

# **IMPACT OF OMEGA-3 FATTY ACIDS ON PERIODONTAL INFLAMMATION**

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



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## **Declaration**

This work contains no material which has been accepted for the award of any other degree in any university or tertiary institution to Boram Park and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the text.

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Boram Park

Dated this ..... day of .....2015

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# Chapter 1. Literature Review

## 1.1 Abstract

**Objective:** To investigate the impact of omega 3 (fish oil supplements) on periodontal inflammation when used as an adjunct to conventional periodontal treatment.

**Methods:** A double-blinded, randomized, placebo-controlled, two centred pilot study of 13 months duration was carried out (n=34). Adults with newly diagnosed chronic periodontitis were recruited according to strict inclusion and exclusion criteria. Participants were randomly allocated (by computer number generator) to consume 6 x 1g capsules per day containing either Soybean oil (control group) or fish oil. Plaque and gingival crevicular fluid (GCF) samples were taken from two worst sites at non-adjacent teeth determined at initial clinical examination appointment. Non-surgical periodontal treatment was carried out once a week for 4 weeks. After 3 months of healing, 4 months data were collected then maintenance appointment was carried out every 3 months. Data at 4 months were used as end point for the purpose of preliminary analysis. Clinical measurements (probing pocket depth (PPD), clinical attachment level (CAL)) as well as the difference in the levels of various inflammatory markers, interleukin (IL)-1 $\beta$ , tumour necrosis factor (TNF)- $\alpha$ , C-reactive protein (CRP) in both plasma and GCF, at baseline (Before treatment) and at 4 months (3 months post treatment) were compared.

**Results:** In this study where fish oil supplements were used as an adjunct to non-surgical periodontal treatment, there were no statistical nor clinically significant differences after 4 months. The level of inflammatory mediators in GCF, IL-1 $\beta$ , TNF- $\alpha$  and CRP, showed no statistically significant differences within placebo nor fish oil group at baseline and 4 months. Unpaired T- test revealed no statistical difference between placebo and fish oil at baseline and 4 months.

**Conclusion:** This study showed no benefit of fish oil supplement as an adjunct to non-surgical periodontal treatment in terms of reduction in PPD and CAL gain as well as suppression of inflammatory markers. However this result could be due to: 1. Short follow up period, 2. Small sample numbers, 3. GCF may not be the best way to measure inflammatory marker level. Further study with larger sample number and longer duration should be carried out.



## 1.2 Periodontitis

Periodontitis is a multifactorial inflammatory disease affecting approximately half of the general population (Eke et al., 2012). It is initiated by bacterial biofilm formation in a susceptible host and is characterised by destruction of the periodontium.

There are multiple factors that influence the predisposition and progression of periodontitis. These include genetic factors, systemic diseases such as diabetes (especially non-insulin dependent diabetes or type II diabetes) and an individual's behavioural factors such as smoking and level of oral hygiene. Although disease cannot be initiated without bacterial biofilm formation, the host response is a key factor that determines the progression of the disease and outcome of treatment.

A number of bacteria have been found to be involved in the initiation and progression of periodontitis (Socransky et al., 1998, Socransky and Haffajee, 2005). These include *Aggregatibacter actinomycetemcomitans* (*A.a*) and *Porphyromonas gingivalis* (*P.g*). Both *A.a* and *P.g* are anaerobic, Gram-negative bacteria. Anaerobic bacteria thrive in subgingival spaces where the oxygen supply is limited and where their habitat is not disturbed. Many anaerobic bacteria are responsible for eliciting a host response, where leucocytes and lymphocytes (T and B cells) are recruited to defend the bacterial attack. The imbalance between pro and anti-inflammatory cytokines produced by leucocytes and lymphocytes result in the destruction of periodontium.

Severe periodontitis affects 10-15% of the general population and has been linked to cardiovascular disease in cross-sectional and cohort studies (Janket et al., 2003, Pussinen et al., 2005). Studies report that elevated cell- and cytokine-mediated markers of inflammation, including C-reactive protein (CRP), fibrinogen and various cytokines, are associated with periodontal diseases. By reducing the progression of periodontal disease, the levels of inflammatory markers common to both diseases (i.e. interleukin-6, tumor necrosis factor- $\alpha$  and C-reactive protein) are decreased, which might, in turn, decrease the severity of vascular disease. Periodontitis is shown to be associated with other systemic diseases such as diabetes, adverse pregnancy outcomes and rheumatoid arthritis (Bartold et al., 2005, Vergnes and Sixou, 2007, Megson et al., 2010, Chee et al., 2013, Kaur et al., 2013, Linden et al., 2013) and therefore controlling inflammation associated with periodontitis may affect the progression of, and help to control, such systemic diseases.

### **1.3 Host Modulatory Therapy**

Host modulatory therapy (HMT) in periodontics comprises systemically or locally delivered pharmaceuticals that are prescribed as adjuncts to conventional periodontal treatment (Salvi and Lang, 2005).

HMT aims to reduce tissue destruction and stabilise or even regenerate the periodontium without impairing normal defence mechanisms or inflammation by modifying or down-regulating destructive aspects of the host inflammatory response and up-regulating the protective or regenerative responses (Williams, 2008, Bhatavadekar and Williams, 2009). Fish oil as a HMT has received more attention since research has shown that omega-3 reduces inflammation (Calder, 2001). For this reason, fish oil has been indicated in the treatment of many chronic conditions with low-grade systemic inflammation including cardiovascular diseases, inflammatory bowel disease (IBD), cancer, and rheumatoid arthritis (James et al., 2010, Wall et al., 2010). The anti-inflammatory properties, together with the good tolerance of omega-3 fatty acids by many patients, has justified investigation of their use in the treatment of periodontal diseases.

### **1.4 What is Fish Oil and Why do we Take it?**

There are three major kinds of omega fatty acids; omega-3, omega-6 and omega-9 fatty acids. Omega-3 is obtained from specific foods, such as fish, walnuts and green leafy vegetables, while omega-6 originates from a diet rich in grains and vegetable oils, and omega-9 comes from animal fat and vegetable oil (Sijben and Calder, 2007, Simopoulos, 2008). In Western diets, omega-6 fatty acids account for the majority of polyunsaturated fatty acids (PUFA) in the food supply. Excessive amounts of omega-6 LC-PUFAs and high omega-6/omega-3 ratio are related to the pathogenesis of many diseases, including cancer (Sonestedt et al., 2008), endometriosis (Khanaki et al., 2012), inflammatory and autoimmune diseases (Simopoulos, 2002), as well as cardiovascular disease (Hayakawa et al., 2012).

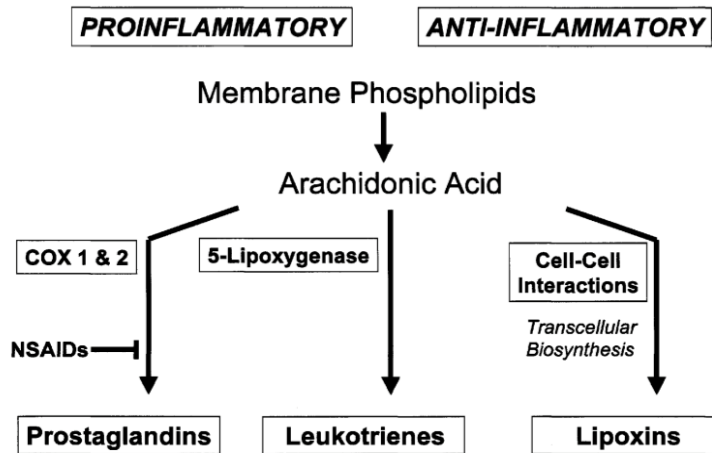
Among the fatty acids, omega-3 PUFA possess the most potent immunomodulatory activities, and among them, those from fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are more biologically potent than  $\alpha$ -linoleic acid (ALA).

Dose-response studies demonstrated that DHA is more effective than EPA, and that diets with combinations of these two omega-3 fatty acids are synergistic (Figueredo et al., 2013). However, it is still unclear what the most effective combinations and doses are for treating different inflammatory conditions.

The omega-6 and omega-3 fatty acids are metabolically distinct and have opposing physiologic functions. When diets are supplemented with omega-3 fatty acids, they partially replace the omega-6 fatty acids in the membranes of almost all cells including erythrocytes, platelets, endothelial cells, monocytes and fibroblasts (Simopoulos, 2002).

Eicosanoids exert complex control over many systems, mainly in inflammation and immunity. These are signalling molecules that are derived from either omega-3 or omega-6 fatty acids and made by oxidation of 20-carbon fatty acids. There are many subgroups of eicosanoids, including prostaglandins (PGs), thromboxanes (TXs), lipoxins, leukotrienes (LTs) and hydroxyl-eicosatetraenoic acids (HETEs). The eicosanoids from omega-6 are generally pro-inflammatory, whereas eicosanoids from omega-3 are more anti-inflammatory. The amounts and balance of omega-3 and omega-6 in an individual's diet will affect the body's eicosanoid-controlled function. Anti-inflammatory drugs such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) act by down-regulating eicosanoid synthesis (Figure 1.1).

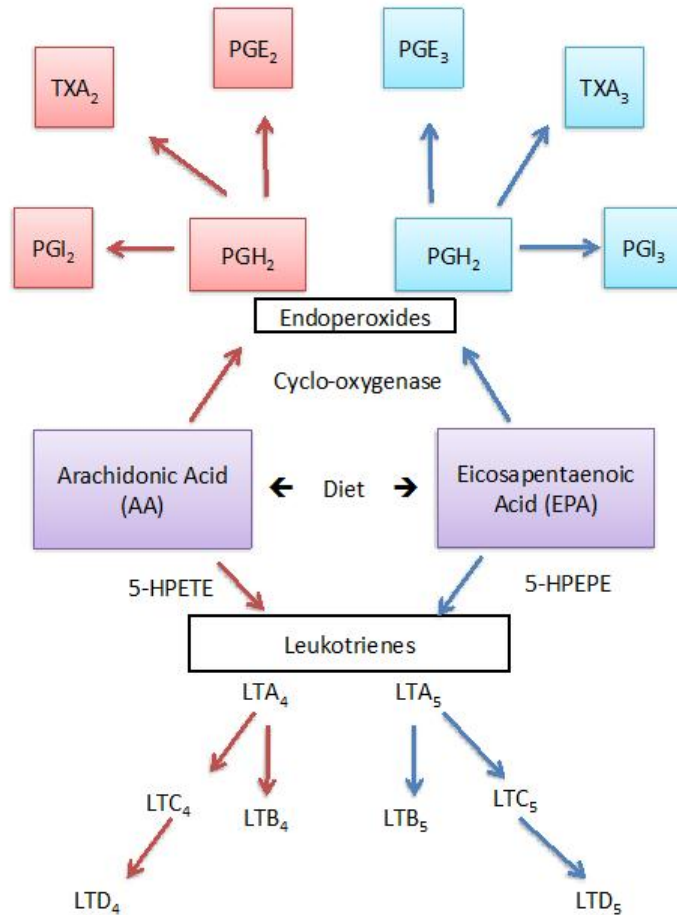
## ROLE OF LIPID MEDIATORS IN INFLAMMATION



**Figure 1.1. Eicosanoids from arachidonic acid.**

Prostaglandins, leukotrienes and lipoxins are produced from arachidonic acid. Anti-inflammatory drugs such as aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) act by down-regulating eicosanoid synthesis (Van Dyke and Serhan, 2003).

Eicosapentaenoic acid, an omega-3 fatty acid, competes with arachidonic acid (AA), an omega-6 fatty acid, for prostaglandin and leukotriene synthesis at the cyclooxygenase and lipoxygenase level (Figure 1.2).



**Figure 1.2. Eicosanoids production from arachidonic acid and eicosapentaenoic acid.** EPA and AA compete for prostaglandin and leukotriene synthesis at cyclo-oxygenase level. (Modified from Simopoulos, 2002).

EPA and DHA from fish or fish oil supplementation of the human diet means less substrate is available for synthesis of eicosanoids from arachidonic acid (Figure 1.2), which leads to (1) a decreased production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) metabolites (Endres et al., 1989, Caughey et al., 1996, Trebble et al., 2003b), (2) a decrease in thromboxane A<sub>2</sub>, a potent platelet aggregator and vasoconstrictor, (3) a decrease in leukotriene B<sub>4</sub> formation, an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence (Lee et al., 1985, Sperling et al., 1993), (4) an increase in thromboxane A<sub>3</sub>, a weak platelet aggregator and a weak vasoconstrictor, (5) an increase in prostacyclin PGI<sub>3</sub>, leading to an overall increase in total prostacyclin by increasing PGI<sub>3</sub> without a decrease in PGI<sub>2</sub> (both PGI<sub>2</sub> and PGI<sub>3</sub> are active vasodilators and inhibitors of platelet aggregation) and (6) an increase in leukotriene B<sub>5</sub>, a weak inducer of inflammation and a weak chemotactic agent (Sperling et al., 1993, Von Schacky et al., 1993, Kobayashi et al., 2001, Simopoulos, 2003, Ramirez-Tortosa et al., 2010).

In most populations, the consumption of omega-3 LC-PUFAs is either insufficient or not efficient in providing adequate tissue levels of EPA and DHA. Consumption of omega-3 polyunsaturated fatty acids, either by fish consumption or through DHA and EPA supplements, has been shown to lower levels of systemic inflammation and the need to use anti-inflammatory drugs. An omega-3 rich diet decreases the level of pro-inflammatory cytokines in blood (Caughey et al., 1996, Trebble et al., 2003a). Omega-3 fatty acids modulate prostaglandin metabolism and decrease triglycerides and in high doses lower cholesterol and have antithrombotic and anti-inflammatory properties (Serhan et al., 2002, Serhan et al., 2004, Serhan et al., 2006). It is extensively documented that in certain clinical settings omega-3 essential fatty acids (EPA and DHA) regulate both innate and acquired immune responses (De Caterina, 2011, Calder, 2013). PUFA also significantly reduces the risk of developing many cancers, stroke and cardiovascular disease, however the precise mechanism of action is still unknown.

Two studies showed significantly higher blood concentrations of omega-6 LC-PUFAs in patients with periodontitis (Requirand et al., 2000, Ramirez-Tortosa et al., 2010). Ramirez-Tortosa et al. (2010) measured plasma fatty acids levels in a group with chronic periodontitis and a control group. Fifty-six patients were included, 30 of which were diagnosed with chronic periodontitis. The level of omega-6 was statistically significantly higher in the periodontitis group whereas the level of omega-3 was not significantly different between periodontitis and control group. Another study by Requirand et al. (2000) evaluated the main serum PUFA and a possible alteration in the levels of AA in 78 periodontitis patients and 27 control patients using gas-chromatography. The level of omega-6 FA was higher and the level of omega-3 FA was lower in periodontitis group. They also noted that the ratios of omega-6/omega-3 were significantly higher in the periodontitis group. In contrast, a more recent study by Figueredo et al. (2013) showed that serum levels of both omega-3 and omega-6 LC-PUFAs were higher in patients with generalised chronic periodontitis compared with those of patients with gingivitis, and there were significant correlations between mean pocket depth and serum concentrations of both LC-PUFAs.

Both the Food and Agriculture Organization, in collaboration with the World Health Organization (FAO/WHO), and the Dietary Guidelines of the US 2010 recommended a combined intake of 250mg/d of EPA and DHA for adults (FAO/WHO, 2009, McGuire, 2011). The American Heart Association (AHA) recommends for the general population a

consumption of fish at least twice a week (Kris-Etherton et al., 2002). Estimating consumption of one portion (125g) of oily fish (2g/100g EPA and DHA) and one portion of lean fish (0.2g/100g), would result in a mean intake of 3g/week or 430mg/d of DHA and EPA. In addition, the National Heart Foundation of Australia released their position statement in 2008 and has recommended that Australian adults consume at least 500mg of omega-3 EPA/DHA every day from oily fish or fish oil supplements.

## **1.5 Acute and Chronic Inflammation**

Acute inflammation is a physiological mechanism that protects the host against local injury (Arita et al., 2005b). Under normal physiological conditions, the inflammatory response is cleared, leading to restoration of normal tissue architecture and function. Defects in the clearance mechanisms are associated with persistent tissue inflammation and eventually result in tissue damage, which causes discomfort and severely compromised normal tissue function (Sun et al., 2007).

In periodontitis, bacteria and their products may initiate the inflammatory response by activating a variety of humoral and cellular mediators. If the host response to an initial bacterial challenge is inadequate then the inflammation continues and becomes chronic, resulting in irreversible destruction of periodontium. Once the inflammatory process is triggered, a variety of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferons (IFNs) and lipid mediators are produced and play a crucial role in inflammatory process (Das, 2011). The imbalance between pro- and anti-inflammatory cytokines ultimately causes tissue damage and destruction of the periodontium. Failure to resolve the inflammation, even after the initial cause of inflammation has been removed, leads to delay in the healing/repair of tissues.

The primary goal of the inflammatory response is to detect and eliminate factors that interfere with homeostasis. A typical inflammatory response consists of four components: inflammatory inducers; the detecting sensors; downstream mediators; and the target tissues that are affected. The type and degree of inflammatory response activated is dependent on the nature of the inflammatory trigger (bacterial, viral or parasitic) and its persistence (Medzhitov, 2010).

Acute inflammatory response is the first line of defence when the host is under attack from bacteria. An acute inflammatory response is, by definition, divided into an initiation phase and a resolution phase. The initiation phase is accompanied by the cardinal signs of inflammation, known as heat, swelling, and pain with eventual loss of function. Oedema, polymorphonuclear neutrophil (PMN) infiltration, and monocyte-macrophage accumulation are the characteristic sequence of events during the initiation of the acute inflammatory response. When acute inflammation is uncontrolled or inappropriately activated, the continued presence of activated leukocytes within tissues without an appropriate termination and clearance of phagocytes is associated with collateral tissue damage and persistence of tissue inflammation. Hence, controlling PMN and macrophage activities could attenuate non-resolving chronic tissue inflammation. Loss of resolution and failure to return tissue to homeostasis is a major cause of human inflammatory pathologies, including arthritis, asthma, cancers, cardiovascular diseases and periodontal diseases. Many initiation-phase pro-inflammatory mediators such as prostaglandins and pro-inflammatory cytokines are used as targets in anti-inflammatory treatments in an attempt to control unwanted excessive inflammation (Flower, 2006, Dinarello et al., 2012). In order to maintain a healthy status, both the initiation of acute inflammation and its resolution must be efficient.

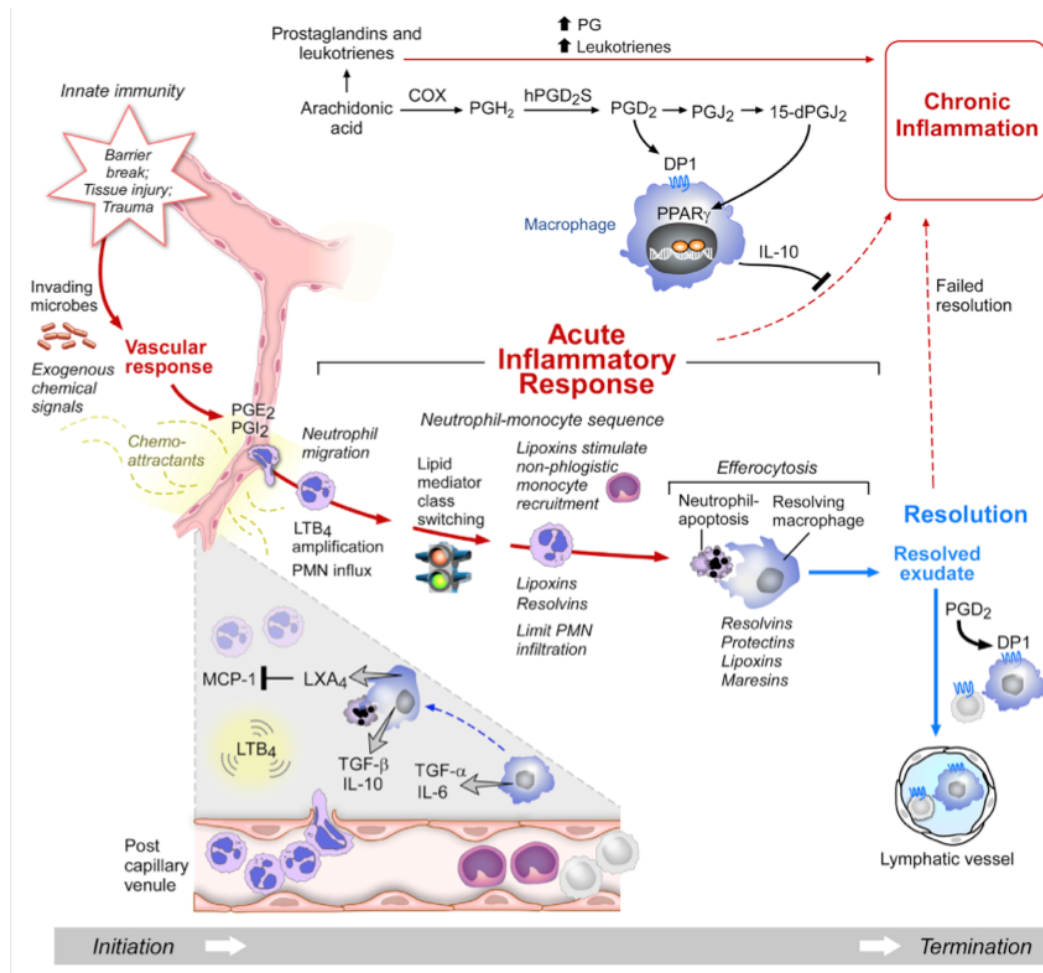
Serhan and colleagues demonstrated that the resolution of inflammatory exudate is an “actively programmed” biochemical process, regulated by the temporal biosynthesis of novel chemical mediators (Spite, 2014). This will be discussed in the next section.

## **1.6 Specialised Pro-resolving Lipid Mediators (SPMs)**

Specialised pro-resolving lipid mediators (SPMs) such as lipoxins, resolvins, protectins and maresins are novel autacoids that resolve inflammation, protect organs, and stimulate tissue regeneration (Bannenberg and Serhan, 2010). These pro-resolving mediators not only counter-regulate inflammatory gene transcription, but also directly block and limit excessive PMN migration and stimulate distinct cellular processes, such as macrophage uptake of apoptotic PMNs, microbes, and cellular debris that are required for tissue homeostasis to be re-established (Figure 1.3) (Serhan et al., 2000, Levy et al., 2001, Serhan et al., 2002, Serhan and Savill, 2005) . Serhan and colleagues also proposed that there is a temporal switch in lipid mediators from the initiation phase to resolution; that is, different lipid mediators are generated at different times during the resolution of the inflammatory

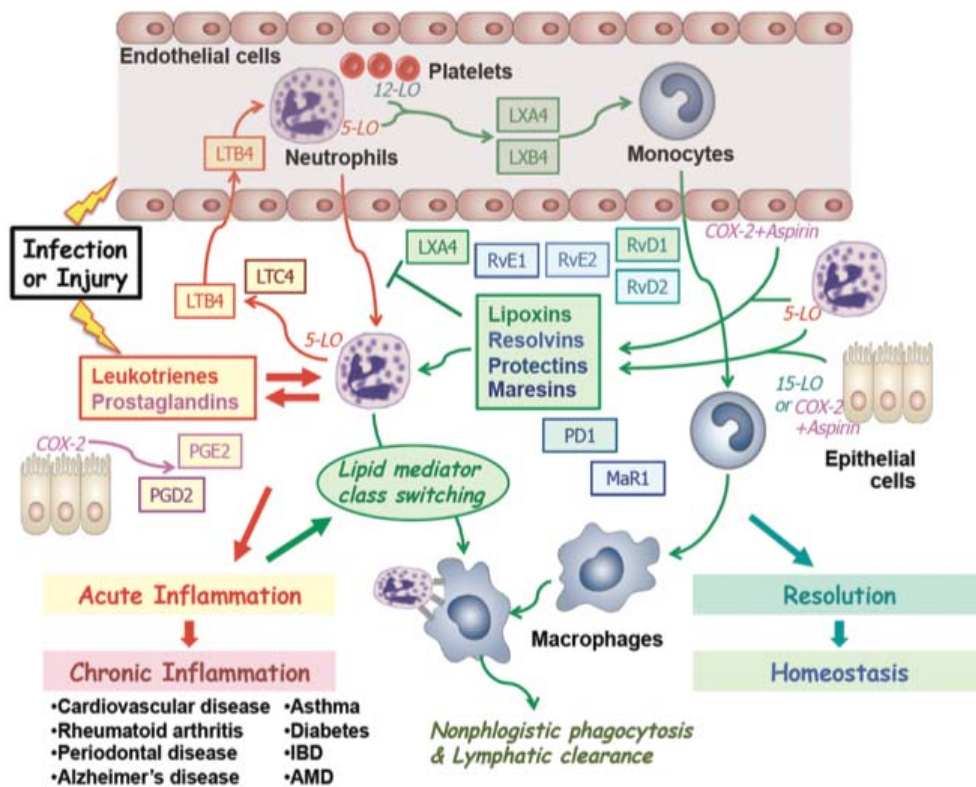


response, and these mediators coincide with distinct cellular traffic and events (Figure 1.4) (Spite et al., 2014). The appearance of specialised pro-resolving lipid mediators (SPMs) increases when macrophages are actively clearing apoptotic PMNs (Dalli and Serhan, 2012).



**Figure 1.3. The role of lipid mediators in resolution of acute inflammatory response.** Specialised pro-resolving lipid mediators (SPMs) such as lipoxins, resolvins, protectins and maresins are generated in acute inflammatory response to resolve inflammation (Buckley et al., 2014).

There are also temporal relationships between different SPM families and individual mediators produced in response to pathogens (e.g. viral versus bacterial) that trigger the production of select host SPMs (Chiang et al., 2012, Koltsida et al., 2013, Morita et al., 2013). This suggests that even within the SPM genus there are distinct roles for individual SPMs and that the complexities of their biosynthesis are just beginning to be appreciated.



**Figure 1.4. Acute inflammation to either chronic inflammation or to resolution.**

Different SPMs are produced at different stages of acute inflammation that either result in resolution or chronic inflammation (Serhan and Petasis, 2011).

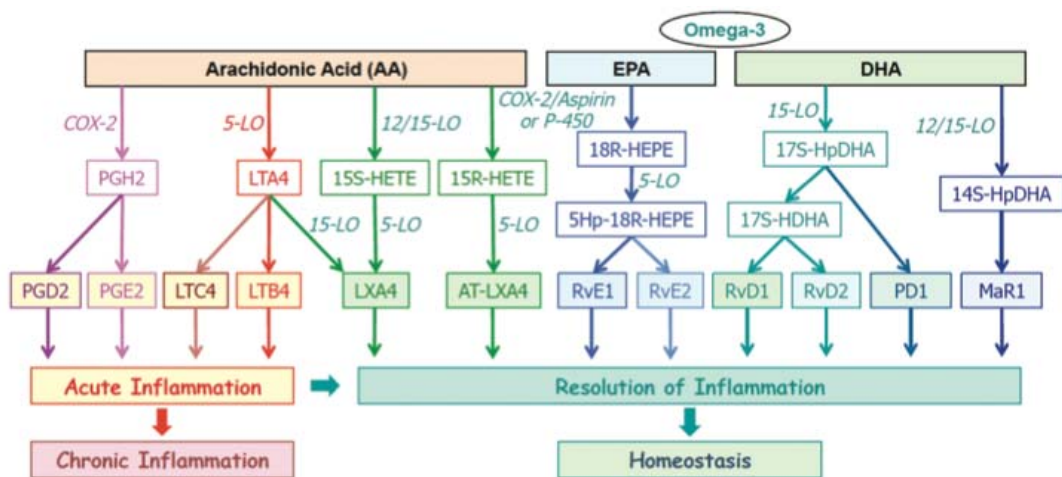
### 1.6.1 Lipoxins

Lipoxins are natural pro-resolving molecules derived from arachidonic acid, omega-6 fatty acids (Das, 2011, Van Dyke, 2011). They have potent anti-inflammatory and resolution actions (Samuelsson et al., 1987, Serhan et al., 1995, Serhan et al., 2000). In the presence of aspirin, more stable, potent and longer acting lipoxins are produced (Serhan et al., 2000), however mediators produced in the absence of aspirin can still evoke anti-inflammatory responses (Serhan and Chiang, 2008).

Lipoxins A4 and B4 were first isolated and identified as inhibitors of PMN infiltration and as stimulators of non-phlogistic (non-inflammatory) recruitment of macrophages (Maddox et al., 1998, Bannenberg et al., 2004). Lipoxin A4 has been found in the gingival crevicular fluid (GCF) of periodontitis patients with active disease, albeit at very low levels (Pouliot et al., 2000).

Three main pathways of lipoxin synthesis have been identified (Figure 1.5) (Serhan and Chiang, 2004). In the first pathway, in human mucosal tissues such as the gastrointestinal

tract, the airways and the oral cavity, sequential oxygenation of arachidonic acid by 15-lipoxygenase and 5-lipoxygenase, followed by enzymatic hydrolysis, leads to the production of lipoxin A4 and lipoxin B4 (Levy et al., 2001). In the second pathway, in blood vessels, 5-lipoxygenase biosynthesises lipoxin A4, and 12-lipoxygenase in platelets produces lipoxin B4. Lipoxin A4 regulates cellular functions through the activation of specific receptors (lipoxin A4 receptor/formyl peptide receptor 2 and G protein-coupled receptor 32); these receptors are expressed by neutrophils and monocytes. A third synthetic pathway is triggered by aspirin. Aspirin promotes the acetylation of cyclooxygenase-2, leading to a change in cyclooxygenase-2 activity and in the chirality of the products, which are termed aspirin-triggered lipoxins. Cells that express cyclooxygenase-2 include vascular endothelial cells, epithelial cells, macrophages and neutrophils. In addition to the synthesis of lipoxin, aspirin also blocks prostaglandin synthesis through acetylation of cyclooxygenase-2, inhibiting inflammation (Serhan and Chiang, 2008).



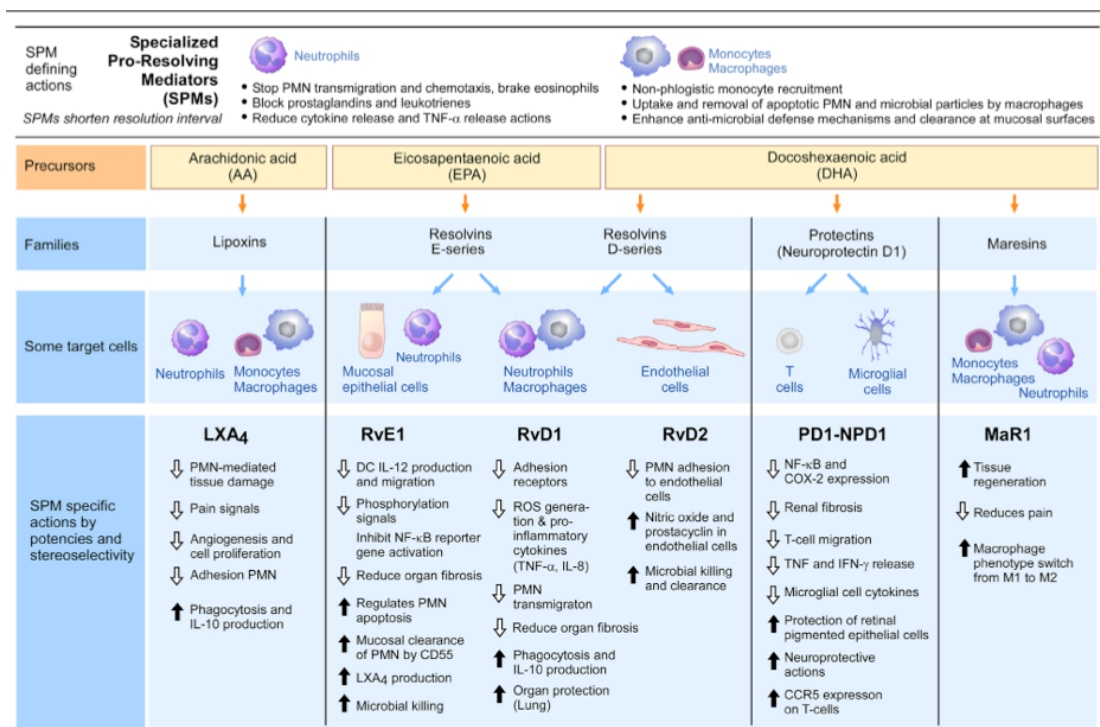
**Figure 1.5. Lipid mediators produced from arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).**

Synthesis and action of various lipid mediators from AA, EPA and DHA (Serhan and Petasis, 2011).

### 1.6.2 Resolvin

Resolvins and protectins, endogenous anti-inflammatory lipid mediators, are generated from precursor omega-3 fatty acids, DHA and or EPA and act to resolve inflammation to minimise tissue damage and maximise tissue protection in acute inflammation (Figure 1.5 and Figure 1.6) (Serhan et al., 2002, Hong et al., 2003, Serhan et al., 2004, Schwab et al., 2007, Keinan et al., 2013). The E-series resolvins (e.g., RvE1, RvE2, and RvE3) are

produced from EPA, and DHA is a precursor of D-series resolvins, protectins, and maresins (Serhan, 2007a, Isobe et al., 2012).



**Figure 1.6. Different types of specialised pro-resolving mediators and their target cells.**

The polyunsaturated fatty acids (AA, EPA, DHA) are converted by leucocytes to different types of SPMs, which in turn act on different target cells (Buckley et al., 2014).

Endogenous resolvins have been shown to accelerate resolution of inflammation in both acute and chronic models of inflammation (Arita et al., 2006, Hasturk et al., 2006). Through both anti-inflammatory and pro-resolution mechanisms, resolvins enhance and restore tissue integrity (Serhan et al., 2007).

Resolvin E1 was first described in inflammatory exudates of the murine dorsal pouch (Serhan et al., 2000). RvE1 acts by reducing neutrophil infiltration and by promoting phagocyte removal, thus speeding resolution of inflammation (Arita et al., 2005a, Serhan, 2007b). Topical application of RvE1 prevented *Porphyromonas gingivalis*-induced periodontal inflammation and alveolar bone loss in an experimental periodontal disease model (Hasturk et al., 2006). At this stage, whether the bone sparing action is entirely mediated through anti-inflammatory and pro-resolution action of RvE1 or whether RvE1 directly acts on bone cells is unanswered. Later Herrera et al. (2008) reported that RvE1 blocks osteoclast differentiation and bone resorption *in vitro*, suggesting a bone-sparing action, which is distinct from RvE1's known anti-inflammatory and pro-resolution actions.

The direct administration of EPA to osteoclast cultures in nanomolar doses results in increased osteoclast formation, whereas administration of RvE1 in similar doses inhibits osteoclast differentiation, suggesting that the bone sparing by EPA observed *in vivo* may not be from a direct action of EPA on osteoclasts, but rather the action of a metabolic derivative of EPA such as RvE1.

RvE1 and RvE2 are also endogenous receptor antagonists for the LTB<sub>4</sub> receptor, BLT-1, which most likely explains their ability to potently regulate PMN trafficking to sites of inflammation (Serhan and Chiang, 2013).

Production of RvE1 is increased in the plasma of individuals taking aspirin or eicosapentaenoic acid, resulting in improvement of the clinical signs of inflammation (Ho et al., 2010, Makriyannis and Nikas, 2011, Oh et al., 2011). Similarly, DHA-derived resolvins, D-series, have been shown to reduce inflammation by decreasing platelet-leukocyte adhesion, and aspirin-triggered DHA conversion produces molecules with dual anti-inflammatory and pro-resolution functions (Serhan, 2009).

### **1.6.3 Protectins**

Protectins are also biosynthesised via a lipoxygenase-mediated pathway from DHA and EPA (Figure 1.6). This pathway converts DHA into a 17S-hydroxyperoxide-containing intermediate that is rapidly taken up by leukocytes and converted into 10,17-Dihydroxydocosahexaenoic acid, known as protectin D1 (Serhan et al., 2006) or neuroprotectin (Bento et al., 2011). The name accounts for the protective actions observed in neural tissues (Mukherjee et al., 2004, Lukiw et al., 2005) and within the immune system. Protectin D1 is also produced by human peripheral blood lymphocytes with a T-helper 2 phenotype; it reduces TNF- $\alpha$  and IFN- $\gamma$  secretion, blocks T-cell migration and promotes T-cell apoptosis (Anderson and Delgado, 2008). Recently, a novel protectin-synthesis pathway, called aspirin-triggered protectin D1, was discovered and has shown a positive interaction with cannabinoid receptor 2 (CB2) and peroxisome proliferator-activated receptor family receptors (Bento et al., 2011, Shinohara et al., 2012).

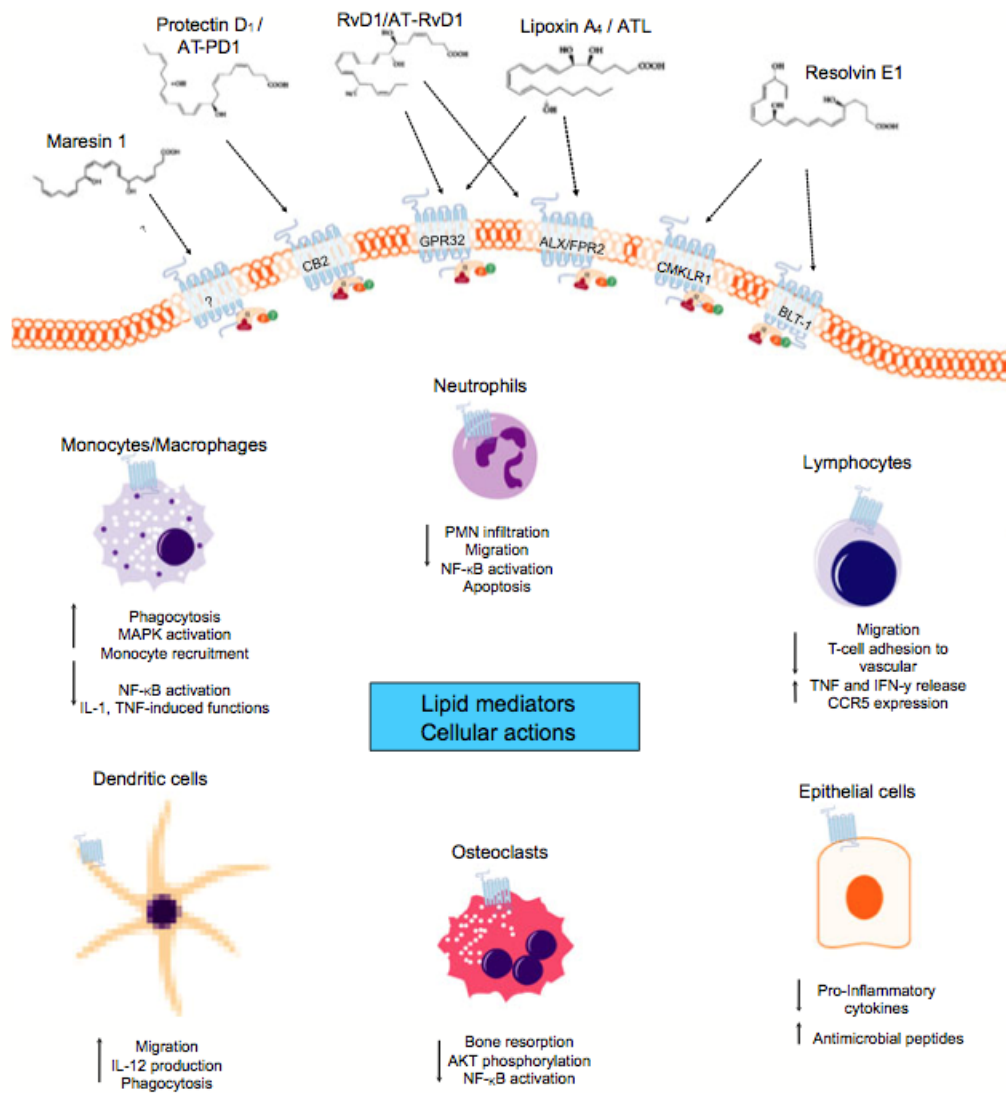
Both protectin D1 and aspirin triggered protectin reduce PMN transmigration through endothelial cells and enhance the clearance (efferocytosis) of apoptotic PMN by human macrophages (Shinohara et al., 2012).

#### 1.6.4 Maresins

Macrophage mediators in resolving inflammation (maresins) were most recently identified as primary molecules biosynthesised from DHA by macrophages with homeostatic functions. There are two types of maresins; maresin 1 (Serhan et al., 2009) and maresin 2 (Deng et al., 2014). A novel pathway of DHA metabolism has been identified in peritonitis models (Serhan, 2009, Serhan et al., 2009, Serhan et al., 2012). Macrophage phagocytosis of apoptotic cells triggers the biosynthesis of resolvin E1, protectin D1, lipoxin A<sub>4</sub> and maresin-1 (Norling and Serhan, 2010). Both maresin-1 and maresin-2 effectively stimulate efferocytosis (burying of dead cells) with human cells, decrease PMN infiltrations and also have regenerative functions (Freire and Van Dyke, 2013, Deng et al., 2014).

The selective interactions between pro-resolution lipid mediators and G protein-coupled receptors have been described in part (Figure 1.7). Binding of Lipoxin A<sub>4</sub> to the receptors lipoxin A<sub>4</sub> receptor formyl peptide 2 and G protein-coupled receptor 32 activates anti-inflammatory process and resolution (Freire and Van Dyke, 2013). Resolvin E1 (agonist) binds to two receptors; chemokine-like receptor 1 and leukotriene B<sub>4</sub> receptor type 1, which has been shown to have positive influences on cell fate in inflammation. Resolvin D1 specifically interacts with both lipoxin A<sub>4</sub> receptor and G protein-coupled receptor 32 on phagocytes, suggesting that each plays a role in resolving acute inflammation. The specific receptor interactions for protectin D1/aspirin-triggered protectin D1 and maresin 1 are yet to be defined; however, protectin D1 lipid mediators have been suggested to target cannabinoid receptor 2, CB2. Interactions between lipid mediators and cognitive receptors were found to influence *in vitro* and *in vivo* cellular functions with cell-type specificity (Figure 1.7) (Freire and Van Dyke, 2013).





**Figure 1.7. Lipid mediators and their receptors.**

Biosynthesized pro-resolution lipid mediators interact selectively with G protein-coupled receptors (Freire and Van Dyke, 2013).

Abbreviations: AKT, protein kinase B; ALX/FPR2, lipoxin A4 receptor; AT-PD1, aspirin-triggered protectin D1; ATL, aspirin-triggered lipoxin; BLT-1, leukotriene B4 receptor type 1; CB2, cannabinoid receptor 2; CCR5, chemokine (C-C motif) receptor 5; chemokine-like receptor 1; GPR32, G protein-coupled receptor 32; IFN- $\gamma$ , interferon gamma; IL, interleukin; MAPK, mitogen-activated protein kinase; NF- $\kappa$ B, nuclear factor kappaB; PNM, polymorphonuclear neutrophil; TNF, tumor necrosis factor.

## 1.7 Possible Mechanisms of Fish Oil to Improve Periodontal Outcomes

### 1.7.1 Reducing Inflammation

Fish oil is postulated to act by reducing production of pro-inflammatory cytokines and eicosanoids. The possibility of using dietary PUFAs to decrease cytokine production is suggested by reports that encapsulated fish oil used as a dietary supplement resulted in suppression of TNF- $\alpha$  and/or IL-1 $\beta$  synthesis (Endres et al., 1989, Meydani et al., 1991,

Caughey et al., 1996). Endres et al. (1989) added 18g of fish oil concentrate per day to a normal western diet for 6 weeks in 9 healthy volunteers. Four patients who did not receive the supplement were used as controls. The production of IL-1 $\beta$  and TNF- $\alpha$  by stimulated blood mononuclear cells *in vitro* was assessed during 4 phases of the study: before the start of omega-3 supplementation, during the 6th week of supplementation, during the 10th and 20th weeks after the end of supplementation. After six weeks of omega-3 supplementation, there was a significant increase in EPA, from 0.7% to 3.8%, with a concomitant decrease of AA, from 13.8% to 8.6%. TNF- $\alpha$  and IL-1 $\beta$  production was also decreased after six weeks of omega-3 supplementation. Ten weeks after the end of omega-3 supplementation, production of TNF- $\alpha$  and IL-1 $\beta$  decreased further. Twenty weeks after the end of supplementation, the production of IL-1 $\beta$ , IL-1 $\alpha$  and TNF- $\alpha$  had returned to the pre-supplement level. The decreased production of IL-1 $\beta$  and TNF- $\alpha$  was accompanied by a decreased ratio of AA to EPA in the membrane phospholipids of mononuclear cells.

In a longer clinical trial by Meydani et al. (1991), 6 young and 6 old healthy women were supplemented with 2.4g of omega-3 for 12 weeks. Blood was collected before and after 1, 2, and 3 months of supplementation. Omega-3 supplementation reduced levels of total IL-1 $\beta$  and TNF- $\alpha$  more dramatically in older women than in young women. The reduction of total IL-1 $\beta$  synthesis was by 48% in young women but by 90% in older women and TNF was reduced by 58% in young and 70% in older women.

Caughey et al. (1996) supplemented 28 subjects for 8 weeks with 9g/day fish oil, which inhibited TNF- $\alpha$  and IL-1 $\beta$  synthesis by 74% and 80% respectively. There was a significant inverse exponential relation between TNF- $\alpha$  or IL-1 $\beta$  synthesis and mononuclear cell content of EPA. Although three trials used different dose of fish oil supplementation and had different study duration, all reported the decrease in IL-1 $\beta$  and TNF- $\alpha$  level after omega-3 supplementation.

Alam et al. (1991) showed that dietary omega-3 fatty acid decreased levels of arachidonic acid (reduced by 56%) in rat gingiva and submandibular salivary glands (SMSG) as well as decreased levels of PGE<sub>2</sub> in SMSG (by 74%) and gingiva (by 83%). They also showed that omega-3 fatty acid fed rats produced significantly less LTC<sub>4</sub> than rats of corn oil fed control group. Since PGE<sub>2</sub> and LTC<sub>4</sub> are believed to be important biochemical mediators of periodontal disease, one may speculate that a diet-induced reduction in these mediators may have a beneficial effect upon the course of periodontal disease. In a human



experimental gingivitis model, Campan et al. (1997) demonstrated decreased gingival levels of AA, PGE<sub>2</sub>, and LTB<sub>4</sub> in those administered with systemic omega-3 fatty acids, whereas levels of these mediators were increased in the placebo group. However, the difference was not statistically significant.

### **1.7.2 Limiting Bone Resorption**

An imbalance between bone forming osteoblasts and bone resorbing osteoclasts results in loss of bone, which is one of the main characteristics of periodontitis. The key factor for osteoclast proliferation and differentiation is signalling through receptor activator of nuclear factor kappaB (RANK) and its ligand (RANKL). RANKL is a member of the tumor necrosis factor superfamily and is expressed by a variety of cells, including osteoblasts, fibroblasts and T cells (Boyle et al., 2003, Redlich and Smolen, 2012). It is expressed in a membrane-bound protein or cleaved into a soluble form (Nakashima et al., 2000, Mizuno et al., 2002). The production of RANKL is regulated in response to the presence of inflammatory cytokines such as TNF- $\alpha$  and IL-1 (Hofbauer and Heufelder, 2001, Caetano-Lopes et al., 2009). The binding of RANKL to its receptor, RANK, on the surface of pre-osteoclasts results in the activation of nuclear factor-kappaB, leading to osteoclast formation.

The benefit of LCn3PUFA supplementation may be mediated in part by improved bone metabolism. Cell culture studies have reported that LCn3PUFA can inhibit osteoclast differentiation, activation and function resulting in reduced levels of RANKL induced by pro-inflammatory cytokine production and intracellular signalling activation (Rahman et al., 2008). These findings have been replicated *in vivo* in rodent models of bone loss and periodontitis where LCn3PUFA supplementation is associated with suppressed expression of inflammatory cytokines, NF- $\kappa$ B activation and osteoclast formation and activation, resulting in less bone resorption (Sun et al., 2003). Furthermore, in *P. gingivalis*-infected rats fed with either fish oil or corn oil for 22 weeks, significantly less alveolar bone resorption was seen in the fish oil group compared with those on a corn oil diet (Kesavalu et al., 2007). A study by Bendyk et al. (2009) showed a positive effect of DHA-rich tuna oil (26% DHA, 6% EPA) in an animal model of periodontitis. After 8 weeks of supplementation, LCn3PUFA levels were substantially higher in oral soft tissues of mice fed tuna oil compared to those fed sunola oil (control); EPA increased from  $1.9 \pm 1.1$  to  $18.0 \pm 2.6$  mg/100g and DHA from  $335 \pm 41$  to  $579 \pm 72$  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 a combined inoculum of *P. gingivalis* and *F. nucleatum* than those fed sunola oil.

A more recent clinical human study investigated the impact of fortified milk containing EPA and DHA in 72 hyperlipidemic patients. After 1 year of replacing regular milk with fortified milk rich in omega-3, there was a significant increase in plasma level of bone formation biomarkers; EPA, DHA, OPG, RANKL (Martin-Bautista et al., 2010). This can be interpreted as intake of omega-3 increases bone formation markers, hence less bone resorption. A pilot study by Kruger et al. (1998) showed that senile osteoporotic women taking supplements of gamma-linolenic acid and EPA had increased bone density over a long period of time. This study suggests that EPA has a beneficial effect on bone metabolism and EPA is safe to be administered for prolonged period of time.

### **1.7.3 Antimicrobial Actions of LCn3PUFA**

Although oral inflammation is primarily triggered by oral bacteria and fungi, little is known about the effect of LCn3PUFA on the survival and growth of these microorganisms. Recently in an *in vitro* study, Huang and Ebersole (2010) were the first to report that, besides their anti-inflammatory effects, LCn3PUFA could have a positive therapeutic effect on oral health via their antibacterial activities. They found that both EPA and DHA exhibited strong antibacterial activity against various oral pathogens, including *S. mutans*, *C. albicans*, and *P. gingivalis* (at 50% inhibitory concentration from 1 to 10µg/ml).

## **1.8 Studies Supporting Fish Oil and Periodontitis**

Whilst the studies conducted in humans to date are limited, there is a growing body of evidence to support the use of fish oil for oral health. Japanese people are known to have a high dietary intake of long-chain omega-3 PUFAs from the high amounts of seafood in their diet (Black et al., 1979). In a cross-sectional study of over 350 adults in Japan, Hamazaki et al. (2006) found a strong positive relationship between the EPA content of erythrocytes and the number of remaining teeth.

Two studies looked at the levels of DHA and EPA and their direct correlation to periodontitis. A longitudinal study by Iwasaki et al. (2010) with fifty-five elderly

participants in community-dwelling showed an inverse, independent relation of dietary DHA intake to the progression of periodontal disease in older people. Subjects involved in this study were not provided with dietary supplements of PUFA, instead the level of PUFA dietary intake was calculated based on the Standard Food Composition Tables in Japan (Iwasaki et al., 2010). Similar supportive cross-sectional data has been reported from the National Health and Nutrition Examination study in the US in which higher dietary intakes of DHA and, to a lesser degree, EPA were associated with lower prevalence of periodontitis in over 9000 adults (Naqvi et al., 2010).

There are several studies looking at the effect of omega-3 supplements in clinical measures of periodontal disease. Some studies showed positive effect whereas some studies failed to show positive effect of omega-3 supplements. An initial short-term pilot study reported by Campan et al. (1997) evaluated the effect of taking 1.8g LCn3PUFA per day for 8 days on clinical measures in healthy volunteers subjected to experimental gingivitis, but they were unable to conclude a benefit. This is not surprising, given the short timeframe for the intervention. Rosenstein et al. (2003) conducted a small pilot study for a longer duration of 12 weeks in thirty patients with periodontitis who were randomised to consume either 3g EPA/day, borage oil combination or placebo (corn oil). Improvements in probing depth were observed with EPA but they did not reach statistical significance. This may be due to the short duration, limited power with small sample number and possibly due to insufficient compliance with supplementation.

Some studies investigated the effect of omega-3 supplementation along with low dose aspirin in treatment of chronic periodontitis (El-Sharkawy et al., 2010, Elkhoul, 2011, Naqvi et al., 2014). El-Sharkawy et al. (2010) examined the effect of supplementation of the diet with fish oil (900mg) and low dose aspirin (81mg) in patients with chronic periodontitis. Forty control patients were treated with scale and root planing (SRP) and another forty test group patients received SRP followed by supplements of fish oil and aspirin. After 3 and 6 months, significant improvements were observed in clinical parameters including reductions in pocket depth and clinical attachment gain. In addition, there were reductions in levels of specific salivary markers for bone resorption, RANKL, and inflammation, matrix metalloproteinase-8 (MMP-8).

Use of omega-3 supplementation as an adjunctive treatment to regenerative therapy of furcation defects also was tested (Elkhoul, 2011). Forty patients with at least a single

grade II furcation defect was enrolled to receive regenerative therapy with decalcified freeze dried bone allograft (DFDBA) with either omega-3 + aspirin or placebo treatment. Clinical parameters at baseline, at 3 months and 6 months following therapy were measured. IL-1 $\beta$  and IL-10 levels in GCF samples were analysed. They reported that at 6 months post treatment, there was a greater mean PPD reduction and gain in CAL in the test group compared with the placebo group. In addition, a significant modulatory effect on the levels of IL-1 $\beta$  and IL-10 compared with placebo was observed.

Whilst these studies demonstrated a benefit in combined therapy of fish oil and aspirin, the results should be carefully interpreted as there was no assessment of compliance with fish oil through measuring erythrocyte incorporation so are not able to attribute this benefit to a specific fatty acid. Moreover these studies were not randomised controlled trials, therefore did not have an aspirin only or fish oil only arm and so it is not clear if aspirin is needed for efficacy. Importantly there has been no adequately powered study to date that has investigated the potential benefit of fish oil as adjunct therapy for periodontitis using a sufficiently high dose of fish oil and a biomarker of compliance.

The most recent double blinded placebo controlled parallel trial by Naqvi et al. (2014) investigated the effect of omega-3 supplements with low dose aspirin without any periodontal treatment. Fifty five adults with moderate chronic periodontitis were randomised to receive either DHA or corn oil capsules for 3 months. All participants received 81mg of aspirin but no other treatment. They concluded that aspirin triggered DHA supplementation significantly improved periodontal outcomes in people with moderate periodontitis.

In contrast, there have also been studies that do not support the use of fish oil in treatment of periodontal disease. Figueredo et al. (2013) showed that serum levels of both omega-3 and omega-6 LC-PUFAs were higher in twenty one patients with untreated generalised chronic periodontitis in comparison to sixteen gingivitis patients. Moreover, significant correlations between mean pocket depth and serum concentrations of both LC-PUFAs were found. Based on this study, Martinez et al. (2014) investigated the impact of periodontal treatment on LC-PUFA serum levels and the effect of dietary omega-3 supplementation on clinical outcome. The test group of 10 patients with generalised chronic periodontitis was treated with scale and root planing (SRP) associated with omega-3 supplementation containing 3g of EPA and DHA per day for 4 months, whereas the

control group of 11 chronic periodontitis patients received SRP plus placebo. The level of docosapentaenoic acid (DPA), EPA, DHA and AA were detected using gas chromatography. In the placebo group, all LC-PUFA levels were reduced significantly whereas in the test group only DPA and AA, which were not present in the supplementation, showed a significant reduction. In addition, in the test group a significant decrease in the ratio of AA/EPA and AA/DHA was observed. In terms of the clinical outcome of treatment, omega-3 supplementation had no effect. Hence this study does not support the notion that adjunctive omega-3 supplementation to non-surgical periodontal therapy is of benefit.

## **1.9 Why Gingival Crevicular Fluid?**

As mentioned earlier, the formation of a bacterial biofilm is necessary but not sufficient to result in periodontitis. The host response is a critical determinant in the development and progression of periodontal disease. One way of measuring the host inflammatory response is measuring the level of biochemical indicators.

Gingival crevicular fluid (GCF) is a serum transudate or more commonly as an inflammatory exudate, which can be collected from the gingival sulcus surrounding the teeth (McCulloch, 1994). GCF contains substances from the host as well as microorganisms from both supragingival and subgingival plaque. The collection of GCF using pre-cut methylcellulose filter paper strips placed in the gingival sulcus is a minimally invasive therefore GCF has been used to measure risk and progression of periodontal disease. The filter strip method of fluid collection can, however, be time consuming and technique sensitive (Lamster and Ahlo, 2007). The sample should be relatively free of plaque, and ideally there should be no contamination by saliva and blood. It has been shown that both plaque and saliva on the test strip can influence the volume of fluid that is collected (Griffiths et al., 1992). Also the strip must remain in the sulcus long enough to obtain an adequate sample of fluid. The objective biochemical assessments by analysis of GCF constituents can provide a critical insight into the cellular response in the periodontium that are transpiring at a site level (Champagne et al., 2003). Among the important host-derived constituents in GCF are markers of inflammation, including enzymes, cytokines, and interleukins. Further, products of tissue breakdown can also be detected in crevicular fluid. The level of these inflammatory mediators may be especially

beneficial for diagnosing current periodontal disease (Sibraa et al., 1991, Kurtis et al., 2005, Sakai et al., 2006, Tuter et al., 2007, Fitzsimmons et al., 2009).

Previous studies have shown that GCF inflammatory mediator levels are highly correlated with the overall individual's systemic inflammatory response (Fitzsimmons et al., 2009, Lambert et al., 2013). The volume of GCF present at a given site may be directly related to tissue inflammation (including vascular permeability and the nature of the inflammatory infiltrate) as well as permeability and ulceration of the crevicular epithelium. Sites characterised as being moderately or severely inflamed demonstrate a greater volume of GCF than less inflamed sites (Ozkavaf et al., 2000). Nevertheless, no studies have yet demonstrated that an increase volume of fluid in the crevice is related to the risk for periodontal tissue destruction.

### **1.10 GCF in Different Stages of Gingival Inflammation**

In the healthy periodontium, microbial plaque is composed mainly of Gram-positive microorganisms. In this situation, the GCF represents a serum exudate, flowing from the gingival tissues into the gingival crevice. In gingivitis, the composition of the microbial plaque changes with an increased presence of gram-negative microorganisms. Gingivitis is dominated by responses from neutrophils hence neutrophil mediators such as LTB<sub>4</sub>, TXB<sub>2</sub>, elastase and collagenases (MMP-8, 10, 19, 24, 32, 59) are identified in GCF samples from gingivitis sites (Champagne et al., 2003). One of the earliest changes associated with transition from health to gingivitis is the increase in the GCF level of the neutrophil chemoattractant IL-8 (Tonetti et al., 1998). In GCF of gingivitis patients, low levels of monocytic products such as IL-1 or TNF are detected.

As plaque matures and increases in mass, it colonises deeper into the gingival crevice further driving the chronic inflammatory response. Gram-negative anaerobes become more predominant as the periodontitis lesion develops. GCF samples from periodontitis sites exhibit both pro- inflammatory and anti-inflammatory cytokines as well as collagen breakdown products (Ebersole et al., 1993).

The inflammatory response at periodontally diseased sites leads to the production of many inflammatory mediators from monocytes including PGE<sub>2</sub>, TXB, IL-1, IL-6 IL-8, TNF- $\alpha$ , as well as tissue degrading enzymes such as collagenase (Champagne et al., 2003). Therefore

levels of these inflammatory mediators in GCF may represent good markers of disease activity at a site-specific level. Elevated GCF levels of neutrophil markers such as neutrophil elastase, beta-glucuronidase, and LTB<sub>4</sub>, may reflect acute episodes of localised tissue destruction (Champagne et al., 2003).

## **1.11 Cytokines**

As described above, once the inflammatory process is triggered, a variety of pro-inflammatory cytokines such as IL-1, IL-6, TNF- $\alpha$ , IFNs and lipid mediators are produced. It is the imbalance between pro- and anti-inflammatory cytokines that results in tissue damage and destruction of the periodontium.

In severe periodontitis there are significant increases in systemic levels of inflammatory mediators in serum (e.g. C-reactive protein [CRP] and pro-inflammatory cytokines) compared with uninflamed sites (Noack et al., 2001, Slade et al., 2003, Loos, 2005). Periodontal treatment can lead to reduction in the serum levels of inflammatory mediators (Iwamoto et al., 2003). Studies in nonhuman primates have confirmed that even when a bacterial challenge is left intact, periodontal tissue destruction can be reduced markedly by drugs that specifically block the production of pro-inflammatory cytokines (Assuma et al., 1998).

### **1.11.1 Prostaglandin E<sub>2</sub>**

Prostaglandins are biological mediators involved in inflammation and have been implicated as stimulators of bone loss (Offenbacher et al., 1984, Offenbacher et al., 1993, Serhan et al., 2008). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), a by-product of arachidonic acid metabolism, is implicated in the pathogenesis of periodontal diseases and suggested to be a molecular marker for disease as the level is enhanced in gingival crevicular fluid, as well as in inflamed periodontal tissues during active disease (Offenbacher et al., 1981, Offenbacher et al., 1986). It is released from various cell membranes by the action of cyclooxygenase (COX). Two COX isoforms, COX-1 and COX-2, have been described. COX-1 is constitutively expressed in nearly all cells whereas COX-2 expression is low or undetectable in most cells but its expression increases dramatically upon stimulation, particularly in cells of the immune system (Tilley et al., 2001). Some of PGE<sub>2</sub>'s effects include increasing inflammatory cell chemotaxis, induction of collagenase release, vasodilation, and osteoclast activation, all of which can directly or indirectly contribute to

the loss of the supporting tissues of the teeth (Raisz, 2005). PGE<sub>2</sub> has a number of pro-inflammatory effects including inducing fever, increasing vascular permeability and vasodilation, and enhancing pain and oedema caused by other agents (Calder, 2006). Studies have demonstrated that PGE<sub>2</sub> induces COX-2 in cultured fibroblasts, and so upregulates its own production of PGE<sub>2</sub>, and induces production of the inflammatory cytokine IL-6 by macrophages (Bagga et al., 2003).

In a human experimental gingivitis model, GCF PGE<sub>2</sub> levels increased at 4 weeks following the cessation of oral hygiene procedures (Heasman et al., 1993). Many studies report that GCF PGE<sub>2</sub> levels are significantly elevated in patients suffering from severe forms of periodontal disease compared to controls and gingivitis patients (Offenbacher et al., 1981, Heasman et al., 1998, Tsai et al., 1998b, Preshaw et al., 1999). In patients diagnosed with moderate to severe periodontitis, Offenbacher et al. (1986) measured a significant correlation between increased PGE<sub>2</sub> concentration in GCF and clinical attachment loss. Other studies also show that high levels of PGE<sub>2</sub> are associated with disease aggressiveness and constitute a reliable indicator of current clinical periodontal destruction (Offenbacher et al., 1984, Tsai et al., 1998a). Using a ligature induced periodontitis model in rhesus monkeys, the changes in GCF levels of PGE<sub>2</sub>, TNF- $\alpha$  and IL-1 $\beta$  were measured over a period of 6 months (Smith et al., 1993). At 2 months there was a statistically significant 3-fold increase at the ligated sites and a 2-fold increase at the non-ligated sites in GCF PGE<sub>2</sub> when compared to baseline levels. IL-1 $\beta$  increased sharply at 2 months and returned to baseline level by 6 months at both ligated and non-ligated sites. TNF- $\alpha$  in GCF was below the limit of detection at all sites throughout the experiment. In addition, both non-surgical and surgical periodontal treatments decreased GCF PGE<sub>2</sub> levels (Alexander et al., 1996, Leibur et al., 1999, Preshaw et al., 1999). These studies indicate that GCF PGE<sub>2</sub> level is a good indicator of inflammatory activity.

Historically, blocking agents have been used to counteract PGE<sub>2</sub> production (Vardar et al., 2005, Tipton et al., 2007). Most of these agents were found to abolish PGE<sub>2</sub> production through complete blockage of COX (Morton and Dongari-Bagtzoglou, 2001). However, since lipoxins promote resolution actively by retarding the entry of new neutrophils to sites of inflammation and promoting neutrophil apoptosis (Singer et al., 2008), inhibition of COX activity may also prevent the generation of the resolution phase by inhibition of lipoxin production (Kantarci and Van Dyke, 2003).



There is a growing interest in using fish oil supplements to reduce PGE<sub>2</sub> levels. Watkins et al. (2000) reported that increasing the ratio of omega-6/omega-3 PUFA by unfavourable dietary intake of PUFA led to increased AA/EPA ratio in bone, which in turn increased PGE<sub>2</sub> production and decreased bone formation in rats. Two studies looked at osteoclast activity after feeding rats with either corn oil or fish oil (Iwami-Morimoto et al., 1999, Sun et al., 2003). Both studies reported a decrease in osteoclast activity after supplementing with fish oil and therefore decrease in alveolar bone resorption. More recently, Spite et al. (2009) treated periodontal ligament cells with RvD1 and showed reduced production of PGE<sub>2</sub>, which may be of relevance for tissue protection.

### **1.11.2 Tumor Necrosis Factor- $\alpha$ and Interleukin-1 $\beta$**

Two cytokines that have major roles in periodontal disease are interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor-  $\alpha$  (TNF- $\alpha$ ) (Nguyen et al., 1991). Production of TNF- $\alpha$ , and IL-1 $\beta$  by monocytes and macrophages is important in response to infection, but overproduction of these cytokines can have adverse effects resulting in irreversible tissue changes. Both cytokines can stimulate a number of processes, including expression of adhesion molecules by a variety of cells, expression of other mediators that facilitate and amplify the inflammatory response, stimulation of MMP and bone resorption. The activity of these cytokines coincides with the loss of periodontal attachment and bone resorption, both critical to the development and progression of periodontitis (Cochran, 2008). Previous studies have found that when antagonists to TNF- $\alpha$  and IL-1 $\beta$  are introduced into experimental animal models of periodontitis there is a reduction in the appearance of both inflammatory cells and the formation of bone-resorbing osteoclasts at the site of infection (Assuma et al., 1998).

Higher serum levels of TNF- $\alpha$  have been reported in periodontitis patients when compared to non-periodontitis control patients (Ramirez-Tortosa et al., 2010). It has been reported that GCF IL-1 levels are elevated with gingival inflammation (Kinane et al., 1992, Gonzales et al., 2001), in all forms of periodontitis compared to health or gingivitis (Salvi et al., 1997) and with the severity of disease (Masada et al., 1990, Liu et al., 1996, Mogi et al., 1999, Ebersole et al., 2000). Within a group of patients with a similar level of disease, differences are detected in GCF IL-1 levels between groups with different IL-1 gene polymorphisms (Engebretson et al., 1999, Shirodaria et al., 2000). Compared to shallow sites in patients with mild/moderate periodontitis, shallow sites in patients with severe

periodontitis demonstrated increased levels of IL-1 $\beta$  (Engebretson et al., 2002). This suggests that GCF IL-1 $\beta$  expression is genetically influenced and is not solely a result of local clinical parameters.

During periods of disease progression, the release of inflammatory mediators increases at all sites within the patient's mouth, at both active and stable sites (Champagne et al., 2003). Although different sites exhibit different disease progression, the host response is more global, and follows the same pattern at all sites within an individual's mouth. A study by Figueredo et al. (1999) reported that GCF IL-1 $\beta$  levels were increased in samples from periodontitis patients, regardless of the severity of disease at the sample site. These authors suggest that GCF IL-1 $\beta$  is characteristic of the patient and that periodontal disease is a disease of the whole mouth, not just of some sites within the mouth.

A study supplementing normal western diet with 18g of fish oil concentrate per day in 9 volunteers showed that omega-3 fatty acid supplementation reduced the ability of monocytes to produce IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  upon stimulation with endotoxin (Endres et al., 1989). The effect was most pronounced 10 weeks after stopping the supplementation and suggests prolonged incorporation of omega-3 fatty acids into a pool of circulating monocytes. Twenty weeks after ending supplementation, the capacity of the monocytes to synthesise IL-1 $\alpha$ , IL-1 $\beta$  and TNF- $\alpha$  returned to the pre-supplement level.

### **1.11.3 C-Reactive Protein**

C-reactive protein (CRP) is a sensitive marker for low-grade inflammation produced by the liver. CRP has been detected in the serum of periodontitis patients, and levels are significantly higher than those of non-periodontitis subjects (Cairo et al., 2008, Higashi et al., 2008, Paraskevas et al., 2008). Although long-term studies evaluating the effect of periodontal treatment on CRP in relation to healthy controls are scarce, available studies show an effect on CRP levels in favour of periodontal treatment (Paraskevas et al., 2008, Offenbacher et al., 2009). CRP has also been detected in the saliva and GCF of periodontitis patients (Tuter et al., 2007, Fitzsimmons et al., 2009, Fitzsimmons et al., 2010). In a recent single-blind, parallel-arm randomised controlled trial by Bokhari et al. (2012) it was shown that in coronary heart disease patients with periodontitis, non-surgical mechanical periodontal therapy significantly reduced serum levels of CRP. A systematic review by Paraskevas et al. (2008) concluded that there is modest evidence of the effect of

periodontal therapy in lowering the level of CRP however, large-scale and conclusive intervention trials investigating the effect of periodontal therapy on CRP levels are necessary.

There are only limited studies available on the effect of fish oil supplements on serum levels of CRP. In a study using an experimental periodontitis model in rats, omega-3 fatty acid administration did not reduce circulating CRP levels (Vardar-Sengul et al., 2006). However this study had limited treatment periods of 14-28 days once periodontitis was induced. This may not have provided enough time for omega-3 fatty acid to be incorporated into cells to have a positive effect on inflammation.

Several human studies have investigated CRP level in relation to cardiovascular diseases and confirmed the animal study by Vardar-Sengul et al., (2006). Madsen et al. (2003) showed no reduction in serum CRP level when healthy volunteers received a supplement of two different doses of n-3 PUFA (either 6.6g per day or 2.0g per day) for a 12 week period. Mori et al. (2003) carried out a double-blind, placebo controlled trial of 49 non-smoking type II diabetic subjects, randomised to 4g daily of EPA, DHA, or olive oil for 6 weeks. This intervention study demonstrated no significant change in CRP, IL-6 or TNF- $\alpha$  following EPA or DHA supplementation. In addition, Geelen et al. (2004) evaluated the effect of n-3 fatty acids in a placebo-controlled, double blinded study in 84 human subjects aged 50-70 years. Capsules with either fish oil or placebo was administered for 12 weeks and there was no difference in serum CRP concentrations.

## **1.12 Concluding Remarks**

Periodontitis is a major public health problem related to many systemic diseases (Baehni and Tonetti, 2010). There are many studies that show that when periodontitis is controlled there is a reduced incidence and severity of systemic diseases (Janket et al., 2005, Raghavendran et al., 2007, Williams et al., 2008).

To our knowledge, this is the first study to evaluate the clinical outcome as well as the level of inflammatory markers when fish oil supplements alone are used as an adjunct to non-surgical periodontal treatment in chronic periodontitis patients. Our hypothesis is that the level of specific periodontitis-related inflammatory markers, in particular, IL-1 $\beta$ , TNF- $\alpha$  and CRP, would be decreased when fish oil supplements rich in the long chain omega-3

polyunsaturated fatty acids (LCn-3PUFA); eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), are used as adjuncts to non-surgical periodontal debridement.

If this is proven to be true, then fish oil could be a cost effective adjunctive therapy to non-surgical periodontal treatment and could be used in the maintenance phase as well. In a bigger scale, fish oil could be used as a relatively cheap, safe and low risk (e.g. minimal side effects) HMT at the population level, which in turn may have a positive effect in prevalence and severity of many systemic diseases.

### **1.13 Aim**

The aim of this study is to determine whether supplementing the diet with fish oil rich in either of the long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA); eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), during standard periodontal treatment can suppress local and/or systemic biomarkers of inflammation.

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# Chapter 2. Impact of Omega-3 Fatty Acids on Periodontal Inflammation

## 2.1 Introduction

Periodontitis is a chronic inflammatory disease induced by microbial colonization at the gingival margin, and modified by host inflammatory and immune responses (Bartold and Van Dyke, 2013). With accumulation of plaque around the gingival margin, bacteria and their products initiate an inflammatory response by activating a variety of humoral and cellular mediators. If the host response to the initial bacterial challenge is inadequate then the inflammation becomes chronic, resulting in irreversible destruction of the periodontium. Once the inflammatory process is triggered, a variety of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interferons (IFNs) and lipid mediators are produced and play a crucial role in the inflammatory process (Das, 2011). The imbalance between the pro- and anti-inflammatory cytokines ultimately causes tissue damage and destruction of periodontium. Failure to resolve the inflammation, even after the initial cause of inflammation has been removed, leads to a delay in the healing/repair of tissues.

Normally periodontitis is treated with non-surgical and/or surgical debridement in order to reduce the bacterial load, which in turn decreases the inflammation. In addition to debridement, local and/or systemic antibiotics may be used in appropriate cases. Although the presence of bacteria is required, bacteria alone cannot initiate periodontitis. A susceptible host is required for periodontitis to become established and progress. Since the host response plays an important role in the progression of periodontal disease, host modulatory therapy (HMT) is becoming of considerable interest. HMT aims to reduce tissue destruction and stabilise or even regenerate the periodontium without impairing normal defence mechanisms or inflammation through modifying or down-regulating destructive aspects of the host inflammatory response and up-regulating the protective or regenerative responses (Williams, 2008, Bhatavadekar and Williams, 2009).

Due to its anti-inflammatory effects and low incidence of side effects, dietary supplementation with fish oil rich in omega fatty acids is one of the host modulatory therapies for inflammatory diseases that has received attention in the past 15 years (Calder, 2001). There are three main types of omega fatty acids; omega-3, -6 and -9. In Western

diets, omega-6 fatty acids account for the majority of polyunsaturated fatty acids (PUFA) in the food supply. Excessive amounts of omega-6 long chain polyunsaturated fatty acids (LC-PUFAs) and a high omega-6/omega-3 ratio are related to the pathogenesis of many diseases, including cancer (Sonestedt et al., 2008), endometriosis (Khanaki et al., 2012), inflammatory and autoimmune disease (Simopoulos, 2002), as well as cardiovascular disease (Hayakawa et al., 2012). Among the three main types of omega-3 fatty acids, those from fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) possess anti-inflammatory properties and they compete with arachidonic acid (AA) in reducing pro-inflammatory eicosanoids (Calder, 2013). Although a report has demonstrated DHA as being more effective than EPA (Figueredo et al., 2013), the most effective combinations and doses for treating different inflammatory conditions are yet to be determined.

Eicosanoids are signalling molecules that are derived from either omega-3 or omega-6 and exert complex control over inflammation. There are many subgroups of eicosanoids including prostaglandins (PGs), thromboxanes (TXs), lipoxins, leukotrienes (LTs) and hydroxyl-eicosatetraenoic acids (HETEs). The eicosanoids from omega-6 are generally pro-inflammatory whereas eicosanoids from omega-3 are predominantly anti-inflammatory. The amounts and balance of omega-3 and omega-6 in an individual's diet will affect the body's eicosanoid-controlled functions.

In most populations, the consumption of omega-3 LC-PUFAs is either insufficient or not effective at providing adequate tissue levels of EPA and DHA. Consumption of omega-3 polyunsaturated fatty acids, either by fish consumption or through DHA and EPA supplements, has been shown to lower levels of systemic inflammation. An omega-3 rich diet is also shown to decrease the level of pro-inflammatory cytokines in blood (Caughey et al., 1996, Trebble et al., 2003).

Omega-3 fatty acid supplementation may help to improve periodontal treatment outcomes by 1) reducing inflammation (Endres et al., 1989, Meydani et al., 1991, Caughey et al., 1996); 2) limiting bone resorption (Sun et al., 2003, Kesavalu et al., 2007, Kesavalu et al., 2009); and 3) antimicrobial action (Huang and Ebersole, 2010).

Resolution of the acute inflammatory response in chronic disease was once considered to be a passive process (Tabas and Glass, 2013). However, there are reports suggesting that resolution of acute inflammation might be an active, programmed response that is "turned



on” (Serhan et al., 2000, Serhan et al., 2002). The omega-3 fatty acids EPA and DHA have been found to be substrates for the biosynthesis of structurally distinct families of signaling molecules, collectively termed specialised pro-resolving mediators (SPMs) (Serhan et al., 2002, Hong et al., 2003). These include resolvins, protectins and maresins. SPMs actively promote resolution and tissue repair without compromising host defence. EPA produces E series resolvins, DHA produces D series resolvins as well as protectins and maresins are derived from DHA by human macrophages. Alongside EPA and DHA derived SPMs, arachidonic acid derived lipoxins play a beneficial role in resolution of inflammation (Chiang et al., 2005).

The hypothesis for the present study is that the level of periodontitis-related inflammatory markers, in particular, IL-1 $\beta$ , TNF- $\alpha$  and CRP, would be decreased when fish oil supplements rich in the long chain omega-3 polyunsaturated fatty acids (LCn-3PUFA); eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), are used as adjuncts to non-surgical periodontal debridement. If this is proven to be true, then fish oil could be used as a relatively cheap, safe and low risk (e.g. few side effects) host modulatory therapy at the specific population level for those who are susceptible to periodontal diseases, either in treatment or maintenance phase, or at a general population level, which in turn may have a positive effect in the prevalence and severity of many systemic diseases.

## **2.2 Materials and Methods**

Systematically healthy volunteers (n=33) aged between 25-80 with newly diagnosed chronic periodontitis were recruited into a double-blinded, randomised, placebo-controlled, two centred pilot study of 13 months duration to investigate the effect of two types of omega-3 supplements (EPA or DHA) compared with placebo (soybean oil) as an adjunct to conventional non-surgical periodontal therapy in chronic periodontitis.

Ethical approval was obtained from the University of Adelaide Human Research Ethics, University of South Australia, South Australian Dental Service (SADS) and Griffith University in Queensland (QLD) (H-037-2010 for University of Adelaide and GU Ref No: DOH/08/12/HREC for Griffith University). The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12610000594022) in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Prior to enrolling participants, the periodontal examiners participated in a calibration training session. Probing depth and recession depth were measured and clinical attachment levels were calculated.

### **2.2.1 Patient Selection**

Participants for this clinical trial of 13 months duration were recruited from the Colgate Australian Clinical Dental Research Centre, University of Adelaide, the South Australian Dental Service as well as Griffith University during the period of March 2012 to November 2013.

### **2.2.2 Subject Inclusion and Exclusion Criteria**

In order to determine eligibility for the study, participants underwent first stage screening that was completed in a brief phone questionnaire (Appendix 1). Lifestyle habits, together with medication and dietary supplement usage, was collected. This was followed by a second stage screening, where the community periodontal index of treatment needs (CPITN) measurement was carried out in order to determine the presence of periodontal disease before baseline full periodontal examination was carried out.

Prior to participation in the study, participants received a written statement of the nature of the study containing a full explanation of the methodology relating to blood, plaque and gingival crevicular fluid (GCF) sampling as well as the potential risks and benefits of the study interventions (Appendix 2). Written informed consent was obtained from all participants by a non-clinical member of the research team before any treatment, and participants were given the opportunity to withdraw from the study at any time.

Participants were included if they were systemically healthy and aged 25-80 years and diagnosed with advanced chronic periodontitis in accordance with the definition established by the Centre for Disease Control and Prevention and American Academy of Periodontology (Page and Eke, 2007). Briefly, participants were required to have two or more interproximal sites with attachment loss  $\geq 6$ mm, not on the same tooth, and one or more interproximal sites with probing pocket depth (PPD) of  $\geq 5$ mm, not on the same tooth.

Participants were excluded if they were current smokers or had quit smoking in the past 6 months, pregnant, type II diabetic or experienced other chronic inflammatory conditions such as rheumatoid arthritis, coronary artery disease, psoriasis or asthma. Individuals with aggressive periodontitis were excluded from the study, as this is difficult to control via conservative management alone. Participants were also excluded if they had less than 20 teeth in total, had received therapy for periodontitis in the past 6 months, had been on antibiotic therapy in the prior 3 months or regularly take more than 1g fish oil per day. Inclusion and exclusion criteria are summarised in Table 2.1.

<b>Inclusion criteria</b>	<b>Exclusion criteria</b>
Age between 25-80 years old	Smoker or had quit smoking in past 6 months
Advanced chronic periodontitis with two or more interproximal sites with attachment loss $\geq 6$ mm, not on the same tooth, and one or more interproximal sites with probing depth of $\geq 5$ mm, not on the same tooth	Less than 20 teeth in total
	Diabetic (Type II)
	Chronic inflammatory conditions
	Aggressive periodontitis
	Taking more than 1g of fish oil per day
	Periodontal therapy in past 6 months
	Antibiotics past 3 months

**Table 2.1. Inclusion and exclusion criteria**

### **2.2.3 DHA and EPA Supplements**

The supplements used for the study were supplied by Novsel Australia (Mudgeeraba, QLD, Australia). Placebo capsules contained 500mg soya bean oil and 2.2 $\mu$ g betacarotene, DHA rich capsules contained 258mg DHA, 66mg EPA and 2.2 $\mu$ g betacarotene and EPA rich capsules contained 27mg DHA, 277mg EPA and 2.2 $\mu$ g betacarotene. Supplement containers were coded and labelled by an independent third party and capsules were opaque and flavoured to ensure that all researchers and participants were blinded to active and control treatments.

Subjects were randomised into three groups (placebo, EPA or DHA). Consecutively treated participants received consecutive numbers and capsules had all been previously randomised into blocks of 10 by an independent third party. A master file held the randomisation code and no-one directly involved in participant contact or data analysis had access to this file. Neither the periodontist nor the participant knew which had been taken to ensure a double-blind approach.

Each subject was randomly assigned to one of the following groups:

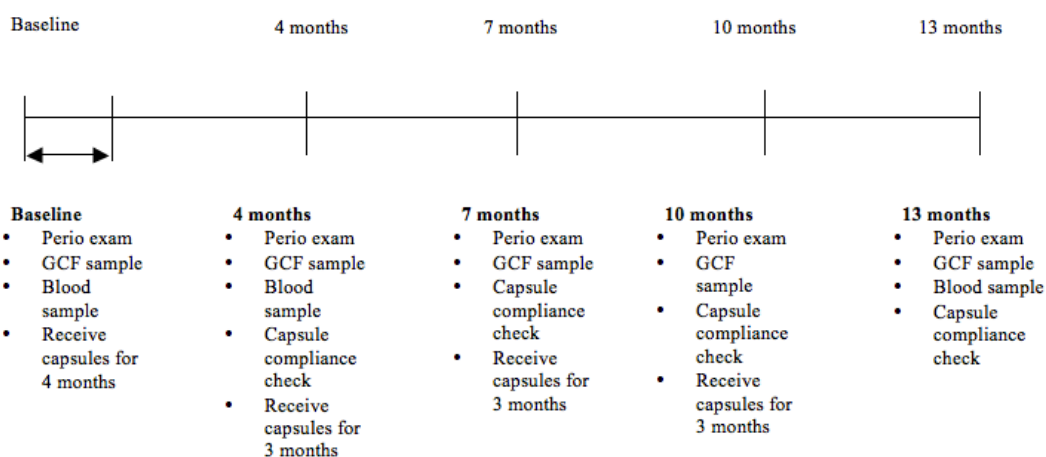
- Fish Oil group – capsules containing either EPA or DHA (providing 3g Long chain omega-3 polyunsaturated fatty acids per day (LCn3PUFA/d))
- Control group – Placebo capsules containing Soybean oil

Capsules were issued at the first non-surgical debridement appointment and participants were instructed to take 6 x 500mg capsules a day at a convenient time to be started on the same day as their first hygiene appointment. Participants were given a 4-5 month supply at one time over the 13 months to ensure regular follow-up and contact.

#### **2.2.4 Time Period**

The baseline visit comprised of a comprehensive periodontal examination as well as collection of GCF and plaque samples. A standard course of non-surgical mechanical debridement of plaque and calculus once a week was carried out for 4 consecutive weeks where possible (Figure 2.1). Participants were recalled every 3 months for further periodontal examination, maintenance clean as well as GCF and plaque sample collection.

For the purpose of this pilot study, only baseline and 4 month (3 months after completion of initial non-surgical periodontal debridement) data were reported.



**Figure 2.1. Timeline of the study.**

### 2.2.5 Non-Surgical Periodontal Therapy

In South Australia, two postgraduate students participating in a three-year doctorate course in periodontology were the clinical operators. They were calibrated at the beginning of the study period. The Intra-class correlation coefficients were between 0.66 and 0.68, which are pretty low. In the SA centre, each patient was treated by the same operator throughout the study time period. Calibration information for operators in QLD centre was not available.

The periodontal condition for all participants was assessed and recorded at the initial examination (baseline). All patients received a course of non-surgical, conservative periodontal treatment under local anaesthetic (at operator's discretion consisting of xylocaine 2% with adrenaline 1:80,000 or articaine hydrochloride 4% with adrenaline 1:100,000) usually completed in four weekly visits. At each appointment oral hygiene instruction was reinforced.

After the completion of non-surgical periodontal therapy re-evaluation appointments were carried out at 3 months post completion of initial phase (4 months in timeline). The participants were then enrolled into further 3 monthly supportive periodontal therapy where full mouth charting and sample collection were performed.

### 2.2.6 GCF and Plaque Sample Collection and Storage

At each appointment a medical history update including changes in any medication and a full mouth periodontal examination were performed. GCF and plaque samples were taken

from the selected sites (two worst sites, in terms of PPD, not neighbouring teeth, identified at baseline) at each of the 5 visits. Participants were instructed not to brush or floss their teeth and not to eat or drink anything except plain water within a period of 4 hours prior to crevicular fluid sampling.

GCF was sampled by the method of Offenbacher et al. (1986) with slight modification (Uematsu et al., 1996). GCF samples were taken after isolating the site with cotton rolls and air drying the site in order to prevent salivary contamination. No plaque was removed to reduce the risk of bleeding before GCF sampling. GCF was collected using PerioPaper™ strips (Oraflow, Plainview, NY, USA), with one strip being used for each collection site. The paper strips were carefully inserted into the gingival crevice, avoiding trauma to gingival tissues and allowed to remain in place for approximately 20 seconds. The volume of GCF collected was measured with a Periotron 8000™ (Oraflow, Plainview, NY, USA). Periotron was calibrated with a clean PerioPaper™ strips at the beginning of each appointment. The paper strips were then wrapped in foil, placed in a sterile 1ml micro-centrifuge tube, and stored at -80°C for later processing and analysis of inflammatory mediator content (Appendix 3).

Subgingival plaque samples were taken from the same sites where GCF samples were collected. Supragingival plaque was carefully removed before subgingival plaque samples were collected. Samples were stored in 200µl of Tris-EDTA (TE) buffer + 200µl of NaOH dissolved in sterile water at -80°C.

### **2.2.7 Blood Sample Collection**

Venous blood samples were collected at baseline, 4 and 13 month time points. Blood samples were collected by venipuncture from the medial cubital vein by a qualified phlebotomist into 10ml tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged to separate plasma. This was removed and stored at -80°C until further analysis.

These samples were used to analyse cytokine levels as well as checking participant's compliance in taking the prescribed capsules. This was determined by measuring the mean levels of LCn3PUFA in erythrocyte membranes in each sample. If there was no significant

difference in mean levels of LCn3PUFA between baseline and 4 months then patient was considered as non-compliant.

### **2.2.8 Assessments of Fatty Acid Profiles**

Levels of LC n-3 PUFA in erythrocytes were analysed following methods adapted from those previously established (Milte et al., 2008, Milte et al., 2011). After blood sample collection in EDTA tubes, erythrocytes were isolated within 2 hours by centrifugation, washed in isotonic saline and stored at -80°C. On the day of extraction, erythrocytes were thawed and lipids extracted with 2:1 chloroform and isopropanol. The resultant pellet was gently resuspended in the buffer and the fatty acids were extracted and assayed by flame-ionization gas chromatography (model GC-20A; Shimadzu, Kyoto, Japan). Individual fatty acids were identified by comparison with known standards (NuChek Prep Inc., Elysian, MN, USA) and reported as a percentage of total fatty acids.

### **2.2.9 Analysis of Inflammatory Mediators**

GCF was eluted by placing paper strips into individual wells of a sterile 96-well microtitre plate (Flow Laboratories, McLean, VA, USA) and adding 240µl of sterile phosphate buffered saline (PBS, pH 7.2) to each well. The plate was then sealed with a foil that was provided in the kit and agitated for 30 minutes at room temperature. The eluted samples were then transferred to 1.5ml microcentrifuge tubes containing a further 240µl of sterile PBS and stored at -80°C until further analysis.

#### *2.2.9.1 C-Reactive Protein*

Human CRP in GCF and plasma was measured using a DuoSet ELISA Development kit (R&D Systems, Minneapolis, USA) in accordance with the manufacturers' protocol. Briefly, microtitre plates were coated with a capture antibody and incubated overnight at room temperature. The following day, wells were washed and blocked with reagent diluent (1% Bovine Serum Albumin) for 1 hour at room temperature. Wells were once again washed before addition of each sample, standard or control (1xPBS for GCF or reagent diluent for plasma samples) and incubated for 2 hours at room temperature. Wells were washed before detection antibody was added to each well and incubated for 2 hours at room temperature. After the wells were washed, Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature avoiding direct light. The wash step was

repeated and substrate solution was added to each well and incubated for 20 minutes at room temperature avoiding the direct light. Finally, a stop solution was added to each well and the optical density was read at 450nm for each well using a Powerwave microplate reader (BioTek Instruments, Winooski, VT, USA). Standard curves were then generated using KC4 Software (BioTek Instruments) and used to determine the CRP concentration in each sample. All samples, standards and controls were assayed in duplicate.

The total amount (pg) of CRP present in GCF samples was determined by multiplying the concentration of CRP (pg/ml) by the final volume that the GCF was eluted in millilitres (0.48ml). The concentration of CRP per  $\mu\text{L}$  of GCF was determined by dividing the total amount of CRP (pg) by the volume of GCF collected ( $\mu\text{L}$ ) as determined using the Periotron 8000<sup>TM</sup> (Oraflow, Plainview, NY, USA).

#### *2.2.9.2 Tumor Necrosis Factor- $\alpha$ and Interleukin-1 $\beta$*

The concentrations of TNF- $\alpha$  and IL-1 $\beta$  in GCF were determined using Magnetic Luminex Performance Assay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. Briefly, each sample, standard or control was added in duplicate to the wells of a microplate containing a microparticle cocktail. The plate was sealed with foil and incubated for 3 hours at room temperature on a horizontal orbital microplate shaker set at  $800 \pm 50$  rpm. A magnet was applied to the bottom of the microplate using a magnetic device designed to accommodate a microplate. The liquid was then removed and each well was washed with wash buffer and the liquid removed again. This step was repeated three times. Diluted Biotin Antibody Cocktail was added to each well and the microplate was sealed with foil and incubated for 1 hour at room temperature on the shaker set at  $800 \pm 50$  rpm. The plate was washed as previously described before addition of Streptavidin-PE and sealed with foil then incubated for 30 minutes at room temperature on the shaker. The plate was washed and microparticles were resuspended by adding wash buffer to each well. The microplate was incubated for 2 minutes at room temperature on the shaker before analysis with a Luminex 200<sup>TM</sup> System (Luminex Corporation, Austin, TX, USA). A standard curve was generated using a five parameter logistic curve for each mediator using xPONENT version 3.1 software (Luminex Corporation). The mean minimum detectable dose (limit of detection) for IL-1 $\beta$  was 0.08pg/ml and TNF- $\alpha$  was 0.29pg/ml.



### **2.2.10 Statistical Analysis**

This is a “proof of principle” pilot study therefore the aim was to recruit a minimum of 30 subjects (15 subjects in each treatment arm).

Subject demographic data including age, height, weight and BMI are presented as mean  $\pm$  standard deviation and Fisher’s exact test was used to determine if there were any statistically significant differences between groups. The distribution of demographic data followed the normal distribution (the bell shape distribution).

The fatty acid levels between groups at both baseline and 4 months were tested by Fishers Exact test. Linear regression models were used to analyse the association of the fatty acids at 4 months with treatment group, controlling for fatty acid levels at baseline. Diagnostics were performed on these regression models and assumptions of a linear regression model were upheld.

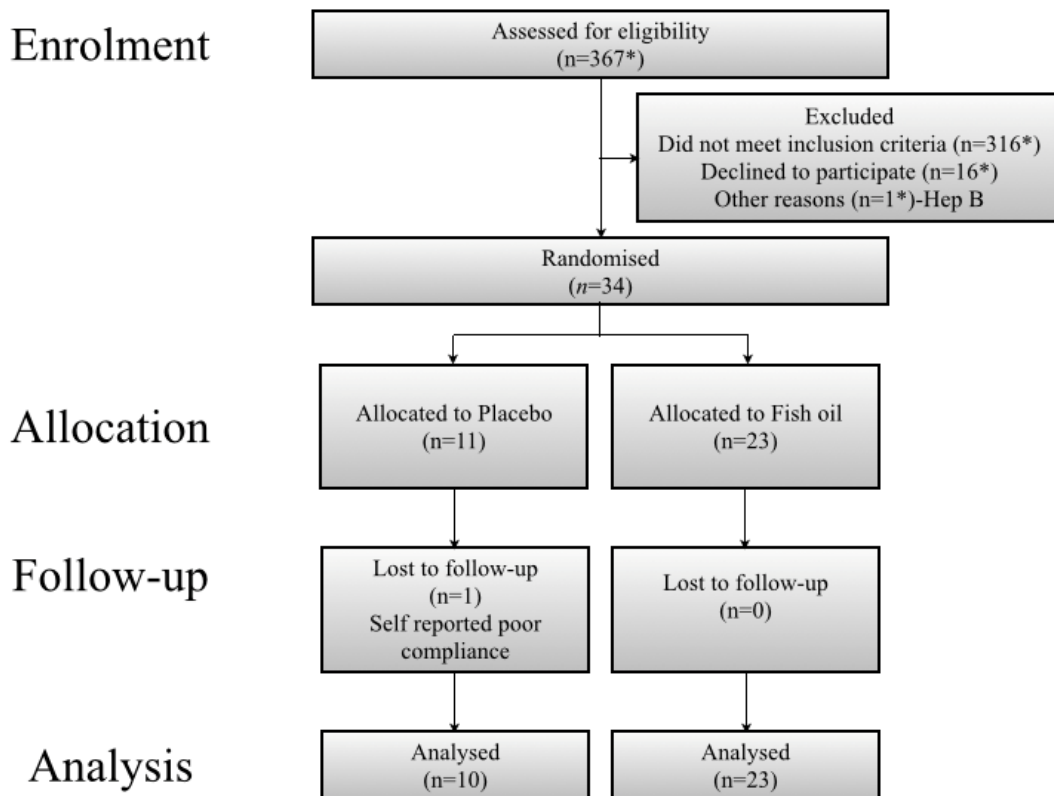
Linear Generalised Estimating Equations (GEE) were used to assess the association of each variable (measurement of cytokine, GCF volume, PPD) at 4 months with treatment group, controlling for each variable at baseline and accounting for clustering on site. Diagnostics were performed on these regression models and assumptions of a linear regression model were only upheld when a logarithmic form of IL1B and CRP plasma (CRPP) outcomes were used. Coefficients were then back transformed (exponentiated) to give desired estimates.

All statistical analyses were performed using the software program SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) and IBM SPSS Statistics ver. 19.0 (IBM Co., Armonk, NY, USA). A p value of less than 0.05 was considered significant.

## **2.3 Results**

Among the 367 people screened for eligibility, 333 people were excluded due to not meeting the study inclusion criteria, declining to participate and Hepatitis B status (Figure 2.2). A total of 34 participants were randomised to either placebo (n=11) or fish oil (n=23) group. The study retention rates were 100% for fish oil group and 97% for placebo group, leaving 33 participants who completed the primary outcome assessment. Only one participant in the placebo group was excluded for analysis because participant reported

poor compliance during the study. At completion of the 4 months follow up, 10 participants in the placebo group and 23 participants in the fish oil group were included for analysis. The second study centre (QLD) did not keep a record of the number of participants screened/excluded and therefore the number included here for screened/excluded was only from one centre (SA).



\* QLD did not keep a record of n screened/excluded/declined to participate

**Figure 2.2. Flow chart of the study design.**

The demographic data indicated that both groups were similar regarding sex, age, height, weight and BMI (Table 2.2). There were higher numbers of female participants who completed the study and the fish oil group was slightly older, with greater weight and BMI but none of these differences were statistically significant. There were no reports of any side effects following ingestion of the capsules.

Characteristics	Placebo N=10	Fish oil N=23	Total N=33	Difference
Gender (Male:Female)	3:7	7:16	10:23	NS* (p=1.00)
Mean Age (years) Range	45 ± 10 (33-58)	55 ± 9 (35-75)	52 ± 10 (33-75)	NS (p=0.38)
Height (cm) Range	167 ± 9 (160-185)	166 ± 9 (152-182)	166 ± 8 (152-185)	NS (p=0.98)
Weight at baseline (kg) Range	68.5 ± 13.2 (49-90.6)	78.3 ± 21.8 (59-159.9)	75.3 ± 19.9 (49-159.9)	NS (p=0.41)
BMI at baseline (kg/m2) Range	24.4 ± 4.0 (17-30)	28.4 ± 6.7 (19.6-48.4)	27.2 ± 6.2 (17-48.4)	NS (p=0.15)
Weight at 4 months (kg) Range	68.1 ± 13.4 (50-88)	74.5 ± 12.7 (59-100)	73.5 ± 13.0 (50-100)	NS (p=0.70)

**Table 2.2. Demographics of subjects recruited into the study.**

Gender is presented as male: female ratio. All other data are represented as mean ±SD. \*Data was analysed by Fisher's Exact test, P<0.05; NS, not significant.

As shown in Table 2.3, there were no statistically significant differences in clinical parameters between the placebo and fish oil groups at baseline. At baseline, the levels of fatty acids measured in plasma samples were similar between the two treatment groups (Table 2.4).

Probing pocket depth				
		Placebo	Fish oil	Difference
Moderate sites (4-6mm PPD)	% of sites	29.06 ± 10.11	26.12 ± 10.84	NS (p=0.50)
	Mean PPD (mm)	4.64 ± 0.15	4.76 ± 0.27	NS (p=0.19)
	Mean CAL (mm)	4.64 ± 0.22	4.76 ± 0.21	NS (p= 0.16)
Deep sites (≥7mm PPD)	% of sites	9.57 ± 11.24	6.17 ± 8.30	NS (p=0.37)
	Mean PPD (mm)	7.56 ± 0.41	7.37 ± 0.38	NS (p=0.26)
	Mean CAL (mm)	7.73 ± 0.24	7.61 ± 0.31	NS (p=0.49)
Full Mouth Bleeding Scores (%)		60.3 ± 24.6	53.4 ± 18.0	NS (p=0.40)

**Table 2.3. Baseline clinical parameters of all study participants.**

All values represented as mean ±SD. Data was analysed by Fisher's Exact test, P<0.05; NS, not significant.

Characteristics	Placebo N=10	Fish oil N=23	Total N=33	Difference
EPA (%)	0.58 ± 0.14	0.80 ± 0.33	0.73 ± 0.30	NS (p=0.71)
DHA (%)	3.84 ± 0.79	3.97 ± 0.98	3.92 ± 0.90	NS (p=0.75)
DPA (%)	2.13 ± 0.22	2.23 ± 0.23	2.20 ± 0.23	NS (p=0.26)
Omega-3 Index (%)	4.43 ± 0.83	4.77 ± 1.18	4.66 ± 1.08	NS (p=0.45)
Total LCn3PUFAs (%)	6.55 ± 0.82	7.00 ± 1.23	6.86 ± 1.12	NS (p=0.33)

**Table 2.4. Baseline fatty acid profile as measured in plasma.**

All fatty acids are presented as the percentage of total fatty acid in erythrocytes membranes. All data are represented as mean ± SD. Data was analysed by Fisher's Exact test,  $P < 0.05$ ; NS, not significant.

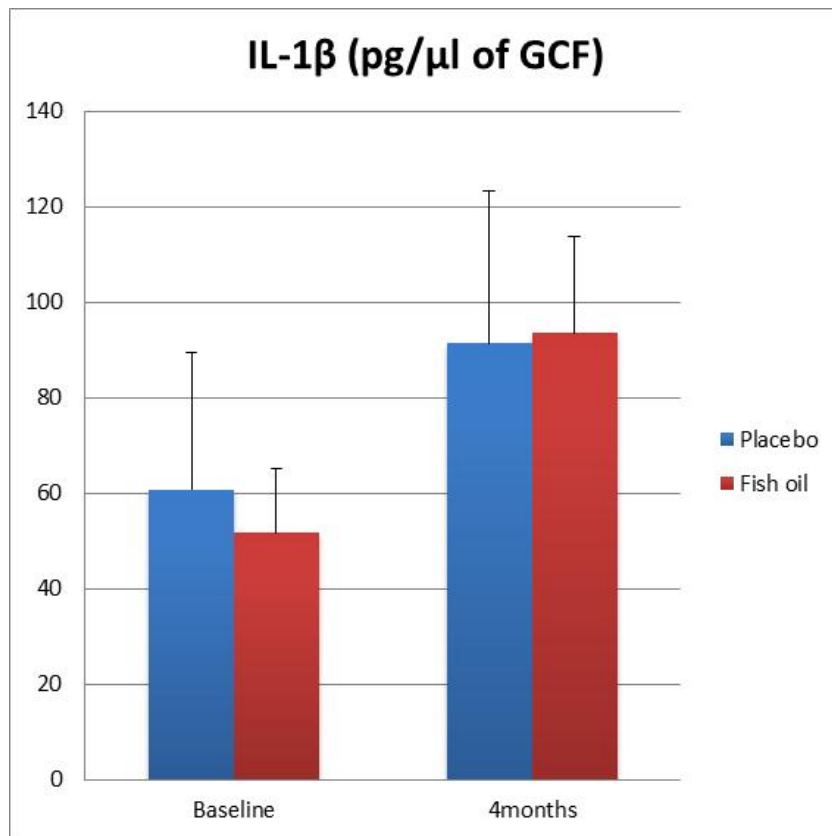
As Table 2.5 shows, there was a statistically significant increase in erythrocyte membrane levels of EPA, DHA, DPA (almost significant), total long chain omega-3 fatty acid and omega-3 index from baseline to 4 months in the fish oil group only. At the end of 4 months there were significant differences between the placebo and fish oil groups in all fatty acid plasma levels ( $p < 0.05$ ).

Group	Fatty acids	At baseline	At 4 months	P value *
Placebo	EPA (%)	0.58 ± 0.14	0.63 ± 0.23	0.63
	DHA (%)	3.84 ± 0.79	3.98 ± 0.75	0.71
	DPA (%)	2.13 ± 0.22	2.02 ± 0.32	0.43
	TLCn3P (%)	6.55 ± 0.82	6.63 ± 0.95	0.86
	N3 index (%)	4.43 ± 0.83	4.60 ± 0.88	0.66
Fish oil	EPA (%)	0.80 ± 0.33	2.32 ± 0.81	<0.0001
	DHA (%)	3.96 ± 0.98	5.97 ± 1.62	<0.0001
	DPA (%)	2.23 ± 0.23	2.66 ± 0.86	0.06
	TLCn3P (%)	7.0 ± 1.23	11.0 ± 1.12	<0.0001
	N3 index (%)	4.77 ± 1.18	8.30 ± 1.34	<0.0001

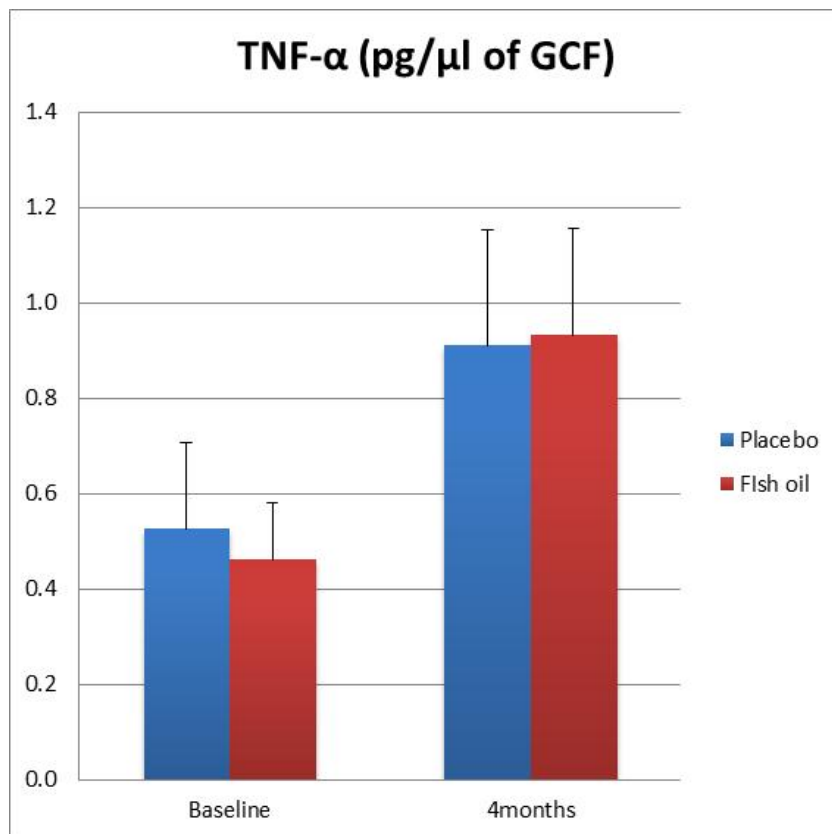
**Table 2.5. Fatty acid levels at baseline and 4 months for both groups presented in % of total fatty acids in the erythrocyte membranes.**

All values represent mean ±SD. Data was analysed by Fisher's Exact test,  $P < 0.05$ ; \*Significantly different between measurements at baseline and 4 months.

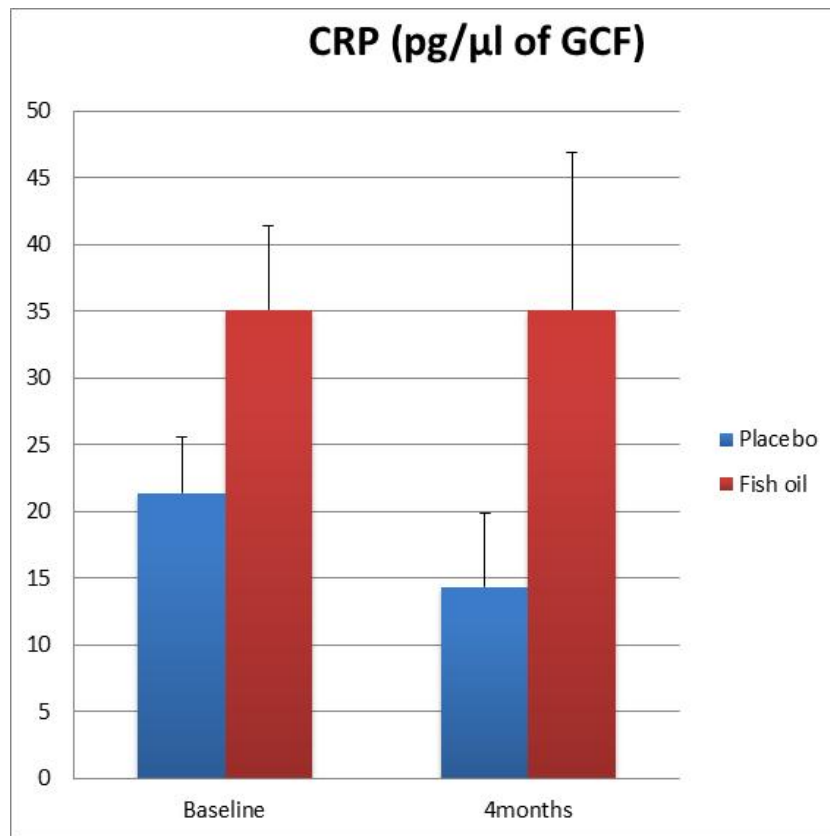
The mean levels of cytokines at baseline and 4 months are presented in Figures 2.3-2.6. The concentration of IL-1 $\beta$  in GCF was increased in both the placebo and fish oil groups at 4 months compared to baseline levels. Levels of IL-1 $\beta$  increased from 60.66 to 91.36 pg/ $\mu$ l of GCF in the placebo group whereas in the fish oil group levels increased from 51.45pg/ $\mu$ l to 93.32pg/ $\mu$ l (Figure 2.3). TNF- $\alpha$  in GCF was also increased from 0.53pg/ $\mu$ l to 0.91pg/ $\mu$ l and 0.46 to 0.93pg/ $\mu$ l for the placebo and fish oil groups respectively (Figure 2.4). However, these were not statistically significant either within the group or between groups ( $p > 0.05$ ). CRP in GCF decreased from 21.34pg/ $\mu$ l to 14.38pg/ $\mu$ l in the placebo group while in the fish oil group CRP GCF remained the same (Figure 2.5). Plasma concentration of CRP also decreased from 2.6 $\mu$ g/ml to 1.34 $\mu$ g/ml in the placebo group whereas in fish oil group CRP plasma level was increased from 1.58 $\mu$ g/ml to 2 $\mu$ g/ml (Figure 2.6). Once again, these differences were not statistically significant ( $p > 0.05$ ).



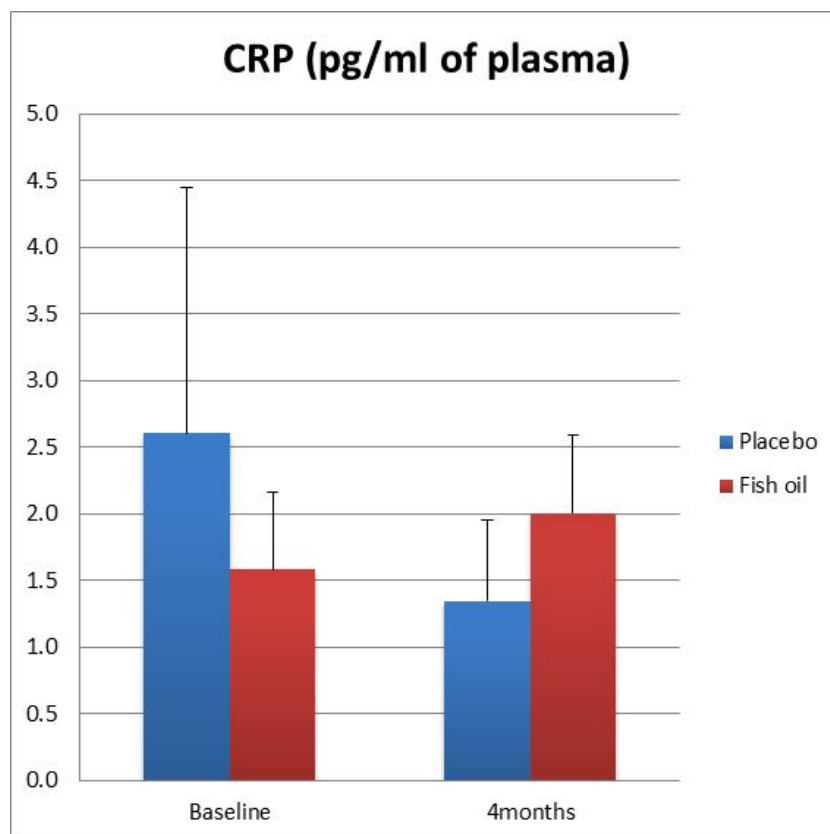
**Figure 2.3. Concentration of IL-1 $\beta$  in GCF samples.**  
 Bars represent the mean  $\pm$  SEM. ( $p > 0.05$ )



**Figure 2.4. Concentration of TNF- $\alpha$  in GCF samples.**  
 Bars represent the mean  $\pm$  SEM. ( $p > 0.05$ )



**Figure 2.5. Concentration of CRP in GCF samples.**  
 Bars represent the mean  $\pm$  SEM. ( $p > 0.05$ )



**Figure 2.6. Concentration of CRP in plasma samples.**  
 Bars represent the mean  $\pm$  SEM. ( $p > 0.05$ )

In the placebo group 90% of the GCF samples had IL-1 $\beta$  levels above the detection level at both baseline and 4 months, whereas for the fish oil group only 30% samples could be detected at both baseline and 4 months. Samples below detection level were not included in analysis. For the majority of samples, TNF- $\alpha$  and CRP in GCF was below the limit of detection in both the placebo and fish oil group at both time points. However for CRP in plasma most of the samples could be detected for both groups at baseline and 4 month (Table 2.6). Due to the limited number of samples with detectable levels of TNF- $\alpha$ , this cytokine was excluded from further statistical analysis.

		Placebo		Fish oil	
		Baseline	4 months	Baseline	4 months
IL-1 $\beta$ GCF	Both sites detected	90%	90%	30%	30%
	Both sites missing	10%	10%	70%	70%
TNF- $\alpha$ GCF	Both sites detected	20%	30%	22%	17%
	Both sites missing	50%	40%	52%	39%
CRP in GCF	Both sites detected	20%	Nil	26%	9%
	Both sites missing	60%	80%	52%	83%
CRP in plasma	Both sites detected	90%	90%	74%	74%
	Both sites missing	10%	10%	26%	26%

**Table 2.6. Percentage of samples with detectable levels of inflammatory markers.**  
Concentration of inflammatory markers in GCF or plasma.

There was no significant association observed between IL-1 $\beta$  concentration ( $p=0.96$ ) or amount ( $p=0.72$ ) and CRP plasma (CRPP) levels ( $p=0.34$ ) at 4 months and treatment group, controlling for baseline levels. There was however, a statistically significant difference for CRP GCF between placebo and fish oil group at 4 months ( $p=0.0027$ ). When comparing the change in CRP GCF with change in CRP plasma, and adjusting for baseline levels of CRP GCF and clustering on site, it was found that only two subjects had a change in CRP GCF value and a change in CRP plasma value, therefore suggesting that there was no significant association between the two variables.



No statistically significant difference in either GCF volume ( $p=0.38$ ) or PPD between placebo and fish oil group at 4 months adjusting for baseline volume and PPD was found ( $p=0.20$ ). In the treatment group, a significant association between changes in volume and changes in PPD, controlling for volume at baseline and accounting for clustering on site was found ( $p=0.02$ ). As for the control group, for every one mm increase in PPD, change in volume decreased by  $0.2\mu\text{l}$  (95% Confidence Interval: -0.4, -0.04). This comparison was statistically significant ( $p=0.02$ ). However, for the fish oil group, there was no significant association between change in PPD and change in volume ( $p=0.85$ ).

The association of cytokines versus change in PPD and treatment group, controlling for cytokine at baseline and accounting for clustering on site showed no statistical significance for IL-1 $\beta$  GCF ( $p=0.97$ ), CRP GCF ( $p=0.42$ ) and CRPP ( $p=0.47$ ).

## **2.4 Discussion**

A double-blinded, randomised, placebo-controlled, two centred pilot study of 13 months duration was carried out to investigate the effect of two types of omega-3 fatty acid dietary supplements (EPA or DHA) compared with placebo (soybean oil) as adjuncts to conventional non-surgical periodontal therapy in chronic periodontitis.

A total of 34 healthy volunteers with chronic periodontitis were randomised into two groups either placebo or fish oil. Initially patients were divided into three groups, placebo, EPA or DHA. However due to the small sample number, DHA and EPA groups were combined into one fish oil group. For the purpose of this preliminary analysis 4 months data was used where 33 out of 34 volunteers were involved in the study.

At baseline, no significant differences in demographic characteristics between placebo and fish oil groups were observed. The fatty acid profile and clinical characteristics were similar between the two treatment groups.

A similar pilot randomised clinical trial with 21 subjects to investigate the effect of DHA and EPA versus placebo as an adjunct to non-surgical periodontal therapy has been reported (Martinez et al., 2014). In this study, compliance was checked by the return of the empty capsule containers and by asking weekly about the intake of supplements. In the present study, the method of checking compliance by counting remaining capsules was

found to be not very reliable, as most patients did not return their bottles. In the present study, blood samples were taken at baseline and 4 months in order to analyse fatty acid levels in erythrocyte membranes to check compliance. Analysis of EPA, DHA, DPA, total long chain omega-3 fatty acid and omega-3 index levels revealed that there was a significant difference in fatty acid levels between placebo and fish oil at 4 months controlling for the levels at baseline ( $p<0.05$ ). Only in the fish oil group, statistically significant increases in fatty acid levels were observed from baseline to 4 months, which confirmed the compliance of participants. However, this change had no significant effect on the levels of inflammatory markers.

It has been reported that intensive non-surgical periodontal treatment under local anaesthetic results in acute short term systemic inflammation (Tonetti et al., 2007). The levels of several inflammatory markers in plasma and serum peaked shortly after treatment, but with a longer follow up period the level was reduced. In the present study, from baseline to 4 months the mean levels of IL-1 $\beta$  and TNF- $\alpha$  increased in both placebo and fish oil groups. However this was not statistically significant. This could be due to acute short-term systemic inflammation as suggested by Tonetti (2007). On the other hand, result of present study is in disagreement with other studies where omega-3 supplementation reduced levels of total IL-1 $\beta$  and TNF- $\alpha$  in plasma (Meydani et al., 1991, Caughey et al., 1996). The duration of each study was similar; however other studies used higher fish oil dosages per day. In the present study, TNF- $\alpha$  levels in the majority of samples were below detection levels for both placebo and fish oil group at both time points; therefore TNF- $\alpha$  was excluded from further statistical analysis. The change in levels of IL-1 $\beta$  was also not significant at 4 months. This could be due to a shorter study period of 4 months. It will be interesting to see if there is any significant change at the end of the 13 months period.

A number of studies, as well as a systematic review have reported reductions in serum CRP levels after conservative periodontal treatment (Paraskevas et al., 2008, Leite et al., 2014). However, the present study did not show any statistically significant change in CRP plasma level after non-surgical periodontal treatment at 4 months. This is in agreement with several studies that showed no effect of omega-3 on serum CRP concentration (Madsen et al., 2003, Mori et al., 2003, Geelen et al., 2004). However, these studies included a different study population to the present study, either healthy volunteers or non-smoking type II diabetics.

CRP plasma levels from baseline to 4 months were increased in the fish oil group, whereas it was decreased in the placebo group. The differences in CRP GCF levels between placebo and fish oil groups at 4 months were statistically significant. Given the anti-inflammatory properties of fish oil, this finding was unexpected, as one would expect the CRP levels to decrease in the fish oil group rather than in the placebo group. It is interesting to note that in the current study, CRP levels in both GCF and plasma decreased in the placebo group only. The possible reason for this is unknown.

Only 2 subjects had a change in CRP GCF value and a change in CRP plasma value therefore the comparison of the changes in CRP GCF with changes in CRP plasma was not possible.

In the majority of participants there was a clinical improvement in terms of PPD after the non-surgical treatment, regardless of the treatment group (data not reported in this thesis). However this observation did not translate to changes in GCF volume. No statistically significant difference in either GCF volume or PPD between placebo and fish oil group at 4 months adjusting for baseline values were observed. Due to increased gingival permeability, GCF volume increases with increased gingival inflammation (Griffiths et al., 1992). Based on this, a decrease in GCF volume with clinical improvement was expected. However, in the present study, the opposite was observed. For every one mm increase in PPD, change in volume decreased by 0.2 $\mu$ l in the placebo group. This comparison was statistically significant. However, for the fish oil group, there was no significant association between change in PPD and change in volume. This could be due to GCF sample techniques or error in measurement of PPD. Another reason could be location of samples in the mouth. Since the two worst sites in terms of PPD at baseline were chosen for sample collection, some samples were taken from anterior teeth and some were taken from posterior teeth. Anterior teeth respond better to periodontal treatment than posterior teeth. This might have had an effect on the clinical outcome PPD, which may have affected the association between PPD and treatment groups.

It has been reported that 2 weeks after periodontal therapy, there is an improvement in clinical parameters (PPD) as well as reduced levels of IL-1 $\beta$  to varying degrees of chronic periodontitis subject (Engebretson et al., 2002). However in the present study there was no statistically significant association between IL-1 $\beta$  or CRP and change in PPD in either

placebo or fish oil groups, controlling for cytokines at baseline and accounting for clustering on site.

Several studies have investigated the role of omega-3 supplements in treatment of periodontal diseases (Campan et al., 1997, Rosenstein et al., 2003, El-Sharkawy et al., 2010, Elkhoul, 2011, Martinez et al., 2014, Naqvi et al., 2014). Some of these studies showed a positive effect of omega-3 supplements and low-dose aspirin in adult chronic periodontitis (El-Sharkawy et al., 2010, Elkhoul, 2011, Naqvi et al., 2014). In contrast, neither a human experimental gingivitis study (Campan et al., 1997) nor a chronic periodontitis study (Martinez et al., 2014) reported any significant effect of omega-3 supplements alone in treatment outcomes.

A study with 80 chronic periodontitis subjects that received SRP followed by dietary EPA+DHA supplementation and 81mg aspirin daily reported significant reductions in pocket depth and significant attachment gain after 3 and 6 months compared to baseline and control group (El-Sharkawy et al., 2010). A more recent study that used DHA supplements with 81mg of aspirin for 3 months without any periodontal treatment reported that there was an increase in DHA in RBC plasma membranes in DHA group but not among the control group (Naqvi et al., 2014). They also reported significant differences in both DHA and control group for GCF hsCRP, IL-1 levels but not in IL-6 or systemic hsCRP levels. Authors concluded that DHA supplementation with low dose of aspirin improved periodontal outcomes in people with periodontitis, indicating its potential therapeutic efficacy.

There are some differences between present study and the above studies. First of all low-dose aspirin was not used in the present study in order to determine the effect of fish oil supplement on its own as an adjunct to non-surgical therapy. Two of above studies included smokers and did not perform any periodontal treatment (Rosenstein et al., 2003, Naqvi et al., 2014). In the present study, smokers were excluded and non-surgical periodontal treatment was carried out before starting the supplements. One study took the GCF samples from the mesio-buccal aspect of first molars in each quadrant (4 samples in total) at baseline and 3 months (Naqvi et al., 2014). However, they did not mention the probing pocket depth of these sites. In the present study the two worst sites on non-neighbouring teeth in terms of probing pocket depth were chosen at baseline and took samples from those sites throughout the study period.

The present study failed to show any additional positive effects of fish oil supplements in the treatment of adult chronic periodontitis. The main difference between the present study and studies that showed a positive effect of omega-3 supplements in addition to periodontal treatment (El-Sharkawy et al., 2010, Elkhoul, 2011) is the low dose of aspirin. When omega-3 supplements are taken with aspirin, aspirin-triggered lipoxin and resolving compounds are produced. These compounds are longer lasting and more potent than endogenous lipoxins and resolvins (Arita et al., 2005). In light of present study's findings, the positive effect of other studies could be due to effect of low-dose aspirin.

The major limitation of the present study is a small sample size. A multi-centred study with a larger sample size would be beneficial to examine the adjunctive effect of omega-3 supplements with or without low-dose aspirin in treating chronic periodontitis. Another limitation of the present study is a short duration of 4 months. The study is ongoing and 13 months data will be available for further analysis in the near future.

Strengths of the current study include strict exclusion criteria (excluding smokers), effective double blinding, randomised controlled design and verifying compliance with plasma samples. Calibration of clinicians involved in the study was carried out at the beginning of the study and for each participant a full periodontal assessment and reinforcement of oral hygiene were conducted at each recall appointment.

In summary, preliminary analysis of the current study showed no benefit of fish oil (EPA and DHA) supplements as an adjunct to non-surgical periodontal treatment (after 4 months) in terms of changes in PPD, CAL and inflammatory markers.

## **2.5 Conclusion**

This pilot study of 4 months duration rejected the null hypothesis of a positive effect for omega-3 supplements (DHA and EPA) as adjuncts to conventional non-surgical periodontal therapy in the treatment of chronic periodontitis. It may be that omega-3 supplementation in combination with low dose of aspirin is required for control of inflammation associated with periodontitis. If this is proven to be effective with further research, then this could be a safe, cheap and low risk protocol for patients in maintenance phase.

Omega-3 supplements could be safely used as a relatively cheap and low risk host modulation therapy at the population level, which in turn may have positive effects in the prevalence and severity of many systemic diseases. Therefore further research with a larger sample number and longer duration is warranted.

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# Appendix 1. Periodontitis and Fish Oil Telephone Screening Questionnaire

Thank you for your interest in the fish oil and dental health study we are conducting.

Hello my name is Dr Alison Coates from UniSA and I am one of the investigators on this project. The study we are conducting is investigating the effects of fish oil as a part of the treatment for people with periodontitis.

I have a few questions to ask to check your eligibility and from there I can explain the study in more detail.

Firstly can I please confirm?

1. Are you aged between 25 & 80 years of age Yes  No
2. Are you a non-smoker Yes  No
3. If you were a smoker previously did you give up at least 6 months ago Yes  No

**If you have answered NO to any of the above questions unfortunately this excludes you from being able to be a participant in this trial. We thank you for your time in completing this questionnaire and for your interest in assisting us with our research.**

If you have answered YES to questions 1 2 & 3 please proceed to the questions following:

4. Are you or could you be pregnant? Yes  No
5. Do you consume more than 1 gram of fish oil per day? Yes  No
6. Have you taken antibiotics in the last three months? Yes  No
7. Have you had anticoagulant therapy in the last 3 months? Yes  No
8. Are you unable to comply with study protocol in anyway? Yes  No
9. Are you taking anti-platelet agents or have any bleeding disorders? Yes  No
10. Do you have gastrointestinal disease (including peptic ulcers)? Yes  No
11. Have you participated in any other study within the last 30 days? Yes  No

**If you have answered YES to any of the above questions numbered 4 to 11 unfortunately this excludes you from being a participant in this trial. We thank you for your time in completing this questionnaire and for your interest in assisting us with our research. It is very much appreciated.**

If you have answered **NO** to questions 4 to 11 please proceed to the questions following?

The next set of questions will help me determine whether **you may have periodontitis**.

12. Do you think that you have gum disease? Yes  No
13. Do your gums bleed during regular brushing or eating? Yes  No
14. Have you noticed your teeth looking longer and the gums around the teeth have receded? Yes  No
15. Have you noticed a build-up of tartar/calculus on your teeth? Yes  No
16. Do any of your teeth feel loose or wobbly? Yes  No
17. Have you ever been told that you have gum problems, gum infection or gum inflammation? Yes  No
18. Have you had therapy for periodontics in the last 6 months? Yes  No
19. Have you noticed a bad smell from your mouth (bad breath)? Yes  No

**From your answers you may be eligible and we could organise a screening appointment for you to confirm whether you have periodontitis, but first let me tell you more about the actual study.**

It is a 13 month study and if eligible you would be asked to take 6 x 500mg capsules containing oil every day. You would be allocated to either fish oil or a vegetable oil randomly (by chance) and both you and the study team will be blinded to what you are taking. All capsules would be provided for you.

You would see a Periodontist at the start of the study for a routine set of appointments for the initial treatment (often this is 3-4 appointments approximately one week apart) involving non-surgical removal of plaque with scaling and cleaning.

You would then have follow-up appointments every 4 months up to 13 months. These appointments would be at the Colgate Dental Research Centre at Adelaide University on Frome Rd.

In addition you would have small fasting blood samples collected at the start, after 4 months and after 13 months and be asked to complete a dietary questionnaire. These tests would be conducted at UniSA, directly opposite the Colgate Dental Research Centre at Adelaide University on Frome Rd.

We would provide a \$25 voucher on **each of these three occasions** to compensate you for your time, as they would be additional time to normal periodontal treatment. There is no cost for the periodontal treatment.

**Now that I have explained the overview of the study would you like me to send out a detailed information sheet to you?**

Contact Details:

Gender            Male                                Female  

Surname \_\_\_\_\_ First Name \_\_\_\_\_

Date of Birth \_\_\_\_\_

Address \_\_\_\_\_  
\_\_\_\_\_

Phone \_\_\_\_\_ Mobile \_\_\_\_\_

Email \_\_\_\_\_

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Office Use Only

Date info sheet forwarded \_\_\_\_\_

Posted      Emailed  

Email sent to Catherine Offler \_\_\_\_\_

Yes                               No

## Appendix 2. Information Sheet

### Fish Oil as an Adjunct for Treatment of Periodontitis

#### **Purpose of Study**

The Universities of Adelaide, South Australia and Griffith University are jointly conducting research on the benefits of fish oil therapy for periodontitis. Periodontitis is a chronic inflammatory condition of the gums resulting from bacterial infection. It afflicts 1 in 5 Australian adults. If it is left untreated the gum tissue and bone that surround and support teeth become seriously damaged, which can result in tooth loss.

Current treatment involves intensive cleaning to remove bacterial plaque and calculus (a hardened form of dental plaque that cannot be removed with a toothbrush), and regular checkups every 3 months.

Recent evidence suggests that long chain omega-3 polyunsaturated fatty acids (omega-3) from fish oil can help reduce inflammation in several chronic diseases. *We are interested in whether regular dietary supplementation with different types of fish oil can improve the outcome when combined with standard periodontal treatment.*

#### **What it involves**

The study will be conducted between the two universities in South Australia and one in Queensland. In South Australia the study is collaboration between The Colgate Research Centre, School of Dentistry, University of Adelaide and the Nutritional Physiology Research Centre, University of South Australia, which is located in the Bonython Jubilee Building at the University's City East Campus on Frome Road, across from the Dental Hospital. The collaborators in Queensland are based in The School of Dentistry and Oral Health, Gold Coast Campus, Griffith University. The principal investigators responsible for the study are Professor Mark Bartold, Dr. Alison Coates, Professor Peter Howe, Dr Toby Hughes, Professor Saso Ivanovski, Dr Stephen Hamlet, Mr Brain Chee and Ms, Boram Park.

The study will commence in July 2010 and we expect to continue recruitment through to December 2014. To be eligible for the study participants will need to have newly diagnosed chronic but not aggressive periodontitis.

126 volunteers are needed to participate in this study.

The table below shows the eligibility criteria for the study;

#### ***Participants and Groups:***

<b>Inclusion Criteria</b>	<b>Exclusion Criteria</b>
<ul style="list-style-type: none"><li>• Aged 25-80 years</li><li>• Advanced chronic periodontitis</li><li>• Non-smoker</li></ul>	<ul style="list-style-type: none"><li>• Person considered by the investigator to be unwilling, unlikely or unable to comprehend or comply with the study protocol</li><li>• Smokers and ex-smokers within the last 2 years</li><li>• Fish oil consumption &gt;1g/d or recently modified i.e. within the last 3 months</li><li>• Therapy for periodontitis in the past 6 months</li><li>• Antibiotic therapy for the past 3 months</li></ul>

	<ul style="list-style-type: none"> <li>• Anticoagulant therapy</li> <li>• Taking other anti-platelet agents; bleeding disorders.</li> <li>• Gastrointestinal disease, including peptic ulcers</li> <li>• Participation in another study within 30 days of commencement of the present study</li> <li>• Pregnant or breastfeeding</li> </ul>
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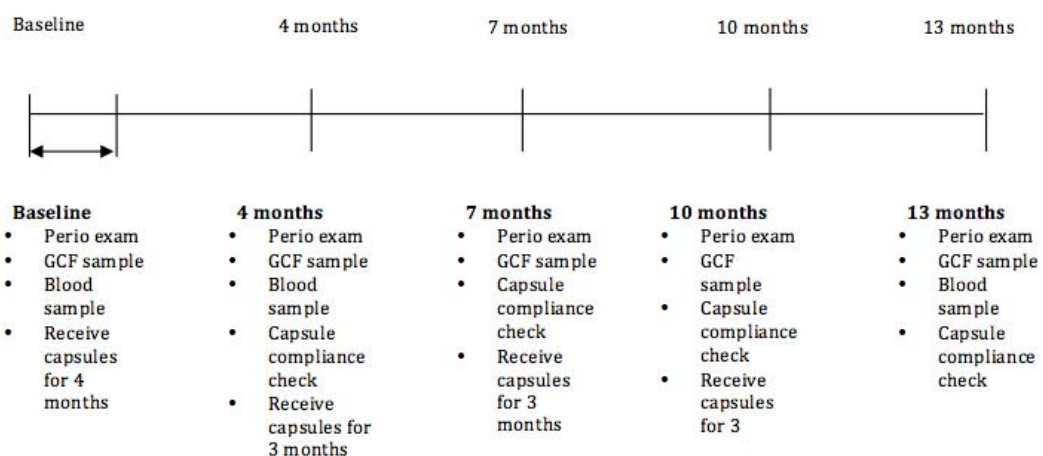
Eligible participants will be randomly allocated (i.e. by chance) to one of three treatment groups undertaking daily supplementation for 13 months as follows:

1. Fish oil rich in the long chain omega-3 fatty acid EPA
2. Fish oil rich in long chain omega-3 fatty acid DHA
3. Vegetable oil (placebo)

All participants will be asked to consume 6 x 500mg capsules each day. The supplements will be identical in appearance and they will be coded and labelled independently so that all researchers and participants are blinded to what is being taken. Participants will be given a supply of capsules at each visit and asked to return the unused capsules at their next visit.

You have been identified during your recent periodontal examination as a suitable candidate for this study. If you agree to participate, you will be asked to visit the Nutritional Physiology Research Centre (opposite the Dental School) where you will be enrolled in the study, allocated to a treatment group and a small blood sample (about 2 teaspoons) will be taken. This should take less than 30mins.

You will then attend the Colgate Research Centre for your first scheduled treatment appointment, after which you will be given capsules to take for the next 4 months. You will return to the Colgate Research Centre for your next 3 treatment visits at weekly intervals and then for follow-up examinations at 3 monthly intervals when you will be issued with further 3 monthly supplies of capsules, according to the schedule below.



At your initial treatment visit, a sample of gingival crevicular fluid (GCF) will be collected. A small area of gingival tissue will be cleaned, isolated with sterile cotton to prevent contamination by saliva and dried with an air syringe then a paper strip will be



inserted in the space between tooth and surrounding gingival tissue for 30 seconds to collect a sample. Participants will then undergo an intensive periodontal treatment involving non-surgical removal of plaque and calculus and a sample of plaque will be collected from the pockets around affected teeth. This treatment will be repeated at weekly intervals for 4 weeks.

Periodontal exams (including scaling and cleaning), will take place every 3 months as per the standard management protocol for periodontitis. Prior to these visits you will be instructed not to brush or floss your teeth, not to eat or drink anything but plain water for 4hrs before your visit.

At each visit information about general health will be recorded, as well as any medications you are taking. Visits at the Colgate Research Centre will take under 1hr.

Participants will be asked to return to the Nutritional Physiology Research Centre at UniSA at both 4 and 13 months to provide another small blood sample.

The blood samples will be used to measure the level of omega-3 fatty acids incorporated into red blood cell membranes and to measure inflammatory biomarkers in circulation. The gingival crevicular fluid (GCF) samples will be used to measure the extent of inflammation localised to the mouth and the plaque samples will be analysed to determine the amount and type of bacteria present.

### **Possible Risks**

All procedures will be carried out by qualified personnel. However, to assist you in making an informed decision, the risks associated with procedures are set out below:

**Blood Sampling:** will be taken by venepuncture. The associated risks are:

- *Infection* - although all of the needles will be sterile and all reasonable precautions will be taken, in any situation involving penetration of the skin there is a slight risk of infection.
- *Blood Clotting* - insertion of a needle into a blood vessel involves a risk of a blood clot forming which can travel through the circulation and block a smaller blood vessel somewhere else. However, the danger of this occurring is considered to be remote.
- *Bruising* - it is possible that you may experience slight bruising around the area where the needle was inserted. This is nothing to worry about as any such bruising should clear up within a few days. Blood thinning agents, such as regular strength aspirin (300mg), and ginko, should not be taken three days prior to sampling.

All procedures are undertaken by experienced staff and in accordance with strict OHS guidelines.

All information collected as part of the study will remain confidential and no information that could lead to identification of any individual will be released. The information collected in this study will be stored on a CD-ROM the Nutritional

Physiology Research Centres' secure data store in the Bonython Jubilee Building, City East Campus in a secure data store for a period of 15 years. Participants in the study may withdraw at any stage without prejudice. Participants will be provided with a copy of their personal results and a summary of the research findings within 6 months of completing the study.

Prior to your participation in the study, we would like to advise your GP of involvement. However, this will only be done with your approval. All participants will receive an honorarium of \$25 worth of store vouchers per visit to the Nutritional Physiology Research Clinic (i.e. visits that are not part of routine treatment) to help cover transport costs.

### **Withdrawal from Study**

Participation in this study is voluntary. You may withdraw from participating in this study at any time and this will in no way jeopardise your dental treatment.

### **Confidentiality**

Your confidentiality is respected at all times. All material collected will be de-identified. There will be no reference made to your name. You will not be identifiable from the sample. The only information collected regarding the sample will be your age and gender.

### **Costs involved**

There are no additional costs involved in participating in this project since the treatment you receive is part of your normal periodontal management.

### **Adverse Events**

In the unlikely event that you suffer an unanticipated adverse reaction to the procedure every effort will be made to see you immediately and deal with whatever problem has arisen at the surgical site

### **Contact Person**

The principal investigator of this project is:

Professor PM Bartold

Contact working hours: 8303 3435

Contact after hours: 8338 7793

**THE UNIVERSITY OF ADELAIDE**  
**HUMAN RESEARCH ETHICS COMMITTEE**

*Document for people who are participants in a research project*

**(1) CONTACTS FOR INFORMATION ABOUT THE PROJECT AND (2)  
INDEPENDENT COMPLAINTS PROCEDURE**

The Human Research Ethics Committee is obliged to monitor approved research projects. In conjunction with other forms of monitoring it is necessary to provide an independent and confidential reporting mechanism to assure quality assurance of the institutional ethics committee system. This is done by providing research participants with an additional avenue for raising concerns regarding the conduct of any research in which they are involved.

The following study has been reviewed and approved by the University of Adelaide Human Research Ethics Committee:

Project title: Fish Oil as an Adjunct for Treatment of Periodontitis

1. If you have questions or problems associated with the practical aspects of your participation in the project, or wish to raise a concern or complaint about the project, then you should consult the project co-ordinator:

*Name: Professor PM Bartold*

*Telephone: 8303 3435*

2. If you wish to discuss with an independent person, matters related to
  - making a complaint; or
  - raising concerns on the conduct of the project; or
  - the University policy on research involving human participants; or
  - your rights as a participant, then

contact the Human Research Ethics Committee's Secretary on phone  
(08) 8303 6028.

THE UNIVERSITY OF ADELAIDE HUMAN RESEARCH ETHICS COMMITTEE  
**STANDARD CONSENT FORM**  
**FOR PEOPLE WHO ARE PARTICIPANTS IN A RESEARCH PROJECT**

1. I, .....(please print name) consent to take part in the research project entitled: **Fish oil as an adjunct treatment for periodontitis**

2. I acknowledge that I have read the attached Information Sheet entitled: **Volunteer Information Sheet - Fish oil as an adjunct treatment for periodontitis**

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. I also consent to the use of dental assessments made during the preceding periodontal examination wherein I was identified as a potential volunteer for the study.

5. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

6. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

7. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

8. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

9. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Information Sheet.

.....

*(signature)* *(date)*

**WITNESS**

I have described to ..... *(name of subject)*  
the nature of the research to be carried out. In my opinion she/he understood the explanation.

Status in Project: .....

Name: .....

.....

*(signature)* *(date)*

## **Appendix 3. Storage of Perio Strips**

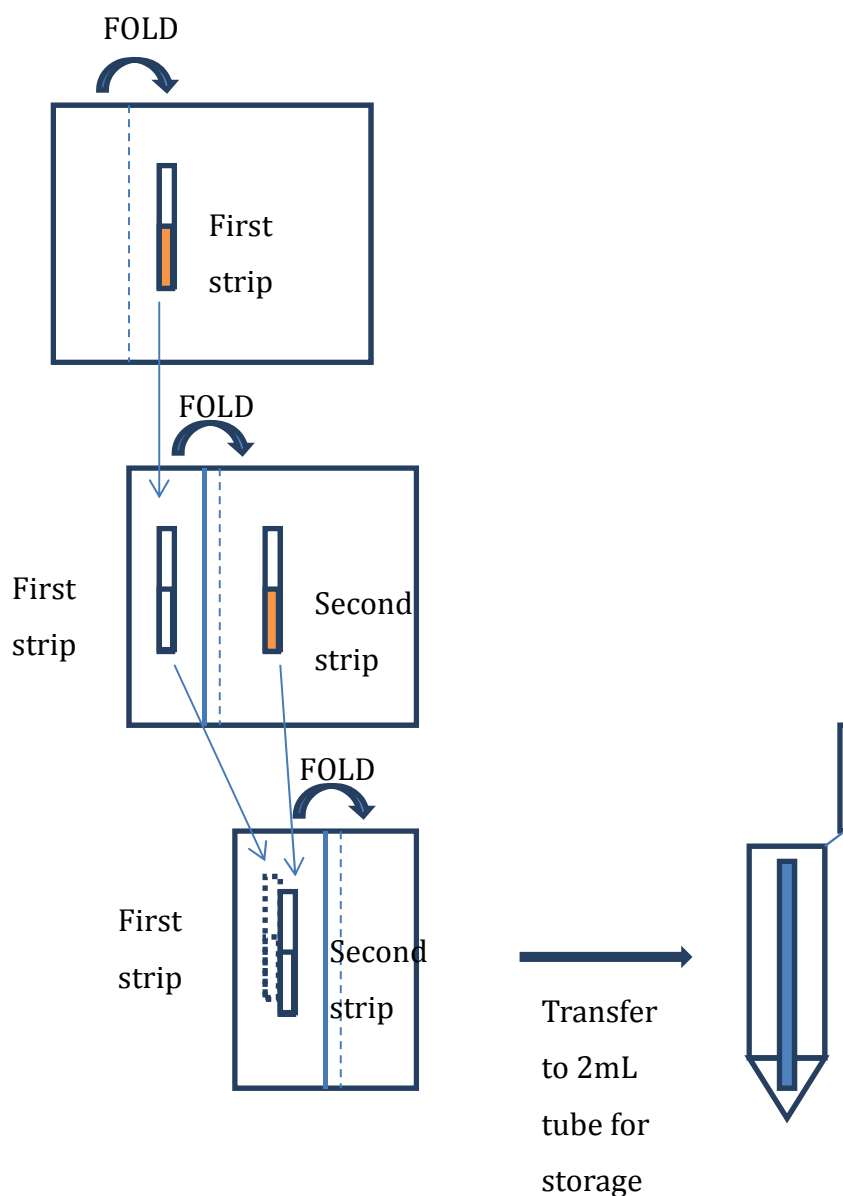
### **Storage of GCF perio strips**

Once GCF is taken, a periotron measurement is taken as quickly as possible to reduce drying of the strip. If the strip dries out too much the periotron measurement will be inaccurate.

The strip is then laid onto a small piece of foil and foil folded over the top. If a second strip has been taken it is laid on the foil next then folded over.

The foil is then stored in a labelled 2mL tube (patient ID and date) and stored at -20°C/-80°C until required.

Remember when unfolded the first strip encountered will be the second strip measured and the second strip will be the first measured.



### **Elution of GCF from perio strips**

To a 96 well plate add 220uL of 1XPBS.

Carefully unfold perio strip(s). Using tweezers transfer one strip to a single well of the 96 well plate and cover with lid.

Incubate the plate @ RT on a microplate mixer for 20 minutes.

To a labelled 1.5mL tube add 220uL of 1XPBS.

Transfer the eluate from the 96 well plate to the 1.5mL tube, total volume of ~440uL

Store at -20°C/-80°C until required.

It is better to elute and use in an ELISA as soon as possible rather than store eluate.

We try to calibrate the periotron monthly. The standard curve generated is used to calculate the volume of GCF which is required for ELISA result analysis.