Enriched Marine Oil Supplements Increase Peripheral Blood Specialized Pro-Resolving Mediators Concentrations and Reprogram Host Immune Responses

A Randomized Double-Blind Placebo-Controlled Study

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RATIONALE: Specialized pro-resolving mediators (SPM—lipoxins, resolvins, protectins, and maresins) are produced via the enzymatic conversion of essential fatty acids, including the omega-3 fatty acids docosahexaenoic acid and n-3 docosapentaenoic acid. These mediators exert potent leukocyte directed actions and control vascular inflammation. Supplementation of animals and humans with essential fatty acids, in particular omega-3 fatty acids, exerts protective actions reducing vascular and systemic inflammation. Of note, the mechanism(s) activated by these supplements in exerting their protective actions remain poorly understood.

OBJECTIVE: Given that essential fatty acids are precursors in the biosyntheses of SPM, the aim of the present study was to establish the relationship between supplementation and peripheral SPM concentrations. We also investigated the relationship between changes in plasma SPM concentrations and peripheral blood platelet and leukocyte responses.

METHODS AND RESULTS: Healthy volunteers were enrolled in a double-blinded, placebo-controlled, crossover study, and peripheral blood was collected at baseline, 2, 4, 6, and 24 hours post administration of placebo or one of 3 doses of an enriched marine oil supplement. Assessment of plasma SPM concentrations using lipid mediator profiling demonstrated a time- and dose-dependent increase in peripheral blood SPM concentration. Supplementation also led to a regulation of peripheral blood cell responses. Here we found a dose-dependent increase in neutrophil and monocyte phagocytosis of bacteria and a decrease in the diurnal activation of leukocytes and platelets, as measured by a reduction in adhesion molecule expression. In addition, transcriptomic analysis of peripheral blood cells demonstrated a marked change in transcript levels of immune and metabolic genes 24 hours post supplementation when compared with placebo.

CONCLUSIONS: Together, these findings demonstrate that supplementation with an enriched marine oil leads to an increase in peripheral blood SPM concentrations and reprograms peripheral blood cells, indicating a role for SPM in mediating the immune-directed actions of this supplement.

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Key Words: leukocyte □ lipoxins □ monocytes □ neutrophils □ platelet

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For Sources of Funding and Disclosures, see page 89.
The role of essential fatty acids in physiology was established in seminal studies by Burr et al., who demonstrated that deficiency in these fats, which till that point were only thought to be a source of energy, was linked with pathology and in severe cases even death. The production of these fatty acids in mammals is limited, thus their uptake from food sources is central to the maintenance of health. Over the years, interest has grown in the potential utility of supplements rich in omega-3 fatty acids in both the maintenance of health and as therapeutics. Despite promising results in experimental systems, clinical studies have yielded apparently conflicting results when these supplements were administered to patients with inflammatory diseases. Indeed, while several studies, including the recently published REDUCE-IT trial (Reduction of Cardiovascular Events With Icosapent Ethyl–Intervention Trial), report protective actions of essential fatty acid supplementation when combined with standard of care treatment, other studies found little to no benefit of omega-3 supplements in regulating the course of inflammatory disease. Of note, an aspect that in clinical settings has received little attention is the identification of biomarker(s) that can be used to provide insights into the potential effectiveness of a given supplement at regulating inflammatory processes. For many years the mechanism by which omega-3 fatty acids were thought to regulate inflammation was by competing for the activity of enzymes involved in the production of arachidonic acid (AA)–derived inflammatory eicosanoids. While this mechanism may contribute to dampening inflammation, recent studies demonstrate that omega-3 fatty acids are converted to bioactive mediators, termed SPM, which potently regulate immune responses. Thus, we investigated the relationship between SPM supplementation and plasma concentrations. We also tested whether SPM regulation was linked with changes in peripheral blood cell biology. Using an enriched marine oil, we found that supplementation leads to time and dose-dependent changes in blood SPM concentrations that are linked with changes in leukocyte and platelet responses as well as a reprogramming of the peripheral blood cell transcriptome. These findings establish a link between plasma SPM concentrations and peripheral blood cell responses, thus, elucidating their potential utility as predictive biomarkers.

Novelty and Significance

What Is Known?
- Omega-3 fatty acids are essential to the maintenance of health.
- Specialized pro-resolving mediators (SPM) are derived from essential fatty acids and promote resolution of inflammation.

What New Information Does This Article Contribute?
- Enriched marine oil supplementation leads to a dose- and time-dependent increase of plasma SPM concentrations.
- Increases in SPM concentrations are correlated with changes in platelet and leukocyte responses, including diurnal activation and bacterial phagocytosis.
- Supplementation reprograms the circulating leukocyte transcriptome.

Nonstandard Abbreviations and Acronyms

| Acronym | Definition                           |
|---------|--------------------------------------|
| AA      | arachidonic acid                     |
| ATP5ME  | ATP synthase membrane subunit E      |
| CD      | cluster of differentiation           |
| DHA     | docosahexaenoic acid                 |
| DPA     | docosapentaenoic acid                |
| EPA     | eicosapentaenoic acid                |
| IFITM3  | interferon-induced transmembrane protein 3 |
| MaR     | maresin                              |
| n-3     | omega-3                              |
| PAF     | platelet aggregating factor          |
| PCTR    | protectin conjugates in tissue regeneration |
| PD      | protectins                           |
| RPL     | L ribosomal protein                  |
| RvD     | D-series resolvin                    |
| RvE     | E-series resolvin                    |
| RvT     | 13-series resolvin                   |
| SPM     | specialized pro-resolving mediators  |
| UQCRB   | ubiquinol-cytochrome c reductase bind- ing protein |

Predictive biomarkers that reflect the clinical efficacy of omega-3 fatty acid supplements are not available, leading to discordant findings on their efficacy at regulating inflammation. Essential fatty acids are converted into bioactive mediators, termed SPM, which potently regulate immune responses. Thus, we investigated the relationship between SPM supplementation and plasma concentrations. We also tested whether SPM regulation was linked with changes in peripheral blood cell biology. Using an enriched marine oil, we found that supplementation leads to time and dose-dependent changes in blood SPM concentrations that are linked with changes in leukocyte and platelet responses as well as a reprogramming of the peripheral blood cell transcriptome. These findings establish a link between plasma SPM concentrations and peripheral blood cell responses, thus, elucidating their potential utility as predictive biomarkers.
defined molecules. These mediators are classified into 4 main families, the lipoxins from AA, the resolvins from EPA, n-3 DPA, and DHA, and the protectins and maresins from n-3 DPA and DHA. The complete stereochemistries for these mediators were established using a total organic synthetic approach coupled with matching of both the physical and biological properties of the synthetic material to that of the endogenous mediators. SPM display potent biological actions in regulating host immune responses to both sterile and infectious insults via the activation of cognate receptors on the target cells. By definition SPM promote the uptake and clearance of apoptotic cells and cellular debris, regulate leukocyte trafficking to the site of inflammation and counter regulate the production of pro-inflammatory mediators, including cytokines and chemokines. In addition, each of the SPM also carries characteristic biological actions. These include, the tissue regenerative actions displayed by maresins and the vasculoprotective actions of RvD (D-series resolvin) 1 and RvD2. Of note, recent studies found that supplementation of healthy volunteers or patients with up to 4 g of omega-3 fatty acids increases plasma SPM concentrations and the ability of peripheral blood leukocytes to phagocytose bacteria. These observations support the hypothesis that the protective actions of omega-3 fatty acids are mediated via the upregulation of SPM biosynthesis. However, the pharmacokinetics and pharmacodynamics of omega-3 supplements in regulating SPM biosynthesis as well as the correlation between changes in tissue SPM concentrations and the regulation of host responses in humans are not well understood.

Therefore, the aim of the present study was to establish the relationship(s) between supplement dose, peripheral blood SPM concentrations, and cellular responses using a novel enriched marine oil preparation. We also aimed to assess the kinetics for these responses to provide insights into the protective mechanisms activated by omega-3 supplements in humans and novel potential biomarkers for determining the effectiveness of omega-3 supplements at regulating host immune responses. To ascertain this, we conducted a double-blinded, crossover, placebo-controlled study in 22 healthy volunteers assessing the temporal regulation of peripheral blood SPM and cellular responses. A placebo cycle was used to control for diurnal patterns which may affect some of the parameters investigated. Results from this study demonstrate that peripheral blood SPM concentrations are rapidly upregulated following supplementation, an action that was correlated with the regulation of peripheral blood leukocyte and platelet responses.

**METHODS**

**Data/Methods Availability**

RNA seq data is available from GEO (accession number: GSE132648; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132648).

All R scripts used for the differential gene expression, gene ontology term enrichment, and Spearman rank correlation analysis, together with the data and expected results (including tables and figures), are found in the GitHub repository: https://github.com/eagomezc/2019_DGE_Correlation_Oil_supplements_study.

**Study Design**

We conducted a double-blind, randomized, crossover, dose escalation placebo-controlled study in healthy volunteers to assess the impact of a single bolus of fatty acid supplementation, given in the form of an enriched marine oil supplement SPM Active produced by Metagenics Inc, at regulating peripheral blood pro-resolving mediator concentrations as well as platelet, neutrophil, and monocyte responses. The study was reviewed by the institutional review board, was granted approval by National Research Ethics Service Committee London—Bromley (16/LO/2182) and was registered on ClinicalTrials.gov. Informed, written consent was obtained before enrolling participants into the study.

**Study**

Participants between the age of 18 and 45 (see Online Table I) were enrolled at the William Harvey Heart Centre where screening, dosing, and blood draws were performed. Blood biochemistries were performed at Barts Hospital, while peripheral blood activation, lipid mediator measurements, and gene expression analysis experiments were performed at William Harvey Research Institute. Participants were enrolled if they met the following criteria: (1) able to provide informed consent; (2) declare not to be taking aspirin, other nonsteroidal anti-inflammatory drugs, other form of medication or omega-3 fatty acid supplements for no less than 2 weeks before screening and for the duration of the participation, (3) abstain from eating oily fish for 2 weeks before each study visit; (4) abstain from alcohol consumption for at least 24 hours before each study visit; and (5) abstain from caffeine before and during study. Participants who regularly eat fish were also enrolled in the study, although they had to abstain from fish consumption for a minimum of 2 weeks.

Participants were excluded from the study if they (1) had a history of chronic disorders, cardiovascular disease (eg, heart disease, stroke), cancer, or diabetes mellitus or significant genetically inherited conditions; (2) were pregnant or breastfeeding; (3) had hypothyroidism; (4) had liver disease in the opinion of the investigator; (5) had any abnormality or pre-existing disease which, in the opinion of the investigator, might either expose the subject to risk, or influence the validity of the results; (6) were women of childbearing potential not taking adequate methods of contraception; (7) inability to read and write in English; (8) had participated in a clinical study of a new chemical entity, biological product or a prescription medicine, or had lost >400 mL blood, within the previous 3 months; (9) were smokers; and (10) had presence or history of drug or alcohol abuse or intake of more than the amount of alcohol in the current guidelines on alcohol consumption. After enrollment, participants were randomly assigned to one of 8 study groups (see Online Table II) as described in the Online Methods and were treated following the schedule outlined in Online Figure I.
RESULTS

Enriched Marine Oil Supplements Upregulate Plasma Lipid Mediator Concentrations

Healthy nonsmoking volunteers aged between 19 and 37 years were recruited between March and September 2017 (see Online Table I volunteer information). After screening, including history taking, resting ECG, physical examination, and routine biochemistry and hematology, eligible volunteers were randomized to one of 8 study groups (see Online Table II for randomization strategy, Online Figure I for study schedule, and Online Figure II for experimental plan). All 22 volunteers completed the study. To investigate the temporal regulation of peripheral blood SPM concentration after supplementation, blood was collected before placebo/supplement administration (0 hour) then 2, 4, 6, and 24 hours post-supplementation. The supplement doses tested were of 1.5, 3, and 4.5 g of total fatty acids of which ≈30 µg per 1.5 g of total fatty acids were composed of unesterified AA (≈3%), EPA (≈46%), n-3 DPA (≈18%), and DHA (≈33%). Of note, the free fatty acid form of these molecules is implicated in SPM biosynthesis. The supplement also contained several SPM precursors including 17-hydroxy-docosahexaenoic acid, 17-HDPA (17-hydroxy-docosapentaenoic acid), and 18-HEPE (hydroxy-eicosapentaenoic acid; Online Table III), while only negligible amounts of these molecules were identified in the Placebo preparation (Online Table III). Volunteers were asked to refrain from consuming oily fish for 14 days and alcohol for 24 hours before the administration of supplement/placebo, attended fasted on the day of administration each time and were given standard breakfast and lunch in the clinical research center. All volunteers had to remain in the center for the first 6 hours post-administration and returned the following morning for a fasting blood collection at 24 hours. If volunteers indulged in either fish or alcohol, their next cycle of testing was delayed by the requisite number of days.

Assessment of peripheral blood lipid mediator concentrations was conducted using liquid chromatography tandem mass spectrometry–based lipid mediator profiling. Identification and quantitation of plasma lipid mediators from the 4 major essential fatty acid metabolomes conducted in accordance with established criteria that include matching retention times and at least 6 diagnostic ions in the MS-MS spectrum. In these plasma samples, we identified mediators from the lipoxygenase and cyclooxygenase-derived bioactive metabolomes that include the DHA and n-3 DPA–derived resolvins (Rv) and protectins (PD). Of note, presupplementation plasma lipid mediator concentrations were comparable among the 4 treatment groups (Online Figure III and Online Table IV).

We next investigated whether fatty acid supplementation regulated peripheral blood lipid mediator profiles. Here, we found a temporal regulation of plasma SPM by enriched marine oil supplementation, which was also dose-dependent (Figure 1). Cumulative plasma pro-resolving lipid mediator concentrations were found to increase at the 2-hour interval in a dose-dependent manner, reaching statistical significance in volunteers given the 3 and 4.5 g doses when compared with both presupplementation concentrations and to lipid mediator concentrations measured at this interval in the placebo group (Figure 1A and Online Table IV). Supplementation was found to increase the circulating concentrations of mediators from all omega-3 fatty acid metabolomes measured, with marked increases in n-3 DPA and the EPA metabolomes including the vasculoprotective RvDn-3 DPA and the RvE (E-series resolvins) Figure 1B, Online Figure IV, and Online Table IV). This increase in peripheral blood SPM concentrations was also linked with a dose-dependent increase in plasma SPM precursor and pathway marker concentrations, including 17-HDHA and 18-HEPE, the biosynthetic precursors to the D-series and E-series resolvins, respectively (Online Figure V).

Furthermore, correlation analysis demonstrated that there was a statistically significant positive correlation between peripheral blood SPM concentrations and marine oil supplement dose at the 2-, 4-, and 6-hour intervals for most SPM families identified and quantified (Online Figure VI). Of note, marine oil supplementation also increased plasma concentrations of the AA-derived lipoxins and leukotriene B4 (Figure 1B, Online Figure IV, and Online Table IV), without significantly altering the concentrations of the AA-derived prostanoids (Online Table IV).

Having observed significant increases in the peripheral blood levels of SPM families after supplementation, we next employed partial least squares discriminant analysis, which generates a regression model based on concentrations of lipid mediators that are differently expressed between the groups, to gain insights into specific mediators that were upregulated following marine oil supplementation. Here we focused on results from the 3.0 and 4.5 g groups and compared them to plasma lipid mediator concentrations in the placebo group. Assessment of the score plots demonstrated a dose-dependent shift in peripheral blood lipid mediator profiles 2- and 4-hour post marine oil supplementation, with lipid mediator concentrations returning to levels comparable to those found in the placebo group at subsequent intervals (Figure 2A and Online Table IV). Assessment of the variable importance in projection scores, which identify the contribution of each mediator in the observed separation between groups, demonstrated an upregulation of mediators from the DHA, n-3 DPA, and EPA bioactive metabolomes post-supplementation. This included increases in MaR (maresin) 2 and MaR2n-3 DPA, the top 2 upregulated mediators at the 2- and 4 hours intervals in both supplement groups, the vasculoprotective RvT3.
Figure 1. Supplementation with an enriched marine oil upregulates peripheral blood specialized pro-resolving mediators (SPM) concentrations in healthy volunteers.

Blood was collected from healthy volunteers pre (0 h) then 2, 4, 6, and 24 h after the administration of 1.5, 3, and 4.5 g of an enriched marine oil supplement or placebo. Plasma was obtained, and lipid mediators were extracted, identified, and quantified using LC-MS/MS based lipid mediator profiling. A, Cumulative SPM (left) and leukotrienes and prostaglandin (right) concentrations. B, Cumulative concentrations for the distinct lipid mediator families identified in plasma. Results are mean, n=22 volunteers. Statistical differences were assessed using 2-way ANOVA and Dunnett post hoc test with P value correction conducted using Benjamini Hochberg correction. cysLT indicates cysteinyl leukotrienes; LTB, metabolome, leukotriene B4, metabolome; LX, lipoxins; MaR, maresins; MaRn-3 DPA, n-3 DPA-derived MaR; MCTR, maresin conjugates in tissue regeneration; PCTR, protectin conjugates in tissue regeneration; PD, protectins; PDn-3 DPA, n-3 DPA-derived PD; PG, prostaglandins; RvD, D-series resolvins; RvDn-3 DPA, n-3 DPA-derived RvD; RvE, E-series resolvins; and RvT, 13-series resolvins.
(13-series resolvin) and RvT4\textsuperscript{9} and PD\textsubscript{1}\textsuperscript{n-3 DPA} that were upregulated at the 2-hour interval together with RvD5\textsubscript{n-3 DPA}\textsuperscript{19} which was upregulated in both supplement groups at the 4-hour interval (Figure 2B). Together these results demonstrate that enriched marine oil supplementation leads to a dose-dependent increase in peripheral lipid mediator concentrations with marked increases in DHA, n-3 DPA, and EPA-derived SPM.

**Marine Oil Supplementation Regulates Diurnal Changes in Adhesion Molecule Expression in Peripheral Blood Platelets, Monocytes, and Neutrophils**

We recently found that n-3 DPA-derived SPM regulate the diurnal activation of peripheral platelets and leukocytes.\textsuperscript{19} Given the increases in peripheral blood SPM, including those derived from n-3 DPA following fatty acid supplementation we next investigated whether these supplements regulated the expression of adhesion molecules on neutrophils, monocytes, and platelets as a measure of cellular activation.\textsuperscript{19} Assessment of adhesion molecules expression on monocytes, which were identified as illustrated in Online Figure VII, demonstrated that supplementation with marine oil led to a dose-dependent decrease in CD11b (cluster of differentiation) expression reaching statistical significance at the 24-hour interval when compared with placebo. Twenty-four hours after enriched marine oil supplementation we also observed a marked decrease in the expression of CD162, the high affinity receptor for CD62P and CD62E (Figure 3A). No differences were observed in the expression of either CD49d or the Fc receptor CD16 (Online Figure VIII).

Expression of CD11b, CD49d, and CD162 on peripheral blood neutrophils was also found to change in a diurnal manner in the placebo group (Figure 3B and Online Figure VIII). Supplementation with enriched marine oils significantly upregulated the expression of both CD11b at the 4-hour interval and that of CD49d at the 24-hour interval when compared with the placebo group; increases that were dose-dependent (Figure 3B). Of note, neutrophil CD11b expression was reduced in the supplement groups at the 24-hour interval when compared with the levels measured in placebo group at the same interval; changes that reached statistical significance in the 3 g dose (Figure 3B).

Given the role that platelet-leukocyte aggregates play in both the perpetuation of inflammation\textsuperscript{21} and organ protection,\textsuperscript{22} we investigated the presence and amounts of these heterotypic cell aggregates by measuring the expression of the platelet marker CD41 on both monocytes and neutrophils. CD41 expression on monocytes was regulated in a dose-dependent manner by supplementation where we observed a reduction in CD41 expression at the 24-hour interval. This reduction was most pronounced in volunteers receiving the 4.5 g dose, although it did not reach statistical significance (P=0.101; Figure 3A). Supplementation, on the contrary, did not reduce neutrophil CD41 expression (Figure 3B).

We next investigated whether enriched marine oil supplementation controlled the expression of activation markers on circulating platelets. To assess platelet activation, we measured the expression of CD62P, the counter ligand to the leukocyte adhesion molecule CD162, together with the expression of CD63 which is upregulated on platelet activation.\textsuperscript{23} Here we found that while the expression of CD62P was not markedly regulated by marine oil supplementation, platelet CD63 levels were significantly reduced at both 2-hour and 24-hour post supplementation in volunteers given 4.5 g of marine oils when compared with volunteers given placebo (Figure 3C).

**Supplementation Regulates Leukocyte and Platelet Responses to Platelet Aggregating Factor**

Having established the role of supplementation on diurnal changes in peripheral blood cell responses ex vivo, we tested whether this also impacted peripheral blood cell responses to an inflammatory stimulus. For this purpose, we incubated whole blood with the pro-inflammatory mediator PAF (platelet aggregating factor), which is implicated in the propagation of vascular inflammation via the increase in leukocytes and platelet aggregation, leukocytes adhesion to the vascular endothelium, increasing vascular permeability and in promoting thrombus formation.\textsuperscript{31} Assessment of adhesion molecule expression in the monocyte population following PAF stimulation demonstrated that supplementation downregulated the expression of CD11b in a dose-dependent manner reaching statistical significance with the 4.5 g dose at the 4- and 24-hour intervals, when compared with placebo values (Figure 3D). Enriched marine oil supplementation also significantly reduced the expression of CD49d, reaching statistical significance at the 6-hour interval in volunteers given 4.5 g of supplement when compared with those that were given placebo (Figure 3D). Monocyte CD16 expression in response to PAF stimulation was upregulated in volunteers given fatty acid supplements (Figure 3D). Marine oil supplementation also regulated neutrophil responses to PAF, where we observed significant increases in the expression of CD49d on neutrophils in all 3 supplement groups which reached statistical significance at the 24-hour interval when compared with the respective placebo values. Of note, we did not observe significant difference in the expression of both CD11b and CD162 on neutrophils following fatty acid supplementation (Figure 3E and Online Figure IX).
Enriched marine oil administration also regulated monocyte-platelet heterotypic aggregates in response to PAF stimulation in a dose-dependent manner. Here we found that supplementation with the 1.5 g dose markedly reduced these aggregates at the 4-hour and 6-hour intervals, although these changes did not reach statistical
Figure 3. Supplementation regulates peripheral blood leukocytes and platelet responses to both diurnal changes and PAF (platelet aggregating factor).

Blood was collected from healthy volunteers pre (0 hour) then 2, 4, 6, and 24 hour after the administration of 1.5, 3, and 4.5 g of an enriched marine oil supplement or placebo. Cell activation in (A) monocytes, (B) neutrophils, and (C) platelets was assessed using fluorescently conjugated antibodies to activation markers and flow cytometry (see Methods for details). Results are mean, n=21 volunteers. (Continued)
significance \((P=0.0553\) and \(0.0831\), respectively) when compared with the respective values obtained after placebo administration (Online Figure IX). Supplementation also regulated platelet response to PAF in a dose-dependent manner with the greatest decreases in platelet CD62P expression observed at the 24-hour interval (Figure 3F).

**Regulation of Bacterial Phagocytosis by Monocytes and Neutrophils**

Given the role of SPM in promoting bacterial clearance by phagocytes, we next investigated whether essential fatty acid supplementation also increased bacterial phagocytosis in peripheral blood monocytes and neutrophils. Here we obtained peripheral blood from volunteers pre- and postadministration of either enriched marine oils or placebo. This was incubated with fluorescently labeled *Escherichia coli* or *Staphylococcus aureus* ex vivo, and phagocytosis was evaluated using flow cytometry. Here we found that supplementation with either 1.5 or 4.5 g of enriched marine oils increases the phagocytosis of *S aureus* by both neutrophils and monocytes (Figure 4A and 4B). Of note, although phagocytosis was increased at the early intervals (2–4 hours) post supplementation the greatest increases were observed at the 24-hour interval in both leukocyte subsets (Figure 4A and 4B) for both of these doses. Enriched marine oil supplementation also regulated the phagocytosis of the gram negative bacterium *E. coli* by neutrophils, but not monocytes, that reached statistical significance at the 24-hour interval in the 3.0 and 4.5 g groups (Figure 4).

**Increases in Peripheral Blood SPM Concentrations Correlate With Changes in Platelet, Monocyte, and Neutrophil Responses**

Since peripheral blood cell responses and SPM concentrations were increased following fatty acid supplementation, we next questioned whether there was a correlation between SPM concentrations and leukocyte and platelet responses. To address this question, we used the area under the curve for mediator concentrations, since it allows us to investigate the influence of cumulative changes in the concentrations of these mediators following supplementation. In this analysis, we focused on those molecules belonging to mediator families that were differentially regulated following supplementation. In this analysis, we focused on those molecules belonging to mediator families that were differentially regulated following supplementation, assessing the correlation between these values and changes in cellular responses at the 24-hour interval. Here we found dose-dependent changes in the correlations between individual SPM concentrations and the expression of cellular markers on platelet, monocyte, and neutrophils (Figure 5). Of note, the largest number of correlations were identified in the 3.0 and 4.5 g groups (Figure 5C and 5D). In these groups, we found that several SPM, including RvD1, RvD2, RvD4, RvD6, and RvD5-13 DPA, negatively correlated with markers of platelet activation CD63 and CD62P. These correlations were observed when assessing the diurnal changes in the expression of these molecules as well as in response to PAF stimulation (Figure 5C and 5D). In volunteers given the higher dose of the marine oil supplement, we also observed negative correlations in the expression of activation markers on leukocytes including a negative correlation between monocyte CD11b and PD1 as well as the expression of CD49d on monocytes and plasma MaR1-3 DPA concentrations.

Towards establishing the mechanism by which marine oil supplements regulate peripheral blood leukocyte and platelet responses, we next assessed the biological actions of mediators found to correlate with changes in adhesion molecule expression or bacterial uptake. Here we found that incubation of peripheral blood with PCTR2 (protectin conjugates in tissue regeneration), RvD1, or RvD4 decreased platelet-monocyte and platelet-neutrophil heterotypic aggregates in response to PAF stimulation, actions that were found to be dose-dependent (Figure 6A and 6B). We also found that these mediators dose-dependently regulated the expression of CD11b on neutrophils and CD63 on platelets (Figure 6B and 6C). Together these findings demonstrate that upregulation of PCTR2, RvD1, or RvD4 following marine oil supplementation contributes to the regulation of peripheral blood cell responses reducing cellular activation in circulating leukocytes and platelets.

**Marine Oil Supplementation Reprgrams Peripheral Blood Cell Transcriptome**

Having observed a regulation of peripheral blood SPM production that was coupled with changes in platelet, monocyte, and neutrophil responses, with the latter changes being most prominent 24 hours after supplementation, we next questioned whether these observations were linked with transcriptional reprogramming of peripheral blood cells. For this purpose, we conducted transcriptomic analysis of peripheral blood cells 24 hours after placebo or 4.5 g of marine oil supplementation.

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**Figure 3 Continued.** Statistical significance was determined using 2-way ANOVA followed by Dunnett post hoc test. **D–F.** Blood was collected from healthy volunteers pre (0 hour) then 2, 4, 6, and 24 hours after the administration of 1.5, 3, and 4.5 g enriched marine oil supplement or placebo. This was then incubated with PAF (100 nmol/L, 30 min; 37°C). Cell activation in **D** monocytes, **E** neutrophils, and **F** platelets was assessed using fluorescently conjugated antibodies to activation markers and flow cytometry. Results are mean, n=21 volunteers. Statistical significance was determined using 2-way ANOVA and Dunnett post hoc test. Gray area is included for visual reference and has no quantitative significance.
Here we found that marine oil supplementation led to the differential regulation of 141 genes that included selectin L (SELL), parkinsonism associated deglycase (PARK7), Rac family small GTPase 2 (RAC2), and S100 calcium binding protein A8 (S100A8; Online Table V and Figure 7A). We next conducted real-time quantitative polymerase chain reaction analysis to validate the expression of a panel of genes found to be upregulated following supplementation and known to be involved in regulating cellular metabolism and immune responses. These included UQCRB (ubiquinol-cytochrome c reductase binding protein), IFITM3 (interferon-induced transmembrane protein 3), and ATP5ME (ATP synthase membrane subunit E). Results of this analysis confirmed the upregulation of this panel of genes (Figure 7B), thus corroborating the results obtained in the transcriptomic analysis. We next interrogated the gene ontology terms and biological pathways that were enriched in peripheral blood cells after supplementation when compared with placebo administration. Gene ontology analysis using genes found to be regulated in the marine oil group demonstrated an enrichment of genes linked with immune responses, leukocyte recruitment and cellular metabolism among others (Figure 7C, Online Figure X, and Online Table VI). This enrichment of immune and metabolism related genes together with signal transduction proteins was confirmed using Reactome database\textsuperscript{24} that identified enrichment of genes within these biological processes, including HLA class II histocompatibility antigen gamma chain (CD74) and IFITM1 (interferon-induced transmembrane protein 1), associated to the adaptive immune system pathways;
Figure 5. Select specialized pro-resolving mediator (SPM) correlate with changes in the expression of peripheral blood cell activation markers 24 h post supplementation.

The area under the curve for plasma SPM concentrations for the duration of the study were correlated with changes in the expression of cellular activation markers when compared with baseline values for peripheral blood from (A) placebo, (B) 1.5 g, (C) 3 g, and (D) 4.5 g supplement groups. Diurnal denotes correlations with changes in adhesion molecule expression in response to diurnal changes. PAF (platelet aggregating factor) denotes correlations with changes in adhesion molecule expression in response to PAF incubation. Red dots indicate a significant negative correlation based on the Spearman Rank test with P value correction conducted using Benjamini Hochberg correction and an adjusted P value of <0.1. Results are representative of n=22 volunteers per group.
and RPL (L ribosomal proteins: 30, 31, 41, among others), associated with metabolism of amino acids and derivatives and rRNA processing pathways (Figure 7D and Online Table VII). Kyoto Encyclopedia of Genes and Genomes pathway analysis highlighted an enrichment of genes linked with RNA transcription, including ribosomal machinery, as well as genes involved in energy production, including oxidative phosphorylation (Online Figure XI and Online Table VIII). Together these findings indicate that upregulation of peripheral blood SPM after marine oil supplementation is linked with transcriptional reprograming of peripheral blood leukocytes.

**DISCUSSION**

In the present study using a placebo-controlled, crossover approach to account for inter-individual variations and a Latin square design to account for carry over effects of marine oil supplementation, we found a time-dependent and dose-dependent increase plasma SPM production after supplementation. Supplementation was found to primarily increase the omega-3-derived PDn-3 DPA, MaRn-3 DPA, and RvE. This increase was linked with a change in the diurnal regulation of peripheral blood leukocyte responses as well as in an increased ability of...
Figure 7. Supplementation leads to transcriptional reprogramming of the peripheral blood cells 24 h after supplement intake. Peripheral blood collected 24 h after either placebo or supplement (4.5 g) intake was subjected to transcriptomic profiling (see methods for details). A, Volcano plot highlighting the significantly upregulated (red) and downregulated (blue) genes in peripheral blood cells from volunteers given 4.5 g of enriched marine oil supplement. Significance was determined using Benjamini Hochberg correction and an adjusted P value of <0.1. B, Validation of a subset of genes found to be upregulated by enriched marine oil supplementation using quantitative polymerase chain. C, Gene ontology term enrichment analysis for biological process of differentially expressed genes from volunteers supplemented with 4.5 g of enriched marine oils. D, Enriched reactome pathways of differentially expressed genes from volunteers supplemented with 4.5 g of enriched marine oils. Results for A, C, and D are representative of n=18 healthy volunteers. Results for B are representative of n=9 volunteers.
Peripheral blood platelets, monocytes and neutrophils to respond to an inflammatory stimulus. Of note, the most pronounced changes in peripheral neutrophil and monocyte responses to both diurnal changes and PAF were observed 24 hours after supplementation pointing to a reprogramming of peripheral blood leukocytes. Transcriptomic analysis of peripheral blood cells further supported this observation given that we observed a significant enrichment of genes involved in immune regulation and peripheral blood cell responses after marine oil supplementation when compared with placebo treated volunteers. Together, these findings indicate that changes in peripheral blood SPM concentrations are linked with a reprogramming of peripheral blood cell responses towards a protective phenotype.

It is now well appreciated that impaired resolution mechanisms are central in the onset and propagation of many inflammatory conditions including cardiovascular disease. We recently found that diurnal regulation of peripheral SPM biosynthesis is an endogenous counterregulatory process that prevents uncontrolled vascular activation of leukocytes and platelets. Indeed, disruption of peripheral blood SPM production in patients with CVD is linked with enhanced peripheral blood cell activation that included an increase in the pro-atherogenic monocyte-platelet heterotypic aggregates and CD11b expression. SPM are also involved in regulating vascular inflammatory responses where alterations in the production of the DHA-derived RvD1, RvD2, and MaR1 are linked with increased vascular leukocyte activation.

Results from the present findings demonstrate that supplementation with marine oils can increase plasma SPM concentrations and reduce peripheral blood monocyte and platelet diurnal activation (Figures 1 through 3). Of note, supplementation was also found to increase peripheral blood platelet-neutrophil heterotypic aggregates as measured by an increase in CD41 expression (Figure 3). This is of interest since these aggregates are an important route for SPM production via transcellular biosynthesis suggesting that they may contribute to the observed increases in SPM production following supplementation.

Current approaches in the treatment of inflammatory conditions involve the blockade of specific pathways which are involved in the propagation of inflammatory responses. This approach is predicated on the presence of the antagonist or inhibitor to the pathway of interest being constantly present at biologically relevant concentrations. Evidence gathered using both animal systems and human primary cells demonstrates that SPM, via engagement of their cognate receptors, initiate signaling cascades that lead to the reprogramming of target cells. This, in turn leads to the activation of a self-perpetuating protective signal which at variance to current therapeutic, does not require the mediator to remain present for its protective biological actions to be sustained.

Results from the present studies support this hypothesis, given that while increases in peripheral blood SPM concentrations were rapid, reaching a maximum around 2-4 hour post supplementation, changes in the responses of peripheral blood monocytes in particular were still present 24 hours after supplementation (Figures 1 through 4). Transcriptomic analysis provides further support to this hypothesis, given that we observed significant changes in the expression of genes that are linked with key pathways in the regulation of host immune responses, including leukocyte tethering, aggregation, and energy generation (Figure 7).

In addition to regulating host immune responses following sterile challenge, SPM are also linked with improving the ability of leukocytes to uptake and clear bacteria. Recent studies demonstrate that RvD1 may reduce the required dose for the clearance of both gram positive and gram negative bacterial infections. The RvD precursor 17-hydroxy docosahexaenoic acid increases resistance to H1N1 (a subtype of influenza A virus) infections via the activation of B-cells antibody production, while the PD metabolome increases the clearance of H1N1 via interfering with the viral replication machinery. In the present studies, we found that increases in peripheral blood SPM were linked with an upregulation of CD49d expression on neutrophils, an adhesion molecule that is linked with enhanced resistance to S aureus infection, and an increased uptake of S aureus by peripheral blood neutrophils (Figure 4). These observations were not limited to gram positive bacteria. Indeed, we found that supplementation dose-dependently increased the clearance of the gram negative bacterium E coli in neutrophils (Figure 4). Thus, these observations suggest that supplementation regulates peripheral blood leukocyte responses to sterile and infectious insults.

The strength of the present study is that it addresses the kinetics of SPM formation after marine oil supplementation and tests the relationship between increasing marine oil concentrations and peripheral blood SPM concentrations. The present study also links changes in peripheral SPM concentrations with a regulation of diurnal peripheral blood platelet and leukocyte activation and assesses the influence of supplementation on peripheral blood cell responses to an inflammatory stimulus, PAF (Figure 4). There are also some limitations that should be considered when evaluating the present findings. The first is that the study population was composed of healthy volunteers aged between 18 and 40 years. Therefore, the present findings may not be generalizable to other age groups. A second limitation is that all the patients were given the supplement in the morning. Given that SPM biosynthetic enzymes are diurnally regulated, as are the different populations of vascular leukocytes, supplementation at different times of day may yield different lipid mediator profiles as well as potentially different biological actions on circulating leukocytes. Since it is now well
established that SPM biosynthetic pathways are altered in disease, essential fatty acids may not be as efficiently converted to bioactive mediators in these patient populations. Despite this defect in SPM biosynthesis, given that this marine oil supplement also contains SPM precursors such as 17'-HDPDA and 7'-HDPDA (Online Table III), it would be anticipated that SPM concentrations would still be elevated by supplementation. Last, the present study focused on the ability of one supplement at regulating peripheral blood SPM concentrations and immune cytokine responses. Given that fatty acid forms may differ between supplements (eg, triglycerides versus ethyl esters) and that this may influence the availability of SPM precursors and substrates for conversion to bioactive mediators, future studies will need to determine the dose response relationships for each supplement form in regulating both peripheral blood SPM concentrations and immune response.

Taken together, these findings demonstrate that supplementation with refined marine oils leads to a rapid upregulation of peripheral blood SPM concentrations and reprogramming of peripheral blood cell responses to sterile and infectious stimuli, changes that were found to persist after SPM concentrations returned back to baseline. We also establish a correlation between specific SPM and the regulation of platelet, monocyte and neutrophil responses thereby providing potential novel biomarkers for establishing the efficacy of marine oil supplementation in controlling host immune response.

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**ORIGINAL RESEARCH**

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J. Dalli designed the experiments and conceived the overall research plan; J. Dalli and D.J. Collier designed the clinical study; P.R. Souza, R.M. Marques, P. De Matteis, E.A. Gomez, and R.A. Colas conducted the experiments and analyzed results; M. Patel, A. Zak, D.J. Collier conducted the clinical study and enrolled patients; all authors contributed to article preparation; and D.J. Collier and J. Dalli contributed to supervision of work.
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