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Effects of Low-Dose Aspirin and Fish Oil on Platelet Function and NF-kappaB in Adults with Diabetes Mellitus

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SUMMARY

Introduction—Many diabetics are insensitive to aspirin's platelet anti-aggregation effects. The possible modulating effects of coadministration of aspirin and fish oil in subjects with diabetes are poorly characterized.

Participants and Methods—Thirty adults with type 2 diabetes mellitus were treated with aspirin 81 mg/d for 7 days, then with fish oil 4g/day for 28 days, then the combination of fish oil and aspirin for another 7 days.

Results—Aspirin alone and in combination with fish oil reduced platelet aggregation in most participants. Five of 7 participants classified as aspirin insensitive 1 week after daily aspirin ingestion were sensitive after the combination. Although some platelet aggregation measures correlated positively after aspirin and fish oil ingestion alone and (in combination) in all individuals, correlation was only observed in those who were aspirin insensitive after ingestion of the combination.

Conclusions—Co-administration of aspirin and fish oil may reduce platelet aggregation more than aspirin alone in adults with diabetes mellitus.

Keywords

Omega-3 fatty acids; eicosapentaenoic acid; docosahexaenoic acid; aspirin; acetylsalicylic acid; platelet function; NF-kappaB; nuclear factor kappa-light-chain-enhancer of activated B cells

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INTRODUCTION

Aspirin has long been a stalwart and inexpensive therapy for the prevention of cardiovascular disease (CVD) as well as in the acute treatment of acute myocardial ischemia [1]. It is well established that low-dose aspirin (81–162 mg/day) has the potential to reduce the rate of recurrent vascular events with a 44% reduction in risk of myocardial infarction [2,3], and is associated with a much lower risk of gastrointestinal bleeding than higher doses [4]. In the primary prevention setting, however, evidence supporting a benefit is much less clear for all individuals [5] and those with diabetes mellitus [6]. Its benefits in preventing CVD have been most widely ascribed to its antiplatelet effects as aspirin reduces the production of the very potent platelet aggregation agonist thromboxane A₂ [4] through the acetylation of cyclooxygenase-1 (COX-1). However, excess thromboxane release has been shown to occur in type 2 diabetic patients with CVD [7].

Many individuals with, and at risk of, CVD events, including those with type 2 diabetes mellitus, do not derive the anticipated anti-platelet aggregation benefits of aspirin. This issue, generally referred to as aspirin insensitivity, is common and associated with an increased risk of CVD events [8]. Unfortunately, aspirin insensitivity is more common in those with diabetes mellitus than for healthy individuals [9]. Krasopoulos, et al. systematically reviewed the frequency of biochemical insensitivity to aspirin, defined by a variety of platelet function assays, within 20 studies including a total of 2930 patients with CVD [8]. Their study concluded that 28% of individuals have biochemical aspirin insensitivity, meaning that its effects on reducing platelet function are absent. The risk for a CVD-related event in individuals with aspirin insensitivity was significantly higher than those with a normal aspirin effect (odds ratio (OR) = 3.85; 95% CI: 3.08 to 4.80), with a higher incidence of death (OR = 5.99; 95% CI: 2.28 to 15.72), and an acute coronary syndrome (OR = 4.06; 95% CI: 2.96 to 5.56). The molecular basis of aspirin insensitivity is poorly understood, although genetic variants including the PLA₁/A₂ polymorphism in the GPIIb/IIIa platelet receptor [10] and platelet activation via pathways that are not modified by aspirin [11] have been suggested to play a role. In addition, a recent study of non-diabetics found that resistance to aspirin's anti-platelet effects could be attributed to enteric coating [12].

Current evidence suggests that patients with aspirin insensitivity do not benefit from other antiplatelet drugs [8]. In addition, the combination of aspirin with other antiplatelet drugs, such as clopidogrel, is associated with a significantly higher risk of major bleeding than with aspirin alone [13–15]. In contrast, emerging evidence from clinical trials and observational studies suggests that combining the omega-3 (ω 3) fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) together with aspirin may be more beneficial than using aspirin alone [16]. EPA and DHA have known anti-inflammatory, anti-platelet aggregation, and CVD preventive effects [17,18]. EPA and DHA may be especially beneficial for individuals with insulin resistance due to the underlying abnormal fatty acid and lipoprotein milieu [19], characterized by elevated circulating free fatty acids, low blood and tissue levels of ω3 fatty acids, and increased concentrations of highly atherogenic oxidized low density lipoprotein (LDL) [19–21].

However, the benefits of combining EPA+DHA with aspirin on platelet function or other inflammatory parameters have not received much attention. We hypothesized that the combination of low-dose aspirin and ω3 fatty acids of fish oil would be more effective than aspirin alone in reducing platelet aggregation and related mechanisms that potentiate the atherosclerotic process in subjects with type II diabetes.

PARTICIPANTS AND METHODS

Participants

We enrolled 30 adults aged 40 to 80 years with type 2 diabetes mellitus based on the criteria from the Executive Committee of the American Diabetes Association Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [22]. These criteria include having symptoms of diabetes plus casual plasma glucose concentration ≥ 200 mg/dl (11.1 mmol/l), a plasma glucose ≥ 126 mg/dl (7.0 mmol/l) after an 8 hr fast, or a 2-hour plasma glucose ≥ 200 mg/dl during an oral glucose tolerance test. Glucose measurements were performed as described by the World Health Organization [22]. Participants could not take vitamins, nutritional supplements, or herbal preparations for the study duration. Exclusion Criteria were: a diagnosis of CVD including coronary heart disease, congestive heart failure, peripheral vascular disease, stroke, or atrial fibrillation; history of malignancy, except subjects who have been disease-free for greater than 10 years, or whose only malignancy has been basal or squamous cell skin carcinoma; history of peptic ulcer or gastrointestinal bleeding in the past 5 years, diagnosed bleeding disorder, use of antiplatelet or antithrombotic therapy, defined as clopidogrel, ticlopidine, cilostazol, dipyridamole, trapidil, warfarin, and argatroban, oral contraceptive use, and daily use of NSAIDs. Other exclusion criteria were a calculated creatinine clearance <60 mg/dL, signs of obstructive hepatic disease, or any other obvious metabolic disease that would influence lipid metabolism, based upon a screening complete blood count and comprehensive metabolic profile, pregnancy, surgery within 30 days of screening, history of drug or alcohol abuse, or current weekly alcohol consumption >14 units/week (1 unit = 1 beer, 1 glass of wine, 1 mixed cocktail containing 1 ounce of alcohol), allergy to aspirin or fish/fish oil, and tobacco use. The use of any diabetes medications was permitted, including insulin.

Protocol

This was an 8-week sequential-therapy clinical trial. The clinical trial timeline for each subject is outlined in Table 1. All study visits were conducted at the University of Rochester's Clinical Research Center (CRC). Our study was designed to examine the acute (4 hour) and chronic (7 day) effects of aspirin in the absence or presence of fish oil supplementation. Over-the-counter standard (not enteric) 81 mg aspirin tablets were used. The fish oil capsules were over-the-counter with each containing 1000 mg of fish oil (OmegaRx brand; Zone Labs, Marblehead, MA) and a dose consisted of 4 capsules (4000 mg). Subjects were required to eat a standardized low-fat meal designed by the CRC dieticians the night before each study visit and to not eat or drink except water for 8 hours prior to each visit. During each study visit where subjects had phlebotomy performed 4 hours after the ingestion of a study agent, a standardized low-fat breakfast designed by the dieticians was provided <30 min post the initial phlebotomy. Less than 30 minutes after the participant finished their breakfast the study agent was ingested. Subjects were permitted to ambulate but not to eat within the CRC. They were asked not to consume any flax seed or fish oil other than the fish oil study agent and to limit fish consumption to no more than twice each week during the entire study. Following a pre-trial 10-day aspirin-free period, a baseline blood sample was obtained (blood draw 1, or BD1) and each participant then ingested a single dose (81 mg) of aspirin. Four hours later, after remaining within the CRC and fasting, a second phlebotomy was performed (BD2) and each participant then took aspirin 81 mg/day for 7 days. After the 7-day treatment, participants returned for a third phlebotomy (BD3) and they then began a 35-day regimen of fish oil (4g/day containing 1600 mg EPA and 800 mg DHA) treatment and discontinued aspirin unless it was prescribed by their doctor. Some participants ($n=8$) were taking aspirin as prescribed by their doctor but they were required to have an aspirin-free period for 10 days prior to their next study visit. Participants then returned for a fourth blood sample (BD4) after 28 days of

fish oil before which they were required to be aspirin-free for at least 10 days. They then ingested a single dose (81 mg) of aspirin, remained in the CRC and fasted, and a fifth phlebotomy (BD5) was obtained 4 hours later. They remained on the fish oil supplement together with 81 mg/day of aspirin for the final 7 days of the study.

The sixth and final blood sample (BD6) was obtained on that final day. Thus BD1-3 were obtained in the absence of fish oil supplementation, whereas BD4-6 were obtained following at least 28 days of fish oil ingestion. BD2 and BD5 reflect the acute effects (4 hrs) of aspirin, whereas BD3 and BD6 reflect the chronic effects (7 days) of aspirin ingestion. Tests performed at all study visits included physical examination, platelet function measurements, serum concentrations of EPA, DHA, leptin and adiponectin, and plasma Thromboxane B2 (TXB2) concentrations. We also measured NF-kappaB activation in peripheral blood mononuclear cells.

Laboratory Methods

Platelet Aggregation Direct Measures—Whole blood electrical impedance platelet aggregation was performed using a Chronolog Whole Blood Lumi-Aggregometer® (Model 560VS) with reagents from Chronolog Corporation (Havertown, PA -USA). Testing was completed within 2 hours of an atraumatic peripheral venous blood collection using the following agonists; ADP at 2.5, 5 and 10 μ M, collagen at 1 and 2 mg/mL, and arachidonic acid at 0.5 mM. Observable inhibition of the normally robust aggregation response to arachidonic acid served as verification that subjects ingested the aspirin. For each concentration of agonist, a 500 μ L aliquot of fresh whole blood anticoagulated with 0.105M sodium citrate was incubated with 500 μ L of normal physiological saline at 37°C for 5 minutes in the presence of a stir bar. A clean electrode was introduced into the cuvette and a baseline measurement of electrical impedance at 20 ohms was recorded. Following addition of different agonists, the aggregation of platelets is measured as a result in the change in impedance over a period of five minutes. The characteristics of the resultant platelet function curve are measured and calculated using Aggro/link software (version 5.1) from Chronolog Corporation.

Thromboxane B2—TXB2 in citrated plasma was analyzed using a competitive enzyme linked immunosorbent assay (EIA) according to manufacturer's instructions (TXB2 EIA Kit, Cayman Chemical Co., Ann Arbor, MI). All reagents were prepared fresh using ultra-pure water, and samples were diluted as necessary in sample buffer to fall within the standard curve (typically 1:2). We found no evidence for interference in the assay using serially diluted plasma (i.e. <20% difference in the final calculated TXB2 concentration), and therefore did not further purify the plasma samples. The limit of detection (LOD) was 7.8 pg/ml.

Adiponectin and Leptin—Adiponectin and leptin were measured in serum samples using commercially available ELISA kits according to the manufacturer's instructions (both from Millipore, Billerica, MA). Adiponectin was measured after 1:500 dilution in assay buffer (LOD 2.5 ng/ml), whereas leptin was measured undiluted (LOD 0.78 ng/ml).

NF-kappaB (NF- κ B)—Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation at room temperature using Lympholyte-H (Cedarlane Labs, Burlington, NC). PBMC nuclear extracts were obtained on ice using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The integrity of fractionation was confirmed in test samples by Western blot using antibodies directed against β actin and YY-1 (not shown). Five micrograms of nuclear extracts were analyzed in duplicate for DNA binding using a

TranAM™ NF- κ B assay kit (Active Motif, Carlsbad, CA). Briefly, samples were incubated for 1 hr at room temperature in wells containing immobilized NF- κ B consensus oligonucleotides, followed by washing and detection using an anti-NF- κ B p65 antibody. Positive controls were nuclear extracts from Jurkat T cells. Binding specificity was confirmed in test samples by demonstrating efficient competition using excess wild-type consensus oligonucleotides, but not mutated oligonucleotides (not shown).

Plasma EPA and DHA Assay—EDTA Plasma and fish oil capsule samples were assayed for EPA/DHA by a liquid chromatography tandem mass spectrometry (LC-MS/MS) approach using an API 4000 triple-quadrupole mass spectrometer (Applied BiosystemMSD Sciex). Briefly, plasma (20 μ l) and capsule (20 μ l) samples were treated with 1 M potassium hydroxide in 95% ethanol at 85 °C for 90 min. The resulting solution was cooled and then neutralized and diluted with 5% formic acid in methanol and mobile phase. The chromatographic separation of EPA/DHA was carried out on a Symmetry C18 column, 2.1 $\times$ 50 mm, 3.5 μ m (Waters, Milford, Massachusetts). The mobile phase consists of 85% methanol and 4 mM ammonium formate containing 0.1% formic acid eluting at 0.4 ml/min. The tandem mass-spectrometer was operated in negative electrospray mode with the ion spray voltage set at 4.0 KV and temperature to 650 °C. EPA/DHA was detected using multiple-reaction monitoring and quantitation achieved through monitoring of mass transition 301.1/257.1 m/z (EPA) and 327.1/283.1 m/z (DHA). All data were acquired and processed with Analyst 1.4.2 software (Applied BiosystemMSD Sciex, Carlsbad, California). The limit of detection was 45 pg for EPA, and 75 pg for DHA.

Statistical Methods

Participants' characteristics were described using relative frequencies for categorical data, and means and standard deviations for continuous data. Based on results from univariate analyses, several measures were logarithm (log) transformed for statistical purposes due to their skewed distribution, including TXB2, adiponectin, leptin, and NF- κ B DNA binding activity. Bivariate associations between platelet function variables and the use of aspirin and EPA+DHA were then performed and plotted. In addition, we performed mixed models to examine platelet function measures among different treatments after controlling for differences in participant characteristics. For direct aggregometry results, the mixed models adjusted for statin use, blood draw, BMI, presence of hypertension (HTN), gender, race, smoking status, alcohol frequency, fish consumption, and education. For the other platelet function results, only blood draw was adjusted for due to small sample size. The unstructured correlation matrix was applied in the mixed models to account for within participant variations. Interaction effects between aspirin and EPA+DHA were estimated and tested in the mixed model. More specifically, the short-term interaction effect of aspirin and EPA+DHA was tested as whether the difference between the second visit (BD2) and the first visit (BD1) was significantly different from the difference between the fifth visit (BD5) and the fourth visit (BD4). Similarly, the long-term interaction effect was tested as whether BD3-BD1 was significantly different from BD6-BD4, and the additional effect of EPA +DHA to short-term aspirin use was tested as whether BD3-BD2 was significantly different from BD6-BD5. We also classified aspirin "insensitivity" by a direct aggregation result with the agonist arachidonic acid as >0 [23] and used a two-sided independent samples T-test to determine the difference between the frequency of this result after the ingestion of aspirin alone and after the ingestion of aspirin + fish oil. Odds ratios and 95% confidence intervals (CI) were calculated for the effect of fish oil on the lack of response to aspirin, which we term "aspirin insensitivity" and tested using the chi-square statistic. To test the effect fish oil had on aspirin insensitivity among individuals who were aspirin insensitivity at four hours and one week of aspirin alone, a single sample t-test for proportions different than zero was used. Finally, we also tested the Pearson correlation coefficient of changes of platelet

function tests with each other for assessing whether the treatments had consistent antithrombotic effects via at least one mechanism. All analyses were performed using SAS version 9.2.3 (SAS Institute, Cary, NC).

RESULTS

The baseline characteristics of the study participants are shown in Table 2. All 30 subjects completed the study. A total of 37 were recruited and 7 did not complete the study due primarily to conflicts between their schedules and study visit requirements. All participants tolerated the aspirin and fish oil study agents. The mean concentrations of DHA and EPA in the study capsules were 406 ± 42 mg/ml and 330 ± 46 mg/ml, respectively. Baseline plasma concentrations averaged 106 ± 48 μ g/ml for DHA and 13 ± 7 μ g/ml for EPA, which increased to 190 ± 65 μ g/ml ($p < 0.0001$) for DHA and 61 ± 26 μ g/ml for EPA ($p < 0.0001$) 28 days after fish oil ingestion (BD4-BD1). Concentrations also increased ($p < 0.0001$) for BD6-BD1 and BD4-BD3, respectively (data not shown).

Table 3 and Figure 1 show the effects of ingesting aspirin alone (BD1-BD3) or aspirin + fish oil (BD4-BD6) on platelet aggregation induced by the three different agonists, namely arachidonic acid (Figure 1A), ADP (Figure 1B), and collagen (Figure 1C). Results in the table are expressed as the change in platelet aggregation (measured in ohms using aggregometry) 4 hours following a one-time ingestion of aspirin (BD2-BD1 vs. BD5-BD4), as well as after 7 days of daily aspirin intake (BD3-BD1 vs. BD6-BD4). In addition, by comparing platelet aggregation at BD3-BD2 vs. BD6-BD5, we also determined whether the effects of aspirin vs. aspirin + fish oil changed over time. Platelet aggregation induced by the agonist arachidonic acid, with a baseline level of 15.9 ohms (95% CI: 14.1 – 17.6), was reduced by aspirin alone and aspirin + fish oil at all time points except after 4 hours when combined with fish oil (Figure 1A). Platelet aggregation induced by a low dose of ADP (2.5 μ M) was modestly reduced, from 11.9 ohms (95% CI: 5.7 – 18.1) at baseline, by acute aspirin ingestion and aspirin + fish oil after 4 hours (Table 3 and Figure 1B). Aggregation induced by higher concentrations of ADP (5 and 10 μ M) was not affected by either aspirin or aspirin + fish oil at any time point (data not shown). Collagen-induced aggregation at low dose (1 μ g) was reduced, from a baseline level of 16.2 ohms (95% CI: 14.0 – 18.4), by aspirin + fish oil after 4 hours ($p < 0.05$) and aspirin as well as aspirin + fish oil after 7 days (Figure 1C). Finally, aggregation with high dose collagen (2 μ g) was reduced by aspirin after 7 days (data not shown) but this was not significantly different than aspirin + fish oil ($p > 0.05$). Aspirin alone tended to be more effective than aspirin + fish oil ($p > 0.05$) between 4 hours and 7 days but these effects were also not significantly different ($p > 0.05$).

Table 4 and Figures 2–3 demonstrate the effects of aspirin and fish oil ingestion on plasma and serum mediators known to influence platelet aggregation. These data are organized similar to those in Table 3 to highlight differences between conditions. Thromboxane B2 concentrations, with an average baseline level of 5.8 pg/mL (95% CI: 4.8 – 6.8), were significantly reduced by aspirin and aspirin + fish oil at each time point after ingestion (Figure 2). Aspirin + fish oil tended to reduce TXB2 more than aspirin alone 4 hours after ingestion, whereas aspirin alone trended toward reduced concentrations more than aspirin + fish oil between 4 hours and 7 days after ingestion. Adiponectin concentrations, averaging 7.4 ng/mL (95% CI: 2.2 – 12.6) at baseline, were not significantly changed by either treatment at any time (data not shown). Leptin concentrations, with an average baseline level of 2.4 ng/mL (95% CI: 1.9 – 2.8), were significantly reduced following acute aspirin ingestion whether or not subjects were taking concomitant fish oil (Figure 3). Interestingly, leptin levels returned to baseline following longer term 7-day aspirin therapy (Figure 3).

We also wanted to analyze the effects of aspirin alone or together with EPA/DHA supplementation on peripheral blood mononuclear cells (PBMC), since these cells are involved in vascular and tissue inflammation. A key transcriptional factor involved in PBMC activation is NF- κ B, which induces pro-inflammatory gene expression. We measured activated NF- κ B in PBMC nuclear lysates using a quantitative DNA binding assay in 10 randomly selected subjects taking aspirin alone or together with EPA/DHA. Baseline NF- κ B DNA binding activity in these subjects was not significantly different from three non-diabetic control subjects (1.46 ± 0.68 vs. 1.58 ± 0.33 O.D. units, respectively). Table 5 and Figure 4 show that PBMC NF- κ B DNA-binding activity was significantly attenuated in diabetic subjects after the acute ingestion of aspirin as well as following 7 days of once/daily dosing. NF- κ B activity was significantly reduced by aspirin after 4 hours and 7 days.

Finally, we analyzed the effects of ingesting fish oil plus aspirin in the subset of subjects who did not exhibit the antiplatelet aggregation effects of aspirin, which we term aspirin insensitivity (see Methods). Among the 23 individuals who were aspirin insensitive 4 hours after ingesting aspirin alone, 2 (8.7%; 95% CI: 0.0%–20.9%; $P=0.1531$) of them became aspirin sensitive 4 hours after ingesting aspirin + fish oil (3 of the 7 sensitive individuals after ingesting aspirin alone for 4 hours became insensitive 4 hours after ingesting of aspirin + fish oil ($P=0.0619$)). Among the 7 individuals who were aspirin insensitive 1 week after ingesting aspirin alone, 5 (71.4%; 95% CI: 29.6%–100.0%; $P=0.0058$) of them became aspirin sensitive 1 week after ingesting aspirin + fish oil (4 of the 23 sensitive individuals after ingesting aspirin alone for 1 week became insensitive after 1 week of aspirin + fish oil ($P=0.0385$)) (See Figure 5). Figure 6 shows platelet aggregation results for each phlebotomy with arachidonic acid as the agonist and platelet function as measured by thromboxane B2 for the 7 individuals who were defined as being aspirin non-responders 1 week after aspirin ingestion. These data suggest that adherence to aspirin therapy was quite good for the 1 week duration. The arachidonic acid data also support a strong effect of the combination of aspirin with fish oil on platelet aggregation with a very narrow standard deviation.

Finally, we conducted correlation analyses in all 30 subjects, and observed that arachidonic acid aggregometry changes 4 hours after aspirin ingestion correlated positively with collagen 1 μ g aggregometry changes (correlation coefficient $\rho=0.65$; $p<0.0001$). After aspirin ingestion for 7 days, no changes in platelet aggregation results correlated ($p>0.05$). After 4 weeks of fish oil ingestion, arachidonic acid result changes correlated positively with collagen 1 μ g changes ($\rho=0.56$; $p=0.001$). Four hours after aspirin ingestion combined with fish oil arachidonic acid result changes correlated positively with collagen 1 μ g changes ($\rho=0.53$; $p=0.003$). After 7 days of aspirin ingestion combined with fish oil arachidonic acid result changes correlated positively with ADP 2.5 μ M changes ($\rho=0.44$; $p=0.015$). All other platelet function test correlations were not significant ($p>0.05$). In the 7 aspirin insensitive subjects, arachidonic acid-induced aggregation 4 hours and 7 days after aspirin ingestion alone and 4 weeks after fish oil ingestion alone did not correlate with any other platelet function tests ($p>0.05$). However, 4 hours after aspirin ingestion combined with fish oil, arachidonic acid result effects positively correlated with collagen 1 μ g changes ($\rho=0.83$; $p=0.02$). Seven days after aspirin ingestion with fish oil arachidonic acid result changes positively correlated with ADP 2.5 μ M changes ($\rho=0.77$; $p=0.04$). All other platelet function correlations in these aspirin insensitive subjects were not significant ($p>0.05$).

DISCUSSION AND CONCLUSION

In this study, we systematically investigated the effects of low-dose aspirin alone or together with fish oil supplementation on platelet aggregation and relevant mediators in adult subjects with type 2 diabetes mellitus. For the study population as a whole, aspirin alone had generally similar effects as aspirin plus fish oil supplementation on platelet aggregation

measures after both 4 hours and 7 days of aspirin ingestion. However, the combination of aspirin plus fish oil appeared to be more effective than aspirin alone in attenuating stimulus-induced platelet aggregation in the subset of subjects who were least sensitive to aspirin alone. In this group of aspirin insensitive individuals more correlation of platelet aggregation reduction effects were observed after the ingestion of both aspirin and fish oil. This suggests that the combined effects on platelet function may be through different mechanisms and more synchronous than with aspirin alone. In addition, we observed novel effects of aspirin on serum leptin levels and circulating leukocytes that we discuss further below.

We found that the effect of aspirin on platelet function in adults with type 2 diabetes mellitus was heterogeneous, and influenced by duration of ingestion and the stimulus used to induce platelet aggregation *in vitro*. Using arachidonic acid and collagen as agonists, inducible platelet aggregation declined progressively between 4 hours and 7 days of ingestion (Table 3 and Figure 1A and 1C). In contrast, ADP induced aggregation was more variable, and the effects of aspirin tended to decrease between 4 hours and 7 days of ingestion (Figure 1B). Platelet aggregation by ADP has been shown to be greater in those with diabetes mellitus than non-diabetic individuals [24] and our results regarding the acute aspirin effect on this aggregation mechanism suggest that it may be time-dependent. Although in general the effects of aspirin alone (BD1-3) were similar to aspirin plus fish oil (BD4-6), close inspection of Table 3 revealed that combination therapy influenced stimulus-induced platelet aggregation under some conditions. For example, the combination of low-dose aspirin and fish oil (BD5-BD4) trended toward attenuated collagen-induced aggregation more than aspirin alone (BD2-BD1) when measured 4 hours following ingestion. The variability in effects and a relatively small sample size limited our ability to be certain as to whether effects between these treatments differed significantly. However, we used a mixed model statistical approach, which allowed us to adjust for the effects of major potential confounders. In a recent study, we reported that combination therapy of aspirin plus fish oil reduced platelet aggregation and thrombogenesis (measured using the PFA-100 closure time metric) whereas each alone did not in a cohort of healthy adults [25]. We suspect that alterations in lipid metabolism influenced the results of combination therapy in the present study of type 2 diabetics, which will need to be followed up in future studies. One consideration is the generation of an oxylipin metabolite of EPA (resolvin E1) generated via the interaction with aspirin, which has been shown to reduce platelet function by down-regulating both thromboxane A₂- and ADP-induced aggregation [26,27], effects that parallel what we observed. In addition, the doses of EPA, DHA, and aspirin we used are similar to those used in humans in whom resolvin E1 generation has been enhanced [28]. Another consideration is the evidence that 81 mg of aspirin per day, but not higher doses, leads to the generation of another oxylipin (15-epi-lipoxin A₄) and its increase in human blood correlates negatively with thromboxane B₂ concentrations [29].

When we restricted our analysis to the subset of subjects with aspirin insensitivity (defined as arachidonic acid-induced aggregation >0 [23]), we observed a striking effect of combination therapy. For example, among 7 individuals who were aspirin insensitive 1 week after ingesting aspirin alone, five of them became aspirin sensitive 1 week after ingesting aspirin + fish oil (Figures 5 & 6). These data suggest that combination therapy may be more effective in diabetic subjects who are least sensitive to monotherapy with aspirin alone. The prevalence and causes of aspirin insensitivity remain poorly understood, and there is currently no consensus about proper terminology in this regard [30]. In our protocol, we observed subjects ingest the first aspirin dose and thus can ensure 100% compliance at the 4 hour time points (BD2 and BD5). In addition, the observation that aspirin insensitive subjects demonstrated marked inhibition of plasma TXB₂ after both 4 hours and 7 days of aspirin ingestion argues against non-compliance as being a major cause of aspirin insensitivity in our study. In addition, the significant increase in plasma EPA and

DHA levels in our study documented adequate compliance with fish oil supplementation during the 28-day intervention. Variability in the anti-platelet effects of aspirin are well-recognized, and are due in part to genetic differences in pathways still being worked out [31–33], as well as effects of enteric coating [12]. Grosser et al considered the possibility that aspirin insensitivity does not exist if aspirin absorption and timing issues are considered. It is important to note, however, that they used cyclooxygenase-1 as the platelet function outcome in healthy individuals [12] whereas we measured platelet aggregation using known agonists in individuals with diabetes mellitus and non-enteric aspirin. Regardless of mechanism(s) involved it has been demonstrated that aspirin insensitivity/resistance is a major risk factor for future cardiac events [8]. Given that arachidonic acid is an omega-6 fatty acid which is known to compete with EPA and DHA for cyclooxygenase metabolism and is metabolized into many proinflammatory cytokines as well as thromboxane [34], a very potent platelet aggregation agonist, our data support the hypothesis that aspirin insensitivity could be described as an EPA+DHA deficiency, at least in subjects with diabetes mellitus. This is of great importance given that the American Diabetes Association, American Heart Association, and American College of Cardiology Foundation all have officially determined that the role of aspirin in the prevention of CVD is unclear [9].

Similar to its effects on agonist-induced platelet aggregation, we observed a consistent inhibitory effect of aspirin on plasma TXB2 levels. Although the effects of aspirin alone vs. aspirin + fish oil were similar, combination therapy in general tended to inhibit plasma TXB2 more than aspirin alone (Table 4).

We also measured serum levels of leptin and adiponectin, two cytokines implicated in metabolic syndrome, type 2 diabetes mellitus, and platelet function. We chose to measure treatment effects on adiponectin as it has been shown to be lower in concentration in humans with insulin resistance than in healthy individuals and has demonstrated antiplatelet effects [35]. Adiponectin was consistently detectable in all subjects, but was not appreciably affected by any treatment condition. Interestingly, we found that the acute (4 hour) ingestion of low-dose aspirin significantly attenuated plasma leptin levels (Figure 3). The inhibitory effect of aspirin on leptin tended to wane with time, and was not appreciably influenced by concomitant fish oil ingestion. To our knowledge, this is the first demonstration that aspirin influences leptin levels, and we are not aware of other data investigating the effects of aspirin on leptin concentrations in humans. Aspirin ingestion has been shown to cause leptin receptor activation [36], and in mouse models prothrombotic effects appear to be mediated by a platelet leptin receptor mechanism [37]. The clinical significance of aspirin-dependent leptin inhibition requires further study.

We found a similar inhibitory effect of low-dose aspirin (with and without fish oil) on NF- κ B activation in circulating peripheral blood mononuclear cells (PBMC). We were only able to analyze a subset of our original cohort due to technical and logistical constraints (n=10 out of 30), but this subset did not differ in important characteristics including age (mean = 57.7 years), gender (50.0% male), BMI (mean = 33.2 kg/m²), smoking status (60.0% never), education (30.0% high school or GED, 40.0% bachelors/ associates degree, 20.0% graduate degree), and blood pressure (systolic: mean = 135.7; diastolic: mean = 79.4). NF- κ B, a protein complex that controls the transcription of DNA, is found in almost all cell types and involved in cellular responses to stimuli such as free radicals, stress, cytokines, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens [38–41]. NF- κ B is a family of seven structurally related transcription factors that play a central role in cardiovascular growth, stress response, and inflammation by controlling gene network expression [39]. NF- κ B activates many target genes crucial to the pathophysiology of the vessel wall, including chemokines, cytokines, and leukocyte adhesion molecules, in addition to genes that regulate cell proliferation and mediate cell survival [42]. Although aspirin ingestion has been

associated with lower expression of NF- κ B in human carotid artery plaques [43], we are not aware of prior studies demonstrating attenuation of NF- κ B activity in PBMC by physiologically relevant doses of aspirin *in vivo*. There was a trend toward further inhibition of NF- κ B when aspirin was combined with fish oil, possibly reflecting the known ability of DHA to inhibit NF- κ B related signal transduction and activation [34,44,45]. However, the dominant effect in our study was seen for low-dose aspirin alone. Future studies investigating the utility of PBMC NF- κ B activation in type 2 diabetes mellitus and metabolic syndrome should prove worthwhile.

In this study we used an aspirin dose of 81 mg each day given that the risk for bleeding is dose-dependent and antiplatelet effects via inhibition of cyclooxygenase has been demonstrated by it and even lower doses [30]. Clinical trial data have supported that the most optimal dose for patients with known coronary artery disease when weighing risks and benefits is between 75 and 100 mg [46]. The ASPECT clinical trial [23,47], the largest serial pharmacodynamic investigation of dose-dependent effects of aspirin on platelet aggregation in individuals with known coronary artery disease, has shown that aspirin effects on arachidonic acid-induced platelet aggregation are an important means of determining aspirin insensitivity in those with known coronary artery disease, but that ADP- and collagen-induced aggregation are also potentially important factors in determining whether aspirin is exerting its antiplatelet effects. Our data regarding effects of ADP and collagen at low dose on aggregation suggest that it may be that aspirin + fish oil exert a greater effect than aspirin alone 4 hours after ingestion. It should be noted that only 26% of the ASPECT study cohort had a diagnosis of diabetes mellitus and no such subgroup analyses are available. However, it is important that the ASPECT study demonstrated individuals with diabetes have greater aspirin reactivity than those without diabetes mellitus. This combined with higher cardiovascular disease event risk in those with diabetes mellitus is why we chose this population. We are not aware of other studies in individuals with diabetes mellitus in which the effects of aspirin and aspirin + fish oil on the same platelet aggregation variables as we used have been investigated.

Watson, et al, in a retrospective study have determined that the risk of major and minor bleeding in patients, most of whom had coronary artery disease, is not statistically different in those taking fish oil (mean 3 g/d) with aspirin and clopidogrel compared to those taking aspirin and clopidogrel alone [48]. We did not observe any significant bleeding in any subject in our short-term study. Interestingly, Watson et al. found that the risk of minor bleeding when EPA and DHA was combined with aspirin and clopidogrel was lower than when aspirin and clopidogrel were taken alone. Also, since the risk of bleeding increases in conjunction with increasing CVD risk and inflammation [49], this observed decreased bleeding risk with EPA and DHA ingestion makes sense given that these fatty acids have anti-inflammatory effects [34,50].

Strengths and Limitations

Although our sample size was small, the cross-over design enhanced statistical power, and we analyzed multiple outcomes at each time point. We also studied carefully characterized diabetic subjects in a controlled research setting where study protocol adherence was strictly documented, and used validated means of measuring platelet aggregation using whole blood. A potential weakness is the relatively small sample size studied. Our mixed model statistical methodology was a strength as it allowed for adjustment of effects for covariates without reducing statistical power. We classified aspirin insensitivity as a lack of complete elimination of platelet aggregation when arachidonic acid was the agonist as this is a common approach due to aspirin's specific effects on aggregation. EPA and DHA are ω 3 fatty acids whereas arachidonic acid is an omega-6 fatty acid and they are known to compete for cyclooxygenase. However, our definition of aspirin insensitivity is one of several other

potential options. Related to this issue, our study was limited by the fact that we do not report the arachidonic acid concentrations or proportions in blood or in the fish oil capsules due to our measurement methodology being LC-MS/MS. Although we tracked EPA and DHA concentrations in plasma before and after the 28 day ingestion and platelet aggregation after aspirin ingestion is reflected by changes with arachidonic acid as the agonist, we cannot confirm that all study participants adhered to the protocol.

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Figure 1. Effects of aspirin with and without fish oil on agonist-induced platelet aggregation
 The effects of aspirin alone (BD1-3) or aspirin + fish oil (BD4-6) on platelet aggregation induced by (A) arachidonic acid, (B) ADP, and (C) collagen. Data are expressed as means and confidence intervals after log transformation (see Methods). Asterisks (*) represent a significant difference from baseline blood draw ($p < 0.05$) using the T-test from a mixed model to adjust for statin use, blood draw, BMI, presence of HTN, gender, race, smoking status, alcohol frequency, fish consumption, and education.

Figure 2. Effects of aspirin with and without fish oil on plasma thromboxane B2
 Plasma TXB2 was suppressed immediately after aspirin ingestion (BD2 and BD5) and fell progressively during both treatment arms. Data and asterisks are as in Figure 1.

Figure 3. Effects of aspirin with and without fish oil on plasma leptin levels

Plasma leptin levels were suppressed immediately after acute aspirin ingestion only (BD2 and BD5). * indicated $p < 0.05$ using a mixed statistical model as in Figure 1.

Figure 4. Effects of aspirin with and without fish oil on NF-kappaB DNA binding activity in peripheral blood mononuclear cells

Peripheral blood mononuclear cells were isolated from a subset of subjects and nuclear extracts analyzed for NF-kB DNA binding activity (see Methods). Log transformed data and asterisks are expressed as in Figure 1.

Figure 5. Combination therapy appears to decrease the numbers of subjects who are aspirin resistant

Frequency distribution of subjects defined as aspirin sensitive or resistant (see text) expressed as percent change from aspirin resistant with aspirin alone to aspirin sensitive with aspirin + fish oil, and vice versa, both 4 hours and 1 week after ingestion. Bars represent percent change and 95% CI.

Figure 6. Effects of aspirin with and without fish oil on the subset of aspirin resistant subjects
Data are arachidonic acid-induced platelet aggregation (A) and plasma thromboxane B2 levels (B) in the 7 aspirin resistant subjects, expressed as after log transformation. * indicates $p < 0.05$ using a mixed model as in Figure 1.

Table 1

Study timeline of activities

	Day	Study Visit	Activity
Aspirin only	1		<ul style="list-style-type: none"> Recruitment and screening Collection of baseline demographic and clinical data
	1–10		<ul style="list-style-type: none"> Aspirin-free period of 10 days prior to Study Visit 1
	11	1	<ul style="list-style-type: none"> Blood draw 1 (baseline, before aspirin) Ingestion single 81 mg dose aspirin Blood draw 2 (4 hours post aspirin ingestion)
	12–17		<ul style="list-style-type: none"> Single 81 mg aspirin/day
Fish oil only	18	2	<ul style="list-style-type: none"> Blood draw 3 (7 days aspirin ingestion) Begin fish oil 4g/day and discontinue aspirin unless subject takes it as prescribed by their doctor
	19–45		<ul style="list-style-type: none"> 28 days of fish oil 4g/day
	36–45		<ul style="list-style-type: none"> Aspirin-free period of 10 days prior to Study Visit 3
Aspirin + fish oil	46	3	<ul style="list-style-type: none"> Blood draw 4 (4 weeks fish oil ingestion, before aspirin) Continue fish oil and a single dose 81 mg aspirin Blood draw 5 (4 hours post aspirin + fish oil ingestion)
	47–52		<ul style="list-style-type: none"> Continue aspirin and fish oil 4g/day
	53	4	<ul style="list-style-type: none"> Blood draw 6 (7 days aspirin + fish oil ingestion) Discontinue fish oil and aspirin

Table 2

Baseline participant characteristics (n = 30)

		Minimum	Maximum
Age, mean (SD)	56.6 (8.9)	40.0	74.5
Male, n (%)	15 (50.0)		
Race, n (%)			
African-American	9 (30.0)		
Asian	2 (6.7)		
Caucasian	17 (56.7)		
Other	2 (6.7)		
Smoking status, n (%)			
Former	11 (36.7)		
Never	19 (63.3)		
Fish intake, n (%)			
<once/month	11 (36.6)		
1–3 times/month	10 (33.3)		
once/week	7 (23.3)		
twice/week	2 (6.7)		
Physical activity (hrs/wk), mean (SD)	5.12 (5.23)		
Metformin use, n (%)	25 (83.3)		
Alcohol consumption, n (%)			
1–3 days/month	2 (6.7)		
1–4 days/week	4 (13.3)		
5 days/week	1 (3.3)		
Education, n (%)			
<high school	2 (6.7)		
High school or GED	6 (20.0)		
Bachelors or associates degree	12 (40.0)		
Graduate degree	6 (20.0)		
Systolic Blood Pressure, mean (SD)	132.9 (14.0)	98.0	171.0
Diastolic Blood Pressure, mean (SD)	76.0 (9.4)	58.0	94.0
BMI, mean (SD)	34.6 (7.5)	22.1	54.0

TABLE 3

Effects of Aspirin and EPA+DHA on Platelet Aggregometry

	4 Hour Effects				7 Day Effects				4 Hour vs 7 Day Values			
	BD2-BD1 vs. BD5-BD4		BD3-BD1 vs. BD6-BD4		BD3-BD1 vs. BD6-BD4		BD3-BD1 vs. BD6-BD4		BD3-BD2 vs. BD6-BD5		BD3-BD2 vs. BD6-BD5	
	BD2-BD1	BD5-BD4	Δ	BD3-BD1	BD6-BD4	Δ	BD3-BD1	BD6-BD4	Δ	BD3-BD2	BD6-BD5	Δ
Arachidonic Acid 0.5 mM	Change	-3.60	-2.10	-1.50	-14.05	-12.79	-1.26	-10.45	-10.69	-10.45	-10.69	0.24
	p-value	0.0202	0.0516	0.2105	<0.0001	<0.0001	0.2220	<0.0001	<0.0001	<0.0001	<0.0001	0.8408
ADP 2.5 μM	Change	-2.39	-2.67	0.28	-1.23	0.88	-2.11	1.16	3.55	1.16	3.55	-2.39
	p-value	0.0247	0.0042	0.8086	0.1064	0.7465	0.4164	0.2123	0.1966	0.2123	0.1966	0.3748
Collagen 1 μg	Change	-1.12	-1.56	0.44	-8.16	-7.58	-0.58	-7.04	-6.02	-7.04	-6.02	-1.02
	p-value	0.3053	0.0378	0.6900	<0.0001	0.0002	0.6748	<0.0001	0.0004	<0.0001	0.0004	0.4255

Results represent differences in platelet aggregometry measures between phlebotomy time points using mixed models, adjusting for statin use, blood draw, BMI, presence of HTN, gender, race, smoking status, alcohol frequency, fish consumption, and education.

TABLE 4

Effects of Aspirin and EPA+DHA on Other Platelet Function Variables

		4 Hour Effects			7 Day Effects			4 Hour vs 7 Day Values		
		BD 2-BD1	BD 5-BD4	Δ	BD 3-BD1	BD 6-BD4	Δ	BD 3-BD2	BD 6-BD5	Δ
Thromboxane B2 (log transformed)	Change	-1.20	-1.64	0.44	-2.42	-2.54	0.12	-1.22	-0.90	-0.32
	p-value	0.0011	<0.0001	0.2250	<0.0001	<0.0001	0.6241	0.0005	0.0011	0.3061
Adiponectin (log transformed)	Change	0.03	-0.03	0.06	0.02	-0.01	0.04	-0.01	0.01	-0.02
	p-value	0.4158	0.5061	0.2906	0.5441	0.7629	0.5235	0.8182	0.7437	0.6180
Leptin (log transformed)	Change	-0.14	-0.13	-0.01	-0.02	0.04	-0.05	0.13	0.17	-0.05
	p-value	0.0017	0.0013	0.8570	0.7529	0.3884	0.4320	0.0425	0.0037	0.5013

Results represent differences in platelet function measures between phlebotomy time points using mixed models, adjusting for statin use, blood draw, BMI, presence of HTN, gender, race, smoking status, alcohol frequency, fish consumption, and education.

TABLE 5

Effects of Aspirin and EPA+DHA on NF-kB (log transformed)

	4 Hour Effects		7 Day Effects		4 Hour vs 7 Day Values	
	BD 2-BD1	BD 5-BD4	BD 3-BD1	BD 6-BD4	BD 3-BD2	BD 6-BD5
Change	-0.16	-0.16	-0.36	-0.35	-0.19	-0.01
p-value	0.0320	0.4408	0.0316	0.1014	0.1514	0.9717

Results represent differences in NFkB between phlebotomy time points using mixed models, adjusting for statin use, blood draw, BMI, presence of HTN, gender, race, smoking status, alcohol frequency, fish consumption, and education.