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

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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 PLASMA FOLLOWING FISH OIL AND ASPIRIN INTAKE

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Background: Unfavorable outcomes following periodontal surgeries can be attributed to impaired resolution mechanisms likely due to decreased levels of specialized pro-resolution molecules (SPM). The current study investigates if SPM substrate pools in platelet-rich plasma preparations (PRP) can be increased by essential fatty acid (EFA) and / or aspirin

supplementation. **Methods:** Nineteen healthy volunteers were randomly assigned to take i) aspirin; ii) EFA; iii) aspirin and EFA. Four hours after intake, the lipid precursor pools in PRP were quantified using combined Liquid Chromatography tandem mass spectrometry (LC-MS/MS) and the data statistically analyzed using ANCOVA. **Results:** Of the 77 metabolites screened, only FFA (18:3) showed a significant interaction effect ($p=0.019$). By itself, neither EFA ($p>0.9$) nor aspirin ($p>0.4$) showed any difference ($P>0.4$). Multiple comparisons could not identify the differences between groups. **Conclusions:** There is inadequate data to support oral supplementation of EFA and /or aspirin to increase SPM levels in PRP.

Keywords: Platelet-rich plasma, specialized pro-resolution molecules, mass spectrometry, precursor lipids, fish oil, aspirin.

Introduction

Disease Burden: Periodontitis is a chronic inflammatory disease initiated by bacterial biofilms that naturally form on the teeth that has been shown to be associated with a variety of systemic diseases including arthritis, Type II diabetes, preeclampsia, conditions associated with preterm low birth weight, and cardiovascular diseases (1). In addition to this link to the systemic diseases, chronic periodontitis by itself is the most prevalent disease affecting almost half of the US adult population (2).

Inflammation in Periodontal Disease: Current molecular basis of the pathogenesis indicates that bacteria are necessary, but not always sufficient to produce disease (3) and the tissue destruction is a result of exaggerated host inflammatory response to the biofilm. The alveolar bone destruction and clinical attachment loss associated with the progression of the diseases is associated with impaired resolution of bacteria-initiated inflammation.

Normal Evolution of Inflammatory Process: Following injury of healthy tissue, a predictable progression of physiologic events unfolds that can be divided into the phases of inflammation, proliferation, and maturation. Each phase is characterized by the sequential elaboration of distinctive cytokines by specific cells. While PMNs drive the acute inflammatory phase, myofibroblasts and macrophages control repair and regeneration that ultimately results in resolution of inflammation.

In the second stage of the inflammatory phase, leukocytes supplant platelets as the dominant cell type, attracted by chemotaxis. White blood cells (WBCs) are the predominant cells for the first 3 days after wounding; their numbers peak at approximately 48 hours. Polymorphonucleocytes (PMNs) are the first to begin bactericidal activities using inflammatory

mediators and oxygen free radical metabolites. However, normal wound healing can occur without PMNs. Another leukocyte, the helper T cell, elaborates interleukin-2 (IL–2). IL-2 promotes further T cell proliferation to augment the immunogenic response to injury.

As PMN leukocytes begin to wane after 24-36 hours, circulating monocytes enter the wound and mature into tissue macrophages. These cells debride the wound on the microscopic level and produce a wide variety of important substances, such as IL-1 and basic fibroblast growth factor (bFGF). IL-1 stimulates the proliferation of inflammatory cells and promotes angiogenesis through endothelial cell replication. bFGF is a chemotactic and mitogenic factor for fibroblasts and endothelial cells. Unlike PMNs, macrophage depletion severely impairs wound healing, as debridement, fibroblast proliferation, and angiogenesis all diminish.

Toward the end of the inflammatory cycle, the evolving milieu of eicosanoids in the wound interact with the cell types present, resulting in fibroblast synthesis of collagen and ground substance (from increased ratio of PGF2α to PGE2). Additionally, the macrophage-derived growth factors are now at optimal levels, strongly influencing the influx of fibroblasts and then keratinocytes and endothelial cells into the wound. As mononuclear cells continue to replace WBCs and macrophages, the proliferative phase begins.

While complete resolution of an acute inflammatory response and return to homeostasis are key processes in maintaining good health, sustained, non-resolving inflammation can lead to fibrosis and loss of function (Figure 1). The key event in this process is the triggering of apoptosis in leucocytes and their subsequent clearance from the site of inflammation by macrophages. It has been shown that continued presence of PMNs is a major cause of non-resolution of inflammation (4).

Injury to vascular tissue initiates the extrinsic coagulation cascade that results in the formation of a fibrin plug to achieve hemostasis. This plug acts as a lattice for the aggregation of platelets, the most common and "signature" cell type of the early inflammatory phase.

Platelets elaborate a number of proinflammatory substances, including AA metabolites such as adenosine diphosphate, tissue growth factor beta (TGF-ß), and platelet-derived growth factors (PDGF). These growth factors act on surrounding cells and stimulate chemotaxis of neutrophils, monocytes, and fibroblasts to the area of injury.

Injured tissues, through activated phospholipase A, simultaneously catalyze arachidonic acids to produce vasoactive prostaglandins and thromboxane, collectively known as eicosanoids. Eicosanoids mediate activity influencing platelet plug formation, vascular permeability, and cellular chemotaxis to influence wound healing.

Arachidonic acid Signaling:

Originally regarded as just membrane constituents and energy storing molecules, lipids are now recognized as potent signaling molecules that regulate a multitude of cellular responses via receptor-mediated pathways, including cell growth and death, and inflammation/infection. AA, a 20-carbon fatty acid is the main eicosanoid precursor and is a basic constituent of all cells. While it is not freely available, AA is released from membrane phospholipid stores through the activity of several phospholipase enzymes (predominantly PLA2), which are activated by various exogenous stimuli including injury. Once in the cytosol, AA can be metabolized via three principal pathways to form an important family of oxygenated products, collectively termed eicosanoids that are released from the source cell and act at nanomolar concentrations in an autocrine/paracrine manner on target cells. Included in this family are prostaglandins (PGs) and

thromboxane (collectively termed prostanoids), formed by cyclooxygenase (COX); leukotrienes (LTs) and lipoxins (LXs) by lipoxygenases (LOX) (5) (Figure 2).

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 (e.g., 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 (6). 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.

Omega-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 (7). 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 (Figure 4). The anti-inflammatory actions of ω-3 fatty acids are, in part, related to reduced leukocyte-derived cytokine formation and modulation of eicosanoid synthesis (8). 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 (10).

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 (11).

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 (12).

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 is 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 (13). Autologous platelet concentrates (APC) are derived from patients' own blood and are processed to enriched the amount of platelets to at least 1,000,000 per micro-liter. The major technique in preparing platelet-rich plasma includes centrifuging whole blood to concentrate platelets. Several different systems are available for use in preparation of PRP. These have either one or two spin cycles, operate at different speeds, may or may not include leukocytes with the platelets, and have different means of activating the platelets. All techniques separates autologous blood into platelets, leukocytes, erythrocytes, and plasma.

Such a high concentration of platelets is expected to be a potent source of endogenous growth factors and cytokines that stimulate healing of bone and soft tissues (14). It was introduced in dentistry by Whitmann and colleagues, who suggested it would enhance surgical healing through the activation of platelets and release of growth factors The efficacy of APC has be shown in a various clinical studies (15-20).

Although there are different recipes for generating APC, we are interested in an FDA approved technologies that are being used routinely in clinical practice: PRP (platelet-rich plasma, Nuo Therapeutics, MD).

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 Appendix A.

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, immunosuppresive 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 Figure 5). After 4 hours after drug administration, the second blood draw was done. Personnel adequately trained in phlebotomy performed the blood draws. At each time, 40 ml of blood was collected in heparinized tubes and centrifuged using Aurix PRP system (Nuo Therapeutics) (Figure 6). This was unique in that the procedure is a soft spin for 1 minute after which the platelet rich plasma fraction was aspirated and mixed with calcium chloride and ascorbic acid following manufacturer's instructions (Figures 7 and 8). After PRP processing, the samples were transferred to labeled tubes with participant's ID number, age, and sex, to protect confidentiality. The labeled specimens were immediately stored at -80oC 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. 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 40^{o} Celsius 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:

FFA(16:1)	FFA(16:2)	FFA(24:1)
FFA(18:1)	FFA(18:2)	FFA(18:3)
FFA(20:0)	FFA(20:1)	FFA(20:3)
FFA(20:4)	FFA(20:5)	FFA(22:4)
FFA(22:5)	FFA(22:6)	FFA(24:0)
LPC(14:0) +AcO	LPC(16:1) +AcO	LPC(16:0) +AcO
LPC(18:0) +AcO	LPC(18:1) +AcO	LPC(18:2) +AcO
LPC(18:3) +AcO	LPC(20:0) +AcO	LPC(20:1) +AcO
LPC(20:2) +AcO	LPC(20:3) +AcO	LPC(20:4) +AcO
LPC(20:5) +AcO	LPC(22:4) +AcO	LPC(22:5) +AcO

LPC(22:6) +AcO	LPE(16:0)	LPE(16:1)
LPE(18:0)	LPE(18:1)	LPE(18:2)
LPE(18:3)	LPE(20:0)	LPE(20:1)
LPE(20:2)	LPE(20:3)	LPE(20:4)
LPE(20:5)	LPE(22:4)	LPE(22:5)
LPE(22:6)	LPG(14:0)	LPG(18:1)
LPG(18:3)	LPG(20:1)	LPG(20:3)
LPG(20:4)	LPG(22:4)	LPG(22:5)
LPG(22:6)	LPI(16:0)	LPI(18:0)
LPI(18:1)	LPI(18:2)	LPI(20:3)
LPI(20:4)	LPS(18:0)	PE(18:2/20:1)
PE(P-16:0/16:0)	PS(18:2/22:6)	SM(14:0)
SM(16:0)	SM(18:0)	SM(18:1)
SM(20:0)	SM(20:1)	SM(22:0)
SM(22:1)	SM(24:0)	SM(24:1)
SM(26:0)	SM(26:1)	

where:

SM=Sphignomyelin

LPC=Lysophosphotidylcholine

LPE=Lysophosphatidylethanolamine

LPG=Lysophosphatidylglycerol

LPI=Lysophosphotidylinositol

LPS-Lysopphosphatidylserine

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 (EFA=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

Sixty participants were screened and 60 found eligible. For this study, 19 of the participant samples were available for analysis. There were 4 participants in group A, 6 in group B, 4 in group C, 5 in group D.

Group differences

There were 77 SPMs screened in the 19 PRP participants. There was 1 SPM with a significant interaction, 6 with an Aspirin effect, 0 with an EFA effect, and 70 with no apparent difference between the groups (Table 1).

Interaction effect

Only FFA(18:3) showed a significant interaction effect (P=0.019). With this metabolomics there was no EFA difference (P>0.9) nor an Aspirin difference (P>0.4). However, the multiple comparison procedure was unable to identify which groups were different (Table 2).

Aspirin effect

There was an Aspirin-related difference in 6 metabolomics. These are summarized in Table 3. In all cases, the no Aspirin groups had a higher level than the Aspirin groups. For instance, in FFA(22:4) the difference was -1.76 (P=0.024).

EFA effect

No EFA-related effect was observed.

No effect evident

There was no difference between the groups found in these lipid precursors: FFA(16:1), FFA(16:2), FFA(18:1), FFA(18:2), FFA(20:0), FFA(20:1), FFA(20:3), FFA(20:4), FFA(20:5),

FFA(22:5), FFA(22:6), FFA(24:0), LPC(14:0) +AcO, LPC(16:0) +AcO, LPC(16:1) +AcO, LPC(18:0) +AcO, LPC(18:1) +AcO, LPC(18:2) +AcO, LPC(18:3) +AcO, LPC(20:0) +AcO, LPC(20:1) +AcO, LPC(20:2) +AcO, LPC(20:3) +AcO, LPC(20:4) +AcO, LPC(20:5) +AcO, LPC(22:4) +AcO, LPC(22:5) +AcO, LPC(22:6) +AcO, LPE(16:0), LPE(16:1), LPE(18:0), LPE(18:1), LPE(18:2), LPE(18:3), LPE(20:0), LPE(20:1), LPE(20:2), LPE(20:3), LPE(20:4), LPE(20:5), LPE(22:5), LPG(18:1), LPG(20:1), LPG(20:3), LPG(20:4), LPG(22:4), LPG(22:5), LPG(22:6), LPI(16:0), LPI(18:0), LPI(18:1), LPI(18:2), LPI(20:3), LPI(20:4), LPS(18:0), PE(18:2/20:1), PE(P-16:0/16:0), PS(18:2/22:6), SM(14:0), SM(16:0), SM(18:0), SM(18:1), SM(20:0), SM(20:1), SM(22:0), SM(22:1), SM(24:0), SM(24:1), SM(26:0), SM(26:1).

DISCUSSION

Wound healing as a result of tissue injury is an active process whereas the body must mount an inflammatory response in order to recruit cells with the ability to repair the site, yet also have the ability to return the body to homeostasis. Failure for this to occur can lead to dysregulated healing events where chronic inflammation persists. Chronic inflammation is known to underlie certain pathologic disease conditions including rheumatoid arthritis, diabetes, and periodontitis.

In periodontal surgery, procedures such as bone augmentation and soft tissue grafts create acute tissue trauma, triggering an inflammatory response. Immediate management of the wound site can improve both the healing process and patient's postoperative experience. Better knowledge of the active process of the resolution of inflammation and healing will help us manage the acute inflammation which occurs after surgery.

Since the discovery of SPMs, they have been at the forefront of research in medicine. SPMs are known to increase in both blood plasma, and platelet concentrates, with the intake of EFA and aspirin (9). Their activity at the wound site is linked to an increase in phagocytic activity, and limitation of polymorphonuclear (PMN) leukocyte recruitment and PMN related tissue damage, which aid in the body's resolution of inflammation, and return to homeostasis. What is less understood are the precursors along these pathways, what impact aspirin and omega 3 may have on them, and whether manipulation of these precursors would provide a longer acting beneficial effect on wound healing when delivered directly to the wound site via PRP.

In this novel experiment, we examined the effects of aspirin and fish oil on levels of lipid precursors to SPMs, in platelet rich plasma. 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.

The data presented in this study includes a population of 19 healthy adult participants. 77 lipid precursor molecules were quantified by analyzing platelet rich plasma, by means of liquid chromatography mass spectrometry, which is used to identify lipid mediators and SPMs in blood plasma and serum. We found no significant EFA effect, one interaction effect when both aspirin and EFA were administered with regard to one lipid precursor, and a significant aspirin effect on 6 lipid precursors.

Although EFA has been found to increase the production of SPMs with a 1400mg dose, it had no significant effect on the lipid precursor pools analyzed in this study population. While we anticipate, based on previous findings, that an increase in SPMs likely occurred, it is probable that their lipid precursors did not have a significant change due to the dose and time examined. It is anticipated that a higher dose administered over a longer timeframe would show a change in these precursor pools.

Aspirin showed a significant effect in 6 lipidomics; FFA 22:4, FFA 22:1, LPE 22:4, LPE 22:6, LPG 14:0, and LPG 18:3. All were significantly decreased compared to baseline values after the intake of aspirin. Analysis of these findings indicate both potentially positive and negative effects depending on the pathway affected.

FFA 22:4 and LPE 22:4 are considered Eicosanoids (Figure 9). The Eicosanoid pathway has several physiological as well as some pathological effects dependent on which path the molecule takes as it competes for enzymes. The effects produced by Eicosanoids range from inhibition of the immune response including fever, allergy, and inflammation, to an increase in vascular permeability, influence on cell signaling, and impact on the systems controlling blood

pressure. FFA 22:4 and LPE 22:4 are lipid molecules following the omega 6 pathway, and are classified as docosatetraenoic acids. These lipid molecules are precursors to arachadonic acid 20:4, which leads to the production of a group of inflammatory molecules including PGD2, PGE2, PGF2, PGI2, TXA4, LTB4, LTC4, LTD4, LTE4 (Figure 10). The effect found in this study indicated that FFA 22:4 and LPE 22:4 were both significantly decreased after the administration of 81mg aspirin, compared to baseline values. This shows that there were less inflammatory lipid precursors present in the PRP four hours after aspirin administration, which is a positive finding. This suggests that a single dose of aspirin may have the ability to alter the pro-inflammatory lipid pathways, and could potentially improve the beneficial clinical effects of PRP at the wound site.

LPE 22:6 was also significantly decreased with the intake of aspirin. This lipid precursor belongs to the omega 3 family, docosahexaenoic acid (DHA). It acts as a precursor to the production of resolvins and other SPMs.

LPG 18:3 is found in the Eicosanoid pathway in both the omega-3 and omega-6 families as linolenic acid. In the omega-3 pathway, alpha linolenic acid is a precursor to SPMs, and provide a beneficial effect at the wound site. In the omega-6 pathway, it exists as gamma linolenic acid, which depending on the enzymes present, could lead to either pro-inflammatory molecules, or resolving molecules. Our analysis could not differentiate which pathway was involved. Further analysis of the same samples is necessary to establish the aspirin effect on LPG 18:3

The remaining significant aspirin effects were established with FFA 22:1 and LPG 14:0, both of which were not associated with the SPM pathways.

CONCLUSIONS

There were 77 lipid precursors screened in the 19 PRP participants. One (FFA 18:3) showed a significant interaction effect with EFA and aspirin, 6 (FFA 22:4, FFA 24:1, LPE 22:4, LPE 22:6, LPG 14:0, LPG 18:3) showed a significant Aspirin effect, 0 had an EFA effect, and 70 with no apparent difference between the groups (Table 1). The aspirin effect significantly lowered 2 pro-inflammatory lipid precursors along the arachadonic acid pathway, significantly decreased 1 lipid precursor in the DHA pathway for the production of SPMs, and significantly decreased lipid precursors in pathways not examined in this study (Figure 11).

The decrease in FFA 22:4 and LPE 22:4 along the Eicosanoid pathway after aspirin intake has never been reported before in literature. It may indicate the potential for storage of anti-inflammatory lipids, which could prove beneficial when delivered directly to a wound site. Additional studies using flux are necessary to understand the exact mechanism.

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Tables

Table 1. ANCOVA results

Lipid	Aspirin	EFA	EFA*Aspirin	Pre
FFA(16:1)	0.2741	0.8750	0.4837	0.5232
FFA(16:2)	0.4828	0.5352	0.1726	0.9169
FFA(18:1)	0.6542	0.8772	0.5452	0.5158
FFA(18:2)	0.9036	0.3524	0.6694	0.1768
FFA(18:3)	0.4046	0.9382	0.0192	0.0009
FFA(20:0)	0.9448	0.9927	0.9107	0.7822
FFA(20:1)	0.7260	0.7178	0.7905	0.0000
FFA(20:3)	0.6457	0.5138	0.1944	0.0003
FFA(20:4)	0.7651	0.6648	0.9918	0.0340
FFA(20:5)	0.9189	0.0748	0.3669	0.0193
FFA(22:4)	0.0239	0.1069	0.9604	0.0013
FFA(22:5)	0.3841	0.6704	0.1161	0.0812
FFA(22:6)	0.9244	0.8103	0.5083	0.2904
FFA(24:0)	0.2107	0.5694	0.2093	0.0324
FFA(24:1)	0.0316	0.4947	0.9779	0.0835
LPC(14:0) +AcO	0.1797	0.7624	0.7013	0.0005
LPC(16:0) +AcO	0.4191	0.9720	0.6622	0.0146
LPC(16:1) +AcO	0.0815	0.6643	0.6097	0.0605
LPC(18:0) +AcO	0.5049	0.8945	0.3742	0.0010
LPC(18:1) +AcO	0.1556	0.8450	0.9647	0.0005
LPC(18:2) +AcO	0.6149	0.9794	0.7394	0.0237
LPC(18:3) +AcO	0.6488	0.9448	0.8138	0.0921
LPC(20:0) +AcO	0.3974	0.4406	0.1158	0.0061
LPC(20:1) +AcO	0.4651	0.6652	0.4413	0.0005
LPC(20:2) +AcO	0.3255	0.8214	0.7532	0.0014
LPC(20:3) +AcO	0.1869	0.9817	0.6862	0.0003
LPC(20:4) +AcO	0.5228	0.9461	0.9053	0.0000
LPC(20:5) +AcO	0.8977	0.6722	0.2875	0.0000
LPC(22:4) +AcO	0.4128	0.5465	0.6387	0.0000
LPC(22:5) +AcO	0.9904	0.7625	0.9632	0.0024
LPC(22:6) +AcO	0.5431	0.5211	0.9001	0.0001
LPE(16:0)	0.3264	0.4743	0.9943	0.0017
LPE(16:1)	0.0846	0.4208	0.2554	0.0066
LPE(18:0)	0.3448	0.9813	0.5346	0.9986
LPE(18:1)	0.5837	0.9580	0.7282	0.1016
LPE(18:2)	0.6302	0.8545	0.4757	0.7568
LPE(18:3)	0.6011	0.7954	0.7323	0.9697

Lipid	Aspirin	EFA	EFA*Aspirin	Pre
LPE(20:0)	0.3612	0.2652	0.1598	0.1459
LPE(20:1)	0.3531	0.4584	0.5329	0.9008
LPE(20:2)	0.2173	0.3068	0.3653	0.0607
LPE(20:3)	0.0853	0.3173	0.9502	0.2665
LPE(20:4)	0.6830	0.7577	0.9971	0.3740
LPE(20:5)	0.2342	0.9895	0.1916	0.2502
LPE(22:4)	0.0290	0.2471	0.2699	0.0002
LPE(22:5)	0.1809	0.5940	0.5642	0.0173
LPE(22:6)	0.0317	0.9657	0.9147	0.0065
LPG(14:0)	0.0016	0.5852	0.5783	0.0006
LPG(18:1)	0.2968	0.9243	0.8990	0.1895
LPG(18:3)	0.0147	0.2093	0.6615	0.0239
LPG(20:1)	0.5030	0.7315	0.5106	0.5234
LPG(20:3)	0.2290	0.7611	0.3150	0.0000
LPG(20:4)	0.4327	0.0692	0.7333	0.7578
LPG(22:4)	0.2145	0.2642	0.4268	0.8392
LPG(22:5)	0.9277	0.5618	0.6018	0.2460
LPG(22:6)	0.1611	0.8231	0.5060	0.6327
LPI(16:0)	0.1476	0.4716	0.2472	0.6713
LPI(18:0)	0.2423	0.2001	0.7050	0.5871
LPI(18:1)	0.3465	0.4029	0.1726	0.6871
LPI(18:2)	0.8090	0.1554	0.1172	0.4930
LPI(20:3)	0.2537	0.1481	0.0807	0.3021
LPI(20:4)	0.8839	0.0948	0.1660	0.5489
LPS(18:0)	0.6652	0.1706	0.9326	0.0042
PE(18:2/20:1)	0.4848	0.4609	0.1968	0.2271
PE(P-16:0/16:0)	0.5258	0.4904	0.9418	0.5678
PS(18:2/22:6)	0.8976	0.8691	0.9328	0.6368
SM(14:0)	0.2439	0.7025	0.5480	0.0000
SM(16:0)	0.5925	0.6788	0.4774	0.0004
SM(18:0)	0.1883	0.7402	0.4524	0.0000
SM(18:1)	0.1854	0.6193	0.5283	0.0000
SM(20:0)	0.2691	0.8538	0.4408	0.0003
SM(20:1)	0.4557	0.5543	0.5394	0.0000
SM(22:0)	0.2453	0.5186	0.4027	0.0045
SM(22:1)	0.1461	0.7441	0.5753	0.0010
SM(24:0)	0.4200	0.5291	0.4747	0.0040
SM(24:1)	0.2971	0.4216	0.4287	0.0001
SM(26:0)	0.4895	0.8924	0.5890	0.0107
SM(26:1)	0.2290	0.8124	0.4225	0.0001

Table 2. Four groups

Label	Effects	EFA	Aspirin	Mean
FFA(18:3)	EFA P=0.938	No	No	54.44[a]
	Aspirin P=0.405	Yes	Yes	52.68[a]
	EFA*Aspirin P=0.019	Yes	No	49.72[a]
		No	Yes	47.68[a]

Table 3. Aspirin differences

Label	Difference	Means
FFA(22:4)	-1.764 (-3.254 to -0.274)	9.719 no Aspirin
	P=0.024	7.955 Aspirin
		9.529 Baseline
FFA(24:1)	-6.629 (-12.576 to -0.682)	30.237 no Aspirin
	P=0.032	23.608 Aspirin
		26.113 Baseline
LPE(22:4)	-1.644 (-3.092 to -0.196)	3.649 no Aspirin
	P=0.029	2.005 Aspirin
		2.614 Baseline
LPE(22:6)	-1.024 (-1.944 to -0.104)	2.915 no Aspirin
	P=0.032	1.891 Aspirin
		1.754 Baseline
LPG(14:0)	-0.057 (-0.088 to -0.026)	0.137 no Aspirin
	P=0.002	0.080 Aspirin
		0.124 Baseline
LPG(18:3)	-0.052 (-0.091 to -0.012)	0.126 no Aspirin
	P=0.015	0.074 Aspirin
		0.105 Baseline

Figures

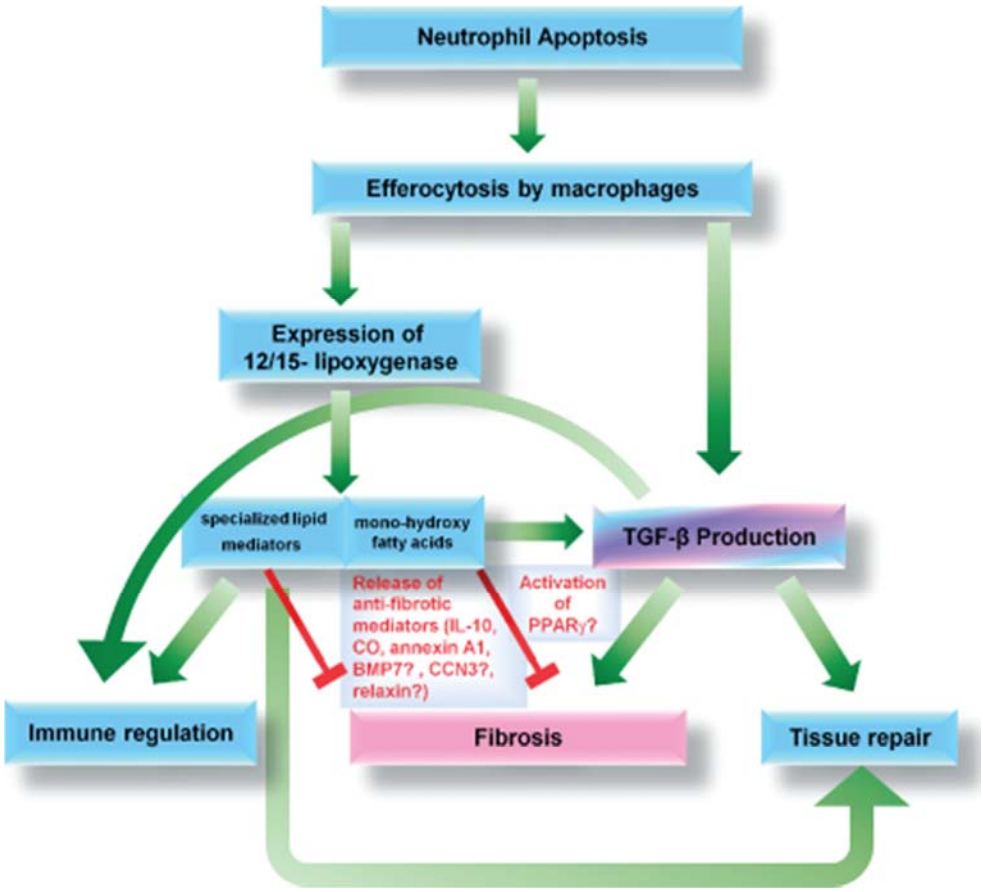

Figure 1. Platelets and Acute Inflammation

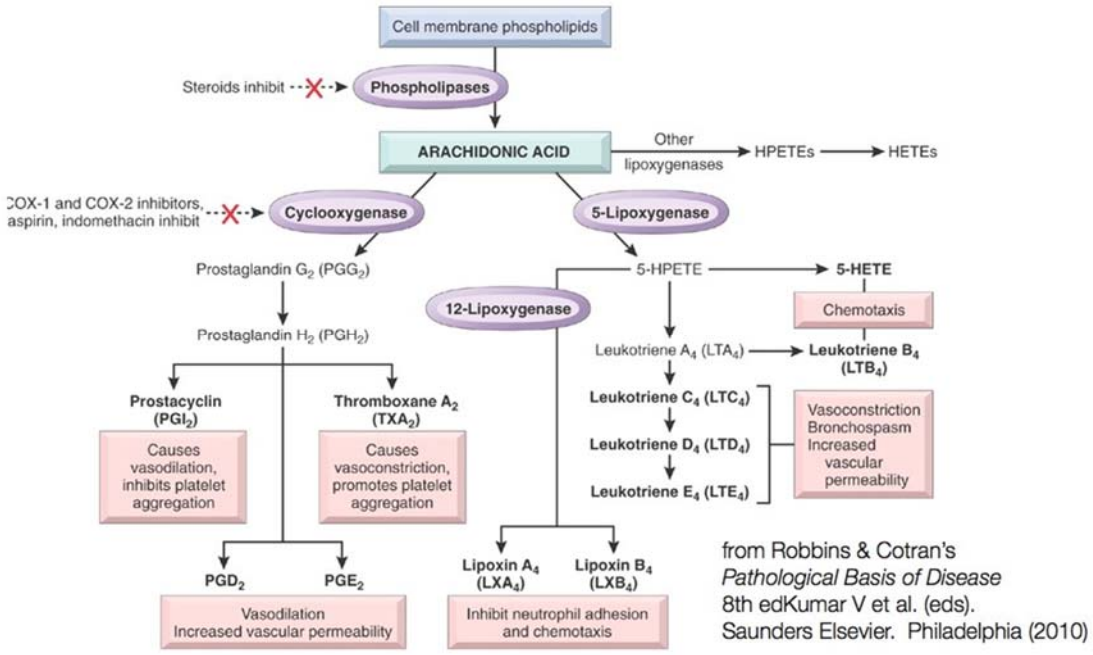

Figure 2. Specialized Pro-resolving Mediators- Lipoxins and Resolvins

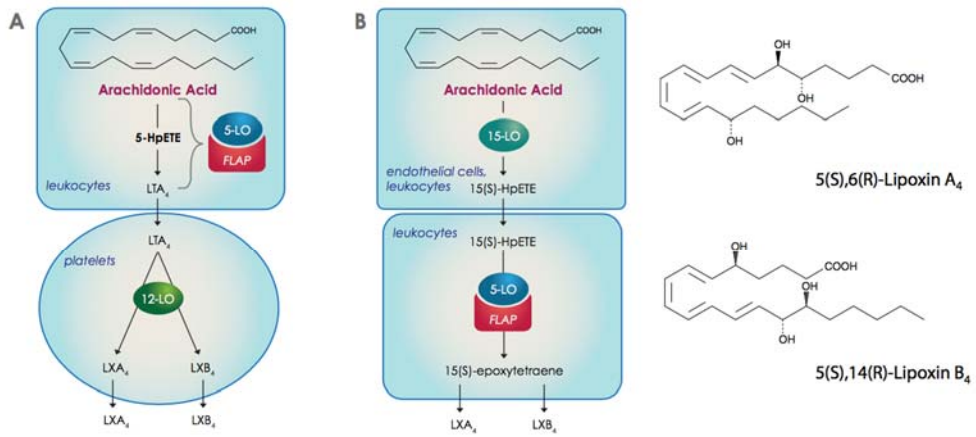

Figure 1. Lipoxin biosynthesis and structures. The synthesis of LXA_4 and LXB_4 involves (A) leukocyte-platelet or (B) endothelial cell/leukocyte-leukocyte cooperativity. (C). LXA_4 and LXB_4 are trihydroxy derivatives of AA.

Figure 3. Lipoxin biosynthesis and structure

Source: https://www.caymanchem.com/news/lipoxins-and-resolvins

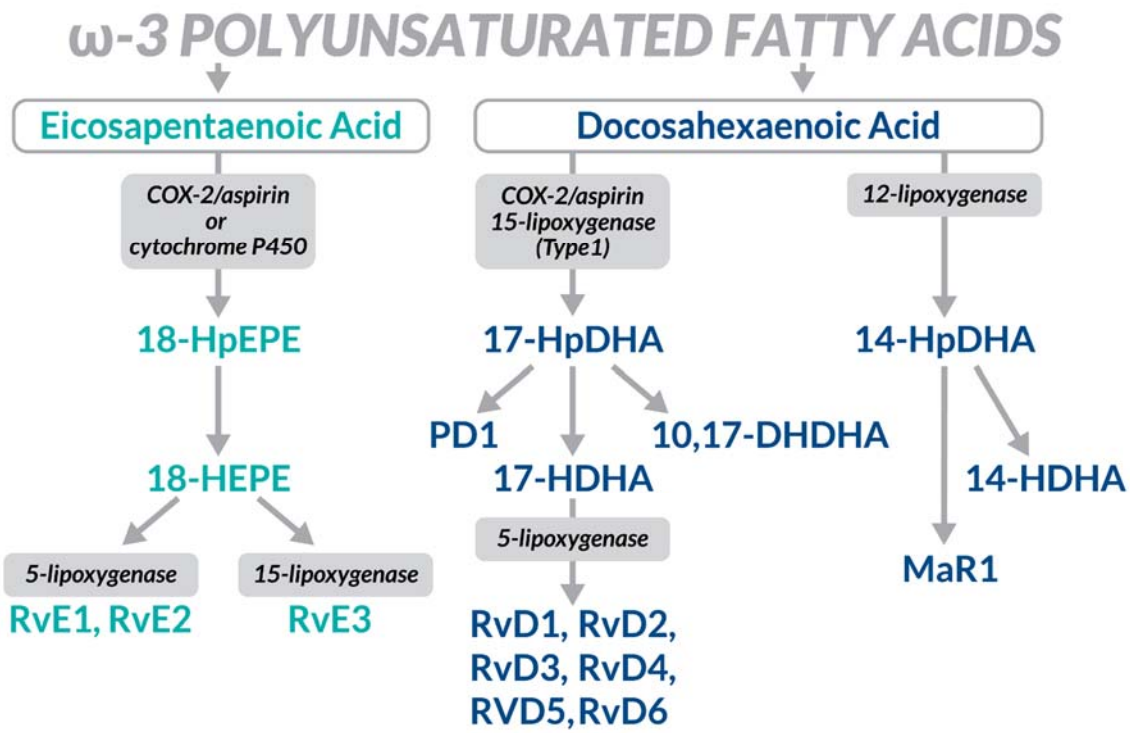

Figure 4. ω-3 Polyunstaurated Fatty Acids

Source: https://www.caymanchem.com/news/measurement-of-lipid-mediators-of-resolution-of-inflammation.

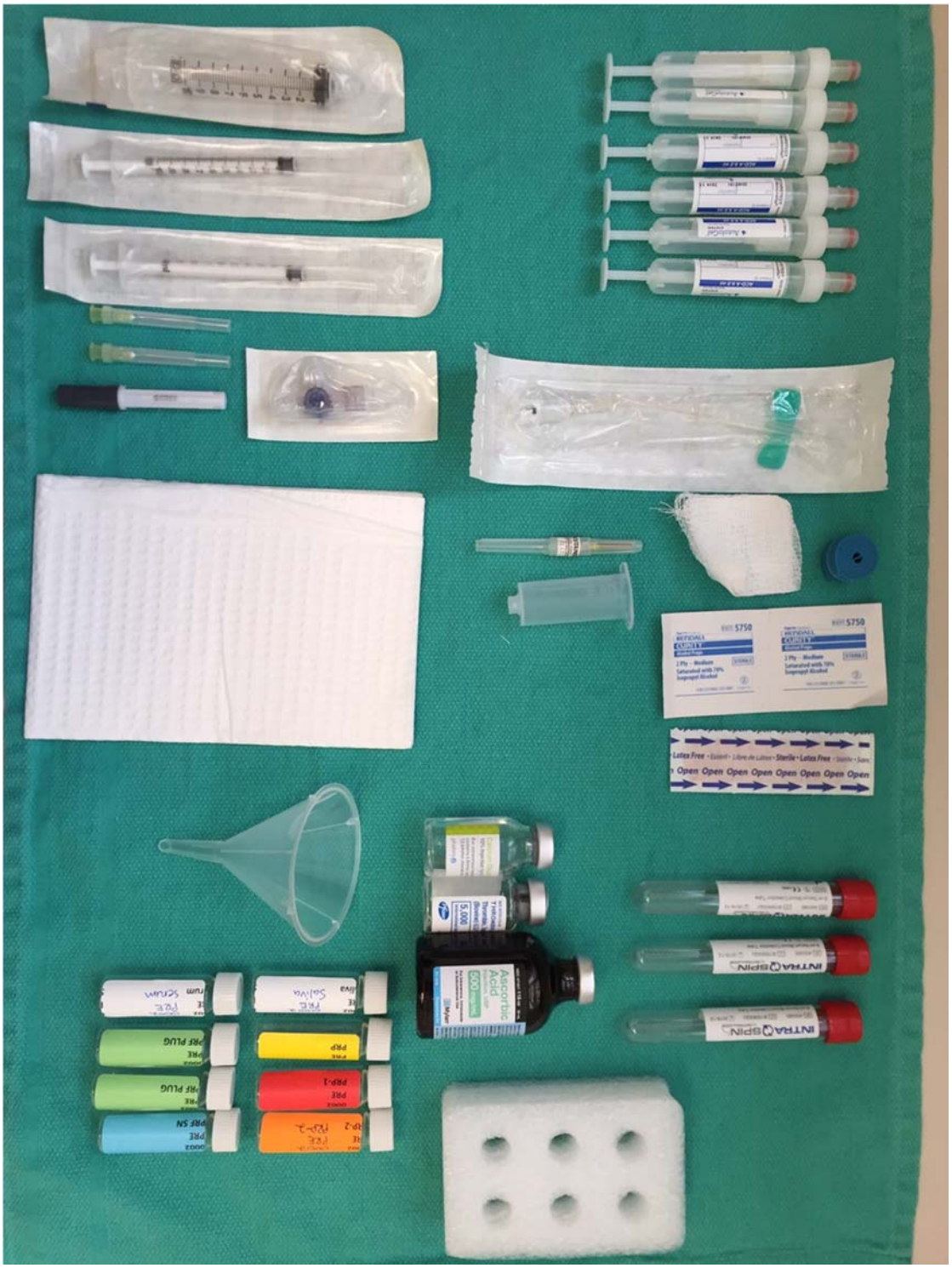

Figure 5. Armamentarium for PRP preparation

Figure 6. Aurix Centrifuge

Source: www.autologel.com

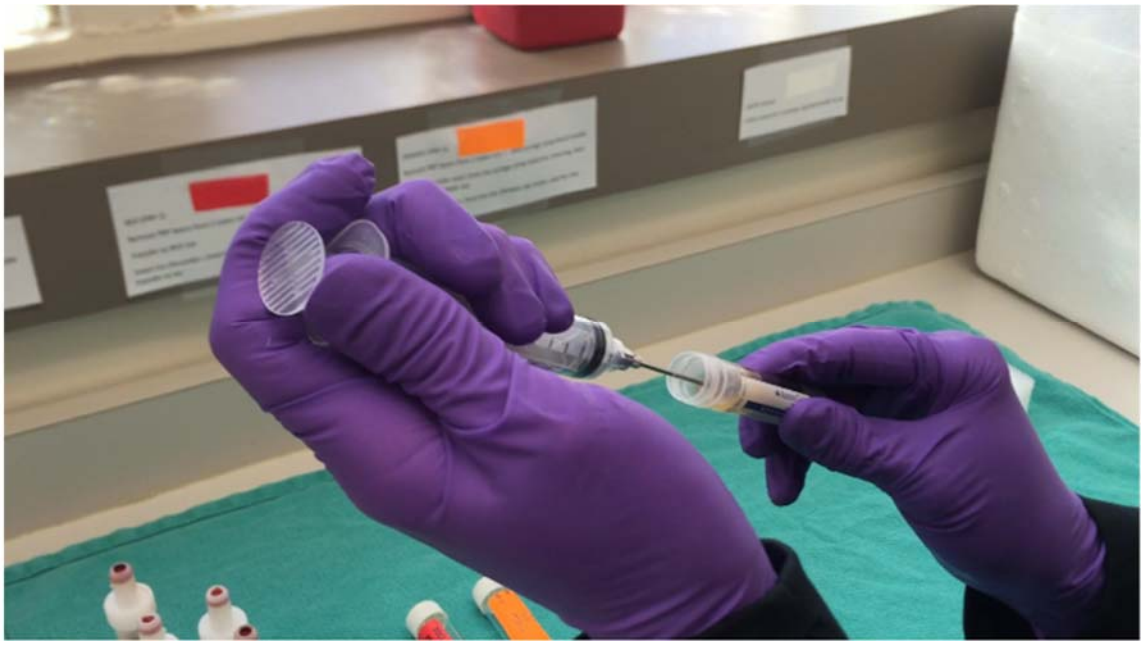

Figure 7. Aspiration of PRP after centrifugation

Figure 8. Addition of Ascorbic Acid to PRP

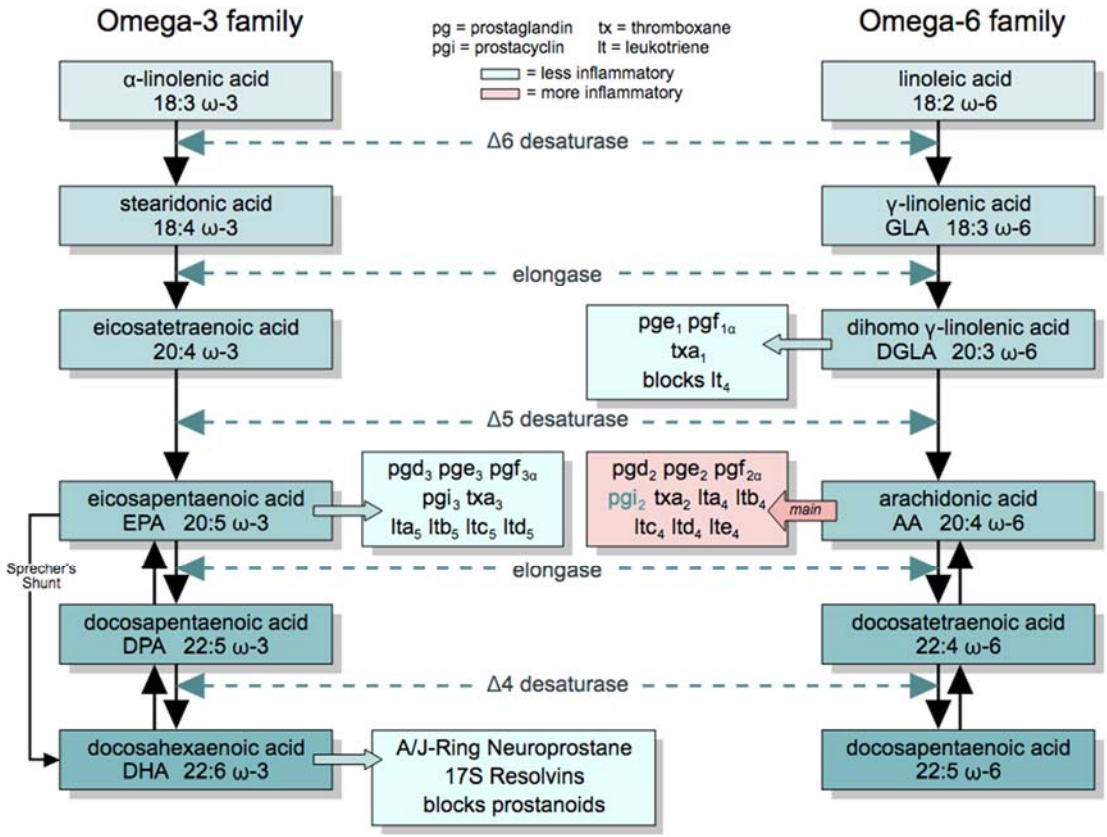

Figure 9. Eicosanoid Signaling Pathways

Source: https://en.wikipedia.org/wiki/Eicosanoid

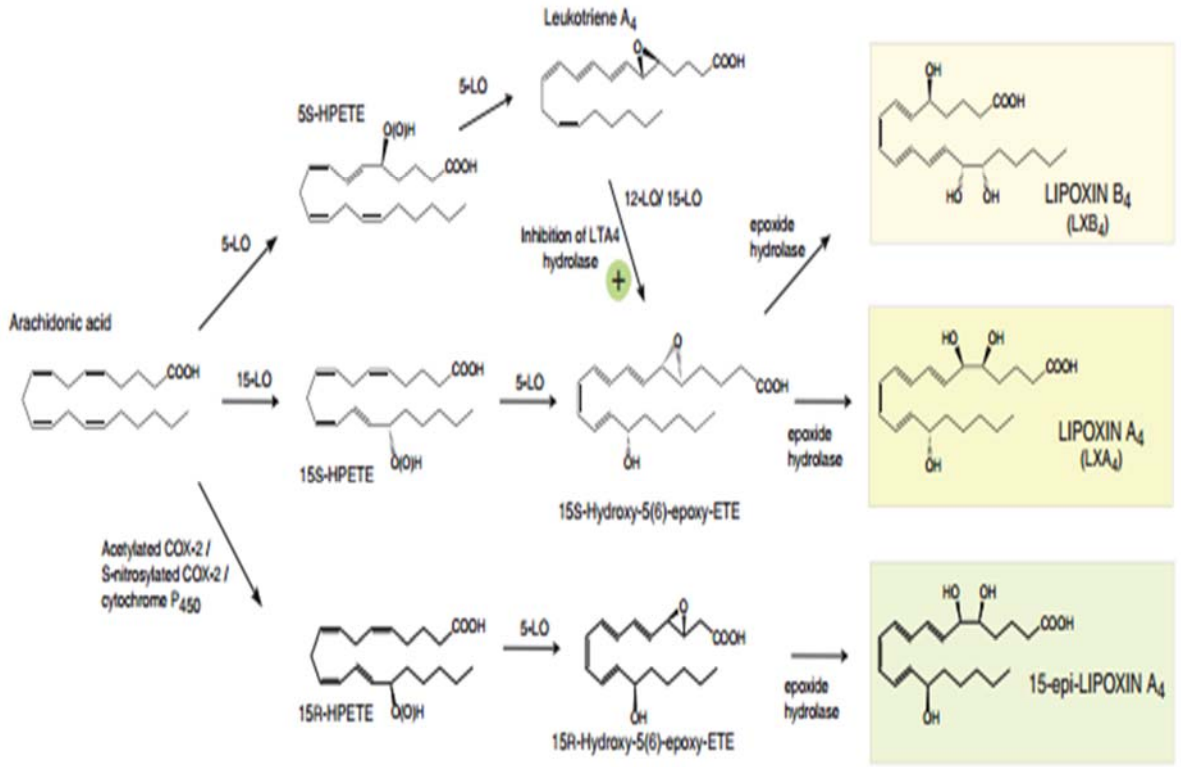

Figure 10. Biosynthesis of lipoxin and aspirin-triggered lipoxins

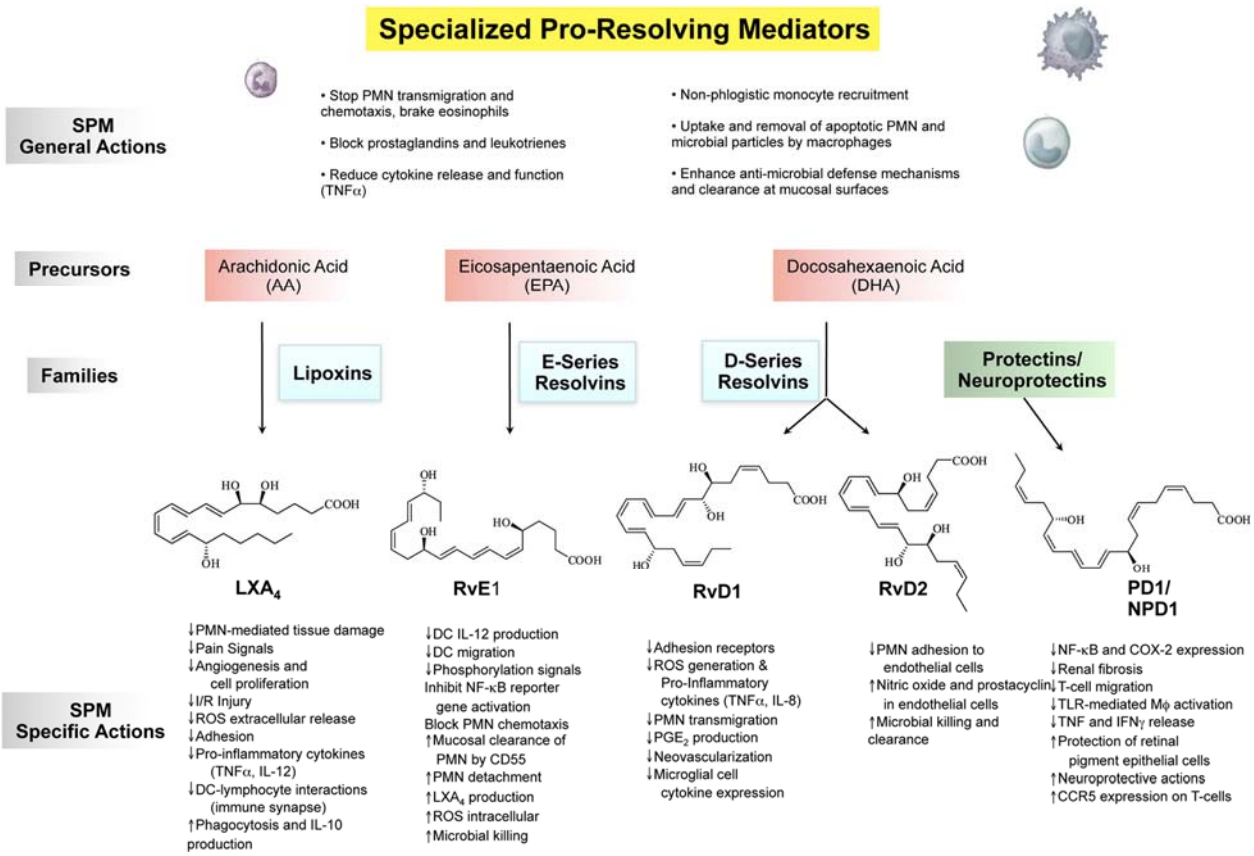

Figure 11. Specialized pro-resolving mediators

Source: http://ajp.amjpathol.org/cms/attachment/445087/3098691/gr3_lrg.jpg

Appendix

Appendix A: Research Protocol for Preparation of PRP

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 and 6 mL into each PRP 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. Place 2 of the PRP tubes labeled whole blood into the freezer for storage
4. Place the remaining 4 PRP tubes into the PRP centrifuge and centrifuge for 1 minute
5. Remove the PRP tubes from the centrifuge and use a 5 mL syringe with a blood needle to take the top PRP layer from two of the PRP tubes. Repeat for the other 2 PRP tubes.
6. Place a stopcock on 1 of the 5 mL syringes and attach the ascorbic acid syringe into the stopcock to mix with the PRP. Use the chart to determine how much ascorbic acid is needed
7. Transfer the PRP and ascorbic acid into a vial labeled AA + Ca + T
8. Transfer the PRP from the remaining 5 mL syringe into the vial labeled Ca + T
9. Release the calcium and thrombin mixture in each of the 1 mL syringes into each vial. Use the chart to determine how much calcium and thrombin mixture is needed.
10. Close the vials and store the samples in the freezer
11. Remove the 2 PRF tubes from the PRF centrifuge
12. Place them onto a clean tray and bring out the Xpression kit which should include a tray, weight, small dish, scalpel, and tweezers.
13. 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.
14. Use a scalpel to remove the red blood cells.
15. Place the weight on top of the PRF layers for 30 seconds.
16. 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.
17. Store all 3 samples into the freezer
18. Clean and disinfect the area.
19. Repeat the protocol when the patient arrives 4-6 hours later.

Appendix B: Study key for preparation of PRP before medication taken, and 4 hours after medication taken.

Color code for preparation tubes:

1. WHITE SALIVA
2. YELLOW PRP: PRP layer extracted after centrifuging
3. RED PRP-1: PRP layer w/ Calcium + Thrombin
4. ORANGE PRP-2: PRP layer w/ Calcium + Thrombin + Ascorbic Acid
5. WHITE SERUM: Serum collected after blood clotted w/o centrifuging
6. 2 GREEN PRF PLUG: PRF plug collected after centrifuging and excision
7. BLUE PRF SN: Supernatant collected after PRF plug was made

Appendix C: 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 D: Aspirin Regimen- Bayer® Low Dose Aspirin 81 mg insert

Drug Facts

Active ingredient (in each tablet) — **Purpose**

Aspirin 81 mg (NSAID)* ..... Pain reliever

*nonsteroidal anti-inflammatory drug

Uses

- for the temporary relief of minor aches and pains or as recommended by your doctor. **Because of its delayed action, this product will not provide fast relief of headaches or other symptoms needing immediate relief.**
- ask your doctor about other uses for Bayer Safety Coated 81 mg Aspirin

Warnings

Reye's syndrome: Children and teenagers who have or are recovering from chicken pox or flu-like symptoms should not use this product. When using this product, if changes in behavior with nausea and vomiting occur, consult a doctor because these symptoms could be an early sign of Reye's syndrome, a rare but serious illness.

Allergy alert: Aspirin may cause a severe allergic reaction which may include:

- hives • facial swelling • asthma (wheezing) • shock

Stomach bleeding warning: This product contains an NSAID, which may cause severe stomach bleeding. The chance is higher if you

- are age 60 or older
- have had stomach ulcers or bleeding problems
- take a blood thinning (anticoagulant) or steroid drug
- take other drugs containing prescription or nonprescription NSAIDs (aspirin, ibuprofen, naproxen, or others)
- have 3 or more alcoholic drinks every day while using this product
- take more or for a longer time than directed

Do not use if you are allergic to aspirin or any other pain reliever/fever reducer

Ask a doctor before use if

- stomach bleeding warning applies to you
- you have a history of stomach problems, such as heartburn
- you have high blood pressure, heart disease, liver cirrhosis, or kidney disease
- you are taking a diuretic
- you have asthma

Ask a doctor or pharmacist before use if you are taking a prescription drug for

- gout • diabetes • arthritis

Stop use and ask a doctor if

- an allergic reaction occurs. Seek medical help right away.
- you experience any of the following signs of stomach bleeding:
  - feel faint • vomit blood • have bloody or black stools
  - have stomach pain that does not get better
- pain gets worse or lasts more than 10 days
- redness or swelling is present
- new symptoms occur
- ringing in the ears or a loss of hearing occurs

If pregnant or breast-feeding, ask a health professional before use. **It is especially important not to use aspirin during the last 3 months of pregnancy unless definitely directed to do so by a doctor because it may cause problems in the unborn child or complications during delivery.**

Keep out of reach of children. In case of overdose, get medical help or contact a Poison Control Center right away.

Directions

- drink a full glass of water with each dose
- adults and children 12 years and over: take 4 to 8 tablets every 4 hours not to exceed 48 tablets in 24 hours unless directed by a doctor
- children under 12 years: consult a doctor

Other information

- save carton for full directions and warnings
- store at room temperature

Inactive ingredients black iron oxide, brown iron oxide, carnauba wax, corn starch, D&C yellow #10 aluminum lake, FD&C yellow #6 aluminum lake, hypromellose, methacrylic acid copolymer type C, polysorbate 80, powdered cellulose, propylene glycol, shellac, sodium lauryl sulfate, triacetin, triethyl citrate

Questions or comments? **1-800-331-4536** (Mon-Fri 9AM – 5PM EST) or www.bayeraspirin.com

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Appendix E: Brand of fish oil used in the study

Appendix F: 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

Vita

Dr. Lisa Turner was born and raised in Richmond, Virginia. She received a Bachelor of Science in Dental Hygiene from Old Dominion University in 2004, a Master of Science in Dental Hygiene from Old Dominion University in 2006, and worked as a dental hygienist in private practice 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. Turner will graduate from Virginia Commonwealth University with a Master of Science in Dentistry, and a Certificate in Periodontics.