

**LONG CHAIN OMEGA-3 FATTY ACIDS AS AN
ADJUNCT TO NON-SURGICAL
PERIODONTAL THERAPY: A RANDOMISED
DOUBLE-BLIND PLACEBO CONTROLLED
TRIAL**

**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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List of Abbreviations

AA	arachidonic acid
ATLs	aspirin-triggered lipoxins
COX-1	cyclo-oxygenase-1
COX-2	cyclo-oxygenase-2
DFDBA	demineralised freeze-dried bone allograft
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
ECM	extracellular matrix
EPA	eicosapentaenoic acid
GLA	gamma-linolenic acid
FAMES	fatty acid methyl esters
ICAM-1	intercellular adhesion molecule-1
IL-1R	human IL-1 receptor type 1
IκB	inhibitory subunit of NF κ B
IP-10	interferon-gamma inducible protein-10
JAK-STAT	janus tyrosine kinase-signal transducer and activator of transcription
MAPK	mitogen activated protein kinase
MaR1	maresin 1
MCP-1/CCL2	monocyte chemotactic protein-1
MIP-1α/CCL3	macrophage inflammatory protein-1 alpha
miRNAs	microRNAs
MMPs	matrix metalloproteinases
mPGES-1	microsomal prostaglandin E synthase-1
NF-κB	nuclear factor kappa B
NSAIDS	non-steroidal anti-inflammatory drugs
LAP	localised aggressive periodontitis
LCn3PUFAs	long-chain n-3 polyunsaturated fatty acids
LOX	lipoygenase
LPS	lipopolysaccharide
LTB₄	leukotriene B ₄
OPG	osteoprotegerin
PAMPS	pathogen-associated molecular patterns

PAHs	polycyclic aromatic hydrocarbons
PCBs	polychlorinated biphenyls
PDL	periodontal ligament
PD1	protectin D1
PGG₂	hydroperoxy endoperoxide
PGF_{2α}	prostaglandin F _{2α}
PGH₂	endoperoxide
PMNLs	polymorphonuclear leukocytes
PPARγ	peroxisome proliferator-activated receptor gamma
RCTs	randomised controlled trials
RvE1	resolvin E1
TGF-β	transforming growth factor-β
Th-2	T-helper 2
TIMPS	tissue inhibitors of metalloproteinases
TLRs	toll-like receptors

Abstract

Background and Aim

Animal studies and early clinical trials suggest a role for long chain omega-3 fatty acids (LCn3PUFAs) in the treatment of periodontal disease due to their anti-inflammatory and pro-resolution actions. The aim of this study was to evaluate the clinical efficacy of fish oil supplementation as an adjunct to non-surgical periodontal therapy in the treatment of advanced chronic periodontitis. Specific objectives were to establish the relative benefit of docosahexaenoic acid (DHA) versus eicosapentaenoic acid (EPA) compared with a placebo.

Materials and Methods

Thirty-four subjects (10 male, 24 female; mean age 50.1) with advanced chronic periodontitis were recruited for this parallel group double-blind placebo-controlled randomised trial. All participants received non-surgical periodontal therapy and were randomly allocated to receive either adjunctive dietary fish oil supplements (equivalent of 2g LCn3PUFA per day) or placebo. Clinical parameters were recorded at baseline, 4, 7, 10 and 13 months. Additionally, erythrocytes were isolated from fasting blood samples to allow assessment of fatty acid biomarkers including EPA, DHA, Omega-3 Index and total LCn3PUFAs. Data for the 4 month follow-up is presented in this initial report.

Results

One participant was lost to follow-up (placebo group), reporting poor compliance with their allocated capsules. Both treatment groups were effective at improving clinical outcomes, demonstrating significant reduction of full-mouth bleeding scores, probing pocket depth reduction and clinical attachment gain. At the 4 month follow-up, no significant difference was seen between groups for the percentage of sites that had ≥ 2 mm gain of clinical attachment ($P = 0.229$) or reduction in probing pocket depth ($P = 0.264$). The mean number of sites with residual pocket depth ≥ 5 mm at follow-up were not significantly different for the test group (6.6%) or placebo group (5.3%) ($P = 0.264$). Additionally, there were no statistically significant differences in clinical parameters for subjects that received supplements containing EPA, DHA or placebo.

Conclusion

Within its limitations, the findings of this study do not support an additional benefit of adjunctive LCn3PUFA supplementation for the treatment of advanced chronic periodontitis. Additionally, no correlation was found between clinical periodontal parameters and fatty acid profiles, and there was no significant difference between EPA and DHA subgroups. There is a need for further research to establish the clinical efficacy of LCn3PUFA as a host modulatory therapy for the treatment of periodontitis, particularly larger multi-centre randomised controlled trials.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Brian Chee

Dated this day of 2015

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Chapter 1. Literature Review of the Role of Inflammation in Periodontitis and the Use of Long Chain Omega-3 Fatty Acids as Host Modulation Therapy

1.1 Introduction

The term ‘inflammation’ is derived from the latin *inflammare* meaning ‘to set on fire’ and its features were first described by the Roman physician Aulus Cornelius Celsus. Acute inflammation arises as an initial physiological response to infection and tissue injury, and is characterised by vasodilation, increased vascular permeability and the infiltration of leucocytes. These processes result in the cardinal signs of *calor* (heat), *dolor* (pain), *rubor* (redness) and *tumor* (swelling), as well as *functio laesa* (loss of function). Several overlapping regulatory mechanisms are in place to minimise host-mediated tissue damage. However, in certain conditions the host inflammatory response can have destructive as well as protective potential.

Periodontitis is a chronic inflammatory condition resulting in progressive destruction of the supporting hard and soft and tissues of the teeth. Accumulation of bacterial biofilm on tooth surfaces initially results in inflammation of the gingival soft tissues known as gingivitis, although under normal physiological conditions improvement in oral hygiene will result in complete resolution of the inflammation (Löe et al., 1965). However, in susceptible individuals, persistence of the bacterial burden can cause a progressive non-resolving lesion and destruction of the periodontal attachment. The progression of periodontal disease to an advanced stage may lead to reduced tooth support with associated aesthetic and functional problems, and ultimately tooth loss.

The prevalence of chronic periodontitis is high, with data from most epidemiological studies indicating that around 30% of the population are affected, with approximately 7-13% experiencing advanced disease (Brown and Löe, 1993). However, surveys may have underestimated the prevalence of periodontitis due to the use of partial-mouth periodontal exam protocols. Recent data from the 2009 and 2010 National Health and Nutrition Examinations Surveys (NHANES) indicates a much higher overall prevalence of periodontitis compared with earlier epidemiological studies (Eke et al., 2012). In a sample of 3,742 adults aged 30 years and older, over 47% had periodontitis, with a distribution of 8.7%, 30% and 8.5% with mild, moderate and advanced disease respectively (Eke et al.,

2012). This study also highlighted the disparity in prevalence within certain ethnic and socio-economic groups, as well as their respective levels of education. Other surveys have reported a range of periodontitis prevalence in different countries (Bourgeois et al., 2007, Robert-Thomson and Do, 2007, Holtfreter et al., 2010). It should also be noted that prevalence is dependent on the case definitions used, and a universally accepted definition of periodontitis has yet to be established (Papapanou, 2012).

1.2 The Role of Bacteria in Periodontitis

Since bacteria are accepted as an essential factor in the initiation of periodontal disease, several hypotheses have been proposed to describe the microbial aetiology. The non-specific plaque hypothesis implicates the overall mass of microbiota as the key factor in the initiation of disease (Theilade, 1986). Although consistent with the features of gingivitis (Løe et al., 1965), this hypothesis fails to adequately explain variation in the clinical presentation of periodontitis, such as differences in disease susceptibility, rate of progression and site distribution. Importantly, there are many cases where the severity of tissue destruction is not consistent with the amount of microbial deposits.

Alternatively, the specific plaque hypothesis places emphasis on a limited number of pathogenic species that express particular virulence factors (Loesche, 1979). Many species are found in high proportions at healthy sites and can be regarded as part of the core resident oral microbiota, including members of the bacterial genera: *Streptococcus*, *Actinomyces*, *Neisseria*, *Haemophilus*, *Veillonella*, *Prevotella* and *Fusobacterium* (Aas et al., 2005, Marsh, 2012). However, several species have also been positively correlated with the development of periodontal disease. Early investigation identified *Actinobacillus actinomycetemcomitans* (later renamed *Aggregatibacter actinomycetemcomitans*) as a specific pathogen in localised juvenile periodontitis (Slots et al., 1980). Reduced levels of this species were also associated with improvement in clinical outcomes following periodontal therapy (Slots and Rosling, 1983, Christersson et al., 1985).

A consensus of the World Workshop in Periodontology later deemed the evidence sufficient to designate *A. actinomycetemcomitans*, *P. gingivalis* and *Tannerella forsythia* (previously *Bacteriodes forsythus*) as periodontal pathogens. This was supported by the work of Socransky et al. (1998) who found that the closely inter-related species of the ‘red complex’, comprising *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella*

*for*sythia were strongly associated with the clinical parameters of pocket depth and bleeding on probing. More recently, Haffajee et al. (2006) found that a reduction in the proportion of the combined red complex species were significantly related to a decrease in mean pocket depth at 12-months post-therapy. Furthermore, decreases in the counts or sites colonised with *T. for*sythia and *P. gingivalis*, as well as decreases in the percentage of red complex species, were significantly associated with attachment level gain over the same follow-up period. In contrast, sites with bleeding on probing had significantly higher counts of all members of the red complex, and nine members of the orange complex (Haffajee et al., 2006).

Most studies that indicate an association between specific microbial species and periodontitis cannot establish causation due to the limitations of cross-sectional data. Moreover, putative periodontal pathogens do not fulfil all the criteria of Koch's postulates that define a causal relationship between an infectious agent and a disease, and are frequently isolated from periodontally healthy subjects (Riep et al., 2009). In this regard, the bacterial species associated with periodontitis have many of the characteristics of opportunistic commensal organisms, rather than true exogenous pathogens. In a recent systematic review (Hujoel et al., 2013), three longitudinal studies were identified that implicated *A. actinomycetemcomitans* as an infectious agent in specific paediatric populations, before the onset of destructive periodontal disease (Van Der Velden et al., 2006, Fine et al., 2007, Haubek et al., 2008). They however failed to identify any longitudinal studies demonstrating significant associations between exposure to specific microorganisms and the onset of periodontitis in previously periodontally healthy adults (Hujoel et al., 2013).

Both the specific and non-specific plaque hypotheses can be considered too simplistic and do not account for the influence of the local environment and host response. The ecological plaque hypothesis was proposed to recognise the dynamic relationship between the resident microbiota and the host in both health and disease (Marsh, 1994). In the latter model, a two-way relationship exists whereby changes in the local environment (e.g. bleeding and increased gingival crevicular fluid flow) can produce a selective pressure for the growth of Gram-negative putative pathogens and favour a shift in the microbial community away from compatibility with health. More recently a novel model of microbial synergy and dysbiosis has been proposed, whereby different members or specific gene combinations within the community fulfil distinct roles that combine to shape and

stabilise a microbiota associated with disease (Hajishengallis et al., 2011, Hajishengallis and Lamont, 2012). A feature of this model is that ‘keystone pathogens’ increase the virulence of the entire microbial community via their interactions with accessory species (Hajishengallis and Lamont, 2012). The host-microbial interaction can be described as a linear process, whereby bacteria only initiate disease and subsequent tissue damage is caused by the host immune response, independent of bacteria. However, a more appropriate model of periodontal disease progression may be characterised by continuous cross-talk between bacteria and host (Gaffen and Hajishengallis, 2008).

1.3 The Role of the Host Response in Periodontitis

Current understanding recognises the multifactorial nature of periodontitis, including the role of host-microbial interactions as well as the influence of environmental and genetic modifiers (Kornman, 2008). Bacteria are thus thought to be essential but not sufficient to cause periodontitis alone, and most periodontal tissue destruction and clinical features are now attributed to a dysregulation of the host immune-inflammatory response (Kornman, 2008, Preshaw and Taylor, 2011).

Following development of the subgingival biofilm, the gingival epithelium interacts with bacteria and their products in what is thought to be initially a non-specific response to the overall mass of biofilm. This results in the expression of a large number of pro-inflammatory cytokines and adhesion molecules, such as intercellular adhesion molecule-1 [ICAM-1], which cause an increase in vascular permeability and migration of leukocytes into the gingival tissues (Page et al., 1997). The recruitment of inflammatory cells is mediated by several chemoattractants including complement products, leukotrienes and chemokines (Kantarci et al., 2003).

The early development of gingival inflammation has the classical features of an acute inflammatory response, with an exudate consisting mostly of neutrophils (Page and Schroeder, 1976, Kornman et al., 1997). These polymorphonuclear leukocytes [PMNLs] migrate to the site of infection by following the chemotactic gradient, forming a first line of defence against the bacterial challenge. Once recruited into the early lesion, neutrophils carry out the killing of microbes by both oxygen-dependent and oxygen-independent mechanisms (Kantarci et al., 2003). The production of antimicrobial products, such as reactive oxygen species by neutrophils, is focused towards the killing of bacteria.

However, their extracellular release can result in ‘bystander’ damage of the surrounding host tissues (Nussbaum and Shapira, 2011).

In most individuals, the protective aspect of the host response will manage the bacterial challenge to the periodontal tissues, with neutrophils and cell-mediated immunity limiting the extent of tissue destruction to allow a return to homeostasis (Gemmell and Seymour, 2004). However, if the microbial challenge and tissue destruction are not adequately managed by the host inflammatory response, the lesion can progress to a state of chronic inflammation. This excessive and uncontrolled inflammation underlies chronic periodontitis and a large number of systemic conditions including obesity, insulin resistance, hypertension, type II diabetes, coronary heart disease and rheumatoid arthritis (Das, 2013).

The initial accumulation of neutrophils into the tissues is followed by the recruitment of macrophages that carry out microbial killing and clearance of apoptotic cells, as well as linking the innate and specific immune response by presenting antigens to T-helper cells. Recruitment of macrophages into the periodontitis lesion further enhances the inflammatory response by the release of more pro-inflammatory cytokines. A cascade effect caused by the activation of resident cells, such as gingival fibroblasts, epithelial cells, keratinocytes, osteoblasts, osteoclasts and endothelial cells, further heighten the inflammatory response by expressing more pro-inflammatory mediators (Sorsa et al., 2006). This ability of macrophages to exacerbate the inflammatory response in periodontitis is indicated by their association with sites of active disease (Silva et al., 2007).

Another feature of periodontitis development is a switch from a primarily T-cell dominated lesion to one involving mostly B-cells and plasma cells (Page and Schroeder, 1976). The dominance of B-cells and plasma cells in the advanced lesion suggests a role for the T-helper cells. Seymour et al. (1993) proposed a model where the immunoregulatory mechanisms underlying periodontitis were primarily governed by T-helper 2 [Th-2] cells and their associated cytokines (IL-4, IL-5 and IL-6), rather than Th-1 phenotype characterised by the secretion of IL-2 and IFN γ . The predominantly Th-2-like response produces the cytokine profile necessary for B-cell proliferation and differentiation, leading to the elevated levels of non-protective antibodies and the on-going production of IL-1 β that contributes to bone resorption (Gemmell and Seymour, 2004). In contrast, other

studies have implicated an increased Th-1 response (Berglundh and Donati, 2005) or combined Th-1 and Th-2 cytokine profiles in the regulation of advanced periodontitis lesions (Berglundh et al., 2002). Furthermore, recent evidence suggests that differences in the Th-1/Th-2 phenotype are not sufficient to explain the progression of the periodontitis lesion, and that a novel pro-inflammatory IL-17 producing T-helper (Th-17) subset may play a significant role (Gaffen and Hajishengallis, 2008). A recent study demonstrated significant over-expression of several specific markers for Th-17 cells in periodontitis subjects compared with a periodontally healthy control group (Adibrad et al., 2012). However, due to a lack of longitudinal data, it is currently difficult to establish a link between destructive periodontal disease and a particular T-helper subset or cytokine profile (Graves, 2008).

In summary, the host response associated with periodontitis is characterised by the infiltration of leucocytes, lymphocytes and the production of pro-inflammatory cytokines such as IL-1 β , TNF- α and IL-6. Therefore, tissue destruction in periodontitis arises largely from a dysregulation of inflammation.

1.4 Mediators of Inflammation

1.4.1 Pattern-recognition Receptors

The inflammatory response to bacterial infection provides the first line of the host defence and is initiated by the detection of the microorganisms by pattern-recognition receptors, such as the Toll-like receptors [TLRs]. This process enables the innate immune system to differentiate between host and pathogen by interacting with unique microbial products that are absent from host cells. These pathogen-associated molecular patterns [PAMPs] include components essential for microbial cell function, although they are not unique to pathogenic organisms (Medzhitov, 2001). It is therefore unknown how the host differentiates between pathogenic and commensal organisms and why TLRs are not continuously activated by commensal microbiota to produce an inappropriate inflammatory response. Low level stimulation by commensal bacteria may in fact be necessary for priming host immunity and maintaining tissue homeostasis (Preshaw and Taylor, 2011). TLRs are predominantly expressed on innate immune cells including neutrophils, macrophages and antigen-presenting dendritic cells (Mahanonda and Pichyangkul, 2007) as well as resident cells of the periodontium including gingival fibroblasts, keratinocytes and epithelium (Uehara and Takada, 2007). The latter reside in the gingival epithelium and have a protective role in producing antimicrobial peptides (α -

and β -defensins), but also secrete a large variety of cytokines (including IL-1 α , IL-1 β , IL-8 and TNF- α) in response to the bacterial challenge (Liu et al., 2010). Moreover, cross-talk between bacteria and gingival epithelial cells and the associated release of pro-inflammatory cytokines and chemokines are likely to have an important role in the initiation and progression of periodontitis (Sandros et al., 2000, Andrian et al., 2006, Stathopoulou et al., 2010).

TLRs on cell surfaces (TLR-1, TLR-2, TLR-4, TLR-5, TLR-6 and TLR-11) are associated with the recognition of microbial membrane components including lipopolysaccharide [LPS], lipoproteins and proteins (Kawai and Akira, 2010), while other TLRs (TLR-7, TLR-8 and TLR-9) are expressed in intracellular compartments and recognise microbial nucleic acid (Kawai and Akira, 2010). Specific microbial cell structures such as *P. gingivalis* fimbriae [FimA] have also been shown to elicit a strong immune response and can be considered as PAMPs (Eskan et al., 2007). Individual TLRs have cell-specific functions and can interact with many structurally unrelated ligands derived from different pathogens, although accessory proteins are sometimes needed (Medzhitov, 2001).

TLR activation leads to the initiation of intracellular signalling cascades and the production of pro-inflammatory mediators (Akira et al., 2006, Freire and Van Dyke, 2013). They are therefore essential for the function of the innate host response, but also have a role in the development of antigen-specific acquired immunity. As well as activation by PAMPs, it is increasingly recognised that TLRs can also interact with endogenous host molecules resulting from cell injury, such as extracellular matrix [ECM] degradation products and heat-shock proteins (Kawai and Akira, 2010). Future research may establish TLR signalling as a pathway by which periodontal inflammation and tissue damage can be exacerbated by a response to localised tissue breakdown products, independent from the response to bacteria (Preshaw and Taylor, 2011).

Other pattern-recognition receptors include the nucleotide-binding oligomerisation domain (NOD)-like receptors that are important in signalling cytokine responses to intracellular infections (Franchi et al., 2009). Members of the NOD-like receptor family are known to promote the secretion of pro-inflammatory cytokines by their involvement in a multi-protein complex called the inflammasome. The overall immune response to a specific bacteria may be determined by the combined activation of several pattern recognition

receptors, and the activity of TLRs may be influenced by other receptors including dectins, NODS and mannose receptors (Gaffen and Hajishengallis, 2008).

The exact role of pattern-recognition receptors in periodontal health and disease has yet to be established, although it is known that TLR and NOD-like receptors are constitutively expressed in gingival fibroblasts and their expression is upregulated during gingival inflammation (Beklen et al., 2008). Furthermore, stimulation of these receptors with chemically synthesised ligands has been shown to significantly upregulate the production of pro-inflammatory cytokines, including IL-6, IL-8 and monocyte chemoattractant protein-1 (Uehara and Takada, 2007). The same authors demonstrated that stimulation of TLRs or NOD1/2 with PAMPS in addition to *P. gingivalis* gingipain had a synergistic effect, leading to the secretion of IL-6 and IL-8 in cultured human monocytes (Uehara et al., 2008). CD14 is another pattern recognition receptor known to act as a co-receptor with TLR-2 and TLR-4. Jin and colleagues (2011) observed membrane-bound CD14 receptors in gingival macrophages, PMNLs and fibroblasts, and found mCD14 expression to be significantly higher in periodontally healthy patients than in patients with chronic periodontitis.

1.4.2 Pro-inflammatory Cytokines

Bacterial-host interaction via pattern recognition receptors can lead to the production of a wide range of pro-inflammatory mediators. This process is initiated and perpetuated by pro-inflammatory cytokines that drive the production of other cytokines, chemokines, prostaglandins and matrix metalloproteinases [MMPs] (Page, 1991). Although they are essential for defence against infection, in the periodontally susceptible individual these mediators can cause a destructive cascade that leads to irreversible periodontal tissue destruction and attachment loss.

Cytokines are a large group of soluble proteins that influence gene regulation by binding to specific receptors on target cells. They represent a diverse group of proteins including the interferons, interleukins, chemokines, mesenchymal growth factors, the tumour necrosis factor family and adipokines (Dinarello, 2007). These signalling molecules are pleotropic, being both produced by and acting on a wide range of different cell types with activity dependent on the target cells. The primary function of cytokines is the modulation of the immune response, and they are known to function and interact in complex overlapping

networks where changes in the level of an individual cytokine does not necessary produce a predictable linear effect (Taylor, 2010, Preshaw and Taylor, 2011). Moreover, cytokines often regulate the expression or receptors of other mediators and their combined action may result in either synergistic or antagonistic effects (Hughes, 1995). Many details of the mechanism by which *in vitro* cytokine networks regulate cell function and tissue turnover in the periodontium have therefore not been elucidated due to the limitations of studying individual cytokines in isolation. Other important homeostatic functions of cytokines include cellular development, homeostasis and tissue repair (Taylor, 2010).

In health, tissue homeostasis is usually maintained because the action of pro-inflammatory cytokines is in equilibrium with anti-inflammatory cytokines and tightly controlled by a number of regulatory mechanisms, including the regulation of their receptor and signalling pathways. For example, the anti-inflammatory cytokine IL-10 is widely expressed in inflamed periodontal tissues and has been shown to modulate tissue destruction by several mechanisms, including the inhibition of TLR or pro-inflammatory cytokine signalling (Yoshimura et al., 2007); upregulation of tissue inhibitors of metalloproteinases [TIMPS] (Garlet et al., 2004); and inhibiting bone loss by promoting the production of osteoprotegerin [OPG] (Zhang and Teng, 2006). The actions of pro-inflammatory cytokines are summarised in Table 1.1.

Cytokine	Pro-inflammatory Action
IL-1 β	<ul style="list-style-type: none"> • Primary mediator of the immune and inflammatory response • Stimulates the production of other pro-inflammatory mediators including PGE₂, Platelet Activating Factor, nitrous oxide • Stimulates osteoclasts by RANKL-dependent and -independent pathways • Stimulates connective tissue turnover by increased expression of MMPs
TNF- α	<ul style="list-style-type: none"> • Primary mediator of the immune and inflammatory response • Enhanced leucocyte recruitment via increased expression of adhesion molecules and chemokines • Stimulates osteoclasts by RANKL-dependent and -independent pathways • Increased expression of COX-2 in endothelial cells
IL-6 family	<ul style="list-style-type: none"> • Key role in acute inflammation • Promotes bone resorption • Stimulates fever, angiogenesis and acute phase protein release • Stimulates T-cell differentiation
IL-17	<ul style="list-style-type: none"> • Increased expression of IL-1β, TNFα, RANKL, chemokines, COX-2 and MMPS
Chemokines	<ul style="list-style-type: none"> • Facilitate leukocyte recruitment

Table 1.1. Action of pro-inflammatory cytokines.
Adapted from Preshaw and Taylor (2011).

In destructive periodontal disease, excessive production of pro-inflammatory cytokines is a major determining factor in the progression and extent of tissue degradation and bone loss (Page, 1991). The pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 have been the most strongly implicated. Likewise, changes in the quantity or function of inflammatory inhibitors such as anti-inflammatory cytokines, receptor antagonists and TIMPS may also contribute to the dysregulation of inflammation (Howells, 1995, Preshaw and Taylor, 2011). Recent investigation has also demonstrated a difference in the levels of IL-11 and IL-17 in gingival tissues affected by gingivitis and periodontitis, suggesting that these cytokines may have a role in the pathogenesis of periodontal disease (Johnson et al., 2004). The *in vitro* activity of these novel pro-inflammatory cytokines was however modest compared with established pro-inflammatory mediators such as IL-1 β and TNF- α , and only weak activation of nuclear factor kappa B [NF- κ B] has been reported (Gaffen and Hajishengallis, 2008).

Pro-inflammatory cytokines can induce osteoclastogenesis and bone resorption by increasing the expression of RANKL while at the same time decreasing OPG production (Cochran, 2008). This is supported by studies that observe higher RANKL/OPG ratios in subjects with periodontitis compared to healthy controls and a decrease in this ratio following periodontal therapy (Buduneli and Kinane, 2011).

In summary, periodontal disease progression and loss of attachment may occur if the balance of pro- and anti-inflammatory processes is disrupted by a combination of interaction with bacteria and host susceptibility due to genetic differences in the immune response.

1.4.2.1 Interleukin-1 Family

There is strong evidence in both human and animal models that supports a role for the interleukin-1 family of cytokines in mediating tissue destruction and bone resorption in periodontitis (Graves, 2008). The IL-1 family encompasses 11 members with overlapping pro-inflammatory functions, although each member is encoded by a separate gene (Dinarello, 2007). There are two important signalling pathways necessary for the secretion of IL-1 β : the first is dependent on TLR receptor activation; the second, via ATP-dependent assembly of inflammasomes (Latz, 2010). Inflammasome activation of caspase-1 mediates

the conversion of pro-IL1- β and pro-IL8 into their mature, biologically active forms (Franchi et al., 2009).

IL-1 β is a potent mediator of periodontal inflammation, primarily produced by macrophages and dendritic cells, but also secreted by gingival fibroblasts, periodontal ligament cells and osteoblasts (Liu et al., 2010). Early research into the role of cytokines demonstrated consistently increased levels of IL-1 β in gingival biopsy samples and GCF of patients with periodontitis, often with a corresponding decrease after treatment (Honig et al., 1989, Masada et al., 1990, Tsai et al., 1995, Gamonal et al., 2000, Yoshinari et al., 2004). Additionally, studies have reported significantly higher levels of IL-1 β in sites that had disease progression compared with stable and healthy sites (Stashenko et al., 1991).

IL-1 β is thought to bring about inflammatory changes indirectly through the production of other inflammatory mediators, including prostaglandin E₂, Platelet Activating Factor and nitrous oxide (Taylor, 2010). Another important pathway by which IL-1 β enhances connective tissue degradation and bone resorption is through the increased expression of MMPs in both gingival fibroblasts and periodontal ligament cells (Birkedal-Hansen et al., 1993). Furthermore, IL-1 β is able to directly stimulate osteoclastogenesis by inducing the increased expression of RANKL in osteoblasts in addition to osteoclast stimulation by RANKL-independent pathways. An *in vitro* study demonstrated that IL-1 β is 15 times more potent than IL-1 α and 500 times more potent than TNF- α at promoting bone resorption (Stashenko, 1987).

The importance of IL-1 β in promoting periodontal tissue destruction is also supported by studies in animal models, where the addition of recombinant human IL-1 β accelerated inflammatory cell invasion and alveolar bone resorption (Koide et al., 1995). Furthermore, the inhibition of IL-1 using human IL-1 receptor type 1 [IL-1R] significantly reduced inflammation, connective tissue attachment loss and bone resorption in an experimental periodontitis model in a non-human primate model (Delima et al., 2002).

1.4.2.2 TNF- α

Tumour necrosis factor- α is a potent pro-inflammatory cytokine predominantly produced by activated T-cells, fibroblasts and macrophages during inflammation. While it has been established that TNF- α is essential for the control of periodontal inflammation, it can also

have the potential to exacerbate the inflammatory response and tissue destruction (Garlet, 2010). Comparable with IL-1 β , TNF- α can also be considered a primary mediator of the immune response, mostly by the induction of secondary inflammatory mediators such as chemokines, adhesion molecules and PGE₂ (Taylor, 2010). This is consistent with the many contradictory protective and destructive functions of pro-inflammatory mediators.

Studies using IL-1 and TNF antagonists have confirmed the inhibition of the inflammatory response, as well as *in vivo* bone loss in animal models (Assuma et al., 1998, Delima et al., 2001). In these studies experimental periodontitis was inhibited by the local injection of soluble receptors to these cytokines, resulting in reduced inflammatory cell infiltrate, reduced connective tissue attachment and pathological bone loss. Blocking the function of these cytokines resulted in 67% reduced osteoclast formation and 60% less bone loss compared to control sites, despite a persistent bacterial challenge (Assuma et al., 1998).

The pro-inflammatory cytokines IL-1 and TNF- α act synergistically to promote bone resorption and can directly influence osteoclast function independent of the RANKL/RANK system, via IL-1R and TLR-4, as well as the TNF receptor-associated factor-2 route (Liu et al., 2010). TNF- α can also contribute to net loss of periodontal tissues by stimulating apoptosis of the matrix-producing fibroblasts and therefore inhibiting repair (Graves and Cochran, 2003).

1.4.2.3 Interleukin-6 and Related Cytokines

There are currently nine members of the IL-6 cytokine family, including IL-1, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, factor, cardiotrophin-1, cardiotrophin-like cytokine, novel neutrophin-1 and IL-27 (Scheller et al., 2014). These cytokines share a common signalling protein, gp130, and therefore have a degree of overlapping activity (Liu et al., 2010). IL-6 is activated by several other cytokines, including IL-1 β and TNF- α (Taylor, 2010). It is expressed in the periodontium by osteoblasts, gingival fibroblasts and periodontal ligament cells (Liu et al., 2010). Studies have reported elevated levels of IL-6 in human gingival fibroblasts and periodontitis lesions (Bartold and Haynes, 1991, Takahashi et al., 1994, Yamazaki et al., 1994). Another study found a correlation between periodontitis severity and total amounts of oncostatin M and IL-6 in GCF (Lin et al., 2005). Important pro-inflammatory properties of IL-6 include the regulation of B-cell and dendritic cell differentiation, determining the

balance of T-cell subsets, as well as stimulating the development of osteoclasts and bone resorption (Taylor, 2010, Preshaw and Taylor, 2011).

A recent study showed enhanced gingival inflammation due to synergistic effects of IL-1 β and IL-6 on gingival fibroblasts through the induction of gp130 expression (Sawada et al., 2013). Furthermore, it has been recently demonstrated that chronic inflammation and autoimmune diseases associated with IL-6 activity are driven by trans-signalling, via a novel agonistic soluble form of IL-6 receptor rather than classic signalling via the membrane-bound IL-6 receptor (Scheller et al., 2014). This discovery has opened up potential for new therapies that selectively target IL-6 trans-signalling with the potential to reduce side effects by preserving protective immune responses (Scheller et al., 2014).

1.4.2.4 Interleukin-17 and Interleukin-23

The production of IL-17 and defective regulation of Th-17 cells are features of chronic inflammation and are prominent in several immuno-inflammatory disorders (Miossec et al., 2009). Moreover, when compared to healthy control sites, the expression of IL-17 is significantly higher in periodontitis lesions, especially in tissues adjacent to bone resorption (Ohyama et al., 2009). IL-17 may contribute to local inflammation by the recruitment and activation of immune cells leading to the increased expression of IL-1 β , TNF α , RANKL and MMPS (Garlet, 2010, Nussbaum and Shapira, 2011, Di Benedetto et al., 2013). Contrary to these findings, Yu et al. (2007) observed reduced PMNL recruitment in the periodontal tissues of IL-17 receptor-deficient mice, associated with significantly more alveolar bone loss. This highlights the dual role of this cytokine and the importance of PMNL recruitment in the initial host inflammatory response. IL-23 is another important cytokine involved in expansion of the Th-17 cell lineage. A recent study found the expression of IL-23 and its receptor is significantly higher in periodontal lesions compared to control sites (Ohyama et al., 2009). Further research is warranted to establish the role of IL-17 and IL-23 in periodontitis.

1.4.2.5 Chemokines

The chemokines are a large group of structurally related heparin-binding proteins characterised by their chemotactic activity (Silva et al., 2007). They are produced by a wide variety of infiltrating and resident (immune and non-immune) cells in the periodontium, such as fibroblasts, endothelial cells, macrophages, osteoclasts, epithelial

cells, neutrophils, monocytes, lymphocytes and mast cells (Graves, 2008). Chemokines have a central role in the inflammatory response by regulating cell migration and proliferation, and coordinating the distribution of leukocyte subsets in the tissues (Preshaw and Taylor, 2011). Some chemokines are constitutively expressed to facilitate normal leukocyte migration, in contrast to the chemokines induced by the pro-inflammatory cytokines (Preshaw and Taylor, 2011). Binding of chemokines to their respective receptors promotes the migration of leukocytes by several actions, including the regulation of cytoskeletal organisation, integrin-dependent adhesion, as well as binding and detachment of cells from their substrate (Silva et al., 2007).

Chemokines have a role in the pathogenesis of periodontal disease in addition to the classic pro-inflammatory cytokines. IL-8/CXCL8 is significantly elevated in inflamed gingival tissues and is preferentially expressed in epithelial cells (Fitzgerald and Kreutzer, 1995). Therefore, by acting as a chemotactic agent for PMNLs, IL-8/CXCL8 directs these phagocytes to migrate through the junctional epithelium and into the sulcus or pocket to meet the bacterial challenge (Tonetti et al., 1998, Silva et al., 2007). This is supported by Tsai et al. (1995) who reported significantly increased levels of IL-8/CXCL8 in the GCF of periodontitis subjects compared with a periodontally healthy control group. Furthermore, the levels of IL-8/CXCL8 were positively related to disease severity and reduced following the initial phase of periodontal treatment (Tsai et al., 1995). IL-8/CXCL8 could therefore contribute to destruction of periodontal tissue by facilitating the accumulation of PMNLs and thereby increasing the degree of neutrophil-associated secondary damage (*q.v.* Section 1.3). However, the important protective role of PMNLs in maintaining periodontal health should be recognised. Yu et al. (2007) observed significantly reduced PMNL recruitment and more alveolar bone loss in mice deficient in murine chemokine receptor. The protective role of neutrophils in the periodontal tissues is also indicated by the advanced periodontal destruction associated with rare disorders in which there are reduced function or number of these cells, such as cyclic neutropenia, Down syndrome, leukocyte adhesion deficiency syndromes, Papillon-Lefèvre syndrome and Chediak-Hygaschi syndrome (Deas et al., 2003).

Monocyte chemotactic protein-1 [MCP-1/CCL2] is the major chemoattractant of macrophages in periodontal diseases (Silva et al., 2007). It is expressed by multiple cells types in inflamed gingiva (Yu and Graves, 1995) and is associated with accumulation of both neutrophils and macrophages in gingivitis and periodontitis (Kornman et al., 1997).

MCP-1/CCL2 is a significant mediator in several other inflammatory diseases such as rheumatoid arthritis, asthma and psoriasis (Xia and Sui, 2009). Significant expression of this chemokine is observed in the gingival tissues of periodontitis patients (Hanazawa et al., 1993).

Interestingly, Garlett et al. (2003) found greater levels of the chemokines macrophage inflammatory protein-1 alpha [MIP-1 α /CCL3] and interferon-gamma inducible protein-10 [IP-10] together with their respective receptors, CCR5 and CXCR3, in gingival biopsies from aggressive periodontitis subjects. In contrast, biopsies from chronic periodontitis subjects were associated with more frequent and higher expression of MCP-1/CCL2 (Garlett et al., 2003).

Chemokines may also contribute to bone resorption in periodontitis through several mechanisms, including promotion of osteoclast differentiation (MCP1-CCL2, IL8/CXCL8), chemotaxis (RANTES/CCL5) and survival (MIP-1 γ CCLP9); as well as inducing osteoclast MMP activity (SDF1 α /CXCL12) (Silva et al., 2007).

1.4.3 Matrix Metalloproteinases

Matrix metalloproteinases are a large group of zinc-dependent structurally related enzymes that degrade extracellular matrix and basement membrane components (Birkedal-Hansen et al., 1993). Recent findings have also revealed that MMPs have additional functions other than the degradation of the ECM, acting on pro-inflammatory cytokines, chemokines and other proteins to regulate several aspects of inflammation and immunity (Parks et al., 2004). MMPs can therefore be considered as modifiers of the host response, and their function related to tissue destruction should not be observed without considering their broader roles in inflammation, tissue remodelling and wound healing (Uitto et al., 2003, Sorsa et al., 2006). The MMP family can be divided into four major subgroups based on their structure and substrate specificity: the collagenases (MMP-1, -8 and -13), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, -10, -11) and the membrane-bound MMPs (MMP-14, -15, -16 and -17) (Sorsa et al., 2004). Other MMPs do not clearly fit these groups, such as matrilysin (MMP-7) and metalloelastase (MMP-12) (Reynolds and Meikle, 1997).

Due to the destructive potential of MMPs, their activity is closely controlled by several mechanisms. Firstly, MMP synthesis is controlled by growth factors, cytokines and transcription factors affecting gene expression (Sorsa et al., 2006). Transforming growth factor- β [TGF- β] can suppress MMP-1 and MMP-8 gene transcription but also induce the expression of the MMP-13 gene, whereas IL1- β and TNF- α have been shown to upregulate MMP-3, -8 and -9 gene expression in gingival fibroblasts, as well as MMP-13 expression in osteoblasts (Sorsa et al., 2006). Furthermore, most MMPs are initially synthesised in an inactive zymogen (enzyme precursor) state, requiring activation by disturbance of the cysteine-zinc interaction known as the 'cysteine switch' (Visse and Nagase, 2003). Proteolytic activation of pro-MMPs is often a step-wise process with additional regulatory mechanisms (Visse and Nagase, 2003). The MMPs are also inhibited by a group of specific endogenous tissue inhibitors of metalloproteinases (TIMPs), as well as non-specific proteinase inhibitors such as α 2-macroglobulin (Visse and Nagase, 2003). The former are the major group of MMP inhibitors, comprising four members (TIMP-1, TIMP-2, TIMP-3 and TIMP-4). TIMPs are synthesised and secreted by most connective tissue cells as well as macrophages (Reynolds and Meikle, 1997).

MMPs have been widely implicated in both soft and hard tissue destruction in chronic periodontitis. The predominant MMPs present in the inflamed gingival tissue, GCF, as well as peri-implant sulcular fluid, are the neutrophil-derived MMP-8 and -9 and epithelial or bone cell-derived MMP-13, rather than the constitutively expressed MMP-1 and MMP-2 (Sorsa et al., 2006, Hernandez-Rios et al., 2009). Studies have also shown that collagenase levels correlate with periodontal disease severity (Uitto et al., 1998) and levels decrease after non-surgical periodontal therapy (Marcaccini et al., 2010). Furthermore, MMP-8 salivary levels have also been investigated as a biomarker with the potential to monitor the severity of periodontal disease and response to therapy (Sexton et al., 2011). Kinney et al. (2014) reported that the level of MMP-8 and MMP-9 and other inflammatory biomarkers in GCF provided a sensitive measure for predicting periodontal disease progression when combined with clinical and microbial outcomes. Another recent investigation of localised aggressive periodontitis showed a reduction in local levels of MMPs following non-surgical debridement with adjunctive antibiotics (Gonçalves et al., 2013). The study also showed a positive correlation of specific MMP levels with clinical treatment outcomes, including mean pocket depth and percentage of residual sites greater than 4 mm (Gonçalves et al., 2013). An association between MMP-8 and TIMP-1 gene

polymorphisms and susceptibility to generalised aggressive periodontitis has also been shown in the Turkish population (Emingil et al., 2013)

1.4.4 Pro-inflammatory Lipid Mediators

Arachidonic acid [AA] is the principle n-6 fatty acid in immune cell phospholipid, and is metabolised by three main classes of enzymes: cyclooxygenases [COX], lipoxygenases [LOX] and p450 epoxygenases (Yates et al., 2014). For AA to become available as a substrate it is first released from cell membrane phospholipid by the enzyme phospholipase A2 (Peters-Golden and Henderson Jr, 2007). The eicosanoid family of AA-derived metabolites are important signalling molecules in inflammation and include the prostaglandins, prostacyclins and thromboxanes. The role of these lipid mediators in causing the cardinal signs of inflammation is summarised in Table 1.2.

Eicosanoids	Pro-inflammatory Action
PGF2 α , TxA ₂ , LTC ₄	Vasoconstriction
LTB ₄	Leukocyte chemotaxis
LTC ₄ , LTD ₄	Vascular permeability
PGE ₂ , PGI ₂ , LTB ₄	Pain and hyperalgesia
PGE ₂ , PGE ₁ , PGE ₂ , PGD ₂	Local heat and systemic fever
PGI ₂ , PGE ₁ , PGE ₂ , PGD ₂	Vasodilation (erythema)
PGE ₂ , LTB ₄	Oedema (swelling)

Table 1.2. Role of eicosanoids in inflammation.
Adapted from (Serhan, 2007).

There are two isoforms of the cyclo-oxygenase enzyme. Cyclo-oxygenase-1 [COX-1] has a physiological role characterised by constitutive activity in nearly all cell types. On the other hand, cyclo-oxygenase-2 [COX-2] activity is normally absent under physiological conditions, but is inducible during inflammation (Vane et al., 1998). Both cyclo-oxygenase enzymes catalyse the first two steps in the biosynthesis of the prostanoids, first oxidising AA to hydroperoxy endoperoxide [PGG₂], which is then reduced to hydroxyl

endoperoxide [PGH₂]. PGH₂ is subsequently converted to PGE₂, PGF₂, PGD_{2α}, PGI₂ and TXA₂ via several additional pathways (Vane et al., 1998). Additionally, AA is also metabolised by the lipoxygenase enzymes to produce the leukotrienes and other products (Peters-Golden and Henderson Jr, 2007).

These AA-derived metabolites have a significant role in inflammation and cause many of its features including fever, hyperalgesia, vasodilation, increased vascular permeability and oedema. Their production is therefore an established target for anti-inflammatory drugs, such as NSAIDs, which act by inhibiting cyclo-oxygenase activity. An outline of eicosanoid synthesis from arachidonic acid is illustrated in Figure 1.1.

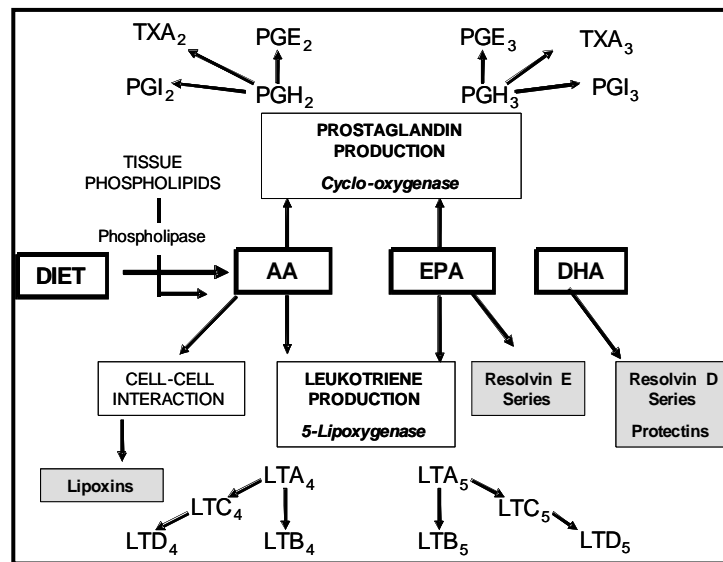


Figure 1.1. Production of eicosanoids and pro-resolution lipid mediators.

1.4.4.1 Prostaglandins

The prostaglandin subclasses D, E, F, G, H and I are thought to be involved with inflammation (Freire and Van Dyke, 2013). PGE₂ in particular has been highlighted as a major pro-inflammatory mediator in periodontal disease. Prostaglandins were first implicated as potent stimulators of alveolar bone resorption through studies of tissue culture in the 1970s (Klein and Raisz, 1970, Goldhaber et al., 1973). The prostaglandins belong to the prostanoid group of lipid mediators, together with the prostacyclins and thromboxanes.

Both IL-1 β and TNF α work synergistically to stimulate the production of PGE₂. These cytokines induce the expression of microsomal prostaglandin E synthase-1 [mPGES-1], the terminal enzyme regulating the synthesis of PGE₂, although IL-1 β is a more potent stimulator (Yucel-Lindberg et al., 2006). PGE₂ induces a wide range of pro-inflammatory effects, including the classical features of fever, as well vasodilation by its effect on vascular smooth muscle. Additionally, prostaglandin E₂ induces both fibroblasts and osteoclasts to express other pro-inflammatory mediators including MMPs, IL-1 β and other cytokines. PGE₂ may also contribute to the sustained inflammatory response by amplifying its own production via the induction of COX-2 expression in a variety of different cell types (Bagga et al., 2003). Although PGE₂ is generally thought to be a potent stimulator of bone resorption, it also appears that it can have contradictory anti-inflammatory effects and can even promote osteogenesis, depending on the cell types and specific receptors it interacts with (Noguchi and Ishikawa, 2007).

The importance of specific T-helper cell subsets in periodontitis pathogenesis is still controversial (*q.v.* Section 1.2). Nevertheless, PGE₂ may also influence periodontal tissue destruction by contributing to TH-17 development via the increased the expression of IL-23 (Gaffen and Hajishengallis, 2008). PGE₂ production is also likely to cause a predominance of the TH-2 response by modulating the production of IL-12 (Noguchi and Ishikawa, 2007, Gaffen and Hajishengallis, 2008).

Elevated levels of PGE₂ in inflamed periodontal tissues have been observed compared to healthy tissues (Ohm et al., 1984). Other studies have also reported significantly higher GCF levels of PGE₂ in patients with periodontal disease compared to healthy controls (Offenbacher et al., 1984, Kardesler et al., 2008). Increased GCF levels of PGE₂ have also been induced using an experimental gingivitis model, with levels doubling after 4 weeks compared with baseline (Heasman et al., 1993). Additionally, Offenbacher et al. (1986) were able to reliably predict periodontal attachment loss using PGE₂ levels in GCF. In the latter study, periodontitis patients with PGE₂ GCF levels two standard deviations above those of the healthy control were 47 times more likely to experience disease progression (Offenbacher et al., 1986). Another recent study also found high sensitivity and specificity of salivary IL-1 and PGE₂ in identifying periodontitis, with a decrease in both mediators after periodontal treatment, supporting their potential use as diagnostic biomarkers (Sánchez et al., 2013).

Prostaglandin $F_{2\alpha}$ [$PGF_{2\alpha}$] levels in gingival tissues and GCF have also been positively correlated with the severity of periodontitis (Vardar et al., 2005). It has been shown to upregulate IL-6 production in IL1- β - and TNF- α - stimulated human gingival fibroblasts, and is possibly involved in the pathogenesis of periodontitis (Noguchi et al., 2001a). Further studies indicate that $PGF_{2\alpha}$ can enhance the production of MMP-1 and ICAM-1 expression in human gingival fibroblasts (Noguchi et al., 1999, Noguchi et al., 2001b).

Further evidence for the role of prostaglandins in periodontal disease comes from clinical trials that investigated the use of non-steroidal anti-inflammatory drugs [NSAIDS] as host-modulatory therapy (*q.v.* Section 1.5).

1.4.4.2 Leukotrienes

Leukotrienes are predominantly produced by inflammatory cells (PMNLs, macrophages and mast cells) and have a wide range of biological effects as well as having a role in inflammatory diseases, including asthma, cardiovascular disease and cancers (Peters-Golden and Henderson Jr, 2007). Leukotrienes promote the accumulation of leukocytes at sites of inflammation, mediating the adhesion, migration, activation and survival of these cells (Peters-Golden and Henderson Jr, 2007). The leukotrienes LTC_4 , LTD_4 and LTE_4 increase vascular permeability and promote hypersensitivity (Schmitz and Ecker, 2008).

Leukotriene B_4 [LTB_4] may contribute to tissue destruction in chronic inflammatory conditions by increasing vascular permeability, mediating the activation and chemotaxis of PMNLs, generation of reactive oxygen species and release of lysosomal enzymes (Schmitz and Ecker, 2008, Das, 2011). The levels of LTB_4 in GCF have been shown to increase with the severity of periodontal disease, with an associated decrease after non-surgical periodontal therapy (Pradeep et al., 2007). LTB_4 has also been shown to play a significant role in alveolar bone loss (Meghji et al., 1988, Vardar et al., 2005), and cell culture studies suggest a direct effect on bone resorption through an increase in osteoclast numbers or activity (Hikiji et al., 2008).

1.5 Anti-inflammatory Approaches in Periodontal Therapy

Conventional periodontal therapy has focused on mechanical debridement to control the bacterial burden that drives the inflammatory response. While this approach is usually effective at reducing inflammation and improving clinical parameters (Cobb and Jeffcoat,

2002), a proportion of patients will be unresponsive to treatment or susceptible to disease progression and subsequent tooth loss (Hirschfeld and Wasserman, 1978, McFall, 1982, Lindhe and Nyman, 1984, Pearlman, 1993). Studies that investigate microbiological outcomes also indicate that it is not possible to completely eliminate putative periodontal pathogens (Renvert et al., 1990, Haffajee et al., 1997, Haffajee et al., 2006) and that recolonisation of the subgingival environment occurs following therapy (Magnusson et al., 1984). The long-term success of conventional periodontal therapy is therefore dependent on regular periodontal maintenance and a high level of oral hygiene to continually disturb the biofilm (Axelsson and Lindhe, 1981, Lindhe and Nyman, 1984, Axelsson et al., 2004).

As well as established therapy directed at controlling the bacterial challenge (mechanical debridement, chemical plaque control, antibiotics) and addressing modifiable risk factors (smoking cessation, diabetic control), host modulation has emerged as an additional therapeutic approach. This strategy is established for the treatment of inflammatory and autoimmune diseases (Kantarci et al., 2006) and aims to reduce tissue destruction and stabilise the periodontium by downregulating the destructive aspects of the host inflammatory response, and by upregulating its protective or regenerative components (Preshaw, 2008).

Current evidence supports the use of therapeutic approaches that target the inflammation associated with periodontitis. The study of subjects using non-steroidal anti-inflammatory drugs for systemic inflammatory conditions suggests that anti-inflammatory agents may be beneficial in the treatment of periodontitis. One case controlled study reported significantly lower gingival index scores and mean pocket depths for the test group taking NSAIDS compared to the control, although there was considerable variation in the drug regime (Waite et al., 1981). This led to an early animal study indicating that the daily administration of indomethacin diminished the initial inflammatory response, delayed the onset of bone resorption and reduced the total amount of bone loss in ligature-induced periodontitis (Nyman et al., 1979). Subsequent research in animals (Williams et al., 1988, Howell et al., 1991, Offenbacher et al., 1992) and humans (Williams et al., 1989, Jeffcoat et al., 1991, Jeffcoat et al., 1995) showed that daily administration of systemic NSAIDS could significantly decrease alveolar bone loss. A meta-analysis by Reddy et al. (2003) also confirmed the ability of NSAIDS to slow the rate of periodontitis, although the need for large multi-centre trials was highlighted. However, some reports also indicate that

periodontitis patients can experience a return to or ‘rebound’ acceleration of the pre-treatment rate of bone loss when these drugs are discontinued (Preshaw, 2008).

Acetylsalicylic acid, also known as aspirin, has long been recognised for its anti-inflammatory, analgesic, antipyretic and antiplatelet action. Cross-sectional data suggests that there may be some benefit of low-dose aspirin in periodontal health, with a small but statistically significant reduction in attachment loss in males aged over 50 years taking aspirin compared to non-aspirin takers (Drouganis and Hirsch, 2001). Limited clinical data also indicates a benefit, with a small split-mouth trial reporting a trend for improved clinical parameters with non-surgical debridement plus adjunctive aspirin (Flemmig et al., 1996). More recently, a daily 325 mg dose of aspirin following debridement was shown to significantly improve the clinical outcomes of smokers compared to a group that received debridement with placebo (Shiloah et al., 2014). Aspirin is unique among the NSAIDs because its actions are not limited to the established blocking of pro-inflammatory eicosanoids. Importantly, modification of the cyclo-oxygenase system by aspirin also leads to generation of the specialised pro-resolution lipid mediators that are usually only produced in the late stages of inflammation (*q.v.* Section 1.7.6).

To date, a wide range of other host modulatory agents have been investigated for use as monotherapy or adjunctive treatment for periodontal disease, including selective COX-2 inhibitors (Bezerra et al., 2000, Yen et al., 2008); bisphosphonates (Reddy et al., 1995, Rocha et al., 2001, Lane et al., 2005); statins (Saver et al., 2007); as well as tetracycline (Golub et al., 1983, Caton et al., 2000) and macrolide antibiotics (Bartold and Van Dyke, 2013, Gannon et al., 2013). Additionally, there is potential for novel therapies that target the RANKL-OPG system (Yuan 2011); pro-inflammatory cytokines (Assuma et al., 1998, Delima et al., 2001); and cell signalling pathways including NF- κ B, mitogen activated protein kinase [MAPK] and janus tyrosine kinase-signal transducer and activator of transcription [JAK-STAT] (Ambili et al., 2005, de Souza et al., 2012). However, many of these drugs are associated with significant adverse side effects that preclude their use in periodontal therapy, including immunosuppression, haemorrhage, gastrointestinal problems, and renal and hepatic impairment (de Souza et al., 2012). Furthermore, the discovery that the resolution phase of inflammation is an active process triggered by endogenous lipid mediators has questioned the use of drugs that act by the non-specific blocking of host immuno-inflammatory pathways (*q.v.* Section 1.7.6).

1.6 Long Chain n-3 Fatty Acids

Dietary polyunsaturated fatty acids are incorporated into the phospholipid of the cell membranes where they act as precursors for lipid mediators involved in cell signalling, gene expression and inflammation as well as contributing to cell membrane integrity and fluidity (Dawson et al., 2014, Silva et al., 2014). The omega-6 polyunsaturated fatty acids contain the n-6 double bond, while the omega-3 variety have both n-6 and n-3 double bonds. The ratio of these fatty acids in the tissues is largely determined by their proportions in the diet, and western diets are typically high in sources of the n-6 fatty acid linoleic acid (soy, corn and sunflower oils) which are converted to arachidonic acid (James et al., 2000, Cleland et al., 2005). Thus, in a typical western diet around 10-20% of fatty acids are arachidonic acid, 0.5-1% eicosapentaenoic acid [EPA] and 2-4% docosahexaenoic acid [DHA] (Dawson et al., 2014).

The long-chain n-3 polyunsaturated fatty acids [LCn3PUFAs] are considered essential dietary components as their synthesis in humans is limited (Yates et al., 2014). Oily fish are a rich source of LCn3PUFAs, with a typical serving of salmon providing 1.5 g of EPA plus DHA, and one typical serving of mackerel providing up to 3 g of these fatty acids (Calder, 2009). Fish and seafood however contribute to only a small proportion of the total fat intake in an average western diet. In a study that assessed the intake of fatty acid levels in the Australian population, fish and seafood contributed only 3% of the total fat intake (Meyer et al., 2003). The mean adult n-3 PUFA intake was 1.36 g per day of which 0.189 g comprised LCn3PUFA (0.056 g of EPA and 0.026 g DPA, 0.106 g DHA). This is in contrast to a much higher mean intake of 10.9 g per day for n-6 PUFAs. Furthermore, median intakes revealed that 50% of the Australian population is consuming no more than 0.03 g of LCn3PUFA per day (Meyer et al., 2003). Much higher intakes have been reported within the population of Japan who consume 1.6 g of LCn3PUFA per day (Sugano, 1996); and the Greenland Eskimo population who derive 14% of total fatty acids as LCn3PUFA per day (Bang et al., 1980). Varying levels of these fatty acids have been reported for the diets of the US and European countries (Astorg et al., 2004).

Current Australian guidelines recommend a daily intake of 500 mg EPA plus DHA for people without cardiovascular disease, while those with a history of cardiovascular should aim for 1 g per day of LCn3PUFAs (NHFA, 2008). The World Health Organisation have also recommended a daily intake of 200-500 mg of EPA and DHA for the prevention of

other chronic diseases (Silva et al., 2014). Typical fish oil preparations are rich sources of LCn3PUFAs and have an important role in achieving these recommendations. The best biomarkers of LCn3PUFA intake are their levels present in functional pools, such as plasma, cell membranes and adipose tissue (Silva et al., 2014). A recent RCT found that the level of DHA and EPA in platelets and mononuclear cells were the most reliable biomarkers to assess changes in intake (Browning et al., 2012). Incorporation of EPA and DHA into human immune cells reaches its peak within 1-4 weeks of increased consumption (Healy et al., 2000, Calder, 2013), while maximal incorporation into erythrocyte phospholipid can take around 6-months (Brown et al., 1991). Measuring the levels of LCn3PUFA acids in erythrocyte membrane phospholipid is widely utilised in the literature to indicate intake and as a method of checking compliance with supplementation.

1.7 Anti-inflammatory Mechanisms of Long-chain n-3 Fatty Acids

Despite a long history of research with long-chain n-3 fatty acids in various systemic diseases, the cellular and molecular mechanisms underlying their host modulatory action are still largely unknown (Serhan, 2007). However, several established and novel mechanisms for their anti-inflammatory action have been described. Interestingly, a recent microbiological study also demonstrated a broad range of antibacterial activity for both EPA and DHA, including the inhibition of putative periodontal pathogens, such as *P. gingivalis*, *F. nucleatum* and *P. intermedia* (Choi et al., 2013). Future therapies based on LCn3PUFAs may therefore offer a combined anti-inflammatory and antimicrobial approach for the treatment of periodontitis.

1.7.1 Effect on Eicosanoid Production

The arachidonic acid-derived eicosanoids have predominantly pro-inflammatory effects. Accordingly, both animal and human studies indicate a positive correlation between the amount of AA in inflammatory cell phospholipid and their ability to produce pro-inflammatory lipid mediators such as PGE₂ and LTB₄ (Kelley et al., 1998, Peterson et al., 1998). However, COX-2 can accept a wide range of alternative fatty acids as substrates (Vane et al., 1998), and an increased dietary intake of long-chain n-3 PUFAs such as EPA and DHA results in a lower proportion of AA in inflammatory cell phospholipid (Calder, 2006). A diet rich in n-3 PUFAs can therefore compete for AA at two levels; firstly for the incorporation into cell membrane phospholipid, reducing the proportion of substrate

available for AA-derived eicosanoids; secondly, by competing as a substrate for the COX and LOX pathways (Bagga et al., 2003).

Furthermore, the utilisation of EPA by the COX and LOX pathways leads to the production of EPA-derived eicosanoids that have reduced pro-inflammatory effects compared to AA-derived eicosanoids. Accordingly, EPA-derived PGE₃ was found to induce significantly lower expression of COX-2 and macrophage IL-6 secretion compared to the PGE₂, possibly due to differences in their half-lives or affinity for the EP receptors (Bagga et al., 2003, Calder, 2013). Thus, while PGE₂ mediated COX-2 expression can result in a positive feedback loop that amplifies the inflammatory response, PGE₃ may attenuate the inflammatory process by decreasing COX-2 activity and PGE₂ synthesis (Bagga et al., 2003). A recent study indicates that AA-derived prostaglandin PGD₂ facilitates neutrophil transmigration across the endothelial cell layer (Tull et al., 2009). However, PGD₃, the alternative series prostaglandin derived from EPA antagonised the PGD₂ receptor (Tull et al., 2009). Dietary EPA therefore has the potential to modulate inflammation by inhibiting neutrophil infiltration into inflamed sites through the action of PGD₃.

The AA-derived LTB₄ exhibits 10- to 30-fold greater chemotactic potency for human neutrophils compared to EPA-derived leukotriene LTB₅ (Goldman et al., 1983). Furthermore, the PMNL chemotaxis induced by LTB₄ is inhibited by the effects of LTB₅ without altering monocyte chemotaxis (Payan et al., 1986).

In summary, the eicosanoids derived from n-6 fatty acids seem to be associated with more pro-inflammatory actions than those derived from n-3 fatty acids. However, the assumption that the biological activity of the EPA-derived eicosanoids is always less than AA-derived products may be misleading since PGE₂ and PGE₃ have shown equivalent inhibition of IL-1 β and TNF α secretion in stimulated mononuclear cells (Miles et al., 2002). Furthermore, PGE₂ and PGD₂ released in the initial phase of inflammation are important in the subsequent resolution by activating the neutrophils class, leading to the production of lipoxins instead of LTB₄ (*q.v* Section 6.1).

1.7.2 Effect on Oxidative Stress

The effect of LCn3PUFAs supplementation on reactive oxygen species [ROS] and antioxidants are considered an important mode of action (Dawson et al., 2014). Reactive oxygen species such as hydrogen peroxide [H₂O₂], superoxide [O₂⁻] and the hydroxyl radical [OH[·]], are highly reactive molecules containing oxygen, formed as metabolic by-products. While low levels of ROS are required for several cell signalling pathways, such as ERK, p38 MAPK and tyrosine phosphatases, their continuous or uncontrolled production can cause cellular damage known as oxidative stress (Galli et al., 2011). Neutrophil generation of ROS through the action of the NADPH oxidase system is a major method of microbial killing but has the potential to cause secondary damage to host tissues (Nussbaum and Shapira, 2011). ROS can damage tissues directly, but may also activate redox-sensitive pro-inflammatory gene transcription factors, such as NF-κB, and activating protein-1 (Chapple and Matthews, 2007). By upregulating RANKL and TNF-α expression via NF-κB activation, ROS play an important role in the formation, survival and resorbing activity of osteoclasts (Galli et al., 2011). Tissue damage by oxidative stress has therefore been implicated as a factor in the pathogenesis of periodontitis. Another study demonstrated that 4 weeks of fish oil supplementation (equivalent of 5.4 g EPA and 3.2 g DHA) decreased superoxide generation in human neutrophils independent of the COX pathway or the release of lysosomal enzymes (Luostarinen and Saldeen, 1996). Similarly, Fisher et al. (1990) reported decreased hydrogen peroxide production by stimulated human monocytes following supplementation with 6 g/day of n-3 PUFAs for 6 weeks. However, other studies indicate that changes in neutrophil and monocyte fatty acid composition resulting from low to moderate amounts of dietary n-3 PUFAs may not be accompanied by changes in ROS production or other cell functions, such as chemotaxis and phagocytosis (Healy et al., 2000, Kew et al., 2003). The effects of LCn3PUFAs on the generation of reactive oxygen species are therefore probably less important than other anti-inflammatory and pro-resolution mechanisms.

1.7.3 Effect on Pro-inflammatory Cytokines

Inhibition of pro-inflammatory cytokines may be one mechanism for the beneficial effects of n-3 fatty acids on chronic-inflammatory diseases. Feeding studies using the rat model indicate that an increase in dietary EPA and DHA can reduce the secretion of pro-inflammatory cytokines in stimulated *ex-vivo* macrophages (Calder, 2009). These findings are confirmed by human studies, where the production of TNF-α and IL-1β by LPS-

stimulated mononuclear cells was significantly reduced by fish oil supplements (Endres et al., 1989, Caughey et al., 1996). These results demonstrate an inverse relationship between EPA content of mononuclear cell phospholipid with the production of these pro-inflammatory cytokines.

While most studies have examined the effects of dietary EPA, a few studies have also shown a reduction in pro-inflammatory cytokine levels with DHA supplementation (Kelley et al., 1999, Vedin et al., 2008). The results of Kelley et al. (1999) suggest less potent anti-inflammatory effects for DHA supplementation (6 g/day for 6 weeks) compared to studies examining EPA, with decreased production of TNF- α and IL-1 β by 20% and 35%, respectively. Conversely, Zhao et al. (2004) showed that DHA decreased TNF- α production to the same extent as EPA (by 40%). Other cell culture studies have confirmed that both EPA and DHA can inhibit the production of the classic pro-inflammatory cytokines IL-1 β , TNF- α and IL-6 in a range of cell types (Miles and Calder, 2012).

1.7.4 Nuclear Factor Kappa B and Other Transcription Factors

There are several mechanisms in which EPA and DHA can modulate the production of pro-inflammatory cytokines at the transcriptional level. NF- κ B has a central role in pro-inflammatory signalling pathways, controlling several cytokines (IL-1, IL-2, IL-6, IL-12, TNF- α), chemokines (IL-8, MIP-1 α , MCP1), adhesion molecules (ICAM, VCAM and E-Selectin) and enzymes (COX-2) (Schmitz and Ecker, 2008, Sculley, 2014). NF- κ B is kept in an inactivated form within the cytoplasm by an inhibitory subunit of NF κ B [I κ B]. In response to external inflammatory stimuli including LPS (via TLR4), I κ B is phosphorylated as part of a signalling cascade, allowing the migration of NF- κ B to the nucleus where it can activate the transcription of target genes (Zhao et al., 2004). Several studies have reported that EPA and DHA can inhibit the production of TNF- α by preventing NF- κ B activation (Lo et al., 1999, Novak et al., 2003, Zhao et al., 2004). This inhibition of NF- κ B activation was associated with decreased I κ B phosphorylation. Additionally, NF- κ B is a redox-sensitive transcription factor that can be activated by low concentrations of hydrogen peroxide (Sanlioglu et al., 2001). Therefore, another way in which n-3 PUFAs can impair NF- κ B activation and associated production of pro-inflammatory mediators is by the reduced production of reactive oxygen species (*q.v.* Section 1.7.2).

Other mechanisms in which n-3 PUFAs can inhibit NF- κ B activation have also been suggested. Recent studies indicate that n-3 PUFAs induce production of another transcription factor with an anti-inflammatory mode of action called peroxisome proliferator-activated receptor gamma [PPAR γ] (Schmitz and Ecker, 2008, Calder, 2013). This transcription factor plays a major role in lipid metabolism and has demonstrated anti-inflammatory effects by directly regulating inflammatory gene expression (Berger and Moller, 2002), as well as interfering with the activation of NF- κ B (Calder, 2013).

A further anti-inflammatory mode of action of LCn3PUFAs is via the cell surface G-protein coupled receptor called GPR120. G-protein coupled receptors have an important role in cell signalling and are highly expressed in pro-inflammatory macrophages (Oh et al., 2010). Activation of this receptor with LCn3PUFAs or a chemical agonist has been shown to inhibit both the TLR2/3/4 and TNF- α pro-inflammatory cascades by impairing NF- κ B activation (Oh et al., 2010). Furthermore, n-3 PUFA-rich diet inhibited LPS-induced tissue inflammation in the mouse model, but the supplementation had no effect in GPR120 knockout mice. Thus, GPR120 is a functional receptor for LCn3PUFAs with the ability to mediate potent anti-inflammatory effects.

1.7.5 Plasma Membrane Organisation

EPA and DHA can influence cell signalling by effecting the physical properties of the plasma membrane, including fluidity, compressibility, phospholipid flip-flop, acyl chain packing, elasticity and lipid domain formation (Shaikh et al., 2012). Additionally, the effect of n-3 PUFAs on early immune cell signalling involves the impaired production of signalling platforms called lipid rafts (Shaikh et al., 2012, Calder, 2013). Lipid rafts are distinct sphingolipid- and cholesterol-rich regions in the membrane that facilitate the interaction of various signalling proteins (Calder, 2013). This spatial organisation and compartmentalisation of signalling proteins within these membrane platforms are thought to be essential for signal transduction (Zeyda and Stulnig, 2006). Consequently, changes in T-cell function associated with lipid rafts alterations have been demonstrated in both cell culture (Stulnig et al., 1998) and animal feeding studies (Fan et al., 2004).

1.7.6 Pro-resolution Lipid Mediators

The study of spontaneously resolving inflammatory lesions has highlighted several discrete and well-defined stages from initiation to resolution, with each stage characterised by

specific classes of eicosanoids. Levy et al. (2001) examined levels of eicosanoids in experimental inflammatory exudates and reported higher levels of the pro-inflammatory mediators PGE₂ and LTB₄ in the early inflammatory lesion which were associated with neutrophil infiltration. Reduced levels of these mediators coincided with higher levels of the lipoxin LXA₄ and an associated reduction in neutrophil recruitment. Furthermore, the administration of a metabolically stable LXA₄ analogue to the experimental exudates early on in the inflammatory response resulted in the cessation of neutrophil infiltration. Taken together, these findings indicate that the profile of lipid mediators changes during the progression of a spontaneously resolving inflammatory lesion, culminating with the production of counter-regulatory mediators that promote resolution. The change in neutrophil phenotype to generate these protective mediators has been termed a 'class switch' (Levy et al., 2001). Aspirin is unique among the NSAIDs because its actions are not limited to the long established blocking of pro-inflammatory eicosanoids. Hence, aspirin also has the ability to generate pro-resolution lipid mediators that are usually only produced in the late stages of inflammation (Serhan, 2005a).

The arachidonic acid-derived lipoxins were the first lipid mediators recognised to have pro-resolution action, and recent research has identified other classes of lipid mediators with anti-inflammatory and pro-resolving actions, including the resolvins, protectins and maresins. The discovery of these pro-resolution lipid mediators represents a paradigm shift in the understanding of the inflammatory response, especially the realisation that resolution is an active biological process, characterised by an agonist-mediated return to tissue homeostasis (Serhan et al., 2007, Van Dyke, 2011). This is in contrast to previous models that involved a passive decrease in pro-inflammatory mediators during the resolution of inflammation. While the current understanding of chronic inflammation recognised the role of excessive pro-inflammatory mediators, the dysregulation of endogenous pro-resolving pathways may also be an important underlying factor in chronic inflammation and periodontitis pathogenesis.

Localised aggressive periodontitis [LAP] is characterised by rapid and advanced attachment loss and bone destruction affecting the first molars and incisors (Armitage, 1999). These clinical features may be in part due to defective neutrophils that exhibit a heightened pro-inflammatory phenotype, resulting in enhanced host-mediated tissue destruction (Kantarci et al., 2003). Although these patients represent a group that is highly susceptible to periodontal tissue destruction, the localised connective tissue destruction

mediated by neutrophils is an integral component of both chronic and aggressive periodontitis (Smith et al., 2010). A recent study by Fredman et al. (2011) observed impaired phagocytotic activity in the macrophages of LAP subjects, although treatment with exogenous RvE1 was able to restore phagocytic activity to a level comparable with periodontally healthy subjects. Aggressive periodontitis was also associated with alterations in the levels of metabolites from the lipoxygenase pathway, with increased synthesis of 5-, 12- and 15-LOX products, suggesting an imbalance between pro-inflammatory and pro-resolving mediator production (Fredman et al., 2011). Taken together, their results confirm that immune cells from LAP patients have a hyperactive phenotype and a reduced capacity to produce pro-resolving mediators. These findings are supported by Elabdeen et al. (2013), who demonstrated a lower ratio of precursors of pro-resolution to pro-inflammatory lipid mediators in the GCF of aggressive periodontitis subjects compared with healthy controls. Further research is therefore warranted to establish the role of impaired resolution pathways and reduced clearance of the inflammatory lesion in the pathogenesis of both aggressive and chronic periodontitis.

Current understanding of the mechanisms and components that underlie the resolution of inflammation has important implications for the development of new therapies that aim to modulate the inflammatory response. Although pro-inflammatory eicosanoids produced by COX-2 are widely regarded as harmful, current anti-inflammatory drugs such as NSAIDs and selective COX-2 inhibitors also impair the endogenous resolution pathways, prolonging the healing process (Schwab et al., 2007, Serhan, 2007). Therefore future host modulation therapies could employ endogenous pro-resolution pathways by using dietary supplementation or pro-resolving mimetics. These therapies should not be restricted by the limitations of anti-inflammatory drugs that act exclusively as antagonists. Specifically, they would achieve greater precision in the targeting of biological pathways, as well as eliminating unwanted immunosuppressive effects (Serhan and Chiang, 2013).

Research of pro-resolving mediators is still in its infancy. Nevertheless, clinical evaluation of resolvins-based therapies has begun for the treatment of several inflammatory diseases, including asthma, inflammatory bowel diseases, rheumatoid arthritis and cardiovascular diseases (Lee, 2012). Although resolvins demonstrate powerful biological activity, they are also relatively unstable and susceptible to rapid metabolic inactivation due to a large number of double bonds and hydroxyl groups (Lee, 2012). This has led to the development of synthetic RvE1 analogues with enhanced resistance to oxidation, and thus

greater potential for clinical use. The analogue 19-(*p*-fluorophenoxy)-RvE1 methyl ester in particular demonstrated greater stability but with similar *in vivo* activity to RvE1 (Zhang and Spite, 2012). Phase II clinical trials have been completed that evaluated a synthetic RvE1 analogue for the treatment of keratoconjunctivitis sicca, with significant improvements reported for the outcomes of this common chronic inflammatory condition (Lee, 2012). Furthermore, Phase I clinical trials have also been completed to examine the safety of orally administered RvE1 analogue in healthy subjects (RX-10001, Resolvyx Pharmaceuticals Inc., Massachusetts, US; Trial identifier NCT00941018; URL: <http://www.clinicaltrials.gov>). Although pro-resolution lipid mediators have not been evaluated for the treatment of periodontitis in humans, dietary supplementation with long-chain n-3 PUFAs and aspirin has been shown to increase circulating levels of resolvins (Serhan et al., 2002). A model of periodontitis and the effects of pro-resolution lipid mediators and other host modulatory agents are illustrated in Figure 1.2.

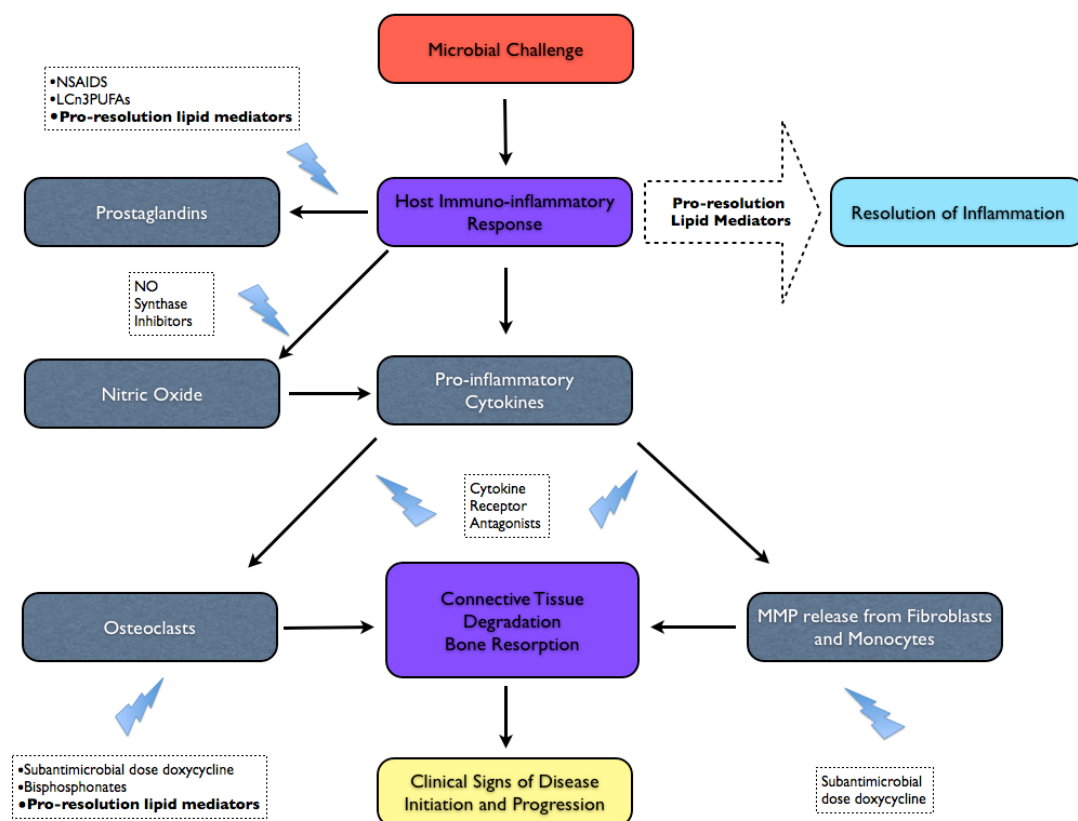


Figure 1.2. Model of periodontitis pathogenesis and host modulation approaches.

1.7.6.1 Lipoxins

As well as the classic eicosanoids, under certain conditions AA is also metabolised by lipoxygenase pathways to form other metabolites: 5-hydroxyeicosatetraenoic acid, 5-oxo-eicosatetraenoic acid and lipoxins (Peters-Golden and Henderson Jr, 2007). Lipoxins are also derived from arachidonic acid but are recognised to have anti-inflammatory and pro-resolving actions, in contrast to the pro-inflammatory eicosanoids previously discussed (Serhan, 2005a) (*q.v.* Section 1.4.4). The activity of lipoxins thus includes modulation of pro-inflammatory cytokine and chemokine and expression, inhibition of PMNL recruitment and enhancing the phagocytosis of apoptotic leucocytes by macrophages (Levy et al., 2001).

Lipoxins comprise two positional isomers, lipoxin (LX) A₄ and LXB₄, and their biosynthesis are carried out by multiple pathways via the action of two or more lipoxygenases. It has been proposed that lipoxins are produced via transcellular biosynthesis during cell-cell interactions, such as those that take place between neutrophils and platelets (Serhan and Sheppard, 1990). Additionally, other routes in which lipoxins are synthesised are by mucosal and vascular cell interactions and membrane phospholipid priming of 15-HETE (Serhan, 2007). The production of lipoxins by the action of 12-lipoxygenase on 5-HPETE is enhanced by the action of aspirin, leading to the synthesis of aspirin-triggered lipoxins [ATLs] such as 15-epi-LXA₄ which show greater activity than their native counterparts (Serhan, 2005b). Studies using a murine model have also demonstrated enhanced production of 15-epi-LXA₄ by statins (Spite and Serhan, 2010).

Serhan et al. (2003) demonstrated reduced inflammation and tissue damage in transgenic rabbits that overexpressed 15-lipoxygenase type 1, producing increased levels of lipoxins. Moreover, the topical application of a stable LX₄ analogue was able to significantly reduce leucocyte infiltration, inflammation and bone loss in a *P. gingivalis*-induced periodontitis model. These findings suggest that lipoxins and the lipoxygenase pathway are potential targets for host modulation therapy in periodontitis (Serhan et al., 2003)

1.7.6.2 Resolvins

Resolvins are potent anti-inflammatory and pro-resolving mediator acids, derived from EPA or DHA. Those produced by the metabolism of EPA are known as the E-series resolvins, whereas the D-series resolvins are derived from DHA. Accordingly, the

enhanced synthesis of resolvins, as well as their increased tissue levels have been demonstrated in human (Mas et al., 2012) and animal feeding studies (Hong et al., 2003) following dietary supplementation with fish oil.

E-series resolvins were the first of these novel di- and tri- hydroxyl containing compounds to be isolated after examining self-limiting inflammatory exudates in the murine model (Serhan et al., 2000). The authors reported changes in the levels of distinct bioactive lipids as the inflammatory lesions progressed and subsequently returned to homeostasis. Human endothelial cells treated with aspirin were found to convert EPA to 18*R*-hydroxy-EPA and 18*S*-hydroperoxy-EPA via COX-2 (Serhan, 2007, Freire and Van Dyke, 2013). These intermediary metabolites were subsequently taken up by human neutrophils and metabolised to resolvin E1 and resolvin E2 by 5-LOX (Serhan, 2007, Freire and Van Dyke, 2013). As monocytes and macrophages are also known to express the 5-LOX enzyme, they could also contribute to resolvin production (Gao et al., 2013). These new compounds later proved to be potent inhibitors of neutrophil transmigration and infiltration *in vivo*.

In contrast to resolvin E1, which accumulates in the resolution phase of an inflammatory lesion, resolvin E2 [RvE2] has been identified during the initiation phase when neutrophils predominate (Oh et al., 2012). This suggests that RvE2 may be protective throughout the initiation and resolution phases of inflammation. Corresponding to other lipid mediators, RvE2 has demonstrated both anti-inflammatory (impaired PMNL chemotaxis) and pro-resolving action (enhanced non-phlogistic clearance by macrophages) (Oh et al., 2012). These results also suggest that RvE2 shares some common receptors with RvE1 and has similar potency in limiting neutrophil infiltration. RvE2 was however only a weak activator of the receptor ChemR23 (Oh et al., 2012).

Six structurally distinct forms of D series resolvins (RvD1-RvD6) have been identified. DHA is converted by the aspirin-modified COX-2 and 15-LOX enzymes to 17*R*-hydroperoxy-DHA and 17*S*-hydroperoxy-DHA respectively (Zhang and Spite, 2012). These products are then converted by 5-LOX via a number of intermediates into resolvin D1 and its aspirin-triggered derivative (Zhang and Spite, 2012). Importantly, both E- and D-series resolvins can also be synthesised in the absence of aspirin by lipoxygenase dependent pathways (Yates et al., 2014).

The individual resolvins demonstrate distinct and diverse biological activity which can be explained by their unique structure and precise stereochemistry (Zhang and Spite, 2012). Further characterisation has also revealed that they target a range of different receptors. Recent studies have revealed that the potent actions of RvE1 were in part due to activation of the specific G-protein coupled receptor ChemR23 which is closely related to the lipoxin A4 receptor ALX/FPR2 (Arita et al., 2007). Binding of the ChemR23 receptor by RvE1 acts directly on dendritic cells, causing reduced migration and IL-12 production, as well as increasing MAPK activation in peripheral blood mononuclear cells. RvE1 was also shown to partially antagonize LTB₄ by interacting with its receptor (BLT1) on neutrophils (Arita et al., 2007). On the contrary, the effects of RvD1 are mediated via interaction with both ALX/FRP2 and G-protein coupled receptor 32 (Freire and Van Dyke, 2013).

The resolvins family has demonstrated profound anti-inflammatory and pro-resolving actions in animal models of systemic inflammatory diseases including periodontitis (*q.v.* Section 1.10.1) arthritis, obesity, diabetes, colitis and asthma (Zhang and Spite, 2012, Calder, 2013). By binding to the aforementioned receptors they demonstrate multiple effects in a range of cell types. However, their activity can be divided into two major functions: a counter-regulatory action that stops pro-inflammatory signals and the promotion of resolution.

Many of the counter-regulatory effects of the resolvins are due to the modulation of neutrophil function, including inhibition of adhesion receptors expression, transmigration, chemotaxis, defensin secretion, as well as the expression of chemokines and their receptors (Zhang and Spite, 2012). Additionally, they promote resolution by the activation of non-phlogistic monocytes and macrophage that have a non-inflammatory phenotype. These resolvins-activated cells show enhanced phagocytosis of apoptotic neutrophils and bacteria, together with increased migration to lymphoid tissue (Schif-Zuck et al., 2011). Moreover, they have a diminished response to TLR ligands as well as pro-inflammatory cytokines and chemokines, and do not express the usual pro-inflammatory mediators (Schif-Zuck et al., 2011). Resolvin enhanced macrophages are therefore important in facilitating clearance of the inflammatory lesion and return to homeostasis, without further exacerbating the host response. The anti-inflammatory and pro-resolution activity of resolvins and other specialised lipid mediators are summarised in Table 1.3.

Pro-resolution Lipid Mediator	Cellular Activity
Resolvin E1	<ul style="list-style-type: none"> • Inhibits neutrophil infiltration • Modulates chemokine/cytokine synthesis • Promotes healing of inflamed tissues and bone regeneration • Enhances phagocytosis • Activates lymphatic removal of phagocytes • Attenuates systemic production of C-reactive protein and interleukin-1 • Reduces eosinophil and lymphocyte recruitment • Regulates adipokines • Decreases inflammatory actions of cyclooxygenase-2 • Attenuate expression of the NF-κB gene • Lowers the number of monocytes • Increases CD55 expression on epithelial cells and PMNL clearance • Rescues impaired phagocytosis in LAP patient macrophages • Prevents rejection of allograft • Activates anti-apoptotic signals
Resolvin D1	<ul style="list-style-type: none"> • Inhibits neutrophil recruitment • Anti-hyperalgesic recruitment • Shortens resolution interval • Reduces oxidative stress-mediated inflammation • Attenuates agonist pain molecules • Induces macrophage phagocytosis • Stimulates the M2 macrophage phenotype • Temporally regulates microRNAs • Enhances microbial clearance • Reduces levels of prostaglandins and leukotrienes
Protectin D1	<ul style="list-style-type: none"> • Decreases inflammatory actions of cyclooxygenase-2 • Inhibits neutrophil infiltration • Modulates chemokine/cytokine synthesis • Regulates macrophage function • Upregulates CCR5 expression on apoptotic leukocytes • Inhibits pain signals
Maresin-1	<ul style="list-style-type: none"> • Reduces neutrophil numbers in exudate • Enhances macrophage phagocytic functions • Decreases PMNL transmigration

Table 1.3. Cellular actions of pro-resolution lipid mediators. Adapted from Freire and Van Dyke (2013).

In addition to the anti-inflammatory and pro-resolution action of resolvins, a direct action on osteoclast development and bone resorption has been demonstrated. Osteoclast cultures derived from mouse bone marrow revealed reduced differentiation and resorption pit formation following treatment with resolvin E1 (Herrera et al., 2008). Further *in vitro* investigation using murine osteoclasts has revealed that RvE1 inhibits the late stages of osteoclast maturation by targeting the osteoclast fusion protein DC-STAMP (Zhu et al., 2013). The binding of RvE1 to its receptors downregulated DC-STAMP expression by 65%, inhibiting the fusion of membranes in osteoclast precursors (Zhu et al., 2013).

A recent study also reported significantly enhanced expression of OPG by RvE1, although levels of RANKL remained unchanged (Gao et al., 2013). Therefore, by restoring a favourable RANKL/OPG ratio, RvE1 can directly affect bone by modulating osteoclast differentiation and bone remodeling. Taken together, these results suggest a potential for pro-resolving mediators as a therapeutic approach for inflammatory diseases with osteoclast-mediated bone destruction, such as periodontitis and arthritis. The cellular actions of resolvins may be responsible for the profound effect on bone regeneration observed in animal models (Hasturk et al., 2007, Gao et al., 2013) (*q.v.* Section 1.10.1).

The emerging field of epigenetics indicates that environmental factors can regulate gene expression by selectively activating or inactivating genes by post-transcriptional DNA methylation, histone modification and microRNAs [miRNAs] (Gomez et al., 2009). The latter offer another level of precise gene control in combination with transcriptional and other regulatory processes and may regulate up to 30% of all genes (Lewis et al., 2005). Environmental factors including dietary modification may therefore have a profound effect on epigenetic modification, influencing disease susceptibility (Bayarsaihan, 2011, Dawson et al., 2014). Recchiuti et al. (2011) recently identified several resolvins-dependent functions of miRNAs by investigating the effect of local Rv1D1 administration in self-limited murine peritonitis. RvD1 administration resulted in reduced PMNL infiltration by 25-50% as well as shortening the resolution interval in zymosan-stimulated inflammation. The differential regulation of specific miRNAs during inflammation and resolution was also highlighted with specific miRNAs associated with the initial inflammation compared with the resolving exudates. Further analysis identified specific RvD1-regulated miRNAs that favoured anti-inflammatory and pro-resolving actions (miR-146b and miR-219).

Regulation of miRNAs is also dependent on the interaction between RvD1 and specific G protein-coupled receptor, GPR32 and ALX/FPR2 (Recchiuti et al., 2011). Target genes for the RvD1-regulated miRNAs were identified as having key roles in inflammation and the immune response, including those associated with cytokines, chemokines and their receptors that are associated with leukocyte trafficking. Increased expression of miR-146b downregulated NF- κ B signaling and resulted in decreased levels of the chemokines IL-8 and RANTES which are important mediators of leukocyte migration. Additionally, miR-219 targeted 5-lipoxygenase and reduced leukotriene production. Thus, the regulation of specific miRNAs by the interaction of RvD1 with its receptors has a role in the resolution of inflammation by altering cytokines and proteins involved in the immune response.

More recently, the same group confirmed the role of RvD1 in the regulation of specific miRNAs (Krishnamoorthy et al., 2012). In this study, RvD1 administration was shown to significantly upregulate miR-208a and miR-219 *in vivo*, using the same transgenic murine model as the previous studies. Nevertheless, further examination of RvD1 interaction with its receptors (ALX/FPR2 and GPR32) using human macrophage highlighted some differences in miRNA expression compared with the *in vivo* murine model (Recchiuti et al., 2011). The authors speculate that these differences may be due to variation in species, signal-to-noise-ratio, or cell type between the experiments. More research is therefore required to elucidate the role of miRNA in the resolution phase of inflammation.

Many of the cited studies that have examined lipid-derived pro-resolution mediators have used murine models of sterile inflammation, particularly the zymogen-induced acute peritonitis model (Serhan et al., 2000, Serhan et al., 2006, Serhan et al., 2009, Zhang and Spite, 2012). However, given the bacterial aetiology that underlies periodontitis it is interesting to establish the influence of pro-resolution mediators on infection. RvE1 has no intrinsic antibacterial activity, and does not impede the growth of *P. gingivalis* as assessed by direct killing experiments (Hasturk et al., 2006). However, pro-resolution lipid mediators are able to exhibit indirect antimicrobial activity by enhancing the host immunoinflammatory response. A recent study by Serhan's group demonstrated the increased phagocytosis of *E. coli* by human macrophages and neutrophils due to RvD1 and RvD5 and protectin D1 [PD1] (Chiang et al., 2012). Furthermore, by using a model of self-limiting murine *E. coli* infection, they showed that these pro-resolving mediators enhance the containment of the infection and lowered the dose of ciprofloxacin required for bacterial clearance (Chiang et al., 2012). Similarly, the clearance of *Staphylococcus aureus* in murine skin infections by vancomycin was also enhanced by the administration of pro-resolution lipid mediators (Chiang et al., 2012). In summary, the beneficial effects of LCn3PUFAs in the treatment of periodontitis seen in preliminary clinical studies may in part be due to a heightened host antimicrobial response caused by resolvins and other pro-resolving lipid mediators.

1.7.6.3 Protectins

Protectins are another group of protective compounds derived from DHA in several immune cells including neutrophils, macrophage and T-cells. PD1 is also referred to as neuroprotectin D1 when produced by neural tissues and was first isolated from human

neutrophils *in vitro* and murine peritonitis exudates *in vivo* (Serhan et al., 2006). The biosynthesis of PD1 occurs via a lipoxygenase-mediated pathway that first produces a 17S-hydroperoxy-DHA intermediate that is rapidly converted into another 16-17-epoxide containing intermediate, before subsequent conversion to the trihydroxy-containing protectin (Hong et al., 2003). In addition to synthesis via the lipoxygenase pathways, an aspirin-triggered form of PD1 can be also be produced by cyclooxygenase and subsequent reactions (Serhan et al., 2006).

Early studies indicated that PD1 is an important mediator in the immune and central nervous system, with additional protective roles in the lung, kidney and liver, as well as retinal and brain tissues (Serhan et al., 2006, Serhan, 2007). Using the zymogen-stimulated murine peritonitis model, PD1 has been shown to significantly inhibit PMNL recruitment *in vivo*, with greater potency than the NSAID indomethacin (Hong et al., 2003, Serhan et al., 2006). Moreover, the local administration of PD1 after the initiation of inflammation was able to impair >90% of further leukocyte infiltration (Serhan et al., 2006). Their findings also confirmed that RvD1 and PD1 have additive but not synergistic actions when administered together, suggesting that they have activate different receptors (Serhan et al., 2006). Comparable with the resolvins, nanogram quantities of PD1 are also shown to increase macrophage ingestion of apoptotic neutrophils *in vivo* and *in vitro*, an important pro-resolution feature (Schwab et al., 2007).

Although PD1 has demonstrated powerful anti-inflammatory and pro-resolution activity, several biological actions have recently been attributed to one of its isomers, protectin DX, which differs in the geometry of the triene double bond (Balas et al., 2014). However, in the peritonitis model, this isomer was less potent than PD1 (Serhan et al., 2006). Further research is therefore needed to evaluate differences in chemical and biological activity between these two isomers.

1.7.6.4 Maresins

A novel resolution pathway has been recently identified that converts DHA into 7,14-dihydroxy-containing compounds via 14-lipoxygenase and several subsequent enzymatic steps (Serhan et al., 2009). One of these products showed potent anti-inflammatory and pro-inflammatory characteristics and was designated maresin 1 [MaR1] to acknowledge its isolation from macrophages. In the murine peritonitis model, MaR1 demonstrated

equivalent potency to RvE1 and PD1 in its ability to reduce neutrophil infiltration and promote phagocytosis in macrophages (Serhan et al., 2009). However, given that the individual resolvins and protectins have unique stereoselective actions and exhibit their anti-inflammatory and pro-resolving activity through specific receptors (Serhan et al., 2006, Arita et al., 2007), it is likely that these most recently discovered mediators also act via their own receptors.

In summary, previously accepted models of inflammation were characterised by passive termination corresponding to the gradual disappearance of local chemotactic stimuli and pro-inflammatory mediators. However, the research by Serhan's group at Harvard University has led to a paradigm shift in the understanding of the inflammatory process. It is now accepted that the resolution of inflammation is mediated by active cellular events and biochemical pathways that are initiated after the acute inflammatory challenge. Current knowledge of the inflammatory process recognises that the return to homeostasis is an active process driven by a new group of bioactive lipid mediators: resolvins, protectins and maresins. These compounds have both counter-regulatory and agonistic actions, which are characterised by the downregulation of neutrophil recruitment and the enhanced clearance of microbial products, apoptotic and necrotic cells from the inflammatory lesion. By promoting the early resolution of inflammation, these mediators can limit secondary tissue damage and oxidative stress associated with inflammation and infection (Serhan et al., 2009). The potent activity of pro-resolution lipid mediators may therefore explain many of the beneficial effects of LCn3PUFAs, and have great potential as new therapeutic approaches that are not limited by the adverse effects associated with current anti-inflammatory medication.

1.8 Fish Oil Therapy and Systemic Inflammatory Diseases

The health benefits of dietary LCn3PUFAs are widely recognised, with a large body of evidence to support their use as a host modulation therapy in the treatment of chronic inflammatory conditions. Currently, there is robust evidence to support the use of fish oil as a dietary intervention in cardiovascular disease and rheumatoid arthritis (for a recent review see Yates et al., 2014). However, there is conflicting data showing a clinical benefit in other conditions such as asthma and inflammatory bowel diseases, indicating that LCn3PUFAs may not be beneficial for all inflammatory conditions.

A large body of research has investigated whether fish consumption and fish oil supplementation can improve the outcomes of treatment for cardiovascular disease. The Diet And Reinfarction Trial (DART) examined the effects of dietary intervention in the secondary prevention of myocardial infarction (MI), and was the first large study of its kind (Burr et al., 1989). This trial reported a 29% decrease in the overall mortality in men who ate oily fish twice a week, corresponding to an equivalent of 500-800 mg/day of omega-3 fatty acids.

The results of the DART trial were later supported by subsequent clinical studies (Singh et al., 1997). In a large multi-centre study including 11,323 patients with a history of myocardial infarction, supplements containing 850-882 mg of EPA and DHA produced a clinically important and statistically significant reduction in the risks for overall and cardiovascular-associated death.

An early meta-analysis including data from 11 trials demonstrated a beneficial effect of both dietary and supplemental n-3 fatty acids, with a reduction in overall mortality, mortality due to MI, as well as sudden death in patients with coronary heart disease (Bucher et al., 2002). In contrast, a more recent meta-analysis found that n-3 fatty acid supplementation had no statistically significant association with a lower risk of all-cause mortality, cardiac death, sudden death, MI or stroke (Rizos et al., 2012). Conflicting data regarding the benefit of n-3 fatty acid supplementation on cardiovascular disease outcomes may be due to variation in the dosage and source of fatty acids, patient compliance with supplementation and/or the confounding effects of statins and other medication. In particular, inclusion of studies that investigate very low doses may lead to underestimation of treatment effect.

Several biological mechanisms have been proposed to explain the cardioprotective effects of fish oil, including triglyceride lowering properties, antiarrhythmic and antithrombogenic effects, inhibition of atherosclerosis and anti-inflammatory properties (Kris-Etherton et al., 2003). The anti-inflammatory properties of LCn3PUFAs are of particular interest since current understanding acknowledges a prominent role of inflammation in the pathophysiology of atherosclerosis. The recruitment of leukocytes and the expression of pro-inflammatory cytokines are features of early atheroma formation, and inhibition of pro-inflammatory mediators has been shown to impair atheroma formation in the murine model (Hansson et al., 2002, Libby, 2002).

Rheumatoid arthritis [RA] is characterised by chronic inflammation of the joint synovium, leading to destruction of cartilage and bone with subsequent joint pain and profound loss of function. Tissue destruction in RA is driven by pro-inflammatory cytokines and arachidonic acid-derived eicosanoids with infiltration of neutrophils, lymphocytes and macrophages into the inflammatory lesion (Yates et al., 2014). It is of interest to establish the effects of LCn3PUFA therapy on the outcomes of RA, given the common pathological features shared with periodontitis (Kaur et al., 2013). Emerging evidence also suggests that common pathological processes, in addition to genetic, environment or behavioural risk factors may underlie an association between the two diseases (de Pablo et al., 2009, Kaur et al., 2013). A recent systematic review including 23 randomised controlled trials [RCTs] reported a consistent but modest clinical benefit of fish oil supplementation on the outcomes of rheumatoid arthritis, including joint swelling and pain, duration of morning stiffness and NSAID sparing effects (Miles and Calder, 2012).

1.9 Safety of Fish Oil Therapy

Current guidelines advise that an intake of up to 3 g per day of LCn3PUFAs is Generally Recognised As Safe (GRAS), considering the effects of omega-3 fatty acids on glycaemic control in diabetics, risk of bleeding and LDL cholesterol. Common but mild side effects of fish oil supplements include gastro-intestinal disturbances, nausea and a fishy aftertaste (Kris-Etherton 2003). Although these side effects are relatively mild, they may affect compliance with supplementation. In the seminal randomised controlled GISSI-Prevenzione trial, 29% of participants had stopped taking the fish oil supplements at the end of the 3.5 year follow-up, although a similar level of compliance was observed in the control group taking vitamin E. The most common side effects reported in this study were gastrointestinal disturbances and nausea, experienced by around 5% of the fish oil group. However, side effects were reported as the reason for discontinuing supplements in only 3.8% of patients in the fish oil group and 2.1% in the vitamin E group.

1.9.1 Increased Risk of Bleeding

As outlined above, LCn3PUFAs compete as a substrate for the cyclo-oxygenase pathways leading into the production of alternative metabolites than the classical eicosanoids (*q.v.* Section 1.4.4). Accordingly, studies have confirmed a reduction in the synthesis of thromboxane A₂ and an associated reduction in platelet aggregation (Ågren et al., 1997).

The effect of LCn3PUFAs have therefore been long been recognised, and an early study in Greenland Eskimos, known for a diet rich in fish, observed a significantly prolonged bleeding time (Bang and Dyerberg, 1980). These observations have led to concerns regarding the increased risk of bleeding in patients taking fish oil supplements, although such concerns may not be supported by clinical data (Bays, 2007). Low level evidence in the form of individual case reports suggest that the close monitoring of these patients is advisable (Buckley et al., 2004, Holbrook et al., 2005). Guidelines from the American Heart Association recommend that patients taking in excess of 3 g of supplemental EPA plus DHA should be regularly reviewed by a physician due to increased risk of excessive bleeding in some individuals (Kris-Etherton et al., 2003). However, a number of clinical trials have demonstrated the safety of high dose LCn3PUFA supplementation, even in patients taking antiplatelet and anticoagulant medication such as aspirin and warfarin (Bays, 2007). A recent objective assessment of the evidence also found no clinically significant risk of bleeding in patients taking LCn3PUFA supplementation (Harris, 2007).

In summary, the benefits of fish oil for the treatment of inflammatory diseases seem to outweigh the hypothetical risk of increased bleeding. Nevertheless, it is prudent to liaise with the patient's physician to allow close monitoring, especially if there is concurrent antiplatelet or anticoagulant medication.

1.9.2 Methyl Mercury and Other Chemical Contaminants

Although beneficial effects of omega-3 fatty acids through the consumption of oily fish have been well documented, there are also concerns regarding potential exposure to chemical pollutants. Several toxic contaminants have been isolated from fish, most notably methyl mercury, dioxins, furans, dioxin-like polychlorinated biphenyls [PCBs] and polycyclic aromatic hydrocarbons [PAHs] (Domingo et al., 2007). However, the processing of fish oil effectively removes contaminants, producing supplements that contain virtually no methyl mercury and very low levels of organochloride contaminants (Kris-Etherton et al., 2003). Less stringently tested products may however contain appreciable amounts (Jacobs et al., 1998).

1.9.3 Association with Cancer Risk

In contrast to the published data supporting a range of health benefits associated with fish oil supplements, a recently published large scale prospective case-controlled cohort study

reported increased prostate cancer risk among men with high blood concentration of long-chain n-3 fatty acids (Brasky et al., 2013). They found the mean percentages of total long-chain n-3 fatty acids were significantly higher in total, low-grade and high-grade prostate cancer case subjects when compared with a sub-cohort matched for age and race. However, these results can be considered contrary to biological plausibility given that the serum n-3 fatty acids levels associated with prostate cancer are equivalent to eating oily fish twice a week. One might therefore expect rates of prostate cancer to be the highest in populations with the highest dietary intake of long-chain n-3 fatty acids, such as the Japanese and Greenland Eskimos (Bang et al., 1980, Sugano, 1996). Moreover, the association is counterintuitive and contradicts currently accepted knowledge about the role of fatty acids and chronic inflammation in carcinogenesis (Fay et al., 1997, Bartsch et al., 1999, Larsson et al., 2004) and other data from case-controlled (Norrish et al., 1999) and cohort studies (Augustsson et al., 2003, Leitzmann et al., 2004, Pham et al., 2009). Another systematic review, including several cohorts from a range of countries and with different demographics, did not support a significant association between omega-3 fatty acids and cancer incidence (MacLean et al., 2006).

To summarise, current evidence suggests that there are negligible serious side effects associated with LCn3PUFA supplementation at the doses suggested for the treatment of periodontal disease. When considering the large number of serious side effects associated with conventional anti-inflammatory approaches, LCn3PUFA supplementation presents a safe and cost-effective new therapy for the adjunctive treatment of periodontal disease.

1.10 Evidence for the Role of Fish Oil in Periodontal Therapy

To identify studies to be included in this literature review, a comprehensive search strategy was carried out using Pubmed and revised appropriately for other databases (Appendix I). No language restrictions were applied to the searches. The database search was also modified to incorporate the Cochrane Highly Sensitive Search Strategy for identifying reports of randomised controlled trials (Higgins and Green, 2011)

The following electronic databases were searched on 1 February 2014.

- Cochrane Oral Health Group Trials Register
- Cochrane Central Register of Controlled Trials (CENTRAL) via the Cochrane Library, to current issue

- Pubmed
- EMBASE

Electronic searches were screened by one reviewer to identify studies of interest, and the reference lists of relevant papers were also searched to identify additional studies. All study types that investigated the effects of omega-3 polyunsaturated fatty acids on periodontal disease were included, such as those using animal models, cross-sectional, longitudinal and intervention designs.

1.10.1 Human *in vitro* and Animal Studies

Several biologically plausible mechanisms by which LCn3PUFA supplementation could protect against alveolar bone destruction in periodontitis have been presented, including downregulation of pro-inflammatory cytokines, changes in eicosanoid profiles and activity related to pro-resolving lipid mediators (Herrera et al., 2008, Miles and Calder, 2012, Gao et al., 2013). LCn3PUFA may regulate inflammation by competing with AA for incorporation into cell membranes and as a substrate for the cyclooxygenase and lipoxygenase pathways. However, for changes in dietary EPA and DHA to be an effective host modulation therapy in periodontal disease, they must first be incorporated into the immune cells and periodontal tissues in sufficient levels to alter AA metabolism. A study using a rat model demonstrated that a diet rich in LCn3PUFAs significantly reduced the AA content in the cell membrane phospholipid of gingival tissues (Alam et al., 1991). This was associated with a subsequent reduction in the endogenous levels of PGE₂ and LTC₄, corresponding to a 6-fold and 20-fold decrease in eicosanoid production, respectively, compared to the control group.

More recent animal studies evaluating a diet rich in LCn3PUFAs have shown a similar reduction in AA-derived eicosanoids. Vardar et al. (2004) evaluated the effects of LCn3PUFA supplementation on LPS-induced experimental periodontitis in rats. Following the administration of LCn3PUFAs they reported reduced gingival tissue levels of PGE₂, PGF_{2α} and LTB₄ comparable with those of healthy tissues. However, both prophylactic and therapeutic doses were ineffective at preventing alveolar bone loss (Vardar et al., 2004, Vardar-Şengül et al., 2006).

These findings are not in agreement with a similar study that examined extended LCn3PUFA supplementation in a *P. gingivalis*-induced rat model (Kesavalu et al., 2006). Elevated serum levels of EPA/DHA were observed following supplementation which were associated with a corresponding reduction in radiographic bone loss compared to the control rats fed corn oil (Kesavalu et al., 2006). Similarly, Bendyk et al. (2009) found that alveolar bone loss was inversely related to tissue levels of LCn3PUFAs in a murine periodontitis model. In this study mice fed a control diet of sunflower oil demonstrated significantly greater alveolar bone loss compared with mice fed a diet of tuna oil (4 times richer in DHA than EPA). Moreover, mice that were fed tuna oil for 8 weeks and inoculated with *P. gingivalis* had 72% less bone loss compared to the control group. Contrasting results reported for these animal feeding studies can be explained by differences in LCn3PUFA dosage and duration, proportion of EPA to DHA, as well as the experimental periodontitis models used.

Several studies have also used *in vitro* cell culture or small animal models to investigate the effects of n-3 PUFA-derived pro-resolution mediators on periodontal tissues and experimental periodontitis. In an *in vitro* study, Hasturk et al. (2006) showed that neutrophils from human subjects with localised aggressive periodontitis were refractory to an aspirin-triggered lipoxin analogue but responded to RvE1. Rabbits with combined ligature and *P. gingivalis*-induced periodontitis treated with topical RvE1 were also shown to have approximately 95% less soft tissue and bone destruction (Hasturk et al., 2006). A subsequent study evaluated RvE1 as a monotherapy in the treatment of established periodontitis in the same rabbit model (Hasturk et al., 2007). After 6 weeks of established experimental periodontitis, animals were randomly assigned to four different treatments including vehicle alone, RvE1, leukotriene B₄ and prostaglandin E₂. In the group treated with topical RvE1, inflammation was completely eliminated, with reduction in probing depths and a return to baseline soft tissue architecture. Histological evaluation also revealed regeneration of bone with restoration of crestal height, elimination of infrabony defects and regeneration of new cementum and bone with an organised periodontal ligament. In this study the control of inflammation resulted in the spontaneous elimination of putative periodontal pathogens including *P. gingivalis* and *A.a.*, even though previous studies failed to demonstrate significant antibacterial actions for RvE1 (Hasturk et al., 2006). This supports the concept that inflammation can impact upon the composition of microbiota in the dental biofilm, with the implication that inflammation precedes the emergence of putative periodontal pathogens (Van Dyke, 2007). This hypothesis is

supported by longitudinal observations of Tanner et al. (2007) where baseline gingivitis was a strong predictor of disease progression, but baseline microbial species were not strongly associated with progressing periodontitis.

1.10.2 Cross-sectional and Longitudinal Studies

Naqvi and colleagues (2010) examined cross-sectional data from NHANES 1999-2004. The survey included 9,182 US adults, of which 1,024 subjects had periodontitis, defined as ≥ 4 mm probing pocket depth plus ≥ 3 mm attachment loss in any mid-facial or mesial site. They found that higher intake of dietary DHA was associated with lower odds of periodontitis (OR 0.78), after adjustment for multiple confounders, including total energy intake, age, sex, race, smoking, diabetes, income, education, pregnancy, BMI and alcohol intake. However, dietary intake of EPA had only a moderate inverse association with periodontitis risk (adjusted OR 0.5). Furthermore, there was no statistically significant additional reduction in the odds of periodontitis, with increased DHA intake between the middle and upper tertile, suggesting that there is no further benefit beyond a moderate intake of this fatty acid. It is of interest that there was no significant additional reduction in the odds of periodontitis for individuals taking fish oil supplements. Moreover, dietary intake of omega-3 PUFA in the middle tertile is relatively low, with ranges of 0-40 mg/day and 0-10 mg/day for DHA and EPA, respectively. These results suggest that a clinically meaningful reduction in the odds of periodontitis can be achieved with only minor changes in diet and small increases in omega-3 PUFAs intake. Another cross-sectional study including 351 participants found a significant negative correlation between tooth loss and the EPA concentrations in red blood cell membranes, after adjusting for confounders (Hamazaki et al., 2006).

Interestingly, studies that evaluated the association between EPA/DHA levels and periodontitis have reported conflicting results. A study including 30 patients with chronic periodontitis found significantly higher n-6 PUFA levels compared to the control group, but there was no difference in n-3 PUFA levels (Ramirez-Tortosa et al., 2010). However, a recent study reported significantly higher levels of both EPA and DHA in periodontitis patients compared to a control group with gingivitis (Figueredo et al., 2013). In contrast, Requirand et al. (2000) reported significantly lower levels of EPA and DHA and higher levels of n-6 fatty acids in patients that had alveolar bone loss compared to the control with no clinical or radiographic signs of bone loss.

Longitudinal data to support a beneficial effect of dietary EPA and DHA in outcomes of periodontal disease are limited. However, one prospective cohort study investigated the relationship between dietary LCn3PUFAs and periodontal disease in a non-institutionalised elderly Japanese population (Iwasaki et al., 2010). After a 5 year follow-up, the groups with the lowest tertile of DHA or EPA intake had significantly fewer teeth when compared with the groups with the highest tertile intakes. Their results indicate that low DHA intake was significantly related to periodontal disease ‘events’ after adjustment for gender, BMI, smoking status, baseline attachment loss and the initial number of teeth. Moreover, subjects with low DHA intake had nearly a 50% higher incidence of clinical attachment loss greater than 3 mm. A trend was also observed between the lowest EPA intake and periodontal disease events, although the relationship was not statistically significant. Thus, the findings of this study suggest a relationship between low dietary DHA intake and periodontal disease progression in an elderly population, although limited by the estimation of intakes using the food frequency questionnaire instead of reliable biomarkers. A subsequent observational cohort study by the same research group suggests that the ratio of n-6 to n-3 PUFAs may be a more significant risk predictor for periodontal disease progression than absolute amounts of each PUFA (Iwasaki et al., 2011). A lower ratio of n-6 to n-3 PUFA has been shown to reduce the risk of cardiovascular disease and other chronic inflammatory diseases (Simopoulos, 2008).

1.10.3 Clinical Studies

There have only been a small number of clinical trials that have investigated LCn3PUFA supplementation as a treatment for periodontal disease, either as a monotherapy or adjunctive to conventional non-surgical therapy or regenerative/reconstructive periodontal surgery. These early studies are limited by small sample sizes.

A pilot randomised controlled trial evaluated n-3 PUFA monotherapy for the treatment of experimental gingivitis (Campan et al., 1997). Following induction of experimental gingivitis by the withdrawal of oral hygiene procedures for 3 weeks, 37 healthy volunteers were randomised to receive either 1.8 g of n-3 PUFA or placebo (olive oil). The level of n-3 PUFA was significantly higher in the gingival tissues samples of the experimental group, however no significant difference was found between groups for the levels of AA, PGE₂, or LTB₄. Although a decrease in gingival inflammation was seen in the

LCn3PUFA group, no significant difference was found between treatment groups. Failure to demonstrate a significant benefit may have been due to the small number of participants or short duration of supplementation.

Rosenstein et al. (2003) conducted a 12 week pilot RCT to evaluate the effect of EPA or gamma-linolenic acid [GLA] (an n-6 PUFAs) supplementation, compared to placebo (corn oil) in 30 subjects with periodontitis. The interventions were evaluated as an adjunctive treatment to oral hygiene instruction as no form of mechanical debridement was provided. A significant improvement in gingival inflammation and probing depth was seen in subjects that received GLA supplementation, but only a trend for improved outcomes was seen for groups that received EPA alone or combined GLA and EPA supplementation. The lack of a statistically significant benefit for EPA supplementation may however have been due to limited study power or poor compliance with supplementation.

Another clinical trial randomly allocated chronic periodontitis subjects to receive either 3g of LCn3PUFAs or placebo (Parulkar et al., 2009). All patients received oral hygiene instruction and were followed up for 28 weeks. For the group taking LCn3PUFAs, there was a significant increase in the amounts of DHA, EPA and total PUFAs in serum and red blood cell membranes at 8 and 16 weeks. However, there were no significant improvements in clinical outcomes associated with these lipid changes. These results suggest that LCn3PUFAs as a monotherapy does not enhance outcomes of periodontitis, or their effect is diminished by the impact of oral hygiene.

A recent RCT randomised 55 subjects with moderate periodontitis to receive either DHA (equivalent to 2000 mg of DHA per day) plus low-dose aspirin (81 mg) or placebo plus low-dose aspirin (Naqvi et al., 2014). All participants received oral hygiene instruction but no mechanical debridement. At the 3 month follow-up, an improvement in probing depth of 2 mm or more was twice as likely in the DHA group (OR = 2.05) compared to the group taking aspirin plus placebo. DHA plus aspirin supplementation also significantly decreased mean probing depths and the number of sites with residual probing depths ≥ 5 mm (OR = 0.62). A significant difference between the DHA and control group was also found for GCF inflammatory markers including highly-sensitive CRP and IL-1 β .

El-Sharkawy et al. (2010) reported significant reductions in pocket depth and significant attachment gain after 3 and 6 months of EPA plus DHA supplementation (900 mg),

combined with low dose (81 mg) aspirin, as an adjunct to non-surgical periodontal therapy. Their clinical trial included 80 patients with advanced chronic periodontitis defined by the presence of ≥ 6 teeth with probing depths > 6 mm, attachment loss ≥ 4 mm and one-third radiographic bone loss. The improvement in clinical parameters involve a significant reduction in the number of pockets > 4 mm. As well as the improvement in clinical parameters, there were also significant reductions in salivary RANKL and MMP-8 levels. However, the study did not have an intervention group consisting of fish oil alone and failed to adequately assess compliance.

Following the control of inflammation by cause-related therapy, the ultimate goal of contemporary periodontal therapy is the complete regeneration of lost periodontal tissues. However, regeneration of the periodontium is unpredictable, requiring insertion of periodontal ligament [PDL] fibres into new cementum and new alveolar bone. Nonetheless, current research suggests that significant regeneration of periodontal tissues is possible with the administration of RvD1 (Hasturk et al., 2007). With the recognition that the action of resolvins are not limited to immune cells, a recent study evaluated their effects on the periodontal ligament (Mustafa et al., 2013). Following the application of RvD1, proliferation of cultured human PDL fibroblasts increased in a dose-dependant manner (Mustafa et al., 2013). Additionally, treatment with RvD1 significantly increased PDL migration in an *in vitro* model of wound closure, compared to the vehicle control. Taken together, these results suggest a beneficial role for pro-resolution lipid mediators in periodontal wound healing and regeneration.

Another recent study evaluated the efficacy of LCn3PUFAs plus low-dose aspirin as an adjunct to regenerative/reconstructive treatment of class II furcation defects (Elkhouli, 2011). Forty patients were randomly allocated to receive demineralised freeze-dried bone allograft [DFDBA] with adjunctive n-3 PUFAs plus low-dose aspirin, or DFDBA with placebo. Supplementation consisted of 3 x 1 g capsules with the equivalent of 30 mg of DHA and 150 mg of EPA. At the 6 month follow-up, the test group had significantly greater reduction in pocket depth (0.9 mm) and gain in clinical attachment (0.6 mm) compared to the control. By the end of the study, there was also a significant reduction in mean gingival index scores and bleeding scores compared to the control group, indicating a clinically meaningful reduction in gingival inflammation. The additional benefits in clinical parameters were also associated with greater reductions in the GCF level of IL-1 β . However, there should be caution when interpreting improvement in probing depth and

clinical attachment for regenerative procedures that use particulate graft materials to reconstruct periodontal defects. Improvement in clinical parameters can represent repair with a long junctional epithelium (Bowers et al., 1989) and fibrous tissue encapsulation of the graft material (Xiao et al., 1996) as opposed to histologically confirmed new attachment.

In summary, animal studies and early clinical trials suggest a role for LCn3PUFAs in the treatment of periodontal disease due to their anti-inflammatory and pro-resolution actions. There is a need for more robust evidence in the form of double-blind placebo controlled trials to establish the clinical efficacy of LCn3PUFAs as an adjunct to conventional periodontal therapy.

1.11 References

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Chapter 2. Long Chain Omega-3 Fatty Acids as an Adjunct to Non-surgical Periodontal Therapy: A Randomised Double-Blind Placebo Controlled Trial

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2.1 Introduction

Periodontitis is a chronic inflammatory disease resulting in progressive destruction of the supporting hard and soft tissues of the teeth. Current understanding recognises the multifactorial nature of periodontitis, including the role of host-microbial interactions as well as the influence of environmental and genetic modifying factors (Kornman, 2008). Bacteria are thought to be essential but not sufficient to cause periodontitis alone, and most periodontal tissue destruction and clinical features are attributed to a dysregulation of the host immuno-inflammatory response leading to chronic non-resolving inflammation (Van Dyke and Serhan, 2003). During disease initiation and progression, a wide range of pro-inflammatory mediators are released that lead to destruction of the periodontal tissues, including cytokines such as IL-1 β , TNF- α and IL-6, matrix metalloproteinases (MMPs) and lipid mediators including the prostaglandins.

Conventional periodontal therapy has focused on mechanical debridement to control the bacterial challenge that initiates the inflammatory response. While this approach is usually effective at reducing inflammation and improving clinical parameters (Cobb and Jeffcoat, 2002), a proportion of patients will be unresponsive to treatment or susceptible to disease progression and subsequent tooth loss (Hirschfeld and Wasserman, 1978, McFall, 1982, Lindhe and Nyman, 1984, Pearlman, 1993). The long-term success of conventional periodontal therapy is also dependent on regular periodontal maintenance and a high level of oral hygiene to continually disturb the biofilm (Axelsson and Lindhe, 1981, Lindhe and Nyman, 1984, Axelsson et al., 2004).

In addition to established therapies directed at controlling the bacterial challenge and addressing modifiable risk factors (smoking cessation, diabetic control), host modulation has emerged as an additional therapeutic approach. This strategy is established for the treatment of systemic inflammatory diseases (Kantarci et al., 2006), and aims to restore homeostasis in the periodontal tissues by downregulating the destructive aspects of the host inflammatory response, and by upregulating its protective or regenerative components (Preshaw, 2008). Long-chain omega-3 polyunsaturated fatty acids (LCn3PUFAs) are promising host-modulatory agents as they target inflammation through several mechanisms including altered eicosanoid synthesis and the modulation of oxidative stress, pro-inflammatory cytokines and transcription factors (Calder, 2013). Moreover, dietary intake of LCn3PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)

leads to the generation of novel bioactive lipid mediators termed resolvins, protectins and maresins (Serhan et al., 2002, Serhan et al., 2009). These lipid mediators promote a return to homeostasis by downregulating neutrophil recruitment and enhancing clearance of microbial products and necrotic cells from the inflammatory lesions. Their activity is therefore characterised by anti-inflammatory as well as agonistic actions that promote the early resolution of inflammation.

Animal studies and early clinical data suggest a role for LCn3PUFA dietary supplementation in the treatment of chronic periodontitis. Our investigation has previously shown an inverse relationship between tissue levels of LCn3PUFAs and alveolar bone loss in a murine periodontitis model (Bendyk et al., 2009). *Porphyromonas gingivalis* inoculated mice fed a diet rich in LCn3PUFAs experienced 72% less alveolar bone resorption compared to the control group fed an n-6 fatty acids rich diet. A recent randomised controlled trial reported a significant decrease in mean probing pocket depths and the number of sites with residual probing depths ≥ 5 mm in participants that received oral hygiene instruction plus combined DHA (2 g/d) and aspirin (81 mg) supplementation compared to a control group that received oral hygiene instruction only (Naqvi et al., 2014). Similarly, another clinical trial reported significant attachment gain and probing pocket depth reduction after 3 months and 6 months of EPA plus DHA supplementation (900 mg), combined with low dose (81 mg) aspirin, as an adjunct to non-surgical periodontal therapy (El-Sharkawy et al., 2010).

The aim of this study was to evaluate the clinical efficacy of fish oil supplementation as an adjunct to non-surgical periodontal therapy in the treatment of advanced chronic periodontitis. Therefore, the null hypothesis tested in this investigation was that adjunctive fish oil supplementation has no additional benefit in clinical periodontal parameters compared to standard non-surgical periodontal therapy alone. Specific objectives were to establish the relative benefit of dietary DHA versus EPA fatty acids compared with a placebo.

2.2 Material and Methods

2.2.1 Experimental Design

A 13 month, parallel group, randomised, placebo-controlled, double-blind, multi-centre clinical trial was undertaken to evaluate the efficacy of fish oil supplementation as an adjunct to non-surgical periodontal therapy in the treatment of advanced chronic

periodontitis. Participants were recruited through the Colgate Australian Clinical Dental Research Centre (University of Adelaide), South Australian Dental Service and Griffith University. The study protocol was approved by the corresponding ethics committees and registered with the Australian New Zealand Clinical Trials Registry (ACTRN1261000594022) prior to subject recruitment. CONSORT guidelines were closely followed to ensure adequate and transparent reporting of this trial (Schulz et al., 2010).

2.2.2 Eligibility

2.2.2.1 Inclusion Criteria

First stage screening was carried out by an initial telephone questionnaire, followed by a clinical examination for subjects that appeared to meet the inclusion criteria. Eligible participants were systemically healthy adults aged between 25-80 years, with a diagnosis of 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). As such, patients had two or more interproximal sites with attachment loss ≥ 6 mm, not on the same tooth, and one or more interproximal sites with probing depths ≥ 5 mm, not on the same tooth. Patients were then asked to complete a questionnaire to allow further assessment of eligibility by capturing information on lifestyle habits, medication and dietary supplement usage. Eligible patients were informed of the potential risks and benefits of the study interventions, and written informed consent was obtained from all participants entering the study.

2.2.2.2 Exclusion Criteria

Patients were excluded if they were: i) current smokers or had quit within the previous 6 months, ii) pregnant, iii) had less than 20 remaining teeth, or iv) had type II diabetes or other chronic inflammatory conditions such as rheumatoid arthritis, coronary artery disease, psoriasis or asthma. Individuals with aggressive periodontitis were excluded from the study as the common standard of care for this condition often involves adjunctive antibiotics. Patients were also excluded if they had received periodontal therapy in the previous 6 months, antibiotic therapy in the previous 3 months, or if they were taking more than 1 g of fish oil per day. Inclusion and exclusion criteria are summarised in Table 2.1.

Inclusion Criteria	Exclusion Criteria
Advanced chronic periodontitis defined as: CAL \geq 6 mm (2 or more interproximal sites) PPD \geq 5 mm (1 or more interproximal sites) Sites not on the same tooth	Aggressive periodontitis
\geq 20 teeth	<20 teeth
25-80 years old	Diabetes
Non-smoker	Chronic inflammatory diseases
	Periodontal therapy in the previous 6 months
	Antibiotic therapy in the previous 3 months
	Current smokers and ex-smokers who had quit within the previous 6 months
	Regularly taking >1 g fish oil/day
	Pregnant or breast feeding

Table 2.1. Inclusion and exclusion criteria

2.2.3 Data Collection

2.2.3.1 Clinical Measurements

All treatment and recording of clinical parameters were carried out by two periodontic registrars (BC, BP) and one qualified periodontist (RL). Clinicians were blind to the allocated treatment groups. The primary clinical outcomes of interest were probing depth reduction (PPD) and clinical attachment gain (CAL) from baseline to 4 months. Full-mouth clinical measurements were taken at baseline, and 4, 7, 10 and 13 months following completion of root surface debridement (Figure 2.1). However, in this initial report only data from the 4 month follow-up will be presented. Probing pocket depth, recession (distance from CEJ or other fixed reference point, such as restoration margin to the gingival margin) and bleeding on probing (BOP), were measured at six sites around each tooth. Measurements were recorded to the nearest 1 mm, with a rounding up convention, using manual periodontal probes (PCP-10, Hu-Friedy, Henry Schein Halas, NSW, Australia). For each tooth site, clinical attachment level was calculated as probing pocket depth plus recession. In addition to the clinical parameters, any adverse events were documented with respect to onset, duration, treatment and relation to study medication and outcome.

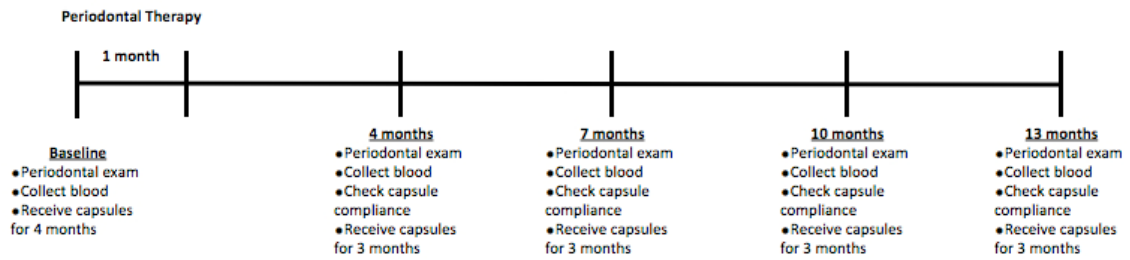


Figure 2.1. Study follow-up.

2.2.3.2 Study Outcomes

The primary outcome measures of the study were the percentage of sites at the patient level demonstrating a ≥ 2 mm reduction in PPD or gain in CAL between baseline and 4-month follow-up.

Secondary outcomes included:

- Mean differences for changes in PPD and CAL (mm) between baseline and 4 month follow-up.
- Number needed to treat (NNT). The number of sites per patient that need to be treated with adjunctive LCn3PUFAs to give a probing depth reduction of ≥ 2 mm at one additional site.
- Number of residual sites with PPD ≥ 5 mm.
- Association between changes in erythrocyte membrane DHA, EPA or omega-3 index and changes in PPD and CAL.

2.2.3.3 Examiner Calibration

To minimise the effect of inter-examiner variation, a training and calibration session was carried out prior to study enrolment to standardise the examination process. The intra-class correlation coefficients were between 0.66 and 0.68, which may be considered ‘moderate’ agreement (Fleiss and Cohen, 1973, Shrout and Fleiss, 1979) (Appendix II). Additionally, each subject was assigned one examiner for the duration of the trial to further minimise the effect of inter-examiner variation.

2.2.4 Procedures

2.2.4.1 Fish Oil Supplementation

Prior to periodontal therapy, participants were randomly allocated to the test or placebo groups using a random number table. Block randomisation was used to ensure balanced groups in terms of baseline probing depths, with 10 subjects assigned to each block. Allocation concealment was achieved with the use of coded and sealed opaque envelopes containing the treatment assigned to each of the participants. Group assignment codes were only accessible to the trial coordinator and broken following completion of data collection.

Test groups received a fish oil capsules providing 2 g of LCn3PUFA per day based on a previous investigation that demonstrated a substantially increased Omega-3 Index (concentration of EPA and DHA in erythrocytes) with this regime (Milte et al., 2008). Participants were required to consume 6 x 500 mg capsules per day containing either soybean oil (control); fish oil rich in EPA; or fish oil rich in DHA (Novasel, QLD, Australia) (Table 2.2). Given the relatively low number of participants recruited, data for the EPA and DHA experimental groups were combined for analysis as a single intervention group to improve statistical power.

Supplement containers were coded and labelled by an independent third party and capsules were identical in appearance and flavour to ensure that all researchers and participants were blinded to active and control treatments. Participants were asked to start taking their allocated capsules after the first visit of periodontal therapy, continuing daily until the 13 month follow-up.

Capsule	DHA	EPA	Other
DHA-rich	258 mg	66 mg	2.2 µg Betacarotene
EPA-rich	27 mg	277 mg	2.2 µg Betacarotene
Placebo	0 mg	0 mg	500 mg soya bean oil + 2.2 µg Betacarotene

Table 2.2. Description of administered capsules.

2.2.4.2 Periodontal Therapy

Participants in the test and control group received identical periodontal therapy, comprised of oral hygiene instruction, full-mouth scaling and root surface debridement. Quadrant-wise treatment was completed over a 4 week period, using Gracey curettes (Hu-Friedy, Henry Schein Halas, NSW, Australia) and ultrasonic instruments (EMS, Henry Schein Halas, NSW, Australia) under local anaesthesia (2% xylocaine with 1:80000 adrenaline, Dentsply, NSW, Australia). Operators were allowed up to one hour per quadrant and treatment was completed when the teeth and root surfaces were considered free from dental plaque and calculus. Clinical parameters were recorded at each follow-up period with the provision of supportive periodontal therapy. These included reinforcement of oral hygiene instruction, removal of plaque and non-surgical re-debridement of any persistent deep or inflamed sites. In the event of significant disease progression (≥ 2 mm attachment loss) or acute periodontal problems, the protocol was to exit participants from the study for appropriate treatment.

2.2.4.3 Assessment of Fatty Acid Profiles

Fasting blood samples were collected by venepuncture and stored in 6 ml EDTA tubes at baseline, 4 and 14 months. Incorporation of LCn3PUFA into erythrocytes was measured in order to check compliance with supplementation of fish oil and to determine whether the altered composition is related to changes in periodontal parameters. The method for determining the relative proportions of fatty acids in erythrocyte phospholipid has been described previously (Milte et al., 2011). In brief, erythrocytes were isolated within two hours of collection by centrifugation, washed in isotonic saline and stored at -80°C . Lipids were extracted from membranes with 2:1 chloroform and isopropanol and subsequently transesterified with acetyl chloride in methanol toluene (4:1v/v) at 100°C for one hour. The resultant fatty acid methyl esters [FAMES] were extracted with 10% potassium carbonate, assayed by gas chromatography and identified by comparing retention time to known lipid standards (GLC-463, Nu-Chek Prep Inc. Elysian, MN, USA).

2.2.5 Statistical Analyses

Sample size was estimated for a two-arm, parallel design study with percentage of pockets improved by ≥ 2 mm PPD as the primary outcome measure. Numbers of affected pockets (≥ 5 mm PPD) at baseline and follow-up were assumed to be normally distributed. Hence,

the percentage change can be treated as a normally distributed variable, enabling sample size to be calculated for an unpaired, between-group T-test. It was determined that a sample size of 64 subjects per treatment arm would provide 80% power to detect a 10% difference between treatment and placebo in the proportion of recovered pockets, assuming that the pooled standard deviation across groups will be 20%. A total sample of 150 subjects would allow for a drop-out rate of up to 20% during the study period. However, this trial was a preliminary pilot study in order to evaluate feasibility and improve sample size estimation. Therefore the aim was to recruit a minimum of 30 subjects, allowing 15 subjects per treatment arm, with LCn3PUFA groups combined.

Mean scores were calculated for probing depths and clinical attachment by summing the scores and dividing by the number of sites for each subject. Subject-level outcomes (i.e. changes in clinical attachment, probing pocket depth and bleeding on probing) were calculated at the 4 month follow-up period by using the mean PPD and CAL scores. Subject-level changes were calculated for the total number and percentage of sites demonstrating a ≥ 2 mm reduction in PPD and ≥ 2 mm gain in CAL between baseline and 4 month follow-up.

Baseline tooth sites were stratified according to disease severity, using probing pocket depth thresholds proposed by previous studies (Caton et al., 2000, Preshaw et al., 2004). According to these criteria, sites with probing pocket depths of 0 to 3 mm were considered normal; sites between 4 and 6 mm were considered mild to moderate disease; and sites ≥ 7 mm were considered severe disease (Table 2.3).

Baseline Disease Severity	Site-level Thresholds
Normal	0-3 mm probing pocket depth
Mild to Moderate Periodontitis	4-6 mm probing pocket depth
Severe Periodontitis	≥ 7 mm probing pocket depth

Table 2.3. Site-level case definition for periodontitis severity.

Intention-to-treat analyses were performed using data obtained from all subjects. Subject-level mean changes between baseline and follow-up were analysed for each group using paired sample *T* tests. The difference was considered statistically significant when the *P*

value was less than 0.05. Prior to analyses, normality was assessed both visually and using the Shapiro-Wilk test.

Subgroup analyses stratified for sites with baseline probing depths between 4-6 mm and ≥ 7 mm were carried out using generalised estimating equations (GEEs) to take into account clustering within patients and teeth. Persistent probing depths ≥ 5 mm at re-evaluation are commonly perceived as needing further treatment. Therefore, a secondary analysis used an analysis of covariance (ANCOVA) model to compare the difference between the fish oil and placebo group for percentage of residual PPD ≥ 5 mm, allowing adjustment for baseline probing depth. If indicated, calculation of the number needed to treat (NNT) was planned to give further clinically useful information regarding the efficacy of adjunctive fish oil supplementation. This is the additional number of sites on average, where adjunctive fish oil would need to be administered, to give a probing depth reduction of ≥ 2 mm at one additional site.

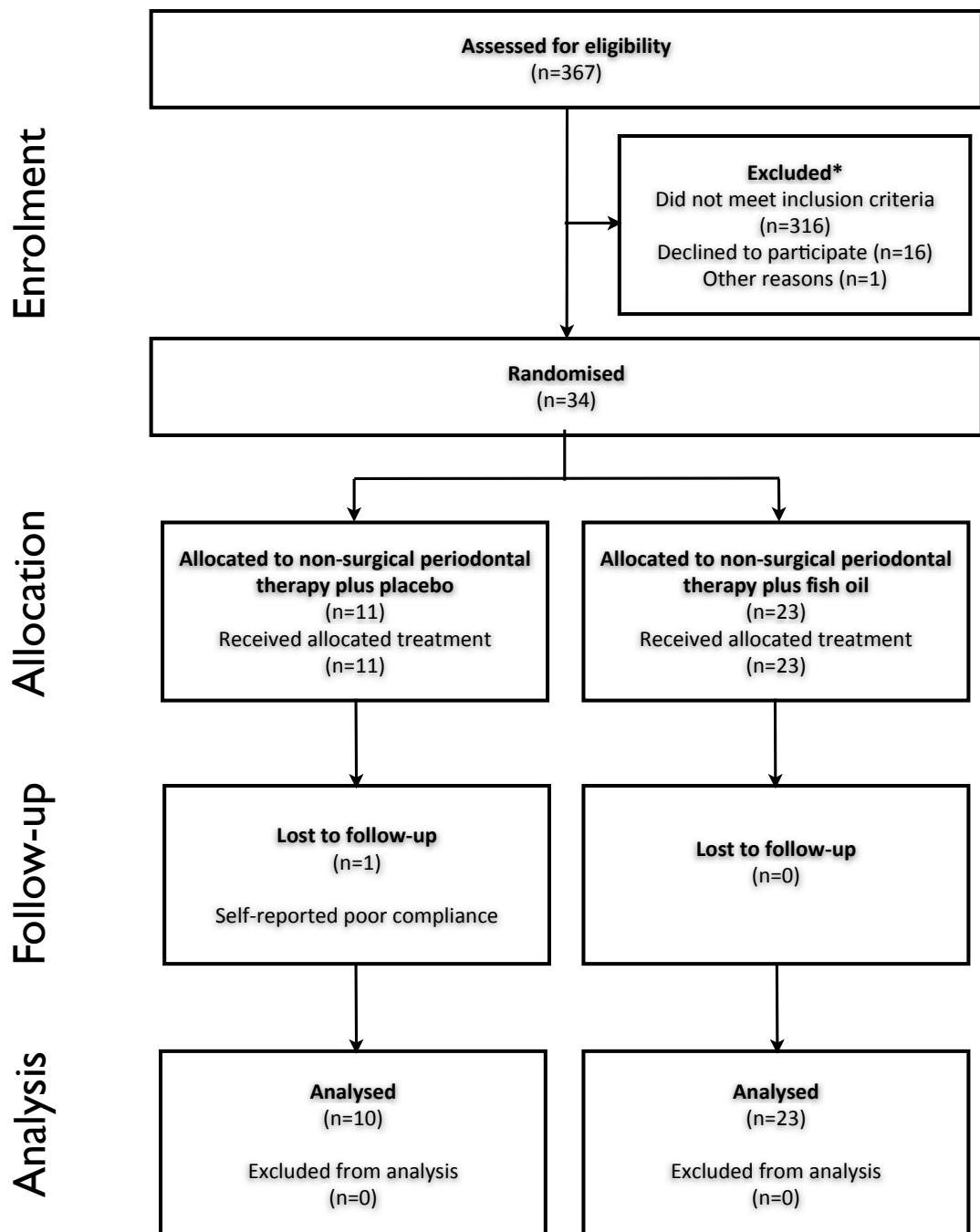
Pearson correlation coefficients were also calculated to determine any relationship between clinical parameters (change in PPD and CAL ≥ 2 mm) and change in EPA, DHA, DPA, omega-3 Index and total LCn3PUFA levels. Analyses were performed using the software program SPSS version 22.0 (Statistical Package for Social Science, SPSS Inc. Chicago, IL, USA) and SAS version 9.3 (SAS Institute Inc. Cary, NC, USA).

2.3 Results

2.3.1 Participants

A total of 367 subjects were screened between August 2010 and November 2013 (Figure 2.2). The main reasons for exclusion from the study were related to medical history, current smoking, periodontal therapy within the last 6 months and current use of fish oil supplements. Of the 34 participants that were randomised, only one subject was lost to follow-up (placebo group), reporting poor compliance with their allocated capsules.

There were no statistically significant differences between groups for baseline demographic characteristics such as age, gender and Body Mass Index [BMI]. Baseline clinical parameters and fatty acid profiles, including omega-3 Index, total LCn3PUFAs as well as levels of EPA, DHA and DPA were also not significantly different between groups (Table 2.4).



*No record of patient assessed for eligibility or exclusion from Griffith University.

Figure 2.2. Participant flow diagram.

Baseline Demographics and Clinical Parameters Mean (\pm SD)	Group 1 Placebo (n=10)	Group 2 Fish Oil (n=23)	Difference
Age (years)	45.10 \pm 9.73	55.00 \pm 8.62	NS (<i>P</i> = 0.382)
Range	33-58	35-77	
Gender (M/F)	3/7	7/16	
Body Mass Index (BMI)	24.39 \pm 3.97	28.37 \pm 6.64	NS (<i>P</i> = 0.152)
Fatty Acid Profile (% of total fatty acids)			
EPA	0.58 \pm 0.14	0.80 \pm 0.33	NS (<i>P</i> = 0.710)
DHA	3.84 \pm 0.79	3.92 \pm 0.98	NS (<i>P</i> = 0.747)
DPA	2.13 \pm 0.22	2.23 \pm 0.23	NS (<i>P</i> = 0.259)
Omega-3 Index	4.43 \pm 0.83	4.77 \pm 1.18	NS (<i>P</i> = 0.446)
Total LCn3PUFAs	6.55 \pm 0.82	7.00 \pm 1.23	NS (<i>P</i> = 0.333)
Moderate Sites (4-6 mm PPD)			
% of sites	29.06 \pm 10.11	26.12 \pm 10.84	NS (<i>P</i> = 0.500)
Mean PPD (mm)	4.64 \pm 0.15	4.76 \pm 0.27	NS (<i>P</i> = 0.185)
Mean CAL (mm)	4.64 \pm 0.22	4.76 \pm 0.21	NS (<i>P</i> = 0.164)
Deep sites (\geq 7 mm PPD)			
% of sites	9.57 \pm 11.24	6.17 \pm 8.30	NS (<i>P</i> = 0.374)
Mean PPD (mm)	7.56 \pm 0.41	7.37 \pm 0.38	NS (<i>P</i> = 0.257)
Mean CAL (mm)	7.73 \pm 0.24	7.61 \pm 0.31	NS (<i>P</i> = 0.485)
Full Mouth Bleeding Scores (%)	60.3 \pm 24.6	53.4 \pm 18.0	NS (<i>P</i> = 0.404)

Table 2.4. Baseline demographic characteristics and clinical parameters.

2.3.2 Efficacy of Periodontal Therapy

Both groups demonstrated a significant reduction in mean probing pocket depth between baseline and 4 months, with a per-subject mean reduction of 0.70 mm (± 0.48) and 0.91 mm (± 0.41) for the test and control group, respectively (Figure 2.3). Moreover, the percentage of sites that experienced a probing depth reduction of 2 mm or more were 26.1% and 19.2% for the test and control group, respectively. The better clinical outcomes observed for the control group were not found to be statistically significant ($P = 0.324$). The overall mean reduction in probing depth reduction is presented in Figure 2.4.

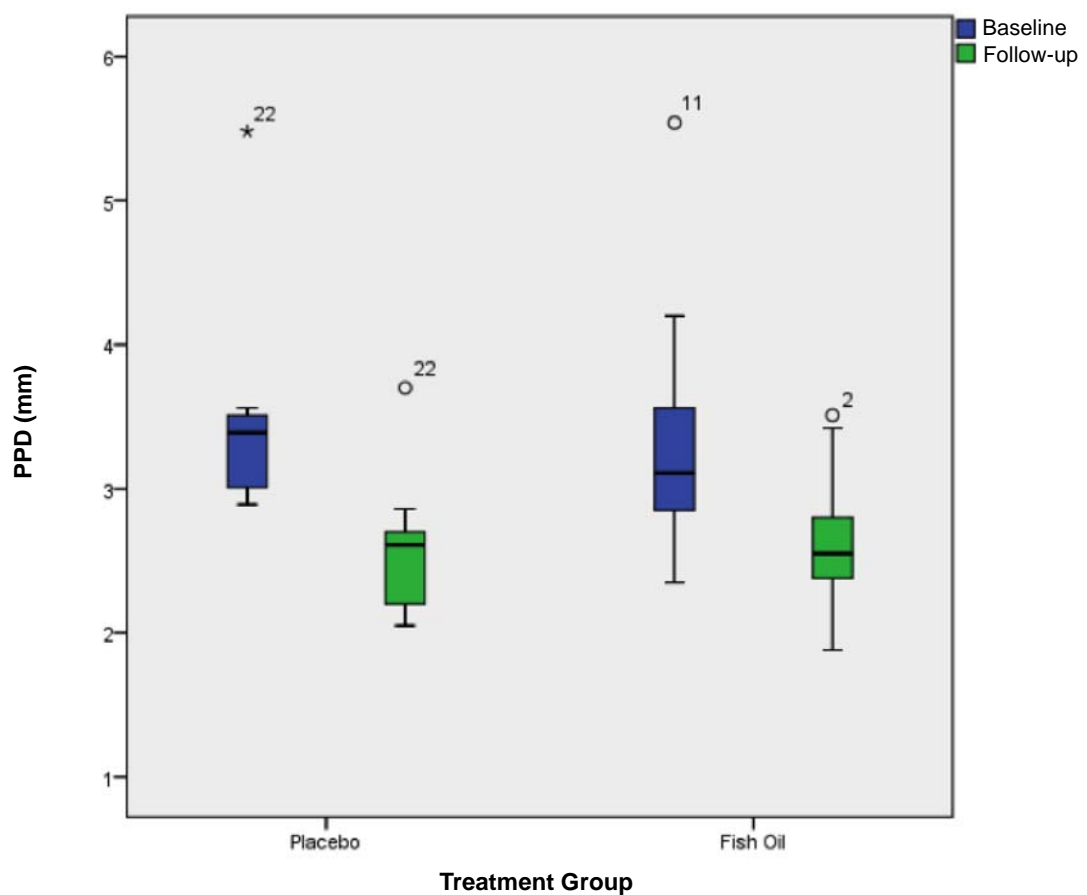


Figure 2.3. Boxplot to show mean probing depths at baseline and follow-up.

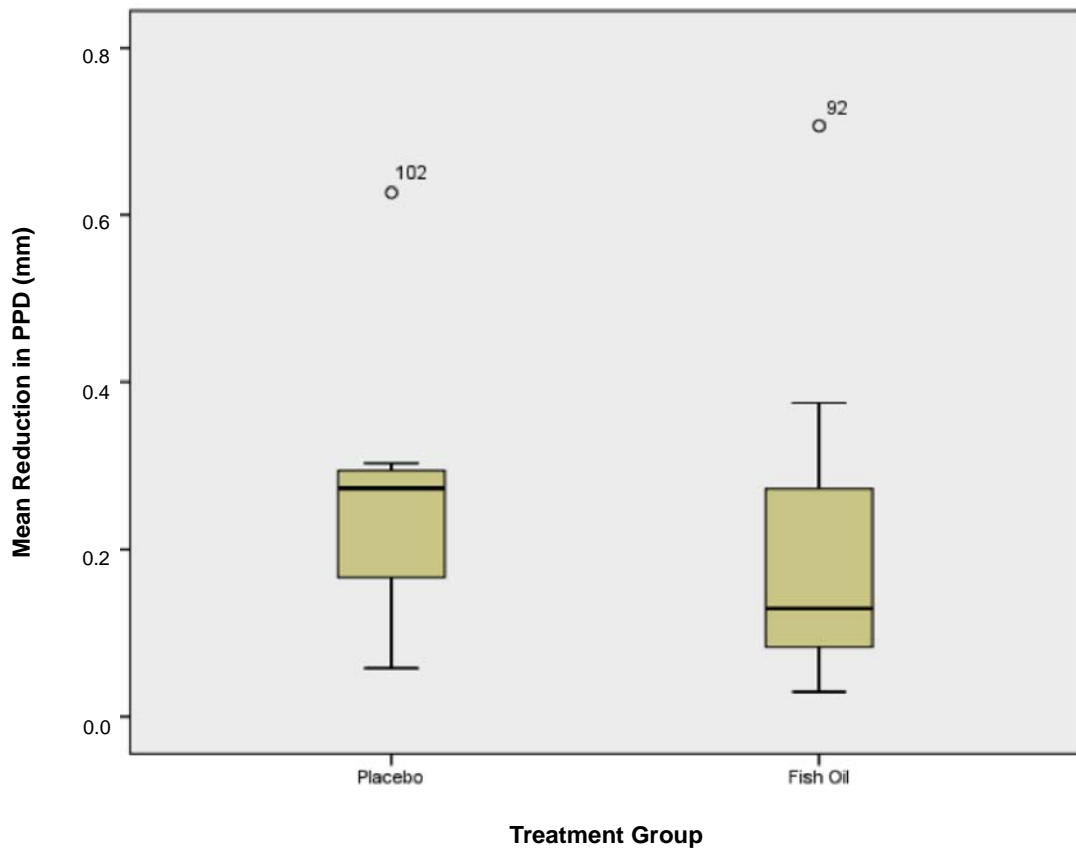


Figure 2.4. Boxplot to show mean reduction in probing depth.

For the group that received adjunctive fish oil supplementation, the mean percentage of sites with ≥ 5 mm probing depth were 21.2% at baseline and 6.6% at the 4 month follow-up. The corresponding percentage of sites with ≥ 5 mm probing depths in the control group were 22.2% at baseline and 5.3% at 4 months. Therefore, following treatment, the mean difference between groups for number of residual pockets ≥ 5 mm was 1.74% (95% CI - 1.41-4.89) after adjusting for baseline probing depths. The difference was not statistically significant ($P = 0.264$). At the 4 month follow-up, the mean number of sites with residual probing depths ≥ 7 mm were $1.86 \pm 4.20\%$ and $1.17 \pm 1.70\%$ for the fish oil and control group, respectively, with no statistically significant difference between groups ($P = 0.552$).

A trend for a greater proportion of sites that gained ≥ 2 mm in clinical attachment was also seen in the control group versus test group. The overall mean percentage of sites that gained ≥ 2 mm in clinical attachment was 29.2% for the placebo group and 21.7% for the test group, respectively. However, this difference was not found to be statistically significant ($P = 0.229$). The differences between the fish oil and control group for changes in probing depth or clinical attachment ≥ 2 mm were assessed using a logistic GEE, with separate models used for sites with baseline probing depth 4-6 mm or ≥ 7 mm. Table 2.6

gives odds ratios, 95% confidence intervals and *P* values for these models. Small but statistically significant differences in favour of the control group were found for change in probing depth and clinical attachment ≥ 2 mm, in sites with baseline probing depths of 4-6 mm (Table 2.5).

Change at Follow-up	Moderate Sites (Baseline PPD 4-6 mm)		Deep Sites (Baselines PPD ≥ 7 mm)	
	Placebo	Fish Oil	Placebo	Fish Oil
PPD reduction ≥ 2 mm % (n)	55.4 (252)*	47.8 (368)	79.8 (71)	83.9 (141)
Mean reduction in PPD (mm) \pm SD	1.65 \pm 2.00	1.46 \pm 1.00	3.11 \pm 3.00	2.93 \pm 3.00
CAL gain ≥ 2 mm % (n)	60.8 (306) †	53.2 (476)	79.8 (79)	75.0 (141)
Mean gain in CAL (mm) \pm SD	1.97 \pm 2.00	1.90 \pm 2.00	3.56 \pm 3.00	2.85 \pm 2.00

Statistically significant difference between groups (* *P* = 0.0350 and † *P* = 0.0286).

Table 2.5. Comparison of treatment groups for reduction in probing pocket depth and gain in clinical attachment.

The difference in attachment levels at baseline and follow-up, and odds ratios for the mean gain in clinical attachment at follow-up are presented in Figures 2.5 and 2.6, respectively.

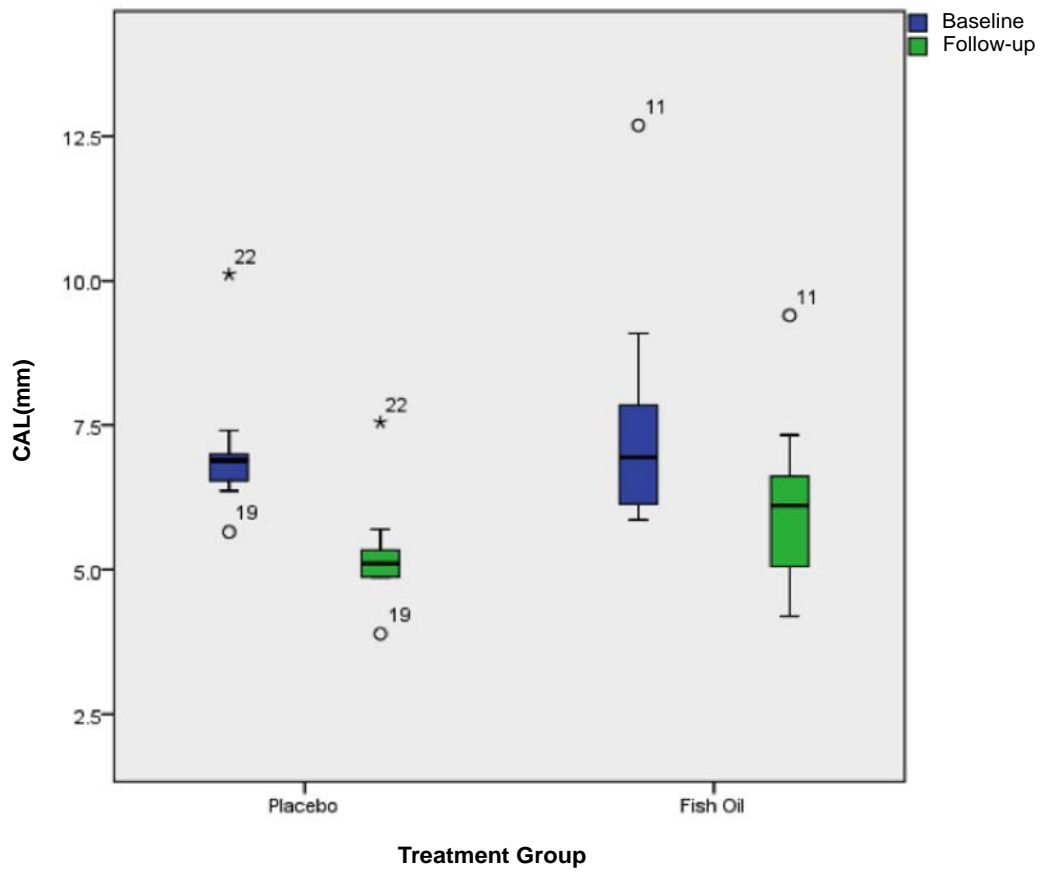


Figure 2.5. Boxplot to show mean clinical attachment at baseline and follow-up.

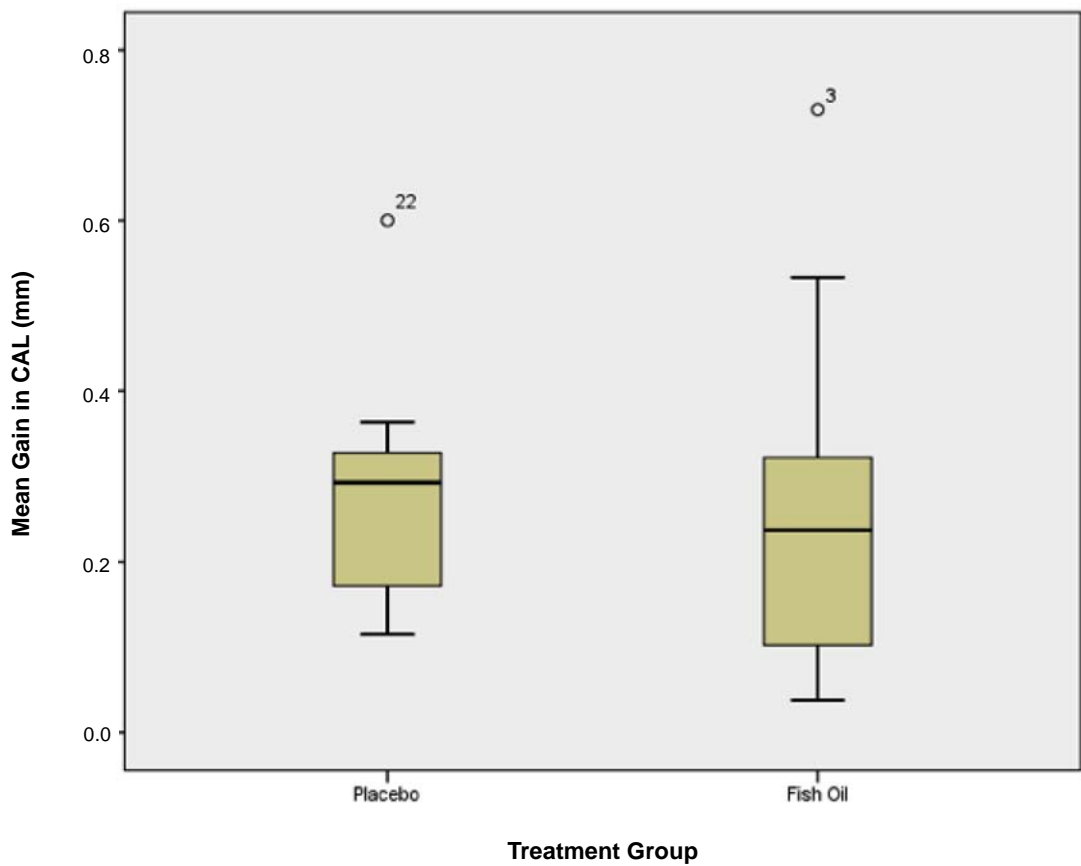


Figure 2.6. Boxplot to show mean gain in clinical attachment at follow-up.

Baseline Periodontitis Severity	Outcome Variable	Predictor	Comparison Variable	Reference Variable	Odds Ratio (95% CI)	P value
Moderate sites (4-6 mm)	Change in PPD ≥ 2 mm	Group	Fish oil	Placebo	0.74 (0.56, 0.98)	0.0350
	Change in CAL ≥ 2 mm	Group	Fish oil	Placebo	0.73 (0.55, 0.97)	0.0286
Deep sites (≥ 7 m)	Change in PPD ≥ 2 mm	Group	Fish oil	Placebo	1.32 (0.62, 2.84)	NS (0.4704)
	Change in CAL ≥ 2 mm	Group	Fish oil	Placebo	0.76 (0.39, 1.50)	NS (0.4266)

Table 2.6. Odds ratios for gain in clinical attachment ≥ 2 mm (based on GEEs).

2.3.2 Safety and Compliance

No drop-outs were attributed to adverse side effects, and there were no adverse side effects reported for subjects that received placebo, EPA or DHA supplementation. In this clinical study capsule counts were not found to be a reliable method of assessing patient compliance with supplementation, as a large proportion of participants failed to return their capsule containers at follow-up. To check compliance with fish oil supplementation, erythrocyte fatty acid profiles were compared between groups (27 participants had blood results at baseline and 4 months). A linear regression model was used to show an association between the percentage of total LCn3PUFA at 4 months (outcome variables) and treatment group (predictor), controlling for baseline total LCn3PUFA. In the placebo group the mean total LCn3PUFA at 4 months was 6.8%, compared with 10.9% in the fish oil group. The placebo group had a mean total LCn3PUFA 4.1% less than the fish oil group at 4 months, controlling for baseline total LCn3PUFA levels (95% CI: -4.7,-3.4). This association was statistically significant ($P < 0.0001$). Linear regression comparing Omega-3 Index was also performed indicating a statistically significant difference ($P < 0.0001$). In contrast, baseline values were not significantly different between groups (Table 2.9, Appendix III)

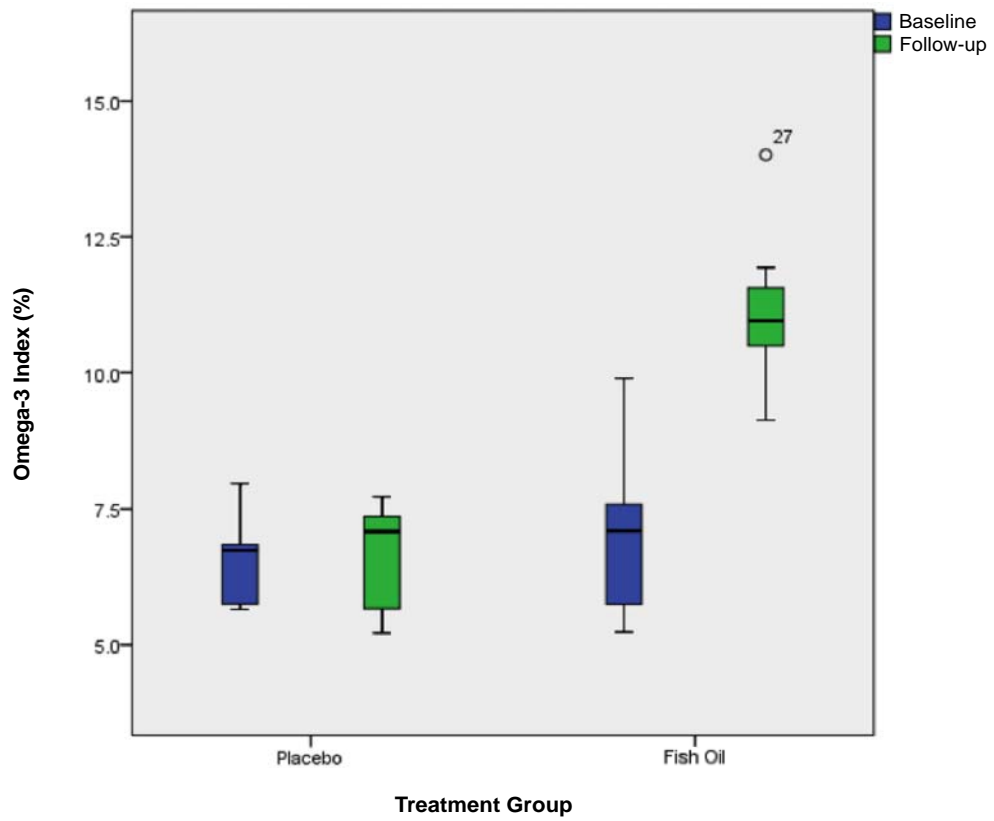


Figure 2.7. Boxplot showing Omega-3 Index at baseline and follow-up.

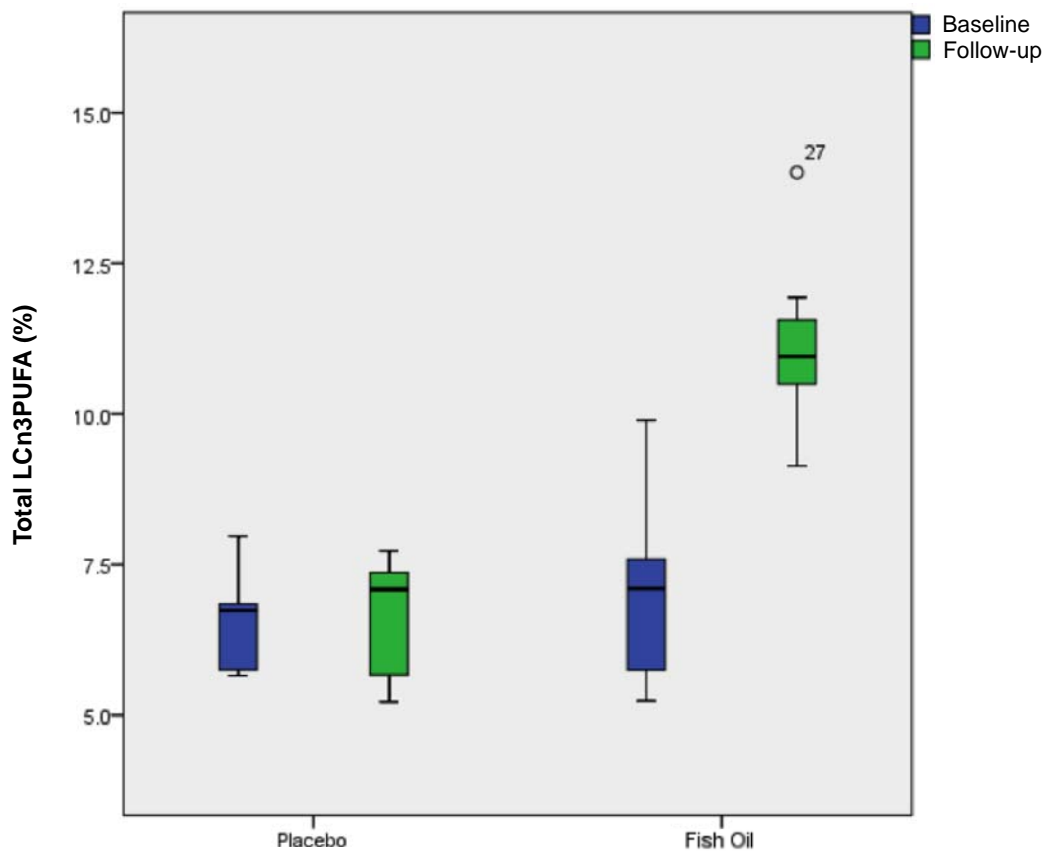


Figure 2.8. Boxplot showing total LCn3PUFA levels at baseline and follow-up.

2.3.3 Correlation Between Clinical Parameters and Fatty Acid Profiles

Pearson correlation analyses were used to identify any linear relationship between changes in fatty acid profiles and changes in clinical periodontal parameters (Appendix III). In Table 2.7, negligible to weak negative correlations can be seen for changes in DHA levels and changes in clinical outcomes. However, there were no statistically significant correlations demonstrated between changes in probing pocket depth and clinical attachment ≥ 2 mm and changes in EPA, DHA, DPA, Omega-3 Index and total LCn3PUFAs.

Fatty Acid Profile (% of total fatty acids)	Change in CAL ≥ 2 mm		Change in PPD ≥ 2 mm	
	Pearson's r	<i>P</i> value	Pearson's r	<i>P</i> value
Change in EPA	-0.091	0.671	-0.51	0.812
Change in DHA	0.086	0.688	0.055	0.799
Change in DPA	-0.207	0.333	-0.176	0.412
Change in Omega-3 Index	0.024	0.912	0.019	0.931
Total LCn3PUFAs	-0.051	0.814	-0.045	0.836

Table 2.7. Correlation between fatty acid profile and clinical periodontal parameters.

2.3.4 Effects of EPA versus DHA

One-way between-group analyses of variance were carried out to determine whether a significant difference exists between EPA and DHA for the percentage of sites with gain in clinical attachment ≥ 2 mm, or the percentage of sites with probing pocket depth reduction ≥ 2 mm. No statistically significant differences were demonstrated between subjects that received supplements containing EPA, DHA or placebo, although a trend was seen for greater changes in both probing depth and clinical attachment for the placebo group (Table 2.8).

Clinical Parameter (Mean ± SD)	EPA group	DHA group	Placebo	P value
% sites with reduction in PPD ≥2 mm	17.3 (±11.0)	22.5 (±21.6)	26.1 (±16.2)	NS (<i>P</i> = 0.542)
% sites with gain in CAL ≥2 mm	18.6 (±12.1)	26.4 (±16.3)	29.2 (±14.3)	NS (<i>P</i> = 0.280)

Table 2.8. Changes in CAL or PPD for subjects that received EPA or DHA.

2.4 Discussion

2.4.1 Summary of Main Results

The aim of this study was to evaluate the clinical benefit of dietary fish oil supplementation as an adjunct to non-surgical debridement for the treatment of chronic periodontitis. Both the test and control group were effective in improving clinical periodontal outcomes including full mouth bleeding scores, probing pocket depth and clinical attachment level. Participants that received adjunctive fish oil or placebo experienced significant and similar reductions in full mouth bleeding scores (mean reductions of $21.3 \pm 14.3\%$ and $22.8 \pm 15.6\%$, respectively, *P* values = 0.00 and 0.02). Both groups however, can be considered to have an undesirable level of residual clinical inflammation, with follow-up mean bleeding scores of $33.2 \pm 14.7\%$ and $37.6 \pm 22.9\%$, respectively. Although this decrease in bleeding on probing 3 months after initial therapy is similar to results of other clinical studies (Badersten et al., 1984b, Loos et al., 1987), bleeding scores between 20% and 30% have been associated with a reduced risk of disease progression (Badersten et al., 1990, Claffey et al., 1990, Joss et al., 1994). However, due to the low positive predictive value of bleeding on probing, Lang et al. (1990) have suggested the alternative use of absence of bleeding as an indicator of periodontal stability. Nonetheless, the impaired platelet function and significantly increased bleeding time in patients taking LCn3PUFA supplementation (Saynor et al., 1983) suggests that bleeding on probing in this cohort of patients may be a less reliable surrogate marker of clinical periodontal inflammation.

In the current study the mean reduction in probing depth for sites with moderate probing depths (4-6 mm) at baseline were 1.65 ± 2.00 mm and 1.46 ± 1.00 mm for the placebo and fish oil group, respectively. As expected, mean probing pocket depths reductions were greater in sites with deep initial probing pocket depths (3.11 ± 3.00 mm and 2.93 ± 3.00 mm for the placebo and fish oil group, respectively). Both groups demonstrated

favourable mean changes in these parameters when compared to averages reported in the literature (Cobb and Jeffcoat, 2002).

Mean changes in probing pocket depth and clinical attachment have been used extensively in the periodontal literature to indicate the efficacy of treatment. Although mean values can be a useful overview of treatment effect, they do not represent changes at individual sites and may actually conceal sites that have a poor response or disease progression. Frequently the reported differences in mean values are less than one millimetre and difficult to relate to the clinical setting (Greenstein and Lamster, 1995). The primary outcomes selected for this study were therefore the percentage of sites that demonstrate a reduction in probing depth or gain in clinical attachment ≥ 2 mm. Periodontal therapy with adjunctive fish oil or placebo demonstrated a clinically significant proportion of sites with probing depth reductions and gain in clinical attachment ≥ 2 mm at follow-up (Table 2.5). The percentage of sites with probing pocket depths ≥ 5 mm following initial therapy was chosen as a secondary outcome, as residual deep pockets have been associated with increased risk of disease progression (Claffey and Egelberg, 1995, Matuliene et al., 2008) and are often regarded by clinicians as needing further treatment. At the 4 month follow-up there were substantial and clinically significant reductions in residual probing depths for both groups (6.6% and 5.3% residual pockets ≥ 5 mm for the fish oil and placebo group, respectively) although there was no significant difference between groups ($P = 0.264$). This level of improvement is in agreement with previous studies that reported between 6% and 10% of residual sites with probing depths of ≥ 6 mm following non-surgical therapy (Badersten et al., 1984a, Lindhe and Nyman, 1985, Serino et al., 2001). Taken together, the results of this study support the efficacy of non-surgical therapy in the treatment of advanced chronic periodontitis for the majority of patients.

In summary the results of this study were not able to show a clinically or statistically significant benefit for adjunctive fish oil supplementation in either probing pocket depth reduction or clinical attachment gain (≥ 2 mm), as well as the number of residual sites with ≥ 5 mm probing depths. This supports the null hypothesis that adjunctive fish oil supplementation has no additional benefit in the treatment of advanced chronic periodontitis compared to standard non-surgical periodontal therapy alone. Additionally, no correlation was found between the proportion of sites with changes in probing depth or clinical attachment ≥ 2 mm and fatty acid biomarkers including EPA, DHA, DPA, total LCn3PUFAs and omega-3 index (Table 2.7). Failure to demonstrate a treatment effect for

LCn3PUFAs could not be attributed to non-compliance in participants that were assigned to fish oil capsules, as indicated by the substantial and highly significant increase in mean total LCn3PUFAs and mean omega-3 index for the test group but not the control (Table 2.9). There is no obvious biologically plausible explanation to explain the small but significantly greater improvement in clinical parameters seen in the placebo group compared with the test group. For example, at moderately deep initial sites, patients in the fish oil group had a 26% reduced odds of achieving ≥ 2 mm probing depth reduction than patients that received the placebo (OR = 0.74, 95% CI: 0.56, 0.98) (Table 2.5). It is also interesting that greater variation in clinical attachment was observed for the test oil group (Figure 2.5).

2.4.2 Agreement and Disagreement with Other Research

A potential role for the treatment of periodontitis with LCn3PUFAs and pro-resolution lipid mediators was highlighted in several experimental periodontitis studies in animal models (Hasturk et al., 2006, Kesavalu et al., 2006, Hasturk et al., 2007, Bendyk et al., 2009). The current investigation is one of the first double-blind randomised placebo controlled trials to evaluate the effect of adjunctive LCn3PUFA supplementation in the treatment of chronic periodontitis. The results are in agreement with another recently conducted pilot RCT that found no additional effect of LCn3PUFAs on the clinical outcomes of periodontal therapy (Martinez et al., 2014). This study compared non-surgical therapy plus dietary supplementation, comprising 3 g/d of EPA plus DHA, with a control group that received SRP plus placebo (Martinez et al., 2014). Other clinical trials have important differences in methodology, having investigated LCn3PUFAs as an adjunct to oral hygiene instruction only (Rosenstein et al., 2003, Parulkar et al., 2009); oral hygiene instruction and DHA plus low-dose aspirin (Naqvi et al., 2014); non-surgical periodontal therapy with adjunctive LCn3PUFA plus low-dose aspirin (El-Sharkawy et al., 2010); or regenerative/reconstructive periodontal surgery with adjunctive LCn3PUFA plus low-dose aspirin (Elkhouli, 2011).

Rosenstein et al. (2003) evaluated the treatment of periodontitis with oral hygiene instruction and supplementation with either EPA, gamma-linolenic acid (GLA, an n-6 fatty acid), combined EPA plus GLA, or olive oil (placebo). A significant improvement in gingival inflammation and probing depth was seen in subjects that received GLA

supplementation, but only a trend for improved outcomes was seen for groups that received EPA alone or combined GLA and EPA supplementation.

Another clinical trial reported significant additional reductions in probing depth and gain in clinical attachment after 3 and 6 months of supplementation with EPA plus DHA (900 mg), combined with low dose aspirin (81 mg), as an adjunct to non-surgical periodontal therapy (El-Sharkawy et al., 2010). As well as the improvement in clinical parameters, there were also significant reductions in salivary RANKL and MMP-8 levels. However, the study did not have an intervention group consisting of LCn3PUFA without aspirin and did not assess compliance using fatty acid biomarkers.

More recently, an RCT found that participants taking daily DHA (2000 mg) plus low dose aspirin (81 mg) were twice as likely to have a reduction in probing depth ≥ 2 mm compared with the control group that received aspirin plus placebo (OR = 2.05) (Naqvi et al., 2014). DHA plus aspirin supplementation also significantly decreased mean probing depths and the number of sites with residual probing depths ≥ 5 mm. Major differences from the current study are that participants received oral hygiene instruction but no mechanical debridement, as well as the addition of low-dose aspirin. The concurrent administration of LCn3PUFAs with aspirin may be a more effective approach to host modulation therapy due to their combined effect on lipid metabolism and inflammation. In addition to the established inhibitory effect on cyclooxygenase (and decrease in arachidonic acid-derived prostaglandin synthesis), aspirin also facilitates the resolution of inflammation by triggering the formation of alternative pro-resolution lipid mediators via different pathways. These aspirin-triggered derivatives are more biologically active and longer lasting than their native forms, and include aspirin-triggered lipoxins and aspirin-triggered resolvins (Arita et al., 2005, Serhan, 2005a). This may account for the difference in results compared with the current investigation. However, given the well-known adverse effects and contraindications associated with aspirin therapy, it is important to establish whether the host modulatory effects of adjunctive LCn3PUFAs can deliver significant improvement independent of the effects of aspirin.

In summary, previous trials that demonstrate a significant improvement in the clinical outcomes of periodontal therapy following treatment with LCn3PUFAs were not supported by the results of this investigation. Study heterogeneity may account for the conflicting results including differences in sample size or baseline periodontitis severity. The

treatment effect of LCn3PUFAs may have also been diminished by the impact of effective non-surgical therapy or the lack of concurrent aspirin administration. The latter may be an important finding, since a clinically significant host modulatory effect of dietary LCn3PUFAs on the periodontium may not be achieved without the generation of aspirin-triggered pro-resolving lipid mediators. Comparisons between studies are also confounded by fish oil regimes that provide different doses of LCn3PUFAs, or proportions of DHA to EPA.

To our knowledge, this is the first study to evaluate the effects of specific fatty acids in the treatment of periodontitis. The test group had a total intake of 2 g LCn3PUFAs per day, with subgroups receiving either predominantly EPA (1600 mg) or predominantly DHA (1600 mg). No statistically significant differences were found between EPA, DHA or placebo for changes in probing pocket depth ($P = 0.542$) or clinical attachment ≥ 2 mm ($P = 0.280$). However, the lack of a statistically significant difference between groups may have been due to the limited sample size.

2.4.3 Limitations and Potential Bias

This trial has a number of strengths, including robust methodology related to blinding, allocation concealment and randomisation, as well as the use of recognised and clearly defined criteria for periodontal disease severity. Analyses were stratified for sites with baseline probing depth of 4-6 mm or ≥ 7 mm as the magnitude of change in probing depth and clinical attachment are related to the severity of the initial disease (Cobb and Jeffcoat, 2002). Multivariate analyses with ANCOVA allowed adjustment for baseline probing pocket depths and may also yield greater study power and reduced type II errors (Tu et al., 2005). Generalised estimating equations were used to account for clustering within patients and teeth as response to treatment is also influenced by individual patient susceptibility, a number of host modifying factors, as well as localised predisposing factors.

There are however several limitations that should be considered. Only qualitative measures of plaque accumulation were recorded and no quantitative plaque indices were used (Silness and L oe, 1964, O'Leary et al., 1972). Although emphasis was placed on achieving a high level of plaque control in all patients, there is the potential for a difference in baseline oral hygiene between test and control group to reduce or enhance the treatment

effect of LCn3PUFA supplementation. Poor plaque control may also be associated with the moderate levels of bleeding on probing at follow-up. However, the improvement in other clinical outcomes for both treatment groups was similar to other studies where low plaque scores were achieved (Lindhe and Nyman, 1985).

Lack of statistical power was probably the main limitation of the current study due to the difficulty in recruiting eligible participants. Given the inclusion criteria of this study, no conclusion can be made about the effects of adjunctive LCn3PUFA therapy in subjects with diabetes and systemic inflammatory diseases, current smokers, patients with aggressive disease or taking concomitant antibiotics. Furthermore, due to the short follow-up period, no assumptions can be made about the long-term effects of adjunctive LCn3PUFA supplementation on the outcomes of periodontal therapy. However, the rapid incorporation of these fatty acids into the cell membranes of inflammatory and immune cells suggests that any effect on the host inflammatory response could be expected within the time period reported. Following ingestion of LCn3PUFA, the incorporation of EPA and DHA into neutrophils reaches a peak after 4 weeks (Healy et al., 2000). Recent studies however indicate that near maximum incorporation of LCn3PUFAs into human mononuclear cells occurs at around 7 days after supplementation, suggesting that changes to immune cell function may occur more quickly than previously considered (Calder 2013). Although most improvement in clinical parameters has usually taken place after a follow-up period of 3 months, additional healing and tissue maturation may take several more months, especially in sites with advanced disease and greater baseline probing depths (Badersten et al., 1984a, Cugini et al., 2000). It would therefore be interesting to examine whether the anti-inflammatory and pro-resolution actions of LCn3PUFAs have a significant effect over a longer duration than investigated in this study.

2.4.4 Implications for Practice and Research

Currently there is not enough evidence to support the use of LCn3PUFAs in the treatment of periodontitis in clinical practice. In view of the conflicting findings from previous studies, further multicentre randomised controlled trials are needed to confirm the efficacy of dietary LCn3PUFA as a periodontal host modulation therapy. If proven effective, evidence-based guidelines for the optimum dose and duration of LCn3PUFA supplementation would need to be determined. Future studies should have longer follow-up and establish the importance of aspirin by comparing the effects of combined

LCn3PUFA plus aspirin with LCn3PUFA plus placebo. Additional evaluation of the relative importance of DHA versus EPA is also warranted.

Several other recommendations can be made for subsequent fish oil studies. Given the variations in LCn3PUFA doses, bioavailability and endogenous interconversion between DHA and EPA, the reporting of reliable fatty acid biomarkers is important to allow meaningful comparisons between clinical trials (Arterburn et al., 2006, Fekete et al., 2009). Another limitation of clinical trials that evaluate pharmacologic-based treatment is that non-compliance in the test group may weaken the ability to detect a significant treatment effect. Fish oil capsules are widely available without a prescription and unplanned supplementation in the control group may also diminish the treatment effect. A standardised and robust method was proposed to assess compliance using established biomarkers of EPA and DHA levels, including total erythrocyte LCn3PUFAs and the Omega-3 Index. The latter is the content of EPA plus DHA, expressed as a percentage of total erythrocyte fatty acid, and has demonstrated low biological variability (Harris and Thomas, 2010).

Data from this pilot study were used to improve sample size estimation so that subsequent trials have sufficient power to demonstrate a significant treatment effect. A sample size calculation was repeated assuming a 6.8% difference between groups for the percentage of sites that demonstrated ≥ 2 mm reduction in probing pocket depth from baseline to 3 month follow-up. Based on an unpaired between-group *T* test and a 0.05 type 1 error, 186 participants would be required to achieve 90% power (Appendix IV). A total sample size of 230 participants would be needed to allow for a drop-out rate of 20%.

2.5 Conclusion

Dietary supplementation with fish oil resulted in a significant increase in erythrocyte phospholipid total LCn3PUFA levels and omega-3 index. Non-surgical periodontal therapy was effective in improving the clinical outcomes of subjects with advanced chronic periodontitis. However, within the limitations of this study the findings do not support an additional benefit of adjunctive LCn3PUFA supplementation and there were no significant differences between EPA and DHA subgroups. There is a need for further research to establish the clinical efficacy of LCn3PUFAs as a host modulatory therapy for the treatment of periodontitis, particularly larger multi-centre randomised controlled trials.

2.6 References

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Appendix I - Electronic Database Search Strategy

Pubmed

<p>Fish oils [MH] OR Fish oil*[TW] OR Fish liver oil*[TW] OR Omega 3[TW] OR n-3 fatty acid*[TW] OR n-3 Polyunsaturated Fatty Acid*[TW] OR n-3 PUFA*[TW] OR Docosahexenoic Acid*[TW] OR Eicosapentanoic Acid*[TW] OR EPA[TW] OR DHA[TW] OR Resolvin[ALL] OR Resolvins[ALL] OR Protectin[ALL] OR Protectins[ALL] OR Lipoxin*[ALL]</p>	<p>Periodontal diseases[MH] OR Periodontitis[TW] OR Periodontal disease*[TW] OR Furcation defect* [TW] OR Gingiv*[TW] OR Peri-implant*[TW] OR Periiimplant*[TW] OR Periodontal atroph*[TW] OR Periodontal pocket*[TW]</p>
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EMBASE

<p>'Fish oil'/SYN OR 'Fish liver' NEXT/1 oil* OR omega 3 fatty acid/SYN OR 'n-3 Fatty Acids' OR 'n-3' NEXT/1 PUFA* OR 'Docosahexenoic Acid'/SYN OR Eicosapentanoic NEXT Acid* OR DHA OR Resolvin OR Resolvins OR Protectin OR Protectins OR Maresin* OR Lipoxin/SYN</p>	<p>Periodontal disease OR Peri-implant* OR Periiimplant* OR</p>
--	---

Appendix II - Interexaminer Agreement

RELIABILITY

```

/VARIABLES=Examiner1 Examiner2
/SCALE('ALL VARIABLES') ALL
/MODEL=ALPHA
/ICC=MODEL(MIXED) TYPE(ABSOLUTE) CIN=95 TESTVAL=0.

```

Case Processing Summary

		N	%
Cases	Valid	168	100.0
	Excluded ^a	0	.0
	Total	168	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.682	2

Intraclass Correlation Coefficient

	Intraclass Correlation ^b	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.519 ^a	.399	.621	3.146	167	167	.000
Average Measures	.683 ^c	.570	.767	3.146	167	167	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.

- The estimator is the same, whether the interaction effect is present or not.
- Type A intraclass correlation coefficients using an absolute agreement definition.
- This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

RELIABILITY

```

/VARIABLES=Examiner1 Examiner3
/SCALE('ALL VARIABLES') ALL
/MODEL=ALPHA
/ICC=MODEL(MIXED) TYPE(ABSOLUTE) CIN=95 TESTVAL=0.

```

Case Processing Summary

		N	%
Cases	Valid	168	100.0
	Excluded ^a	0	.0
	Total	168	100.0

a. Listwise deletion based on all variables in the procedure.

Reliability Statistics

Cronbach's Alpha	N of Items
.683	2

Intraclass Correlation Coefficient

	Intraclass Correlation ^b	95% Confidence Interval		F Test with True Value 0			
		Lower Bound	Upper Bound	Value	df1	df2	Sig
Single Measures	.494 ^a	.352	.610	3.157	167	167	.000
Average Measures	.661 ^c	.521	.757	3.157	167	167	.000

Two-way mixed effects model where people effects are random and measures effects are fixed.

- a. The estimator is the same, whether the interaction effect is present or not.
- b. Type A intraclass correlation coefficients using an absolute agreement definition.
- c. This estimate is computed assuming the interaction effect is absent, because it is not estimable otherwise.

Appendix III - Statistical Analyses

T-tests to compare percentage of sites with ≥ 2 mm gain in CAL between groups

The TTEST Procedure

Variable: Delta_CAL (Delta_CAL)

Randomisation	N	Mean	Std Dev	Std Err	Minimum	Maximum
B: Placebo	9	0.2920	0.1429	0.0476	0.1146	0.6000
A: Fish Oil	17	0.2174	0.1455	0.0353	0.0385	0.5333
Diff (1-2)		0.0746	0.1446	0.0596		

Randomisation	Method	Mean	95% CL Mean	Std Dev	95% CL Std Dev
B: Placebo		0.2920	0.1822 0.4019	0.1429	0.0965 0.2737
A: Fish Oil		0.2174	0.1426 0.2922	0.1455	0.1084 0.2214
Diff (1-2)	Pooled	0.0746	-0.0485 0.1977	0.1446	0.1129 0.2012
Diff (1-2)	Satterthwaite	0.0746	-0.0506 0.1998		

Method	Variances	DF	t Value	Pr > t
Pooled	Equal	24	1.25	0.2229
Satterthwaite	Unequal	16.68	1.26	0.2255

T-tests to compare percentage of sites with ≥ 2 mm reduction in PPD between groups

The TTEST Procedure

Variable: Delta_poc (Delta_poc)

Randomisation	N	Mean	Std Dev	Std Err	Minimum	Maximum
B: Placebo	9	0.2606	0.1620	0.0540	0.0577	0.6267
A: Fish Oil	17	0.1922	0.1662	0.0403	0.0298	0.7067
Diff (1-2)		0.0684	0.1648	0.0679		

Randomisation	Method	Mean	95% CL Mean	Std Dev	95% CL Std Dev
B: Placebo		0.2606	0.1361 0.3851	0.1620	0.1094 0.3103
A: Fish Oil		0.1922	0.1068 0.2777	0.1662	0.1238 0.2529
Diff (1-2)	Pooled	0.0684	-0.0718 0.2086	0.1648	0.1287 0.2292
Diff (1-2)	Satterthwaite	0.0684	-0.0739 0.2107		

Method	Variances	DF	t Value	Pr > t
Pooled	Equal	24	1.01	0.3242
Satterthwaite	Unequal	16.792	1.01	0.3246

ANCOVA model to compare number of residual pockets ≥ 5 mm between groups at follow-up

The MEANS Procedure

Randomisation	N Obs	Variable	Mean	Std Dev
B: Placebo	11	vis_PD50	22.1538715	16.2224170
		vis_PD54	1.1	7.7735074
			1.2	5.2984599
A: Fish Oil	23	vis_PD50	21.2044153	13.8468021
		vis_PD54	6.6419474	5.4116412

ANCOVA model to compare changes in mean probing depth between groups at follow-up

ANCOVA MODEL: INVESTIGATING CHANGE IN MEAN POCKET DEPTH AT 4M * RANDOMISATION ACCOUNTING FOR BASELINE SCORES

The GLM Procedure
Least Squares Means

Randomisation	vis_PD54 LSMEAN	Standard Error	H0:LSMEAN=0	H0:LSMean1=LSMean2
			Pr > t	Pr > t
A: Fish Oil	2.67793708	0.05879428	<.0001	0.1901
B: Placebo	2.53795676	0.08552781	<.0001	

Randomisation	vis_PD54 LSMEAN	95% Confidence Limits	
A: Fish Oil	2.677937	2.556848	2.799026
B: Placebo	2.537957	2.361809	2.714105

Pearson Correlation analyses for fatty acid profiles and gain in CAL ≥ 2 mm

Sites that gained CAL ≥ 2 mm and change in EPA levels.

Correlations

		Delta_CAL	EPA_D
Delta_CAL	Pearson Correlation	1	-.091
	Sig. (2-tailed)		.671
	N	26	24
EPA_D	Pearson Correlation	-.091	1
	Sig. (2-tailed)	.671	
	N	24	28

Sites that gained CAL ≥ 2 mm and change in DHA levels.

Correlations

		Delta_CAL	DHA_D
Delta_CAL	Pearson Correlation	1	.086
	Sig. (2-tailed)		.688
	N	26	24
DHA_D	Pearson Correlation	.086	1
	Sig. (2-tailed)	.688	
	N	24	28

Sites that gained CAL ≥ 2 mm and change in DPA levels.

Correlations

		Delta_CAL	DPA_D
Delta_CAL	Pearson Correlation	1	-.207
	Sig. (2-tailed)		.333
	N	26	24
DPA_D	Pearson Correlation	-.207	1
	Sig. (2-tailed)	.333	
	N	24	28

Sites that gained CAL ≥ 2 mm and change in omega-3 index.

Correlations

		Delta_CAL	n3_Index_D
Delta_CAL	Pearson Correlation	1	.024
	Sig. (2-tailed)		.912
	N	26	24
n3_Index_D	Pearson Correlation	.024	1
	Sig. (2-tailed)	.912	
	N	24	28

Sites that gained CAL ≥ 2 mm and change in total LCn3PUFAs

Correlations

		Delta_CAL	TLCn3P_D
Delta_CAL	Pearson Correlation	1	-.051
	Sig. (2-tailed)		.814
	N	26	24
TLCn3P_D	Pearson Correlation	-.051	1
	Sig. (2-tailed)	.814	
	N	24	28

Pearson Correlation analyses for fatty acid profiles and reduction in PPD ≥ 2 mm

Sites with ≥ 2 mm PPD reduction and change in EPA levels.

Correlations

		Delta_poc	EPA_D
Delta_poc	Pearson Correlation	1	-.051
	Sig. (2-tailed)		.812
	N	26	24
EPA_D	Pearson Correlation	-.051	1
	Sig. (2-tailed)	.812	
	N	24	28

Sites with ≥ 2 mm PPD reduction and change in DHA levels.

Correlations

		Delta_poc	DHA_D
Delta_poc	Pearson Correlation	1	.055
	Sig. (2-tailed)		.799
	N	26	24
DHA_D	Pearson Correlation	.055	1
	Sig. (2-tailed)	.799	
	N	24	28

Sites with ≥ 2 mm PPD reduction and change in DPA levels.

Correlations

		Delta_poc	DPA_D
Delta_poc	Pearson Correlation	1	-.176
	Sig. (2-tailed)		.412
	N	26	24
DPA_D	Pearson Correlation	-.176	1
	Sig. (2-tailed)	.412	
	N	24	28

Sites with ≥ 2 mm PPD reduction and change in omega-3 index.

Correlations

		Delta_poc	n3_Index_D
Delta_poc	Pearson Correlation	1	.019
	Sig. (2-tailed)		.931
	N	26	24
n3_Index_D	Pearson Correlation	.019	1
	Sig. (2-tailed)	.931	
	N	24	28

Sites with ≥ 2 mm PPD reduction and change in total LCn3PUFAs

Correlations

		Delta_poc	TLCn3P_D
Delta_poc	Pearson Correlation	1	-.045
	Sig. (2-tailed)		.836
	N	26	24
TLCn3P_D	Pearson Correlation	-.045	1
	Sig. (2-tailed)	.836	
	N	24	28

One-way between group ANOVA comparing change in clinical attachment and probing depth ≥ 2 mm for subgroups that received DHA, EPA or placebo

Descriptives

Delta_CAL

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	9	.29211	.142814	.047605	.18233	.40189	.115	.600
2.00	9	.18567	.121075	.040358	.09260	.27873	.038	.357
3.00	8	.26437	.163007	.057632	.12810	.40065	.051	.533
Total	26	.24673	.144364	.028312	.18842	.30504	.038	.600

1=placebo, 2=EPA, 3=DHA

ANOVA

Delta_poc

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.035	2	.017	.629	.542
Within Groups	.636	23	.028		
Total	.670	25			

1=placebo, 2=EPA, 3=DHA

ANOVA

Delta_CAL

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.055	2	.027	1.346	.280
Within Groups	.466	23	.020		
Total	.521	25			

Descriptives

Delta_poc

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1.00	9	.26067	.161960	.053987	.13617	.38516	.058	.627
2.00	9	.17322	.110491	.036830	.08829	.25815	.060	.375
3.00	8	.22525	.216492	.076541	.04426	.40624	.030	.707
Total	26	.21950	.163754	.032115	.15336	.28564	.030	.707

Independent T-test comparing baseline and follow-up total n-3 PUFAs

Group Statistics					
	1=placebo, 2=Fish oil	N	Mean	Std. Deviation	Std. Error Mean
TLCn3P_0	1.00	9	6.5524	.82157	.27386
	2.00	19	7.0001	1.23111	.28244
TLCn3P_4mth	1.00	9	6.6273	.94974	.31658
	2.00	18	10.9549	1.11651	.26316

10.95-7.00=3.95 increase from baseline for fish oil group
 3.95/7.00 x 100 =56.43% increase in fish oil group

6.63-6.55= 0.08 increase from baseline in placebo
 0.08/6.55 x 100= 1.22% increase in placebo group

Independent Samples Test

	Levene's Test for Equality of Variances	t-test for Equality of Means								
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
TLCn3P_0	Equal variances assumed	1.086	.307	-.987	26	.333	-.44761	.45367	-1.38015	.48493
	Equal variances not assumed			-1.138	22.670	.267	-.44761	.39341	-1.26209	.36687
TLCn3P_4mth	Equal variances assumed	.000	.999	-9.944	25	.000	-4.32761	.43519	-5.22390	-3.43133
	Equal variances not assumed			10.512	18.679	.000	-4.32761	.41168	-5.19026	-3.46496

Independent T-test comparing baseline and follow-up Omega-3 index

Group Statistics					
	1=placebo, 2=Fish oil	N	Mean	Std. Deviation	Std. Error Mean
n3_Index_0	1.00	9	4.4250	.82948	.27649
	2.00	19	4.7659	1.18489	.27183
n3_Index_4mth	1.00	9	4.6043	.88188	.29396
	2.00	18	8.2953	1.34354	.31668

8.295-4.7659 =3.529 increase in fish oil group
 3.529/4.7659 x 100 =74.05%

4.604-4.425 =0.179 increase in placebo group
 0.179/4.425 x 100 =4.05%

Appendix III - Supplemental Tables

4 month Follow-up (% of total fatty acids)	Group 1 Placebo		Group 2 Fish Oil		Difference
	Mean	95% CI	Mean	95% CI	
Total LCn3PUFA	6.81	6.30, 7.31	10.86	10.50, 11.22	<0.0001
Omega-3 Index	4.72	4.05, 5.40	8.34	7.76, 8.71	<0.0001

Table 2.9. Difference in fatty acid profile at follow-up

Appendix IV - Sample Size Calculation

Power calculation for CAL gain of at least 2 mm

The POWER Procedure

Two-Sample t Test for Mean Difference

Fixed Scenario Elements

Distribution	Normal
Method	Exact
Mean Difference	0.0746
Standard Deviation	0.1446
Nominal Power	0.8
Number of Sides	2
Null Difference	0
Alpha	0.05

Computed N Per Group

Actual Power	N Per Group
0.800	60

Power calculation for PD reduction of at least 2 mm

The POWER Procedure

Two-Sample t Test for Mean Difference

Fixed Scenario Elements

Distribution Normal

Method Exact

Mean Difference 0.0684

Standard Deviation 0.1648

Nominal Power 0.8

Number of Sides 2

Null Difference 0

Alpha	0.05
-------	------

Computed	N	Per
Group		

Actual Power	N	Per
	Group	

0.804	93	
-------	----	--