VASCULAR AND ANTITHROMBOTIC EFFECTS OF OMEGA-3 FATTY ACIDS IN MAN

BY

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ABSTRACT

Background Dietary intake of omega-3 fatty acids is associated with a reduction in cardiovascular events. The mechanisms for this are uncertain and previous studies investigating effects on vascular function and thrombosis have produced inconsistent results. We aimed to study the effect of omega-3 fatty acids on endothelial vasomotor function, endogenous fibrinolysis and platelet activation in man.

Methods Firstly, we assessed the effect of dietary intervention with fish rich in marine-derived omega-3 fatty acids on platelet activation in 14 healthy volunteers, using flow cytometric analysis of platelet-monocyte aggregates. Secondly, we investigated the effect of dietary intervention on both platelet-monocyte aggregates and arterial stiffness in a single-blind randomised crossover trial with 30 healthy volunteers. Thirdly, we performed double-blind randomised controlled trials to assess the effect of dietary supplementation with omega-3 fatty acid capsules on endothelial function, endogenous fibrinolysis and platelet-monocyte aggregates in 20 healthy smokers and 20 patients with stable ischaemic heart disease. Endothelial function was assessed using venous occlusion plethysmography during intra-arterial infusions of Substance P, acetylcholine and sodium nitroprusside. Endogenous fibrinolysis was assessed by measuring stimulated release of tissue plasminogen activator (t-PA) from the forearm vasculature during substance P infusion.

Results Dietary intervention with fish rich in marine-derived omega-3 fatty acids reduced platelet-monocyte aggregates in healthy volunteers (16.0±9.0% versus 24.8±10.9%, P<0.01). Dietary intervention with walnuts rich in plant-derived omega-3 fatty acids did not affect platelet-monocyte aggregates or arterial stiffness in healthy volunteers. Supplementation with omega-3 fatty acid capsules increased endothelium-dependent vasodilatation (P=0.032) and stimulated t-PA release (P<0.01) in healthy smokers. There was no effect on platelet-monocyte aggregates. Supplementation with omega-3 fatty acids did not affect endothelium-dependent vasodilatation, t-PA release or platelet-monocyte aggregates in patients with stable ischaemic heart disease. There was a marked increase in plasma concentrations of marine-derived omega-3 fatty acids during dietary intervention with oil-rich fish and supplementation with omega-3 fatty acid capsules (P<0.0001).

Discussion We have demonstrated that dietary intervention with oil-rich fish reduces platelet-monocyte aggregates in healthy volunteers. Dietary intervention with walnuts did not have any effect on platelet-monocyte aggregates or arterial stiffness. Supplementation with omega-3 fatty acids improved endothelial function and endogenous fibrinolysis in healthy smokers, but had no effect on platelet-monocyte aggregates. Omega-3 fatty acids had did not affect endothelial function, endogenous fibrinolysis or platelet-monocyte aggregation in patients with ischaemic heart disease.
This thesis is dedicated to

my parents Nasim and Shahida Din, my sister Farhat Din, and my wife
Shamaila Din for all their support and encouragement during my research
studies.
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<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DART</td>
<td>The Diet and Reinfarction Trial [1989]</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FBF</td>
<td>Forearm blood flow</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FL</td>
<td>Fluorescence parameter</td>
</tr>
<tr>
<td>GRP-P</td>
<td>glucose-regulated protein precursor</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor type 1</td>
</tr>
</tbody>
</table>
PE          Phycoerythrin

PPACK       D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone

SD          Standard deviation

SEM         Standard error of the mean

t-PA         Tissue plasminogen activator
DECLARATION

I hereby declare that this thesis is my own work and effort and that it has not been submitted elsewhere for any award. Where others have contributed, they have been acknowledged.

Jehangir Nasim Din

April 2013
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This thesis would not have been possible without the guidance and support of several individuals who contributed to the completion of this work.

Foremost, I would like to express my deep gratitude to my supervisors Professor David Newby and Dr Andrew Flapan. Their continued support, enthusiasm, encouragement and patience have been invaluable at every stage of my research.

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Professor Rudolph Riemersma provided helpful advice throughout on fatty acids and assisted me with the ELISAs performed in Chapter 3. Karin Lyall performed all the plasma fatty acid analyses for this work and I am extremely grateful for her technical input and general support.

The vascular studies were performed in the Clinical Research Facility at the Royal Infirmary of Edinburgh. I would like to thank all the research nursing staff in the unit who assisted throughout and in particular Sharon Cameron, Lesley Breen and Finny Paterson. I am grateful to Drs Nick Cruden and Simon Robinson who taught me how to perform venous occlusion plethysmography. I also thank Pamela Dawson at the haematology laboratory in the Royal Infirmary of Edinburgh who performed the TPA/PAI-1 analyses for Chapters 5 and 6.

The omega-3 fatty acid supplements and placebo were packaged and blinded by the Pharmacy at the Royal Infirmary of Edinburgh, and I would like to thank Hazel Milligan for her support with this. I am grateful to Richard Roden at Eurocaps who kindly provided the placebo olive oil capsules without charge.

Several medical students assisted with volunteer recruitment and performed some of the flow cytometry in this research under my direct supervision. I would like to thank Alasdair Jessop, Christopher Valerio, Syed Aftab, Alastair Jubb and Rachel Archer for their contribution.

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CONTRIBUTORSHIP STATEMENT

Chapter 2: Experiments 1, 3 and 6 were performed by Scott Harding. I designed and performed experiments 2, 4 and 7.

Chapter 3: Subject recruitment and flow cytometry was performed by Christopher Valerio, Scott Harding and myself. Christopher Valerio obtained and delivered oil-rich fish to the study participants. Measurement of soluble CD40L and soluble P-selectin was performed by myself and Professor Rudolph Riemersma. I organised the plasma fatty acid analysis which was performed by Karin Lyall.

Chapter 4: Recruitment of participants was performed primarily by Syed Aftab and Alasdair Jubb. The study was conducted by myself with assistance from Syed Aftab and Alasdair Jubb. All flow cytometry was performed by myself (or by Syed Aftab under my direct supervision). All pulse waveform analysis was performed by Alasdair Jubb with guidance from me. Food diary analysis was done by Lize Van Rensburg.

Chapter 5: Recruitment of participants was performed by myself and Rachel Archer. I performed all vascular studies. All flow cytometry was performed by myself (or by Rachel Archer under my direct supervision). Plasma fatty acid analysis was performed by Karin Lyall. Analysis of TPA/PAI-1 was performed by Pam Dawson.

Chapter 6: I recruited all participants, performed all vascular studies and flow cytometry. Plasma fatty acid analysis was performed by Karin Lyall. Analysis of TPA/PAI-1 was performed by Pam Dawson.
The laboratory based and clinical research work in this thesis took part between February 2003 and June 2006, and all results were available in May 2009. There were several logistical challenges which have contributed to the delay in completion of this thesis, which are outlined below. I take full responsibility for these delays, and the details below are purely to provide the reader with some explanation and context of the timelines involved.

1. There were difficulties in obtaining a suitable placebo for the studies in Chapters 5 and 6. We required an olive oil placebo matched to our omega 3 capsule (Omacor), and with the same amount of α-tocopherol. We finally got agreement from Solvay pharmaceuticals to provide the 10,000 olive oil placebo capsules (via Eurocaps Ltd) in October 2003. Subsequently, our hospital pharmacy had to fill the blinded containers of omega 3 fatty acids or placebo, and we were not able to recruit for the main studies in Chapters 5 and 6 until at least 12 months after I started my research time.

2. Although there was no expiry on the olive oil placebo capsules from Eurocaps, our hospital pharmacy decided (without prior notice) that they would not be able to dispense the capsules in 2005, as they invoked an arbitrary expiration date of their own. Recruitment of new volunteers for the omega 3 supplement studies had to cease for a period of approximately 4 months. I had to send a sample of capsules back to the manufacturer to be tested before our pharmacy agreed to dispense the placebo again. This unforeseen hiatus had an important impact as it delayed completion of the clinical omega 3 studies. My research time finished in June 2006 when I was due to return to full time clinical training. This delay meant that I had to complete a few of the final study visits (each study period being a total of 16 weeks) after I had returned to full time clinical work.

3. Having returned to clinical work, I was posted outside Edinburgh within a few months and it became a logistical challenge to organise and transfer all plasma samples from the university freezers for fatty acid and TPA/PAI-1 analysis. This unfortunately led to further delay and I managed to get all plasma fatty acid results for the studies in Chapters 5 and 6 by October 2008 (kindly performed by Karin Lyall). I received all TPA/PAI-1 results in May 2009 (kindly performed by Pam Dawson). I was then able to analyse these and present all the results contained in this thesis at the University of Edinburgh Final MD/PhD Series, Centre for Cardiovascular Sciences (Queens Medical Research Institute, 18th June 2009).

4. I elected to pursue publication of my results in peer-reviewed journals as a priority, before completion of this thesis. In hindsight, this was unwise and contributed to further delay. However, I was keen for our research findings to be presented and published as promptly as possible. Therefore, whilst there has been a significant delay in submission of this thesis, it is important to note...
that the results have been presented to the wider scientific community in a timely fashion.

Chapter 3
Oral presentation at the 75th European Atherosclerosis Society Congress. Prague, April 2005.
• First submission to Circulation in November 2006 and rejected.

Chapter 4
Poster presentation at the European Society of Cardiology Congress, Stockholm, September 2005
• Submitted to Journal of the American College of Cardiology in March 2007. Rejected without review.
• Submitted to American Journal of Clinical Nutrition March 2007. Rejected after review, invited to resubmit April 2007, but we declined as they wished further dietary analysis which we did not have.
• Submitted to Clinical Nutrition November 2008. Invited to resubmit after peer-review in February 2009, but we declined as they wished further food diary data which we did not have.

Chapter 5
Poster presentation at American Heart Association Scientific Sessions, Los Angeles, 9th November 2012.
Paper: 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 Feb;99(3):168-74. [Received 22 August 2012, Revised 26 October 2012, Accepted 30 October 2012, Published Online First, 26 November 2013]
• Submitted to *Circulation* September 2011. Rejection after review October 2011.
• Submitted to *Journal of the American College of Cardiology* November 2011. Rejected after review January 2012, but invited to resubmit as a de novo manuscript after extensive revision. Resubmitted after major revision in March 2012, but rejected as one reviewer still not satisfied.
• Submitted to *European Heart Journal* April 2012. Rejected without review as Editor felt more suitable for specialist journal.
• Submitted to *Arteriosclerosis, Thrombosis and Vascular Biology* May 2012. Rejected after review May 2012, but invited to resubmit revised manuscript including additional experimental work. Decision not to resubmit as additional experimental work requested vague even after we sought clarification.

**Chapter 6**

• Submitted to *Journal of the American Heart Association* August 2012. Rejected without review.
CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Omega-3 fatty acids from fish and fish oils can protect against coronary heart disease. Both health professionals and the public are increasingly interested in their role in the prevention and management of coronary heart disease. In this era of multiple pharmacological treatments for coronary heart disease many feel that simple dietary interventions or nutritional supplements may be a more natural and acceptable method of providing benefits.

Several areas of uncertainty remain. Some studies have produced conflicting results and there have been increasing concerns about environmental contamination of certain fish. The optimal intake of omega-3 fatty acids is not firmly established nor is their mechanism of action fully understood.

1.1.1 OMEGA-3 POLYUNSATURATED FATTY ACIDS

The association between omega-3 fatty acids and cardiovascular disease originates from the observation that the Greenland Inuit had low mortality from coronary heart disease despite a diet rich in fat. In the 1970s the Danish investigators Bang and Dyerberg suggested that this could be due to the high omega-3 fatty acid content of the Inuit diet [Dyerberg et al 1975] which consisted largely of fish, seal and whale (Figure 1.1).

Omega-3 fatty acids, along with omega-6 fatty acids, are essential polyunsaturated fatty acids (Figures 1.2 and 1.3). There is an abundance of omega-6 fatty acids in
Figure 1.1 Greenland Inuit gutting a seal in the early 1900s. Their diet consisted largely of fish, whale, seal and walrus resulting in a high intake of omega-3 fatty acids. Copyright Arctic Institute, used with permission.
Fatty acids are saturated, monounsaturated, or polyunsaturated. There are two types of polyunsaturated fatty acid—the omega-6 and the omega-3 fatty acids. The omega-6 fatty acids are available mainly from vegetable oils. Three types of omega-3 fatty acid exist: α-linolenic acid is available from certain plants but eicosapentaenoic acid and docosahexaenoic acid must be obtained from marine sources.
Figure 1.3 Structures of the two classes of polyunsaturated fatty acids. The omega-3 fatty acids have their first double bond at the third carbon molecule from the methyl (CH3) end of the fatty acid, whereas the omega-6 fatty acids have their first double bond at the sixth carbon molecule. The chemical names for each fatty acid are also given: the number of carbon atoms is given first, followed by the number of double bonds and the position of the first double bond. Omega-6 linoleic acid can be desaturated in certain plants to form omega 3 α-linolenic acid. Whereas linoleic acid is mainly converted into arachidonic acid, α-linolenic is elongated and desaturated into eicosapentaenoic acid and then docosahexaenoic acid.
the Western diet mainly from vegetable oils rich in linoleic acid [Kris-Etherton et al 2000]. However, humans lack the necessary enzymes to convert omega-6 to omega-3 fatty acids, and the latter must be obtained from separate dietary sources. While α-linolenic acid (ALA) is available from certain plants, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are derived from fish and fish oils.

1.2 EPIDEMIOLOGICAL AND OBSERVATIONAL STUDIES

Most studies have shown an inverse association between fish consumption and the risk of coronary heart disease [Kromhout et al 1985; Daviglus et al 1997; Hu et al 2002]. Furthermore, both fish consumption [Albert et al 1998] and higher blood concentrations of omega-3 fatty acids [Albert et al 2002] are associated with a reduced risk of sudden death. However, some studies [Ascherio et al 1995; Morris et al 1995] have not found a relationship between fish intake and coronary heart disease. These inconsistencies could be due to differences in methodology, study populations or fish. Importantly, most studies showing no association were in populations with an already moderate fish intake, potentially masking any relationship. Overall, fish consumption appears to be beneficial and a systematic review of 11 prospective cohort studies concluded that fish intake significantly reduced coronary heart disease mortality in higher-risk populations [Marckmann and Gronbaek 1999].
1.3 CLINICAL INTERVENTION TRIALS

Several trials have assessed the effects of fish and fish oil supplements on coronary heart disease, mainly after myocardial infarction (Table 1.1). The Diet and Reinfarction Trial (DART) randomised 2,033 men with a recent myocardial infarction to three dietary interventions [Burr et al 1989]. Patients who received fish advice had a 29% relative reduction in total mortality during the 2-year follow-up (P<0.05), mainly due to a reduction in coronary heart disease deaths. The open-label GISSI-Prevenzione trial [1999] randomised 11,324 patients post-myocardial infarction to either: a daily ~850 mg omega-3 fatty acid capsule, 300 mg vitamin E, both or neither. After 3.5 years, subjects randomised to fish oil capsules had a relative risk reduction of 15% in the composite primary end point of total mortality, non-fatal myocardial infarction and stroke (P=0.023). There was also a reduction in the relative risk of cardiovascular death by 30% (P=0.024) and in sudden death by 45% (P=0.01). These benefits were apparent within just 4 months of randomisation [Marchioli et al 2002].

Two smaller secondary prevention trials have also assessed the effects of omega-3 fatty acids. In an Asian population, patients with a suspected myocardial infarction randomised to fish oil capsules, experienced a significant reduction in coronary heart disease mortality after 1 year compared to placebo [Singh et al 1997]. However, a Norwegian study reported no benefit in post-myocardial infarction patients given fish oil capsules versus placebo after 1.5 years [Nilsen et al 2001]. This may have been

[1] It should be noted that serious doubts have been raised concerning the validity of research performed by R B Singh, including suspicions of research fraud [BMJ. Jul 30, 2005; 331(7511): 281–288]. On balance, we included this study as no specific concerns have been raised relating to this paper which remains on Medline and has never been withdrawn, retracted, or had an expression of concern.
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Intervention</th>
<th>Intervention events (%)</th>
<th>Control events (%)</th>
<th>ARR (%)</th>
<th>RRR (%)</th>
<th>NNT</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet and Reinfarction Trial, UK (1989)</strong></td>
<td>Randomised, controlled 2 year follow-up, 2033 men post-MI</td>
<td>fish meal twice weekly or fish oil capsules if unable to tolerate fish (1.5 g/d)</td>
<td>7.7*</td>
<td>11.4</td>
<td>3.7</td>
<td>32.5</td>
<td>27</td>
<td>prior to routine use of secondary prevention therapy such as Aspirin, β-blockers, statins</td>
</tr>
<tr>
<td><strong>Indian Experiment of Infarct Survival, India (1997)</strong></td>
<td>Randomised, double-blind placebo-controlled 1 year follow-up, 360 patients post-MI</td>
<td>fish oil (EPA+DHA 1.8 g/d) or mustard seed oil (ALA 2.9 g/d)</td>
<td>11.4*</td>
<td>22</td>
<td>10.6</td>
<td>48.2</td>
<td>10</td>
<td>small size, high mortality, not applicable to Western populations</td>
</tr>
<tr>
<td><strong>GISSI-Prevenzione Trial, Italy (1999)</strong></td>
<td>Randomised, controlled 3.5 yr follow-up, 11324 patients post-MI</td>
<td>fish oil (EPA+DHA 0.85 g/d)</td>
<td>4.8**</td>
<td>6.8</td>
<td>2</td>
<td>29.7</td>
<td>50</td>
<td>not blinded, no placebo</td>
</tr>
<tr>
<td><strong>Nilsen et al, Norway (2001)</strong></td>
<td>Randomised, double-blind placebo-controlled 1.5 year follow-up, 300 patients post-MI</td>
<td>fish oil (EPA+DHA 3.5 g/d)</td>
<td>5.3</td>
<td>5.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>small size, reasonable intake of fish amongst general population</td>
</tr>
</tbody>
</table>

*p<0.01 between control and intervention groups; **P=0.024 between control and intervention groups.

ARR - absolute risk reduction; RRR - relative risk reduction; NNT - number needed to treat to prevent one event; MI - myocardial infarction; CHD - coronary heart disease; EPA - eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA - α-linolenic acid
due to the high habitual fish consumption among the general population in that area, with omega-3 supplementation conferring no additional benefit.

A trial of 3,114 men with angina unexpectedly found that individuals advised to eat oily fish, and particularly those given fish oil capsules, had a higher risk of cardiac death (11.5% versus 9%, \( P=0.02 \)) [Burr et al 2003]. The investigators speculated that this may have arisen from risk compensation or other changes in patient behaviour. More recent clinical trials have yielded inconsistent results. Whilst some demonstrated improved outcomes with omega-3 fatty acids [Yokoyama et al 2007; GISSI-HF Investigators 2008] others found no evidence of benefit [Galan et al 2010; Kromhout et al 2010; Rauch et al 2010].

**1.4 MECHANISM OF ACTION**

Whilst the weight of evidence outlined above supports a protective effect of omega-3 fatty acids on coronary heart disease, the mechanisms through which they confer these benefits remain unclear. Omega-3 fatty acids have several potentially cardioprotective effects, although the relative contribution of each of these is not fully understood.

**1.4.1 ARRHYTHMIAS**

The benefits of fish oils were originally thought to be due to their antithrombotic effects, but recent evidence has suggested that the predominant effect may be anti-arrhythmic. In the GISSI-Prevenzione trial [1999] the decrease in mortality was
largely due to a reduction in sudden death and, as in DART [Burr et al 1989], there was no reduction in non-fatal myocardial infarction. Fish oil supplementation increases heart rate variability in post-myocardial infarction patients, which correlates with a lower risk of mortality and malignant arrhythmia [Christensen et al 1996]. In animal models fish oil protects against ventricular fibrillation after surgical coronary artery occlusion [Leaf et al 2003]. The addition of EPA or DHA can prevent or terminate pharmacologically-induced arrhythmias in cultured neonatal rat cardiomyocytes [Leaf et al 2003]. However subsequent studies in patients with implantable cardioverter defibrillators have failed to demonstrate a direct anti-arrhythmic effect [Brouwer et al 2009].

1.4.2 THROMBOSIS
Platelet activation and deposition at sites of unstable plaque rupture promotes thrombus formation, and these critical events have become a common therapeutic target in acute coronary syndromes. However, the effects of omega-3 fatty acids on platelet function and thrombosis are controversial. Large doses reduce platelet aggregation, but smaller amounts have modest platelet inhibitory effects [Mori et al 1997]. Omega-3 fatty acids have inconsistent effects on fibrinolysis and little effect on blood coagulability [Kristensen et al 2001]. Therefore, although omega-3 fatty acids have an antithrombotic effect, its relevance to the mortality reduction seen with lower doses is unclear.
1.4.3 Atherosclerosis

Omega-3 fatty acids may also influence the atherosclerotic process. Fish oil fed to experimental animals can protect against atherosclerotic plaque progression [Weiner et al 1986; Davis et al 1987]. In humans with coronary heart disease, omega-3 fatty acid supplementation versus placebo for 2 years resulted in modest improvements in atherosclerosis as assessed by angiography [von Schacky et al 1999]. These effects may be due to a reduction in lipids, inflammation, or reduced growth factor production and suppression of smooth muscle cell proliferation [Connor 2000]. An important recent study randomised patients awaiting carotid endarterectomy to fish oil capsules, sunflower oil capsules or control until surgery and then assessed plaque morphology [Thies et al 2003]. Omega-3 fatty acids were readily incorporated into atherosclerotic plaques in the fish oil group, and these plaques were more likely to have thick fibrous caps and less inflammatory infiltrate. These features suggest a plaque less vulnerable to rupture and indicate that fish oils may be important in establishing plaque stability.

1.4.4 Inflammation

Inflammation has a central role in the development and progression of coronary artery disease. Omega-3 fatty acids have recognised anti-inflammatory actions that may contribute to their beneficial cardiac effects. Omega-6 fatty acids can be converted into arachadonic acid and then metabolised into the omega-6 eicosanoids [Heller et al 1998] (Figure 1.4). These cellular mediators enhance platelet aggregation and are generally pro-inflammatory. Consumption of omega-3 fatty acids increases cell membrane EPA that competes with arachadonic acid for
Fig 1.4 Synthesis of eicosanoids from omega-6 and omega-3 fatty acids. Arachidonic acid and eicosapentaenoic acid compete for the cyclo-oxygenase and lipoxygenase enzymes for conversion into eicosanoids. Those derived from arachidonic acid are pro-inflammatory and pro-aggregatory, whereas those derived from omega 3 fatty acids are anti-inflammatory and inhibit platelet aggregation. AA – arachidonic acid. EPA – eicosapentaenoic acid.
enzymatic conversion into its own metabolites, the omega-3 derived eicosanoids. These are less active and can partially oppose or antagonise the pro-inflammatory actions of the omega-6 eicosanoids.

Independent of effects on eicosanoid metabolism, fish oils suppress pro-inflammatory cytokines and reduce cell adhesion molecule expression [De Caterina et al 2000]. These are critical in recruiting circulating leucocytes to the vascular endothelium, an important event in the pathogenesis of atherosclerosis and inflammation. These effects may be mediated through actions on intracellular signalling pathways leading to reduced activation of transcription factors such as nuclear factor kappa B [De Caterina et al 2000]. However, the precise effects of omega-3 fatty acids on these fundamental cellular processes and their potential impact on coronary heart disease are yet to be completely delineated.

1.4.5 ENDOTHELIAL FUNCTION
Abnormal endothelial function is found in individuals with cardiovascular risk factors or established coronary heart disease. Omega-3 fatty acids have direct effects on endothelial vasomotor function. Higher levels are associated with improved brachial artery dilatation in young adults with cardiovascular risk factors, suggesting a protective effect on endothelial function [Leeson et al 2002]. In hyperlipidaemic men omega-3 fatty acid supplementation improved systemic arterial compliance [Nestel et al 2002] and DHA supplementation increased vasodilator responses in the human forearm [Mori et al 2000].
1.4.6 **Blood pressure**

Fish oils can produce modest reductions in blood pressure, possibly through their effects on endothelial function discussed above. A recent meta-analysis of 36 randomised trials found a reduction in systolic blood pressure of 2.1 mmHg and diastolic blood pressure of 1.6 mmHg [Geleijnse *et al.* 2002]. However, most trials used relatively high doses of fish oils (~3.6 g/day) and the effects of lower intakes of omega-3 fatty acids, such as those in the secondary prevention trials, remain to be established.

1.4.7 **Triglyceride lowering**

Omega-3 fatty acids reduce triglyceride levels in a dose-dependent manner, with intakes of ~4 g/day lowering serum triglycerides by 25-30% [Harris 1997]. Their effect on cholesterol is small and of uncertain clinical significance. Higher doses (3-5 g/day) can be used in the treatment of hypertriglyceridaemia. There is only a small reduction in triglycerides at the lower doses used in the GISSI-Prevenzione trial [1999] (~1 g/day) and it therefore seems unlikely that this effect alone could be responsible for the coronary heart disease benefits.

1.5 **Clinical implications**

Omega-3 fatty acids from fish or fish oil supplements should be considered in the secondary prevention regime of post-myocardial infarction patients. Patients should consume ~1 g/day of EPA+DHA, preferably by increasing their intake of oily fish to at least two servings per week. Fish oil capsules may be considered for those unable
to tolerate fish or effectively change their diet. Proven pharmaceutical-grade capsules should be prescribed rather than encouraging over-the-counter supplements.

Recent American Heart Association guidelines have gone further, supporting the use of fish oil supplements for patients with ‘documented’ coronary heart disease [Kris-Etherton et al 2002]. However, we believe more evidence is required before considering fish oil supplements for patients with coronary heart disease outwith the specific post-myocardial infarction population. Others have argued that fish oil supplements should not be recommended routinely for post-myocardial infarction patients until more definitive evidence is available [Grundy 2003].

No trials have assessed the effects of fish oils on coronary heart disease risk in primary prevention, and therefore explicit recommendations for this group cannot be made at present. Such a trial may prove impractical in terms of numbers required. However, based on evidence from epidemiological and observational studies the consumption of (preferably oily) fish at least twice weekly should be encouraged as part of a balanced diet. Current consumption and dietary sources of EPA+DHA are shown in Table 1.2.

Any recommendations regarding fish and fish oils should be balanced against safety issues. Side effects such as fishy aftertaste are uncommon, and gastrointestinal upset is infrequent at moderate intakes [Kris-Etherton et al 2002]. Some reports suggest that fish oil may worsen glycaemic control in diabetes, but two meta-analyses found no significant adverse effect [Friedberg et al 1998; Montori et al 2000]. Furthermore,
TABLE 1.2 Omega-3 fatty acid content of selected fish and seafood

<table>
<thead>
<tr>
<th></th>
<th>EPA+DHA content (g) per 100 g serving of fish (edible portion)</th>
<th>Amount of fish (g) required to provide ~1 g EPA+DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuna (fresh)</td>
<td>0.28-1.51</td>
<td>66-357</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>1.28-2.15</td>
<td>42.5-70.9</td>
</tr>
<tr>
<td>Mackerel</td>
<td>0.4-1.85</td>
<td>54-250</td>
</tr>
<tr>
<td>Atlantic herring</td>
<td>2.01</td>
<td>50</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>1.15</td>
<td>87</td>
</tr>
<tr>
<td>Sardines</td>
<td>1.15-2</td>
<td>50-87</td>
</tr>
<tr>
<td>Halibut</td>
<td>0.47-1.18</td>
<td>85-213</td>
</tr>
<tr>
<td>Tuna (canned)</td>
<td>0.31</td>
<td>323</td>
</tr>
<tr>
<td>Cod</td>
<td>0.28</td>
<td>357</td>
</tr>
<tr>
<td>Haddock</td>
<td>0.24</td>
<td>417</td>
</tr>
<tr>
<td>Catfish</td>
<td>0.18</td>
<td>556</td>
</tr>
<tr>
<td>Flounder/Sole</td>
<td>0.49</td>
<td>204</td>
</tr>
<tr>
<td>Oyster</td>
<td>0.44</td>
<td>227</td>
</tr>
<tr>
<td>Shrimp</td>
<td>0.32</td>
<td>313</td>
</tr>
<tr>
<td>Scallop</td>
<td>0.2</td>
<td>500</td>
</tr>
<tr>
<td>Cod liver oil capsule</td>
<td>0.19</td>
<td>5</td>
</tr>
<tr>
<td>Omacor (Pronova)</td>
<td>0.85</td>
<td>1</td>
</tr>
</tbody>
</table>

EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid.

Omega-3 content varies markedly depending on species, season, diet, packaging and cooking methods and the figures above are, therefore, rough estimates. Adapted from: [Kris-Etherton et al 2002].
a recent prospective cohort study found that higher consumption of omega-3 fatty acids was associated with a lower incidence of coronary heart disease and mortality in diabetic women [Hu et al 2003]. There have been concerns regarding adverse effects on low-density lipoprotein (LDL) cholesterol and oxidative stress, but increases in LDL cholesterol are modest whilst studies into oxidative stress have been contradictory [Harris et al 2003]. Overall, these effects are unlikely to be dominant given the apparent cardiac benefits of omega-3 fatty acids. More specific concerns regarding dietary fish relate to environmental contaminants, and a recent study suggested that mercury in fish may attenuate their cardioprotective effects [Guallar et al 2002]. Contaminants accumulate in larger, predatory fish and consumption of a variety of fish should minimise any possible adverse effects [Kris-Etherton et al 2002].

1.6 AIMS

The aims of the thesis are to determine whether oily fish or fish oil supplements can enhance endothelial vasomotor and fibrinolytic function in healthy smokers and patients with a recent myocardial infarction. In addition, we wish to establish whether fish oils have an antithrombotic action as determined by a reduction in the recently described phenomenon of platelet-monocyte binding. The latter has direct relevance to platelet and monocyte function and activation: central aspects of atherothrombosis.
1.7 ORIGINAL HYPOTHESES

We hypothesise that:

1. Marine-derived omega-3 fatty acids reduce platelet-monocyte aggregation in healthy volunteers (Chapter 3).

2. Plant-derived omega-3 fatty acids reduce platelet-monocyte aggregation and arterial stiffness in healthy volunteers (Chapter 4).

3. Omega-3 fatty acid supplements improve endothelial vasomotor and fibrinolytic function and platelet-monocyte aggregates in cigarette smokers (Chapter 5).

4. Omega-3 fatty acid supplements improve endothelial vasomotor and fibrinolytic function and platelet-monocyte aggregates in patients with a recent myocardial infarction (Chapter 6).
CHAPTER 2

FLOW CYTOMETRIC ANALYSIS OF CIRCULATING PLATELET-MONOCYTE AGGREGATES IN WHOLE BLOOD:
METHODOLOGICAL CONSIDERATIONS

Harding SA, Din JN, Sarma J, Jessop A, Weatherall M, Fox KA, Newby DE.
Flow cytometric analysis of circulating platelet-monocyte aggregates in whole blood: methodological considerations.
2.1 SUMMARY

Platelet-monocyte aggregates are increasingly being used to quantify platelet activation. The variables that influence platelet-monocyte aggregates have not been well defined. We sought to determine the effect of blood collection, handling and processing techniques on detected levels of platelet-monocyte aggregates using a flow cytometric assay. Whole blood was labelled with anti-cluster of differentiation (CD)14-phycoerythrin (PE) and anti-CD42a-fluorescein isothiocyanate (FITC). Thereafter, samples were fixed and red cells lysed. Analysis was performed with the flow cytometer initially triggering on light scatter and then on fluorescence parameter (FL)-2 to identify CD14-PE positive monocytes. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. The effect of collection, handling and processing techniques on this assay were assessed. Anticoagulation with heparin (20.1±2.0%), D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone (PPACK) (16.8±1.9%), sodium citrate (12.3±1.6%) and ethylenediaminetetraacetic acid (EDTA) (9.5±1.0%) resulted in markedly different levels of platelet-monocyte aggregation (P<0.0001). Platelet-monocyte aggregation was higher in samples obtained from intravenous cannulae compared to those obtained by venepuncture (13.8±2.4% versus 20.9±3.9%, P=0.03). For every 10 minutes of delay prior to processing platelet-monocyte aggregates increased by 2.8% (P=0.0001) in PPACK anticoagulated blood and 1.7% (P=0.01) in citrate anticoagulated blood. Erythrocyte lysis together with fixation does not affect platelet-monocyte aggregation. Platelet-monocyte aggregates remained stable over 24 hours when fixed and stored at 4°C. Multiple handling and processing factors may affect platelet-monocyte aggregation.
We recommend the measurement of platelet-monocyte binding on samples collected by direct venepuncture, using a direct thrombin inhibitor as the anticoagulant and minimising the time delay before sample fixation.

2.2 INTRODUCTION

Platelets play a key role in mediating inflammatory and thrombotic responses during both health and disease. Once activated, circulating platelets bind to leukocytes, predominantly monocytes, to form platelet-leukocyte aggregates [Jungi et al 1986; Larsen et al 1989; Rinder et al 1991]. This is initiated largely through the binding of platelet surface P-selectin on activated platelets to its leukocyte counter-receptor, P-selectin glycoprotein ligand-1 [Hamburger and McEver 1990; Palabrica et al 1992; Sarma et al 2002]. Platelet-leukocyte aggregates are then stabilised by several mechanisms, including the binding of leukocyte membrane-activated complex-1 (CD11b/CD18) and platelet glycoprotein Ib [Simon et al 2000].

Flow cytometric analysis of platelet surface expression of P-selectin has previously been considered to be the ‘gold standard’ marker of platelet activation. However, in vivo activated platelets rapidly lose their surface platelet glycoprotein Ib whilst continuing to circulate and function [Michelson et al 1996]. Recently it has been demonstrated that platelet-monocyte aggregates remain detectable in peripheral blood for significantly longer and are a more sensitive marker of in vivo platelet activation than platelet-surface P-selectin expression [Michelson et al 2001].

Given that platelet-monocyte aggregates can be readily quantified using flow
cytometric analysis and the high sensitivity of the assay it is not surprising that there has been rapid growth in the use of this assay to quantify platelet activation. In the last year alone over 40 papers assessing platelet-monocyte or platelet-leukocyte aggregates have been published.

The high sensitivity of the assay leaves it vulnerable to artefactual in vitro activation and reliable laboratory methods are essential to ensure accurate and consistent results. A number of variables including choice of anticoagulant, sample collection, handling and processing techniques may potentially affect the assessment of platelet-monocyte aggregates and vary markedly between published studies. While several flow cytometric protocols have been described [Li et al 1997; Hagberg and Lyberg 2000; Barnard et al 2003], the influence of the above factors on measured levels of platelet-monocyte aggregation remains incompletely characterised. In the present study, we sought to determine the effect of blood collection, handling and processing techniques on detected levels of platelet-monocyte aggregates using a simple two-colour flow cytometric assay.

2.3 METHODS

2.3.1 SUBJECTS AND BLOOD COLLECTION

These studies were performed between February 2003 and June 2006. Peripheral venous blood was obtained from healthy volunteers, aged between 18 and 35 years, who were taking no medication. Ethical approval was obtained from the local Research Ethics Committee and all subjects provided written informed consent. Blood was drawn by clean venepuncture of a large antecubital vein using a 19-gauge
needle, unless otherwise stated. Care was taken to ensure a smooth blood draw without venous stasis. Unless otherwise stated, blood was collected into tubes containing the direct thrombin inhibitor PPACK (75 μM PPACK, Cambridge Bioscience Ltd, Munro House, Trafalgar Way, Bar Hill, Cambridge CB23 8SQ, UK). Tubes were gently inverted to ensure mixing of whole blood with anticoagulant.

2.3.2 ANTIBODIES AND OTHER REAGENTS

All chemicals were obtained from Sigma Chemical Company (The Old Brickyard New Road, Gillingham, Dorset SP8 4XT, UK) unless otherwise stated. Fluorescein isothiocyanate-conjugated CD42a (glucose-regulated protein precursor (GRP-P), immunoglobulin (Ig)G1), and control IgG1 were obtained from Serotec Ltd (Endeavour House Langford Lane, The Langford Business Park, Kidlington, Oxfordshire OX5 1GE, UK). Phycoerythrin-conjugated CD14 (Tuk-4, IgG2a) was obtained from Dako Cytomation (Cambridge House, St Thomas Place, Ely, Cambridgeshire CB7 4EX, UK). FACS-Lyse was obtained from Becton Dickinson (The Danby Building, Edmund Halley Rd, Oxford OX4 4DQ, UK).

2.3.3 IMMUNOLABELLING AND FLOW CYTOMETRY

Aliquots of whole blood (50 μL) were incubated with anti-CD14-PE, anti-CD42a-FITC and isotype-matched controls for 20 minutes at room temperature. Thereafter, samples were fixed and the red cells lysed by the addition of 500 μL of FACS-Lyse solution unless otherwise stated. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm laser (Beckman Coulter, Oakley Court,
Kingsmead Business Park, London Road, High Wycombe HP11 1JU, UK) within 4 hours unless otherwise stated. A medium flow setting was used to minimise leukocyte-platelet coincident events. Monocytes were identified based on their forward and side scatter characteristics and then by triggering on FL-2 to identify CD14-PE positive monocytes and exclude large granular lymphocytes. For each measurement a minimum of 2,500 monocytes were collected. Platelet-monocyte aggregates were defined as monocytes positive for CD42a (Figure 2.1). All results are expressed as percentage of positive cells. Analyses were performed using EXPO32 software (Beckman Coulter, Oakley Court, Kingsmead Business Park, London Road, High Wycombe HP11 1JU, UK).

2.3.4 EXPERIMENTAL DESIGN

Using the above flow cytometric protocol, with modifications where appropriate, we conducted a series of six studies to assess the effects of different blood collection and sample handling methods on the measured level of circulating platelet-monocyte aggregates.

Protocol 1: Choice of anticoagulant

To determine the effect of different anticoagulants on platelet-monocyte aggregation, blood from 8 healthy volunteers was incubated for 5 minutes with sodium citrate (0.106 M; Sarstedt Monovette, SARSTEDT Ltd, 68 Boston Road, Beaumont Leys, Leicester LE4 1AW, UK), lithium heparin (16 IU/mL; Sarstedt Monovette), EDTA (1.6 mg/mL; Sarstedt Monovette) and PPACK. All samples were then labelled with appropriate monoclonal antibodies,
Fig 2.1 Platelet-monocyte aggregates were assessed in whole blood using two-colour flow cytometry. Monocytes were labelled with CD14-PE and platelets with CD42a-FITC. A, Representative forward and side scatter properties of leukocytes in whole blood. Monocytes were gated based on their characteristic scatter properties. B, A histogram of events in gate A was created, with a second gate placed over the CD14-bright monocytes. A total of 2500 CD14-bright monocytes were collected for analysis. C, A quadrant plot of CD14-bright monocytes against an appropriate IgG1 isotype control was used to set a marker to allow for non-specific binding and autofluorescence. D, A second quadrant plot of CD14-bright monocytes against CD42a was then used to determine the percentage of monocytes positive for CD42a. CD – cluster of differentiation; PE – phycoerythrin; FITC – fluorescein isothiocyanate; Ig – immunoglobulin.
and fixed before flow cytometric analysis as described above.

**Protocol 2: Effect of serial sampling via intravenous cannula**

We determined the effect of serial sampling of whole blood through an intravenous cannula on platelet-monocyte aggregation. A group of 8 healthy volunteers underwent serial blood sampling through both repeated venepuncture or through an intravenous cannula. Blood was drawn at baseline, 30 minutes, 50 minutes and 85 minutes. Repeated venepuncture was performed from large antecubital veins using 19-gauge needles. Sampling from intravenous cannulae was performed after a 17-gauge cannula had been inserted into a large antecubital vein. When sampling from the cannula the first 2-5 mL of blood were discarded and after each blood draw the cannula was flushed with 10 mL of normal saline. Only cannulae which allowed a smooth blood draw after insertion were accepted. Blood samples taken at each time point were labelled with appropriate monoclonal antibodies before fixation and flow cytometric analysis as described above.

**Protocol 3: Time delay prior to immunolabelling**

To study the effect of time delay between sample draw and sample preparation on spontaneous *in vitro* platelet-monocyte aggregation, blood was collected from 8 healthy volunteers and anticoagulated with sodium citrate or PPACK. After intervals of 0, 10, 20, 30 and 60 minutes individual aliquots were labelled with monoclonal antibodies. Samples were then fixed and taken for flow cytometric analysis as described above.
**Protocol 4: Effect of erythrocyte lysis**

In order to determine the effect of erythrocyte lysis on platelet-monocyte aggregation, blood from 8 healthy volunteers was anticoagulated in PPACK before incubation in duplicate with appropriate monoclonal antibodies as above. After 20 minutes duplicate samples underwent either a) fixation and erythrocyte lysis by the addition of 500 µL of 1:10 FACS-lysing solution or b) fixation with no erythrocyte lysis by the addition of 500 µL of 1% formaldehyde. Flow cytometric analysis in the lysis group was performed as described above. As it is not possible to distinguish readily leukocyte subpopulations in whole blood without erythrocyte lysis, unlysed samples were collected using a live gate for CD14 positive events. Monocytes were subsequently identified on the basis of their characteristic scatter properties. As unlysed whole blood results in a very cell-rich preparation passing the laser, samples were processed with a low flow rate to minimise the risk of coincident events. The time to collect 1,000 CD14 positive events was recorded for both lysed and unlysed samples.

**Protocol 5: Samples stability after fixation**

We assessed the effects of time delay on the stability of fixed samples prior to flow cytometric analysis. Blood from 8 healthy volunteers was anticoagulated with PPACK and labelled with appropriate monoclonal antibodies prior to fixation as above. Aliquots were then stored at 4°C. These samples were then analysed by flow cytometry to determine the level of platelet-monocyte aggregation at the following time points: immediately, 4 hours, 8 hours, 12 hours and 24 hours.
Protocol 6: Reproducibility

Blood was drawn from 8 healthy volunteers and anticoagulated in PPACK. After 5 minutes, five individual aliquots of blood were each incubated with appropriate monoclonal antibodies for 20 minutes before fixation and subsequent flow cytometric analysis to determine the level of platelet-monocyte aggregation. The coefficient of variation was then derived.

2.3.5 Statistical Analysis

Continuous variables are reported as mean±standard error of the mean (SEM). Statistical analyses were performed using Student’s t-test, two-way analysis of variance (ANOVA) or linear regression where appropriate. In order to account for the repeated measures when analysing the effect of sampling technique, time delay and fixation on platelet-monocyte aggregation a form of linear regression was fitted using mixed linear models. Statistical significance was taken at 5%. Calculations were performed using SAS version 8.2 (SAS Institute Inc, 100 SAS Campus Drive, Cary, NC 27513-2414, USA).

2.4 RESULTS

2.4.1 Choice of Anticoagulant

The type of anticoagulant used has a marked effect on the measured platelet-monocyte aggregate levels (Figure 2.2). Platelet-monocyte aggregation was increased in blood anticoagulated with heparin (20.1±2.0%) compared to PPACK (16.8±1.9%, P=0.01), sodium citrate (12.3%±1.6%, P<0.0001) or EDTA.
Fig 2.2 Effect of anticoagulant on platelet-monocyte aggregation. Platelet-monocyte aggregation was assessed in blood anticoagulated with either heparin, PPACK, sodium citrate or EDTA. Data are presented as mean±SEM (n=8). *P=0.01, **P=0.002, ***P=0.05. PPACK – D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone; EDTA – ethylenediaminetetraacetic acid; SEM – standard error of the mean.
Levels of platelet-monocyte aggregates were reduced in blood anticoagulated with sodium citrate or EDTA compared to PPACK (P<0.01).

2.4.2 BLOOD COLLECTION: SAMPLING VIA INTRAVENOUS CANNULA COMPARED WITH VENEPUNCTURE

At baseline (time 0), samples obtained from intravenous cannulae had a significantly higher level of platelet-monocyte aggregation compared to those obtained by venepuncture (20.9±3.9% versus 13.8±2.4%, P=0.03; Figure 2.3). The levels of platelet-monocyte aggregation increased steadily when repeated samples were taken from an intravenous cannula (1.5% per 10-minute interval, 95% confidence interval 0.6-2.4%, P=0.001) but did not change with time when serial sampling was performed by venepuncture (0.3% per 10-minute interval, 95% confidence interval -0.6-1.2%, P=0.47; Figure 2.3).

2.4.3 EFFECT OF TIME DELAY BETWEEN COLLECTION AND IMMUNOLABELLING

The percentage of platelet-monocyte aggregates in unfixed samples increased in a time-dependent manner (Figure 2.4). Based on a mixed linear model that allowed the PPACK group have a different (greater) variance the percentage of platelet-monocyte aggregates in blood anticoagulated with PPACK increased by 2.8% (95% confidence interval 2.20-3.4%, P=0.01) for every 10 minutes of delay while in blood anticoagulated with sodium citrate there was a slower rate of increase 1.7% (95% confidence interval 0.1-2.9%, P=0.01) every 10 minutes. However, the difference between these two slopes did reach statistical significance (P=0.1).
Fig 2.3 Effect of blood sampling technique on platelet-monocyte aggregates. Blood was drawn at baseline, 30 minutes, 50 minutes and 85 minutes using serial venipuncture (squares) or serial sampling from an intravenous cannula (triangles) and platelet-monocyte aggregates were assessed. Data are presented as mean±SEM (n=8). *P=0.03, P=<0.001. SEM – standard error of the mean.
Fig 2.4 Effect of time delay prior to processing on platelet-monocyte aggregates. Immunostaining and processing of whole blood samples was delayed for intervals of 0, 10, 20, 30 and 60 min following blood collection. For every 10 min of delay prior to processing platelet-monocyte aggregates increased by 2.8% (95% CI 2.2 to 3.4% P=0.0001) in PPACK anticoagulated blood (squares) and 1.7% (95% CI 0.1 to 2.9%, P=0.01) in citrate anticoagulated blood (triangles). Data are reported as mean±SEM (n=8). CI – confidence interval; PPACK - D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone; SEM – standard error of the mean.
2.4.4 Effect of erythrocyte lysis on platelet-monocyte aggregation

Erythrocyte lysis had no effect on platelet-monocyte aggregation (16.9±2.8% versus 16.1±2.5%, P=0.4). There was a strong correlation between levels of platelet-monocyte aggregation from lysed and unlysed samples (r=0.97, P<0.0001). Bland-Altman analysis also revealed good agreement between the two methods (mean bias 0.72%, 95% limit of agreement -3.6-0%). Collection times were shorter using erythrocyte lysis (67±10 seconds versus 149±23 seconds, P= 0.02).

2.4.5 Sample stability and reproducibility after fixation

In the fixed samples stored at 4°C platelet-monocyte aggregation did not alter over the 24-hour period (P=0.26; Figure 2.5). The interassay coefficient of variation was calculated for samples anticoagulated with PPACK using the standard method describe above (n=8). The mean coefficient of variation for the percentage of platelet-monocyte aggregates was 7.8%.

2.5 DISCUSSION

The quantification of platelet-monocyte aggregates is a sensitive measure of platelet activation and has become widely used in clinical research. In the current study we report a simple reproducible method of assessing platelet-monocyte aggregates and identify a number of factors which may substantially influence the levels of platelet-monocyte aggregation.
Fig 2.5 Stability of fixed platelet-monocyte aggregates over 24 h. Platelet-monocyte aggregation measured in fixed samples did not differ over 24 h when stored at 4°C (P=0.26). Data are reported as mean ± SEM (n=8). SEM – standard error of the mean.
Several methods for the flow cytometric analysis of platelet-leukocyte aggregates have been described. In the present study, we utilised a two-colour whole blood technique incorporating erythrocyte lysis and fixation to quantify platelet-monocyte aggregates. This methodology has been widely used for the assessment of platelet-monocyte aggregation [Sarma et al 2002; Barnard et al 2003; Xiao and Theroux 2004]. We choose to assess platelet-monocyte aggregates rather than platelet-leukocyte aggregates or platelet-neutrophil aggregates as work by Michelson and colleagues had previously suggested that platelet-monocyte aggregates may be a more robust indicator of in vivo platelet activation [Michelson et al 2001].

We utilised methodology incorporating erythrocyte lysis as it allows efficient and accurate discrimination of leukocyte subpopulations, the ease of which such assays can be performed and the cytometric problems that are avoided [Barnard et al 2003]. Li et al [1997] have previously suggested that red cell lysis can lead to artificial increases in platelet-leukocyte aggregation. Although they demonstrated increased platelet-leukocyte aggregation with erythrocyte lysis, their lysis technique involved considerable additional sample manipulation including repeated washing and centrifugation with no fixation. Notably, the authors acknowledged that the marked artefacts observed with their lysis protocol were likely to have been predominantly due to repeated centrifugation rather than a major effect of lysis. In contrast, we avoided any additional centrifugation or washing steps that could activate platelets or monocytes when assessing the effect of erythrocyte lysis. In addition, our protocol included fixation at the time of erythrocyte lysis which would minimise the opportunity for platelet activation by adenosine diphosphate released from
erythrocytes. Our results suggest that red cell lysis along with immediate fixation does not increase platelet-monocyte aggregation and is less time consuming to perform.

The current study demonstrates that the type of anticoagulant used has a marked effect on the measured levels of platelet-monocyte aggregation. We have previously demonstrated that platelet-monocyte binding is largely calcium-dependent and therefore it was not surprising that the calcium chelating anticoagulants, EDTA and trisodium citrate, reduced levels of platelet-monocyte aggregates [Sarma et al 2002]. We and others have also previously demonstrated that unfractionated heparin activates platelets and increases platelet-monocyte aggregation by a P-selectin mechanism [Thomson et al 1973; Salzman et al 1980; Henrich et al 1988; Harding et al 2006]. In contrast, direct thrombin inhibitors such as PPACK provide reliable anticoagulation without activating platelets [Harding et al 2006]. Thus, we prefer the use of a direct thrombin inhibitor such as PPACK that maintains physiological calcium concentrations and does not cause platelet activation.

Our study also demonstrates that platelet-monocyte aggregates increase in a time-dependent manner in vitro. This increase occurred in both blood anticoagulated with PPACK or citrate. The rate of increase appeared to be slower in samples anticoagulated with citrate compared to PPACK. It is therefore important to perform processing as soon as possible and to standardise this time interval. In situations where there may be a substantial delay before immunostaining and fixation is performed, it may be more appropriate to use citrate as the anticoagulant.
Following immunostaining and fixation, samples stored at 4°C were stable for 24 hours. If methods without fixation are used then it would be important to analyse samples immediately after immunostaining to minimise artefactual *in vitro* increases in platelet-monocyte aggregates. However, this is often difficult to achieve in practice as there may be multiple samples to run, limited access to a flow cytometer or separation between the sites where samples are collected and the flow cytometric analysis is performed.

Handling and processing techniques affect the measurement of platelet activation and platelet-monocyte aggregation. These factors should be standardised and fully described in the description of study methods. Where possible, we recommend (i) avoiding taking blood samples through intravenous cannulae, (ii) using a direct thrombin inhibitor as an anticoagulant, (iii) minimising and standardising time delays between blood collection and processing, and (iv) storing fixed samples stored at 4°C prior to analysis.
CHAPTER 3

EFFECT OF DIETARY INTERVENTION WITH OIL-RICH FISH ON PLATELET-MONOCYTE AGGREGATION

3.1 SUMMARY

Dietary intake of fish rich in omega-3 fatty acids is associated with a reduction in cardiovascular events. The mechanisms for this are uncertain and previous studies investigating effects on platelet function have produced inconsistent results. Platelet-monocyte aggregation is a sensitive marker of platelet activation and may contribute to the initiation and progression of atherothrombosis. This study assessed the effect of dietary intervention with oily fish on platelet-monocyte aggregation in healthy subjects. Fourteen subjects had their diet supplemented with 500 g of oil-rich fish per week for 4 weeks. A control group of 14 subjects received no dietary intervention over a 4-week period. Platelet-monocyte aggregates were assessed with flow cytometry. Dietary intervention with fish led to an increase in omega-3 fatty acids in plasma phospholipids (14.2±3.4% versus 5.8±1.3%, P<0.001). In contrast to the control group, platelet-monocyte aggregates were reduced by 35% following dietary intervention with oily fish (16.0±9.0% versus 24.8±10.9%, P<0.01), and returned to basal levels 4 weeks after discontinuation of supplementation. There was an inverse correlation between platelet-monocyte aggregation and plasma omega-3 fatty acid concentrations (r=-0.421, P=0.006). There were no changes in the plasma markers of platelet activation, soluble P-selectin or soluble CD40 ligand. We have demonstrated, for the first time, that dietary intervention with oil-rich fish reduces platelet-monocyte aggregation in man. Our results suggest that reduced platelet activation provides a potential mechanism through which fish oils confer their cardiovascular preventative benefits.
3.2 INTRODUCTION

Consumption of fish oil may protect against cardiovascular disease [Din et al 2004]. Data from observational studies suggest that fish intake is inversely associated with fatal coronary heart disease [He et al 2004], and randomised controlled trials of dietary fish or fish oil supplementation after myocardial infarction demonstrate a reduction in mortality [Burr et al 1989; GISSI-Prevenzione trial 1999]. These effects are believed to be due to the high amounts of omega-3 fatty acids, EPA and DHA, present in fish oil. Eicosapentaenoic acid and DHA must be obtained from dietary sources and recent guidelines from the American Heart Association recommend the consumption of oily fish at least twice weekly for individuals with and without coronary heart disease [Kris-Etherton et al 2002]. However, not all randomised controlled trials have shown a benefit [Hooper et al 2006], and the underlying mechanisms through which cardiac protection might be conferred remain uncertain.

The effects of fish oil on platelet function and thrombosis are controversial. Although fish oils were originally thought to act by reducing platelet aggregation through effects on eicosanoid metabolism [Dyerberg et al 1978], subsequent studies have produced contradictory results [Knapp 1997]. Alternative mechanisms of action have been proposed including effects on arrhythmias, inflammation, endothelial function and atherosclerotic plaque stability [De Caterina et al 2000; Mori et al 2000; Leaf et al 2003; Thies et al 2003]. Indeed, it has been suggested that there is little evidence for a major antithrombotic effect of fish oils at the moderate doses shown to reduce mortality in the secondary prevention trials [Kristensen et al 2001].
Most previous studies investigating the effects of fish oils on platelet function have relied on *in vitro* platelet aggregometry and plasma assays of soluble markers of platelet activation [Knapp 1997]. These techniques are poorly suited to the detection of potentially subtle effects induced by dietary changes. Reproducibility is unsatisfactory and the tests are difficult to standardise [Michelson *et al* 2000]. Platelet aggregometry can only measure *ex vivo* changes in platelet reactivity to a single external stimulus, and sample manipulation required for both methods leaves them particularly vulnerable to artefactual *in vitro* activation.

Modern flow cytometric techniques allow platelets to be analysed directly in their physiological environment of whole blood with minimal sample manipulation. Circulating platelet-monocyte aggregates, formed by the binding of activated platelets to leukocytes via a P-selectin dependent mechanism [Jungi *et al* 1986], can be readily measured in this way and have emerged as a highly sensitive marker of platelet activation [Michelson *et al* 2001]. The adhesion of activated platelets to monocytes also has important functional consequences, and can induce the expression of cytokines, chemokines, adhesion molecules and tissue factor [Neumann *et al* 1997]. Consistent with these biological effects, circulating platelet-monocyte aggregates appear to promote atherosclerotic lesion formation and are increased in stable coronary heart disease and acute coronary syndromes [Sarma *et al* 2002; Huo *et al* 2003].
We therefore investigated whether supplementing the diet of healthy individuals with a moderate intake of oil-rich fish would reduce platelet activation and platelet-monocyte aggregation.

3.3 METHODS

3.3.1 STUDY PARTICIPANTS AND DESIGN

Twenty-eight healthy male volunteers aged between 21 and 28 years were enrolled into the study. Exclusion criteria included those taking regular medication, those with clinical evidence of atherosclerotic vascular disease, hypertension, diabetes mellitus, hypercholesterolaemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency. Ethical approval was obtained from the lothian Research Ethics Committee and all subjects provided written informed consent.

Fourteen volunteers were asked to eat 500 g of mackerel per week for 4 weeks (equivalent to approximately 1 g per day of EPA+DHA). The fish intake was calculated to provide similar amounts of omega-3 fatty acids to those shown to reduce mortality. The remaining 14 participants constituted a control group and received no dietary intervention for a 4-week period. Blood was drawn from all participants at baseline and at the end of the 4-week period, and a further sample was drawn from the dietary intervention group 4 weeks after cessation of fish intake. Subjects in the fish supplementation group kept 3-day weighed food diaries at
baseline, during fish supplementation and during the washout period to assess changes in dietary intake during the study period.

3.3.2 Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with sodium citrate (0.106 M), EDTA (1.6 mg/mL) and the direct thrombin inhibitor PPACK. Whole blood anticoagulated with PPACK was immunolabelled within 5 minutes of phlebotomy for subsequent flow cytometric analysis of platelet-monocyte aggregates. Plasma was prepared from blood anticoagulated with sodium citrate and EDTA by centrifugation (1500 x g for 30 minutes). To minimise ex vivo platelet activation, blood was centrifuged within 5 minutes of collection and separated immediately. Plasma samples were stored at -40°C until analysis.

3.3.3 Flow cytometry

The following reagents were used: FITC-conjugated CD42a (GRP-P, IgG1), and isotype control IgG1 were obtained from Serotec Ltd, PE-conjugated CD14 (Tuk-4, IgG2a) was obtained from Dako Cytomation and FACS-Lyse was obtained from Becton Dickinson. Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with anti-CD14, anti-CD42a and isotype-matched controls for 20 minutes at room temperature. Thereafter, samples were fixed and the red cells lysed by the addition of 500 μL of FACS-Lyse solution. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser within 4 hours of labelling. Samples were initially analysed with the flow cytometer
triggered on light scatter and then by triggering on FL-2 to identify CD14-PE positive monocytes. For each measurement 2,500 cells were collected. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. All results are expressed as percentage of positive cells. Analyses were performed using EXPO32 software. The mean coefficient of variation for the percentage of platelet-monocyte aggregates is 7.8%.

3.3.4 Measurement of soluble CD40 ligand and soluble P-selectin
Soluble CD40 ligand and soluble P-selectin concentrations were measured in plasma prepared from blood anticoagulated with sodium citrate. Plasma soluble CD40 ligand and soluble P-selectin concentrations were determined with commercially available enzyme-linked immunosorbant assays (eBioscience Ltd, 2nd Floor, Titan Court, 3 Bishop Square, Hatfield AL10 9NA, UK, detection limit of 0.095 ng/mL, coefficient of variation 4%; and R&D Systems, 19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK, detection limit 0.5 ng/mL, coefficient of variation 5.6%; respectively).

3.3.5 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 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, 610 Wharfedale Road, IQ Winnersh, Wokingham, Berkshire RG41 5TP, UK). 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%.

3.3.6 Statistical Methods
Continuous variables are reported as mean±standard deviation (SD). Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons, or two-tailed Student’s t-tests where appropriate. Based on preliminary data we calculated that a sample size of 13 subjects in each group would give 80% power of detecting a 6% difference in platelet-monocyte aggregates at a significance level of 5%. All calculations were performed using GraphPad Prism (GraphPad Software Inc, 2236 Avenida de la Playa, La Jolla, California 92037, USA). Statistical significance was taken at 5%.

3.4 Results

3.4.1 Baseline Characteristics
Baseline characteristics were similar between the fish supplementation and control groups (Table 3.1). Study participants were young and there were no differences in body mass index, smoking status, or lipid profile.
<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>Fish Supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=14</td>
<td>n=14</td>
</tr>
<tr>
<td>Age, years</td>
<td>24±3</td>
<td>22±1</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24±2</td>
<td>23±4</td>
</tr>
<tr>
<td>Current smokers</td>
<td>3/14</td>
<td>4/14</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.5±1.1</td>
<td>4.3±0.9</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.3±0.3</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Chol:HDL chol ratio</td>
<td>3.6±1.3</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.6±1.0</td>
<td>2.3±0.6</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.5±1.8</td>
<td>1.6±1.0</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD.
HDL - high-density lipoprotein; Chol - cholesterol; LDL - low-density lipoprotein; SD - standard deviation.
3.4.2 Effect of fish intervention on plasma phospholipid fatty acid composition

The plasma phospholipid fractions of omega-3 and omega-6 fatty acids did not differ between groups at baseline. Dietary intervention with fish led to an increase in the percentage of total omega-3 fatty acids (14.2±3.4% versus 5.8±1.3%, P<0.001), EPA (5.2±2.1% versus 1.0±0.3%, P<0.001; Figure 3.1A) and DHA (7.5±1.5% versus 3.5±1.0%, P<0.001; Figure 3.1B) at 4 weeks compared with baseline. Four weeks after cessation of the fish diet there was a reduction in the percentage of both EPA (1.8±0.7%) and DHA (4.7±1.1%), although DHA concentrations remained significantly higher than baseline (Figure 3.1A, 3.1B). There was no change in the percentage of ALA, a plant-derived omega-3, during dietary fish intervention (Figure 3.2). As the proportion of EPA and DHA increased during the fish diet, there was a corresponding reduction in the percentage of arachidonic acid (8.2±0.3% versus 9.9±0.5%, P<0.01), linolenic acid (20.1±0.8% versus 23.1±0.6%, P<0.01), and oleic acid (12.0±0.4% versus 13.7±0.4%, P<0.001) at 4 weeks compared with baseline (Table 3.2). There were no changes in the plasma phospholipid levels of omega-3 or omega-6 fatty acids in the control group during the 4-week period.
Fig 3.1 Percentage omega-3 fatty acids in plasma phospholipids in the fish intervention group and control group over the study duration. * P<0.001 versus baseline, † P<0.001 versus 4 weeks. Statistical analyses for the fish intervention group were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons. Statistical analyses for the control group used a 2-tailed Students t-test. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALA, α-linolenic acid, ANOVA – analysis of variance.
Fig 3.2 Effect of dietary intervention with oil-rich fish on platelet-monocyte aggregation. Data are shown for the fish intervention group at baseline, after 4 weeks of dietary supplementation with mackerel 500 g weekly, and 4 weeks after cessation of fish. Data are also shown for the control group at baseline and 4 weeks (no dietary intervention). Statistical analyses for the fish intervention group were performed using one-way ANOVA with repeated measures and Bonferroni's post-tests for multiple comparisons. Statistical analyses for the control group used a 2-tailed Students t-test. NS – not significant; ANOVA – analysis of variance.
3.4.3 Nutritional composition and lipid profile during fish intervention

Analysis of the 3-day weighed food diaries in the fish intervention group revealed no differences in intake of energy, protein, fat, carbohydrate or fibre during the study period (Table 3.3). In terms of mineral intake, there was an increase in iodine ($P<0.0001$) and selenium ($P=0.025$) consumption during the fish diet. Vitamin intake was also determined, and revealed an increase in consumption of vitamin D ($P<0.0001$) and vitamin $B_{12}$ ($P=0.0004$) during the fish intervention period. Although there was higher vitamin A intake during the washout period after the cessation of dietary fish this was of borderline statistical significance ($P=0.05$). Dietary intervention with fish did not affect total cholesterol, high-density lipoprotein (HDL) cholesterol, LDL cholesterol or triglyceride levels (data not shown).

3.4.4 Effect of fish intervention on platelet-monocyte aggregation

Platelet-monocyte aggregation was reduced by 35% following 4 weeks dietary intervention with oil-rich fish (16.1±9.0% versus 24.8±10.9%, $P<0.01$; Figure 3.2). Platelet-monocyte aggregates returned to baseline levels 4 weeks after cessation of the fish intervention (24.9±9.6% versus 24.8±10.9%, $P=NS$; Figure 3.2). There was no change in platelet-monocyte aggregation in the control group over the corresponding 4-week period (18.7±6.9% versus 21.5±6.7%, $P=NS$; Figure 3.2). There were inverse correlations between levels of platelet-monocyte aggregation in the fish intervention group and the plasma phospholipid percentages of EPA
### TABLE 3.2 Percentage fatty acid composition of plasma phospholipids

<table>
<thead>
<tr>
<th></th>
<th>Fish Intervention Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 weeks</td>
</tr>
<tr>
<td><strong>Palmitic acid (16:0)</strong></td>
<td>28.9±1.3</td>
<td>28.0±0.9</td>
</tr>
<tr>
<td><strong>Stearic acid (18:0)</strong></td>
<td>13.4±0.9</td>
<td>13.6±1.1</td>
</tr>
<tr>
<td><strong>Oleic acid (18:1 n-9)</strong></td>
<td>13.7±1.5</td>
<td>12.0±1.3‡</td>
</tr>
<tr>
<td><strong>Linoleic acid (18:2 n-6)</strong></td>
<td>23.1±2.2</td>
<td>20.1±3.1§</td>
</tr>
<tr>
<td><strong>Arachidonic acid (20:4 6)</strong></td>
<td>9.9±1.8</td>
<td>8.2±1.0§</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD. *P<0.05 versus baseline, †P<0.01 versus 4 weeks, ‡P<0.001 versus baseline, § P<0.01 versus baseline, || P<0.001 versus 4 weeks. In the fish intervention group statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons; in the control group statistical analyses were performed using Student’s t-tests.

SD - standard deviation; ANOVA - analysis of variance.
### TABLE 3.3 Daily nutritional intake in fish supplementation group

<table>
<thead>
<tr>
<th></th>
<th>Fish Intervention Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td><strong>Energy (kcal)</strong></td>
<td>2530±651</td>
</tr>
<tr>
<td><strong>Protein (g)</strong></td>
<td>91±20</td>
</tr>
<tr>
<td><strong>Carbohydrate (g)</strong></td>
<td>322±98</td>
</tr>
<tr>
<td><strong>Total fat (g)</strong></td>
<td>82±34</td>
</tr>
<tr>
<td>- Saturated fat (g)</td>
<td>28±13</td>
</tr>
<tr>
<td>- Monounsaturated fat (g)</td>
<td>24±12</td>
</tr>
<tr>
<td>- Polyunsaturated fat (g)</td>
<td>13±7</td>
</tr>
<tr>
<td><strong>Fibre (g)</strong></td>
<td>15±5</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
</tr>
<tr>
<td>- Sodium (mg)</td>
<td>3476±986</td>
</tr>
<tr>
<td>- Potassium (mg)</td>
<td>3365±1086</td>
</tr>
<tr>
<td>- Calcium (mg)</td>
<td>968±293</td>
</tr>
<tr>
<td>- Magnesium (mg)</td>
<td>353±120</td>
</tr>
<tr>
<td>- Iron (mg)</td>
<td>13±5</td>
</tr>
<tr>
<td>- Zinc (mg)</td>
<td>10±2</td>
</tr>
<tr>
<td>- Selenium (μg)</td>
<td>65±27</td>
</tr>
<tr>
<td>- Iodine (μg)</td>
<td>100±35</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>- Vitamin A (μg)</td>
<td>558±395</td>
</tr>
<tr>
<td>- Vitamin D (μg)</td>
<td>1.7±0.9</td>
</tr>
<tr>
<td>- Vitamin E (μg)</td>
<td>7±3</td>
</tr>
<tr>
<td>- Vitamin B1 (mg)</td>
<td>1.9±0.7</td>
</tr>
<tr>
<td>- Vitamin B2 (mg)</td>
<td>2.2±0.9</td>
</tr>
<tr>
<td>- Vitamin B6 (mg)</td>
<td>2.5±1.0</td>
</tr>
<tr>
<td>- Vitamin B12 (μg)</td>
<td>3.6±1.1</td>
</tr>
<tr>
<td>- Folate (μg)</td>
<td>362±166</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD. * P<0.05 versus 4 weeks, † P<0.001 versus baseline, ‡ P<0.001 versus 4 weeks, § P<0.01 versus 4 weeks. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons.

SD - standard deviation; ANOVA - analysis of variance.
(r=-0.364, P=0.018), DHA (r=-0.45, P=0.003), and total omega-3 fatty acids (r=-0.421, P=0.006).

3.4.5 EFFECTS OF FISH INTERVENTION ON SOLUBLE CD40 LIGAND AND SOLUBLE P-SELECTIN

Plasma concentrations of soluble P-selectin did not change after the dietary fish intervention (46.4±11.1 ng/mL versus 51.8±10.8 ng/mL, P=NS) or subsequent washout period (48.1±12.7 ng/mL versus 51.8±10.8 ng/mL, P=NS). There was also no change in concentrations of soluble CD40 ligand during fish intervention (1.9±2.0 g/mL after 4 weeks versus 1.9±2.0 g/mL at baseline, P=NS) or washout (1.9±2.1 g/mL at 8 weeks in fish group versus 1.9±2.0 g/mL at baseline, P=NS). In the control group there were no differences in either soluble P-selectin (42.3±8.2 ng/mL versus 44.0±10.3 ng/mL, P=NS) or soluble CD40 ligand (1.9±1.7 ng/mL versus 1.8±1.8 ng/mL, P=NS) at 4 weeks compared with baseline.

3.5 DISCUSSION

We have demonstrated, for the first time, that dietary intervention with oil-rich fish is associated with a reversible reduction in platelet-monocyte aggregation in man. There was an inverse correlation between platelet-monocyte aggregation and the plasma levels of EPA and DHA, consistent with the effects being related to the omega-3 content of oily fish. Our results suggest that reduced platelet activation could represent an important mechanism through which dietary fish confer their putative cardiovascular benefits.
Platelet activation has a central role in the pathogenesis of atherothrombosis, and the adhesion of activated platelets to monocytes has important pro-inflammatory consequences. Furthermore, platelet-monocyte aggregates promote the formation of atherosclerotic lesions in Apolipoprotein E-deficient (ApoE−/−) mice via the delivery of platelet-derived mediators to the monocyte surface and vessel wall [Huo et al 2003]. 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 [Sarma et al 2002]. Therefore, a reduction in platelet-monocyte aggregation could reflect a mechanism by which dietary fish might influence critical events in the progression of atherosclerosis and its complications.

Previous research investigating the effects of fish oils on platelet function has largely focused on platelet aggregometry and measurements of thromboxane synthesis [Knapp 1997]. These studies have produced inconsistent results, and the effects of fish oil on platelet activation have remained unclear. However, the techniques used suffer from poor reproducibility and the sample manipulation required makes them vulnerable to artefactual platelet activation. Plasma assays of platelet release products are also indirect measures of platelet activation at best. Furthermore, there is uncertainty about the relation of ex vivo platelet reactivity assessed by traditional aggregometry and actual platelet-vascular interactions in vivo. In contrast, the flow cytometric analysis of platelet-monocyte aggregates in whole blood represents a highly sensitive and physiologically relevant measure of platelet activation [Michelson et al 2001]. Modern flow cytometric techniques allow the detection of
specific activation-dependent changes in the platelet surface and as well as their interactions with other cells. They are therefore able to provide important mechanistic insights into platelet and leukocyte responses. Indeed, our results are consistent with a previous report which used flow cytometric analysis of platelet activation and demonstrated reductions in platelet surface P-selectin and glycoprotein IIb/IIIa expression after EPA supplementation [Nomura et al 2003].

Over recent years reduced platelet activation has fallen somewhat out of favour as a primary explanation for the cardiac benefits of fish oils [Kristensen et al 2001; Leaf et al 2003; Lee et al 2005], perhaps because of the inconsistent results and technical limitations in assessing platelet function. Instead, it has been suggested that an anti-arrhythmic effect may be dominant because of the reduction in sudden death observed in clinical trials [GISSI-Prevenzione trial 1999; Leaf et al 2003]. However, clear evidence of a direct anti-arrhythmic effect in humans is lacking [Cleland et al 2004; Leaf et al 2005; Raitt et al 2005; Brouwer et al 2006]. The mechanism through which fish oils confer their benefits therefore remains unclear, and our findings raise the possibility of potentially important and previously unrecognised effects on platelet and leukocyte function. Such mechanisms remain consistent with reduced sudden death, as most are triggered by coronary thrombosis after rupture of a vulnerable atherosclerotic plaque.

We did not observe any effect of dietary fish intervention on the plasma markers of platelet activation, soluble P-selectin and soluble CD40 ligand. Although one report has demonstrated a reduction in soluble P-selectin with high dose omega-3 fatty
acids [Eschen et al 2004], our results are consistent with the majority of published studies which find no effect on soluble P-selectin [Johansen et al 1999; Woodman et al 2003; Leaf et al 2005]. We found that dietary fish supplementation did not alter soluble CD40 ligand, in keeping with the only previous study to assess the effects of omega-3 fatty acids on this biomarker [Aarsetoy et al 2006]. This lack of effect may reveal the limitations of plasma assays of soluble markers of platelet activation. These are vulnerable to artefactual in vitro activation as a result of the separation procedures required, and cannot assess activation-dependent events on the cell surface. In comparison with the measurement of platelet-monocyte aggregates, these assays may not have the sensitivity required to detect the potentially subtle effects expected in dietary intervention studies. However, we cannot fully exclude a type 2 error as the study was powered to assess differences in platelet-monocyte aggregates and may have been underpowered to detect differences in circulating markers of platelet activation.

Previous studies report that anti-platelet effects of fish oils have only been apparent at very high doses, with little evidence for a major antithrombotic effect with more practical intakes [Kristensen et al 2001]. However, the reduction in platelet-monocyte aggregation in the present study was achieved with moderate amounts of fish, similar to those shown to reduce mortality in secondary prevention trials [Burr et al 1989; GISSI-Prevenzione trial 1999]. The cardiovascular effects of oil-rich fish are believed to be primarily due to the high levels of omega-3 fatty acids present, and the inverse correlations demonstrated between plasma EPA and DHA and platelet-monocyte aggregation are in keeping with this hypothesis. However, this does not
prove causation and it is possible that accompanying nutritional changes may have contributed to the observed effects. There are other nutrients in fish such as vitamin D which may potentially affect platelet activation or function. Currently, there are no studies looking at the effect of vitamin D on platelet-monocyte aggregation and further research would be required to explore this. Although we found a clear reduction in platelet-monocyte aggregation after 4 weeks of fish consumption, our study was limited to three time points. It is not clear whether the effect on platelet-monocyte aggregation might have become apparent at an earlier stage, and further studies with analyses at weekly time points would be of considerable interest.

It should be noted that while the fish intake in this study was calculated to provide similar amounts of omega-3 fatty acids to those shown to reduce mortality, this is an approximation as oil content can vary markedly with species, season, diet, and packaging and cooking methods. However, the marked increase in plasma phospholipid concentrations of omega 3 fatty acids during fish intervention demonstrates a clear increase in omega 3 levels. The participants in the fish intervention and control groups were different and therefore the baseline platelet-monocyte aggregate levels differed. This is the reason we presented the data as mean values in each group, rather than difference from before and after treatment.

3.5.1 CONCLUSION
We have demonstrated that dietary intervention with moderate amounts of oil-rich fish can reduce platelet-monocyte aggregation in man. Platelet-monocyte aggregates
are sensitive markers of platelet activation and contribute to the initiation and progression of atherosclerosis. This could represent a previously unreported mechanism through which fish oils might confer their potential cardiovascular benefits. Most previous studies assessing the effects of fish oils on platelet function have used older techniques with limited reproducibility and physiological relevance. We suggest a renewed research focus into the actions of fish oils on inflammation and thrombosis using modern research tools to investigate interactions between platelets, leukocytes and the vasculature. Establishing the mechanism through which fish and fish oils confer their potential preventative benefits may help guide the application and development of future therapeutic interventions.
CHAPTER 4

EFFECT OF MODERATE WALNUT CONSUMPTION ON
LIPID PROFILE, ARTERIAL STIFFNESS, AND
PLATELET ACTIVATION IN HUMANS

Din JN, Aftab SM, Jubb AW, Carnegy FH, Lyall K, Sarma J, Newby DE, Flapan AD.
Effect of moderate walnut consumption on lipid profile, arterial stiffness and platelet activation in humans.
A large intake of walnuts may improve lipid profile and endothelial function. The effect of moderate walnut consumption is not known. We investigated whether a moderate intake of walnuts would affect lipid profile, arterial stiffness and platelet activation in healthy volunteers. Thirty healthy males were recruited into a single-blind, randomised controlled crossover trial of 4 weeks dietary walnut supplementation (15 g/day) and 4 weeks control (no walnuts). Arterial stiffness was assessed using pulse waveform analysis to determine the augmentation index and augmented pressure. Platelet activation was determined using flow cytometry to measure circulating platelet-monocyte aggregates. There were no differences in lipid profile after 4 weeks of walnut supplementation compared with control. Dietary intake of ALA was increased during the walnut diet (2.1±0.4 g/day versus 0.7±0.4 g/day, P<0.0001). There were no differences in augmentation index or augmented pressure during walnut supplementation. Walnut supplementation did not affect platelet-monocyte aggregation. Dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in man. Our results suggest that the potentially beneficial cardiac effects of walnuts may not be apparent at lower and more practical levels of consumption.
4.2 INTRODUCTION

Frequent consumption of nuts may be protective against coronary heart disease [Hu and Stampfer 1999]. Nuts are rich in unsaturated fatty acids and contain potentially beneficial compounds including antioxidants, L-arginine, fibre, and folic acid [Kris-Etherton et al 1999]. Walnuts are unique because they also contain high levels of ALA, a plant-derived omega-3 fatty acid that may confer additional cardiac protection [de Lorgeril and Salen 2004]. Dietary intervention studies have demonstrated that a large intake of walnuts can reduce LDL cholesterol [Banel and Hu 2009]. In addition, walnuts may improve endothelial function in patients with hyperlipidaemia or diabetes [Ros et al 2004; Ma et al 2010]. However, the precise mechanisms through which walnuts confer their putative cardiac benefits remains uncertain.

The endothelium plays a critical role in the regulation of vascular smooth muscle tone, thrombosis and inflammation. Vascular smooth muscle tone is an important determinant of central arterial pressure and arterial stiffness. Large artery stiffness, wave reflections and central pulse pressure are inversely associated with endothelial function and can be measured non-invasively using pulse waveform analysis [McEniery et al 2006]. The endothelium also regulates local platelet activation and aggregation through the release of paracrine factors, such as nitric oxide and prostacyclin. Platelet-leukocyte aggregates are highly sensitive markers of platelet activation that are closely related to impaired endothelial vasomotor function [Robinson et al 2006]. They contribute to the development of atherothrombosis and
are elevated in coronary heart disease and acute coronary syndromes [Sarma et al 2002].

Marine-derived long chain omega-3 fatty acids reduce arterial compliance and platelet activation [Din et al 2004; Din et al 2008]. We therefore hypothesised that dietary intervention with walnuts, rich in plant-derived omega-3 fatty acids, would reduce arterial stiffness and platelet activation in humans. Previous intervention studies have asked participants to consume large amounts of walnuts (30-108 g or approximately 8-27 whole walnuts daily) providing up to 50% of the total dietary fat intake. This proportion of fat energy from walnuts may be too high to be considered practical, and no previous trials have assessed the effect of a more realistic intake of walnuts. In the present study we examined the effect of a moderate intake of walnuts on lipid profile, arterial stiffness and platelet activation.

4.3 SUBJECTS AND METHODS

4.3.1 STUDY PARTICIPANTS AND DESIGN

Thirty healthy male volunteers were enrolled into the study. Exclusion criteria included those taking regular medication, those with clinical evidence of atherosclerotic vascular disease, hypertension, diabetes mellitus, hypercholesterolaemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency. Ethical approval was obtained from the lothian Research Ethics Committee and all subjects provided
written informed consent. This study took place between August 2003 and February 2004.

Participants were randomised to 4 weeks of walnut supplementation (15 g/day) or 4 weeks of control (no walnuts) in a single-blind randomised crossover trial. The randomization schedule was created and held by a co-researcher with no involvement in this study. All researchers performing data collection in this study were blinded to treatment allocation. The walnut intake was calculated to provide levels of ALA similar to the current American Heart Association recommendations and trials showing a reduction in cardiac events with diets rich in ALA [de Lorgeril et al 1994; Kris-Etherton et al 2002]. Lipid profile, platelet-leukocyte aggregation, and arterial stiffness were assessed at the end of each 4-week period. Investigators were blinded as to whether participants were in the walnut intervention or control period.

4.3.2 DIETARY INTERVENTION

Participants were provided with walnuts at the beginning of the dietary intervention phase and asked to consume 15 g of walnuts daily in addition to their habitual diets. No other specific dietary advice was provided and there were no restrictions on calorie or fat intake. For the control phase of the study, participants were simply asked to continue with their usual diets. Similar to previous studies, we did not include a washout period between diets as diet-induced lipoprotein changes stabilise in less than 4 weeks [Kris-Etherton and Dietschy 1997]. Participants completed a 3-day weighed food diary to assess intake of omega-3 fatty acids during each phase of the study.
4.3.3 BLOOD COLLECTION PROTOCOL

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with the direct thrombin inhibitor PPACK. Whole blood was then immunolabelled within 5 minutes of phlebotomy for subsequent flow cytometric analysis of platelet-monocyte aggregates.

4.3.4 FLOW CYTOMETRY

The following reagents were used: FITC-conjugated CD42a (GRP-P, IgG1), and isotype control IgG1 were obtained from Serotec Ltd, PE-conjugated CD14 (Tuk-4, IgG2a) was obtained from Dako Cytomation and FACS-Lyse was obtained from Becton Dickinson. The methods are as previously described [Harding et al 2007]. Briefly, aliquots of whole blood were incubated with anti-CD14, anti-CD42a and isotype-matched controls. Thereafter, samples were fixed and the red cells lysed. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser. Samples were initially analysed with the flow cytometer triggered on forward scatter and then by triggering on FL-2 to identify CD14 positive monocytes. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. Neutrophils were identified and gated based on their scatter properties only. Platelet-neutrophil aggregates were defined as neutrophils positive for CD42a. Whilst we measured platelet-monocyte and platelet neutrophil aggregates, our primary measure was platelet-monocyte aggregates as this has been demonstrated to be a more sensitive marker of platelet activation [Michelson et al; 2001]. All results
are expressed as percentage of positive cells. Analyses were performed using EXPO32 software.

4.3.5 **Pulse waveform analysis**

Arterial stiffness was measured non-invasively with the SphygmoCor system (AtCor Medical Unit 11, West Ryde Corporate Centre, 1059-1063 Victoria Rd, West Ryde, NSW 2114, Australia). Measurements were taken in a quiet, temperature controlled room after subjects had been in the recumbent position for a 15-minute rest period. Peripheral pressure waveforms were obtained using applanation tonometry of the radial artery with a pressure sensitive micromanometer (Millar Instruments Inc, 6001 Gulf Freeway Houston, TX 77023, USA). A generalised transfer function was used to derive the corresponding aortic pressure waveform. The first systolic peak of this waveform is a result of left ventricular ejection and the second systolic peak is caused by wave reflection from the periphery. The augmented pressure was defined as the difference between the first and second systolic peaks. The augmentation index was the augmented pressure expressed as a percentage of the pulse pressure. Peripheral blood pressure measurements were performed using an automated upper arm blood pressure monitor (Omron 705IT, Omron Healthcare, Opal Drive, Fox Milne Milton Keynes, MK 15 0DG, UK).
4.3.6 STATISTICAL METHODS

Continuous variables are reported as mean±SD. Statistical analyses were performed using two-tailed Student's *t*-tests. All calculations were performed using GraphPad Prism. Statistical significance was taken at 5%.

4.4 RESULTS

4.4.1 BASELINE CHARACTERISTICS

Study participants were young (23±3 years) and had a normal body mass index (24.5±2.3 kg/m²). Systolic blood pressure (117±9 mmHg), diastolic blood pressure (60±7 mmHg), total cholesterol (4.57±1.06 mmol/L), LDL cholesterol (2.70±0.92 mmol/L), HDL cholesterol (1.32±0.24 mmol/L) and triacylglycerol concentrations (1.22±0.58 mmol/L) were within normal limits. Two subjects were cigarette smokers and the average alcohol intake was within recommended limits (15±10 units alcohol per week). In terms of ethnic origin, 22 participants were North European Caucasians, 5 were Indian Asians and 3 were of Far East Asian origin.

4.4.2 EFFECT OF WALNUT SUPPLEMENTATION ON LIPID PROFILE AND INTAKE OF OMEGA-3 FATTY ACIDS

There were no differences in lipid profile after 4 weeks of walnut supplementation compared with 4 weeks of control diet (Table 4.1). Dietary intake of ALA was markedly increased during the walnut diet (2.1±0.4 g/day *versus* 0.7±0.4 g/day; Figure 4.1). There were no changes in consumption of the marine-derived omega-3 fatty acids EPA (0.2±0.2 g/day *versus* 0.2±0.3 g/day) or DHA (0.2±0.3 g/day *versus*...
0.3±0.3 g/day) during walnut supplementation compared with control (Figure 4.1). There was a small increase in the percentage of plasma phospholipid ALA during the walnut intervention, but this did not reach statistical significance (0.27±0.1% baseline versus 0.29±0.1% after walnut intervention versus 0.26±0.1% after control; P=0.4).

4.4.3 Effect of walnut supplementation on measures of arterial stiffness

Dietary intervention with walnuts did not affect heart rate, peripheral blood pressure, or central aortic pressures compared with control (Table 4.2). There were also no differences observed in augmentation index (-6.6±6.5% versus -8.4±6.3%; Figure 4.2), augmented pressure (-2.2±2.1 mmHg versus -2.7±2.4 mmHg; Figure 4.2), or time to wave reflection (171±20 ms versus 176±18 ms) during walnut supplementation.
TABLE 4.1  Effect of walnut supplementation on serum lipid profile

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Walnuts</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td>4.37±1.01</td>
<td>4.25±0.99</td>
<td>0.11</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mmol/L)</strong></td>
<td>2.46±0.85</td>
<td>2.30±0.84</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mmol/L)</strong></td>
<td>1.24±0.19</td>
<td>1.24±0.26</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>Chol:HDL chol ratio</strong></td>
<td>3.47±0.85</td>
<td>3.39±0.85</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/L)</strong></td>
<td>1.45±0.74</td>
<td>1.55±0.93</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Data are reported as mean±SD.
LDL - low-density lipoprotein; HDL - high-density lipoprotein; Chol - cholesterol; SD - standard deviation.
Figure 4.1 Dietary intake of omega-3 fatty acids during intervention with walnuts and control period. EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; ALA - \( \alpha \)-linolenic acid. Data are reported as mean±SD. SD – standard deviation.
### TABLE 4.2  Haemodynamic effects of walnut supplementation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Walnuts</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate</strong> (beats per minute)</td>
<td>64±8</td>
<td>68±11</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Peripheral systolic blood pressure</strong> (mmHg)</td>
<td>117±8</td>
<td>120±10</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Peripheral diastolic blood pressure</strong> (mmHg)</td>
<td>62±5</td>
<td>63±8</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Peripheral pulse pressure</strong> (mmHg)</td>
<td>54±8</td>
<td>57±9</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Mean arterial pressure</strong> (mmHg)</td>
<td>77±6</td>
<td>79±8</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Central aortic systolic pressure</strong> (mmHg)</td>
<td>96±7</td>
<td>98±8</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Central aortic diastolic pressure</strong> (mmHg)</td>
<td>63±5</td>
<td>64±8</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Data are reported as mean±SEM.
SEM - standard error of the mean.
Figure 4.2 Effect of dietary intervention with walnuts on augmentation index (A) and augmented pressure (B). Median (horizontal line), interquartile range (box) and 95% confidence intervals (bars).
4.4.4 Effect of walnut supplementation on platelet-leukocyte aggregation

Walnut supplementation did not affect platelet-monocyte aggregation (18.5±7.2% versus 19.5±7.2%; Figure 4.3) or platelet-neutrophil aggregation (5.2±1.8% versus 5.5±2.2%; Figure 4.3).

4.5 DISCUSSION

We have demonstrated that dietary supplementation with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy subjects. This is in contrast with previous studies which have shown that heavy consumption of walnuts as part of a low-fat or ‘Mediterranean’ diet can reduce LDL cholesterol and improve endothelial function. The present study is the first dietary intervention trial to assess the efficacy of moderate rather than large walnut consumption on markers of cardiovascular risk.

Data on the effects of walnut consumption on lipid profile have been inconsistent. Whilst several randomised trials have found a reduction in total or LDL cholesterol with walnuts [Sabate et al 1993; Zambon et al 2000; Iwamoto et al 2002; Ros et al 2004; Tapsell et al 2004; Torabian et al 2010], others have shown no effect [Chisholm et al 1998; Morgan et al 2002; Mukuddem-Petersen et al 2007; Spaccarotella et al 2008; Tapsell et al 2009]. Overall, a meta-analysis of 11 trials found that high walnut-enriched diets reduced total and LDL cholesterol by 4.9% and 6.7%, respectively [Banel and Hu 2009].
Platelet-monocyte aggregates

Platelet-neutrophil aggregates

$P = 0.4$

$P = 0.5$

Control  Walnut  Control  Walnut

**Fig 4.3** Effect of dietary intervention with walnuts on platelet-monocyte and platelet-leukocyte aggregates. Data are reported as mean±SD. SD – standard deviation.
The most likely explanation for the lack of effect on lipid parameters in our study is the lower amount of walnuts consumed (15 g daily) compared to previous trials. Studies demonstrating reductions (6-16%) in serum LDL cholesterol concentrations with walnuts have required participants to consume between 40-84 grams of walnuts per day [Sabate et al 1993; Zambon et al 2000; Iwamoto et al 2002; Ros et al 2004]. Furthermore, in these studies walnuts isocalorically replaced other fat containing foods as part of low fat, low cholesterol or ‘Mediterranean’ style diets, whilst our study participants were free-living. It is not possible to know whether the beneficial lipid effects in previous studies were specifically due to walnut intake or the replacement of other sources of dietary fat.

Most previous trials have investigated patients with hyperlipidaemia, and the relatively low baseline cholesterol in our study population may have contributed to the neutral lipid results. A recent trial found that lipid-lowering with walnut supplementation was only evident in patients with higher cholesterol levels; there was no benefit in those with baseline cholesterol concentrations <4.94 mmol/L [Torabian et al 2010]. The only two trials in normolipidaemic patients where walnuts reduced cholesterol levels required a large intake of >50 grams per day [Sabate et al 1993; Iwamoto et al 2002]. It is possible that supplementation with a smaller amount of walnuts is not sufficient to alter lipid parameters in a young, low-risk, normolipidaemic population.

The present study is the first trial to assess the effect of walnut consumption on arterial stiffness and platelet activation. We have previously demonstrated that
dietary intervention with fish rich in marine-derived omega-3 fatty acids reduces platelet-monocyte aggregates, a highly sensitive marker of platelet activation [Din et al 2008]. However, supplementation with walnuts rich in plant-derived omega-3 fatty acids did not reduce platelet-monocyte aggregates, and there was also no change in the augmentation index or augmented pressure. Although we did not study and cannot exclude an effect with heavy walnut intake, our results indicate that any potential cardiac benefits of moderate walnut consumption are unlikely to be mediated through effects on platelet activation or arterial stiffness.

The large amount of walnuts consumed in many of the previous dietary intervention trials provided up to 20% of total energy and 55% of daily total fat intake. There have been concerns that this proportion of fat energy from walnuts may be too high to be practical or sustainable in a non-research setting [Feldman 2002; Banel and Hu 2009]. In 2004, the Food and Drug Administration issued a qualified health claim for walnuts in response to a petition from the California Walnut Commission [Food and Drug Administration 2004. Center for Food Safety and Applied Nutrition, Maryland, USA]. Their report recognised that the clinical studies used high daily walnut intakes and that there were no data from which to extrapolate beneficial effects to lower amounts. The present study provides some data to address these concerns, and suggests that a smaller and more practical intake of walnuts may not be sufficient to improve lipid parameters or cardiovascular risk markers.

A potential limitation in our trial is the lack of a run-in phase before randomisation, or washout period between cross-over. All eligible subjects were randomised and an
intention-to-treat analysis performed in order to maximise the applicability and external validity of the study. Similar to previous studies, we did not include a washout period between diets as diet-induced lipoprotein changes stabilise in less than 4 weeks [Kris-Etherton and Dietschy 1997]. However, whilst there were no differences between the diets at the end of each 4 week period, both the walnut intervention and control diets reduced cholesterol concentrations and platelet-leukocyte aggregates compared with baseline. These changes may reflect a modification in participants’ dietary habits after randomisation, independent of their dietary assignment, and could have reduced our ability to detect subtle differences between diets. We also cannot fully exclude carry-over effects from the first study period into the second.

A further limitation is the lack of an increase in plasma phospholipid concentrations of ALA during walnut intervention. This could reflect either low ALA content in our walnuts, a walnut dose too small to have biologic effects, or lack of adherence to the dietary intervention by study participants. Therefore, it remains possible that a dietary intervention which significantly increased plasma ALA could have modified our outcome measures. Further studies using either larger walnut intakes or richer sources of ALA are required to clarify this issue.

4.5.1 CONCLUSION
We have demonstrated that dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy male volunteers. This is in contrast to previous studies in which large amounts of walnuts,
as part of isocaloric low fat diets, were found to reduce serum LDL cholesterol concentrations and improve endothelial function. Our results suggest that the potentially beneficial effects of walnuts on lipid parameters and cardiovascular biomarkers may not be apparent at lower and more practical levels of consumption. Further studies are necessary to determine whether larger amounts of walnuts might affect arterial stiffness or platelet activation. Longer trials are required to establish if the higher levels of walnut consumption associated with improved lipid profiles or endothelial function can be sustained over time and reduce clinical end points.
CHAPTER 5

EFFECT OF OMEGA-3 FATTY ACID SUPPLEMENTATION ON ENDOTHELIAL FUNCTION, ENDOGENOUS FIBRINOLYSIS AND PLATELET ACTIVATION IN CIGARETTE SMOKERS

5.1 SUMMARY

The mechanisms through which omega-3 fatty acids reduce adverse cardiac events remain uncertain. Previous studies investigating effects on endothelial function, fibrinolysis and platelet function have been inconsistent. We investigated the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in healthy cigarette smokers; a group at increased risk of myocardial infarction. Twenty cigarette smokers were recruited into a randomised, double-blind, placebo-controlled, crossover trial of omega-3 fatty acid supplementation (2 g/day for 6 weeks). Peripheral blood was taken for analysis of platelet and monocyte activation, and forearm blood flow (FBF) was assessed in a subset of 12 smokers during intrabrachial infusions of acetylcholine, substance P and sodium nitroprusside. Stimulated plasma tissue plasminogen activator (t-PA) concentrations were measured during substance P infusion. All vasodilators caused dose-dependent increases in FBF (P<0.0001). Compared to placebo, omega-3 fatty acid supplementation led to greater endothelium-dependent vasodilatation with acetylcholine and substance P (P=0.0032 and P=0.056). Substance P caused a dose-dependent increase in plasma t-PA concentrations (P<0.0001) that was greater after omega-3 fatty acid supplementation (P=0.029). Omega-3 fatty acids did not affect platelet-monocyte aggregation, platelet P-selectin or CD40L, or monocyte CD40. We have demonstrated for the first time that omega-3 fatty acids augment acute endothelial t-PA release and improve endothelial vasomotor function in cigarette smokers; a population at high-risk of cardiovascular events. These findings suggest
that improved endogenous fibrinolysis and endothelial function may represent important mechanisms through which omega-3 fatty acids confer their potential cardiovascular benefits.

5.2 INTRODUCTION

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease [Din et al 2004]. Whilst omega-3 fatty acids have been associated with several biological actions, the actual mechanisms through which they confer any cardiac benefits are uncertain. In recent years, an effect on ventricular arrhythmias has been thought to be central due to the reduction in sudden death observed in clinical trials [Marchioli et al 2002; Leaf et al 2003]. However, subsequent studies have failed to clearly demonstrate a direct anti-arrhythmic effect [Brouwer et al 2009]. 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 [Bowker et al 2003].

The endothelium controls vascular tone and blood flow, but also regulates thrombosis through the production of factors that influence fibrinolysis and platelet activation [Widlansky et al 2003]. The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi and is regulated by the profibrinolytic factor, t-PA, and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1) [Oliver et al 2005]. The rapid release of t-PA from the endothelium is critical, with thrombus
dissolution being more effective if t-PA is incorporated early during thrombus formation [Fox et al 1985].

Endothelial cells also contribute to thrombosis through the release of paracrine factors which mediate platelet function, and there is an inverse relationship between endothelial vasomotor function and platelet activation [Robinson et al 2006]. Activated platelets can bind to leukocytes via a P-selectin-dependent mechanism to form platelet-leukocyte aggregates [Jungi et al 1986]. Interactions between platelets, leukocytes and endothelial cells can also be modulated by the CD40 receptor and its ligand which are co-expressed by most cell types, including activated platelets and monocytes [Schonbeck et al 2001]. Formation of platelet-leukocyte aggregates or ligation of CD40 can mediate an array of pro-inflammatory and prothrombotic effects [Neumann et al 1997; Schonbeck et al 2001], promoting leukocyte adhesion to activated endothelial cells and thereby contributing to endothelial injury and atherothrombosis [Huo et al 2003].

Although dietary supplementation with omega-3 fatty acids has been found to improve endothelial function in several patient groups [Tagawa et al 1999; Goodfellow et al 2000; Mori et al 2000; Khan et al 2003; Morgan et al 2006], not all studies have demonstrated a beneficial effect [Woodman et al 2003; Skulas-Ray et al 2011]. The effects of omega-3 fatty acids on fibrinolysis and platelet activation have been inconsistent with little evidence supporting a major antithrombotic action [Kristensen et al 2001]. However, previous studies investigating fibrinolysis have measured basal plasma t-PA concentrations, which do not reflect the local capacity for acute endothelial t-PA release [Hrafinkelsdottir et al 2004]. The assessment of
acute endothelial t-PA release is of greater pathophysiological relevance, and indeed predicts the future risk of cardiovascular events [Robinson et al 2007]. Whilst we have previously demonstrated that dietary intervention with oil-rich fish reduces platelet-monocyte aggregation [Din et al 2008], the effect of omega-3 fatty acid supplements on platelet-monocyte aggregation or the CD40/CD40 ligand system has not been studied.

Cigarette smokers, a group at considerably increased risk of acute myocardial infarction [Teo et al 2006], display a marked attenuation of acute t-PA release in the peripheral and coronary circulation [Newby et al 1999; Newby et al 2001; Pretorius et al 2002]. They also demonstrate increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system [Harding et al 2004]. We therefore aimed to investigate the effect of omega-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation in healthy cigarette smokers.

5.3 METHODS

5.3.1 STUDY PARTICIPANTS
Twenty healthy cigarette smokers (5-25 cigarettes/day) aged between 20 and 45 years participated in the study. 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, asthma,
hypertension, on prescribed medication, diabetes, hyperlipidaemia, renal or hepatic failure, vascular disease or any intercurrent illness likely to be associated with an inflammatory response.

5.3.2 STUDY DESIGN
This was a prospective, double-blind, placebo-controlled, randomised crossover trial. Subjects were randomised to receive either omega-3 fatty acid supplements (2 g/day Omacor capsules, Pronova Biopharma, Vollsveien 6, 1366 Lysaker, Norway) or matching placebo capsules (2 g/day Olive oil capsules, Eurocaps Limited, Unit B-D, Crown Business Park, Tredegar, Gwent NP22 4EF) 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% EPA and 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. A subset of 12 participants also underwent measurement of FBF and endogenous fibrinolysis at the end of each treatment period.

5.3.3 BLOOD COLLECTION PROTOCOL
Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with EDTA (1.6 mg/mL) and the direct thrombin inhibitor PPACK. Whole blood anticoagulated with PPACK was immunolabelled within 5 minutes 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 -70°C until analysis.

5.3.4 FLOW CYTOMETRY

The following reagents were used: FITC-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), PE-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls were obtained from Serotec Ltd. Phycoerythrin-conjugated CD154 (TRAP1, IgG1), PE-conjugated CD14 (Tuk-4, IgG2a), PE-conjugated CD 62P (IE3, IgG2a), and their appropriate isotype controls was obtained from Dako Cytomation. FACS-Lyse was obtained from Becton Dickinson. Aliquots of whole blood (60 µL) anticoagulated with PPACK were incubated with appropriate antibodies and their isotype-matched controls for 20 minutes at room temperature. To evaluate platelet-monocyte aggregates and CD40 on monocytes, samples were fixed and the 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 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 EXPO32 software.
5.3.5 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% butyrate hydroxytoluene as an antioxidant (Folch extraction). Phospholipids were isolated by solid-phase extraction using aminopropyl silica columns and fatty acids converted into methyl esters by transmethylation with 0.5M sodium methoxide. Fatty acid methyl ester analysis was performed with an HP-INNOWAX capillary column. 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%.

5.3.6 Vascular studies

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

All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle (Coopers Needle Works Limited, 261-265 Aston Lane, Birmingham, West Midlands B20 3HS, UK) under controlled conditions. After a 30-minute baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-
dependent vasodilator that does not release t-PA; Merck Biosciences, Boulevard Industrial Park, Padge Road, Beeston, Nottingham NG9 2JR, UK; Substance P at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator that releases t-PA; Clinalfa, Bachem AG, Hauptstrasse 144, 4416 Bubendorf, Switzerland); and sodium nitroprusside at 2, 4, and 8 μg/min (endothelium-independent vasodilator that does not release t-PA; David Bull Laboratories, Spartan Close, Tachbrook Park, Warwick CV34 6RS, UK) were infused for 6 minutes at each dose. The three vasodilators were separated by 20-minute saline infusions and given in a randomised order.

Forearm blood flow was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges as described previously [Newby et al 1997]. Venous cannulae (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, 6025 Nicolle St, Ventura, California CA 93003, USA) for t-PA assays and into citrate (BD Vacutainer, Becton Dickinson) for PAI-1 assays. Samples were kept on ice before being centrifuged at 2000 g for 30 minutes 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, Brunner Str. 67 1230, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, 155 Rue d'Eragny, 95000 Neuville-Sur-Oise, France) concentrations were determined by enzyme-linked immunosorbent assays. Haematocrit was determined by capillary tube centrifugation at baseline.
5.3.7 DATA ANALYSIS AND STATISTICAL METHODS

Continuous variables are reported as mean±SEM. Forearm plethysmographic data were analysed as described previously [Newby et al 1997]. Estimated net release of plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused FBF) and the concentration difference between the infused and non-infused arms [Newby et al 1997]. Statistical analyses were performed using one-way and two-way ANOVA with Bonferroni’s post-tests for multiple comparisons where appropriate. All calculations were performed using GraphPad Prism. Statistical significance was taken at 5%.

5.4 RESULTS

5.4.1 BASELINE CHARACTERISTICS

Participants were young with normal blood pressure, fasting glucose and lipid profiles (Table 5.1). Smokers had a mean cigarette consumption of 15±5 cigarettes per day over a mean period of 9±2 years (8±2 pack years).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>135 ± 4</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>81 ± 2</td>
</tr>
<tr>
<td>Heart rate (beats per minute)</td>
<td>67 ± 2</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Chol:HDL chol ratio</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>4.6 ± 0.1</td>
</tr>
<tr>
<td>Cigarettes per day</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>Total pack years</td>
<td>8 ± 2</td>
</tr>
</tbody>
</table>

Data are reported as Mean ± SEM.

LDL - low-density lipoprotein; HDL - high-density lipoprotein; Chol - cholesterol; SEM - standard error of the mean.
5.4.2 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 (2.7±0.3% versus 1.2±0.1%, P<0.0001) and placebo (2.7±0.3% versus 1.2±0.1%, P<0.0001; Figure 5.1A). There was also an increase in DHA compared with baseline (4.3±0.2% versus 3.2±0.3%, P<0.0001) and placebo (4.3±1.0% versus 3.0±0.2%, P<0.0001; Figure 5.1B). As the proportion of EPA and DHA increased with omega-3 fatty acid supplementation, there was a corresponding reduction in the percentage of arachadonic acid compared with baseline (8.7±0.3% versus 9.9±0.4%, P<0.05) and placebo (8.7±1.4% versus 9.6±0.3%, P<0.05; Table 5.2). There was no effect on ALA, linoleic acid, palmitic acid, stearic acid or oleic acid with either omega-3 fatty acid supplements or olive oil placebo (Table 5.2).

5.4.3 Effect of omega-3 fatty acid supplementation on lipid profile

Supplementation for 6 weeks with omega-3 fatty acids did not affect total cholesterol, LDL cholesterol, HDL cholesterol or triglycerides (Table 5.3).

5.4.4 Effect of omega-3 fatty acid supplementation on vasomotor function

Omega-3 fatty acid supplementation did not have any effect on systolic blood pressure (132±3 mmHg versus 128±3, P=0.2), diastolic blood pressure (78±2 mmHg versus 76±2 mmHg, P=0.06) or heart rate (67±4 beats per minute versus 70±2 beats per minute, P=0.6) compared with placebo. During forearm vascular studies
Figure 5.1. 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; ANOVA - analysis of variance.
TABLE 5.2 Effect of omega-3 fatty acid supplementation on plasma phospholipid fatty acid composition

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Omega-3</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-linolenic acid</td>
<td>0.3±0.02</td>
<td>0.3±0.02</td>
<td>0.3±0.03</td>
<td>0.8</td>
</tr>
<tr>
<td>Arachadonic acid</td>
<td>9.9±0.4</td>
<td>8.7±0.3</td>
<td>9.6±0.3</td>
<td>0.002</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>24.5±0.7</td>
<td>23.2±0.7</td>
<td>24.2±0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>28.3±0.3</td>
<td>28.6±0.4</td>
<td>28.6±0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>13.0±0.3</td>
<td>13.0±0.3</td>
<td>13.0±0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>13.3±0.5</td>
<td>13.0±0.5</td>
<td>13.5±0.5</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Mean±SEM. Data analysed using one-way ANOVA. SEM - standard error of the mean; ANOVA - analysis of variance.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Omega-3</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate</strong> (beats per minute)</td>
<td>67±2</td>
<td>67±4</td>
<td>70±2</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Systolic blood pressure</strong> (mmHg)</td>
<td>134±5</td>
<td>132±3</td>
<td>128±3</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure</strong> (mmHg)</td>
<td>81±2</td>
<td>78±2</td>
<td>76±2</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Total cholesterol</strong> (mmol/L)</td>
<td>4.4±0.2</td>
<td>4.7±0.3</td>
<td>4.7±0.3</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>LDL cholesterol</strong> (mmol/L)</td>
<td>2.6±0.2</td>
<td>2.6±0.2</td>
<td>2.7±0.3</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>HDL cholesterol</strong> (mmol/L)</td>
<td>1.2±0.1</td>
<td>1.3±0.1</td>
<td>1.3±0.1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Chol:HDL chol ratio</strong></td>
<td>3.8±0.3</td>
<td>3.7±0.3</td>
<td>4.0±0.4</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Triacylglycerol</strong> (mmol/L)</td>
<td>1.3±0.2</td>
<td>1.3±0.2</td>
<td>1.5±0.2</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Mean±SEM. Data analysed using one-way ANOVA.
LDL - low-density lipoprotein; HDL - high-density lipoprotein; Chol - cholesterol; SEM - standard error of the mean; ANOVA - analysis of variance.
substance P, acetylcholine, and sodium nitroprusside led to a dose-dependent increase in absolute FBF (P<0.0001 for all). Omega-3 fatty acid supplementation led to increased endothelium-dependent vasodilatation with acetylcholine and substance P compared with placebo (P=0.0032 and P=0.056; Figure 5.2). There was no effect on endothelium-independent vasodilatation with sodium nitroprusside (P=0.77; Figure 5.2).

5.4.5 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 and antigen concentrations after both omega-3 fatty acid supplementation and placebo (P<0.0001; Table 5.4). The increase in plasma t-PA activity was greater after omega-3 fatty acid supplementation compared with placebo (P=0.0290; Table 5.4). Net release of t-PA activity was also greater after omega-3 fatty acid supplementation compared with placebo (P=0.0294; Figure 5.3). Omega-3 fatty acids did not affect PAI-1 concentrations (Table 5.4).

5.4.6 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 5.5).
Figure 5.2 Effect of omega-3 fatty acids on absolute forearm blood flow in response to endothelium-dependent and endothelium-independent vasodilators. Statistical analysis two-way ANOVA and Bonferroni’s post-tests for multiple comparisons. NS – not significant; ANOVA – analysis of variance.
TABLE 5.4 Effect of omega-3 fatty acid supplementation on plasma t-PA activity concentrations

<table>
<thead>
<tr>
<th>Substance P pmol/min</th>
<th>Omega-3 fatty acids</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td><strong>t-PA activity (mL⁻¹)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>0.39±0.07</td>
<td>0.47±0.07</td>
</tr>
<tr>
<td>Infused arm</td>
<td>0.45±0.06</td>
<td>0.85±0.12</td>
</tr>
<tr>
<td><strong>t-PA antigen (ng mL⁻¹)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>6.44±0.60</td>
<td>6.38±0.58</td>
</tr>
<tr>
<td>Infused arm</td>
<td>6.88±0.43</td>
<td>6.79±0.41</td>
</tr>
<tr>
<td><strong>PAI-1 activity (ng mL⁻¹)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.61±0.41</td>
<td>1.56±0.39</td>
</tr>
<tr>
<td>Infused arm</td>
<td>1.59±0.40</td>
<td>1.38±0.35</td>
</tr>
<tr>
<td><strong>PAI-1 antigen (ng mL⁻¹)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>29.57±4.59</td>
<td>28.85±3.95</td>
</tr>
<tr>
<td>Infused arm</td>
<td>29.79±3.81</td>
<td>28.30±3.39</td>
</tr>
<tr>
<td><strong>Net t-PA antigen release</strong></td>
<td>(ng 100 mL⁻¹) of tissue (mm⁻¹)</td>
<td>1.00±0.90</td>
</tr>
</tbody>
</table>

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

* t-PA activity: Dose response P=0.0001, Omega-3 fatty acids versus placebo; P=0.029 (infused arm).
* t-PA antigen: Dose response P=0.0001, Omega-3 fatty acids versus placebo; P=0.44 (infused arm).
* PAI-1 activity: Dose response P=0.99, Omega-3 fatty acids versus placebo; P=0.063 (infused arm).
* PAI-1 antigen: Dose response P=0.90, Omega-3 fatty acids versus placebo; P=0.35 (infused arm).
* Net t-PA antigen: Dose response P=0.001, Omega-3 fatty acids versus placebo; P=0.15 (infused arm).
* t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type 1; SEM - standard error of the mean; ANOVA - analysis of variance.
Figure 5.3 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. t-PA – tissue plasminogen activator; ANOVA – analysis of variance.
### TABLE 5.5 Effect of omega 3-fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand system

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Omega-3</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-monocyte aggregates (%)</td>
<td>19.5±2.0</td>
<td>18.8±2.0</td>
<td>15.9±1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Platelet-neutrophil aggregates (%)</td>
<td>8.1±2.1</td>
<td>4.9±0.7</td>
<td>4.7±0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Platelet-surface expression of P-selectin (%)</td>
<td>4.5±0.9</td>
<td>3.7±0.4</td>
<td>3.8±0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Platelet-surface expression of CD40L (%)</td>
<td>3.1±0.4</td>
<td>2.7±0.3</td>
<td>2.7±0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Monocyte expression of CD40 (%)</td>
<td>44.3±1.9</td>
<td>43.9±2.7</td>
<td>41.0±2.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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

CD - cluster of differentiation; SEM - standard error of the mean; ANOVA - analysis of variance.
DISCUSSION

To our knowledge, we have demonstrated for the first time that dietary supplementation with omega-3 fatty acids can augment acute endothelial t-PA release in cigarette smokers. We have also shown that omega-3 fatty acids improve endothelial vasomotor function in this high-risk population. These findings suggest that improved endogenous fibrinolysis and endothelial function may represent important mechanisms through which omega-3 fatty acids confer their cardiovascular benefits.

The effect of omega-3 fatty acids on fibrinolysis has been controversial, and the present study provides important new insight. Whilst some studies have reported a beneficial impact on fibrinolytic parameters [Mehta et al 1988; Smith et al 1989], others have found an adverse effect [Fumeron et al 1991; Spannagl et al 1991; Boberg et al 1992] or no effect [Hellsten et al 1993; Toft et al 1997; Finnegan et al 2003; Woodman et al 2003]. Results have varied widely and it has been concluded that omega-3 fatty acids are unlikely to influence the fibrinolytic system [Knapp HR 1997; Kristensen et al 2001].

However, previous studies have only measured basal plasma t-PA concentrations, which do not reflect the local capacity for acute endothelial t-PA release [Hrafnkelsdottir et al 2004]. It is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. The increased risk of myocardial infarction in cigarette
smokers is believed to be at least partly due to impaired acute endogenous t-PA release [Newby et al 1999; Newby et al 2001]. Using an established model of acute endothelial t-PA release which predicts cardiovascular outcome [Newby et al 1997; Robinson et al 2007], we have demonstrated that omega-3 fatty acids supplementation improves acute endogenous fibrinolytic capacity in cigarette smokers.

We have also demonstrated that omega-3 fatty acid supplementation improves endothelial vasomotor function in healthy cigarette smokers. Previous studies have found a beneficial effect on endothelial function in a variety of populations including healthy volunteers [Chin et al 1993; Khan et al 2003], patients with hyperlipidaemia [Goodfellow et al 2000; Mori et al 2000], diabetes mellitus [McVeigh et al 1993], coronary artery disease [Tagawa et al 1999; Haberka et al 2011] and heart failure [Morgan et al 2006]. However, not all studies have shown an improvement in endothelial function [Woodman et al 2003; Wong et al 2010; Skulas-Ray et al 2011]. It is difficult to identify a clear reason for discrepancies in study results as there is significant variability in trial design, and it seems likely that inconsistent results are due to differing study populations, concomitant medications, or omega-3 fatty acid doses. The present study extends the existing data as it is the first to investigate the effect of omega-3 fatty acids on endothelial function in healthy cigarette smokers, a high-risk group with known endothelial dysfunction.

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. Impaired endothelial-dependent vasodilatation in the forearm vascular bed in response to intra-arterial acetylcholine infusion is a powerful and independent predictor of cardiovascular events over 5 years [Lind et al 2011]. Endogenous fibrinolytic capacity, as measured by stimulated endothelial release of t-PA from the forearm vasculature in response to substance P infusion has also been shown to predict outcome [Robinson et al 2007].

Several clinical trials of dietary fish or omega-3 fatty acid supplements have demonstrated beneficial effects on mortality or cardiac events in a variety of patient groups [Burr et al 1989; GISSI-Prevenzione trial 1999; Yokoyama et al 2007; GISSI-HF Investigators 2008]. Although some trials have not shown a benefit [Rauch et al 2010], this may reflect differences in the disease populations studied. The mechanisms through which omega-3 fatty acids may confer cardiac benefits remain uncertain, and a presumed effect on ventricular arrhythmias has not been borne out by subsequent studies in patients with implantable cardioverter-defibrillators [Brouwer et al 2009]. However, an improvement in endogenous fibrinolytic capacity and endothelial function, as observed in the present study, may represent a mechanism through which omega-3 fatty acids can reduce the risk of coronary thrombosis and subsequent myocardial infarction. This may be of particular importance in cigarette smokers, who demonstrate a marked predisposition to acute thrombosis rather than vulnerable plaque rupture in cases of sudden death in patients with coronary disease [Burke et al 1997].
Cigarette smokers demonstrate increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system [Harding et al 2004], and we have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation [Din et al 2008]. 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 [Aarsetoy et al 2006; Din et al 2008].

5.5.1 CONCLUSION

We have 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. These distinct but complementary measures of vascular function may represent important mechanisms through which omega-3 fatty acids confer their cardiovascular benefits. Omega-3 fatty acids had no effect on platelet activation or the CD40/CD40 ligand system at the doses used in the current study.
CHAPTER 6

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

6.1 SUMMARY

The mechanisms through which omega-3 fatty acids reduce adverse cardiac events remain uncertain. Previous studies investigating effects on endothelial function, fibrinolysis and platelet function have been inconsistent. 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. Twenty patients with a previous myocardial infarction were recruited into a randomised, double-blind, placebo-controlled, crossover trial of omega-3 fatty acid supplementation (2 g/day for 6 weeks). Peripheral blood was taken for flow cytometric analysis of platelet and monocyte activation, and FBF was assessed during intrabrachial infusions of acetylcholine, substance P and sodium nitroprusside. Stimulated t-PA concentrations were measured during substance P infusion. All vasodilators caused dose-dependent increases in FBF (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. 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.
6.2 INTRODUCTION

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease [Din et al 2004]. Clinical trials have demonstrated beneficial effects on mortality or cardiac events in patients with coronary heart disease [Burr et al 1989; GISSI-Prevenzione trial 1999; Yokoyama et al 2007]. However, the actual 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 [Marchioli et al 2002; Leaf et al 2003], subsequent studies have failed to clearly demonstrate an anti-arrhythmic effect [Brouwer et al 2009]. 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 [Bowker et al 2003].

The endothelium controls vascular tone and blood flow, but also regulates thrombosis through the production of factors that influence fibrinolysis and platelet activation [Widlansky et al 2003]. The endogenous fibrinolytic system is responsible for the dissolution of arterial thrombi and is regulated by the endothelium-derived profibrinolytic factor, t-PA, and its inhibitor, PAI-1 [Oliver et al 2005]. The rapid release of t-PA from the endothelium is critical, with thrombus dissolution being more effective if t-PA is incorporated early during thrombus formation [Fox et al 1985].
Endothelial cells regulate thrombosis through the release of paracrine factors that mediate platelet function, and there is an inverse relationship between endothelial vasomotor function and platelet activation [Robinson et al 2006]. Activated platelets can bind to leukocytes via a P-selectin-dependent mechanism [Jungi et al 1986], and these interactions can also be modulated by the CD40 receptor and its ligand [Schonbeck et al 2001]. Formation of platelet-leukocyte aggregates or ligation of CD40 can mediate an array of pro-inflammatory and prothrombotic effects, promoting leukocyte adhesion to activated endothelial cells and thereby contributing to endothelial injury and atherothrombosis [Huo et al 2003].

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 [Din et al 2013]. Previously, we have shown that dietary fish intake reduces platelet-monocyte aggregation in man [Din et al 2008]. We therefore hypothesised 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.

6.3 METHODS

6.3.1 STUDY PARTICIPANTS
Twenty patients with a myocardial infarction at least 3 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.

6.3.2 STUDY DESIGN

This was a prospective, double-blind, placebo-controlled, randomised crossover trial. Subjects were randomised to receive either omega-3 fatty acid supplements (2 g/day Omacor capsules) or matching placebo capsules (2 g/day olive oil capsules) 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% EPA and 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 FBF and endogenous fibrinolysis at the end of each treatment period.
6.3.3 Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with EDTA and the direct thrombin inhibitor PPACK. Whole blood anticoagulated with PPACK was immunolabelled within 5 minutes 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 -70°C until analysis.

6.3.4 Flow cytometry

The following reagents were used: FITC-conjugated CD42a (GRP-P, IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), PE-conjugated CD40 (LOB7/6, IgG1), and their appropriate isotype controls 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, and FACS-Lyse. Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with appropriate antibodies and their isotype-matched controls for 20 minutes 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 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 EXPO32 software.

6.3.5 **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% butyraldehyde hydroxytoluene as an antioxidant (Folch extraction). Phospholipids were isolated by solid-phase extraction using aminopropyl silica columns 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. 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%.

6.3.6 **VASCULAR STUDIES**

Studies were carried out in a quiet temperature controlled room (22–25°C). Subjects fasted for 6 hours prior to the study and avoided caffeine and alcohol for the preceding 24 hours. Blood pressure and heart rate were recorded throughout the study using an automated non-invasive oscillometric sphygmomanometer.
All subjects underwent brachial artery cannulation with a 27-standard wire gauge steel needle under controlled conditions. After a 30-minute baseline saline infusion, acetylcholine at 5, 10, and 20 µg/min (endothelium-dependent vasodilator that does not release t-PA), substance P at 2, 4, and 8 pmol/min (endothelium-dependent vasodilator that releases t-PA) and sodium nitroprusside at 2, 4, and 8 µg/min (endothelium-independent vasodilator that does not release t-PA) were infused for 6 minutes at each dose. The 3 vasodilators were separated by 20-minute saline infusions and given in a randomised order.

Forearm blood flow was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges as described previously [Newby et al 1997]. Venous cannulae (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 for t-PA assays and into citrate for PAI-1 assays. Samples were kept on ice before being centrifuged at 2000 g for 30 minutes at 4°C. Platelet-free plasma was decanted and stored at -80°C before assay. Plasma t-PA antigen and activity and PAI-1 antigen and activity concentrations were determined by enzyme-linked immunosorbent assays. Haematocrit was determined by capillary tube centrifugation at baseline.

6.3.7 Data analysis and statistical methods
Continuous variables are reported as mean±SEM. Forearm plethysmographic data were analysed as described previously [Newby et al 1997]. Estimated net release of
plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused FBF) and the concentration difference between the infused and non-infused arms [Newby et al 1997]. Statistical analyses were performed using one-way and two-way ANOVA with Bonferroni’s post-tests for multiple comparisons where appropriate. All calculations were performed using GraphPad Prism. Statistical significance was taken at 5%.

6.4 RESULTS

6.4.1 BASELINE CHARACTERISTICS

Patients were relatively young and well treated in terms of blood pressure control and lipid profile (Table 6.1). 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 angiotensin-converting enzyme (ACE) inhibitors, and over half had undergone revascularisation post-myocardial infarction.
<table>
<thead>
<tr>
<th><strong>Baseline Characteristics</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years</strong></td>
<td>53±3</td>
</tr>
<tr>
<td><strong>Body mass index (kg/m²)</strong></td>
<td>28±1</td>
</tr>
<tr>
<td><strong>Systolic blood pressure (mmHg)</strong></td>
<td>137±5</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure (mmHg)</strong></td>
<td>78±3</td>
</tr>
<tr>
<td><strong>Heart rate (beats per minute)</strong></td>
<td>60±2</td>
</tr>
<tr>
<td><strong>Total cholesterol (mmol/L)</strong></td>
<td>4.2±0.2</td>
</tr>
<tr>
<td><strong>LDL cholesterol (mmol/L)</strong></td>
<td>2.3±0.2</td>
</tr>
<tr>
<td><strong>HDL cholesterol (mmol/L)</strong></td>
<td>1.1±0.1</td>
</tr>
<tr>
<td><strong>Chol:HDL chol ratio</strong></td>
<td>3.8±0.2</td>
</tr>
<tr>
<td><strong>Triacylglycerol (mmol/L)</strong></td>
<td>1.6±0.2</td>
</tr>
<tr>
<td><strong>Fasting glucose (mmol/L)</strong></td>
<td>5.4±0.1</td>
</tr>
<tr>
<td><strong>Time from MI (months)</strong></td>
<td>16±4</td>
</tr>
<tr>
<td><strong>Revascularisation post-MI (%)</strong></td>
<td>56%</td>
</tr>
<tr>
<td><strong>Current or ex-smoker (%)</strong></td>
<td>61%</td>
</tr>
<tr>
<td><strong>Hypertension (%)</strong></td>
<td>11%</td>
</tr>
<tr>
<td><strong>Diabetes mellitus (%)</strong></td>
<td>0%</td>
</tr>
<tr>
<td><strong>Hyperlipidemia (%)</strong></td>
<td>78%</td>
</tr>
<tr>
<td><strong>Family history of premature coronary heart disease (%)</strong></td>
<td>33%</td>
</tr>
<tr>
<td><strong>Medical therapy</strong></td>
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<tr>
<td><strong>Aspirin (%)</strong></td>
<td>100%</td>
</tr>
<tr>
<td><strong>Clopidogrel (%)</strong></td>
<td>11%</td>
</tr>
<tr>
<td><strong>ACE-inhibitor/Angiotensin-receptor blocker (%)</strong></td>
<td>56%</td>
</tr>
<tr>
<td><strong>Beta-blocker (%)</strong></td>
<td>78%</td>
</tr>
<tr>
<td><strong>Statin (%)</strong></td>
<td>100%</td>
</tr>
</tbody>
</table>

Data are reported as Mean±SEM.
LDL - low-density lipoprotein; HDL - high-density lipoprotein; Chol - cholesterol; MI - myocardial infarction; ACE - angiotensin-converting enzyme; SEM - standard error of the mean.
6.4.2 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 6.1A). 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 6.1B). There was a reduction in the plasma phospholipid percentage of arachadonic acid, but no effect on ALA, linoleic acid, palmitic acid, stearic acid or oleic acid with either omega-3 fatty acid supplements or olive oil placebo (Table 6.2).

6.4.3 Effect of omega-3 fatty acid supplementation on lipid profile

Supplementation for 6 weeks with omega-3 fatty acids did not affect total cholesterol, LDL cholesterol, HDL cholesterol or triglycerides (Table 6.3).

6.4.4 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 6.3). During forearm vascular studies substance P, acetylcholine, and sodium nitroprusside led to a dose-dependent increase in absolute FBF (P<0.0001 for all).
Figure 6.1 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. NS – not significant; EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; ANOVA – analysis of variance.
### TABLE 6.2 Effect of omega-3 fatty acid supplementation on plasma phospholipid fatty acid composition

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

Mean±SEM. Data analysed using one-way ANOVA. SEM - standard error of the mean; ANOVA - analysis of variance.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Omega-3</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate (beats per minute)</strong></td>
<td>60±3</td>
<td>60±2</td>
<td>60±2</td>
<td>0.9</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>131±4</td>
<td>125±4</td>
<td>130±4</td>
<td>0.2</td>
</tr>
<tr>
<td>Diastolic blood pressure (mm Hg)</td>
<td>77±3</td>
<td>74±2</td>
<td>74±3</td>
<td>0.5</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.3±0.3</td>
<td>3.9±0.3</td>
<td>4.0±0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.5±0.2</td>
<td>2.2±0.3</td>
<td>2.2±0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>1.1±0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>Chol:HDL chol ratio</td>
<td>3.8±0.3</td>
<td>3.5±0.3</td>
<td>3.5±0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/L)</td>
<td>1.5±0.2</td>
<td>1.3±0.1</td>
<td>1.5±0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mean±SEM. Data analysed using one-way ANOVA.
LDL - low-density lipoprotein; HDL - high-density lipoprotein; Chol - cholesterol; SEM - standard error of the mean; ANOVA - analysis of variance
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 6.2), or endothelium-independent vasodilatation with sodium nitroprusside (P=0.9; Figure 6.2).

6.4.5 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 6.4). Omega-3 fatty acid supplementation did not affect plasma t-PA activity, t-PA antigen or PAI-1 concentrations compared with placebo (Table 6.4). There was no difference in net release of t-PA activity after omega-3 fatty acid supplementation compared with placebo (P=0.94; Figure 6.3).

6.4.6 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 6.5).
Fig 6.2 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. ANOVA = analysis of variance.
<table>
<thead>
<tr>
<th>Substance P pmol/min</th>
<th>Omega-3 fatty acids</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>t-PA activity (IU mL⁻¹)</td>
<td>0.39±0.08</td>
<td>0.45±0.09</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>0.45±0.07</td>
<td>0.52±0.08</td>
</tr>
<tr>
<td>Infused arm</td>
<td>0.38±0.08</td>
<td>0.83±0.16</td>
</tr>
<tr>
<td>t-PA antigen (ng mL⁻¹)</td>
<td>11.78±1.29</td>
<td>12.01±1.00</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>13.45±1.40</td>
<td>12.93±1.70</td>
</tr>
<tr>
<td>Infused arm</td>
<td>11.90±1.45</td>
<td>13.98±1.33</td>
</tr>
<tr>
<td>PAI-1 activity (ng mL⁻¹)</td>
<td>1.77±0.53</td>
<td>1.84±0.43</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.44±0.29</td>
<td>1.38±0.26</td>
</tr>
<tr>
<td>Infused arm</td>
<td>2.33±0.86</td>
<td>2.18±0.61</td>
</tr>
<tr>
<td>PAI-1 antigen (ng mL⁻¹)</td>
<td>39.51±9.22</td>
<td>40.84±7.08</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>45.06±7.09</td>
<td>43.33±6.45</td>
</tr>
<tr>
<td>Infused arm</td>
<td>37.64±8.36</td>
<td>38.83±6.25</td>
</tr>
<tr>
<td>Net t-PA antigen release (ng 100 mL⁻¹) of tissue mm⁻¹</td>
<td>0.23±0.51</td>
<td>-0.28±4.7</td>
</tr>
<tr>
<td></td>
<td>-0.87±1.1</td>
<td>-0.84±2.82</td>
</tr>
</tbody>
</table>

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). t-PA - tissue plasminogen activator; PAI-1 - plasminogen activator inhibitor type 1; SEM - standard error of the mean; ANOVA - analysis of variance.
Figure 6.3 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. t-PA – tissue plasminogen activator; ANOVA – analysis of variance.
<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Omega-3</th>
<th>Placebo</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet-monocyte aggregates (%)</td>
<td>21.2±3.9</td>
<td>23.6±4.2</td>
<td>23.0±4.1</td>
<td>0.7</td>
</tr>
<tr>
<td>Platelet-neutrophil aggregates (%)</td>
<td>4.9±1.0</td>
<td>6.7±1.2</td>
<td>6.7±1.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Platelet-surface expression of P-selectin (%)</td>
<td>3.9±0.9</td>
<td>5.0±1.0</td>
<td>4.3±1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Platelet-surface expression of CD40L (%)</td>
<td>3.6±0.4</td>
<td>3.4±0.3</td>
<td>3.4±0.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Monocyte expression of CD40 (%)</td>
<td>52.5±5.0</td>
<td>46.8±3.4</td>
<td>47.4±2.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Mean±SEM. Data analysed using one-way ANOVA.
CD - cluster of differentiation; SEM - standard error of the mean; ANOVA - analysis of variance
6.5 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 [Burr et al 1989; GISSI-Prevenzione trial 1999]. 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 [Tagawa et al 1999; Mori et al 2000; Din et al 2008; Din et al 2013].

Omega-3 fatty acids have previously been shown to have inconsistent effects on endothelial function [Egert et al 2011]. Whilst some studies have reported beneficial
effects in a variety of populations including healthy volunteers [Chin et al 1993; Khan et al 2003], patients with hyperlipidaemia [Goodfellow et al 2000; Mori et al 2000], diabetes mellitus [McVeigh et al 1993], cigarette smokers [Din et al 2013] and heart failure [Morgan et al 2006], others have not found any improvement [Woodman et al 2003; Wong et al 2010; Skulas-Ray et al 2011]. Our findings are in contrast to previous studies in coronary heart disease which demonstrated an improvement in endothelial function with omega-3 fatty acids [Tagawa et al 1999; Tagawa et al 2002; Haberka et al 2011]. These discrepancies could be partly due to differences in study populations or concomitant medication. However, the previous studies were all either not randomised 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 [Kristensen et al 2001]. Whilst some studies have reported a beneficial impact on fibrinolytic parameters [Mehta et al 1988; Smith et al 1989], others have found an adverse effect [Spannagl et al 1991] or no effect [Hellsten et al 1993; Toft et al 1997; Finnegan et al 2003; Woodman et al 2003]. However, previous studies have only measured basal plasma t-PA concentrations that do not reflect the local capacity for acute endothelial t-PA release [Hrafinkelsdottir et al 2004; Oliver et al 2005]. 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 [Newby et al 1997; Robinson et al 2007], but found no effect of omega-3 fatty acid supplementation on acute endogenous fibrinolytic capacity in coronary heart disease.

There are several possible mechanisms which may account for the lack of effect omega-3 fatty acids have on endothelial function and endogenous fibrinolysis observed in the present coronary heart disease population compared with our previous study in cigarette smokers [Din et al 2013]. The patients in the present study were all well treated with modern cardioactive medication known to influence endothelial vasomotor function [Treasure et al 1995; Mancini et al 1996]. In contrast, patients in previous studies demonstrating improved endothelial function [Tagawa et al 1999; Tagawa et al 2002] and cardiac outcomes [Burr et al 1989; GISSI-Prevenzione Investigators 1999] with omega-3 fatty acids were much less likely to be taking HMG CoA reductase inhibitors or ACE 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 [Galan et al 2010; Kromhout et al 2010; Rauch et al 2010].
However, concomitant medication cannot 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 [Newby et al 2002]. Angiotensin-converting enzyme inhibitors only augment bradykinin-induced t-PA release; they do not affect t-PA release stimulated by substance P [Witherow et al 2002]. 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 [Sarma et al 2002]. We have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation [Din et al 2008]. 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 [Aarsetoy et al 2006; Din et al 2008].

6.5.1 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.
CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS
7.1 INTRODUCTION

Dietary fish or fish oil supplements containing omega-3 fatty acids may protect against cardiovascular disease. Whilst omega-3 fatty acids have been associated with several biological actions, the actual mechanisms through which they confer any cardiac benefits are uncertain. Although an effect on ventricular arrhythmias has been thought to be central due to the reduction in sudden death observed in clinical trials, subsequent studies have failed to clearly demonstrate an anti-arrhythmic effect. An alternative mechanism may therefore be an effect on the vascular endothelium and thrombosis, as acute myocardial infarction due to plaque rupture and subsequent coronary thrombosis remains the most common cause of sudden cardiac death.

Although dietary supplementation with omega-3 fatty acids has been found to improve endothelial function in several patient groups, not all studies have demonstrated a beneficial effect, and their effects on fibrinolysis and platelet activation have been inconsistent. However, previous studies investigating fibrinolysis have measured basal plasma t-PA concentrations, which do not reflect the local capacity for acute endothelial t-PA release. The assessment of acute endothelial t-PA release is of greater pathophysiological relevance, and indeed predicts the future risk of cardiovascular events. Most previous studies investigating effects on platelet function have relied on crude measures of platelet activation, poorly suited to the detection of potentially subtle effects induced by dietary changes, rather than modern flow cytometric techniques which allow platelets to be analysed.
directly in their physiological environment of whole blood with minimal sample manipulation.

We therefore conducted a series of investigations to assess the effects of omega-3 fatty acids on endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation.

7.2 EFFECT OF DIETARY INTERVENTION WITH OILY FISH ON PLATELET-MONOCYTE AGGREGATES

We have demonstrated, for the first time that dietary intervention with oil-rich fish is associated with a reversible reduction in platelet-monocyte aggregation in man. There was an inverse correlation between platelet-monocyte aggregation and the plasma levels of EPA and DHA, consistent with the effects being related to the omega-3 content of oily fish. Our results suggest that reduced platelet activation could represent an important mechanism through which dietary fish confer their putative cardiovascular benefits. Most previous studies assessing the effects of fish oils on platelet function have used older techniques with limited reproducibility which are poorly suited to the detection of subtle dietary effects. Modern flow cytometric techniques allow platelets to be analysed directly in their physiological environment of whole blood, and circulating platelet-monocyte aggregates represent a highly sensitive marker of platelet activation. Further work is required to determine if the observed effects are due to omega-3 fatty acids or some other nutrient present in fish. This could be achieved with a study comparing an oil-rich fish diet (rich in
omega-3 fatty acids) with both a lean fish diet (not rich in omega-3 fatty acids) and omega-3 fatty acid supplement.

7.3 EFFECT OF DIETARY INTERVENTION WITH WALNUTS ON PLATELET-MONOCYTE AGGREGATES AND ARTERIAL STIFFNESS

We have demonstrated that dietary supplementation with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy subjects. This is in contrast with previous studies which have shown that heavy consumption of walnuts as part of a low-fat or 'Mediterranean' diet can reduce LDL cholesterol and improve endothelial function. The present study is the first dietary intervention trial to assess the efficacy of moderate rather than large walnut consumption on markers of cardiovascular risk. Further work is required to establish if improvements in platelet-monocyte aggregates or arterial stiffness would be seen with larger amounts of walnut consumption. The effect of other sources of plant-derived ALA, for example canola oil, must also be investigated. Finally, the effect of plant sources rich in ALA on vascular function and thrombosis should also be assessed in patients with cardiovascular risk factors or disease.
7.4 EFFECT OF OMEGA-3 FATTY ACIDS ON ENDOTHELIAL FUNCTION AND ENDOGENOUS FIBRINOLYSIS IN SMOKERS

To our knowledge, we have demonstrated for the first time that dietary supplementation with omega-3 fatty acids can augment acute endothelial t-PA release in cigarette smokers. Previous studies have only measured basal plasma t-PA concentrations, which do not reflect the local capacity for acute endothelial t-PA release, and it is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. In addition, we have shown that omega-3 fatty acids improve endothelial vasomotor function in this high-risk population. Our findings suggest that improved endogenous fibrinolysis and endothelial function may represent important mechanisms through which omega-3 fatty acids confer potential cardiovascular benefits in cigarette smokers. Further work is required to establish the underlying processes by which omega-3 fatty acids improve endothelial function and t-PA release in this population, and whether these benefits would also be evident with dietary fish rather than supplements.

7.5 EFFECT OF OMEGA-3 FATTY ACIDS ON ENDOTHELIAL FUNCTION AND ENDOGENOUS FIBRINOLYSIS IN PATIENTS WITH PREVIOUS MYOCARDIAL INFARCTION

We 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. 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 or endogenous fibrinolysis. The explanation for a lack of effect in coronary heart disease compared with our findings in cigarette smokers is uncertain. It is possible that endothelial function cannot be further improved by the addition of omega-3 fatty acids in patients already treated with optimal modern medical therapy, and older patients may have a dysfunctional endothelium and fibrinolytic system less responsive to dietary interventional measures. Additional work is required to clarify whether omega-3 fatty acids have distinct effects in different population groups.

7.6 LACK OF EFFECT OF OMEGA-3 FATTY ACIDS ON PLATELET-MONOCYTE AGGREGATION OR CD40/CD40 LIGAND

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. While we have demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation, we did not observe any effect of omega-3 fatty acid supplements on these measures of platelet and monocyte activation in either cigarette smokers or patients with coronary heart disease. 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.

7.7 FUTURE DIRECTIONS

This research raises several important questions which could be explored further with appropriately designed translational and clinical studies. Firstly, work needs to be focused on establishing the ideal amount and formulation of omega-3 fatty acids that might confer cardiac benefits. Different effects may be imparted by dietary fish versus omega-3 supplements, and the optimal amounts of EPA, DHA and ALA remains uncertain.

Secondly, research is required to understand the underlying mechanisms whereby dietary fish reduces platelet-monocyte aggregation and omega-3 supplements improve endothelial function and endogenous fibrinolysis. The effect on endothelial function is thought to be partly due to the ability to modify eicosanoid biosynthesis, resulting in the formation of leukotrienes, thromboxanes and novel mediators which favour vasodilatation and reduce vasoconstriction and inflammation. Improvements in endothelial function with omega-3 fatty acids are also largely, but not exclusively, mediated by nitric oxide. Consistent with an important role for nitric oxide are the observations that omega-3 fatty acids increase endogenous nitric oxide production, and induce structural changes in plasma membrane microdomains leading to increased endothelial nitric oxide synthase activity. Factors regulating acute endothelial t-PA release are poorly understood, but may involve G proteins and
increased intracellular calcium concentrations, whilst the role of nitric oxide is unclear. Although omega-3 fatty acids influence calcium availability by modulating cell membrane ion channels, and can signal via G protein coupled receptors, further research is required to establish whether the increased t-PA release we observed is mediated by effects on these cellular processes.

Thirdly, our research suggests that omega-3 fatty acids may have diverse effects in different populations. It is not clear if this is due to concomitant cardiac medication in certain patient groups, or distinct biological responses between healthy volunteers and individuals with cardiovascular risk factors or disease. Clearly, it may not be appropriate to extrapolate effects seen in one group to another, and on-going work will be needed to delineate these different responses and investigate the underlying reasons for them.

Finally, further clinical trials are required to clarify the effect of omega-3 fatty acids on cardiac outcomes. Although older clinical trials demonstrated that dietary fish and omega-3 fatty acid supplements reduced mortality in patients with heart disease, several recent clinical trials have yielded inconsistent results. A large Japanese trial randomised 18,645 patients with hyperlipidaemia to 1800 mg of EPA and statin versus statins alone [Yokoyama et al 2007]. At a mean follow-up of 4.6 years those in the EPA group had a 19% relative reduction in major adverse cardiac events, largely driven by a reduction in unstable angina. The GISSI Heart Failure Study randomised 6975 patients with chronic heart failure to 1g daily of omega 3 fatty acids or placebo and followed them for a median of 3.9 years [GISSI-HF
Investigators 2008]. Patients in the omega 3 group had significant reduction in all-cause mortality (27% vs 29%; P=0.04) and admission to hospital for cardiovascular reasons (57% vs 59%; P=0.009).

However, not all contemporary trials have shown a benefit from omega 3 fatty acids. The Alpha-Omega Trial enrolled 4837 patients with a previous myocardial infarction, and randomised to 376 mg per day of EPA+DHA versus placebo or ALA as margarine [Kromhout et al 2010]. There was no difference in major cardiovascular events after a median of 3.7 years. The OMEGA Trial randomised 3851 patients with a recent myocardial infarction to 1 g per day or omega 3 fatty acids or placebo, and found no difference in cardiac outcomes after 1 year of follow-up [Rauch et al 2010]. Finally, the SU.FOL.OM3 trial randomised 2501 patients with a prior history of cardiovascular or cerebrovascular disease to 600 mg daily EPA+DHA or placebo, and again found no effect on major cardiac events after 4.2 years [Galan et al 2010]. The reason for these discordant results is uncertain. It has been suggested that the recent trials have all been underpowered for the detection of a mortality reduction, and certainly the OMEGA and SU.FOL.OM3 had lower than expected event rates which resulted in <20% power to detect a 25% relative reduction in mortality. Nevertheless, the recent clinical trial data suggests that omega-3 fatty acid supplementation may not be able to reduce cardiac events in coronary heart disease patients already on optimal modern medical therapy. However, there could be benefit in individuals with cardiac risk factors but not established disease. Their overall risk may still be modifiable with specific dietary
interventions and further clinical and population studies would be required to explore this in more detail.

7.8 CONCLUSIONS

We have performed a series of studies to investigate the effects of omega-3 fatty acids on vascular function and thrombosis. We have demonstrated that dietary intervention with oily fish rich in EPA and DHA reduces platelet-monocyte aggregates in healthy volunteers, whereas intervention with walnuts rich in ALA did not have any effect on platelet-monocyte aggregates or arterial stiffness. Supplementation with omega-3 fatty acids improved endothelial function and endogenous fibrinolysis in cigarette smokers, but had no effect on platelet-monocyte aggregates. However, omega-3 fatty acid supplements did not affect endothelial function, endogenous fibrinolysis or platelet-monocyte aggregation in patients with ischaemic heart disease. Major strengths of our study are the use of a highly sensitive measure of platelet activation and a robust model to simultaneously assess both endothelial vasomotor tone and endogenous fibrinolysis: two important and complementary measures of vascular function. Our results indicate that dietary fish and omega-3 fatty acids can have beneficial effects on platelet-monocyte aggregation, endothelial function and endogenous fibrinolysis, and these may represent important mechanisms through which omega-3 fatty acids confer any potential cardiovascular benefits. However, these effects were not seen in all groups studied and omega-3 fatty acids may have diverse effects in different populations. Further work is required to investigate these differences. Establishing the mechanism
through which omega-3 fatty acids confer any preventative benefits may help guide the application and development of future therapeutic interventions. In this era of multiple pharmacological interventions, the benefits of simple dietary interventions and nutriceutical supplements may provide a more acceptable and natural method of providing secondary preventative benefits.
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PUBLICATIONS
PUBLICATIONS


Clinical review

Science, medicine, and the future

Omega 3 fatty acids and cardiovascular disease—fishing for a natural treatment

Jehangir N Din, David E Newby, Andrew D Flapan

Omega 3 fatty acids from fish and fish oils can protect against coronary heart disease. This article reviews the evidence regarding fish oils and coronary disease and outlines the mechanisms through which fish oils might confer cardiac benefits.

Summary points

- Coronary heart disease is still the most common cause of death in the United Kingdom.
- Omega 3 fatty acids from fish and fish oils can protect against coronary heart disease.
- There is evidence to support the use of fish or fish oil supplements after myocardial infarction.
- The mechanisms by which fish oils confer their benefits are not fully understood.
- Unravelling these mechanisms may identify novel therapeutic targets and could help guide the development of future treatments for coronary heart disease.
- Future trials may identify other patients who could benefit, such as those with stable angina, risk factors for coronary heart disease, or left ventricular dysfunction.

Sources and selection criteria

We searched PubMed for relevant articles by using the key words “fish,” “fish oils,” “omega 3 fatty acids,” and “cardiovascular disease.” References identified in the search are on bmj.com.

Omega 3 polyunsaturated fatty acids

The association between omega 3 fatty acids and cardiovascular disease was established following the observation that the Greenland Inuit had low mortality from coronary heart disease despite a diet that is rich in fat. In the 1970s the Danish investigators Bang and Dyerberg proposed that this could be because of the high content of omega 3 fatty acid in the Inuit diet, which consisted largely of fish, seal, and whale (fig 1).

Fig 1 Greenland Inuit gutting a seal in the early 1900s. Their diet consisted largely of fish, whale, seal, and walrus, resulting in a high intake of omega 3 fatty acids. Copyright Arctic Institute, used with permission from Leif Vanggaard, Arctic Institute.
Recent trial after advice. Patients who received advice had a relative reduction in total mortality of 29% during the two year follow up (P < 0.05), mainly because of a reduction in deaths from coronary heart disease. The open label Gruppo Italiano per lo Studio della Sopravivenza nell'Infarto Miocardico (Gruppo Italiano per la Prevenzione Olistica) trial randomised 31,114 patients after myocardial infarction to either a daily capsule of about 850 mg omega 3 fatty acids, 300 mg vitamin E, both, or neither. After 3.5 years participants randomised to fish oil capsules had a reduction in relative risk of 15% in the composite primary end point of total mortality, non-fatal myocardial infarction, and stroke (P = 0.023). The relative risk of cardiovascular death was also reduced, by 30% (P = 0.024), and of sudden death by 45% (P = 0.01). These benefits were apparent within just four months of randomisation.

Two smaller secondary prevention trials have also assessed the effects of omega 3 fatty acids. In an Asian population patients with a suspected myocardial infarction randomised to fish oil capsules experienced a significant reduction in mortality from coronary heart disease after one year compared with placebo. However, a Norwegian study reported no benefit in patients after myocardial infarction who were given fish oil capsules compared with placebo after 1.5 years. This may have been because of the high habitual fish consumption among the general population in that area, with omega 3 supplementation conferring no additional benefit.

A recent trial of 31,114 men with angina unexpectedly found that individuals advised to eat oily fish, and particularly those given fish oil capsules, had a higher risk of cardiac death than people not given advice to eat fish (11.5% v 9%, P = 0.02). The investigators speculated that this may have arisen from risk compensation or other changes in patients’ behaviour. Several flaws in this study weakened the validity of the results, and they should be viewed with caution until more evidence becomes available.

Epidemiological and observational studies

Most studies have shown an inverse association between fish consumption and the risk of coronary heart disease. Furthermore, both consumption of fish and higher blood concentrations of omega 3 fatty acids are associated with a reduced risk of sudden death. However, some studies have not found a relation between intake of fish and coronary heart disease. These inconsistencies could be due to differences in methods, study populations, or fish. Importantly, most studies showing no association were in populations with an already moderate fish intake, potentially masking any relation. Overall, fish consumption seems to be beneficial, and a systematic review of 11 prospective cohort studies concluded that fish intake notably reduced mortality due to coronary heart disease in populations at increased risk.

Clinical intervention trials

Several trials have assessed the effects of fish and fish oil supplements on coronary heart disease, mainly after myocardial infarction (table 1). The diet and reinfarction trial (DARI) randomised 2093 men with a recent myocardial infarction to three dietary interventions. Patients who received advice on fish had a relative reduction in total mortality of 29% during the two year follow up (P < 0.05), mainly because of a reduction in deaths from coronary heart disease. The open label Gruppo Italiano per lo Studio della Sopravivenza nell’Infarto Miocardico (GISSI-Prevenzione) trial randomised 11,324 patients after myocardial infarction to either a daily capsule of about 850 mg omega 3 fatty acids, 300 mg vitamin E, both, or neither. After 3.5 years participants randomised to fish oil capsules had a reduction in relative risk of 15% in the composite primary end point of total mortality, non-fatal myocardial infarction, and stroke (P = 0.023). The relative risk of cardiovascular death was also reduced, by 30% (P = 0.024), and of sudden death by 45% (P = 0.01). These benefits were apparent within just four months of randomisation.

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Mechanism of action

Although the weight of evidence outlined above supports a protective effect of omega 3 fatty acids on coronary heart disease, the mechanisms through which they confer these benefits remain unclear. Omega 3 fatty acids have several potentially cardioprotective effects (Box 1), although the relative contribution of each of these is not fully understood.

Arrhythmias

The benefits of fish oils were originally thought to be due to their antiarrhythmic effects, but recent evidence has indicated that the predominant effect may be antiarrhythmic. In the GISSI-Prevenzione trial the decrease in mortality was largely due to a reduction in sudden death, and, as in DART, no reduction in the rate of non-fatal myocardial infarction occurred. Fish oil supplementation increases heart rate variability in patients after myocardial infarction, which correlates with a lower risk of mortality and malignant arrhythmia. In animal models fish oil protects against ventricular fibrillation after surgical occlusion of a coronary artery. The addition of eicosapentanoic acid or docosahexanoic acid can prevent or terminate pharmacologically induced arrhythmias in cultured cardiomyocytes from newborn rats. However, studies are necessary to show a direct antiarrhythmic effect in humans and trials are currently under way in patients with implantable defibrillators.

Thrombosis

Activation of platelets and their deposition at sites of unstable plaque rupture promotes thrombus formation, and these critical events have become a common therapeutic target in acute coronary syndromes. However, the effects of omega 3 fatty acids on platelet function and thrombosis are controversial. Large doses reduce platelet aggregation, but smaller amounts have modest platelet inhibitory effects. Omega 3 fatty acids have inconsistent effects on fibrinolysis and little effect on blood coagulability. Therefore, although omega 3 fatty acids have an antithrombotic effect, its relevance to the mortality reduction seen with lower doses is unclear.

Atherosclerosis

Omega 3 fatty acids may also influence the atherosclerotic process. Fish oil fed to experimental animals protects against progression of atherosclerotic plaques. In humans with coronary heart disease omega 3 fatty acid supplementation versus placebo for two years resulted in modest improvements in atherosclerosis as assessed by angiography. These effects may be due to a reduction in lipids, inflammation, production of growth factor, or suppression of smooth muscle cell proliferation. An important recent study randomised patients awaiting carotid endarterectomy to fish oil capsules, sunflower oil capsules, or control until surgery and then assessed morphology of the plaque. Omega 3 fatty acids were readily incorporated into atherosclerotic plaques in the fish oil group, and these plaques were more likely to have thin fibrous caps and less inflammatory infiltrate. These features imply a plaque that is less vulnerable to rupture and indicate that fish oils may be important in establishing stability of the plaque.

Inflammation

Inflammation has a central role in the development and progression of coronary artery disease. Omega 3

Table 1 Effect of marine derived omega 3 fatty acids on death from coronary heart disease in secondary prevention of myocardial infarction

<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Intervention</th>
<th>Intervention events (%)</th>
<th>Control events (%)</th>
<th>Absolute risk reduction (%)</th>
<th>Relative risk reduction (%)</th>
<th>No needed to treat</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet and reinfarction trial</td>
<td>Randomised, controlled, two year follow up, 2033 men after myocardial infarction</td>
<td>Fish meal once weekly or fish oil capsules if unable to tolerate fish (1.3 g/d)</td>
<td>7.7*</td>
<td>11.4</td>
<td>3.7</td>
<td>32.5</td>
<td>27</td>
<td>Before routine use of secondary prevention treatment such as aspirin, p blockers, statins</td>
</tr>
<tr>
<td>Indian experiment of interest survival</td>
<td>Randomised double-blind placebo controlled, one year follow up, 300 patients after myocardial infarction</td>
<td>Fish oil (EPA+DHA 1.8 g/d) or mustard seed oil (ALA 2.9 g/d)</td>
<td>11.4*</td>
<td>22</td>
<td>10.6</td>
<td>48.2</td>
<td>10</td>
<td>Small size, high mortality, may not be applicable in Western populations</td>
</tr>
<tr>
<td>GISSI-Prevention trial, Italy</td>
<td>Randomised, controlled, 3.5 year follow up, 11,324 patients after myocardial infarction</td>
<td>Fish oil (EPA+DHA 0.85 g/d)</td>
<td>4.8**</td>
<td>6.8</td>
<td>2</td>
<td>29.7</td>
<td>50</td>
<td>Not blinded, no placebo</td>
</tr>
<tr>
<td>Nilsen et al</td>
<td>Randomised double-blind placebo controlled, 1.5 year follow up, 330 patients after myocardial infarction</td>
<td>Fish oil (EPA+DHA 3.5 g/d)</td>
<td>5.3</td>
<td>5.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Small size, reasonable intake of fish among general population</td>
</tr>
</tbody>
</table>

*P<0.001 between control and intervention groups. **P=0.024 between control and intervention groups. EPA=eicosapentanoic acid, DHA=docosahexanoic acid, ALA=linolenic acid.
fatty acids have recognised anti-inflammatory actions that may contribute to their beneficial cardiac effects. Omega 6 fatty acids can be converted into arachidonic acid and then metabolised into the omega 6 eicosanoids (fig 4). These cellular mediators enhance platelet aggregation and are generally pro-inflammatory. Consumption of omega 3 fatty acids increases eicosapentaenoic acid in the cell membrane. This competes with arachidonic acid for enzymatic conversion into its own metabolites, the omega 3 derived eicosanoids. These are less active and can partly oppose or antagonise the pro-inflammatory actions of the omega 6 eicosanoids.

Independent of the effects on the metabolism of eicosanoids fish oils suppress pro-inflammatory cytokines and reduce expression of cell adhesion molecules. These are critical in recruiting circulating leukocytes to the vascular endothelium, an important event in the pathogenesis of atherosclerosis and inflammation. These effects may be mediated through actions on intracellular signalling pathways, leading to reduced activation of transcription factors such as NF-kB. However, the precise effects of omega 3 fatty acids on these fundamental cellular processes and their potential impact on coronary heart disease are yet to be delineated completely.

Endothelial function
Abnormal endothelial function is found in individuals with cardiovascular risk factors or established coronary heart disease. Omega 3 fatty acids have direct effects on endothelial vasomotor function. Higher concentrations are associated with improved dilation of the brachial artery in young adults with cardiovascular risk factors, which implies a protective effect on endothelial function. In hyperlipidaemic men omega 3 fatty acid supplementation improved systemic arterial compliance and supplementation with docosahexaenoic acid increased vasodilator responses in the human forearm.

Blood pressure
Fish oils can produce modest reductions in blood pressure, possibly through their effects on endothelial function discussed above. A recent meta-analysis of 36 randomised trials found a reduction in systolic blood pressure of 2.1 mm Hg and in diastolic blood pressure of 1.6 mm Hg. However, most trials used relatively high doses of fish oils (3.6 g/day), and the effects of lower intakes of omega 3 fatty acids, such as those in the secondary prevention trials, remain to be established.

Triglyceride lowering
Omega 3 fatty acids reduce triglyceride concentrations in a dose dependent manner, with intakes of about 4 g per day lowering serum triglycerides by 25-30%. Their effect on cholesterol is small and of uncertain clinical importance. Higher doses (3-5 g/day) can be used in the treatment of hypertriglyceridemia.

Box 2: Recommendations for intake of omega 3 fatty acid
- Patients without documented coronary heart disease: Eat a variety of (preferably oily) fish at least twice weekly. Include oils and foods rich in a linoleic acid.
- Patients with documented coronary heart disease: Consume 1 g of eicosapentaenoic and docosahexaenoic acid daily, preferably from oily fish. Supplements could be considered in consultation with a doctor.
- Patients with hypertriglyceridaemia: Take 2-4 g of eicosapentaenoic acid and docosahexaenoic acid daily, provided as capsules under a doctor's care.
These are the recommendations of the American Heart Association.

Box 3: Consumption and sources of marine derived omega 3 fatty acids
- Current consumption of marine derived omega 3 fatty acids is low, at 0.1-0.2 g/day. An expert US panel of nutrition scientists has recommended an intake of 0.65 g/day whereas the British Nutrition Foundation's recommendation is 1.2 g/day. Secondary prevention trials after myocardial infarction indicate that consumption of 0.5-1.8 g/day of eicosapentaenoic and docosahexaenoic acid from fish or fish oil supplements may be beneficial. Intake of marine derived omega 3 fatty acids can be increased through diet or with fish oil supplements. Oily fish such as mackerel, herring, tuna, salmon, sardines and trout are rich sources of eicosapentaenoic and docosahexaenoic acid (table 2), and two to three servings per week should provide approximately 1 g/day omega 3 fatty acids. Lean fish such as cod or haddock have smaller amounts, and fried fish (for example, from fast food establishments or frozen products) contains minimal amounts of omega 3 fatty acids.
- Concerns about the depletion of fish stocks will become more pressing if the benefits of fish oils are confirmed beyond the population after myocardial infarction, as this may result in an unsustainable increase in demand. Alternative strategies to increase omega 3 intake include supplementing animal feed with fish oil to augment the omega 3 content of eggs, meat, and milk. Available foods can also be enriched in eicosapentaenoic and docosahexaenoic acid, although they may impart a fishy aroma or flavour. A different approach independent of an adequate supply of fish oil would involve using modern biotechnology to genetically modify certain plants species, thereby producing plants and plant oils rich in eicosapentaenoic and docosahexaenoic acid.

Fig 4 Synthesis of eicosanoids from omega 6 and omega 3 fatty acids. Arachidonic acid and eicosapentaenoic acid compete for the cyclo-oxygenase and lipoxigenase enzymes for conversion into eicosanoids. Those derived from arachidonic acid are pro-inflammatory and pro-aggregatory, whereas those derived from omega 3 fatty acids are anti-inflammatory and inhibit platelet aggregation.
Table 2 Content of omega 3 fatty acids of selected fish and seafood (adapted from the guidelines of the American Heart Association)\(^\text{13}\)

<table>
<thead>
<tr>
<th>Fish</th>
<th>EPA/DHA content (g) per 100 g serving of fish (wet weight)</th>
<th>Amount of fish (in g) required to provide 1 g EPA/DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuna (fresh)</td>
<td>0.28-1.51</td>
<td>66-357</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>1.28-2.15</td>
<td>42.5-70.9</td>
</tr>
<tr>
<td>Mackerel</td>
<td>0.4-1.85</td>
<td>64-259</td>
</tr>
<tr>
<td>Atlantic herring</td>
<td>2.01</td>
<td>50</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>1.15-2</td>
<td>50-67</td>
</tr>
<tr>
<td>Sardine</td>
<td>1.15-2</td>
<td>50-67</td>
</tr>
<tr>
<td>Haddock</td>
<td>0.47-1.18</td>
<td>88-213</td>
</tr>
<tr>
<td>Tuna (canned)</td>
<td>0.31</td>
<td>323</td>
</tr>
<tr>
<td>Cod</td>
<td>0.28</td>
<td>357</td>
</tr>
<tr>
<td>Haddock</td>
<td>0.24</td>
<td>417</td>
</tr>
<tr>
<td>Calfish</td>
<td>0.18</td>
<td>506</td>
</tr>
<tr>
<td>Flounder or sole</td>
<td>0.49</td>
<td>204</td>
</tr>
<tr>
<td>Oyster</td>
<td>0.44</td>
<td>227</td>
</tr>
<tr>
<td>Shrimp</td>
<td>0.32</td>
<td>313</td>
</tr>
<tr>
<td>Scallop</td>
<td>0.2</td>
<td>500</td>
</tr>
<tr>
<td>Cod liver oil capsule</td>
<td>0.19</td>
<td>5</td>
</tr>
<tr>
<td>Omega-3 (Pronova)</td>
<td>0.85</td>
<td>1</td>
</tr>
</tbody>
</table>

EPA= eicosapentanoic acid; DHA= docosahexanoic acid.

Men of patients after myocardial infarction. Patients should consume about 1 g/day of eicosapentanoic acid and docosahexanoic acid, preferably by increasing their intake of oily fish to at least two servings per week. Fish oil capsules may be considered for those unable to tolerate fish or change their diet effectively. Approved pharmaceutical grade capsules should be prescribed rather than encouraging over the counter supplements.

Recent guidelines from the American Heart Association (Box 2) have gone further, supporting the use of fish oil supplements for patients with "documented" coronary heart disease.\(^\text{23}\) However, we believe that more evidence is required before considering fish oil supplements for patients with coronary heart disease outside the specific indications of myocardial infarction. Others have argued that fish oil supplements should not be recommended routinely for patients after myocardial infarction until more definitive evidence is available.\(^\text{13}\)

No trial has assessed the effects of fish oils on risk of coronary heart disease in primary prevention, and therefore explicit recommendations for this group cannot be made currently. Such a trial may prove impractical in terms of the numbers required. However, on the basis of evidence from epidemiological and observational studies the consumption of (preferably oily) fish at least twice weekly should be encouraged as part of a balanced diet. Box 3 and table 2 show current consumption and dietary sources of eicosapentanoic and docosahexanoic acid.

Any recommendations regarding fish and fish oils should be balanced against safety issues. Side effects such as fishy aftertaste are uncommon, and gastrointestinal upset is infrequent at moderate intakes.\(^\text{23}\) Some reports show that fish oil may worsen glycaemic control in diabetes, but two meta-analyses found no adverse effects.\(^\text{24,25}\) Furthermore, a recent prospective cohort study found that a higher consumption of omega 3 fatty acids was associated with a lower incidence of coronary heart disease and mortality in diabetic women.\(^\text{12,13}\) Concerns have been raised regarding adverse effects on low density lipoprotein (LDL) cholesterol and oxidative stress, but increases in LDL cholesterol are modest and studies into oxidative stress have been contradictory.\(^\text{24,25}\) Overall these effects are unlikely to be dominant given the apparent cardiac benefits of omega 3 fatty acids. More specific concerns regarding dietary fish relate to environmental contaminants, and a recent study showed that mercury in fish may attenuate their cardioprotective effects.\(^\text{23}\) Contaminants accumulate in larger, predatory fish, and consumption of a variety of fish should minimise any possible adverse effects.\(^\text{23}\)

**Future directions**

Despite advances in our understanding of the cardioprotective effects of fish oils in the past three decades, many issues remain unresolved. A double blind, placebo controlled trial of fish oil capsules in patients after myocardial infarction is required, and further trials are needed in individuals with risk factors for coronary heart disease or with heart failure. The specific effects of eicosapentanoic acid versus docosahexanoic acid on risk of coronary heart disease and the relative merits of oily fish compared with fish oil

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**Clinical review**

Clinical Nutrition Foundation. Charitable organisation promoting healthy eating through impartial interpretation of nutritional knowledge and advice (www.nutrition.org.uk/)

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**Additional educational resources**

**Websites**

- www.nal.usda.gov/fnic/foodcomp/ - USDA Nutrient Data Laboratory, a database with online search function to find the omega-3 contents of various foods
- www.foodstandardsgovuk/multimedia/pdfs/4is40_2003.pdf - Food Standards Agency UK, provides data on mercury content of various fish and advice on safe consumption for pregnant women and women of childbearing potential.

**Reviews**

- *American Journal of Clinical Nutrition* 2000;71(suppl 1) - dedicated to fish oils and omega-3 fatty acids. Twenty articles covering a range of subjects from current intake and biological actions through to effects on a wide range of medical conditions.

**For patients**

Omega 3 Information Service. A wealth of well balanced information about omega 3 fatty acids, including their effects on medical conditions apart from coronary heart disease (www.omega-3info.com/)

British Nutrition Foundation. Charitable organisation promoting healthy eating through impartial interpretation of nutritional knowledge and advice (www.nutrition.org.uk/)
capsules also require further investigation. In addition to trials with clinical end points, research efforts should be focused on understanding the mechanisms by which fish oils might confer cardiac benefits. This will allow us not only to refine the clinical applications of fish oils but hopefully also to identify other therapeutic targets and help guide the development of future treatments for coronary heart disease.

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Competing interests None declared.


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Corrections and clarifications

Editor's Choice and the editor "An extreme failure of concordance"

In his Editor's Choice of 11 October, Richard Smith wrote about the failure of communication between a Hungarian family in California with a daughter with severe epilepsy and the California healthcare system—as described in a book from which we published an extract in the same issue (as a "filler," p 807). Unfortunately, Smith said that Lia had now died. He was wrong to say this; she is still alive. Annie Fadiman, the author of the book (The Spirit Catches You and You Fall Down) has asked us to make clear that "Lia suffered profound neurological damage after an episode of status epilepticus and that the parents thought that the doctors and their drugs had injured rather than helped." In the filler, we also misspelled the first name of the book's author and introduced a typo to apostrophe into the word "Frictions." We apologize for these errors to all concerned.

Communicating risks of the population level: application of population impact numbers

Richard F Heller and colleagues have reported an error in their Education and Debate article (15 November, pp 1102-5) that was due to a little-recognized problem in calculating population attributable risk for multiple levels of exposure. This led to an overestimation of population attributable risk in table 2, which shows the impact of blood cholesterol concentration on premature death from coronary heart disease (p 1102). However, this does not alter the general conclusion drawn from the table (that the population level of cholesterol concentrations of 5.2-6.4 mmol/l and of 6.5-7.8 mmol/l is larger than that of concentrations above 7.8 mmol/l) or the substance of the article. Full details of the correct calculations and the corrected table 2 appear on bmj.com (http://bmj.bmjournals.com/cgi/content/full/327/7424/1162-DC1).

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Flow cytometric analysis of circulating platelet-monocyte aggregates in whole blood: Methodological considerations

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Summary
Platelet-monocyte aggregates are increasingly being used to quantify platelet activation. The variables that influence platelet-monocyte aggregates have not been well defined. We sought to determine the effect of blood collection, handling and processing techniques on detected levels of platelet-monocyte aggregates using a flow cytometric assay. Whole blood was labelled with anti-CD14-PE and anti-CD42a-FITC. Thereafter, samples were fixed and red cells lysed. Analysis was performed with the flow cytometer initially triggering on light scatter and then on FL-2 to identify CD14-PE positive monocytes. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. The effect of collection, handling and processing techniques on this assay were assessed. Anticoagulation with heparin (20.1 ± 2.0%), PPACK (16.8 ± 1.9%), sodium citrate (12.3 ± 1.6%) and EDTA (9.5 ± 1.0%) resulted in markedly different levels of platelet-monocyte aggregation (P<0.0001). Platelet-monocyte aggregation was higher in samples obtained from intravenous cannulae compared to those obtained by venepuncture (20.9 ± 3.9% vs. 13.8 ± 2.4%, P=0.03). For every 10 minutes of delay prior to processing platelet-monocyte aggregates increased by 2.8% (P=0.0001) in PPACK anticoagulated blood and 1.7% (P=0.01) in citrate anticoagulated blood. Erythrocyte lysis together with fixation does not affect platelet-monocyte aggregation. Platelet-monocyte aggregates remained stable over 24 hours when fixed and stored at 4°C. Multiple handling and processing factors may affect platelet-monocyte aggregation. We recommend the measurement of platelet-monocyte aggregates on samples collected by direct venepuncture, using a direct thrombin inhibitor as the anticoagulant and minimising the time delay before sample fixation.

Keywords
Platelet activation, leukocytes, methods, flow cytometry

Introduction
Platelets play a key role in mediating inflammatory and thrombotic responses during both health and disease. Once activated, circulating platelets bind to leukocytes, predominantly monocytes, to form platelet-leukocyte aggregates (1–3). This is initiated largely through the binding of platelet surface P-selectin on activated platelets to its leukocyte counter-receptor, P-selectin glycoprotein ligand-1 (PSGL-1) (4–6). Platelet-leukocyte aggregates are then stabilized by several mechanisms, including the binding of leukocyte Mac-1 (CD11b/CD18) and platelet glycoprotein Ibα (7).

Flow cytometric analysis of platelet surface expression of P-selectin has previously been considered to be the "gold standard" marker of platelet activation. However, in-vivo activated platelets rapidly lose their surface P-selectin whilst continuing to circulate and function (8). Recently, it has been demonstrated that platelet-monocyte aggregates remain detectable in peripheral blood for significantly longer and are a more sensitive marker of in-vivo platelet activation than platelet-surface P-selectin expression (9).

Given that platelet-monocyte aggregates can be readily quantified using flow cytometric analysis and the high sensitivity of the assay it is not surprising that there has been an rapid growth in the use of this assay to quantify platelet activation. In the last year alone over 40 papers assessing platelet-monocyte or platelet-leukocyte aggregates have been published.

The high sensitivity of the assay leaves it vulnerable to artificial in-vitro activation, and reliable laboratory methods are essential to ensure accurate and consistent results. A number of
variables including choice of anticoagulant, sample collection, handling and processing techniques may potentially affect the assessment of platelet-monocyte aggregates and vary markedly between published studies. While several flow cytometric protocols have been described (10–12), the influence of the above factors on measured levels of platelet-monocyte aggregation remains incompletely characterised. In the present study, we sought to determine the effect of blood collection, handling and processing techniques on detected levels of platelet-monocyte aggregates using a simple two-colour flow cytometric assay.

Materials and methods

**Subjects and blood collection**

Peripheral venous blood was obtained from healthy volunteers, aged between 18 and 35 years, who were taking no medication. Ethical approval was obtained from the local research ethics committee and all subjects provided written informed consent. Blood was drawn by clean venepuncture of a large antecubital vein using a 19-gauge needle, unless otherwise stated. Care was taken to ensure a smooth blood draw without venous stasis. Unless otherwise stated, blood was collected into tubes containing the direct thrombin inhibitor, D-Phenylalanine-L-prolyl-L-arginine chloromethyl ketone (PPACK, Cambridge Biosciences). Tubes were gently inverted to ensure mixing of whole blood with anticoagulant.

**Antibodies and other reagents**

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Fluorescein isothiocyanate (FITC)-conjugated CD42a (GR-P; IgG1), and control IgG1 were obtained from Serotec Ltd (Oxford, UK). Phycoerythrin (PE)-conjugated CD14 (Tuk-4, IgG2a) was obtained from DakoCytomation (Buckinghamshire, UK). FACSLyse was obtained from Becton-Dickinson (Cowley, UK).

**Immunolabelling and flow cytometry**

Immunolabelling and flow cytometry were performed in whole blood to avoid centrifugation and washing steps which can lead to artifactual platelet activation. Aliquots of whole blood (50 μl) were incubated with anti-CD14-PE, anti-CD42a-FITC and isotype matched controls for 20 minutes (min) at room temperature. Thereafter, samples were fixed and the red cells lysed by the addition of 500 μl of FACS-Lyse solution (Becton Dickinson) unless otherwise stated. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm laser (Beckman Coulter, High Wycombe, UK) within 4 hours (h) unless otherwise stated. A medium flow setting was used to minimize leukocyte-platelet coincident events. Monocytes were identified based on their forward and side scatter characteristics and then by triggering on FL-2 to identify CD14-PE positive monocytes and exclude large granular lymphocytes. For each measurement a minimum of 2,500 monocytes were collected. Platelet-monocyte aggregates were defined as monocytes positive for CD42a (Fig. 1). All results are expressed as percentage of positive cells.

![Flow cytometric analysis of platelet-monocyte aggregates in whole blood](image-url)
Analyses were performed using EXPO32 software (Beckman Coulter, High Wycombe, UK).

Experimental design
Using the above flow cytometric protocol, with modifications where appropriate, we conducted a series of six studies to assess the effects of different blood collection and sample handling methods on the measured level of circulating platelet-monocyte aggregates.

Protocol 1: Choice of anticoagulant
To determine the effect of different anticoagulants on platelet-monocyte aggregation, blood from eight healthy volunteers was incubated for 5 min with sodium citrate (0.106 M, Sarstedt Monovette), lithium heparin (16 IU/ml, Sarstedt Monovette), EDTA (1.6 mg/ml, Sarstedt Monovette) and PPACK (75 μM, Haematologic Technologies Inc). All samples were then labelled with appropriate monoclonal antibodies, and fixed before flow cytometric analysis as described above.

Protocol 2: Effect of serial sampling via intravenous cannula
We determined the effect of serial sampling of whole blood through an intravenous cannula on platelet-monocyte aggregation. A group of eight healthy volunteers underwent serial blood sampling through both repeated venepuncture or through an intravenous cannula. Blood was drawn at baseline, 30 min, 50 min and 85 min. Repeated venepuncture was performed from large antecubital veins using 19-gauge needles. Sampling from intravenous cannulae was performed after a 17-gauge cannula had been inserted into a large antecubital vein. When sampling from the cannula the first 2 – 5 ml of blood were discarded, and after each blood draw the cannula was flushed with 10 ml of normal saline. Only cannulae which allowed a smooth blood draw after insertion were accepted. Blood samples taken at each time point were labelled with appropriate monoclonal antibodies before fixation and flow cytometric analysis as described above.

Protocol 3: Time delay prior to immunolabelling
To study the effect of time delay between sample draw and sample preparation on spontaneous in-vitro platelet-monocyte aggregation, blood was collected from eight healthy volunteers and anticoagulated with sodium citrate (0.106 M, Sarstedt Monovette) or PPACK (75 μM, Haematologic Technologies Inc). After intervals of 0, 10, 20, 30 and 60 min individual aliquots were labelled with monoclonal antibodies. Samples were then fixed and taken for flow cytometric analysis as described above.

Protocol 4: Effect of erythrocyte lysis
In order to determine the effect of erythrocyte lysis on platelet-monocyte aggregation, blood from eight healthy volunteers was anticoagulated in PPACK before incubation in duplicate with appropriate monoclonal antibodies as above. After 20 min duplicate samples underwent either a) fixation and erythrocyte lysis by the addition of 500 μl of 1:10 FACS-lysing solution (Becton Dickinson) or b) fixation with no erythrocyte lysis by the addition of 500 μl of 1% formaldehyde. Flow cytometric analysis in the lysis group was performed as described above. As it is not possible to distinguish readily leukocyte subpopulations in whole blood without erythrocyte lysis, unlysed samples were collected using a live gate for CD14-positive events. Monocytes were subsequently identified on the basis of their characteristic scatter properties. As unlysed whole blood results in a very cell-rich preparation passing the laser, samples were processed with a low flow rate to minimise the risk of coincident events. The time to collect 1,000 CD14-positive events was recorded for both lysed and unlysed samples.

Protocol 5: Sample stability after fixation
We assessed the effects of time delay on the stability of fixed samples prior to flow cytometric analysis. Blood from eight healthy volunteers was anticoagulated with PPACK and labelled with appropriate monoclonal antibodies prior to fixation as above. Aliquots were then stored at 4°C. These samples were then analyzed by flow cytometry to determine the level of platelet-monocyte aggregation at the following time points: immediately, 4 h, 8 h, 12 h and 24 h.

Protocol 6: Reproducibility
Blood was drawn from eight healthy volunteers and anticoagulated in PPACK. After 5 min, five individual aliquots of blood were each incubated with appropriate monoclonal antibodies for 20 min before fixation and subsequent flow cytometric analysis to determine the level of platelet-monocyte aggregation. The coefficient of variation was then derived.

Statistical analysis
Continuous variables are reported as mean ± standard error of the mean. Statistical analyses were performed using Student’s t-test, two-way ANOVA or linear regression where appropriate. In order to account for the repeated measures when analysing the effect of sampling technique, time delay and fixation on platelet-monocyte aggregation a form of linear regression was fitted using mixed linear models. Statistical significance was taken at 5%. Calculations were performed using SAS version 8.2 (SAS Institute, Cary, NC, USA).

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Figure 2: Effect of anticoagulant on platelet-monocyte aggregation. Platelet-monocyte aggregation was assessed in blood anticoagulated with either heparin, PPACK, sodium citrate or EDTA. Data are presented as mean ± SEM (n=8). *P<0.01, **P<0.002, ***P<0.05.
Results

Choice of anticoagulant

The type of anticoagulant used had a marked effect on the measured platelet-monocyte aggregate levels (Fig. 2). Platelet-monocyte aggregation increased in blood anticoagulated with heparin (20.1 ± 2.0%) compared to PPACK (16.8 ± 1.9%, P=0.01), sodium citrate (12.3% ± 1.6%, P=0.0001) or EDTA (9.5% ± 1.0% P=0.0001). Levels of platelet-monocyte aggregates were reduced in blood anticoagulated with sodium citrate or EDTA compared to PPACK (P<0.01).

Blood collection: Sampling via intravenous cannula compared with venepuncture

At baseline (time 0), samples obtained from intravenous cannulae had a significantly higher level of platelet-monocyte aggregation compared to those obtained by venepuncture (20.9 ± 3.9% vs. 13.8 ± 2.4%, P=0.03; Fig. 3). The levels of platelet-monocyte aggregation increased steadily when repeated samples were taken form an intravenous cannula (1.5% per 10 min interval, 95% CI 0.6 to 2.4%, P=0.001) but did not change with time when serial sampling was performed by venepuncture (0.3% per 10 min interval, 95% CI -0.6 to 1.2%, P=0.47) (Fig. 3).

Effect of time delay between collection and immunolabelling

The percentage of platelet-monocyte aggregates in unfixed samples increased in a time-dependent manner (Fig. 4). Based on a mixed linear model that allowed the PPACK group to have a different (greater) variance the percentage of platelet-monocyte aggregates in blood anticoagulated with PPACK increased by 2.8% (95% CI 2.2 to 3.4%, P<0.0001) in PPACK anticoagulated blood (closed squares) and 1.7% (95% CI 0.1 to 2.9%, P=0.01) in citrate anticoagulated blood (closed triangles). Data are reported as mean ± SEM (n=8).

Effect of erythrocyte lysis on platelet-monocyte aggregation

Erythrocyte lysis had no effect on platelet-monocyte aggregation (16.9 ± 2.8% vs. 16.1 ± 2.5%, P=0.4). There was a strong correlation between levels of platelet-monocyte aggregation from lysed and unlysed samples (r=0.97, P<0.0001). Bland-Altman analysis also revealed good agreement between the two methods (mean bias 0.72%, 95% limit of agreement -3.6 to 5.0%). Collection times were shorter when using erythrocyte lysis (67 ± 10 seconds vs. 149 ± 23 seconds, P=0.02).

Sample stability and reproducibility after fixation

In the fixed samples stored at 4°C platelet-monocyte aggregation did not alter over the 24 h period (P=0.26, Fig. 5). The interassay coefficient of variation was calculated for samples anticoagulated with PPACK using the standard method described above (n=8). The mean coefficient of variation for the percentage of platelet-monocyte aggregates was 7.8%.
Discussion

The quantification of platelet-monocyte aggregates is a sensitive measure of platelet activation and has become widely used in clinical research. In the current study we report a simple reproducible method of assessing platelet-monocyte aggregates and identify a number of factors which may substantially influence the levels of platelet-monocyte aggregation.

Several methods for the flow cytometric analysis of platelet-leukocyte aggregates have been described. In the present study, we utilised a two-colour whole blood technique incorporating erythrocyte lysis and fixation to quantify platelet-leukocyte aggregates. This methodology has been widely used for the assessment of platelet-monocyte aggregation (6, 12-15). We chose to assess platelet-monocyte aggregates rather than platelet-leukocyte aggregates or platelet-neutrophil aggregates as work by Michelson et al. had previously suggested that platelet-monocyte aggregates may be a more robust indicator of in-vivo platelet activation (9).

We utilised methodology incorporating erythrocyte lysis as it allows efficient and accurate discrimination of leukocyte subpopulations, the ease of which such assays can be performed and the cytometric problems that are avoided (12). Li et al. (10) have previously suggested that red cell lysis can lead to artificial increases in platelet-leukocyte aggregation. Although they demonstrated increased platelet-leukocyte aggregation with erythrocyte lysis, their lysis technique involved considerable additional sample manipulation including repeated washing and centrifugation with no fixation. Notably, the authors acknowledged that the marked artefacts observed with their lysis protocol were likely to have been predominantly due to repeated centrifugation rather than a major effect of lysis. In contrast, we avoided any additional centrifugation or washing steps that could activate platelets or monocytes when assessing the effect of erythrocyte lysis. In addition, our protocol included fixation at the time of erythrocyte lysis which would minimise the opportunity for platelet activation by ADP released from erythrocytes. Our results suggest that red cell lysis along with immediate fixation does not increase platelet-monocyte aggregation and is less time consuming to perform.

The current study demonstrates that the type of anticoagulant used has a marked effect on the measured levels of platelet-monocyte aggregation. We and others have previously demonstrated that unfractionated heparin activates platelets and increases platelet-monocyte aggregation by a P-selectin-dependent mechanism (16-19). The anticoagulant activity of EDTA and citrate is achieved through chelation of divalent metal cations.

We have previously demonstrated that cation chelation with both EDTA and EGTA markedly reduces platelet-leukocyte interactions in vitro (6). Residual binding in conditions of cation chelation is likely to be attributable to sub-total cation chelation, of relevance when working with platelets that carry a large reservoir of divalent cations (20). Furthermore, we can also demonstrate a reduction in platelet-neutrophil binding with EDTA and citrate (data not shown), in keeping with work from Simon et al. (7), demonstrating the important effects of cation chelation upon Mac-1/CD11b mediated binding events. Finally, it should be noted that standard protocols for leukocyte density gradient centrifugation utilise sodium citrate specifically to reduce platelet adherence to leukocytes in vitro (21). Thus, it is not surprising that the calcium-chelating anticoagulants, EDTA and trisodium citrate, were associated with lower levels of platelet-monocyte aggregates. In contrast, direct thrombin inhibitors such as PPACK provide reliable anticoagulation without cation chelation or causing platelet activation (16). For these reasons we prefer the use of PPACK over the other anticoagulants.

Our study also demonstrates that platelet-monocyte aggregates increase in a time dependent manner in vitro. This increase occurred in blood anticoagulated with either PPACK or citrate. It is therefore important to perform processing as soon as possible and to standardise this time interval. The rate of increase in platelet-monocyte aggregates appeared to be slower in samples anticoagulated with citrate. Therefore, in situations where there may be a substantial delay prior to immunostaining and fixation it may be appropriate to use citrate as the anticoagulant in an attempt to reduce in vitro platelet-monocyte aggregate formation.

Following immunostaining and fixation, samples stored at 4°C were stable for 24 h. If methods without fixation are used then it would be important to analyse samples immediately after immunostaining to minimise artefactual in-vitro increases in platelet-monocyte aggregates. However, this is often difficult to achieve in practice as there may be multiple samples to run, limited access to a flow cytometer or separation between the sites where samples are collected and the flow cytometric analysis is performed.

Handling and processing techniques affect the measurement of platelet activation and platelet-monocyte aggregation. These factors should be standardised and fully described in the description of study methods. Where possible, we recommend i) avoiding taking blood samples through intravenous cannulae, ii) using a direct thrombin inhibitor as an anticoagulant, iii) minimising and standardising time delays between blood collection and processing, and iv) storing fixed samples at 4°C prior to analysis.

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Harding et al. Platelet-monocyte aggregates

Dietary intervention with oil rich fish reduces platelet-monocyte aggregation in man

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Abstract

Background: Dietary intake of fish rich in omega-3 fatty acids is associated with a reduction in cardiovascular events. The mechanisms for this are uncertain and previous studies investigating effects on platelet function have produced inconsistent results. Platelet-monocyte aggregation is a sensitive marker of platelet activation and may contribute to the initiation and progression of atherothrombosis. This study assessed the effect of dietary intervention with oily fish on platelet-monocyte aggregation in healthy subjects.

Methods: Fourteen subjects had their diet supplemented with 500 g of oil-rich fish per week for 4 weeks. A control group of 14 subjects received no dietary intervention over a 4-week period. Platelet-monocyte aggregates were assessed with flow cytometry.

Results: Dietary intervention with fish led to an increase in omega-3 fatty acids in plasma phospholipids (14.2 ± 3.4% versus 5.8 ± 1.3%, \( P < 0.001 \)). In contrast to the control group, platelet-monocyte aggregates were reduced by 35% following dietary intervention with oily fish (16.0 ± 9.0% versus 24.8 ± 10.9%, \( P < 0.01 \)), and returned to basal levels 4 weeks after discontinuation of supplementation. There was an inverse correlation between platelet-monocyte aggregation and plasma omega-3 fatty acid concentrations (\( r = -0.421, P = 0.006 \)). There were no changes in the plasma markers of platelet activation, soluble P-selectin or soluble CD40 ligand.

Conclusions: We have demonstrated, for the first time, that dietary intervention with oil-rich fish reduces platelet-monocyte aggregation in man. Our results suggest that reduced platelet activation provides a potential mechanism through which fish oils confer their cardiovascular preventative benefits.

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Keywords: Oily fish; Omega-3 fatty acids; Platelet activation; Platelet-monocyte aggregates; Cardiovascular disease

1. Introduction

Consumption of fish oil may protect against cardiovascular disease [1]. Data from observational studies suggest that fish intake is inversely associated with fatal coronary heart disease [2], and randomized controlled trials of dietary fish or fish oil supplementation after myocardial infarction demonstrate a reduction in mortality [3,4]. These effects are believed to be due to the high amounts of omega-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), present in fish oil. EPA and DHA must be obtained from dietary sources and recent guidelines from the American Heart Association recommend the consumption of oily fish at least twice weekly for individuals with and without coronary heart disease [5]. However, not all randomised controlled trials have shown a benefit [6], and the underlying mechanisms through which cardiac protection might be conferred remain uncertain.

The effects of fish oil on platelet function and thrombosis are controversial. Although fish oils were originally thought
to act by reducing platelet aggregation through effects on eicosanoid metabolism [7], subsequent studies have produced contradictory results [8]. Alternative mechanisms of action have been proposed including effects on arrhythmias, inflammation, endothelial function and atherosclerotic plaque stability [9–12]. Indeed, it has been suggested that there is little evidence for a major antithrombotic effect of fish oils at the moderate doses shown to reduce mortality in the secondary prevention trials [13].

Most previous studies investigating the effects of fish oils on platelet function have relied on in vitro platelet aggregometry and plasma assays of soluble markers of platelet activation [8]. These techniques are poorly suited to the detection of potentially subtle effects induced by dietary changes. Reproducibility is unsatisfactory and the tests are difficult to standardize [14]. Platelet aggregometry can only measure ex vivo changes in platelet reactivity to a single external stimulus, and sample manipulation required for both methods leaves them particularly vulnerable to artefactual in vitro activation.

Modern flow cytometric techniques allow platelets to be analyzed directly in their physiological environment of whole blood with minimal sample manipulation. Circulating platelet-monocyte aggregates, formed by the binding of activated platelets to leukocytes via a P-selectin dependent mechanism [15], can be readily measured in this way and have emerged as a highly sensitive marker of platelet activation [16]. The adhesion of activated platelets to monocytes also has important functional consequences, and can induce the expression of cytokines, chemokines, adhesion molecules and tissue factor [17]. Consistent with these biological effects, circulating platelet-monocyte aggregates appear to promote atherosclerotic lesion formation and are increased in stable coronary heart disease and acute coronary syndromes [18,19].

We therefore investigated whether supplementing the diet of healthy individuals with a moderate intake of oil-rich fish would reduce platelet activation and platelet-monocyte aggregation.

2. Methods
2.1. Study participants and design

Twenty-eight healthy male volunteers aged between 21 and 28 years were enrolled into the study. Exclusion criteria included those taking regular medication, those with clinical evidence of atherosclerotic vascular disease, hypertension, diabetes mellitus, hypercholesterolemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency. Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects provided written informed consent.

Fourteen volunteers were asked to eat 500 g of mackerel per week for 4 weeks (equivalent to approximately 1 g per day of EPA + DHA). The fish intake was calculated to provide similar amounts of omega-3 fatty acids to those shown to reduce mortality. The remaining 14 participants constituted a control group and received no dietary intervention for a 4-week period. Blood was drawn from all participants at baseline and at the end of the 4-week period, and a further sample was taken from the dietary intervention group 4 weeks after cessation of fish intake. Subjects in the fish supplementation group kept 3-day weighed food diaries at baseline, during fish supplementation and during the washout period to assess changes in dietary intake during the study period.

2.2. Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with sodium citrate (0.106 M, Sarstedt Monovette), 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 of platelet-monocyte aggregates. Plasma was prepared from blood anticoagulated with sodium citrate and EDTA by centrifugation (1500 × g for 30 min). To minimize ex vivo platelet activation, blood was centrifuged within 5 min of collection and separated immediately. Plasma samples were stored at –40 °C until analysis.

2.3. Flow cytometry

The following reagents were used: fluorescein isothiocyanate (FITC)-conjugated CD42a (GRP-P, IgG1), and isotype control IgG1 were obtained from Serotec Ltd. (Oxford, UK), phycoerythrin (PE)-conjugated CD14 (Tuk-4, IgG2a) was obtained from Dako Cytomation (Buckinghamshire, UK) and FACS-Lyse was obtained from Becton-Dickinson (Cowley, UK). Aliquots of whole blood (60 μL) anticoagulated with PPACK were incubated with anti-CD14, anti-CD42a and isotype matched controls for 20 min at room temperature. Thereafter, samples were fixed and the red cells lysed by the addition of 500 μL of FACS-Lyse solution. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK) within 4 h of labelling. Samples were initially analysed with the flow cytometer triggered on light scatter and then by triggering on FL-2 to identify CD14-PE positive monocytes. For each measurement 2500 cells were collected. Platelet-monocyte aggregates were defined as monocytes positive for CD42a. All results are expressed as percentage of positive cells. Analyses were performed using EXPO 32 software (Beckman Coulter, High Wycombe, UK). The mean coefficient of variation for the percentage of platelet-monocyte aggregates is 7.8%.
2.4. Measurement of soluble CD40L and soluble P-selectin

Soluble CD40L and soluble P-selectin concentrations were measured in plasma prepared from blood anticoagulated with sodium citrate. Plasma soluble CD40L and soluble P-selectin concentrations were determined with commercially available enzyme-linked immunosorbant assays (Bender MedSystems, detection limit of 0.095 ng/mL, coefficient of variation 4%; and R&D Systems, detection limit 0.5 ng/mL, coefficient of variation 5.6%; respectively).

2.5. 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-methane-methanol (2:1) containing 0.005% butydated 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%

2.6. Statistical methods

Continuous variables are reported as mean ± standard deviation. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons, or two-tailed Student’s t-tests where appropriate. All calculations were performed using GraphPad Prism (Graph Pad Software). Statistical significance was taken at 5%.

3. Results

3.1. Baseline characteristics

Baseline characteristics were similar between the fish supplementation and control groups (Table 1). Study participants were young and there were no differences in body mass index, smoking status, or lipid profile.

3.2. Effect of fish intervention on plasma phospholipid fatty acid composition

The plasma phospholipid fractions of omega-3 and omega-6 fatty acids did not differ between groups at baseline.
Dietary intervention with fish led to an increase in the percentage of total omega-3 fatty acids (14.2 ± 3.4% versus 5.8 ± 1.3%, P<0.001), EPA (5.2 ± 2.1% versus 1.0 ± 0.3%, P<0.001; Fig. 1A) and DHA (7.5 ± 1.5% versus 3.5 ± 1.0%, P<0.001; Fig. 1B) at 4 weeks compared with baseline. Four weeks after cessation of the fish diet there was a reduction in the percentage of both EPA (1.8 ± 0.7%) and DHA (4.7 ± 1.1%), although DHA concentrations remained significantly higher than baseline (Fig. 1A and B). There was no change in the percentage of α-linolenic acid, a plant derived omega-3, during dietary fish intervention (Fig. 1C). As the proportion of EPA and DHA increased during the fish diet, there was a corresponding reduction in the percentage of arachidonic acid (8.2 ± 0.3% versus 9.9 ± 0.5%, P<0.01), linolenic acid (20.1 ± 0.8% versus 23.1 ± 0.6%, P<0.01), and oleic acid (12.0 ± 0.4% versus 13.7 ± 0.4%, P<0.001) at 4 weeks compared with baseline (Table 2). There were no changes in the plasma phospholipid levels of omega-3 or omega-6 fatty acids in the control group during the 4-week period.

3.5. Nutritional composition and lipid profile during fish intervention

Analysis of the 3-day weighed food diaries in the fish intervention group revealed no differences in intake of energy, protein, fat, carbohydrate or fibre during the study period (Table 3). In terms of mineral intake, there was an increase in iodine (P<0.0001) and selenium (P=0.025) consumption during the fish diet. Vitamin intake was also determined, and revealed an increase in consumption of vitamin D (P<0.0001) and vitamin B12 (P=0.0004) during the fish intervention period. Although there was higher vitamin A intake during the washout period after the cessation of dietary fish this was of borderline statistical significance (P=0.05). Dietary intervention with fish did not affect total cholesterol, HDL-cholesterol, LDL-cholesterol or triglyceride levels (data not shown).

3.4. Effect of fish intervention on platelet-monocyte aggregation

Platelet-monocyte aggregation was reduced by 35% following 4 weeks dietary intervention with oil-rich fish (16.1 ± 9.0% versus 24.8 ± 10.9%, P<0.01; Fig. 2). Platelet-monocyte aggregates returned to baseline levels 4 weeks after cessation of the fish intervention (24.9 ± 9.6% versus 24.8 ± 10.9%, P=NS; Fig. 2). There was no change in platelet-monocyte aggregation in the control group over the corresponding 4-week period (18.7 ± 6.9% versus 21.5 ± 6.7%, P=NS; Fig. 2). There were inverse correlations between levels of platelet-monocyte aggregation in the fish intervention group and the plasma phospholipid percentages of EPA (r = −0.364, P=0.018), DHA (r = −0.45, P=0.003), and total omega-3 fatty acids (r = −0.421, P=0.006).
Table 3

<table>
<thead>
<tr>
<th>Daily nutritional intake in fish supplementation group</th>
<th>Fish intervention group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Baseline</td>
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<td>Energy (kcal)</td>
<td>2530 ± 651</td>
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<tr>
<td>Protein (g)</td>
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<td>Carbohydrate (g)</td>
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<td>Saturated fat (g)</td>
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<td>31 ± 15</td>
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<tr>
<td>Fibre (g)</td>
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<td>15 ± 8</td>
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Minerals

| Sodium (mg)                                          | 3476 ± 986 | 3870 ± 1574 | 3188 ± 680 | 3480 ± 986 | 3870 ± 1574 | 3188 ± 680 |
| Potassium (mg)                                       | 3365 ± 1086 | 3290 ± 1247 | 3315 ± 1235 | 3370 ± 1124 | 3290 ± 1247 | 3315 ± 1235 |
| Calcium (mg)                                         | 968 ± 293 | 930 ± 349 | 936 ± 267 | 970 ± 293 | 930 ± 349 | 936 ± 267 |
| Magnesium (mg)                                       | 353 ± 120 | 348 ± 140 | 307 ± 98 | 354 ± 120 | 348 ± 140 | 307 ± 98 |
| Iron (mg)                                            | 13 ± 5 | 14 ± 6 | 14 ± 8 | 14 ± 5 | 14 ± 6 | 14 ± 8 |
| Zinc (mg)                                            | 10 ± 2 | 9 ± 4 | 8 ± 2 | 10 ± 2 | 9 ± 4 | 8 ± 2 |
| Selenium (µg)                                        | 65 ± 27 | 90 ± 37 | 62 ± 25 | 66 ± 27 | 90 ± 37 | 62 ± 25 |
| Iodine (µg)                                          | 100 ± 35 | 192 ± 44 | 111 ± 53 | 100 ± 35 | 192 ± 44 | 111 ± 53 |

Vitamins

| Vitamin A (µg)                                       | 558 ± 395 | 442 ± 145 | 703 ± 434 | 560 ± 395 | 442 ± 145 | 703 ± 434 |
| Vitamin D (µg)                                       | 1.7 ± 0.9 | 7.0 ± 2.2 | 3.1 ± 2.7 | 1.7 ± 0.9 | 7.0 ± 2.2 | 3.1 ± 2.7 |
| Vitamin E (mg)                                       | 7 ± 3 | 6 ± 3 | 7 ± 4 | 7 ± 3 | 6 ± 3 | 7 ± 4 |
| Vitamin B₁ (mg)                                      | 1.9 ± 0.7 | 1.9 ± 0.6 | 2.1 ± 0.7 | 1.9 ± 0.7 | 1.9 ± 0.6 | 2.1 ± 0.7 |
| Vitamin B₂ (mg)                                      | 2.2 ± 0.9 | 2.3 ± 0.9 | 2.1 ± 0.9 | 2.2 ± 0.9 | 2.3 ± 0.9 | 2.1 ± 0.9 |
| Vitamin B₆ (mg)                                      | 2.5 ± 1.0 | 2.9 ± 1.3 | 2.5 ± 1.0 | 2.5 ± 1.0 | 2.9 ± 1.3 | 2.5 ± 1.0 |
| Vitamin B₁₂ (µg)                                     | 3.6 ± 1.1 | 7.0 ± 1.8 | 4.3 ± 3.2 | 3.6 ± 1.1 | 7.0 ± 1.8 | 4.3 ± 3.2 |
| Folate (µg)                                          | 362 ± 166 | 354 ± 173 | 316 ± 165 | 362 ± 166 | 354 ± 173 | 316 ± 165 |

Values are reported as mean ± S.D. Statistical analyses were performed using one-way ANOVA with repeated measures and Bonferroni’s post-tests for multiple comparisons.

1\(^{1}\) P<0.05 vs. baseline.
2\(^{2}\) P<0.001 vs. baseline.
3\(^{3}\) P<0.001 vs. 4 weeks.
4\(^{4}\) P<0.01 vs. 4 weeks.

3.5. Effects of fish intervention on soluble CD40 ligand and soluble P-selectin

Plasma concentrations of soluble P-selectin did not change after the dietary fish intervention (46.4 ± 11.1 ng/mL versus 51.8 ± 10.8 ng/mL, P = NS) or subsequent washout period (48.1 ± 12.7 ng/mL versus 51.8 ± 10.8 ng/mL, P = NS). There was also no change in concentrations of soluble CD40 ligand during fish intervention (1.9 ± 2.0 ng/mL versus 1.9 ± 2.0 ng/mL, P = NS) or washout (1.9 ± 2.1 ng/mL versus 1.8 ± 1.8 ng/mL, P = NS). In the control group there were no differences in either soluble P-selectin (42.3 ± 8.2 ng/mL versus 44.0 ± 10.3 ng/mL, P = NS) or soluble CD40 ligand (1.9 ± 1.7 ng/mL versus 1.8 ± 1.8 ng/mL, P = NS) at 4 weeks compared with baseline.

4. Discussion

We have demonstrated, for the first time, that dietary intervention with oily-rich fish is associated with a reversible reduction in platelet-monocyte aggregation in man. There was an inverse correlation between platelet-monocyte aggregation and the plasma levels of EPA and DHA, consistent with the effects being related to the omega-3 content of oily fish. Our results suggest that reduced platelet activation could represent an important mechanism through which dietary fish confer their putative cardiovascular benefits.

Platelet activation has a central role in the pathogenesis of atherothrombosis, and the adhesion of activated platelets to monocytes has important pro-inflammatory consequences. Furthermore, platelet-monocyte aggregates promote the formation of atherosclerotic lesions in ApoE⁻/⁻ mice via the delivery of platelet-derived mediators to the monocyte surface and vessel wall [18]. 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 [19]. Therefore, a reduction in platelet-monocyte aggregation could reflect a mechanism by which dietary fish might influence critical events in the progression of atherosclerosis and its complications.

Previous research investigating the effects of fish oils on platelet function has largely focused on platelet aggregometry and measurements of thromboxane synthesis [8]. These studies have produced inconsistent results, and the effects of fish oil on platelet activation have remained unclear. However, the techniques used suffer from poor reproducibility and the sample manipulation required makes them vulnerable to artificial platelet activation. Plasma assays of platelet release products are also indirect measures of platelet activation at best. Furthermore, there is uncertainty about the relation of ex vivo platelet reactivity assessed by traditional aggregometry and actual platelet-vascular interactions in vivo. In contrast, the flow cytometric analysis of platelet-monocyte aggregates in whole blood represents a highly sensitive and
physiologically relevant measure of platelet activation [16]. Modern flow cytometric techniques allow the detection of specific activation-dependent changes in the platelet surface and as well as their interactions with other cells. They are therefore able to provide important mechanistic insights into platelet and leukocyte responses. Indeed, our results are consistent with a previous report which used flow cytometric analysis of platelet activation and demonstrated reductions in platelet surface P-selectin and glycoprotein IIb/IIIa expression after EPA supplementation [20].

Over recent years reduced platelet activation has fallen somewhat out of favor as a primary explanation for the cardiovascular benefits of fish oils [9,13,21], perhaps because of the inconsistent results and technical limitations in assessing platelet function. Instead, it has been suggested that an anti-arrhythmic effect may be dominant because of the reduction in sudden death observed in clinical trials [4,9]. However, clear evidence of a direct anti-arrhythmic effect in humans is lacking [22–25]. The mechanism through which fish oils confer their benefits therefore remains unclear, and our findings raise the possibility of potentially important and previously unrecognized effects on platelet and leukocyte function. Such mechanisms remain consistent with reduced sudden death, as most are triggered by coronary thrombosis after rupture of a vulnerable atherosclerotic plaque.

We did not observe any effect of dietary fish intervention on the plasma markers of platelet activation, soluble P-selectin and soluble CD40 ligand. Although one report has demonstrated a reduction in soluble P-selectin with high dose omega-3 fatty acids [26], our results are consistent with the majority of published studies which find no effect on soluble P-selectin [22,27,28]. We found that dietary fish supplementation did not alter soluble CD40 ligand, in keeping with the only previous study to assess the effects of omega-3 fatty acids on this biomarker [29]. This lack of effect may reveal the limitations of plasma assays of soluble markers of platelet activation. These are vulnerable to artifactual in vitro activation as a result of the separation procedures required, and cannot assess activation-dependent events on the cell surface. In comparison with the measurement of platelet-monocyte aggregates, these assays may not have the sensitivity required to detect the potentially subtle effects expected in dietary intervention studies.

Previous studies report that anti-platelet effects of fish oils have only been apparent at very high doses, with little evidence for a major anti-thrombotic effect with more practical intakes [13]. However, the reduction in platelet-monocyte aggregation in the present study was achieved with moderate amounts of fish, similar to those shown to reduce mortality in secondary prevention trials [3,4]. The cardiovascular effects of oil-rich fish are believed to be primarily due to the high levels of omega-3 fatty acids present, and the inverse correlations demonstrated between plasma EPA and DHA and platelet-monocyte aggregation are in keeping with this hypothesis. However, this does not prove causation and it is possible that accompanying nutritional changes may have contributed to the observed effects. Although we found a clear reduction in platelet-monocyte aggregation after 4 weeks of fish consumption, our study was limited to three time points. It is not clear whether the effect on platelet-monocyte aggregation might have become apparent at an earlier stage, and further studies with analyses at weekly time points would be of considerable interest.

5. Conclusion

We have demonstrated that dietary intervention with moderate amounts of oil-rich fish can reduce platelet-monocyte aggregation in man. Platelet-monocyte aggregates are sensitive markers of platelet activation and contribute to the initiation and progression of atherosclerosis. This could represent a previously unreported mechanism through which fish oils might confer their potential cardiovascular benefits. Most previous studies assessing the effects of fish oils on platelet function have used older techniques with limited reproducibility and physiological relevance. We suggest a renewed research focus into the actions of fish oils on inflammation and thrombosis using modern research tools to investigate interactions between platelets, leukocytes and the vasculature. Establishing the mechanism through which fish and fish oils confer their potential preventative benefits may help guide the application and development of future therapeutic interventions.

Acknowledgements

We would like to thank Shona Johnstone for technical assistance with flow cytometry, and Lianne Milligan for performing analysis of the weighed food records. JD was supported by a British Heart Foundation Project Grant (PG/2003/009).

References

ORIGINAL ARTICLE

Effect of moderate walnut consumption on lipid profile, arterial stiffness and platelet activation in humans

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Background/Objectives: A large intake of walnuts may improve lipid profile and endothelial function. The effect of moderate walnut consumption is not known. We investigated whether a moderate intake of walnuts would affect lipid profile, arterial stiffness and platelet activation in healthy volunteers.

Subjects/Methods: A total of 30 healthy males were recruited into a single-blind randomized controlled crossover trial of 4 weeks of dietary walnut supplementation (15 g/day) and 4 weeks of control (no walnuts). Arterial stiffness was assessed using pulse waveform analysis to determine the augmentation index and augmented pressure. Platelet activation was determined using flow cytometry to measure circulating platelet-monocyte aggregates.

Results: There were no differences in lipid profile after 4 weeks of walnut supplementation compared with control. Dietary intake of α-linolenic acid was increased during the walnut diet (2.1 ± 0.4 g/day versus 0.7 ± 0.4 g/day, P<0.0001). There were no differences in augmentation index or augmented pressure during walnut supplementation. Walnut supplementation did not affect platelet-monocyte aggregation.

Conclusions: Dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in man. Our results suggest that the potentially beneficial cardiac effects of walnuts may not be apparent at lower and more practical levels of consumption.

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Keywords: walnuts; lipids; platelet activation; arterial stiffness; cardiovascular disease

Introduction

Frequent consumption of nuts may be protective against coronary heart disease (Hu and Stampfer, 1999). Nuts are rich in unsaturated fatty acids and contain potentially beneficial compounds including antioxidants, L-arginine, fibre and folic acid (Kris-Etherton et al., 1999). Walnuts are unique because they also contain high levels of α-linolenic acid, a plant-derived omega-3 fatty acid that may confer additional cardiac protection (de Lorgeril and Salen, 2004). Dietary intervention studies have demonstrated that a large intake of walnuts can reduce low-density lipoprotein (LDL) cholesterol (Banel and Hu, 2009). In addition, walnuts may improve endothelial function in patients with hyperlipidaemia or diabetes (Ros et al., 2004; Ma et al., 2010). However, the precise mechanisms through which walnuts confer their putative cardiac benefits remain uncertain.

The endothelium has a critical role in the regulation of vascular smooth muscle tone, thrombosis and inflammation. Vascular smooth muscle tone is an important determinant of central arterial pressure and arterial stiffness. Large artery stiffness, wave reflections and central pulse pressure are inversely associated with endothelial function and can be measured noninvasively using pulse waveform analysis (McEniery et al., 2006). The endothelium also regulates local
platelet activation and aggregation through the release of paracrine factors, such as nitric oxide and prostacyclin. Platelet-leukocyte aggregates are highly sensitive markers of platelet activation that are closely related to impaired endothelial vasomotor function (Robinson et al., 2006). They contribute to the development of atherothrombosis and are elevated in coronary heart disease and acute coronary syndromes (Sarma et al., 2002).

Marine-derived long chain ω-3 fatty acids reduce arterial compliance and platelet activation (Din et al., 2004, 2008). We therefore hypothesized that dietary intervention with walnuts, rich in plant-derived ω-3 fatty acids, would reduce arterial stiffness and platelet activation in humans. Previous intervention studies have asked participants to consume large amounts of walnuts (30–108 g or ~8–27 whole walnuts daily) providing up to 50% of the total dietary fat intake. This proportion of fat energy from walnuts may be too high to be considered practical, and no previous trials have assessed the effect of a more realistic intake of walnuts. In the present study we examined the effect of a moderate intake of walnuts on lipid profile, arterial stiffness and platelet activation.

Subjects and methods

Study participants and design

A total of 30 healthy male volunteers were enrolled into the study. Exclusion criteria included those taking regular medication, those with clinical evidence of atherosclerotic vascular disease, hypertension, diabetes mellitus, hypercholesterolaemia, an intercurrent illness likely to be associated with an acute phase inflammatory response, and renal or hepatic insufficiency. Ethical approval was obtained from the Lothian Research Ethics Committee and all subjects provided written informed consent.

Participants were randomized to 4 weeks of walnut supplementation (15 g/day) or 4 weeks of control (no walnuts) in a single-blind randomized crossover trial. Block randomization was used and investigators were blinded as to whether participants were in the walnut intervention or control period. The walnut intake was calculated to provide levels of α-linolenic acid similar to those in the current American Heart Association recommendations and trials showing a reduction in cardiac events with diets rich in α-linolenic acid (de Lorgeril et al., 1994; Kris-Etherton et al., 2002). Lipid profile, platelet-leukocyte aggregation and arterial stiffness were assessed at the end of each 4-week period.

Dietary intervention

Participants were provided with walnuts at the beginning of the dietary intervention phase and asked to consume 15 g of walnuts daily in addition to their habitual diets. No other specific dietary advice was provided and there were no restrictions on calorie or fat intake. For the control phase of the study, participants were simply asked to continue with their usual diets. Similar to previous studies, we did not include a washout period between diets as diet-induced lipoprotein changes stabilize in <4 weeks (Kris-Etherton and Dietschy, 1997). Participants completed a 3-day weighed food diary to assess intake of ω-3 fatty acids during each phase of the study. Subsequent dietary analysis was performed by a qualified dietician using the Compli@t Nutritional Analysis Software program (Nutrition Systems, Banbury, UK), enabled with McCance and Widdowson’s reference food composition tables.

Blood collection protocol

Peripheral venous blood was drawn from a large antecubital vein with a 19-gauge needle and anticoagulated with the direct thrombin inhibitor D-phenylalanine-L-prolyl-L-arginine chloromethyl ketone (75 μmol, Cambridge Biosciences, Cambridge, UK). Whole blood was then immunolabelled within 8 min of phlebotomy for subsequent flow cytometric analysis of platelet–monocyte aggregates.

Flow cytometry

The following reagents were used: fluorescein isothiocyanate-conjugated CD42a (GRP-P, IgG1) and isotype control IgG1 (obtained from Serotec Ltd., Oxford, UK), phycoerythrin-conjugated CD14 (Tuk-4, IgG2a; obtained from Dako, Cytomation, Buckinghamshire, UK) and FACS-Lyse (obtained from Becton-Dickinson, Cowley, UK). The methods are as previously described (Harding et al., 2007). Briefly, aliquots of whole blood were incubated with anti-CD14, anti-CD42a and isotype-matched controls. Thereafter, samples were fixed and the red cells lysed. Samples were analysed using a Coulter EPICS XL flow cytometer equipped with a 488 nm wavelength laser (Beckman Coulter, High Wycombe, UK). Samples were initially analysed with the flow cytometer triggered on forward scatter and then by triggering on FL2 to identify CD14-positive monocytes. Platelet–monocyte aggregates were defined as monocytes positive for CD42a. All results were expressed as percentage of positive cells.

Analyses were performed using EXPO 32 software (Beckman Coulter). The mean coefficient of variation for the percentage of platelet–monocyte aggregates was 7.8%.

Pulse waveform analysis

Arterial stiffness was measured non-invasively with the SphygmoCor system (AtCor Medical, New South Wales, Australia). Measurements were taken in a quiet, temperature-controlled room after subjects had been in the recumbent position for a 15-min rest period. Peripheral pressure waveforms were obtained using applation tonometry of the radial artery with a pressure-sensitive micromanometer (Millar Instruments, Houston, TX, USA). A generalized transfer function was used to derive the corresponding aortic
pressure waveform. The first systolic peak of this waveform is a result of left ventricular ejection and the second systolic peak is caused by wave reflection from the periphery. The augmented pressure was defined as the difference between the first and second systolic peaks. The augmentation index was the augmented pressure expressed as a percentage of the pulse pressure. Our reproducibility measures demonstrate within-observer differences of 0.4 ± 1.5% for the augmentation index and 0.3 ± 0.6 mm Hg for augmented pressure. Peripheral blood pressure measurements were performed using an automated upper arm blood pressure monitor (Omron 705IT, Omron Healthcare, Milton Keynes, UK).

Statistical methods
Continuous variables are reported as mean ± s.d. Statistical analyses were performed using two-tailed Student's t-tests. All calculations were performed using GraphPad Prism (Graph Pad Software, La Jolla, CA, USA). Statistical significance was taken as P < 0.05.

Results
Baseline characteristics
Baseline characteristics for the study population are given in Table 1. Study participants were young and had a normal body mass index, blood pressure and lipid profile. Two subjects were cigarette smokers and the average alcohol intake was within recommended limits (15 ± 10 units per week). In terms of ethnic origin, 22 participants were North European Caucasians, 5 were Indian Asians and 3 were of Far East Asian origin.

Effect of walnut supplementation on lipid profile and intake of n-3 fatty acids
Total and LDL cholesterol were lower than baseline after both the walnut intervention and control diets (P < 0.01), but there were no differences in lipid profile between groups at the end of each 4-week period (Table 2).

Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.5 ± 2.3</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>117 ± 9</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>4.57 ± 1.06</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>2.70 ± 0.92</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.32 ± 0.24</td>
</tr>
<tr>
<td>Triacylglycerol, mmol/l</td>
<td>1.22 ± 0.58</td>
</tr>
<tr>
<td>Augmentation index, %</td>
<td>−5.3 ± 6.9</td>
</tr>
<tr>
<td>Augmented pressure, mm Hg</td>
<td>−1.8 ± 2.5</td>
</tr>
<tr>
<td>Platelet-monocyte aggregates, %</td>
<td>22.0 ± 6.8</td>
</tr>
<tr>
<td>Platelet-neutrophil aggregates, %</td>
<td>7.2 ± 3.0</td>
</tr>
</tbody>
</table>

Table 2 Effect of walnut supplementation on serum lipid profile

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Walnuts</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>4.37 ± 1.01</td>
<td>4.25 ± 0.99</td>
<td>0.11</td>
</tr>
<tr>
<td>change from baseline, mmol/l</td>
<td>−0.33 ± 0.39</td>
<td>−0.20 ± 0.37</td>
<td></td>
</tr>
<tr>
<td>LDL cholesterol, mmol/l</td>
<td>2.46 ± 0.85</td>
<td>2.30 ± 0.84</td>
<td>0.10</td>
</tr>
<tr>
<td>change from baseline, mmol/l</td>
<td>−0.40 ± 0.47</td>
<td>−0.24 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
<td>1.24 ± 0.19</td>
<td>1.24 ± 0.26</td>
<td>0.85</td>
</tr>
<tr>
<td>change from baseline, mmol/l</td>
<td>−0.09 ± 0.19</td>
<td>−0.08 ± 0.15</td>
<td></td>
</tr>
<tr>
<td>Chol:HDL chol ratio</td>
<td>3.47 ± 0.85</td>
<td>3.39 ± 0.85</td>
<td>0.63</td>
</tr>
<tr>
<td>change from baseline, mmol/l</td>
<td>−0.13 ± 0.61</td>
<td>−0.05 ± 0.62</td>
<td></td>
</tr>
<tr>
<td>Triacylglycerol, mmol/l</td>
<td>1.45 ± 0.74</td>
<td>1.55 ± 0.93</td>
<td>0.61</td>
</tr>
<tr>
<td>change from baseline, mmol/l</td>
<td>0.33 ± 0.86</td>
<td>0.23 ± 0.59</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein. Mean values are represented as ± s.d.

Table 3 Haemodynamic effects of walnut supplementation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Walnuts</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats per min</td>
<td>64 ± 8</td>
<td>69 ± 11</td>
<td>0.08</td>
</tr>
<tr>
<td>Peripheral systolic blood pressure, mm Hg</td>
<td>117 ± 8</td>
<td>120 ± 10</td>
<td>0.07</td>
</tr>
<tr>
<td>Peripheral diastolic blood pressure, mm Hg</td>
<td>62 ± 5</td>
<td>63 ± 6</td>
<td>0.06</td>
</tr>
<tr>
<td>Peripheral pulse pressure, mm Hg</td>
<td>54 ± 8</td>
<td>57 ± 9</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>77 ± 6</td>
<td>79 ± 8</td>
<td>0.29</td>
</tr>
<tr>
<td>Central aortic systolic pressure, mm Hg</td>
<td>96 ± 7</td>
<td>98 ± 8</td>
<td>0.09</td>
</tr>
<tr>
<td>Central aortic diastolic pressure, mm Hg</td>
<td>63 ± 5</td>
<td>64 ± 8</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Mean values are represented as ± s.d.

Dietary intake of α-linolenic acid was markedly increased during the walnut diet (2.1 ± 0.4 versus 0.7 ± 0.4 g/day; P < 0.0001). There were no changes in consumption of the marine-derived α-3 fatty acids eicosapentaenoic acid (0.2 ± 0.2 versus 0.2 ± 0.3 g/day) or docosahexaenoic acid (0.2 ± 0.3 versus 0.3 ± 0.3 g/day) during walnut supplementation compared with control.

Effect of walnut supplementation on measures of arterial stiffness
Dietary intervention with walnuts did not affect heart rate, peripheral blood pressure or central aortic pressures compared with control (Table 3). Measures of arterial stiffness did not change from baseline after either walnut supplementation or control, and there were no differences observed in augmentation index (−6.6 ± 6.5 versus −8.4 ± 6.3%) or augmented pressure (−2.2 ± 2.1 versus −2.7 ± 2.4 mm Hg) or at the end of each 4-week period (Figure 1).

Table 4 Haemodynamic effects of walnut supplementation

<table>
<thead>
<tr>
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<td>0.54</td>
</tr>
</tbody>
</table>

Mean values are represented as ± s.d.
found a been have cardiovascular risk. rather than intervention dietary endothelial function. The improve 'Mediterranean' heavy This is in moderate intake of walnuts does We have demonstrated that dietary supplementation with walnuts control and versus ±1.8 (5.2 differences in platelet-monocyte aggregation (18.5 ± 7.2 versus 19.5 ± 7.2%) or platelet-neutrophil aggregation (5.2 ± 1.8 versus 5.5 ± 2.2%) between walnut intervention and control diets at the end of each 4-week period (Figure 2).

Discussion

We have demonstrated that dietary supplementation with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy subjects. This is in contrast with previous studies that have shown that heavy consumption of walnuts as part of a low-fat or 'Mediterranean' diet can reduce LDL cholesterol and improve endothelial function. The present study is the first dietary intervention trial to assess the efficacy of moderate rather than large walnut consumption on markers of cardiovascular risk.

Data on the effects of walnut consumption on lipid profile have been inconsistent. Several randomized trials have found a reduction in total or LDL cholesterol with walnuts (Sabate et al., 1993; Zambon et al., 2000; Iwamoto et al., 2002; Ros et al., 2004; Tapsell et al., 2004; Torabian et al., 2010), whereas others have shown no effect (Chisholm et al., 1998; Morgan et al., 2002; Mursudd-Petersen et al., 2007; Spaccaretta et al., 2008; Tapsell et al., 2009). Overall, a meta-analysis of 11 trials found that high walnut-enriched diets reduced total and LDL cholesterol by 4.9 and 6.7%, respectively (Banel and Hu, 2009).

The most likely explanation for the lack of effect on lipid parameters in our study is the lower amount of walnuts consumed (15 g daily) compared with previous trials. Studies demonstrating reductions (6–16%) in serum LDL cholesterol concentrations with walnuts have required participants to consume between 40 and 84 g of walnuts per day (Sabate et al., 1993; Zambon et al., 2000; Iwamoto et al., 2002; Ros et al., 2004). Furthermore, in these studies walnuts isocalorically replaced other fat-containing foods as part of low-fat, low-cholesterol or 'Mediterranean' style diets, whereas our study participants were free-living. It is not possible to know whether the beneficial lipid effects in previous studies were specifically because of walnut intake or the replacement of other sources of dietary fat.

Most previous trials have investigated patients with hyperlipidaemia, and the relatively low baseline cholesterol in our study population may have contributed to the neutral lipid results. A recent trial found that lipid lowering with walnut supplementation was only evident in patients with higher cholesterol levels; there was no benefit in those with baseline cholesterol concentrations < 4.94 mmol/l (Torabian et al., 2010). The only two trials in normolipidaemic patients where walnuts reduced cholesterol levels required a large intake of >50 g per day (Sabate et al., 1993; Iwamoto et al., 2002). It is possible that supplementation with a smaller amount of walnuts is not sufficient to alter lipid parameters in a young, low-risk, normolipidaemic population.

The present study is the first trial to assess the effect of walnut consumption on arterial stiffness and platelet activation. We have previously demonstrated that dietary intervention with fish rich in marine-derived ω-3 fatty acids reduces platelet-monocyte aggregates, a highly
sensitive marker of platelet activation (Din et al., 2008). However, supplementation with walnuts rich in plant-derived ω-3 fatty acids did not reduce platelet-monocyte aggregates, and there was also no change in the augmentation index or augmented pressure. Although we did not study and cannot exclude an effect with heavy walnut intake, our results indicate that any potential cardiac benefits of moderate walnut consumption are unlikely to be mediated through effects on platelet activation or arterial stiffness.

The large amount of walnuts consumed in many of the previous dietary intervention trials provided up to 20% of total energy and 55% of daily total fat intake. There have been concerns that this proportion of fat energy from walnuts may be too high to be practical or sustainable in a non-research setting (Feldman, 2002; Banel and Hu, 2009). In 2004, the Food and Drug Administration issued a qualified health claim for walnuts in response to a petition from the California Walnut Commission (Food and Drug Administration, Center for Food Safety and Applied Nutrition, 2004). Their report recognized that the clinical studies used high daily walnut intakes and that there were no data from which to extrapolate beneficial effects to lower amounts. The present study provides some data to address these concerns, and suggests that a smaller and more practical intake of walnuts may not be sufficient to improve lipid parameters or cardiovascular risk markers.

Our study has potential limitations that should be recognized. First, the sample size is relatively small. However, the power of the study is augmented by the crossover design and was considered sufficient to detect important changes in outcome measures. Previous studies demonstrating improved lipid parameters and endothelial function with walnuts have had fewer participants. We therefore believe it is unlikely that our neutral results are because of a lack of power. Second, we cannot fully exclude a lack of adherence to the assigned diet as a cause of our neutral findings. Although the weighed food diary analysis suggested complete adherence, this is a self-reported and imperfect measure of compliance.

Finally, we did not include a run-in period prior to randomization. All eligible subjects were randomized and an intention-to-treat analysis performed in order to maximize the applicability and external validity of the study. Although there were no differences between the diets at the end of each 4-week period, both the walnut intervention and control diets reduced cholesterol concentrations and platelet-leukocyte aggregates compared with baseline. These changes may reflect a modification in participants’ dietary habits after randomization, independent of their dietary assignment, and could have reduced our ability to detect subtle differences between diets.

Conclusions

We have demonstrated that dietary intervention with a moderate intake of walnuts does not affect lipid profile, arterial stiffness or platelet activation in healthy male volunteers. This is in contrast to previous studies in which large amounts of walnuts, as part of iso-caloric low-fat diets, were found to reduce serum LDL cholesterol concentrations and improve endothelial function. Our results suggest that the potentially beneficial effects of walnuts on lipid parameters and cardiovascular biomarkers may not be apparent at lower and more practical levels of consumption. However, we cannot exclude the possibility that the neutral results are because of either small study size or lack of adherence to the walnut intervention. Further studies are necessary to determine whether larger amounts of walnuts might affect arterial stiffness or platelet activation. Longer trials are required to establish if the higher levels of walnut consumption associated with improved lipid profiles or endothelial function can be sustained over time and reduce clinical end points.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to the Clinical Research Facility at the Royal Infirmary for assistance with pulse wave analysis. We thank Lize Van Rensburg for performing the food diary analysis. We also thank Shona Johnstone for assistance with flow cytometry. JND was supported by the British Heart Foundation Grant (PG/03/009).

References


Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in male cigarette smokers

Jehangir N Din,1 Rachel M Archer,1 Scott A Harding,2 Jaydeep Sarma,3 Karin Lyall,1 Andrew D Flapan,4 David E Newby1

ABSTRACT
Objective The effects of ω-3 fatty acids on endothelial function, fibrinolysis and platelet function are uncertain. We investigated the effects of ω-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and platelet and monocyte activation in healthy cigarette smokers; a group at increased risk of myocardial infarction.

Design, setting, participants Twenty cigarette smokers were recruited into a randomised, double-blind, placebo-controlled, crossover trial of ω-3 fatty acid supplementation.

Intervention ω-3 fatty acid supplements (2 g/day) or placebo for a 6-week period.

Main outcome measures Peripheral blood was taken for analysis of platelet and monocyte activation, and forearm blood flow (FFB) was assessed in a subset of 12 smokers 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 FBF (p<0.0001). Compared with placebo, ω-3 fatty acid supplementation led to greater endothelium-dependent vasodilatation with acetylcholine and substance P (p=0.0032 and p=0.036). Substance P caused a dose-dependent increase in plasma t-PA concentrations (p<0.0001) that was greater after ω-3 fatty acid supplementation compared with placebo (8.8±2.3 IU ml−1 vs 3.6±1.1 IU ml−1, p=0.029). ω-3 fatty acids did not affect platelet-monocyte aggregation, platelet P-selectin or CD40L, or monocyte CD40.

Conclusions We have demonstrated for the first time that ω-3 fatty acids augment acute endothelial t-PA release and improve endothelial vasomotor function in cigarette smokers. Improved endogenous fibrinolysis and endothelial function may represent important mechanisms through which ω-3 fatty acids confer potential cardiovascular benefits.

INTRODUCTION
Dietary fish or fish oil supplements containing ω-3 fatty acids may protect against cardiovascular disease.1 While ω-3 fatty acids have been associated with several biological actions, the actual mechanisms through which they confer any potential cardiac benefits are uncertain. In recent years, an effect on ventricular arrhythmias has been thought to be central due to the reduction in sudden death observed in clinical trials.2 3 However, subsequent studies have failed to clearly demonstrate a direct anti-arrhythmic effect.4 5 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.6

The endothelium controls vascular tone and blood flow, but also regulates 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).7 8 The rapid release of t-PA from the endothelium is critical, with thrombus dissolution being more effective if t-PA is incorporated early during thrombus formation.9

Endothelial cells regulate thrombosis through the release of paracrine factors that mediate platelet function, and there is an inverse relationship between endothelial vasomotor function and platelet activation.10 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, promoting leukocyte adhesion to activated endothelial cells and thereby contributing to endothelial injury and atherothrombosis.13

Cigarette smokers, a group at considerably increased risk of acute myocardial infarction,14 display a marked attenuation of acute t-PA release in the peripheral and coronary circulation.15 16 They also demonstrate increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system.17 We therefore aimed to investigate the effect of ω-3 fatty acid supplementation on endothelial vasomotor function, endogenous fibrinolysis, and markers of platelet and monocyte activation in healthy cigarette smokers.

METHODS
Study participants Twenty healthy male cigarette smokers (>5 cigarettes/day) aged between 20 and 45 years participated in the study. All subjects gave written consent.
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 more than 1 fish meal per week, asthma, hypertension, on prescribed medication, diabetes, hyperlipidaemia, renal or hepatic failure, vascular disease or any intermittent illness likely to be associated with an inflammatory response.

**Study design**

This was a prospective, double-blind, placebo-controlled, randomised crossover trial. Subjects were randomised to receive either α-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 α-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 α-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. A subset of 12 participants also underwent measurement of forearm blood flow (FBF) 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-H-sarminolyl-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×g for 30 min). Plasma samples were stored at −70°C until analysis.

**Flow cytometry**

The following reagents were used: fluorescein isothiocyanate (FITC)-conjugated CD42a (GR-P; IgG1), FITC-conjugated CD14 (UCHM1, IgG2a), phycoerythrin (PE)-conjugated CD40 (L0B7/6, IgG1), and their appropriate isotype controls (Becton; Oxford, UK) as well as PE-conjugated CD154 (TRA1, IgG1), PE-conjugated CD14 (TIK-4, IgG2a), PE-conjugated CD62P (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 h 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 300 μl of plasma using dichloro-methane-methanol (2:1 containing 0.005% butyric hydroxytoluene as an antioxidant (Pollich 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, acetylsalicylic acid 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 μmol/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 three vasodilators were separated by 20-min saline infusions and given in a randomised order.

FBF was measured in infused and non-infused arms by venous occlusion plethysmography with mercury-in-silicone elastomer strain gauges as described previously. Venous cannulas (17-gauge) were inserted into large subcutaneous veins of the antecubital fossa of all 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 Zymotest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Oise, France) concentrations were determined by ELISAs. Haematocrit was determined by capillary tube centrifugation at baseline.

**Data analysis and statistical methods**

Continuous variables are reported as mean±SEM. 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%. 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,
Endothelial function

giving 90% power to detect a 5% difference in mean platelet-monocyte aggregation at a significance level of 5%. Forearm plethysmographic data were analysed as described previously. Estimated net release of plasma t-PA, has been defined previously as the product of the infused forearm plasma flow (based on the mean haematocrit and the infused FBF) and the concentration difference between the infused and noninfused arms. Statistical analyses were performed using one-way and two-way analysis of variance (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 (GraphPad Software).

RESULTS
Baseline characteristics
Participants were young with normal blood pressure, fasting glucose and lipid profiles (table 1). Smokers had a mean cigarette consumption of 15±5 cigarettes per day over a mean period of 9±2 years (8±2 pack years).

Effect of ω-3 fatty acid supplementation on plasma phospholipid fatty acid composition
Dietary supplementation with ω-3 fatty acids led to a marked increase in EPA as a percentage of plasma phospholipids compared with both baseline (2.7±0.3% vs 1.2±0.1%, p<0.0001) and placebo (2.7±0.3% vs 1.2±0.1%, p<0.0001; figure 1A). There was also an increase in DHA compared with baseline (4.3±0.2% vs 3.2±0.3%, p<0.0001) and placebo (4.3±1.0% vs 3.0±0.2%, p<0.001; figure 1B). As the proportion of EPA and DHA increased with ω-3 fatty acid supplementation, there was a corresponding reduction in the percentage of arachidonic acid compared with baseline (8.7±0.3% vs 9.9±0.4%, p<0.05) and placebo (8.7±1.4% vs 9.6±0.3%, p<0.05). There was no effect on α-linolenic acid, linoleic acid, palmitic acid, stearic acid or oleic acid with either ω-3 fatty acid supplements or olive oil placebo (see online supplementary appendix).

Effect of ω-3 fatty acid supplementation on lipid profile
Supplementation for 6 weeks with ω-3 fatty acids did not affect total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides (see online supplementary appendix).

Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th>N=20</th>
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<tr>
<td></td>
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<tr>
<td>Age, years</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
</tr>
<tr>
<td>Heart rate, beats per minute</td>
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<tr>
<td>Total cholesterol, mmol/l</td>
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<tr>
<td>LDL cholesterol, mmol/l</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/l</td>
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<tr>
<td>Cholesterol/HDL cholesterol ratio</td>
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<tr>
<td>Triglycerides, mmol/l</td>
</tr>
<tr>
<td>Fasting glucose, mmol/l</td>
</tr>
<tr>
<td>Cigarettes per day</td>
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<tr>
<td>Total pack years</td>
</tr>
</tbody>
</table>

Mean±SEM.

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Effect of ω-3 fatty acid supplementation on vasomotor function
ω-3 fatty acid supplementation did not have any effect on systolic blood pressure (132±3 vs 128±3 mm Hg; p=0.2), diastolic blood pressure (78±2 vs 76±2 mm Hg; p=0.06) or heart rate (67±4 bpm vs 70±2 bpm; p=0.6) compared with placebo. During forearm vascular studies substance P, acetylcholine and sodium nitroprusside led to a dose-dependent increase in absolute FBF (p<0.0001 for all). ω-3 fatty acid supplementation led to increased endothelium-dependent vasodilatation with acetylcholine and substance P compared with placebo (p=0.0032 and p=0.056; figure 2). There was no effect on endothelium-independent vasodilatation with sodium nitroprusside (p=0.77; figure 2).

Effect of ω-3 fatty acid supplementation on stimulated t-PA activity
Substance P infusion caused a dose-dependent increase in plasma t-PA activity and antigen concentrations after both ω-3 fatty acid supplementation and placebo (p<0.0001; table 2). The increase in plasma t-PA activity was greater after ω-3 fatty acid supplementation compared with placebo (p=0.0290; table 2). Net release of t-PA activity was also greater after ω-3 fatty acid supplementation compared with placebo (p=0.029; figure 3). ω-3 fatty acids did not affect PAI-1 concentrations (table 2).

Effect of ω-3 fatty acid supplementation on platelet-monocyte aggregation and CD40/CD40 ligand
Supplementation with ω-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 (see online supplementary appendix).

DISCUSSION
To our knowledge, we have demonstrated for the first time that dietary supplementation with ω-3 fatty acids can augment acute endothelial t-PA release in cigarette smokers. In addition, we have shown that ω-3 fatty acids improve endothelial vasomotor function in this high-risk population. These findings suggest that improved endogenous fibrinolysis and endothelial function may represent important mechanisms through which ω-3 fatty acids confer their potential cardiovascular benefits.

The effect of ω-3 fatty acids on fibrinolysis has been controversial, and the present study provides important new insights. While some studies have reported a beneficial impact on fibrinolytic parameters, others have found an adverse effect or no effect. Results have varied widely and it has been concluded that ω-3 fatty acids are unlikely to influence the fibrinolytic system. However, previous studies have only measured basal plasma t-PA concentrations that do not reflect the local capacity for acute endothelial t-PA release. It is the rapid endogenous release of t-PA from the endothelium which regulates the dissolution of thrombus and is of greater pathophysiological relevance. The increased risk of myocardial infarction in cigarette smokers is believed to be at least partly due to impaired acute endogenous t-PA release. Using an established model of acute endothelial t-PA release that predicts cardiovascular outcome, we have demonstrated that ω-3 fatty acid supplementation improves acute endogenous fibrinolytic capacity in cigarette smokers.

We have also demonstrated that ω-3 fatty acid supplementation improves endothelial vasomotor function in healthy cigarette smokers. Although dietary supplementation with ω-3 fatty acids...
Figure 1  Percentage ω-3 fatty acids in plasma phospholipids at baseline, during ω-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 ω-3 fatty acid supplementation on absolute forearm blood flow in response to endothelium-dependent and endothelium-independent vasodilators. Statistical analyses were performed using two-way ANOVA and p values comparing responses to each vasodilator during ω-3 fatty acids supplementation and placebo are illustrated. There were no differences between ω-3 fatty acids and placebo at individual drug doses using Bonferroni’s post-tests for multiple comparisons.
acids has been found to improve endothelial function in some populations, not all studies have demonstrated a beneficial effect.\textsuperscript{23} It is difficult to identify a clear reason for discrepancies in study results as there is significant variability in trial design, and it seems likely that inconsistent results are due to differing study populations, concomitant medications, or \(\omega-3\) fatty acid doses. The present study extends the existing data as it is the first to investigate the effect of \(\omega-3\) fatty acids on endothelial function in healthy cigarette smokers, a high-risk group with known endothelial dysfunction.

The mechanism through which \(\omega-3\) fatty acids might improve endothelial function and endogenous fibrinolysis is uncertain.

Their effect on endothelial function is thought to be partly due to the ability to modify eicosanoid biosynthesis, resulting in the formation of leukotrienes, thromboxanes and novel mediators which favour vasodilatation and reduce vasoconstriction and inflammation.\textsuperscript{24} Improvements in endothelial function with \(\omega-3\) fatty acids are also largely, but not exclusively, mediated by nitric oxide. Consistent with an important role for nitric oxide are the observations that \(\omega-3\) fatty acids increase endogenous nitric oxide production,\textsuperscript{25} and induce structural changes in plasma membrane microdomains leading to increased endothelial nitric oxide synthase activity.\textsuperscript{26} Factors regulating acute endothelial t-PA release are poorly understood, but may involve G proteins and increased intracellular calcium concentrations, while the role of nitric oxide is unclear.\textsuperscript{7} Although \(\omega-3\) fatty acids influence calcium availability by modulating cell membrane ion channels, and can signal via G protein coupled receptors,\textsuperscript{24} further research is required to establish whether the increased t-PA release we observed is mediated by effects on these cellular processes.

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. Although we have measured endothelial function in the microvascular rather than large arteries, impaired endothelium-dependent vasodilatation in the forearm resistance arteries in response to intra-arterial acetylcholine is a powerful and independent predictor of cardiovascular events over 5 years.\textsuperscript{27} Endogenous fibrinolytic capacity, as measured by stimulated endothelial release of t-PA from the forearm vasculature in response to substance P infusion has also been shown to predict outcome.\textsuperscript{28}

Several clinical trials of dietary fish or \(\omega-3\) fatty acid supplements have demonstrated beneficial effects on mortality or cardiac events in a variety of patient groups.\textsuperscript{2,3} The mechanisms through which \(\omega-3\) fatty acids may confer cardiac benefits remain uncertain, and a presumed effect on ventricular

Table 2 Effect of \(\omega-3\) fatty acid supplementation on plasma t-PA activity concentrations

<table>
<thead>
<tr>
<th>Substance P pmol/min</th>
<th>(\omega-3) fatty acids</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>t-PA activity, IU ml(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>0.39±0.07</td>
<td>0.47±0.07</td>
</tr>
<tr>
<td>Infused arm</td>
<td>0.45±0.06</td>
<td>0.85±0.12</td>
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<tr>
<td>t-PA antigen, ng ml(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>6.44±0.60</td>
<td>6.38±0.58</td>
</tr>
<tr>
<td>Infused arm</td>
<td>6.88±0.43</td>
<td>6.79±0.41</td>
</tr>
<tr>
<td>PAI-1 activity, pmol/min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.61±0.41</td>
<td>1.56±0.39</td>
</tr>
<tr>
<td>Infused arm</td>
<td>1.59±0.40</td>
<td>1.38±0.35</td>
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<tr>
<td>PAI-1 antigen, ng ml(^{-1})</td>
<td></td>
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<tr>
<td>Non-infused arm</td>
<td>29.57±4.59</td>
<td>28.85±3.95</td>
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<tr>
<td>Infused arm</td>
<td>29.79±3.81</td>
<td>28.30±3.59</td>
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<tr>
<td>Net t-PA antigen release</td>
<td></td>
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<tr>
<td>ng 100 ml(^{-1}) of tissue mm(^{-1})</td>
<td>1.00±0.90</td>
<td>2.79±2.53</td>
</tr>
</tbody>
</table>

Table 2 Effect of \(\omega-3\) fatty acid supplementation on plasma t-PA activity concentrations

Mean±SEM. Data analysed using two-way ANOVA with Bonferroni's post-tests for multiple comparisons.

P<0.001 compared to placebo.

Effect of \(\omega-3\) fatty acid supplementation on plasma t-PA activity concentrations.

Figure 3 Net release of plasma t-PA activity with \(\omega-3\) fatty acid supplementation and placebo. Statistical analyses were performed using two-way ANOVA, with \(p<0.029\) comparing the response with substance P infusion during \(\omega-3\) fatty acids supplementation and placebo. \(\sim p<0.01\) for substance P 8 pmol/min using Bonferroni's post-tests for multiple comparisons.
Endothelial function

Disclosures None.
Competing interests None.
Ethics approval Lothian Regional Ethics Committee.
Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

STUDY LIMITATIONS
Our study participants were relatively young healthy male smokers. Women were not recruited as endothelial function can be modulated by changing hormonal patterns during the menstrual cycle, potentially making interpretation of results difficult. Aging is associated with progressive endothelial dysfunction, and there may be gender differences in vascular endothelial responses. Further research is required to establish if our findings are applicable to older adults, women, or patients with cardiovascular disease.

CONCLUSIONS
We have demonstrated that ω-3 fatty acid supplements improve endogenous fibrinolysis and endothelial function in healthy cigarette smokers, a group at high risk of adverse cardiac events. These distinct but complementary measures of vascular function may represent important mechanisms through which ω-3 fatty acids confer their potential cardiovascular benefits. ω-3 fatty acids had no effect on platelet activation or the CD40/CD40 ligand system at the doses used in the current study.

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Contributors Conception and design or analysis and interpretation of data: JND, RMA, KL, SAH, JS, ADF, DEN. Drafting of the manuscript or revising it critically for important intellectual content: JND, JS, ADF, DEN. Final approval of the manuscript submitted: All authors. Responsible for overall content as guarantor: JND.

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Arrhythmias has not been borne out by subsequent studies in patients with implantable cardioverter-defibrillators. However, an improvement in endogenous fibrinolytic capacity and endothelial function, as observed in the present study, may represent a mechanism through which ω-3 fatty acids can reduce the risk of coronary thrombosis and subsequent myocardial infarction. This may be of particular importance in cigarette smokers, who demonstrate a marked predisposition to acute thrombosis rather than vulnerable plaque rupture in cases of sudden death in patients with coronary disease.

Cigarette smokers demonstrate increased platelet-monocyte aggregation and upregulation of the CD40/CD40 ligand system, and we have previously demonstrated that moderate intake of oil-rich fish can significantly reduce platelet-monocyte aggregation. However, we did not observe any effect of ω-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 ω-3 fatty acids, and we cannot exclude a dose-effect of ω-3 fatty acids on platelet activation. ω-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 ω-3 fatty acids or dietary fish on soluble CD40 ligand.

The ω-3 fatty acid supplement used in this study is a highly purified, pharmaceutical grade preparation containing concentrated ω-3 fatty acid ethyl esters. The selected dose was chosen to be similar to that seen to reduce cardiac events in clinical trials, and lower than doses which decrease triglyceride concentrations. There are many commercially available ω-3 fatty acid supplements, with wide variability in the content and composition of fatty acids. Therefore, the results of the present study cannot be extrapolated to other ω-3 preparations or doses.

We have demonstrated that ω-3 fatty acid supplements improve endogenous fibrinolysis and endothelial function in healthy cigarette smokers, a group at high risk of adverse cardiac events. These distinct but complementary measures of vascular function may represent important mechanisms through which ω-3 fatty acids confer their potential cardiovascular benefits. ω-3 fatty acids had no effect on platelet activation or the CD40/CD40 ligand system at the doses used in the current study.

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Contributors Conception and design or analysis and interpretation of data: JND, RMA, KL, SAH, JS, ADF, DEN. Drafting of the manuscript or revising it critically for important intellectual content: JND, JS, ADF, DEN. Final approval of the manuscript submitted: All authors. Responsible for overall content as guarantor: JND.

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Endothelial function


Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in male cigarette smokers

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BMJ Open  Effect of ω-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 Flapan4

ABSTRACT
Objective: The mechanisms through which ω-3 fatty acids reduce adverse cardiac events remain uncertain. We aimed to investigate the effect of ω-3 fatty acid supplementation on endothelial 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: 20 male patients with a previous myocardial infarction.

Intervention: ω-3 Fatty acid supplementation (2 g/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 (FBF) 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 FBF (p<0.001). ω-3 Fatty acid supplementation did not affect endothelium-dependent vasodilation with acetylcholine and substance P compared with placebo (p=0.5 and 0.9). Substance P caused a dose-dependent increase in plasma t-PA concentrations (p<0.001), which was not affected by ω-3 fatty acid supplementation (p=0.9). ω-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 ω-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 ω-3 fatty acids in coronary heart disease are unlikely to be mediated through effects on these systems.

INTRODUCTION
Dietary fish or fish oil supplements containing ω-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 antiarrhythmic 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 leucocytes through a P-selectin-dependent mechanism,11 and these interactions can also be modulated by the CD40 receptor and its ligand.12 Formation of platelet–leucocyte 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 ω-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, hypothesised that ω-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 3 months previously were recruited to participate in the study. Myocardial infarction was defined as any of the following: typical clinical history, ECG changes (Q waves in 2 contiguous leads) or elevation of cardiac markers (CKMB or troponin). All participants gave written informed consent. Exclusion criteria included dietary fish allergy or intolerance, consumption of more than one 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, randomised crossover trial. Participants were randomised to receive either ω-3 fatty acid supplements (2 g/day, Omacor capsules, Pronova, Norway) or matching placebo capsules (2 g/day olive oil capsules; Eurocaps Ltd, Gwent, Australia) 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 ω-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 randomisation schedule was generated by an investigator not involved in the study, and securely kept in the Royal Infirmary of Edinburgh Pharmacy. The ω-3 fatty acid capsules contained 85–88% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as ethyl esters in a ratio of 1:2.1. The ω-3 fatty acid capsules as well as olive oil placebo contained 4 mg α-tocopherol. All 20 participants 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 withdrew from the study; one was withdrawn after being admitted with unstable angina and a second was lost to follow-up. A subset of 12 participants also underwent measurement of forearm blood flow (FBF) 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 EDTA (1.6 mg/mL, Sarstedt Monovette) and the direct thrombin inhibitor d-phenylalanine-l-proplylarginine 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 (1500g for 30 min). Plasma samples were stored at −70°C until analysis.

Flow cytometry

The following reagents were used: fluorescein 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 CD62P (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 h 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).
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 dichloromethane-methanol (2:1) containing 0.005% butyryl hydroxytouline 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 transesterification 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 conducted in a quiet temperature controlled room (22–25°C). Participants 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, Kyoho, Japan).

All participants underwent brachial artery cannulation with a 27-gauge 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; Chinalfa, 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 three vasodilators were separated by 20-min saline infusions and given in a randomised order.

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 2000g 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 Assay; Elisa Kit; Technoclone, Vienna, Austria) and PAI-1 antigen and activity (Elitest PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, Neuville-Sur-Orse, France) concentrations were determined by ELISAs. Haematocrit was determined by capillary tube centrifugation at baseline.

Data analysis and statistical methods
Continuous variables are reported as means±SE 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%.10 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 3%. Forearm plethysmographic data were analysed 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 haematocrit and the infused FBF) and the concentration difference between the infused and non-infused 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 online 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 and 16 months, respectively. Patients were on standard medical therapy including aspirin, β-blockers, statins and ACE-inhibitors, and over half had undergone revascularisation post-MI.

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

Effect of ω-3 fatty acid supplementation on lipid profile

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

**Effect of ω-3 fatty acid supplementation on vasomotor function**

ω-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 FBF (p<0.0001 for all). Compared with placebo, ω-3 fatty acid supplementation did not affect endothelium-dependent vasodilation in response to acetylcholine or substance P (p=0.5 and 0.9; figure 1), or endothelium-independent vasodilation with sodium nitroprusside (p=0.9; figure 1).

**Effect of ω-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 ω-3 fatty acid supplementation and placebo (p<0.0001; table 3). ω-3 Fatty acid supplementation did not affect plasma TPA activity, TPA antigen or PAI-1 concentrations compared to placebo (table 3). There was no difference in net release of t-PA activity after ω-3 fatty acid supplementation compared to placebo (p=0.94; figure 2).

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

Supplementation with ω-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 current study has demonstrated that dietary supplementation with ω-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 ω-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 current 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 ω-3 fatty acids. The dose and duration of therapy with ω-3 fatty acids are also likely to have been appropriate. We used 2 g/day of ω-3 fatty acids which is similar to the amount shown to reduce mortality in secondary prevention trials. Although we cannot exclude an effect with a longer...
ω-3 Fatty acids have previously been shown to have inconsistent effects on endothelial function. Although some studies have reported beneficial effects in a variety of populations including healthy volunteers,22 patients with hyperlipidaemia,21 23 diabetes mellitus24 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 ω-3 fatty acids.20 29 30 These discrepancies could be partly due to differences in study populations or concomitant medication. However, previous studies were all either not randomised 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 ω-3 fatty acids on endothelial vasomotor function in coronary heart disease; we therefore believe that our study design and findings are likely to be robust.

We also found that ω-3 fatty acids did not augment endogenous fibrinolysis in coronary heart disease. Previous results have varied widely and it has been concluded that ω-3 fatty acids are unlikely to influence the fibrinolytic system.31 While some studies have reported a beneficial impact on fibrinolytic parameters,32 33 others have found an adverse effect34 or no effect.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 20 but found no effect of ω-3 fatty acid supplementation on acute endogenous fibrinolytic capacity in coronary heart disease.

There are several possible explanations for the lack of effect of ω-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 function20 29 and cardiac outcomes2 3 with ω-3 fatty acids were much less likely to be taking HMG CoA reductase inhibitors or ACE inhibitors. It is conceivable that endothelial function cannot be further improved by the addition of ω-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 ω-3 fatty acid supplementation.42–44

However, concomitant medication may not fully explain the neutral effects on endogenous fibrinolysis. While lipid-lowering therapy improves endothelial...
vasomotor function, it has not been found to influence acute t-PA release.\textsuperscript{43} ACE inhibitors only augment bradykinin induced t-PA release; they do not affect t-PA release stimulated by substance P.\textsuperscript{44} Therefore, there may be other factors that explain why ω-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.\textsuperscript{15} We have previously demonstrated that moderate intake of oily-rich fish can significantly reduce platelet-monocyte aggregation.\textsuperscript{18} However, we did not observe any effect of ω-3 fatty acid supplements on these measures of platelet and monocyte activation in the current study. It is possible that our previous results were due to another active ingredient in oily fish rather than ω-3 fatty acids, and we cannot exclude a dose-effect of ω-3 fatty acids on platelet activation. ω-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 ω-3 fatty acids or dietary fish on soluble CD40 ligand.\textsuperscript{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.\textsuperscript{15,19} 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 ω-3 fatty acids. Second, as we did not measure fatty acids at the beginning of the second treatment stage we cannot fully exclude the possibility of some carryover of EPA or DHA into the early placebo phase in the group receiving ω-3 fatty acids first. However, we feel any such effect would be modest and unlikely to alter the study outcomes.

Table 3  Effect of ω-3 fatty acid supplementation on plasma t-PA activity concentrations.

<table>
<thead>
<tr>
<th>Substance P, pmol/min</th>
<th>ω-3 Fatty acids</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>t-PA activity, IU mL(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>0.39±0.08</td>
<td>0.45±0.09</td>
</tr>
<tr>
<td>Infused arm</td>
<td>0.38±0.08</td>
<td>0.83±0.16</td>
</tr>
<tr>
<td>t-PA antigen, ng mL(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>11.78±1.29</td>
<td>12.01±1.0</td>
</tr>
<tr>
<td>PAI-1 activity, ng mL(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.77±0.53</td>
<td>1.84±0.43</td>
</tr>
<tr>
<td>Infused arm</td>
<td>2.33±0.66</td>
<td>2.18±0.61</td>
</tr>
<tr>
<td>PAI-1 antigen, ng mL(^{-1})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>39.51±9.22</td>
<td>40.84±7.08</td>
</tr>
<tr>
<td>Infused arm</td>
<td>37.64±8.36</td>
<td>38.83±6.25</td>
</tr>
<tr>
<td>Net t-PA antigen release, ng mL(^{-1})</td>
<td>0.23±0.51</td>
<td>0.28±0.47</td>
</tr>
</tbody>
</table>

Mean±SEM. Data analysed using two-way analysis of variance.

Tissue plasminogen activator (t-PA) activity: dose response p<0.0001. ω-3 Fatty acids versus placebo; p=0.83 (infused arm).

T-PA antigen: dose response p=0.7. ω-3 Fatty acids versus placebo; p=0.80 (infused arm).

Plasminogen-activator inhibitor type 1 (PAI-1) activity: dose response p=0.94. ω-3 Fatty acids versus placebo; p=0.17 (infused arm).

PAI-1 antigen: dose response p=0.67. ω-3 Fatty acids versus placebo; p=0.40 (infused arm).

Net t-PA antigen: dose response p=0.02. ω-3 Fatty acids versus placebo; p=0.62 (infused arm).

- Omega 3 fatty acids
- Placebo

Figure 2  Net release of plasma tissue plasminogen activator activity with ω-3 fatty acid supplementation and placebo. Statistical analyses two-way analysis of variance and Bonferroni’s post-tests for multiple comparisons.
CONCLUSIONS
We have demonstrated that n-3 fatty acid supplementation does not affect endothelial function, endogenous fibrinolitic 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 endothelial vasomotor tone as well as endogenous fibrinolysis: two important and complementary measures of vascular function. Our results suggest that any potential cardiac benefits conferred by n-3 fatty acids in this patient group are unlikely to be mediated by effects on endothelial function or the fibrinolitic system.

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Contributors JD, KL, SH, JS, AF and DN were involved in conception and design or analysis and interpretation of data. JD, JS, AF and DN were responsible for drafting of the article or revising it critically for intellectual content. All authors were involved in the final approval of the article to be submitted.

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Competing interests None.

Ethics approval Lothian Regional Ethics Committee. The study was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki.

Prevention and peer review Not commissioned; externally peer reviewed.

Data sharing statement No additional data are available.

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Effect of ω-3 fatty acid supplementation on endothelial function, endogenous fibrinolysis and platelet activation in patients with a previous myocardial infarction: a randomised controlled trial

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