EFFECT OF OMEGA 3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN PATIENTS WITH A PREVIOUS MYOCARDIAL INFARCTION

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003054
Article Type:	Research
Date Submitted by the Author:	14-Apr-2013
Complete List of Authors:	Din, Jehangir; University of Edinburgh, Cardiology Sarma, Jaydeep Harding, Scott Lyall, Karin Newby, David; University of Edinburgh, Centre for Cardiovascular Sciences Flapan, Andrew; Royal Infirmary of Edinburgh, Cardiology
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Nutrition and metabolism
Keywords:	Coronary heart disease < CARDIOLOGY, NUTRITION & DIETETICS, VASCULAR MEDICINE

EFFECT OF OMEGA 3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN PATIENTS WITH A PREVIOUS MYOCARDIAL INFARCTION

Jehangir N Din[1], Jaydeep Sarma[2], Scott A Harding[3], Karin Lyall[1], David E Newby[1], Andrew D Flapan[4]

[1]Centre for Cardiovascular Sciences, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, United Kingdom; [2]North West Heart Centre, Wythenshawe Hospital, Manchester, United Kingdom; [3]Department of Cardiology, Wellington Hospital, Wellington, New Zealand; and [4]Edinburgh Heart Centre, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom.

WORD COUNT

2801

KEY WORDS

Omega 3 fatty acids, endothelial function, endogenous fibrinolysis, platelet activation,

AUTHOR FOR CORRSPONDENCE

Dr Jehangir N Din

University of Edinburgh

The Chancellor's Building

49 Little France Crescent

Edinburgh EH16 4SB

UNITED KINGDOM

Telephone: +44 131 242 1850

Fax: +44 131 242 6422

E-mail: jehangirdin@hotmail.com

Abstract

Objective – The mechanisms through which omega-3 fatty acids reduce adverse cardiac events remain uncertain. We aimed to investigate the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Design – Randomised, double-blind, placebo-controlled, crossover trial.

Setting – Academic cardiac centre.

Participants - Twenty male patients with a previous myocardial infarction.

Intervention - Omega-3 fatty acid supplementation (2g/day for 6-weeks) versus olive oil placebo.

Outcome measures - Peripheral blood was taken for analysis of platelet and monocyte activation, and forearm blood flow was assessed in a subset of 12 patients during intrabrachial infusions of acetylcholine, substance P and sodium nitroprusside. Stimulated plasma tissue plasminogen activator (t-PA) concentrations were measured during substance P infusion.

Results - All vasodilators caused dose-dependent increases in forearm blood flow (P<0.0001). Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation with acetylcholine and substance P compared with placebo (P=0.5 and P=0.9). Substance P caused a dose-dependent increase in plasma t-PA concentrations (P<0.0001), which was not affected by omega-3 fatty acid supplementation (P=0.9). Omega-3 fatty acids did not affect platelet-monocyte aggregation, platelet P-selectin or CD40L, or monocyte CD40.

Conclusions – We have demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function, endothelial t-PA release or platelet and monocyte activation in patients with coronary heart disease. Cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on these systems.

Article Summary

Article focus

- The mechanisms through which omega-3 fatty acids may reduce adverse cardiac events remain uncertain.
- We have previously demonstrated that omega-3 fatty acids improve endothelial function and endogenous fibrinolysis in healthy cigarette smokers.
- The present study investigated the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Key messages

- Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation, acute tPA release, or platelet and monocyte activation in patients with coronary heart disease.
- Any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function, the endogenous fibrinolytic system, or platelet activation.

Strengths and limitations of this study

- Randomised, double-blind, placebo-controlled crossover design.
- Use of an established and robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function.
- Limitations: modest sample size.

Funding statement

JD was supported by a British Heart Foundation Project Grant (PG/2003/009). DEN is supported by the British Heart Foundation. The Wellcome Trust Clinical Research Facility is supported by NHS Research Scotland (NRS) through NHS Lothian.

Competing interests

None.

Introduction

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease.[1] Clinical trials have demonstrated beneficial effects on mortality or cardiac events in patients with coronary heart disease.[2-4] However, the mechanisms through which they confer any cardiac benefits are uncertain. Although an effect on ventricular arrhythmias has been thought to be important due to an observed reduction in sudden death,[5,6] subsequent studies have failed to clearly demonstrate an anti-arrhythmic effect.[7] An alternative mechanism may therefore be an effect on the vascular endothelium, as acute myocardial infarction due to plaque rupture and subsequent coronary thrombosis remains the most common cause of sudden cardiac death.[8]

The endothelium regulates vascular tone and blood flow, and mediates thrombosis through the production of factors that influence fibrinolysis and platelet activation. The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi and is regulated by the endothelium-derived profibrinolytic factor, tissue plasminogen activator (t-PA), and its inhibitor, plasminogen-activator inhibitor type 1 (PAI-1).[9] The rapid release of t-PA from the endothelium is vital, with thrombus dissolution being more effective if t-PA is incorporated early during thrombus formation.[10]

Endothelial cells regulate thrombosis through the release of paracrine factors that mediate platelet function. Activated platelets can bind to leukocytes via a P-selectin dependent mechanism,[11] and these interactions can also be modulated by the CD40 receptor and its ligand.[12] Formation of platelet-leukocyte aggregates or ligation of CD40 can mediate an array of proinflammatory and prothrombotic effects, thereby contributing to endothelial injury and atherothrombosis.[13]

Patients with coronary heart disease demonstrate impaired endothelial function,[14] in addition to increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system.[15,16] We have recently demonstrated that omega-3 fatty acid supplements improve endogenous fibrinolysis and endothelial function in healthy cigarette smokers, a group at high risk of adverse cardiac events.[17]

Previously, we have shown that dietary fish intake reduces platelet-monocyte aggregation in man.[18] We therefore hypothesized that omega-3 fatty acid supplementation would improve endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation in patients with coronary heart disease.

Methods

Study participants

Twenty patients with a myocardial infarction at least three months previously were recruited to participate in the study. Myocardial infarction was defined as any two of: typical clinical history, electrocardiographic changes (Q waves in 2 contiguous leads) or elevation of cardiac markers (CKmB or troponin). All subjects gave written informed consent and the study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Exclusion criteria included dietary fish allergy or intolerance, consumption of > 1 fish meal per week, renal or hepatic failure, or any intercurrent illness likely to be associated with an inflammatory response.

Study design

This was a prospective, double-blind, placebo-controlled, randomized crossover trial. Subjects were randomized to receive either omega-3 fatty acid supplements (2 g/day, Omacor capsules, Pronova, Norway) or matching placebo capsules (2 g/day olive oil capsules, Eurocaps Limited, Gwent) for a 6-week period. After a 4-week washout phase, participants crossed over to the opposite treatment arm for a further 6-week period. The omega-3 fatty acid capsules contained 85-88% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters in a ratio of 1.2:1. Both the omega-3 fatty acid capsules and olive oil placebo contained 4 mg α-tocopherol. All 20 subjects had peripheral blood taken for fasting lipid profile, plasma fatty acid analysis and flow cytometric analysis of platelet activation at baseline and at the end of each treatment period. Two patients dropped out of the study: one was withdrawn after being admitted with unstable angina and a second patient was lost to follow-up. A subset of 12 participants also underwent measurement of forearm blood flow and endogenous fibrinolysis at the end of each treatment period.

Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with ethylene diamine tetra-acetic acid (EDTA; 1.6 mg/mL, Sarstedt Monovette) and the direct thrombin inhibitor D-Phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75 μM, PPACK, Cambridge Biosciences). Whole blood anticoagulated with PPACK was immunolabelled within 5 min of phlebotomy for subsequent flow cytometric analysis. Plasma was prepared from blood anticoagulated with sodium EDTA by centrifugation (1500 x *g* for 30 min). Plasma samples were stored at -70ºC until analysis.

Flow cytometry

The following reagents were used: fluorscein isothiocyanate (FITC)-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), phycoerythrin (PE)-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls (Serotec Ltd; Oxford, UK) as well as PE-conjugated CD154 (TRAP1, IgG1), PE-conjugated CD14 (Tuk-4, IgG2a), PE-conjugated CD 62P (IE3, IgG2a), and their appropriate isotype controls (Dako Cytomation; Buckinghamshire, UK) and FACS-Lyse (Becton-Dickinson; Cowley, UK). Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with appropriate antibodies and their isotype matched controls for 20 min at room temperature. To evaluate platelet-monocyte aggregates and CD40 on monocytes, samples were fixed and red cells lysed by the addition of 500 μL of FACS-Lyse solution. To evaluate platelet surface P-selectin and CD40 ligand, samples were fixed with 1% paraformaldehyde. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK) within 6 hours of labelling. Monocytes and platelets were identified by gating for CD14 and CD42a positive cells respectively. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. Analyses were performed using EXPO 32 software (Beckman Coulter, High Wycombe, UK).

Plasma fatty acid analysis

The fatty acid composition of plasma phospholipids was determined from blood anticoagulated with EDTA. Total lipids were recovered from 500 µL of plasma using dichloro-metane-metanol (2:1) containing 0.005% butyrated hydroxytoluene as an antioxidant (Folch extraction). Phospholipids were isolated by solid-phase extraction using aminopropyl silica columns (IST International), and fatty acids converted into methyl esters by transmethylation with 0.5 M sodium methoxide. Fatty acid methyl ester analysis was performed with an HP-INNOWAX capillary column (Agilent Technologies). Peaks were identified by comparison of retention times with known fatty acid methyl ester standards and quantified using an internal standard. Plasma total phospholipid fatty acids were expressed as the individual fractions of fatty acids and fatty acid groups as relative values (% of total fatty acids). The mean coefficient of variation for the assay was 2.4%

Vascular studies

Studies were carried out in a quiet temperature controlled room (22–25 °C). Subjects fasted for 6 h prior to the study and avoided caffeine and alcohol for the preceding 24 h. Blood pressure and heart rate were recorded throughout the study using a semi-automated non-invasive oscillometric sphygmomanometer (OMRON 705 IT, Kyoto, Japan).

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-min baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences), substance P at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator that releases t-PA; Clinalfa, Switzerland) and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories) were infused for 6 min at each dose. The 3 vasodilators were separated by 20-min saline infusions and given in a randomized order.

Forearm blood flow (FBF) was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in–silicone elastomer strain gauges as described previously.[19] Venous

cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during infusion of each dose of substance P and collected into acidified buffered citrate (Stabilyte tubes, Biopool International; for t-PA assays) and into citrate (BD Vacutainer; for PAI-1 assays). Samples were kept on ice before being centrifuged at 2000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by enzyme-linked immunosorbent assays. Haematocrit was determined by capillary tube centrifugation at baseline.

Data analysis and statistical methods

Continuous variables are reported as mean ± standard error of the mean. The pre-specified primary endpoint was endothelial vasomotor and fibrinolytic function. The sample size of n=12 was based on power calculations derived from previous studies giving 90% power to detect a 17% difference in the mean t-PA release at a significance level of 5%.[19] The pre-specified secondary endpoint was platelet and monocyte activation. The sample size of n=20 was based on power calculations derived from previous studies, giving 90% power to detect a 5% difference in mean platelet-monocyte aggregation at a significance level of 5%. Forearm plethysmographic data were analyzed as described previously.[17] Estimated net release of plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms.[19] Statistical analyses were performed using one-way and two-way ANOVA with Bonferroni's post-tests for multiple comparisons where appropriate. The statistical methods for each analysis are detailed in the relevant Figure and Table legends. All calculations were performed using GraphPad Prism (Graph Pad Software).

Results

Baseline characteristics

Patients were relatively young and well treated in terms of blood pressure control and lipid profile (Table 1). The mean and median times from myocardial infarction were 12 months and 16 months, respectively. Patients were on standard medical therapy including aspirin, beta-blockers, statins and ACE-inhibitors, and over half had undergone revascularization post-MI.

Effect of omega 3 fatty acid supplementation on plasma phospholipid fatty acid composition

Dietary supplementation with omega-3 fatty acids led to a marked increase in EPA as a percentage of plasma phospholipids compared with both baseline (3.7±0.4% versus 2.0±0.2%, $P<0.0001$) and placebo (3.7±0.4% versus 1.7±0.1%, $P<0.0001$; Figure 1A). There was also an increase in DHA compared with baseline (5.6±0.2% versus 4.8±0.3%, $P<0.01$) and placebo (5.6±0.2% versus 4.4±0.3%, $P<0.001$; Figure 1B). There was a reduction in the plasma phospholipid percentage of arachadonic acid, but no effect on alpha-linolenic acid, linoleic acid, palmitic acid, stearic acid or oleic acid with either omega-3 fatty acid supplements or olive oil placebo (Table 2).

Effect of omega 3 fatty acid supplementation on lipid profile

Supplementation for 6 weeks with omega 3 fatty acids did not affect total cholesterol, low-density liproprotein cholesterol, high-density liproprotein cholesterol or triglycerides (Table 3).

Effect of omega 3 fatty acid supplementation on vasomotor function

Omega-3 fatty acid supplementation did not have any effect on systolic blood pressure, diastolic blood pressure or heart rate compared with placebo (Table 3). During forearm vascular studies substance P, acetylcholine, and sodium nitroprusside led to a dose-dependent increase in absolute forearm blood flow ($P<0.0001$ for all). Compared with placebo, omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation in response to acetylcholine and substance P

(*P*=0.5 and *P*=0.9; Figure 2), or endothelium-independent vasodilatation with sodium nitroprusside (*P*=0.9; Figure 2).

Effect of omega-3 fatty acid supplementation on stimulated t-PA activity

Substance P infusion caused a dose-dependent increase in plasma t-PA activity concentrations after both omega-3 fatty acid supplementation and placebo (*P*<0.0001; Table 4). Omega-3 fatty acid supplementation did not affect plasma TPA activity, TPA antigen or PAI-1 concentrations compared with placebo (Table 4). There was no difference in net release of t-PA activity after omega-3 fatty acid supplementation compared with placebo (*P*=0.94; Figure 3).

Effect of omega-3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand

Supplementation with omega-3 fatty acids did not have any effect on platelet-monocyte aggregation, platelet-neutrophil aggregation, platelet surface expression of P-selectin or CD40L, or monocyte expression of CD40 (Table 5).

Discussion

The present study has demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function or endothelial t-PA release in patients with coronary heart disease. There is also no effect on markers of platelet or monocyte activation. These findings suggest that any cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on endothelial function, endogenous fibrinolysis or platelet activation.

We do not believe the lack of effect on outcome measures in the present study is likely to have been due to poor compliance. The assessment of plasma phospholipid fatty acid composition confirmed substantial increases in the percentage of both EPA and DHA during supplementation with omega-3 fatty acids. The dose and duration of therapy with omega-3 fatty acids are also likely to have been appropriate. We used 2 grams per day of omega-3 fatty acids which is similar to the amount shown to reduce mortality in secondary prevention trials.[2,3] Although we cannot exclude an effect with a longer duration of therapy, 6 weeks of supplementation caused a large increase in the plasma phospholipid content of omega-3 fatty acids and has previously been long enough to demonstrate clear effects on vascular function and platelet activation.[20-22]

Omega-3 fatty acids have previously been shown to have inconsistent effects on endothelial function. Whilst some studies have reported beneficial effects in a variety of populations including healthy volunteers,[22] patients with hyperlipidaemia,[21,23] diabetes mellitus,[24] and heart failure,[25] others have not found any improvement.[26-28] Our findings are in contrast to previous studies in coronary heart disease which demonstrated an improvement in endothelial function with omega-3 fatty acids.[20,29-30] These discrepancies could be partly due to differences in study populations or concomitant medication. However, the previous studies were all either not randomized or double-blinded, and lacked a control group or placebo. Indeed, our trial is the first double-blinded, placebo-controlled trial investigating the effect of omega-3 fatty acids on endothelial vasomotor function in coronary heart disease; we therefore believe our study design and findings are likely to be robust.

We also found that omega-3 fatty acids did not augment endogenous fibrinolysis in coronary heart disease. Previous results have varied widely and it has been concluded that omega-3 fatty acids are unlikely to influence the fibrinolytic system.[31] Whilst some studies have reported a beneficial impact on fibrinolytic parameters,[32-33] others have found an adverse effect[34] or no effect.[26,35-37] However, previous studies have only measured basal plasma t-PA concentrations that do not reflect the local capacity for acute endothelial t-PA release.[9,38] It is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. We used an established model of acute endothelial t-PA release that predicts cardiovascular outcome,[19,39] but found no effect of omega-3 fatty acid supplementation on acute endogenous fibrinolytic capacity in coronary heart disease.

There are several possible explanations for the lack of effect omega-3 fatty acids on endothelial function and endogenous fibrinolysis observed in the present coronary heart disease population. The patients were all well treated with modern cardio-active medication known to influence endothelial vasomotor function.[40-41] In contrast, patients in previous studies demonstrating improved endothelial function[20,29] and cardiac outcomes[2-3] with omega-3 fatty acids were much less likely to be taking HMG CoA reductase inhibitors or angiotensin-converting enzyme inhibitors. It is conceivable that endothelial function cannot be further improved by the addition of omega-3 fatty acids in coronary heart disease patients already treated with modern medical therapy. This possibility is supported by the most recent large clinical trials which found a low rate of cardiac events in patients on optimal medical therapy post-myocardial infarction, which could not be improved with omega-3 fatty acid supplementation.[42-44]

However, concomitant medication may not fully explain the neutral effects on endogenous fibrinolysis. Whilst lipid-lowering therapy improves endothelial vasomotor function, it has not been found to influence acute t-PA release.[45] Angiotensin-converting enzyme inhibitors only augment bradykinin induced t-PA release; they do not affect t-PA release stimulated by substance P.[46]

Therefore, there may be other factors to explain why omega-3 fatty acid supplementation can improve endogenous fibrinolytic capacity in healthy cigarette smokers but not in patients with coronary heart disease. Perhaps the most likely explanation is that the coronary heart disease group was considerably older and may have a dysfunctional endothelium and fibrinolytic system less responsive to dietary interventional measures.

Circulating platelet-monocyte aggregates are increased in stable coronary heart disease and acute coronary syndromes, consistent with an important role in both the development of atherosclerotic lesions and in acute thrombosis.[15] We have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation.[18] However, we did not observe any effect of omega-3 fatty acid supplements on these measures of platelet and monocyte activation in the present study. It is possible our previous results were due to another active ingredient in oily fish rather than omega-3 fatty acids, and we cannot exclude a dose-effect of omega-3 fatty acids on platelet activation. Omega-3 fatty acids also had no effect on monocyte expression of CD40 or platelet surface CD40 ligand, consistent with previous studies which found no effect of either omega-3 fatty acids or dietary fish on soluble CD40 ligand.[18,47]

Conclusions

We have demonstrated that omega-3 fatty acid supplementation does not affect endothelial function, endogenous fibrinolytic capacity or markers of platelet and monocyte activation in patients with stable coronary heart disease. A major strength of our study is the use of a robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function. Our results suggest that any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function or the fibrinolytic system.

Funding

None

Competing Interests

None

Data sharing

No additional unpublished data from this study.

Contributorship

1) Conception and design or analysis and interpretation of data: JD, KL, SH, JS, AF, DN

2) Drafting of the manuscript or revising it critically for intellectual content: JD, JS, AF, DN

3) Final approval of the manuscript submitted: All authors.

References

1. Din JN, Newby DE, Flapan AD. Omega 3 fatty acids and cardiovascular disease - fishing for a natural treatment. *BMJ* 2004;328:30-5.
2. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 1989;2:757-61.
3. GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354:447-55.
4. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, Shirato K; Japan EPA lipid intervention study (JELIS) Investigators. *Lancet*. 2007; 369:1090-8.

5. Marchioli R, Barzi F, Bomba E, Chieffo C, Di Gregorio D, Di Mascio R, *et al*. Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation* 2002;105:1897-903
6. Leaf A, Kang JX, Xiao YF, Billman GE. Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation* 2003;107:2646-52.
7. Brouwer IA, Raitt MH, Dullemeijer C, Kraemer DF, Zock PL, Morris C, Katan MB, Connor WE, Camm JA, Schouten EG, McAnulty J. Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators. *Eur Heart J* 2009;30:820-6.
8. Bowker TJ, Wood DA, Davies MJ, Sheppard MN, Cary NR, Burton JD, Chambers DR, Dawling S, Hobson HL, Pyke SD, Riemersma RA, Thompson SG. Sudden, unexpected cardiac or unexplained death in England: a national survey. *QJM*. 2003;96:269-79.
9. Oliver JJ, Webb DJ, Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans, *Arterioscler Thromb Vasc Biol* 2005; 25: 2470–2479.
10. Fox KA, Robison AK, Knobb RM *et al*. Prevention of coronary thrombosis with subthrombolytic doses of tissue type plasminogen activator. *Circulation* 1985; 72:1346-1354.
11. Jungi TW, Spycher MO, Nydegger UE, Barandun S. Platelet-leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* 1986;67:629-36.
12. Schonbeck U, Lippy P. CD40 signaling and plaque instability. *Circ Res.* 2001; 89: 1092–1103
13. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61-7
14. Zeiher AM, Drexler H, Wollschlager H, Just H. Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991;83:391-401.
15. Sarma J, Laan CA, Alam S, Jha A, Fox KA, Dransfield I. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* 2002; 105: 2166-71.

16. Tousoulis D, Antoniades C, Nikolopoulou A, Koniari K, Vasiliadou C, Marinou K, Koumallos N, Papageorgiou N, Stefanadi E, Siasos G, Stefanadis C. Interaction between cytokines and sCD40L in patients with stable and unstable coronary syndromes. *Eur J Clin Invest*. 2007;37:623-8.

17. Din JN, Archer RM, Harding SA, Sarma J, Lyall K, Flapan AD, Newby DE. Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in male cigarette smokers. *Heart*. 2013; 99:168-74.

18. Din JN, Harding SA, Valerio CJ, Sarma J, Lyall K, Riemersma RA, Newby DE, Flapan AD. Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. *Atherosclerosis* 2008;197:290-6.

19. Newby DE, Wright RA, Ludlam CA, Fox KA, Boon NA, Webb DJ. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost.* 1997; 78: 1242–1248

20. Tagawa H, Shimokawa H, Tagawa T, Kuroiwa-Matsumoto M, Hirooka Y, Takeshita A. Long-term treatment with eicosapentaenoic acid augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilatation in patients with coronary artery disease. *J Cardiovasc Pharmacol*. 1999 Apr;33(4):633-40.

21. Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. *Circulation* 2000;102:1264-9.

22. Chin JP, Gust AP, Nestel PJ, Dart AM. Marine oils dose-dependently inhibit vasoconstriction of forearm resistance vessels in humans. *Hypertension* 1993;21(1):22-8.

23. Goodfellow J, Bellamy MF, Ramsey MW, Jones CJ, Lewis MJ. Dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia. *J Am Coll Cardiol*. 2000;35:265-70.

24. McVeigh GE, Brennan GM, Johnston GD, McDermott BJ, McGrath LT, Henry WR, Andrews JW, Hayes JR. Dietary fish oil augments nitric oxide production or release in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1993;36:33-8.

25. Morgan DR, Dixon LJ, Hanratty CG, El-Sherbeeny N, Hamilton PB, McGrath LT, Leahey WJ, Johnston GD, McVeigh GE. Effects of dietary omega-3 fatty acid supplementation on

endothelium-dependent vasodilation in patients with chronic heart failure. *Am J Cardiol*. 2006;97:547-51.

26. Woodman RJ, Mori TA, Burke V, Puddey IB, Barden A, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis* 2003;166:85-93.
27. Wong CY, Yiu KH, Li SW, Lee S, Tam S, Lau CP, Tse HF. Fish-oil supplement has neutral effects on vascular and metabolic function but improves renal function in patients with Type 2 diabetes mellitus. *Diabet Med* 2010;27:54-60.
28. Skulas-Ray AC, Kris-Etherton PM, Harris WS, Vanden Heuvel JP, Wagner PR, West SG. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. *Am J Clin Nutr*. 2011;93:243-52.
29. Tagawa T, Hirooka Y, Shimokawa H, Hironaga K, Sakai K, Oyama J, Takeshita A. Long-term treatment with eicosapentaenoic acid improves exercise-induced vasodilation in patients with coronary artery disease. Hypertens Res. 2002;25:823-9.
30. Haberka M, Mizia-Stec K, Mizia M, Janowska J, Gieszczyk K, Chmiel A, Zahorska-Markiewicz B, Gąsior Z. N-3 polyunsaturated fatty acids early supplementation improves ultrasound indices of endothelial function, but not through NO inhibitors in patients with acute myocardial infarction: N-3 PUFA supplementation in acute myocardial infarction. *Clin Nutr* 2011;30:79-85.
31. Kristensen SD, Iversen AM, Schmidt EB. n-3 polyunsaturated fatty acids and coronary thrombosis. *Lipids* 2001;36 Suppl:S79-82.
32. Smith P, Arnesen H, Opstad T, Dahl KH, Eritsland J. Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo. *Thromb Res* 1989;53:467-74.
33. Mehta J, Lawson D, Saldeen TJ. Reduction in plasminogen activator inhibitor-1 (PAI-1) with omega-3 polyunsaturated fatty acid (PUFA) intake. *Am Heart J* 1988;116(5 Pt 1):1201-6.

34. Spannagl M, Drummer C, Fröschl H, von Schacky C, Landgraf-Leurs MM, Landgraf R, Schramm W. Plasmatic factors of haemostasis remain essentially unchanged except for PAI activity during n-3 fatty acid intake in type I diabetes mellitus. *Blood Coagul Fibrinolysis*. 1991;2:259-65.

35. Finnegan YE, Howarth D, Minihane AM, Kew S, Miller GJ, Calder PC, Williams CM. Plant and marine derived (n-3) polyunsaturated fatty acids do not affect blood coagulation and fibrinolytic factors in moderately hyperlipidemic humans. *J Nutr* 2003;133:2210-3.

36. Hellsten G, Boman K, Saarem K, Hallmans G, Nilsson TK. Effects on fibrinolytic activity of corn oil and a fish oil preparation enriched with omega-3-polyunsaturated fatty acids in a long-term study. *Curr Med Res Opin*. 1993;13:133-9.

37. Toft I, Bønaa KH, Ingebretsen OC, Nordøy A, Jenssen T. Fibrinolytic function after dietary supplementation with omega3 polyunsaturated fatty acids. *Arterioscler Thromb Vasc Biol*. 1997;17:814-9.

38. Hrafnkelsdottir T, Gudnason T, Wall U, Jern C, Jern S. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost* 2004; 2: 1960–8.

39. Robinson SD, Ludlam CA, Boon NA, Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol* 2007; 27**:** 1651–6.

40. Treasure CB, Klein JL, Weintraub WS, Talley JD, Stillabower ME, Kosinski AS, Zhang J, Boccuzzi SJ, Cedarholm JC, Alexander RW. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. *N Engl J Med* 1995; 332:481–7.

41. Mancini GB, Henry GC, Macaya C, O'Neill BJ, Pucillo AL, Carere RG, Wargovich TJ, Mudra H, Lüscher TF, Klibaner MI, Haber HE, Uprichard AC, Pepine CJ, Pitt B. Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease: the TREND (Trial on Reversing ENdothelial Dysfunction) study. *Circulation* 1996; 94: 258–65.

42. Rauch B, Schiele R, Schneider S, Diller F, Victor N, Gohlke H, Gottwik M, Steinbeck G, Del Castillo U, Sack R, Worth H, Katus H, Spitzer W, Sabin G, Senges J; OMEGA Study Group. OMEGA, a

randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction. *Circulation*. 2010; 122(21):2152-9

43. Galan P, Kesse-Guyot E, Czernichow S, Briancon S, Blacher J, Hercberg S; SU.FOL.OM3 Collaborative Group. Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial. *BMJ*. 2010 Nov 29;341:c6273
44. Kromhout D, Giltay EJ, Geleijnse JM; Alpha Omega Trial Group. n-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med*. 2010; 363:2015-26.
45. Newby DE, Witherow FN, Wright RA, Bloomfield P, Ludlam CA, Boon NA, Fox KA, Webb DJ. Hypercholesterolaemia and lipid lowering treatment do not affect the acute endogenous fibrinolytic capacity in vivo. *Heart* 2002; 87: 48 –53.
46. Witherow FN, Dawson P, Ludlam CA, Fox KA, Newby DE. Marked bradykinin-induced tissue plasminogen activator release in patients with heart failure maintained on long-term angiotensin-converting enzyme inhibitor therapy. *J Am Coll Cardiol*. 2002; 40: 961-6.
47. Aarsetoy H, Brugger-Andersen T, Hetland O, Grundt H, Nilsen DW. Long term influence of regular intake of high dose n-3 fatty acids on CD40-ligand, pregnancy-associated plasma protein A and matrix metalloproteinase-9 following acute myocardial infarction. *Thromb Haemost* 2006;95:329-36.

Acknowledgements

We are grateful to the Clinical Research Facility at the Royal Infirmary of Edinburgh.

Authorship

1) Conception and design or analysis and interpretation of data: JD, KL, SH, JS, AF, DN

2) Drafting of the manuscript or revising it critically for intellectual content: JD, JS, AF, DN

3) Final approval of the manuscript submitted: All authors.

.

Figure Legends

Figure 1. Percentage omega-3 fatty acids in plasma phospholipids at baseline, during omega-3 fatty acid supplementation and placebo. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni's post-tests for multiple comparisons. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Figure 2. Effect of omega-3 fatty acid supplementation on absolute forearm blood flow in response to endothelium-dependent and endothelium-independent vasodilators. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

Figure 3. Net release of plasma t-PA activity with omega-3 fatty acid supplementation and placebo. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

TABLE 1. Baseline Characteristics

Age, years	53±3
Body mass index, kg/m^2	28±1
Systolic blood pressure, mm Hg	137±5
Diastolic blood pressure, mm Hg	78±3
Heart rate, beats per minute	60±2
Total cholesterol, mmol/L	4.2±0.2
LDL cholesterol, mmol/L	2.3±0.2
HDL cholesterol, mmol/L	1.1±0.1
Chol:HDL chol ratio	3.8±0.2
Triacylglycerol, mmol/L	1.6±0.2
Fasting glucose, mmol/l	5.4±0.1
Time from MI, months	16±4
Revascularization post-MI, %	56%
Current or ex-smoker, %	61%
Hypertension, %	11%
Diabetes mellitus, %	0%
Hyperlipidemia, %	78%
Family history of premature coronary heart disease, %	33%
Medical therapy	
Aspirin, %	100%
Clopidogrel, %	11%
ACE-inhibitor/Angiotensin-receptor blocker, %	56%
Beta-blocker %	78%
Statin, %	100%

Mean±SEM.

TABLE 2. Effect of omega-3 fatty acid supplementation on plasma phospholipid fatty acid composition

	Baseline	Omega-3	Placebo	P value
Alpha-linolenic acid	0.3±0.01	0.3±0.02	0.3±0.03	0.3
Arachadonic acid	12.5±0.4	11.0±0.3	11.6±0.5	0.0005
Linoleic acid	18.8±0.6	19.0±0.6	20.0±0.6	0.1
Palmitic acid	28.2±0.4	27.9±0.3	28.2±0.3	0.6
Stearic acid	13.8±0.3	14.1±0.2	13.9±0.2	0.4
Oleic acid	13.3±0.5	13.0±0.4	13.8±0.5	0.1

Mean±SEM. Data analysed using 1-way ANOVA.

TABLE 3. Effect of omega-3 fatty acid supplementation on blood pressure and lipid profile

	Baseline	Omega 3	Placebo	P value
Heart rate, beats per minute	60±3	60±2	60±2	0.9
Systolic blood pressure, mm Hg	131±4	125±4	130±4	0.2
Diastolic blood pressure, mm Hg	77±3	74±2	74±3	0.5
Total cholesterol, mmol/L	4.3±0.3	3.9±0.3	4.0±0.2	0.1
LDL cholesterol, mmol/L	2.5±0.2	2.2±0.3	2.2±0.1	0.2
HDL cholesterol, mmol/L	1.1±0.1	1.1±0.1	1.1±0.1	0.4
Chol:HDL chol ratio	3.8±0.3	3.5±0.3	3.5±0.2	0.3
Triacylglycerol, mmol/L	1.5±0.2	1.3±0.1	1.5±0.1	0.5

Mean±SEM. Data analysed using 1-way ANOVA.

TABLE 4. Effect of omega 3 fatty acid supplementation on plasma t-PA activity concentrations

	Omega-3 fatty acids				Placebo			
Substance P pmol/min	**0**	**2**	**4**	**8**	**0**	**2**	**4**	**8**
t-PA activity, IU mL^{-1}								
Non-infused arm	0.39±0.08	0.45±0.09	0.54±0.12	0.64±0.14	0.45±0.07	0.52±0.08	0.60±0.09	0.65±0.11
Infused arm	0.38±0.08	0.83±0.16	1.12±0.23	1.67±0.38	0.43±0.07	0.78±0.10	1.09±0.11	1.26±0.15
t-PA antigen, ng mL^{-1}								
Non-infused arm	11.78±1.29	12.01±1.0	12.69±1.08	12.83±1.49	13.45±1.40	12.93±1.70	13.08±1.80	12.37±1.27
Infused arm	11.90±1.45	13.98±1.33	13.63±1.12	14.86±1.40	12.55±1.10	12.85±1.44	13.45±1.35	13.97±1.55
PAI-1 activity, ng mL^{-1}								
Non-infused arm	1.77±0.53	1.84±0.43	1.80±0.42	1.64±0.45	1.44±0.29	1.38±0.26	1.39±0.47	1.34±0.44
Infused arm	2.33±0.86	2.18±0.61	2.21±0.69	1.92±0.63	1.69±0.46	1.64±0.41	1.54±0.39	1.49±0.39
PAI-1 antigen, ng mL^{-1}								
Non-infused arm	39.51±9.22	40.84±7.08	39.99±6.62	38.48±5.79	45.06±7.09	43.33±6.45	44.41±6.67	44.26±7.03
Infused arm	37.64±8.36	38.83±6.25	41.71±5.74	40.26±7.32	48.89±8.25	42.65±6.59	43.12±6.60	40.61±6.46
Net t-PA antigen release ng 100 mL^{-1} of tissue mm^{-1}	0.23±0.51	-0.28±4.7	3.92±1.8	8.41±2.94	-0.87±1.1	-0.84±2.82	0.94±4.22	8.10±3.67

Mean±SEM. Data analysed using 2-way ANOVA.

t-PA activity: Dose response $P<0.0001$. Omega-3 fatty acids versus placebo; $P=0.83$ (infused arm).

t-PA antigen: Dose response $P=0.7$. Omega-3 fatty acids versus placebo; $P=0.60$ (infused arm).

PAI-1 activity: Dose response $P=0.94$. Omega-3 fatty acids versus placebo; $P=0.17$ (infused arm).

PAI-1 antigen: Dose response $P=0.67$. Omega-3 fatty acids versus placebo; $P=0.40$ (infused arm).

Net t-PA antigen: Dose response $P=0.02$. Omega-3 fatty acids versus placebo; $P=0.62$ (infused arm).

TABLE 5. Effect of omega 3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand system

	Baseline	Omega 3	Placebo	P value
Platelet-monocyte aggregates, %	21.2±3.9	23.6±4.2	23.0±4.1	0.7
Platelet-neutrophil aggregates, %	4.9±1.0	6.7±1.2	6.7±1.1	0.1
Platelet-surface expression of P-selectin, %	3.9±0.9	5.0±1.0	4.3±1.0	0.3
Platelet-surface expression of CD40L, %	3.6±0.4	3.4±0.3	3.4±0.3	0.9
Monocyte expression of CD40, %	52.5±5.0	46.8±3.4	47.4±2.5	0.5

Mean±SEM. Data analysed using 1-way ANOVA.

Figure 1

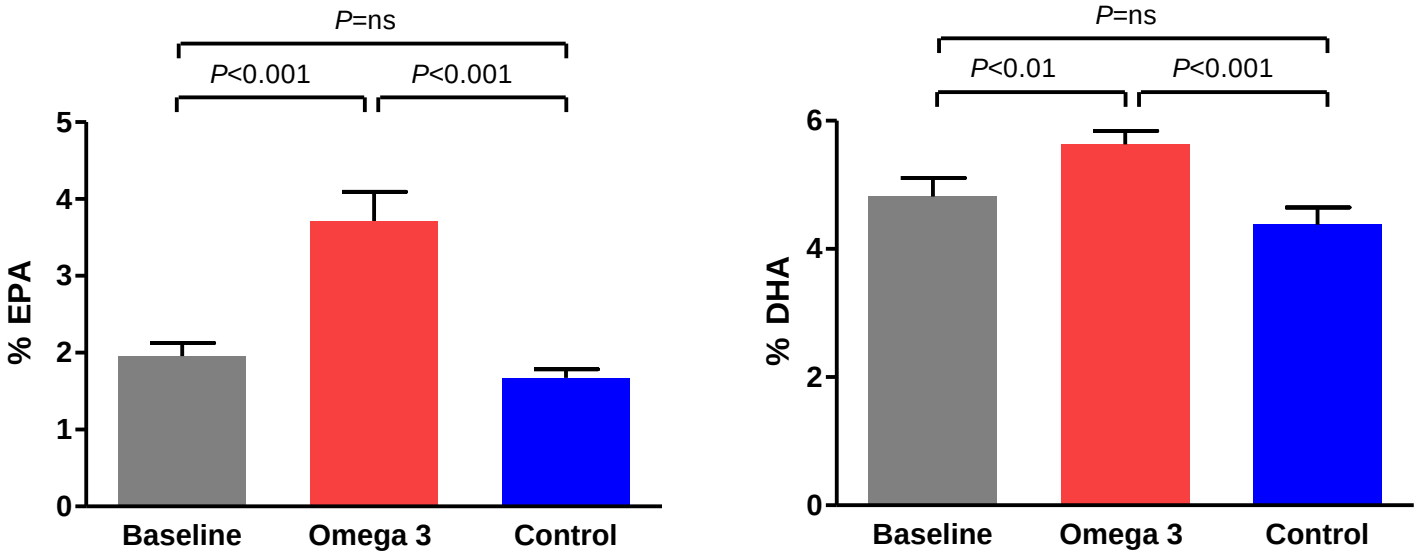

P=ns
P<0.001
P<0.001
% EPA
5
4
3
2
1
0
Baseline
Omega 3
Control
P=ns
P<0.01
P<0.001
% DHA
6
4
2
0
Baseline
Omega 3
Control

Figure 2

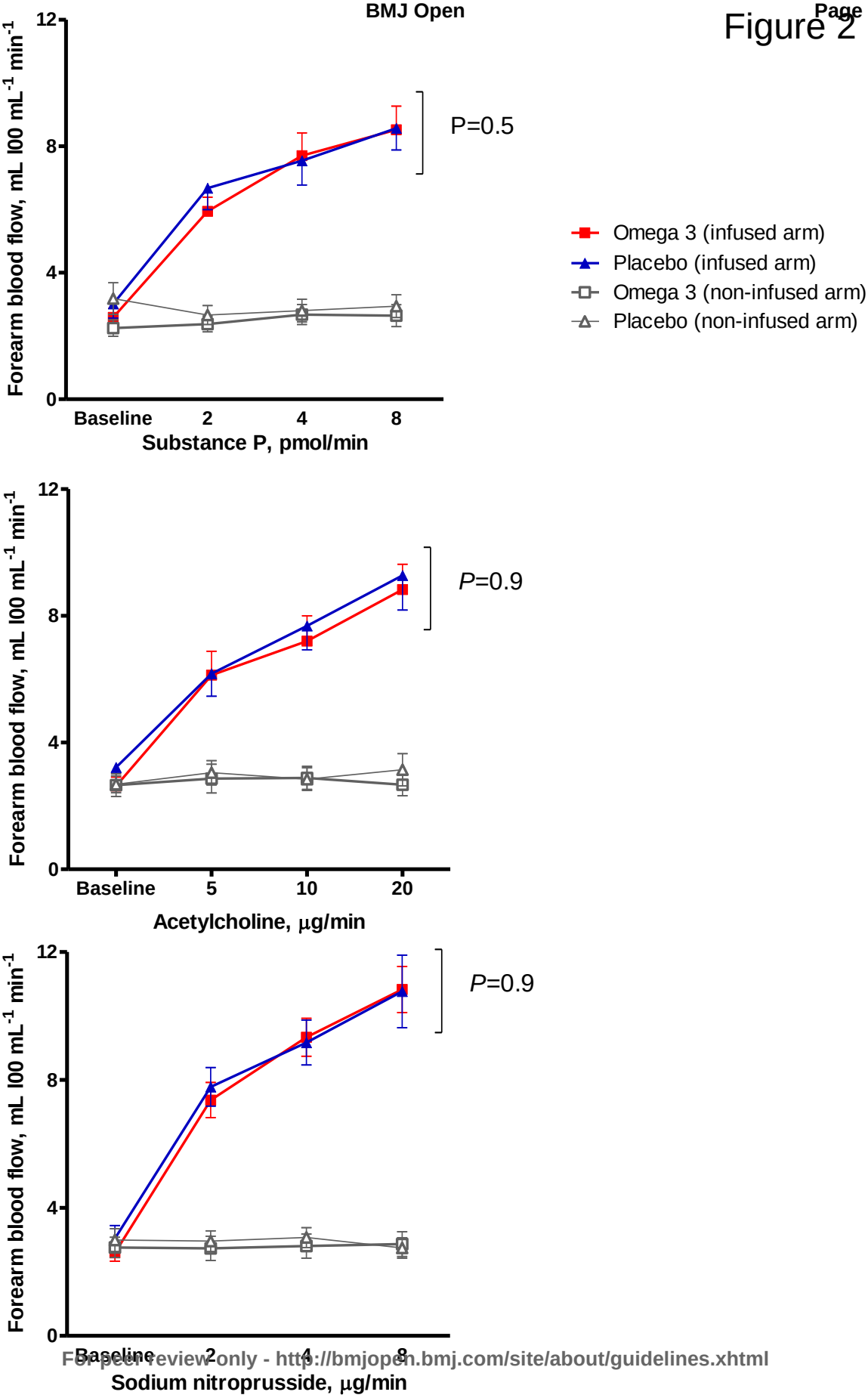
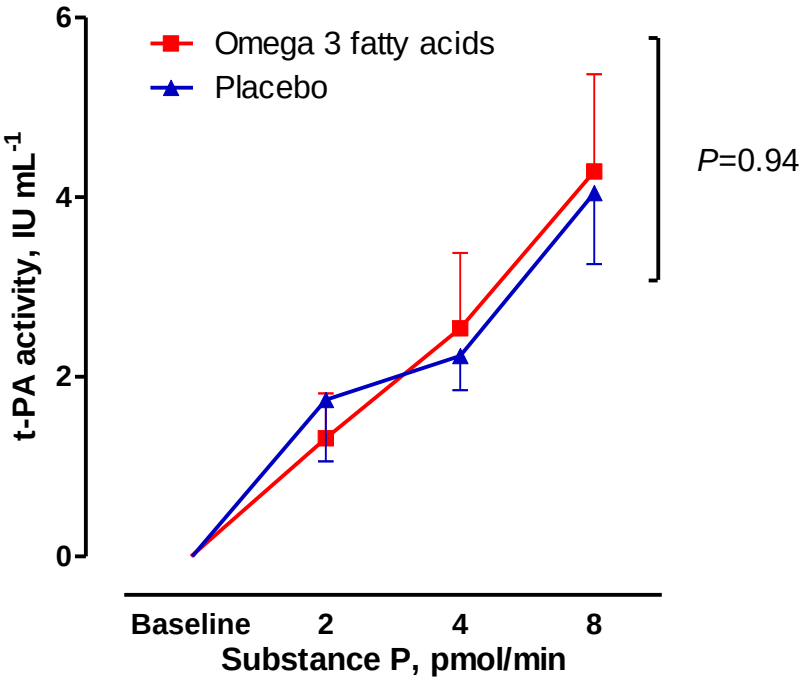


Figure 3


Omega 3 fatty acids
Placebo
P=0.94
t-PA activity, IU mL-1
0
2
4
6
Baseline
2
4
8
Substance P, pmol/min

BMJ open

EFFECT OF OMEGA 3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN PATIENTS WITH A PREVIOUS MYOCARDIAL INFARCTION: A RANDOMISED CONTROLLED TRIAL

Journal:	*BMJ Open*
Manuscript ID:	bmjopen-2013-003054.R1
Article Type:	Research
Date Submitted by the Author:	05-Jul-2013
Complete List of Authors:	Din, Jehangir; University of Edinburgh, Centre for Cardiovascular Sciences Sarma, Jaydeep; Wythenshawe Hospital, North West Heart Centre Harding, Scott; Wellington Hospital, 3Department of Cardiology Lyall, Karin; University of Edinburgh, 1Centre for Cardiovascular Sciences Newby, David; University of Edinburgh, Centre for Cardiovascular Sciences Flapan, Andrew; Royal Infirmary of Edinburgh, Cardiology
Primary Subject Heading:	Cardiovascular medicine
Secondary Subject Heading:	Nutrition and metabolism
Keywords:	Coronary heart disease < CARDIOLOGY, NUTRITION & DIETETICS, VASCULAR MEDICINE

EFFECT OF OMEGA 3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN PATIENTS WITH A PREVIOUS MYOCARDIAL INFARCTION: A RANDOMISED CONTROLLED TRIAL

Jehangir N Din[1], Jaydeep Sarma[2], Scott A Harding[3], Karin Lyall[1], David E Newby[1], Andrew D Flapan[4]

[1]Centre for Cardiovascular Sciences, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, United Kingdom; [2]North West Heart Centre, Wythenshawe Hospital, Manchester, United Kingdom; [3]Department of Cardiology, Wellington Hospital, Wellington, New Zealand; and [4]Edinburgh Heart Centre, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom.

WORD COUNT 2801

ClinicalTrials.gov Identifier: NCT01888211

KEY WORDS

Omega 3 fatty acids, endothelial function, endogenous fibrinolysis, platelet activation,

AUTHOR FOR CORRSPONDENCE

Dr Jehangir N Din

University of Edinburgh

The Chancellor's Building

49 Little France Crescent

Edinburgh EH16 4SB

UNITED KINGDOM

Telephone: +44 131 242 1850

Fax: +44 131 242 6422

E-mail: jehangirdin@hotmail.com

Abstract

Objective – The mechanisms through which omega-3 fatty acids reduce adverse cardiac events remain uncertain. We aimed to investigate the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Design – Randomised, double-blind, placebo-controlled, crossover trial.

Setting – Academic cardiac centre.

Participants - Twenty male patients with a previous myocardial infarction.

Intervention - Omega-3 fatty acid supplementation (2g/day for 6-weeks) versus olive oil placebo.

Outcome measures - Peripheral blood was taken for analysis of platelet and monocyte activation, and forearm blood flow was assessed in a subset of 12 patients during intrabrachial infusions of acetylcholine, substance P and sodium nitroprusside. Stimulated plasma tissue plasminogen activator (t-PA) concentrations were measured during substance P infusion.

Results - All vasodilators caused dose-dependent increases in forearm blood flow (P<0.0001). Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation with acetylcholine and substance P compared with placebo (P=0.5 and P=0.9). Substance P caused a dose-dependent increase in plasma t-PA concentrations (P<0.0001), which was not affected by omega-3 fatty acid supplementation (P=0.9). Omega-3 fatty acids did not affect platelet-monocyte aggregation, platelet P-selectin or CD40L, or monocyte CD40.

Conclusions – We have demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function, endothelial t-PA release or platelet and monocyte activation in patients with coronary heart disease. Cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on these systems.

Article Summary

Article focus

- The mechanisms through which omega-3 fatty acids may reduce adverse cardiac events remain uncertain.
- We have previously demonstrated that omega-3 fatty acids improve endothelial function and endogenous fibrinolysis in healthy cigarette smokers.
- The present study investigated the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Key messages

- Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation, acute tPA release, or platelet and monocyte activation in patients with coronary heart disease.
- Any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function, the endogenous fibrinolytic system, or platelet activation.

Strengths and limitations of this study

- Randomised, double-blind, placebo-controlled crossover design.
- Use of an established and robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function.
- Limitations: modest sample size.

Introduction

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease.[1] Clinical trials have demonstrated beneficial effects on mortality or cardiac events in patients with coronary heart disease.[2-4] However, the mechanisms through which they confer cardiac benefits are uncertain. Although an effect on ventricular arrhythmias has been thought to be important due to an observed reduction in sudden death,[5,6] subsequent studies have failed to clearly demonstrate an anti-arrhythmic effect.[7] An alternative mechanism may therefore be an effect on the vascular endothelium, as acute myocardial infarction due to plaque rupture and subsequent coronary thrombosis remains the most common cause of sudden cardiac death.[8]

The endothelium regulates vascular tone and blood flow, and mediates thrombosis through the production of factors that influence fibrinolysis and platelet activation. The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi and is regulated by the endothelium-derived profibrinolytic factor, tissue plasminogen activator (t-PA), and its inhibitor, plasminogen-activator inhibitor type 1 (PAI-1).[9] The rapid release of t-PA from the endothelium is vital, with thrombus dissolution being more effective if t-PA is incorporated early during thrombus formation.[10]

Endothelial cells regulate thrombosis through the release of paracrine factors that mediate platelet function. Activated platelets can bind to leukocytes via a P-selectin dependent mechanism,[11] and these interactions can also be modulated by the CD40 receptor and its ligand.[12] Formation of platelet-leukocyte aggregates or ligation of CD40 can mediate an array of proinflammatory and prothrombotic effects, thereby contributing to endothelial injury and atherothrombosis.[13]

Patients with coronary heart disease demonstrate impaired endothelial function,[14] in addition to increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system.[15,16] We have recently demonstrated that omega-3 fatty acid supplements improve endogenous fibrinolysis and endothelial function in healthy cigarette smokers, a group at high risk of adverse cardiac events.[17]

Previously, we have shown that dietary fish intake reduces platelet-monocyte aggregation in man.[18] We therefore hypothesized that omega-3 fatty acid supplementation would improve endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation in patients with coronary heart disease.

Methods

Study participants

Twenty patients with a myocardial infarction at least three months previously were recruited to participate in the study. Myocardial infarction was defined as any two of: typical clinical history, electrocardiographic changes (Q waves in 2 contiguous leads) or elevation of cardiac markers (CKmB or troponin). All subjects gave written informed consent and the study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Exclusion criteria included dietary fish allergy or intolerance, consumption of > 1 fish meal per week, renal or hepatic failure, or any intercurrent illness likely to be associated with an inflammatory response. The first patient was randomised in December 2004 and the last study visit took place in June 2006. There were logistical delays in the analysis of frozen plasma samples and the final data became available for analysis in June 2009.

Study design

This was a prospective, double-blind, placebo-controlled, randomized crossover trial. Subjects were randomized to receive either omega-3 fatty acid supplements (2 g/day, Omacor capsules, Pronova, Norway) or matching placebo capsules (2 g/day olive oil capsules, Eurocaps Limited, Gwent) for a 6-week period. After a 4-week washout phase, participants crossed over to the opposite treatment arm for a further 6-week period. The omega 3 fatty acid supplements and placebo were packaged and dispensed in identical containers by the Royal Infirmary of Edinburgh Pharmacy. All study participants and investigators were blinded to the study allocation. The randomization schedule was generated by an investigator not involved in the study, and securely kept in the Royal Infirmary of Edinburgh Pharmacy. The omega-3 fatty acid capsules contained 85-88% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters in a ratio of 1.2:1. Both the omega-3 fatty acid capsules and olive oil placebo contained 4 mg α-tocopherol. All 20 subjects had peripheral blood taken for fasting lipid profile, plasma fatty acid analysis and flow cytometric analysis of platelet activation at baseline and at the end of each treatment period. Two patients dropped out of the study:

one was withdrawn after being admitted with unstable angina and a second patient was lost to follow-up. A subset of 12 participants also underwent measurement of forearm blood flow and endogenous fibrinolysis at the end of each treatment period.

Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with ethylene diamine tetra-acetic acid (EDTA; 1.6 mg/mL, Sarstedt Monovette) and the direct thrombin inhibitor D-Phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75 μM, PPACK, Cambridge Biosciences). Whole blood anticoagulated with PPACK was immunolabelled within 5 min of phlebotomy for subsequent flow cytometric analysis. Plasma was prepared from blood anticoagulated with sodium EDTA by centrifugation (1500 x *g* for 30 min). Plasma samples were stored at -70ºC until analysis.

Flow cytometry

The following reagents were used: fluorscein isothiocyanate (FITC)-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), phycoerythrin (PE)-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls (Serotec Ltd; Oxford, UK) as well as PE-conjugated CD154 (TRAP1, IgG1), PE-conjugated CD14 (Tuk-4, IgG2a), PE-conjugated CD 62P (IE3, IgG2a), and their appropriate isotype controls (Dako Cytomation; Buckinghamshire, UK) and FACS-Lyse (Becton-Dickinson; Cowley, UK). Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with appropriate antibodies and their isotype matched controls for 20 min at room temperature. To evaluate platelet-monocyte aggregates and CD40 on monocytes, samples were fixed and red cells lysed by the addition of 500 μL of FACS-Lyse solution. To evaluate platelet surface P-selectin and CD40 ligand, samples were fixed with 1% paraformaldehyde. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK) within 6 hours of labelling. Monocytes and platelets were identified by gating for CD14 and CD42a positive cells respectively. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. Analyses were performed using EXPO 32 software (Beckman Coulter, High Wycombe, UK).

Plasma fatty acid analysis

The fatty acid composition of plasma phospholipids was determined from blood anticoagulated with EDTA. Total lipids were recovered from 500 µL of plasma using dichloro-metane-metanol (2:1) containing 0.005% butyrated hydroxytoluene as an antioxidant (Folch extraction). Phospholipids were isolated by solid-phase extraction using aminopropyl silica columns (IST International), and fatty acids converted into methyl esters by transmethylation with 0.5 M sodium methoxide. Fatty acid methyl ester analysis was performed with an HP-INNOWAX capillary column (Agilent Technologies). Peaks were identified by comparison of retention times with known fatty acid methyl ester standards and quantified using an internal standard. Plasma total phospholipid fatty acids were expressed as the individual fractions of fatty acids and fatty acid groups as relative values (% of total fatty acids). The mean coefficient of variation for the assay was 2.4%

Vascular studies

Studies were carried out in a quiet temperature controlled room (22–25 °C). Subjects fasted for 6 h prior to the study and avoided caffeine and alcohol for the preceding 24 h. Blood pressure and heart rate were recorded throughout the study using a semi-automated non-invasive oscillometric sphygmomanometer (OMRON 705 IT, Kyoto, Japan).

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-min baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences), substance P at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator that releases t-PA; Clinalfa, Switzerland) and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories) were infused for 6 min at each dose. The 3 vasodilators were separated by 20-min saline infusions and given in a randomized order.

Forearm blood flow (FBF) was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in–silicone elastomer strain gauges as described previously.[19] Venous

cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during infusion of each dose of substance P and collected into acidified buffered citrate (Stabilyte tubes, Biopool International; for t-PA assays) and into citrate (BD Vacutainer; for PAI-1 assays). Samples were kept on ice before being centrifuged at 2000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by enzyme-linked immunosorbent assays. Haematocrit was determined by capillary tube centrifugation at baseline.

Data analysis and statistical methods

Continuous variables are reported as mean ± standard error of the mean. The pre-specified primary endpoint was endothelial vasomotor and fibrinolytic function. The sample size of n=12 was based on power calculations derived from previous studies giving 90% power to detect a 17% difference in the mean t-PA release at a significance level of 5%.[19] The pre-specified secondary endpoint was platelet and monocyte activation. The sample size of n=20 was based on power calculations derived from previous studies, giving 90% power to detect a 5% difference in mean platelet-monocyte aggregation at a significance level of 5%. Forearm plethysmographic data were analyzed as described previously.[17] Estimated net release of plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms.[19] Statistical analyses were performed using one-way and two-way ANOVA with Bonferroni's post-tests for multiple comparisons where appropriate. The statistical methods for each analysis are detailed in the relevant Figure and Table legends. All calculations were performed using GraphPad Prism (Graph Pad Software).

Results

Baseline characteristics

Participant flow through the study including a CONSORT diagram is included in the Supplementary File. Patients were relatively young and well treated in terms of blood pressure control and lipid profile (Table 1). The mean and median times from myocardial infarction were 12 months and 16 months, respectively. Patients were on standard medical therapy including aspirin, beta-blockers, statins and ACE-inhibitors, and over half had undergone revascularization post-MI.

Effect of omega 3 fatty acid supplementation on plasma phospholipid fatty acid composition

Dietary supplementation with omega-3 fatty acids led to a marked increase in EPA as a percentage of plasma phospholipids compared with both baseline (3.7±0.4% versus 2.0±0.2%, $P<0.0001$) and placebo (3.7±0.4% versus 1.7±0.1%, $P<0.0001$; Table 2). There was also an increase in DHA compared with baseline (5.6±0.2% versus 4.8±0.3%, $P<0.01$) and placebo (5.6±0.2% versus 4.4±0.3%, $P<0.0001$; Table 2). We did not detect any carry-over of EPA or DHA after 6 weeks of placebo in the group who had omega-3 fatty acids first (data not shown). There was a reduction in the plasma phospholipid percentage of arachidonic acid, but no effect on alpha-linolenic acid, linoleic acid, palmitic acid, stearic acid or oleic acid with either omega-3 fatty acid supplements or olive oil placebo (Table 2).

Effect of omega 3 fatty acid supplementation on lipid profile

Supplementation for 6 weeks with omega 3 fatty acids did not affect total cholesterol, low-density liproprotein cholesterol, high-density liproprotein cholesterol or triglycerides (data not shown).

Effect of omega 3 fatty acid supplementation on vasomotor function

Omega-3 fatty acid supplementation did not have any effect on systolic blood pressure, diastolic blood pressure or heart rate compared with placebo (data not shown). During forearm vascular studies substance P, acetylcholine, and sodium nitroprusside led to a dose-dependent increase in absolute

forearm blood flow (P<0.0001 for all). Compared with placebo, omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation in response to acetylcholine or substance P (P=0.5 and P=0.9; Figure 1), or endothelium-independent vasodilatation with sodium nitroprusside (P=0.9; Figure 1).

Effect of omega-3 fatty acid supplementation on stimulated t-PA activity

Substance P infusion caused a dose-dependent increase in plasma t-PA activity concentrations after both omega-3 fatty acid supplementation and placebo (P<0.0001; Table 3). Omega-3 fatty acid supplementation did not affect plasma TPA activity, TPA antigen or PAI-1 concentrations compared with placebo (Table 3). There was no difference in net release of t-PA activity after omega-3 fatty acid supplementation compared with placebo (P=0.94; Figure 2).

Effect of omega-3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand

Supplementation with omega-3 fatty acids did not have any effect on platelet-monocyte aggregation, platelet-neutrophil aggregation, platelet surface expression of P-selectin or CD40L, or monocyte expression of CD40 (data not shown).

Discussion

The present study has demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function or endothelial t-PA release in patients with coronary heart disease. There is also no effect on markers of platelet or monocyte activation. These findings suggest that any cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on endothelial function, endogenous fibrinolysis or platelet activation.

We do not believe the lack of effect on outcome measures in the present study is likely to have been due to poor compliance. The assessment of plasma phospholipid fatty acid composition confirmed substantial increases in the percentage of both EPA and DHA during supplementation with omega-3 fatty acids. The dose and duration of therapy with omega-3 fatty acids are also likely to have been appropriate. We used 2 grams per day of omega-3 fatty acids which is similar to the amount shown to reduce mortality in secondary prevention trials.[2,3] Although we cannot exclude an effect with a longer duration of therapy, 6 weeks of supplementation caused a large increase in the plasma phospholipid content of omega-3 fatty acids and has previously been long enough to demonstrate clear effects on vascular function and platelet activation.[20-22]

Omega-3 fatty acids have previously been shown to have inconsistent effects on endothelial function. Whilst some studies have reported beneficial effects in a variety of populations including healthy volunteers,[22] patients with hyperlipidaemia,[21,23] diabetes mellitus,[24] and heart failure,[25] others have not found any improvement.[26-28] Our findings are in contrast to previous studies in coronary heart disease which demonstrated an improvement in endothelial function with omega-3 fatty acids.[20,29-30] These discrepancies could be partly due to differences in study populations or concomitant medication. However, the previous studies were all either not randomized or double-blinded, and lacked a control group or placebo. Indeed, our trial is the first double-blinded, placebo-controlled trial investigating the effect of omega-3 fatty acids on endothelial vasomotor function in coronary heart disease; we therefore believe our study design and findings are likely to be robust.

We also found that omega-3 fatty acids did not augment endogenous fibrinolysis in coronary heart disease. Previous results have varied widely and it has been concluded that omega-3 fatty acids are unlikely to influence the fibrinolytic system.[31] Whilst some studies have reported a beneficial impact on fibrinolytic parameters,[32-33] others have found an adverse effect[34] or no effect.[26,35-37] However, previous studies have only measured basal plasma t-PA concentrations that do not reflect the local capacity for acute endothelial t-PA release.[9,38] It is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. We used an established model of acute endothelial t-PA release that predicts cardiovascular outcome,[19,39] but found no effect of omega-3 fatty acid supplementation on acute endogenous fibrinolytic capacity in coronary heart disease.

There are several possible explanations for the lack of effect omega-3 fatty acids on endothelial function and endogenous fibrinolysis observed in the present coronary heart disease population. The patients were all well treated with modern cardio-active medication known to influence endothelial vasomotor function.[40-41] In contrast, patients in previous studies demonstrating improved endothelial function[20,29] and cardiac outcomes[2-3] with omega-3 fatty acids were much less likely to be taking HMG CoA reductase inhibitors or angiotensin-converting enzyme inhibitors. It is conceivable that endothelial function cannot be further improved by the addition of omega-3 fatty acids in coronary heart disease patients already treated with modern medical therapy. This possibility is supported by the most recent large clinical trials which found a low rate of cardiac events in patients on optimal medical therapy post-myocardial infarction, which could not be improved with omega-3 fatty acid supplementation.[42-44]

However, concomitant medication may not fully explain the neutral effects on endogenous fibrinolysis. Whilst lipid-lowering therapy improves endothelial vasomotor function, it has not been found to influence acute t-PA release.[45] Angiotensin-converting enzyme inhibitors only augment bradykinin induced t-PA release; they do not affect t-PA release stimulated by substance P.[46]

Therefore, there may be other factors to explain why omega-3 fatty acid supplementation can improve endogenous fibrinolytic capacity in healthy cigarette smokers but not in patients with coronary heart disease. Perhaps the most likely explanation is that the coronary heart disease group was considerably older and may have a dysfunctional endothelium and fibrinolytic system less responsive to dietary interventional measures.

Circulating platelet-monocyte aggregates are increased in stable coronary heart disease and acute coronary syndromes, consistent with an important role in both the development of atherosclerotic lesions and in acute thrombosis.[15] We have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation.[18] However, we did not observe any effect of omega-3 fatty acid supplements on these measures of platelet and monocyte activation in the present study. It is possible our previous results were due to another active ingredient in oily fish rather than omega-3 fatty acids, and we cannot exclude a dose-effect of omega-3 fatty acids on platelet activation. Omega-3 fatty acids also had no effect on monocyte expression of CD40 or platelet surface CD40 ligand, consistent with previous studies which found no effect of either omega-3 fatty acids or dietary fish on soluble CD40 ligand.[18,47]

Our study has potential limitations that should be acknowledged. First, the sample size is relatively small which raises the possibility of a type II error due to lack of power. However, the sample size was based on separate power calculations for the vascular function and the platelet monocyte studies, and we have previously detected modest changes in these outcome measures with similar sample sizes.[17,18] Although it is possible we lacked power to detect very small changes, we believe the study had sufficient power to detect any clinically relevant effects of omega-3 fatty acids. Secondly, as we did not measure fatty acids at the beginning of the second treatment stage we cannot fully exclude the possibility of some carry-over of EPA or DHA into the early placebo phase in the group receiving omega-3 fatty acids first. However, we feel any such effect would be modest and unlikely to alter the study outcomes.

Conclusions

We have demonstrated that omega-3 fatty acid supplementation does not affect endothelial function, endogenous fibrinolytic capacity or markers of platelet and monocyte activation in patients with stable coronary heart disease. A major strength of our study is the use of a robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function. Our results suggest that any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function or the fibrinolytic system.

References

1. Din JN, Newby DE, Flapan AD. Omega 3 fatty acids and cardiovascular disease - fishing for a natural treatment. *BMJ* 2004;328:30-5.
2. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 1989;2:757-61.
3. GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354:447-55.
4. Yokoyama M, Origasa H, Matsuzaki M, et al. Japan EPA lipid intervention study (JELIS) Investigators. *Lancet*. 2007; 369:1090-8.
5. Marchioli R, Barzi F, Bomba E, *et al*. Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation* 2002;105:1897-903
6. Leaf A, Kang JX, Xiao YF, et al. Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation* 2003;107:2646-52.
7. Brouwer IA, Raitt MH, Dullemeijer C, et al. Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators. *Eur Heart J* 2009;30:820-6.
8. Bowker TJ, Wood DA, Davies MJ, et al. Sudden, unexpected cardiac or unexplained death in England: a national survey. *QJM*. 2003;96:269-79.
9. Oliver JJ, Webb DJ, Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans, *Arterioscler Thromb Vasc Biol* 2005; 25: 2470–2479.
10. Fox KA, Robison AK, Knobb RM *et al*. Prevention of coronary thrombosis with subthrombolytic doses of tissue type plasminogen activator. *Circulation* 1985; 72:1346-1354.

11. Jungi TW, Spycher MO, Nydegger UE, et al. Platelet-leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* 1986;67:629-36.
12. Schonbeck U, Lippy P. CD40 signaling and plaque instability. *Circ Res.* 2001; 89: 1092–1103
13. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61-7
14. Zeiher AM, Drexler H, Wollschlager H, et al. Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991;83:391-401.
15. Sarma J, Laan CA, Alam S, et al. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* 2002; 105: 2166-71.
16. Tousoulis D, Antoniades C, Nikolopoulou A, et al. Interaction between cytokines and sCD40L in patients with stable and unstable coronary syndromes. *Eur J Clin Invest*. 2007;37:623-8.
17. Din JN, Archer RM, Harding SA, et al. Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in male cigarette smokers. *Heart*. 2013; 99:168-74.
18. Din JN, Harding SA, Valerio CJ, et al. Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. *Atherosclerosis* 2008;197:290-6.
19. Newby DE, Wright RA, Ludlam CA, et al. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost.* 1997; 78: 1242–1248
20. Tagawa H, Shimokawa H, Tagawa T, et al. Long-term treatment with eicosapentaenoic acid augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilatation in patients with coronary artery disease. *J Cardiovasc Pharmacol*. 1999 Apr;33(4):633-40.
21. Mori TA, Watts GF, Burke V, et al. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. *Circulation* 2000;102:1264-9.

22. Chin JP, Gust AP, Nestel PJ, et al. Marine oils dose-dependently inhibit vasoconstriction of forearm resistance vessels in humans. *Hypertension* 1993;21(1):22-8.

23. Goodfellow J, Bellamy MF, Ramsey MW, et al. Dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia. *J Am Coll Cardiol*. 2000;35:265-70.

24. McVeigh GE, Brennan GM, Johnston GD, et al. Dietary fish oil augments nitric oxide production or release in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1993;36:33-8.

25. Morgan DR, Dixon LJ, Hanratty CG, et al. Effects of dietary omega-3 fatty acid supplementation on endothelium-dependent vasodilation in patients with chronic heart failure. *Am J Cardiol*. 2006;97:547-51.

26. Woodman RJ, Mori TA, Burke V, et al. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis* 2003;166:85-93.

27. Wong CY, Yiu KH, Li SW, et al. Fish-oil supplement has neutral effects on vascular and metabolic function but improves renal function in patients with Type 2 diabetes mellitus. *Diabet Med* 2010;27:54-60.

28. Skulas-Ray AC, Kris-Etherton PM, Harris WS, et al. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. *Am J Clin Nutr*. 2011;93:243-52.

29. Tagawa T, Hirooka Y, Shimokawa H, et al. Long-term treatment with eicosapentaenoic acid improves exercise-induced vasodilation in patients with coronary artery disease. Hypertens Res. 2002;25:823-9.

30. Haberka M, Mizia-Stec K, Mizia M, et al. N-3 polyunsaturated fatty acids early supplementation improves ultrasound indices of endothelial function, but not through NO inhibitors in patients with acute myocardial infarction: N-3 PUFA supplementation in acute myocardial infarction. *Clin Nutr* 2011;30:79-85.

31. Kristensen SD, Iversen AM, Schmidt EB. n-3 polyunsaturated fatty acids and coronary thrombosis. *Lipids* 2001;36 Suppl:S79-82.

32. Smith P, Arnesen H, Opstad T, et al. Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo. *Thromb Res* 1989;53:467-74.

33. Mehta J, Lawson D, Saldeen TJ. Reduction in plasminogen activator inhibitor-1 (PAI-1) with omega-3 polyunsaturated fatty acid (PUFA) intake. *Am Heart J* 1988;116(5 Pt 1):1201-6.

34. Spannagl M, Drummer C, Fröschl H, et al. Plasmatic factors of haemostasis remain essentially unchanged except for PAI activity during n-3 fatty acid intake in type I diabetes mellitus. *Blood Coagul Fibrinolysis*. 1991;2:259-65.

35. Finnegan YE, Howarth D, Minihane AM, et al. Plant and marine derived (n-3) polyunsaturated fatty acids do not affect blood coagulation and fibrinolytic factors in moderately hyperlipidemic humans. *J Nutr* 2003;133:2210-3.

36. Hellsten G, Boman K, Saarem K, et al. Effects on fibrinolytic activity of corn oil and a fish oil preparation enriched with omega-3-polyunsaturated fatty acids in a long-term study. *Curr Med Res Opin.* 1993;13:133-9.

37. Toft I, Bønaa KH, Ingebretsen OC, et al. Fibrinolytic function after dietary supplementation with omega3 polyunsaturated fatty acids. *Arterioscler Thromb Vasc Biol*. 1997;17:814-9.

38. Hrafnkelsdottir T, Gudnason T, Wall U, et al. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost* 2004; 2: 1960–8.

39. Robinson SD, Ludlam CA, Boon NA, et al. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol* 2007; 27**:** 1651–6.

40. Treasure CB, Klein JL, Weintraub WS, et al. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. *N Engl J Med* 1995; 332:481–7.

41. Mancini GB, Henry GC, Macaya C, et al. Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease: the TREND (Trial on Reversing ENdothelial Dysfunction) study. *Circulation* 1996; 94: 258–65.

42. Rauch B, Schiele R, Schneider S, et al. OMEGA Study Group. OMEGA, a randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction. *Circulation*. 2010; 122(21):2152-9

43. Galan P, Kesse-Guyot E, Czernichow S, et al. SU.FOL.OM3 Collaborative Group. Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial. *BMJ*. 2010 Nov 29;341:c6273

44. Kromhout D, Giltay EJ, Geleijnse JM; Alpha Omega Trial Group. n-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med*. 2010; 363:2015-26.

45. Newby DE, Witherow FN, Wright RA, et al. Hypercholesterolaemia and lipid lowering treatment do not affect the acute endogenous fibrinolytic capacity in vivo. *Heart* 2002; 87: 48 –53.

46. Witherow FN, Dawson P, Ludlam CA, et al E. Marked bradykinin-induced tissue plasminogen activator release in patients with heart failure maintained on long-term angiotensin-converting enzyme inhibitor therapy. *J Am Coll Cardiol*. 2002; 40: 961-6.

47. Aarsetoy H, Brugger-Andersen T, Hetland O, et al. Long term influence of regular intake of high dose n-3 fatty acids on CD40-ligand, pregnancy-associated plasma protein A and matrix metalloproteinase-9 following acute myocardial infarction. *Thromb Haemost* 2006;95:329-36.

Acknowledgements

We are grateful to the Clinical Research Facility at the Royal Infirmary of Edinburgh.

Authorship

1) Conception and design or analysis and interpretation of data: JD, KL, SH, JS, AF, DN

2) Drafting of the manuscript or revising it critically for intellectual content: JD, JS, AF, DN

3) Final approval of the manuscript submitted: All authors.

Funding statement

JD was supported by a British Heart Foundation Project Grant (PG/2003/009). DEN is supported by the British Heart Foundation. The Wellcome Trust Clinical Research Facility is supported by NHS Research Scotland (NRS) through NHS Lothian.

Competing interests

None.

Data sharing

No additional unpublished data from this study.

.

Figure Legends

Figure 1. Effect of omega-3 fatty acid supplementation on absolute forearm blood flow in response to endothelium-dependent and endothelium-independent vasodilators. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

Figure 2. Net release of plasma t-PA activity with omega-3 fatty acid supplementation and placebo. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

TABLE 1. Baseline Characteristics

Age, years	53±3
Body mass index, kg/m^2	28±1
Systolic blood pressure, mm Hg	137±5
Diastolic blood pressure, mm Hg	78±3
Heart rate, beats per minute	60±2
Total cholesterol, mmol/L	4.2±0.2
LDL cholesterol, mmol/L	2.3±0.2
HDL cholesterol, mmol/L	1.1±0.1
Chol:HDL chol ratio	3.8±0.2
Triacylglycerol, mmol/L	1.6±0.2
Fasting glucose, mmol/l	5.4±0.1
Time from MI, months	16±4
Revascularization post-MI, %	56%
Current or ex-smoker, %	61%
Hypertension, %	11%
Diabetes mellitus, %	0%
Hyperlipidemia, %	78%
Family history of premature coronary heart disease, %	33%
Medical therapy	
Aspirin, %	100%
Clopidogrel, %	11%
ACE-inhibitor/Angiotensin-receptor blocker, %	56%
Beta-blocker %	78%
Statin, %	100%

Mean±SEM.

TABLE 2. Effect of omega-3 fatty acid supplementation on plasma phospholipid fatty acid composition

	Baseline	Omega-3	Placebo	*P* value
Eicosapentaenoic acid, EPA	2.0±0.2	3.7±0.4	1.7±0.1	*P*<0.0001
Docosahexaenoic acid, DHA	4.8±0.3	5.6±0.2	4.4±0.3	*P*<0.0001
Alpha-linolenic acid	0.3±0.01	0.3±0.02	0.3±0.03	0.3
Arachidonic acid	12.5±0.4	11.0±0.3	11.6±0.5	0.0005
Linoleic acid	18.8±0.6	19.0±0.6	20.0±0.6	0.1
Palmitic acid	28.2±0.4	27.9±0.3	28.2±0.3	0.6
Stearic acid	13.8±0.3	14.1±0.2	13.9±0.2	0.4
Oleic acid	13.3±0.5	13.0±0.4	13.8±0.5	0.1

Mean±SEM. Data analysed using 1-way ANOVA. P values in the table are for the difference between the three means. P values for individual comparisons are below.
EPA: baseline vs omega-3, *P*<0.0001; baseline vs placebo, P=NS; omega-3 vs placebo, *P*<0.0001.
DHA: baseline vs omega-3, *P*<0.01; baseline vs placebo, *P*=NS; omega-3 vs placebo, *P*<0.0001.
Arachidonic acid: baseline vs omega-3, *P*<0.001; baseline vs placebo, *P*=0.05; omega-3 vs placebo, *P*=NS.

TABLE 3. Effect of omega 3 fatty acid supplementation on plasma t-PA activity concentrations

	Omega-3 fatty acids				Placebo			
Substance P pmol/min	**0**	**2**	**4**	**8**	**0**	**2**	**4**	**8**
t-PA activity, IU mL^{-1}								
Non-infused arm	0.39±0.08	0.45±0.09	0.54±0.12	0.64±0.14	0.45±0.07	0.52±0.08	0.60±0.09	0.65±0.11
Infused arm	0.38±0.08	0.83±0.16	1.12±0.23	1.67±0.38	0.43±0.07	0.78±0.10	1.09±0.11	1.26±0.15
t-PA antigen, ng mL^{-1}								
Non-infused arm	11.78±1.29	12.01±1.0	12.69±1.08	12.83±1.49	13.45±1.40	12.93±1.70	13.08±1.80	12.37±1.27
Infused arm	11.90±1.45	13.98±1.33	13.63±1.12	14.86±1.40	12.55±1.10	12.85±1.44	13.45±1.35	13.97±1.55
PAI-1 activity, ng mL^{-1}								
Non-infused arm	1.77±0.53	1.84±0.43	1.80±0.42	1.64±0.45	1.44±0.29	1.38±0.26	1.39±0.47	1.34±0.44
Infused arm	2.33±0.86	2.18±0.61	2.21±0.69	1.92±0.63	1.69±0.46	1.64±0.41	1.54±0.39	1.49±0.39
PAI-1 antigen, ng mL^{-1}								
Non-infused arm	39.51±9.22	40.84±7.08	39.99±6.62	38.48±5.79	45.06±7.09	43.33±6.45	44.41±6.67	44.26±7.03
Infused arm	37.64±8.36	38.83±6.25	41.71±5.74	40.26±7.32	48.89±8.25	42.65±6.59	43.12±6.60	40.61±6.46
Net t-PA antigen release ng 100 mL^{-1} of tissue mm^{-1}	0.23±0.51	-0.28±4.7	3.92±1.8	8.41±2.94	-0.87±1.1	-0.84±2.82	0.94±4.22	8.10±3.67

Mean±SEM. Data analysed using 2-way ANOVA.

t-PA activity: Dose response $P<0.0001$. Omega-3 fatty acids versus placebo; $P=0.83$ (infused arm).

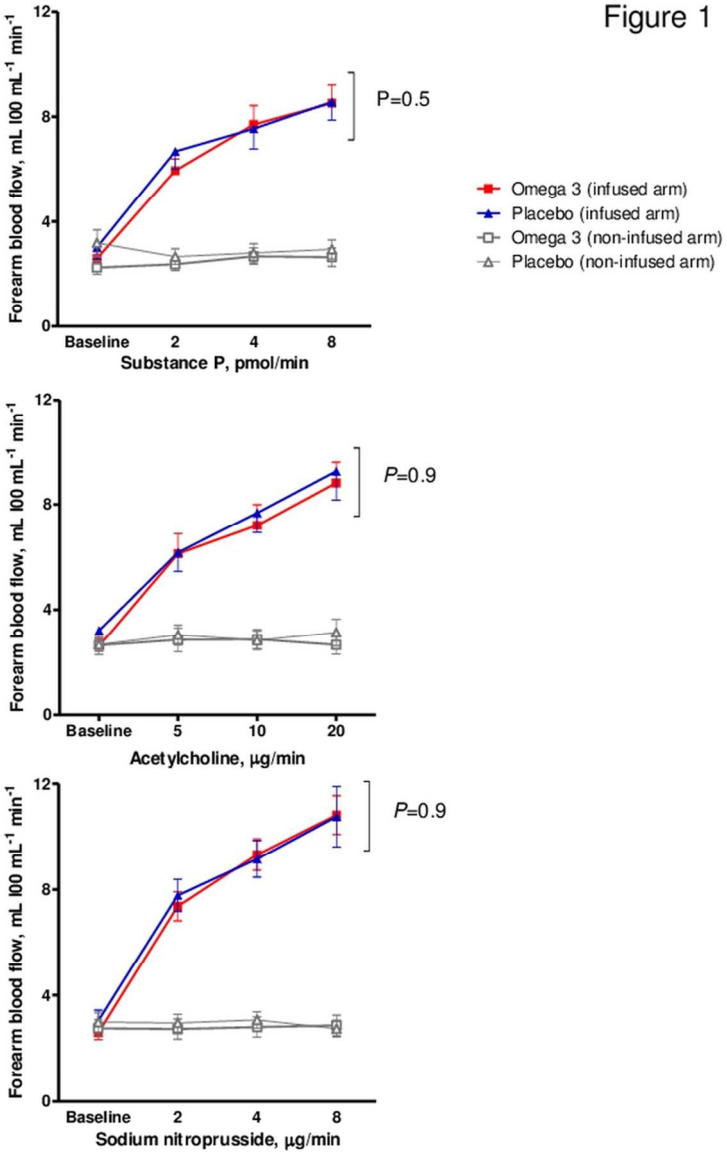
t-PA antigen: Dose response $P=0.7$. Omega-3 fatty acids versus placebo; $P=0.60$ (infused arm).

PAI-1 activity: Dose response $P=0.94$. Omega-3 fatty acids versus placebo; $P=0.17$ (infused arm).

PAI-1 antigen: Dose response $P=0.67$. Omega-3 fatty acids versus placebo; $P=0.40$ (infused arm).

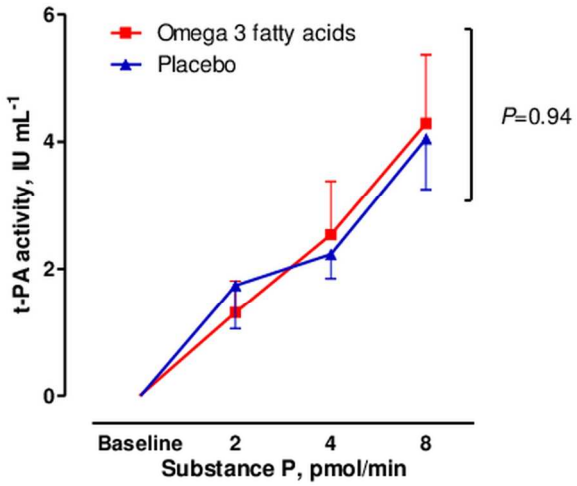
Net t-PA antigen: Dose response $P=0.02$. Omega-3 fatty acids versus placebo; $P=0.62$ (infused arm)

Figure 1



90x119mm (300 x 300 DPI)

Figure 2



186x139mm (300 x 300 DPI)

EFFECT OF OMEGA 3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN PATIENTS WITH A PREVIOUS MYOCARDIAL INFARCTION: A RANDOMISED CONTROLLED TRIAL

Jehangir N Din[1], Jaydeep Sarma[2], Scott A Harding[3], Karin Lyall[1], David E Newby[1], Andrew D Flapan[4]

[1]Centre for Cardiovascular Sciences, University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, United Kingdom; [2]North West Heart Centre, Wythenshawe Hospital, Manchester, United Kingdom; [3]Department of Cardiology, Wellington Hospital, Wellington, New Zealand; and [4]Edinburgh Heart Centre, Royal Infirmary of Edinburgh, Edinburgh, United Kingdom.

WORD COUNT 2801

ClinicalTrials.gov Identifier: NCT01888211

KEY WORDS

Omega 3 fatty acids, endothelial function, endogenous fibrinolysis, platelet activation,

AUTHOR FOR CORRSPONDENCE

Dr Jehangir N Din

University of Edinburgh

The Chancellor's Building

49 Little France Crescent

Edinburgh EH16 4SB

UNITED KINGDOM

Telephone: +44 131 242 1850

Fax: +44 131 242 6422

E-mail: jehangirdin@hotmail.com

Abstract

Objective – The mechanisms through which omega-3 fatty acids reduce adverse cardiac events remain uncertain. We aimed to investigate the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Design – Randomised, double-blind, placebo-controlled, crossover trial.

Setting – Academic cardiac centre.

Participants - Twenty male patients with a previous myocardial infarction.

Intervention - Omega-3 fatty acid supplementation (2g/day for 6-weeks) versus olive oil placebo.

Outcome measures - Peripheral blood was taken for analysis of platelet and monocyte activation, and forearm blood flow was assessed in a subset of 12 patients during intrabrachial infusions of acetylcholine, substance P and sodium nitroprusside. Stimulated plasma tissue plasminogen activator (t-PA) concentrations were measured during substance P infusion.

Results - All vasodilators caused dose-dependent increases in forearm blood flow ($P<0.0001$). Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation with acetylcholine and substance P compared with placebo ($P=0.5$ and $P=0.9$). Substance P caused a dose-dependent increase in plasma t-PA concentrations ($P<0.0001$), which was not affected by omega-3 fatty acid supplementation ($P=0.9$). Omega-3 fatty acids did not affect platelet-monocyte aggregation, platelet P-selectin or CD40L, or monocyte CD40.

Conclusions – We have demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function, endothelial t-PA release or platelet and monocyte activation in patients with coronary heart disease. Cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on these systems.

Article Summary

Article focus

- The mechanisms through which omega-3 fatty acids may reduce adverse cardiac events remain uncertain.
- We have previously demonstrated that omega-3 fatty acids improve endothelial function and endogenous fibrinolysis in healthy cigarette smokers.
- The present study investigated the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in patients with coronary heart disease.

Key messages

- Omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation, acute tPA release, or platelet and monocyte activation in patients with coronary heart disease.
- Any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function, the endogenous fibrinolytic system, or platelet activation.

Strengths and limitations of this study

- Randomised, double-blind, placebo-controlled crossover design.
- Use of an established and robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function.
- Limitations: modest sample size.

Funding statement

JD was supported by a British Heart Foundation Project Grant (PG/2003/009). DEN is supported by the British Heart Foundation. The Wellcome Trust Clinical Research Facility is supported by NHS Research Scotland (NRS) through NHS Lothian.

Competing interests

None.

Introduction

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease.[1] Clinical trials have demonstrated beneficial effects on mortality or cardiac events in patients with coronary heart disease.[2-4] However, the mechanisms through which they confer ~~any~~ cardiac benefits are uncertain. Although an effect on ventricular arrhythmias has been thought to be important due to an observed reduction in sudden death,[5,6] subsequent studies have failed to clearly demonstrate an anti-arrhythmic effect.[7] An alternative mechanism may therefore be an effect on the vascular endothelium, as acute myocardial infarction due to plaque rupture and subsequent coronary thrombosis remains the most common cause of sudden cardiac death.[8]

The endothelium regulates vascular tone and blood flow, and mediates thrombosis through the production of factors that influence fibrinolysis and platelet activation. The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi and is regulated by the endothelium-derived profibrinolytic factor, tissue plasminogen activator (t-PA), and its inhibitor, plasminogen-activator inhibitor type 1 (PAI-1).[9] The rapid release of t-PA from the endothelium is vital, with thrombus dissolution being more effective if t-PA is incorporated early during thrombus formation.[10]

Endothelial cells regulate thrombosis through the release of paracrine factors that mediate platelet function. Activated platelets can bind to leukocytes via a P-selectin dependent mechanism,[11] and these interactions can also be modulated by the CD40 receptor and its ligand.[12] Formation of platelet-leukocyte aggregates or ligation of CD40 can mediate an array of proinflammatory and prothrombotic effects, thereby contributing to endothelial injury and atherothrombosis.[13]

Patients with coronary heart disease demonstrate impaired endothelial function,[14] in addition to increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system.[15,16] We have recently demonstrated that omega-3 fatty acid supplements improve endogenous fibrinolysis and endothelial function in healthy cigarette smokers, a group at high risk of adverse cardiac events.[17]

Previously, we have shown that dietary fish intake reduces platelet-monocyte aggregation in man.[18] We therefore hypothesized that omega-3 fatty acid supplementation would improve endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation in patients with coronary heart disease.

Methods

Study participants

Twenty patients with a myocardial infarction at least three months previously were recruited to participate in the study. Myocardial infarction was defined as any two of: typical clinical history, electrocardiographic changes (Q waves in 2 contiguous leads) or elevation of cardiac markers (CKmB or troponin). All subjects gave written informed consent and the study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki. Exclusion criteria included dietary fish allergy or intolerance, consumption of > 1 fish meal per week, renal or hepatic failure, or any intercurrent illness likely to be associated with an inflammatory response. The first patient was randomised in December 2004 and the last study visit took place in June 2006. There were logistical delays in the analysis of frozen plasma samples and the final data became available for analysis in June 2009.

Study design

This was a prospective, double-blind, placebo-controlled, randomized crossover trial. Subjects were randomized to receive either omega-3 fatty acid supplements (2 g/day, Omacor capsules, Pronova, Norway) or matching placebo capsules (2 g/day olive oil capsules, Eurocaps Limited, Gwent) for a 6-week period. After a 4-week washout phase, participants crossed over to the opposite treatment arm for a further 6-week period. The omega 3 fatty acid supplements and placebo were packaged and dispensed in identical containers by the Royal Infirmary of Edinburgh Pharmacy. All study participants and investigators were blinded to the study allocation. The randomization schedule was generated by an investigator not involved in the study, and securely kept in the Royal Infirmary of Edinburgh Pharmacy. The omega-3 fatty acid capsules contained 85-88% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters in a ratio of 1.2:1. Both the omega-3 fatty acid capsules and olive oil placebo contained 4 mg α-tocopherol. All 20 subjects had peripheral blood taken for fasting lipid profile, plasma fatty acid analysis and flow cytometric analysis of platelet activation at baseline and at the end of each treatment period. Two patients dropped out of the study:

one was withdrawn after being admitted with unstable angina and a second patient was lost to follow-up. A subset of 12 participants also underwent measurement of forearm blood flow and endogenous fibrinolysis at the end of each treatment period.

Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with ethylene diamine tetra-acetic acid (EDTA; 1.6 mg/mL, Sarstedt Monovette) and the direct thrombin inhibitor D-Phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75 μM, PPACK, Cambridge Biosciences). Whole blood anticoagulated with PPACK was immunolabelled within 5 min of phlebotomy for subsequent flow cytometric analysis. Plasma was prepared from blood anticoagulated with sodium EDTA by centrifugation (1500 x *g* for 30 min). Plasma samples were stored at -70ºC until analysis.

Flow cytometry

The following reagents were used: fluorscein isothiocyanate (FITC)-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), phycoerythrin (PE)-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls (Serotec Ltd; Oxford, UK) as well as PE-conjugated CD154 (TRAP1, IgG1), PE-conjugated CD14 (Tuk-4, IgG2a), PE-conjugated CD 62P (IE3, IgG2a), and their appropriate isotype controls (Dako Cytomation; Buckinghamshire, UK) and FACS-Lyse (Becton-Dickinson; Cowley, UK). Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with appropriate antibodies and their isotype matched controls for 20 min at room temperature. To evaluate platelet-monocyte aggregates and CD40 on monocytes, samples were fixed and red cells lysed by the addition of 500 μL of FACS-Lyse solution. To evaluate platelet surface P-selectin and CD40 ligand, samples were fixed with 1% paraformaldehyde. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK) within 6 hours of labelling. Monocytes and platelets were identified by gating for CD14 and CD42a positive cells respectively. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. Analyses were performed using EXPO 32 software (Beckman Coulter, High Wycombe, UK).

Plasma fatty acid analysis

The fatty acid composition of plasma phospholipids was determined from blood anticoagulated with EDTA. Total lipids were recovered from 500 µL of plasma using dichloro-metane-metanol (2:1) containing 0.005% butyrated hydroxytoluene as an antioxidant (Folch extraction). Phospholipids were isolated by solid-phase extraction using aminopropyl silica columns (IST International), and fatty acids converted into methyl esters by transmethylation with 0.5 M sodium methoxide. Fatty acid methyl ester analysis was performed with an HP-INNOWAX capillary column (Agilent Technologies). Peaks were identified by comparison of retention times with known fatty acid methyl ester standards and quantified using an internal standard. Plasma total phospholipid fatty acids were expressed as the individual fractions of fatty acids and fatty acid groups as relative values (% of total fatty acids). The mean coefficient of variation for the assay was 2.4%

Vascular studies

Studies were carried out in a quiet temperature controlled room (22–25 °C). Subjects fasted for 6 h prior to the study and avoided caffeine and alcohol for the preceding 24 h. Blood pressure and heart rate were recorded throughout the study using a semi-automated non-invasive oscillometric sphygmomanometer (OMRON 705 IT, Kyoto, Japan).

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-min baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA; Merck Biosciences), substance P at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator that releases t-PA; Clinalfa, Switzerland) and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories) were infused for 6 min at each dose. The 3 vasodilators were separated by 20-min saline infusions and given in a randomized order.

Forearm blood flow (FBF) was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in–silicone elastomer strain gauges as described previously.[19] Venous

cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossae of both arms. Blood (10 mL) was withdrawn simultaneously from each arm at baseline and during infusion of each dose of substance P and collected into acidified buffered citrate (Stabilyte tubes, Biopool International; for t-PA assays) and into citrate (BD Vacutainer; for PAI-1 assays). Samples were kept on ice before being centrifuged at 2000 *g* for 30 min at 4°C. Platelet-free plasma was decanted and stored at –80°C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by enzyme-linked immunosorbent assays. Haematocrit was determined by capillary tube centrifugation at baseline.

Data analysis and statistical methods

Continuous variables are reported as mean ± standard error of the mean. The pre-specified primary endpoint was endothelial vasomotor and fibrinolytic function. The sample size of n=12 was based on power calculations derived from previous studies giving 90% power to detect a 17% difference in the mean t-PA release at a significance level of 5%.[19] The pre-specified secondary endpoint was platelet and monocyte activation. The sample size of n=20 was based on power calculations derived from previous studies, giving 90% power to detect a 5% difference in mean platelet-monocyte aggregation at a significance level of 5%. Forearm plethysmographic data were analyzed as described previously.[17] Estimated net release of plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and noninfused arms.[19] Statistical analyses were performed using one-way and two-way ANOVA with Bonferroni's post-tests for multiple comparisons where appropriate. The statistical methods for each analysis are detailed in the relevant Figure and Table legends. All calculations were performed using GraphPad Prism (Graph Pad Software).

Results

Baseline characteristics

Participant flow through the study including a CONSORT diagram is included in the Supplementary File. Patients were relatively young and well treated in terms of blood pressure control and lipid profile (Table 1). The mean and median times from myocardial infarction were 12 months and 16 months, respectively. Patients were on standard medical therapy including aspirin, beta-blockers, statins and ACE-inhibitors, and over half had undergone revascularization post-MI.

Effect of omega 3 fatty acid supplementation on plasma phospholipid fatty acid composition

Dietary supplementation with omega-3 fatty acids led to a marked increase in EPA as a percentage of plasma phospholipids compared with both baseline (3.7±0.4% versus 2.0±0.2%, $P<0.0001$) and placebo (3.7±0.4% versus 1.7±0.1%, $P<0.0001$; Table 2). There was also an increase in DHA compared with baseline (5.6±0.2% versus 4.8±0.3%, $P<0.01$) and placebo (5.6±0.2% versus 4.4±0.3%, $P<0.0001$; Table 2). We did not detect any carry-over of EPA or DHA after 6 weeks of placebo in the group who had omega-3 fatty acids first (data not shown). There was a reduction in the plasma phospholipid percentage of arachidonic acid, but no effect on alpha-linolenic acid, linoleic acid, palmitic acid, stearic acid or oleic acid with either omega-3 fatty acid supplements or olive oil placebo (Table 2).

Effect of omega 3 fatty acid supplementation on lipid profile

Supplementation for 6 weeks with omega 3 fatty acids did not affect total cholesterol, low-density liproprotein cholesterol, high-density liproprotein cholesterol or triglycerides (data not shown).

Effect of omega 3 fatty acid supplementation on vasomotor function

Omega-3 fatty acid supplementation did not have any effect on systolic blood pressure, diastolic blood pressure or heart rate compared with placebo (data not shown). During forearm vascular studies substance P, acetylcholine, and sodium nitroprusside led to a dose-dependent increase in absolute

forearm blood flow (P<0.0001 for all). Compared with placebo, omega-3 fatty acid supplementation did not affect endothelium-dependent vasodilatation in response to acetylcholine ~~and~~ or substance P (P=0.5 and P=0.9; Figure 1), or endothelium-independent vasodilatation with sodium nitroprusside (P=0.9; Figure 1).

Effect of omega-3 fatty acid supplementation on stimulated t-PA activity

Substance P infusion caused a dose-dependent increase in plasma t-PA activity concentrations after both omega-3 fatty acid supplementation and placebo (P<0.0001; Table 3). Omega-3 fatty acid supplementation did not affect plasma TPA activity, TPA antigen or PAI-1 concentrations compared with placebo (Table 3). There was no difference in net release of t-PA activity after omega-3 fatty acid supplementation compared with placebo (P=0.94; Figure 2).

Effect of omega-3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand

Supplementation with omega-3 fatty acids did not have any effect on platelet-monocyte aggregation, platelet-neutrophil aggregation, platelet surface expression of P-selectin or CD40L, or monocyte expression of CD40 (data not shown).

Discussion

The present study has demonstrated that dietary supplementation with omega-3 fatty acids does not affect endothelial vasomotor function or endothelial t-PA release in patients with coronary heart disease. There is also no effect on markers of platelet or monocyte activation. These findings suggest that any cardiac benefits conferred by omega-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on endothelial function, endogenous fibrinolysis or platelet activation.

We do not believe the lack of effect on outcome measures in the present study is likely to have been due to poor compliance. The assessment of plasma phospholipid fatty acid composition confirmed substantial increases in the percentage of both EPA and DHA during supplementation with omega-3 fatty acids. The dose and duration of therapy with omega-3 fatty acids are also likely to have been appropriate. We used 2 grams per day of omega-3 fatty acids which is similar to the amount shown to reduce mortality in secondary prevention trials.[2,3] Although we cannot exclude an effect with a longer duration of therapy, 6 weeks of supplementation caused a large increase in the plasma phospholipid content of omega-3 fatty acids and has previously been long enough to demonstrate clear effects on vascular function and platelet activation.[20-22]

Omega-3 fatty acids have previously been shown to have inconsistent effects on endothelial function. Whilst some studies have reported beneficial effects in a variety of populations including healthy volunteers,[22] patients with hyperlipidaemia,[21,23] diabetes mellitus,[24] and heart failure,[25] others have not found any improvement.[26-28] Our findings are in contrast to previous studies in coronary heart disease which demonstrated an improvement in endothelial function with omega-3 fatty acids.[20,29-30] These discrepancies could be partly due to differences in study populations or concomitant medication. However, the previous studies were all either not randomized or double-blinded, and lacked a control group or placebo. Indeed, our trial is the first double-blinded, placebo-controlled trial investigating the effect of omega-3 fatty acids on endothelial vasomotor function in coronary heart disease; we therefore believe our study design and findings are likely to be robust.

We also found that omega-3 fatty acids did not augment endogenous fibrinolysis in coronary heart disease. Previous results have varied widely and it has been concluded that omega-3 fatty acids are unlikely to influence the fibrinolytic system.[31] Whilst some studies have reported a beneficial impact on fibrinolytic parameters,[32-33] others have found an adverse effect[34] or no effect.[26,35-37] However, previous studies have only measured basal plasma t-PA concentrations that do not reflect the local capacity for acute endothelial t-PA release.[9,38] It is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. We used an established model of acute endothelial t-PA release that predicts cardiovascular outcome,[19,39] but found no effect of omega-3 fatty acid supplementation on acute endogenous fibrinolytic capacity in coronary heart disease.

There are several possible explanations for the lack of effect omega-3 fatty acids on endothelial function and endogenous fibrinolysis observed in the present coronary heart disease population. The patients were all well treated with modern cardio-active medication known to influence endothelial vasomotor function.[40-41] In contrast, patients in previous studies demonstrating improved endothelial function[20,29] and cardiac outcomes[2-3] with omega-3 fatty acids were much less likely to be taking HMG CoA reductase inhibitors or angiotensin-converting enzyme inhibitors. It is conceivable that endothelial function cannot be further improved by the addition of omega-3 fatty acids in coronary heart disease patients already treated with modern medical therapy. This possibility is supported by the most recent large clinical trials which found a low rate of cardiac events in patients on optimal medical therapy post-myocardial infarction, which could not be improved with omega-3 fatty acid supplementation.[42-44]

However, concomitant medication may not fully explain the neutral effects on endogenous fibrinolysis. Whilst lipid-lowering therapy improves endothelial vasomotor function, it has not been found to influence acute t-PA release.[45] Angiotensin-converting enzyme inhibitors only augment bradykinin induced t-PA release; they do not affect t-PA release stimulated by substance P.[46]

Therefore, there may be other factors to explain why omega-3 fatty acid supplementation can improve endogenous fibrinolytic capacity in healthy cigarette smokers but not in patients with coronary heart disease. Perhaps the most likely explanation is that the coronary heart disease group was considerably older and may have a dysfunctional endothelium and fibrinolytic system less responsive to dietary interventional measures.

Circulating platelet-monocyte aggregates are increased in stable coronary heart disease and acute coronary syndromes, consistent with an important role in both the development of atherosclerotic lesions and in acute thrombosis.[15] We have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation.[18] However, we did not observe any effect of omega-3 fatty acid supplements on these measures of platelet and monocyte activation in the present study. It is possible our previous results were due to another active ingredient in oily fish rather than omega-3 fatty acids, and we cannot exclude a dose-effect of omega-3 fatty acids on platelet activation. Omega-3 fatty acids also had no effect on monocyte expression of CD40 or platelet surface CD40 ligand, consistent with previous studies which found no effect of either omega-3 fatty acids or dietary fish on soluble CD40 ligand.[18,47]

Our study has potential limitations that should be acknowledged. First, the sample size is relatively small which raises the possibility of a type II error due to lack of power. However, the sample size was based on separate power calculations for the vascular function and the platelet monocyte studies, and we have previously detected modest changes in these outcome measures with similar sample sizes.[17,18] Although it is possible we lacked power to detect very small changes, we believe the study had sufficient power to detect any clinically relevant effects of omega-3 fatty acids. Secondly, as we did not measure fatty acids at the beginning of the second treatment stage we cannot fully exclude the possibility of some carry-over of EPA or DHA into the early placebo phase in the group receiving omega-3 fatty acids first. However, we feel any such effect would be modest and unlikely to alter the study outcomes.

Conclusions

We have demonstrated that omega-3 fatty acid supplementation does not affect endothelial function, endogenous fibrinolytic capacity or markers of platelet and monocyte activation in patients with stable coronary heart disease. A major strength of our study is the use of a robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function. Our results suggest that any potential cardiac benefits conferred by omega-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function or the fibrinolytic system.

References

1. Din JN, Newby DE, Flapan AD. Omega 3 fatty acids and cardiovascular disease - fishing for a natural treatment. *BMJ* 2004;328:30-5.
2. Burr ML, Fehily AM, Gilbert JF, et al. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet* 1989;2:757-61.
3. GISSI-Prevenzione Investigators. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354:447-55.
4. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, Shirato K; Japan EPA lipid intervention study (JELIS) Investigators. *Lancet*. 2007; 369:1090-8.
5. Marchioli R, Barzi F, Bomba E, Chieffo C, Di Gregorio D, Di Mascio R, *et al*. Early protection against sudden death by n-3 polyunsaturated fatty acids after myocardial infarction: time-course analysis of the results of the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione. *Circulation* 2002;105:1897-903
6. Leaf A, Kang JX, Xiao YF, Billman GE. Clinical prevention of sudden cardiac death by n-3 polyunsaturated fatty acids and mechanism of prevention of arrhythmias by n-3 fish oils. *Circulation* 2003;107:2646-52.
7. Brouwer IA, Raitt MH, Dullemeijer C, Kraemer DF, Zock PL, Morris C, Katan MB, Connor WE, Camm JA, Schouten EG, McAnulty J. Effect of fish oil on ventricular tachyarrhythmia in three studies in patients with implantable cardioverter defibrillators. *Eur Heart J* 2009;30:820-6.
8. Bowker TJ, Wood DA, Davies MJ, Sheppard MN, Cary NR, Burton JD, Chambers DR, Dawling S, Hobson HL, Pyke SD, Riemersma RA, Thompson SG. Sudden, unexpected cardiac or unexplained death in England: a national survey. *QJM*. 2003;96:269-79.
9. Oliver JJ, Webb DJ, Newby DE. Stimulated tissue plasminogen activator release as a marker of endothelial function in humans, *Arterioscler Thromb Vasc Biol* 2005; 25: 2470–2479.

10. Fox KA, Robison AK, Knobb RM *et al*. Prevention of coronary thrombosis with subthrombolytic doses of tissue type plasminogen activator. *Circulation* 1985; 72:1346-1354.

11. Jungi TW, Spycher MO, Nydegger UE, Barandun S. Platelet-leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. *Blood* 1986;67:629-36.

12. Schonbeck U, Lippy P. CD40 signaling and plaque instability. *Circ Res.* 2001; 89: 1092–1103

13. Huo Y, Schober A, Forlow SB, et al. Circulating activated platelets exacerbate atherosclerosis in mice deficient in apolipoprotein E. *Nat Med* 2003;9:61-7

14. Zeiher AM, Drexler H, Wollschlager H, Just H. Modulation of coronary vasomotor tone in humans: progressive endothelial dysfunction with different early stages of coronary atherosclerosis. *Circulation* 1991;83:391-401.

15. Sarma J, Laan CA, Alam S, Jha A, Fox KA, Dransfield I. Increased platelet binding to circulating monocytes in acute coronary syndromes. *Circulation* 2002; 105: 2166-71.

16. Tousoulis D, Antoniades C, Nikolopoulou A, Koniari K, Vasiliadou C, Marinou K, Koumallos N, Papageorgiou N, Stefanadi E, Siasos G, Stefanadis C. Interaction between cytokines and sCD40L in patients with stable and unstable coronary syndromes. *Eur J Clin Invest*. 2007;37:623-8.

17. Din JN, Archer RM, Harding SA, Sarma J, Lyall K, Flapan AD, Newby DE. Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in male cigarette smokers. *Heart*. 2013; 99:168-74.

18. Din JN, Harding SA, Valerio CJ, Sarma J, Lyall K, Riemersma RA, Newby DE, Flapan AD. Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man. *Atherosclerosis* 2008;197:290-6.

19. Newby DE, Wright RA, Ludlam CA, Fox KA, Boon NA, Webb DJ. An in vivo model for the assessment of acute fibrinolytic capacity of the endothelium. *Thromb Haemost.* 1997; 78: 1242–1248

20. Tagawa H, Shimokawa H, Tagawa T, Kuroiwa-Matsumoto M, Hirooka Y, Takeshita A. Long-term treatment with eicosapentaenoic acid augments both nitric oxide-mediated and non-nitric oxide-mediated endothelium-dependent forearm vasodilatation in patients with coronary artery disease. *J Cardiovasc Pharmacol*. 1999 Apr;33(4):633-40.

21. Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. *Circulation* 2000;102:1264-9.

22. Chin JP, Gust AP, Nestel PJ, Dart AM. Marine oils dose-dependently inhibit vasoconstriction of forearm resistance vessels in humans. *Hypertension* 1993;21(1):22-8.

23. Goodfellow J, Bellamy MF, Ramsey MW, Jones CJ, Lewis MJ. Dietary supplementation with marine omega-3 fatty acids improve systemic large artery endothelial function in subjects with hypercholesterolemia. *J Am Coll Cardiol*. 2000;35:265-70.

24. McVeigh GE, Brennan GM, Johnston GD, McDermott BJ, McGrath LT, Henry WR, Andrews JW, Hayes JR. Dietary fish oil augments nitric oxide production or release in patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia*. 1993;36:33-8.

25. Morgan DR, Dixon LJ, Hanratty CG, El-Sherbeeny N, Hamilton PB, McGrath LT, Leahey WJ, Johnston GD, McVeigh GE. Effects of dietary omega-3 fatty acid supplementation on endothelium-dependent vasodilation in patients with chronic heart failure. *Am J Cardiol*. 2006;97:547-51.

26. Woodman RJ, Mori TA, Burke V, Puddey IB, Barden A, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis* 2003;166:85-93.

27. Wong CY, Yiu KH, Li SW, Lee S, Tam S, Lau CP, Tse HF. Fish-oil supplement has neutral effects on vascular and metabolic function but improves renal function in patients with Type 2 diabetes mellitus. *Diabet Med* 2010;27:54-60.

28. Skulas-Ray AC, Kris-Etherton PM, Harris WS, Vanden Heuvel JP, Wagner PR, West SG. Dose-response effects of omega-3 fatty acids on triglycerides, inflammation, and endothelial function in healthy persons with moderate hypertriglyceridemia. *Am J Clin Nutr*. 2011;93:243-52.

29. Tagawa T, Hirooka Y, Shimokawa H, Hironaga K, Sakai K, Oyama J, Takeshita A. Long-term treatment with eicosapentaenoic acid improves exercise-induced vasodilation in patients with coronary artery disease. Hypertens Res. 2002;25:823-9.

30. Haberka M, Mizia-Stec K, Mizia M, Janowska J, Gieszczyk K, Chmiel A, Zahorska-Markiewicz B, Gąsior Z. N-3 polyunsaturated fatty acids early supplementation improves ultrasound indices of endothelial function, but not through NO inhibitors in patients with acute myocardial infarction: N-3 PUFA supplementation in acute myocardial infarction. *Clin Nutr* 2011;30:79-85.
31. Kristensen SD, Iversen AM, Schmidt EB. n-3 polyunsaturated fatty acids and coronary thrombosis. *Lipids* 2001;36 Suppl:S79-82.
32. Smith P, Arnesen H, Opstad T, Dahl KH, Eritsland J. Influence of highly concentrated n-3 fatty acids on serum lipids and hemostatic variables in survivors of myocardial infarction receiving either oral anticoagulants or matching placebo. *Thromb Res* 1989;53:467-74.
33. Mehta J, Lawson D, Saldeen TJ. Reduction in plasminogen activator inhibitor-1 (PAI-1) with omega-3 polyunsaturated fatty acid (PUFA) intake. *Am Heart J* 1988;116(5 Pt 1):1201-6.
34. Spannagl M, Drummer C, Fröschl H, von Schacky C, Landgraf-Leurs MM, Landgraf R, Schramm W. Plasmatic factors of haemostasis remain essentially unchanged except for PAI activity during n-3 fatty acid intake in type I diabetes mellitus. *Blood Coagul Fibrinolysis*. 1991;2:259-65.
35. Finnegan YE, Howarth D, Minihane AM, Kew S, Miller GJ, Calder PC, Williams CM. Plant and marine derived (n-3) polyunsaturated fatty acids do not affect blood coagulation and fibrinolytic factors in moderately hyperlipidemic humans. *J Nutr* 2003;133:2210-3.
36. Hellsten G, Boman K, Saarem K, Hallmans G, Nilsson TK. Effects on fibrinolytic activity of corn oil and a fish oil preparation enriched with omega-3-polyunsaturated fatty acids in a long-term study. *Curr Med Res Opin.* 1993;13:133-9.
37. Toft I, Bønaa KH, Ingebretsen OC, Nordøy A, Jenssen T. Fibrinolytic function after dietary supplementation with omega3 polyunsaturated fatty acids. *Arterioscler Thromb Vasc Biol*. 1997;17:814-9.
38. Hrafnkelsdottir T, Gudnason T, Wall U, Jern C, Jern S. Regulation of local availability of active tissue-type plasminogen activator in vivo in man. *J Thromb Haemost* 2004; 2: 1960–8.
39. Robinson SD, Ludlam CA, Boon NA, Newby DE. Endothelial fibrinolytic capacity predicts future adverse cardiovascular events in patients with coronary heart disease. *Arterioscler Thromb Vasc Biol* 2007; 27**:** 1651–6.

40. Treasure CB, Klein JL, Weintraub WS, Talley JD, Stillabower ME, Kosinski AS, Zhang J, Boccuzzi SJ, Cedarholm JC, Alexander RW. Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease. *N Engl J Med* 1995; 332:481–7.

41. Mancini GB, Henry GC, Macaya C, O'Neill BJ, Pucillo AL, Carere RG, Wargovich TJ, Mudra H, Lüscher TF, Klibaner MI, Haber HE, Uprichard AC, Pepine CJ, Pitt B. Angiotensin-converting enzyme inhibition with quinapril improves endothelial vasomotor dysfunction in patients with coronary artery disease: the TREND (Trial on Reversing ENdothelial Dysfunction) study. *Circulation* 1996; 94: 258–65.

42. Rauch B, Schiele R, Schneider S, Diller F, Victor N, Gohlke H, Gottwik M, Steinbeck G, Del Castillo U, Sack R, Worth H, Katus H, Spitzer W, Sabin G, Senges J; OMEGA Study Group. OMEGA, a randomized, placebo-controlled trial to test the effect of highly purified omega-3 fatty acids on top of modern guideline-adjusted therapy after myocardial infarction. *Circulation*. 2010; 122(21):2152-9

43. Galan P, Kesse-Guyot E, Czernichow S, Briancon S, Blacher J, Hercberg S; SU.FOL.OM3 Collaborative Group. Effects of B vitamins and omega 3 fatty acids on cardiovascular diseases: a randomised placebo controlled trial. *BMJ*. 2010 Nov 29;341:c6273

44. Kromhout D, Giltay EJ, Geleijnse JM; Alpha Omega Trial Group. n-3 fatty acids and cardiovascular events after myocardial infarction. *N Engl J Med*. 2010; 363:2015-26.

45. Newby DE, Witherow FN, Wright RA, Bloomfield P, Ludlam CA, Boon NA, Fox KA, Webb DJ. Hypercholesterolaemia and lipid lowering treatment do not affect the acute endogenous fibrinolytic capacity in vivo. *Heart* 2002; 87: 48 –53.

46. Witherow FN, Dawson P, Ludlam CA, Fox KA, Newby DE. Marked bradykinin-induced tissue plasminogen activator release in patients with heart failure maintained on long-term angiotensin-converting enzyme inhibitor therapy. *J Am Coll Cardiol*. 2002; 40: 961-6.

47. Aarsetoy H, Brugger-Andersen T, Hetland O, Grundt H, Nilsen DW. Long term influence of regular intake of high dose n-3 fatty acids on CD40-ligand, pregnancy-associated plasma protein A and matrix metalloproteinase-9 following acute myocardial infarction. *Thromb Haemost* 2006;95:329-36.

Acknowledgements

We are grateful to the Clinical Research Facility at the Royal Infirmary of Edinburgh.

Authorship

1) Conception and design or analysis and interpretation of data: JD, KL, SH, JS, AF, DN

2) Drafting of the manuscript or revising it critically for intellectual content: JD, JS, AF, DN

3) Final approval of the manuscript submitted: All authors.

.

Figure Legends

~~**Figure 1.** Percentage omega-3 fatty acids in plasma phospholipids at baseline, during omega-3 fatty acid supplementation and placebo. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni's post-tests for multiple comparisons. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.~~

Figure 1. Effect of omega-3 fatty acid supplementation on absolute forearm blood flow in response to endothelium-dependent and endothelium-independent vasodilators. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

Figure 2. Net release of plasma t-PA activity with omega-3 fatty acid supplementation and placebo. Statistical analyses two-way ANOVA and Bonferroni's post-tests for multiple comparisons.

TABLE 1. Baseline Characteristics

Age, years	53±3
Body mass index, kg/m^2	28±1
Systolic blood pressure, mm Hg	137±5
Diastolic blood pressure, mm Hg	78±3
Heart rate, beats per minute	60±2
Total cholesterol, mmol/L	4.2±0.2
LDL cholesterol, mmol/L	2.3±0.2
HDL cholesterol, mmol/L	1.1±0.1
Chol:HDL chol ratio	3.8±0.2
Triacylglycerol, mmol/L	1.6±0.2
Fasting glucose, mmol/l	5.4±0.1
Time from MI, months	16±4
Revascularization post-MI, %	56%
Current or ex-smoker, %	61%
Hypertension, %	11%
Diabetes mellitus, %	0%
Hyperlipidemia, %	78%
Family history of premature coronary heart disease, %	33%
Medical therapy	
Aspirin, %	100%
Clopidogrel, %	11%
ACE-inhibitor/Angiotensin-receptor blocker, %	56%
Beta-blocker %	78%
Statin, %	100%

Mean±SEM.

TABLE 2. Effect of omega-3 fatty acid supplementation on plasma phospholipid fatty acid composition

	Baseline	Omega-3	Placebo	*P* value
Eicosapentaenoic acid, EPA	2.0±0.2	3.7±0.4	1.7±0.1	*P*<0.0001
Docosahexaenoic acid, DHA	4.8±0.3	5.6±0.2	4.4±0.3	*P*<0.0001
Alpha-linolenic acid	0.3±0.01	0.3±0.02	0.3±0.03	0.3
Arachidonic acid	12.5±0.4	11.0±0.3	11.6±0.5	0.0005
Linoleic acid	18.8±0.6	19.0±0.6	20.0±0.6	0.1
Palmitic acid	28.2±0.4	27.9±0.3	28.2±0.3	0.6
Stearic acid	13.8±0.3	14.1±0.2	13.9±0.2	0.4
Oleic acid	13.3±0.5	13.0±0.4	13.8±0.5	0.1

Mean±SEM. Data analysed using 1-way ANOVA. P values in the table are for the difference between the three means. P values for individual comparisons are below.
EPA: baseline vs omega-3, *P*<0.0001; baseline vs placebo, P=NS; omega-3 vs placebo, *P*<0.0001.
DHA: baseline vs omega-3, *P*<0.01; baseline vs placebo, *P*=NS; omega-3 vs placebo, *P*<0.0001.
Arachidonic acid: baseline vs omega-3, *P*<0.001; baseline vs placebo, *P*=0.05; omega-3 vs placebo, *P*=NS.

TABLE 3. Effect of omega-3 fatty acid supplementation on blood pressure and lipid profile

	Baseline	Omega 3	Placebo	P value
Heart rate, beats per minute	60±3	60±2	60±2	0.9
Systolic blood pressure, mm Hg	131±4	125±4	130±4	0.2
Diastolic blood pressure, mm Hg	77±3	74±2	74±3	0.5
Total cholesterol, mmol/L	4.3±0.3	3.9±0.3	4.0±0.2	0.1
LDL cholesterol, mmol/L	2.5±0.2	2.2±0.3	2.2±0.1	0.2
HDL cholesterol, mmol/L	1.1±0.1	1.1±0.1	1.1±0.1	0.4
Chol:HDL chol ratio	3.8±0.3	3.5±0.3	3.5±0.2	0.3
Triacylglycerol, mmol/L	1.5±0.2	1.3±0.1	1.5±0.1	0.5

Mean±SEM. Data analysed using 1-way ANOVA.

TABLE 3. Effect of omega 3 fatty acid supplementation on plasma t-PA activity concentrations

	Omega-3 fatty acids				Placebo			
Substance P pmol/min	**0**	**2**	**4**	**8**	**0**	**2**	**4**	**8**
t-PA activity, IU mL^{-1}								
Non-infused arm	0.39±0.08	0.45±0.09	0.54±0.12	0.64±0.14	0.45±0.07	0.52±0.08	0.60±0.09	0.65±0.11
Infused arm	0.38±0.08	0.83±0.16	1.12±0.23	1.67±0.38	0.43±0.07	0.78±0.10	1.09±0.11	1.26±0.15
t-PA antigen, ng mL^{-1}								
Non-infused arm	11.78±1.29	12.01±1.0	12.69±1.08	12.83±1.49	13.45±1.40	12.93±1.70	13.08±1.80	12.37±1.27
Infused arm	11.90±1.45	13.98±1.33	13.63±1.12	14.86±1.40	12.55±1.10	12.85±1.44	13.45±1.35	13.97±1.55
PAI-1 activity, ng mL^{-1}								
Non-infused arm	1.77±0.53	1.84±0.43	1.80±0.42	1.64±0.45	1.44±0.29	1.38±0.26	1.39±0.47	1.34±0.44
Infused arm	2.33±0.86	2.18±0.61	2.21±0.69	1.92±0.63	1.69±0.46	1.64±0.41	1.54±0.39	1.49±0.39
PAI-1 antigen, ng mL^{-1}								
Non-infused arm	39.51±9.22	40.84±7.08	39.99±6.62	38.48±5.79	45.06±7.09	43.33±6.45	44.41±6.67	44.26±7.03
Infused arm	37.64±8.36	38.83±6.25	41.71±5.74	40.26±7.32	48.89±8.25	42.65±6.59	43.12±6.60	40.61±6.46
Net t-PA antigen release ng 100 mL^{-1} of tissue mm^{-1}	0.23±0.51	-0.28±4.7	3.92±1.8	8.41±2.94	-0.87±1.1	-0.84±2.82	0.94±4.22	8.10±3.67

Mean±SEM. Data analysed using 2-way ANOVA.

t-PA activity: Dose response $P<0.0001$. Omega-3 fatty acids versus placebo; $P=0.83$ (infused arm).

t-PA antigen: Dose response $P=0.7$. Omega-3 fatty acids versus placebo; $P=0.60$ (infused arm).

PAI-1 activity: Dose response $P=0.94$. Omega-3 fatty acids versus placebo; $P=0.17$ (infused arm).

PAI-1 antigen: Dose response $P=0.67$. Omega-3 fatty acids versus placebo; $P=0.40$ (infused arm).

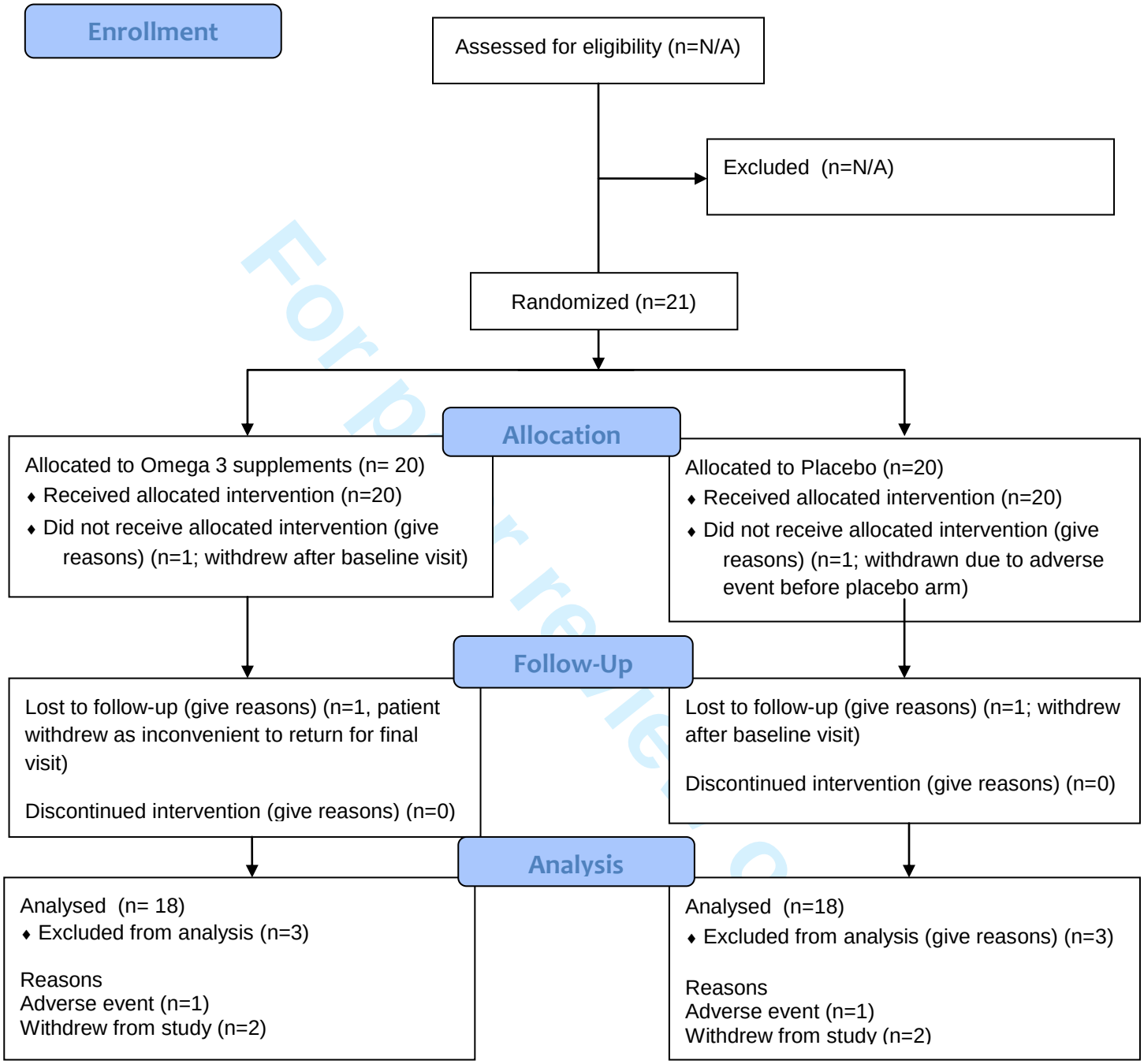
Net t-PA antigen: Dose response $P=0.02$. Omega-3 fatty acids versus placebo; $P=0.62$ (infused arm).

TABLE 5. Effect of omega-3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand system

	Baseline	Omega-3	Placebo	P value
Platelet-monocyte aggregates, %	21.2±3.9	23.6±4.2	23.0±4.1	0.7
Platelet-neutrophil aggregates, %	4.9±1.0	6.7±1.2	6.7±1.1	0.1
Platelet-surface expression of P-selectin, %	3.9±0.9	5.0±1.0	4.3±1.0	0.3
Platelet-surface expression of CD40L, %	3.6±0.4	3.4±0.3	3.4±0.3	0.9
Monocyte expression of CD40, %	52.5±5.0	46.8±3.4	47.4±2.5	0.5

Mean±SEM. Data analysed using 1-way ANOVA.

CONSORT 2010 Flow Diagram



At the time the study was performed, we unfortunately did not record the number of patients screened for eligibility or excluded before randomization for our vascular function studies. Generally, around 30% of patients approached agreed to participate in the invasive venous plethysmography studies.

Three patients were not included in the final analysis. One patient complained of chest pain when he arrived for his second visit (after completing the omega-3 intervention arm) and was admitted with suspected unstable angina. He underwent coronary intervention and made an uncomplicated recovery. An adverse event was logged and the patient withdrawn from the study. The event was reviewed by the Chief Investigator and Independent Advisor and felt unlikely to be related to the omega-3 supplements. Two patients withdrew from the study because they found returning for subsequent visits inconvenient.

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	Page 1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	Page 2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	Page 5-6
	2b	Specific objectives or hypotheses	Page 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	Page 7
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	None
Participants	4a	Eligibility criteria for participants	Page 7
	4b	Settings and locations where the data were collected	Page 9
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	Page 7-11
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	Page 7-11
	6b	Any changes to trial outcomes after the trial commenced, with reasons	None
Sample size	7a	How sample size was determined	Page 11
	7b	When applicable, explanation of any interim analyses and stopping guidelines	None
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	Page 7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	Page 7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	Page 7
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	Page 7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	Page 7

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	Not applicable
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	Page 11
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	Page 11
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	Supplementary file
	13b	For each group, losses and exclusions after randomisation, together with reasons	Supplementary file
Recruitment	14a	Dates defining the periods of recruitment and follow-up	Page 7
	14b	Why the trial ended or was stopped	Page 7
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Page 24
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Supplementary file
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	Page 12-13
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Not applicable
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Page 12-13
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	Supplementary file
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	Page 16
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	Page 16
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	Page 14-16
Other information			
Registration	23	Registration number and name of trial registry	Clinical Trials.gov
Protocol	24	Where the full trial protocol can be accessed, if available	Clinical Trials.gov
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Page 4

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.