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**ARTICLE**

# Supplementation with omega-3 or omega-6 fatty acids attenuates platelet reactivity in postmenopausal women

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## Abstract

Postmenopausal women are at increased risk for a cardiovascular event due to platelet hyperactivity. There is evidence suggesting that ω-3 polyunsaturated fatty acids (PUFAs) and ω-6 PUFAs have cardioprotective effects in these women. However, a mechanistic understanding of how these fatty acids regulate platelet function is unknown. In this study, we supplemented postmenopausal women with fish oil (ω-3 fatty acids) or evening primrose oil (ω-6 fatty acids) and investigated the effects on their platelet activity. The effects of fatty acid supplementation on platelet aggregation, dense granule secretion, and activation of integrin αIIbβ3 at basal levels and in response to agonist were tested in postmenopausal women following a supplementation and washout period. Supplementation with fish oil or primrose oil attenuated the thrombin receptor PAR4-induced platelet aggregation. Supplementation with ω-3 or ω-6 fatty acids decreased platelet dense granule secretion and attenuated basal levels of integrin αIIbβ3 activation. Interestingly, after the washout period following supplementation with primrose oil, platelet aggregation was similarly attenuated. Additionally, for either treatment, the observed protective effects post-supplementation on platelet dense granule secretion and basal levels of integrin activation were sustained after the washout period, suggesting a long-term shift in platelet reactivity due to fatty acid supplementation. These findings begin to elucidate the underlying mechanistic effects of ω-3 and ω-6 fatty acids on platelet reactivity in postmenopausal women. Hence, this study supports the beneficial effects of fish oil or primrose oil supplementation as a therapeutic intervention to reduce the risk of thrombotic events in postmenopausal women. https://clinicaltrials.gov/ct2/show/NCT02629497.

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Adriana Yamaguchi and Livia Stanger authors contributed equally to this work.

**Study Highlights**

**WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?**
Postmenopausal women are at increased risk for cardiovascular disease due to platelet hyperactivity. Evidence suggests that ω-3 and ω-6 fatty acids have cardioprotective effects in postmenopausal women. There is limited information about how ω-3 and ω-6 fatty acids regulate platelet function in postmenopausal women.

**WHAT QUESTION DID THE STUDY ADDRESS?**
Does supplementation with ω-3 or ω-6 fatty acids provide long-term cardiovascular protection for postmenopausal women through attenuation of platelet reactivity and minimizing the risk for thrombosis?

**WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?**
Treatment of postmenopausal women with fish oil and evening primrose oil results in both acute and chronic protection from thrombosis beyond the duration of supplementation due to decreased integrin activation on the platelet surface and decreased positive feedback as a result of reduced granule secretion from the platelet.

**HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?**
Due to the significant disparity in thrombotic risk in postmenopausal women compared to men, supplementation with fish oil or evening primrose oil represents a minimally invasive and safe method for preventing blood clots.

# INTRODUCTION

The risk of cardiovascular disease (CVD) in women has been underestimated due to the grouping of women irrespective of age, resulting in the conclusion that women are protected against CVD.[1] Premenopausal women have innate cardiovascular protection, which is lost during the menopausal transition, when their risk for CVD, myocardial infarction, stroke, and thrombotic events surpasses that of the male population in the same age range.[2,3] This has long been considered to be due to the abrupt fall in endogenous estrogen production.[2] However, the Women's Health Initiative study followed several clinical studies that evaluated the effects of hormone replacement therapy (HRT) on cardiovascular protection of postmenopausal women, all of which reported no positive or pathological effects of HRT.[4,5] In addition to the loss of innate protection, postmenopausal women experience several phenotypic and metabolic changes during the menopausal transition, affecting body weight, adipose tissue distribution, energy expenditure, and insulin secretion and sensitivity.[6,7] These changes have been associated with a rise in blood pressure and cholesterol levels,[1] and the development of diabetes, atherosclerosis, and/or hypertension, which are major risk factors for CVD and thrombosis.[6,7] As a result of the increased life-span, postmenopausal women have become a growing percentage of the population at an increased risk for a thrombotic event.[2]

Platelets play an essential role in cardiovascular health by modulating hemostatic and thrombotic responses to vascular injuries in blood vessels.[8,9] Aberrant platelet activation is a major contributor to pathological thrombosis, leading to stroke and myocardial ischemia.[10,11] Notably, postmenopausal women have increased expression of markers of platelet activation, including P-selectin expression and activation of integrin αIIbβ3.[12] Although dual antiplatelet therapy consisting of aspirin and a $P2Y_{12}$ receptor antagonist is clinically used to attenuate platelet activation,[13] studies have indicated that a sex-related response to antiplatelet drugs exists.[1,14] Additionally, studies have shown that women have a lower reduction in CVD events than men, despite antiplatelet therapy.[14,15]

Polyunsaturated fatty acids (PUFAs) are able to regulate platelet function and hemostasis.[16–18] In postmenopausal women, increased serum levels of omega-3 (ω-3) and omega-6 (ω-6) PUFAs were associated with both a lower incidence of ischemic stroke[19] and inversely associated with coronary heart disease risk.[20] Moreover, the ingestion of ω-3 fatty acids has been directly associated with protection against the progression of coronary atherosclerosis and stenosis in postmenopausal women with coronary artery disease.[21] Although these studies suggest that ω-3 and ω-6 fatty acids may have cardioprotective effects, there are limited data on the regulatory effects of dietary supplementation with these fatty acids on platelet activity in postmenopausal women.

In this study, we sought to determine whether supplementation with ω-3 or ω-6 fatty acids attenuates platelet reactivity in postmenopausal women. In addition, we aimed to determine the mechanism by which platelet function is altered in postmenopausal women supplemented with these fatty acids. Therefore, we conducted

a randomized, double-blind, two-period crossover trial, consisting of a 60-day supplementation period followed by a 14-day washout period in between and at the end of the study.

## METHODS

### Study design

#### Subject enrollment and retention

This study was approved by the University of Michigan Institutional Review Board (IRB) and all subjects enrolled in this study completed a written consent. Healthy postmenopausal women were recruited from the Ann Arbor community and diabetic postmenopausal women were recruited from the University of Michigan Medical Center. All subjects were recruited under study protocols approved by the University of Michigan IRB between November 2015 and March 2017. Enrollment criteria included postmenopausal women, defined by the absence of a menstrual cycle for 12 consecutive months, aged 40–70 years old that were healthy or diagnosed with type 2 diabetes mellitus. Exclusion criteria included inability or refusal to provide consent, history of a blood related disorder, or personnel under the direct supervision of the principal investigator of the study. The sample-size calculation was powered for outcomes related to fold-changes in αIIbβ3 activation. A sample size of $N = 40$ would be required to provide at least 95% power to detect a difference by analysis of variance and at least 80% power to detect a pairwise comparison difference in means of size two, even if the standard deviation is as large as 3.5. A total of 90 postmenopausal women were enrolled to participate in the study. Subjects were randomly assigned to groups who received daily supplementation with either omega-3 (ω-3) or omega-6 (ω-6) fatty acid supplementation with a subsequent crossover. Prior to the start of the study, subjects were required to refrain from taking dietary supplements for 14 days, fish and plant oils for 30 days, and nonsteroidal anti-inflammatory drugs and aspirin for 7 days. On day 0, blood was drawn prior to the beginning of treatment and demographic information was collected for each patient, including age, sex, race, body mass index (BMI), and medications.

#### Fatty acid supplements

Nature's Bounty fish oil supplements were used for this study due to their commercial availability and subsequent translatability to over-the-counter use. Due to the fact that fatty acid supplements are classified as nutraceuticals, they are deemed safe for human use. The ω-3 fish oil supplement (1000 mg gel capsule), contained 300 mg (30%) ω-3 fatty acids, including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and other fatty acids. The ω-6 enriched supplement, known as evening primrose oil (1000 mg gel capsule), contained 730 mg cis-linoleic acid and 90 mg (9%) gamma-linolenic acid (GLA). Because platelets exist in the blood for 7–10 days, in order to assess acute versus chronic effects on the platelet of fatty acid supplementation, following each supplementation period, a 14-day washout period was initiated to allow all of the platelets that had been directly exposed to the supplement to be cleared from the blood. Each treatment course lasted 60 days with the 14-day washout period in between, as well as a 14-day washout at the end of the study. The supplement was ingested twice daily for a total of 2 g/day, and blood was drawn when subjects returned on day 60, at the end of treatment. Subjects returned to have blood drawn after the 14-day washout period.

### Measurement of fatty acid content in the platelet

Fatty acid methyl ester (FAME) samples were prepared as follows. Platelet pellets were resuspended to 1 ml with anhydrous 3 N MeOH/HCl, sonicated and transferred to Teflon screw-capped glass vials. 2.5 μl of a 10 mg/ml solution of Heptadecenoic acid in anhydrous methanol was added to each vial as an internal standard. The samples were placed in a dry bath at 90°C for 1 h. The FAME samples were extracted using 2 ml of n-hexane three times, dried under nitrogen and then reconstituted in 100 μl of n-hexane. The FAME samples were analyzed by injecting 5 μl of each sample onto a gas chromatograph mass spectrometer (Hewlett Packard 5890 Series II) equipped with a 30-m, 0.25 mm high polar phase capillary column (Restek-2330) and a 7673 controller and injector. The oven temperature started at 70°C and ended at 210°C with a 20°C/min increase. A FAME reference standard (NU-Chek Prep, GLC M13-N) was injected immediately before and after the unknown samples to identify peaks by their retention time and mass spectra. FAME samples not found in this standard mix were identified through their mass spectra alone.

### Preparation of washed human platelets

Whole blood was collected from subjects via venipuncture of the antecubital vein into vacutainers containing

sodium citrate (3.2%; Greiner Bio-One). Platelet-rich plasma (PRP) was obtained through centrifugation at 200 g for 10 min with no brake. The PRP was then treated with acid citrate dextrose (2.5% sodium citrate tribasic, 1.5% citric acid, and 2.0% D-glucose) and apyrase (0.02 U/ml) and centrifuged at 2000 g for 10 min with no brake, thereby pelleting the platelets. The platelets were resuspended in Tyrode's buffer (10 mM N-2-hydroxyethylpiperazine-N9-2-ethanesulfonic acid, 12 mM sodium bicarbonate, 127 mM sodium chloride, 5 mM potassium chloride, 0.5 mM monosodium phosphate, 1 mM magnesium chloride, and 5 mM glucose), and brought to a concentration of $3 \times 10^8$ platelets/ml, which was confirmed using a complete blood cell counter (Hemavet 950FS; Drew Scientific).

## Platelet aggregation

Washed platelets from subjects (250 μl at $3 \times 10^8$ platelets/ml) were stimulated with various concentrations of protease-activated receptor 1 activating peptide (PAR1-AP; 0.5, 1, 2.5, and 5 μM), protease-activated receptor 4 activating peptide (PAR4-AP; 10, 25, 50, and 75 μM), thrombin (0.1, 0.25, 0.5, and 1 nM), or collagen (0.125, 0.25, 0.5, and 1 μg/ml), without the addition of extracellular calcium. The concentration range used for each agonist was based on a known concentration response range for the ligand-receptor response in platelets. Aggregation of the subjects' platelets was measured pre-supplementation and post-supplementation with fish oil or primrose oil, as well as post-washout. Platelet aggregation was measured in a lumi-aggregometer (Model 700D; Chrono-log). Light transmission was monitored in real time for 10 min at 37°C under stirring conditions (1100 rpm).

## Flow cytometry

Washed platelets (50 μl at $3 \times 10^8$ platelets/ml) from subjects' pre-supplementation, post-supplementation, and post-washout were incubated with 3 μl of PAC-1 (BD Pharmingen), an active αIIbβ3-specific fluorescein isothiocyanate (FITC)-conjugated antibody, protected from light at 37°C for 10 min prior to stimulation with PAR1-AP, PAR4-AP, thrombin, or convulxin (CVX). CVX is a direct activator of the collagen receptor GPVI. Reactions were stopped by adding 50 μl of 2% paraformaldehyde and samples were incubated and protected from light at room temperature for 10 min. Fluorescence intensity was measured via flow cytometry (Accuri C6; BD Biosciences).

## Dense granule secretion

Adenosine triphosphate (ATP) release was measured from washed platelets (245 μl at $3 \times 10^8$ platelets/ml) as a surrogate for dense granule secretion, as previously described.[22] Prior to activation, washed platelets were incubated with 5 μl of Chrono-Lume luciferin-luciferase reagent (CHRONO-LOG), an ATP-sensitive dye, protected from light at 37°C for 1 min. Platelets were stimulated with PAR1-AP (0.5, 1, 2.5, and 5 μM), PAR4-AP (10, 25, 50, and 75 μM), thrombin (0.1, 0.25, 0.5, and 1 nM), or collagen (0.125, 0.25, 0.5, and 1 μg/ml) under stirring conditions. Fluorescence was measured in real-time using a Lumi-Aggregometer (CHRONO-LOG Model 700D).

## Statistical analyses

Unpaired and paired two-tailed Student's $t$-tests and mixed-effects analyses with Dunnett's multiple comparison post-test were performed using the Prism GraphPad Software for analysis. The statistical test used in each assay is noted in the figure legend. The data represent mean ± SEM for platelet aggregation, flow cytometry, and dense granule secretion. The data represent mean ± standard deviation (SD) for subjects' baseline characteristics and measurement of platelet fatty acid content.

# RESULTS

## Study population

Ninety postmenopausal women were screened and enrolled in the study; diabetic subjects were found through a registry at the University of Michigan that allowed us to screen for eligibility, and healthy subjects responded to a flier indicating the criteria that needed to be met in order to participate in the trial. A total of 78 subjects completed the study, with 12 subjects dropping out (Figure S1). All 12 subjects that were discontinued dropped out voluntarily during the study due to physician recommendation (3) or noncompliance (8), whereas one subject elected to drop out without indicating a specific reason. The demographic and clinical characteristics of the subjects completing the study are described in Table 1. The majority of the women were White (84.6%), the mean age was 60.67 ± 4.69 years, and the mean BMI was 29.37 ± 6.60 kg/m$^2$, which is considered overweight (BMI = 25–30 kg/m$^2$).[23] Approximately 50% of the postmenopausal women were being treated for diabetes, hypertension, and/or dyslipidemia. In addition, 39.7% of the subjects were taking neurological medications and

**TABLE 1**  Subject baseline characteristics

| N | 90 |
|---|---|
| Mean age (years) | 60.83 ± 4.77 (51–70) |
| Mean BMI (kg/m2) | 29.80 ± 6.73 (16.8–48.5) |
| Race | |
| White | 76 (84.4%) |
| African American | 11 (12.2%) |
| Other | 3 (0.03%) |
| Medication use | |
| Antihypertensive | 43 (47.78%) |
| ACE inhibitors | 24 (26.67%) |
| ARBs | 12 (13.33%) |
| Diuretics | 21 (23.33%) |
| Vasodilators | 12 (13.33%) |
| β-Blockers | 16 (17.78%) |
| α-adrenergic agonists | 1 (1.11%) |
| Dyslipidemia | 42 (46.67%) |
| Statins | 41 (45.46%) |
| Cholesterol absorption inhibitors | 1 (1.11%) |
| Fibrates | 3 (3.33%) |
| Neurological | 39 (43.33%) |
| Antidepressants | 45 (50%) |
| Sedatives | 6 (6.67%) |
| Anxiolytics | 2 (2.22%) |
| Anticonvulsants | 14 (15.56%) |
| Antipsychotics | 2 (2.22%) |
| Narcotics | 5 (5.56%) |
| Immunomodulatory | 28 (31.11%) |
| Immunosuppressors | 7 (7.78%) |
| Antihistamines | 20 (22.22%) |
| Anti-inflammatory | 7 (7.78%) |
| Anti-diabetic | 45 (50%) |
| Incretin mimetics | 3 (3.33%) |
| Sulfonylureas | 8 (8.89%) |
| Fast-acting insulin | 9 (10%) |
| Long-acting insulin | 10 (11.11%) |
| DPP-4 Inhibitors | 5 (5.56%) |
| Biguanides | 36 (40%) |
| Baseline CBC (n = 88) | |
| WBC (K/µl) | 6.37 ± 2.41 (3.26–18.3) |
| RBC (K/µl) | 5.39 ± 0.44 (4.55–6.6) |
| MVC (fl) | 84.54 ± 5.67 (66.6–100.2) |
| PLT (K/µl) | 339.09 ± 73.24 (200–548) |
| MPV (fl) | 9.42 ± 2.11 (0–21.1) |

*Note*: Subject baseline characteristics shown as number (percentage) for race and medication use, and mean ± standard deviation (SD) for age, BMI, and CBC. Range is given in parenthesis.

Abbreviations: BMI, body mass index; CBC, complete blood count; MCV, mean corpuscular volume; MPV, mean platelet volume; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

34.6% were prescribed immunomodulatory medications. At baseline, the mean complete blood count values were found to be within the normal range.

## Fish oil or evening primrose oil supplementation affects platelet ω-3 and ω-6 fatty acids content in postmenopausal women

To determine if supplementing postmenopausal women with fish oil or evening primrose oil affected the ω-3 and/or ω-6 fatty acid content in platelets, the fatty acid content was assessed at baseline, following the 60 days of supplementation and after a 14-day washout period (Tables S1 and S2). Supplementation with fish oil increased the levels of the ω-3 fatty acids, DPA (≈0.4 µg, 0.204 to 0.6), and DHA (≈0.5 µg, 0.234–0.726). The content of the ω-6 fatty acids, GLA and DGLA, was also higher after supplementation. GLA levels ranged from 0.148 µg at baseline to 0.431 µg post-supplementation, whereas DGLA levels were increased ≈0.2 µg (0.232–0.411) post-supplementation (Figure 1b). Following the 60-day supplementation with evening primrose oil, an increase in the levels of $DPA_{\omega-3}$ and DHA was observed, ranging from 0.204 to 0.526 µg and from 0.234 to 0.650 µg, respectively, when compared to baseline. Additionally, whereas GLA levels increased (≈0.3 µg, 0.148–0.429), the content of DGLA was higher (≈0.1 µg, 0.232–0.341) post-supplementation (Figure 1c). The increased content of ω-3 and ω-6 fatty acids in the platelet was sustained after 14 days of washout, following either fish oil or primrose oil supplementation. Following the washout period after supplementation with fish oil, the levels of $DPA_{\omega-3}$ and DHA were higher (≈0.5 µg, 0.204–0.677 and ≈0.6 µg, 0.234–0.815, respectively) compared to baseline. The levels of the ω-6 fatty acids, GLA and DGLA, also increased following washout. Whereas GLA levels ranged from 0.148 µg at baseline to 0.249 µg, DGLA content increased from 0.232 µg at baseline to 0.330 µg post-washout. The levels of the ω-3 and ω-6 fatty acids were also observed to be higher following the 14-day washout after supplementation with evening primrose oil compared to baseline. The content of $DPA_{\omega-3}$ and DHA was increased (≈0.4 µg, 0.204–0.571 and ≈0.5 µg, 0.234–0.742, respectively). In addition, whereas GLA content was 0.311 µg post-washout versus 0.148 µg at baseline, DGLA levels were 0.294 µg post-washout versus 0.232 µg at baseline. Regarding whether supplementation affected other fatty acids from the ω-3 and ω-6 families, we did not observe a significant difference in the platelet content of the ω-3 ALA and the ω-6 AA following supplementation or washout (Tables S1 and S2). The level of the ω-6 DPA fatty acid was not analyzed due to co-migration with a
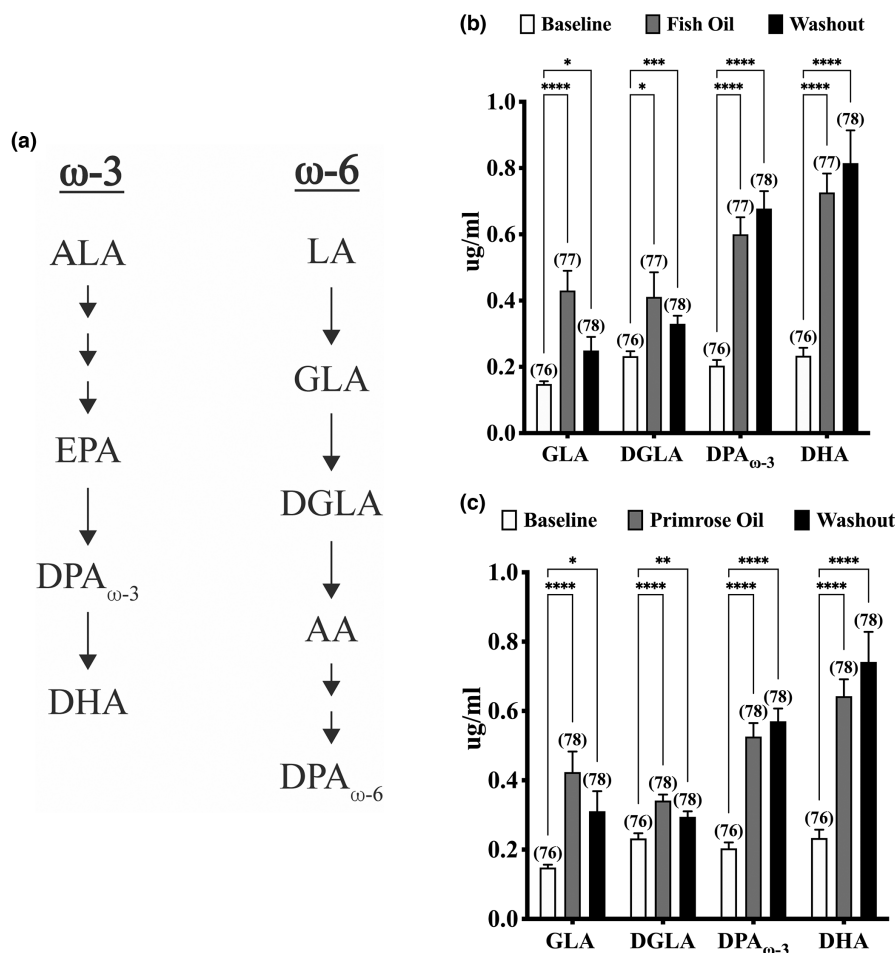
**FIGURE 1** Fish oil or evening primrose oil supplementation enhances ω-3 and ω-6 fatty acid content in platelets. (a) Schematic figure of major fatty acids produced by the precursor ω-3 α-linolenic fatty acid (ALA) and the precursor ω-6 linoleic fatty acid (LA). (b) Platelets from subjects supplemented with fish oil and (c) platelets from subjects supplemented with evening primrose oil were measured at pre-supplementation (baseline), after 60 days of supplementation (fish oil or primrose oil) and after 14-day washout period (washout) for docosapentaenoic acid ω-3 (DPAω-3), docosahexaenoic acid (DHA), γ-linolenic acid (GLA), and dihomo-γ-linolenic acid (DGLA). Numbers are given in parenthesis. The data represent mean ± SD. A mixed-effects analysis with Dunnett's multiple comparison post-hoc test was performed. Asterisks indicate statistically significant differences between groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

contaminating fatty acid, as observed through mass spectrometry analysis, but it is reported in Tables S1 and S2.

## Fish oil supplementation in postmenopausal women attenuates PAR4-AP-induced platelet aggregation

The effect of fish oil supplementation on agonist-induced platelet aggregation was assessed following completion of the 60-day supplementation period. To assess potential changes in platelet activity, platelet function was measured at three timepoints, (1) pre-supplementation, (2) post-supplementation, and (3) post-washout (Figure 2). Washed platelets were stimulated with increasing concentrations of the thrombin PAR1-AP (0.5, 1, 2.5, and 5 μM), PAR4-AP (10, 25, 50, and 75 μM), thrombin (0.1, 0.25, 0.5, and 1 nM),

or collagen (0.5, 1, 2.5, and 1 μg/ml). Following supplementation, PAR4-induced platelet aggregation was attenuated at 10 and 25 μM. Whereas at 10 μM, aggregation decreased from 8.65% at baseline to 2.51% post-supplementation, at 25 μM, a reduction from 33.74% to 21.67% was observed post-supplementation compared to baseline. Additionally, platelets stimulated with low levels (10 μM) of PAR4-AP showed a decrease in aggregation (8.65–2.45%) after post-washout period compared to baseline (Figure 2b).

## Primrose oil supplementation in postmenopausal women attenuates PAR-4-induced platelet aggregation

Similar to the protocol described above for fish oil supplementation, washed platelets were prepared following evening
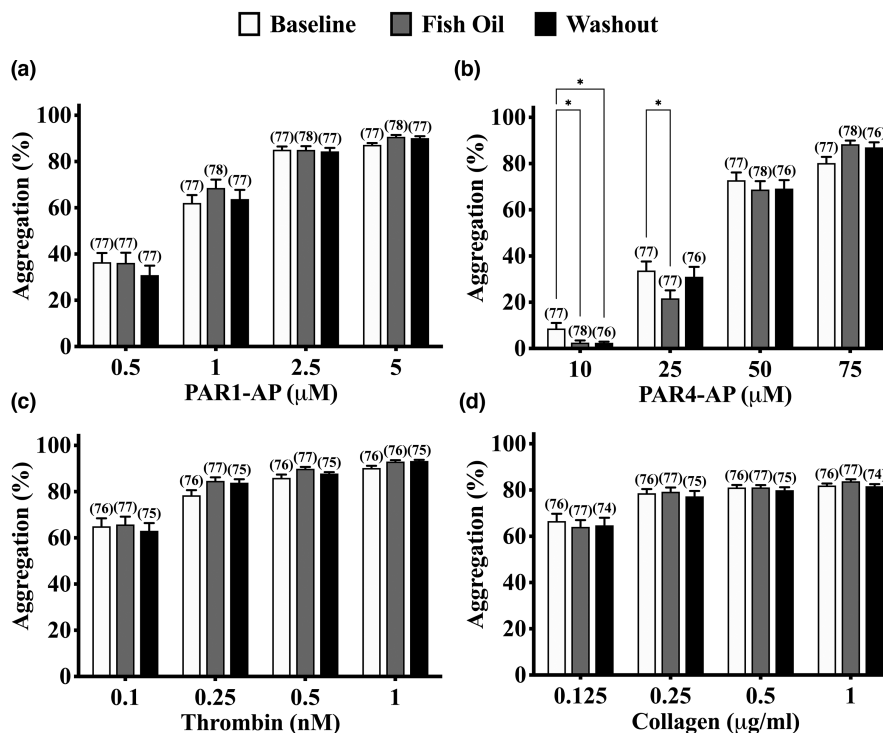
**FIGURE 2** Fish oil supplementation in postmenopausal women attenuates PAR4-induced platelet aggregation. Platelet aggregation was tested at pre-supplementation (baseline), after 60 days of supplementation (fish oil), and after a 14-day washout period (washout) in the presence of a variety of agonists (a) PAR1-AP, (b) PAR4-AP, (c) thrombin, or (d) collagen. Numbers are given in parenthesis. The data represent mean $\pm$ SEM. A mixed-effects analysis with Dunnett's multiple comparison post-hoc test was performed. Asterisks indicate statistically significant differences between groups (*$p < 0.05$).

primrose oil supplementation with blood being obtained pre-supplementation, post-supplementation, and post-washout. Washed platelets were stimulated with PAR1-AP, PAR4-AP, thrombin, or collagen and the degree of aggregation was measured (Figure 3). When the subject samples were stimulated with low levels (10 or 25 μM) of PAR4-AP, platelets displayed a significant decrease in aggregation post-supplementation (Figure 3b). Whereas at 10 μM, supplementation attenuated aggregation (8.65%–1.10%) compared to baseline, at 25μM, platelet aggregation decreased from 33.74% at baseline to 22.31% post-supplementation. No difference in the level of platelet aggregation was observed following stimulation with PAR1-AP, thrombin, or collagen in any of the conditions tested.

## Basal activity of integrin αIIbβ3 is lowered following supplementation and washout, but attenuation in agonist-induced αIIbβ3 activation is only observed post-washout

In order to assess if fish oil or primrose oil supplementation attenuates integrin αIIbβ3 activation on platelets, active αIIbβ3 was measured by flow cytometry in unstimulated platelets and in response to agonist with PAC1, an antibody known to bind only to active αIIbβ3. Post-supplementation, the basal levels of active αIIbβ3 showed a marked decrease in both fatty acid supplementation groups (Figure 4a,b). Compared to baseline, fish oil supplementation reduced αIIbβ3 activation by 23% (from 1051 to 810.36 MFI), whereas supplementation with evening primrose oil reduced integrin activation by 28% (1051–760.74 MFI), compared to baseline. After the 14-day washout period, the significant decrease observed for active αIIbβ3 was maintained for both fatty acid supplementation groups (Figure 4c,d). Following the washout period after either fish oil or primrose oil supplementation, αIIbβ3 activation was lower by 30% (from 1051 to 729.96 MFI and 1051 to 729.58 MFI, respectively) compared to baseline. The effects of fatty acid supplementation were also assessed in response to stimulation by different agonists. Post-washout following supplementation with fish oil, active αIIbβ3 was attenuated in response to PAR1-AP and PAR4-AP (Figure 4e). Whereas with PAR1-AP, αIIbβ3 activation was attenuated by 20% (2484.48–1980.86 MFI), upon PAR4-AP exposure, integrin activation was reduced by 13% (3716.06–3231.43 MFI) compared to baseline. The attenuation of αIIbβ3 activation observed in platelets post-washout following supplementation with evening primrose oil ranged from 16% for CVX (4049.32–3396.44 MFI),
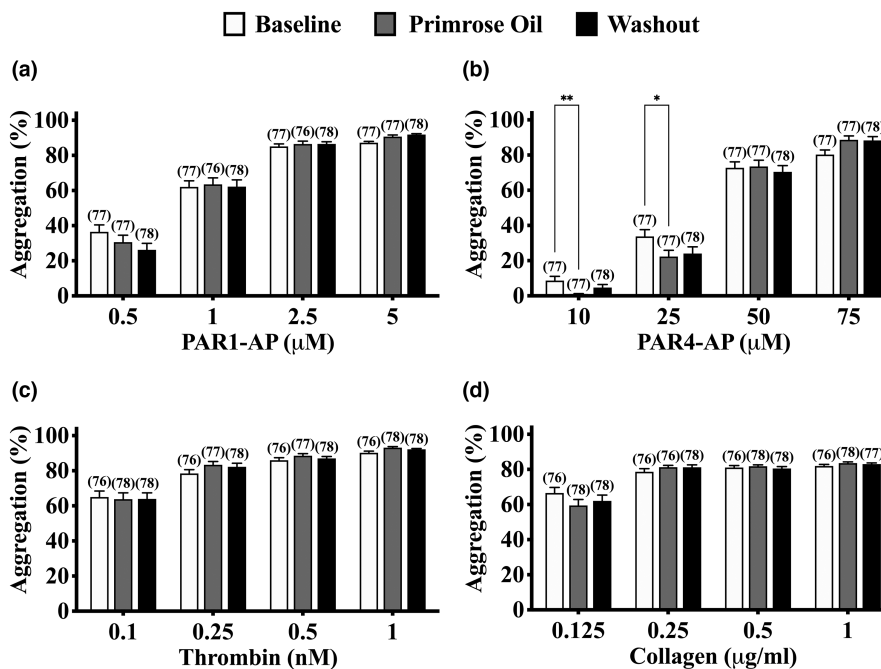
**FIGURE 3** Primrose oil supplementation in postmenopausal women attenuates PAR4-induced platelet aggregation. Platelet aggregation was tested at pre-supplementation (baseline), after 60 days of supplementation (primrose oil), and after a 14-day washout period (washout) in the presence of a variety of agonists (a) PAR1-AP, (b) PAR4-AP, (c) thrombin, or (d) collagen. Numbers are given in parenthesis. The data represent mean $\pm$ SEM. A mixed-effects analysis with Dunnett's multiple comparison post-hoc test was performed. Asterisks indicate statistically significant differences between groups (*$p < 0.05$, **$p < 0.01$).

17% for PAR4-AP (3716.06–3078.06 MFI) to 41% (2484.48–1457.41 MFI) in response to PAR1-AP, compared to baseline (Figure 4f).

## Postmenopausal women exhibit attenuated dense granule secretion when supplemented with fish oil

To assess if ω-3 fatty acid supplementation attenuates platelet dense granule release, washed platelets from subjects' post-supplementation were stimulated with varying concentrations of PAR1-AP, PAR4-AP, thrombin, or collagen. A statistically significant decrease in ATP secretion was observed for all agonists both post-supplementation and post-washout (Figure 5).

## Postmenopausal women exhibit attenuated dense granule secretion when supplemented with evening primrose oil

Subjects were assessed for the degree of dense granule secretion prior to and following supplementation with evening primrose oil. Following evening primrose oil supplementation, subjects exhibited a statistically significant decrease in ATP secretion when stimulated with

PAR1-AP, PAR4-AP, thrombin, or collagen (Figure 6). This effect was preserved for all agonists following the washout period.

## DISCUSSION

Platelet hyperactivity is a critical contributor to the increased risk for cardiovascular diseases observed in postmenopausal women.[24] In this study, we demonstrated that supplementing postmenopausal women with ω-3 or ω-6 PUFAs attenuated platelet reactivity. Previously, the Women's Health Initiative study showed that increased ω-3 phospholipid levels in the plasma of postmenopausal women were associated with lower risk of coronary heart disease.[20] In addition, clinical studies with postmenopausal women that received supplementation with fish oil indicated cardioprotective effects through the inhibition of lipid peroxidation[25] and low-density lipoprotein oxidation.[26] However, how the ω-3 and ω-6 PUFA regulation of platelet function contributes to the protection of postmenopausal women from a thrombotic event remains unclear.

The increased risk of postmenopausal women for a cardiovascular event can be explained mechanistically due to the fact that their platelets exhibit an increase in many biochemical end points of platelet activation, including the active form of the platelet integrin αIIbβ3 and P-selectin
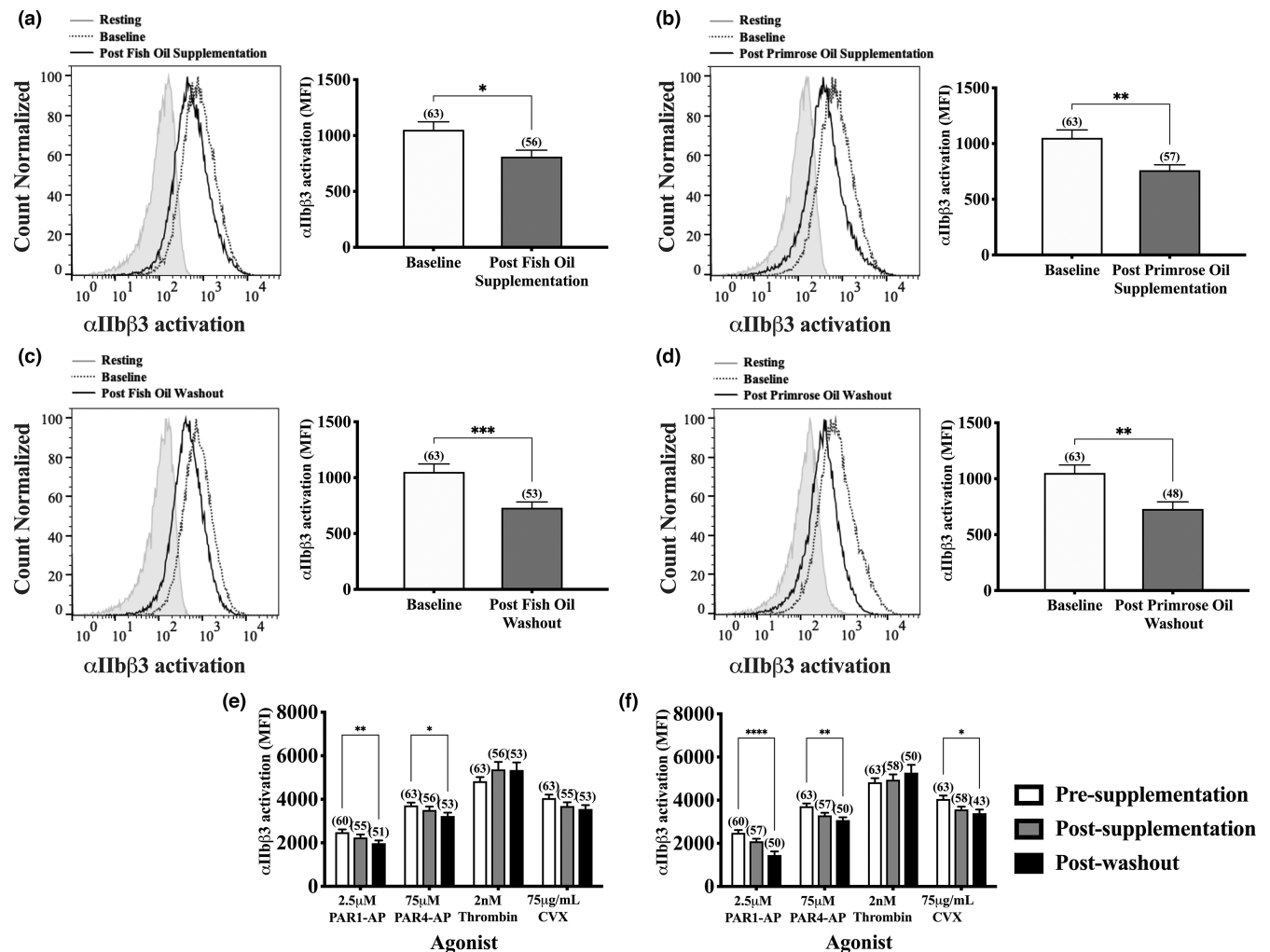
**FIGURE 4** Basal activity of integrin αIIbβ3 is lowered following supplementation and washout, but attenuation in agonis-induced αIIbβ3 activation is only observed post-washout. Basal activation of αIIbβ3 was measured before supplementation (baseline), after 60 days of supplementation with either (a) fish oil (post fish oil supplementation) or (b) evening primrose oil (post primrose oil supplementation) and (c, d) after a 14-day washout period (post fish oil washout AND post primrose oil washout). Agonist-induced αIIbβ3 activation was assessed at same timepoints for (e) fish oil and (f) primrose oil supplementation. Numbers are given in parenthesis. The data represent mean ± SEM. An unpaired two-tailed $t$-test was performed to determine significance. Asterisks indicate statistically significant differences between groups ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). MFI refers to mean fluorescence intensity.

expression.[12,27] We have demonstrated that supplementation with either fish oil or evening primrose oil decreased levels of basal integrin αIIbβ3 activation in postmenopausal women (Figure 4). However, we did not observe an effect on platelet P-selectin expression following supplementation with either fish oil or evening primrose oil (data not shown). Integrin αIIbβ3 is the dominant integrin on the surface of platelets and is essential for normal platelet function, including adhesion, spreading, and aggregation.[28] Importantly, increased αIIbβ3-mediated platelet activation is involved in pathological thrombosis[29] and is the target for an entire class of antiplatelet agents used in the clinic.[30,31] The data presented here supports that supplementing postmenopausal women with ω-3 or ω-6 fatty acids attenuates their basal platelet hyperactivity state.[24]

Furthermore, we have demonstrated that post-washout agonist-induced αIIbβ3 activation was attenuated following supplementation with fish oil or primrose oil. Taken together, these findings suggest that supplementation with ω-3 or ω-6 fatty acids may further contribute to lowering the chronic risk of a thrombotic event leading to myocardial infarction or stroke in postmenopausal women.

Platelet dense granules play a critical role in hemostasis and thrombosis.[32,33] Agonist-induced platelet activation releases dense granule contents, including small molecules such as 5-HT, epinephrine, ATP, and ADP.[34] These bioactive molecules, together with synthesized thromboxane $A_2$ (TxA$_2$), act in a feedforward manner on circulating platelets and contribute to propagating the activation and aggregation of platelets at the site of injury.[35] Whereas a previous
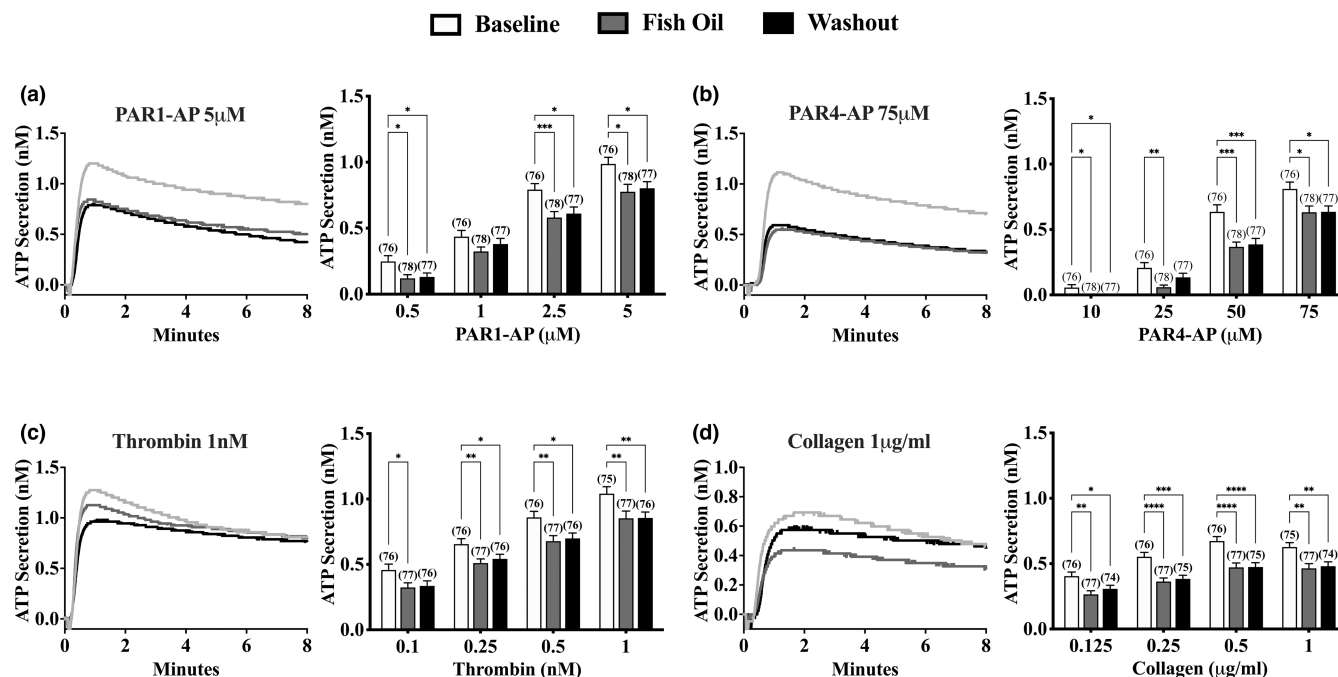
**FIGURE 5** Postmenopausal women exhibit attenuated dense granule secretion when supplemented with fish oil. ATP secretion were assessed at pre-supplementation (baseline), post-supplementation (fish oil), and after a 14-day washout period (washout) in the presence of (a) PAR1-AP, (b) PAR4-AP, (c) thrombin, or (d) collagen. Numbers are given in parenthesis. The data represent mean $\pm$ SEM. A mixed-effects analysis with Dunnett's multiple comparison post hoc test was performed. Asterisks indicate statistically significant differences between groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

study with postmenopausal women supplemented with the ω-3 PUFA, DHA, demonstrated that DHA reduced basal formation of $TxA_2$,[36] we show for the first time that ω-3 (Figure 5) and ω-6 (Figure 6) fatty acids regulate the positive feedback signaling that sustains platelet activation by lowering platelet dense granule secretion.

Following activation, phospholipids on the plasma membrane are metabolized to form free fatty acids.[37] The cleavage of the phospholipid produces substrates for de novo eicosanoid formation through metabolism by either cyclooxygenase-1 (COX-1) or 12-lipoxygenase (12-LOX).[38] A number of lipid substrates exist which may be oxidized by 12-LOX to form unique eicosanoids that can regulate platelet function.[22,39] This study has shown that supplementation with either fish oil or primrose oil enhances the ω-3 and ω-6 fatty acid content in platelets of postmenopausal women (Figure 1). The shift in substrate availability of these fatty acids through supplementation may represent a preferable approach in treating platelet dysfunction in postmenopausal women who are at increased risk for thrombosis. Notably, the levels of AA were not altered by supplementation with either fish oil or evening primrose oil (Tables S1 and S2). Because GLA is a precursor of AA, the increase in AA levels would be expected, in particular, following supplementation with evening primrose oil. However, a previous study has shown that supplementing subjects with GLA (1.5–6 g/day) for 3 weeks

resulted in an enrichment of DGLA, but no change in neutrophil AA levels.[40] Furthermore, in the same study, the extension of the supplementation period to 12 weeks with 3 g/day of GLA did not change the release of AA and only increased the release of DGLA from stimulated neutrophils. Additionally, studies have indicated that platelets have larger elongase activity relative to Δ-5-desturase activity, as the neutrophils.[41] Therefore, it is possible that platelets rapidly elongate GLA to DGLA, which decreases their ability to desaturate DGLA to AA.

In this study, we have shown that supplementation attenuated platelet reactivity in postmenopausal women. Consistent with our findings, several clinical studies have reported the cardioprotective effects of EPA.[42–44] Additionally, our findings are supported by a recent study from our group in which chronic supplementation with DHA inhibited mouse platelet aggregation and attenuated integrin αIIbβ3 activation[45] through the formation of 12-LOX-derived bioactive oxylipins. DHA is readily oxidized by 12-LOX in platelets, producing 11(S)-hydroxydocosahexaenoic acid (11-HDHA) and 14(S)-hydroxydocosahexaenoic acid (14-HDHA) by glutathione peroxidases.[37,46–48] Our group has shown that 11-HDHA and 14-HDHA play a role in the regulation of platelet activation and thrombosis,[45] suggesting that the increased DHA content in platelets observed in this study following supplementation, may favor the metabolism of ω-3 oxylipins, which might help to synergistically
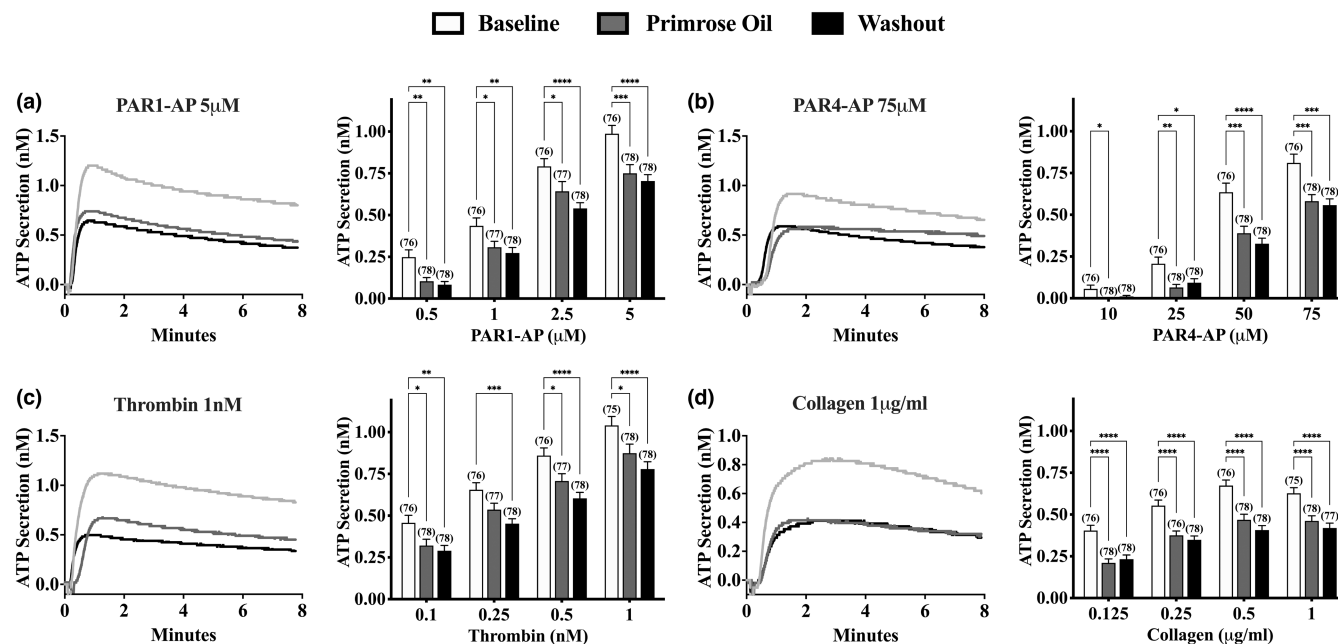
**FIGURE 6** Postmenopausal women exhibit attenuated dense granule secretion when supplemented with evening primrose oil. ATP secretion were assessed at pre-supplementation (baseline), post-supplementation (primrose oil), and after a 14-day washout period (washout) in the presence of (a) PAR1-AP, (b) PAR4-AP, (c) thrombin, or (d) collagen. Numbers are given in parenthesis. The data represent mean ± SEM. A mixed-effects analysis with Dunnett's multiple comparison post hoc test was performed. Asterisks indicate statistically significant differences between groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

regulate platelet reactivity in postmenopausal women. In addition, we have shown that supplementation with either fish oil or primrose oil results in increased ω-6 GLA and DGLA levels in platelets (Figure 1). Our group has previously demonstrated that DGLA is a substrate for platelet 12-LOX, generating the oxylipin 12(S)-hydroxyeicosatetraenoic (12-HETrE).[16] Consistent with the data presented in this intervention study, our group has previously shown that the acute addition of 12-HETrE attenuated human platelet reactivity in vitro through the inhibition of platelet aggregation, attenuation of integrin αIIbβ3 activation and a decrease in dense granule secretion.[16,49]

There are limited published data regarding the effects of ω-3 or ω-6 fatty acids on platelet aggregation in postmenopausal women. Vericel et al.[36] demonstrated that supplementing postmenopausal women with DHA attenuated collagen-induced platelet aggregation. Using two endogenous agonists, collagen and thrombin, and activating peptides PAR1-AP and PAR4-AP, we have shown that supplementation with fish oil or evening primrose oil attenuates PAR4-induced platelet aggregation in postmenopausal women (Figures 2 and 3).

Thrombopoiesis occurs primarily in the bone marrow.[50] It involves the differentiation of hematopoietic stem cells into mature megakaryocytes, which shed long-branching cytoplasmic protrusions called proplatelets.[9] Following the elaboration of proplatelets, platelets are assembled and released in the blood.[51] Platelets have a short lifespan,

circulating in the blood for 7 to 10 days, prior to elimination in the spleen and liver.[9] In order to eliminate all platelets exposed to either supplement, subjects had a washout period of 14 days. Interestingly, we have demonstrated that the increased content of ω-3 and ω-6 fatty acids in platelets and the regulatory effects on platelet reactivity is sustained following the post-washout period. Although more studies are necessary to further elucidate this effect, these observations suggest that supplementation might have a long-term effect through the regulation of newly formed platelets. These findings may point to a role of these fatty acids in the early stages of platelet biogenesis from the megakaryocyte. However, the sustained increased levels of ω-3 and ω-6 fatty acids in platelets following the washout period is a limitation of the study and suggests that a longer period of washout may be required in future studies to return the fatty acid levels to baseline.

Our study has certain limitations. Although we did not exclude postmenopausal women with comorbidities and/or diseases from our cohort, we are aware that almost half of the women were taking medications that may have had an effect on platelet function (Table 1). Because there is a frequent debate in clinical studies on how best to handle variables in the study design and findings, we made the decision not to exclude this group in the context of the high incidence of metabolic changes that affect postmenopausal women's health,[6,7] which may lead to the use of those medications. Furthermore, in practice, supplements will be

taken in addition to medications, supporting the criteria used in this study. Although the observations were consistent across almost 80 women who completed the study, the small sample size does not allow for assessment of a shift in clinical outcomes. Additionally, the exclusion of some samples was due to the variability of available blood between subject draws with patient samples resulting in different N numbers per group/assay, which limits the statistical power of the results.

Anti-aggregatory effects were observed when platelets were stimulated by low concentrations of PAR4-AP. Therefore, a further study is warranted to determine whether supplementation might have an effect on platelet aggregation under physiological conditions, such as determining the effects on aggregation in PRP. For this study, although PRP may be more appropriate to represent physiological conditions, the use of washed platelets allowed us to determine whether supplementation with ω-3 or ω-6 fatty acids have a direct effect on isolated platelets. A shorter period of supplementation has already been shown to reduce platelet aggregation only induced by very low concentration of agonist (collagen at 0.05 μg/ml).[36] However, because we demonstrated in the current study that both supplementations decrease biochemical end points of platelet activation (integrin αIIbβ3 and granule secretion), it is possible that a longer period of supplementation might be required to further elicit more robust attenuation of platelet aggregation. For the current study, a 60-day supplementation period was determined based on previous studies from our group in which we have demonstrated that at least 30 days of supplementation was required to increase mouse platelet levels of ω-3 and ω-6 fatty acids,[52] and to attenuate mouse platelet aggregation, integrin αIIbβ3 activity, and α-granule secretion.[45]

Based on the fact that cardiovascular disease disproportionally affects Black individuals relative to White individuals,[53] it would be informative to conduct a subanalysis of our data based on race. However, as a limitation of the study, there is inadequate diversity to make a conclusion regarding the effects of ω-3 or ω-6 fatty acids supplementation on platelet reactivity based on race.

In conclusion, this study supports the claim that supplementation with ω-3 and ω-6 fatty acids might offer postmenopausal women additional protection from cardiovascular diseases. These findings represent a better understanding of how modulating levels of ω-3 and ω-6 fatty acids through dietary supplementation reduces platelet reactivity in these women. Importantly, based on the fact that postmenopausal women have heightened cardiovascular morbidity and mortality due to thrombotic diseases,[2,54] this study may help to further develop minimally invasive therapeutic options to attenuate the risk of postmenopausal women suffering a major cardiovascular event.

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