The effect of dietary Omega-3 polyunsaturated fatty acids on experimental periodontitis lesions in the mouse

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

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Although the articles contained are multi-authored and contribution was greatly appreciated, their input was mainly advisory and I carried out all of the laboratory procedures and bulk of the writing.

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ABSTRACT

Periodontitis is an infective disease caused predominantly by gram negative anaerobic bacteria. However it is apparent that alveolar bone loss, which characterises periodontitis, is a result of the host inflammatory response to pathogenic bacteria, not the infectious agents directly. Omega-3 polyunsaturated fatty acids (O-3 PUFAs) are recognised, and used widely, for their anti-inflammatory effects. Evidence is emerging that their oxygenated derivatives are key chemical mediators in the resolution of inflammation. We hypothesised that dietary supplementation with fish oil rich in the O-3 PUFA docosahexaenoic acid would modify inflammatory reactions within the periodontium and thus reduce alveolar bone loss in mice infected with periodontopathic bacteria.

Eighty mice were fed experimental diets containing either 10% tuna oil (40) or a sunola oil (40) which contained no traceable O-3 PUFAs for 57 days. After two weeks each dietary set was split into four groups of ten mice, with these groups being inoculated with either

a) *Porphyromas gingivalis*
b) *P. gingivalis* and *Fusobacterium nucleatum* (combined inoculum)
c) Carboxymethylcellulose (control) or
d) No inoculations (control).

Of the twenty mice which received no inoculations, half were sacrificed after fifteen days and half at the end of the experiment to enable comparative fatty acid analysis of the oral soft tissues. Results demonstrated that eicosapentoic acid and docosahexaenoic acid were found in significantly higher proportions in the oral soft tissues of mice fed a tuna oil diet, and that animals receiving this diet exhibited an average of 54 - 72% less alveolar bone resorption in response to the different bacterial infections. Irrespective of diet, the combined inoculum resulted in slightly more alveolar resorption than *P. gingivalis* alone. The findings of this study suggest that fish oil dietary supplementation may have potential benefits as a host modulatory agent in the adjunctive management of periodontitis. Given its advantages in terms of safety, cost effectiveness and widespread availability, this dietary supplement warrants further research in human trials to assess its ability to modulate alveolar bone loss in individuals with periodontitis.

Diet, nutrition and periodontal disease
A literature review

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

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1.1 Introduction

Immunological competence and nutritional status are two of the most important determinants of morbidity and mortality in humans. Periodontitis is an infective disease, the extent and progression of which is influenced dramatically by the host response. Malnutrition has been consistently shown to impair innate and adaptive defences of the host, including phagocytic function, cell-mediated immunity, the complement system, secretory antibody production and secretion and action (Enwonwu *et al* 2002).

It is very likely that a relationship exists between nutritional status and the periodontal condition. However to date there is a paucity of uniform, convincing evidence, because of a general lack of study into specific areas (i.e. specific nutrients, minerals, etc), vast heterogeneity in the studies which have been performed (human, animal, experimental, cell culture, cross-sectional and case reports), different outcome measures (gingivitis, periodontitis, pocket depth, clinical attachment loss, radiographic bone loss, tooth loss, periodontal treatment), varied mechanisms of dietary/nutritional assessment and finally disagreement and a degree of confusion with regard to nomenclature and definitions (terms such as malnutrition, undernutrition, vitamin deficient etc). What follows is a review of the current literature, with regards to the relationship between periodontal diseases and both severe malnutrition (such as is seen predominantly in conditions of poverty in developing nations) and inadequate nutritional status (a less serious deficiency/excess in one or more important dietary components which is common in western societies like Australia).

1.2 Nomenclature

In this paper, **malnutrition** is defined as the "pathophysiologic consequences of the ingestion of inadequate, excessive or unbalanced amounts of nutrients" (WHO 2005). **Severe malnutrition** encompasses gross deficiencies in essential macronutrients and micronutrients

which predispose to nutrient deficiency disease and a high prevalence of various infections. Globally the most prevalent form of severe malnutrition is **protein energy malnutrition (PEM)**. PEM is a deficiency in protein or energy intake, or both, and covers clinical conditions including: failure to thrive, kwashiorkor, marasmus, cachexia, phthisis, nutritional dwarfism and nutritional or famine oedema (Tomkins *et al* 2000). More than 150 million children worldwide suffer from PEM, with most of these children residing in Sub-Saharan Africa and Southern Asia (Enwonwu 2002). Most of the estimated 33,000 children who die each day are classified as protein energy malnourished (Whitney and Rolfes 2002). In Australia PEM is exceedingly rare, seen mainly at the extremes of age (cases of neglect) and in individuals suffering from anorexia nervosa. However the possibility of a previous history of PEM/other severe malnutrition needs to be borne in mind by periodontists when assessing the periodontal status of refugees from developing nations.

Adequate nutritional status is more than the absence of nutrient deficiency disease. It is a broad concept, which infers that an individual can achieve a food intake sufficient to meet their requirements for specific nutrients to support optimal health and well-being. Numerous health organisations around the world publish **Recommended Daily Intake (RDI)** tables, guidelines to lower and sometimes upper limits for a range of important vitamins and minerals.

Individuals whose daily dietary intake consistently means they fall below minimum levels or above maximum levels for one or more nutrients can be classified as having **inadequate nutritional status (INS).** Groups most at risk of INS include those with malabsorption syndromes such as Crohn's or coeliac disease, alcoholics, people with eating disorders, those with HIV and the elderly. However data published in 2002 by the U.S Department of Agriculture showed that on any given day, 41% of subjects did not eat any fruit, 82% did not eat cruciferous vegetables, 72% did not eat vitamin C rich fruits or vegetables, 80% did not

eat vitamin A rich fruit or vegetables, and 64% did not eat high fibre grain food, like cereal (Basiotis *et al* 2002).

Secondary INS may also be present in individuals with impaired use or loss of ingested nutrients (eg menorrhagia and iron deficiency in females).

At a population level, overweight and obesity are measured by **body mass index (BMI)**. This is calculated as Weight (in kg)/ Height (in m)^2.

A Caucasian Australian is considered to be **underweight** if BMI <20, within the desirable weight range if BMI is 20-25, **overweigh**t if BMI is >25 and **obese** if BMI is >30. These ranges vary among different ethnicities.

The biggest nutritional problem facing developed countries is the alarming rise in people classified as either overweight or obese. In Australia in recent times this trend has received widespread attention from the media and public health sector. It was estimated in 2001 that 30% of Australians were overweight at that time and a further 20% obese, with the incidence of these conditions increasing. It was forecast that by 2010, 70% of Australians would be overweight or obese (NHMRC 2001). It is relatively common for a person to be obese and still have inadequate nutritional status, as food is mainly chosen on the basis of taste, cost, convenience and psychological gratification, largely ignoring the needs of providing nutrients to the body. Hence individuals who are obese may be ingesting large quantities of nutrient poor, calorie and saturated fat dense foods, as the USDA data on the previous page may indicate (Neiva and Steigenga 2003).

1.3 Severe malnutrition and periodontal disease

Several decades ago, it was reported that gingivitis and periodontitis, particularly aggressive forms in children and young adults, were far more prevalent and severe in socioeconomically disadvantaged communities in the third world than in western societies (Enwonwu and Edozien 1970, Macgregor 1980, Löe *et al* 1986).

Albandar *et al* (2002) showed that 199 out of 690 (28.8%) Ugandans between the ages twelve and twenty five years showed clinical signs of early-onset periodontitis. Acute necrotising gingivitis, which is relatively uncommon in the western world, is still frequently encountered in Sub Saharan Africa and it disproportionately affects impoverished children residing in unsanitary surroundings who are generally severely immunocompromised by malnutrition and various infections. The reported prevalence varies significantly but may be as high as 27.6% in certain rural communities (Arendorf *et al* 2001, Taiwo 1996).

Part of this high prevalence of early-onset periodontitis (EOP) and acute necrotising gingivitis (ANG) in developing countries is likely due to poor oral hygiene (Cohen *et al* 2002, Taiwo 1993). Racial differences in the gingival flora, particularly with reference to recovery of specific anaerobic microorganisms have also been reported (Craig *et al* 2001, Sawyer *et al* 1986). A far higher incidence of HIV infection in African/Southern Asian children probably also contributes to the high ANG figures (Contreras *et al* 1997). However studies in India (Pindborg *et al* 1967), Africa (Enwonwu and Edozien 1970, Enwonwu 1985) and South America (Jemenez and Baer 1975) have all suggested that EOP and ANG are far more prevalent in the impoverished and "visibly malnourished". Their findings suggest that prolonged malnutrition could modify the response of periodontal tissues to local irritants, an observation consistent with experimental data published by Stahl *et al* (1955) over fifty years ago which showed increased loss of alveolar bone and connective tissue in rats subject to PEM.

It is probable that severe malnutrition exerts an influence on the progress of inflammatory periodontal diseases in numerous ways:

1.3.1 Changes in oral microbial ecology

The formation, composition and development of dental plaque can all be influenced by dietary and salivary components. Antibodies and other secretory and serum derived protective

factors work to impede bacterial attachment while promoting the maintenance of an appropriate ecological balance amongst oral commensal organisms (Enwonwu 1994).

Both the volume and physico-chemical properties of saliva are altered in malnourished individuals. Reddy *et al* (1976) demonstrated that children suffering from severe protein energy malnutrition have far lower secretory IgA concentrations in their saliva than adequately nourished children, a finding supported by McMurray *et al* (1977) looking at secretory immunity in malnourished Colombians. Agarwal *et al* (1984) also found that serum and salivary arginase activity decreased markedly, with arginase levels proportional to the severity of PEM. In severely malnourished states bacteria-agglutinating glycoprotein in saliva is reduced, which may favour dental plaque formation (Johansson *et al* 1994).

Sawyer *et al* (1986) carried out studies of oral microflora in well nourished and PEM children in Western Nigeria. Their data suggest that PEM children, in comparison with their age matched, well-fed ethnic counterparts, have a higher prevalence of various potentially pathogenic oral microorganisms. The anaerobes most frequently isolated from the malnourished children included periodontopathogens *Porphyromonas gingivalis, Prevotella intermedia* and in juvenile periodontitis *Aggregatibacter actinomycetemcomitans*.

The causative mechanisms behind this higher prevalence of periodontopathogens in malnourished individuals are unclear. Perhaps the greater availability of free arginine in saliva, due to the lack of arginase in malnourished patients (discussed above) acts as a source of energy for the abovementioned periodontopathogens, which have been shown to form ATP by the arginase deaminase pathway (Levy and Eisenberg 1992).

Increased arginine will also raise salivary and plaque pH, which may promote bacterial overgrowth.

Increased salivary steroid levels have been reported in children suffering PEM (Enwonwu *et al* 1999). Steroids, particularly glucocorticoids, are likely to play an important role in higher periodontopathogen counts in malnourished individuals as they provide nutritional support.

These raised steroid levels may also demonstrate an altered and ultimately less effective immune response (Melnick *et al* 02). Psychologic stress, which has been consistently shown to result in higher salivary cortisol levels, also invariably accompanies severely malnourished states. Severe ascorbic acid (vitamin C) deficiency results in the depletion of ascorbate in the adrenal glands and promotes prominent synthesis and release of the glucocorticoids, resulting in significantly increased salivary glucocorticoid levels (Laney *et al* 1990, Melnick *et al* 1988, Enwonwu 1995).

There are also suggestions that viruses, particularly measles (still very prevalent in many developing nations) and the herpesviruses (including Epstein-Barr virus, Human Cytomegalovirus and Herpes Simplex) promote subgingival overgrowth of periodontopathic bacteria through impairment of oral immunity (Slots and Contreras 2000). In western populations, a correlation between infection with these viruses and the prevalence of ANG has been demonstrated. Human Cytomegalovirus infection is extremely common in many African nations (Contreras *et al* 1997).

1.3.2 The inflammatory response, tissue destruction and healing

There is striking evidence that the pathogenesis of periodontitis is largely dependent on the host's response to microbial factors (Van Dyke and Serhan 2003).

Cytokines are critical chemical mediators of the inflammatory response, the synthesis, release and actions of which can be markedly altered by nutrition related factors (Grimble 1992). As a result severely malnourished individuals are more susceptible to a variety of opportunistic infections (Aaby 2007).

Protein energy malnutrition markedly impairs cytokine production and function, hence the poor prognosis of infections in such individuals.

The cytokines Interleukin-1 (IL-1), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumour Necrosis Factor α (TNFα) and Interferon γ (IFNγ) are key components of the cytokine

cascade in periodontitis, as well as the TNF receptors and the IL-1 receptor antagonist (Enwonwu *et al* 2002). IL-1 is of primary importance to the outcome of bacterial infections, with one of its numerous actions being the induction of synthesis of the neutrophil chemotactic cytokine IL-8 by monocytes, fibroblasts and endothelial cells (Van Dyke and Serhan 2003). In protein deficient states the ability of stimulated monocytes to release IL-1 and the hepatic and hypothalamic responses to the released IL-1 are impaired (Klasing *et al* 1988). Similar alterations in IL-1 production and action have also been demonstrated in individuals with major deficiencies in zinc and magnesium, and this may be one of the mechanisms by which malnutrition leads to diminished immunocompetence (Woodward and Filiteau 1990).

In severely malnourished individuals there is a marked increase in blood levels of the soluble receptors of tumour necrosis factor α (sTNFR-pp55, sTNFR-p75) and IL-1 receptor antagonist, as well as IL-10 and IL-4. IL-10 is an anti-inflammatory cytokine secreted predominantly by macrophages and other cells that inhibit transcription of many proinflammatory cytokines, chemokines and inflammatory enzymes, in part through an inhibitory effect on the DNA binding heterodimeric protein Nuclear Factor κB (NF-κB) (Barnes 1998). Levels of TNFα, IL-1 and IFNγ are significantly reduced in severe malnutrition (Grimble 2001).

We do not yet have a full understanding of the way in which changes in blood cytokine levels affect the prevalence and severity of periodontal disease in the severely malnourished. However Enwonwu (2002) proposed that the reduced production and subsequent secretion of TNFα and IL-1 may impair the expression of the adhesion molecules ICAM-1, VCAM-1 and ELAM-1 on endothelial cells, thus restricting the binding of neutrophils, monocytes, eosinophils and T-lymphocytes to these molecules.

The acute phase response (APR) is a non-specific protective reaction to tissue injury, regulated by the hepatic production of various acute phase proteins. In children suffering from

malnutrition the acute phase response to infections is significantly reduced (Doherty *et al* 1993); an observation which has serious periodontal ramifications given the central role of the acute phase response in promoting tissue healing (Jennings *et al* 1992).

Neutrophils are the hallmark cells of the early inflammatory response to periodontal pathogens (Page and Schroeder 1976). The importance of this non-specific defence cell in the pathogenesis of periodontitis is dramatically highlighted by the prominent and usually rapid periodontal destruction seen in individuals with systemic diseases characterised by either an innate or an induced abnormality in neutrophil number or function (Hart *et al* 1994). Secondary neutropenia is a common feature in PEM children and individuals with severe vitamin C deficiency.

Certain nutrients act as either antioxidants or key contributors in the production of antioxidant enzymes. Any substance is defined as antioxidant if, when present at lower concentrations than those of an oxidable substrate, it significantly delays or prevents the oxidation of the substrate (Battino *et al* 2002). In periodontitis the majority of periodontal tissue destruction is caused by an inappropriate host response to gram negative periodontopathogens and their products. More specifically, "a lack of homeostatic balance between proteolytic enzymes (e.g. neutrophil elastase) and their inhibitors (e.g. α_1-antitrypsin) and reactive oxygen species (ROS) and the antioxidant defense systems that protect and repair vital tissue, cell, and molecular components is believed to be responsible" (Chapple and Matthews 2007). In PEM there is a marked reduction in the action of antioxidant enzymes with tissue depletion of antioxidant nutrients vitamin E, zinc, vitamin B_c and vitamin C (Mandal *et al* 2006). The resultant imbalance between antioxidants and reactive oxygen species leads to oxidative stress, with reactive oxygen species (ROS) capable of damaging DNA and proteins, initiating lipid peroxidation, oxidising important enzymes such as antiproteases and stimulating proinflammatory cytokine release through depleting intracellular thiol compounds and activating Nuclear Factor kappa beta (NF-κB) (Chapple and Matthews 2007). It has been

demonstrated that during phagocytosis, potent oxidative species (including O_2^-, H_2O_2, HOCL and the OH radical) are released by neutrophils in the sulcular epithelium and underlying connective tissues. When they are not limited into phagocytosis vacuoles, these oxidative species react with several biological components leading to host cell damage. These events, if not adequately controlled or neutralised, can contribute to periodontal tissue destruction by chronic PMN stimulation (Diab-Ladki *et al* 2003). The damage mediated by ROS can be mitigated by antioxidants via three separate mechanisms; scavenging of free radicals as they form, sequestering transition metal ions and catalysing oxidation (Ritchie and Kinane 2003).

In 2004, Wei *et al* evaluated three GCF markers for oxidative stress (glutathione peroxidase, lactoferrin and myeloperoxidase), and the proinflammatory cytokine IL-1ß. They found that the total amounts of IL-1ß and the three markers for oxidative stress were higher in periodontitis sites and gingivitis sites than healthy sites, and were positively correlated with plaque index, gingival index, probing depth and probing attachment level. The authors suggest that the imbalance between the levels of myeloperoxidase/IL-1β and glutathione peroxidase/lactoferrin could result in tissue damage by ROS in periodontitis which is initiated and perpetuated by the chronic insults of periodontopathogens.

Other features of severe malnutrition, which have been suggested in the literature as having the potential to adversely influence the prognosis of periodontal infections, include an inverted helper-suppressor T-cell ratio and histaminemia (Enwonwu 1994).

Finally, the synthesis and/or the availability of numerous factors and nutrients critical to wound healing and repair, including TGF-β, essential amino acids, essential fatty acids, dietary energy, zinc, calcium, retinol and ascorbate as well as acute phase proteins, may be grossly compromised in the severely malnourished. Subsequently, the response to treatment is often impaired in severely malnourished individuals and secondary infections are more common (Enwonwu 2002).

1.4 Inadequate nutritional status

1.4.1 Dietary calcium and vitamin D (serum 25-hydroxyvitamin D3)

Calcium and phosphorus are the two major components of bone hydroxyapatite, with calcium less widely available in the food supply of humans. Vitamin D is an essential hormone, the functions of which include aiding the absorption of calcium from the intestine and regulation of calcium metabolism. An inadequate daily calcium intake is a major modifiable risk factor for osteopenia and osteoporosis, as calcium and vitamin D intakes that meet or exceed minimum daily levels have been shown to slow the rate of bone loss from the hip and total body in middle-aged/elderly men and women (Dawson-Hughes *et al* 1997). Pathologic bone resorption is a common denominator for osteopenia/osteoporosis and periodontitis.

Mild calcium deficiency (minimally below RDI) is quite common in western civilisations. A recent study in Geelong, Australia showed that 76% of women consume less than the recommended daily allowance of calcium, and furthermore, 14% have less than the most minimal requirement of 300 mg/day, leaving them in negative calcium balance and at risk of bone loss (Pasco *et al* 2005).

Sunlight is the main source of vitamin D, accounting for around 90% of our intake (Holick 2002). Only a few foods (e.g. fish with a high fat content) naturally contain significant amounts of vitamin D, and therefore in Australia margarine, some brands of milk and milk products are currently fortified with vitamin D. The Geelong Osteoporosis Study detected mild or moderate deficiency in more than one in three women surveyed in summer, and this rose to one in two in winter (Pasco *et al* 2001). Even in regions exposed to more sunshine, such as south-east Queensland, Western Australia and New South Wales, nearly a third of men and women have mild to moderate vitamin D deficiency (McGrath *et al* 2001). Almost all patients in aged care facilities in Australia have at least a mild vitamin D deficiency (Ebeling *et al* 2005).

Most animal and human studies looking at calcium intake, bone mineral density, clinical parameters of periodontitis and/or tooth loss have provided a rationale for hypothesising that low dietary intake of calcium is a risk factor for periodontal disease, with the notable exception of Weyant *et al* (1999), who suggested no significant correlation between bone mineral density and periodontitis in humans.

In 1969 Oliver investigated the effect of calcium and vitamin D deficiencies on the periodontal tissues in rats. When the diet was deficient in both supplements bone changes resembling osteoporosis were observed. However when the diet was adequate in calcium but low in vitamin D, there were no obvious effects on the periodontal tissues. These observations showed a possible influence of calcium-deficient diets on susceptibility to periodontitis progression. Abe *et al* (1989) also observed alveolar bone changes in rats on a calcium deficient diet, and reported higher osteoclastic activity in calcium deficient animals compared with controls, even over a period as short as seven days. Osborn *et al* (1977) evaluated dietary calcium intake among periodontal patients. In a private practice setting the authors analysed nutrient intake via 5 day diet diaries obtained from 100 patients. They concluded that that calcium intake below the RDI was common in periodontal patients, although given the very high prevalence of inadequate calcium intake in western populations demonstrated by Pasco *et al* (2005) this is not surprising.

An early small uncontrolled study by Spiller (1971) proposed that calcium supplements could reverse clinical indicators of periodontal disease and alveolar bone loss, however Uhrbom and Jacobson (1984) in a larger and more thoroughly controlled trial did not reproduce those findings, concluding that periodontal bone loss is irreversible and calcium supplementation does not alter this.

More recently, Nishida *et al* (2000a) analysed the dietary intake surveys and periodontal examinations of over 12 000 adults from the third U.S National Health and Nutrition Examination Survey (NHANES III). The relationship between low dietary calcium intake and

increased levels of periodontal disease showed an estimated odds ratio of 1.84 (95% Confidence interval 1.36 to 2.48) for young males, 1.99 (95% CI 1.34 to 2.97) for young females and 1.9 (CI 1.41 to 2.55) for the older group of males. In females a dose response pattern was also evident, with a 54% greater risk of periodontal disease for the lowest levels of dietary calcium intake and 27% greater risk in females who took moderate levels of dietary calcium, as compared to those who took 800mg or more dietary calcium a day (RDI baseline in Australia is 800 mg). Dietrich *et al* (2005) looked at the NHANES III data to evaluate the association between gingival inflammation and serum concentrations of vitamin D. They found that sites in subjects in the highest serum vitamin D quintile were 20% less likely to bleed on probing than sites in the lowest serum vitamin D quintile. They found the association to be quite linear over the entire vitamin D range, and to be consistent across sex, racial groupings and users/nonusers of vitamin and mineral supplementation. The authors concluded that vitamin D might reduce susceptibility to gingival inflammation through its anti-inflammatory effects.

Krall *et al* (2001) published results of a large longitudinal placebo controlled trial, in which test group subjects took daily dietary calcium (500 mg) and vitamin D (700 IU) supplementation. There was 60% less tooth loss in the test group than the placebo controls after five years – although this study did not adjust for many known confounding factors in periodontal disease. Also in 2001, Krall published results of a large long term prospective study in male war veterans and showed the number of teeth with progression of alveolar bone loss over a seven year period was significantly lower in men whose calcium intake was at least 1000 mg per day, compared to men with a calcium intake below this level.

In 2004, Dietrich *et al* published an analysis on the NHANES III data and found that serum vitamin D concentrations were significantly and inversely associated with attachment loss in men and women fifty years and older (no correlations below 50 years). They concluded that

low serum Vitamin D concentrations might be associated with periodontal disease, irrespective of bone mineral density.

To date a definitive causal link between low dietary calcium or vitamin D, osteopenia/osteoporosis and human periodontal diseases has not been established. The potential linking mechanism may be the bone-resorptive process. The uncoupling of normal bone homeostasis, primarily due to increased osteoclastic activity rather than decreased osteoblastic activity, may be the pathophysiological mechanism. In particular the pro-inflammatory cytokine IL-6 (the action of which is regulated by vitamin D) is thought to play a significant role in stimulating bone resorption. In normal homeostasis, IL-6 production by osteoblasts stimulates osteoclastic bone resorption. It has been hypothesised (Reddy 2001) that many of the effects on bone mineral density may be modulated through IL-6. For instance, oestrogen decreases IL-6 production, thus decreasing osteoclastic activity, and having a beneficial effect on bone mineral density.

Nishida *et al* (2000a) suggested that decreased dietary calcium may significantly alter calcium:phosphorus ratios and could cause increased parathyroid hormone release, leading to greater osteoclastic activity.

However, these hypotheses leave questions unanswered. While they provide a potential explanation for decreased bone mineral density (BMD) and perhaps loss of alveolar bone height, they do not explain how or why there is loss of soft tissue attachment to the tooth. Some authors have suggested decreased alveolar BMD increases the rate at which alveolar bone is lost during active stages of periodontitis (Hildebolt *et al* 2005). While this conclusion seems plausible, more investigation is required in this area.

Vitamin D also has anti-inflammatory properties, including inhibition of C-Reactive Protein (CRP). Elevated CRP levels have been correlated with individuals with extensive periodontal disease (Slade *et al* 2000, Taylor *et al* 2006). Associations between vitamin D receptor polymorphisms and early onset periodontitis have also been described (Hennig *et al* 1999).

1.4.2 Vitamin B complex

The vitamin B complex refers to the group of all known essential water-soluble vitamins except for vitamin C. These include thiamine (B_1), riboflavin (B_2), Niacin (B_3), pantothenic acid (B_5), pyroxidine (B_6), biotin, folic acid (vitamin B_9) and the cobalamins (B_{12}).

The structure of each member of the B complex is unique, performing specific functions often unrelated to other B group vitamins.

Given evidence in the wider medical literature (Partridge *et al* 1998) that nutrient supplements can positively influence wound healing processes, Neiva *et al* (2005) looked at the effect of vitamin B supplementation on the response to periodontal access flap surgery in patients with moderate to severe chronic periodontitis. Fifteen patients were instructed to take one vitamin B capsule per day for a month, which contained 50 mg thiamin, 50 mg riboflavin, 50 mg niacin, 50 mg pantothenic acid, 50 mg pyroxidine, 50 μg biotin, 50 μg cobalamin and 400 mcg of folate. A further fifteen patients took a placebo tablet for the month. Both groups experienced comparable reductions in pocket depth, however changes in mean clinical attachment level were significantly more favorable in the subjects who took vitamin B supplementation, in both shallow and deeper sites.

1.4.2.1 Vitamin B_9 (folate)

Folate is needed to replicate DNA. Thus folate deficiency hinders DNA synthesis and cell division, affecting clinically sites of rapid cell turnover (e.g healing wounds and the creation of bone marrow). As RNA and protein synthesis are not hindered in folate deficient states, large red blood cells called megaloblasts are produced, resulting in megaloblastic anaemia. The Australian population's folate intake is marginal, and vegans are particularly at risk of folate deficiency. Certain drugs, including anticonvulsants and oral contraceptives may impair

folate absorption, while anticonvulsants have also been shown to increase the rate of folate metabolism (Swain and St Clair 1997).

Vogel in 1976 evaluated the effects of vitamin B_9 supplementation in humans, and showed that in untreated patients over a 30 day period, 4 mg of Vitamin B_9 daily led to less gingival inflammation (compared with controls who took placebo). However, there were only 15 patients in the B_9 group and as this group were not treated, no comment could be made on whether vitamin B_9 supplementation positively alters the healing response. It has been demonstrated convincingly that smokers are more susceptible to periodontitis (Bergstrom and Preber 1994). Erdemir and Bergstrom (2006) found that among adults with chronic periodontitis, the serum folic acid level is lower in smokers than non-smokers. In a population based cross sectional study Yu *et al* (2007) examined the NHANES III data of 848 individuals sixty years and over, who completed a periodontal examination and a laboratory test for serum folate levels. After adjusting for a variety of variables, a low serum folate level was independently associated with periodontal disease in older adults.

Dreizen *et al* (1977) showed that a severe deficiency of Vitamin B_9 in the diet of monkeys was characterised orally by a stomatitis highlighted by necrotising gingivitis and periodontitis, and by an ulcerative and atrophic glossitis. Taguchi (1989) demonstrated that use of a folate supplemented toothpaste in monkeys caused a greater reduction in gingival inflammation - measured by blood flow, bacterial counts and GCF volume - than a placebo paste, and an equal reduction when compared with a 0.5% chlorhexidine paste. Thomson and Pack (1982) conducted a controlled double blind study in which women in their 8^{th} month of pregnancy were divided into groups and given:

a) Placebo mouthwash and placebo tablets.
b) Placebo mouthwash and one 5mg folate tablet daily.
c) Placebo tablets and rinsed with folate mouthwash twice daily for one minute.

Subjects in group C showed a highly significant improvement in the gingival index, despite the plaque index remaining basically unchanged. In group B changes in gingival health were not significantly different from group A. The authors concluded that vitamin B_9 could improve the resistance of the gingival tissues to dental plaque.

Pack later conducted two further studies looking at the effect of Vitamin B_9 on the gingival tissues of non-pregnant adults. The first (Pack 1984) showed a significant reduction in GI scores when a folate mouthrinse was used, but not when systemic supplements were given. The second study (Pack 1986) was carried out in a similar fashion to Loe *et al*'s classic "Experimental gingivitis in man" clinical trial from 1965 and showed no significant affect of Vitamin B_9 mouthrinse on gingival inflammation.

Thus there may be a weak association between low serum folate levels and periodontitis. A decrease in serum folate may be a consequence of cigarette smoking and contribute to the higher prevalence of periodontitis in these individuals. Across the literature there is some evidence that folate mouthrinses may have some mild anti-gingivitis properties, but of a magnitude which does not compare favourably to currently used chemical plaque control agents and thus does not warrant their widespread use. Systemic supplementation alone has generally shown minimal or no periodontal benefit.

Credible periodontal literature investigating the link between periodontal disease and deficiencies in other B group vitamins is scant. However at a theoretical level it is plausible that deficiencies in these vitamins may alter immunity or impair the healing response, thus impacting on susceptibility to disease.

1.4.2.2 Vitamin B_1 (thiamin)

A deficiency in thiamin hinders the ability of cells to generate energy, thus delaying wound healing. In western societies thiamin deficiency was not uncommon in the 1930's and prior as

it was often removed during the milling of grains. However today in Australia it is extremely rare, as it is present in large amounts in most grains and cereals.

Chronic thiamin deficiency can lead to severe neurological symptoms and to cardiovascular and musculature defects (Winston *et al* 2000).

1.4.2.3 Vitamin B_2 (riboflavin)

Riboflavin deficiencies are rare in the western world, however oral manifestations of riboflavin deficiency include angular stomatosis and glossitis. Riboflavin is an essential precursor of the catabolic metabolic pathways that produce ATP.

1.4.2.4 Vitamin B_3 (niacin)

Niacin is a precursor for NAD+, which is also essential in the production of ATP. Glossitis is an accepted oral symptom of niacin deficiency. In Australia niacin deficiency is rare and mainly seen in chronic alcoholics.

1.4.2.5 Vitamin B_5 (pantothenic acid)

Deficiency is exceptionally rare. Pantothenic acid is an essential nutrient needed to form co-enzyme A, and is critical in the metabolism and synthesis of carbohydrates, proteins and fats.

1.4.2.6 Vitamin B6 (pyridoxine)

The requirement for pyroxidine in the diet is proportional to the level of protein consumption. Pyridoxine is needed for protein and fatty acid metabolism, the formation of red blood cells and certain brain chemicals. It influences brain processes and development, immune function and steroid hormone activity. Deficiencies in pyroxidine are rare and usually seen in patients with a general deficiency in B complex vitamins (Neiva and Steigenga 2003).

1.4.2.7 Vitamin B7 (biotin)

Biotin is a critical vitamin in cell growth, the production of fatty acids and fat and amino acid metabolism. Biotin not only assists in various metabolic chemical conversions, but also helps to transfer carbon dioxide and plays an important role in the maintenance of stable serum glucose levels. Deficiency is extremely rare, as intestinal bacteria generally produce above the body's daily requirement. For this reason, statutory agencies in many countries, including the Australian Department of Health and Aging, do not prescribe a recommended daily intake.

1.4.2.8 Vitamin B_{12} (cobalamin)

Vitamin B_{12} is involved in the production and maintenance of body cells, mental ability and the breaking down of fatty and amino acids. Vitamin B_{12} has a close relationship with folate, as both depend on the other to exert correct function. Pernicious anaemia is a megaloblastic anaemia caused by vitamin B_{12} deficiency. The anaemia results from impaired DNA synthesis due to a block in purine and thymidine biosynthesis (Andres *et al* 2001).

1.4.3 Vitamin C

Vitamin C (ascorbic acid) is a highly effective antioxidant which is also a critical enzymatic cofactor for the biosynthesis of collagen, with a severe vitamin C deficiency effecting the hydroxylation of proline. Clinically it helps maintain skin elasticity, aids iron absorption and improves resistance to infection (Mazzotta *et al* 1994). Severe vitamin C deficiency is known as scurvy. A main feature of the disease is bleeding from all mucous membranes, and sufferers experience severe pain and may become immobilised. Vitamin C deficiency is well known to lead to a severe periodontal syndrome called “scorbutic gingivitis,” which is characterised by ulcerative gingivitis with spontaneous bleeding and gingivae with a purplish appearance (Touyz 1997). Subsequent to scorbutic gingivitis, a very aggressive form of periodontitis may develop characterised by rapid periodontal pocket development and tooth

exfoliation. Scurvy was once a relatively common disease in individuals who had no access to citrus fruit (e.g. amongst crew members on long sea voyages or soldiers in trench warfare), however it is exceedingly rare in present day Australia.

Currently of greater interest to clinicians is whether subclinical vitamin C deficiency (consistently below RDI recommendations) may have an adverse effect on the periodontium – and to date, scientific findings have been relatively incoherent. Multiple early studies (Barros and Witkop 1963, Vogel and Weschler 1979, Woolfe et al 1980) failed to show any significant relationship between vitamin C and periodontal disease. Leggott *et al* (1986) conducted an investigation into the effect of systemic levels of vitamin C on periodontal health assessing plasma ascorbic acid concentrations (potentially more reliable than estimated dietary intakes used to estimate vitamin C status in aforementioned earlier studies). Eleven healthy, non-smoking men aged 19-28 years of age ate a rotating 7 day diet adequate in all nutrients but low in vitamin C. The authors concluded that measures of gingival inflammation, particularly gingival bleeding, were directly related to ascorbic acid status (there were no changes in plaque accumulation or pocket depth). In 1991 Leggott *et al* went on to describe the relationship between a range of vitamin C intakes, periodontal status and the composition of the subgingival microflora in men who lived for thirteen weeks in a nutrition suite in which they underwent controlled periods of ascorbic acid depletion and repletion. When the different vitamin C intakes and serum concentrations were compared there were no significant changes in plaque accumulation, pocket depth or attachment level. However the authors found that gingival bleeding increased significantly after periods of vitamin C depletion and returned to baseline values after the repletion period.

It has been reported in the literature that the use of ‘mega doses’ of vitamin C (additional supplements in patients who already meet the recommended daily intake) does not exert a beneficial effect on the gingival response to initial therapy (Woolfe *et al* 1984). In line with

this, mega doses of vitamin C do not alter neutrophil chemotaxis or responses to experimental gingivitis (Vogel *et al* 1986).

Nishida *et al* (2000b) analysed NHANES III data to study the role of dietary vitamin C as a contributing risk factor for periodontal disease. Using multiple logistic regression analysis, a very weak but statistically significant relationship between reduced dietary Vitamin C and increased risk for periodontal disease for the overall population was found. The odds ratio was 1.3 (95% CI 1.14 to 1.65) for those taking 0 to 29 mg of Vitamin C per day and 1.16 (95% CI 1.02 to 1.33) for those taking 100 to 179mg per day when compared with individuals taking 180 mg or more per day. Both past and present smokers taking less vitamin C showed a greater increase in risk of periodontal disease. Corroborative results were produced by Amarasena *et al* (2005), who looked at the relationship between clinical attachment levels and serum vitamin C in community dwelling elderly Japanese. Multiple logistic regression analysis revealed serum vitamin C levels to have a weak but statistically significant relationship with periodontitis.

Aside from the effects of vitamin C deficiency on collagen formation previously discussed, other postulated mechanisms by which subclinical vitamin C deficiency might potentially have an adverse affect on periodontal status include:

a) Alteration of neutrophil ability and function due to impaired chemotactic ability (Nishida *et al* 2000b).
b) Alteration of salivary composition, particularly with regards to increased levels of glucocorticoids, which may favour overgrowth of certain periodontopathogens (Enwonwu 2002).
c) Depletion of ascorbate reduces the resistance to oxidative stress, as it is a powerful scavenger of oxygen free radicals. Ascorbate also protects against oxidants in cigarette smoke, and it regenerates the antioxidant α-tocopherol from the tocopherol radical that forms at membrane surfaces.

It has also been reported in the literature that the use of 'mega doses' of vitamin C (additional supplements in patients who already meet the recommended daily intake) does not exert a beneficial effect on the gingival response to initial therapy (Woolfe *et al* 1984). In line with this, mega doses of vitamin C do not alter neutrophil chemotaxis or responses to experimental gingivitis (Vogel *et al* 1986).

In light of the current literature the link between mild vitamin C deficiency and periodontitis risk seems weak at most. There is some evidence in the literature that deficiency may influence gingivitis, seen clinically as more prevalent bleeding in response to plaque bacteria. Vitamin C supplementation has not been shown to have a positive effect in the management of periodontitis.

1.4.4 Other antioxidants

The aberrant host response to periodontopathogens and their products seen in periodontitis is characterised by exaggerated inflammation, involving the release of proteolytic enzymes and reactive oxygen species. Studies by Panjamurthy *et al* (2005) and Sugano *et al* (2000) have shown increased levels of biomarkers of tissue damage induced by reactive oxygen species in periodontitis patients. In inflamed periodontal tissues, antioxidant enzymes also appear to be up-regulated in response to the oxygen free radical challenge (Akalin *et al* 2005), and extracellular antioxidant scavengers are depleted (Brock *et al* 2004). Garg *et al* (2006) also found that gingival tissue levels of antioxidants are far lower in non-smokers than smokers, and this may partially explain the mechanism by which periodontitis is more prevalent and severe among smokers (Grossi *et al* 1996).

Tissue damage may be caused directly by reactive oxygen species, or indirectly by activation of nuclear factor kappa beta which alters the cytokine response profile (Chapple *et al* 1997).

Chapple *et al* (2007) performed multiple logistic regression analysis of the NHANES III data to determine whether serum antioxidant concentrations were associated with an altered risk

for periodontitis. The authors found that the total serum antioxidant level was inversely associated with periodontitis, with the association remaining stronger in more severe cases of periodontitis. This association was significant in both smokers and never smokers.

1.4.4.1 Vitamin E (α-tocopherol)

Vitamin E is an antioxidant with limited tissue mobility, which terminates the free radical chain reaction and stabilises membrane structure. Vitamin E also potentiates selenium, which is an important element in the formation of immunoglobulins. As with vitamin C, no statistically significant differences have been found in the plasma vitamin E levels of individuals with and without chronic periodontitis (Slade *et al* 1976). However some small studies of gingival tissue (animal and human) have suggested a mitigating effect of vitamin E on periodontal inflammation and collagen breakdown, with lower gingival levels of vitamin E found amongst individuals with periodontal disease compared to healthy controls (Offenbacher *et al* 1990, Cohen *et al* 1993).

In Australia, even mild vitamin E deficiency is rare. However there has been debate in the medical literature over whether 'mega doses' of vitamin E might provide enhanced defence against reactive oxygen species (Haidara *et al* 2006). Meydani *et al* (2005) also reported enhanced proliferation of T cells and depressed function of the pro-inflammatory eicosanoid prostaglandin E_2 in individuals taking large doses of vitamin E daily.

1.4.4.2 Coenzyme Q10

Coenzyme Q10 - a benzoquinone - is a vitamin like substance found in all human cells with an important role in ATP production, and its potential therapeutic properties are beginning to attract more attention in the scientific literature. This powerful antioxidant is a 'key ingredient' in many skin care products, and a recent cell culture study by Figuero *et al* (2006)

suggested that the catabolic effects of nicotine could be reversed by higher tissue levels of coenzyme Q10.

1.4.4.3 Grape seed Proanthocyanodins

Proanthocyanodins have been reported to possess a wide range of biologic properties against oxidative stress. Houde *et al* (2006) investigated the effect of these grape seed proanthocyanidins (GSE) on the production of reactive oxygen metabolites by murine macrophages stimulated by lipopolysaccharides of various periodontopathogens. GSE strongly decreased ROS production and the authors call for further research into the use of GSE's as a potential agent in the prevention of periodontitis.

1.4.5 Omega-3 polyunsaturated fatty acids

Omega-3 polyunsaturated fatty acids (O-3 PUFAs) are essential fatty acids that must be derived from dietary sources such as fish oil. The major polyunsaturated fatty acid components are eicosapentoic acid (EPA) and docosahexaenoic acid (DHA). In therapeutics, O-3 PUFAs have been demonstrated to have a range of desirable properties including anti-atherogeneitcity, platelet anti–aggregation, anti-arrhythmia, hypolipidemic and anti-inflammatory properties, whilst showing a negligible adverse reaction profile (James and Cleland 1997).

Periodontal tissue destruction occurs as a result of an aberrant host immunoinflammatory response to periodontopathogenic bacteria and their by-products. Metabolism of arachidonic acid (AA), which is released from cell membrane phospholipids, produces numerous eicosanoids believed to be amongst the most important mediators of this tissue destruction (Offenbacher *et al* 1996).

A range of virulence factors produced by gram negative anaerobes, including lipopolysaccharides, promote arachidonic acid metabolism via the cyclo-oxygenase and lipoxygenase pathways, producing a range of prostaglandins (PG), thromboxanes (Tx) and leukotrienes. Offenbacher *et al* (1993) suggested prostaglandin E_2 is the most potent mediator of alveolar bone loss in periodontitis. In 1974, Goodson *et al* demonstrated a ten-fold increase in PGE_2 levels in inflamed periodontal tissue compared with healthy tissue. Offenbacher *et al* (1992) have found that PGE_2 levels are higher in the GCF of patients with gingivitis and particularly periodontitis, and have suggested it may act as a predictive marker of further periodontal attachment loss.

PGF_2 is also found at higher levels in the GCF and gingival tissues of patients with periodontitis (Offenbacher *et al* 1999). It enhances interleukin 6 levels, stimulates osteoclast induction and matrix metalloproteinase-1 production (Noguchi 2001a, 2001b) and thus can be hypothesised to play an active role in connective tissue destruction seen in active periodontitis. Leukotriene B_4, an AA metabolite derived via the lipoxygenase pathway, has been thought to contribute to tissue destruction via cellular degranulation and superoxide generation. Many other AA metabolites have important pro-inflammatory roles.

Omega-3 polyunsaturated fatty acids have been demonstrated to compete with arachidonic acid as substrates for the cyclooxygenase and lipoxygenase pathways, reducing the synthesis of AA metabolites. Offenbacher *et al* (1990) showed that EPA or DHA could inhibit the production of PGE_2 to an extent similar to ibuprofen when added to human homogenoates. Therapies which reduce the synthesis of pro-inflammatory AA mediators by blocking the cyclooxygenase and lipoxygenase pathways, e.g non steroidal anti-inflammatory drugs (NSAIDs), have proven beneficial in the treatment of both experimental gingivitis and clinical periodontitis (Salvi *et al* 1997). However, continued use of these agents for this purpose must be weighed against their significant side effect profile, particularly with regards to gastric ulceration.

O-3 PUFA metabolism also produces modified end products from both the cyclooxygenase and lipoxygenase pathways which are less inflammatory. Cyclooxygenase metabolism of EPA results in PGI_3, which has strong anti-inflammatory properties that include preventing platelet aggregation and promoting vasodilation, and thromboxane A_3 which has markedly reduced pro-thrombotic and pro-inflammatory effects compared with TXA_2. Metabolism of EPA via the 5-lipoxygenase pathway results in the production of LTB_5, which has markedly reduced chemotactic potency compared to the AA derived LTB_4 and LTC_4.

EPA and DHA have very recently been discovered to be mobilised within inflammatory tissue exudates in a temporally orchestrated process, and biosynthesised to different classes of bioactive compounds, termed resolvins and protectins, which act as local chemical regulators believed to be critical in the resolution of inflammation (Serhan 2006). The E series Resolvins which utilise fish oil derived EPA as a precursor were identified first from self limiting and resolving murine exudates (Hong *et al* 2003). Among them, Resolvin E1 (RvE1) was demonstrated to promote resolution by hindering the recruitment and migration of white blood cells in the inflammatory phase.

In a ligature combined with *P. gingivalis* application experimentally induced periodontitis model in rabbits, Hasturk *et al* (2006) demonstrated that animals treated topically with the EPA derived resolvin E1showed four times less periodontal bone loss than control animals not treated with resolvin E1. Histology suggested resolvin E1 suppressed the activity of osteoclasts, thus potentially inhibiting bone loss. This finding is supported by the earlier work of Sun *et al* (2003) who found EPA and DHA inhibited osteoclast activation *in vitro*. Hasturk *et al* (2006) also demonstrated that human neutrophils derived from patients with localised aggressive periodontitis respond to RvE1.

Following the discovery of RvEs, D series resolvins (RvDs) and docosatrienes (termed protectins), which are derived via DHA biosynthesis, have been described. RvDs have been demonstrated to block tumor necrosis factor α (TNFα) induced IL-1ß transcripts in microglial

cells (Hong *et al* 2003) and act as regulators which limit the infiltration of neutrophils into inflamed tissues (Marcheselli *et al* 2003, Schwab and Serhan 2006).

Of the docosatriene derived family, 10,17S-docosatriene which is commonly referred to as protectin D1 (PD1) has proved a potent regulator of neutrophil influx in both laboratory and clinical studies. Serhan *et al* (2006) demonstrated that PD1 was a potent regulator of PMN's, reducing PMN infiltration by approximately 40% at 1 ng in mice in a murine peritonitis model. PD1 seems to complement the actions of RvE1 and to reduce neutrophil infiltrate in the early phases of inflammation (Serhan *et al* 2006).

Exogenously administered DHA is converted to both the D series resolvins and protectin D1 and their actions in concert have recently been demonstrated to reduce the number of infiltrating leukocytes and block toll like receptor-mediated activation of macrophages in acute kidney injury (Duffield *et al* 2006). DHA administration alone has been found to result in a more marked reduction in prostaglandin production by various cell types compared with EPA alone (Roman *et al* 2006). Weldon *et al* (2007) investigated the differential effects of isolated pure EPA and pure DHA on cytokine expression and nuclear factor kappa B (NF-kappaB) activation in human THP-1 monocyte-derived macrophages. Pre-treatment of cells with EPA and DHA significantly decreased lipopolysaccharide (LPS)-stimulated THP-1 macrophage TNFα, IL-1ß and IL-6 production compared to control cells. Both O-3 PUFAs tested reduced TNFα, IL-1ß and IL-6 mRNA expression. In all cases, the effect of DHA was significantly more potent than that of EPA.

Dietary supplementation with Omega-3 polyunsaturated fatty acids has been shown to hinder neutrophil adherence, reduce synthesis of a range of pro-inflammatory cytokines which includes IL-1α, IL-1ß, IL-2, TNF-α, IL-6 and granulocyte macrophage colony stimulating factor, decrease oxidative stress and subsequent tissue damage, and diminish T-lymphocyte proliferation and reactivity (Rosenstein *et al* 2003).

Because of these properties, dietary supplementation with O-3 PUFAs has become an accepted, although underutilised, prevention and treatment modality for numerous conditions and diseases. These include the prevention of cardiovascular disease, the specific use of Omega-3 supplements for lowering triglyceride levels and the treatment of rheumatoid arthritis. Because of their membrane stabilisation and anti-inflammatory properties, Omega-3 fatty acids are being investigated for their potential to prevent preterm labour in pregnant women, as well as early investigations as to their potential uses in a wide range of conditions, including asthma, inflammatory bowel disease, depression and neurological disorders.

The periodontal literature investigating the potential effects of O-3 PUFAs in periodontal disease is fairly minimal but growing. Alam *et al* (1991) showed that rats fed a diet rich in O-3 PUFAs for eight weeks exhibited suppressed gingival levels of AA, PGE_2 and Leukotriene C4. Campan *et al* (1997) used a human experimental gingivitis model to study the effect of dietary O-3 PUFAs. Thirty-seven healthy volunteers, who discontinued routine oral hygiene measures, were treated with either fish oil (6g of 30% O-3 PUFAs) or placebo (olive oil) for eight days. The results showed a significant decrease in the gingival index in the Omega-3 treated group. The gingival tissue levels of AA, PGE_2, and LTB_4 were found to be reduced in the treated group and increased in the control group, but without statistical significance being demonstrated. Eberhard *et al* (2002) investigated the effect of an O-3 PUFA mouthrinse on bleeding on probing and GCF flow in a human experimental gingivitis model in 11 adults. Their results with this topical application were less promising. A small, but not statistically significant reduction in BOP scores and GCF flow rates at the end of the 21 day study period were observed.

Vardar *et al* (2004) investigated the effect of systemic O-3 PUFAs on an endotoxin (LPS) induced palatal periodontal abscess model in rats. Omega-3 supplementation was given to one group as a treatment modality alone (only for 15 days after periodontitis was induced) while another group received O-3 PUFAs prophylactically in addition to treatment (for 15 days

before periodontitis then also 15 days after). The gingival tissue levels of PGE_2, PGF_2, LTB_4 and platelet activating factor were then analysed and measured by enzyme immunoassay and radioimmunoassay. The authors found that PGE_2 levels were five times greater in the LPS control than in either prophylactic plus therapeutic or therapeutic only O-3 PUFA fed rats. There were significant decreases in the gingival tissue levels of all of the above mediators compared with LPS only controls, with the prophylactic plus therapeutic group showing slightly greater reductions than the therapeutic administration only group.

In a follow up study, Vardar-Sengul *et al* (2006) evaluated the effects of the same Omega-3 treatment regimes on experimental alveolar bone loss in the endotoxin (LPS) induced periodontal abscess model. The authors found that there was no difference in alveolar bone loss seen between test and control groups.

In difference to the previous result but using a model more relevant to chronic periodontitis, Kesavalu *et al* (2006) investigated the effect of O-3 PUFAs in rats using recurring oral gavage with *P. gingivalis* to induce alveolar bone loss over time. The control group received a corn oil diet, while the test group received a high fish oil diet for 22 weeks. The authors found that the Omega-3 fed rats showed significantly less alveolar bone loss around both maxillary and mandibular teeth, and that bone levels in these rodents were similar to control rats that had not been inoculated with *P. gingivalis*.

As previously eluded to, the potential role of non-steroidal anti-inflammatory drugs as modulators of the host inflammatory response has been an area of considerable interest in the periodontal literature. Vardar *et al* (2005) demonstrated in the same rat model described for their previous two experiments that O-3 PUFA supplementation resulted in a slightly greater reduction in the four tested inflammatory mediators than celecoxib (COX-2 inhibitor) supplementation. This supports the earlier finding by Offenbacher *et al* (1990) that EPA or DHA can inhibit the production of PGE_2 to an extent similar to ibuprofen. Given the inherent safety, cost efficiency and potential collateral benefits of 'fish oil' administration compared

with NSAIDs, the role of Omega-3 polyunsaturated fatty acids in the prevention/treatment of periodontal disease warrants further investigation.

1.4.6 Zinc

Humans with zinc deficiency have been reported to exhibit a large variety of symptoms – including impaired growth, anaemia, dermatitis, mental lethargy and delayed wound healing (Endre et al 1990). However there is minimal evidence available which examines the effect of subclinical zinc deficiency specifically on the periodontal tissues. Joseph et al 1981 found structural alterations in the oral epithelium of zinc-deficient rabbits including flattened filiform papillae showing parakeratosis on the tongue, parakeratosis in the cheek (normally nonkeratinised epithelium) and hyperplasia of the lip epidermis.

Orbak et al 2007 fed rats a zinc deficient diet and found significantly higher mean plaque index scores and gingival index scores in the deficient rats compared with control rats fed a normal diet. More apthous ulcerations were found in the zinc deficient group.

Periodontal disease specific research investigating the effects of nutritional deficiency in other essential vitamins and minerals is sparse. Given our growing understanding of the importance of the host response in periodontitis progression, further micronutrients, which play important roles in the human immune response and in wound healing, could potentially impact on periodontitis progression. Certainly a number of micronutrients not mentioned previously (e.g vitamin A and iron) have been reported in the medical literature as impacting upon various aspects of innate and adaptive immunity and investigation into the periodontal ramifications of these deficiencies may produce results of interest.

1.4.7 Inadequate nutritional status – concluding remarks

The use of dietary vitamin and mineral supplements, herbs and so on by consumers is growing at a rapid rate. A 2002 report published by the US Food and Drug Authority states that 60% of adults in the US use dietary supplements on a regular basis, and one in four regularly use herbs. Brownie and Rolfe (2004) found that 43% of randomly selected Australians aged 65 years and over reported the use of some form of supplement. The majority of supplements were consumed regularly (daily or every few days) and for an extended period (more than three years).

Several dietary supplements have widely accepted systemic actions. In some circumstances dietary supplements may be beneficial, and in cases of continued dietary vitamin intake below RDI levels, vitamin supplementation can play an important role in the achievement of adequate nutritional status. However there is often scant scientific evidence to support claims made by manufacturers of 'natural health products', in an industry which in Australia is not subject to the rigorous trials and proof of efficacy required for pharmaceuticals. There is also a growing body of evidence demonstrating that many herbs and dietary supplements have pharmacological activities that have adverse interactions when taken with prescription medications. Supplements (particularly herbs) that are metabolised using the P450 enzyme pathway have been shown to have a potentially life threatening effect by interacting with some of the many drugs metabolised in this manner (McDonough 2003).

The evidence in the periodontal literature linking subclinical vitamin deficiencies to periodontal disease is scant, and further research is required to draw more definitive conclusions or implement valid practical recommendations. Studies have generally been small, and given that chronic periodontitis is a disease of long duration, long term controlled clinical trials with strategies to deal with the myriad confounding factors in humans are needed. Large sample sizes will also be necessary to detect differences in clinical parameters, which in the case of a subclinical deficiency of a single nutrient might be quite small.

In an attempt to address this sample size issue, a series of papers have been published since the turn of the century analysing the US NHANES III data (over 12,000 participants). However the nutritional information supplied by cross sectional analysis of the NHANES data must be viewed with caution, as it has generally been gathered either by analysing a short dietary diary or a point in time blood test prior to clinical examination. To extrapolate such short 'point in time analyses' to a realistic measure of the patient's nutritional status over a lifetime (or at least the decades over which chronic periodontitis develops) leaves scope for gross inaccuracy.

The greatest amount of periodontal evidence dealing with inadequate nutrition to date concerns the link between periodontitis and low dietary calcium or vitamin D intake (accepted major risk factors for osteoporosis), which is more evident in older age groups. As these are quite common marginal deficiencies in western countries this is an area which certainly warrants more study. Currently available scientific evidence tends to suggest a link between periodontal disease progression and inadequate intake of both nutrients - however currently a firm conclusion can not be drawn, and there is certainly insufficient evidence to date to conclude that calcium supplementation as an adjunct to periodontal treatment in patients with a marginal deficiency will hinder periodontitis progression. However one could certainly argue that in these patients increased calcium intake is associated with negligible risk and it may have general health benefits irrespective of the relationship between calcium supplementation and periodontal disease.

Evidence on B complex vitamins suggest that subclinical niacin and folate deficiencies are associated with gingival inflammation, and topical application (toothpastes, mouthrinses etc) of these vitamins may have an effect in reducing this inflammation. However there is no evidence that such topical measures are more efficacious in reducing inflammation than currently available methods (e.g. chlorhexidine or triclosan mouthrinses). This pool of data is still small and quite dated, with studies not addressing whether such topical measures are

useful for individuals who aren't deficient in these vitamins. In light of this it is telling that mouthrinses containing folate are not commercially marketed.

Looking at the literature on whole, evidence linking subclinical B vitamin deficiencies and increased susceptibility to periodontitis or disease progression is not overwhelming, although recent research looking specifically at serum folate levels have suggested a weak but significant correlation. Neiva *et al* (2005) also recently suggested supplementation with the myriad of B group vitamins may improve healing in response to surgical periodontal therapy.

The link between absolute vitamin C deficient states (scurvy) and periodontitis is widely accepted, however the literature regarding the effect of subclinical vitamin C deficiency on periodontitis has shown mixed results, and if a link exists it is very mild. Subclinical deficiencies may alter the gingival inflammatory response to plaque. In Australia and most other developed countries, the vast majority of people have a vitamin C intake which exceeds the baseline RDI value, and mega doses have been shown to have no beneficial effect on periodontal health.

Vitamin E deficiency is rare, but there has been a large amount of conflicting recent research in the wider medical literature into whether mega doses of vitamin E are beneficial in preventing ROS damage. This could conceivably have implications in adjunctive treatment for periodontitis.

From a prevention and treatment point of view Omega-3 polyunsaturated fatty acids deserve further research. Their proven and understood anti-inflammatory effects along with wide and easy availability and negligible side effect profile mean they could theoretically be future candidates for use as a long term adjunct to conventional periodontal therapy in patients who are not responding well to conventional treatment, or in whom a host modulation approach may be deemed beneficial. However evidence to date is preliminary and confined mainly to animal models.

In summation, given the discussed effect of various vitamin deficiencies on multiple aspects of systemic immune function, and the importance of the inflammatory/immune response in the initiation and progression of periodontitis, it is plausible that some subclinical deficiencies may be risk factors for periodontitis. If long term, large scale, prospective studies which minimised the potential influence of confounding variables showed this assumption to be correct, making use of such findings effectively in a clinical setting would be an important pursuit likely to require a multidisciplinary team approach.

1.5 Obesity

Diet, the quality and quantity of the foods we eat, is the major determinant of body mass index. Nearly three decades ago, obesity was noted to contribute to the severity of periodontal disease in rats (Perlstein *et al* 1977). The authors of this study suggested that intimal periodontal blood vessel thickening, causing decreased blood flow and a less effective inflammatory response, was the causative pathway.

More recently, obesity has been looked at as a potential periodontitis risk factor in human studies. In 2001, Saito *et al* studied 643 healthy, dentate Japanese adults. Waist-hip ratio, BMI and body fat percentage were shown to be significant risk factors for periodontitis after adjusting for confounding variables. In a study of older adults, Ritchie *et al* (2001) found that increased BMI was associated with gingival bleeding, and periodontal disease was associated with self reported weight gain. Al-Zahrani *et al* (2003) looked at obesity (BMI and waist circumference) and periodontal disease in young, middle aged and older adults. A significant association between the measures of body fat and periodontal disease was found among the younger adults, but not middle aged or older subjects. The adjusted OR for individuals 18-34 yrs with a BMI > 30 having periodontal disease was 1.76 (95% CI 1.19 to 2.61) -compared with subjects in the same age group with BMI in the desired range. Young subjects with a

high waist circumference had an odds ratio of 2.27 (95% CI 1.48 to 3.49) for periodontal disease.

Wood *et al* (2003) used the NHANES III data to assess any potential relationship between obesity and periodontal disease. Significant correlations between BMI, waist-hip ratio and periodontitis were observed. The authors concluded that "the study strengthens arguments that periodontal disease and certain obesity associated systemic illnesses are related, with abnormal fat metabolism possibly being an important factor."

Alabdulkarim *et al* (2005) in another cross sectional study found that after statistically adjusting for age, gender, smoking, employment, diabetes, marital status and number of teeth present, obese subjects were 1.86 times (95% CI 1.55 to 3.63) more likely to have periodontitis, as measured radiographically by alveolar bone loss, than those within the desirable weight range. The authors conclude that prevention and management of obesity may be considered to promote better systemic and periodontal health. In support of this, Nishida *et al* (2005) found that high BMI had a significantly stronger association with periodontitis than alcohol consumption, though still far weaker than cigarette smoking.

Dalla Vecchia *et al* (2005), while finding some significant associations in some study groups between obesity and periodontitis, found no associations in any groups between overweight and periodontitis.

In a case control study, Reeves *et al* (2006) found that both weight and waist circumference were associated with periodontitis, but the association varied in different age groups. Adolescents between 17 and 21 years had a proportionally increased risk of periodontitis as waist diameter and weight increased. The authors suggested that periodontitis might follow a similar pattern to a range of other chronic conditions that originate early in life and are related to central adiposity. However this finding was refuted by Linden *et al* (2007), who looked at the association between obesity and periodontitis in British 60-70 year olds. The authors

concluded that obesity was associated with periodontitis (OR=1.77, 95% CI 1.2 – 2.63), however high BMI levels early in life did not predict periodontitis in later life.

All of these studies were observational in nature and subsequently there is the potential for confounding of results by other health behaviours. There is also limited information regarding the temporal sequence between obesity and periodontitis, with the evidence we do have seemingly in conflict (Linden *et al* 2007, Reeves *et al* 2006).

The pathophysiology of the relationship has not been fully elucidated, however recent evidence has demonstrated that adipose tissue secretes a variety of molecules which alter the metabolism of the entire body, contributing to low grade systemic inflammation. Leptin, IL-6, TNF-α, adipsin, complement factor C3, angiotensinogen and plasminogen activator inhibitor-1 are all secreted by adipose tissue, with many of these molecules released proportionally to the degree of adiposity (Montague and Rahilly, 2000). IL-6 and TNFα in turn stimulate production of the acute phase proteins (such as C-Reactive protein) within the liver, possibly contributing to the hyper-inflammatory response seen in periodontitis (Van Dyke and Serhan 2003).

Tomofuji *et al* (2006) fed rats a high cholesterol diet, and found that this altered the host response to bacterial pathogens and their by products in an experimentally induced periodontitis model. The pro-inflammatory cytokine response was markedly different between high cholesterol diet rats and regular diet controls. Mitochondrial 8-hydroxydeoxyguanosine was also present in greater proportions in the high cholesterol rats. The authors suggest that excess oxidative tissue damage induced by a high cholesterol diet could potentiate and increase pro-inflammatory cytokine production by fibroblasts.

1.5.1 Type II diabetes - An indirect effect of diet on periodontal disease

Diabetes mellitus is a chronic disease with systemic manifestations. Increased risk of several diseases, including periodontitis, is particularly associated with poorly controlled diabetes. Altered vascular permeability, microangiopathies and a compromised host response increase the risk of periodontal disease, and impair the wound healing response post treatment (Grossi and Genco 1998). Among genetic and environmental factors, obesity is the strongest risk factor for impaired insulin sensitivity (Nishimura and Murayama 2001). Obese people require more insulin to maintain blood glucose homeostasis than individuals with a healthy weight. Eventually, pancreatic production of endogenous insulin cannot meet the high requirement for insulin, and patients develop the characteristic phenotype of Type II diabetes. In Australia, over 80% of individuals with Type II diabetes are classified as either overweight or obese at the time of diagnosis, and the prevalence of both overweight/obese and Type II diabetes is rising alarmingly (NHMRC 2006).

While not yet fully conclusive, some evidence suggests the relationship between periodontal disease and Type II diabetes is bi-directional, with individuals who exhibit advanced and active periodontal disease also exhibiting less metabolic control of the diabetic state (Grossi and Genco 1998, Nishimura and Murayama 2001, Mealey and Oates 2006).

Improved periodontal health in individuals with both Type I and II diabetes may lead to improved metabolic control and reduce the risk of further sequelae of diabetes (Taylor 2001).

Genco *et al* (2005) looked at the relationship between insulin resistance, obesity and periodontal disease using the NHANES III data. They found that BMI was positively related to the severity of periodontal attachment loss, and that insulin resistance (IR) likely mediated some of this relationship as obese individuals with a high IR exhibited a 48% greater risk for severe periodontal disease than overweight individuals with a low IR.

1.6 Alcohol

In Australia, alcohol consumption is part of most people's life (90% consume alcohol at least monthly). 11% of males and 6% of females drink daily (Australian Institute of Health and Welfare figures for the 2004 National Drug Household Survey). In this survey 10% of males and 9% of females drank alcohol in a pattern deemed to put them at a high risk of long-term harm.

Relatively few studies have examined the possible relationship between alcohol intake and periodontitis. An early study by Movin (1981) observed increased prevalence and severity of periodontal disease among patients with cirrhosis of the liver. The authors attributed this to an unhygienic lifestyle and poor oral hygiene which is often associated with individuals who are frequently intoxicated. In Finland, Sakki *et al* (1995) confirmed that heavy alcohol consumption is associated with poor oral hygiene practices, and also found an increased incidence of gingivitis and periodontitis in these individuals. Novacek *et al* (1995) reported worse periodontal conditions in alcoholic patients with and without liver cirrhosis than in non-alcoholic controls, and Shizukuishi *et al* (1998) reported a significant association between moderate periodontal disease and alcoholism among Japanese factory workers by bivariate analysis. More recent findings from the Genco group (Tezal *et al* 2001) showed a positive relationship between the alcohol consumption (as evaluated through self-report questionnaires) of 1371 subjects aged 25 to 74 and more severe attachment loss and gingival bleeding. Alcohol consumption of >5 drinks per week was associated with increased attachment loss, (odds ratio of 1.36, 95% CI 1.02 to 1.8), compared with consumption of <5 drinks per week. The odds ratio was modestly stronger when 10 drinks per week were used as a threshold (1.44, 95% CI 1.04 to 2.0). Wine beer and spirit intakes all had similar associations with periodontitis risk. It should be noted that the ranges for alcohol consumption (≥5 or ≥10 drinks per week) in this study are low. An intake of 5-10 standard drinks per week encompasses a huge number of 'social drinkers'. Most numeric definitions of alcoholism

suggest a threshold of at least 4 standard drinks per day (≥28 drinks per week). Thus this study is not just referring to increased periodontitis risk amongst heavy alcohol users, but rather its results suggest an increased periodontitis risk for a very large percentage of the adult Australian population.

This group (Tezal *et al* 2004) published another paper in 2004 in which they looked at adults over 20 years who took part in the NHANES III surveys. This included a questionnaire from all participants in which they estimated the number of drinks per day on a drinking day, and then the number of drinking days per week. These two figures were multiplied together to give the average number of drinks per week. This method does leave scope for inaccuracy, and as with other retrospective studies current alcohol intake does not necessarily give an accurate measure of lifelong alcohol intake. A significant linear relationship was found between number of drinks per week (dpw) and clinical attachment levels. Odds ratios were 1.22 (95% CI 1.02 to 1.47) for 5-9 dpw, 1.39 (95% CI 1.13 to 1.71) for 10-14 dpw, 1.54 (95% CI 1.22 to 1.93) for 15-19 dpw and 1.67 (95% CI 1.25 to2.23) for ≥20 dpw when compared to the 0-5 dpw group.

Pitiphat *et al* (2003), in a prospective study, examined the association between alcohol consumption and periodontitis among 39,461 male health professionals aged 40 to 75 years who were free of periodontitis at the start of the study. Alcohol intake was assessed at baseline and updated every four years by a food frequency questionnaire. Periodontal disease status was self-reported, which even amongst a population of health professionals is a source for error. Age, smoking, diabetes, time period and BMI were all adjusted for by multivariate analysis. During the twelve years of follow up, there were 2125 cases of periodontitis. Compared with non-drinkers, the odds ratio for periodontitis among men reporting usual alcohol intake of 0.1-4.9 g per day was 1.24 (95% CI 1.09 to 1.42), 5.0 to 14.9 g per day was 1.18 (95% CI 1.04 to 1.35), 15 to 29.9 g per day was 1.18 (95% CI 1.01 to 1.38) and ≥ 30g per day 1.27 (95% CI 1.08 to 1.49). One standard drink is 10 g of alcohol. The authors

concluded that alcohol is an independent modifiable risk factor for periodontitis, however this conclusion is based on evidence which has not accounted for many plausible confounding factors and there are numerous potential sources for error – thus this conclusion must be interpreted with caution.

More recent research has been less conclusive regarding a relationship between periodontal disease and alcohol intake. Okamoto *et al* (2006) in a longitudinal study of 1332 Japanese males investigated the links between cigarette smoking, alcohol consumption and periodontitis. While smoking was a strong risk factor for periodontitis progression and tooth loss, alcohol consumption was unrelated to periodontal disease. Shimazaki *et al* (2005) found that drinkers who on average consumed more than 15g of alcohol per day did have a significantly increased risk for widespread periodontal disease, defined as more than one third of teeth with PD ≥4 mm, as compared to non drinkers. Conversely, in periodontitis patients with less widespread pocketing (<35% of teeth), alcohol consumption was not related to periodontitis.

Finally, Nishida *et al* (2004) assessed whether alcohol consumption and $ALDH_2$ genotypes were associated with periodontitis. In humans, alcohol is first oxidised by alcohol dehydrogenase into acetaldehyde, which is then oxidised by aldehyde dehydrogenase into acetate. Asian populations frequently have polymorphisms in such alcohol-metabolising enzymes as $ALDH_2$, which play a central role in the alcohol hypersensitivity observed in a large proportion of the Asian population.

Lifestyle factors were examined by questionnaire, and the percentage of pocket depths ≥3.5 mm was used as a parameter for periodontitis. Multiple logistic analyses showed that alcohol consumption (>33 g per day) was significantly associated with periodontitis, with an odds ratio of 2.77 (95% CI 1.29 to 5.98). Alcohol consumption below 33 g per day was not significantly associated with periodontitis.

There are several plausible biological explanations which may explain a detrimental effect of alcohol on periodontal disease.

a) Alcohol impairs neutrophil function and may cause complement deficiencies (Tezal *et al* 2004).
b) Evidence from in vitro studies has suggested that alcohol may suppress osteoblastic activity while promoting osteoclastic activity, stimulating bone resorption. (Pitiphat *et al* 2003).
c) With high alcohol intakes, monocytes have been shown to release more pro-inflammatory cytokines. Offenbacher *et al* (1996) demonstrated that monocytic release of IL-1, IL-6 and TNF-α in the gingival crevice is associated with periodontitis progression.

On the whole, the literature fairly uniformly suggests that heavy alcohol consumption/alcoholism is a modifiable risk factor for periodontitis, although far more mild than smoking or uncontrolled diabetes. Poor oral hygiene in this group is likely responsible for a portion of this increased risk, but it is likely that an altered systemic response to bacterial challenge also plays a role. The relationship between moderate alcohol consumption and periodontitis is unclear, with mixed results appearing in the literature. Again, further large scale, methodologically sound prospective research which limits the potential influence of confounding variables is required.

1.7 Conclusions

Severe malnutrition indisputably has major deleterious effects on multiple aspects of immunity and wound healing capability, and as such it is not surprising that a considerable weight of evidence strongly demonstrates that grossly undernourished individuals are more susceptible to the periodontal diseases – particularly in their more advanced and aggressive forms. Severe malnutrition is very uncommon in Australia, however this predisposing factor

should still be borne in mind - particularly when investigating the causes of periodontitis in patients who have migrated from less developed nations.

On a day-to-day basis in Australia, periodontists will also commonly see patients who are subclinically deficient in folate, calcium and vitamin D. Some evidence to date suggests that inadequate nutritional status with regard to numerous vitamins and minerals may have a deleterious effect on periodontal health – and numerous plausible causative mechanisms have been postulated to explain such an effect. However many gaps still exist in the literature, and to date there does not exist a volume of sound scientific research which allows strong conclusions to be made about the potential negative influences of subclinical deficiency in any single nutrient on the periodontium. Further large scale, carefully designed and controlled research is required. The currently available, early evidence exploring these links suggests further research into the periodontal effects of mild vitamin D, calcium, folate and vitamin C deficiencies is particularly warranted. In addition, a growing body of recent research suggests that heavy alcohol intake and obesity are both risk factors for periodontitis – albeit weak risk indicators in comparison to long established environmental risk factors such as cigarette smoking.

Both the diet and dietary supplement industries in Australia are large and expanding rapidly. In addition current legislation allows manufacturers and retailers to make bold and often poorly substantiated claims about the efficacy of various products. Thus it is incumbent on members of the profession to have a sound knowledge of the literature and basic nutrition guidelines in order to educate and inform patients of possible associations between periodontitis and the various deficiencies and excesses, and limitations or risks of supplementation with various agents freely available ‘over-the-counter’.

Early investigations of Omega-3 polyunsaturated fatty acids on the other hand have shown promise – and further research into this essential dietary fatty acid as a safe method of modulating the host inflammatory response to plaque bacteria is warranted.

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The effect of dietary Omega-3 polyunsaturated fatty acids on experimental periodontitis lesions in the mouse

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

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2.1 Abstract

NOTE: This abstract has been moved to page vii of the electronic copy of the thesis held by the University of Adelaide Library.

2.2 Introduction

In human periodontitis, the vast majority of periodontal tissue destruction occurs as the result of an exuberant host immunoinflammatory response to periodontopathic bacteria and their metabolites – rather than as a direct action of bacterial products themselves. A range of these bacterial virulence factors stimulate the metabolism of arachidonic acid (AA), which is released from cell membrane phospholipids and then metabolised via the cyclo-oxygenase and lipoxygenase pathways to produce prostaglandins and leukotrienes. Some of these resultant eicosanoids are believed to be amongst the most important mediators of tissue destruction in periodontitis. Prostaglandin E_2 (PGE_2) is present in higher levels in the gingival crevicular fluid and periodontal tissues of patients with active periodontitis compared to healthy subjects (Offenbacher et al 1993). In addition to enhancing the activity of a range of other pro-inflammatory cytokines, PGE_2 stimulates the induction of osteoclasts and the production of matrix metalloproteinase-1, and therefore is likely to play an active role in the destruction of periodontal connective tissues and alveolar bone (Offenbacher 1996). Leukotriene B_4 (LTB_4) is believed to contribute to periodontal tissue destruction via cellular degranulation and superoxide generation. A range of other arachidonic acid metabolites, such as thromboxane A_2 (TxA_2), have marked pro-inflammatory and pro-thrombotic effects.

Eicosapentoic acid (EPA) and docosahexaenoic acid (DHA) are the two major Omega-3 polyunsaturated fatty acids (O-3 PUFAs) found in high quantities in fish oil, and have been demonstrated to competitively inhibit the production of arachidonic acid metabolites via the cyclo-oxygenase and lipoxygenase pathways, thus reducing the synthesis of pro-inflammatory AA derived prostaglandins and leukotrienes (Simopoulos 02).

Various medical solutions e.g. non-steroidal anti-inflammatory drugs (NSAID), which block AA metabolite production and thus reduce this pro-inflammatory burden, have proven beneficial in both the treatment of experimental gingivitis and periodontitis (Heasman *et al* 1994, Williams *et al* 1988). However long term NSAID administration (both selective, such as celecoxib, and non-selective, such as ibuprofen) is associated with a significant risk of side effects, particularly in the gastrointestinal tract. These effects are largely dose-dependent, and in many cases are severe enough to pose the risk of ulcerative perforation, upper gastrointestinal bleeding, and even death (Leong and Chan 2006). Up to 20% of NSAID patients experience dyspepsia, and NSAID-associated upper gastrointestinal adverse events are estimated to result in over one hundred thousand hospitalisations and nearly seventeen thousand deaths per year in the United States. NSAID's account for 43% of drug-related emergency visits (Green 2001). These side effects are caused by both indirect and direct irritation of the gastrointestinal tract by NSAIDs. Low pH active molecules may directly irritate the gastric mucosa; and inhibition of the enzyme cyclo-oxygenase-1 reduces the levels of a range of protective prostaglandins in the gut. Cyclo-oxygenase-2 selective NSAIDs have been demonstrated to produce fewer gastric adverse drug reactions, however relatively recent concerns about potential cardiac side effects have reduced the scope of their use (Moore *et al* 2006).

In contrast, the side effect profile of O-3 PUFA administration is minimal, with prophylactic dietary fish oil supplementation demonstrated to possess anti-ulcerative properties in the gastrointestinal tract in response to experimental challenge by various ulcerative and necrotizing agents (al-Harbi *et al* 1995).

In addition to reducing arachidonic acid metabolites, O-3 PUFA metabolism produces modified end products of both the cyclo-oxygenase and lipoxygenase pathways, such as PGI_3, which have potent anti-inflammatory properties. O-3 PUFAs are also precursors to

oxygenated derivatives termed resolvins and protectins which are emerging as key chemical mediators in the resolution of inflammation.

Fish oil has been demonstrated to hinder neutrophil adherence and cellular function and to reduce synthesis of a wide range of pro-inflammatory cytokines including interleukins 1, 2 and 6 (IL-1, IL-2, IL-6) and tumour necrosis factor alpha (TNF-α) which are implicated in active periodontal disease (Calder 2006). It has also been shown to diminish T-lymphocyte proliferation and reactivity and increase host anti-oxidant capacity, thus limiting tissue damage resulting from oxidative stress (Mori and Bellin 2004).

As a result of these anti-inflammatory actions and a positive safety profile, O-3 PUFA dietary supplementation has become an accepted therapeutic strategy in the management of chronic inflammatory diseases such as rheumatoid arthritis and cardiovascular disease (Smith and Sahyoun 2005). Research into their potential benefits in a wide range of inflammatory conditions continues to grow.

Within the periodontal literature there has been growing interest shown in the potential preventive or therapeutic effects of O-3 PUFA in the management of periodontal diseases. Alam *et al* (1991) showed that rats fed a diet rich in O-3 PUFA for eight weeks exhibited suppressed gingival levels of arachidonic acid and the pro-inflammatory eicosanoids PGE_2 and Leukotriene C_4. Vardar *et al* (2004) investigated the effect of systemic O-3 PUFA on bone loss in an experimental *E. coli* lipopolysaccharide (LPS) induced palatal periodontal abscess model in rats. In this study Omega-3 supplementation was given to one group as a treatment modality alone while another group received O-3 PUFAs prophylactically prior to endotoxin injections in addition to treatment. The gingival tissue levels of PGE_2, PGF_2, LTB_4 and platelet activating factor were then analysed and measured by enzyme immunoassay and radioimmunoassay. The results showed that PGE_2 levels were five times greater in the LPS controls than in either the prophylactic plus therapeutic or therapeutic only O-3 PUFA rats. The fish oil diet groups demonstrated significant decreases in the gingival tissue levels of all

of the above mediators compared with LPS only controls, with the prophylactic plus therapeutic group showing slightly greater reductions than the therapeutic administration only group.

In a follow up study using the same abscess model, Vardar *et al* (2005) found that O-3 PUFA supplementation resulted in a slightly greater reduction in the same four tested pro-inflammatory mediators than celecoxib (selective COX-2 inhibiting NSAID) supplementation. This added support to the earlier finding by Offenbacher *et al* (1990) that EPA or DHA can inhibit the production of PGE_2 to an extent similar to the non-selective NSAID ibuprofen.

In a ligature combined with *P. gingivalis* inoculation experimental periodontitis model in rabbits, Hasturk *et al* (2006) demonstrated that animals treated topically with the EPA-derived resolvin E1 showed four times less periodontal bone loss than control animals not treated with resolvin E1. Histology of the periodontal tissues suggested resolvin E1 suppressed the activity of osteoclasts, thus potentially inhibiting bone loss. This finding supported the earlier work of Sun *et al* (2003) who found EPA and DHA inhibited osteoclast activation *in vitro*.

Vardar-Sengul *et al* (2006) hypothesised that given the reduction in a number of pro-inflammatory cytokines and eicosanoids closely linked to periodontitis seen when rodents were fed a high O-3 PUFA diet, this diet may be protective against alveolar bone loss. Utilising an *E. coli* LPS experimental abscess model in rats, the authors found that while osteocalcin levels were higher in the O-3 PUFA groups no significant difference existed in the amount of alveolar bone loss seen between animals fed a high fish oil diet and controls fed a regular diet.

In contrast to this finding, Kesavalu *et al* (2006) examined the effect of O-3 PUFAs in an experimental periodontitis model in which oral inoculation with *P. gingivalis* was used to induce alveolar bone loss in rats. The control group received a corn oil diet, while the test group received a high fish oil diet for 22 weeks. The authors found that the Omega-3 fed rats

had high serum concentrations of EPA and DHA and showed significantly less alveolar bone loss around both maxillary and mandibular teeth compared with corn oil diet controls. They found that bone levels in these test rodents were similar to that of rats that had not been inoculated with *P. gingivalis*.

To date most studies have investigated the effects of a fish oil diet high in EPA with a low proportion of DHA. However, Kelley *et al* (1999) examined the effect of a high DHA diet on human peripheral blood mononuclear cells (PBMNC). Men on a high DHA diet showed a decrease in PBMNC arachidonic acid composition of almost 50%, and a 60 - 75% reduction in LTB_4 and PGE_2. Natural killer cell activity and *in vitro* secretion of interleukin 1beta (IL-1β) and TNF-α, two pro-inflammatory cytokines heavily implicated in periodontitis progression (Graves and Cochran 2003), were significantly reduced by DHA feeding. Roman *et al* (2006), in an *in vitro* study on decidual cells from human placentae challenged with IL-1β, also found that DHA supplementation decreased PGE_2 and PGF_2 production by approximately 60% more than EPA supplementation.

Given that the two studies to date exploring this relationship (Vardar-Sengul *et al* 2006, Kesavalu *et al* 2006) have produced conflicting results, the primary intent of this study was to further investigate the effect of a high Omega-3 polyunsaturated diet on alveolar bone loss in a rodent experimental periodontitis model. Since recent evidence demonstrates DHA has an effect on reducing pro-inflammatory prostaglandin synthesis, high DHA concentration tuna oil was chosen as the O-3 PUFA dietary supplement for this study.

As an adjunct to the primary study we also investigated the effect of combined use of *F. nucleatum* and *P. gingivalis* in the induction of experimental periodontitis lesions to determine whether a synergistic pathogenic relationship in active periodontitis was evident. *F. nucleatum* is a ubiquitous member of the subgingival plaque in inflamed sites and periodontitis lesions (Rogers 1998). *P. gingivalis* is accepted as a principle aetiologic agent in periodontitis (Proceedings of the 1996 World Workshop in Periodontics). Both *F. nucleatum*

and *P. gingivalis* produce a variety of metabolites, which if active in the confines of the host periodontal environment could result in prolonged pathogenic host inflammatory responses leading to connective tissue destruction and bone loss. In humans, *F nucleatum* colonises subgingival plaque prior to *P. gingivalis*, and high levels of *F. nucleatum* have been shown in frequent close association with *P. gingivalis* in patients with adult periodontal disease (Goodson *et al* 1984, Roques *et al* 2000). It has been demonstrated that *F. nucleatum* produces environmental conditions conducive to the establishment of acid sensitive, obligate anaerobic pathogens such as *P. gingivalis* (Diaz *et al* 2002).

Evidence exists in the literature that sequential colonisation and coaggregation between *F. nucleatum* and the later coloniser *P. gingivalis* could potentially modulate the immune response at humoral, cellular and molecular levels (Choi B *et al* 2000, Choi J *et al* 2000, Gemmell *et al* 2002, Gemmell *et al* 2004, Rosen and Sela 2006), with *F. nucleatum* suggested as having a role in 'priming' immune cells.

In a soft tissue abscess model, Feuille *et al* (1996) infected mice with *P. gingivalis* and *F. nucleatum* strains, and elicited lesions of various sizes as a function of the infective dose. Compared with *P. gingivalis* alone, primary infection with *F. nucleatum* and *P. gingivalis* resulted in significantly greater lesion sizes. Infection with *F. nucleatum* and *P. gingivalis* simultaneously or *F. nucleatum* administered within 4 hrs prior to *P. gingivalis* infection significantly enhanced the ability of *P. gingivalis* to form large phlegmonous lesions. Ebersole *et al* (1997) supported these findings of a synergistic effect between the two species demonstrating production of a larger soft tissue abscess.

Thus in the current study we also examined whether periodontal lesions induced in mice by repeated inoculation with a combination of *F. nucleatum* and *P. gingivalis* would result in greater hard tissue destruction than *P. gingivalis* alone in an established murine experimental periodontitis model.

2.3 Materials and methods

2.3.1 Ethics

The study was approved by the Institute of Medical and Veterinary Science (IMVS) Animal Ethics Committee of Adelaide, South Australia in December 2005 for a three year period. All experiments were carried out according to the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

2.3.2 Animals

Eighty female Balb/c mice 6 - 8 weeks old were purchased from the IMVS Veterinary Services Division and housed in the IMVS animal house in temperature controlled rooms. The mice in this study received water and food *ad libitum* throughout the entire experimental period.

2.3.3 Diet

Two separate diets were prepared:

a) Sunola control diet: consisting of standard soft powdered chow (containing predominantly wheat and barley – full composition available at www.specialtyfeeds.com) prepared by Specialty Feeds (Western Australia, Australia) with 10% by weight Sunola oil (Goodman Fielder®, New South Wales, Australia) added and evenly distributed. Gas chromatography analysis of the sunola oil demonstrated 0.00% of either DHA or EPA present.

b) Tuna test diet: consisting of standard chow prepared by Specialty Feeds with 10% by weight NuMega® HiDHA® 25N tuna oil (NuMega Ingredients, Brisbane, Australia)

added. Omega-3 polyunsaturated fatty acids comprised 35% of total fatty acids of which 25.5% was DHA and 6.3% EPA.

Fresh dietary mixtures were prepared every second day and kept in refrigerated conditions overnight before use the following morning in order to minimise peroxidative damage.

All groups of mice received their respective diets for the entire experimental period.

2.3.4 Study design

2.3.4.1 Fatty acid uptake into oral tissues over the experimental period

Mice were divided into eight groups of ten animals. Two groups – one receiving sunola feed and one receiving tuna feed - received no inoculations. Five mice from each of these two groups were sacrificed at experimental day 15 and their oral soft tissues collected for analysis of polyunsaturated fatty acid concentrations. The other seventy animals were sacrificed at the end of the experimental period (day 57), with the oral soft tissues of forty animals that had received bacterial inoculations then collected for fatty acid analysis.

2.3.4.2 Experimental groups

The remaining ten animals in the two groups receiving no inoculations were sacrificed at day 57 and were analysed by methods described below for comparison of alveolar bone levels with the carboxymethylcellulose inoculation groups to ensure the validity of our control inoculations. Of the remaining six groups of mice three groups received sunola feed and three groups received tuna feed. Within each feed type, one group acted as an inoculation sham control receiving 2% w/v pre-reduced, carboxymethylcellulose (CMC) only, one group received *P. gingivalis* inoculations and one group received combined *P. gingivalis/F. nucleatum* inoculations (referred to as the combined inoculum).

2.3.4.3 Antibiotic pre-treatment prior to bacterial inoculation

From day 5, all groups received kanamycin (1mg/ml) *ad libitum* for a 7 day period in their drinking water to suppress the oral microbiota. Mice were then rested for three days before inoculations commenced.

2.3.4.4 Bacterial preparation

P. gingivalis W50 (W83) and *F. nucleatum* ATCC 10953 were maintained on anaerobic blood agar plates, at 37^{o} C in an atmosphere of $N_2/CO_2/H_2$ (90:5:5).

Following incubation for 72 hrs, anaerobic blood agar (ABA) plates were removed from the anaerobic jar and 1.5 ml of 2% w/v pre-reduced, carboxymethylcellulose was placed onto each plate. Using a sterile, disposable spreader (Technoplas, Adelaide, South Australia), bacteria were scraped from the surface of the ABA plate to create a suspension of bacteria in CMC. The bacterial suspension was then removed with a Pasteur pipette and placed in a 1.5 ml eppendorf tube so that the headspace was kept to a minimum. Inoculation of mice occurred within 30 minutes of cell harvesting.

Cell density was estimated to be greater than 10^{11} organisms/ml as optical density (560 nm) was >5.0.

Dual inoculums containing both *P. gingivalis* and *F. nucleatum* were prepared separately as stated above. The combination inoculum was prepared by adding equal volumes (0.5 ml) of both preparations to an eppendorf tube and the suspension was then mixed carefully by aspiration with a Pasteur pipette.

2.3.4.5 Sequence of Inoculations

The sequence of inoculations began three days following the end of kanamycin administration (on experimental day 15) in the six experimental groups previously described. Mice were

inoculated according to the technique described by Baker *et al* (1999) with 100µl of inoculum containing either:

a) 2% carboxymethyl cellulose alone,

a) 10^{10} colony forming units per 100µl of live *P. gingivalis* in 2% carboxymethyl cellulose, or

b) $5x10^9$ colony forming units of live *P. gingivalis* and $5x10^9$ colony forming units of live *F. nucleatum* per 100 µl in 2% carboxymethyl cellulose, giving a total bacterial concentration of 10^{10} colony forming units per 100 µl.

The inoculations were administered by oral gavage into the posterior portion of the oral cavity around the maxillary molar teeth. The first sequence occurred four times over 8 days and then this regime was repeated 2 weeks later (second sequence). During the fortnight between the inoculation sequences the animals were inoculated twice weekly and these biweekly inoculations continued after the second sequence until the end of the experimental period (day 57). In total each mouse received sixteen inoculations. After each inoculation mice were held without food or water for one hour to minimise elimination of the inoculant from the oral cavity.

2.3.4.6 Tissue collection and preparation

Ten mice were euthanased at day 15 and the remaining 70 mice at day 57 by CO_2 inhalation and cervical dislocation. Approximately 80 mg of oral cavity soft tissue was collected from each animal by sharp dissection of the palatal gingiva, palatal mucosa, maxillary and mandibular buccal gingiva and oral mucosa and the tongue. These tissue samples were stored individually and 'snap frozen' in liquid nitrogen, then kept at -70º Celsius until tissue assays were performed. The heads were removed and the overlying fur and skin were dissected away using a scalpel blade. The heads and remaining soft tissue attachments were soaked in 1% NaOH solution for ten days to remove any residual soft tissue debris. At this stage the

mandibular and maxillary portions of the skulls became dissociated. Following defleshing the skulls and detached mandibles were thoroughly washed and allowed to dry in a controlled 40ºC heat environment. The maxillary portion of the skull was used to assess bone loss around the left and right side upper molars. To assist this analysis the maxillary portion of the skull was stained with 1% aqueous methylene blue to identify the cemento-enamel junction and alveolar bone crest of the maxillary molar teeth.

2.3.4.7 Imaging

For imaging purposes, the skulls were mounted on a rotatable and lockable stage. This allowed all specimens to be loaded and positioned identically so that there could be proper comparative analysis between the specimens within groups and between different groups. Bone loss analysis was conducted on the buccal surface of all 3 upper molars on the left and right side in a method similar to that described by Alayan *et al* (2006).

The buccal surfaces of all the upper molar teeth were digitally imaged using a Leica MZ16FA stereo microscope (Leica Microsystems, Wetzlar, Germany) and 32x magnification. The captured images were then further analysed using ImageJ® software (US National Institutes of Health, Maryland, USA) which helped to calculate the exposed buccal surface area from the cemento-enamel junction to the alveolar bone crest and bound by the mesiobuccal and distobuccal line angles for each of the 3 upper molars. This area was calculated for the left and right side and compared between the different experimental groups. This area does not give a strict quantitative assessment of bone loss, as it does not take into account the area occupied by the oral soft tissues, i.e. connective tissue and epithelium between the CEJ and the alveolar crest (an area commonly referred to as the 'biologic width'). However, increases in the area between the cemento-enamel junction and alveolar crest between groups sacrificed at the same age and timepoint were taken to represent loss of crestal alveolar bone.

Morphometric analysis of this nature has been shown an accurate and effective method of measuring alveolar bone loss in the mouse model (Li and Amar 2007).

The bone loss calculations were also repeated on all specimens from each group by an independent, blind observer who had no knowledge of the experimental groups tested in this study.

2.3.5 O-3 PUFA tissue assays

Oral tissue samples were defrosted and added to a glass/glass homogeniser, with 200 µl of internal standard C23:0 (Sigma T6543) then made up as 1 mg/ml in chloroform was added to the homogenation. Working in a fume hood, 3 ml chloroform:methanol 2:1 plus butylhydroxytoluene (15 mg/ml) was also added to the flask and the tissue gently homogenised.

The homogenate (150µl) was transferred to a glass methylation tube, and 2 ml of methanol toluene (4:1 v/v) added.

A positive displacement pipettor was used to slowly add 200 µl of acetyl chloride down the side of the tube, vortexing continuously. Tubes were sealed immediately with Teflon tape and capped, then heated for one hour at 100ºC in a heating block. Tubes were cooled with water, then 3 ml of K_2CO_3 (10%) added and vortexed. Tubes were centrifuged for five minutes (Hettich centrifuge) at 4000 rpm at 4ºC. The upper toluene phase (300µl) was transferred to a gas chromatograph vial with insert and these tubes stored at room temperature. A batch file was set up on the gas chromatograph in real time analysis and then 1 µl from the final volume was injected onto a Shimadzu Gas Chromatograph (GC) 20A BPX70 capillary column with a split ratio of 5:1 (Shimadzu, Melbourne, Australia).

The GC data was then analysed as per GC software package #211. Data was compared by peak area to the peak area of the internal standard peak.

2.3.6 Statistics

Data was assessed for outliers using a z score analysis. No significant outliers were observed in the raw data set. The data was also assessed for normality and was neither significantly skewed or kurtosed.

Alveolar bone loss was modelled using a generalised linear mixed model run in SAS 9.1.3 (SAS Institute Inc, North Carolina, USA) including the fixed effects of diet, inoculant, tooth, side and the interaction of diet by inoculation. Rat identification was fitted as a random effect in the model. Post hoc t-tests were conducted on all class variables and adjusted for multiple testing. The level of statistical significance was set at $p<0.05$.

2.4 Results

2.4.1 The effect of the tuna oil diet on the oral soft tissue content

The tuna oil diet induced marked changes in the O-3 PUFA content of the intraoral soft tissues. Table 1 demonstrates highly significant differences in the levels of DHA and EPA between mice fed the tuna oil and sunola oil diets. The type of bacterial inoculation received (*P. gingivalis* alone or *P. gingivalis/F. Nucleatum*) had no significant effect on tissue fatty acid levels.

Fatty Acids	**Sunola+*Pg***	**Tuna+*Pg***	**Sunola+*Pg/Fn***	**Tuna+*Pg/Fn***
18:2 Lineolic Omega 6	310±54*	148±30*	216±43*	116±8*
20:5 EPA Omega-3	2.0±1.4¤	19.5±2.7¤	1.7±0.7¤	16.5±1.2¤
22:6 DHA Omega-3	336±25^	621±75^	333±54^	536±38^

Table 1. Mean oral tissue polyunsaturated fatty acid concentrations (mg/100g) in mice sacrificed at 57 days. *, ¤ and ^ denote significantly higher tissue concentrations of

lineolic, eicosapentoic and docosahexaenoic acid respectively between different groups receiving the same inoculation regime and different diets. See appendix for P values.

Significant differences were observed between groups which received the same inoculation regime but different diets. Groups fed sunola oil exhibited higher tissue concentrations of lineolic omega 6 fatty acid (significant differences denoted by *), while the tuna oil feed resulted in far greater detectable levels of EPA (significant differences denoted by ¤) and DHA (significant differences denoted by^). All values are presented as mean tissue concentrations in mg/100g ± standard errors.

Between groups on different diets a highly significant inverse correlation was observed between both increased EPA and DHA levels in isolation and combination and alveolar bone loss. Within individual groups of mice on the same dietary regime and receiving the same inoculant no significant dose/response relationships were observed between tissue levels of either EPA, DHA or both and alveolar bone levels. This is possibly explained by the relatively high tuna oil concentration fed to the mice and the subsequent high tissue levels observed in all mice in the tuna feed groups, which may meet or exceed the concentration required for maximum therapeutic effect. Intra-group bone loss was relatively consistent within all experimental groups.

Markedly increased O-3 PUFA concentrations in the oral tissues were also observed in five mice from each dietary regime that were sacrificed at day 15, prior to commencement of bacterial inoculations. These levels were statistically no different to those found in the oral tissues of twenty mice from each dietary regime analysed after sacrifice at the end of the experiment (see Table 2).

Fatty Acids	Sunola	Sunola	Tuna	Tuna
Day of Sacrifice	15	57	15	57
20:5 EPA Omega 3	2.2±1.0	1.9±1.1	16.8±2.2	18.0±2.6
22:6 DHA Omega 3	401±10.8	335±41.1	555±31	579±72.3

Table 2. Mean oral soft tissue concentrations (mg/100g) of eicosapentoic acid (EPA) and docosahexaenoic acid (DHA) irrespective of inoculant at the two different time intervals. Five mice in each dietary group were given respective feeds for fifteen days and then sacrificed. Forty animals sacrificed at the end of the experimental period on day 57 had oral soft tissue polyunsaturated fatty acid assays performed. There were no statistically significant differences within dietary groupings between EPA and DHA concentrations at 15 and 57 days. All values are shown as mean tissue concentration in mg/100g ± standard errors.

2.4.2 Alveolar bone loss

At the end of the experimental period there were no statistically significant differences in the area between the CEJ and alveolar bone crest (mm^2) between animals receiving CMC inoculations and animals which were not inoculated, irrespective of diet ($p>0.05$).

Table 3 presents data on the mean area per tooth in mm^2, calculated as the area between the cementoenamel junction and the alveolar crest and bounded by the mesial and distal line angles, in groups receiving different combinations of diet and inoculant. Part of this area represents the space occupied by supracrestal connective tissue and epithelium, commonly referred to as the biologic width (Gargiulo et al 1961) – demonstrated in Figure 1. Figure 2 presents this data in graphical form.

NOTE: This figure is included on page 77 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1. The biologic width is the sum of the connective tissue and the junctional epithelium (courtesy of Padbury Jr *et al* 2003).

	Sunola	**Tuna**
No Inoculations (0.5 ml/group)	0.110±0.01	0.112±0.01
Carboxymethylcellulose	0.120±0.006	0.123±0.008
P. gingivalis	0.154±0.006*^	0.129±0.008*
P. gingivalis* and *F. nucleatum	0.165±0.008*^	0.141±0.008*^

Table 3. Mean area per tooth (mm2) between the alveolar crest, cementoenamel junction and mesial and distal line angles in mice

Mean area of bone loss per tooth in groups of mice receiving different combinations of diet and inoculation. These figures are inclusive of the space referred to as the 'biologic width'. Bone loss is given in mm2 ± the standard error of the mean adjusted for inter 'tooth type' variation.

***denotes a statistically significant difference in bone loss between groups receiving the same type of bacterial inoculations but different diets**

^denotes groups receiving bacterial inoculations which demonstrated significantly more bone loss than both CMC and no inoculation controls

See Appendix for P values.

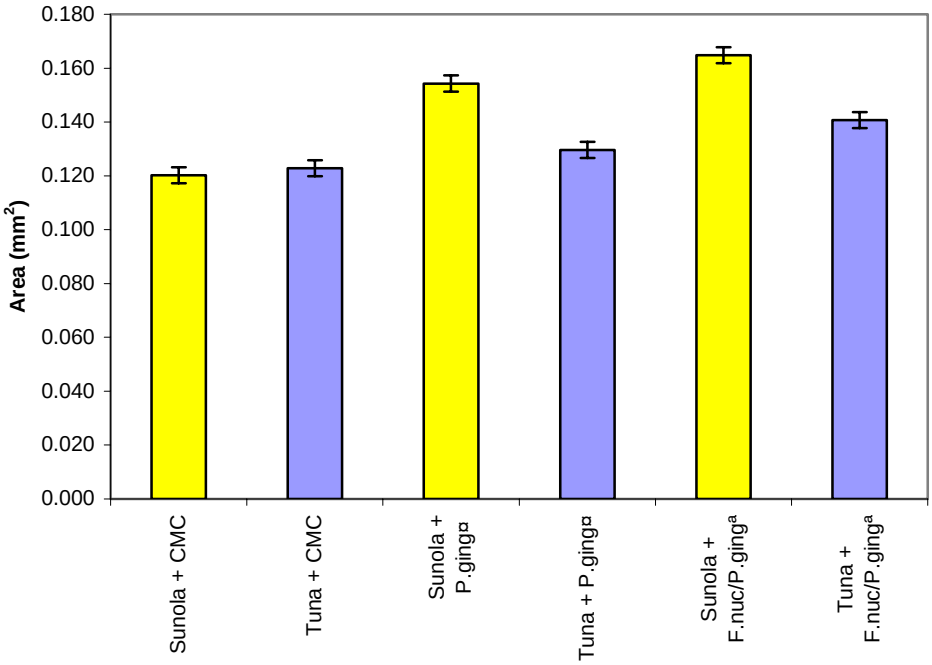

Figure 2. Mean area per tooth between the alveolar crest and CEJ in all groups receiving inoculations. ¤ denotes statistically significant differences ($p<0.05$) between diet groupings in mice receiving the *P.gingivalis* inoculation regime, while [a] denotes statistically significant differences between diet groupings in mice receiving inoculations with *P.gingivalis* combined with *F.nucleatum*

The y axis represents the total buccal area between the CEJ and the alveolar crest (mm^2) per tooth in the six study groups which were fed either a tuna oil or sunola oil rich diet and then either received control (CMC) inoculations or had experimental periodontitis induced by infection with *P. gingivalis* or a combination of *P. gingivalis* and

***F. nucleatum*. Columns represent means and vertical bars represent one positive or negative standard error from the mean adjusted fort inter-molar type variation.**

Mice infected with either *P. gingivalis* W50 (W83) alone or a combination of *P. gingivalis* W50 (W83) and *F. nucleatum* ATCC 10953 and fed a sunola oil diet demonstrated a significantly larger area between the CEJ and alveolar crest, indicative of greater bone loss in this group ($p<0.05$) than mice inoculated with carboxymethylcellulose only (sham inoculations) or not inoculated. In both the *P. gingivalis* and *P. gingivalis* + *F. nucleatum* inoculation groups, mice fed a diet of tuna oil demonstrated significantly less bone loss than animals which received the same inoculation program but were fed sunola oil. Mice fed the tuna oil diet and then inoculated with *P. gingivalis* showed only a mildly greater area of bone loss than control mice inoculated with CMC, with these bone loss differences not reaching statistical significance.

Figure 3 demonstrates the mean area of alveolar bone loss per tooth, using the mean area of bone loss for mice fed the control diet and receiving CMC (sham) inoculations as a baseline in order to account for the space naturally occupied by supracrestal connective tissues and epithelium, the biologic width. A diet of 10% tuna oil reduced alveolar bone loss by an average of 72% in *P. gingivalis* infected mice and 54% in the *P. gingivalis* + *F. nucleatum* infected mice compared with groups which underwent the same inoculation regime and were maintained on a sunola oil diet. Representative photographs of the de-fleshed jaws can be seen in Figures 4 and 5 on the following pages.

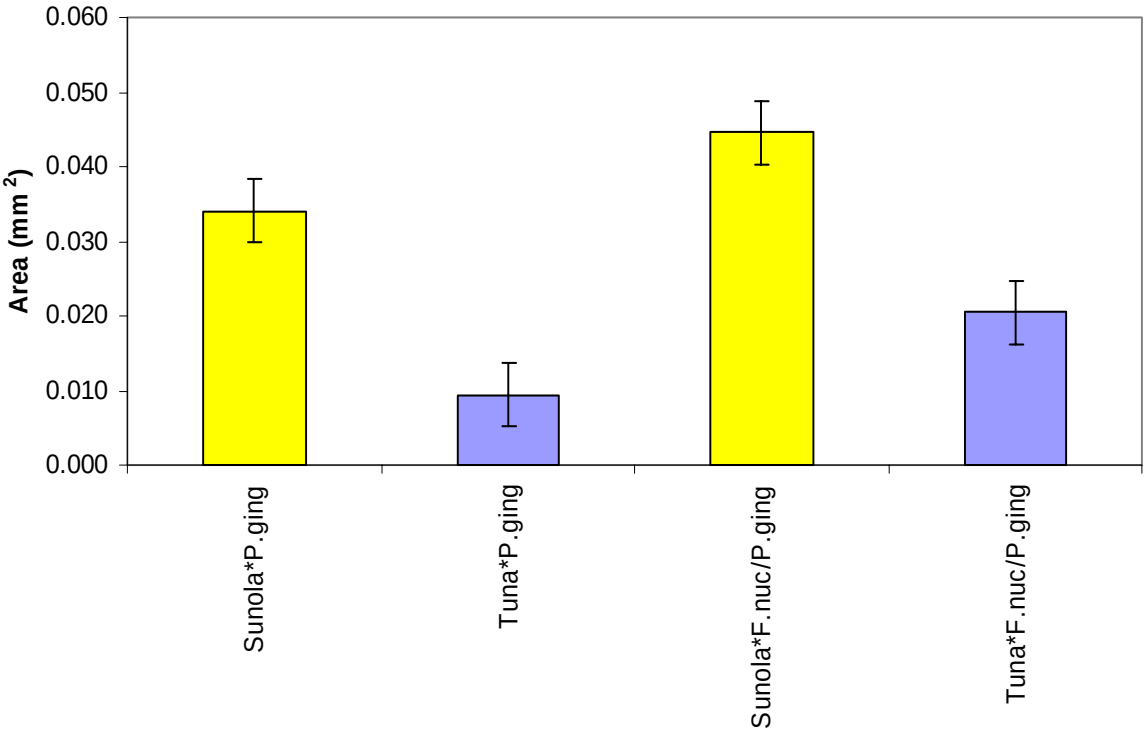

Figure 3. Mean bone loss per tooth (mm^2) in animals receiving bacterial inoculations.

Mice receiving the combined inoculum showed more bone loss than mice receiving *P.ging* alone in respective diet groups. The tuna oil diet markedly reduced bone loss irrespective of inoculation type ($p<0.05$). Columns represent mean area per tooth and vertical bars represent one positive or negative standard error from the mean adjusted fort inter-molar type variation.

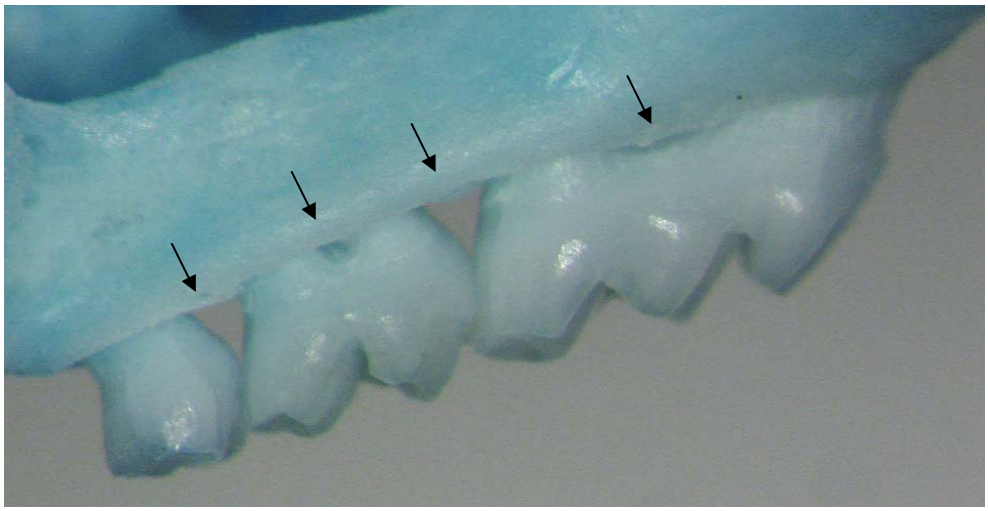

Figure 4.

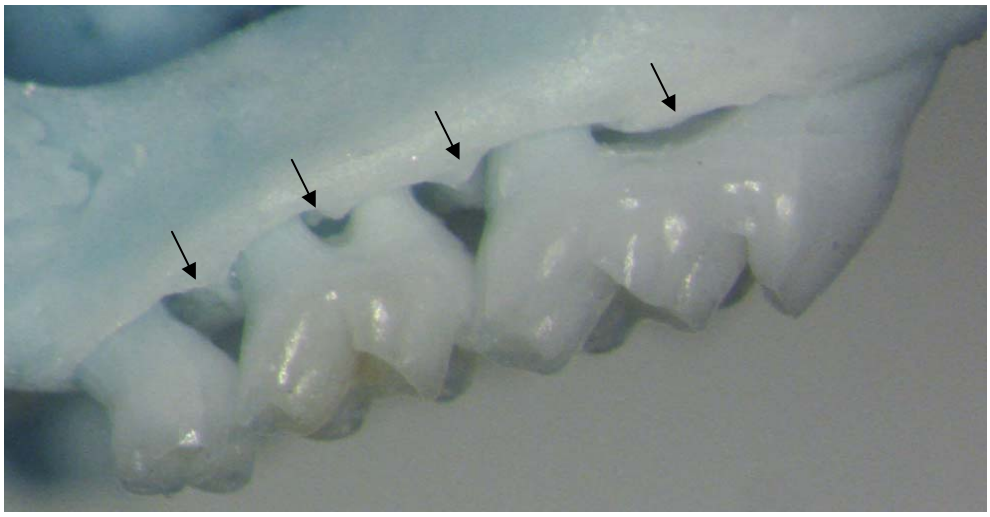

Figure 5.

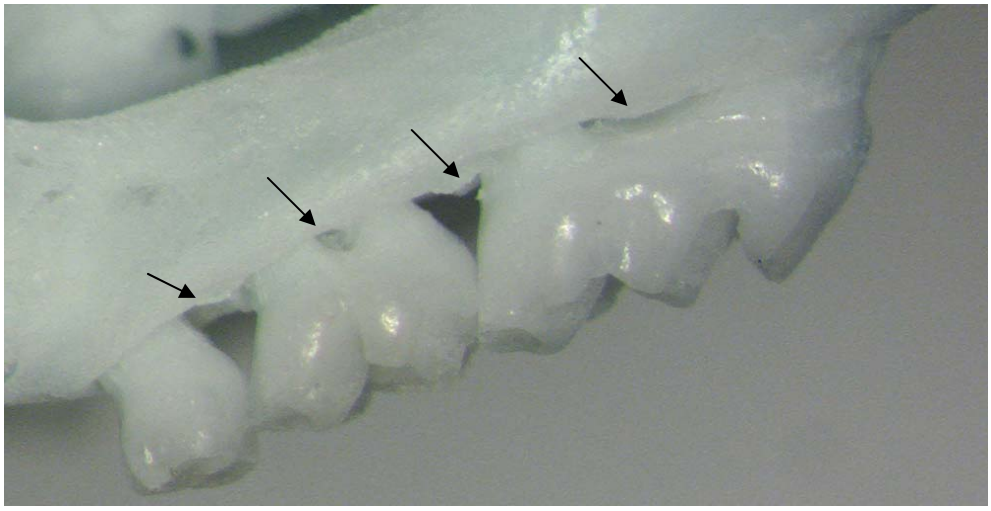

Figure 6.

Figures 4, 5 & 6. Right hand side posterior region of the maxilla showing three molar teeth (arrows demarcate the areas of difference in bone loss most discernable by eye). The animals in figures 4, 5 and 6 received sunola oil diet and CMC inoculations, sunola oil diet and P.gingivalis inoculations and tuna oil diet and *P.gingivalis* inoculations respectively. There is significantly less bone loss evident in Figure 5 than Figure 6. On average the tuna oil fed mice showed an area of bone loss 72% less than mice fed sunola oil with *P.gingivalis* inoculum.

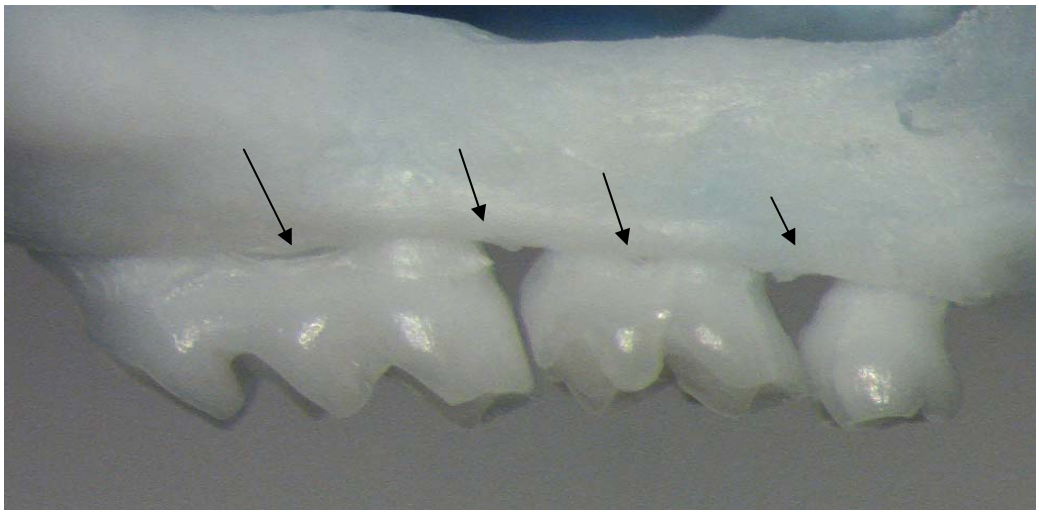

Figure 7

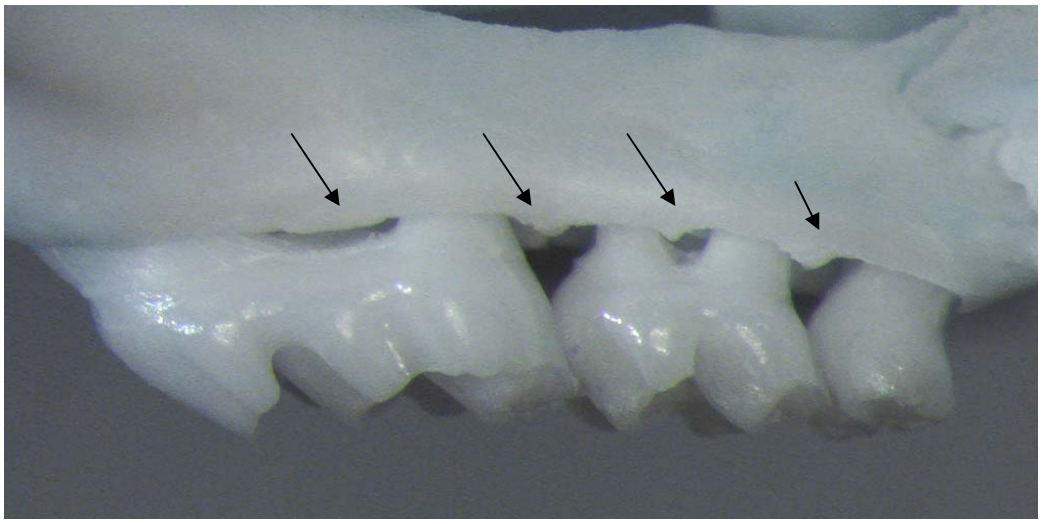

Figure 8

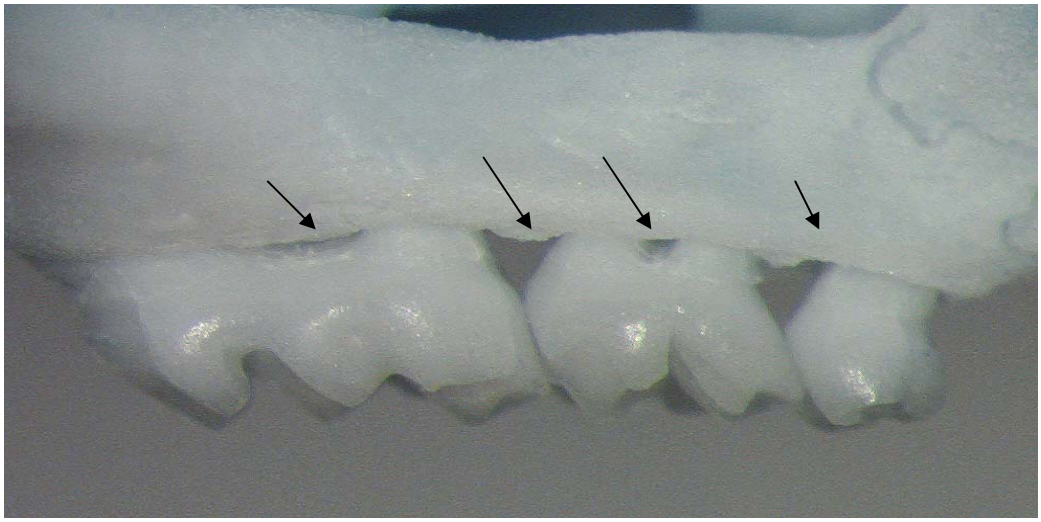

Figure 9

Figures 7, 8, & 9. Left hand side posterior region of the maxilla showing three molar teeth. The animals in figures 7, 8 and 9 received sunola oil diet and CMC inoculations, sunola oil diet and *P. gingivalis* + *F. nucleatum* inoculations and tuna oil diet and *P. gingivalis* + *F. nucleatum* inoculations respectively. Note significantly less bone loss in Figure 9 than Figure 8.

2.4.3 Effect of inoculation with a combination of *P. gingivalis* W50 (W83) and *F. nucleatum* ATCC 10953

Inoculation with the combination of *P. gingivalis* W50 (W83) and *F. nucleatum* ATCC 10953 resulted in a modest (mean 8% greater area per animal) but statistically significant increase in the area of alveolar bone loss observed ($p<0.05$). These differences remained significant irrespective of whether dietary regime was considered (see Appendix).

2.4.4 Accuracy

Bone loss calculations were also carried out by an independent observer who had no knowledge of the experimental groups tested in this study. Statistical analysis of the two observers' readings was assessed by paired Student's t-test, with significance levels at ($p<0.05$). No significant difference was found between bone loss calculations by the two observers.

3. Discussion

The results of this study demonstrate that dietary supplementation with tuna oil significantly increases the oral soft tissue concentration of the two main Omega-3 polyunsaturated fatty acids, EPA and DHA. In concordance with previous evidence (Croft *et al* 1986, Campan *et al* 1996) these raised tissue levels were established after two weeks of dietary supplementation,

with no significant differences noted between concentrations of either EPA or DHA at two weeks and the end of the experimental period.

Kesavalu *et al* (2006) demonstrated increased EPA and DHA levels in the serum of mice fed a fish oil diet and suggested that their uptake could be expected by both resident cells and immunoinflammatory cells in gingival tissues. Our findings in assays of tissues derived from the gingiva, oral mucosa and the tongue support this statement.

The anti-inflammatory actions of fish oil suppress production of pro-inflammatory arachidonic acid metabolites in the gingival tissues and may also inhibit the action of osteoclasts in bone resorption. (Vardar *et al* 2004, Kanzaki *et al* 2002, Kim *et al* 2005, Noguchi *et al* 2007, Sun *et al* 2003)

The results of our study indicated that a high tuna oil diet for eight weeks resulted in an average area of alveolar bone loss 72% less than corresponding controls fed a diet devoid of traceable levels of O-3 PUFAs, in an established and well accepted murine experimental periodontitis model inoculating with *P. gingivalis* (O'Brien-Simpson *et al* 2000). These results are comparable to the findings of Kesavalu *et al* (2006) who reported that a fish oil diet inhibited alveolar bone resorption in a similar *P. gingivalis* induced experimental periodontitis model in rats, and that "this dietary supplementation strategy appeared to have a magnitude of effect such that the O-3 PUFA groups had alveolar bone levels comparable with those in uninfected animals".

In the current study, mice infected with a combination of *P. gingivalis* and *F. nucleatum* also demonstrated greatly reduced alveolar bone loss when fed the tuna oil diet. Within these groups a mean 54% reduction in the area of bone loss was seen compared with mice fed sunola oil. Whilst this percentage is lower than that observed for *P. gingivalis* alone, this is due to the greater amount of bone loss seen in mice that received the combined inoculation regime. In real terms an almost identical mean area of bone loss (mm^2) was prevented in the

tuna oil groups in the combined inoculation mice as the animals infected with *P. gingivalis* alone.

In some contrast with the results of the current study and the results published by Kesavalu *et al*, Vardar-Sengul *et al* (2006) found that alveolar bone loss was greater in fish oil fed rats than control animals inoculated with saline in an *E. coli* endotoxin induced periodontal abscess model, but the differences were small and not statistically significant. This lack of a statistically significant effect may be partially explained by the daily O-3 PUFA dosage used in that study, which was only a small fraction of that added to the rodent feed both in the current study and the report by Kesavalu and co-workers. In the present study a 10% tuna oil diet was used. In rodent studies across the medical literature dietary concentrations in the range of 5 - 25% fish oil w/w are commonly used (Chiu *et al* 2007, Petursdottir and Hardardottir 2007) and believed to be within the optimal therapeutic range. The pathophysiology of bone loss in the endotoxin induced periodontal abscess model used by these authors also differs from that in our experimental periodontitis model in which mice were gavaged with whole live bacteria. In the abscess model only one bacterial virulence factor was used, with this lipopolysaccharide derived from *E. coli*, an organism not considered to be a major periodontal pathogen.

Previous studies in the periodontal literature investigating the potential therapeutic effects of O-3 PUFAs have evaluated fish oils which are high in EPA but relatively low in DHA. Recently evidence has been published highlighting the anti-inflammatory effects of docosahexaenoic acid, particularly with regards to decreasing PGE_2 production (Roman *et al* 2006). Other researchers have demonstrated that DHA directly binds to intracellular receptors and is incorporated within the nuclear membrane, leading to regulation and modification of gene transcription (Kitajka *et al* 2002, Vanden Heuvel 2004), while evidence supporting the importance of DHA derived resolvins and protectins in the resolution of inflammation

(Serhan 2006) is expanding. Thus a tuna oil with a high DHA:EPA ratio was chosen as the active therapeutic agent investigated in this experiment.

Recently it has become apparent that different fatty acid precursors, such as EPA and DHA, can be mobilised within tissue exudates in a temporally orchestrated process and biosynthesised to different classes of bioactive compounds (such as resolvins and protectins) which act as local chemical regulators of inflammation.

Hasturk *et al* (2006) have recently demonstrated that topical application of RvE1 in rabbit periodontitis confers a dramatic and almost complete protection against bone loss in an experimentally induced periodontitis model. The same authors also demonstrated that human neutrophils derived from patients with localised aggressive periodontitis respond to RVE1.

Subsequent to the discovery of EPA derived E series resolvins, D series resolvins (RvDs) and docosatrienes (termed protectins), which are derived via DHA biosynthesis, have been heavily investigated. Amongst their anti-inflammatory actions RvDs have been demonstrated to block tumour necrosis factor α induced IL-1ß transcripts in microglial cells (Hong *et al* 2003) and act as regulators which limit the infiltration of neutrophils into infamed tissues (Marcheselli *et al* 2003, Schwab and Serhan 2006, Serhan et al 2006).

Exogenously administered DHA (as was utilised in this study) is converted to both the D series resolvins and protectin D1 and their combined actions have been shown to reduce the number of infiltrating leukocytes and block Toll like receptor-mediated activation of macrophages in acute kidney injury (Duffield *et al* 2006). DHA administration alone results in a more marked reduction in prostaglandin production by various cell types compared to EPA (Roman *et al* 2006), while Weldon *et al* (2007) found that DHA reduced TNFα, IL-1ß and IL-6 mRNA expression by macrophages to a significantly greater degree than that of EPA.

The results of the current study show a significant reduction in alveolar bone loss in rats fed a diet high in DHA. Further investigation into the anti-inflammatory effects of high DHA:EPA ratio fish oil supplementation in the periodontal field is warranted, particularly given the emerging evidence demonstrating the effect of its metabolites in triggering the resolution of inflammation.

Scientific evidence demonstrating the health benefits of O-3 PUFA supplementation has been available for over thirty years. Omega-3 supplementation is widely acknowledged and advocated by government and professional bodies world wide for use in the prevention of cardiovascular diseases, to specifically lower triglyceride levels and to aid in the management of rheumatoid arthritis (Cleland and James 2002, Heart Foundation of Australia 2007).

In addition, a rapidly expanding body of research into the immuno-modulatory effects of Omega-3 polyunsaturated fatty acid supplementation in a range of inflammatory human diseases is evident. Substantial promising results have been published exploring the potential benefits of O-3 PUFAs in the management of ulcerative colitis, asthma, cystic fibrosis and the prevention of preterm labour in pregnant women (Calder 2006).

The determination that periodontal tissue breakdown is primarily due to the host response to certain bacteria has created great interest in methods of modulating this response. Such an approach may be a particularly beneficial adjunct to therapy in individuals with aggressive forms of disease and disease which proves refractory to conventional periodontal treatment. A large range of agents that modify various aspects of disease pathogenesis have been tested, however currently only one systemically administered agent is commercially available specifically for adjunctive use in the treatment of periodontitis - sub-antimicrobial dose doxycycline (Golub et al 2001).

Fish oil has many potential advantages for use as a supplement in host modulation therapy. It is widely available for purchase ‘over the counter’, has potential collateral health benefits and is relatively inexpensive. In contrast with many other agents which have been proposed as

possible candidates for systemic use in host modulation, large scale long term data suggests that O-3 PUFA supplementation is essentially free of side effects (Mozzaffarian and Rimm 2006). Its use in pregnant women and young children has been evaluated and shown to be safe, with EPA and DHA common and internationally approved additives to baby formula for their roles in enhancing neural development. Some concerns have been raised over the potential for increased consumption of certain toxins present in fish oil such as methyl mercury, however recent reports have suggested that it is highly unlikely that the risk of exposure to toxins would outweigh the potential benefits of O-3 PUFA supplementation (Mozzaffarian and Rimm 2006, UK Food Standards Agency 2004). The microencapsulated tuna oil used for many types of supplements and fortified products (including the oil used in this study) is also purified to remove pollutants.

Thus, given the promising evidence to date in animal models, human clinical trials are now necessary to further explore the potential benefits of systemic O-3 PUFA supplementation in individuals with periodontitis.

This study also demonstrated a small but significant increase in alveolar bone loss when mice were infected with a combination of *P. gingivalis* and *F. nucleatum* compared with *P. gingivalis* alone, despite mice in the combined inoculum groups only receiving half the dose of *P. gingivalis* as the *P. gingivalis* alone groups. *F. nucleatum* coaggregates with many bacteria including *P. gingivalis* and has been demonstrated to act as a bridge between early colonisers and the late colonising 'red complex' in subgingival plaque (Socransky and Haffajee 2005). *F. nucleatum* has been shown to exhibit biological activities which promote bone resorption (Zubery *et al* 1998). The mechanisms of interaction between these bacterial species has not been fully elucidated, however there exists the possibility of a synergistic pathogenic relationship. Kesavalu *et al* (2007) reported that in a rat model infection *F. nucleatum* alone did not induce significant alveolar bone loss compared with uninfected control animals, and that when included in an inoculum with *Bacteroides forsythus*, *P.*

gingivalis, and *Treponema denticola*, the three members of the pathogenic 'red complex' described by Socransky *et al* (1998), results demonstrated no more alveolar bone loss than infection with the members of the red complex alone.

4. Conclusion

Dietary supplementation with high dose docosahexaenoic acid tuna oil causes a marked elevation of O-3 PUFA levels in oral soft tissues. This diet reduced the amount of alveolar bone loss in a murine experimental periodontitis model compared to a control diet containing no Omega 3 PUFAs. Given recent advances in our understanding of the way Omega-3 fatty acids may exert their anti-inflammatory effects, further human research to evaluate the possible benefits of their use in patients with periodontitis is required. Hypothetically fish oil, as a dietary adjunct, is a very attractive candidate for use as a host modulating agent in patients with periodontitis.

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6. Appendix – Statistical Analyses

The UNIVARIATE Procedure

Variable: Area (Area)
Moments

N	*420*	*Sum Weights*	*420*
Mean	0.13432143	Sum Observations	56.415
Std Deviation	0.08058651	Variance	0.00649419
Skewness	0.62422267	Kurtosis	-0.6995954
Uncorrected SS	10.298807	Corrected SS	2.72106361
Coeff Variation	59.9952729	Std Error Mean	0.00393222

Basic Statistical Measures

Location		*Variability*	
Mean	0.134321	Std Deviation	0.08059
Median	0.118500	Variance	0.00649
Mode	0.054000	Range	0.36400
		Interquartile Range	0.13900

Tests for Location: Mu0=0

Test		*Statistic*	*p Value*	
Student's t	t	34.15919	Pr > |t|	<.0001
Sign	M	209.5	Pr >= |M|	<.0001
Signed Rank	S	43995	Pr >= |S|	<.0001

Tests for Normality

Test	*Statistic*		*p Value*	
Shapiro-Wilk	W	0.92401	Pr < W	<0.0001
Kolmogorov-Smirnov	D	0.108913	Pr > D	<0.0100
Cramer-von Mises	W-Sq	1.703249	Pr > W-Sq	<0.0050
Anderson-Darling	A-Sq	10.7357	Pr > A-Sq	<0.0050

Quantiles (Definition 5)

Quantile	*Estimate*
100% Max	0.3640
99%	0.3200
95%	0.2850
90%	0.2595
75% Q3	0.1980
50% Median	0.1185
25% Q1	0.0590

Variable: Area (Area)
Quantiles (Definition 5)

Quantile	*Estimate*
10%	0.0460
5%	0.0390
1%	0.0290
0% Min	0.0000

Extreme Observations

Lowest		*Highest*	
Value	*Obs*	*Value*	*Obs*
0.000	214	0.320	94
0.023	192	0.321	136
0.025	12	0.322	172
0.027	273	0.340	160
0.029	15	0.364	82

The Mixed Procedure

Model Information

Data Set	*WORK.LOSS*
Dependent Variable	Area
Covariance Structure	Variance Components
Subject Effect	ID
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	*Levels*	*Values*
Tooth	3	1 2 3
Side	2	L R
Diet	2	Sunola Tuna
Innoculation	4	CMC F.nuc/P.ging Nothing
		P.ging

Dimensions

Covariance Parameters	2
Columns in X	20
Columns in Z Per Subject	1
Subjects	70
Max Obs Per Subject	6

Number of Observations

Number of Observations Read	420
Number of Observations Used	420
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	-1763.88498030	
1	2	-1765.15046333	0.00000012
2	1	-1765.15061387	0.00000000

Convergence criteria met.

Covariance Parameter Estimates

Cov Parm	*Subject*	*Estimate*
ID	ID	9.518E-9
Residual		0.000682

Fit Statistics

-2 Res Log Likelihood	-1765.2
AIC (smaller is better)	-1761.2
AICC (smaller is better)	-1761.1
BIC (smaller is better)	-1756.7

Solution for Fixed Effects

Effect	*Side*	*Diet*	*Innoculation*	*Tooth*	*Estimate*	*Standard Error*	*DF*	*t Value*
Intercept					0.05556	0.004508	347	12.33
Diet		Sunola			0.02475	0.005238	347	4.73
Diet		Tuna		0	.	.	.	
Innoculation			CMC		-0.00665	0.005454	347	-1.22
Innoculation			F.nuc/P.ging		0.01129	0.005678	347	1.99
Innoculation			Nothing		-0.02370	0.006524	347	-3.63
Innoculation			P.ging	0		.	.	.
Tooth				1	0.1779	0.003121	347	57.00
Tooth				2	0.06577	0.003121	347	21.07
Tooth				3	0	.	.	.
Side	L				-0.01455	0.002548	347	-5.71

Solution for Fixed Effects

Effect	*Side*	*Diet*	*Innoculation*	*Tooth*	*Pr > |t|*
Intercept					<.0001
Diet		Sunola			<.0001
Diet		Tuna			.
Innoculation			CMC		0.2234
Innoculation			F.nuc/P.ging		0.0475
Innoculation			Nothing		0.0003
Innoculation			P.ging		.
Tooth				1	<.0001
Tooth				2	<.0001
Tooth				3	.
Side	L				<.0001

Solution for Fixed Effects

Effect	*Side*	*Diet*	*Innoculation*	*Tooth*	*Estimate*	*Standard Error*	*DF*	*t Value*
Side	R			0		.	.	.
Diet*Innoculation		Sunola	CMC		-0.02741	0.007303	347	-3.75
Diet*Innoculation		Sunola	F.nuc/P.ging		-0.00091	0.007536	347	-0.12
Diet*Innoculation		Sunola	Nothing		-0.02084	0.008989	347	-2.32
Diet*Innoculation		Sunola	P.ging	0		.	.	.
Diet*Innoculation		Tuna	CMC	0		.	.	.
Diet*Innoculation		Tuna	F.nuc/P.ging	0		.	.	.
Diet*Innoculation		Tuna	Nothing	0		.	.	.
Diet*Innoculation		Tuna	P.ging	0		.	.	.

Solution for Fixed Effects

Effect	*Side*	*Diet*	*Innoculation*	*Pr > |t|*
Side	R			
Diet*Innoculation		Sunola	CMC	0.0002
Diet*Innoculation		Sunola	F.nuc/P.ging	0.9035
Diet*Innoculation		Sunola	Nothing	0.0210
Diet*Innoculation		Sunola	P.ging	.
Diet*Innoculation		Tuna	CMC	.
Diet*Innoculation		Tuna	F.nuc/P.ging	.
Diet*Innoculation		Tuna	Nothing	.
Diet*Innoculation		Tuna	P.ging	.

Type 3 Tests of Fixed Effects

	Num	*Den*		
Effect	*DF*	*DF*	*F Value*	*Pr > F*
Diet	1	347	18.26	<.0001
Innoculation	3	347	44.11	<.0001
Tooth	2	347	1661.43	<.0001
Side	1	347	32.59	<.0001
Diet*Innoculation	3	347	6.68	0.0002

Least Squares Means

Effect	*Diet*	*Innoculation*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Diet	Sunola	0.1372	0.001975	347	69.47	<.0001	
Diet	Tuna	0.1248	0.002146	347	58.15	<.0001	
Innoculation		CMC	0.1215	0.002545	347	47.76	<.0001

Least Squares Means

Effect	*Diet*	*Innoculation*	*Estimate*	*Standard Error*	*DF*	*t Value*	*Pr > |t|*
Innoculation		F.nuc/P.ging	0.1527	0.002709	347	56.37	<.0001
Innoculation		Nothing	0.1078	0.003653	347	29.51	<.0001
Innoculation		P.ging	0.1419	0.002619	347	54.18	<.0001
Diet*Innoculation	Sunola	CMC	0.1202	0.003406	347	35.29	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	0.1646	0.003545	347	46.44	<.0001
Diet*Innoculation	Sunola	Nothing	0.1097	0.005124	347	21.42	<.0001
Diet*Innoculation	Sunola	P.ging	0.1543	0.003463	347	44.55	<.0001
Diet*Innoculation	Tuna	CMC	0.1229	0.003781	347	32.49	<.0001
Diet*Innoculation	Tuna	F.nuc/P.ging	0.1408	0.004097	347	34.37	<.0001
Diet*Innoculation	Tuna	Nothing	0.1058	0.005208	347	20.32	<.0001
Diet*Innoculation	Tuna	P.ging	0.1295	0.003930	347	32.95	<.0001

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*Estimate*	*Standard Error*	*DF*
Diet	Sunola		Tuna		0.01246	0.002916	347
Innoculation		CMC		F.nuc/P.ging	-0.03119	0.003717	347
Innoculation		CMC		Nothing	0.01376	0.004452	347
Innoculation		CMC		P.ging	-0.02036	0.003652	347
Innoculation		F.nuc/P.ging		Nothing	0.04495	0.004548	347

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*t Value*	*Pr > |t|*
Diet	Sunola		Tuna		4.27	<.0001
Innoculation		CMC		F.nuc/P.ging	-8.39	<.0001
Innoculation		CMC		Nothing	3.09	0.0022
Innoculation		CMC		P.ging	-5.58	<.0001
Innoculation		F.nuc/P.ging		Nothing	9.88	<.0001

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*Adjustment*	*Adj P*
Diet	Sunola		Tuna		Tukey	<.0001
Innoculation		CMC		F.nuc/P.ging	Tukey-Kramer	<.0001
Innoculation		CMC		Nothing	Tukey-Kramer	0.0115
Innoculation		CMC		P.ging	Tukey-Kramer	<.0001
Innoculation		F.nuc/P.ging		Nothing	Tukey-Kramer	<.0001

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*Estimate*	*Standard Error*	*DF*
Innoculation		F.nuc/P.ging		P.ging	0.01083	0.003768	347
Innoculation		Nothing		P.ging	-0.03412	0.004495	347
Diet*Innoculation	Sunola	CMC	Sunola	F.nuc/P.ging	-0.04444	0.004916	347
Diet*Innoculation	Sunola	CMC	Sunola	Nothing	0.01047	0.006153	347
Diet*Innoculation	Sunola	CMC	Sunola	P.ging	-0.03406	0.004857	347
Diet*Innoculation	Sunola	CMC	Tuna	CMC	-0.00266	0.005089	347
Diet*Innoculation	Sunola	CMC	Tuna	F.nuc/P.ging	-0.02060	0.005328	347
Diet*Innoculation	Sunola	CMC	Tuna	Nothing	0.01439	0.006222	347
Diet*Innoculation	Sunola	CMC	Tuna	P.ging	-0.00931	0.005201	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	Nothing	0.05491	0.006231	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	P.ging	0.01038	0.004956	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	CMC	0.04178	0.005183	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	F.nuc/P.ging	0.02384	0.005418	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	Nothing	0.05883	0.006300	347
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	P.ging	0.03513	0.005293	347
Diet*Innoculation	Sunola	Nothing	Sunola	P.ging	-0.04454	0.006184	347
Diet*Innoculation	Sunola	Nothing	Tuna	CMC	-0.01313	0.006368	347
Diet*Innoculation	Sunola	Nothing	Tuna	F.nuc/P.ging	-0.03108	0.006561	347
Diet*Innoculation	Sunola	Nothing	Tuna	Nothing	0.003915	0.007306	347
Diet*Innoculation	Sunola	Nothing	Tuna	P.ging	-0.01978	0.006458	347
Diet*Innoculation	Sunola	P.ging	Tuna	CMC	0.03140	0.005127	347
Diet*Innoculation	Sunola	P.ging	Tuna	F.nuc/P.ging	0.01346	0.005365	347
Diet*Innoculation	Sunola	P.ging	Tuna	Nothing	0.04845	0.006254	347
Diet*Innoculation	Sunola	P.ging	Tuna	P.ging	0.02475	0.005238	347
Diet*Innoculation	Tuna	CMC	Tuna	F.nuc/P.ging	-0.01794	0.005576	347
Diet*Innoculation	Tuna	CMC	Tuna	Nothing	0.01705	0.006436	347
Diet*Innoculation	Tuna	CMC	Tuna	P.ging	-0.00665	0.005454	347
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	Nothing	0.03499	0.006626	347
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	P.ging	0.01129	0.005678	347
Diet*Innoculation	Tuna	Nothing	Tuna	P.ging	-0.02370	0.006524	347

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*t Value*	*Pr > |t|*
Innoculation		F.nuc/P.ging		P.ging	2.88	0.0043
Innoculation		Nothing		P.ging	-7.59	<.0001
Diet*Innoculation	Sunola	CMC	Sunola	F.nuc/P.ging	-9.04	<.0001
Diet*Innoculation	Sunola	CMC	Sunola	Nothing	1.70	0.0897
Diet*Innoculation	Sunola	CMC	Sunola	P.ging	-7.01	<.0001
Diet*Innoculation	Sunola	CMC	Tuna	CMC	-0.52	0.6016
Diet*Innoculation	Sunola	CMC	Tuna	F.nuc/P.ging	-3.87	0.0001
Diet*Innoculation	Sunola	CMC	Tuna	Nothing	2.31	0.0214
Diet*Innoculation	Sunola	CMC	Tuna	P.ging	-1.79	0.0742
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	Nothing	8.81	<.0001

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*t Value*	*Pr > |t|*
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	P.ging	2.09	0.0370
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	CMC	8.06	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	F.nuc/P.ging	4.40	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	Nothing	9.34	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	P.ging	6.64	<.0001
Diet*Innoculation	Sunola	Nothing	Sunola	P.ging	-7.20	<.0001
Diet*Innoculation	Sunola	Nothing	Tuna	CMC	-2.06	0.0399
Diet*Innoculation	Sunola	Nothing	Tuna	F.nuc/P.ging	-4.74	<.0001
Diet*Innoculation	Sunola	Nothing	Tuna	Nothing	0.54	0.5924
Diet*Innoculation	Sunola	Nothing	Tuna	P.ging	-3.06	0.0024
Diet*Innoculation	Sunola	P.ging	Tuna	CMC	6.12	<.0001
Diet*Innoculation	Sunola	P.ging	Tuna	F.nuc/P.ging	2.51	0.0126
Diet*Innoculation	Sunola	P.ging	Tuna	Nothing	7.75	<.0001
Diet*Innoculation	Sunola	P.ging	Tuna	P.ging	4.73	<.0001
Diet*Innoculation	Tuna	CMC	Tuna	F.nuc/P.ging	-3.22	0.0014
Diet*Innoculation	Tuna	CMC	Tuna	Nothing	2.65	0.0084
Diet*Innoculation	Tuna	CMC	Tuna	P.ging	-1.22	0.2234
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	Nothing	5.28	<.0001
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	P.ging	1.99	0.0475
Diet*Innoculation	Tuna	Nothing	Tuna	P.ging	-3.63	0.0003

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*Adjustment*	*Adj P*
Innoculation		F.nuc/P.ging		P.ging	Tukey-Kramer	0.0222
Innoculation		Nothing		P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	CMC	Sunola	F.nuc/P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	CMC	Sunola	Nothing	Tukey-Kramer	0.6860
Diet*Innoculation	Sunola	CMC	Sunola	P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	CMC	Tuna	CMC	Tukey-Kramer	0.9995
Diet*Innoculation	Sunola	CMC	Tuna	F.nuc/P.ging	Tukey-Kramer	0.0033
Diet*Innoculation	Sunola	CMC	Tuna	Nothing	Tukey-Kramer	0.2900
Diet*Innoculation	Sunola	CMC	Tuna	P.ging	Tukey-Kramer	0.6270
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	Nothing	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Sunola	P.ging	Tukey-Kramer	0.4209
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	CMC	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	F.nuc/P.ging	Tukey-Kramer	0.0004
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	Nothing	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	F.nuc/P.ging	Tuna	P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	Nothing	Sunola	P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	Nothing	Tuna	CMC	Tukey-Kramer	0.4418
Diet*Innoculation	Sunola	Nothing	Tuna	F.nuc/P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	Nothing	Tuna	Nothing	Tukey-Kramer	0.9995
Diet*Innoculation	Sunola	Nothing	Tuna	P.ging	Tukey-Kramer	0.0480

Differences of Least Squares Means

Effect	*Diet*	*Innoculation*	*Diet*	*Innoculation*	*Adjustment*	*Adj P*
Diet*Innoculation	Sunola	P.ging	Tuna	CMC	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	P.ging	Tuna	F.nuc/P.ging	Tukey-Kramer	0.1951
Diet*Innoculation	Sunola	P.ging	Tuna	Nothing	Tukey-Kramer	<.0001
Diet*Innoculation	Sunola	P.ging	Tuna	P.ging	Tukey-Kramer	<.0001
Diet*Innoculation	Tuna	CMC	Tuna	F.nuc/P.ging	Tukey-Kramer	0.0303
Diet*Innoculation	Tuna	CMC	Tuna	Nothing	Tukey-Kramer	0.1424
Diet*Innoculation	Tuna	CMC	Tuna	P.ging	Tukey-Kramer	0.9257
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	Nothing	Tukey-Kramer	<.0001
Diet*Innoculation	Tuna	F.nuc/P.ging	Tuna	P.ging	Tukey-Kramer	0.4910
Diet*Innoculation	Tuna	Nothing	Tuna	P.ging	Tukey-Kramer	0.0077

7. Related Abstracts Accepted for Presentation

7.1 DENTAL SCHOOL RESEARCH DAY 2007

Poster no Oral Presentation yes

Title of study: The Effect of Dietary Omega-3 Polyunsaturated Fatty Acids (Fish Oil) on Murine Experimental Periodontitis

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Abstract::Periodontitis is an infective disease caused predominantly by Gram negative anaerobic bacteria. However it is becoming apparent that alveolar bone loss, which characterises periodontitis, is a result of the host inflammatory response to pathogenic bacteria, not the infectious agents directly. Omega 3 polyunsaturated fatty acids (O-3 PUFAs) are recognised, and used widely, for their anti-inflammatory effects. Evidence is emerging that their oxygenated derivatives are key chemical mediators in the resolution of

inflammation. We hypothesised that dietary supplementation with a fish oil rich in the O-3 PUFA docosahexaenoic acid would modify those inflammatory reactions which lead to periodontal bone loss in mice infected with periodontopathic bacteria.
Eighty mice were fed experimental diets containing either 10% tuna oil (40) or a sunola oil (control) (40) which contained no traceable O-3 PUFAs for 57 days. After two weeks each dietary set was split into four groups of ten mice, with these groups being inoculated with either
a) *P.gingivalis* b) *P.gingivalis* and *F.nucleatum* (combined inoculum)
c) Carboxymethylcellulose (control) or d) no inoculations (control). Of the twenty mice which received no inoculations, half were sacrificed after fifteen days and half at the end of the experiment to enable comparative fatty acid analysis of the oral soft tissues. Results demonstrated that eicosapentoic acid and docosahexaenoic acid were found in significantly higher proportions in the oral soft tissues of mice fed a tuna oil diet, and that animals receiving this diet exhibited an average of 54-72% less alveolar bone resorption in response to bacterial infection. Irrespective of diet, the combined inoculum resulted in slightly more alveolar resorption than *P.gingivalis* alone. The findings of this study suggest that fish oil dietary supplementation may have potential benefits as a host modulatory agent in the adjunctive management of periodontitis. Given its advantages in terms of safety, cost effectiveness and widespread availability, this dietary supplement warrants further research in human trials to assess its ability to modulate alveolar bone loss in individuals with periodontitis.

7.2 Nutrition Society of Australia Annual Scientific Meeting 2007

Dietary omega-3 polyunsaturated fatty acids counteract murine experimental periodontitis

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Background - Periodontitis is an infective disease caused predominantly by Gram negative anaerobic bacteria. However it is becoming apparent that alveolar bone loss, which characterises periodontitis, results from the host inflammatory response to pathogenic bacteria and not the infectious agents directly. Omega-3 polyunsaturated fatty acids (PUFA) have recognised anti-inflammatory effects and their oxygenated derivatives are key mediators in resolving inflammation.

Objective - To test the hypothesis that dietary supplementation with tuna fish oil rich in the omega-3 PUFA docosahexaenoic acid would reduce alveolar bone loss in mice inoculated with periodontopathic bacteria.

Design - 80 mice were fed experimental diets containing either 10% tuna oil (HiDHA; NuMega Ingredients, Australia) or sunola oil (placebo) for 57 days. After two weeks each dietary set was split into four groups of ten mice, which were a) untreated or inoculated with b) *Porphyromonas gingivalis,* c) *P.gingivalis* and *Fusobacterium nucleatum,* d) carboxymethylcellulose (treatment control). Oral cavity soft tissues were taken from mice at sacrifice for gas chromatographic determination of fatty acid composition. The maxilla was removed, stained with methylene blue and digitally imaged to assess bone loss around the upper molars.

Outcomes - Omega-3 PUFA levels were substantially higher in oral soft tissues of mice fed tuna oil compared to those fed sunola oil. Eicosapentaenoic acid increased from 1.9 ± 1.1 to 18.0 ± 2.6 mg/100g and docosahexaenoic acid from 335 ± 41 to 579 ± 72 mg/100g. Mice fed tuna oil exhibited 72% less alveolar bone resorption in response to infection with *P.gingivalis* and 54% less bone resorption following the combined inoculum than those fed sunola oil

(P<0.05). **Conclusions** - Fish oil dietary supplementation may have potential benefits as a host modulatory agent in the prevention and/or adjunctive management of periodontitis.