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Effects of supplemental long-chain omega-3 fatty acids and erythrocyte membrane fatty acid content on circulating inflammatory markers in a randomized controlled trial of healthy adults

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ABSTRACT

The long-chain omega-3 polyunsaturated (n-3 PUFA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), may have anti-inflammatory effects. We evaluated the dose-response effect of EPA+DHA supplementation on circulating TNF-α, IL-6, and CRP and explored associations between red blood cell (RBC) membrane PUFA content and TNF-α, IL-6, and CRP. Young adults with low fish intake ($n=116$) received one of five doses (0, 300, 600, 900, or 1,800 mg/d EPA+DHA) for 5 months. There were no significant effects of supplemental EPA+DHA on IL-6 or CRP; however, there was a marginal treatment effect for TNF-α ($p<0.08$). At baseline, higher quartiles of RBC DHA were associated with lower TNF-α ($p=0.001$); higher quartiles of arachidonic acid were associated with higher TNF-α ($p=0.005$). EPA+DHA supplementation had no dose-response effect on TNF-α, IL-6, or CRP in healthy young adults; however, associations between inflammatory markers and RBC PUFA warrant further investigation.

1. Introduction

Chronic inflammatory diseases are increasingly prevalent and typically managed by costly medical therapies that have significant side effects. The important role of diet in reducing low grade inflammation and thereby modulating the risk of chronic diseases, including cardiovascular disease (CVD), is supported by observational data [1]. In particular, the potential anti-inflammatory effect of increased dietary long-chain omega-3 (n-3) PUFA—specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—is demonstrated by mechanistic research [2] and population studies in which higher EPA+DHA intake is associated with lower levels of inflammation [3–6]. However, randomized controlled trials with n-3 PUFA supplementation have yielded mixed results in terms of both inflammatory markers and CVD outcomes [7–16].

Clinically evaluating the effects of n-3 PUFA interventions on inflammatory status is difficult due to considerable acute variations in inflammatory markers caused by factors unrelated to the study intervention. Blood concentrations of the pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as well as the acute phase reactant, C-reactive protein (CRP), are used as markers of systemic inflammatory status because they provide quantitative assessments of ongoing inflammatory processes [17,18]. However, acute elevations in these markers can be caused by unavoidable stimuli in the daily lives of research participants, including unreported acute illness [19], changes in physical activity [20], and psychosocial stressors [21]. This poses a significant challenge for evaluating the effects of interventions by introducing extreme changes in inflammatory markers that are not due to treatment effects. Excluding these acutely elevated values according to pre-defined thresholds may enable more accurate assessment of the relationship between n-3 PUFA and chronic inflammation.

Another approach to evaluate links between PUFA and inflammation is utilizing blood biomarkers of fatty acid intake [22]. The EPA+DHA content of the RBC membrane is a useful biomarker of dietary n-3 PUFA intake as it reflects dietary intake over the course

Abbreviations: AA, arachidonic acid; ALA, alpha-linolenic acid; CRP, c-reactive protein; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; IL-6, interleukin-6; LA, linoleic acid; n-3, omega-3; n-6, omega-6; PUFA, polyunsaturated fatty acid; TNF-α, tumor necrosis factor alpha

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of the RBC lifespan (~120 d) and correlates with concentrations in tissues and other cells [23–25]. Inverse associations between RBC n-3 PUFA content and inflammatory marker concentrations have been reported [26–28]. However, the relationships between the content of individual n-3 and n-6 PUFA in RBC membranes and concentrations of circulating inflammatory markers is not completely understood. In addition, whether changes in RBC PUFA relate to changes in inflammatory status has not been determined. Despite this, fish oil supplementation across a range of non-pharmacological doses (<2 g/d) is commonly used in the U.S. for anti-inflammatory purposes [29,30]. Understanding the links between supplemental n-3 PUFA intake, tissues levels, and markers of inflammation is important given inadequate EPA+DHA intake in the U.S. and the increasing burden of chronic inflammatory diseases.

We recently reported that EPA+DHA supplementation dose-dependently increased RBC EPA+DHA content and that additional factors, such as body weight and baseline RBC EPA+DHA, further explained variability in the RBC EPA+DHA response [31]. In the present study, we evaluated the dose-response effects of 0, 300, 600, 900, and 1800 mg/d EPA+DHA on circulating markers of inflammation (TNF-α, IL-6, and CRP) in healthy young adults. We also explored the relationships between both the baseline content and post-supplementation changes in RBC n-3 and n-6 PUFA content with circulating inflammatory marker concentrations, and assessed the potential of n-3 PUFA supplementation to alter circulating white blood cell populations as an additional immunoregulatory effect [32].

2. Methods

2.1. Study design and intervention

Healthy young adults ($n=125$) between the ages of 20–45 y and with BMI between 20–30 kg/m^2 reporting no or low habitual oily fish consumption (<4 servings per month) and not taking n-3 PUFA supplements were recruited. Additional exclusion criteria included use of n-3 PUFA supplemented foods in the past 3 months, history of diabetes, serious medical conditions, smoking, chronic anti-inflammatory medications, planning to change dietary habits, and pregnant, nursing, or planning a pregnancy. Details of the study recruitment and screening have been reported previously [31]. The study was approved annually by the Pennsylvania State University Institutional Review Board.

Each participant was randomly assigned to one of five doses (0, 300, 600, 900, 1800 mg) daily of EPA+DHA as fish oil supplements, using soybean oil as the placebo (Nordic Naturals, Watsonville, CA) for approximately 5 months. The intervention provided nutritionally-achievable doses of EPA+DHA in triglyceride form equivalent to that which could be obtained by consumption of oily fish. The fatty acid profile of the supplements has been described in detail [31]. The fish oil capsules contained approximately 20% EPA, 2% docosapentaenoic acid (DPA), and 13% DHA. Participants agreed to maintain their weight, activity level, usual (limited) fish consumption, and not take any other n-3 PUFA supplements during the study. Daily log sheets and monthly check-ins were completed to verify compliance. Participants returned to the Pennsylvania State University Clinical Research Center every 8 weeks to receive new supplies, return empty containers, and provide completed log sheets.

2.2. Blood sample collection

At the beginning of the study and after the treatment period, participants reported to the Clinical Research Center after a 12-h overnight fast to provide a blood sample by venipuncture. Whole blood was centrifuged at 1500*g* for 15 min at 4 °C. Serum was collected and stored at −80 °C until they were analyzed, except for the complete blood count (CBC), which was measured in EDTA anticoagulated whole blood samples using a Coulter LH 750 with VCS technology (Quest Diagnostics, Pittsburgh, PA). Red blood cells (RBC) were collected following separation from plasma by centrifugation and frozen at −80 °C until analyzed. Fatty acid analysis was performed as described previously [31].

2.3. Inflammatory marker concentrations

Serum concentrations of TNF-α and IL-6 were measured using high-sensitivity ELISA kits (R&D Systems, Minneapolis, MN) in duplicate (CV < 10%). Serum high-sensitivity CRP was measured by latex-enhanced immunonephelometry (Quest Diagnostics; assay CV < 8%).

2.4. Statistical analysis

Statistical analyses were performed using Minitab (version 16.2, Minitab, State College, PA). Analytes that were assayed in duplicate (i.e., TNF-α and IL-6) were averaged before analysis. Fit statistics were assessed for each variable to identify any outliers and to test assumptions of normality. Subjects with acute inflammation (i.e., baseline or endpoint TNF-α and IL-6 values >3.0 ng/L or CRP values >3.0 mg/L), were identified as outliers and secondarily removed from the analysis to ensure that analysis was performed on samples from the target population of non-inflamed adults [33,34]. A natural log transformation was applied to baseline and endpoint values of IL-6 and CRP because of non-normal distribution (skew > 1), and further analysis of associations and treatment effects were performed on the transformed values.

Independent two-sample *t* tests were used to assess sex differences in baseline inflammatory marker concentrations. Associations between baseline inflammatory marker concentrations and BMI, body weight, blood pressure, and age were assessed using Pearson correlation tests.

General linear models were used to test the effects of treatment on inflammatory marker concentrations and CBC measures. Baseline values were included as covariates. We present 4 models for the effect of supplementation on TNF-α, IL-6, and CRP concentrations: unadjusted (model 1), adjusted for baseline value (model 2), adjusted for baseline value, sex, and age (model 3), and adjusted for baseline value, sex, age, blood pressure, and body weight (model 4). Tukey-adjusted *P*-values were used for *post-hoc* comparisons between treatment groups, with adjusted $p<0.05$ considered significant.

Baseline RBC membrane content of n-3 PUFA (alpha-linolenic acid [ALA], EPA, DPA, DHA) and n-6 PUFA (linoleic acid [LA], arachidonic acid [AA]) were assessed as quartiles and compared with circulating inflammatory marker concentrations using ANOVA. Tukey-adjusted *P*-values were used for *post-hoc* comparisons between quartiles. Multiple regression models, with baseline inflammatory markers as the response variable and RBC PUFA as predictor variables, also were conducted to test for the combined effects of RBC PUFA on baseline inflammatory marker concentrations and to guard against spurious findings in the models that examined the relationships between baseline inflammatory marker concentrations and single RBC PUFA quartiles. Changes in RBC membrane content were compared with changes in inflammatory marker concentrations using Pearson correlation tests. Change scores were calculated as the end of supplementation value minus baseline value. Scatterplots were generated to illustrate exploratory analyses of continuous relationships between baseline RBC PUFA content and inflammatory marker concentrations with