapes and reduction in cell attachment and viability (Poggi *et al* 2002).

Human gingival fibroblasts have also been found to participate in the host response by releasing inflammatory cytokines (Dongari-Bagtzoglou and Ebersole 1998). Human gingival fibroblasts taken from diseased sites of chronic periodontitis patients produce greater amounts of cytokine in vitro than cells from healthy sites (Almasri *et al* 2007, Dongari-Bagtzoglou and Ebersole 1998, Wendell and Stein 2001). High doses of nicotine and either *E. coli* or *P. gingivalis* lipopolysaccharide also have synergistic effects in upregulating cytokine production by human gingival fibroblasts (Wendell and Stein 2001). This supports the theory of fibroblast heterogeneity, initially proposed by Ko *et al* (1977), who demonstrated that subpopulations of human gingival fibroblasts were responsive to prostaglandin.

1.10.5.2 Periodontal ligament fibroblasts

Nicotine has an inhibitory effect on periodontal ligament fibroblast growth and attachment to tissue culture plates (James *et al* 1999). Nicotine also negatively affects the functions of periodontal ligament fibroblasts which included proliferation, attachment, alkaline phosphatase production and chemotaxis (Giannopoulou *et al* 1999). It has been reported

that proliferation of periodontal ligament fibroblasts is inhibited by nicotine in a dose-dependent manner (Chang *et al* 2002, Giannopoulou *et al* 1999).

In general, nicotine suppresses fibroblast functions. It is not possible to estimate the in vivo potential of these effects based upon the data from these in vitro experiments, which usually test high levels of nicotine and do not take the other noxious compounds into account. Nevertheless, it is likely that smoke products will affect fibroblast recruitment and adhesion to root surfaces.

1.10.6 Endothelial cells

Apart from long term chronic effect of smoking on vasculature in periodontal tissues, the negative effects of smoking on endothelial cells and possible implication with atherosclerosis have been well established (Blann and McCollum 1993, Cacciola *et al* 2007, Pittilo 2000). Therefore, OPG production by endothelial cells is most likely reduced by smoking.

1.11 Gingival crevicular fluid

To date, periodontal diagnostic procedures rely heavily on traditional methods utilised in combination with the continuous assessment philosophy. It can be very subjective and at times unreliable. Therefore, there is always a need for diagnostic tests that provide more accurate assessment of periodontal disease activity than currently available methods.

GCF contains a rich array of cellular and biochemical mediators that reflect the metabolic status of periodontal disease (Page 1992). GCF is an easily and non-invasively collected medium for assessing changes in the periodontal tissues (Curtis *et al* 1989). Numerous components have been studied for their potential as markers of periodontal breakdown, but none of these have fulfilled the ideal requirement of a diagnostic test even though studies on GCF extend over a period of about 50 years (Loos and Tjoa 2005). Considering periodontitis is characterised by bone loss and attachment loss, markers specific to bone matrix such as deoxypyridinium (Giannobile *et al* 1995), chondroitin sulfate (Smedberg *et al* 1993) and osteocalcin (Bullon *et al* 2005) have been studied in GCF as potential indicators of disease activity. Although some seem promising as potential markers, more studies are required before they become useful clinical tests.

Considering periodontitis is characterised by bone loss and attachment loss, a marker specific to bone matrix may be a reliable indicator of disease activity. RANKL which is involved in regulation of osteoclastogenesis in bone remodelling and inflammatory osteolysis is found in elevated levels in GCF in periodontal disease (Vernal *et al* 2004). The ratio of the concentration of RANKL to that of OPG was also found to be significantly higher for patents with periodontal disease (Bostanci *et al* 2007a, Mogi *et al* 2004). This could potentially be a useful biological marker.

It is unlikely that one test will fulfil the needed specificity and sensitivity required for accurate diagnosis and disease prediction. In fact, a battery of tests that are based on bacterial etiology, genetic susceptibility, host response, and metabolic events associated with the development, progression and resolution of periodontal disease will be used in conjunction with additional measures of anatomical changes. This would then provide an accurate picture of past disease activity, current disease status, likelihood of future breakdown and response to treatment (Bartold and Narayanan 1998).

Researchers have been searching actively for a marker for periodontitis in GCF. The goal is to develop a simple chair side 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.

Some studies express GCF concentrations and some "total amounts" of a substance per unit sample time. The latter has been recommended in studies that attempt to identify markers of active disease (Chapple *et al* 1999, Lamster *et al* 1986). However, for studies that address pathogenesis, it is vital to measure GCF volumes and examine concentrations as well as total amounts of the analyte under investigation. This is because studies have shown a significant relationship between pocket depth and GCF volume and the latter is a major confounder of analyte concentration in association studies of periodontitis (Brock *et al* 2004).

Finally, the comparative contribution of serum- and tissue-derived products in a GCF sample is impossible to determine. Persson *et al* (1999) showed that smokers had significantly lower resting GCF flow rate. In addition, Bergstrom and Preber (1986) also showed that the increase in GCF during experimental gingivitis was less in smokers. McLaughlin *et al* (1993) demonstrated that smoking caused an immediate effect of a transient increase in GCF flow rate. On smoking cessation, Morozumi *et al* (2004) showed that GCF flow was greater at 5 days after quitting.

It would seem logical to assume that factors associated with periodontitis would be higher in smokers than non-smokers. However, most GCF studies have shown lower levels of cytokines associated with smoking. This could therefore be related to lower levels of inflammation.

1.12 Conclusions

Periodontitis is a multifactorial disease which involves complex interactions between bacteria and host response, modified by environmental factors. Smoking is clearly one of the major risk factors for chronic periodontitis. It increases the risk of having periodontitis and the effect is dose-dependent. Numerous studies have been conducted to elucidate the mechanisms involved in pathogenesis of smoking-related periodontitis. Smoking is able to shift the host's reparative processes towards a more destructive state leading to tissue degradation. While headways have been made in various areas concerning the molecular pathogenesis of periodontitis, one area which is of increasing importance is the involvement of the RANKL/OPG axis in bone remodelling and bone loss in periodontitis. Bone loss is one of the most striking diagnostic features of periodontitis and smokers suffer from more severe alveolar bone loss. It is possible that smoking-related periodontitis is partly related to homeostatic disturbance along the RANKL/OPG pathway.

RANKL and OPG are expressed by multiple cells in periodontal tissues. Numerous studies have shown higher levels of RANKL and lower levels of OPG in periodontitis patients as compared with healthy controls. Furthermore, an increased RANKL:OPG ratio appears to be a reliable indicator for bone loss with greater ratio leading to greater bone loss. The reparative processes may well be reflected by OPG and the destructive or inflammatory processes may be reflected by RANKL. Therefore, it is possible that cigarette smoking may affect the cells in periodontal tissues in such a way that RANKL production is

increased and OPG production is decreased. More studies are required to elucidate this further. Nevertheless, the interactions of smoking with periodontal pathogens and gene polymorphisms should not be overlooked considering the multifactorial nature of periodontitis.

Bone remodelling and bone resorption involves a complex molecular interplay between RANKL and OPG. Therefore, the RANKL:OPG ratio may be bone-specific which makes it a promising disease marker in smokers and non-smokers. If the disease activity is reflected in the expression of RANKL and OPG, then treatment of periodontitis could be targeted to modulate this pathway. Studies have been carried out to explore the possibility of using OPG in blocking bone loss in periodontitis. However, more studies are required to determine the most efficacious therapeutic approach on this molecular interaction in smokers and non-smokers. Nevertheless, smoking cessation remains one of the most effective ways of controlling smoking-related periodontitis.

The RANKL/OPG axis presents an exciting area in the field of periodontal research. Understanding the influence of smoking on the axis is important in terms of diagnostics and therapeutic inventions. Furthermore, it would contribute to the understanding of the pathogenesis of periodontitis.

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Chapter 2.

EFFECT OF SMOKING ON CONCENTRATIONS OF RANKL AND OPG IN HUMAN GINGIVAL CREVICULAR FLUID

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2.1 Abstract

Background and Objective: Smoking is one of the major risk factors for chronic periodontitis. However, the mechanisms involved in tissue degradation due to cigarette smoking are not clear. Receptor activator of nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG) are a system of molecules that regulate bone resorption. The aim of this study was to compare the levels of soluble RANKL (sRANKL), OPG and their relative ratio in GCF among periodontitis patients with varying smoking histories.

Material and Methods: GCF samples were collected from 149 periodontitis patients who were never smokers (n=58), former smokers (n=39) and current smokers (n=52). sRANKL and OPG concentrations in GCF were measured by enzyme-linked immunosorbent assays.

Results: sRANKL, OPG and their relative ratio were not statistically significant among the never smokers, former smokers and current smokers. However, OPG was significantly reduced and subsequently the sRANKL:OPG ratio was significantly increased in the high pack-years group as compared with never smokers. The positive correlation between pack-years and sRANKL:OPG ratio was statistically significant even after adjusting for age and current smoking status.

Conclusion: Increased lifetime exposure to cigarette smoking above a minimum threshold suppresses OPG production and leads to increased sRANKL:OPG. This may partially explain increased bone loss in smoking-related periodontitis.

2.2 Introduction

Smoking is recognised as one of the major risk factors for chronic periodontitis (Borrell and Papapanou 2005). It is associated with degradation of the periodontal tissues leading to attachment loss, bone loss and eventually tooth loss if left untreated (Johnson and Hill 2004). In several studies, smokers' risk of having periodontitis is 5 to 6 times as great as non-smokers (Bergstrom 2004). The relative risk is particularly higher among younger populations (Linden and Mullally 1994). A clear dose-response relationship between periodontitis and smoking has also been reported by several studies (Alpagot *et al* 1996, Grossi *et al* 1995, Martinez-Canut *et al* 1995). Furthermore, smokers suffer from a higher incidence of tooth loss even in periodontally treated and well-maintained populations (McGuire and Nunn 1999).

Numerous studies have attempted to explain the mechanisms of action of smoking in the pathogenesis of periodontitis, but they are still poorly understood. So far, the effects of smoking on subgingival microflora (Haffajee and Socransky 2001, Umeda *et al* 1998, Zambon *et al* 1996), gingival vasculature (Bergstrom *et al* 1988, Clarke *et al* 1981, Rezavandi *et al* 2002), neutrophils (Gustafsson *et al* 2000, Ryder *et al* 1998, Soder *et al* 2002, Sorensen *et al* 2004), serum IgG (Gunsolley *et al* 1997, Quinn *et al* 1998, Quinn *et al* 1996) and circulating levels of cytokines (Bostrom *et al* 1998, Giannopoulou *et al* 2003, Petropoulos *et al* 2004) have been reported. Although the findings from these studies are conflicting, they indicate that smoking may interfere with several reparative and destructive factors in the pathogenesis of periodontitis.

One of the main diagnostic features of periodontitis is alveolar bone loss. Numerous studies have shown that smokers have more severe alveolar bone loss than non-smokers (Meinberg *et al* 2001, Payne *et al* 2000). In addition, smokers also suffer from more progression of bone loss than non-smokers in longitudinal studies (Bergstrom *et al* 2000a, Jansson and Lavstedt 2002). Smoking is also related to higher incidence of localised alveolitis (Sweet and Butler 1979) and delayed alveolar healing in extraction sockets (Pinto *et al* 2002). This relationship is interesting as smoking not only affects alveolar bone, it also affects bone in general. Smoking has been reported to impair osseous healing, affect bone mineral (Benson and Shulman 2005, Hollinger *et al* 1999) and lower mineral density in postmenopausal women (Rapuri *et al* 2000).

Bone remodelling is a coupled process between bone formation and bone resorption. It is regulated by molecular interactions between receptor activator of nuclear factor κB ligand (RANKL), its cellular receptor, RANK and the decoy receptor, osteoprotegerin (OPG) (Kong *et al* 1999). This has been demonstrated in disease models such as rheumatoid arthritis and periodontitis (Firestein 2003, Taubman *et al* 2005). RANKL and OPG are both members of the tumour necrosis factor family. They are expressed by multiple cells in periodontal tissues such as fibroblasts, endothelial cells and osteoblasts. Their expression is up-regulated by inflammatory cytokines such as IL-1 and TNF-α (Hasegawa *et al* 2002, Lacey *et al* 1998, Quinn *et al* 2000, Sakata *et al* 1999).

In the presence of macrophage colony-stimulating factor, RANKL is required for osteoclast differentiation. RANKL binds directly to RANK on the surface of pre-osteoclasts and osteoclasts, stimulating both the differentiation of osteoclast progenitors and activity of mature osteoclasts (Burgess *et al* 1999, Hsu *et al* 1999, Lacey *et al* 1998, Matsuzaki *et al* 1998). On the other hand, OPG is the naturally occurring inhibitor of osteoclast differentiation. It binds to RANKL with high affinity and blocks RANKL from interacting with RANK (Lacey *et al* 1998, Yasuda *et al* 1998). Overall, bone resorption occurs as a result of the uncoupled process in bone remodelling and it can be reflected in an increased RANKL/OPG ratio. The increased ratio could be due to increased RANKL, decreased OPG or the combination of both.

In gingival tissues, periodontitis patients exhibit higher expression of RANKL (Bostanci *et al* 2007b, Cesar-Neto *et al* 2007, Crotti *et al* 2003, Garlet *et al* 2004, Liu *et al* 2003, Vernal *et al* 2004) and lower expression of OPG (Crotti *et al* 2003, Liu *et al* 2003) compared with healthy controls. Similar results have been reported for gingival crevicular fluid (GCF) (Bostanci *et al* 2007a, Lu *et al* 2006, Mogi *et al* 2004, Vernal *et al* 2004). As a consequence, RANKL/OPG ratios are found to be increased in periodontitis patients compared with healthy controls (Bostanci *et al* 2007a, Bostanci *et al* 2007b, Liu *et al* 2003, Mogi *et al* 2004). Vernal *et al* (2004) studied the GCF levels of RANKL in relation to disease progression in a group of untreated periodontitis patients. They showed that higher levels of RANKL were associated with active sites as compared with inactive sites. In all the studies mentioned above, there was no reporting on the proportions of smokers. Over the last 2 years, three studies have been published investigating the effect of smoking on the expression of RANKL and OPG (Buduneli *et al* 2008, Cesar-Neto *et al* 2007, Lappin *et al* 2007). The studies have shown consistently that smoking has an inverse relationship on

the expression on OPG. Considering smoking is one of the major risk factors in chronic periodontitis, more studies are warranted to determine the effect of smoking on the modulation of RANKL and OPG expression.

Therefore, for this study we hypothesized that among periodontitis patients, smokers would have higher RANKL:OPG ratio. This could explain in part the greater amount of bone loss in smokers compared to non-smokers. This could either be related to lower lowers of OPG or higher levels of RANKL in smokers compared with non-smokers. GCF contains a rich array of cellular and biochemical mediators that reflect the metabolic status of periodontal disease. It is an easily and non-invasively collected medium for assessing changes in periodontal tissues. We aimed to measure the levels of OPG, sRANKL and their relative ratio in GCF of periodontitis patients and to investigate the relationship between these mediators and cigarette smoking.

2.3 Aims and hypothesis

Considering RANKL and OPG are the key regulators of alveolar bone loss in periodontitis and the severity of periodontal breakdown is increased in smokers, we hypothesise that the RANKL:OPG ratio is higher in smokers compared to non-smokers, among periodontitis patients

The aims of this project are to:

- investigate the GCF levels of RANKL and OPG in a group of periodontitis patients
- relate history of smoking to the GCF levels of RANKL and OPG, and their relative ratio

2.4 Materials and methods

2.4.1 Subject Selection

Periodontitis patients attending clinics at the Adelaide Dental Hospital were invited to participate in the study. They were a convenience sample of patients who were either treated or untreated, recruited between January 2007 and April 2008. Written and informed consent were obtained from each subject before enrolment in the study. Complete medical histories were obtained after the enrolment. The exclusion criteria included pregnant women and sites with evidence of suppuration or clinical or radiographic evidence of

endodontic pathology. This study was approved by the Human Ethics Research Committee of The University of Adelaide.

The smoking history was collected by means of self-reporting following a standardised questionnaire (Appendix 2.1). Based on this questionnaire, patients were then classified as either current smokers (regular daily smokers); former smokers (previous regular smokers who had ceased the habit); and never smokers, (never smoked cigarettes). The life time smoking exposure of former and current smokers was expressed as pack-years: calculated as the number of cigarettes smoked per day multiplied by the number of years the patient had smoked, divided by 20 (a standard pack of cigarettes). The life time exposure of these former and current smokers was subsequently categorised into ≥20 pack-years, <20 pack-years and 0 pack-years.

All subjects underwent a clinical periodontal examination and the presence and severity of periodontitis was ascertained from the documentation in each subject's dental records and assessment of past dental radiographs. Using the modified Hugoson and Jordan (1982) classification, subjects were divided based on the following criteria: no discernible radiographic evidence of bone loss (representing no periodontal disease, P0), proximal bone loss reaching at most one third of normal bone height (mild periodontal disease, P1), proximal bone loss between one third and two thirds of normal bone height (moderate periodontitis, P2), or proximal bone loss more than two thirds of normal bone height (severe periodontitis, P3).

2.4.2 Site selection and GCF sampling

Each subject had GCF samples taken from the two periodontal sites that had the deepest periodontal pockets. These sites were selected after full-mouth probing measurements of probing depth (PD) at 6 sites per tooth. The probing was done at the same visit that GCF was sampled. The probing pocket depth and gingival recession of the sampled sites were recorded. The GCF was sampled by previously published methods (Offenbacher *et al* 1986) with slight modification (Uematsu *et al* 1996). All clinically detectable supragingival plaque was removed without touching the gingiva in order to minimise contamination of the paper strips by plaque. The sites under study were isolated with cotton rolls and gently dried with an air syringe. A saliva ejector was used to avoid salivary contamination if necessary. Paper strips (Periopaper, Harco, Tustin, CA, USA) were

carefully inserted 1 mm into the gingival crevice for 1 minute. The presence or absence of blood on the paper strips was also recorded. The volume of GCF collected was measured with a Periotron 8000 (Harco, Tustin, CA, USA). The Periotron readings were then converted through a calibration curve to obtain the volume (GCF Vol). The calibration curve was constructed according to the manufacturer's instructions which involve recording Periotron readings corresponding to set volumes of saline. One paper strip was used for each collection site. The paper strips from the individual sites were stored at -20°C for later processing.

GCF was extracted from the paper strips with phosphate buffered saline, pH 7.2, and collected following centrifugation. The eluted samples from each individual strip were pooled for each patient and were stored at -20°C for Enzyme Linked Immunosorbent Assays (ELISA) testing later.

2.4.3 Soluble RANKL (sRANKL) and OPG analysis in GCF

The amount of sRANKL and OPG in GCF was determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA for OPG and Biomedica, Vienna for sRANKL) in accordance with the manufacturers' instructions. The lower detection thresholds for the OPG and sRANKL ELISA kits were 50.8 pg/ml and 1.6 pg/ml respectively. Calculation of sRANKL and OPG concentrations in GCF was performed by dividing the total amount by volume of the samples. Data were reported as the amount of sRANKL or OPG per microlitre of GCF (pg/μl of GCF).

2.4.4 Statistical analysis

Histograms were generated to depict the distribution of sRANKL and OPG and descriptive statistics were generated. The data were markedly skewed, particularly for OPG where 74.8% of samples were below the detection threshold. For statistical analysis, the data therefore were log-transformed for individual mediators (Figures 2.1 - 2.4):

$$\text{Transformed sRANKL} = \log([\text{sRANKL}] + 0.003)+6$$

$$\text{Transformed OPG} = \log([\text{OPG}] + 0.98) + 0.1$$

Where: [RANKL] = concentration of RANKL; the constant 0.003 was halfway between zero and the minimum-observed concentration of RANKL; the constant 6 moved the log-transformed data curve sufficiently to the right to create positive values.

[OPG] = concentration of OPG; the constant 0.98 was halfway between zero and the minimum-observed concentration of RANKL; the constant 0.1 moved the log-transformed data curve sufficiently to the right to create positive values.

Constants were used in these formulas so that a mathematically meaningful ratio of the transformed data could be computed that did not have negative values. The ratio of transformed variables represented the third dependent variable used in the analysis:

$$\text{sRANKL:OPG ratio} = \frac{\text{transformed sRANKL}}{\text{transformed OPG}}$$

Additional descriptive statistics were generated for smoking history and demographic characteristics of subjects.

Statistical analysis was undertaken to test hypotheses about the relationship between cigarette consumption and three dependent variables: transformed sRANKL, transformed OPG and the sRANKL:OPG ratio. It was expected the mediators could be influenced separately by the qualitative experience of cigarette smoking (current, former, never) and the quantitative amount of exposure (pack-years), multivariate statistical models were constructed separately for each dependent variable. Age was expected to be associated with the mediators and with smoking, so it was included as a covariate in the models. Because mediators were measured at two sites per subjects, generalised estimating equations were used to adjust for clustering of observations within subjects. This was achieved in SAS proc genmod assuming an independent working correlation matrix. Each dependent variable was modelled as a linear variable using the identity link function. Statistical significance of parameter estimates was based on Wald's tests and 95% confidence intervals calculated using empirical standard error estimates, and $p \leq 0.05$ was considered to be statistically significant.

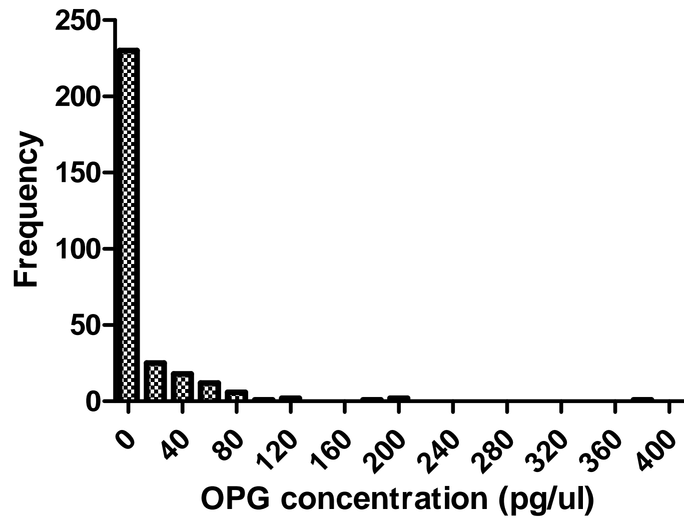

Figure 2.1 Frequency distribution of OPG concentration.

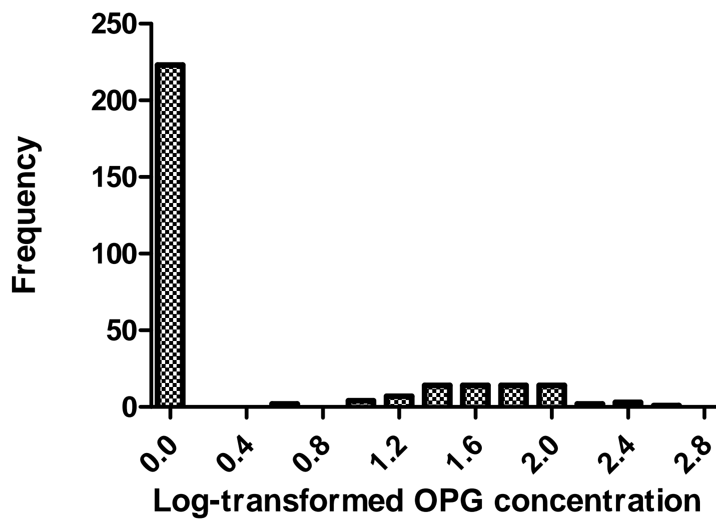

Figure 2.2 Frequency distribution of log-transformed OPG concentration.

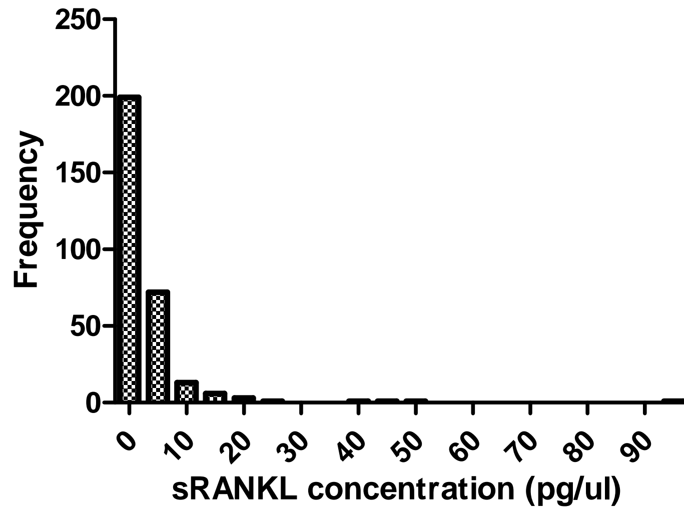

Figure 2.3 Frequency distribution of sRANKL concentration.

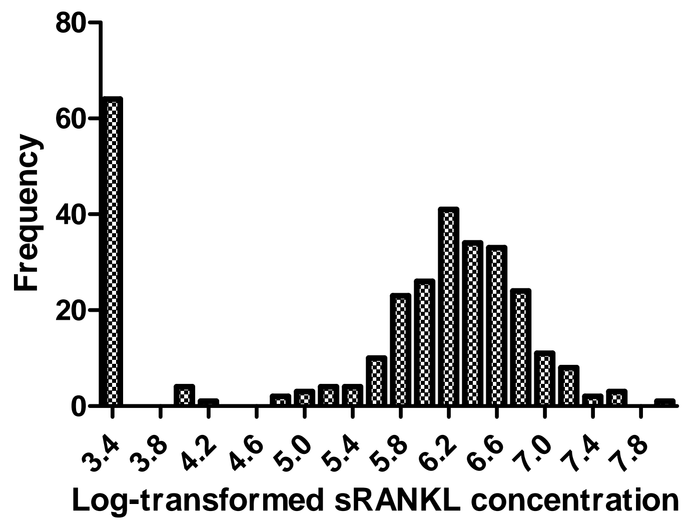

Figure 2.4 Frequency distribution of log-transformed sRANKL concentration.

2.5 Results

	Never smoker	Former smoker	Current smoker
Number of subjects	58	39	52
No. of Female:Male	42:16	19:20	32:20
Age (years) (±SD)	60 (±15)	57 (±15)	49 (±10)
PPD (mm) (±SD)	4.9 (±1.4)	5.4 (±1.4)	5.1 (±1.4)
REC (mm) (±SD)	1.1 (±1.1)	1.2 (±1.3)	1.3 (±1.1)
GCF Volume (µl) (±SD)	1.13 (±0.59)	1.16 (±0.58)	1.01 (±0.62)
P1 (%)	19 (33)	8 (21)	12 (23)
P2 (%)	22 (38)	14 (36)	22 (42)
P3 (%)	17 (29)	17 (44)	18 (35)
Mean cigarettes/day (±SD)	0	16.4 (±11.7)	16.5 (±7.8)
Mean duration of smoking (years) (±SD)	0	21.3 (±13.3)	28.0 (±10.7)
Pack-years (±SD)	0	18.3 (±16.1)#	22.8 (±15.1)

SD = sample standard deviation; PPD=probing pocket depth, REC=gingival recession

Pack-years data were missing for one patient, the calculation was based on 38 subjects.

Table 2.1 Demographic parameters of subjects and clinical parameters of the sampling areas in the study group

2.5.1 Population demographics

There were a total of 149 patients recruited into the study with 56 males and 93 females in the age range of 26-86 years (Table 2.1). They were stratified into 3 groups: <45, 45-<65 and ≥65. There were 37, 66 and 46 in those three age groups respectively. The numbers of current smokers, former smokers and never smokers in the populations under study were 52, 39 and 58 respectively.

2.5.2 Never smokers

The never smoker group included 42 females and 16 males who never smoked cigarettes (Table 2.1). These patients ranged in age from 27 to 83 years (mean age = 60 years). There were 33%, 38% and 29% in the P1, P2 and P3 categories respectively.

2.5.3 Former smokers

The former smoker group included 19 females and 20 males who had ceased smoking at the time of recruitment (Table 2.1). These patients ranged in age from 34 to 81 years (mean age: 57 years). There were 21%, 36% and 44% in the P1, P2 and P3 categories respectively. The mean previous consumption and duration were 16.4 cigarettes per day and 21.3 years respectively. The mean smoking-free time of former smokers since smoking cessation was 13.5 years. The mean life time exposure for former smoker groups was 18.3 pack-years with the calculation based on 38 subjects.

2.5.4 Current smokers

The current smoker group included 32 females and 20 males who were daily cigarette smokers on the day of enrolment (Table 2.1). These patients range in age from 33 to 69 years (mean age = 49 years). There were 23%, 42% and 35% in the P1, P2 and P3 categories respectively. The mean consumption and duration of current smokers were 16.5 cigarettes per day and 28 years respectively. The mean life time exposure for the current smokers was 22.8 pack-years.

2.5.5 Clinical findings of periodontal sites

The demographic and clinical data are shown in Table 2.1. The mean PD and the mean recession of the sampled sites appeared to be increased in current and former smokers as compared with never smokers. In addition, the GCF volume appeared to be decreased in current smokers compared with never smokers and former smokers. Statistical analyses were not performed on the clinical data as they were not the primary measures of this study.

	Never-smoker	< 20 pack-years	≥ 20 pack-years
Number of subjects	58	51	39
Mean age (years)	60	52	54
Former smokers			
(Number of subjects)		24	15
Current Smokers			
(Number of subjects)		27	24

Table 2.2 Demographic parameters of subjects according to pack-years in the study group.

The data in Table 2.2 show that based on pack-years, 51 patients smoked less than 20 pack-years while 39 patients smoked equal or greater than 20 pack-years. The mean age in the <20 pack-year and ≥20 pack-year groups were 52 and 54 years respectively. There were 24 former smokers and 27 current smokers in the low pack-years group (<20 pack-years) while there were 15 formers and 24 current smokers in the high pack-years group (≥20 pack-years). The mean smoking-free time of former smokers who had smoked ≥20 pack-years was 7.9 years and for those former smokers who had smoked <20 pack-years was 16.6 years.

2.5.6 sRANKL and OPG concentrations

Out of the total of 298 sites analysed, sRANKL and OPG were detectable in the GCF collected from 234 and 75 sites respectively.

2.5.6.1 Age

Age (years)	N†	Log-transformed OPG	Log-transformed sRANKL	Log-transformed sRANKL:OPG
<45	37	0.6	5.8	68.4
45-<65	65	0.8	5.0	54.1
≥65	46	1.7	5.2	42.9*

* Significant difference compared to the <45 group (p=0.01) - Wald's test.

† Number of subjects

Table 2.3 sRANKL and OPG concentrations in GCF between different age groups (person-level analysis).

The data were analysed based on the age group as outlined in Table 2.3. The log-transformed OPG concentration was increased with increasing age. The log-transformed sRANKL concentration was lower in the 45-<65 and ≥65 groups than in the <45 people. Neither of these relationships was statistically significant. However, the log-transformed sRANKL:OPG was significantly lower in the ≥65 age group as compared with the <45 age group (p=0.01).

2.5.6.2 Smoking status and lifetime exposure (Pack-Years)

	N†	**Log-transformed OPG**	**Log-transformed sRANKL**	**Log-transformed sRANKL:OPG**
Never smoker	58	1.3	5.0	47.0
Former smoker	38	0.8	5.8	65.5
Current smoker	52	0.8	5.2	54.0
<20 pack-years	51	1.0	5.3	51.5
≥20 pack-years	39	0.6*	5.5	68.4#

* Significant difference from never smoker groups (p=0.05) - Wald's test.

Significant difference from never smoker groups (p=0.03) -Wald's test.

† Number of subjects

Table 2.4 sRANKL and OPG concentrations in GCF between different smoking groups and pack-years groups (person-level analysis).

When subjects were categorised by smoking status, the log transformed OPG concentration tended to be lower in the former and current smokers compared to never smokers (Table 2.4). The opposite was seen with the log-transformed sRANKL concentration where higher concentrations were measured in GCF of the former and current smokers compared to never smokers. As a consequence, the sRANKL:OPG ratio also appeared to be higher in the former and current smokers compared to never smokers. However, none of these relationships were statistically significant (Table 2.4).

Apart from the smoking status, the subjects were also divided arbitrarily into high and low pack-years groups in order to take the quantitative amount of smoking into consideration.

The log transformed OPG concentration decreased with increasing pack-years with the value significantly lower in the high pack-years group (≥20 pack-years) compared with the never-smoker group ($p=0.05$) (Figure 2.1). Although the log transformed sRANKL appeared increased with increased pack-years but the difference was not statistically significant. Consequently, the log-transformed sRANKL:OPG ratio was significantly higher in the high pack-years group (≥20 pack-years) as compared with the never-smoker group ($p=0.03$) (Table 2.4). In the multivariate analysis adjusting for age and smoking status, log transformed sRANKL:OPG ratio remained significantly higher in the high pack-years group compared with low pack-years group ($p=0.03$). The periodontal diease severity was not included in the multivariate analysis it is a parameter indicative of disease outcome rather than disease process.

2.6 Discussion

Smoking is considered one of the most important environmental risk factors in modifying periodontitis. A key pathway in bone destruction involves the RANK/RANKL/OPG axis. Therefore, this study aimed to explore the interrelationship between smoking and the RANKL/OPG axis in modulating the manifestation of periodontitis.

To our knowledge, there are only three studies published to date that have looked specifically at the effect of smoking on serum levels, salivary levels and gene expression of RANKL and OPG in periodontitis patients (Buduneli *et al* 2008, Cesar-Neto *et al* 2007, Lappin *et al* 2007). However, the present study is the first to examine the effect of smoking on the levels of RANKL and OPG in GCF. The study by Lappin *et al* (2007) measured both RANKL and OPG concentrations in serum and found that periodontitis patients who were also current smokers had reduced levels of OPG and higher RANKL:OPG ratio as compared with periodontitis patients who were never smokers. Furthermore, the OPG levels were negatively correlated with pack-years. Another group detected gene expression in gingival tissues for both mediators in periodontitis patients and showed similar results, but the influence of pack-years was not considered (Cesar-Neto *et al* 2007). More recently, Buduneli *et al* (2008) measured the saliva concentrations of RANKL and OPG in smokers and non-smokers with periodontitis. They reported that the salivary OPG concentration was significantly lower and the sRANKL:OPG ratio was higher in smokers than non-smokers. Furthermore, OPG concentration also correlated negatively with pack-years.

In the current study comparing the three smoking groups, the concentrations of OPG, sRANKL and sRANKL:OPG ratio were not significantly different between the groups. When pack-years were taken into consideration, significant differences could be found between the high pack-years group and the never smoker group for OPG and the sRANKL:OPG ratio. There was a negative correlation between OPG concentration in GCF and pack-years and a positive correlation between sRANKL:OPG ratio and pack-years. Furthermore, the high pack-year group showed a significantly higher sRANKL:OPG ratio compared with the low pack-years group even after adjusting for age and current smoking status. The present data are in agreement with, and build on the findings from similar studies in serum, saliva and gingival tissues (Buduneli *et al* 2008, Cesar-Neto *et al* 2007, Lappin *et al* 2007). This finding may indicate that greater alveolar bone loss among smokers in periodontitis patients could be related to the suppression of OPG expression by cigarette smoking, leading to higher sRANKL:OPG ratio compared with never smokers. In addition, the influence of smoking on the concentrations of the two mediators, particularly OPG is dependent on the quantitative amount of cigarette exposure not the qualitative experience of smoking. Our data also suggests that a minimum threshold of lifetime exposure is required before cigarette smoking starts to have an effect on OPG levels and sRANKL:OPG ratio. As mentioned previously, the negative correlation between saliva and serum levels of OPG and pack-years has been reported (Lappin *et al* 2007, Buduneli *et al* 2008). Although Cesar-Neto *et al* (2007) did not consider pack-years in their analysis, it could be argued that it was the high lifetime exposure to cigarette smoking in their study that led to the reduced gene expression of OPG and increased RANKL:OPG ratio, not because of the current smoking status of the subjects. This is because the study only included smokers who had smoked ≥1 pack/day for at least 10 years with the mean number of pack-years in their smokers group being 26.5.

Based on the three age groups, the sRANKL:OPG ratio was significantly lower in the older people compared with the younger people. This finding may indicate that the progression of alveolar bone loss is more evident among the younger periodontitis patients as compared with the older periodontitis patients. While this may appear to be counter-intuitive as prevalence of periodontitis naturally increases with age (Albandar 2002, Albandar *et al* 1999), alveolar bone loss as a result of periodontitis is mostly irreversible, so epidemiological measures of periodontitis mostly capture cumulative experience of disease. Nevertheless, age is an important factor in identifying individuals susceptible to periodontitis progression. The amount of bone loss in relation to patient's age may be a

good predictor of future disease progression. A young individual with aggressive periodontitis is considered at higher risk of disease progression than an older individual with the same amount of disease (Heitz-Mayfield 2005). It can be proposed that the greater susceptibility to progression of alveolar bone loss in the younger group may be reflected in the sRANKL:OPG ratio. Interestingly, current smokers in this study were younger than former smokers and never smokers. However, the multivariate analysis showed age as an independent factor in influencing sRANKL:OPG ratio. Therefore, further studies are warranted to study the relationships between sRANKL:OPG ratio and periodontal disease susceptibility.

One of the shortcomings of our study is that 74.8% and 21.5% of the samples were below the detection threshold for the OPG and sRANKL ELISA kits respectively. This is a common occurrence reported in the literature (Arikan *et al* 2008, Lu *et al* 2006). It supports the need for the development of high-sensitivity assays. In addition, the high number of samples below the detection threshold for OPG could be related to the low levels of OPG in GCF in our present periodontitis populations. This is consistent with the previous findings that periodontitis patients have lower GCF levels of OPG compared with healthy controls (Mogi *et al* 2004, Lu *et al* 2006, Bostanci *et al* 2007a).

The mean probing depths and recession values of the sample sites appeared greater among the former and current smokers compared with never smokers. In addition, the proportions of current and former smokers who had lost alveolar bone of more than one third of root length (P2 and P3) seemed to be higher than never smokers. Although statistical analyses of these data were not performed, the observed patterns were consistent with the previous findings reporting that smokers have greater attachment loss (Grossi *et al* 1994), greater gingival recession (Calsina *et al* 2002, Martinez-Canut *et al* 1995) and greater alveolar bone loss (Grossi *et al* 1995). In addition, the GCF volume tended to be decreased in current smokers as compared with never smokers, but increased with smoking cessation in former smokers. This is consistent with the findings of previous studies (Bergstrom and Preber 1986, Morozumi *et al* 2004, Persson *et al* 1999).

From a clinical perspective, the present data correlates with the findings that smokers with a history of high pack-years suffer from more severe periodontitis compared with never smokers. In a cross-sectional study, increased exposure of smoking in terms of pack-years was found to be significantly correlated with probing depth (Alpagot *et al* 1996) and

alveolar bone loss (Grossi *et al* 1995). In addition, a minimum threshold of tobacco usage is necessary before it increases susceptibility to periodontal disease (Alpagot *et al* 1996, Martinez-Canut *et al* 1995). Once again, this demonstrates the dose-response relationship between cigarette smoking and periodontitis.

Interestingly, the high pack-years group considered in our study included 24 current smokers and 15 former smokers. This may indicate that the modulating effect of smoking on OPG and sRANKL:OPG remains even after smoking cessation. In a cross-sectional study involving 257 dentally aware adults, there was a lack of significant difference between former and current smokers in terms of periodontal bone height (Bergstrom *et al* 2000b). The mean smoking free time in their study was 12.5 years. Considering the mean smoking free time for former smokers who had smoked ≥20 pack-years in our study was 7.9 years, it is possible that the suppressive effect of smoking on OPG and subsequently increased sRANKL:OPG, remains after smoking cessation for several years. Although smoking cessation may bring resolution to periodontal soft tissue reactions as reported by Bergstrom *et al* (2000), the risk of losing further alveolar bone may still remain elevated for some time. In a 10-year longitudinal study also by Bergstrom *et al* (2000a), former smokers were found to have significant periodontal bone height reduction over the 10-year period although the change was towards the levels of never smokers. In addition, a report on periodontal risk assessment (Lang and Tonetti 2003) only considered individuals who had ceased smoking for at least 5 years as former smokers. Future studies are required to determine the risk of further disease progression in terms of alveolar bone loss after smoking cessation. Based on the present data, the short-term beneficial effects of smoking cessation on alveolar bone loss remain uncertain.

Overall, the reduced concentrations of OPG and subsequently increased sRANKL:OPG ratio in this study may partially explain the bone loss in smoking-related periodontitis. The mechanism by which OPG levels are reduced in GCF is yet to be elucidated. It can be speculated that the cells which produce OPG may be negatively affected by the direct and indirect effects of nicotine and other chemicals found in cigarettes leading to reduced OPG production. In addition to the reported harmful effects of nicotine on human osteoblasts (Walker *et al* 2001, Yuhara *et al* 1999), it has also been demonstrated that nicotine and LPS combined lead to reduced OPG production by osteoblasts in a dose-dependent manner (Tanaka *et al* 2006). Apart from osteoblasts, OPG is also produced by periodontal ligament (PDL) cells, gingival fibroblasts, endothelial cells and epithelial cells (Hofbauer *et al* 1998,

Kanzaki *et al* 2002, Sakata *et al* 1999). The negative effect of smoking on the function and proliferation of these cells may also contribute to the reduced OPG expression in smokers (Blann and McCollum 1993, Cattaneo *et al* 2000, Chang *et al* 2002, Giannopoulou *et al* 1999, Giannopoulou *et al* 2001, James *et al* 1999, Tipton and Dabbous 1995). Overall, OPG producing cells may be affected by the direct detrimental effects of cigarette smoking and the indirect adverse effects of the altered immuno-inflammatory response induced by cigarette smoking. Therefore, the resultant increased sRANKL:OPG ratio may then lead to an imbalance in tissue homeostasis and tissue degradation.

Considering periodontitis is a multifactorial disease, the modulating effect of smoking on OPG could be related to the composition of the subgingival microflora. In vitro studies have shown that periodontopathogens such as *P. gingivalis* and *A. actinomycetemcomitans* are able to affect RANKL and OPG mRNA expression by human PDL cells and gingival fibroblasts (Belibasakis *et al* 2005a, Belibasakis *et al* 2005b, Kiji *et al* 2007, Yamamoto *et al* 2006). In addition, the influence of genetics in the modulation of OPG expression by smoking is always possible. A recent study in a Korean population showed that two OPG gene polymorphisms were more associated with periodontitis patients compared to control subjects (Park *et al* 2008). Therefore, future studies are required to elucidate these complex interactions.

In conclusion, the results of the present study indicate that increased lifetime exposure to cigarette smoking above a minimum threshold suppresses OPG production and leads to an increased sRANKL:OPG ratio. This may partially explain increased bone loss in smoking-related periodontitis, and may open up avenues for future diagnostic applications and therapeutic interventions in periodontitis particularly for smokers.

2.7 Appendix 2.1

Questionnaire

Sample ID No:

Clinician:

Patient's Details

Name:

URL:

DOB:

Gender:

Medical History:

____

Has the patient had antibiotics treatment or non-steroidal anti-inflammatory therapy in the 6 months? Y / N

Treatment status: untreated / ongoing active treatment / supportive periodontal therapy

When did the patient last have periodontal treatment?

____

Periodontal Diagnosis:

____

Smoking History

Does the patient smoke cigarettes? Y / N

If yes, how many cigarettes per day? How long has the patient been smoking?

____

If no, has the patient ever smoked? Y / N

If yes, when did the patient stop smoking?

____

Sample Collection

Sites 1:

GCF Volume:

Presence of blood: Y / N

Probing Depth:

Recession:

Site 2:

GCF Volume:

Presence of blood: Y / N

Probing Depth:

Recession:

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Chapter 3.

FUTURE DIRECTIONS

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3.1 Future directions

From cross-sectional studies, there is increasing evidence of involvement of the RANKL/OPG axis in the pathogenesis of periodontitis in smokers and non-smokers. Future studies should be longitudinal in nature in order to establish the association of RANKL:OPG ratio with onset and progression of periodontitis. The influence of age is of particular importance and it should be adjusted in all future studies. This may help to explain the heterogeneity of susceptibility to onset and progression of periodontitis among smokers and non-smokers. Not only that, the quantitative amount of cigarette smoking in terms of pack-years should always be taken into consideration in future studies. Considering the multifactorial nature of periodontitis, the interactions of genetics and periodontal pathogens with the RANKL/OPG axis should be established further in smokers and non-smokers. Therefore, it is worthwhile to study the relative contribution of OPG gene polymorphisms and periodontal pathogens in future studies involving RANKL and OPG.

The RANKL and OPG levels in different forms of periodontitis, and changes of the levels subsequent to different periodontal therapies, should also be the focus of future studies. It would be interesting to determine whether RANKL and OPG levels could reflect the response of individuals to periodontal treatment. This may be of significance in smokers as the majority of refractory cases are smokers (MacFarlane *et al* 1992). In fact, studies are urgently required to explore the effect of smoking cessation on refractory periodontitis in current smokers. In addition, the risk of progression of alveolar bone loss, particularly in former smokers with periodontitis, should be determined further in relation to history of pack-years. It is possible that the risk may remain elevated for several years after smoking cessation.

The feasibility of a diagnostic test based on OPG and RANKL should be tested in the hopes of providing more accurate assessment of periodontal disease activity than currently available methods. Considering periodontitis is characterised by bone loss and attachment loss, a marker specific to bone matrix may be a reliable indicator of disease activity. RANKL and OPG, which are involved in regulation of osteoclastogenesis in bone remodeling and inflammatory osteolysis, could potentially be useful biological markers in both smokers and non-smokers with periodontitis. Nevertheless, it is unlikely that one single test based on OPG or RANKL will fulfill the needed specificity and sensitivity. In

fact, a battery of tests should be used based on bacterial etiology, genetic susceptibility, host response, and metabolic events associated with the development, progression and resolution of periodontal disease. In addition to the tests, measures of anatomical changes are also required to provide an accurate picture of past disease activity, current disease status, likelihood of future breakdown and response to treatment (Bartold and Narayanan 1998).

Tissue degradation in periodontitis has been related to an imbalance between Th1 and Th2 cytokines (Alayan *et al* 2007). There have been ongoing debates on whether Th1 or Th2 is more significantly related to periodontal disease progression or periodontal health and vice versa (Ebersole and Taubman 1994, Gemmell and Seymour 2004). As OPG expression correlates with the expression of Th2-type cytokines such as IL-4 and IL-10 (Garlet *et al* 2004), it can be speculated that IL-4 and IL-10 expression would be down-regulated by cigarette smoking. Indeed, IL-4 levels in GCF and IL-10 in gingival tissues have been found to be lower in smokers as compared with non-smokers (Cesar-Neto *et al* 2007, Giannopoulou *et al* 2003). Further studies are required to clarify the inter-relationships between OPG and the Th2-type cytokines. Not only that, the relative contribution in terms of OPG expression by individual cells in periodontal tissues should also be determined. With the full understanding of the pathway, the possibility of targeted delivery of treatment can then be established.

Considering that smoking suppresses OPG production and this may be one mechanism which leads to more alveolar bone loss in smokers, host modulation targeting OPG could be feasible and warrants further investigation. Use of the decoy receptor OPG to inhibit RANKL function has been investigated in mice orally infected with *A. actinomycetemcomitans* (Mahamed *et al* 2005, Teng *et al* 2000). Both studies showed significantly reduced numbers of osteoclasts and alveolar bone loss following the OPG treatment. Similarly, local injection of OPG has also been found to inhibit B-cell mediated periodontal bone resorption (Han *et al* 2006). Recently, the use of human recombinant OPG-Fc fusion protein in blocking bone loss has also been reported in an experimental periodontitis model (Jin *et al* 2007). Nevertheless, smoking cessation remains one of the most effective ways of controlling smoking-related periodontitis and should form part of a periodontal treatment plan.

In summary, periodontitis is a multifactorial disease with complex interactions between host response and plaque biofilm, further modified by genetics and environmental factors. Smoking is one of the major environmental risk factors for chronic periodontitis and it affects the tissue homeostasis by interfering with several reparative and destructive factors in the pathogenesis of periodontitis. The RANKL/OPG pathway clearly forms part of the equation. This understanding would strengthen our abilities to manage smoking-related periodontitis in terms of diagnostics and therapy.

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