

## Acute supplementation with eicosapentaenoic acid reduces platelet microparticle activity in healthy subjects

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### Abstract

**Background:** Dietary supplementation with omega-3 fatty acids has been associated with reduced incidence in thrombotic events. In addition, administration of n-3 polyunsaturated fatty acids (PUFAs) has been shown to rectify elevated platelet microparticle (MP) number and procoagulant activity in post myocardial infarction patients. However, it is unknown whether supplementation can alter these parameters in healthy individuals and if such effects are immediate or require long-term supplementation. We have previously demonstrated a gender-specific effect of LCn-3PUFA supplementation on platelet aggregation in healthy human subjects. Here we extend these findings to include the acute effects of supplementation with EPA- or DHA-rich oils on circulating MP levels and activity in healthy subjects.

**Design:** A placebo-controlled trial was conducted in healthy males and females ( $n=30$ ). MP activity, MP levels and platelet aggregation were measured at 0 and 24 h postsupplementation with either a placebo or EPA- or DHA-rich oil.

**Results:** Both EPA and DHA effectively reduced platelet aggregation at 24 h postsupplementation relative to placebo ( $-13.3\%$ ,  $P=.006$  and  $-11.9\%$ ,  $P=.016$ , respectively), but only EPA reduced MP activity ( $-19.4\%$ ,  $P=.003$ ). When grouped by gender, males showed a similar reduction in both platelet aggregation and MP activity ( $-20.5\%$ ,  $P=.008$ ;  $-22\%$ ,  $P=.008$ ) following EPA, while females showed significantly reduced platelet aggregation ( $-13.7\%$ ,  $P=.04$ ) but not MP activity after DHA only.

**Conclusion:** EPA and DHA exert gender-dependent effects on platelet aggregation and platelet MP activity, but not on MP levels. With respect to thrombotic disease risk, males may benefit more from EPA supplementation.

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**Keywords:** Eicosapentaenoic acid; Procoagulant activity; Microparticles; Platelet aggregation

### 1. Introduction

Microparticles (MPs) are small, phospholipid-rich, membrane-bound fragments shed from stimulated or apoptotic cells. Derived from various cell types, they differ in their antigenic composition and functional properties, which are specific to their cellular origin and the cellular processes triggering their formation [1]. MPs circulate at low levels in the bloodstream of healthy individuals, but are increased in a variety of pathological states. The most abundant are the platelet-derived MPs, less than 1.0  $\mu\text{m}$  in diameter and constituting approximately 70% to 90% of all circulating MPs [2].

Platelet MPs were first described by Wolf as tiny membrane fragments known as 'platelet dust' that were released upon platelet activation but possessing comparable procoagulant activity as activated intact platelets [3]. In a resting platelet, the negatively charged phospholipids are distributed in the inner membrane layer.

When platelets become activated, the nature of the membrane is disrupted, and these phospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are relocated to the outer membrane and exposed [4]. Exposure of PS provides a negatively charged surface to bind coagulation factors and allows the assembly of the prothrombinase complex where prothrombin is cleaved into thrombin [5]. With respect to activated platelets, MPs are formed from the phospholipid-rich outer platelet membrane, thus providing additional phospholipid surface for assembly of various enzyme complexes of the coagulation cascade [6]. Since the discovery of MP, further investigation has revealed diverse roles in coagulation, cellular interactions and cell signalling. They are able to transfer their procoagulant potential to target cells [7], bind to the surface antigens of other cells and by doing so induce intracellular signalling pathways [8]. With further insight, platelet MPs are now acknowledged as bioactive vascular effectors, a storage pool for procoagulant phospholipids and a key modulator of the hemostatic balance. Indeed, elevated levels of circulating platelet MPs are observed in diseases of vascular involvement and high platelet aggregation

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activity [9], and platelet aggregation which is initiated by platelet activation is an early event in the development of thrombosis [10]. Hence, platelet MPs are now considered as one of the reliable markers of platelet hyperactivity in determining the risk of cardiovascular and thrombotic diseases.

Dietary supplementation with long-chain omega-3 polyunsaturated fatty acids (LCn-3PUFAs; eicosapentaenoic acid, EPA; docosahexaenoic acid, DHA) has been associated with reduced incidences in thrombotic events and significant cardiovascular risk reduction [11–14]. Many of the cardioprotective effects are attributed to the incorporation of LCn-3PUFAs into the phospholipid membranes of platelets and endothelial cells [15]. Here, EPA and DHA compete with arachidonic acid as a substrate for cyclooxygenase to reduce formation of proaggregatory eicosanoids [16–21]. Indeed, the platelet antiaggregatory effects of LCn-3PUFA have been reported [16,19] in long-term supplementation studies. We have also recently demonstrated that LCn-3PUFAs effectively reduce platelet aggregation over 24 h in healthy human subjects [22]. Furthermore, the antiaggregatory effects observed were dependent on gender and the different concentration ratios of the LCn-3PUFAs (EPA vs. DHA).

Whether supplementation with LCn-3PUFA affects platelet MP generation or MP activity in healthy male and female subjects has not been studied. Furthermore, it is also not known whether circulating platelet MP levels correlate with MP activity in healthy individuals.

The aim of the present investigation was thus to investigate the effects of acute supplementation with EPA- or DHA-rich oils on circulating platelet MP levels and procoagulant activity with respect to platelet aggregation and gender in healthy human participants.

## 2. Study design and methods

### 2.1. Participants

This study was a blinded placebo-controlled intervention trial. A total of 30 healthy participants completed the study: 15 males and 15 females recruited from the general community of Newcastle, NSW, Australia. Exclusion criteria were diagnosed non-insulin-dependent diabetes; insulin resistance; impaired glucose tolerance; cardiovascular or hematological disorders; body mass index (BMI) greater than 35 kg/m<sup>2</sup>; taking aspirin, antiplatelet medication or nonsteroidal anti-inflammatory drugs. Participants were also excluded if they had consumed fish oil supplements or consumed more than two seafood servings/week. Participants were asked to complete a medical questionnaire and 24-h food recall and to follow a diet low in tomatoes and seafood prior to the study day. All participants provided written informed consent according to governmental regulations concerning the ethical use of human volunteers. Approval for the study was granted by the Human Research Ethics Committee of the University of Newcastle, Australia, and the study has been registered in the Australian New Zealand Clinical Trials Registry (ACTRN12609000164291). The study was conducted in accordance with the Declaration of Helsinki.

All work was conducted in the research unit at the Medical Sciences Building, University of Newcastle. All participants attended the research unit on three separate occasions to consume a different supplement on each occasion with a minimum 1-week washout period in between. Participants were blinded to consume a single dose of 2×1-g capsules containing either placebo (sunola oil) or EPA-rich oil (EPAX 5510 TG/N) providing 1 g EPA with an EPA/DHA ratio of 5:1 or DHA-rich oil (EPAX 1050 TG/N) providing 1 g DHA with an EPA/DHA ratio of 1:5. During each visit, a fasting blood sample was collected prior to supplementation and 24 h following supplementation. The participants were asked not to consume foods containing any seafood or tomato products and limit physical activity until the final 24-h sample was collected.

### 2.2. Blood analysis

Venous blood was collected into Vacutainer tubes containing 3.2% sodium citrate following a <10-h fast. Whole citrate blood was analyzed 20 min after collection for platelet aggregation assays. Platelet aggregation and lag time (time taken to initiate aggregation) was measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Havertown, PA). Whole blood (500 µL) was diluted with an equal volume of phosphate-buffered saline. Samples were pre-incubated for 6 mins at 37°C prior to stimulation with collagen (5 µg/mL). Platelet activity was then monitored for 6 minutes and the area under the aggregation curve (AUC) calculated. For measurement of PMP activity and PMP numbers, citrate blood was centrifuged at 3000g for 10 min to obtain platelet-free plasma, and aliquots were stored at –80°C until further analysis. Samples were thawed at 37°C within 15 min prior to testing. Fasting blood

was collected into EDTA and lithium heparin tubes for analysis of full blood count and hormonal levels (testosterone, oestradiol), respectively. Samples were analyzed by Hunter New England Area Health Pathology Services (NSW, Australia) using standard analytical techniques.

### 2.3. Platelet aggregation assays

Platelet aggregation and lag time (time taken to initiate aggregation) were measured using a Chronolog 560ca whole blood aggregometer (Chronolog-Log, Havertown, PA, USA) according to the method of Cardinal and Flower [23]. Whole blood (500 µL) was diluted with an equal volume of phosphate-buffered saline (PBS). Samples were preincubated for 6 min at 37°C prior to stimulation with collagen (5 µg/mL). Platelet activity was then monitored for 6 min, and the area under the aggregation curve (AUC) was calculated. Reduction of platelet aggregation was determined by calculating the change in AUC from blood samples collected 24 h postsupplementation with baseline aggregation values and expressed as a percentage change. Measurement variation was minimized by having the same trained technician process all samples using the same equipment.

### 2.4. Measurement of MP activity

The procoagulant activity of MPs was measured using the Zymuphen MP-Activity enzyme-linked immunosorbent assay kit (HYPHEN BioMed, Neuville-sur-Oise, France) following the manufacturer's instructions. In this assay, Annexin V is used for capturing MPs expressing PS (procoagulant activity) where MP activity in the sample was determined by the binding to Annexin V in the wells. The unbound particles were washed away, and Factor Xa-Va mixture and prothrombin were added. Exposure of phospholipid surface of the Annexin V bound MPs allowed the activation of prothrombin to thrombin. Thrombin was measured via cleavage of a chromogenic thrombin substrate, and the absorbance was read at 405 nm with a correction at 690 nm. The amount of thrombin generation is directly related to the phospholipid concentration in the plasma. The results were compared to a standard curve of known MP concentration expressed in nM PS equivalent. All samples were analyzed in duplicate, and samples from one participant were determined in the same series to avoid bias due to assay variability. Only replicates with a coefficient of variation <15 were used in the final analysis.

### 2.5. Flow cytometry

Platelet MP was identified and quantified by flow cytometry with specific platelet (CD41a) surface markers. All reagents and solutions used were sterile and filtered (0.2-µm filter). A 30-µL aliquot of platelet-free plasma was incubated at room temperature for 15 min with 10 µL of CD41a-PE (Clone HIP8, BD Biosciences, CA, USA) and 10 µL of Annexin V-FITC (BD PharMingen, San Diego, CA, USA) as a marker of activation/PS expression. The sample was incubated for 30 min at room temperature and then diluted with 500 µL of PBS and stained with 30-µL fluorospheres (Flow-Count, Beckman Coulter Inc., CA, USA). A known number of 10-µm enumeration beads (Flow-count Fluorospheres; Beckman Coulter, Fullerton, CA, USA) were added prior to analysis, and data were analyzed using a BD FACS Canto flow cytometer and CXP software (BD Biosciences, CA, USA) according to a standardized method [24]. The MP gate, based on a particle size of <1.0 µm, was used for identification of MP. Events in the MP gate were assessed for labeling with antibody-positive events to distinguish true events from background noise. The number of CD41+ Annexin V bound MP was identified as MP expressing procoagulant activity.

Microparticles were quantified using a modified Combes method [25] and enumerated by using the formula:

$$(\text{MP count}/\text{bead count}) \times (\text{bead concentration}/\mu\text{L}) = \text{MP count}/\mu\text{L}$$

### 2.6. Statistical analysis

Statistical analyses were performed using PASW Statistics software (version 18; SPSS Inc., Chicago, IL, USA). Sample size was calculated using means, standard deviation and power calculations from our previous data for comparison of platelet aggregation between males and females using a two-tailed *t* test, an alpha of 0.05 and a power of 0.90. All data are presented as mean±S.E.M. Preliminary assumption testing was conducted to check for normality, linearity, outliers and homogeneity of variance with no serious violations observed for all test variables within gender and LCn-3PUFA group. Statistical significance was assessed using one-way analysis of variance (ANOVA) and the *t* test for paired data. Comparisons between gender groups and different LCn-3PUFA groups were made with general linear models using univariate analysis and post hoc Tukey tests. Bivariate correlations were Pearson product moment coefficients. A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

Table 1  
Characteristics of study participants

|                                      | Combined (n=30)  | Male (n=15)      | Female (n=15)    | P value <sup>a</sup> |
|--------------------------------------|------------------|------------------|------------------|----------------------|
| Age (years)                          | 43.7±1.5         | 40.1±2.1         | 47.4±1.9         | .014                 |
| BMI (kg/m <sup>2</sup> )             | 26.1±0.6         | 27.6±0.6         | 24.6±0.8         | .006                 |
| Platelet count (×10 <sup>9</sup> /L) | 256±4.8          | 237±6.1          | 275±6.2          | <.001                |
| Platelet aggregation (AUC)           | 56.4±1.5         | 53.1±2.4         | 59.7±1.9         | .037                 |
| Lag time (s)                         | 60.4±0.03        | 68.0±0.06        | 52.7±0.04        | .043                 |
| MP activity (nM)                     | 23.05±1.1        | 23.0±1.76        | 23.1±1.37        | NS                   |
| CD41+ Annexin V (MP/μl) <sup>b</sup> | 13.7 (11.6–15.7) | 14.0 (11.3–16.7) | 13.4 (10.2–16.6) | NS                   |
| Testosterone (nmol/L)                | 7.8±0.78         | 14.7±0.56        | 1.06±0.08        | <.001                |
| Oestradiol (pmol/L)                  | 144.2±21.5       | 75.0±7.4         | 213.4±39.8       | .001                 |

Values are mean±S.E.M. NS, not significant.

<sup>a</sup> P values were obtained using independent-samples *t* test for males vs. females.

<sup>b</sup> Values reported are ×10<sup>3</sup>; lower and upper quartiles are reported in parentheses.

### 3. Results

#### 3.1. Baseline demographics

All 30 participants completed each treatment (placebo, *n*=30; EPA, *n*=30; DHA, *n*=30). Significant differences in the baseline characteristics were observed between the male and female participants. Females in the study were older, and majority were of postmenopausal age. Males had significantly greater BMI (kg/m<sup>2</sup>), longer lag time (s) and higher levels of testosterone. Females had significantly higher platelet count, higher levels of oestradiol, as well as significantly greater baseline platelet aggregation than males. Despite this, there were no significant gender differences in platelet MP activity or CD41+ Annexin V bound MP (Table 1).

#### 3.2. Relationship between platelet and MP parameters

In the combined population, platelet aggregation was found to be inversely correlated to lag time ( $r=-.765$ ,  $P<.001$ ) and positively correlated to platelet count ( $r=.224$ ,  $P<.05$ ) and MP activity ( $r=.318$ ,  $P=.002$ ), but not to the number of CD41+ Annexin V bound MP (Table 2).

When the data were separated into the gender groups, only males continued to demonstrate the positive correlation between platelet aggregation and MP activity ( $r=.456$ ,  $P=.002$ ) (Fig. 1). In addition, lag time was inversely correlated to platelet MP activity ( $r=-.308$ ,  $P=.039$ ) in this group. In contrast, only the females demonstrated a positive relationship between the number of CD41+ Annexin V bound MP and MP activity ( $r=-.395$ ,  $P=.011$ ).

#### 3.3. Effect of LCn-3PUFA supplementation on platelets and platelet-derived MPs

When the cohort was analyzed as a whole, supplementation with either EPA or DHA was effective at reducing platelet aggregation ( $-13.3\%$ ,  $P=.006$ ;  $-11.9\%$ ,  $P=.016$ , respectively) relative to placebo (Fig. 2). However, only EPA-rich oil produced a decrease in MP activity ( $-19.4\%$ ,  $P=.003$ ), and neither oil resulted in changes to MP numbers (Fig. 3).

When separated by gender, the response to supplementation was differential amongst the male and female subjects. In males, the mean lag time increased by 29.5% (60 vs. 79 s) at 24 h post EPA supplementation. In addition, an inverse relationship was observed between platelet aggregation activity at 24 h post EPA supplementation with testosterone levels ( $r=-0.443$ ,  $P=.04$ ).

EPA was effective only in males, with  $-20.5\%$  ( $P=.008$ ) reduction in platelet aggregation and a similar reduction in MP activity ( $-22\%$ ,  $P=.008$ ), whereas DHA was not significantly effective relative to placebo (Fig. 3). In addition, the mean lag time increased by 29.5% (60 vs. 79 s). In contrast, DHA was effective in reducing platelet aggregation in the female subjects only ( $-13.7\%$ ), but did not significantly affect any of the other parameters. The number of CD41+ Annexin V bound MP was also not affected following either EPA or DHA supplementation in either gender group.

### 4. Discussion

This study demonstrates for the first time that a single dose of EPA-rich oil significantly inhibits platelet MP activity in parallel with a reduction in platelet aggregation, while supplementation with DHA-rich oils reduces platelet aggregation independent of MP activity. The present finding that EPA and DHA affect platelet-derived MP activity differentially strengthens our previous findings of gender-specific platelet aggregation response.

In the combined study population, both EPA and DHA effectively reduced platelet aggregation to a similar extent when compared with placebo. When separated into the male and female groups, the aggregation response pattern was divided between the gender groups such that the antiaggregatory effects of EPA was owing to the male subjects and the effect of DHA was unique to the female subjects. The demonstration of such a striking response pattern clearly suggested differences in the mechanistic pathways whereby these LCn-3PUFAs exert their antiaggregatory effects.

Since platelet aggregation is initiated following platelet activation and platelet activation results in the release of procoagulant MP, we studied the effects of LCn-3PUFA on circulating platelet MP levels and activity. Indeed, our study findings indicate platelet MPs as a potential pathway whereby LCn-3PUFAs may differentially modulate the hemostasis.

Table 2  
Correlations between platelet and MP parameters in the total cohort (*n*=30)

|                      | MP activity               | MP number                | Lag time                   |
|----------------------|---------------------------|--------------------------|----------------------------|
| Platelet aggregation | $r=.318$ , $P$ value=.002 | $r=-.210$ , $P$ value=NS | $r=-.765$ , $P$ value<.001 |
| Lag time             | $r=-.185$ , $P$ value=NS  | $r=.139$ , $P$ value=NS  | –                          |
| MP number            | $r=-.196$ , $P$ value=NS  | –                        | –                          |

Results are bivariate correlation and Pearson coefficient.

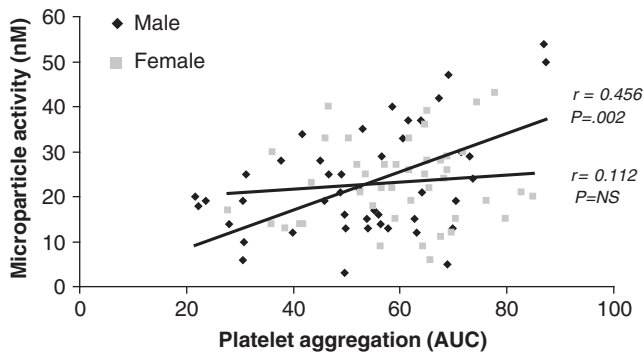


Fig. 1. Gender differences in the relationship between baseline aggregation and MP activity. Results are bivariate correlation and Pearson coefficient for AUC and MP procoagulant activity of baseline sample.

Our findings clearly demonstrate that a single oral dose of EPA-rich oils decreases platelet MP activity in healthy subjects, whereas supplementation with DHA-rich oils is not effective. EPA significantly reduced platelet MP activity by  $-19.4\%$  ( $P=.003$ ) 24 h following supplementation relative to placebo. When the MP data were segregated into the male and female groups, a gender-biased response was once more observed such that the effect of EPA to reduce MP activity was predominantly owing to the male subjects. In males, there was a  $-22\%$  ( $P=.008$ ) reduction in MP activity, whereas in females, the effects of EPA were no longer significant relative to placebo. Interestingly, there were no effects on the number of CD41 + Annexin V bound MP following supplementation, indicative that the antiaggregatory effects of EPA are via the inhibition of the procoagulant MP activity but with no effects on circulating MP levels in plasma. Perhaps, this could be considered more favorable given that the mechanism of MP clearance from the circulation is not known in humans. Therefore, decreasing levels of circulating MP would not be of further benefit if the clearance rate is rapid in humans. Microparticle clearance has been reported to be rapid in animal studies regardless of treatment. Platelet MPs are cleared from the circulation within 30 min following infusion in mice and in less than 10 min in rabbits [26]. The implication that endogenous platelet MPs are rapidly cleared from the circulation suggests that they are generated continuously to maintain baseline levels as they continue to circulate in healthy individuals in the absence of trauma [2]. In our study, reduction in MP activity but not in MP levels suggests that the new platelet MPs generated following EPA supplementation are less procoagulant, possibly due to the incorporation of EPA into the

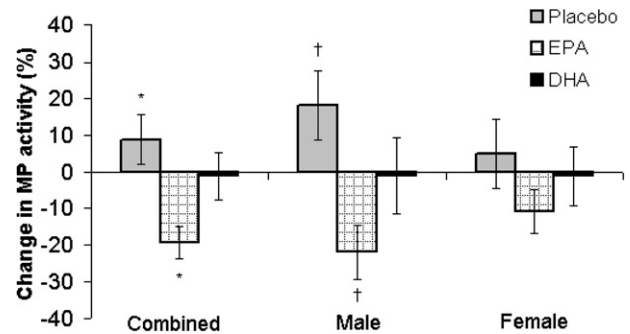


Fig. 3. Effect of LCN-3PUFA on MP activity in males and females. Results are mean MP % change using one-way ANOVA with post hoc Tukey tests; values sharing a common symbol (\*, †) are significantly different.  $P < .05$ .

platelet membrane. The kinetics of the incorporation of LCN-3PUFAs into plasma phospholipids and platelets have been reported in the GISSI-Prevenzione Study. An early and rapid increase of EPA was found in platelets and plasma phospholipids after 1 week following a low dose of LCN-3PUFA, while DHA levels were not affected in platelets [27]. Indeed, EPA is preferentially incorporated into platelets [28] compared with DHA. Of further interest, reports have shown that, in males, EPA incorporation into plasma lipids and platelet phospholipids increased two- to fivefold higher than DHA following fish oil supplementation [28–30].

Consistent with the finding that MP activity was reduced in parallel with platelet aggregation in males, MP procoagulant activity was positively associated with platelet aggregation in the male population only. Furthermore, lag time (time taken to initiate aggregation) was inversely associated with platelet MP activity, and indeed, lag time increased by 29.5% (60 vs. 79 s) in males following EPA supplementation. Lag time is reflective of prothrombin time, and this is important given that MP activity is initiated following the exposure of PS where prothrombin is cleaved into thrombin [5]. The increased lag time in parallel with the decreased MP activity and platelet aggregation observed in our study suggests a potential pathway whereby EPA may reduce platelet aggregation via incorporation of EPA into PS, thus increasing prothrombin time. Indeed, EPA has been reported to be incorporated in PS following EPA supplementation in healthy male subjects [31]. It is also interesting to note that Thorngren and coworkers have reported that an EPA-enriched diet significantly increased prothrombin time and bleeding time in a population of healthy male individuals [32]. The observed effects of EPA in our study may be owing to the possibility that EPA is more effectively incorporated into platelets in males than females, while females are able to retain DHA more efficiently. Furthermore, analysis of plasma fatty acids in our study cohort also revealed that circulating DHA levels were increased only in female subjects 24 h following DHA supplementation (unpublished data). Indeed, females have a greater capacity to synthesize DHA compared to males [33–35]. Estrogens have been shown to increase DHA synthesis, while testosterone has been reported to decrease DHA concentrations [36]. Higher synthesis and/or retention of DHA in females compared to males may explain the observed greater inhibition of platelet aggregation in females.

Gender differences observed in platelet aggregation could also be due to the interaction between sex hormones to influence circulating levels of EPA/DHA and thus their mechanisms of action. It has been suggested that the effects of DHA are surface mediated [29,37], thus possibly acting upon surface cell adhesion molecules (CAMs) or receptors. It has been reported that DHA content of granulocytes is negatively associated with intercellular CAM-1 (ICAM-1) levels, while granulocyte content of EPA is not correlated with ICAM-1 or vascular CAM-1 levels [38]. Furthermore, P-selectin has been reported to be inversely associated with estrogen levels [39]; indeed, females are

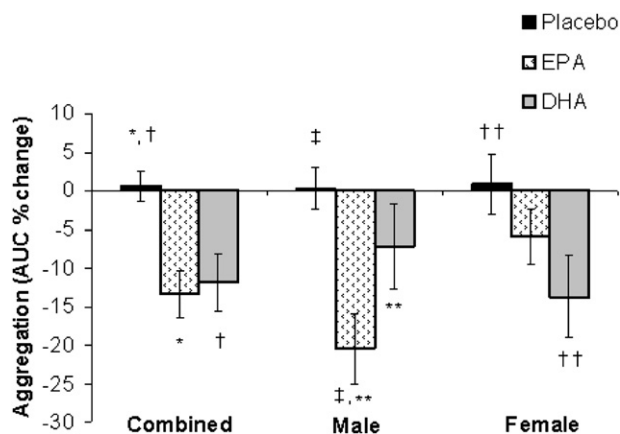


Fig. 2. Effect of LCN-3PUFA on platelet aggregation in males and females. Results are mean AUC % change using one-way ANOVA with post hoc Tukey tests; values sharing a common symbol (\*, †, ††, \*\*, †††) are significantly different.  $P < .05$ .



reported to have significantly lower levels of CAM expression [40,41] and higher levels of DHA [42].

Particularly striking was that a positive relationship was observed between the number of CD41+ Annexin V bound MP and platelet aggregation in the female population. This suggests that, in females, platelet aggregation is predominately dependant on the level of circulating MPs. Further supporting this observation, circulating MP levels were not affected following EPA supplementation; hence, platelet aggregation was also not reduced. Females in our study had a higher platelet count, which may have also allowed for increased MP generation with respect to increased platelet numbers. MP numbers were not affected following DHA supplementation in females, which suggests that DHA reduces platelet aggregation in females independent of MP numbers and activity.

The lack of effect of EPA to reduce platelet aggregation in females is further strengthened by the finding that MP activity was not affected in females following EPA supplementation. Furthermore, DHA was not effective in reducing MP activity or bearing any relationship with baseline MP activity in both male and female subjects. These findings confirm two things: (a) DHA acts to reduce platelet aggregation differentially, and (b) DHA is not effective to reduce platelet aggregation in males.

No other studies have investigated the differential effects of LCn-3PUFA supplementation on platelet MP in healthy individuals; however, combined EPA/DHA supplementation in patients with previous myocardial infarction has been shown to significantly reduce both the number and procoagulant activity of platelet-derived MPs over a 12-week period [43]. It is possible that while MP activity can be immediately reduced via incorporation of LCn-3PUFA into the platelet membrane, the production of MPs may take longer to stabilize. Alternatively, the increased levels of MPs in this group of patients may be the result of a pathological condition that simply does not exist in a healthy cohort, and thus treatment with LCn-3PUFA will not reduce MP levels below a normal range. We are presently investigating this hypothesis with a longer intervention trial.

The mechanisms by which acute supplementation with EPA/DHA-rich oil alters platelet aggregation or MP activity are not clear. Since EPA/DHA levels were significantly increased following supplementation for 24 h, it is likely that at least some of EPA/DHA may have made their way into platelet membranes via exchange process and/or direct uptake. To date, no study has examined the acute effects of EPA/DHA supplementation on fatty acid incorporation into platelet membranes. This is an important point that warrants examination in future studies.

Though the precise mechanism involved in the protection against thrombotic disease risk cannot be elucidated, our study findings clearly indicate that both EPA and DHA reduce platelet aggregation in males and females differentially. The novel finding that EPA inhibits MP activity in male subjects further strengthens our previous findings of the gender-specific platelet aggregation response of EPA vs. DHA. Hence, our study suggests that supplementation with EPA but not DHA inhibits platelet MP activity in parallel with a reduction in platelet aggregation in a gender-specific manner. Our finding that EPA-rich oils inhibit procoagulant MP activity is important given that thrombosis remains one of the leading causes of mortality in developed countries and platelet MPs are now recognized as pathogenic markers of thrombotic disease.

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M.P. participated in the conception, study design, participant recruitment, and sample and data analysis. L.L.F. and M.S. were involved in the sample and data analysis and provided significant advice. M.L.G. was involved in the design and coordination of the study and provided significant advice and consultation. All of the authors contributed to the revisions and subsequent drafts and reviewed the final version of the manuscript. The authors declare no conflict of interest.

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