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Effects of aspirin in combination with EPA and DHA on HDL-C cholesterol and ApoA1 exchange in individuals with type 2 diabetes mellitus

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ABSTRACT

Background/synopsis: Low-dose aspirin is an effective drug for the prevention of cardiovascular disease (CVD) events but individuals with diabetes mellitus can be subject to 'aspirin resistance'. Thus, aspirin's effect in these individuals is controversial. Higher blood levels of seafood-derived omega-3 polyunsaturated fatty acids (ω3) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) also have beneficial effects in reducing risk of CVD events but few studies have examined the interaction of plasma EPA and DHA with aspirin ingestion.
Objective/purpose: Our study examined the combinatory effects of EPA, DHA, and aspirin ingestion on HDL-cholesterol (HDL-C) and apoA-I exchange (shown to be associated with CVD event risk).
Methods: 30 adults with Type 2 diabetes mellitus ingested aspirin (81 mg/day) for 7 consecutive days, EPA + DHA (2.6 g/day) for 28 days, then both for 7 days. Plasma was collected at baseline and at 5 subsequent visits including 4 h after each aspirin ingestion. Mixed model methods were used to determine HDL-C-concentrations and apoA-I exchange compared to the baseline visit values. LOWESS curves were used for non-linear analyses of outcomes to help discern change patterns, which was followed by piecewise linear functions for formal testing of curvilinear relationships.
Results: Significant changes ($p < 0.05$) compared to baseline in both HDL-C-concentrations and apoA-I exchange were present at different times. After 7 days of aspirin-only ingestion, apoA-I exchange was significantly modified by increasing levels of DHA concentration, with increased apoA-I exchange observed up until log(DHA) of 4.6 and decreased exchange thereafter ($p = 0.03$). These LOWESS curve effects were not observed for EPA or HDL-C ($p > 0.05$). Aspirin's effects on apoA-I exchange were the greatest when EPA or DHA concentrations were moderate compared to high or low. Comparison of EPA, DHA, and EPA + DHA LOWESS curves, demonstrated that the majority of the effect is due to DHA.
Conclusion: Our results strongly suggest that plasma concentrations of EPA and DHA influence aspirin effects on lipid mediators of CVD event risk where their concentrations are most beneficial when moderate, not high or low. These effects on HDL-C cholesterol and apoA-I exchange are novel. Personalized dosing of DHA in those who take aspirin may be a beneficial option for patients with type 2 diabetes mellitus.

1. Introduction

Type 2 diabetes mellitus is a growing public health concern, present in about 9.3% of the United States population [1]. Current trends indicate that the magnitude of the problem will continue to rise in coming years with a projected increase of 165% for the years 2000 through 2050 [2]. This disorder is a serious health concern and is currently the

7th leading cause of death in the US [3]. In addition, it contributes to numerous cardiovascular complications including cardiovascular disease deaths, hypertension, stoke and dyslipidemia [4,5].

Prophylactic dosing of aspirin (81–162 mg/day) is strongly supported in the cardiovascular literature as having a therapeutic effect on a range of cardiovascular conditions with minimal side effects [6–10]. It is associated with reduced cardiovascular events in those with coronary

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artery disease [11] and is indicated for use in thrombosis patients [12]. Aspirin has also been associated with having a protective influence on cholesterol metabolism. It is believed these cardiovascular benefits stem from aspirin's ability to reduce the synthesis of thromboxane A2, a platelet agonist. However, the beneficial effects of aspirin in the type 2 diabetes mellitus (T2DM) subset of the cardiovascular risk population is controversial [11,13]. This population is prone to "aspirin resistance", rendering the beneficial effects of aspirin on platelet function un-measureable. As a result, aspirin has been interpreted as having no direct cardiovascular benefit in T2DM patients [14,15].

While the physiological effects of aspirin have been widely investigated, few studies demonstrate the role of ω3 fatty acids when used in combination with aspirin, particularly prophylactic dosing of fish oil. Fish oil is also strongly regarded as having positive cardiovascular effects [16–18]. Among its beneficial effects are those that are anti-inflammatory and tissue-protective [18]. Our study aimed to examine the individual and combined effects of aspirin and fish oil ingestion on 2 cholesterol transport markers that predict cardiovascular disease events. They are high density lipoprotein (HDL-C) concentration [19] and HDL-apolipoprotein (apo)A-I exchange (HAE) [20] within adults with type 2 diabetes mellitus. Given the extensive evidence supporting the benefits of both fish oil and aspirin, we hypothesized that the interaction of the two will have a greater influence on HDL metabolism than either one alone.

2. Participants and methods

2.1. Patients

The study sample was comprised of 30 adults aged 40–80 who met criteria for diagnosis of type 2 diabetes mellitus based on the Executive Committee of the American Diabetes Association Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus criteria [21]. Distribution of participant characteristics can be found in Table 1, as previously described [22]. Individuals were excluded if they

Table 1
Distribution of study participants (N = 30).

		Min.	Max.
Age, mean (SD)	56.6 (8.9)	40.0	74.5
Male, n (%)	15 (50)		
Race, n (%)			
Caucasian	17 (56.6)		
African-American	9 (30.0)		
Asian	2 (6.7)		
Other	2 (6.7)		
Education, n (%)			
< High school	2(6.7)		
High school or GED	9 (30.0)		
Bachelor's or associate's	13 (43.3)		
Graduate degree	6 (20.0)		
Smoking status, n (%)			
Former	11 (36.7)		
Never	19 (63.3)		
Fish intake, n (%)			
< Once/month	11 (36.7)		
1–3 times/month	10 (33.3)		
Once/week	7 (23.3)		
Twice/week	2 (6.7)		
Physical activity (h/week), mean (SD)	5.1 (5.2)		
BMI, mean (SD)	34.6 (7.5)	22.1	54.0
Metformin use, n (%)	25 (83.3)		
Alcohol intake, n (%)			
None	23 (76.7)		
1–3 days/month	2 (6.7)		
1–4 days/week	4 (13.3)		
≥ 5 days/week	1 (3.3)		
Systolic BP, mean (SD)	132.9 (14.0)	98.0	171.0
Diastolic BP, mean (SD)	76.0 (9.4)	58.0	94.0

Table 2
Schedule of study activities.

Study visit	Day	Regimen	Visit activities
1	1	Aspirin only	• Recruitment and Screening • Baseline demographics
	1–10		• 10-day aspirin free period
	11		• **Blood draw 1** (baseline) • Ingestion of 81 mg aspirin • **Blood draw 2** post (4 h post aspirin ingestion)
2	12–17	Fish oil only	• Continue aspirin dosing 81 mg/day
	18		• **Blood draw 3** (7 days aspirin ingestion) • Begin fish oil 4 g/day and discontinue aspirin unless otherwise noted by doctor
3	19–45	Aspirin + Fish oil	• 28 days of fish oil 4 g/day
	46		• **Blood draw 4** (4 weeks fish oil ingestion, before aspirin) • Continue fish oil and single dose 81 mg aspirin • **Blood draw 5** (4 h post aspirin + fish oil ingestion)
4	47–52		• Continue aspirin and fish oil 4 g/day
	53		• **Blood draw 6** (7 days aspirin + fish oil ingestion) • Discontinue fish oil and aspirin

had a diagnosed CVD; recent history of malignancy, peptic ulcer, or bleeding disorder; use of antiplatelet or antithrombotic therapy, oral contraceptives, or NSAIDs; signs of any metabolic disease that would influence lipid metabolism; allergy to aspirin or fish oil; pregnancy; drug or alcohol abuse and tobacco use. Patients who enrolled were asked to refrain from flax seed oil, supplements and other fish oil containing products not including the study capsules. Fish intake was to be limited to 3 servings/week.

2.2. Protocol

This was an 8-week sequential-therapy clinical trial. Our study was designed to investigate the acute and chronic effects of prophylactic aspirin dosing individually and in combination with fish oil supplements. Acute was defined as 4 h and chronic was defined as 7 days. Standard 81 mg over-the counter aspirin tablets and 1000 mg over-the-counter fish oil capsules (OmegaRx brand; Zone Labs, Marblehead, MA) were used. One fish oil dose was defined as 4000 mg. Each capsule contained 400 mg EPA and 200 mg of DHA. All subjects underwent a 10-day aspirin washout period following the screening visit. A complete timeline of events can be found in Table 2 and Fig. 1. Clinical Research Center dieticians provided subjects with a low-fat meal plan for the night preceding each study visit. They were also instructed not to eat or drink with the exception of water for 8 h prior to the visit. Study visit 1 took place after a 10-day aspirin free period and consisted of a venipuncture to obtain baseline values. A second venipuncture (blood draw 2) was performed at 4 h post ingestion of the first aspirin dose. Subjects were instructed to continue aspirin dosing for 7 days, at the end of which a third venipuncture (blood draw 3) was taken. Following the third blood draw subjects began a 35-day regime of fish oil (1600 mg EPA and 800 mg DHA). Patients were then instructed to discontinue aspirin. This study allowed for the continuation of aspirin per the subject's doctor if needed with a 10 day washout period, however all subjects were able to discontinue aspirin as planned. Those patients were provided with instructions to have a 10-day aspirin free period before the next visit. At day 28, a fourth venipuncture (blood draw 4) was performed to obtain values for fish oil only. Subjects were then dosed with aspirin (81 mg) again and received a fifth venipuncture (blood draw 5) for the acute combination of aspirin and fish oil, and a final venipuncture (blood draw 6) for the chronic effects after 7 days of combined aspirin and fish oil. Thus, the first three blood draws did not
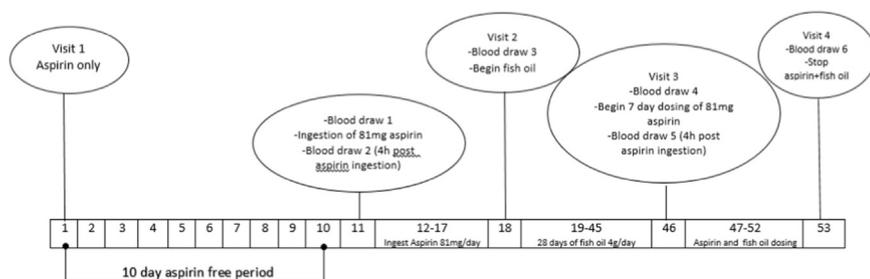
Fig. 1. Timeline of dosing regimen, study visits and blood draws.

include fish oil supplementation while blood draws 4 through 6 contained fish oil supplementation for at least 28 days.

This study was approved by the Research Subject Review Board at the University of Rochester and registered with Clinicaltrials.gov (NCT01181882).

2.3. Laboratory methods

2.3.1. HDL-apoA-I exchange (HAE) measurement

HAE was measured using electron paramagnetic resonance (EPR) to quantify the binding of exogenous, spin-labeled apoA-I (HAE probe) to HDL in plasma as previously described in prior studies [20,23]. Freshly thawed plasma was mixed 1:4 with PBS (20 mM phosphate, 150 mM NaCl, pH 7.4) and 24% w/v PEG 6000 (Sigma) was added to a final concentration of 4%. Samples were centrifuged at 13,000 rpm for 10 min in a tabletop centrifuge at 4 °C to remove apoB-containing lipoproteins. The clarified plasma was then mixed with 3 mg/mL spin-labeled apoA-I in a 3:1 ratio and drawn into an EPR-compatible borosilicate capillary tube (VWR).

EPR measurements were performed with a Bruker eScan EPR spectrometer outfitted with temperature controller (Noxygen). Samples were incubated for 15 min at 37 °C and scanned at 37 °C. The peak amplitude of the nitroxide signal from HAE probe in the sample (3462–3470 Gauss) was compared to the peak amplitude of a proprietary internal standard (3507–3515 Gauss) provided by Bruker. The internal standard was contained within the eScan spectrometer cavity and did not contact the sample. Since the y-axis of an EPR spectrum is measured in arbitrary units, measuring the sample against a fixed internal standard facilitates normalization of sample response. HAE activity represents the sample: internal standard signal ratio at 37 °C. The % (maximal) HAE activity was calculated by comparing HAE activity to a standard curve of HAE probe: internal standard signal ratio, wherein HAE probe is in conformations ranging from fully lipid-free to fully lipid-bound. All samples were read in triplicate and averaged.

2.3.2. 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 API4000 triple-quadrupole mass

spectrometer (Applied Biosystem MSD Sciex) as described previously [22,24]. Briefly, all data were acquired and processed with Analyst 1.4.2 software (Applied Biosystem MSD Sciex, Carlsbad, California). The limit of detection was 45 pg for EPA, and 75 pg for DHA.

2.4. Statistical methods

Distributions of characteristics of participants at baseline as shown in Table 2 were generated using frequencies for categorical variables and means and standard deviations (SD) for continuous variables. Blood concentrations of apoA-I and HDL-C outcomes were log transformed to help stabilize their skewed distributions. The changes in concentration relative to baseline as well as between blood draws were calculated. Both apoA-I and HDL-C treatment effects were analyzed after controlling for statin use, blood draw, body mass index (BMI), hypertension, gender, race, smoking status, alcohol use, fish intake, and education level. The effects of each study agent on concentrations were calculated by subtracting baseline concentrations (blood draw 1, BD1) from follow-up concentration blood draws.

The log-transformed linear combinations of EPA and DHA were used as the exposure of interest both individually and combined. A locally weighted scatterplot-smoothing (LOWESS) curve was first fit to the scatterplot of the score of the apoA-I or HDL-C concentration vs. EPA, DHA and EPA + DHA concentrations to study variability in slope and discern change patterns (e.g., V-shaped nature of the relationship) as well as to suggest the location of the inflection point. LOWESS curves were generated for each change in outcome following aspirin ingestion. When V-shaped curvilinear relationships were apparent by the LOWESS curve, piecewise linear regression was followed to formally test whether the slopes before and after the inflection point were different.

3. Results

Table 1 describes the participants. Table 2 describes the schedule of study activities. Fig. 1 provides supplemental information on dosing regimen, blood draws and study visits. 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 mg/ml for DHA and 13 ± 7 mg/ml for EPA, which
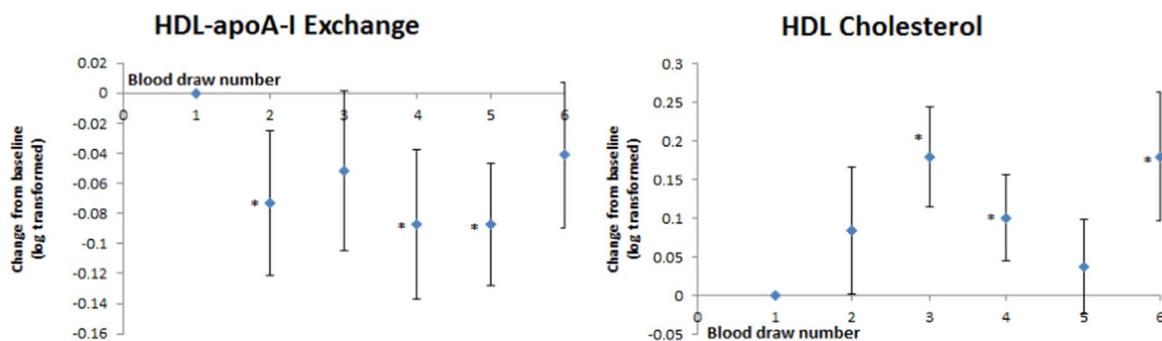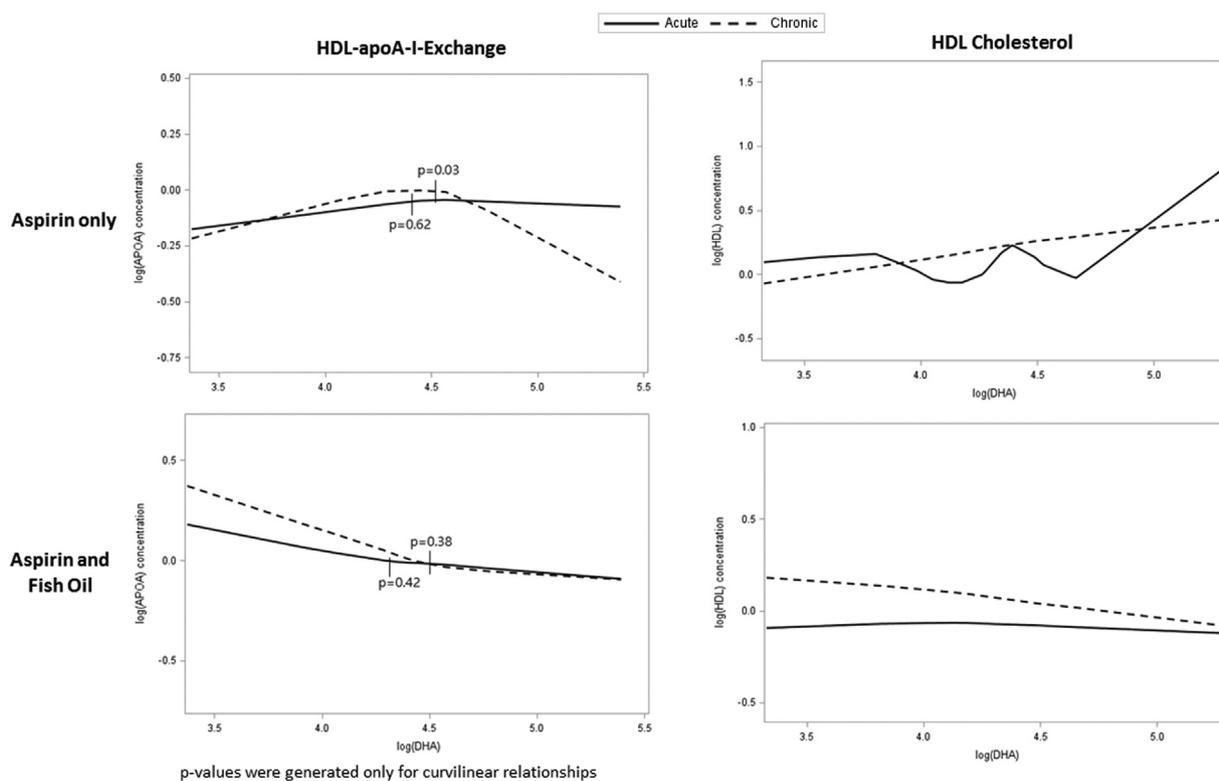

Fig. 2. Change from baseline of log transformed HAE and log transformed HDL-C concentrations by blood draw. The boxes are mean changes and bars are standard deviations. Asterisks are present for those outcomes that were statistically significant ($p < 0.05$).

Fig. 3. Acute and seven day effects of aspirin and aspirin + fish oil ingestion on HAE and HDL-C dependent on DHA concentrations. Acute (4 h) and chronic (7 days) effects of 81 mg aspirin and 81 mg aspirin + fish oil accounting for DHA plasma concentration only. The y-axis scale is the log-transformed HAE and HDL-C concentration. The x-axis is the log-transformed DHA concentration. Each LOWESS curve corresponds to the effect of aspirin (acute and chronic) as well as aspirin + Fish oil (acute and chronic) on DHA.

increased to 190 ± 65 mg/ml (p < 0.0001) for DHA and 61 ± 26 mg/ml for EPA (p < 0.0001) 28 days after fish oil ingestion [22]. Fig. 2 displays forest plots for the log transformed mean difference from baseline for HAE and HDL-C concentrations by blood draw. Each blood draw is representative of the exposure of interest for that blood draw (i.e., blood draw 3 is representative of the chronic effects, after 7 days of aspirin only). Significant reductions in HAE were present comparing blood draw 1 vs. blood draw 2, blood draw 1 vs. blood draw 4 and blood draw 1 vs. blood draw 5. Relative to baseline, these represent 4 h after aspirin ingestion, 28 days of fish oil ingestion only, and combined fish oil and 4 h after aspirin ingestion, respectively. Significant increases in HDL-C concentration were observed for blood draw 1 vs. blood draw 3, blood draw 1 vs. blood draw 4 and blood draw 1 vs. blood draw 6. Relative to baseline, these represent 7 days of aspirin ingestion, 28 days of fish oil ingestion only, and combined 7 days of fish oil and aspirin ingestion, respectively. Only blood draw 1 vs. blood draw 4 was found to be significant for both the HDL-C and apoA-I exchange concentrations.
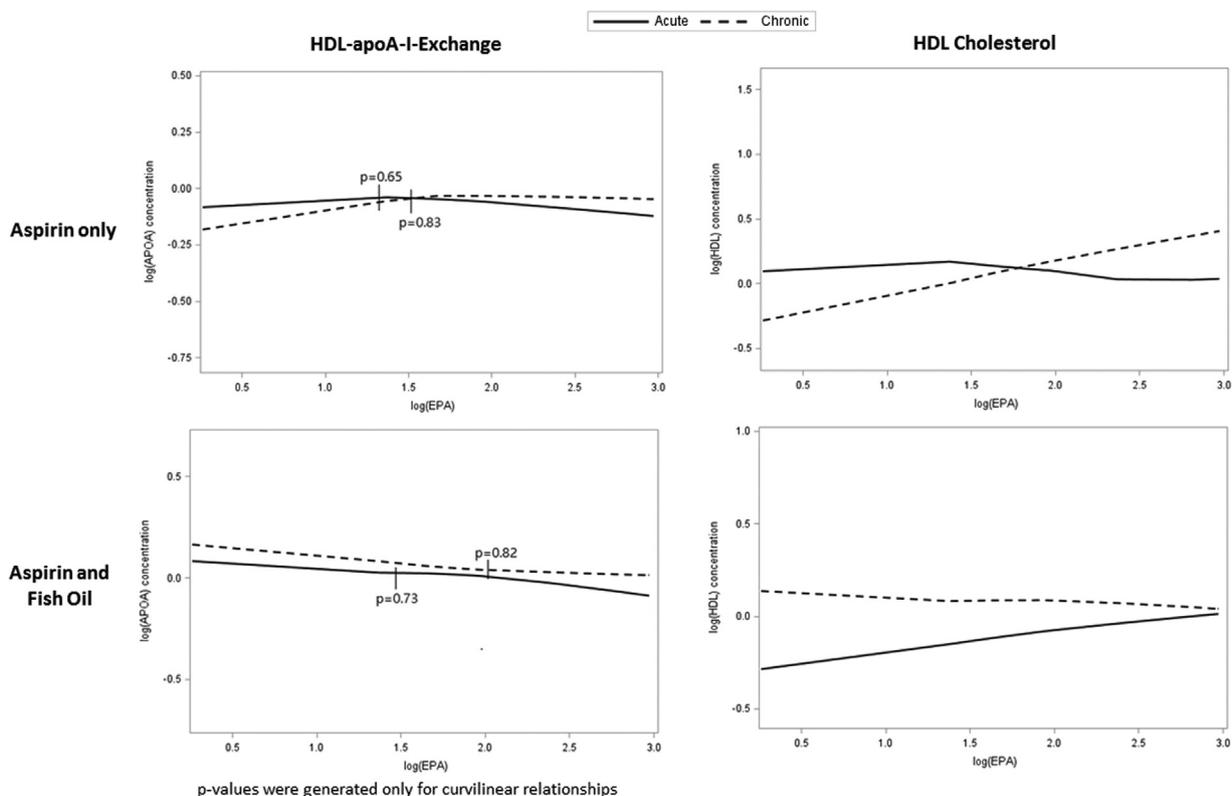
The LOWESS curves in Figs. 3–5 illustrate the log-transformed relationships of DHA, EPA, and combined EPA + DHA, respectively, for HAE and HDL-C concentrations. The inflection in the curve was analyzed using a piecewise function to determine the point at which the greatest change in slope occurs. In Fig. 3, a significant change in slope was observed for HAE after 7 days of aspirin-only ingestion, meaning that when DHA is analyzed alone the effects of 7 days of aspirin ingestion has a significant effect on HDL function. This relationship was not observed for the aspirin and fish oil combination nor for HDL-C after aspirin ingestion only and combined aspirin and fish oil ingestion. EPA alone did not generate any significant changes for either exposure. Similar to DHA, the combined effects of EPA + DHA demonstrated a significant relationship after 7 days of aspirin use only. Once again, this relationship was not observed in the aspirin and fish oil combination or for either of the HDL-C exposures. Comparison of all three LOWESS curves (Figs. 3–5) seem to suggest that DHA drives most of the effect,

particularly when viewing the individual EPA and DHA curves in relation to the combined EPA + DHA curves.

4. Discussion and conclusion

In this study, we systematically investigated the effects of low-dose aspirin and ω3 polyunsaturated fatty acid ingestion on HDL-C and apoA-I exchange as well as the interactive effect of plasma levels of EPA and DHA on aspirin's effects on HDL-C cholesterol concentrations and HAE in adults with type 2 diabetes mellitus. Significant decreases in HAE after acute aspirin ingestion, fish oil consumption for 28 days, and after aspirin was added acutely after this fish oil ingestion, occurred. In addition, 7-days of aspirin ingestion, chronic ingestion of fish oil, and both ingested for 7 days and 28 days, respectively, led to increasing concentrations of HDL-C. The effects of aspirin on HAE was dependent on DHA plasma concentrations with increases in HAE activity occurring only when both aspirin and DHA concentrations were moderate (not high or low), whereas no such dependency was present for EPA.

The effects of aspirin on reverse cholesterol metabolism pathway associated markers (HDL-C and HAE) in individuals with type 2 diabetes mellitus are both potentially important and are novel. We are not aware of prior studies that have examined the interactions of aspirin and these ω3 this way, most notably the influence they have on HAE. However, observations reported here are consistent with our prior study in which the effects of aspirin on lysophosphatidic acids (highly atherogenic) and other platelet function agonist biomarkers using the same study population [22]. Given the relative concern of aspirin resistance in this population, these results offer a promising start for future public health interventions. Individuals with type 2 diabetes mellitus are particularly predisposed to "resist" the CVD beneficial effects of aspirin, including reduced incidence of death and acute coronary syndrome [25–28]. In addition, it is intriguing that the effect of aspirin on these outcomes is dependent on DHA but not EPA concentrations, as both of these ω3 fatty acids are metabolized via cyclooxygenase 1, as is

Fig. 4. Acute and seven day effects of aspirin and aspirin + fish oil ingestion on HAE and HDL-C dependent on EPA concentrations. Acute (4 h) and chronic (7 days) effects of 81 mg aspirin and 81 mg aspirin + fish oil accounting for EPA plasma concentration only. The y-axis scale is the log-transformed HAE and HDL-C concentration. The x-axis is the log-transformed EPA concentration. Each LOWESS curve corresponds to the effect of aspirin (acute and chronic) as well as aspirin + fish oil (acute and chronic) on EPA.
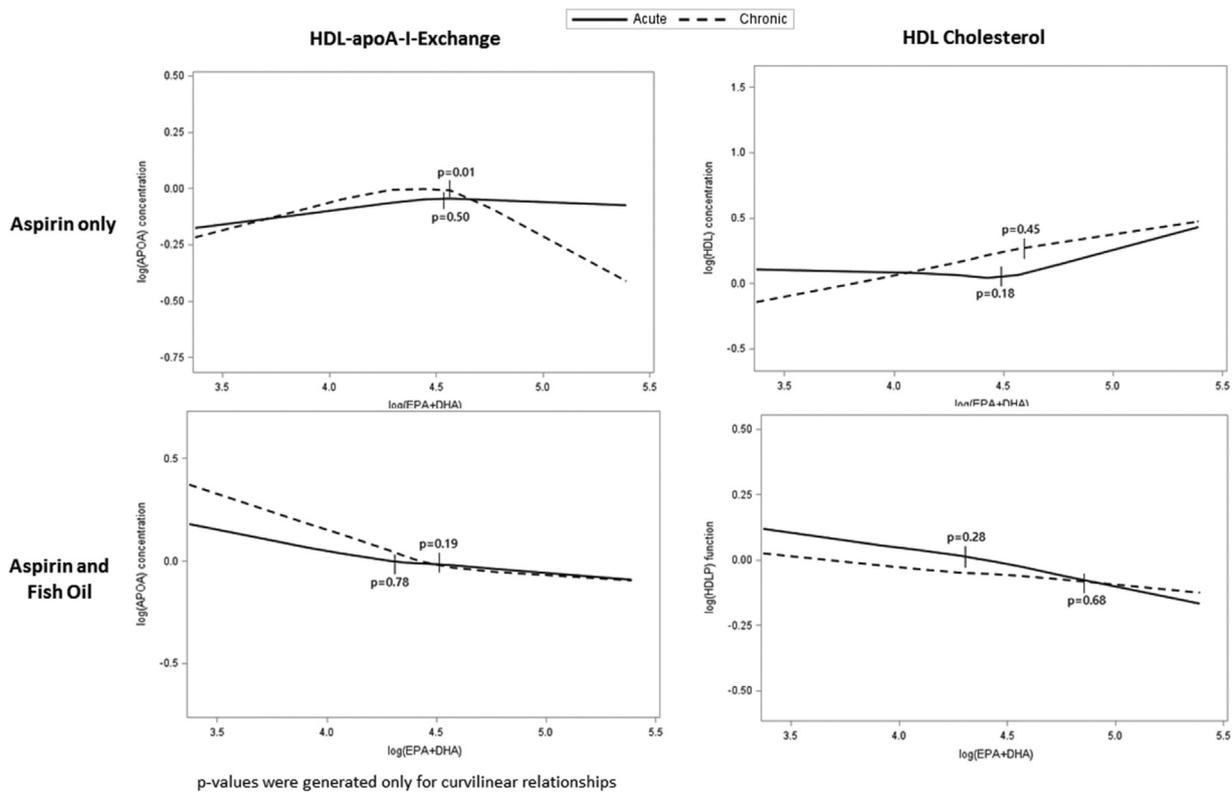
Fig. 5. Acute and seven day effects of aspirin and aspirin + fish oil ingestion on HAE and HDL-C dependent on EPA + DHA concentrations. Acute (4 h) and chronic (7 days) effects of 81 mg aspirin and 81 mg aspirin + fish oil accounting for combined EPA + DHA plasma concentrations. The y-axis scale is the log-transformed HAE and HDL-C concentration. The x-axis is the log-transformed EPA + DHA concentration. Each LOWESS curve corresponds to the effect of aspirin (acute and chronic) as well as aspirin + Fish oil (acute and chronic) on combined EPA + DHA concentrations.

aspirin [29]. The causal mechanism(s) underlying the interaction between low-dose aspirin and these ω3 fatty acids is unknown but deserves future research given the importance of aspirin and seafood consumption in healthy cardiovascular regimens [10,18].

ApoA-I is the major protein component of HDL and is essential for HDL biogenesis and function. A key atheroprotective function of apoA-I is its ability to exchange on and off of HDL particles [30–32]. Lipid-poor/lipid-free apoA-I is the preferred substrate of ATP binding cassette transporter A1 (ABCA1) [30,33,34]. Since apoA-I is not expressed in the intima but arrives there associated with HDL, apoA-I exchange is a critical step to ABCA1-mediated cholesterol efflux and de novo HDL biogenesis [35]. ApoA-I undergoes significant conformational change when exchanging between HDL-bound and lipid-free states [32] and this shift in conformation can be reliably quantified by electron paramagnetic resonance (EPR) or fluorescent methods using strategically positioned EPR or fluorescent labels in apoA-I [20,33]. In this study aspirin and DHA interact to influence HAE. The presence of an optimal moderate dose for DHA in the context of aspirin treatment suggests that DHA and aspirin influence the same factor(s) that have a direct effect on HDL's ability to exchange apoA-I. In this study changes in HAE do not appear to coincide with HDL-C, consistent with the notion that HDL-C is not a good proxy measure of this aspect of HDL function. How DHA and aspirin increases HAE and what parameters of HDL are mutually influenced by both to lower HAE is of considerable future interest. It would be intriguing if this effect can be reproduced in vitro, leading to the possibility of identifying optimal DHA and aspirin dosing, without the need for trial and error screening of patients.

Our study has numerous strengths but is not without limitations. The study sample represented a small number of adult individuals with T2DM. However, despite this small sample size the sequential design of this trial allows for baseline values that serve as each individual's own control thereby limiting confounding and increasing statistical power. While the possibility of measurement error cannot be ruled out, all blood specimens were processed using the same analytic machine in each separate laboratory for the analysis of EPA/DHA, HDL-C, and HAE. While the EPA + DHA assay did not account for other fatty acids, such as arachidonic acid (a ω6-PUFA) it did use a robust technique for measuring EPA and DHA absolute concentrations. Our participants can be considered representative of the general adult population of individuals with T2DM. Thus, concentrations are likely to be representative of other such individuals with high cardiovascular disease risk.

Conclusions from this study include the following: 1) low-dose aspirin has significant effects on HDL-C and HAE, which are major elements of the reverse cholesterol metabolism pathway; 2) its effects are enhanced by moderate plasma concentrations of DHA; 3) its effects are not enhanced by levels of EPA; and 4) the etiology of these results are unknown. These conclusions can lead to many future research directions including translating the interactions from bench to bedside by examining the interplay of aspirin with other factors (nutritional status, nutritional supplement consumption and other therapeutic interventions) in the prevention of CVD events. Basic science research focused on the metabolic mechanism(s) underlying the influence of these factors on aspirin's effect on CVD risk. Findings from this study highlight the benefits of precision medicine to identify optimal conditions for the application of interventions in the metabolic & nutritional context of the patient.

Summary

Many individuals with diabetes mellitus do not have cardiovascular disease (CVD) event reduction when taking aspirin. Many have termed this "resistance". The precise cause of this public health challenge is not known. Low-dose aspirin and the seafood-derived omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have effects on the cyclooxygenase pathway which can reduce platelet

function. HDL-cholesterol (HDL-C) is associated with CVD risk where ApoA-I is the major protein component of HDL-C and is essential for HDL-C biogenesis and function. A key atheroprotective function of apoA-I is its ability to exchange on and off with HDL-C particles. We have found in this study that aspirin has statistically significant effects on HDL-C concentrations and apoAI exchange when levels of DHA are moderate in plasma of adults with type 2 diabetes mellitus (T2DM). These results have potential implications for future research in which the mechanism for this correlation is investigated.

Sources of support

Disclosures

Trial registration

Clinicaltrials.gov: NCT01181882.

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