Impact of an SPM Enriched Omega-3 Fatty Acid Dietary Supplement on Periodontal Regeneration

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ABSTRACT

Specialized pro-resolving lipid mediators (SPMs) biosynthesized from essential polyunsaturated fatty acids (PUFA) promote resolution of inflammation and enhance stem cell proliferation and differentiation leading to tissue regeneration. SPMs are derived from lipoxygenase-driven conversion of long-chain PUFA, such as arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid. Periodontitis is an inflammatory disease of the supporting structures of the teeth that involves an interplay between microbial biofilm and the host immune response leading to inflammatory-immune destruction of connective tissue attachment and bone. Regeneration of hard and soft tissues lost to periodontal disease is limited and not predictable. Flap surgery, enamel matrix proteins, bone and soft tissue grafting have been employed to regenerate periodontal tissues lost to disease, generally with poor outcomes.

Prior studies have shown that Resolvin E1 enhances resolution of inflammation, restores horizontal and vertical bone defects and produces significant bone regeneration. Prior work in the pig demonstrated that Benzo-Lipoxin A_4 ($BLXA_4$), a stable analog mimetic of lipoxin A_4, encapsulated in nanoparticle liposomes, significantly improved surgical outcomes and improved regeneration of bone. The aim of this study was to determine whether a dietary supplement that contains the omega-3 fatty acids EPA and DHA, and is enriched with the precursors for SPMs, enhances periodontal regeneration. Interproximal periodontal defects 6 mm in depth were created using burs in 3 female adult Göttingen Miniature Pigs, the defects were created in all 4 quadrants around second and fourth premolars and were maintained by placement of sterile orthodontic wires. The sites were left untreated for 40 days to stabilize periodontal disease and ligatures were thereafter removed, and established, stable periodontal disease was surgically treated 40 days later. Each Pig was assigned to a treatment group 40 days after ligature removal; surgical debridement only (negative control), surgical debridement with delivery of 0.5 µg of $BLXA_4$ in a lipid nanoparticle solution to each of eight treatment sites (two per quadrant) using a P20 micro-pipette (positive control), and surgical debridement and daily dietary supplementation with an enriched omega-3 supplement (Lipinova®, Solutex) (3ml, added to a small portion of regular food or Gatorade) until the end of the study. Animals were euthanized 90 days later and jaws harvested. MicroCT scans were performed before jaws samples were decalcified for histological analyses. Blood samples were obtained from each pig for metabolipidomic analysis by LC-MS/MS. Three-dimensional bone analysis with Micro CT

revealed significant reduction in defect depth in the Diet and BLX_4 groups compared to control (ANOVA, $p< 0.005$). Metabololipidomics analysis revealed a significant shift in SPM profiles in the $BLXA_4$ and diet groups compared to control. This study in a large animal model demonstrates that dietary supplementation with omega-3 PUFA enhances bone regeneration to a similar level to a very low dose of the active molecule, $BLXA_4$. SPMs are known to activate wound healing with tissue regeneration and directly improve bone healing and regeneration. Dietary supplementation with a specific omega-3 PUFA preparation that is enriched with SPM precursors exhibited statistically significant improvement in tissue healing and regeneration.

1.INTRODUCTON

1.1 Periodontal disease

Periodontal disease is a significant public health issue worldwide. In the United States (US), it has been estimated to affect 143.8 million adult Americans 30 years or older [1]. Among US dentate adults in the age group 30-79 years, the prevalence of periodontal disease was 42.2%; 7.8% had severe periodontitis while 34.4 had mild to moderate periodontitis [1]. Periodontitis is an inflammatory disease of supporting structures of the teeth: the alveolar bone, periodontal ligament and cementum, and involves an interplay between the microbial biofilm that accumulates on the teeth and the host immune response that leads to host-mediated destruction of supporting structures of the tooth including connective tissue attachment and bone [2, 3]. The presence of microbial biofilm alone is not sufficient to trigger periodontitis; disease occurs when there is an imbalance between the microbial biofilm and host response caused by a heightened inflammatory state that leads to destruction of supporting structures of the tooth [4, 5].

Commensal microflora in the form of organized bacteria biofilms colonize the exposed crown of the tooth in the mouth and the gingival sulcus and is known as supragingival and subgingival dental plaque, respectively. Periodontal disease usually starts with gingivitis, which is an inflammation of the gingiva resulting from chronic exposure to bacterial biofilm present in the mouth. The initial immune response in gingivitis is dominated by neutrophils, and over time the lesion becomes mature with an influx of B cells and plasma cells. In advanced gingivitis, there is loss of gingival collagen; but the lesion is reversible at this stage with plaque removal [3, 6].

The biofilm associated with periodontitis is more complex and dominated by more gram-negative bacteria. Gram-negative bacterial pathobionts overgrow in response to excess inflammation representing a shift to a more pathogenic flora that incorporates other Gram-negative pathogens causing more inflammation. If uninterrupted, this cycle of increasing inflammation and the formation of a mature immunologic lesion leads to destruction of tissues beyond the gingival unit to periodontal supporting tissues (periodontitis)[3]

P. gingivalis is a gram negative, obligate, anaerobic, asaccharolytic organism that has been implicated in the etiopathogenesis of periodontal disease. *P. gingivalis* derives energy from collagen break down products (amino acids) provided by the host through inflammation using an array of proteolytic enzymes, the gingipains [3, 7]. It is a very proinflammatory organism *in situ* and has been the focus of considerable study in the etiology of periodontitis. Irreversible loss of connective tissue attachment and alveolar bone marks progression to

periodontal disease. At this stage, plaque removal does not result in return to tissue homeostasis with regeneration of lost tissues [3, 6].

Bacteria are beyond any reasonable doubt the specific etiology of gingivitis, and periodontitis has been associated with overgrowth of a subset of bacteria pathobionts; however, overwhelming evidence has shown that it is the uncontrolled host inflammatory and immune response that drives the tissue destruction associated with periodontal disease [3, 8].

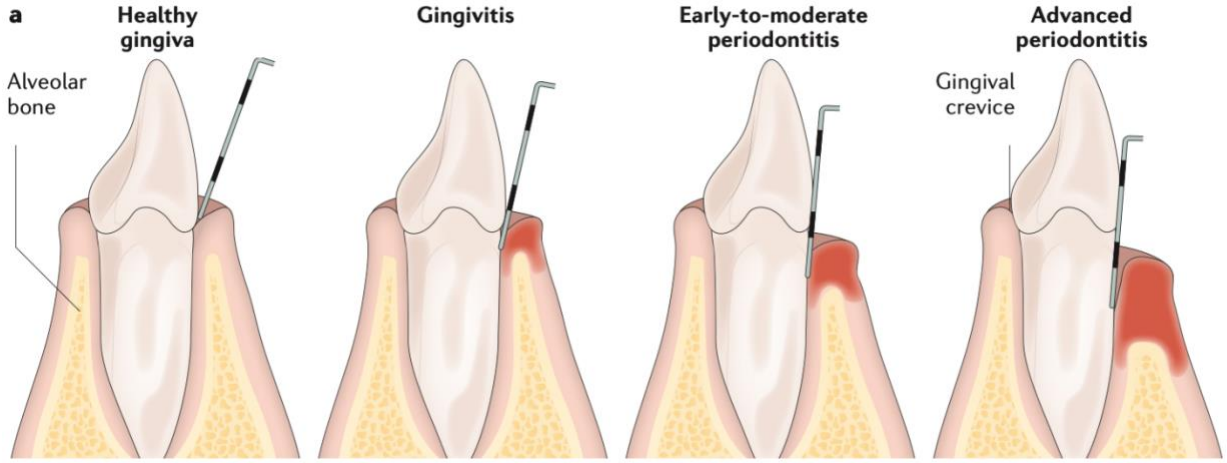

Figure 1.1 Stages of Periodontitis- healthy gingiva, gingivitis, early-to-moderate periodontitis and advanced periodontitis [5]

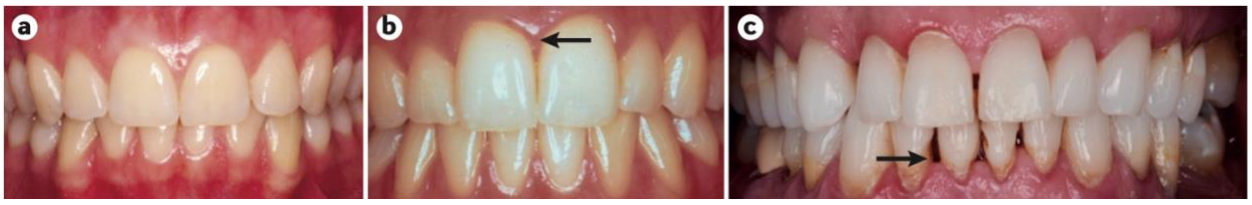

Figure 1.2a-c. Healthy and diseased periodontium. a. Healthy periodontal tissues. b. Early gingival inflammation (gingivitis; arrow) c. Clinical appearance of chronic periodontitis, with tissue loss and deep periodontal 'pockets' that are a hallmark of disease (arrow) [5]

Periodontitis is difficult to treat and can only be controlled with active and invasive periodontal therapy to prevent further loss of the periodontal tissues. Regeneration of hard and soft tissues lost to periodontal disease is limited and not predictable. Flap surgery, enamel matrix protein, bone and tissue grafting are different methods that have been employed to regenerate periodontal tissues lost to disease with relatively poor and incomplete outcomes [3, 9, 10]. There is a critical need for new therapeutics for regeneration of periodontal tissues lost

to disease. Fundamental knowledge of the processes that regulate stem cell function is essential to develop novel therapeutics and regenerative approaches for the treatment of periodontitis. Multipotent stem cells and progenitor cell function in a well-coordinated, reciprocal stem cell niche that is coordinated by signaling biomolecules, stromal and immune cells within the structure of the tissue matrix [11-14].

1.2. Specialized Pro-Resolving Mediators

The essential role of inflammation in health and disease was recognized many years ago but the detailed molecular mechanisms and biological events that regulate the progression and resolution of inflammation were elucidated more recently [15]. Recent studies have provided solid evidence that the resolution of inflammation is not a passive process, but a biosynthetically active process that is regulated by biochemical mediators and receptor-signaling pathways that are driven by specialized pro-resolving mediators (SPMs) [16, 17]. Pioneering work by Serhan and his group established that active resolution of inflammation is coordinated by specific endogenous lipid mediators, biosynthesized from essential polyunsaturated fatty acids (PUFA) that are called specialized pro-resolving lipid mediators (SPMs) [16, 18, 19]. The SPM genus includes lipoxins (LX), resolvins (Rv), protectins (PD), and maresins (MaR). They derive from lipoxygenase (LO)-driven conversion of PUFA, i.e. lipoxins from arachidonic acid (AA), E series resolvins from eicosapentaenoic acid (EPA) and D-series resolvins, protectins and maresins from docosahexaenoic acid (DHA) that promptly appear in inflammatory exudates [18, 20, 21]. SPMs have been shown to actively promote infection clearance and prevent recurrence of infection. They are also involved in stem cell proliferation and differentiation leading to a pro-regenerative environment and eventual tissue regeneration [7, 22].

The localized acute inflammatory response is a part of the host's normal protective response to tissue injury and invasion by microbial pathogens and serves a protective function to the host. However, uncontrolled host inflammatory response that is excessive and does not resolve can result in chronic and systemic inflammatory disorders [18]. Diseases such as periodontal disease, cardiovascular disease, inflammatory bowel disease, diabetes mellitus (type II), rheumatoid arthritis, asthma and Alzheimer's disease, which are relatively common and difficult to treat, have been linked to uncontrolled, excessive or chronic inflammation. Resolution of inflammation is an actively regulated process and not just the decay of pro-inflammatory mediators [18, 19] and thus, is amenable to therapeutic manipulation.

Focusing on lipid mediators of inflammation, following tissue injury or microbial infection, pro-inflammatory lipid mediators, such as leukotrienes (LTs) and prostaglandins (PGs) are released, and these mediators unveil a series of signaling cascades with the goal of destroying the invading pathogens and repairing damaged tissue. The biosynthesis and release of the potent chemotactic agent leukotriene B_4 (LTB_4) promotes the recruitment of neutrophils (PMNs) to the inflamed tissue, while the formation of prostaglandins E_2 and D_2 (PGE_2 and PGD_2) further accelerates the inflammatory process, ultimately resulting acute inflammation [18, 19]. Neutrophils are present mainly in inflamed or injured tissues and their effective elimination is a prerequisite for complete resolution of an inflammatory response. The ideal outcome of active inflammation is its complete resolution. Despite its critical host-protective function, acute inflammation is not sustainable over a prolonged period, giving rise to disruptive conditions of chronic inflammation that may be responsible for the pathogenesis of a wide range of diseases that can be attributed to a failure of resolution [3, 6, 7].

It is now widely accepted that uncontrolled acute inflammation can lead to tissue injury, chronic inflammation, scarring and tissue fibrosis, significantly preventing healing, regeneration and reconstruction [3]. In chronic osteolytic inflammatory diseases such as periodontal disease, microbiome dysbiosis from sustained microbial challenge and failure of endogenous resolution of inflammation results in irreversible tissue destruction[3].

Each family of SPMs exerts specialized actions, including blocking neutrophil recruitment, limiting neutrophil mediated tissue damage, enhancing bacteria phagocytosis and killing, promoting the recruitment and activation of monocytes, and mediating the nonphlogistic phagocytosis and lymphatic clearance of apoptotic neutrophils by resolving (M2-like) macrophages. SPMs have other functions such as regulating autoreactive T cells, monocytes and macrophages; receptor-mediated control of osteoclast and osteoblast function in wound healing and bone regeneration, regulation of stem cells [23-27] that differentiate into fibroblasts, and osteoblasts that function in wound healing and bone regeneration [17]. SPMs regulate macrophage phenotype favoring prohealing (M2-like) over proinflammatory (M1-like) macrophages [28]. SPM receptors are expressed on human periodontal stem cells, and the activation of these receptors by SPMs significantly enhances the proliferation, migration and wound healing capacity of human periodontal ligament stem cells [27, 29]. Eventually, through the combined actions of these mediators, the resolution of inflammation is completed, and

homeostasis is reached [17, 29].

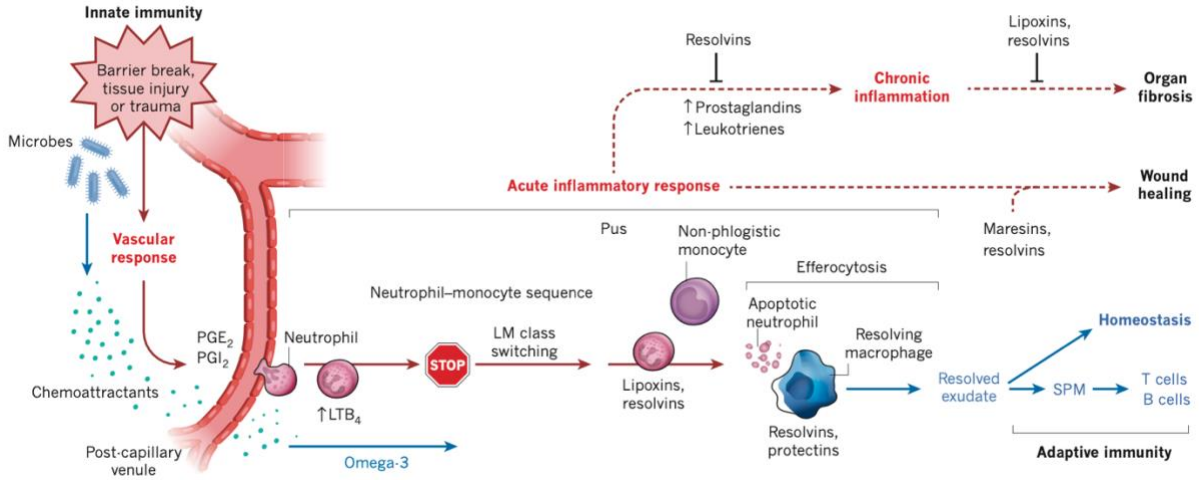

Figure 1.3. Lipid mediators in the acute inflammatory response, resolution and other outcomes[17]

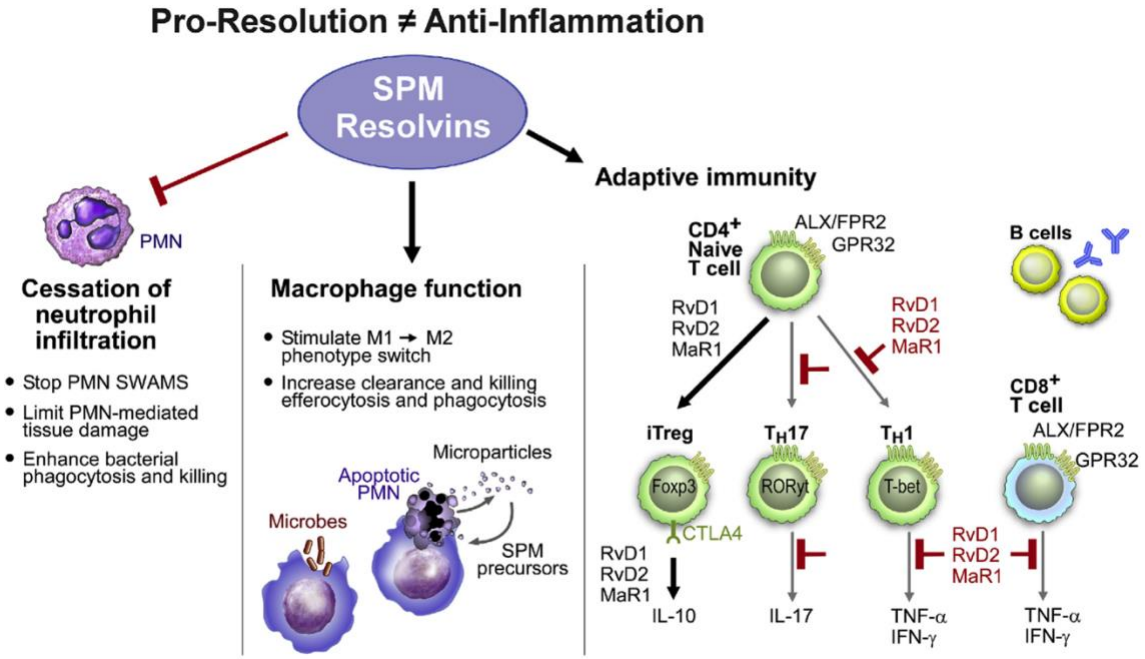

Figure 1.4. Actions of SPMs: Pro-Resolution and Anti-Inflammation Are Not Equivalent Mechanisms. SPMs limit PMN and stimulate macrophage functions. SPM also directly act on the adaptive immune cells, including T cells and B cells [19]

1.3. Fatty Acids

Fatty acids (FA) are the major components of membrane lipids and most often contain 12 to 24 carbon atoms forming hydrocarbon chains. They can be saturated (without double bonds), monounsaturated (one double bond) and polyunsaturated (with two or more double bonds). The number of double bonds determines the biologic properties of the lipids; shorter and saturated FAs are found in fats that are solid at room temperature, whereas longer and more unsaturated FAs form lipids that are liquid at room temperature (referred to as oils). The order of numbering carbon atoms in FAs starts with the carbon in the carboxyl group (COOH), which is designated as C1, and the carbon atom furthest from the COOH group is defined by the letter omega – ω or n [30-32].

Essential Fatty Acids (EFA) are important constituents of the cell membrane and contribute to the regulation of membrane properties like fluidity, flexibility, permeability and modulation of membrane-bound proteins. They cannot be endogenously synthesized by the body and must be obtained from the diet; hence termed essential. There are two types of naturally occurring EFAs in the body, the ω-6 series derived from cis-linoleic acid (LA, 18:2) and the ω-3 series derived from α-linolenic acid (ALA, 18:3) [31]. EFAs are polyunsaturated fatty acids (PUFAs) because they contain two or more double bonds, they have at least two carbon-to-carbon double bonds in a hydrophobic hydrocarbon chain, which typically includes X-Y carbon atoms and terminates in a carboxylic acid group. There are at least four independent families of PUFAs, depending on the parent fatty acid from which they are synthesized. The “ω-3” series are derived from ALA (18:3, ω-3); the “ω-6” series are derived from cis-LA (18:2, ω-6); the “ω-9” series are derived from oleic acid (18:1, ω-9); and the “ω-7” series derived from palmitoleic acid (PA, 16:1, ω-7). Only LA and ALA are EFAs. Both parent EFA are metabolized to long chain polyunsaturated fatty acids (LCPUFA) of 20 and 22 carbon atoms, respectively [17, 31, 32].

A wide variety of short-lived regulatory molecules such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT), together called eicosanoids, arise from LCPUFA, notably dihomo-γ-linolenic acid (20:3ω6; DLA), arachidonic acid (20:4ω6; AA), and eicosapentaenoic acid (20:5ω3; EPA). They are involved in inflammatory and anti-viral reactions, endothelial integrity and many more biologic activities[30, 33, 34]. Cyclooxygenase (COX) action on AA generates prostaglandin E_2 (PGE_2), an inflammatory mediator, prostacyclin I_2 (PGI_2), responsible for blood vessel dilation and thromboxane A_2 (TXA_2) responsible for activation of blood platelet aggregation and vasospasm [35]. Lipoxygenase (LOX) action leads

to formation of 4-series of leukotrienes, they play crucial roles in the development and maintenance of the inflammatory response. Eicosapentaenoic acid (EPA, C20: 5ω-3) is also metabolized by cyclooxygenase and lipoxygenase to generate 3-series prostanoids and 5-series leukotrienes of different properties, mostly anti-inflammatory (PGE_3, LTA_5, LTB_5, LTC_5, LTD_5), antiaggregatory (TXA_3) and vasodilative (PGI_3) [35].

Prostanoids and leukotrienes are mobilized as initial response to tissue injury or inflammation. This response ideally lasts for a relatively short time and is a beneficial process that serves to eliminate threatening factors. Resolution of inflammation is an important active stage and is mediated by products of the omega-6 and omega-3 acid metabolism. Lipoxygenases (LOX: LOX-5, LOX-15 and LOX-12) convert AA, EPA and DHA into lipid mediators such as lipoxin A_4 and B_4 (LXA_4 and LXB_4 – arising from AA), E-series resolvins (RvE1 – RvE3) – generated from EPA, and D-series resolvins (RvD1 – RvD6) generated from DHA that actively eliminates the inflammatory process [30, 34, 36].

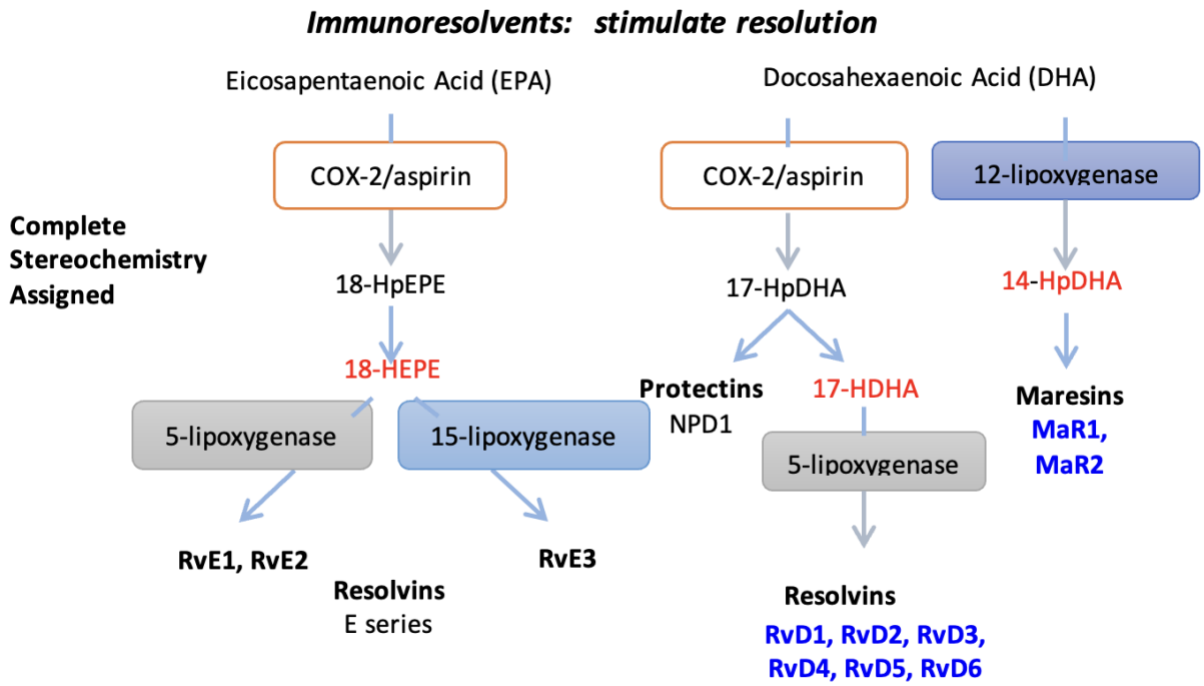

Figure 1.5. Resolution of inflammation Pathways

The catalytic domain of COX-2 is modified by aspirin acetylation, blocking prostaglandin biosynthesis, but it remains active and synthesizes 15R-hydroxyeicosatetraenoic acid (15R-HETE) from AA, 18(R)-hydroxyeicosapentaenoic acid (18R-HEPE) from EPA and 17R-hydroxydocosahaxanoeic acid(17R-HDHA) from DHA in cells carrying COX-2 [33, 35]. These products can be transformed by human neutrophils in vitro to aspirin-triggered lipoxins, resolvins and protectins. Each has potent actions, stopping human neutrophil migration and enhancing macrophage clean up [19, 30].

Cytochrome P450 is a family of heme-containing monooxygenases that can metabolize AA into epoxyeicosatrienoic acids (EETs). Hydration of EETs with soluble epoxide hydrolase (sEH) generates less biologically active metabolites, dihydroxyeicosatrienoic acids (DHETs) [37].

Henrique et al [38] reported that the inhibition of soluble epoxide hydrolase leads to production of SPMs and promotes macrophage plasticity. In the study, Mice were treated with either sEH inhibitor, EETs or a combination of sEH inhibitor and EETs two hours before ligature placement to induce experimental periodontitis. Pharmacological inhibition of sEH significantly prevented alveolar bone loss in a dose-dependent manner. There was significant prevention of bone loss starting from 100 $ng.kg^{-1}$. Treatment with EETs did not alter disease progression compared with non- treated animals. combination of pharmacological sEH inhibition and EETs did not improve the outcome observed with pharmacological sEH inhibition, although it blocked bone resorption. The study also examined the expression of osteolysis, using markers of periodontal disease that characterize the disease phenotype. Consistent with bone resorption analysis, gingival mRNA expression of IL-17A was downregulated with pharmacological sEH inhibition and the combination with EETs. Although EETs did not prevent bone resorption, there was a clear effect on IL-17A expression, a well-known inflammatory cytokine that activates osteoclast activity. Expression of RANKL was also decreased by sEH inhibition and the combination but not by EETs alone. Levels of osteoprotegerin (OPG) did not show significant differences between the non-treated animals and the treated groups. However, when the ratio of RANKL/OPG was calculated, the combination treatment provided the lowest balance, whereas the periodontal disease group and treatment with EETs alone exhibited the highest proportions ($P < 0.05$). These findings taken together from the Henrique study revealed that pharmacological sEH inhibition prevented bone resorption that is associated with a downregulation of gene expression of osteolytic factors.

The study by Henrique et al also revealed that pharmacological inhibition of sEH altered the SPM lipid profile and synthesis in saliva, compared with untreated animals with periodontitis.

Individually, pharmacological sEH inhibition and the combination with EETs significantly stimulated the release of RvE1, RvE2 and LXA4. Lipidomic analysis revealed that sEH inhibition augmented levels of LXA4, RvE1, and RvE2 concomitant with up-regulation of LTB4R1, CMKLR1/ChemR23, and ALX/FPR2 SPM receptors. Notably, there was an impact on gingival macrophage plasticity suggesting an inflammation resolving phenotype with sEH inhibition. In bone marrow derived macrophages, sEH inhibition reduced inflammatory macrophage activation, and resolving macrophages were triggered to produce SPMs.

SPMs are potent bioactive lipid mediators derived from AA, EPA, DPA and DHA that were first identified after being isolated from inflammatory exudates [17]. They shorten the inflammatory resolution interval by limiting the recruitment of neutrophils, stimulating phagocytosis of bacteria and macrophage efferocytosis, orchestrating the events involved and amplifying anti-inflammatory actions that promote healing [17]. SPMs stimulate resolution of inflammation through cell-surface G-protein coupled receptor (GPCRs). Each SPM activates one or more specific GPCR in a stereospecific manner, leading to generation of downstream signals and ultimately pro-resolving functions. Lipoxin A_4 (LXA_4) and D-series resolvins act on the ALX/FPR2 receptor; while select D-series resolvins act on the DRV1/GPR32 and DRV2/ GPR18 receptors. RvE1 and RvE2 signal via a GPCR related to chemokine receptors [29, 39].

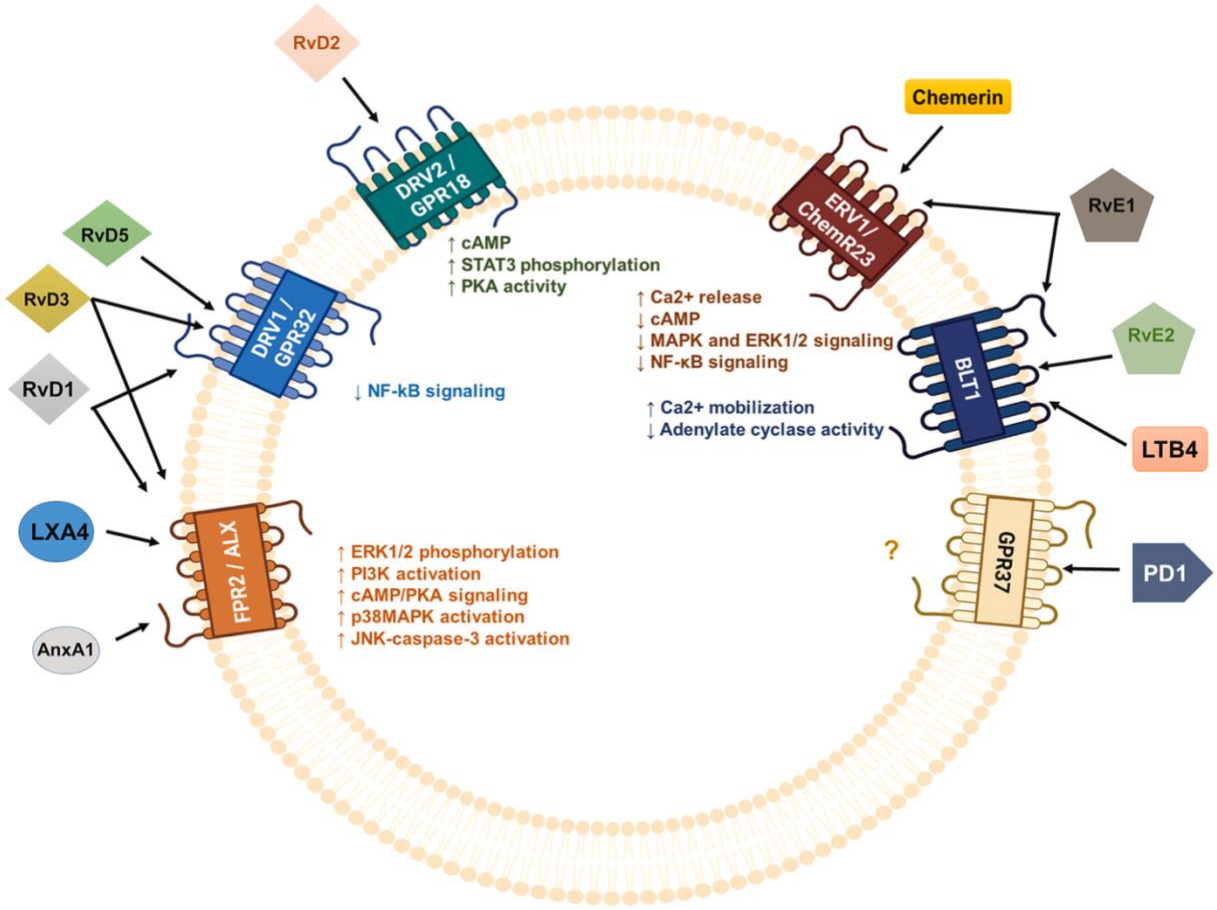

Figure 1.6. SPM receptors and their associated ligands. SPM receptors are GPCRs activated by multiple ligands and activating different signaling mechanisms [29].

1.4. SPMs in Periodontal Regeneration

Hasturk et al [40] evaluated the impact of local application of RvE1 on ligature induced periodontitis in Rabbits. Animals were divided into four groups; ligatures were tied around mandibular second premolars of rabbits for 6 weeks, stimulating an environment for growth of an exogenously added pathogen, *Porphyromonas gingivalis*. The second group were ligatured without the microbial challenge and the third received neither ligature nor microbial challenge. The fourth group received systemically administered metronidazole to kill *P. gingivalis*. This was to compare the effect of endogenous anti-inflammatory mediator RvE1 vs. antimicrobial therapy with metronidazole. Topical treatment with RvE1 at ~4 µg per tooth three times per week at the site of the ligature inhibited tissue and bone damage by >95%. There was prominent leukocyte infiltrates and bone loss in specimens from non-RvE1 treated animals, while no neutrophils or tissue damage was observed in RvE1-treated animals. In addition, within the bone lacunae,

there were large number of TRAP-positive cells in *P. gingivalis*-infected animals while RvE1-treated and control specimens contained few detectable TRAP-positive cells. These results show that RvE1 protects from neutrophil and osteoclast mediated tissue and bone destruction respectively[40].

In another study to examine the impact of RvE1 on soft and hard tissue destruction in ligature induced periodontitis in New Zealand White Rabbits by Hasturk et al [7]; Periodontal disease was induced with ligatures for 6 weeks in all groups. One group was sacrificed at 6 weeks to determine baseline disease, while four other groups then entered treatment for an additional 6 weeks, RvE1 as a monotherapy; vehicle-alone (95% ethanol) as a placebo control; and two structurally related lipid mediators, LTB_4 and PGE2, as alternative monotherapies. The progression of disease was unaffected by placebo therapy, and progression was significantly more severe when either LTB_4 or PGE_2 was used. Topical RvE1 treatment resulted in essentially complete resolution of periodontal inflammation and restoration of both soft and hard tissues clinically. The irregular, edematous and hyperemic appearance of soft tissues was also resolved, and no clinical signs of inflammation were identifiable in RvE1-treated rabbits, and they demonstrated bone regrowth to pre-disease levels.

Quantitative morphologic assessments revealed that administration of topical RvE1 (4 g/tooth) resulted in statistically significant bone regrowth (95%) compared with baseline periodontitis. Both the infrabony (vertical) defects and horizontal bone loss were completely restored. There was tooth mobility Teeth in all groups due to destruction of periodontal attachment and local inflammation which became severe by 12 weeks in all groups except the RvE1 treatment group where the teeth exhibited essentially no mobility at 12 wk. This study supports the hypothesis that RvE1 can act as a therapeutic agent in periodontal disease and lead to restoration of lost soft tissue and bone [7].

1.5. Dietary PUFAs in Immunity and Inflammation

Dietary supplementation with fish oils containing EPA and DHA results in an increase in blood levels of PUFAs, and a concomitant decrease in AA levels. Direct intake of various PUFAs such as EPA and DHA alter the cell membrane fatty acid composition, which in effect modulates cell/tissue response to infection, injury and inflammatory events. Increased dietary intake of GLA, DGLA, and EPA/DHA substantially decreases the inflammatory response [32]. Fish Oil supplementation markedly alters the FA composition of both neutrophils and lymphocytes, the percentages of AA and linoleic acids are decreased with a proportional increase in the percentage of Omega-3 FAs [41].

The oxidation of AA by cyclooxygenase is competitively inhibited by Omega-3 FA. In addition, EPA acts as substrate for both cyclooxygenase and 5-lipoxygenase, which results in production of eicosanoids with different biological properties to those produced from arachidonic acid metabolism. The incorporation of EPA and DHA into inflammatory cell membranes occurs in a dose dependent manner whilst outcompeting AA. As a result, less substrate AA becomes available for the synthesis of inflammatory eicosanoids by inflammatory cells decreasing their production of anti-inflammatory mediators [41-44]. Increased availability of EPA and DHA in cell membranes from dietary supplementation not only leads to a decrease in production of inflammatory eicosanoids, but an alternate family of mediators are produced including EPA derived eicosanoids, endocannabinoids, and SPMs (E-series and D-series resolvins, protectins and maresins) [41, 45, 46]. Dietary supplementation with fish oil rich in DHA stimulates an increase in phagocytic activity by 62% and 145% in neutrophils and monocytes, respectively. Neutrophil chemotactic response is increased by 128%. The rate of lymphocyte proliferation and production of reactive oxygen species by neutrophils was also increased [41]. Binding of Resolvin E1 to its GPCR chemokine-like receptor (ChemR23) attenuates TNF-stimulated NF-κB activation and inhibition of the synthesis of pro-inflammatory cytokines [47, 48].

The Omega-3 family of SPMs are potent anti-inflammatory mediators and were discovered as distinct EPA- and DHA- derived mediators. Lipid mediator class switching from eicosanoids to lipoxins signals ‘stop’ and begins the end of the acute inflammatory response [17]. Distinct SPMs facilitate the resolution of inflammation by limiting neutrophil influx, enhancing phagocytosis and efferocytosis as well as accelerating tissue regeneration and repair [32, 46]. Lipoxins and resolvins stimulate the recruitment of non-phlogistic monocytes which clear apoptotic neutrophils and remove cellular debris. SPMs counter-regulate pro-inflammatory chemical mediators, reducing the magnitude and duration of inflammation; they stimulate re-epithelialization, wound healing and tissue regeneration [17].

1.6 Dietary SPMs and Treatment of Periodontitis

Dietary SPMs, especially when taken with low dose aspirin, increase blood levels of SPMs and improve periodontal disease outcomes. A study by El Sharkawy et al. [49] investigated the impact of daily dietary supplementation with omega-3 fatty acid and low dose aspirin on chronic periodontitis. Eighty subjects in good systemic health with advanced chronic periodontitis were enrolled in the study and divided into two groups. The first group received a placebo following standard non-surgical periodontal therapy (scaling and root plaining), while the second group received dietary supplementation with 3 g fish oil (Each capsule contained 900 mg fish oil (EPA/DHA 30%) and 100 mg wheat-germ oil.) and 81 mg aspirin daily, following standard non-surgical periodontal therapy (scaling and root planing). The study period was 26 weeks. Clinical assessments were made, and saliva samples were collected at baseline and 3 and 6 months by a calibrated periodontist who was blinded by groups. At baseline there was no significant difference in PD and CAL, however there were significant differences at 6 months when both groups were compared. Furthermore, a significant shift to lower PD and greater CAL gain was noted for the omega-3 + aspirin group compared to the placebo control group. The percentage of pockets with PD <4 mm was 49.1% in the placebo group at 3 months versus 74.7% in the omega-3 + aspirin group; at 6 months, the percentage in the control group was 54.7% versus 79.5% in the omega-3 treatment group. The salivary RANKL levels (picograms per milliliter) and MMP-8 levels (nanograms per milliliter) of both groups at different time intervals were compared. There was no statistically significant difference between the two groups at baseline ($P > 0.05$). At 3 and 6 months, there was a statistically significant reduction in RANKL and MMP-8 concentrations in saliva in the omega-3 + aspirin group ($P < 0.01$).
This clinical proof-of-principle study by El Sharkawy et al. showed that 900 mg of EPA + DHA in a fish-oil dietary supplement administered daily in conjunction with 81-mg aspirin for 6 months significantly improved the outcome of standard SRP using PD and CAL as outcome measures. In addition, specific salivary markers of bone resorption (RANKL) and inflammation (MMP-8) were significantly reduced in the dietary supplement group.

Normal fish oil preparations contain omega-3s but are not enriched with SPMs. The study reported here employed a unique product (LIPINOVA-11TG ™) that contains normal fish oil enriched with SPM precursors. It contains eicosapentaenoic acid (226.2mg/g) & docosahexaenoic acid (321.1mg/g) concentrate reconstituted in triglyceride form and enriched with 17-HDHA (117.1 mg/kg), 18-HEPE (72.1 mg/kg) and 14-HDHA (54.3 mg/kg).

1.7 Metabolism of PUFAs

More than 90% of FAs consumed through the diet are absorbed into cells via FA transporters [50]. After absorption, they are converted to FA acyl-CoA thioesters, which act as substrates for three metabolic pathways: beta oxidation pathway for ATP production; synthesis of triglycerides, cholesterol esters and polar lipids (phospholipids and sphingolipids) and elongation/desaturation reactions generating long chain PUFAs from the initial C18 precursors. Essential FAs are important constituents of cell membranes and are precursors to eicosanoids which have many vital biologic functions. The incorporation of PUFAs in cell membranes contributes to their fluidity, which plays an important role in determining correct hormone-receptor binding [31]. Linoleic Acid (LA) and Alpha Linoleic acid (ALA) are metabolized by the same set of enzymes in a series of alternating steps of desaturation (removal of two hydrogen atoms and insertion of an extra double bond) and elongation (addition of two carbon atoms), a process that takes place in the endoplasmic reticulum [32, 51].

Omega-3 and Omega-6 PUFAs compete for the same desaturation enzymes. Hence, alterations of the ALA/LA ratio will affect the composition of their long chain metabolite. The desaturase enzymes show preference for FAs from the various series in the order ω3>ω6>ω9. The ∆6- and ∆5-desaturases are generally considered to be rate limiting in the metabolism of EFAs and their activity is controlled by dietary and genetic factors. Essential co-factors required for ∆6 desaturase include Magnesium, Zinc and Vitamin B6. LA is desaturated to form g-linolenic acid (GLA, 18:3 o-6) that in turn is elongated to form dihomo-GLA (DGLA, 20:3 o-6), the precursor of 1 series of prostaglandins (PGs). DGLA is also converted to form AA (20:4 o-6), the precursor of 2 series of PGs, thromboxanes (TXs) and 4 series leukotrienes (LTs). ALA is metabolized to eicosapentaenoic acid (20:5 o-3), the precursor of 3 series of PGs, TXs, and 5 series LTs. EPA is also converted to form docosahexaenoic acid (22:6 o-3). DGLA, GLA, EPA, DHA and AA are all long chain PUFAs, and they are more biologically potent than LA and ALA [32, 51].

Resolvins and protectins were the first two families of bioactive mediators synthesized from Omega-3 PUFAs, Resolvin E1 can be produced *in vitro* recapitulating the *in vivo* events, by treating human vascular endothelial cells in a hypoxic environment with aspirin. These cells convert EPA to18R-hydroperoxyeicosapentaenoic acid (18R-HPEPE) and release 18R hydroxyeicosapentaenoic acid (18R-HEPE), which is rapidly transformed by activated human neutrophil 5-lipoxygenase to Resolvin E1. Microbial and mammalian cytochrome P450 enzymes convert EPA into 18-HEPE, which can be transformed by human neutrophils to resolvin E1 and resolvin E2 [20, 52].

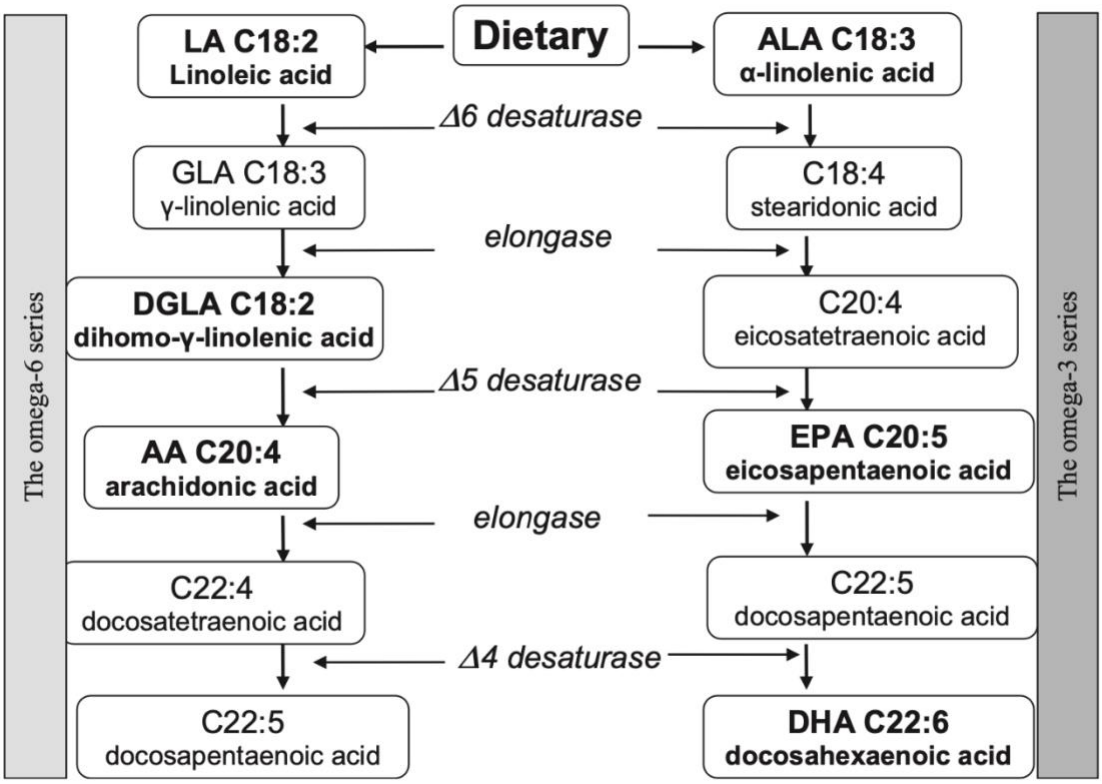

FIGURE 1.7. Pathways of biosynthesis of unsaturated fatty acids omega-6 and -3 series

Endogenous DHA is converted in vivo via lipoxygenase-initiated mechanisms to the 17S-hydroxy-containing series of four resolvins, known as resolvin D1–resolvin D4. Studies in mice that were administered aspirin and DHA have shown that in the presence of aspirin, human recombinant COX2 converts DHA into a 13-hydro(peroxy)-containing product; the oxygen at carbon position 13 switches to position 17 with an R configuration to generate aspirin triggered 17R D-series resolvins[20, 48, 53].

Protectins are biosynthesized via a lipoxygenase-mediated pathway that converts DHA to a 17S-hydroperoxide-containing intermediate11, which is rapidly converted by human leukocytes into a 16(17)- epoxide that is enzymatically opened in these cells into a 10,17-dihydroxy-containing anti-inflammatory molecule (10,17-diHDHA), Protectin D1. Maresins (MaRs) are produced by macrophages via initial lipoxygenation at the carbon-14 position by lipoxygenation and insertion of molecular oxygen, producing a 13S,14S-epoxide-MaR intermediate that is enzymatically converted to the MaR family members MaR1 and MaR2[23, 48, 53, 54].

New strategies and tools are needed to ensure long-term function and survival of regenerated tissues in periodontal disease. Previous research has relied heavily on rodents; however, the human host response, microbiome, and jaw size and function differ substantially between humans and rodents. To understand the modulation of disease-related infection, inflammation, and tissue destruction and optimize structural and functional regenerated tissues, large animal models are required. Hypothesis testing with appropriate large animal models and integration of innovative approaches to regulate inflammation will ensure there is development of new strategies for regeneration of functional complex multi-tissue structures. The unanswered question remains, which is whether n-3 dietary supplements are metabolized in sufficient quantity to expect clinical effects in periodontal regeneration and whether a marketed preparation that is enriched with SPMs provides added benefit

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1.8 Benzo-Lipoxin A_4 ($BLXA_4$)

Lipoxins are products of lipoxygenase- lipoxygenase interactions, they are trihydroxy products of arachidonic acid that actively drive resolution of inflammation; they are typified by lipoxin A_4 (LXA_4). This bioactive molecule is an endogenous anti-inflammatory agent with pro-resolution action, it is an arachidonic acid product of the interaction between platelets and neutrophils or neutrophil conversion of 15S-hydroxy-eicosatetraenoic acid released from activated monocytes and epithelial cells. Lipoxin A_4 inhibits neutrophil chemotaxis, adherence, and transmigration and stimulates macrophage phagocytosis of apoptotic neutrophils and enhances chemotaxis of monocytes/macrophages [55, 56].

Benzo-Lipoxin A_4 ($BLXA_4$) is a stable analog mimetic of lipoxin A_4. In comparison with native LXA_4, the methyl-ester of $BLXA_4$ was found to be stable in an *in-vitro* stability assay system containing eicosanoid oxido-reductase, and this analog was most effective at inhibiting polymorphonuclear neutrophil infiltration in a murine peritonitis model. $BLXA_4$ is synthesized with an o-6 R hydroxyl group. In addition, a benzene ring was used to replace the labile tetraene backbone present in LXA_4 because of the inherent chemical instability of the conjugated tetraene system. This structural modification enabled a highly convergent and stereoselective synthesis and provided conformational rigidity [55, 56].

Hasturk et al. [56] conducted a phase 1 clinical trial to determine the safety and preliminary efficacy of $BLXA_4$ in patients with gingival inflammation. The study product, $BLXA_4$ containing oral rinse, received an Investigational New Drug designation (IND #: 107061) from the FDA prior to study initiation. The topical oral rinse dosage form of $BLXA_4$ consisted of drug substance prepared at a concentration of 1.0 mM in an aqueous vehicle solution. The full

chemical name of the $BLXA_4$ drug substance is (5S, 6R, E)-methyl 5,6-dihydroxy-8-(2-((R, E)-3-hydroxyoct-1-enyl) phenyl) oct-7-enoate (9, 12-Benzo LXA_4).

With regards to changes in gingival inflammation, the study revealed that $BLXA_4$ topically applied in a mouthwash to inflamed oral/periodontal tissues demonstrated significant clinical benefits. In the study, gingival inflammation measured with two separate methods (modified gingival index and bleeding on probing) was markedly reduced as was pocket depth. Furthermore, subjects with pocket depth ≥6 mm showed a greater reduction (-1.23 ± 0.4 mm) with a 1.22 ± 0.6 mm clinical attachment gain in the $BLXA_4$ group compared to placebo group (-0.71 ± 0.3 mm in PD with a clinical attachment gain of 0.72 ± 0.3mm). Specific SPMs including lipoxins (LXA4, LXB4), resolvins (RvE1, RvD1-RvD6), protectins (PD1) and maresins (MaR1, MaR2) responsible for a range of cellular and biochemical events in resolution of inflammation, and their precursors (15-HETE, 18- HEPE, 17-HDHA), were upregulated in the serum of $BLXA_4$-treated participants.

Van Dyke et al [2] reported the use of nano-proresolving medicines (NPRM) containing a novel lipoxin analog (benzo-lipoxin A_4, $BLXA_4$) to promote regeneration of hard and soft tissues irreversibly lost to periodontitis in the Hanford miniature pig. Pigs have more similarities to humans compared to other commonly used laboratory animals in terms of anatomy, physiology, immunology, and oral structures and they are specifically suitable for regenerative studies. Interproximal periodontal defects 6 mm in depth were created using a bur from the cemento-enamel junction (CEJ) and extending to the mid-interproximal buccolingually in all 4 quadrants around the second and fourth premolars and defects were maintained by placing sterile orthodontic wires below the CEJ and the twisted ends placed in the surgical defects. A notch was placed at the defect base, and measurements (from CEJ to crestal bone and to the base of the defect) were recorded.

Defects were treated with $BLXA_4$ alone (10 µg of 2.1 µM solution), NPRM alone, or NPRM containing $BLXA_4$ (NPRM-$BLXA_4$). Four defects were treated with each regimen and surgery alone was the negative control. In this study, NPRM-BLXA4 dramatically reduced inflammatory cell infiltrate into chronic periodontal disease sites treated surgically and dramatically increased new bone formation and regeneration of the periodontal organ. The results reveal that NPRM-$BLXA_4$ is a mimetic of endogenous resolving mechanisms with potent bioactions that offers a new therapeutic tissue-engineering approach for the treatment of chronic osteolytic inflammatory diseases such as periodontitis.

More recently, Serhan and co-workers demonstrated that repeated treatment with lower doses of SPMs also has clinical effects [57]. Thus, in this work, we used a concentration of $BLXA_4$ for this experiment that is 100-fold lower than the dose from the prior mini-pig study.

2. HYPOTHESIS AND PURPOSES OF THIS RESEARCH

SPMs stop neutrophil infiltration while attracting nonphlogistic monocytes to the lesion which phagocytose apoptotic neutrophils without contributing to further inflammation or soft and bone tissue destruction. They modulate inflammatory response by shifting the response to more rapid resolution and effectively preventing the chronic phase thereby promoting tissue regeneration. Uncontrolled inflammation is a major obstacle to tissue regeneration and reconstruction of both diseased and injured tissues resulting in further tissue injury, tissue scarring and fibrosis. Persistent signals promoting inflammation disrupt stem cell activity, whereas specific anti-inflammatory signals enhance stem cell activity [1,2]. In successful regeneration, mesenchymal stem cells assume an anti-inflammatory phenotype. The goal of this study was to determine if controlling local inflammation with dietary supplementation with SPM enriched omega-3 PUFAs fosters regeneration.

The Central hypothesis is that dietary supplementation with SPM-enriched omega-3 PUFAs will activate resolution of inflammation pathways producing mediators to promote periodontal regeneration.

To test our hypothesis, we propose the following specific aims:

Specific aim 1: Determine whether dietary supplementation with SPM-enriched EPA/DHA enhances periodontal regeneration.

Specific aim 2: Determine the impact of the dietary supplementation on systemic (blood) levels of pro-inflammatory and proresolving lipid mediators.

3. MATERIALS AND METHODS

3.1 Göttingen Miniature Pig

The Institutional Animal Care Committee (IACUC) at Massachusetts General Hospital (MGH) reviewed and approved the animal protocol prior to commencement of this study - **2022N000103**. The study conformed to ARRIVE guidelines for pre-clinical animal studies and all procedures employed in this study complied with the Association for Assessment and Accreditation of Laboratory Animal Care International–accredited guidelines

The *in vivo* experiment was performed using the Göttingen Miniature Pig following the protocol of Van Dyke et al [2]. Three adult female Pigs (12 months old, 40 kilograms; Pain and distress -category D) were purchased from Marshall Bioresources and acclimatized for 7 days before the start of clinical procedures. They were housed in metal pens separately and the pens were maintained at 24 °C ± 2 °C and 55% relative humidity and fed a standard diet and tap water *ad libitum*.

There were 3 treatments for this experiment (One Pig for each treatment, eight sites per Pig):

Group 1-Surgical debridement + Standard diet

Group 2-Surgical debridement + SPM-enriched Omega-3 PUFA dietary supplement

Group 3-Surgical debridement + $BLXA_4$ + Standard diet

3.2 Surgical Procedures

3.21 Induction of chronic periodontal defects:

Animals were premedicated with Telazol (4 to 6 mg/kg intramuscularly [IM]) and xylazine (2.2 mg/kg IM) and maintained by isoflurane 3% to 4% for induction and 0.5% to 2% for maintenance. Local anesthesia (2% lidocaine with 1:100,000 epinephrine–0.5 carpule/quadrant) was administered for hemostasis. Mucoperiosteal flaps were elevated with vertical releasing incisions. Interproximal periodontal defects were created using burs (6 mm in depth from the cemento-enamel junction [CEJ] and extending to the mid-interproximal buccolingually). Sterile orthodontic wires were positioned below the CEJ and the twisted ends placed in the surgical defects in all 4 quadrants around the second and fourth premolars in all animals. Flaps were repositioned covering the ligatures using 4-0 Vicryl sutures (Ethicon, Inc., Blue Ash, OH). Animals were fed a soft diet for 6 weeks to stimulate plaque accumulation. Oral examinations including digital photographs were repeated at 2 and 4 weeks, and ligatures were removed at 6 weeks. The sites were left untreated 40 days (± 3 days) to stabilize the periodontal disease and bone loss obtained and to eliminate the self-healing.

3.22 Collection of Blood Samples

Blood samples (up to 20ml) were collected via external jugular vein prior to the treatment/ second surgery and on the day of euthanasia [2, 56].

3.23 Blood plasma collection

Blood was collected in an anticoagulant tube with ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1500xg for 10min at 4°C. Three distinct layers were visible; top layer clear-straw color; plasma, middle layer; buffy coat and bottom layer; concentrated erythrocytes With a pipette, plasma was carefully aspirated (~3-4ml) from the tube and transferred to a clean 15ml centrifuge tube leaving a small amount of plasma above the buffy coat and not disturbing the buffy coat. The tube containing the plasma was centrifuged a second time at 1000-1500xg for 10min at 4°C. The plasma was removed and dispensed into 200ul aliquots, leaving a small amount of plasma (1-2mm) remaining in the 15ml tube in case of remaining buffy coat contaminant.

3.24 Blood serum collection

Whole blood was collected in a red-topped tube and allowed to coagulate for a minimum of 30 to a maximum of 60 minutes at room temp. Centrifuged at 1500xg for 10min in a refrigerated centrifuge. Following centrifugation immediately carefully aspirate the liquid component (serum) was carefully aspirated and dispensed into 200ul aliquots in sterile tubes. Any remaining serum was evenly distributed between the tubes, leaving a small amount of serum (1-2mm) above the coagulated buffy coat.

3.3 Experimental Protocol

Day 0 – General Anesthesia.

1. Two bony defects were created per quadrant for each pig (2 x 4 = 8 defects per pig) and wire ligatures tied around teeth to induce and maintain chronic periodontal disease
2. Pigs were started on a soft diet to stimulate plaque accumulation.

Day 14 - Anesthesia: Minor Procedure

Follow-up: intra-oral pictures and examination

Day 28 - Anesthesia: Minor Procedure

Follow-up: intra-oral pictures and examination

Day 42 - **Anesthesia**: Minor Procedure

Ligatures were removed.

Day 82 - **General Anesthesia**

Group 1-Surgical debridement + Started Animal on regular diet after surgery

Group 2-Surgical debridement + Started Animal on regular diet with SPM-enriched omega-3 dietary supplements

Group 3-Surgical debridement + $BLXA_4$ + regular diet

Surgery + 14 days - Anesthesia: Minor Procedure

Follow-up: intra-oral pictures and examination

Surgery + 90 days – General Anesthesia

Euthanasia

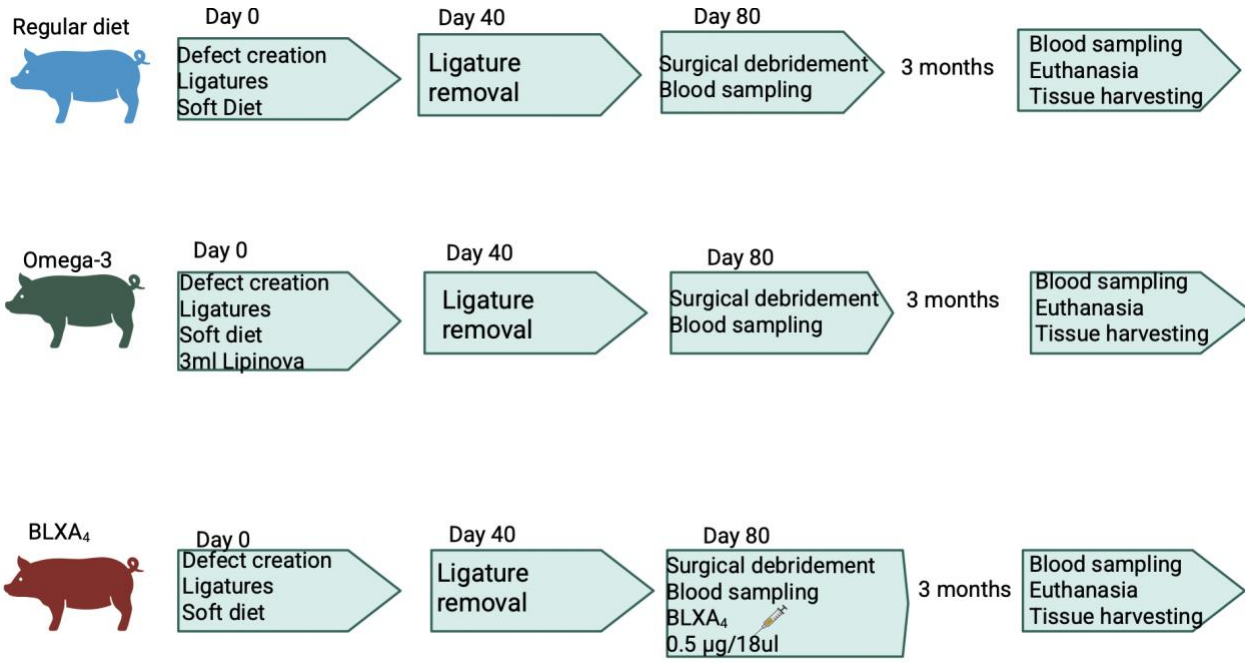


Figure 8 Flowchart of the Experiment

3.4 Clinical Measurements

Standard periodontal measures were made with a UNC probe with Williams' markings by a single examiner, including probing pocket depth and clinical attachment level at baseline, at 3 months and at end of the study (euthanasia). Miniature pigs had a baseline dental examination after induction of periodontal defects to categorize the presence and extent of disease. The oral examination included a detailed survey of buccal mucosa, floor of the mouth, tongue, hard and soft palate, gingiva, and teeth before and after treatment as a safety measure. The following parameters were quantified.

Probing Depth- Probing pocket depth is defined as the distance in millimeters from the gingival margin to the base of the sulcus/pocket. It was measured on six surfaces per tooth (distobuccal, midbuccal, mesiobuccal, distolingual, midlingual, and mesiolingual) of study teeth.

Bleeding on Probing- The number of sites with BOP was recorded (presence or absence of bleeding following probing expressed as percent bleeding sites).

Dental Plaque Assessment- Plaque was assessed on three surfaces per tooth: buccal, mesiobuccal and lingual [58]. A score of 0: visible plaque absent; 1: visible plaque present; or Y: cannot be assessed.

3.5 Dietary Supplements

The dietary supplement utilized in these experiments was a marketed fish oil preparation that is an SPM-enriched omega-3 PUFA, as measured by EPA and DHA content (Trade name: Lipinova® (Solutex) at the manufacturer's recommendation dose. It contains 18-HEPE, 14-HDHA, and 17-HDHA in addition to 400 mg EPA + DHA. Group 2 animals in addition to regular diet, received Lipinova® (Solutex) (3ml, added to a small portion of regular food or Gatorade) after surgical treatment (Day 80 after defect creation) given daily until the end of the study.

3.6 Benzo-Lipoxin A_4 ($BLXA_4$)

3.61 $BLXA_4$ lipid nanoparticle delivery

$BLXA_4$ lipid nanoparticle solution was provided by Dr. Charles Serhan's laboratory. It was freshly prepared the day before and stored at 4°C until delivery. Approximately 0.5 µg per site in 18 µl of the $BLXA_4$ lipid nanoparticle solution was delivered to each of eight treatment sites (two per quadrant) using a P20 pipette. It was delivered following degranulation of defects and pre-suturing.

3.62 $BLXA_4$ Intragingival Injection

To maintain the local level of proresolving $BLXA_4$ for 2 weeks after surgery, 10 micrograms per site of $BLXA_4$ was delivered in 2.5 microliters of normal saline diluted from a concentrated stock solution (Vinresol, 8.75 mg/ml ethanol solution to eight treatment sites (two per quadrant) via intragingival route using a microliter syringe (Hamilton #65 (87943) with a 34-gauge needle (Hamilton 207434-10)) under sedation at the time of 14-day follow-up.

3.7 PROCESSING OF SAMPLES AFTER EUTHANASIA

Jaw samples from all animals were fixed in 10% formalin immediately after harvesting for a minimum of 1 week at 4^0C with agitation. The solution was changed 72 hours after placement in 10% formalin. Samples were transferred after 1 week to PBS (1x) for Micro-CT scanning.

3.71 Micro-CT*:*

μCT analysis was performed as described in previous publications [59, 60]. Samples were placed in a standardized sample holder and scanned using a high-resolution micro-CT system (Scanco Medical) at a spatial resolution of 5.376 μm (Voxel dimension) with 1536 x 1536-pixel matrices. After scanning, the two-dimensional image data was stored in the Digital Imaging and Communications in Medicine (DICOM) format, transferred to a computer, and three-dimensional reconstruction and analyses performed. To reduce the size of data for computation, mandibular and maxillary regions were cropped and saved from the obtained consecutive micro-tomographic slice images as volume of interest using Amira software (Visualization Sciences Group) [61].

The volume of new bone was measured by constructing an interproximal cone within a digital box with a fixed size (6 (high) X 4 (diameter at the base)) positioned in the same location for all samples using anatomical landmarks. Tissue type was segmented using a global thresholding procedure (bone tissue = 3741). The newly regenerated bone was cropped from preexisting bone and root structure by applying a cylindrical divider at the base of the previous bone defect. The total and newly formed bone volume was calculated and presented as mean and standard deviation of voxel units calculated by AMIRA.

3.72 Lipidomics: All liquid samples (e.g. plasma and serum) were used as such or after dilution in phosphate buffer, pH 7.2. Tissue samples and cells were homogenized in phosphate buffer using a bead homogenizer (Precellys, Bertin Technologies). The typical ratio of solid sample to buffer was 1:9.[62, 63]

Fatty acyl lipidomic analysis:

Samples (adjusted to a maximum volume of 1-2 ml) were spiked with a mixture of internal standards consisting of 15(S)-HETE-d_8, Leukotriene B_4-d_4, Resolvin D2-d_5, 14(15)-EpETrE-d_{11}, and prostaglandin E_1-d_4 (5 ng each) for recovery and quantitation and mixed thoroughly. The samples were then extracted for PUFA metabolites using C18 extraction columns. Briefly, the internal standard spiked samples were applied to conditioned C18 cartridges, washed with water followed by hexane and dried under vacuum. The cartridges were eluted with 0.5 ml methanol. The eluate was dried under a gentle stream of nitrogen. The residue was re-dissolved in 50 μl methanol-25 mM aqueous ammonium acetate (1:1) and subjected to LC-MS analysis.

HPLC was performed on a Prominence XR system (Shimadzu) using Luna C18 (3μ, 2.1x150 mm) column. The mobile phase consists of a gradient between A: methanol-water-acetonitrile (10:85:5 v/v) and B: methanol-water-acetonitrile (90:5:5 v/v), both containing 0.1% ammonium acetate.

The gradient program with respect to the composition of B is as follows: 0-1 min, 50%; 1-8 min, 50-80%; 8-15 min, 80-95%; and 15-17 min, 95%. The flow rate was 0.2 ml/min. The HPLC eluate was directly introduced to ESI source of QTRAP5500 mass analyzer (SCIEX) in the negative ion mode with following conditions: Curtain gas, GS1, and GS2: 35 psi, Temperature: 600 ˚C, Ion Spray Voltage: -2500 V, Collision gas: low, Declustering Potential: -60 V, and Entrance Potential: -7 V. The eluate was monitored by Multiple Reaction Monitoring (MRM) method to detect unique molecular ion – daughter ion combinations for each of the 125 transitions (to monitor a total of 156 lipid mediators). The MRM was scheduled to monitor each transition for 120 s around the established retention time for each lipid mediator. Optimized Collisional Energies (18 – 35 eV) and Collision Cell Exit Potentials (7 – 10 V) were used for each MRM transition. Mass spectra for each detected lipid mediator were recorded using the Enhanced Product Ion (EPI) feature to verify the identity of the detected peak in addition to MRM transition and retention time match with the standard. The data were collected using Analyst 1.6.2 software and the MRM transition chromatograms were quantitated by MultiQuant software (both from SCIEX). The internal standard signals in each chromatogram were used for normalization for recovery as well as relative quantitation of each analyte[62, 63]

3.73 Histology

Harvested jaw sections were decalcified with Formical-2000 (Stat lab) for a minimum of 6 weeks, separated into 2 segments staying as far as possible from the region of interest (defect) and continue decalcification for further 2-3 weeks. After decalcification, sections were washed in 1X PBS 30 minutes, 3 times followed by processing for paraffin. Samples were dehydrated through an ascending ethanol concentration (50%, 70%, 90%) finally followed by 100% ethanol two times. Each step is changed 16h-24h undertaken in 4^0C overnight.

After ethanol dehydration, specimens were cleared in xylene for 15 minutes and then infiltrated with a mixture of xylene and paraffin (1:1) for 1 hour at 60^0C oven, followed by 100% paraffin for 48hrs three times in a 60 C oven. Sections were then embedded in paraffin and 6 um thick sections made from each block to assess new bone formation.

4 RESULTS

4.1 Induction of Periodontal defects

Interproximal periodontal defects 6 mm in depth were created using burs after induction of anesthesia in all 4 quadrants around second and fourth premolars, two defects per quadrant and a total of eight defects per pig. (Fig 4.1)

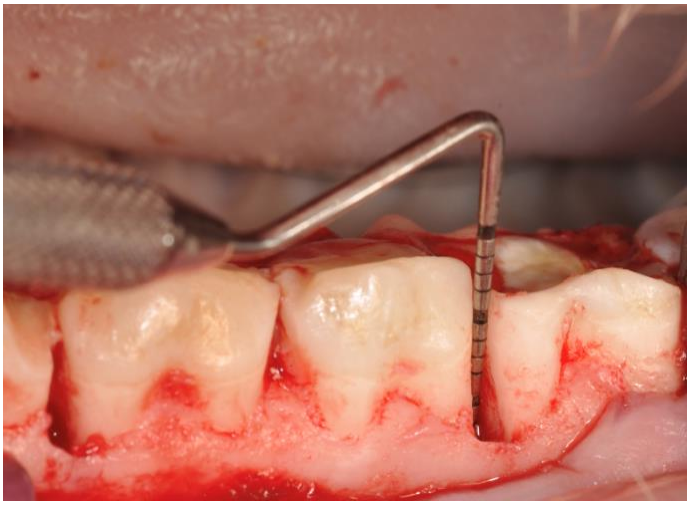


Fig 4.1 Mucoperiosteal flaps were elevated with vertical relieving incisions and 6 mm Interproximal periodontal defects were made with burs around two premolar teeth in each quadrant

4.2 Maintenance of Periodontal defects

Interproximal periodontal defects were stabilized and maintained by placement of sterile orthodontic wires. (Fig 4.2a). Animals were fed with soft diet to stimulate plaque accumulation around ligatures for 6 weeks after which the ligature is removed. The sites were left untreated for an additional 40 days to stabilize the periodontal disease and bone loss obtained and to eliminate the self-healing aspect of the bone defects. The sites were debrided after 40 days to eliminate granulation tissue (Fig 4.2b)

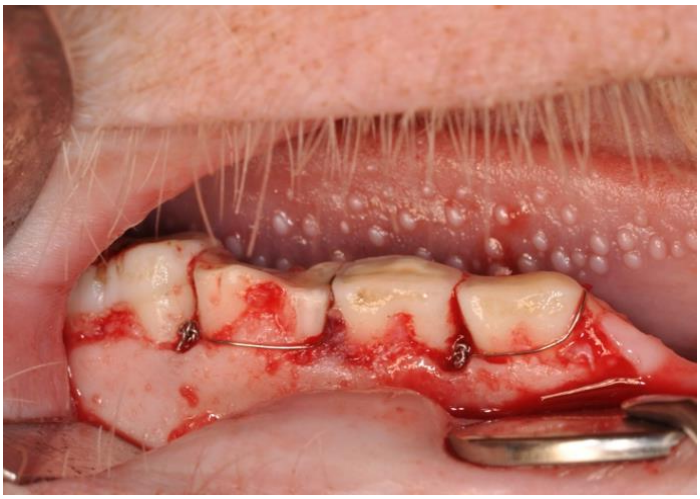


Fig 4.2a The defects were made chronic by placement of sterile orthodontic wire ligature. This method created non-healing, progressing periodontitis with sufficiently large vertical defects and horizontal bone loss.

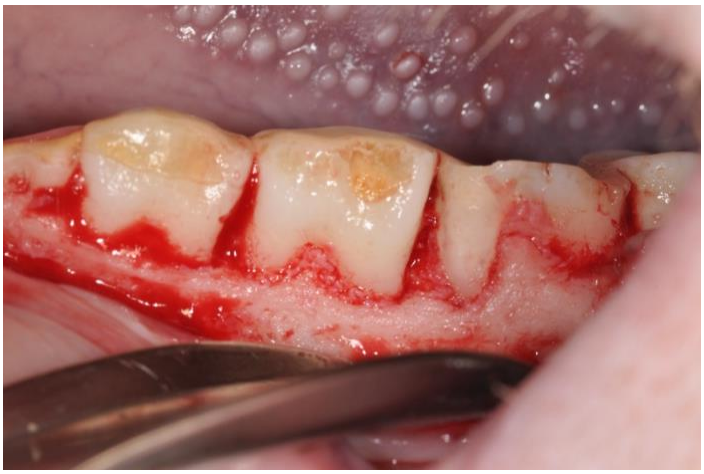


Fig 4.2b Teeth were debrided after ligature removal

4.3 Clinical Parameters -

Pocket Depth: In the control group that had regular diet with surgical debridement, Pocket depth (PD) at baseline was an average of 2.8 mm which increased to 2.9 mm at end of the study. In the Omega-3 Supplement group, PD at baseline was an average of 2.78 mm with a slight reduction to 2.33 mm at end of study while in the $BLXA_4$ group, the PD at baseline was 2.79 mm with a slight reduction to 2.41 mm (Figure 4.3)

Bleeding on Probing: The full mouth bleeding score at baseline was 13% for the Regular diet group and it increased to 45% at end of study. Similarly at Baseline full mouth bleeding score for Omega-3 diet group was 30% but increased to 60% at end of study, while the full mouth bleeding score for the $BLXA_4$ group increased from 10% to 43%. (Table 1)

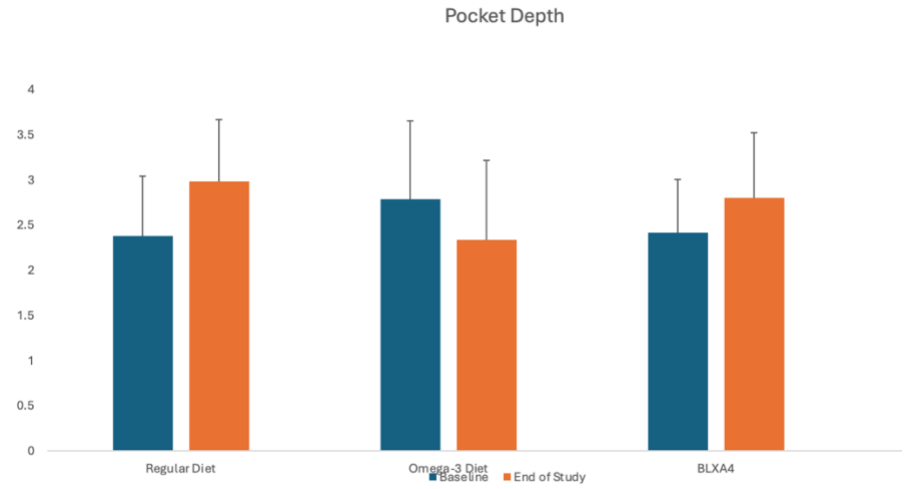


Fig 4.3 There was slight reduction in Pocket Depth in the Omega-3 Diet and $BLXA_4$ group

***Dental Plaque Assessment*:**

The full mouth plaque assessment for the Regular diet group increased from 71% at baseline to 94% at end of study. In the Omega-3 diet group, full mouth plaque assessment reduced from 97% at baseline to 90% at the end of the study, while in the $BLXA_4$ group, full mouth plaque assessment increased from 89% at baseline to 94% at the end of the study (Table 1).

Table 1

Groups	Full Mouth Bleeding Score %		Full Mouth Plaque Score %	
	Baseline	**End of Study**	**Baseline**	**End of Study**
Regular Diet	**13**	**45**	**71**	**94**
Omega-3 Diet	**30**	**60**	**97**	**90**
$BLXA_4$	**10**	**43**	**89**	**94**

Table-1: The full mouth bleeding score increased from baseline to end of the study in all the groups as well as full mouth plaque score except for the Omega-3 Diet group where there was a slight reduction.

4.4. Linear Measurement of Bone loss with Micro CT

Bone loss was 4.5 mm in the control group, 3.2 mm in the BLX_4 group and 2.7 mm in the enriched omega-3 supplement group. The difference was statistically significant (Analysis of variance *P<0012) (Figure 4.4). There was no significant difference between the $BLXA_4$ Nanoparticle group and Omega-3 Supplement Group, but both were superior to the control.

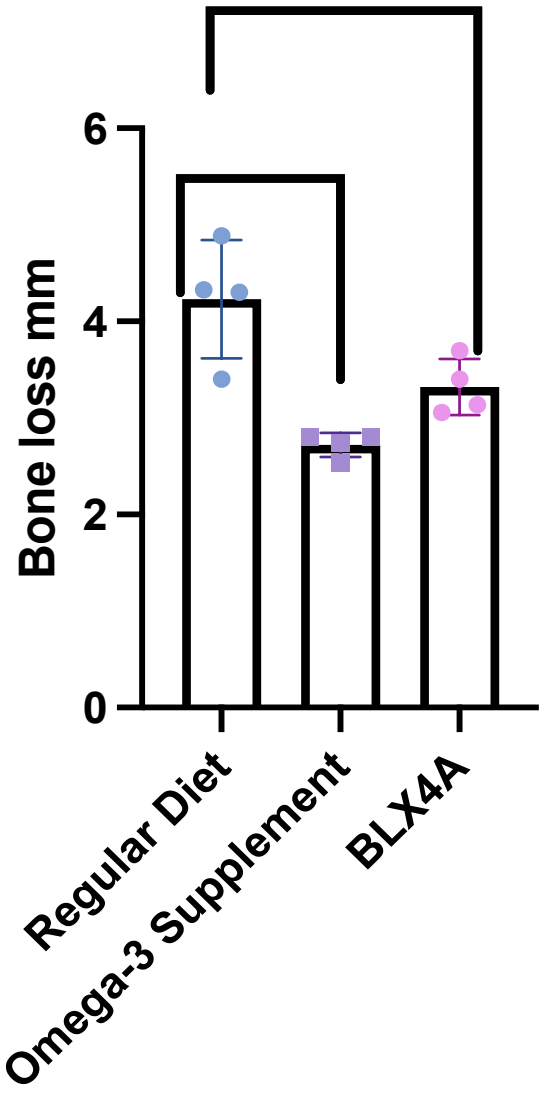


Figure 4.4 The Omega-3 Supplement group had the least bone loss, while the Control group had the highest bone loss. The difference was significant (Analysis of variance *P<0012)

4.5 Measurement of Bone volume/Total Volume with AMIRA

There were significant differences in bone gain between the enriched omega-3 supplement group, $BLXA_4$ group and control group. Dietary supplementation with enriched omega-3 supplement led to statistically significant gain of bone compared to the control group that had regular diet. Quantification of bone volume revealed markedly greater bone gain with omega-3 dietary supplementation, Fig 4.5. Note that the notch in the root placed at the time of surgery at the bone level is no longer visible in the Omega-3 supplement samples Fig 4.6 a, b.

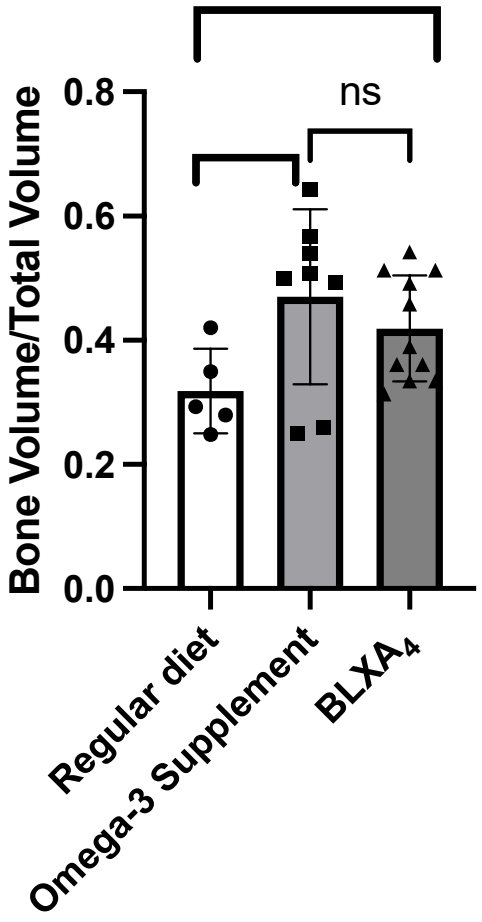


Figure 4.5 Omega-3 dietary supplementation induced greater bone formation (*P<0.01, analysis of variance)

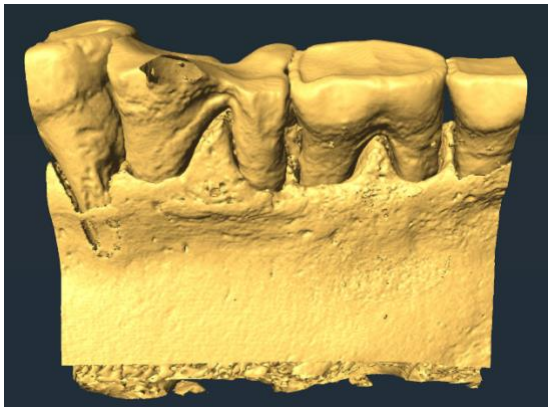
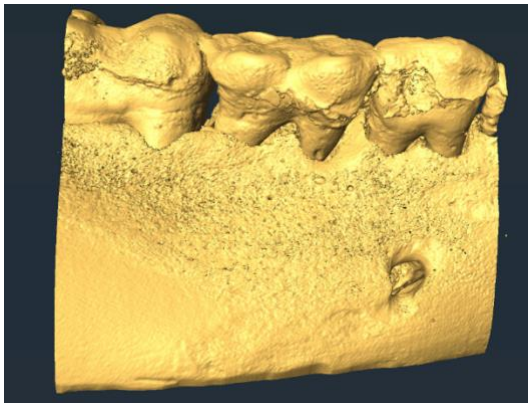
A  **B** 

Figure 4.6a and b, Three-dimensional reconstruction of µCT of the control group (a) with regular diet and Omega-3 supplement group (b) to provide visual references for changes induced by Lipinova. Note that bone irregularities are resolved and root notches placed at the time of surgery are covered by new bone in the Omega-3 supplement specimen.

4.6 Lipidomics

LM levels were investigated using LM metabololipidomics. Serum levels of RvD3, RvD5, AT-RvD3, PGF1a, PGF2a, TXB2, 15-HETE, PGD3 and LTB5 were measured. These revealed that dietary supplementation with enriched Omega-3 markedly upregulates systemic levels of proresolving mediators such as RvD3, RvD5, AT-RvD3, 13-HDoHE and 17-HDoHE, while proinflammatory prostaglandins such as TXB2 and PGF2a were down regulated, (Figures 4.7 and 4.8-10.

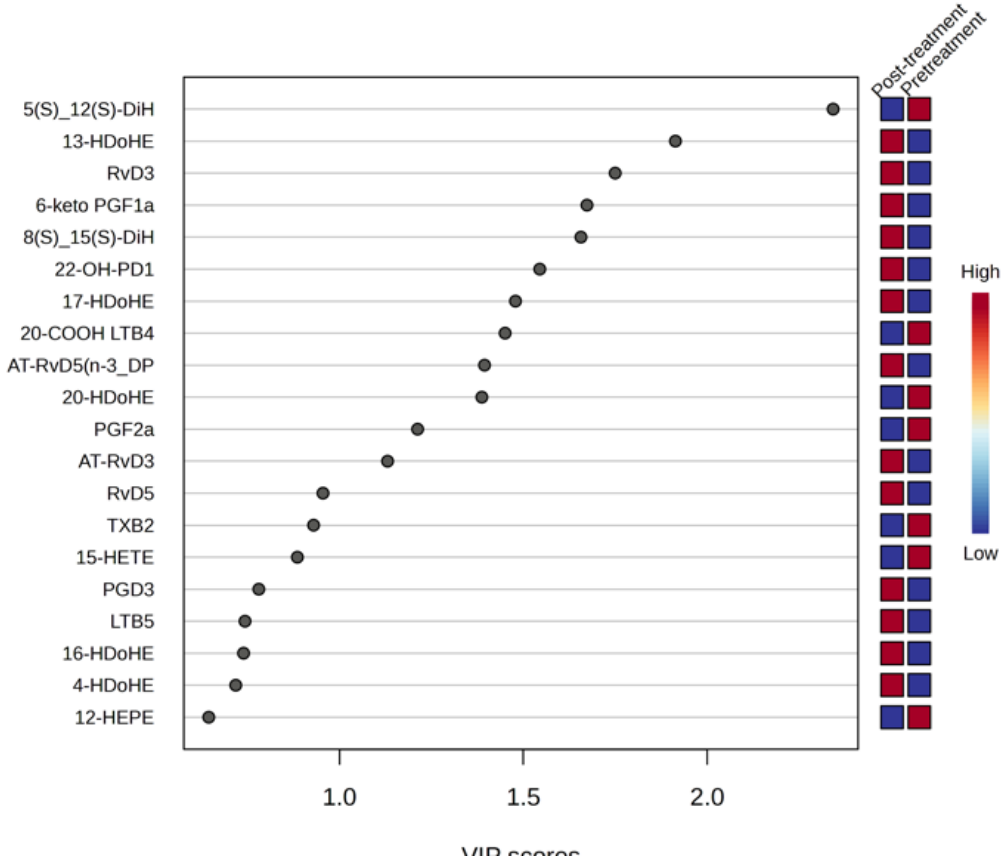


Figure 4.7 Proresolving mediators were upregulated and proinflammatory mediators downregulated in the enriched Omega-3 group

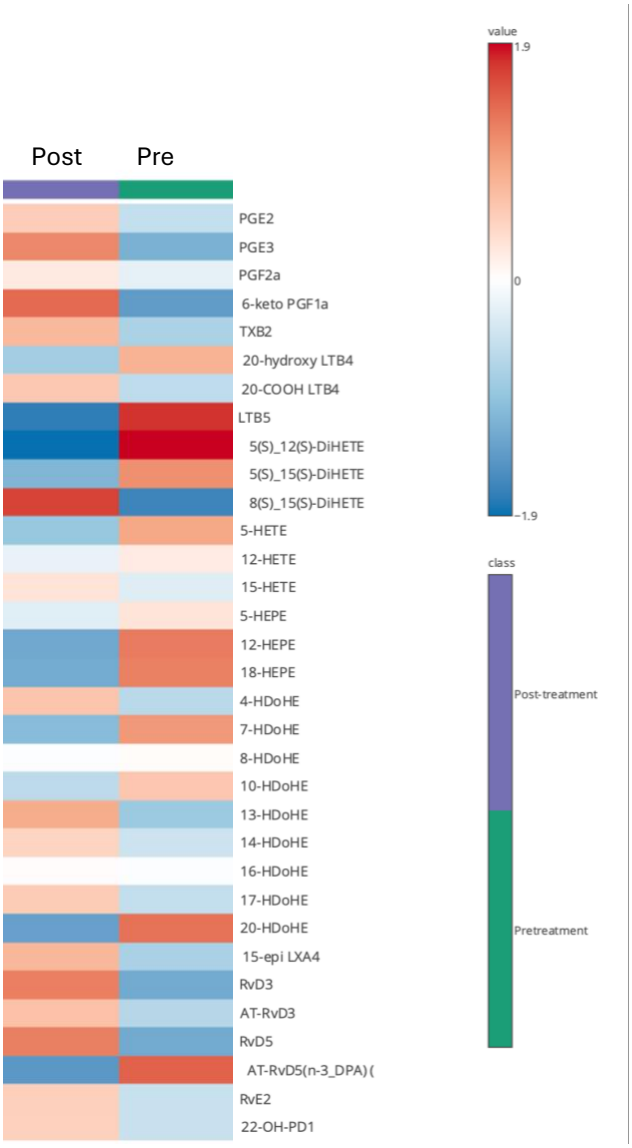


Figure 4.8a Control group Metabololipidomics analysis revealed a shift in SPM profiles from Pro-inflammatory to Pro-resolving in the control group

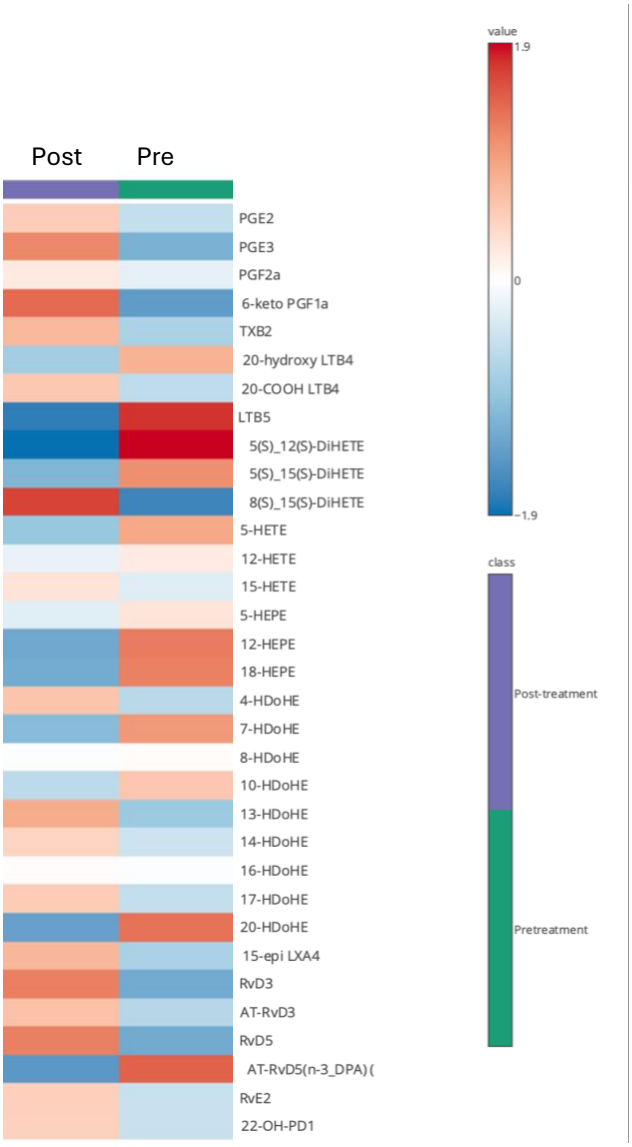


Figure 4.8b Omega-3 Supplement group. Metabololipidomics analysis revealed a significant shift in SPM profiles from pro-inflammatory to Pro-resolving in the Omega-3 Supplement group

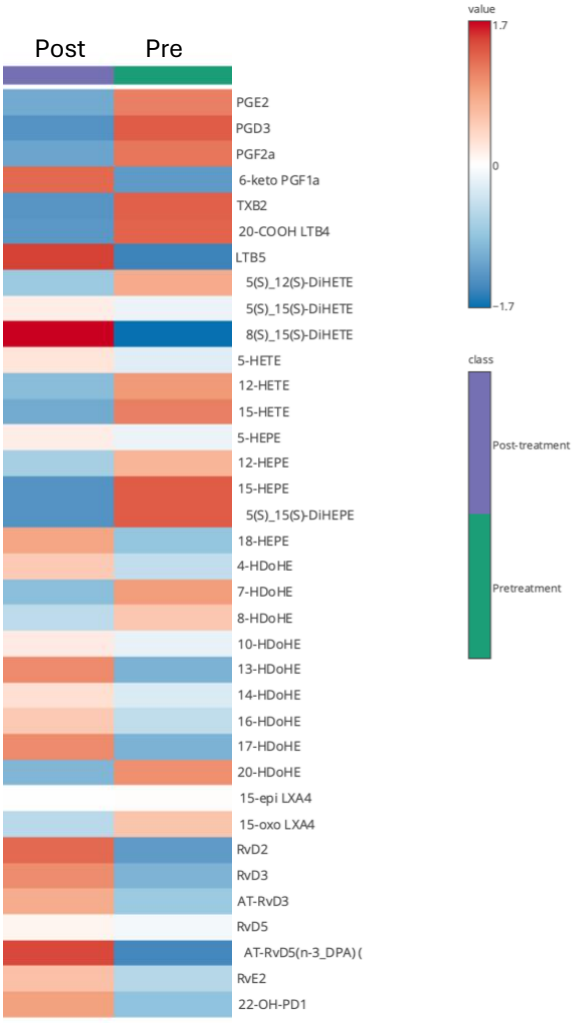


Figure 4.8c $BLXA_4$ group. Metabololipidomics analysis revealed a significant shift in SPM profiles from Pro-inflammatory to Proresolving in the $BLXA_4$ group.

4.7 Histopathology

In the control group (standard diet), the notch placed at the time of surgery was farthest from soft tissue and bone crest, while in the Omega-3 supplement group the notch was covered with new bone. In addition, there was significant new bone regeneration in the omega-3 supplement and $BLXA_4$ groups compared to the control group that had the standard diet (Figure 4.9).

Representative images from the most coronal aspect of the bone crest reveals good quality bone in Omega-3 supplement and $BLXA_4$ groups compared to the control group (Figure 4.10).

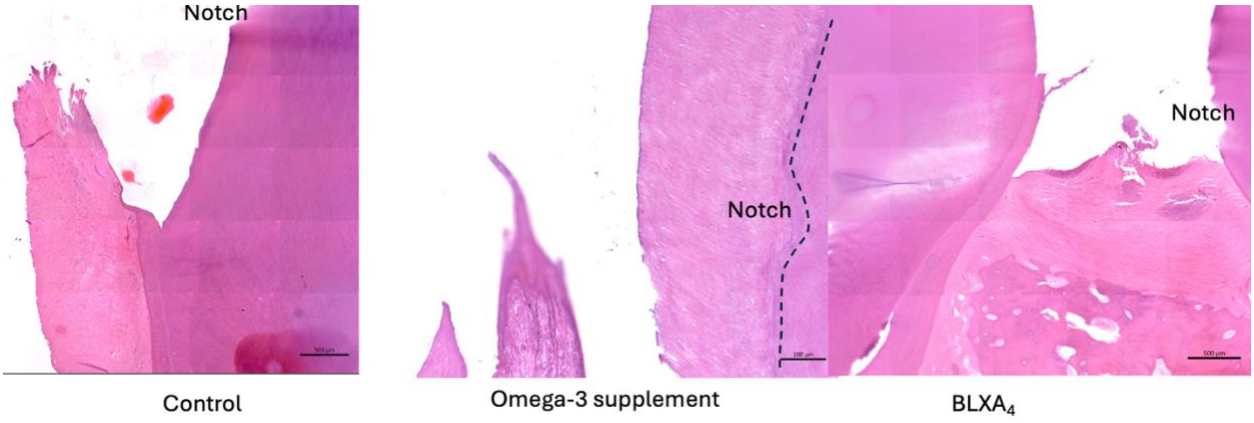


Figure 4.9 Images show the notches placed at time of surgery in relation to soft tissue and crest of bone (20X magnification). At this magnification, in the control group, the position of the soft tissue and bone crest is apical to the field of view. In the Omega-3 supplement group, there is filled with mineralized tissue in the area where the notch was placed at surgery (dashed lines) covering the notch, demonstrating bone regrowth coronal to the notch. In the $BLXA_4$ group, the distance between the notch and bone crest is minimal compared to the control due to bone infill and the soft tissue is at the level of the notch.

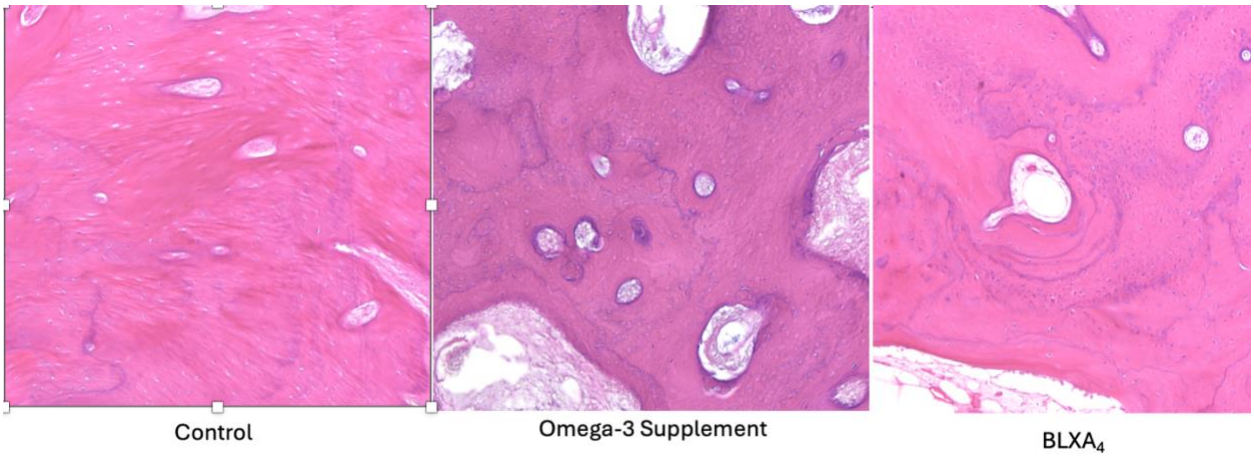


Figure 4.10 Images from the coronal aspect of the bone crest shows regeneration of new bone in the Omega-3 supplement and $BLXA_4$ groups compared to the control group that shows fibrous connective tissue.

5.0 Discussion

In this work, we report that dietary supplementation with an SPM precursor enriched preparation of omega-3 PUFA significantly improves outcomes of periodontal surgery. This study in a large animal model demonstrates that dietary supplementation with SPM precursor enriched omega-3 PUFA enhances bone regeneration in the treatment of chronic periodontal disease to a similar level as a low dose of the bioactive molecule $BLXA_4$ when compared to the control group that had only surgical debridement. Observations from this study reveal that SPM precursor enriched omega-3 dietary supplementation leads to a significant increase in bone volume compared to the untreated control group as well as a shift to synthesis of proresolving mediators from pro-inflammatory mediators. In addition, SPMs were metabolized in sufficient quantity to have a profound effect on resolution of inflammation and regeneration of the periodontal organ. SPM precursor enriched omega-3 PUFA dietary supplementation with Lipinova in this large animal model enhanced regeneration as evidenced by significant new bone height and volume compared with the control group.

Omega-3 dietary supplementation in addition had a significant impact on biosynthesis of systemic proresolving mediators; it promoted endogenous biosynthesis of proresolving lipid mediators and dampened the production of proinflammatory mediators. Lipidomics analysis revealed that systemic levels of proresolving mediators such as RvD3, RvD5, AT-RvD3, 13-HDoHE and 17-HDoHE were upregulated post-treatment, while proinflammatory prostaglandins such as TXB_2 and PGF_{2a} were down regulated. These findings support the hypothesis that dietary supplementation with concentrated HEPE/HDHA resolvin precursors in EPA/DHA

enriched fish oil enhances periodontal regeneration and upregulates systemic levels of proresolving lipid mediators.

Periodontitis is an inflammatory disease of the supporting structures of the teeth; cementum, alveolar bone and periodontal ligament, that is initiated by bacteria that form a biofilm on the surface of the teeth. The pathogenesis of the disease is a result of complex interactions between the biofilm and the host response that results in dysbiosis of the microbiome and dysregulation of the inflammatory response [7]. While bacteria are undoubtedly the cause of periodontitis, overwhelming evidence has revealed that it is uncontrolled inflammatory and immune responses that drive the tissue destruction. Lipid mediators play an important part in the pathogenesis of periodontitis [6].

Uncontrolled inflammation is a major impediment to tissue engineering, regeneration and reconstruction of both diseased and injured tissues resulting in further tissue injury, tissue scarring and fibrosis. The resolution of inflammation was defined in modern molecular terms very recently. Importantly, the resolution of inflammation is now believed to be an active process and a completely distinct process from anti-inflammation. This is because, in addition to serving as agonists to stop and reduce neutrophil infiltration to inflamed tissues, pro-resolution molecules promote the uptake and clearance of apoptotic cells and microorganisms by macrophages in inflamed sites and stimulate the antimicrobial activities of mucosal epithelial cells. The resolution of inflammation is accompanied by an active class switch in lipid mediators that predominate in exudates. Mediators, such as classic prostaglandins and leukotrienes, which activate and amplify the cardinal signs of inflammation, are replaced by resolvins and lipoxins which actively promote resolution of inflammation[21].

These families of endogenous pro-resolution molecules are not anti-inflammatory or immunosuppressive; they function in the resolution of inflammation by activating specific mechanisms to promote homeostasis. The proresolving molecules stop neutrophil infiltration, limit tissue damage by neutrophils, enhance microbial killing and cause apoptosis of neutrophils without inflammation while attracting nonphlogistic monocytes to the lesion which phagocytose apoptotic neutrophils and clear necrotic debris without contributing to further inflammation or tissue damage. By binding to G-protein coupled receptors on CD4 cells, they stimulate production of IL-10 and inhibit production of IL-17, TNF- α and IFN- γ [17]. Stem cell activity is disrupted by persistent signals promoting inflammation, whereas specific anti-inflammatory signals enhance stem cell activity. In successful regeneration, mesenchymal stem cells assume an anti-inflammatory phenotype [19].

This study employed a large animal model (Gottingen miniature pig), a marked improvement over other periodontal models in translational research such as mice and rabbits. The pigs are suitable for laboratory housing and have more similarities to humans in terms of jaw size, anatomy, physiology, immunology, and other oral structures and have been shown to be specifically suitable for regenerative studies [64]. In this study, a nonhealing, chronic periodontal lesion in the Gottingen miniature swine was used to mimic the human situation including an omnivorous diet and jaw motions during chewing. The miniature swine represents a significant advance in translational research that targets novel approaches to inflammatory diseases [2].

Results from this study are similar to results from Van Dyke et al [2] in which they utilized Nano Proresolving medicine (NRPM) to regenerate periodontal tissues. The study was done in Hanford miniature swine and had four treatment groups: $BLXA_4$ alone (10 µg of 2.1 µl solution), NPRM alone, or NPRM containing $BLXA_4$ (NPRM-bLXA4) while the control group only had surgical debridement. In contrast, our study utilized 0.5 ug per site in 18 µl of the $BLXA_4$ lipid nanoparticle solution delivered to each of eight treatment sites. In addition, there was an additional 10 µg per site of $BLXA_4$ delivered in 2.5 microliters (Vinresol, 8.75 mg/ml ethanol solution) in PBS to the eight treatment sites via intragingival route using a microliter syringe (Hamilton #65 (87943) with a 34-gauge needle (Hamilton 207434-10)) under sedation at the time of 14-day follow-up to prolong the delivery of the $BLXA_4$. This was based on a recent study that showed that repetitive low dosing with SPMs activate immune and resolution pathways that lead to regeneration [57].

In the NPRM and $BLXA_4$ study by Van Dyke et al [2], the volume of bone regeneration obtained in the NPRM + $BLXA_4$ group was similar to volume of bone regeneration in the omega-3 diet group in our study, and even though the present study employed a significantly less potent dosage of $BLXA_4$; there was significant bone regeneration compared to the control group that had only regular diet. This reinforces the reports by Serhan et al [57] that low doses of SPMs can ensure resolution of inflammation and lead to regeneration of lost periodontal tissues.

The probing pocket depths in this study showed a reduction in the Omega-3 diet and $BLXA_4$ group, while it was slightly increased in the Regular diet group similar to observations from Van Dyle et al [2]. This slight reduction may be because of excessive plaque accumulation from the soft diet of the animals. However, the treatment groups still showed a reduction in pocket depth post-treatment compared to the Regular diet group.

Our study also revealed a significant shift from pro-inflammatory lipid mediators to proresolving lipid mediators post-treatment; dietary supplementation with enriched omega-3 markedly

upregulates systemic levels of proresolving mediators such as RvD3, RvD5, AT-RvD3, 13-HDoHE and 17-HDoHE, while proinflammatory prostaglandins such as TXB_2 and PGF_{2a} were down regulated, this result is similar to report by Van Dyke et al. in which local application of $BLXA_4$ increased arachidonic acid and ω-3 polyunsaturated fatty acid–derived mediators including endogenous lipoxins and resolvins. Concurrently, proinflammatory prostaglandins PGE_2 and PGD_2 were decreased.

Our study employed an SPM enriched omega-3 dietary supplement that contained eicosapentaenoic acid and docosahexaenoic acid concentrate; 400 mg EPA + DHA enriched with 18-HEPE, 14-HDHA, and 17-HDHA. (**LIPINOVA-11TG™),** and our observations are similar to reports from a study by El Sharkawy et al [49] in a double masked clinical study that investigated the effect of dietary supplementation with fish oil\(900 mg EPA +DHA) and 81 mg aspirin daily in healthy subjects with advanced periodontitis. They reported significant reduction in probing depth and significant increase in attachment gain as well as reduction in salivary RANKL and MMP-8 levels in subjects who had dietary supplementation with fish oil compared with controls. Dietary supplementation with Omega -3 PUFA may be employed to treat advanced periodontal disease in patients with good systemic health.

6.0 Conclusion

SPMs activate wound healing with tissue regeneration and directly improve bone healing and regeneration. Dietary supplementation with a specific omega-3 PUFA preparation that is enriched with SPM precursors exhibited statistically significant improvement in tissue healing and regeneration.

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