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Profiling Precursor Lipids for Specialized Pro-Resolution Molecules in Platelet-Rich Fibrin Following Fish Oil and Aspirin Intake

Danielle M. McCormack

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Profiling Precursor Lipids for Specialized Pro-Resolution Molecules in Platelet-Rich Fibrin Following Fish Oil and Aspirin Intake

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

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Abstract

PROFILING PRECURSOR LIPIDS FOR SPECIALIZED PRO-RESOLUTION MOLECULES IN PLATELET-RICH FIBRIN FOLLOWING FISH OIL AND ASPIRIN INTAKE

by

Danielle M. McCormack, MSD

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Dentistry at Virginia Commonwealth University.

Virginia Commonwealth University, 2017

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Background: Current research has demonstrated that aspirin and fish oil (EFA) increase plasma levels of specialized pro-resolution molecules (SPMs). This study investigates their effects on SPM precursor pools in platelet rich fibrin (PRF). Methods: Twenty healthy volunteers were randomly assigned to take aspirin; EFA or aspirin and EFA. Four hours later, SPM precursor levels were quantified using combined Liquid Chromatography tandem mass spectrometry. The differences between the groups: Aspirin (yes or no), EFA (yes or no), were analyzed by ANCOVA, testing for group differences after covarying out the baseline value. Results: There were 4 significant interactions, 1 with an aspirin effect, 2 with an EFA effect, and 64 with no
difference between the groups. The significant interaction effect was found for the following lipidome: LPE(20:4), LPI(16:1), LPI(18:1), and LPI(20:3). Aspirin decreased the LPG(16:4) levels, and EFA decreased the LPE(22:5) and PG(16:0/18:0) lipidomes. **Conclusions:** Some SPM precursor pools in PRF were increased following supplementation.

*Keywords:* Platelet-rich fibrin, specialized pro-resolution molecules, mass spectrometry, precursor lipids, EFA, aspirin.
**Introduction**

Periodontal Disease is a chronic inflammatory disease: It is becoming increasingly clear that bacteria are necessary, but not always sufficient to produce disease (1) and the tissue destruction is a result of exaggerated host inflammatory response to the biofilm. In addition to being associated with a variety of systemic diseases, (2) chronic periodontitis is the most prevalent disease affecting almost half of the US adult population (3).

**Acute Inflammation and Its Resolution**

In response to injury or infection, the protective program of acute inflammation and its complete and timely resolution are critical for the restoration of tissue homeostasis. This highly coordinated and synergistic program combines the distinct actions of multiple cell types to achieve pathogen eradication and subsequent tissue repair. The acute inflammatory response can be divided into 2 general phases: initiation and resolution (4, 5).

Initiation is marked by:

a. Tissue edema resulting from increased blood flow and permeability of the microvasculature; processes that are mediated, in part, by lipid mediators (ie, leukotrienes and prostaglandins) and other vasoactive products (ie, histamine and bradykinin).

b. Subsequently, polymorphonuclear neutrophils (PMN) migrate to the area to defend against microbial invasion. Drawn to the site of injury by chemical signals including proinflammatory lipid mediators, PMN traverse the vasculature through precise interactions with endothelial adhesion receptors and subsequently engulf and degrade pathogens within phagolysosomes.

The resolution phase is already being enacted at this early point as:
a. The influx of PMN is halted at a level appropriate for the insult and is accompanied by their timely apoptosis.

b. Monocytes subsequently infiltrate the tissue where they differentiate into macrophages that avidly respond to the so-called find-me and eat-me signals (ie, nucleotides and externalized phosphatidylserine) released or presented by apoptotic cells such as PMN.

c. Uptake of apoptotic cells by macrophages (ie, efferocytosis) is an anti-inflammatory process associated with decreased production of inflammatory mediators, thus coupling the initiation of inflammation with its ultimate resolution.

d. The timely clearance of microbes and apoptotic cells is required to prevent bystander tissue damage and to set the stage for tissue repair and regeneration, allowing for the return to homeostasis.

e. Active clearance of apoptotic cells is a key defining feature of resolution, as failed clearance can lead to cellular necrosis and exacerbated inflammation beyond the initial insult, impeding tissue repair.

Critical to progressing from initiation to resolution is the temporal switch in lipid mediators that are biosynthesized by leukocytes in the tissue, a process known as lipid mediator class switching (Figure 1). Interruption of this process at any point (ie, prolonged leukocyte recruitment and survival, impairments in apoptotic cell removal, and alterations in macrophage phenotype switching) could potentially lead to chronic inflammation with resultant tissue damage, excessive fibrosis, and loss of function (5, 6).

Resolution of inflammation is an active, agonist-mediated, well-orchestrated return of tissue homeostasis. It is important to realize that anti-inflammation and resolution are completely independent mechanisms that affect the final outcome. While anti-inflammation is a
pharmacologic intervention, resolution is biologic pathways that restore tissue homeostasis. A growing body of research suggests that chronic inflammatory periodontal disease is at least involves a failure of resolution pathways to restore homeostasis.

For several years, anti-inflammatory agents were used for prevention and management of inflammatory diseases. However anti-inflammation is not the same as resolution. Once the inflammatory response is initiated, a continuous cascade of events takes place during which the body attempts to eliminate invaders through proinflammatory actions of cells and their products. The symptoms of inflammation are relieved primarily due to catabolism of proinflammatory mediators.

Recently, the possibility of using potent ‘resolution agonists’ to orchestrate the return of the tissue to homeostasis and rescue resolution deficits has been proposed and is promising (Figure 2). Existing data demonstrate that natural pathways of resolution can be used to limit inflammation and promote healing and regeneration with a minimal risk of side effects. Within this context, lipid mediators represent a paradigm change in the management of periodontal diseases (7).

Platelets and Acute Inflammation:
Platelets or thrombocytes are one of the important components of blood that play very important role in hemostasis, inflammation and regeneration. Normal platelet counts in whole blood are in the range of 150,000 to 400,000 per micro-liter. Autologous platelet concentrates (APC) are derived from patients' own blood and are processed to enrich the amount of platelets to at least 1,000,000 per micro-liter. Such a high concentration of platelets is expected to be a potent source of endogenous peptide growth factors and cytokines like PDGF, IGF, VEGF and FGF that stimulate healing of bone and soft tissues (8).
In contrast to anti-inflammatory effects of growth factors, its resolution is orchestrated by a family of lipid molecules called specialized pro-resolving mediators (SPM) including lipoxins, resolvins, maresins and protectins (9),(10).

**Specialized Pro-resolving Mediators- Lipoxins and Resolvins:**

Lipoxins (LX): are trihydroxy-products derived from arachidonic acid (AA) through the cooperative interaction of diverse cell types in the inflammatory milieu- neutrophils, eosinophils, monocytes, platelets, and endothelial cells. The dense clustering of different cell types presents a unique situation for lipid handling. In contrast to the synthesis of protein mediators (ie, cytokines), lipid mediators can be produced along enzyme pathways that involve multiple cells, in a process known as transcellular biosynthesis (Figure 3). Under the action of cytosolic phospholipase A, membrane AA is immediately secreted into the extracellular milieu, where it is taken up and used by nearby cells. In this way, the inflamed tissue becomes a specialized organ for lipid metabolism, producing the types and amounts of lipid mediators needed to promote or resolve inflammation.

There are two major routes of LX production from AA in humans. The type of lipoxygenase that initiates AA oxygenation can distinguish these, and these are distributed differently by cell type. The first pathway involves the insertion of molecular oxygen at C-5 by 5-lipoxygenase (5-LO) in concert with the 5-LO activating protein (FLAP) to produce 5-hydroperoxyeicosatetraenoic acid (5-HpETE), which is further metabolized by 5-LO to produce the intermediate, leukotriene A (LTA). This pathway is conducted solely by leukocytes, since the distribution of 5-LO is largely restricted to these cell types. LTA4 is readily transferred to adjacent cells, usually leading to its processing to other LTs (11). However, adherent platelets, via 12-LO, will convert LTA4, donated by leukocytes, to LXA4 and LXB4. The second major
route of LX biosynthesis involves the initial conversion of AA to 15(S)-HpETE by 15-LO. Following secretion, 15(S)-HpETE is taken up by either neutrophils or monocytes and rapidly converted through a 5-LO/ FLAP dependent mechanism to LXA4 and LXB4.

Resolvins (Rv) are endogenous chemical mediators that are biosynthesized from the major ω-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), denoted E series (RvE) and D series (RvD) resolvins, respectively. Like LX, Rv can be produced through transcellular cooperation, initiated by enzymes in epithelial cells and completed by adjacent leukocytes. Conceptually, the substrates EPA and DHA are released from membrane phospholipids, metabolized in a transcellular fashion, and secreted in amounts sufficient to reverse the course of inflammation. Ideally, this must happen throughout the inflamed tissue, at a time that appropriately follows the elimination of the insult, which caused the inflammation, continuing on to a return to homeostasis.

ω-3 fatty acids, Aspirin and SPM: Epidemiological, clinical, and animal studies provide substantial support that the long chain ω-3 fatty acids from fish and fish oils, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) improve outcomes associated with inflammation (Figure 4) (12). The mechanisms by which ω-3 fatty acids exert their protection are still emerging but likely include alterations in cell membrane composition and effects on gene expression and receptors regulating signaling. The anti-inflammatory actions of ω-3 fatty acids are, in part, related to reduce leukocyte-derived cytokine formation and modulation of eicosanoid synthesis (13) (Figure 5). As mentioned above, resolution of inflammation is accomplished by SPMs derived from EPA and DHA, known as E-series and D-series resolvins, respectively. Aspirin irreversibly acetylates cyclooxygenase-2 (COX-2), inhibiting its ability to produce prostanoids. However, acetylated COX-2 can metabolize AA to 15(R)-HETE, which may then
be processed to the “aspirin-triggered” LX by 5-LO. Thus, epimers of LXA₄ and LXB₄ can be produced following aspirin treatment. Similar to LX, aspirin-triggered Rv epimers are produced by the acetylated COX-2/5-LO pathway.

Clinical studies involving supplementation of omega-3 fatty acids and detection of SPM from human plasma samples using targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) have showed that the plasma levels of SPMs can be accurately quantified using this technology and that these levels were increased within 4 hours of omega-3 and aspirin intake (9). In another recent study, healthy volunteers were given ω-3 fatty acid supplements for seven days and then randomized to receive aspirin or placebo in addition to ω-3 fatty acids during the last two days. The study showed that ω-3 fatty acid supplementation for five days increased plasma levels of 18-HEPE, 17-HDHA, 14-HDHA, and RvE1. Aspirin taken in addition to ω-3 fatty acids did not differentially affect any SPM. However, aspirin significantly reduced the ratio of R- to S-isomers of 17-HDHA (14).

In a trial on 74 patients with chronic renal disease randomized to ω-3 fatty acids or coenzyme Q10 (CoQ) for eight weeks, it was found that ω-3 Fatty acids significantly increased plasma levels of 18-HEPE, 17-HDHA, and RvD1 while CoQ had no effect on any plasma SPM. Regression analysis showed the increase in 18-HEPE and 17-HDHA following ω-3 fatty acids was associated with a change in platelet EPA and DHA, respectively (15).

Patients with metabolic syndrome who started with reduced plasma concentrations of the precursors of the E- and D- series resolvins had their plasma E-series resolvins increased following ω-3 fatty acid supplementation and the addition of aspirin did not alter any of the plasma SPMs in diseased and control subjects (16).
**Autologous Platelet Concentrates:** Platelets or thrombocytes are one of the important components of blood that play very important role in hemostasis, inflammation and regeneration. Normal platelet counts in whole blood are in the range of 150,000 to 400,000 per micro-liter. The efficacy of platelets is due to degranulation of alpha and dense granules where a variety of growth factors responsible for regeneration is stored (17). Autologous platelet concentrates (APC) are derived from patients' own blood and are processed to enrich the amount of platelets to at least 1,000,000 per micro-liter.

One form of autologous platelet concentrate is platelet-rich fibrin (PRF), which is a second-generation platelet concentrate that was developed in France by Choukroun that is being used routinely in clinical practice (18, 19). The processing technique of PRF requires minimum amount of blood to be harvested (10 ml) and has some advantages compared to platelet-rich plasma (PRP) such as the simplified technique for preparation/application, minimal expense, and lack of biochemical modification (no anticoagulant or bovine thrombin is required).

Considering the fact that platelet-rich fibrin (PRF) once applied is allowed to stay in contact with the wound surface and stimulate a slow release of growth factors for 7 days and up to 28 days in vitro (20, 21), it is important to know if the PRF has sufficient capacity to provide a continuous supply of precursors for the continuous production of SPMs. In this study, we seek to investigate the levels of precursors of SPM in PRF and the effect of administering essential fatty acid or aspirin on the total levels of the precursors of these signaling lipids.
Materials and Methods

Protocol:
The clinical study was undertaken after prior approved by the Institutional Review Board of Virginia Commonwealth University (Study ID: HM20002473). Informed consent was obtained from each participant. All experiments listed in this protocol involve handling of human blood and/or blood products and appropriate personal protective equipment need to be worn at all times. The waste were considered as biohazard and disposed of according to regulations. The protocol for the entire procedure is listed in the Appendix 1.

Study Design:
Sixty study participants were recruited who satisfy the following inclusion/exclusion criteria.

Inclusion Criteria:
1. Healthy adults (>18 years of age)
2. Non-pregnant
3. Weight > 110 pounds

Exclusion Criteria:
1. History of smoking in the past 2 years
2. Individuals with known medical conditions and currently receiving treatment for the same
3. History of anti-coagulant, immunosuppressive or antibiotic therapy in the last 6 months
4. History of non-steroidal anti-inflammatory drug use (Advil, Tylenol) in the past 2 days or Aspirin in the past 10 days
5. History of essential fatty acid (fish oil supplement) intake in the past month
6. Known allergic to fish oil components or nuts

Once written informed consent was obtained from the participant, the subject opened a sealed envelope to determine the group he/she will fall into. The research coordinator assigned the subjects into any one of the four groups with 15 participants each:

Group A: Control

Group B: Subjects taking one softgel of fish oil supplement (1400 mg, Sundown Naturals, NY)

Group C: Subjects taking one tablet of Bayer low-dose (81 mg) aspirin

Group D: Subjects with both EFA supplement (1.4 g) and aspirin (81 mg) 2 hours apart

**Blood draw protocol:**

Each patient had a total of 2 blood draws and the initial draw was done to establish the baseline. After 4 hours after drug administration, the second blood draw was done. Personnel adequately trained in phlebotomy performed the blood draws. At each time, 10 ml of blood was collected in red top tubes and centrifuged in IntraSpin System (Intra-lock, FL) (Figure 6). The tubes were centrifuged at 2700 rpm for 12 minutes at room temperature. The PRF membrane was separated from the clot and was compressed using the Xpression Box for 30 sec (Figure 7). The PRF plugs were transferred to labeled tubes with participant’s ID number, age, and sex, to protect confidentiality. The labeled specimens were immediately stored at -80°C freezer to prevent degradation until ready for analysis. When all samples were collected, the samples were thawed and analyzed using the protocol described below.

**Sample preparation for lipid extraction:**

Lipids were extracted from the biological material using a modified Bligh and Dyer method (22). Briefly, to 200μl of PRP 1ml of methanol containing the required internal standards were added followed by the addition of 0.5 ml of chloroform. The monophasic mixture thus obtained was
incubated at 4°C overnight. Depending on the analysis carried out, the extracted lipids were either further purified using solid phase extraction or used as is following dilution to achieve the target concentration in the linear dynamic range.

**Lipid Quantification using LC-MS/MS:**

These specialized pro-resolving mediators were analyzed using targeted LC MS/MS methods using Shimadzu Nexera UPLC and a hybrid triple quadrupole linear ion trap (AB SCIEX 6500), or via untargeted analysis using either LC MS/MS or shotgun lipidomics (AB SCIEX 5600). The available lipid panel allowed quantification of following lipids:

<table>
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<tr>
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<th>FFA(16:2)</th>
<th>FFA(24:1)</th>
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<td>FFA(18:3)</td>
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<td>FFA(20:5)</td>
<td>FFA(22:4)</td>
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<tr>
<td>FFA(22:5)</td>
<td>FFA(22:6)</td>
<td>FFA(24:0)</td>
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<tr>
<td>LPC(14:0) +AcO</td>
<td>LPC(16:1) +AcO</td>
<td>LPC(16:0) +AcO</td>
</tr>
<tr>
<td>LPC(18:0) +AcO</td>
<td>LPC(18:1) +AcO</td>
<td>LPC(18:2) +AcO</td>
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<tr>
<td>LPC(18:3) +AcO</td>
<td>LPC(20:0) +AcO</td>
<td>LPC(20:1) +AcO</td>
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<td>LPC(20:2) +AcO</td>
<td>LPC(20:3) +AcO</td>
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<td>LPC(22:4) +AcO</td>
<td>LPC(22:5) +AcO</td>
</tr>
<tr>
<td>LPC(22:6) +AcO</td>
<td>LPE(16:0)</td>
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<td>LPS(18:0)</td>
<td>PE(18:2/20:1)</td>
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<td>PS(18:2/22:6)</td>
<td>SM(14:0)</td>
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<tr>
<td>SM(16:0)</td>
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<tr>
<td>SM(26:0)</td>
<td>SM(26:1)</td>
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</table>

where:

SM=Sphingomyelin
LPC=Lysophosphotidylcholine
LPE=Lysophosphatidylethanolamine
LPG=Lysophosphatidylglycerol
LPI=Lysophosphatidylinositol
LPS=Lysophosphatidylserine
PS=Phosphatidylserine
FFA=Free Fatty Acids
PE= Phosphatidylethanolamine
The number designation is for the following (XX:Y):

XX = number of carbon atoms in the acyl chain. Also referred to as the chain length.
Y = number of double bonds in the acyl chain.

**Statistical analysis:**

Participants were randomly assigned to one of four group intervention groups using a two-by-two design (ETA=No or Yes and Aspirin=No or Yes) and there was a specimen collection at baseline and post-intervention. The statistical method used to analyze this question was an analysis of covariance (ANCOVA) with the following effects: Baseline covariate, Aspirin, EFA, and the EFA*Aspirin interaction. There are thus three questions of interest.

- The p-value associated with the Aspirin effect answers this question: Is the post-intervention mean of the Aspirin=No groups different than the post-intervention mean of the Aspirin=Yes groups, after covarying out the patients’ baseline? Question: Does Aspirin have a simple effect?

- The p-value associated with the EFA effect answers this question: Is the post-intervention mean of the EFA=No groups different than the post-intervention mean of the EFA=Yes groups, after covarying out the patients’ baseline? Question: Does EFA have a simple effect?

- The p-value associated with the EFA*Aspirin interaction answers this question: Does the effect of EFA differ in the Aspirin=No and Aspirin=Yes groups, after covarying out the patients’ baseline. Question: Is the effect of EFA and Aspirin complicated?

In statistical analysis, the answer to the interaction question is inspected first. And so, the results will be reported according to the following patterns:
• 4 Treatments: In those lipid precursors where there is a significant EFA*Aspirin interaction, all possible treatment group differences will be inspected by Tukey’s HSD multiple-comparison procedure and the differences summarized.

• Aspirin: In those lipid precursors where there is no significant interaction, but there is a significant Aspirin effect, the two Aspirin groups will be compared.

• EFA: In those lipid precursors where there is no significant interaction, but there is a significant EFA effect, the two EFA groups will be compared.

• No effect: In those lipid precursors with no significant interaction effects and no significant main effects, the lipid precursors will be listed.

All analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary NC). Statistical significance was declared at alpha = 0.05. (Unfortunately, in this small study) No correction for multiple comparisons was applied.
Results

Flow of Participants

For this study, 20 of the participant samples were available for analysis. There were 5 participants in group A, 6 in group B, 4 in group C, and 5 in group D (Figure 8).

Significant interactions

There were 71 SPM screened in the 20 PRF subjects. There were 4 SPMs with a significant interaction, 1 with an Aspirin effect, 2 with an EFA effect, and 64 with no apparent difference between the groups.

Interaction effect

There was a significant interaction effect for these SPMs: LPE(20:4), LPI(16:1), LPI(18:1), and LPI(20:3). These results are shown in Table 1 where the means for the four treatment groups are ordered by size in the right-hand column. Means sharing the same superscript were not significantly different.

The pattern that is evident for LPE(20:4) is that there is no significant EFA difference (P=0.061), no significant Aspirin difference (P=0.458) but a significant interaction (P=0.046). That is, there is some evidence for a difference between the four groups but the pattern is not simple. As may be seen by the fact that all four means share the same “a” superscript, the multiple comparison procedure cannot identify which of the groups were different.

The pattern for LPI(16:1) is clearer. The EFA=No Aspirin=No group has a significantly higher mean LPI(16:1) than any of the other three groups.

The pattern for LPI(18:1) and LPI(20:3) is that the EFA=No Aspirin=NO group is only significantly higher than the EFA=No Aspirin=Yes group. And the other two groups were not different from any of the other groups.
**Aspirin effect**

The only significant Aspirin effect was evident in LPG(16:1).

**EFA effect**

A significant EFA effect was evident in LPE(22:5) and PG(16:0/18:0). The Aspirin and EFA effects are shown in Table 2. For LPG(16:1) the no Aspirin group had higher levels than the Aspirin groups (a difference of -0.04, P=0.044). For LPE(22:5) the no EFA group had lower mean than the EFA group (a difference of 16.3, P=0.050). For PG(16:0/18:0) the no EFA groups had higher mean than the EFA group (a difference of -3.6, P=0.016).

**No effect evident**

Discussion

Acute inflammation is the body’s natural response to tissue injury and the failure to resolve inflammatory processes can lead to persistent low-grade inflammation. This chronic inflammation is associated with many diseases including periodontal disease, diabetes, rheumatoid arthritis, and inflammatory bowel disease (23). During periodontal surgery, soft tissue and bone augmentation procedures create acute tissue trauma and in turn trigger an inflammatory response. Immediate management of the wound site at time of surgery can improve both the healing process and patient’s postoperative pain. To help manage the acute inflammation that occurs after surgery, better knowledge of the active process of resolution of inflammation and healing are needed.

There are many different cell types and signaling pathways involved in wound healing and it has been established over the past few decades that lipid signaling pathways play a dominant role (24). Serhan has shown that resolution of inflammation is not a passive process but an active one that is mediated by specialized pro-resolution molecules (SPMs) (25). SPM activity has been linked to cessation of polymorphonuclear leukocyte (PMN) recruitment to the wound site, and enhancement of macrophage uptake of debris, bacteria, and apoptotic cells to aid in inflammatory resolution and return to homeostasis. It has also been demonstrated that SPMs are increased in blood plasma and platelet concentrates with the intake of aspirin and essential fatty acids (EFA). This is due to increasing omega-3 fatty acids EPA and DHA triggering the “classical” pathway for SPM production as well as the “aspirin-trigger” pathway biosynthesis of specific epimers of these mediators. What is less understood are the lipid precursors along these pathways and whether manipulation of these precursors would provide a longer acting beneficial effect on wound healing.
Autologous platelet concentrates (APC) are currently being employed in the field of regenerative medicine because of the abundance of peptide growth factors and cytokines. Platelet-rich fibrin (PRF) brought about a huge advance in the evolution of platelet concentrates. Whereas platelet-rich plasma (PRP) undergoes rapid activation with the use of thrombin that can cause fibrinogen polymerization to be incomplete and result in friable fibrin gels, PRF forms a dense fibrin membrane with entrapped platelets that allows the slow release of growth factors over a longer period of time (19, 20, 26). The preparation of L-PRF allows for the processes of fibrinogen polymerization, platelet enhancement, and activation to occur simultaneously in one tube without the use of anticoagulant, bovine thrombin, or calcium chloride. This method of concentrating platelets not only simplifies the process when compared to PRP, but also significantly decreases costs. One important consideration in generating consistent L-PRF membranes is the speed of blood collection and immediate centrifugation of tubes, typically within a minute. If either harvesting of blood or transfer to centrifuge is prolonged, then the result is formation of a small incoherent, friable mass of fibrin with unknown content. Once the PRF clot is formed, it can be compressed into membranes and/or plugs to be utilized for surgical application.

Although aspirin and fish oil have been shown to increase the levels of SPMs when examined in both whole blood and platelet concentrates, the levels of their lipid precursor molecules have never been investigated before now. Considering that PRF is able to stimulate a slow release of growth factors, it is important to know if the PRF has sufficient capacity to provide a continuous supply of precursors for the continuous production of SPMs and if administering essential fatty acid or aspirin has an effect on the total levels of the precursors of these bioactive lipids.
In this pilot study, lipids were measured in multiple forms that can be precursors to SPMs. Whereas free fatty acids may be used as immediate precursors, lysophospholipids are stored precursors that need an enzyme to be activated prior to potential SPM conversion. This stored form of lipid precursors has the potential to be cleaved and increase the available free fatty acids to be used as an immediate source for potential conversion to SPMs. The data presented in this experiment includes a population of 20 healthy adult participants. Seventy-one lipid precursor molecules were quantified by analyzing platelet-rich fibrin plugs, by means of liquid chromatography mass spectrometry. We found four lipid precursors with significant interactions when both aspirin and EFA were administered, one with an Aspirin effect, and two with a significant EFA effect.

A significant interaction effect for LPE(20:4), LPI(16:1), LPI(18:1), and LPI(20:3) are shown in Table 1. LPE(20:4) is derived from a phosphatidylethanolamine, which is typical of cell membranes. This lipid precursor was seen to have a significant interaction when taking both aspirin and fish oil (P=0.046) but only a pattern towards significance when comparing mean values. The physiological significance of human plasma LPE remains unknown (15). The higher average of molecule LPI (16:1), LPI(18:1), and LPI(20:3) in the control groups compared to the groups receiving an intervention, indicates an overall increase in their metabolism with treatment. Both LPE and LPI are lysophospholipids derived from phospholipids, which are bioactive lipid molecules that make act as signaling mediators. In order to understand what signaling effects these lysophospholipids are contributing to, it would be necessary to know what free fatty acid chain was cleaved to contribute to either proinflammatory or anti-inflammatory pathways and would need further analysis.
The only significant Aspirin effect was evident in LPG(16:1), in which LPG is a precursor for synthesis of phosphatidylglycerol (PG). A significant EFA effect was evident in LPE(22:5) and PG(16:0/18:0). The increase in LPE(22:5) is noteworthy and has never been reported before and may indicate an increased metabolism of arachidonic acid to a longer chain omega-6 docosapentaenoic acid that does not give rise to inflammatory lipids. It can also indicate a buildup of precursor lipid omega-3 docosapentaenoic acid, for conversion to DHA, which is a precursor for SPM. Either way, this leads to a decrease in inflammation. Additional studies using flux analysis are needed to understand the exact mechanism.

The most notable limitations of this study are the sample size taken to perform the pilot study and not having the ability to analyze all samples taken. This was due to having limited available access to liquid chromatography tandem mass spectrometry equipment. Lipid precursors may have also been available in the supernatant that was compressed from the PRF plug and was not included in this pilot study analysis. Another limitation may be due to only giving a single dose of aspirin or fish oil. It is possible that more lipid precursors did not have a significant change due to the dose and time examined. It may be anticipated that a higher dose administered over a longer timeframe would show a change in these precursor pools.
Conclusions

In this pilot study, findings indicate that the pattern for LPI(16:1) for EFA=No, Aspirin=No group has a significantly higher mean LPI(16:1) than any of the other three groups. This indicates a higher average of molecule LPI (16:1) in the control group compared to the groups receiving an intervention, indicating a metabolism of this molecule during treatment.

The pattern for LPI(18:1) and LPI(20:3) is that the EFA=No, Aspirin=No group is only significantly higher than the EFA=No, Aspirin=Yes group. This also demonstrates higher levels LPI(18:1) and LPI(20:3) in the control group than compared to the aspirin group and again indicates an overall increase in their metabolism with treatment. The decrease in LPI with these treatments have never been reported before and is worth investigating further due to their signaling potential.

The increase in LPE(22:5) is noteworthy and has never been reported before. It may indicate an increased metabolism of arachidonic acid to a longer chain omega-6 docosapentaenoic acid that does not give rise to inflammatory lipids. It can also indicate a build up of precursor lipid omega-3 docosapentaenoic acid, for conversion to DHA, which is a precursor for SPM. Either way, this leads to a decrease in inflammation. Additional studies using flux analysis are needed to understand the exact mechanism.

Conflict of Interest Disclosure: The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
References


### Table 1. Differences between the four treatment groups

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<th>Lipid</th>
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<th>Aspirin</th>
<th>Mean</th>
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Table 3. ANCOVA results, P-values for each Lipid

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<th>EFA Pre</th>
<th>EFA* Aspirin</th>
<th>Pre</th>
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<td>FFA(16:1)</td>
<td>0.1134</td>
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<td>0.2148</td>
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<td>0.6863</td>
<td>0.0779</td>
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<td>0.6492</td>
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<tr>
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<td>EFA*</td>
<td>Pre</td>
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Figure 1: Chronology of events in acute inflammation and its resolution.

Source: Adapted from Circulation Research. 2016;119:113-130
Figure 2: A Schematic showing the paradigm shift in the approach towards treating chronic unresolved inflammation including periodontitis.
Source: http://www.eumbrella.org/timer_objectives.html
Figure 3: Transcellular biosynthesis of SPM during platelet-neutrophil interaction.
Figure 4: Table of SPM mediators.

Source: Serhan C. Novel lipid mediators and resolution mechanisms in acute inflammation: To resolve or not? Am J Pathol. 2010 Oct;177(4):1576-1591
Figure 5: Essential fatty acid production and metabolism to form eicosanoid signaling molecules, some of which are pro-inflammatory and others pro-resolving.

Source: https://en.wikipedia.org/wiki/Eicosanoid
Figure 6: A. Armamentarium for the PRF preparation with labeled tubes for sample collection. B is the centrifuge used for PRF preparation (Intra-Lock, FL)
Source: http://www.dentalimplants.clinic/services/surgical-procedures
Figure 7: Clinical steps involved in generating PRF.
A: Blood draw; B: Different layers of PRF in a tube; C: Preparing the membrane to form a plug; D: Compressed plug ready for storage.
Figure 8: Flow chart of study protocol.
Appendices

Appendix 1: Chair-side PRF Research Protocol

Before blood draw

1. Prepare the materials basket which should include: a phlebotomy kit, goggles, 6 pre-labeled PRP tubes, 3 pre-labeled PRF tubes, 3 5 mL syringes, goggles, 1 stopcock, 1 blood needle, 3 1 mL syringes with needles, ascorbic acid, calcium chloride, thrombin, and 5 glass vials with labels

2. Use a 5 mL syringe to draw 5 mL of calcium chloride and inject into the thrombin vial. Mix and let it sit until ready to use

3. Load 0.5 mL of ascorbic into a 1 mL syringe

4. Load 0.5 mL of the calcium chloride and thrombin mixture into the 2 other 1 mL syringes and leave for use after obtaining the blood samples

5. Meet with the subject and have them fill out the screening form

6. If the subject is eligible, review the participant consent form with the subject and if they wish to participate in the study have he/she sign the consent form with you as witness

7. Write the subjects name onto the data sheet and let them know if they are in group A, B, C or D

8. Inform the subject how to take their medication accordingly

9. Ask the subject if they are ready for the blood draw or if they would like to schedule for another day
**During the blood draw**

1. Bring out the phlebotomy kit which should include: 1 butterfly needle, 6 pre-labeled PRP tubes, 3 pre-labeled PRF tubes, gauze, 1 band aid, 1 test tube rack, 1 tourniquet, and 1 alcohol swab.
2. Put on gloves and goggles and ask the patient which arm they prefer to get the blood drawn from.
3. Wrap the tourniquet tightly around their desired arm and locate the vein you wish to use.
4. Disinfect the area using the alcohol swab and allow 30 seconds for the area to dry.
5. Proceed to inject the butterfly needle and draw 8 mL of blood into each PRF tube.
6. Mix the contents of each tube by inverting the tube 5-8 times then place each tube on the rack.
7. Remove the tourniquet and place some gauze on the injected area, before removing the butterfly needle.
8. Apply pressure with the gauze until the bleeding has stopped or apply a band aid.
9. Thank and discharge the patient, confirming that they will be returning in 4-6 hours.
10. Clean/disinfect the patient area.

**After the blood draw**

1. Place 1 of the PRF tubes labeled whole blood into the freezer for storage. Place the remaining two PRF tubes in opposite sides of the PRF centrifuge.
2. Press the green button and centrifuge for 12 minutes.
3. Remove the 2 PRF tubes from the PRF centrifuge.
4. Place them onto a clean tray and bring out the Xpression kit which should include a tray, weight, small dish, scalpel, and tweezers.
5. Use tweezers to remove the PRF layer from each of the tubes and place them on the Xpression tray. Make sure the PRF layers are directly above the small dish.

6. Use a scalpel to remove the red blood cells.

7. Place the weight on top of the PRF layers for 30 seconds.

8. Collect the PRF pellets and place them into separate labeled vials and then collect the liquid that is now in the small dish and transfer it into a separate labeled vial.

9. Store all 3 samples into the freezer.

10. Clean and disinfect the area.

11. Repeat the protocol when the patient arrives 4-6 hours later.
Appendix 2: Study key for preparation of PRF before medication taken and 4 hours after medication taken.

Color code for preparation tubes:

1. WHITE: Serum collected after blood clotted w/o centrifuging
2. 2 GREEN PRF PLUG: PRF plug collected after centrifuging and excision
3. BLUE PRF SN: Supernatant collected after PRF plug was made
Appendix 3: Recruitment Flyer

Virginia Commonwealth University
Healthy Volunteers Wanted for a Research Study
Effects of Dietary Essential Fatty Acids and Low-dose Aspirin on Specialized Pro-resolving Lipid Mediators in Autologous Platelet Concentrate Gels

The purpose of this research is to investigate the effects of two commonly available medications: fish-oil supplement and baby aspirin, on certain lipid (fat) components of blood necessary for wound healing. We will use sophisticated lab methods to accurately measure the blood levels of these fats in healthy volunteers after taking EFA (fish-oil pill) and baby aspirin. The information from this research will allow us to determine if taking either fish-oil or baby aspirin or both will have beneficial effects on wound healing following surgery. The research is conducted under the direction of Dr. Parthasarathy Madurantakam, Philips Institute of Oral Health Research, VCU School of Dentistry.

The study will comprise of 60 participants and will involve two blood draws (60 ml each, approximately 4 tbsp) following clinically approved protocols, 4 hours apart. Your participation in this study will be limited to one half day and you will not be compensated for your participation.

To be eligible, you should be a healthy adult (> 18 years of age and not having any known medical condition), non-pregnant and weigh at least 110 lbs. In addition, you will not be eligible to participate if you have:

- History of smoking in the past 2 years
- History of anti-coagulant, immunosuppresive or antibiotic therapy in the last 6 months
- History of non-steroidal anti-inflammatory drug use (Advil, Tylenol or Aspirin) in the past 1 month
- History of fish-oil supplement (essential fatty acid) intake in the past month
- Known allergy to fish-oil components or nuts

If you are interested and want to participate in this study or if you have any questions, please contact

Kimberly Hollaway at klhollaway@vcu.edu or 804-828-4553

Dr. Madurantakam at madurantakap@vcu.edu or 804-828-9353
Appendix 4: IRB approval and Consent form

This Box for IRB Office Use Only – Do Not Delete or Revise  Template Rev Date: 6-19-14

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM TITLE: Effects of Dietary Essential Fatty Acids and Low-dose Aspirin on Specialized Pro-resolving Lipid Mediators in Saliva and Autologous Platelet Concentrate Gels

VCU IRB PROTOCOL NUMBER: HM20002473 INVESTIGATOR: Dr. Parthasarathy Madurantakam

You have been approached by the study doctor or the study staff because you have responded to our request to volunteer in the above-mentioned study. If any information in this consent document is not clear to you, please feel free to talk to the doctor or the staff involved in the study. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.

In this consent form, “you” always refers to the research participant.

PURPOSE OF THE STUDY

The purpose of this research is to investigate the effects of two commonly available medications: fish-oil supplement and baby aspirin, on certain components in blood and saliva necessary for wound healing. The information from this research will allow us to determine if taking either fish-oil or baby aspirin or both has a beneficial effect on wound healing following surgical procedures.

DESCRIPTION OF THE STUDY

It has been recently found that presence of adequate amounts of certain kind of fats is critical for normal healing after surgery or trauma. Studying the blood levels of these fats was not possible because precision instruments were not available. We will use the recent technological advances to accurately measure the blood levels of these fats in healthy volunteers after taking EFA (fish-oil pill) and baby aspirin. In addition, we will explore the potential of using saliva as an alternate body fluid to blood to detect these fats.

Your participation will be limited to one day and we expect to enroll 60 study participants.

PROCEDURES

On the day of your scheduled appointment, a member of the research team will meet with you to go over the details of the study and the informed consent process. You can use this opportunity to ask any questions related to the study; seek more time to decide or may choose to not participate in the study.

Once you sign the informed consent document, you will have the two procedures done: 1. We will collect 4 ml of your saliva (that is normally secreted, unstimulated) into a
plastic tube 2. A trained professional will draw 60 ml of your blood (4 tablespoons). This amount is well within the established safety limits. After the blood draw procedure, you will open a sealed envelope to determine which group you would belong. The study has 4 groups based on the type of medications the participants will take:

Group A will not take any pills

Group B will take one softgel of fish oil supplement (1400 mg, Sundown Naturals, NY) and a second blood draw 4 hours later

Group C will take one tablet of Bayer low-dose (81 mg) aspirin and a second blood draw 4 hours later

Group D will take fish-oil supplement first, low-dose aspirin 2 hours later and a second blood draw 2 hours later

All your saliva and blood samples (baseline and at 4 hours) will be collected in a container that contains your study ID number, age, sex and the timing of blood draw (time 0 or time 4hrs). No personally identifiable information will be attached to the specimen and no one working in the laboratory will know the source.

RISKS AND DISCOMFORTS

There are no known risks of saliva collection other than the possibility of you experiencing mild discomfort due to dryness of the mouth.

Possible side effects associated with having blood drawn may involve:

- Headache
- Bruising and discoloration at the site
- Pain
- Dizziness
- Prolonged bleeding

Currently, there is no evidence that aspirin and fish-oil interact to produce any complications.

**BENEFITS TO YOU AND OTHERS** This is not a treatment study, and you are not expected to receive any direct benefits from your participation in the study. The information from this research study may lead to better treatment in the future for people who undergo surgery. **PAYMENT FOR PARTICIPATION** In order to compensate for your time and procedures you are going through, we will pay you a total sum of $30 by cash. This money will be paid at the end of second blood draw and saliva collection. No compensation will be provided if you do not come back for the second appointment. In such circumstance, you will no longer be
considered a study participant and your previously collected sample will not be used in the analysis.  **ALTERNATIVE** Your alternative is not to participate in the study.

**CONFIDENTIALITY**

Potentially identifiable information about you will consist of names, email addresses and phone numbers. The investigator, research coordinator and the residents who work on this study will have access to this information. At the end of the blood draw procedure, the blood will be transferred into a separate container and labeled with a study ID number, your age, sex and the time of sample collection (time 0 or time 4 hours). No personally identifiable information will be attached to the specimen and hence no one working with the specimen in the laboratory will know the source.

The research coordinator will store your identifiable information and link it to the study ID number. This information will be kept separately in a locked research area and only the coordinator and the PI will have access to this information. The personal identifiable information and the study code will be destroyed at the end of the study while the consent forms will be kept for a period of five years from the conclusion of the study in accordance to VCU policies.

Although results of this research may be presented at meetings or in publications, identifiable personal information pertaining to participants will not be disclosed.

**QUESTIONS**

If you have any questions, complaints, or concerns about your participation in this research, contact:

**Dr. Parthasarathy Madurantakam**

Assistant Professor  Philips Institute, Room 4130  1101 East Leigh Street, Richmond, VA 23298 Telephone: (804) 828 9353  Email: madurantakap@vcu.edu

and/or

**Ms. Kimberly Hollaway**

Research Coordinator  521 North 11th Street, Richmond, Virginia 23298 Phone: (804) 828-4553  Email: klhollaway@vcu.edu

The researcher/study staff named above is the best person(s) to call for questions about your participation in this study.

If you have general questions about your rights as a participant in this or any other research, you may contact:

Office of Research  Virginia Commonwealth University 800 East Leigh Street, Suite 3000 P.O. Box 980568  Richmond, VA 23298  Telephone: (804) 827-2157
Contact this number for general questions, concerns, or complaints about research. You may also call this number if you cannot reach the research team or if you wish to talk to someone else. General information about participation in research studies can also be found at http://www.research.vcu.edu/irb/volunteers.htm.

Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

CONSENT

I have been provided with an opportunity to read this consent form carefully. All of the questions that I wish to raise concerning this study have been answered.

By signing this consent form, I have not waived any of the legal rights or benefits, to which I otherwise would be entitled. My signature indicates that I freely consent to participate in this research study. I will receive a copy of the consent form once I have agreed to participate.

Participant Name, printed

Participant Signature Date

Name of Person Conducting Informed Consent Date Discussion / Witness

Signature of Person Conducting Informed Consent Date Discussion / Witness

Principal Investigator Signature (if different from above) Date

Approved by the VCU IRB on 5/4/2015
Appendix 5: Aspirin Regimen- Bayer® Low Dose Aspirin 81 mg insert
Appendix 6: Brand of fish oil used in the study
Appendix 7: Recruitment Flyer

Virginia Commonwealth University

Healthy Volunteers Wanted for a Research Study

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- History of fish-oil supplement (essential fatty acid) intake in the past month
- Known allergy to fish-oil components or nuts

If you are interested and want to participate in this study or if you have any questions, please contact

Kimberly Hollaway at klhollaway@vcu.edu or 804-828-4553
Dr. Madurantakam at madurantakap@vcu.edu or 804-828-9353
Vita

Dr. Danielle McCormack was born in Miami, Florida, and grew up in Port Charlotte, Florida. She received a Bachelor of Science in Biology from Florida State University in 2009 before attending Virginia Commonwealth University School of Dentistry, where she earned a Doctor of Dental Surgery in 2014. She is a member of the American Dental Association, Virginia Dental Association, and the American Academy of Periodontology. Dr. McCormack will graduate from Virginia Commonwealth University with a Master of Science in Dentistry, and a Specialty Certificate in Periodontics.