The Role of Omega-3 Fatty Acids and Aspirin in the Prevention of Cardiovascular disease in Diabetes

and

Biochemical effectiveness of Omega-3 Fatty Acids and Aspirin in the ASCEND trial

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Medicine (M.D.) (Res)

Final version

Statement of originality

I, Theingi Aung, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with or supported by others that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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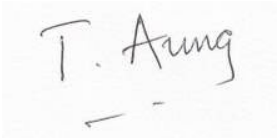
Signature: 

Date: 15th May 2017

Candidates' contribution to the work described herein and acknowledgments

I was employed as a full-time Clinical Research Fellow at the Clinical Trial Service Unit (CTSU) of the University of Oxford. CTSU is world-renowned for its research into the causes, prevention, and treatment of cardiovascular disease in 2010. Within my three-year fellowship, I worked on 3 large randomised trials: ASCEND, HPS2-THRIVE, and HPS3-REVEAL. The latter two trials provided me with general skills and knowledge relating to the management of clinical aspects of large-scale trials, but my main area of work was on the ASCEND (A Study of Cardiovascular Events iN Diabetes) trial, under the supervision of principal investigators: Professor Jane Armitage and Associate Professor Louise Bowman. ASCEND is a randomised controlled trial comparing the safety and efficacy of aspirin and of omega-3 fatty acids for the primary prevention of major cardiovascular events in people with diabetes.

I commenced working during the recruitment phase of ASCEND. Over the course of my fellowship, I have spoken to several hundred trial participants by telephone to address any concerns pertinent to their clinical safety and emotional wellbeing, and to maintain compliance with the trial medication as much as reasonably possible. Information provided during these conversations, along with participants' response to 6 monthly follow-up questionnaires was represented in adverse event records, which I was responsible for reviewing. I also created participant newsletters. The latter

provided a platform from which I was able to directly address current themes raised by the popular media or scientific journals which had a potential to impact on the study's compliance. I also used the newsletter to introduce participants to members of the study team at CTSU, who would otherwise be anonymous to them by virtue of the trial's mail-based design. Each newsletter required ethical approval before they were sent to participants. I prepared the substantial amendment documents and sought to gain a favorable opinion in a timely fashion. I also was responsible for writing requisite progress reports for regulatory bodies and an independent Data Monitoring Committee. Furthermore, I wrote standard operating procedures (SOPs) and the first draft of the Data Analysis Plan for the ASCEND trial. Alongside these responsibilities in ASCEND, I trained research nurses on the background rationale and methods and provided day-to-day clinical guidance for the HSP2-THRIVE and HPS3-REVEAL trials. I gained experience of adjudicating cardiovascular outcomes as part of the HSP2-THRIVE study. Finally, I provided mentorship, on-the-job training, and ongoing support to new clinical research fellows who joined CTSU after me. Through my fellowship, I have therefore developed a thorough understanding of trial management, research methodologies, and leadership.

This thesis represents a sub-study of the ASCEND trial for which I was primarily responsible. In particular, I reviewed the literature to select a laboratory method to measure the biochemical effects of study medication which could be used as an indirect indicator of compliance with study medication, within the constraints of a mail-based trial. Based on this, I

coordinated a post-randomisation blood and urine analysis of a biomarker of aspirin's anti-platelet efficacy in this diabetic population. I also measured levels of omega-3 fatty acids as a means of demonstrating compliance with this treatment. A novel aspect of this work involved the collection of samples by mail which necessitated finding suitable analytes that would be stable in whole blood or urine during transport in the post. In the early stages of the sub-study, I wrote a protocol amendment and sought approval from the Multicentre Research Ethics Committee (REC reference no. 03/8/087). Once approval was granted in 2011, I worked with colleagues at CTSU to modify blood and urine test kits which had been used to collect lipid and HbA1c data at an entry to the trial, for the purpose of this project. I also developed documents including a participant consent form for the blood collection, a participant information leaflet, and a GP information letter. When blood samples were returned to CTSU, I worked closely with administrative colleagues and laboratory staff who tracked receipt of the samples and analysed them, respectively. Following this, I led analyses of those data with support from the trial statistician; the final results of which are described in this thesis.

I also carried out a systematic review and tabular data meta-analysis of the trials of omega-3 fatty acid for prevention of cardiovascular disease. This involved setting up the Omega-3 FA Treatment Trialists' Collaboration (OTTC), which was supervised by Professor Robert Clarke, who also works at CTSU. I independently located and critically reviewed the relevant published literature. I requested unpublished data from the authors of the

included published trials, led the analysis of the data once received, and collaborated with principal investigators of the participating trials. I wrote the first draft of the meta-analysis paper.

The writing of this thesis has been my own work. It is, however, important to acknowledge that such an undertaking would not have been possible without the support of the entire study team of the ASCEND at CTSU and my supervisors Professor Jane Armitage, Professor Robert Clarke, Professor Louise Bowmen and Professor David Leslie. Last but not the least, I want to thank you to my husband, Win, and without his understanding and support over last 7 years, I wouldn't have been able to complete this thesis.

Abstract:

Background

The role of aspirin (100 mg daily) and omega-3 fatty acids (FA) (1 g daily) for primary prevention of cardiovascular disease in diabetes is being investigated in the 2x2 factorial design ASCEND trial. To support the interpretation of the trial's efficacy findings, it is important to compare self-reported compliance by participants with measures of the biochemical effects of each intervention. The previous data on the effect of supplementation with omega-3 FA on coronary heart disease is uncertain.

Methods

The ASCEND trial randomly allocated 15480 people with diabetes (94% type 2 DM) who do not already have diagnosed occlusive arterial disease to receive aspirin or placebo and to omega-3 FA or placebo. Blood and urine samples were collected by mail at baseline and after 3 years follow-up. The effectiveness of aspirin to suppress urinary thromboxane B2 (UTxB2), a marker of platelet activity, and, of omega-3 FA supplements to increase red cell membrane omega-3 index were assessed. A systematic review of previous trials of omega-3 FA was conducted to summarize the prior evidence for the effects of omega-3 FA supplements on major vascular events (MVEs).

Results

Aspirin reduced UTxB2 levels by 67% (63-70%) ($p < 0.0001$) compared with placebo, from 3453 pg/mg (95% CI 3061-3895) at baseline to 1190 pg/mg (1100-1287) on those allocated to aspirin during the trial. During follow-up, the omega-3 index increased by 33% (95% CI 26%-39%) in those allocated omega-3 FA compared to placebo ($p<0.0001$). The meta-analysis of previous studies of omega-3 FA showed no effect on MVEs (HR 0.97; [0.93-1.01]) overall or in any pre-specified sub-groups.

Conclusions

Low dose aspirin and omega-3 FA are biochemically effective at reducing UTxB2 and increasing the omega-3 index, respectively. Previous trials show that supplementation with omega-3 FA had no significant effect on MVEs. The results of the ASCEND trial, assessing the effects of both aspirin and omega-3 FA on MVEs, will be available in 2018.

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Chapter 1: Introduction

Chapter 2: Background

Abbreviations and trial acronyms

AGEs	advanced glycation end products
ALA	α-Linolenic acid
ASCEND	A Study of Cardiovascular Events iN Diabetes
BP	Blood pressure
CHD	Coronary heart disease
CI	Confidence interval
CKD	Chronic Kidney disease
COX 1	Cycloxygenase-1
CTSU	Clinical Trial service unit
CV	Coefficient of variation
CVD	Cardiovascular disease
DFU	Diabetic foot ulcer
DHA	Docosahexaenoic acid
DM	Diabetes mellitus
DME	Diabetic macular oedema
DR	Diabetic retinopathy
GFR	Glomerular filtration rate
ELIZA	Enzyme-linked immunoassay
EPA	Eicosapentaenoic acid
ERFC	Emerging risk factor collaboration
ESRD	End-stage renal disease
GP	General practices
HDL	High-density lipoprotein
HR	Hazard ratio
LDL	Low-density lipoprotein
MVE	Major vascular event
NSAIDs	Nonsteroidal anti-inflammatory drugs
Omega-3 FA	Omega-3 fatty acid
Omega-3 index	Omega-3 fatty acid index
OTTC	The Omega-3 FA Treatment Trialists' Collaboration
PUFA	Polyunsaturated fatty acid
PVD	Peripheral vascular disease
RBC	Red blood cell
ROS	Reactive oxygen species
RR	Risk ratio
TG	Triglyceride
TxA2	Thromboxane A2
UTxB2	Urinary thromboxane B2
VLDL	Very low-density lipoprotein
WHO	World Health Organisation

1 Introduction

This thesis represents a sub-study of the ASCEND trial for which I was primarily responsible. I also carried out a systematic review and tabular data meta-analysis of the trials of omega-3 fatty acid (FA) for prevention of cardiovascular disease. This involved setting up the Omega-3 FA Treatment Trialists' Collaboration (OTTC). The two main objectives of my thesis were:

1. To investigate the reliability of questionnaire-based, self-reported compliance of study medications in the large mail-based ASCEND trial.
2. To review the literature on the effect of omega-3 FA supplementation for the prevention of cardiovascular disease.

1.1 The aims of the thesis

The aims of this thesis were:

To review the epidemiology of diabetes mellitus (DM) and the current strategies for reduction of cardiovascular disease risk in people with DM.

To examine the baseline characteristics of 15,480 participants in the ASCEND trial; describe details of follow-up and current levels of compliance with study treatments, and methods for maintaining it.

To assess the biochemical effects on various biomarkers of treatment with either aspirin or placebo, and omega-3 fatty acids (FA) or placebo in a random sample after 3 years follow-up and to relate results to reported compliance.

To assess the known effects of omega-3 FA supplementation on components of major vascular events (MVE) (coronary heart disease, stroke, and revascularisation), cancer, all-cause mortality overall, and on MVE in pre-specified sub-groups, by undertaking a meta-analysis of published randomised trials.

1.2 How these aims will be addressed in the thesis

Background: Chapter 2 includes a detailed literature review of the global burden of diabetes and its cardiovascular complications, evidence-based current prevention and treatment strategies for the reduction of cardiovascular disease (CVD) in diabetes.

Methodology: The first part of chapter 3 describes the ASCEND trial and the substudy which forms the basis of this thesis. This includes the baseline characteristics of 15,480 participants in the ASCEND trial, a description of the details of follow-up, current levels of compliance and methods for maintaining compliance.

The second part of chapter 3 describes the methods of my substudy to investigate the biochemical effects of aspirin, in order to demonstrate that it is delivering its expected anti-platelet effect in this diabetic population, and of omega-3 FA in order to show that blood levels have increased as expected. The results of the biochemical effects of each intervention will be compared to self-reported compliance from follow-up questionnaires.

Results: A systematic review of randomised trials of omega-3 FA was conducted to summarize the prior evidence for effects of omega-3 FA supplements on MVEs. Chapter 4 describes results of the meta-analysis of completed trials on the Omega-3 FA Treatment Trialists' Collaboration (OTTC). This has involved writing to Principal Investigators, seeking summary data from 10 collaborating trials involving nearly 78,000 participants.

The results of biochemical measurements for the effectiveness of aspirin and omega-3 FA in ASCEND will form the second part of chapter 4. The results of laboratory analysis of omega-3 Index and urinary thromboxane B2 (UTxB2) are also described in chapter 4.

Discussion and conclusion: A summary of the findings and contribution of this thesis to the literature is discussed in chapter 5.

2 Background

2.1 Diabetes mellitus and its complications

Diabetes mellitus (DM) is a chronic disorder of glucose metabolism. It is associated with long-term macrovascular and microvascular complications and is associated with significant morbidity, mortality, socio-economic impact, and health care costs (1, 2). Macrovascular complications include cardiovascular disease, peripheral arterial disease, and cerebrovascular disease. Microvascular complications include retinopathy, nephropathy, and neuropathy.

This chapter reviews the epidemiology of diabetes mellitus and its vascular complications, current strategies and treatment options, and prevention strategies for cardiovascular disease focusing on the role of aspirin and omega-3 FA.

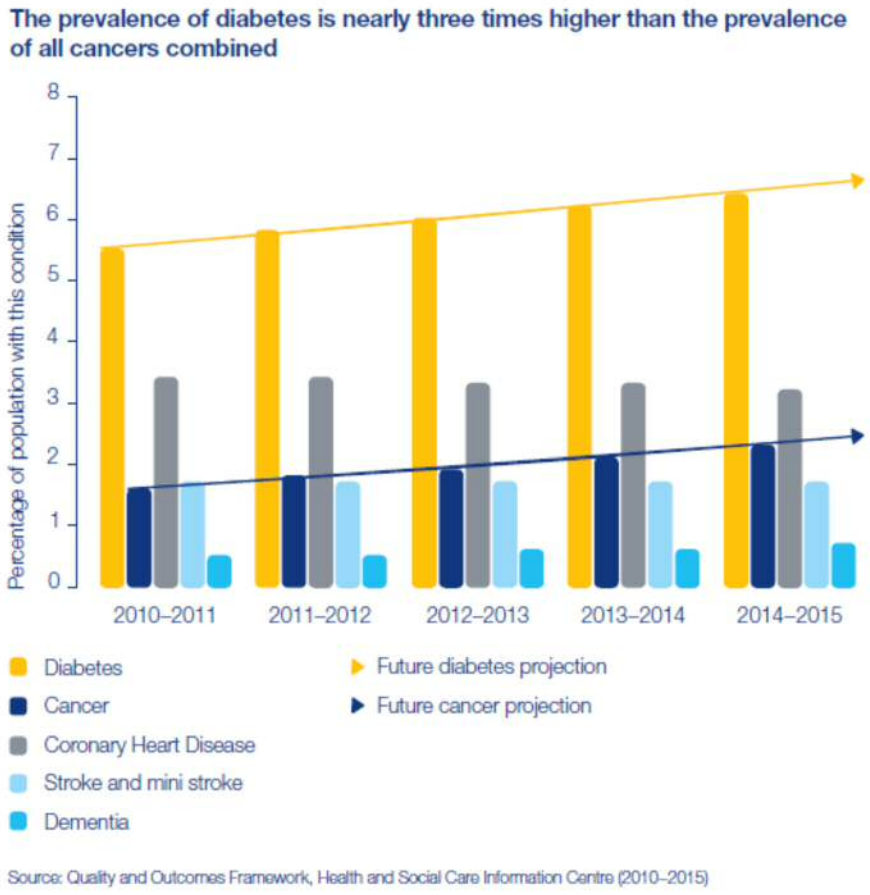
2.1.1 **Epidemiology and the disease burden of diabetes mellitus**

The prevalence of DM is increasing rapidly and DM has become a global epidemic which represents a major health burden in both developed and developing countries (3). The reasons behind this increase are multifactorial including the combined effects of changing lifestyles, rising levels of obesity

and inactivity, increasing lifespan, and improved detection of the disease. The number of people with diabetes has risen from 108 million in 1980 to 422 million in 2014 (4). The global prevalence of diabetes among adults over 18 years of age has increased from 4.7% in 1980 to 8.5% in 2014. If present trends continue, between 2010 and 2030, there will be a 69% increase in the prevalence of diabetes in developing countries and a 20% increase in developed countries (5). In 2013, it was reported that 382 million people had diabetes; but this number is expected to increase to 592 million by 2035 (6). A systematic analysis of health examination surveys and epidemiological studies (representing 370 country-years and 2.7 million people surveyed) also confirmed that the prevalence of diabetes is rising globally, driven mainly by the population growth and aging (3). A particular cause of concern is the dramatic rise of type 2 diabetes in children and adolescents (International Diabetes Federation -IDF Diabetes Atlas report) with a longer exposure to the disease and increased risk of complications.

The number of people living with diabetes in the UK reached above 4 million in early 2016 according to GP- data described by the Diabetes UK. A further 5 million people in England are at high risk of developing diabetes (State of the Nation Report July 2016, Diabetes UK). The prevalence of DM is nearly three times higher than all cancers combined (7) (Figure 2.1). If current trends persist, one in ten people will develop DM in 2034.

Figure 2.1: Prevalence of diabetes compared to other diseases (Adapted from State of the nation report 2016 (Diabetes UK))



Prolonged exposure to hyperglycaemia is associated with an increased risk of atherosclerosis and is the primary determinant of diabetic complications (pathophysiology of prolonged hyperglycaemia will be discussed in section 2.2 of this chapter). Patients with diabetes are at high risk for several cardiovascular disorders (8): including coronary heart disease, cardiomyopathy and congestive heart failure (9, 10). Cardiovascular

complications are the leading causes of diabetes-related morbidity and mortality (10, 11).

Many studies indicate that diabetes is an independent risk factor not only for cardiac disease but also for other vascular complications including stroke, sight-threatening retinopathy, kidney disease, and peripheral vascular disease and foot complications leading to diabetic foot ulcers, limb amputations, cognitive decline and dementia, and overall increased mortality (11).

A meta-analysis of data from 102 prospective studies coordinated by the Emerging Risk Factor Collaboration (ERFC), which included data from over 690,000 participants (with 52,765 fatal or non-fatal cardiovascular outcomes and 8.49 million person-years at risk) demonstrated that having diabetes was independently associated with about a two-fold excess risk for developing vascular disease (cardiovascular disease, cerebrovascular disease, and peripheral vascular disease [PVD]) compared to no diabetes and after adjusting for conventional risk factors (coronary heart disease HR 2.00 (95% CI 1.83-2.19); ischaemic stroke HR 2.27 (1.95-2.65); vascular death HR 1.73 (1.08-1.26)) (8). The hazard ratios (HR) for coronary heart disease with diabetes were significantly higher in women HR 2.59 (2.29-2.93) than in men HR 1.89 (1.73-2.06) (8).

There is also an increased incidence of ischaemic stroke in people with diabetes. A different meta-analysis of data from 64 cohort studies (775,385

individuals, 12,539 fatal and non-fatal strokes) reported that the relative risk of stroke associated with diabetes was 2·28 (95% CI 1.93-2.69) in women and 1.83 (1.60-2.08) in men (12). Data suggest there is also a significant variation by age and that the excess vascular risk is most prominent in younger age group (e.g. before age of 55) (13).

The risks of PVD are higher in people with diabetes, PVD occurs earlier and is often more severe and diffuse than in people without diabetes. The presence of PVD results in higher risks of claudication, ischaemic ulcers, gangrene, and amputation. PVD is also a marker for generalized atherosclerosis and a strong predictor of ischaemic cardiovascular events. Individuals with diabetes have a 2 to 4-fold increased risk of PVD (14). Overall mortality from PVD tends to be higher in people with diabetes compared to people without diabetes (51.7 vs. 25.6%, OR 3.1, P = 0.002), and in one study, diabetic patients who died were younger at presentation than non-diabetic patients (64.7 +/- 11.4 vs. 71.1 +/- 8.7 years, P = 0.04) and the duration and severity of diabetes correlated with the incidence and extent of their PVD (15).

Having diabetes is also associated with an increased risk of premature mortality compared with those without the disease. For example on average, a 50-year old with diabetes but with no history of any vascular disease is about 6 years younger at the time of death than a counterpart without diabetes. The ERFC quotes a HR of 1.8 (95% CI 1.71-1.9) for death from any cause; and HR 2.32 (95% CI 2.11 to 2.56) for death from a vascular cause

(11). The crude overall death rates, as reported in Emerging Risk Factor Collaboration study, were higher among participants with diabetes than among those without diabetes: 29 per 1000 person-years vs 12 per 1000 person-years for men, respectively, and 23 per 1000 person-years vs 7 per 1000 person-years for women, respectively (11). The Multiple Risk Factor Intervention Study (MRFIT) screening data study reported that the absolute risks of CVD death were higher for men with diabetes than non-diabetic men in every age stratum, by ethnic background, and by risk factor level—overall three times higher after adjustment for age, race, income, serum cholesterol level, systolic blood pressure and reported number of cigarettes/day (P <0.0001) (16). A more recent study also has highlighted that mortality among persons with type 2 diabetes as compared with that in the general population varied greatly depending on age, glycemic control, and renal complications. A recent Swedish study which included more than 90% of all the people with type 2 diabetes in Sweden reported that overall, 77,117 of 435,369 patients with diabetes (17.7%) died, as compared with 306,097 of 2,117,483 controls (14.5%) (adjusted hazard ratio HR, 1.15; 95% confidence interval [CI], 1.14 to 1.16) at the mean follow up of 4.6 years. The rate of cardiovascular death was 7.9% among patients versus 6.1% among controls (HR 1.14; 95% CI,1.13 to 1.15) (17). Hence this suggests that the excess risk of mortality in type 2 diabetes has reduced over time compared to the result of MRFIT study. Not only in type 2 diabetes, another Swedish nationwide population-based observational cohort study (with median follow-up of 8.3 and 8.9 years) recently reported for individuals with type 1 diabetes and controls, 1500 (4.5%) and 1925 (1.2%) respectively, experienced non-fatal AMI or died

from CHD, adjusted HR 4.07 (95% CI 3.79 to 4.36). This excess risk increased with younger age, female sex, worse glycaemic control and severity of renal complications (18). Diabetes was estimated to cause 4.6 million deaths worldwide in 2011, and 10% of vascular deaths in developed countries were attributable to DM (estimated 325,000 deaths per year) (8). The WHO projects that DM will be the 7th leading cause of death worldwide in 2030 (19).

Diabetic retinopathy (DR) and diabetic macular oedema (DME) are common complications in people with diabetes and associated with significant disability. DR is an important cause of blindness and accounts for 2.6% of global blindness (20). DR is accountable for 0.8 million cases of blindness and 3.7 million cases of visual impairment with a significant increasing trend from 1990 to 2010 (21). DR is a long-term complication of diabetes mellitus and after 15 years of having the disease, 10% of people with diabetes develop severe visual impairment and of those, about 2% will go blind (19). In patients with type 1 DM, the cumulative 14-yr incidence of visual impairment and blindness were 12.7% and 2.4% respectively (22). An individual participant data analysis (of >22 000 individuals with diabetes) reported that the prevalence of any DR was about 35%, proliferative DR of about 7% and DME of about 7%. Translated globally, this means that approximately 93 million people with diabetes have some degree of DR, 17 million have proliferative DR and 21 million have DME worldwide (23). With increased awareness of the risk factors for retinopathy, early diagnosis (with a national screening program for DR) and treatment of retinopathy as well as

its risk factors, the rate of progression of DR to proliferative diabetic retinopathy (PDR) and severe visual loss has decreased since 1985 in the United States (24). However, the increasing prevalence of DM suggests that this diabetes-related blindness and visual impairment will continue to cause a significant impact on the health and an economic burden. Fortunately, control of metabolic abnormalities of diabetes can slow the progression of DR.

Diabetes has also become the most common cause of chronic kidney disease (CKD) and end-stage renal disease (ESRD) in the US and Europe (25) and 10-20% of people with diabetes die from renal failure (19). The prevalence of various degrees of CKD among people with type 2 DM increased to 40% in the US in the 1999-2006 National Health and Nutritional Examination Survey. Diabetic nephropathy is characterised by albuminuria (microscopic or overt) and a reduced glomerular filtration rate (GFR). Microalbuminuria is an important clinical finding in patients with diabetes as it is associated with progression to CKD. CKD is also increasingly recognised as an independent risk factor for cardiovascular disease (26). A low GFR (<60 ml/min/1.73 m^2) is also known to be an independent risk factor for cardiovascular events and death (27). CKD is also associated with an increased risk of cardiovascular disease, ESRD, and mortality (28).

Diabetic foot disease represents a composite of pathologies including peripheral neuropathy, vascular insufficiency, and infection. About 50% of diabetic patients develop peripheral neuropathy and as many as 20% have neuropathy at the time of diagnosis (29). People with diabetes are 25-times

more likely to lose a leg because of diabetic foot problems than those without this condition and 85% of amputations are preceded by active foot ulcers (30). Diabetic foot disease causes significant pain, sensory loss, gait disturbance, falls, foot deformity, foot ulceration and amputation and not only impacts on patients' lives but also on the lives of family members and society in general. The lifetime risk of a person with diabetes developing a foot ulcer is thought to be as high as 25% (31). The nature of diabetic foot ulcers (DFU) is that they tend to be recurrent (>50% after 3 years) and need long-term structured multi-disciplinary care (30). One meta-analysis, including 3619 events of all-cause mortality during 81,116 person-years of follow-up, reported that DFU were associated with an increased risk of all-cause mortality (RR 1.89, CI 1.6-2.2) compared with diabetic patients without a history of DFU (32).

People with diabetes have an increased risk of dementia and cognitive impairment (33). In 1996, the Rotterdam study reported strong associations of dementia with diabetes treated with insulin (odds ratio: 3.2, 95% confidence interval: 1.4-7.5). The associations were strongest for vascular dementia but were also observed for Alzheimer's disease. These associations were independent of smoking, body mass index, atherosclerosis, blood pressure and antihypertensive drug treatment (34). An add-on project of the Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen Detected Diabetes in Primary Care (ADDITION) study in the Netherlands reported in that modest cognitive decrements were already present at the early stage of type 2 diabetes (35). Individuals with diabetes

had a 1.2 to 1.5-fold greater decline in cognitive function compared with those without diabetes. When assessed by the Mini-Mental State Exam and the Digit Symbol Span tests, a diagnosis of diabetes increased the odds of cognitive decline by 1.2-fold (95% CI 1.05-1.4) and 1.7-fold (95% CI 1.3-2.3), respectively. Diabetes also increased the odds of future dementia by 1.6-fold (95% CI 1.4-1.8) compared to people without diabetes and also people with diabetes have a greater rate of decline in cognitive function and a greater risk of cognitive decline (36). The precise mechanism by which diabetes causes cognitive decline are not fully understood but may include vascular disease and alterations in glucose, insulin, and amyloid metabolism (37). Degeneration of neurons in the brain, impaired regional blood supply, and genetic predisposition are all involved in diabetes-associated dementia or cognitive impairments (38). Further high-quality studies are needed to establish the contribution of vascular disease and other comorbidities to dementia.

2.1.2 **Health-care economic costs**

With a rising prevalence of the disease, the diabetic population consumes a disproportionate share of health resources not only because of the disease itself but also because of the associated complications that are described earlier. The health-care costs worldwide for diabetes include: (i) the direct costs of the disease (hospitalisation, treatment of complications); (ii) indirect costs associated with lost production; (iii) intangible costs (pain, anxiety, inconvenience and generally lower quality of life, not only for the patient but also of family and friends); and (iv) costs for the prevention of diabetes. The

estimated total economic cost of diagnosed diabetes in 2012 in the United States was $245 billion ($176 billion for direct medical costs and $69 billion in reduced productivity), a 41% increase from the previous estimate of $174 billion (in 2007 dollars) (39). On average, people diagnosed with DM, have a medical expenditure that is approximately 2.3-times higher than it would be in the absence of diabetes (40). A study from 5 European countries (France, Germany, Italy, Spain and the UK) reported that the estimated total annual costs for diabetes for 2010 ranged from €5.45 billion (Spain) to €43.2 billion (Germany); across these 5 EU countries the total direct health care cost for people with diabetes was estimated to be €90 billion (41). The WHO estimates that the overall direct healthcare costs of DM (excluding other indirect costs) range from 2.5% to 15% of annual health care budgets depending on local diabetes prevalence and the treatment options available worldwide (WHO fact sheet No 236) (4). In the UK, it is currently estimated that the NHS spends about £10 billion every year which is about 10% of the NHS budget. The combined direct and indirect cost associated with diabetes is about £23.7 billion. This figure is predicted to rise with increasing prevalence of the disease to £39.8 billion by 2035-36 (7).

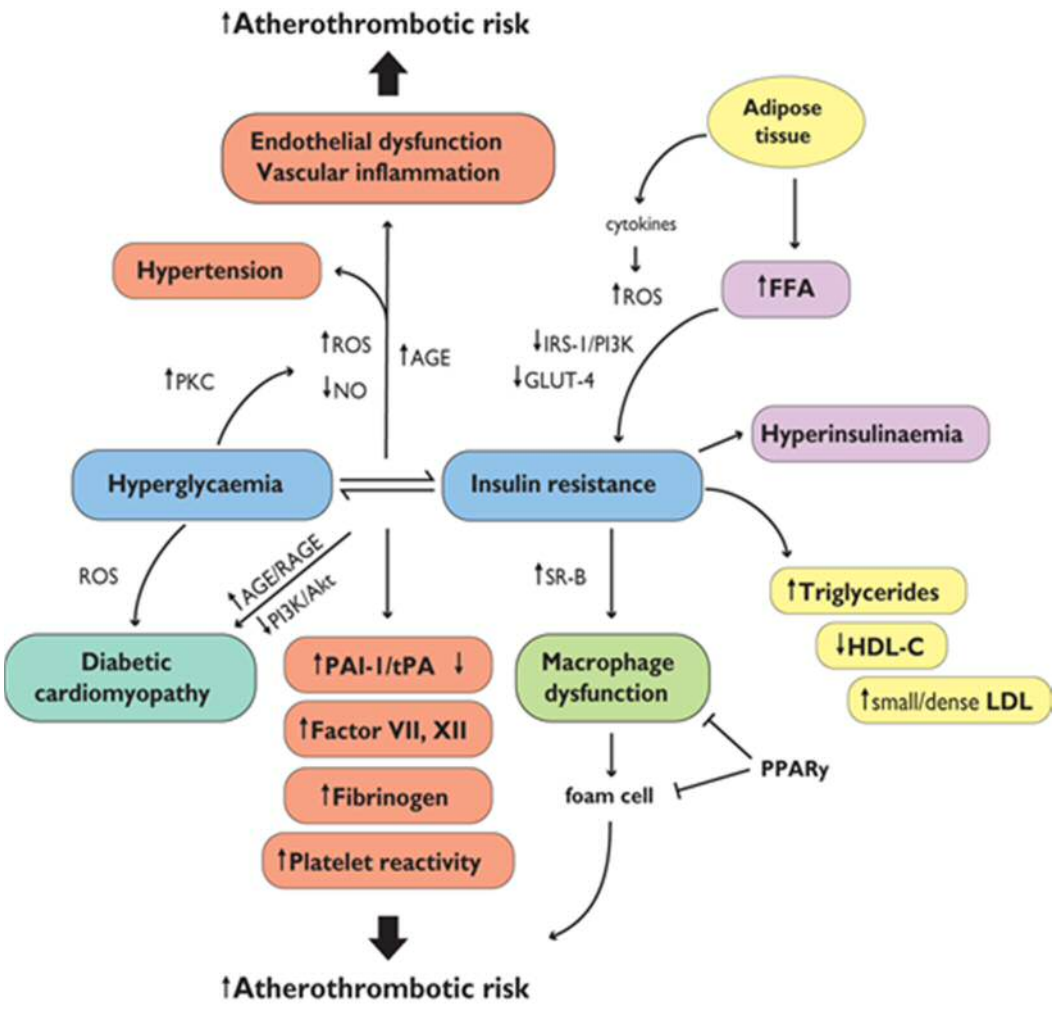
2.2 Pathophysiology of diabetes mellitus

Plasma glucose concentrations are maintained within normal narrow range through tightly regulated insulin secretion and its action in peripheral tissues. Endogenous glucose production is inhibited to maintain normal glucose level (42). Type 2 DM is the predominant form of diabetes and accounts for about

90% of all diabetes cases with increasing prevalence (43). The abnormal glucose homeostasis in DM is mainly due to inadequate secretion of insulin, and/or peripheral resistance to secreted insulin, to match metabolic needs (44). Chronic hyperglycaemia triggers changes in cellular metabolism causing tissue damage leading to long-term diabetic complications (45). The extent of diabetic tissue damage is also determined by genetic determinants of individual susceptibility (46). The presence of independent accelerating factors, such as hypertension and dyslipidaemia, also cause atherosclerosis. Excessive caloric intake, lack of exercise and a sedentary lifestyle lead to obesity which is also a major risk factor for developing DM (47, 48).

Figure 2.2 summarizes a schematic representation of the metabolic basis of increased atherosclerosis risk in diabetes. Hyperglycaemia and insulin resistance (IR) increase atherothrombotic risks via endothelial dysfunction by decreasing nitric oxide (NO), increasing advanced glycated end-products (AGE) and reactive oxygen species (ROS pathway) which lead to vascular inflammation and a prothrombotic state (increased thrombosis and reduced fibrinolysis) (45). Adipose tissue releases free fatty acids (FFAs) and cytokines which directly impair insulin sensitivity by increasing FFA-induced ROS production, reducing activation of insulin receptor substrate 1 (IRS-1) and phosphatidylinositide 3-kinase (PI3K-Akt) signaling, leading to down-regulation of insulin-responsive glucose transporter 4 (GLUT-4) resulting in IR (49, 50).

Figure 2.2: Hyperglycaemia, insulin resistance, and cardiovascular disease (50)



(AGE = advanced glycated end-products; FFA = free fatty acids; GLUT-4 = glucose transporter 4; HDL-C = high-density lipoprotein cholesterol; LDL = low-density lipoprotein particles; NO = nitric oxide; PAI-1 = plasminogen activator inhibitor-1; PKC = protein kinase C; PPARγ = peroxisome proliferator-activated receptor γ; PI3K = phosphatidylinositide 3-kinase; RAGE = AGE receptor; ROS = reactive oxygen species; SR-B = scavenger receptor B; tPA = tissue plasminogen activator)

The mechanisms which cause tissue damage by hyperglycaemia have been previously thoroughly investigated and reported with different pathways described including (1) increased flux of glucose through the polyol pathway; (2) increased intracellular formation of AGEs; (3) increased expression of the receptor for AGEs and its activating ligands; (4) activation of protein kinase (PK) C isoforms; and (5) overactivity of the hexosamine pathway (46). However, overproduction of superoxide by the mitochondrial electron-transport chain is now believed to be the underlying single mechanism causing tissue damage by hyperglycaemia (46). Increased ROS by intracellular hyperglycaemia activates pathogenic signaling pathways which in turn decrease mitochondrial biogenesis (51). This oxidative stress has an impact on susceptible pancreatic islets (β cells), adipocytes and peripheral tissues which in turn cause impaired insulin secretion by islet cells and IR in adipocytes and peripheral tissues (52). Inadequate insulin secretion and IR lead to hyperglycaemia (both post-prandial and type 2 DM with chronic hyperglycaemia) which acts as a feedback mechanism for the development of oxidative stress which causes IR (Figure 2.3) (52).

Figure 2.3 Mechanism of hyperglycaemia and dyslipidaemia-induced inflammation for the development of IR and type 2 diabetes (52)

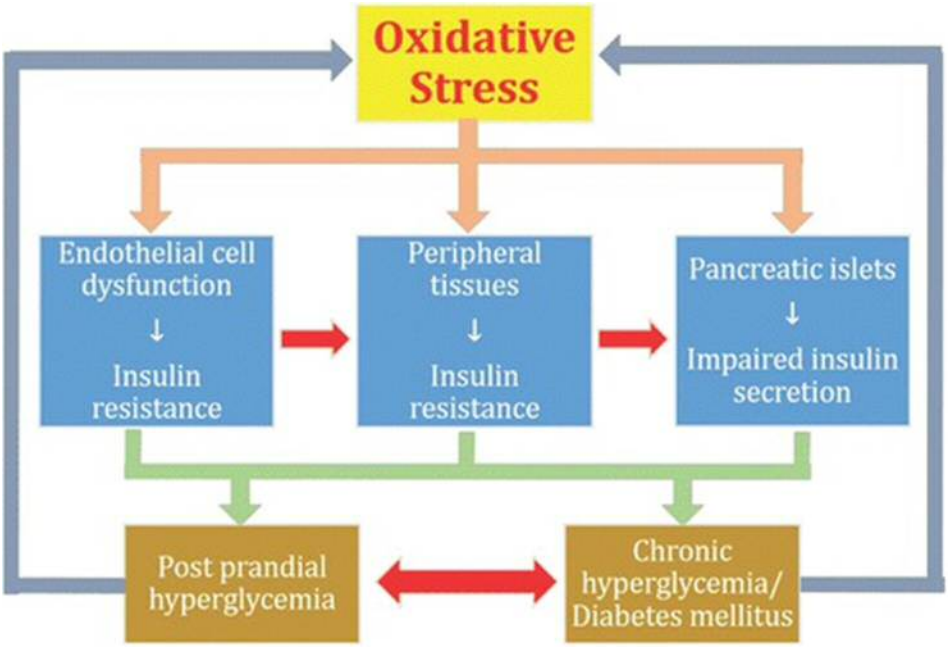
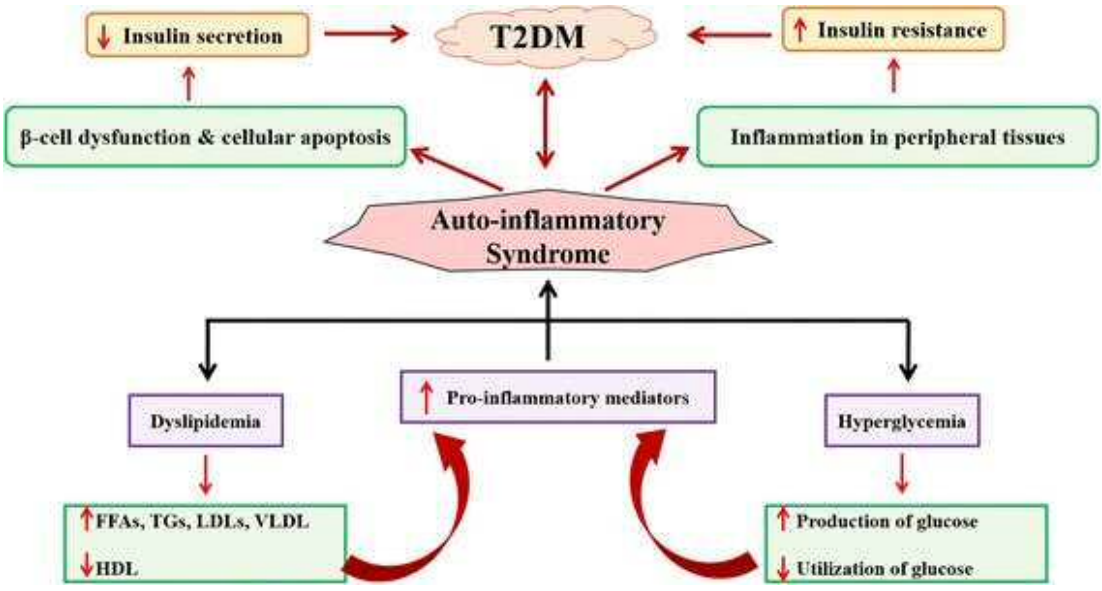


Figure 2.4: Mechanism of hyperglycaemia: Impaired insulin secretion and increased insulin resistance in type 2 diabetes (52)

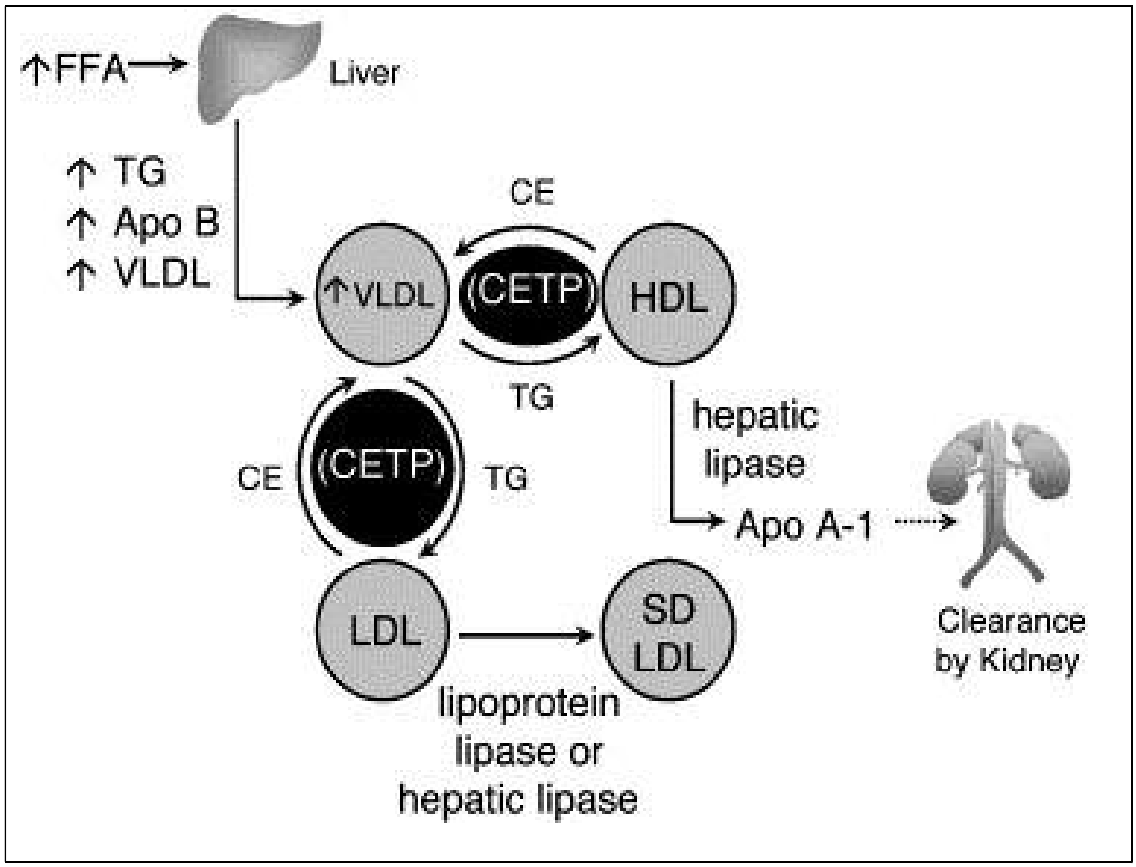


Chronic IR stimulates pancreatic secretion of insulin, generating a complex phenotype that includes progressive beta cell dysfunction (49). Insulin deficiency in diabetes results in disruption of the regulation of glucose production in the liver (imbalance between gluconeogenesis, glycogenolysis, and loss of hepatic autoregulation of glucose), glucose uptake from muscle and the release of fatty acids from adipose tissue (53). Risk factors that are associated with peripheral IR include visceral fat deposition, obesity/high BMI, genetic factors, and conditions of abnormal hormone production such as Cushing's syndrome, acromegaly, and polycystic ovary syndrome. Long-term hyperglycaemia leads to diabetic endothelial dysfunction which is regarded as an important factor in the pathogenesis of diabetic vasculopathy, including CHD (54).

Lipid changes associated with diabetes mellitus are attributed to increased free fatty acid influx secondary to IR, although the precise mechanism of diabetic dyslipidaemia is not clear. Dyslipidaemia in diabetes includes various patterns of lipid abnormalities, however, three main features constitute the diabetic dyslipidaemia triad including i) high levels of plasma triglycerides (TG); ii) low high-density lipoprotein (HDL) cholesterol levels; and iii) high levels of small dense low-density lipoprotein (LDL) cholesterol particles (55). IR causes increased breakdown of TG in the adipocytes which increase the release of fatty acids into the circulation. This increases fatty acid delivery to the liver resulting in increased synthesis of TG and very low-density lipoprotein (VLDL). Increased VLDL dissociates apolipoprotein A-I from HDL leading to quick clearance of free apolipoprotein A-I causing a

reduction in HDL. This also reduces the protective effect of HDL on atherogenesis and thereby increases the likelihood of developing lipid-laden plaques (56). Moreover, activation of lipase activity increases the synthesis of inflammatory mediators, and greater abdominal adiposity promotes the generation of small dense LDL particles. These are more likely to undergo oxidative modification, leading to a greater susceptibility to foam cell formation and propagation of atherosclerotic plaque (Figure 2.5).

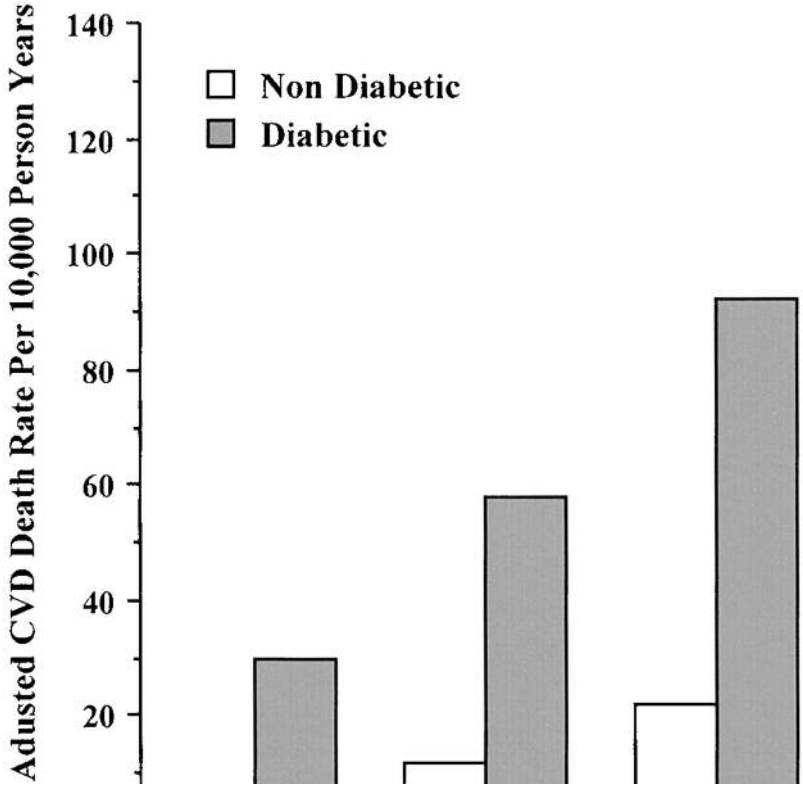
Fig. 2.5: Dyslipidaemia: Abnormal lipoprotein synthesis due to excess FFA availability in liver because of insulin resistance in Type 2 DM (56)



2.3 Modifiable risk factors for cardiovascular disease in addition to diabetes

Cardiovascular disease (CVD) is a major cause of death globally (4). There are well-recognized risk factors for CVD, including both non-modifiable risk factors (age, sex, ethnicity, social-economic status and family history), and modifiable risk factors (blood pressure, blood lipids, smoking, obesity, lifestyle). The risks of incident CVD or CVD mortality are directly proportional to the number of risk factors (Figure 2.6) (16). Hence this chapter also includes a brief description of effects of modifiable risk factors for CVD.

Figure 2.6: Age-adjusted CVD death rate by presence of number of risk factors (smoking, dyslipidaemia, hypertension) with and without diabetes (16)



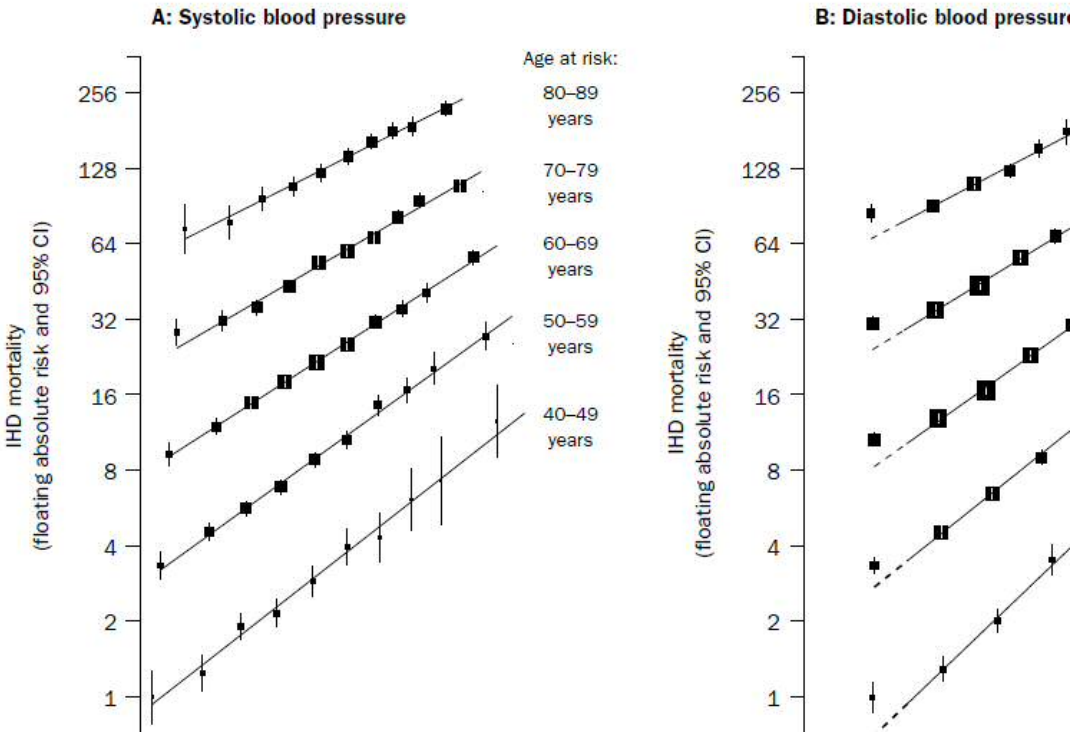
Blood pressure

Hypertension is a major global health problem. It is also a major risk factor for cardiovascular disease. About 970 million people worldwide suffer from hypertension according to the World Health Organization data (4). One recent study on the prevalence of hypertension by measured conventional blood pressure (BP) and 24-hour ambulatory BP (N=6546, aged 40 to 79 years, in 10 cohorts on 3 continents) reported that the overall prevalence of hypertension is as high as 49.3% (range between cohorts, 40.0%–86.8%) for conventional hypertension (conventional BP ≥140/90 mm Hg) and 48.7% (35.2%–66.5%) for 24-hr ambulatory hypertension (ambulatory BP ≥130/80 mm Hg) (57). Socio-economic factors influence the prevalence in different cohorts in this study. Another study reported that in 2010, 31.1% (95% CI, 30.0%–32.2%) of the world's adults had hypertension; 28.5% (95% CI 27.3%–29.7%) in high-income countries and 31.5% (95% CI 30.2%–32.9%) in low- and middle-income countries (58). With increasing awareness and treatment, from 2000 to 2010, the age-standardized prevalence of hypertension reduced by 2.6% in high-income countries, but increased by 7.7% in low- and middle-income countries. Diabetes and hypertension are common coexisting conditions and over 50% of people with diabetes mellitus, either type 1 or 2, ultimately develop hypertension as a vascular complication (59).

Uncontrolled high BP leads to a variety of changes in the myocardial structure, coronary vessels and the conduction system which results in the development of left ventricular hypertrophy (LVH), coronary artery disease

(CAD), abnormalities of the conduction system and systolic or diastolic dysfunction of the myocardium. Such anatomical changes manifest clinically as angina or myocardial infarction, cardiac arrhythmias (especially atrial fibrillation), and congestive heart failure (CHF). Studies have clearly shown a continuous log-linear relationship between blood pressure (BP) and cardiovascular risk in the whole population (both diabetic and non-diabetic) (60-62). Each difference of 20 mm Hg SBP is associated with more than a twofold difference in the stroke death rate and with twofold differences in the death rates from IHD and from other vascular causes (61). Throughout middle and old age, blood pressure is strongly and directly related to vascular mortality (Figure 2.7).

Figure 2.7: Ischaemic heart disease (IHD) mortality rate in each decade of age versus blood pressure at the start of that decade (61)



Lipids

In addition to hyperglycaemia and hypertension, dyslipidaemia is a modifiable cardiovascular risk factor for patients with or without diabetes and remains largely uncontrolled (63). In the Prospective Studies Collaboration meta-analysis a 1 mmol/L lower total cholesterol was associated with about a halving in the risk of IHD mortality at ages 40-49 years (hazard ratio 0.44 [95% CI 0.42-0.48]), and about a third (0.66 [0.65-0.68]), and a sixth (0.83 [0.81-0.85]) lower IHD mortality in both sexes at ages, 50-69, and 70-89 years, respectively (62). In the prospective meta-analysis of data from 90 056 participants in 14 randomised trials of statins, it was reported that a 1 mmol/L lower LDL cholesterol was associated with a 9% proportional reduction in all-cause mortality in people with diabetes (rate ratio [RR] 0·91, 99% CI 0·82–1·01; p=0·02) compared to the 13% reduction in those without diabetes (0·87, 0·82–0·92; p<0·0001) (64). The relationship between the absolute reductions in LDL cholesterol achieved and the proportional reductions in the incidence of coronary and other major vascular events in both people with and without DM are already established.

Smoking

Smoking is another major cause of cardiovascular disease, responsible for about one-third of all cardiovascular death in Western populations and is the most common preventable cause of cardiovascular morbidity and mortality (65). Smoking is associated with a 2 to 4-fold higher risk of coronary heart disease, a >70% higher risk of death from coronary heart disease, and an

elevated risk of sudden death (65). Many epidemiological studies have reported that smoking in both men and women substantially increases the incidence of cardiovascular disease (66-71).

Obesity

The prevalence of obesity has increased over time. According to the Health Survey for England in 2012-2014, 7 out of 10 men and 6 out of 10 women in the UK are overweight or obese. The trends (Figure 2.8) indicate that the prevalence of obesity continues to rise. Obesity is a chronic disorder associated with an increased risk of CHD and this is likely to be mediated via combined effects on lipids, blood pressure, and diabetes. Obesity, particularly in association with high waist circumference, indicating visceral fat deposition, and high body mass index (BMI), are independent risk factors for coronary heart disease (CHD) and diabetes. It was reported that marginal (5 lb) to moderate (11 to 22 lb) weight gain in adulthood increases the risk of chronic disease and negatively affects CHD risk status (72). Men and women with BMI >30 kg/m^2 have about twice the risk of CHD compared to the people with BMI 18.5 to 22.9 kg/m^2 (73). A collaborative analysis of 57 prospective studies in 894 576 participants reported that in both sexes, mortality was lowest at BMI of about 22.5-25 kg/m^2. Above this range, each 5 kg/m^2 higher BMI was on average associated with about 30% higher overall mortality: 40% for vascular mortality (HR 1.41 [1.37-1.45]) and 60-120% for diabetes (74).

Figure 2.8: Trends in obesity prevalence among adults (Health survey for England 1993-2014), Adults 16+ year of age, BMI obesity ≥ 30 kg/m^2

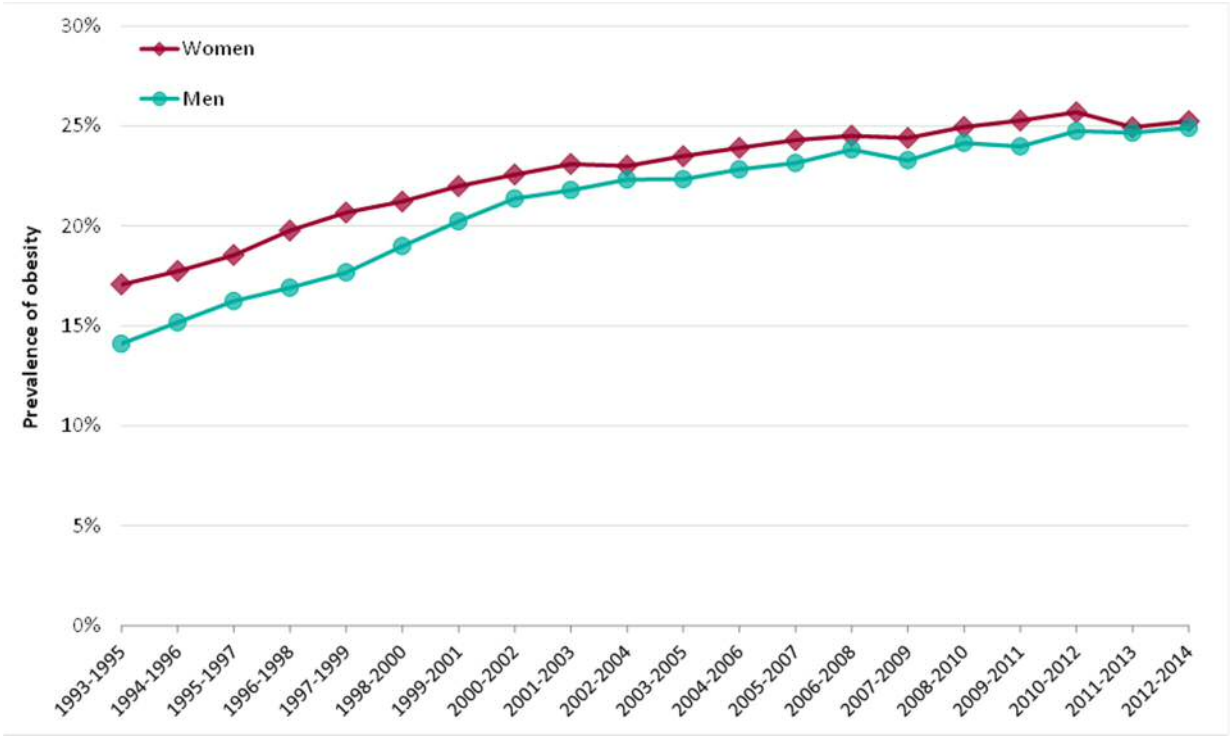
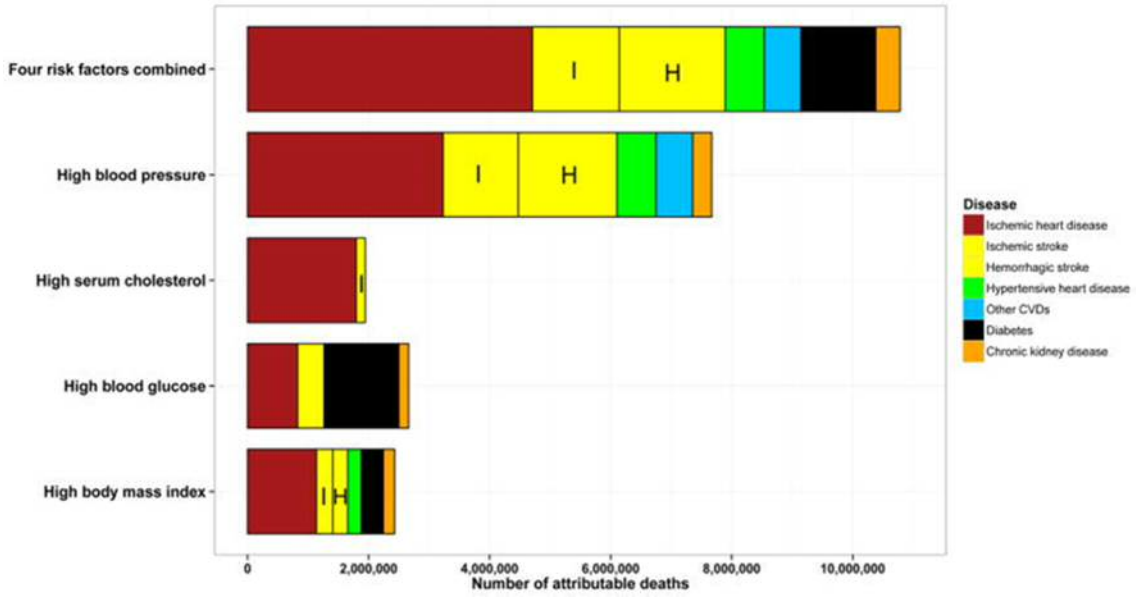


Figure 2.9 describes deaths attributable to the individual and combined effects of high body mass index, blood pressure, cholesterol, and glucose in 2010, by disease (1). Overall 44% of deaths attributable to the combined effects of these risk factors in 2010 were from IHD, 30% from stroke, and 11% from diabetes. Direct diabetes deaths accounted for only 46% of deaths due to non-optimal glucose control, with the remainder having IHD, ischaemic stroke, or CKD as the underlying causes of death (1).

Figure 2.9: Deaths attributable to the individual and combined effects of high body mass index, blood pressure, cholesterol, and glucose in 2010, by disease (1)



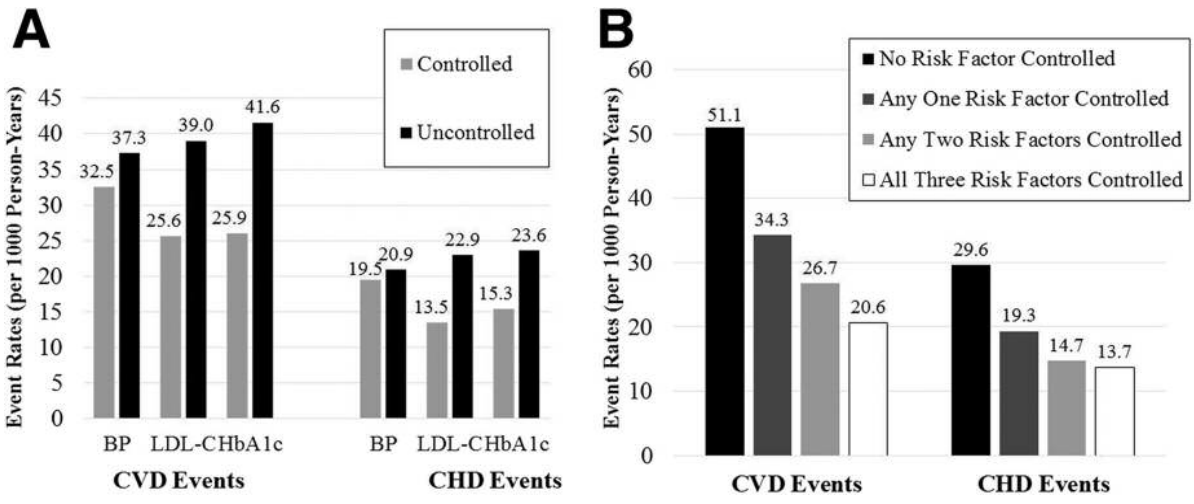
2.4 Current strategies aimed at reducing risk of cardiovascular disease in diabetes

With management and intensification of treatment for these modifiable cardiovascular risk factors over the last few decades, it has been reported that the risk of developing CHD among DM patients is falling (75). The Framingham Heart Study investigated the incidence rate of CVD among people with or without DM. Rates were compared between 1950-1966 and 1977-1995. From the earlier to the later period (there was a 50% reduction in the incidence of CVD events among people with diabetes, although the absolute risk of having CVD in people with diabetes was still 2-fold greater than in people without diabetes (75). Overall in the United States, the

estimated 10-year risk of developing CHD among adults with DM has improved significantly from 1999-2000 to 2007-2008 (76). Sustained efforts at improving risk factors should further benefit the cardiovascular health of people with diabetes (77).

One recent study assessing the effects of multifactorial risk factor control in diabetes and cardiovascular disease showed that patients with diabetes who were at target levels for HbA1c (<53 mmol/mol), BP (<130/80 mmHg), and LDL-C (<2.6 mmol/L) had substantially (~60%) lower risks for CVD and CHD than persons who were not at target levels for those risk factors (78) (Figure 2.10). For each individual risk factor, persons at target levels had lower CVD event rates than those who were not at target levels (78).

Figure 2.10: Unadjusted CVD and CHD event rates per 1,000 person-years for subjects with DM, by status of being at target level for individual risk factors BP, LDL-C, and HbA1c (A) and by the number of risk factors at target levels (B) (78)



2.4.1 **Optimisation of glycaemic control and cardiovascular outcome in diabetes**

The landmark United Kingdom Prospective Diabetes Study (UKPDS) randomised participants to intensive versus standard glucose control and was able to assess the long-term impact of a 0.9% lower HbA1c on all-cause mortality in 4585 patients with type 2 DM. It reported that each 1% reduction in mean HbA1c was associated with a 21% reduction in the risk of death related to diabetes, and the incidence of myocardial infarction and microvascular complications were reduced by 14% and 37% respectively (79). The relative risk reduction persisted for several years even after stopping intensive glucose treatment in a 10-year follow-up of the UKPDS study (risk reduction for myocardial infarction by 15% and death from any cause by 13% for the sulphonyl-insulin group) (80). However, three other large RCTs, the Action to Control Cardiovascular Risk in Diabetes (ACCORD), Action in Diabetes and Vascular Disease Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE), and the Veterans Administration Diabetes Trial (VADT) did not show that intensive blood glucose control, achieving HbA1c levels less than 7%, significantly reduced the risk of cardiovascular events compared to controls but only achieved modest risk reduction of about 10% (81-83). It was also reported to be associated with a significant increase in hypoglycaemic episodes (RR 2.03 (1.46-2.81) (84). One possible explanation for discrepant findings between UKPDS and more recent trials is that the latter were targeting much lower HbA1C level.

Several meta-analyses of the randomised trials investigating the effect of intensive glycaemic control on cardiovascular outcomes have also reported mixed results over the last few years. There is a reasonable consensus that intensive glucose control reduces the risk of microvascular complications (retinopathy, nephropathy, and neuropathy) in both type 1 and 2 diabetes patients (See Table 2.1). Most meta-analyses report a 10-15% proportional reduction in non-fatal myocardial infarction (MI) with intensive glucose control compared to standard control (84-87). However, there was no clear effect on stroke, cardiovascular death or all-cause mortality in the intensive glucose control group compared to standard control group (88-90). However, compared to the standard glucose control, intensive glucose control was associated with more serious hypoglycaemia (84). Another meta-analysis investigating similar outcomes (which included five prospective randomised controlled trials, 33,040 participants) showed that intensive versus standard glycaemic control significantly reduced coronary events without an increased risk of death (85). Similar results were reported in another meta-analysis (which included 13 studies with 34,533 patients) with a risk reduction of 15% for non-fatal MI, however, the benefit on all-cause mortality was uncertain (89). The HbA1c target range for each diabetic individual requires consideration of several factors (e.g. comorbidity, capacity for self-care, family and social support system) and assessment of the patient's risk for hyperglycaemia-related complications versus the risks of therapy. The individualization of HbA1c targets has become a treatment goal for glycaemic control (91).

Table 2.1: Summary table of meta-analyses of trials assessing the effect of intensive glucose control on cardiovascular events in diabetes mellitus

Meta-analysis (Author and year)	No. of studies included	Studies included	HbA1C difference between intensive vs. standard treatment groups	No. of participants	Effects of Intensive glycaemic control		
					MI	all-cause Mortality	Cardiovascular death
Ray *et al*. (2009)(85)	5	UKPDS 33, UKPDS 34, PROactive, ADVANCE, VADT, ACCORD	0.90%	33040	OR 0.83 (0.75-0.93)	OR 1.02 (0.87-1.19)	
Kelly *et al*. (2009)(84)	4	UKPDS 33, UKPDS 34, ACCORD, ADVANCE, VADT	0.5 to 1.4%	27802	OR 0.89 (0.75-0.93)	OR 0.98 (0.84-1.15)	
Mannucci *et al.* (2009)(86)	5	UKPDS 33, UKPDS 34, PROactive, ACCORD, ADVANCE,VADT	0.90%	32632	OR 0.86 (0.78-0.93)	OR 0.98 (0.77-1.23)	
Turnbull *et al*. (2009)(87)	4	UKPDS 33, ACCORD, ADVANCE, VADT	0.66 to 1.16%	27049	HR 0.85(0.76-0.94)	HR 1.04 (0.9-1.2)	
Wu *et al*. (2010)(88)	6	VACSDM, Kumamoto,UKPDS A, UKPDS B,VADT, ACCORD, ADVANCE	0.90%	28065	RR 0.92 (0.87-0.98)	RR 0.95 (0.8-1.12)	RR 1.10 (0.79-1.53)

Boussageon *et al*. (2011)(89)	13	UGDP (1975/76), UGDP, Kumamoto, Veteran, UKPDS, PROactive, Dargie, ACCORD, ADVANCE, VADT, HOME	NA	34533	RR 0.85 (0.74-0.96)	RR 1.04 (0.91-1.19)	RR 1.11 (0.86-1.43)
Hemmingsen *et al*. (2011)(90)	14	ACCORD, ADVANCE, Bagg,Becker, IDA,Jaber,Kumamoto, Lu, REMBO, Service, UGDP, UKPDS, VA CSDM, VADT	NA	28614	RR 0.85 (0.76-0.95)	RR 1.02 (0.91-1.13)	RR 1.11 (0.92-1.35)

2.4.2 Optimising blood pressure and cardiovascular outcomes

The UKPDS randomised people with diabetes and hypertension into a sub-study which evaluated the effect of intensive versus less intensive antihypertensive therapy (92). Treatment of hypertension was the first intervention proven to reduce cardiovascular risk in people with diabetes. Overall in the UKPDS, more intensive therapy significantly reduced the risk of stroke by 44% (95% CI 11-65, p=0.013) and there was also a 21% lower (-7-41) risk of MI, although the latter did not achieve statistical significance. More recently the ADVANCE study randomised over 11,000 people with type 2 diabetes to treatment with a fixed combination of an angiotensin-converting enzyme (ACE) inhibitor/diuretic versus placebo (93). In those allocated active treatment the relative risk of a major macrovascular or microvascular event was reduced by 9% (0-17, p=0.04). A reduction in macrovascular events alone was similar but not independently significant (HR 0.92; 0.81-1.04, p=0.16). The UKPDS also demonstrated that lowering BP reduced the progression of retinopathy and the development of albuminuria (92).

A meta-analysis of individual data from one million adults in 61 prospective studies clearly showed that having a lower BP was associated with a lower risk of mortality from ischaemic heart disease and other vascular causes (61). The risk of cardiovascular events was 2-fold higher for every 20/10

mmHg higher level of systolic blood pressure (SBP). A recent meta-analysis of 40 RCTs trials (100 354 participants) determined the association between BP-lowering treatment and vascular disease in type 2 DM (94). Lowering SBP by 10 mmHg was associated with a significantly lower risk of mortality (relative risk (RR), 0.87; 95% CI, 0.78-0.96); including a reduction in risk of cardiovascular events, coronary heart disease, stroke, retinopathy and albuminuria (94).

Treatment of hypertension in people with diabetes has been shown to improve cardiovascular outcomes. However, the benefits of intensive BP lowering (SBP 120-130 mmHg or DBP 80 mmHg) are inconclusive. There is still a debate about how low a BP target should be when treating hypertension in people with diabetes. Recent studies have examined whether intensive BP lowering will further prevent cardiovascular events compared with a standard BP target (SBP 140-160 mmHg, DBP 85-100 mmHg). The ACCORD BP trial investigated the effect of intensive antihypertensive therapy and did not demonstrate a significant reduction in the primary cardiovascular outcome or the rate of death from any cause, despite the fact that there was a significant and sustained difference between the intensive-therapy group and the standard-therapy group in mean systolic blood pressure (95). The SPRINT trial (Systolic Blood Pressure Intervention Trial), involving a longer duration of treatment and a larger number of participants demonstrated benefits of intensive management of SBP to a target <120 mmHg compared with managing SBP to a target of less than 140 mm Hg. Intensive management of SBP to a target of <120 mm Hg reduced risks of

complications of high blood pressure, including heart attacks, heart failure, and stroke, by 25 percent and lowered the risk of death by 27 percent (96). Because of clear superior benefit, the trial was recommended to stop early by the Data Monitoring Committee. A systematic review reported that the use of intensive compared with standard blood pressure targets was associated with a small reduction in the risk of stroke, but no evidence of a reduced risk for all-cause mortality or myocardial infarction. However, this review involved a relatively small number of trials (N=5) with a short duration of follow-up (97). NICE guidelines recommend adding medications if lifestyle advice does not reduce blood pressure to below 140/80 mmHg (below 130/80 mmHg if there is kidney, eye or cerebrovascular damage).

There are several different classes of medication available for treatment of high BP including beta blockers, calcium channel blockers, ACE inhibitors, angiotensin receptor blockers and thiazides diuretics. The use of BP-lowering drugs for the prevention of cardiovascular disease was investigated in a meta-analysis of 147 RCTs, comprising data from 958 000 people (98). It reported that all the classes of BP- lowering drugs have a similar effect, in general, reducing cardiovascular events. The NICE guideline recommends a once-daily, generic angiotensin-converting enzyme (ACE) inhibitor as the first-line antihypertensive medication in diabetic population except for people of African or Caribbean family origin, where a calcium channel blocker should be used as first-line antihypertensive therapy. An angiotensin-converting enzyme inhibitor (ACE-I) or an angiotensin-receptor-blocker (ARB) is the preferred drug of choice for treatment of hypertension in people with diabetes

at high risk of cardiovascular disease (99-102). Recently developed drugs, such as glucagon-like peptide -1 (GLP-1) receptor agonists and Sodium-glucose co-transporter-2 (SGLT2) inhibitors, also have hypotensive actions to use in diabetics with hypertension. SGLT2 inhibitors and GLP-1 receptor agonists also suppress the onset and progression of cardiovascular disease, as well as diabetic nephropathy (103).

2.4.3 **Optimising lipid profiles**

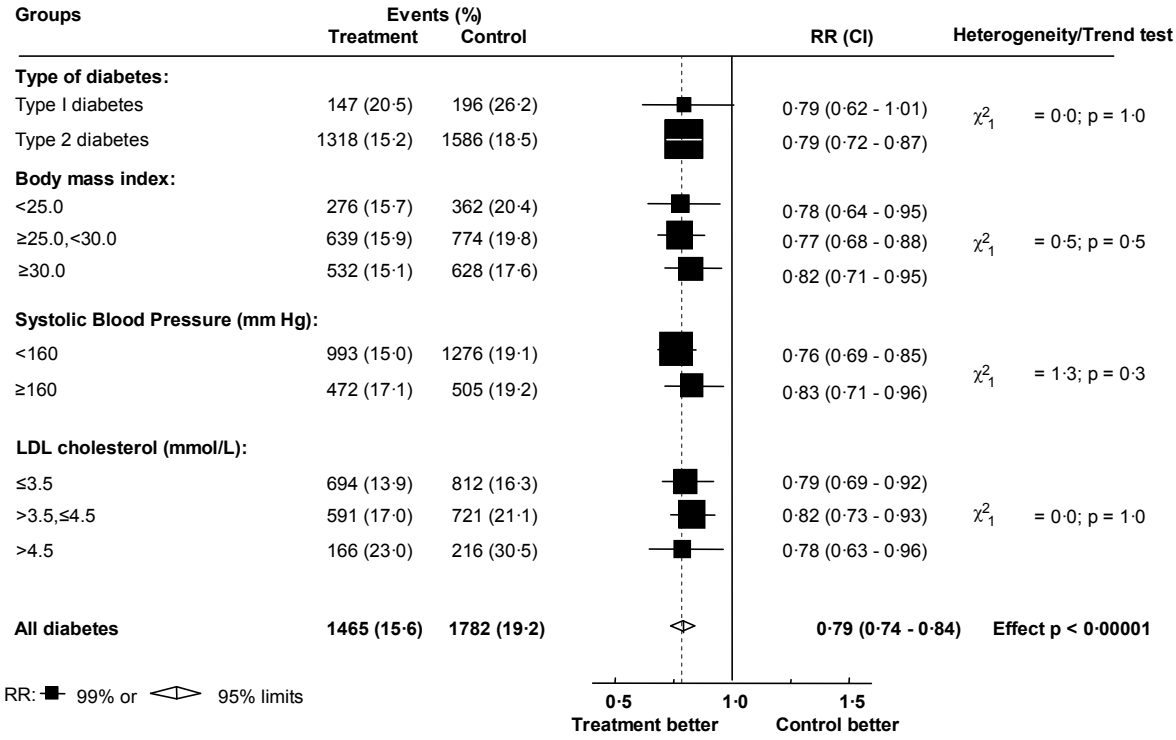
Cholesterol-lowering, particularly using statins, is also of clear and proven benefit in reducing cardiovascular risk in people with diabetes (104, 105). Many observational studies have shown that a continuous positive (log-linear) relationship between coronary heart disease and blood cholesterol level, and randomised trials have shown this to be causal (64, 104).

The Heart Protection Study (HPS) included 5963 people with diabetes, about half of whom did not have diagnosed vascular disease at baseline. Overall in HPS, allocation to simvastatin 40mg daily reduced LDL-cholesterol by an average of 1 mmol/L and among the participants with diabetes there was a highly significant 22% (95% CI 13-30; $p < 0.0001$) reduction in major vascular events (106). Other RCTs have also shown that lowering LDL-cholesterol can significantly reduce the incidence of CHD among participants with diabetes (107).

An individual participant meta-analysis including 14 RCTs of statins in 90,056 individuals showed that lowering LDL-cholesterol by 1 mmol with a statin can safely reduce the 5-year incidence of major cardiovascular events (including major coronary events, coronary revascularisation, and stroke) by about one-fifth (104). The 19% proportional reduction in CHD death per mmol/L LDL-cholesterol reduction translated into 14 fewer deaths per 1000 among participants with pre-existing CHD. This meta-analysis indicated that the proportional reduction in risk of major vascular events was approximately related to the absolute reduction in LDL-cholesterol achieved with statin treatment in both diabetic and non-diabetic participant groups (64). The role of fibrates added to a statin was also investigated in the ACCORD lipid trial, but the findings did not support the routine combined use of a fibrate and a statin in diabetic patients (108).

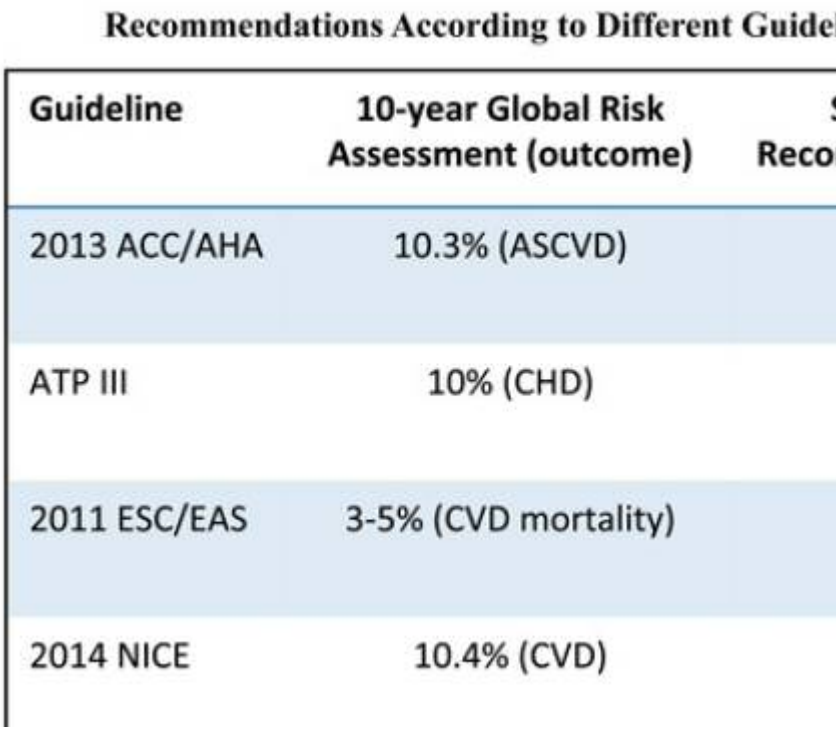
In the Cholesterol Treatment Trialists' Collaborative meta-analyses of data from over 90,000 individuals in 14 trials, 21% of participants had diabetes at baseline (64, 104). The incidence of major vascular events in this group was reduced by 21% (99% CI 14-28; $p<0.0001$) per 1 mmol/L lower LDL-cholesterol concentration (Figure 2.11).

Figure 2.11: Proportional effects on major vascular events per mmol/L reduction in LDL-cholesterol by baseline prognostic factors in participants with diabetes (64)



Current guidelines advocate prescribing statins using an individualized approach according to calculated cardiovascular risks (Figure 2.12) (109).

Figure 2.12: Recommendation of statin use per current guidelines with cardiovascular risk assessment (109)



Recommendations According to Different Guide

Guideline	10-year Global Risk Assessment (outcome)	Reco
2013 ACC/AHA	10.3% (ASCVD)	
ATP III	10% (CHD)	
2011 ESC/EAS	3-5% (CVD mortality)	
2014 NICE	10.4% (CVD)	

The NICE guidelines recommend that use of statins depends on the individualised assessment including blood tests. The assessment before recommending statin therapy for the prevention of cardiovascular disease includes assessment of smoking status, alcohol consumption, blood pressure, body mass index or other measure of obesity, total cholesterol, non‐HDL cholesterol, HDL cholesterol and triglycerides, HbA1c, renal function and eGFR, transaminase level (fatty liver), and thyroid‐stimulating hormone. For the primary prevention of CVD to people with type 2 diabetes who have a

10% or greater 10‑year risk of developing CVD, statin therapy is recommended (NICE guideline CG 181).

2.4.4 **Lifestyle and BMI modification**

Aspects of lifestyle, especially less physical activity, and more sedentary behavior play a major role in the risk of developing diabetes and, in turn, the risk of CVD (110). Lifestyle intervention is an affordable, safe and feasible approach for the prevention of CVD. Previous large trials, including the Diabetes Prevention Program (DPP), the Finnish Diabetes Prevention Study (DPS), the Indian Diabetes Prevention Programme (IDPP) and the Da Qing study clearly showed that interventions with diet and exercise can prevent or delay diabetes incidence and that these interventions were cost-effective in high-risk populations (111-116). A recent review and meta-analysis which included 18 observational studies with 794 577 participants showed that higher levels of sedentary (sitting) time (comparing the greatest sedentary time compared with the lowest) was associated with an increased risk of diabetes (RR 2.12 95% confidence interval (CI) 1.61-2.78), CVD (RR 2.47 95% CI 1.44-4.24) and a 90% increase in the risk of cardiovascular mortality (HR 1.90 95% CI 1.36-2.66) (117). The Look AHEAD study (N= 5145 overweight or obese patients with type 2 diabetes) randomly assigned individuals into an intensive lifestyle intervention that promoted weight loss through reduced caloric intake and increased physical activity (intervention group) or to receive diabetes support and education (control group). Although

primary cardiovascular outcomes were not statistically significant (HR in the intervention group 0.95, 95% CI 0.83-1.09, P =0.51) at median follow-up of 9.6 years, weight loss was greater in the intervention group than in the control group throughout the study (8.6% vs. 0.7% at 1 year; 6.0% vs. 3.5% at study end). The intensive lifestyle intervention produced greater reductions in HbA1C and greater initial improvements in fitness and all cardiovascular risk factors (118). A meta-analysis investigating the effects of all lifestyle interventions vs standard advice (HR 0.51, 95% CI 0.44 to 0.60, P<0.001) reported a relative 49% reduction in risk of developing diabetes(119). Hence a healthy diet, regular physical activity, maintaining a healthy body weight and avoiding tobacco appear to prevent or delay the onset of type 2 DM and its complications including prevention of CHD (19). Newer pharmacological treatments are available, such as the medical therapy orlistat, non-surgical endoscopic intervention (gastric balloon, endo-barrier) and bariatric procedures (Gastric bypass surgery, sleeved gastrectomy) for people with morbid obesity. The Swedish Obese Subjects (SOS) study reported, at 15 years, weight loss (percent of total body weight) was 27 ± 12% for gastric bypass, and 13 ± 14% for gastric banding compared with a slight weight gain for control subjects (120). Long-term medical (nonsurgical) weight loss rarely exceeded 8% of excess weight loss (118). Bariatric surgery in overweight and obese people with diabetes improves glycaemic control and is even reported to diabetes remission in the early phase of the disease (121). Other weight management measures including very low-calorie liquid diet (VLCD) has been proven to be beneficial after 1 year, mean (±SD) weight changes of 11.4 ± 9.1 kg with the VLCD, -6.8 ± 6.4 kg with the LCD, and -5.1 ± 5.9 kg

with the restricted normal food diet (122) and the amount of weight loss with those diet plans was comparable with or without diabetes (123).

In summary, more than half of the reduction in cardiovascular mortality in the last three decades has been attributed to population-level changes in cardiovascular risk factors, primarily reductions in cholesterol and blood pressure levels and smoking. However, other major risk factors, such as obesity, type 2 diabetes, and aging of the population have contributed to an increased absolute number of CVD events. Recently, the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS) reviewed the available evidence and set a reasonable and cost-effective target approach for cardiovascular disease prevention and Figure 2.13 describe the treatment target and goals for managing modifiable risk factor for cardiovascular disease.

Figure 2.13: Treatment target goals for cardiovascular disease prevention adopted from 2016 ESC/EAS Guidelines (124)

Treatment targets and goals for cardiovascular disease preven

Smoking	No exposure to tobacco in any form.
Diet	Healthy diet low in saturated fat with a focus on whole grain products, vegetables, fruit and fish.
Physical activity	2.5–5 h moderately vigorous physical activity per week or 30–60 min most days.
Body weight	BMI 20–25 kg/m^2, waist circumference <94 cm (men) and <80 cm (women).
Blood pressure	<140/90 mmHg[a]
Lipids LDL-C is the primary target[b]	**Very high-risk: LDL-C <1.8 mmol/L (70 mg/dL)** or a reduction of at least 50% if the baseline[b] is between 1.8 and 3.5 mmol/L (70 and 135 mg/dL).
	High-risk: LDL-C <2.6 mmol/L (100 mg/dL) or a reduction of at least 50% if the baseline[b] is between 2.6 and 5.2 mmol/L (100 and 200 mg/dL).
	Low to moderate risk: LDL-C <3.0 mmol/L (115 mg/dL).
	Non-HDL-C secondary targets are <2.6, 3.4 and 3.8 mmol/L (100, 130 and 145 mg/dL) for very high-, high- and moderate-risk subjects, respectively.
	HDL-C: no target, but >1.0 mmol/L (40 mg/dL) in men and >1.2 mmol/L (48 mg/dL) in women indicates lower risk.
	TG: no target but <1.7 mmol/L (150 mg/dL) indicates

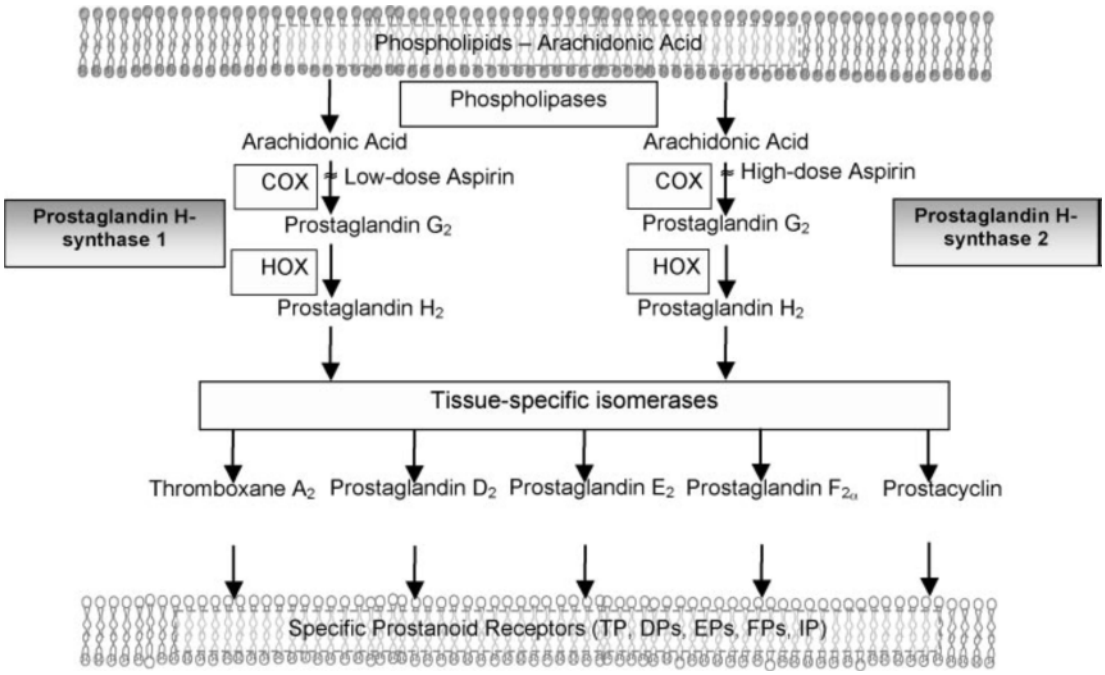
2.5 Role of aspirin and omega-3 FA for primary prevention of cardiovascular disease in diabetes

2.5.1 **Aspirin and prevention of cardiovascular disease**

Platelets are anucleated blood cells formed by fragmentation of megakaryocyte's cytoplasm, with a maximum circulating life span of 10 days in humans (125). Approximately 10^{11} platelets are produced each day with a capacity to increase up to 10 times under conditions of increased demand (125). Platelets are a vital component of physiological haemostasis, with the capacity to adhere to and accumulate at the site of an injured blood vessel wall and to activate the release of biochemical factors to stop bleeding such as cytokines, chemokines and growth factors. For example, activated platelets synthesize prostanoids such as thromboxane A2 from arachidonic acid released from cell membrane phospholipids. Aspirin irreversibly acetylates platelet prostaglandin H-synthase 1 or cyclooxygenase-1 (COX 1) or H-synthase 2 (COX-2) (Figure 2.14) (126). This results in a reduction in thromboxane A2 production (TxA2) which is a potent vasoconstrictor and promoter of platelet aggregation. TxA2 rapidly metabolises to thromboxane B2 which is then excreted via the urine. It was previously reported that aspirin inhibits platelet COX 1 activity by about 95% (127). Platelets produce 70% of the thromboxane in the human body and the remaining 30% is produced by extra-platelet sources such as monocytes and macrophages in the setting of acute inflammation (128). By virtue of these actions, aspirin mediates

beneficial anti-inflammatory and anti-platelet effects which may have important roles in preventing atherosclerotic risk in individuals with diabetes (see Section 2.2 and Figure 2.2 of this chapter).

Figure 2.14: Arachidonic acid metabolism and mechanism of action of aspirin (126)



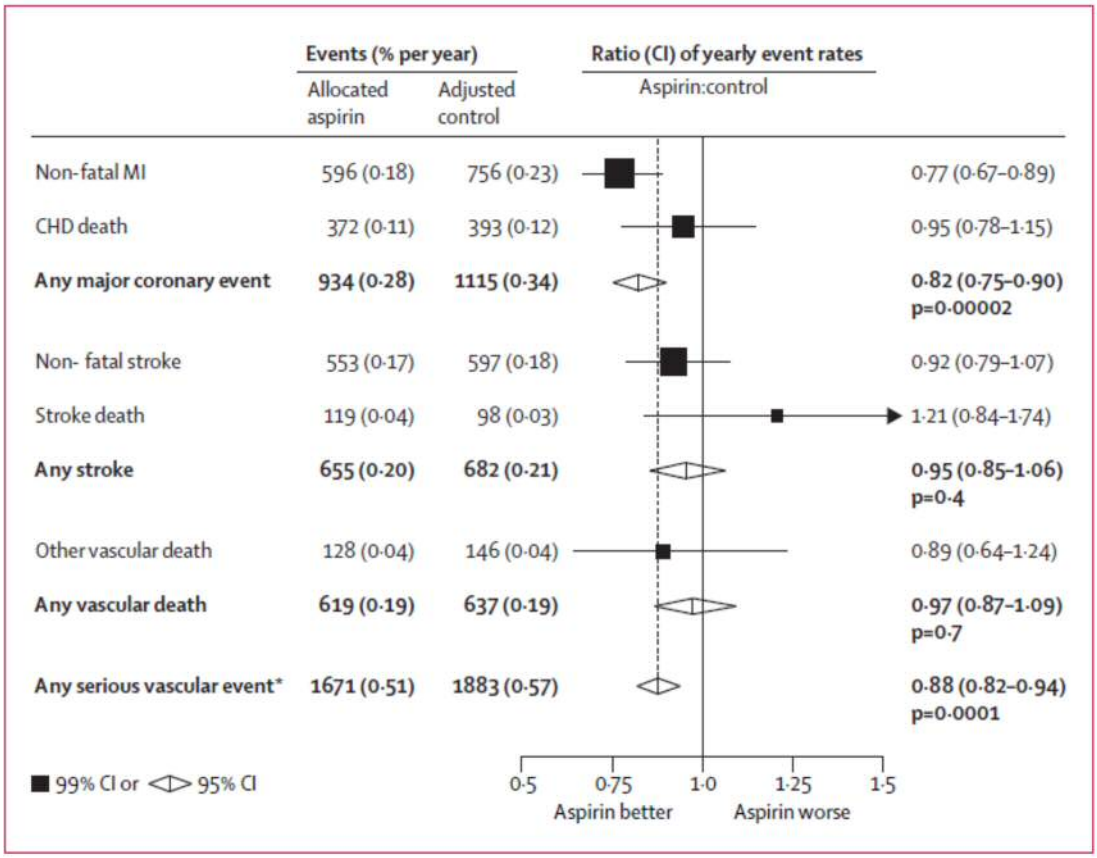
For the secondary prevention of cardiovascular disease, it is well established that aspirin reduces CVD risk and the proportional benefits appear to be similar whether or not such individuals have diabetes (129). Antiplatelet therapy is used both for the short-term treatment of patients after an acute coronary syndrome event and for secondary prevention in high-risk patients who have had a previous MI, stroke or transient ischaemic attack (TIA). Aspirin is the most commonly used antiplatelet drug prescribed and a dose of 75-325 mg is as effective as higher doses for long-term treatment. The

collaborative meta-analysis of RCTs of antiplatelet therapy in high-risk patients involving 287 studies and 135,000 participants compared antiplatelet therapy versus control using an outcome measure of major vascular events: (i.e. non-fatal MI, non-fatal stroke or vascular death). The results showed that allocation to antiplatelet therapy reduced the combined outcome of any major vascular event by about one quarter; non-fatal MI was reduced by one-third, non-fatal stroke by one-quarter and vascular mortality by one-sixth. It also showed that the absolute benefits in these high-risk patients substantially outweighed the absolute risks of major bleeding (130).

However, the majority of people with diabetes (70-80%) do not have clinically established occlusive arterial disease and it is currently unclear whether they should take aspirin for primary prevention of major vascular events (MVEs). A meta-analysis of individual participant data from six primary prevention RCTs, (involving 95,000 individuals, 660,000 person-years, and 3554 serious vascular events) showed that aspirin is of uncertain net value (131). Aspirin allocation showed a 12% proportional reduction in major vascular events but vascular mortality did not differ significantly (Figure 2.15). In contrast, aspirin allocation increased the risk of major gastrointestinal and extracranial bleeding (131). Although diabetic patients are at higher vascular risk and may, therefore, have more to gain from aspirin's anti-platelet effects, they are also at greater risk of bleeding than non-diabetic patients (131) and aspirin will increase this risk. Other meta-analyses of aspirin for primary prevention of CVD results suggest a modest (~9%) relative reduction in risk for CVD events, however, ≥2-fold increase relative risk of bleeding, mainly from the

gastrointestinal system (132-134). However, the majority of people with diabetes do not have established occlusive arterial disease. In this intermediate-risk group, it is unclear whether they should take aspirin for the primary prevention of cardiovascular disease. Hence there is a need for more randomised evidence to explore aspirin's safety and efficacy in this context. The large ASCEND study was established to address this question and is the largest ongoing primary prevention trial of aspirin in diabetes.

Figure 2.15: Serious vascular events in primary prevention trials-proportional effects of aspirin allocation (131)



2.5.2 **Aspirin resistance**

Aspirin reduces the risk of cardiovascular disease by about 25% in patients with vascular disease, which is believed to result from its ability to reduce clotting via its antiplatelet effect (131). The term “aspirin resistance” has been used in the literature to describe variability in the response to aspirin’s antiplatelet activity especially in patients with diabetes. Aspirin resistance is thought to be due to a failure to adequately suppress thromboxane generation resulting in an increased risk of occlusive events. Although the exact causes are still unclear, the possible mechanisms include rapid platelet turnover, possible extra-platelet sources of thromboxane A production, and drug interactions, for example, NSAIDs competing with aspirin for a common molecular action site (126). In aspirin resistance, the assumption is that aspirin either fails to suppress TxA2 production or incompletely inhibits COX-1 leading to the faster recovery of platelet COX-1 activity (135). The implication of faster recovery of COX-1 activity is that once-daily dosing may not be adequate to achieve sustained suppression of COX-1 and that some patients may need a twice-daily regimen in order to maintain complete suppression of TxA2 production (136).

Aspirin irreversibly acetylates platelet prostaglandin H-synthase 1 or cyclooxygenase-1 (COX 1) or H-synthase 2 (COX-2) (as described earlier Figure 2.9). The half-life of TxA2 is very short (about 30 seconds). It is rapidly converted to its stable metabolite, thromboxane B2 (TxB2) (137). Trials have been conducted to assess aspirin resistance using urinary thromboxane B2

(UTxB2) as a biomarker of in-vivo thromboxane generation. In the Heart Outcomes Prevention Evaluation (HOPE) Study, UTxB2 was measured in 488 people treated with aspirin who had a myocardial infarction, stroke, or cardiovascular death during 5 years of follow-up and in 488 sex- and age-matched control subjects also receiving aspirin who did not have an event (138). It was reported that among aspirin-treated patients at high risk of cardiovascular events, persistent thromboxane generation predicts the risk of the cardiovascular outcomes. It was also suggested that high UTxB2 can prospectively identify aspirin resistance patients (138). The Leukotrienes and Thromboxane In Myocardial Infarction (LTIMI) study (n=60+119) also evaluated the relationship between UTxB2 and MVEs in patients with acute myocardial infarction (AMI) (139). The study found that UTxB2 levels predicted the 1-year cumulative MVEs in AMI patients and they provide prognostic information on the left ventricular performance (139). This recent trial result also supported the utility of UTxB2 measurements as a predictive biomarker of MVEs in high-risk patients.

It has been suggested that the clinical efficacy of low-dose aspirin in patients with diabetes may be substantially lower than in people without diabetes (140-142). In two small studies, there was reduced response to antiplatelet activity in people with diabetes. Using a platelet function analyser (PFA)-100, both studies measured levels of TxB2, platelet aggregation (PA), and platelet COX-1 and COX-2 expression. There was a suggestion of a higher dose of aspirin being required by people with DM to achieve full TxA2 suppression and to have a desired cardioprotective effect (143, 144). Differing levels of

UTxB2 were measured before and after aspirin therapy in people with type 2 DM, indicating the biological variability of TxA2 suppression by aspirin (145). It was also thought that there is the faster recovery of inhibition of prostaglandin synthesis from arachidonic acid (AA-dependent platelet function) and rapid platelet turnover in diabetes (146). However, a further study from the same research group concluded that variability in the recovery of platelet COX activity after low-dose aspirin occurs in patients with and without diabetes and is related more to BMI (147).

A single-centre, randomised, prospective double-blind study examined the effect of 2-week treatment periods with aspirin 100 mg once daily, 200 mg once daily or 100 mg twice daily in people with diabetes without a pre-existing cardiovascular event (148). The UTxB2 reduction was greater with 100 mg twice daily dosing in type 2 diabetes compared with once-daily dosing prompting the need for the further trials evaluating the effects of twice-daily dosing for prevention of cardiovascular outcomes (148).

Although some studies report a lack of suppression of COX2 by aspirin in diabetes, others report no difference in UTxB2 measurement in people with or without diabetes after taking aspirin. One study reported (n=161) that aspirin treatment inhibited UTxB2 by 72.5% in diabetic patients and by 75.1% in controls and in that study there was no statistically significant difference between diabetic and control patients in the proportion labelled as poor aspirin responders defined by their UTxB2/mg creatinine ratio being higher than 1500 pg (127). Our own small pilot study (n=11) showed similar results

with a change in UTxB2 after aspirin of 68.15% ± 8.65 (mean ± SD) in healthy volunteers.

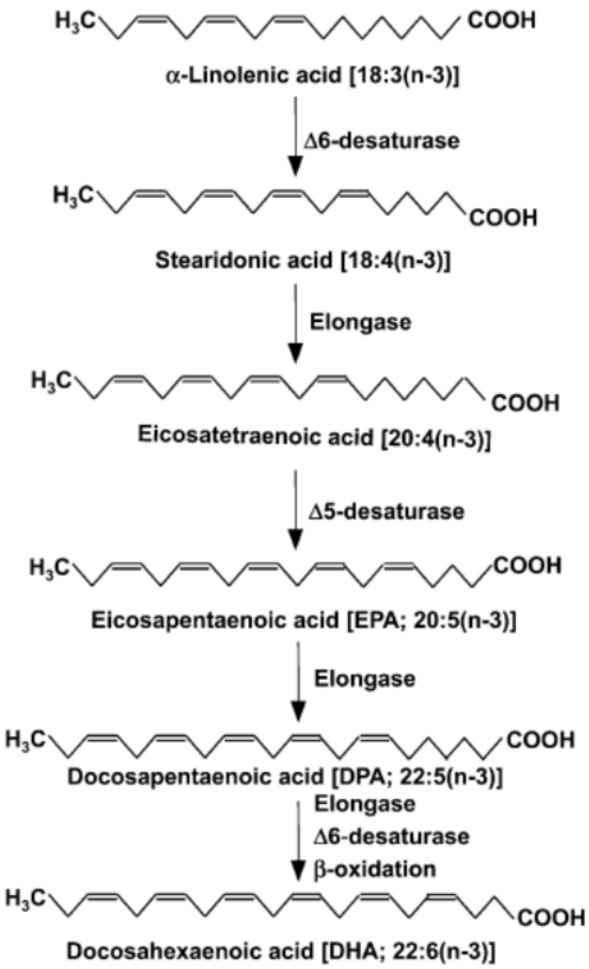
Therefore, it would be helpful to know that TxA2 is adequately suppressed in patients taking aspirin in ASCEND to help inform the interpretation and generalizability of the results in patients with diabetes. The pilot study in ASCEND also represents an opportunity to assess the time-dependent recovery of platelet COX-1 activity during once-daily dosing of aspirin, by profiling the concentration of aspirin metabolites over time. Further discussion on the plausible markers of compliance with aspirin and measurement of urinary thromboxane B2 in ASCEND will be described in the methodology chapter, section 3.4.

2.5.3 **Omega-3 FA and prevention of cardiovascular disease**

Omega-3 FAs are n-3 polyunsaturated fatty acids (3-PUFA) that humans are unable to synthesize de novo. The principal n-3 PUFAs [eicosapentaenoic acid (EPA, 20:5n–3) and docosahexaenoic acid (DHA, 22:6n–3)] are either synthesized from α-linolenic acid (ALA, 18:3n-3) (Figure 2.16) or absorbed from the diet as a pre-formed nutrient. ALA is an essential fatty acid for humans and cannot be synthesized from saturated fatty acids. Humans are unable to insert a double bond at the C-15 position of a fatty acid carbon chain because of a lack of the desaturase enzyme (149). Oily fish and various other seafoods are rich in omega-3 FA. Many dietary

recommendations encourage people to consume fish meals twice a week (preferably containing oily fish). Omega-3 FA is consumed by individuals for prevention of cardiovascular and other disease outcomes, but whether such supplements are beneficial is uncertain.

Figure 2.16: Biosynthesis of docosahexaenoic acid (DHA) from alpha-linolenic acid (ALA) (Adopted from Philip Calder, Journal of Nutrition (150))



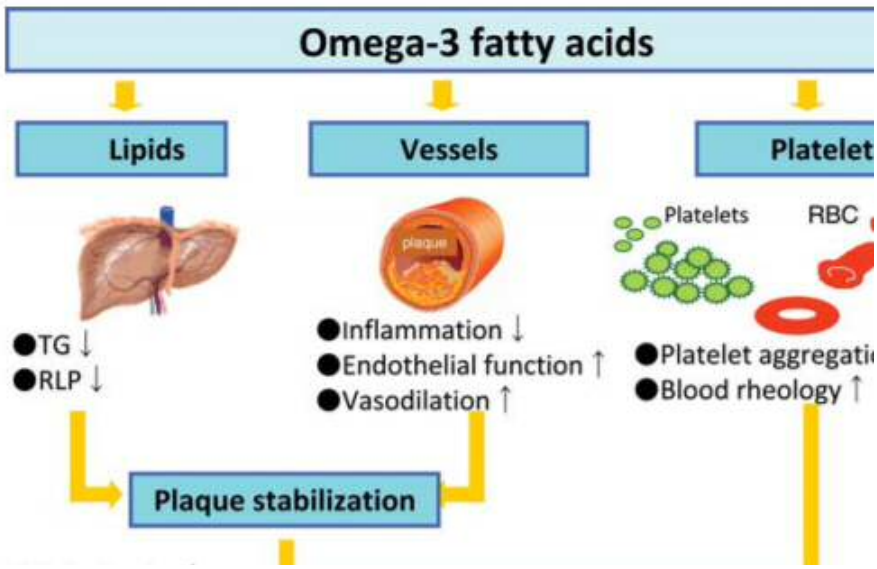
The possible link between 3-PUFA and the prevention of CVD was first recognised in the 1940s after very low rates of CHD were observed among

Greenland Eskimos compared to native Danish populations (151). Greenland Eskimos typically consumed a greater intake of dietary fish compared to the Danes, despite high total fat intakes in both groups. Since then the possible cardioprotective effects of 3-PUFA or fish intake have been tested in various secondary prevention trials published before ASCEND was designed, which suggested a modest benefit of supplementation with omega-3 FA (152-154).

2.5.3.1 Possible effects of omega-3 FA on cardiovascular disease

The possible effects of omega-3 FA on cardiovascular disease have been extensively investigated. Previous reports suggest that omega-3 FA can increase arrhythmic thresholds, reduce blood pressure, improve arterial and endothelial function, reduce platelet aggregation, and has effects on lipids(155). Figure 2.17 summarises the beneficial cardiovascular protective effects of omega-3 FA on lipids, blood vessels, and platelets.

Figure 2.17: Beneficial effects of omega-3 fatty acids. TG, triglycerides; RLP, remnant lipoproteins; RBC, red blood cells (155)



Triglycerides and lipoproteins

It was first reported that fish oil has an effect on lipoproteins in 1977 after a scientist put himself on an Inuit diet which had very high content of omega-3 FA for 100 days (156). A reduction in the triglyceride-rich VLDL cholesterol was observed, along with an increase in HDL-cholesterol. The dose-dependent effect of omega-3 FA to lower triglycerides (TG) is now well recognised (157). Various possible mechanisms for lowering TGs have been proposed. It appears that reduction of the fatty acid delivery to the liver reduces denovo lipogenesis, which is the process of converting carbohydrates into fat and is an important step in reducing the fatty acid availability for TG synthesis (158).

A systematic review and meta-analysis of 23 RCTs in type 2 DM (with a mean treatment duration of about 9 weeks with a mean dose of omega-3 FA of 3.5 g/day) concluded that omega-3 FAs significantly reduced plasma levels of TG by 25% (mean: 0.45 mmol/L), VLDL-cholesterol by 36% (mean: 0.07 mmol/L) and VLDL-TG by 39.7% (mean: 0.44 mmol/L), and a slightly increased LDL-c (mean: 5.7% (159). Another meta-analysis of 26 trials also reported a similar TG-lowering effect with a reduction by almost 30% (160). Recently the possible different effects of DHA and EPA on cardiovascular outcomes were investigated given their different effects on lipoproteins (161, 162). A systematic review and meta-analysis of randomised placebo-controlled trials of monotherapy with EPA (n=10), DHA (n=17) or EPA versus DHA (n=6) reported that although both EPA and DHA reduce triglyceride, DHA increased LDL-c, while EPA non-significantly reduced LDL-c (161).

However, it was not clear what the underlying mechanism might be or what the significance of these differences might be on clinical outcomes in clinical trials. However, neither of these meta-analyses explored the effects of higher doses of omega-3 FA on lipoprotein levels. In hypertriglyceridemic patients, supplementation with omega-3 FA lowers triglycerides level modestly and omega-3 FA can be considered as a reasonable therapeutic strategy in these individuals to prevent cardiovascular disease.

Effects of omega-3 FA on blood glucose

Early non-randomised studies in individuals with type 2 diabetes suggested that omega-3 FA might have an impact on glycemic control. One study used a high dose of omega-3 FA (8g/day) for 8 weeks duration in people with diabetes and reported that after omega-3 FA supplementation, fasting plasma glucose levels increased by 22% (p=0.005) and meal-stimulated glucose increased by 35% (P=0.036) (163). Similar result was reported in a small study (N=6) after 1 month of dietary omega-3 FA supplement (164). in a prospective study (36,328 women, mean age: 54.6 y, follow-up from 1992 to 2008) suggested an increased risk of type 2 diabetes with the intake of long-chain omega-3 fatty acids, especially with higher intakes (>/= 0.20 g omega-3/d or >/= 2 servings of fish/d) (165).The diagnosis of diabetes in this trial was self-reported. Since then, RCTs were done for further investigation on this aspect and, a meta-analysis of 26 RCTs reported that the use of omega-3 FA has no adverse effects on HbA1c in people with diabetes treated with omega-3 FA supplementation. Fasting blood glucose levels were slightly increased with in non-insulin dependent DM (0.43 mmol/l [95% CI,

0.00-0.87], P = 0.06) and were significantly lower in insulin dependent DM subjects (-1.86 mmol/l [95% CI, -3.1 to -0.61], P < 0.05) (160). Another systematic review included 18 RCTs with a mean duration of 12 weeks with doses ranging from 3 to 18 g/day reported no significant effects of omega-3 FA on glycaemic control (166). A Cochrane review including 23 RCTs confirmed that there were no statistically significant effects of omega-3 FA on glycaemic control or on fasting insulin concentration (167). A recent review and meta-analysis included 540 184 individuals also came to a similar conclusion that omega-3 FA intake was not associated with an increased incidence of type 2 diabetes (168). Hence, although there were some suggestion that omega-3 FA supplementation in people with diabetes can increase blood glucose in early non-randomised and observational trials, many RCTs, reviews and meta-analysis do not suggest this but show no effects on HbA1c or blood sugar level in people with diabetes.

Effects of omega-3 FA on cardiac rhythm

There are suggestions of effects of omega-3 FA on ventricular arrhythmia, sudden cardiac death, and atrial fibrillation (AF) mainly post-operative AF. Animal studies suggested both direct and indirect anti-arrhythmic effects of omega-3 FA mediated by anti-oxidant, anti-inflammatory, and anti-fibrotic properties (169). Some animal and epidemiological evidence suggests an inverse association between the incidence of sudden cardiac death and high dietary omega-3 FA intake (170). These observations prompted trials to assess the possible anti-arrhythmic effects of omega-3 FA have been investigated since. In a meta-analysis of 27 animal studies, DHA and EPA

appeared to have protective effects against ventricular tachycardia and ventricular fibrillation in ischaemia but not on reperfusion-induced arrhythmias (171).

Several clinical trials which examined the anti-arrhythmic effect of omega-3 FA have reported results. The hypothesis that fish consumption may protective against sudden cardiac death was derived from the DART trial which showed a 33% reduction in cardiac mortality in people who consumed at least two portions of fatty fish weekly. One population-based case-control study suggested that omega-3 FA level in the RBC membrane was directly related to a reduced rate of primary cardiac arrest (172). However, further RCTs have failed to confirm these results. The Omega-3 Fatty Acids for Prevention of Post-operative Atrial Fibrillation (OPERA) RCT (N=1516 patients scheduled for cardiac surgery), where perioperative supplementation with omega-3 FA compared with placebo, reported no reduction of the risk of postoperative AF (173). Another RCT (N=200) was conducted to determine whether omega-3 FA supplementation may have beneficial antiarrhythmic effects in people with a history of sustained ventricular tachycardia (VT) or ventricular fibrillation (VF) and reported that among patients with a recent episode of sustained ventricular arrhythmia and an ICD, omega-3 FA supplementation does not reduce the risk of VT/VF but the possible signal that this could be pro-arrhythmic (174). Other RCTs including the Study on Omega-3 Fatty Acids and Ventricular Arrhythmia (SOFA) RCT (175) investigating the effect of omega-3 FA on ventricular arrhythmias in people with ICD devices and meta-analysis of RCTs, those did not show any

reduction in ICD discharge but indicated heterogeneous responses among those people to fish-oil supplementation (176). The heterogeneity results of the effect of omega-3 FA from earlier animal and observational studies and later RCTs could result from the different underlying pathophysiological mechanisms of cardiac arrhythmias (*e.g.* ischaemia, reperfusion, scarring, and inflammation) and different background diet and intake of fish. In summary, the effect of supplementation of omega-3 FA on prevention of cardiac arrhythmias is still uncertain and it is still difficult to predict or confirm the exact pathophysiological pathway affected by the supplementation in the myocardium. Recent RCTs on cardiovascular outcome not directed to the anti-arrhythmic effect of omega-3 FA will be discussed in the OTTC meta-analysis section of this thesis (section 4.1).

Possible anti-thrombotic effects

Omega-3 FA has been shown to have a wide range of antiatherosclerotic and antithrombotic effects in animal and human studies. Omega-3 FA causes a reduction in synthesis of thromboxane A2, a potent promoter of platelet aggregation, and an increase in the formation of thromboxane A3, which is a weak platelet aggregation factor (177). Omega-3 FAs at a very high dose may increase bleeding time (e.g. 15 g/day) but up to 3g/day appears to be safe in clinical practice and without any untoward effects (178). Recent study also reported the safe consumption of omega-3 FA, even at short-term doses up to 10 g/day or consumed for up to 52 weeks above 1.5 g/day, in selected vulnerable populations such as subjects with gastrointestinal cancer or patients in an intensive care unit (177). Several clinical trials have

investigated whether omega-3 FA alters fibrin clot properties in patients undergoing percutaneous coronary intervention (OMEGA-PCI clot) and they reported favorable antithrombotic effects induced by omega-3 FA (179).

Omega-3 FA supplementation and ASCEND

The possible mechanisms of omega-3 FA, theoretically, should be beneficial in the pathogenesis of atherosclerosis in diabetes. Review of the literature as discussed earlier, demonstrated shows that omega-3 FA consistently lower elevated plasma TG levels in a dose-dependent fashion; smaller effects on increasing plasma levels of HDL; no consistent effects on other lipids, plasma glucose level, anti-arrhythmia or plaque stabilization parameters but no bleeding risks have been identified. However, uncertainty remains about their role in primary prevention. Since the ASCEND trial was started, several large RCTs have been reported which have investigated the role of omega-3 FA in the prevention of cardiovascular disease but results have been conflicting (180, 181). To help clarify the role of omega-3 FA supplementation for cardiovascular disease prevention, a tabular data meta-analysis of RCTs of omega-3 FA supplementation was undertaken. This meta-analysis forms the first part of the results chapter of this thesis.

2.5.4 Biochemical effectiveness of study medications

Monitoring the biochemical effectiveness of trial medication is helpful during an RCT to ensure that the intervention is producing its expected effect(s) thus allowing the trial to address its hypothesis. The thesis includes a literature

review of the biochemical methods used in ASCEND to investigate the biochemical effectiveness of aspirin and omega-3 FA.

3 Methodology

3.1 Overview of the ASCEND trial: methods, baseline characteristics, monitoring and plans to ensure good compliance

In light of the uncertainty around the value of aspirin and omega-3 FA for primary prevention in diabetes, “A Study of Cardiovascular disease iN Diabetes” known as ASCEND was established with funding from the British Heart Foundation. ASCEND is a large mail-based, ongoing randomised trial of aspirin 100 mg daily and/or omega-3 FA 1 g daily (Figure 3.1) (182). The Anti-Thrombotic Trialists’ (ATT) collaborative meta-analysis of previous trials found that high doses of 500-1500 mg aspirin daily (which are more gastrotoxic) were no more effective than lower doses of 75-100 mg/day either in direct comparisons or in indirect comparisons (130). As a consequence, daily doses of 75-150 mg are generally preferred for long-term treatment as protection against serious vascular events in high-risk patients. Hence, a daily dose of 100 mg aspirin was selected to use in ASCEND. In the large GISSI Prevenzione trial, 90% of participants were taking aspirin, but no excess of bleeding was observed with the addition of 1 g omega-3 FA daily. The only side-effects reported in that open-label study were a slight fishy after-taste and some gastrointestinal disturbances, but only 3.8% of participants stopped their omega-3 FA supplements because of these side effects (153). For ASCEND, a daily dose of approximately 1 g of omega-3 FA (0.4 g EPA and 0.3 g DHA were used (as in GISSI), which can be

conveniently provided in 1 capsule of the concentrated preparation (with matching placebo capsules containing olive oil). ASCEND aims to demonstrate whether aspirin and/or omega-3 FAs, safely reduces the risk of cardiovascular events in individuals with diabetes who do not already have diagnosed occlusive arterial disease (183). Most prevalent cases of diabetes in young and middle age are unlikely to have a history of vascular disease. A recent US survey reported that an estimated 27% of adults with diabetes had CVD, and an additional 71% have one or more CVD risk factors (184). Aspirin was used regularly by 37% of those with CVD and by 13% of those without CVD (184). The people with diabetes entered into the UK Prospective Diabetes Study were aged between 25 and 65 (mean age 52 years) and only 33% had either an abnormal ECG or retinopathy, but the remainder did not have any clinical cardiovascular disease (185). Hence when ASCEND was planned, it was estimated that up to 70% of people with diabetes who were young or middle-aged did not have established a clinical diagnosis of cardiovascular disease. Between 2005 and 2011, 15 480 diabetic individuals from around the UK were randomised and are being followed up with a planned average duration of 7.5 years, concluding in late 2017. This chapter provides a summary overview of the trial's design including its recruitment methods, follow-up procedures, and measures in place for maintaining compliance. (Detailed background to the trial, including sample size calculations, are provided in the ASCEND protocol including in the appendix of this thesis)

Figure 3.1 Factorial design of ASCEND trial

	Aspirin Tablets	**Placebo Tablets**	
Omega-3 FA capsules	3750 Aspirin + Omega-3 FA	3750 Omega-3 FA	Subtotal 1: 7500 **Omega-3 FA**
Placebo capsules	3750 Aspirin	3750 Neither	Subtotal 2: 7500 **Placebo**
	Subtotal A: 7500 **Aspirin**	Subtotal B: 7500 **Placebo**	

3.1.1 **Recruitment methodology**

3.1.1.1 Central coordination and local coordination

The Clinical Trial Service Unit (CTSU) at Oxford University coordinated ASCEND and had overall responsibility for the administration and analysis of the trial. CTSU was responsible for obtaining Multicentre Research Ethics Committee approval; for the identification (with the assistance of the local medical collaborators and GPs), of potentially eligible participants; for obtaining any relevant permissions to invite suitable patients to participate; for all initial invitations of participants, subsequent randomisation and follow-up by mail; and finally for the provision of a 24-hour Freefone telephone

giving clinical or administrative support to participants, their relatives or carers, or healthcare professionals involved in their care

3.1.1.2 Identification of participants

The main challenge of recruitment was to identify a large number of potentially suitable individuals to be able to randomise at least 15,000 eligible patients. Randomisation of 15,000 patients with a follow-up of 7.5 years should provide robust statistical power (i.e. >90% at 2p<0.05) to detect plausible risk reductions of 12-15% with an estimated annual vascular event rate of 1.3 % (Further details about power calculations are provided in the ASCEND protocol in the appendix of this thesis). Potential study participants with diabetes were sought from 3 main sources: 1) diabetes registers, 2) trial databases and 3) general practice registers. Consultants from around the UK were invited to collaborate and allow invitation of potentially eligible individuals from locally held diabetes registers (such as those held for retinopathy screening or for service provision). Other people with diabetes were identified from among the populations taking part in the Heart Protection Study and other trials that were coordinated by CTSU. In order to streamline the invitation process, the contact details of potentially eligible people were sought electronically whenever possible, to allow central mailings in the name of the local doctor. This approach enabled large numbers to be recruited and was more efficient and cost-effective than mailings sent from individual centres or practices. It also facilitated over-selection of certain groups (e.g. older individuals) to ensure an appropriate balance of different subtypes of the participants. The third source of potential

participants came from general practices. Randomised participants were also able to recommend any friend or relative they thought might be eligible and interested in participating in the study. Finally, people with diabetes could volunteer themselves if they heard about the study from any source.

In addition, with the collaboration of the Diabetes Research Network (DRN) and the Primary Care Research Network (PCRN), general practices (GP) agreed to send pre-assembled invitation packs to people on their locally held registers. Responses from participants were collected on questionnaires, which were returned to the coordinating centre. The data were entered into the coordinating centre computer (following an operating procedure for data handling). Overall recruitment status by route is summarised in Table 3.1. A total of 423,403 potentially eligible individuals were invited via the different routes of recruitment, of whom 29% (121,254 people) returned a screening questionnaire to the coordinating centre. About two-thirds of those who responded declined to join the trial and a further 14,000 did not meet the eligibility criteria. After review of the questionnaire data, 26,462 participants (6% of those originally invited) were willing and eligible to join ASCEND and entered the 2-month run-in period. About 40% of all patients who entered the run-in dropped out before randomization. Around half of those who dropped out prior to randomisation had no clinical reason to stop the trial but simply declined to continue, highlighting the value of a run-in period in the trial design. Without this pre-randomization phase, many such withdrawals may have occurred shortly after randomization, resulting in a substantial reduction in the statistical power of the trial. Towards the end of the 2-month run-in,

randomization questionnaires were sent to 22,579 patients. Of these, 15,480 people returned a completed questionnaire, indicating that they were still willing and eligible to be randomised into ASCEND. Overall 3.7% of all individuals invited were randomised into ASCEND (Table 3.1).

3.1.1.3 Summary of recruitment

The detailed methodology is described in the trial protocol of the ASCEND trial (183) (Figure. 3.2 summarises practical procedures for recruitment into ASCEND). To facilitate recruitment, the trial design was straightforward with simple inclusion and exclusion criteria. Double sided A3 questionnaires were used for screening, randomisation, and follow-up. Potentially suitable patients who were identified from any source were invited to take part by letter. The invitation letter also enclosed an information leaflet and a Screening questionnaire that was intended to help the coordinating centre determine eligibility and to confirm consent to participate, along with a Freepost return envelope. Preliminary eligibility for the pre-randomisation run-in phase was based on information provided on the completed Screening questionnaire (i.e. diagnosis of diabetes, no history of diagnosed occlusive arterial disease, no contraindication to regular aspirin and signed a consent to participate). Once those forms were returned, database algorithms and review by clinical staff were used to confirm eligibility to take part.

Run-in packs of medication were sent to all eligible patients. ASCEND had a placebo run-in phase, which used placebo aspirin tablets and placebo

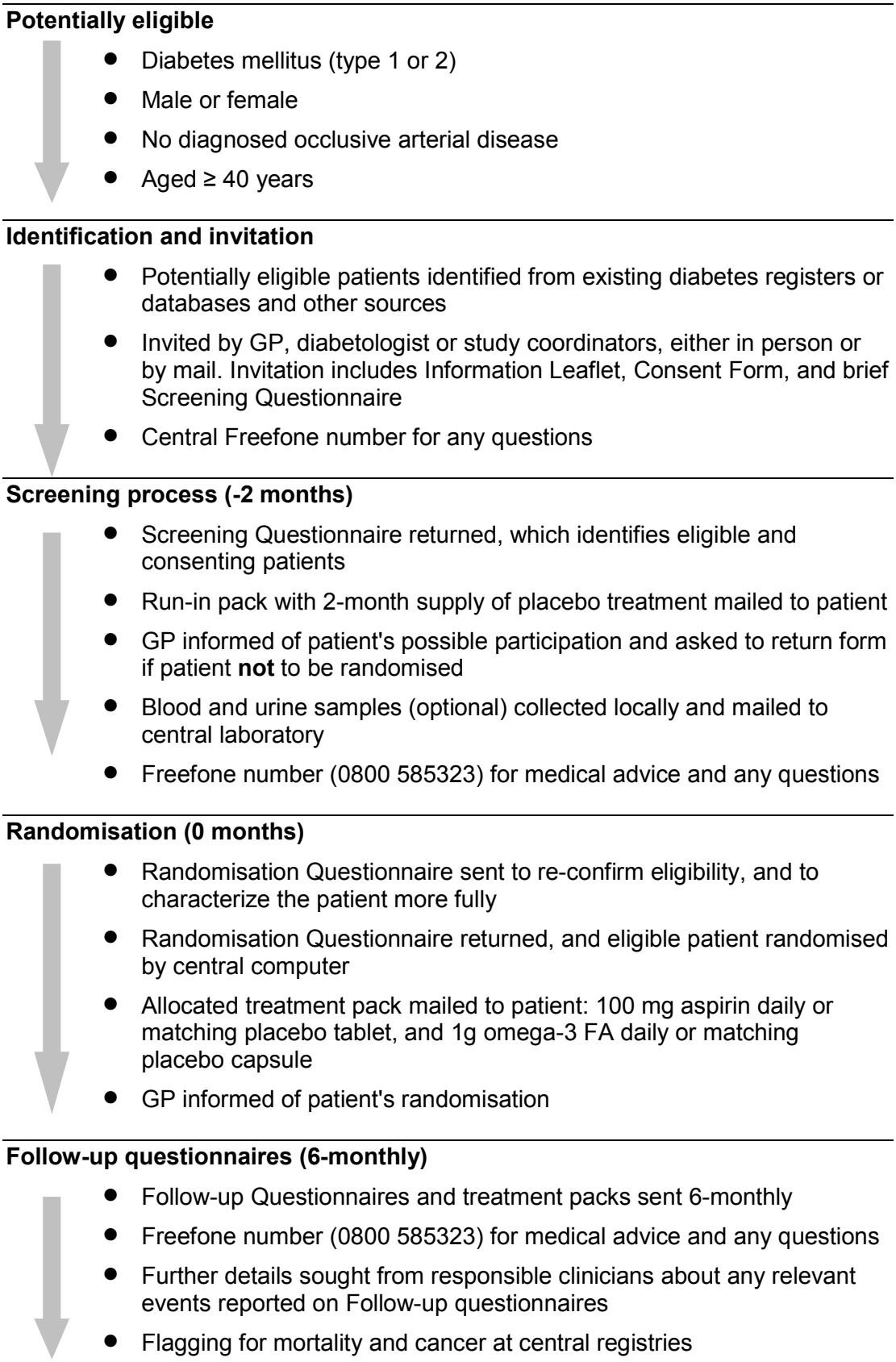
omega-3 FA capsules. The purpose of this was to ensure that only those patients who were compliant with study treatment and still enthusiastic about participating were randomised into the trial. About 2-4 weeks after the run-in pack was sent, participants were sent an optional blood and urine sampling kit, and asked to take this kit to their general practice for sample collection. Those samples were then mailed to the central laboratory in the containers provided. A supplementary information leaflet was provided and separate consent sought for this 5-10ml blood and urine collection which allowed baseline stratification by important biochemical prognostic variables. About 2 months after the run-in pack was sent, a randomisation questionnaire was mailed. If participants were still willing and eligible, they were randomised to the trial.

Table 3.1: Overall Recruitment status by route

	Centrally-held Register	GP practices (Local register)	Others *	Total
Invitations sent	300188	120875	2223	423286
Forms returned	101323	19930	1262	122507
Patients enter Run-in	16104	9741	635	26480
Drop out during Run-in	5009	2496	144	7648
Randomisation form sent	13488	8542	558	22588
Patients randomised	9013 (3.0%)	6037 (5.0%)	430 (19.3%)	15480 (3.7%)

*HPS study database/self-referral/Friend and Family

Figure 3.2 Summary of practical procedures in trial recruitment



3.1.2 **Baseline characteristics**

The baseline characteristics of the 15,480 randomised ASCEND participants are summarized in Table 3.2. A total of 15,480 people with diabetes throughout the UK, with an average age of 63 years (mean (SD) 62.8 (9.2)) were randomised into ASCEND between 2005 and 2011. Of these, 96% were Caucasian, 63% were men (9684 participants), and 94% (14 559 participants) had type 2 diabetes. The diagnosis of diabetes was based on a broad clinical definition involving age of diagnosis, use of insulin within one year of diagnosis and BMI. About 46% (7201participants) are obese with BMI (kg/m^2) ≥30. The mean duration of diabetes before randomisation was 9.8 years (SD 9.4). At randomisation, 8% (1280 participants) were reported as current smokers, 62% (9534 participants) reported having hypertension, and 20% (3023 participants) reported having diabetic retinopathy. Prior to joining ASCEND, 36% (5508 participants) were taking regular aspirin. Both they and their GP were happy for them to stop it in order to participate in the trial. Table 3.3 shows the principal non-study treatments being used at randomisation. During run-in, baseline blood and urine sample were sent. 74% of all randomised patients returned their samples. Among those, the mean HbA1c was 55 mmol/mol or 7.2% (SD 1.2), the mean total cholesterol level was 4.2 mmol/L (SD 0.9), and mean eGFR was 94 (SD 34) (Table 3.2).

Table 3.2: Baseline characteristics of 15,480 randomised patients

Sex		
Male	9684	(63%)
Female	5796	(37%)
Age (years)		
<50	1090	(7%)
≥50, <60	4501	(29%)
≥60, <70	6247	(40%)
≥70	3643	(24%)
Mean age (SD)	62.8	(9.2)
Ethnic Origin		
White	14935	(96%)
Diabetes type 2	14559	(94%)
BMI (kg/m^2)		
<25	2250	(15%)
≥25, <30	5529	(36%)
≥30	7201	(46%)
Mean BMI (SD)	30.7	(6.2)
Duration of diabetes (years)		
<5	4891	(31%)
≥5,<10	4334	(28%)
≥10, <20	3538	(23%)
>20	1862	(12%)
Mean duration of diabetes (SD)	9.8	(9.4)
Other Cardiovascular Risk Factors		
Reported Hypertension	9534	(62%)
Current Smoker	1280	(8%)
Diabetic retinopathy	3023	(20%)
Biochemical measurement		
Mean HbA1c (%) (SD)	7.2	(1.2)
Mean total cholesterol (mmol/L) (SD)	4.2	(0.9)
Mean eGFR (SD)	94	(34)
Albuminuria present	1603	(10%)

Table 3.3: The principal non-study treatments being used at randomisation

Treatment	**N**	**(%)**
Insulin	3931	25%
Metformin	10093	65%
Sulphonylurea	4145	27%
ACE inhibitor	6625	43%
ARB	2634	17%
Beta-blocker	2024	13%
Ca channel blocker	3773	24%
Statin	11653	75%
Thiazide or related diuretics	2930	19%
Aspirin (at screening)	5508	36%

ACE inhibitor = angiotensin converting enzyme inhibitor, Ca channel blocker = calcium channel blocker, ARB =angiotensin receptor blocker

It was recorded that only 205 individuals (1.6% of all participants in ASCEND) reported taking Omega-3 FA supplementation at randomisation as participants were asked to avoid any form of fish oil supplementation during run-in and in the trial.

3.1.3 **Strategies to ensure good compliance in ASCEND**

The success of RCTs is highly dependent on both maintaining compliance with the study treatments and ensuring complete follow-up of all participants irrespective of whether they are still continuing to take their allocated study treatments in order to ensure unbiased complete ascertainment of all

relevant study outcomes. Maintaining compliance with study treatment by participants can be the most labour-intensive and difficult phase of trials particularly for long-term mail-based trials like ASCEND.

During a trial, it was important to monitor the degree of compliance with treatment: a) to understand whether the statistical power of the study to address its aims is being maintained; b) to develop and implement strategies for improving it if it is falling to unacceptable levels; and c) to allow any treatment effect to be interpreted in the light of the in-trial compliance. Poor compliance can have a major adverse effect on the statistical power of a study to detect a clinically relevant treatment effect. For a clinical endpoint study, such as ASCEND, the statistical power is inversely proportional to the square of the compliance, indicating that relatively small losses of compliance can have a large impact on the study power.

3.1.3.1 Previous successful experience of conducting cost-effective randomised trials by mail

Both aspirin and omega-3 FA are widely available and used, the hazards are low and well characterised, and neither requires biochemical monitoring. Several large RCTs have been conducted using mailed drug supply and follow-up, including the CTSU-coordinated British Doctors' Study (186) and the (first) US Physicians Health Study (187) of aspirin for the prevention of MI. Other large studies (188, 189) of either aspirin or various supplements

being conducted entirely by mail in the US: the (second) US Physicians' Health Study II, the Women's Antioxidant Study (WACS) and the Women's Health Study (WHS) also run by mail. Experience from these studies shows that with information leaflets, consent forms and questionnaires, good response rates and compliance can be achieved and reliable information about medical events gathered. In addition, the 24-hour Freefone service established by CTSU for other large heart disease trials allows study participants to discuss any aspects of the study with experienced clinical staff and so helps ensure good compliance and the early identification of serious problems.

3.1.4 Follow-up methods in ASCEND

3.1.4.1 6-Monthly follow-up questionnaires sent by mail (with telephone back-up)

Follow-up questionnaires were sent to all ASCEND participants on a 6-monthly basis. The questionnaire included questions on cardiovascular events, other serious adverse events (including bleeding episodes), compliance with study treatment and use of relevant non-study treatments. Questions on the questionnaire were designed in collaboration with patient forums to ensure that the wording was easily understandable. Supplies of the participant's allocated study treatment are also mailed with the follow-up questionnaires 6-monthly. All randomised patients were encouraged to return their questionnaire, but if forms were not returned, up to 2 mailed reminders

are then sent. If there is still no response, following reminders, a study administrator telephoned the patient in order to complete the Follow-up questionnaire. At the time of writing, about 86% of live participants had returned a follow-up form within the last year and 89% within the last 18 months. Additional efforts were used, including direct contact of those participants who did not return questionnaires to ensure that as many as possible were accounted for and ensure complete follow-up. The remaining participants were being followed-up via their general practitioner. In addition, data were sought on all hospital admissions and on deaths and cancers from the Health and Social Care Information Centre and other central registries. All randomised patients were flagged through the Office for National Statistics and other central registries for death, cancer and other relevant events. Consequently, unbiased cause-specific mortality and site-specific cancer incidence data for all patients could be obtained, independent of whether they were still complying with study medication or responding to questionnaires (190).

3.1.4.2 Maintaining compliance in ASCEND

The follow-up questionnaire included questions about compliance over the previous 6 months. Participants were asked how regularly they have taken their tablets (aspirin or placebo) or capsules (omega-3 FA or placebo) in the last 6 months with the options of every day, most days, only occasionally or never. The first two categories (every day and most days) were defined as being “compliant” and last two as “non-compliant”. Once forms arrived back

to CTSU, they were electronically scanned and reviewed by administrative staff. If queries arose they were also reviewed by clinical staff. I have reviewed several thousand follow-up forms which dealt with clinical queries and recorded relevant reported events in the study database. Keeping in touch with participants is crucial. In order to maintain enthusiasm for the trial and encourage compliance, I have been responsible for writing 6 monthly newsletters which were also mailed to participants. Each newsletter updated participants about the study, reassured patients about alarmist reports about aspirin or fish oils in the popular media, introduced team members from CTSU, and presented interesting stories that were invited from other trial participants. One example was when there were media reports on the use of aspirin for prevention of colon cancer, the number of telephone calls from participants of ASCEND increased and I prepared a special edition of the newsletter with a summary of available evidence on this subject at that time. Emphasis was always placed on the importance of continuing trial medication and reporting adverse events, and contact details were provided for participants to call CTSU to discuss any concerns with a member of the team.

By the estimated mid-point of the study (45 months), over two-thirds (69%) of those allocated aspirin or placebo remained compliant (i.e. taking treatment most days) with their allocated treatment (which is comparable with other studies in the Antithrombotic Trialists' (ATT) Collaboration). For the omega-3 FA or placebo capsules, the proportion reporting taking study treatment most days was higher at 78%. Of those who have stopped their study aspirin or

placebo, about 40% gave no specific reason except wishing to stop (many of whom have been in the study for several years), a further 17% were taking aspirin for a clinical reason (some of whom will have had a primary endpoint or report angina), 8% were taking aspirin by choice but without a clear clinical indication, 7% reported a variety of other symptoms as reasons for non-compliance, 6% have upper gastrointestinal symptoms and 6% are taking a contra-indicated drug (mainly anti-coagulants), the remainder give a variety of other medical or non-medical reasons for discontinuation.

Based on self-reports of compliance with the study medications, the estimated adherence (having taken treatment on every day/most days) across both active and placebo aspirin groups was 88% at 1 year, 80% at 2 and 72% at 3 years after randomisation; and for omega-3 FA or placebo capsules 91%, 86% and 80% at 1, 2 and 3 years respectively. However, it was recognized that self-reports of compliance can be subject to social desirability bias and recall bias. Given this, objective indicators of compliance may be helpful, such as measuring biomarkers of drug effects. Importantly, by triangulating sources of compliance data in this way, ASCEND will provide realistic estimates of compliance which will help inform the interpretation and generalizability of the results at the end of the study. This leads to my project on evaluating the biochemical effectiveness of the trial medications in ASCEND.

3.2 Evaluation of the biochemical effectiveness of omega-3 FA and aspirin in ASCEND

The aim of this project was to assess the biochemical effects of aspirin, in order to demonstrate that it is having its expected biochemical effect in this diabetic population and of omega-3 FA in order to show that blood levels have increased as expected at a median follow-up of 3.5 years. Being a mail-based trial, it is important to compare self-reported compliance by participants to measures of the biochemical effect of each intervention in the trial follow-up phase. If the results of this sub-study show that mail-based self-reported compliance and measured biochemical compliance are the same, the final results of ASCEND would have a meaningful impact of study medication without reporting bias from participants. Recruitment started in April 2005 and was completed in August 2011. A total of 15,480 patients were randomised and median duration of follow-up was about 3.5 years in 2012 (with a planned median follow-up of at least 7 years). During a placebo run-in phase, baseline blood and urine samples were collected by mail and 74% of randomised participants provided samples. The study protocol stated that as well as asking all participants routinely about their compliance with allocated study treatments, biochemical effects will be assessed in a random sample of participants at intervals during the study. A randomly selected subset of randomised participants (10%) was sent a kit for blood and urine collection.

3.2.1 **Sample selection**

A total number of 1800 participants (10% as stated and aimed from the study protocol) for follow-up sampling was selected randomly from different arms of study medications (50% of those from the treatment group and 50% from the placebo group), and from that 74% of participants who provided baseline sample with the aim to interpret the follow-up sample results in light of baseline value for comparison if necessary.

3.2.1 **Sample collection and processing**

During 2012, blood and urine sampling kits (Figure 3.3) which contained a 10ml EDTA blood sample and two containers of urine (one 10ml container and one 5ml container without preservative) were sent to 1800 randomly selected randomised participants, along with an information leaflet, instruction letter and consent form. Ethics committee approval was applied and granted for this purpose. The participants were asked to go to their local GP practice for the blood test and blood and urine samples were collected using the sampling kit provided. The samples were then posted in the mailing container provided along with the completed consent form to the coordinating centre laboratory in Oxford. The CTSU Wolfson Laboratories are accredited by the United Kingdom Accreditation Service (UKAS) to ISO 17025:2005 (General requirements for the competence of testing and calibration laboratories) which demonstrates technical competence for a defined list of tests and the operation of a laboratory quality management system.

Figure 3.3: Picture of mailing sampling kit sent out for blood and urine collection in the ASCEND follow-up sample collection.

In total 1288 samples were returned (1265 returned blood and urine samples, 6 blood only, 17 urine only, 512 did not return any samples). On arrival at the central laboratory, an aliquot of whole blood was removed for the HbA1c assay. The remainder of the blood sample was centrifuged and plasma extracted for the pre-specified assays (total- and HDL-cholesterol, apolipoproteins A1 and B, cystatin C) as in the protocol to see the study medications were not having impacts on other factors that might be relevant to the cardiovascular outcome in ASCEND. The remaining plasma and red blood cells were aliquoted into barcoded cryovials and frozen at -80°C. The urine samples were also aliquoted into barcoded cryovials and frozen at -80°C.

I investigated appropriate and reliable biomarkers for assessing aspirin and omega-3 FA compliance in the context of the ASCEND trial. The design of ASCEND imposes some practical constraints regarding which biomarkers can be reliably assessed. In designing my compliance sub-study, compromises needed to be made to take account of the fact that direct contact with the participants was not possible. A novel aspect of this work is the collection of samples by mail so a significant challenge has been finding suitable analytes that might be stable in whole blood or urine during transport in the post. The appropriate biomarkers for aspirin and omega-3 FA will be discussed in detail in section 3.3. As part of the compliance sub-study during the follow-up phase, information about non-study treatment use, blood pressure, and BMI were asked in addition to information about compliance from the randomly selected participants. Analyses of HbA1c, total cholesterol, HDL-cholesterol, Apo A1, Apo B, and cystatin C) and urinary microalbumin/creatinine ratio) were undertaken on the follow-up samples in addition to the measurements of the biochemical effectiveness of trial medications.

3.3 Omega-3 fatty acid biomarkers

Monitoring of biochemical effectiveness of trial medications during the follow-up phase of the trial is needed for the reliable interpretation of the trial results as previously described in section 2.5. It is particularly relevant in a long-term mail-based trial like ASCEND which will last for more than 7 years and where the estimated compliance relies on self-reported questionnaires. In clinical trials investigating omega-3 FA as an intervention, one of the practical difficulties could be the lack of a generally accepted biomarker that reflects the biochemical efficacy of its intake. This section reviews the available biomarkers for omega-3 FA intake, describes the suitable methodology of a most appropriate biomarker to use in the context of ASCEND.

3.3.1 **Literature review of methods for omega-3 fatty acid biomarkers**

One systematic review including 41 studies, evaluated the utility of different biomarkers of omega-3 FA intake and reported that eighteen different biomarkers had been used to measure the change in omega-3 FA level after taking supplements (191). Total plasma lipid DHA (used in six RCTs) and plasma phospholipid DHA (used as a biomarker in 21 studies) both reflected the supplement dose and were considered useful indicators of DHA status. Plasma triacylglycerol DHA, plasma cholesteryl ester DHA and plasma non-esterified fatty acid DHA were also used in a small number of trials. Different

stages of red blood cell (RBC), platelet and peripheral blood mononuclear cell phospholipid DHA were also used in a small number of trials. Granulocyte, neutrophil, neutrophil phospholipid, peripheral blood mononuclear cell, LDL, and HDL were also used as sources of measurement of DHA in a small number of trials. Hence there is a range of useful biomarkers which can reflect omega-3 FA intake/supplementation. The most commonly used ones involve red blood cells (RBCs), plasma, and plasma phospholipids (PL).

Given the long half-life of RBCs (about 120 days), it has been suggested that FA levels in RBC may better reflect long-term intake of particular FAs and therefore be a more appropriate biomarker to use to than plasma FA levels (192). DHA measurement in both plasma and RBC correlated with intake, but RBC DHA concentration was more strongly associated with long-term intake (193). To have a greater power and more reliable results, a biomarker with low biological variability would be preferred. Theoretically, RBC membrane omega-3 FA measures are likely to be relatively stable because of their esterified status in the membrane. Plasma contains lipoprotein-associated FAs (cholesteryl esters, triglycerides, and phospholipids) as well as non-esterified FAs, and plasma FA composition may be more variable because of varying levels of types and amount of lipoproteins. A study was conducted which reported the rate and extent of which EPA and DHA were incorporated into RBC membranes in an 18 –month controlled trial (194). The proportion of EPA in RBC increased after just 3 days of supplementation. The incorporated half-life of EPA was reported as 28 days and the concentration

of EPA plateaued at 6 months, the RBC concentrations reflected the intake over the previous month. When supplementation was stopped, levels of FA came down at approximately the same rate at which they had risen and had fallen to about 50% of peak values after 1 month.

A study which compared biological variability of omega-3 FA measurements in RBC, plasma and plasma phospholipids (PL), confirmed that RBC omega-3 FA measurement showed the lowest with-in subject coefficient of variation (CV) (4.1 ±1.9%) compared with whole plasma (CV 15.0 ± 6.4%) or plasma PL (14.5 ± 8.4%) (192). A prior meal lowered the plasma EPA and DHA proportions (expressed as a percent of total FAs). The consumption of a meal that does not contain long-chain omega-3 FA (but does contain other FAs) will dilute the plasma FA pool with non-omega-3 FAs, lowering the relative content of omega-3 FA. But RBC omega-3 FA composition is not altered acutely by a meal, which is an advantage as a fasting sample is not required (192). Most of the FAs in RBCs are esterified in membrane PLs and are relatively stable in contrast with plasma which contains lipoprotein-associated FAs and non-esterified FAs. Hence plasma FA composition is more variable than FAs in RBC since there are many FA carrier molecules. RBC omega-3 FA measurement showed the lowest biological variability and was unaffected by a prior meal, Hence it was possible to use nonfasting blood samples to obtain reliable RBC omega-3 FA concentration and this would be the preferred sample type in which to assess omega-3 FA status in ASCEND. The limitation of this study was that the composition of the meal or the exact timing of the blood collection after the meal were not critically

considered as their goal was to replicate the normal variability observed in clinical practice.

A study looking at the adherence to nutritional advice to increase EPA and DHA showed that measurement of the percentage of DHA in RBCs characterizes adherence to EPA and DHA intakes in long-term intervention following the dietary advice, while plasma measurements of the percentage of EPA and DHA and dietary assessment reflect short-term increases in EPA and DHA intakes (195). Rapid incorporation of EPA into plasma and erythrocyte lipids, and DHA into plasma lipids makes measures of EPA and DHA susceptible to acute compensatory EPA and DHA intake just prior to blood collection during follow-up of long-term trials. The study excluded individuals with 4% Omega-3 FA index, existing CVD or diabetes mellitus and those consuming nutraceuticals containing EPA and DHA. The study was not blinded and knowing that blood samples would be measured at each study visit could have led to compensatory adherence immediately prior to the study visits.

In the substudy of ASCEND, there was an option of measuring both plasma and RBC omega-3 index as a comparison of the two fractions. However, with a limited amount of blood collected (one ETDA tube) and the need to spare plasma for analysis of other laboratory tests that were pre-specified in the protocol, this option was not adopted. Moreover, based on the review of the literature, it appeared that measuring RBC omega-3 FA levels in ASCEND samples was the most appropriate method to use given the stability, reduced

variability and no requirement to use fasting samples for the purpose of long-term adherence of study medication.

The main challenge of collecting mailed based samples was the stability of analytes during postal shipment from the general practice to the laboratory at the CTSU. With the previous experience of collecting blood and urine samples at the baseline in the ASCEND, the CTSU laboratory team recorded of the majority of samples (>90%) arrived in the laboratory within 7 days at baseline sampling process. Hence the analytes to be measured needed to be stable at room temperature for at least 7 days.

3.3.2 **Red cell membrane omega-3 index**

The omega-3 index is the EPA and DHA content of the red blood cells (RBCs) expressed as a percent of total identified RBCs FA (196) (OMEGAQUANT, 2009-2011 document). The omega-3 index is a biomarker of n-3 FA status and correlates with EPA and DHA supplementation in a dose-dependent manner (197). A randomised, placebo-controlled, double-blind, parallel-group study in 125 people assessed 5 doses of EPA+DHA (0, 300, 600, 900, 1800 mg) given daily for 5 months as fish oil supplements (Nordic Naturals). The omega-3 index was used to assess the level of FA uptake at baseline and at the end of the trial. The results showed that omega-3 FA supplements increased the omega-3 index in a dose-dependent manner. The omega-3 index increased by 121% (from 4.3% to 9.5%) for the 1800 mg/day dose group, 75% for the 900 mg/day dose group, 59% for the 600 mg/day dose group, and 44% for the 300 mg/day dose group (197).

In the same trial, RBCs FA composition was analysed according to the HS-Omega-3 index® methodology using dried blood spot preparation for easy transport to their laboratory in the USA. Briefly, fatty acid methyl esters were generated from RBCs by acid transesterification with boron trifluoride and analysed by gas chromatography using a GC2010 Gas Chromatograph (Shimadzu Corporation, Columbia, MD) equipped with an SP2560, 100-m column (Supelco, Bellefonte, PA). Fatty acids were identified by comparison with a standard mixture of fatty acids characteristic of RBCs. The omega-3 index is the EPA+DHA content of RBCs expressed as a percentage of total identified fatty acids. The factors influencing the omega-3 index were investigated and independent determinants positively associated with the omega-3 index were age, a history of high cholesterol whilst negatively associated factors were being a current smoker and triglycerides (198): marine-derived n-3 FA supplementation explained two-thirds of the variability in response to RBCs EPA+DHA content, and several factors beyond dose (i.e., body weight, baseline omega-3 index, age, physical activity, and sex) added more precision to the predictive model (197). The CTSU Wolfson laboratory had no experience of measuring FAs and did not have gas chromatography machine on site and hence I set up a collaboration with Harris Laboratory at Sioux Falls, SD 57106, USA to analyse the omega-3 index for collected samples.

3.3.3 **Sample size calculation**

Power calculation Formula

The basic power formula for testing whether the difference in mean level between two treatment arms with equal numbers n per arm, is > dmin is: (d-dmin) $\sqrt{(n / 2)} / s > (za + zb)$, where d is the expected difference and s is the SD of a basic observation.

The simplest basic observation is an individual observation, off treatment in one arm and on-treatment in the other. However, depending on the reproducibility, it may be more powerful to make the basic observation a difference between an on-trial observation and a baseline observation off treatment. If r is the self-correlation, and s is the SD of an individual observation then the SD of the difference between two values is: $s \sqrt{(2(1-r))}$.

Given a fixed total number of samples n within each arm, which can be used either: (1) wholly for on-trial observations; or (2) as n/2 measurements of paired off and on-trial measurements for an individual, the standard errors of the means in an arm are:

(1) $sa=s/\sqrt{n}$

(2) $sa=s \sqrt{(4(1-r)/n)}$

(2) will give a lower standard error if

$\sqrt{(4(1-r))} <1$

which evaluates to $r>0.75$

Power formula with paired measurements

Where “si” is the SD of the contribution from one individual, which may be either a single measurement or the difference between a pair of measurements at different times, and ni is the number of individuals within each arm:

(d-dmin) $\sqrt{}$(ni/ 2) / si > (za + zb)

Thus, for the paired measurements, with this becomes

(d-dmin) $\sqrt{}$(ni / 2) / s $\sqrt{}$ (2(1-r)) > (za + zb)

(d-dmin) $\sqrt{}$ni / s $\sqrt{}$ (4(1-r)) > (za + zb)

(so with r=0.75, this reduces back to the standard formula of (d-dmin) $\sqrt{}$(n/ 2) / s > (za + zb), with ni= n/2)

Estimates from the literature: EPA+DHA measurement after supplementation of omega-3 FA

EPA and DHA levels in red blood cells, whole plasma, and plasma phospholipids in 20 healthy volunteers were tested weekly over 6 weeks by Harris group. The within-subject coefficients of variation (Mean±SD) were 4.1%±1.9%, 15.9%±6.4%, and 14.5%±8.4%, respectively (RBC vs. others, $p<0.001$). RBC omega-3 index showed the lowest biological variability and was not altered in the fed state (199) with the change in the red cell membrane omega-3 index of up to 46%.

The change in the red cell membrane omega-3 FA was also studied in the subset of the Framingham Offspring study (200). The RBC omega-3 index increased by 41% (95% CI: 31 to 52%) in 38 individuals who were taking fish oil supplements, but in 253 participants not taking fish oil, the proportion of RBC EPA+DHA did not change. The SD of measurements of pre and post-supplementation with omega-3 FA calculated from the text and figure 1 of the published paper are given in Table 3.4 (200).

Table 3.4: Sample size and study power calculation data for omega-3 FA

Point	SE of log	SD	r
On supplement-Baseline (n=38)	0.042	0.26	
On supplement-post supplementation (n=38)	0.036	0.22	
Diff, ratio of CI for on treatment to off treatment	0.038	0.23	~0.5
No supplementation post randomisation, n=253	0.0176	0.28	

It is expected, from the literature review, that there might be a 41% difference with an SD of log EPA+DHA of 0.28. Therefore with the funding allowance of analyzing the total number of 152 (38 in each of the treatment sub-groups), the study was powered to show a difference of at least 20% (with an expected 41% effect). This equates to the power to detect a non-zero effect

with a 21% (i.e. 41%-20%) expected effect. Stored samples were retrieved from the deep freezer using a unique sample identifiable code for each sample in a subgroup of 152 patients in the ASCEND study. Dry blood spots were prepared from those blood samples and transported to the OmegaQuant laboratory, Bill Harris's laboratory in South Dakota. Omega-3 index was measured using the dried blood spot technology which was simple and easy for transport worldwide. In addition to EPA (20:5n-3) and DHA (22:6n-3), the other 22 fatty acids (by class as listed in the table below) are identified. The sum of these 24 fatty acids constituted the total fatty acid content of the blood. All of the FAs which were assayed are listed in Table 3.5. Data in my thesis is presented as a percentage of the total fatty acid content, with the all fatty acids summing to 100% for all 152 samples.

Table 3.5: Free fatty acids analysed from dry blood spot testing

Saturated FA	**Cis monounsaturated FA**	**Trans unsaturated FA**	**Cis n-6 PUFA**	**Cis n-3 PUFA**
Myristic C14:0	Palmitoleic C16:1n7	Palmitelaidic C16:1n7t	LA Linoleic C18:2n6	ALA alpha-Linolenic C18:3n3
Palmitic C16:0	Oleic C18:1n9	Elaidic C18:1t	GLAgamma-Linolenic C18:3n6	Eicosapentaenoic C20:5n3
Stearic C18:0	Nervonic C24:1n9	Linoelaidic C18:2n6t	Eicosadienoic C20:2n6	Docosapentaenoic - n3 C22:5n3
Arachidic C20:0			DGLA Dihomo-g-linolenic C20:3n6	
Behenic C22:0			AA Arachidonic C20:4n6	
Lignoceric C24:0			Docosatetraenoic C22:4n6	
			Docosapentaenoic - n6 C22:5n6	

3.3.4 **Method of analysis**

The results presented in this chapter considered EPA+DHA (the Omega-3 Index) fatty acids only. Log values of each fatty acid were analysed as the results follow log-normal distributions. Pearson (Self) correlation coefficients between fatty acids at baseline and on trial were calculated in the placebo arm. Differences in log fatty acids between the active and placebo omega-3 arms were investigated using generalised linear models. The means of the log variables were exponentiated to give geometric means. The modeled estimate of the effect of omega-3 FA was exponentiated and converted to give % difference.

In summary, long-term omega-3 FA compliance in ASCEND was assessed using the omega-3 index. Samples were measured at two different time points; at baseline and during the trial in both trial arms (treatment and placebo arm). The results are presented in next chapter.

3.4 Measurement of biochemical effects of aspirin

Aspirin irreversibly acetylates platelet prostaglandin H-synthase-1 or cyclooxygenase-1 (COX 1) or H-synthase 2 (Cox-2) (as described earlier in figure 2.11). This results in a reduction in thromboxane A2 production (TxA2) which is a potent vasoconstrictor and promoter of platelet aggregation. The half-life of thromboxane A2 is very short (about 30 seconds). It is rapidly

converted to its stable metabolite, thromboxane B2 (TxB2) (137). Ideally, to measure whether aspirin is being taken and exerting its desired effect, we would measure aspirin blood levels. However, to date, there are no reliable assays to measure blood levels of aspirin.

After decades of innovative attempts to assess aspirin response by measuring stable biochemical specific metabolite, thromboxane B2, there are now reliable methods which can be used for both serum and urine (201-203). However, in ASCEND, there are significant obstacles to measuring serum thromboxane as it requires careful sample collection, incubation at 37°C for about 1 hour, immediate centrifugation for 5 min (127), immediate separation and the storage of serum at least below -20°C. This requires a clinic setting with onsite facilities to process the sample after collection, which was not possible in ASCEND which is run mainly by mail. Urinary thromboxane B2 (UTxB2) was therefore chosen as the preferred method for use in ASCEND despite some recognised limitations. UTxB2 has the advantage of being stable in urine over several days hence allowing collection of the sample and dispatch to the central laboratory by mail. Difficulties of assessing COX-1 inhibition attributable to aspirin using UTxB2 include: the influence of body mass index (BMI) (145); smoking (204); the possibility that individuals with diabetes behave differently from those without, physiological factors (age and renal function); pathological factors (kidney, liver and metabolic disorder, obesity, myeloproliferative neoplasm); genetic variants; drug-drug interaction (e.g. NSAIDs); development of resistance or tolerance; and compliance with the medication. Consequently, although not an ideal measure because of

these factors, measuring UTxB2 does provide an opportunity to assess the degree of platelet inhibition being seen in those randomised to aspirin compared to placebo in the ASCEND trial. The process of randomisation and the interpretation of means across the groups (rather than focusing on individual's values) imply that despite the limitations useful information can be obtained. For these reasons, other trials have also used UTxB2 as a biomarker of COX inhibition due to aspirin (138, 139, 145).

3.4.1 Measurement of Urinary Thromboxane B2 in ASCEND

For the ASCEND trial, the AspirinWorks test kit was used to measure thromboxane B2 (UTxB2) in urine samples. The AspirinWorks test kit is an enzyme-linked immunoassay (ELIZA) to determine the level of 11-dehydro thromboxane B2 in the urine sample to see the platelet response to aspirin ingestion. Validation of the assays was done in the CTSU laboratory before being used to measure trial samples.

3.4.2 Assay validation process for UTxB2 measurement

3.4.2.1 Accuracy

In the absence of a certified reference material or an external quality assurance scheme, the kit quality control (QC) sample was used to test for accuracy. The three levels of kit QC were tested (four times) over two days and the results fell within the range supplied by the manufacturer when using the semi-Log processing method. For the Log-log processing method, the QC levels 2 and 3 fell within the range stated by the manufacturer, but one

individual result for level 1 fell outside of the manufacturer's range but the mean of the duplicates fell within the manufacturer's range. To improve accuracy based on these findings it was decided to run the samples in duplicate.

3.4.2.2 Precision

The precision test was carried out using the kit Quality Control (QC) material for both within-batch precision and between batch precision. The manufacturer states in the kit insert that the precision data has been obtained using the mean values of duplicate samples and was used 106 data points to gain the precision data. Both within-batch and between batch precision improved with duplicate samples as recommended compared to individual sample results.

3.4.2.3 Reportable range (linearity)

The detection range stated by the manufacturer was 300 – 4000 pg/mL and any sample with a result higher than 4000 pg/mL were retested with the appropriate dilution. Due to the limited space available on the plates, it was not possible to run six dilutions in triplicate. The linearity was checked by running a high sample (above the highest standard) diluted with the sample diluent (provided with the kit) to obtain a series of samples that were run in duplicate. The results were plotted and the percentage recovery calculated for each level (observed/expected x 100%). Limited data was produced for linearity due to the lack of plates available for the validation. The samples

were analysed in duplicate. The observed results were plotted against the expected results in Figure 3.4 (A- Semi-log and B-log-log). Both processing methods have been used. All sample results were included in the graphs, but note that the 1 in 4 dilutions (M) were used to generate the expected results for both processing methods.

Figure 3.4 (A) Linearity plot (Semi-Log) showing reportable range for UTxB2 measurement in ASCEND

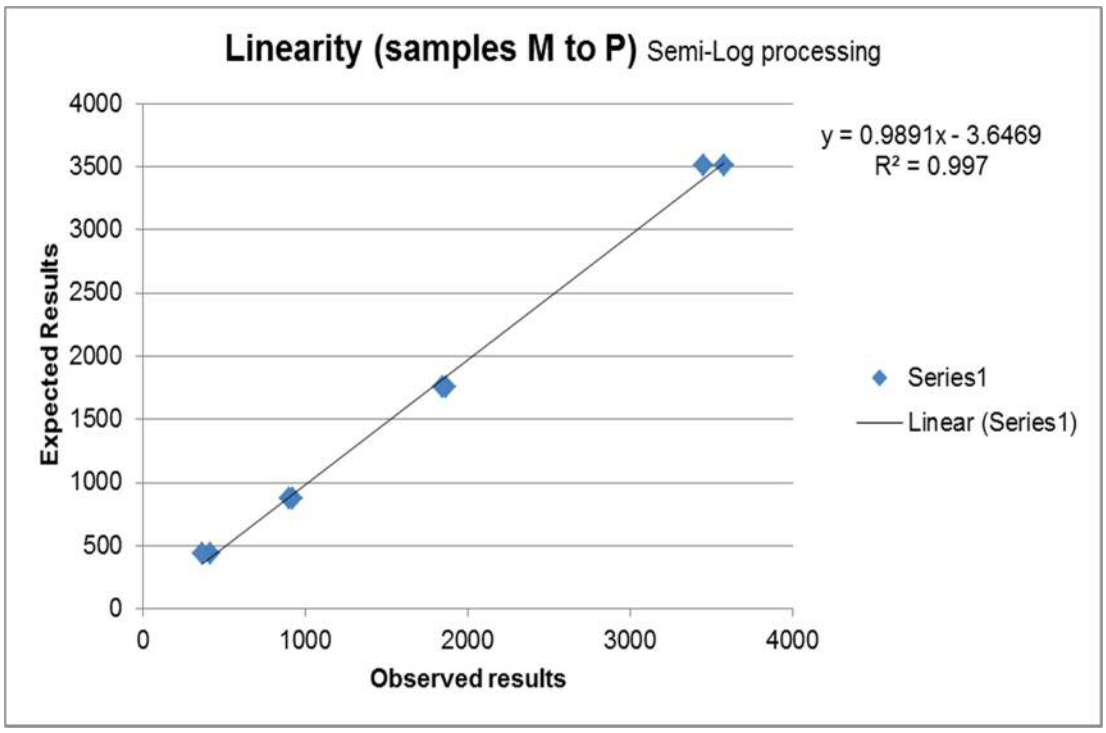
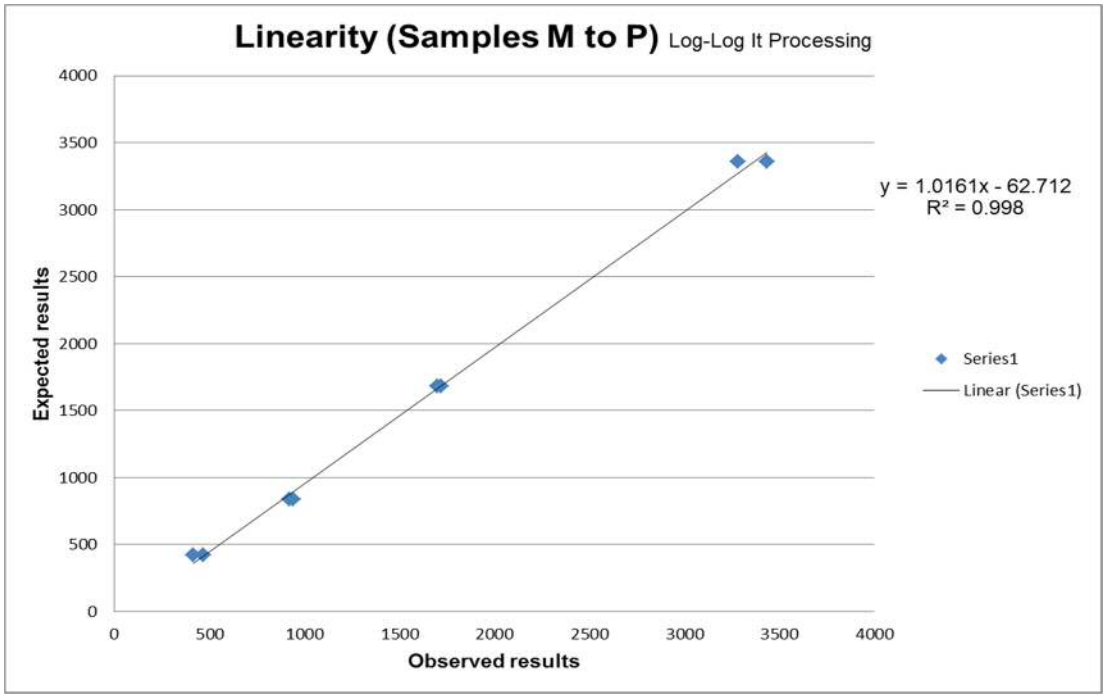


Figure 3.4 (B) Linearity plot (Log-Log) showing reportable range for UTxB2 measurement in ASCEND



The Semi-Log processing produced a recovery from between 88.6% to 105.5%, while the Log-Log processing for the same samples produced a recovery from between 101% to 110.9%. The linearity was acceptable for both types of processing as they fall between 80% and 120%.

3.4.2.4 Patient sample range

The possible patient sample range was checked using sets of samples that have been obtained from healthy volunteers (n=11) in our trial centre as a pilot project to help understand UTxB2 behaviour after aspirin ingestion. The first sample was obtained at time zero (approximately 8 am) before taking

150 mg of aspirin (300 mg aspirin preparation was available for this pilot project and 150 mg (half of 300 mg) was used) a second sample was collected 12 hours (approximately 8 pm) after the aspirin was taken and a third sample was collected 24 hours (approximately 8 am the following day) after the aspirin was taken. Some blank samples were also obtained from volunteers who had not taken aspirin in the 48 hours prior to giving the sample. The samples were placed at 4°C before being aliquoted and frozen at -80°C.

It was expected that most samples corresponding to time 0 would give a result >1500pg UTxB2/mg creatinine (i.e. representing no suppression of TxA production so high UTxB2) and the subsequent time points would show suppression and a result of ≤1500pg UTxB2/mg creatinine, therefore, demonstrating the effect of the aspirin. All volunteers showed a change in the level of UTxB2, but three had anomalous results. One had a high UTxB2 level, even after taking the aspirin with it not going below the 1500pg UTxB2/mg creatinine cut off, and two had levels below 1500pg UTxB2/mg creatinine at time zero, although they also decreased after taking aspirin. For these 2 individuals, the zero time point appeared to be from someone who had already taken aspirin but may have been due to other interfering substances (Table 3.6, Figure 3.5 A and B).

Table 3.6: Results of UTxB2 measurement in the pilot study (n=11) of ASCEND

Sample ID	Mean 0 Hrs	Mean 12Hrs	Mean 24Hrs	% change 12Hrs v 0Hrs	% change 24Hrs v 0Hrs
A1	1791.8	904.4	433.30	-49.5	-75.8
A2	2638.9	831.0	606.47	-68.5	-77.0
A3	2405.0		553.44		-77.0
A4	2876.8	577.6	578.96	-79.9	-80.5
A5	5297.6	2274.6	2067.13	-57.1	-61.0
A6	2848.9	640.1	740.19	-77.5	-74.0
A7	1743.0	480.0	500.37	-72.5	-71.3
A8	866.1	339.9	359.56	-60.8	-58.5
A9	2810.8	672.4	623.27	-76.1	-77.9
A10	1275.7	450.5	497.89	-64.7	-60.97
A11	2471.6	599.5	965.32	-75.7	-60.94
Overall Mean	2456.9	777.0	720.54	-68.2	-70.44
Overall SD	1159.3	552.6	474.86	10.0	8.35
Overall CV	47.2	71.1	65.90	-14.7	-11.85

Figure 3.5 (A) The change in UTxB2 measurements after taking aspirin (Semi-Log data)

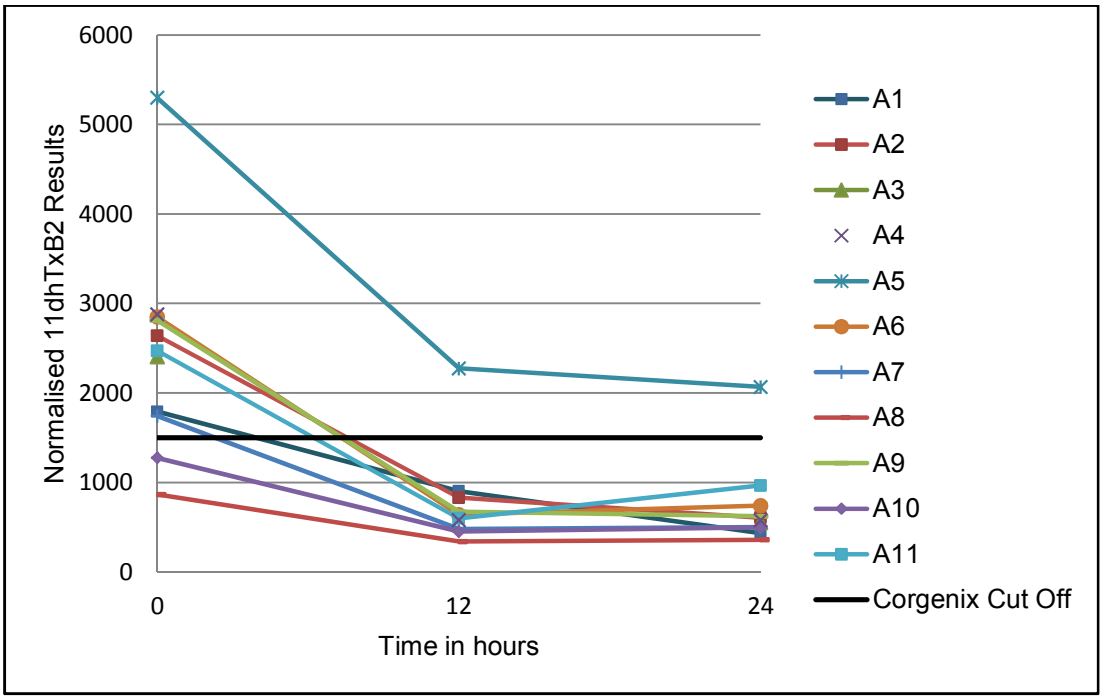
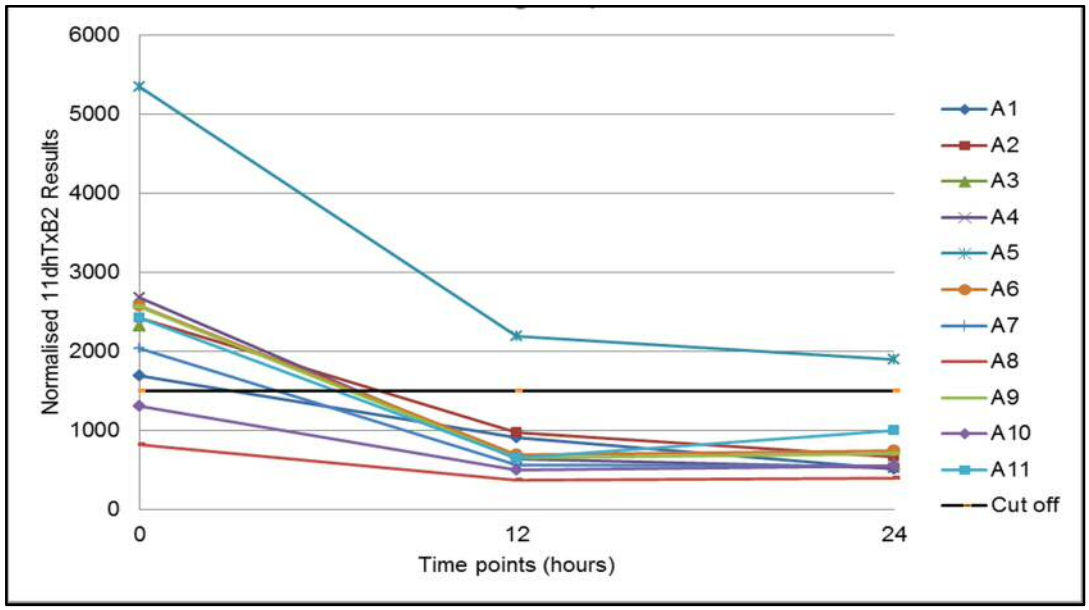


Figure 3.5 (B) The change in UTxB2 measurements after taking aspirin (Log-Log data)



The overall mean reduction of UTxB2 at 12 hr after 150 mg of aspirin ingestion in the pilot study was 68.23% with SD 10.01, and of 70.44% with overall SD of 8.35 at 24 hr post-aspirin ingestion (Table 3.5). As a result of the pilot study, it was decided to analyse the samples at baseline and during the trial to better assess any changes due to treatment allocation.

3.4.2.5 Comparing preserved and non-preserved samples

After deciding to analyse samples from two-time points (i.e. baseline and in-trial), we investigated whether using chlorostat preservative in the urine sample had any impact on the analysis. The aim was to assay an individual's baseline and follow-up samples on the same plate (to minimise variation between runs) to assess changes in the level of UTxB2. The baseline urine sample had been collected without chlorostat preservative, therefore, a comparison between samples with and without preservative was required. The manufacturer recommends the use of preservatives as compulsory. However, after contacting the product specialist (personal communication) from the company, the reason of compulsory use was unclear and at Mayo Clinic, the non-preserved samples are routinely used. The use of preservative tablets for the collection of urine samples varied from different centres, for example, the Cleveland heart laboratory does not use preservatives for their sample collection. Samples from 3 volunteers were divided so that half the sample was in a tube without preservative and the other half had a preservative added. Each sample (both with and without

preservative) was then analysed and no significant differences on UTxB2 measurement were observed between samples with or without preservative tablets. The results are displayed in Table 3.7 A and B.

Table 3.7 (A) Semi-Log Preserved vs. non-preserved sample

Mean result			
	With Preservative	Without Preservative	% Difference from Preserved sample
1	2080.47	1980.87	4.79
2	971.65	912.15	6.12
3	859.51	738.22	14.11

Table 3.7 (B) Log-Log it preserved vs. non-preserved sample

Mean result			
	With Preservative	Without Preservative	% Difference from Preserved sample
1	1968.6	1889.34	4.03
2	1077.04	1012.99	5.95
3	949.54	813.05	14.37

3.4.2.6 Sample suitability

The samples that were to be tested were mailed to the laboratory using the standard postal service, then aliquoted upon receipt and stored at ≤-80°C. The baseline samples were stored in liquid nitrogen tanks for several years before retrieval. The follow-up samples were stored at ≤-80°C for less than 1 year. The follow-up samples had undergone a freeze/thaw cycle before being analysed for UTxB2. To check that this extra freeze/thaw did not have an effect on the samples, two aliquots (a and b) were taken and stored at -80°C. The 'b' sample was removed and defrosted at room temperature for 3 hours before being returned to the freezer. Then both the 'a' and 'b' aliquots were analysed on the same plate to compare the effects of the extra freeze/thaw on the sample. In total 28 samples were analysed but 6 required repeating at a different dilution, so only 22 sample results were used in the comparison. The results are displayed in Figures 3.6 A and B) below; the twice defrosted sample (b) plotted against the once defrosted sample (a).

Figure 3.6 (A) Semi-log plot showing freeze and thaw data for UTxB2 measurements in ASCEND

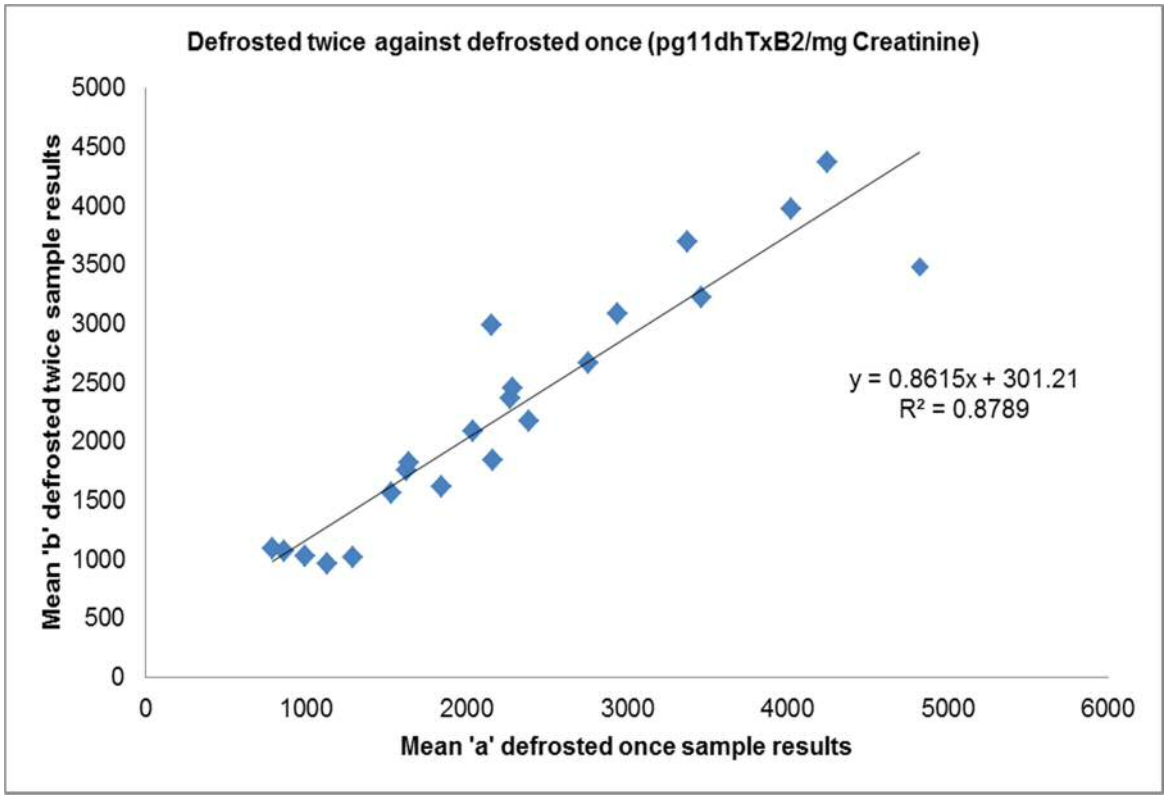
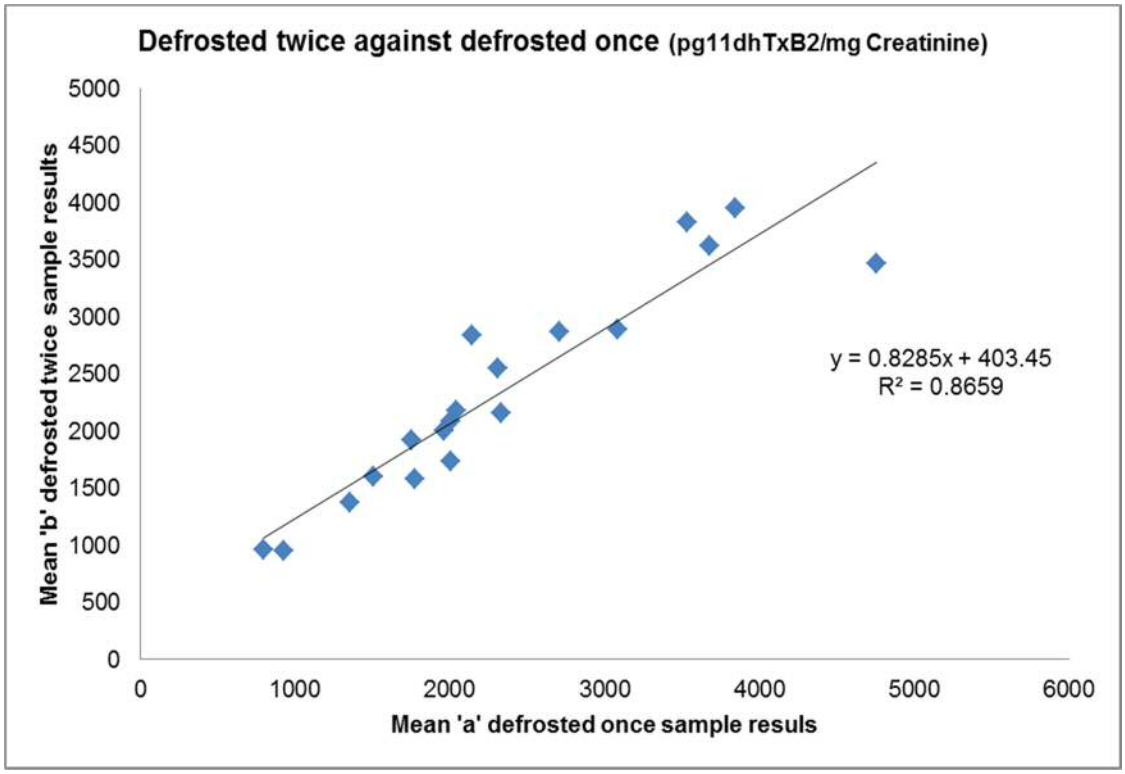


Figure 3.6 (B) Semi-log plot showing freeze and thaw data for UTxB2 measurements in ASCEND



For the semi-Log processing method, there was a mean change of 1.81%.

For the Log-Log processing method, there was a mean change of 2.43%.

3.4.2.7 High Creatinine results

From inspection of the raw data for samples that yielded unexpected results (false positive or false negative), there was some concern that a high level of creatinine in the sample might have interfered with the UTxB2 assay. As less than 2% of ASCEND patients had high creatinine levels at baseline this was not thought to have a material impact on the collected sample.

3.4.2.8 Stability in mail-based sample

The experiments on the stability of UTxB2 for up to 7 days in 12 subjects, incubating the samples between 22 and 24 °C, e.g. in the 'worse' conditions of temperature during shipment of the samples was done in collaboration with Professor Carlo Patrono's research laboratory in Italy and the results are shown below. The results indicate that there was no sign of degradation of the UTxB2 during this time frame at the set temperatures (Figure 3.7) and data from the manufacturer states that analytes were stable in samples stored at room temperature for 14 days (Table 3.8).

Figure 3.7 Stability of sample with duration of storage at temperature 22-24'C (n=12)

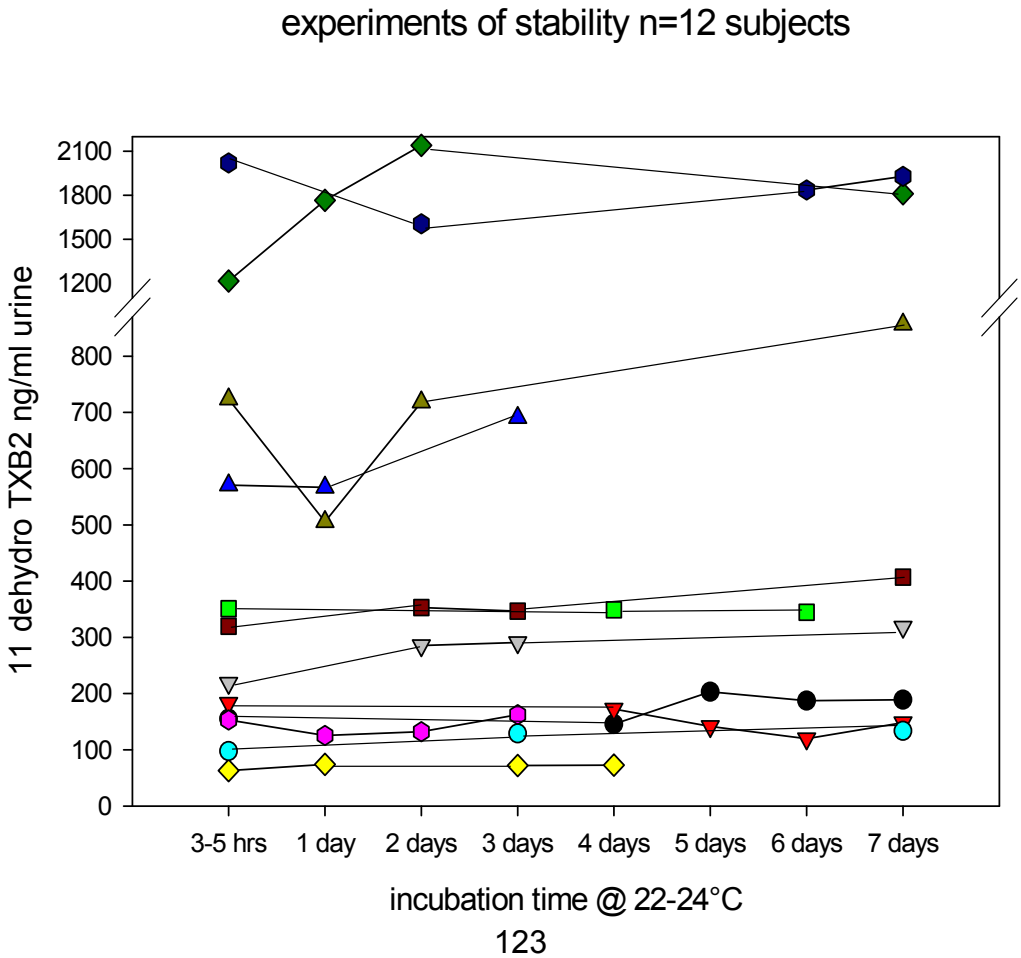


Table 3.8 Data from Manufacturer: Sample storage study at room temperature until 14 days.

ODs RT

DATE:	2006-08-30	2006-09-01	2006-09-06	2006-09-13			
TIME:	time zero	day 2	day 7	day 14	Mean	SD	%CV
REF SOLN 5000	0.338	0.320	0.320	0.326	0.326	0.009	2.7%
REF SOLN 2500	0.488	0.467	0.462	0.466	0.471	0.011	2.4%
REF SOLN 1250	0.688	0.674	0.663	0.678	0.675	0.010	1.5%
REF SOLN 625	0.893	0.874	0.875	0.889	0.882	0.010	1.1%
REF SOLN 312.5	1.051	1.037	1.042	1.057	1.046	0.009	0.9%
REF SOLN 156.25	1.137	1.140	1.172	1.172	1.155	0.019	1.7%
B_0	1.295	1.295	1.309	1.298	1.299	0.007	0.5%
Blank	0.112	0.113	0.117	0.124	0.116	0.005	4.7%
Sample #109	0.876	0.850	0.880	0.859	0.866	0.014	1.6%
Sample #112	0.837	0.826	0.842	0.879	0.846	0.023	2.7%
Sample #107	0.578	0.581	0.581	0.590	0.582	0.005	0.9%
Sample #111	0.491	0.472	0.468	0.491	0.480	0.012	2.5%
Sample #108	0.489	0.476	0.487	0.495	0.487	0.008	1.7%

3.4.2.9 Method of collection

Urinary albumin and creatinine ratio and UTxB2 were measured using a competitive enzyme-linked immunoassay (ELISA) method using Corgenix UTxB2 test kit AspirinWorks (Manufacturer's CV 5-8%). The baseline samples from the same subset of patients (which have been stored in liquid nitrogen) were analysed for UTxB2 and RBC membrane omega-3 index using the same methodology. The laboratory in Oxford used a number of internal and external quality control procedures and follows a standard operating procedure (in accordance with Good Laboratory Practice guidelines). Confirmed assay results were transferred to the central ASCEND database and linked to the patients' other data. (Sample collection and processing have been described earlier in section 3.2.1)

3.4.3 **Sample size calculation for UTxB2**

The statistical power to detect differences between the randomised groups depends on the distribution of UTxB2, its reproducibility in samples taken some years apart from the same individual, and the effect of aspirin on it. Ames *et al*. (2012), reported UTxB2 post-aspirin [mean (± SD)] in people with diabetes of 996 ± 845 pg/mg creatinine; and at baseline of 3665 ± 2465 (127). As the SDs were approximately proportional to the means, this suggests an approximately lognormal distribution. On this basis, these results lead to estimates for the SD of log (UTxB2) of 0.8 and 0.7 (127). These estimates were also corroborated by reports from Eikelboom group of the SD of log (UTxB2) of 0.7 to 0.8 (205). We assumed that the SD of the log-transformed data was the same for both groups. We assumed a self-correlation over the time period from baseline to follow-up of 0.75. The Ames results suggest that aspirin reduces UTxB2 by 100 x (1-996/3665) i.e. ~70%. With 80% compliance in ASCEND, thus, a ~60% reduction might be expected. However, there was considerable uncertainty around these estimates and so the power calculations also allowed for a smaller reduction in UTxB2 of 45%.

Table 3.9: Sample size calculation with power for UTxB2 measurement

N per arm (n)	Percent reduction	Percent reduction to detect	Percent reduction to detect as a change in log (d_del)	Self-correlation (r)	SD of log (s)	Power at p=0.05
75	60%	25%	-0.6	0.75	0.8	~99%
75	45%	25%	-0.3	0.75	0.8	~90%

* Using the formula

$Zb=abs((d\_del\sqrt{n})/(s\sqrt{(4(1-r))}))-Za$

where Za=1.96

As described in section 3.4.2, the overall mean reduction of UTxB2 at 12 hr after 150 mg of aspirin ingestion in the pilot study was 68.23% with SD 10.01, and of 70.44% with overall SD of 8.35 at 24 hr post-aspirin ingestion. With 75 individuals per arm (assayed at two-time points), the power to detect a reduction of at least 25%, was >99% with a predicted reduction of 60% and about 90% with a predicted reduction of 45% (Table 3.9).

Hence UTxB2 concentration (measured in pg/ml) at baseline and on trial in a subgroup of 152 patients in the ASCEND study were analysed with an attempt to answer the biochemical effectiveness of aspirin ingestion. Those randomly selected 152 samples were retrieved from storage after arriving back to the CTSU laboratory after sample collection. Patients were randomly selected within treatment arm from the group who had urine samples

available at both time points, to ensure equal numbers per randomised arm. Two duplicate samples per patient were to be analysed at each time point, thus giving four results per person.

An additional research question was raised, as discussed earlier (section 2.5), whether in diabetic patients the duration of platelet COX inhibition after aspirin ingestion is shorter than in people without diabetes. To investigate this a further sample (n=199) were selected from patients who were part of the mid-study sample and who reported taking aspirin either more than 12 hours before their urine sample or between 0 and 12 hours earlier. These additional UTxB2 assays analyses were done only on samples from the mid-study but not on baseline levels. In each case, duplicate samples were analysed on the same plate.

3.4.3.1 Data completeness

UTxB2 concentrations are given as pg/ml in the majority of cases. Low results are recorded as <150 pg/ml and high levels as above assay range or >10000 pg/ml. Of the 152 patients in the random sample, 144 (95%) had at least one in-trial result within assay range and 146 (96%) had at least one baseline result within assay range. The corresponding numbers for those with both results within assay range are 139 (91%) in-trial and 134 (88%) baseline, with 123 (81%) having all four possible results within the assay range. In 4 cases, one of the baseline results was unsuccessful due to a

plate error (all were on the same plate). Of the 199 patients in the additional sample, 193 (97%) had both on-trial results within assay range and the remaining 6 had both assay results reported as “above range”.

3.4.4 **Method of analysis**

The mean of the two duplicate UTxB2 concentrations was calculated at each time point. The Log of this value was analysed as the results followed a log-normal distribution. Pearson (Self) correlation coefficients between duplicates of the same sample and between log UTxB2 at baseline and on trial were calculated. Differences in log UTxB2 between the active and placebo aspirin arms were investigated using generalised linear models. The means of the log variables were exponentiated to give geometric means. Differences in mean logs were exponentiated to give ratios of geometric means which were then converted to percentage differences.

3.4.4.1 Correlations and choice of analysis

The self-correlation between baseline and on-trial log UTxB2 in the 60 placebo aspirin arm patients with all four results within the assay range is 0.39 ($p=0.002$). As the self-correlation is <0.5, the analysis of on-trial values is more valid than analysing the change between baseline and on-trial. Log UTxB2 concentrations between the duplicates of the same sample (where both are within assay range) are highly correlated: 0.95 ($p<0.0001$, $n=134$) for baseline samples, 0.95 ($p<0.0001$, $n=139$) for on-trial samples in the

original dataset and 0.99 ($p<0.0001$, n=193) for on-trial samples in the additional dataset.

3.5 Chapter summary

The ASCEND trial is a large mail-based 2x2 factorial design randomised trial for diabetic patients who do not have established cardiovascular disease exploring the role of aspirin and/or omega-3 fatty acid for primary prevention of cardiovascular disease. The trial follow-up is mainly by self-reported follow-up questionnaires with backup telephone follow-up.

To be able to interpret the trial results meaningfully, it is important to know the study medications are taken as self-reported compliance. Adherence to trial medication and hence biochemical confirmation of the effectiveness of study medication is vital to have meaningful study results.

As ASCEND is mail-based without any study clinic visits, and because samples needed to withstand the postal service, this limited what was possible. This needed careful consideration while taking into account the most reliable measurement with limited sample collection. Creating a sample collection kit was another challenge in order to have the right amount and type of stable sample posted in the mail. Working with the ASCEND team, I designed this sub-study, working closely with members of the CTSU laboratory on the assay validation, designing the mail-based sample

collection kit, collaborating with primary care physicians (GPs) to obtain sample collections and worked with a statistician to analyse the data when it arrived in the study database. I submitted an ethics committee application for the sub-study which was approved. With the findings from the laboratory investigations, into UTxB2, samples were analyzed in duplicate at each different time point (baseline and in-trial) to compare the percentage change in both treatment and placebo arm. Omega-3 index was measured with a novel dried blood spot method at the Omegaquant laboratory.

4 Results

4.1 Literature review and meta-analysis of the cardiovascular effects of omega-3 fatty acids

Many trials assessing the effects of omega-3 FA on cardiovascular events have been conducted and reported conflicting results (153, 154, 180, 206-212). As part of my study, I undertook a literature review and meta-analysis of published and some unpublished results from 10 RCTs of the effects of omega-3 fatty acid (FA) supplementation on risk of major vascular events (MVEs) (coronary heart disease, stroke and revascularisation), cancer and all-cause mortality, overall and on MVEs in pre-specified sub-groups.

Ecological studies among the Inuit people prompted interest in research into the effects of omega-3 FA for prevention of cardiovascular disease, omega-3 FA becomes the research of interest for its possible role in cardio protection for decades. Since 1985, results of many prospective cohort studies on fish consumption and cardiovascular disease have been published showing a protective effect on cardiovascular disease. In the Japan Public Health Center-based Study, the relative risk (RR) of non-fatal MI was 0.43 (95% CI 0.23–0.81) in participants with a median fish consumption of 180 g per day compared with participants with a consumption of 23 g per day (213). In addition to the promising observational studies that are described in section 2.5.3, many research groups around the world have undertaken RCTs to

assess if omega-3 FA supplementation had a beneficial effect on cardiovascular disease (Selective characteristic of RCTs are described in Table 4.7-4.9). Several systematic reviews and meta-analyses of these RCTs of the primary or secondary prevention of omega-3 FA on cardiovascular disease have also been reported. A summary of the published meta-analyses is shown in Table 4.1.

A meta-analysis from 2002 included 11 RCTs (15,806 participants with coronary heart disease, minimum 6 months follow-up) published between 1966 and 1999, concluded that dietary and supplementation of n-3 PUFA may decrease overall mortality, mortality due to myocardial infarction, and sudden death in patients with coronary heart disease; the risk reduction for mortality was statistically significant (214). The risk ratio (RR) of nonfatal myocardial infarction in patients who were on n-3 PUFA-enriched diets compared with control diets or placebo was 0.8 (95% confidence interval [CI]: 0.5 to 1.2), and RR of fatal myocardial infarction was 0.7 (95% CI: 0.6 to 0.8). In 5 trials, sudden death was associated with a RR of 0.7 (95% CI: 0.6 to 0.9), and RR of overall mortality was 0.8 (95% CI: 0.7 to 0.9) (214). However, several trials in this meta-analysis were small (N=59 to 600), the exceptions being The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial (N=11324 whereas total N=15608) and diet and reinfarction trial (DART) trial (N=2033), and many had re-stenosis as an endpoint (152, 153). These analyses did not differentiate between dietary omega-3 FA intake and supplements. This may be important as different forms of supplements may have different effects, for example, alpha-linolenic

acid (ALA) - a plant-derived omega-3 FA - is only poorly converted to EPA and DHA.

In 2006, Lee Hooper published a meta-analyses which included 48 RCTs of omega 3 intake for ≥ 6 months in adults (with or without risk factors for cardiovascular disease) with data on a relevant outcome (36,913 participants with or without established cardiovascular disease) and 41 cohort studies in a systematic review of the effects of omega-3 FA on mortality, cardiovascular disease, cancer and bleeding events (215). The findings of this analyses differed from previous analysis (214) and found no strong evidence of a clear effect on total mortality or combined cardiovascular events. They reported non-significant reductions in the risk of total mortality (RR 0.87, 95% CI 0.73 to 1.03) and combined cardiovascular events (0.95 CI, 0.82 to 1.12) in participants allocated additional omega-3 FA. The duration of intervention was only ≥ 6 months and this may not have been long enough to detect any effects of supplementation on clinical outcomes of interest. It also adopted a different methodology from the previous meta-analysis as cohort studies those estimated omega-3 intake and related this to clinical outcomes during at least 6 months were also included in this meta-analysis and studies with participants with or without risk factors for cardiovascular disease were also included. Hence differences in study design may explain the discrepant results between the results of previous meta-analyses.

In 2009, Marik and Varon carried out a systematic review of RCTs assessing whether dietary supplements of EPA and DHA decrease cardiovascular

events. Placebo-controlled RCTs that evaluated effects of omega-3 FA on clinical cardiovascular end points (cardiovascular death, sudden death, and nonfatal cardiovascular events) and all-cause mortality were included in this meta-analysis (N=11 trials, 39 044 participants with recent myocardial infarction, those with an implanted cardioverter defibrillator, and those with heart failure, peripheral vascular disease, and hypercholesterolemia). It was prespecified that included trials used supplements of EPA/DHA for at least 1 year and the primary end point was cardiovascular death (216) (Table 4.1). The average dose of EPA/DHA was 1.8 ± 1.2 g/day and the mean duration of follow-up was 2.2 ± 1.2 years. It was concluded that dietary supplementation with omega-3 FA for ≥ 1 year, reduced the risk of cardiovascular deaths (odds ratio [OR]: 0.87, 95% CI: 0.79–0.95) sudden cardiac death (OR: 0.87, 95% CI: 0.76–0.99), all-cause mortality (OR: 0.92, 95% CI: 0.85–0.99), and nonfatal cardiovascular events (OR: 0.92, 95% CI:0.85–0.99). The results were similar to those of Hooper's meta-analysis that was reported 3 years earlier using different methodology. Marik and Varon excluded a trial by Burr *et al* because treatment allocation was not blinded and the dose of omega-3 FA was not standardized. Since then, several additional RCTs of omega-3 FA supplements have been conducted (207-210, 217).

In 2012, a further meta-analysis (14 RCTs, 20,485 participants with a history of cardiovascular disease) concluded that there was insufficient evidence for a secondary preventive effect of omega-3 FA supplements on cardiovascular events (218) and supplementation with omega-3 FA did not reduce the risk of overall cardiovascular events (RR, 0.99; 95% CI, 0.89-1.09). However, it

reported a small reduction in cardiovascular death (RR, 0.91; 95% CI, 0.84-0.99) with omega-3 FA supplementation. This raised the question of whether there were differential effects of omega-3 FA on fatal versus non-fatal cardiovascular disease outcomes. The authors excluded two large open-label trials (the GISSI-Prevenzione study (N=11323) (153) and the Japan EPA Lipid Intervention study (JELIS) (N=18645) (206) which both suggested beneficial effects of omega-3 FA in the primary analysis. Hence, although a lot of research has focused on the role of omega-3 FA for the primary and secondary prevention of cardiovascular disease, there is still no clear evidence and some doubt persists about whether there are beneficial effects and some large trials are still on-going (183, 219, 220). Since starting the ASCEND trial, 8 large clinical trials have been completed and reported.

To help resolve some of the uncertainty, the Omega-3 FA Treatment Trialists' Collaboration (OTTC) was set up under the supervision of Professor Robert Clarke, to combine data from all the large randomised trials assessing the relevance of omega-3 FA for the prevention of CVD. The aims of the meta-analysis were to assess the effects of omega-3 fatty acids on components of MVE, including coronary heart disease, stroke and revascularisation, cancer and all-cause mortality overall; and on MVE in pre-specified sub-groups. Summary (study-level) trial data from trials were sought from the investigators for this collaboration using a prespecified protocol and analysis plan. A collaborative meta-analysis of these trials should ensure that reliable evidence emerges about the effects of omega-3 FA on the risk of

cardiovascular outcomes and help with the interpretation of results from ASCEND in due course.

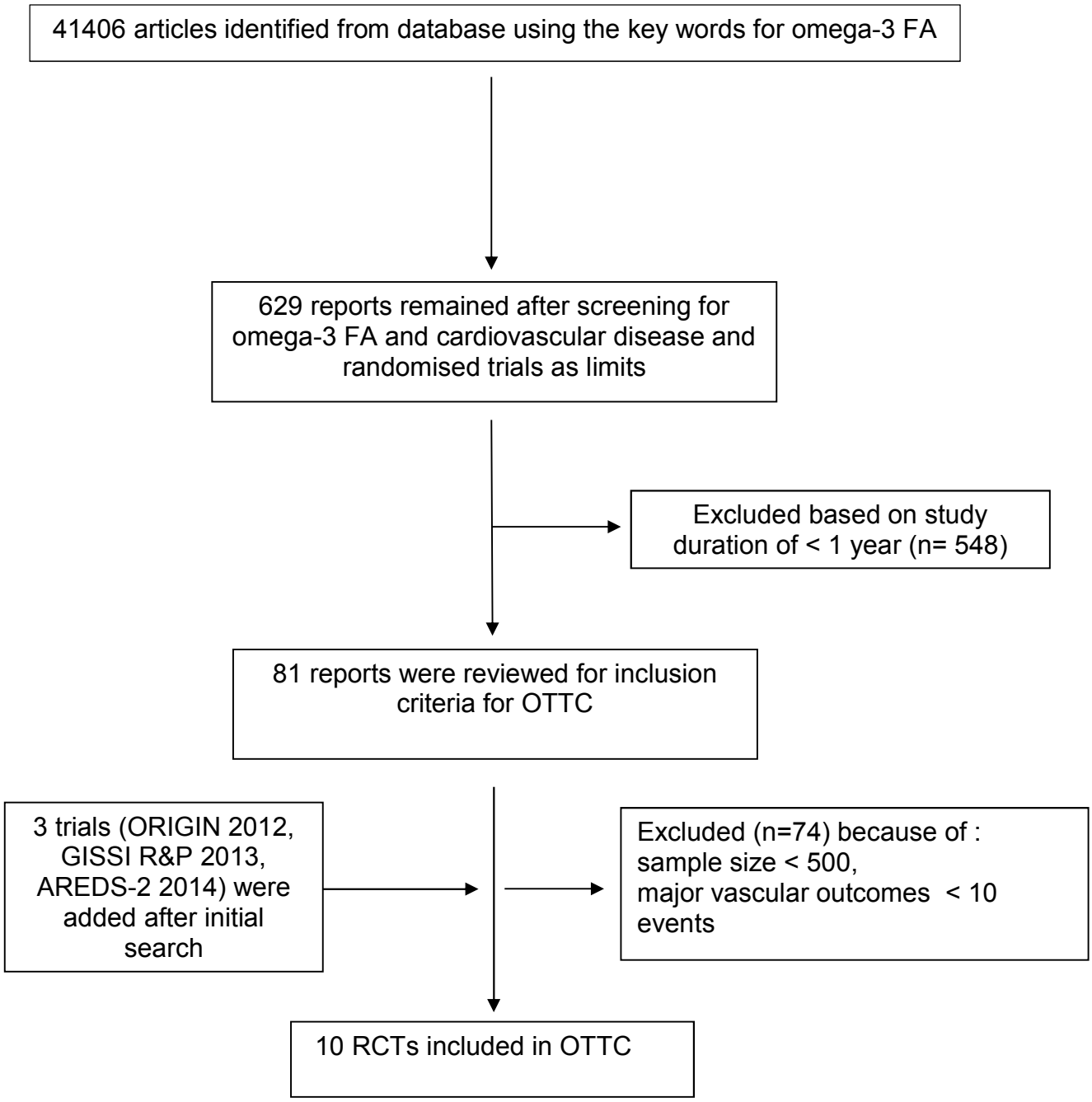
Table 4.1: Summary table of the results of published meta-analyses comparison with OTTC

Meta-analysis (Reference)	No. of trials	No. of patients	All-cause mortality RR (95% CI)	MVEs RR (95% CI)	Sudden cardiac death-RR (95% CI)	Cardiovascular death RR (95% CI)	Fatal MI RR (95% CI)	Non-Fatal MI RR (95% CI)
OTTC 2017 (221)	**10**	**77,906**	**0.96 (0.92-1.01)**	**0.97 (0.93-1.01)**		**0.94 (0.82-1.07)**		**0.97 (0.87-1.08)**
Rizos (222)	20	68,680	0.96 (0.91-1.02)		0.87 (0.75-1.01)	0.91 (0.85-0.98)		0.89 (0.76-1.04)
Kotwal (223)	20	63,030	0.95(0.86-1.04)	0.96 (0.9-1.03)		0.86 (0.75-0.99)		
Kwak (218)	14	20,485	0.96 (0.89-1.14)	0.99 (0.89-1.09)	0.93 (0.66-1.30)	0.91 (0.84-0.99)	0.87 (0.67-1.13)	0.86 (0.65-1.14)
Chen (224)	10	33,429			0.96 (0.84-1.10)			
Filion (225)	29	35,144	0.88(0.64-1.03)					
Zhao (226)	8	20,997			0.43 (0.20-0.91)			
Marik (216)	11	39,044	0.92 (0.85-0.99)	0.92 (0.85–0.99)	0.87 (0.76-0.99)	0.87 (0.79-0.95)		
Hooper (215)	48	36,913	0.87 (0.73-1.03)	0.95 (0.82-1.12)				
Bucher (214)	11	15,806	diet-0.7 (0.6-0.9), supplementation (suppl)-0.8 (0.7-0.9)		suppl-0.7 (0.6-0.9)		Diet-0.5 (0.3-1.1), suppl-0.8 (0.7-0.9)	Diet-0.7 (0.1-3.2), suppl-0.8 (0.55-1.2)

4.1.1 **The Omega-3 Treatment Trialists' meta-analysis of large randomised trials of omega-3 FA supplementation**

The Omega-3 FA Treatment Trialists' Collaboration (OTTC) combined data from selected large RCTs (at least 500 patients randomised and duration at least 12 months of supplementation) assessing the effect of omega-3 FA supplements for the prevention of CVD in adults (Detailed inclusion criteria described below Figure 4.1 and Table 4.2). The present analysis included the results from 10 large RCTs (153, 154, 180, 206-212) (involving 77 906 individuals) completed and reported between 1999 and 2016. A collaborative meta-analysis of these trials should ensure that reliable evidence emerges about the effects of omega-3 FA on cardiovascular disease outcomes. The pre-specified aims of this meta-analysis were to assess the effects of supplementation with omega-3 FA on (i) fatal CHD, non-fatal MI, stroke, major vascular events, cancer and all-cause mortality; and (ii) major vascular events in various sub-groups.

Figure 4.1: Screening and selection of included trials for the OTTC meta-analysis



4.1.1.1 Identification of trials

The aim of the search was to identify all relevant RCTs assessing the effects of omega-3 FA supplementation on pre-specified vascular outcomes. Potentially eligible trials according to pre-specified eligibility criteria as per OTTC were identified by a range of methods, including computer-aided literature searches on electronic databases and scrutiny of the reference lists of trials. The search was done in March 2011. We reviewed the bibliographies of all selected articles and review articles that included information of omega-3 FA and cardiovascular disease. Summary of the literature search are provided in Figure 4.1 and search terms are described below.

Step 1: The key words related to Omega-3 FA used for the search were omega, omega 3, omega 3 fatty, omega 3 fatty acids, omega-3 polyunsaturated, omega-3 polyunsaturated fatty acid, omega-3 fish oil, omega fatty acid, eicosapentaenoic acid or EPA, docosahexaenoic acid or DHA (N=41406 articles were retrieved).

Step 2: Search terms were restricted further to cardiovascular outcomes and for randomised trials (N=629 trials remained)

Step 3: Limit to treatment duration minimum of 12 months after abstract review (N=81 trials remained)

Step 4: 7 RCTs remaining after full-text review to include in OTTC

Step 5: 3 trials added after the initial literature search those were published after March 2011 (Origin 2012, GISSI-R and P 2013 and ARDS-2 2014)

Full paper reviews were done to some trials included in the previous meta-analysis but excluded in OTTC and the reason for exclusion was given in table 4.2.

Table 4.2: Selected details of excluded trials and reasons for exclusion for OTTC meta-analysis

Trial (year)	No. randomized	Duration (years)	Insufficient size	Insufficient duration	Irrelevant outcomes
Garbagnati (2009)	38	1	+	-	-
Sacks (1995)	59	2	+	-	-
Gajos (2010)	63	0.1	+	+	-
Leng (1999)	120	2	-	+	-
Milner (1989)	194	0.5	+	+	-
Raitt (2015)	200	2	+	-	+
Svensson (2006)	206	2	+	-	-
von Shacky (1999)	223	1	+	-	+
Singh (1977)	230	1	+	-	-
Grundt (2004)	300	2	+	-	-
Leaf (2015)	402	1	+	-	+
Johansen (1999)	500	0.5	-	+	-
Eritsland (1995)	511	1	-	-	+
Brower (2006)	546	1	-	-	+
Holman (2008)	778	0.3	-	+	-

4.1.1.2 Trials assessing effects on vascular outcomes

After review of previously published meta-analysis results, 6 months intervention with omega-3 FA did not show any meaningful clinical outcome (215) however when this was increased to 1 year in subsequent analysis, variable results were reported (216). One of the aims for performing this meta-analysis was to examine the effects of completed large (N>500) RCTs where omega-3 FA supplementation was used as intervention similar to ASCEND (study duration >1 year with MVE outcomes). With this background, we prespecified strict eligibility criteria in order to identify RCTs with sufficient cardiovascular outcomes to include in OTTC. The endpoints for OTTC were adopted from ASCEND outcomes and subgroups were predefined in the OTTC protocol before the start of the literature search. During the OTTC paper submission, subgroup analysis for statin use was requested from a reviewer. All eligible trials which fulfilled predetermined criteria were included in this meta-analysis without excluding whether they were primary or secondary cardiovascular prevention trials. However, the presence or absence of prior CHD was added in a prespecified subgroup. The results of the population-based case-control and prospective cohort studies suggest a protective effect of omega-3 FA on fatal cardiac events (cardiac arrest and sudden death) and Zhao meta-analysis reported the possible beneficial effects of omega-3 FA on sudden death (Table 4.1 and anti-arrhythmic section of omega-3 FA section 2.5.3). Hence, the analysis also included prespecified assessment of separate effects on fatal and nonfatal events.

Randomised trials for prevention of CVD were eligible for inclusion in this collaborative meta-analysis if the trial had:

(i) Sample size of at least 500 participants

(ii) Study duration of at least 1 year

(iii) MVE outcome of at least 10 events and

(iv) Randomised to omega-3 FA supplements or control (open or control).

Table 4.3: Cardiovascular and non-vascular endpoints for meta-analysis

Coronary Heart Disease (CHD)	Non-fatal MI Coronary death Sudden Death Total CHD
Stroke (and stroke sub-types)	Fatal stroke Non-fatal stroke Total stroke* Ischaemic stroke Haemorrhagic stroke Unclassified stroke
Revascularisation	Coronary Non-coronary Any revascularisation
Any Major Vascular Events (MVEs)	
Total Cancer**	
Mortality	Vascular death Non-vascular death All-cause mortality

* excluding transient ischaemic attack (TIA)

** excluding non-fatal non-melanoma skin cancer

Table 4.3 and 4.4 summarizes the vascular outcomes to be assessed in this meta-analysis. The primary comparisons were:

(i) Coronary heart disease(CHD) (Non-fatal myocardial infarction, coronary death, total coronary heart disease)

(ii) Stroke excluding transient ischaemic attack (TIA) (fatal or non-fatal stroke, total stroke with classification of ischaemic, haemorrhagic or unclassified)

(iii) Revascularisation (coronary, non-coronary or any vascularisation)

(iv) Major Vascular Event (MVE) – 1st occurrence of any major coronary event (MCE), stroke or coronary or non-coronary revascularization (coronary revascularization included coronary artery bypass grafting or coronary angioplasty (with or without stent insertion); and non-coronary revascularization includes carotid endarterectomy or carotid artery angioplasty, repair of an aortic aneurysm, peripheral arterial surgery, or angioplasty);

(v) Total cancer, excluding non-fatal non-melanoma skin cancer and

(vi) Mortality (vascular death, non-vascular death, all-cause mortality).

Table 4.4: Definition of events examined in the meta-analysis

Outcome	**Definition**
Major Vascular Event (MVE)	A composite of non-fatal myocardial infarction or coronary death; non-fatal or fatal stroke; or any revascularisation procedure (including coronary or non-coronary angioplasty or grafting)
Coronary Revascularisation	Coronary artery bypass graft, coronary angioplasty (PTCA with or without stent insertion)
Non-Coronary Revascularisation	Arterial surgery including leg artery bypass procedure, aortic or other aneurysm repair or carotid surgery or stenting
Stroke	Fatal or non-fatal stroke (not including transient cerebral ischaemic attack). I64 (ICD 10 codes)
Cancer	Total cancer excluding non-fatal non-melanoma skin cancer
Myocardial Infarction	I21 (ICD 10 code)
Cardiac Death	I46.9 (ICD 10 code)

Table 4.5: Prespecified subgroups for cardiovascular events

Age at randomisation	<65 65+
Sex	Male Female
Prior CHD	Yes No
Prior stroke	Yes No
Prior diabetes	Yes No
Total cholesterol	< 5.0 mmol/L ≥ 5.0 mmol/L
Triglycerides	≤ 1.7 mmol/L > 1.7 mmol/L
HDL-cholesterol	≥ 1.0 mmol/L < 1.0 mmol/L
LDLcholesterol*	< 3.0 mmol/L ≥ 3.0 mmol/L

* calculated or measured LDL-cholesterol

4.1.1.3 Statistical analysis

In 1985, Peto and colleagues reported an alternative method to the usual Mantel-Haenszel method for pooling odds ratios across the strata of two-by-two tables. The Peto odds ratio (POR) method is not mathematically equal to the classical odds ratio but is approximately equivalent (227). It uses an inverse variance approach and utilizes an approximate method of estimating the log odds ratio using different weights. An alternative way of viewing POR method is as a sum of 'O – E' statistics. Here, O is the observed number of events and E is an expected number of events in the experimental intervention group of each study. The approximation used in the computation of the log odds ratio works well when effects of the intervention are small (i.e. odds ratios close to one), events are not particularly common and the studies have similar numbers in experimental and control groups. (Cochrane handbook-5-1.cochrane.org/chapter_9/9_4_4).

POR works well for rare events as it does not need correction for zero counts. Furthermore, POR is also employed with dichotomous events based on time-to-event analyses. For the calculation of relative measures such as risk ratio (RR) and odds ratio (OR) in a single study, additional approaches are required for zero counts in any group. Also, POR method can be used to combine studies with dichotomous outcomes in studies using time-to-event analyses where log-rank tests have been used In the case of zero counts in any one treatment arm, POR can be calculated without continuity correction,

and is currently the relative effect estimation method of choice for binary data with rare events (228).

The Peto odds ratio can cause bias if there are substantial differences in the number of participants between the treatment and control groups or other unbalanced randomization (229, 230). The Mantel-Haenszel fixed-effects odds-ratio and logistic regression (standard, exact or Firth penalized likelihood logistic regression) provide reliable results for studies involving more common event rates.

In OTTC, the included trials had randomised controlled designs and had approximately equal number of participants in the treatment and control groups and the effects of treatment were small. Hence after considering the available methods and detailed discussions with statisticians, POR was adopted instead of the Mantel-Haenszel fixed effects odds-ratio.

The effect of treatment in each trial was analysed separately and summary statistics were calculated for each trial. For each trial, the “observed minus expected” statistic (O−E) and its variance (V) from the number of participants who developed the relevant endpoint, and the total number of participants in each treatment group were calculated, using standard formulae for 2×2 contingency tables. These (O−E) values, one from each trial, were summed to produce a grand total (G), with variance (V) equal to the sum of their separate variances. The value exp (G/V) is Peto’s “one step” estimate of the rate ratio (RR), and its continuity corrected 95% confidence interval is given

by exp (G/V ± (0.5/V + 1.96/√V) (19). Rate ratios (RR) were given with 95% confidence intervals (CI) for the overall results and with 99% CI (replacing 1.96 in the formula above by 2.58) for the results of individual trials or subgroups). Heterogeneity between the different subgroups was assessed by calculating S−(G2/V), where S is the sum of (O−E)2/V for each trial (or sub-grouping), and testing this statistic against a χ2 distribution with degrees of freedom equal to one less than the number of sub-groups.

Additional analyses of the primary outcomes assessed the effects of treatment on major vascular events in pre-defined subgroups, including age, sex, prior CHD, prior stroke, prior diabetes, blood lipids (total cholesterol, triglyceride, HDL-cholesterol, calculated or measured LDL-cholesterol), and design of trials (open-label or blinded trials). In interpreting subgroup results, the chief emphasis was placed on the overall results unless there was strong evidence of heterogeneity ($p<0.001$).

Standardized data request forms were sent to principal investigators of selected trials (selected according to previously described eligibility criteria) After having secured the agreement for collaboration in this meta-analysis from the trials, the completed tabular data sets were then returned to central office at Clinical Trial Services Unit, the University of Oxford for data entry and analysis.

4.1.2 **Results of the meta-analysis of omega-3 FA**

4.1.2.1 Characteristics of individual trials

Data were obtained on a total of 10 trials involving 77,906 participants (153, 154, 181, 206-210, 231, 232) that met the inclusion criteria, ranging in size from 563 to 18,645 participants. The characteristic of each trial included in OTTC meta-analysis are described in table 4.7 and 4.8. The risk of bias assessment of included trials is listed in table 4.6. Eight trials had a double-blind design and used placebo control and two trials had an open design (153, 206). Combinations of omega-3 FA (polyunsaturated fatty acid ethyl esters of eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) were used in all except one trial (206) that tested 1.8 g daily of EPA alone. The daily dose of omega-3 FA supplements ranged from 0.4 to 2.4 g/day (weighted mean 1.1 g/day). The mean duration of treatment in individual trials varied from 1 to 6.2 years (weighted mean 4.4 years). About two-thirds of the participants were male and the mean age at entry was 64 years. About 62% of participants had a prior history of CHD, 30% had a prior stroke, and 38% had prior diabetes. Among 77,906 individuals, there was a total of 11,088 major vascular events, (including 2276 with non-fatal MI, 1599 with CHD death, 1713 with stroke, and 6603 with revascularisation events. Data were available on the effect of treatment by prior use of statin therapy in 6 trials involving 47 548 participants (207-209, 212, 231, 233).

Table 4.6: Risk of bias assessment of included trials in OTTC meta-analysis

	Selection bias	**Performance bias**		**Detection bias**	**Attention bias**
Trial name (year)	Random sequence generation	Blinding of participants	Blinding of personnel	Blinding of outcome	Incomplete follow-up
DOIT (2010)	0	0	0	0	0
AREDS-2 (2014)	0	0	0	0	0
SU-FOL-OM3 (2010)	0	0	0	0	0
JELIS (2007)	0	1	1	0	0
ALPHA OMEGA (2010)	0	0	0	0	0
GISSI-HF (2008)	0	0	0	0	0
OMEGA (2010)	0	0	0	0	0
GISSI R&P (2013)	0	0	0	0	0
ORIGIN (2012)	0	0	0	0	0
GISSI-P (1999)	0	2	2	0	0

Low risk of bias	0
Unclear risk of bias	1
High risk of bias	2

Table 4.7: Characteristic of included trials

Trial	Number randomised	Trial design	Placebo control	Supplement	Dose of Omega-3 FA (g/day)	Male (%)	Duration Mean (years)	Age Mean (SD) (years)	Prior CHD (%)	Prior stroke (%)	Prior diabetes (%)	Statin use (%)
DOIT (2010)	563	Blind	Y	EPA+DHA	2.4	100	3	70 (3)	24	7	8	-
AREDS-2 (2014)	4203	Blind	Y	EPA+DHA	0.65	43	-	74 (-)	-	-	-	-
SU-FOL-OM3 (2010)	2501	Blind	Y	EPA+DHA	0.6	79	4.7	61 (-)	75	26	18	83
JELIS (2007)	18645	Open	N	EPA	1.8	31	4.6	61 (8)	-	-	16	100
ALPHA OMEGA (2010)	4837	Blind	Y	EPA+DHA	0.4	78	3.3	69 (6)	100	7	21	85
GISSI-HF (2008)	6975	Blind	Y	EPA+DHA	1	78	3.9	67 (11)	50	5	28	-
OMEGA (2010)	3818	Blind	Y	EPA+DHA	1	75	1	64 (-)	22	6	27	94
R&P (2013)	12505	Blind	Y	EPA+DHA	1	62	5	64 (-)	30	5	60	44
ORIGIN (2012)	12536	Blind	Y	EPA+DHA	1	65	6.2	64 (8)	65	87	88	54
GISSI-P (1999)	11323	Open	N	EPA+DHA	1	85	3.5	59 (11)	100	-	19	-
Total	77906				1.13	62	4.4	64	62	30	38	

Table 4.8: Detail characteristics of included trials in OTTC meta-analysis

Trials (Published journal and year)	Country	Study design	Number randomised	Participants characteristics	Intervention of Omega 3 FA	Dose of Omega-3 FA (g/day)	Follow-up (years)	Primary outcomes
DOIT (Eur J Cardiovasc Prev Rehabil, 2010)	Norway	Double-blind	563	Elderly men with long-standing hypercholesterolemia with or without coronary heart disease	Omega-3 FA	2.4	3	Measures of atherosclerosis progression: IMT, Pulse Wave Propagation Time, Circulating biomarkers
AREDS-2 (JAMA Intern Med 2014)	USA	Double-blind	4203	Persons aged 50 to 85 with bilateral intermediate AMD or advanced AMD in 1 eye	Factorial design, Omega-3 FA /macular xanthophylls	0.65	4.8	Composite outcome of myocardial infarction, stroke, and cardiovascular death
SU-FOL-OM3 (BMJ 2010)	France	Double-blind	2501	individuals with a history of myocardial infarction, unstable angina, or ischaemic stroke	Factorial design, Omega-3 FA and 5-methyltetrahydrofolate (560 µg), vitamin B-6 (3 mg), and vitamin B-12 (20 µg)	0.6	4.7	Major cardiovascular events (composite of non-fatal myocardial infarction, stroke, or death from cardiovascular disease).
JELIS (Lancet 2007)	Japan	Open	18645	Individuals with a total cholesterol of 6.5 mmol/L or greater	EPA	1.8	4.6	Major coronary events, (sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events)
ALPHA OMEGA (NEJM 2010)	Netherlands	Double-blind	4837	Individuals with History of a myocardial infarction who were receiving state-of-the-art antihypertensive, antithrombotic, and lipid-modifying therapy	Factorial design Omega-3 FA (EPA + DHA) and ALA	0.4	3.3	Major cardiovascular events, (fatal and nonfatal cardiovascular events and cardiac interventions)

GISSI-HF (Lancet 2008)	Italy	Double-blind	6975	Individuals with chronic heart failure (New York Heart Association class II-IV)	EPA+DHA	1	3.9	Death or hospital admission for cardiovascular reasons
OMEGA (Circulation 2010)	Germany	Double-blind	3818	Survivors of acute myocardial infarction	EPA+DHA	1	1	Sudden cardiac death
GISSI R&P (Trials 2013)	Italy	Double-blind	12505	High cardiovascular risk because of a cardiovascular disease other than myocardial infarction, or multiple risk factors (at least four major risk factors in non-diabetic patients and one in diabetics)	EPA+DHA	1	5	Cardiovascular mortality (including sudden death) and hospitalization for cardiovascular reasons
ORIGIN (NEJM 2012)	Canada	Double-blind	12536	Individuals with high risk for cardiovascular events and had impaired fasting glucose, impaired glucose tolerance, or diabetes	Factorial design, Omega-3 FA and insulin glargine	1	6.2	Death from cardiovascular causes
GISSI-P (Lancet 1999)	Italy	Open	11323	Survivors of recent (< or = 3 months) myocardial infarction	Omega-3 FA and vitamin E	1	3.5	Death, non-fatal myocardial infarction, and stroke

Table 4.9 Distribution of events in individual trials

Trial	Number randomised	Non-fatal MI	CHD death	Any CHD	Stroke	Revascularisation	MVE
DOIT (2010)	563	12	11	23	17	24	64
AREDS-2 (2014)	4203	70	18	88	84	117	421
SU-FOL-OM3 (2010)	2501	61	18	78	67	351	427
JELIS (2007)	18645	145	60	201	328	413	586
ALPHA OMEGA (2010)	4837	115	138	248	101	408	663
GISSI-HF (2008)	6975	200	45	236	225	268	677
OMEGA (2010)	3818	141	57	208	35	975	1075
R&P (2013)	12505	476	158	634	37	-	1478
ORIGIN (2012)	12536	600	615	1215	650	1762	2571
GISSI-P (1999)	11323	456	479	893	169	2285	3102
All	77906	2276	1599	3824	1713	6603	11064

4.1.2.2 Effects on CHD and on other major vascular events

There were 5480 any major vascular events in the omega-3 FA treatment group and 5584 events in the control group in total. Figure 4.2 shows that allocation to omega-3 FA had no effect on the rate ratios for any coronary heart disease (CHD) (rate ratio 0.96; [95% CI 0.90-1.02]), including CHD death (0.94; 95% CI 0.82-1.07) or non-fatal MI (0.97 [95% CI 0.87-1.08]). Allocation to omega-3 FA had no effect on the rate ratios (95% CI) for stroke (1.03 [0.93-1.13]) including ischaemic (1.03 [0.88-1.21]) or haemorrhagic stroke (1.07 [95% CI 0.76-1.51]). Likewise, allocation to omega-3 FA had no effect on the rate ratios (95% CI) for revascularisation events (0.99 [0.94-1.04]) including coronary (1.00 [0.93-1.07]) or non-coronary (0.92 [0.75-1.13]). Overall allocation to omega-3 FA had no effect on the rate ratios (95% CI) for major vascular events (0.97 [0.93–1.01]).

This meta-analysis also showed no significant heterogeneity between the results of individual trials for non-fatal MI, CHD death, any CHD or major vascular events (Figure 4.3). A funnel plot for subtypes of CHD and for MVEs is shown in figure 4.4.

Figure 4.2 Effects of supplementation with omega-3 fatty acid on major vascular events

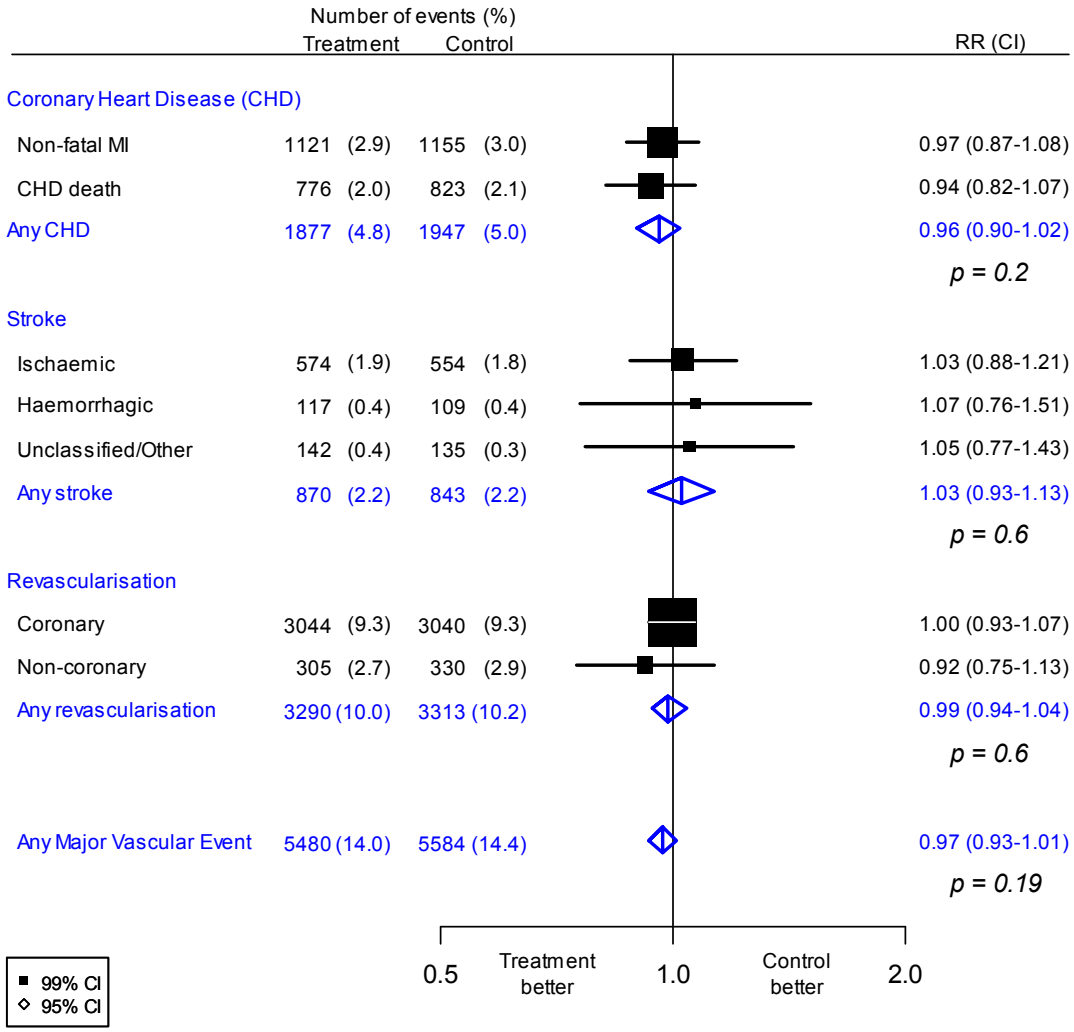


Figure 4.3: Effects of supplementation with omega-3 fatty acid on subtypes of CHD and on major vascular events by trials

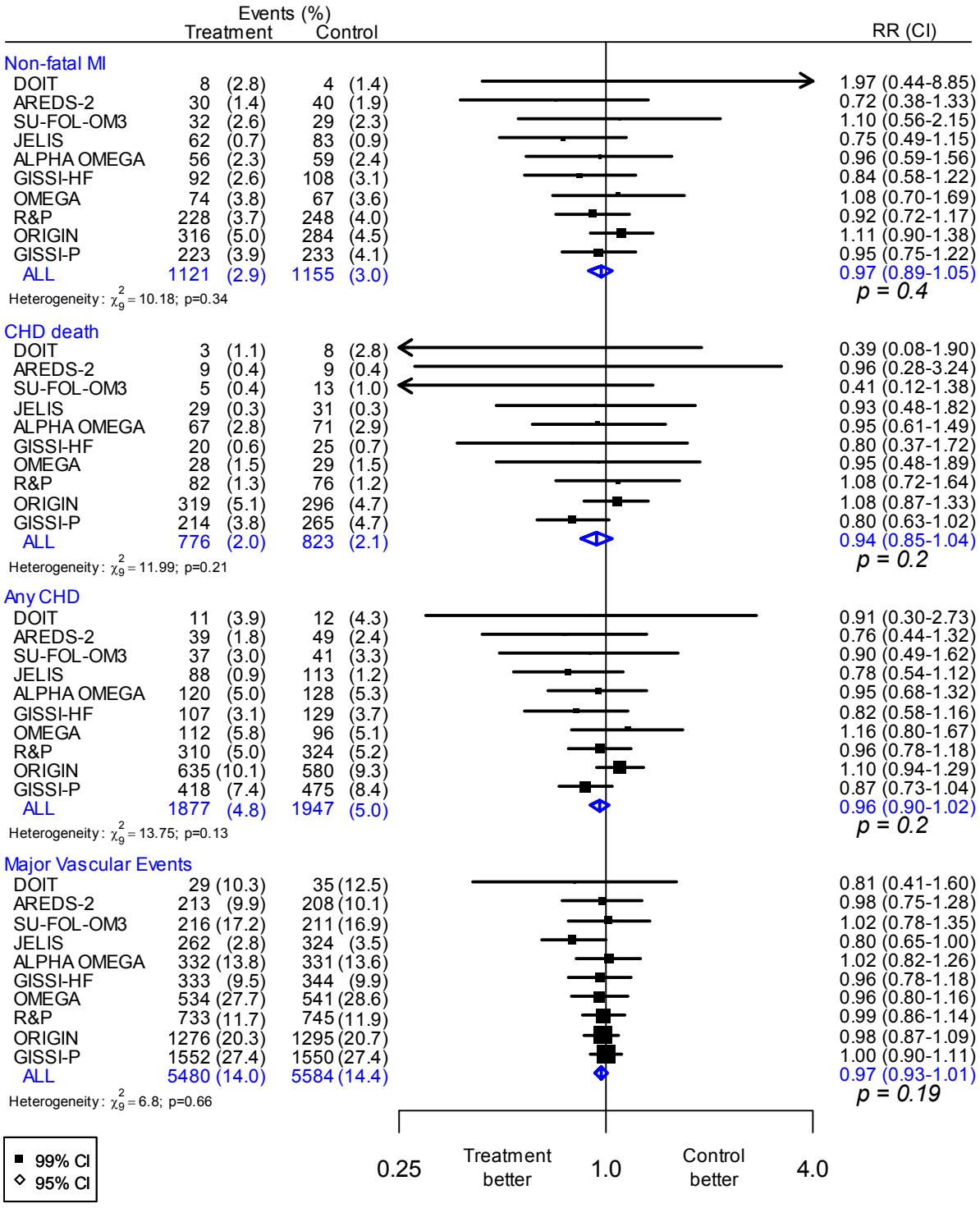


Figure 4.4: Funnel plots for subtypes of CHD and for major vascular events

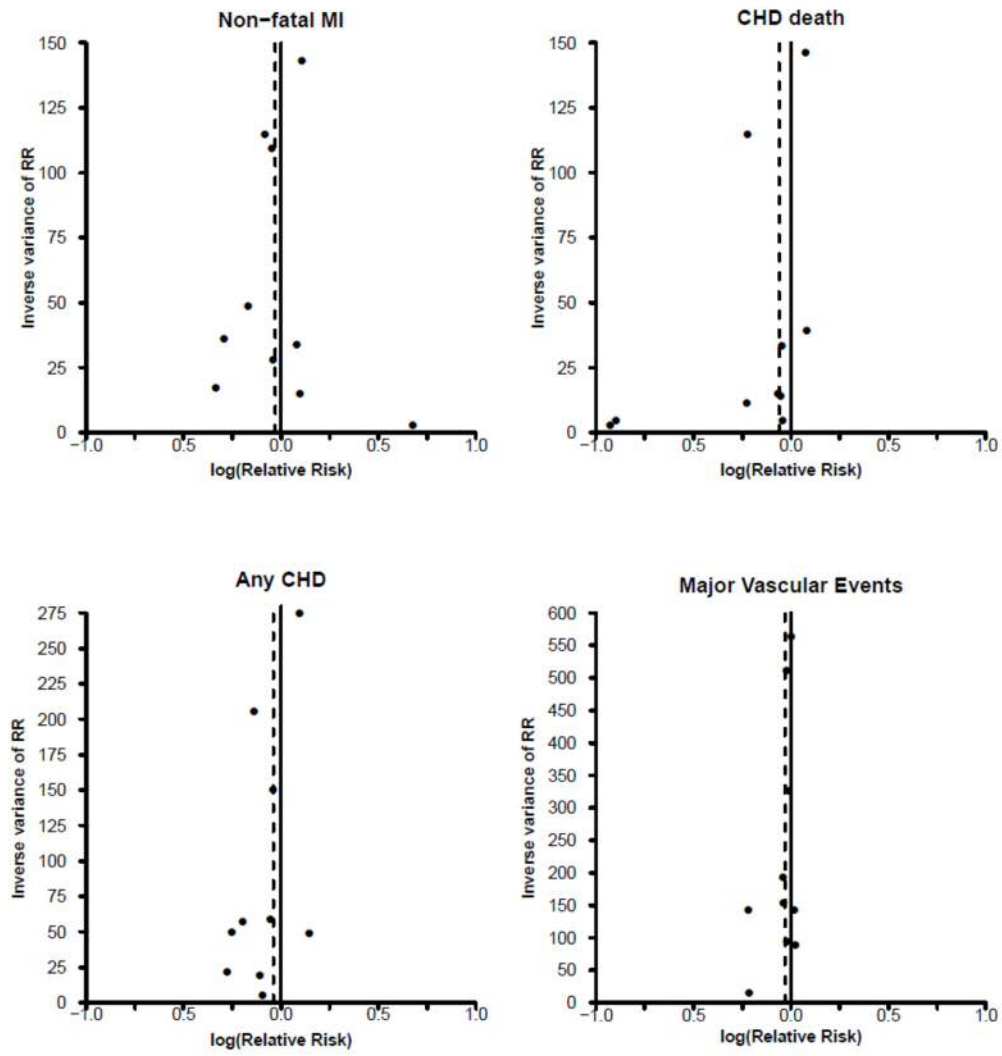


Figure 4.4 showed the funnel plot for subtypes of CHD and for major vascular events however with pre-specified eligibility criteria in the OTTC meta-analysis, this type of analysis has limited value for any meaningful explanation. Figure 4.5 shows that allocation to omega-3 FA had no significant effect on major vascular events in any of the pre-specified subgroups, including those defined by sex, prior CHD, prior diabetes, pre-treatment levels of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides or prior use of statin therapy, albeit there was weak

evidence of heterogeneity in the effects of age (unadjusted p=0.03) and by prior stroke (p=0.03), respectively. While it was not possible to assess the effects of treatment by race, the results were unaltered after exclusion of the JELIS trial which was conducted in a Japanese population (Figure 4.6).

Figure 4.5 Effects of supplementation with omega-3 fatty acid on major vascular events in pre-specified subgroups

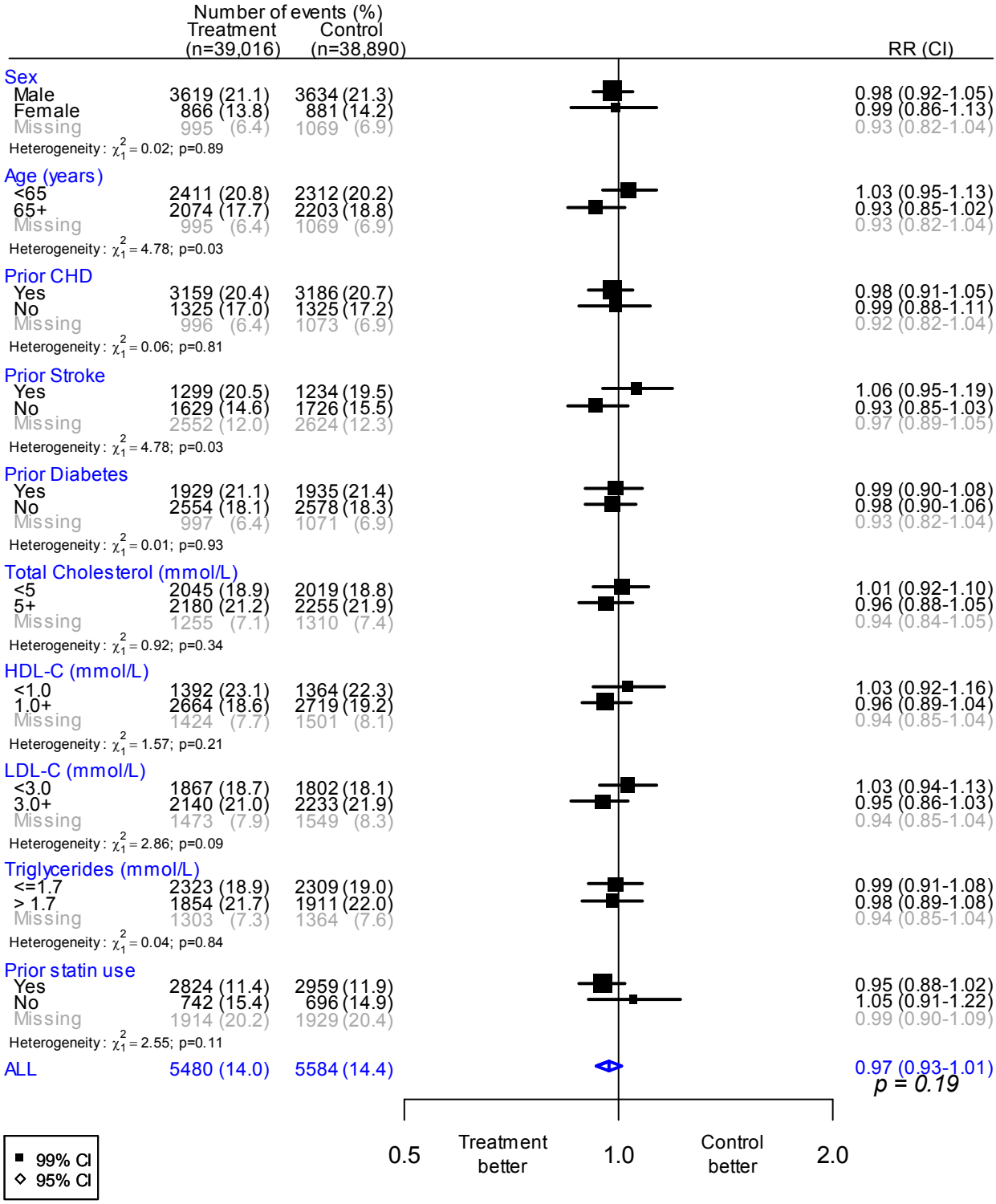


Figure 4.6 Effects of supplementation with omega-3 fatty acid on CHD and MVE by trials excluding JELIS

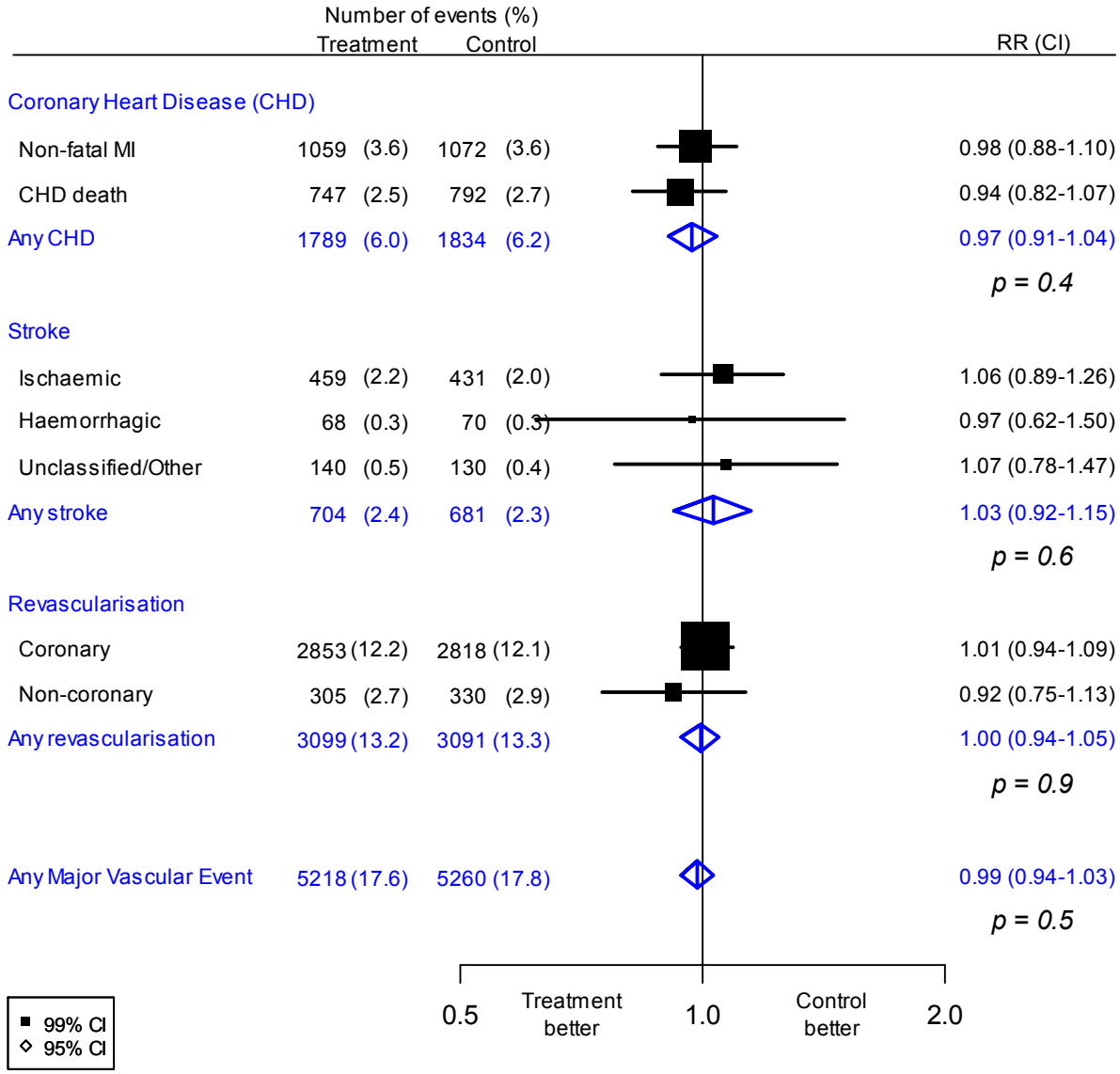
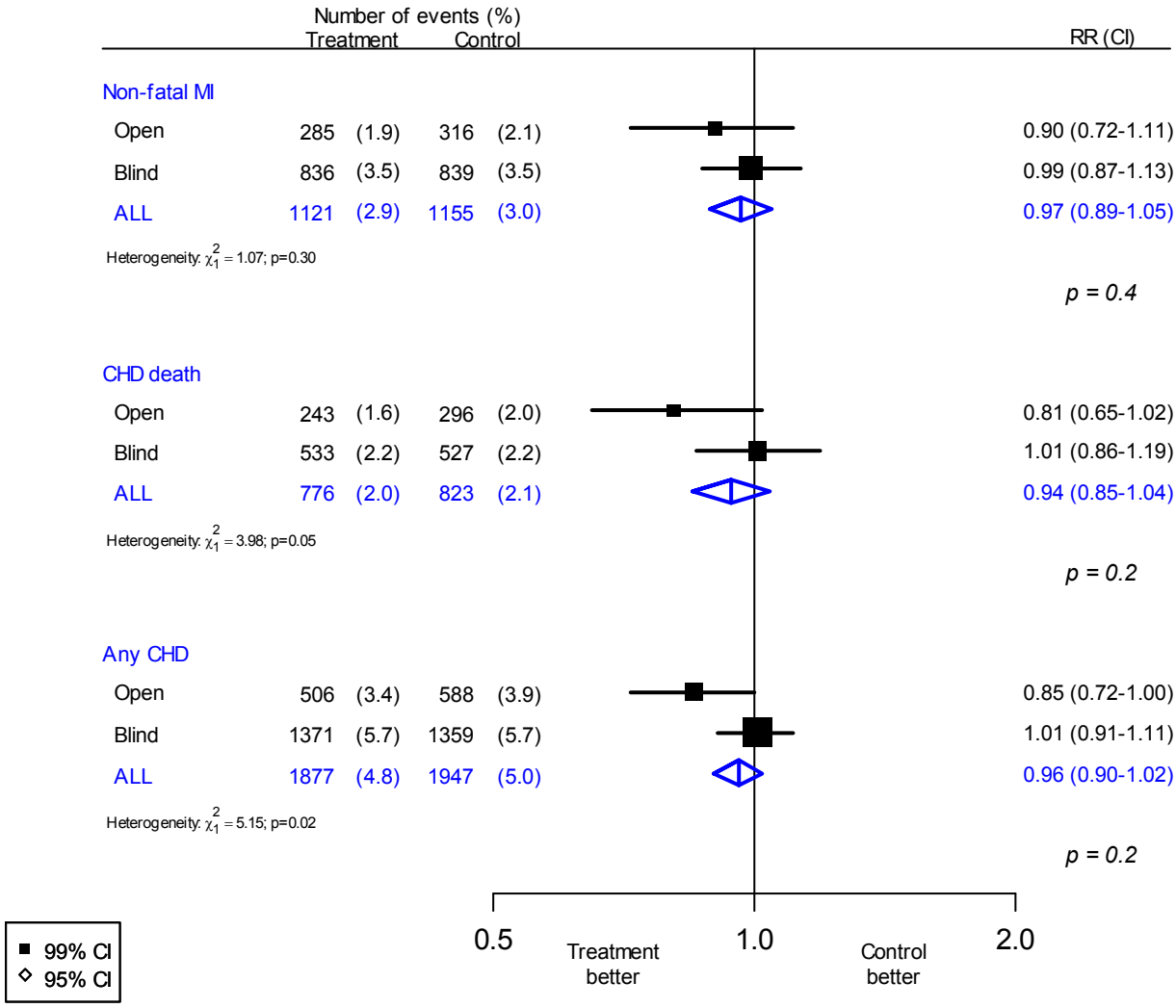


Figure 4.7 shows that allocation to omega-3 FA intervention had no significant effect on non-fatal MI, CHD death or overall CHD in the trials that used either an open and blind design. However, there was also some weak evidence of heterogeneity between the results from open versus blinded trials for all CHD (RR

0.85 [95% CI 0.72-1.00] vs 1.01 [0.91-1.11]; unadjusted p=0.02), but not for either fatal CHD or non-fatal myocardial infarction, respectively.

Figure 4.7 Effects of supplementation with Omega-3 fatty acid on fatal and non-fatal vascular events by trial design (Open vs blinded)



Importantly, allocation to omega-3 FA intervention had no significant effects on the rate ratios (95% CI) for cancer (1.01 [0.94-1.08]) (Figure 4.8) or total mortality (0.96 [0.92-1.01]) (Figure 4.9).

Figure 4.8 Effects of supplementation with omega-3 fatty acid on cancer

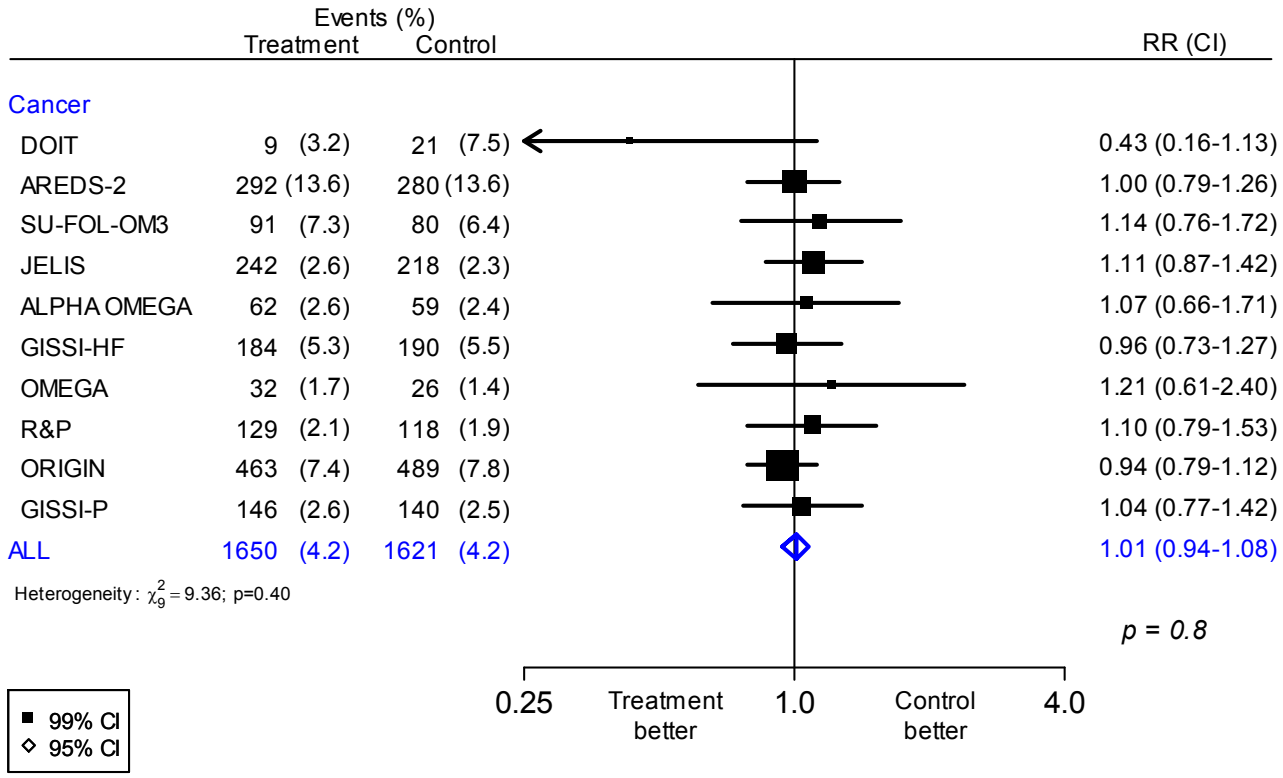
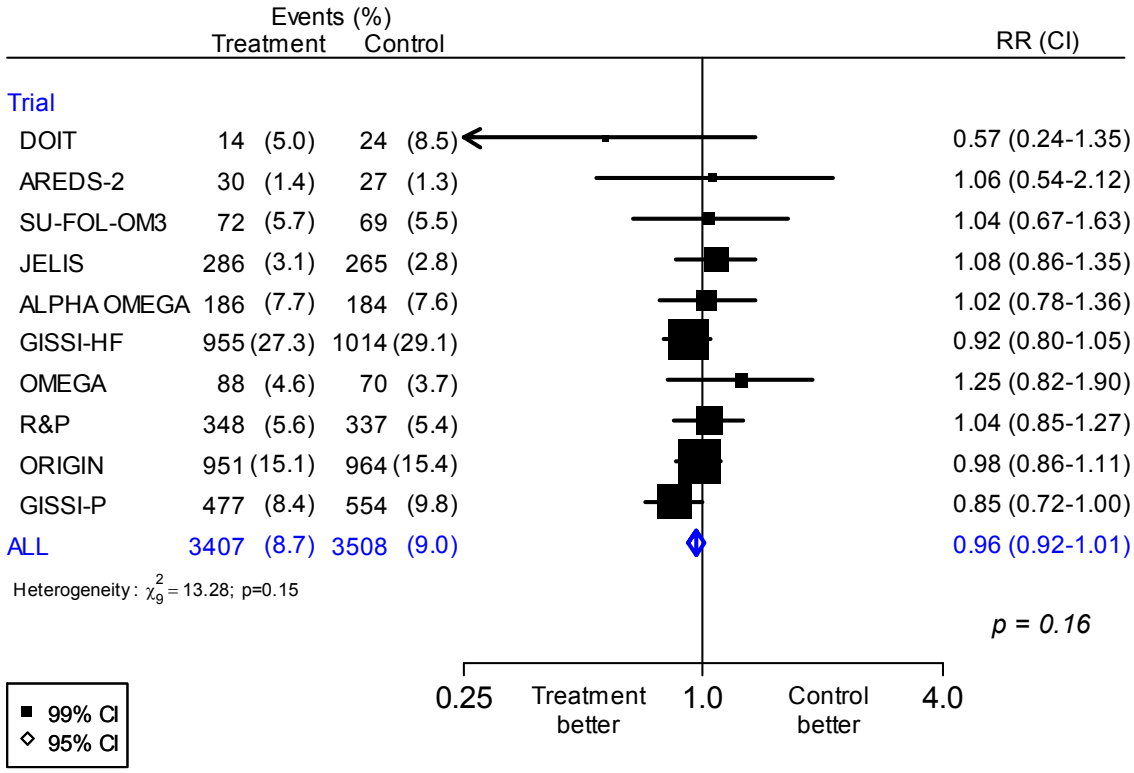


Figure 4.9 Effects of supplementation with omega-3 fatty acid on total mortality



4.2 Results of biochemical effects of omega-3 FA

In the ASCEND trial, blood spots from baseline and follow-up samples from 152 participants were sent to the Harris laboratory in the USA for measurement of omega-3 FA index and results are given below. At baseline the geometric mean (95% CI) omega-3 FA index was 7.11 (6.79-7.45) among the 76 participants subsequently allocated active omega-3 FA and 6.59 (6.25-6.94) among the 76 subsequently allocated placebo. These increased to 9.1 (8.79-9.41) during the trial (Table 4.10) in those allocated active omega-3 FA and were 6.54 (6.2-6.91) during the trial in those allocated placebo capsules. This represented a 33% (26%-39%) increase compared to placebo among those allocated active omega-3 FA capsules and was highly statistically significant p <0.0001 (Table 4.10 and 4.11).

Table 4.10: Omega-3 Index (% of total fatty acids) on-trial allowing for baseline Omega-3 index (Overall) in ASCEND substudy

	Active Omega-3 arm			Placebo Omega-3 arm			Percent increase on trial in Omega-3 arm vs. Placebo arm	P-value	P-value for hetero-
		Geometric mean (95% CI)			Geometric mean (95% CI)				
Arm	**N**	**Baseline**	**On-trial**	**N**	**Baseline**	**On-trial**	**(95% CI)**	**for effect**	**geneity**
Overall	76	7.11 (6.79 to 7.45)	9.10 (8.79 to 9.41)	76	6.59 (6.25 to 6.94)	6.54 (6.20 to 6.91)	33% (26% to 39%)	<0.0001	
Aspirin allocation									
Active aspirin	38	7.13 (6.71 to 7.57)	9.10 (8.71 to 9.50)	38	6.45 (5.96 to 6.98)	6.48 (6.00 to 7.00)	32% (23% to 41%)		0.8
Placebo aspirin	38	7.10 (6.62 to 7.62)	9.09 (8.63 to 9.59)	38	6.73 (6.27 to 7.21)	6.61 (6.12 to 7.14)	33% (24% to 42%)		
Compliance with brown capsules									
No	1	7.77	6.79	3	6.60 (5.35 to 8.14)	7.07 (5.84 to 8.57)	-13% (-38% to 20%)		0.01
Yes	70	7.08 (6.74 to 7.43)	9.19 (8.87 to 9.52)	73	6.59 (6.24 to 6.95)	6.52 (6.17 to 6.90)	35% (28% to 41%)		
Baseline Omega-3 index									
High (8%+)	23	8.77 (8.40 to 9.16)	9.72 (9.09 to 10.40)	15	9.22 (8.70 to 9.78)	8.98 (8.36 to 9.65)	11% (1% to 22%)		<0.0001
Low (<8%)	53	6.50 (6.21 to 6.80)	8.84 (8.52 to 9.17)	61	6.06 (5.81 to 6.33)	6.05 (5.77 to 6.35)	41% (33% to 48%)		

	Active Omega-3 arm			Placebo Omega-3 arm			Percent increase on trial in Omega-3 arm vs. Placebo arm (95% CI)	P-value for effect	P-value for hetero-geneity
		Geometric mean (95% CI)			Geometric mean (95% CI)				
Arm	**N**	**Baseline**	**On-trial**	**N**	**Baseline**	**On-trial**			
Fatty acid supplement at baseline									
No	64	7.11 (6.74 to 7.49)	9.08 (8.75 to 9.41)	69	6.47 (6.13 to 6.84)	6.44 (6.08 to 6.81)	33% (27% to 40%)		0.4
Yes	12	7.16 (6.58 to 7.80)	9.21 (8.33 to 10.18)	7	7.79 (6.92 to 8.77)	7.72 (6.78 to 8.79)	25% (9% to 44%)		
Type of fatty acid supplement at baseline									
Cod liver oil capsule	10	7.09 (6.40 to 7.84)	9.07 (8.06 to 10.22)	5	7.41 (6.89 to 7.97)	7.13 (6.52 to 7.79)	32% (15% to 50%)		0.5
Omega-3/fish oil supplement	2	7.54 (7.17 to 7.93)	9.89 (9.47 to 10.33)	2	8.83 (5.95 to 13.10)	9.43 (7.38 to 12.04)	18% (-8% to 51%)		

Percent increase and p-values are from Generalised Linear Models and allow for baseline Omega-3 index

Note: No patient had fatty acid supplements noted on their on-trial blood sample form

Allocation to aspirin had no significant effect on change in the omega-3 FA index with supplements (p for heterogeneity = 0.80). Only 4 participants (1 active and 3 on placebo) among those selected for omega-3 index measures reported non-compliance with their omega-3 FA capsules or placebo, providing limited power to assess differences. However, as expected, the omega-3 index did not rise significantly from baseline in the one non-compliant participant.

The effects on the omega-3 index were not significantly influenced by self-reported omega-3 fatty acid supplement use at baseline (Table 4.10), but only 15 participants reported such use. For those reporting baseline use, there was still a 25% (9%-44%) increase in the index, but this was not significantly different from the overall results (p for heterogeneity 0.5). By contrast, those with a baseline omega-3 FA index >8% (i.e. a high baseline level), only increased by a 11% (1% to 22%) during the trial versus placebo compared with a 41% (33% to 48%) increase vs placebo in those with a low baseline omega-3 FA index <8% (p for heterogeneity <0.001).

Tables 4.11 and 4.12 show the impact of accounting for baseline FA index and baseline use of omega-3 supplementation but does not change the overall message.

Table 4.11: Omega-3 Index on-trial allowing for baseline index (No fatty acid use reported at baseline) in ASCEND substudy

	Active Omega-3 arm			Placebo Omega-3 arm			Percent increase on-trial in Omega-3 arm vs. Placebo arm	P-value for effect	P-value for hetero-geneity
		Geometric mean (95% CI)			Geometric mean (95% CI)				
Arm	**N**	**Baseline**	**On-trial**	**N**	**Baseline**	**On-trial**	**(95% CI)**		
Overall	64	7.11 (6.74 to 7.49)	9.08 (8.75 to 9.41)	69	6.47 (6.13 to 6.84)	6.44 (6.08 to 6.81)	33% (26% to 39%)	<0.0001	
Aspirin allocation									
Active aspirin	33	7.12 (6.65 to 7.61)	9.04 (8.62 to 9.48)	35	6.35 (5.84 to 6.90)	6.37 (5.87 to 6.90)	33% (23% to 43%)		0.8
Placebo aspirin	31	7.10 (6.54 to 7.70)	9.11 (8.62 to 9.63)	34	6.60 (6.14 to 7.10)	6.51 (6.00 to 7.06)	34% (25% to 45%)		
Compliance with brown capsules									
No	1	7.77	6.79	3	6.60 (5.35 to 8.14)	7.07 (5.84 to 8.57)	-13% (-38% to 22%)		0.01
Yes	59	7.04 (6.66 to 7.45)	9.17 (8.84 to 9.52)	66	6.47 (6.11 to 6.85)	6.41 (6.04 to 6.80)	36% (29% to 43%)		
Baseline Omega-3 index									
High (8%+)	21	8.75 (8.34 to 9.17)	9.57 (8.93 to 10.26)	13	9.20 (8.67 to 9.77)	8.95 (8.29 to 9.66)	10% (-1% to 21%)		<0.0001
Low (<8%)	43	6.42 (6.09 to 6.77)	8.84 (8.50 to 9.20)	56	5.97 (5.71 to 6.24)	5.96 (5.67 to 6.26)	43% (35% to 51%)		

Percent increase and p-values are from Generalised Linear Models and allow for baseline Omega-3 index Note: No patient had fatty acid supplements noted on their on-trial blood sample form.

Table 4.12: Omega-3 Index on-trial allowing for baseline index (fatty acid use reported at baseline) in ASCEND substudy

	Active Omega-3 arm			Placebo Omega-3 arm			Percent increase on-trial in Omega-3 arm vs. Placebo arm		
		Geometric mean (95% CI)			Geometric mean (95% CI)				
Arm	**N**	**Baseline**	**On-trial**	**N**	**Baseline**	**On-trial**	**(95% CI)**	**P-value for effect**	**P-value for hetero-geneity**
Overall	12	7.16 (6.58 to 7.80)	9.21 (8.33 to 10.18)	7	7.79 (6.92 to 8.77)	7.72 (6.78 to 8.79)	33% (26% to 39%)	<0.0001	
Aspirin allocation									
Active aspirin	5	7.21 (6.39 to 8.15)	9.48 (8.44 to 10.65)	3	7.72 (7.21 to 8.26)	8.01 (7.70 to 8.33)	25% (4% to 51%)		0.8
Placebo aspirin	7	7.12 (6.29 to 8.07)	9.01 (7.71 to 10.54)	4	7.85 (6.33 to 9.73)	7.51 (5.93 to 9.52)	30% (11% to 53%)		
Compliance with brown capsules									
Yes	11	7.26 (6.64 to 7.93)	9.26 (8.31 to 10.33)	7	7.79 (6.92 to 8.77)	7.72 (6.78 to 8.79)	28% (13% to 44%)		
Baseline Omega-3 index									
High (8%+)	2	9.03 (8.49 to 9.59)	11.50 (10.78 to 12.27)	2	9.35 (7.06 to 12.40)	9.21 (6.89 to 12.31)	28% (-1% to 65%)		1.0
Low (<8%)	10	6.84 (6.37 to 7.34)	8.80 (7.98 to 9.71)	5	7.24 (6.83 to 7.68)	7.20 (6.51 to 7.96)	27% (10% to 47%)		
Type of fatty acid supplement at baseline									
Cod liver oil capsule	10	7.09 (6.40 to 7.84)	9.07 (8.06 to 10.22)	5	7.41 (6.89 to 7.97)	7.13 (6.52 to 7.79)	32% (15% to 50%)		0.5

	Active Omega-3 arm			Placebo Omega-3 arm			Percent increase on-trial in Omega-3 arm vs. Placebo arm (95% CI)	P-value for effect	P-value for hetero-geneity
		Geometric mean (95% CI)			Geometric mean (95% CI)				
Arm	**N**	**Baseline**	**On-trial**	**N**	**Baseline**	**On-trial**			
Omega-3/fish oil supplement	2	7.54 (7.17 to 7.93)	9.89 (9.47 to 10.33)	2	8.83 (5.95 to 13.10)	9.43 (7.38 to 12.04)	18% (-8% to 51%)		

Percent increase and p-values are from Generalised Linear Models and allow for baseline Omega-3 index

Note: No patient had fatty acid supplements noted on their on-trial blood sample form.

4.3 Results of biochemical effects of aspirin

Among the participants randomly sampled allocated to receive active aspirin, the geometric mean (95% CI) of UTxB2 concentration at baseline was 3453 (3069-3895) pg/mg (n=70) which decreased to 1190 (1100-1287) pg/mg (n=167) during the trial (Table 4.10). Among those allocated the placebo white tablet, UTxB2 was 3499 (3114-3931) pg/mg (n=69) at baseline and 3607 (3312-3929) pg/mg (n=170) during the trial. This represented 67% (63-70%) decrease compared to placebo among those allocated active aspirin and was highly statistically significant with a p-value of <0.0001 (Table 4.10).

Allocation to omega-3 FA in both active and placebo aspirin groups had no significant effect on change in UTxB2 concentrations (p for heterogeneity =0.80). Only 14 participants (6 active aspirin and 8 on placebo) among those selected for UTxB2 measures reported non-compliance with aspirin or placebo providing limited power to say anything useful. However, as expected, UTxB2 did not reduce significantly from baseline in the 6 non-compliant participants. The 8 non-compliant participants in placebo groups reduced UTxB2 indicating that they may have been on prescribed aspirin after they had stopped taking trial tablets.

These results were not influenced by reported time since taking their aspirin or placebo tablet in compliant patients. In those who reported taking their tablet between 0-12 hours prior to the urine sample, the reduction in UTxB2

was 67% (63-72%) and in those who reported taking their treatment 12-24 hours prior to sampling the reduction was 70% (64-74%) (Table 4.10) P =0.6.

These results were not influenced by self-reported non-study NSAID use at baseline nor by on-trial supplement use or baseline UTxB2 results (high (>3000) and low (0-3000)) (Table 4.10)

Figure 4.11 gives the results of creatinine-adjusted UTxB2 in-trial and at baseline by allocation to active vs placebo aspirin and non-study NSAID use.

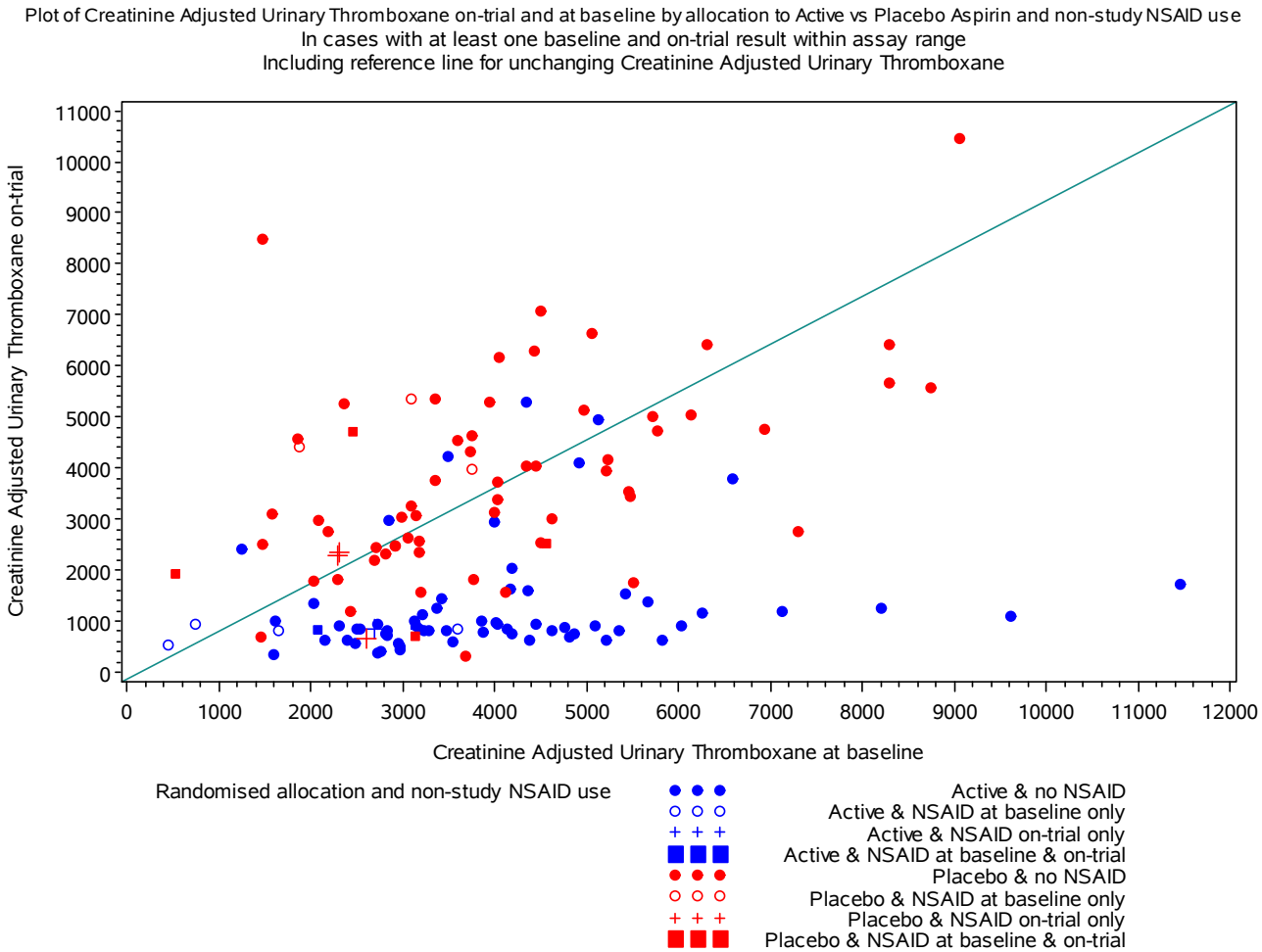
Table 4.13: Main results of measurement of urinary Thromboxane B2 in ASCEND substudy

	Active Aspirin arm				Placebo Aspirin arm				Percent decrease on-trial in Aspirin arm vs. Placebo arm (95% CI)	P-value for effect (on-trial)	P-value for hetero-geneity (on-trial)
	Baseline		On-trial		Baseline		On-trial				
Arm	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)			
Overall	70	3453 (3061-3895)	167	1190 (1100-1287)	69	3499 (3114-3931)	170	3607 (3312-3929)	67% (63% to 70%)	<0.0001	
Omega-3 allocation											
Active Omega	35	3294 (2750-3945)	77	1070 (967-1184)	36	3303 (2787-3914)	86	3337 (2957-3765)	68% (62% to 72%)		0.8
Placebo Omega	35	3620 (3083-4252)	90	1303 (1163-1460)	33	3726 (3181-4364)	84	3907 (3468-4402)	67% (61% to 71%)		
Compliance with white tablets											
No	6	2344 (1108-4959)	6	2053 (998-4223)	8	3243 (2141-4912)	8	1449 (730-2874)	-42% (-143% to 17%)		<0.0001
Yes	64	3581 (3205-4000)	161	1166 (1081-1257)	59	3465 (3067-3915)	160	3783 (3497-4091)	69% (65% to 72%)		
Time since white tablet last taken(in compliant patients)											
0-12 hours	49	3581 (3129-4097)	99	1127 (1024-1242)	43	3180 (2758-3667)	93	3489 (3166-3845)	67% (63% to 72%)		0.6
12-24 hours	9	3669 (2887-4662)	56	1259 (1106-1432)	11	4283 (3451-5316)	62	4168 (3643-4769)	70% (64% to 74%)		
Baseline thromboxane (Original dataset only)											
High (>3000)	45	4534 (4153-4949)	45	1180 (997-1396)	46	4541 (4160-4957)	46	3574 (2999-4258)	67% (58% to 74%)		0.7
Low (0-3000)	25	2115 (1770-2528)	25	761 (624-927)	23	2077 (1783-2419)	23	2472 (1945-3143)	69% (57% to 78%)		
Non-study NSAID at baseline											
No	64	3725 (3368-4120)	153	1191 (1096-1294)	62	3658 (3271-4091)	153	3699 (3384-4044)	68% (63% to 71%)		0.2
Yes	6	1538 (804-2943)	14	1181 (939-1485)	7	2357 (1386-4009)	17	2877 (2176-3803)	59% (40% to 72%)		
Non-study NSAID on-trial											
No	67	3497 (3087-3962)	160	1194 (1101-1295)	62	3685 (3297-4119)	157	3780 (3473-4114)	68% (64% to 72%)		0.05
Yes	3	2597 (2047-3295)	7	1094 (920-1301)	7	2208 (1340-3637)	13	2053 (1477-2853)	48% (16% to 68%)		
Compliance with white tablets and non-study NSAID on-trial											
Non-compliant and no NSAID	6	2344 (1108-4959)	6	2053 (998-4223)	7	3260 (2019-5264)	7	1603 (752-3418)	-28% (-121% to 26%)		<0.0001
Non-compliant and NSAID	0		0		1	3131	1	713			
Compliant and no NSAID	61	3638 (3245-4079)	154	1169 (1081-1265)	53	3670 (3282-4105)	148	3946 (3652-4264)	70% (66% to 73%)		
Compliant and NSAID	3	2597 (2047-3295)	7	1094 (920-1301)	6	2083 (1172-3702)	12	2242 (1653-3040)	52% (23% to 70%)		
Unknown and no NSAID	0		0		2	6309 (4736-8407)	2	3117 (2439-3984)			
Non-study NSAID at baseline and/or on-trial											
Neither	63	3744 (3381-4146)	151	1193 (1097-1298)	59	3738 (3333-4192)	150	3765 (3449-4110)	68% (64% to 72%)		0.3
Baseline only	4	1195 (494-2893)	9	1212 (858-1712)	3	2793 (1866-4178)	7	4105 (3033-5555)	70% (50% to 82%)		

	Active Aspirin arm				Placebo Aspirin arm				Percent decrease on-trial in Aspirin arm vs. Placebo arm (95% CI)	P-value for effect (on-trial)	P-value for hetero-geneity (on-trial)
	Baseline		On-trial		Baseline		On-trial				
Arm	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)	N	Geometric mean (95% CI)			
On-trial only	1	2700	2	1015 (690-1494)	3	2398 (2214-2596)	3	1528 (675-3460)	41% (-51% to 77%)		
Both	2	2547 (1695-3826)	5	1127 (911-1394)	4	2076 (822-5240)	10	2243 (1567-3211)	50% (12% to 71%)		

Percent decrease and p-values are from Generalised Linear Models (adjusted for original versus additional dataset) On-trial Creatinine Adjusted Urinary Thromboxane, Adjusted for original versus additional dataset, unadjusted for baseline Creatinine Adjusted Urinary Thromboxane.

Figure 4.10: Creatinine adjusted UTxB2 on trial and at baseline by allocation to active vs placebo aspirin and non-study NSAID use



4.4 Summary of results

The OTTC meta-analysis of tabular data from 10 large RCTs involving 77,906 participants demonstrated that allocation to omega-3 FA supplements for an average duration of 4.4 years had no effect on fatal CHD, non-fatal MI, stroke, revascularisation events or any major vascular events. Importantly, this meta-analysis also showed no beneficial effect on major vascular events in any particular subgroups. Likewise, the present meta-analysis indicated no evidence of any hazards of omega-3 FA supplements on cancer or on all-cause mortality.

The biochemical effects of study medications were consistent with the expected effects. Allocation to omega-3 FA supplements was associated with a 33% increase in the omega-3 FA index (consistent with the previous report) with no effect among allocated placebo. Similarly, allocation to active aspirin was associated with the expected reduction in UTxB2 illustrating the suppression of TxA2 resulting from COX-1 inhibition. Allocation to aspirin significantly reduced UTxB2 even up to 24 hours after ingestion with no evidence of any loss of effect. There was no interference of allocation of aspirin on omega-3 FA biochemical effectiveness or vice visa. These effects were only apparent in participants who reported that they were compliant with their allocated treatments.

5 Discussion and Conclusion

5.1 Discussion of findings from the Omega-3 Treatment Trialists' meta-analysis of large randomised trials

This meta-analysis of 10 large RCTs involving 77,906 participants demonstrated that allocation to omega-3 FA supplements for an average duration of 4.4 years had no effect on fatal CHD, non-fatal MI, stroke, revascularisation events or any major vascular events. Importantly, this meta-analysis also showed no beneficial effect on major vascular events in any particular subgroups. Likewise, the present meta-analysis indicated no evidence of any hazards of omega-3 FA supplements on cancer or on all-cause mortality.

The chief strength of this study was the availability of tabular data extracted by the trial Principal Investigators for all trials included in this meta-analysis (with the exception of the JELIS trial (206), where published data were used). The definitions of events were more directly comparable than in previous meta-analyses of the published results (214, 218, 222). Secondly, this meta-analysis had strict eligibility criteria for selection of trials, involving comparisons of omega-3 FA supplementation trials, involving 500 or more participants, and a treatment duration of at least one year. Thirdly, this meta-analysis also examined effects of treatment in pre-specified subgroups of major vascular events.

The reasons for the discrepant results of the previous trials of omega-3 FA supplements on fatal and non-fatal CHD events are not fully understood. In contrast with the null findings for most recent trials e.g. the Alpha omega trial, the Omega trial and SU-FOL-OM3, the older GISSI-P trial (153) reported a 14% reduction in major vascular events, chiefly due to a reduction in cardiac deaths and the JELIS trial reported a 19% (95% CI 5-31%) reduction in major CHD events, albeit based on only 586 events, chiefly due to a reduction in non-fatal CHD events. Both the GISSI-P and the JELIS trial used an open-label design where placebo capsules were lacking. In the GISSI-P trial for patients surviving a recent MI, supplementation of omega-3 FA 900 mg per day reduced significantly fatal CVD by 30%, fatal CAD by 35%, and sudden death by 45%. In the GISSI-HF trial, where heart failure patients were included, fatal CVD was only reduced by 10%, sudden death non-significantly by 7%, and first hospital admissions for ventricular arrhythmias significantly by 28% with the same dose of omega-3 FA supplementation. The JELIS trial showed the reduction of only major coronary events (fatal and non-fatal CAD, unstable angina, percutaneous coronary intervention, and coronary artery bypass grafting) with 1.8 g intake of EPA. Another possible explanation in addition to different study design could be that whether differences in prior disease, use of statins or other secondary prevention treatments may explain some of the conflicting results of individual trials (characteristic of included trials as described in section 4.1.2). Participants in the more recent trials were very well treated not only by antithrombotics but also by antihypertensives and statins. Compared with the recent trials, the treatment level with statins was low in the GISSI-P trial (29%). This could be

the reason for the high risk of fatal CAD and sudden death in the GISSI-P trial compared with the Alpha Omega Trial (85%), the omega trial (94%) and the SU-FOL-OM3 (83%). Previous reports suggested that the effects of omega-3 FA treatment may vary by prior use of statins (234, 235). For example, the Alpha Omega trial reported that low dose omega-3 FA reduced the risk of major vascular events in patients with prior MI not treated with statins (235). Importantly, the present meta-analysis demonstrated no heterogeneity of the effects of omega-3 FA on CHD death or non-fatal MI between the individual trials. The present meta-analysis also reported no differential effects of omega-3 FA on major vascular events by subgroups of those with or without prior cardiovascular disease or diabetes, or those with below or above average lipid levels or by prior use of statin therapy. The results of the present meta-analysis were unaltered with or without the inclusion of the JELIS trial (206), in which all were also treated with statins.

Although several possible mechanisms of effects of omega-3 FA on fatal CHD have been suggested, including anti-arrhythmic effects, anti-thrombotic effects, anti-inflammatory effects, plaque stabilisation and vasodilatation or triglycerides lowering effects (234, 236), these trials were unable to evaluate any of these mechanisms. It is possible that the discrepant results of the observational studies of n-3 fatty acid biomarkers and fatal CHD (237), and the effects on fatal CHD in this meta-analysis of the RCTs may reflect confounding by the healthy lifestyle of individuals with high versus low plasma levels of biomarkers of omega-3 FA intake in the observational studies. Results of observational prospective cohort studies and randomized

trials published before the year 2000 demonstrated that diets with higher amounts of omega-3 FA or supplements with omega-3 FA reduced cardiovascular mortality. The American Heart Association Guidelines, that people with established coronary arterial disease should be advised to take 900–1000 mg of omega-3 FA (EPA–DHA combined) per day. Most recently reported RCTs with null findings of omega-3 FA supplementation on cardiovascular outcomes did not have dietary fish intake data to investigate whether dietary intake or therapeutic supplements are the best source of omega-3 fatty acids is yet to be determined. Alternatively, the absence of any beneficial effect of omega-3 FA on MVE may reflect an insufficient dose of omega-3 FA used in the existing trials.

Previous meta-analyses of omega-3 FA trials (214, 218, 222, 238), were limited by being incomplete (214), including trials of dietary advice to increase fish consumption (214, 222) or by failing to distinguish effects of omega-3 FA on fatal versus non-fatal CHD outcomes (214, 218, 222, 238). Importantly, the results of the present meta-analysis differ from previous meta-analyses which suggested that omega-3 FA had a beneficial effect on fatal CHD (218, 222). In contrast, the present meta-analysis restricted to all eligible trials and tabular data supplied by individual trialists (except for the JELIS trial (206), reported no significant effect of omega-3 FA supplements on fatal CHD events. The present meta-analysis reported weak evidence of heterogeneity between the results of open and blinded trials for any CHD, which may reflect either chance or greater compliance in the open than in the blinded trials. An individual participant data meta-analysis (IPD) may have a

greater chance of detecting effects of omega-3 FA supplements on subtypes of fatal CHD (i.e. sudden death or ventricular arrhythmias) in a wider range of subgroups, but the overall null results of the present meta-analysis provide little encouragement for such an approach. Moreover, when I carried out OTTC as part of my MD project during my research time out of clinical training, I aimed to complete within the allocated time frame of 2 years and hence tabular meta-analysis methodology was adopted over IPD. In contrast to the previous meta-analyses of these trials, the present meta-analysis, involving 5 years of treatment and 3271 incident cancer events, indicated no adverse effects of omega-3 FA on cancer incidence. While these findings confirmed the safety of omega-3 FA on overall risk of cancer, no data were available on site-specific cancer. An additional limitation of the present meta-analysis involved the use of tabular rather than individual-level data. Hence, it was not possible to conduct additional analyses of the effects of omega-3 FA on major vascular events in subgroups by smoking or other cardiovascular risk factors. However, since the overall results and results in subtypes of disease were null, it is very unlikely that the results of a meta-analysis using individual-level data would differ from that using study-level data. Time-to-event analyses require individual-level data, but the results of such analyses are also unlikely to differ from those obtained using the Peto observed minus expected statistic (O-E) and its variance methodology used in this meta-analysis.

The 95% CI in the present meta-analysis of 10 trials, involving 78,906 high-risk individuals, including 11,000 major vascular events and 3824 CHD

events, cannot exclude a 7% lower risk of MVEs and a 10% lower risk of CHD for the effects of omega-3 FA supplements. Several ongoing large RCTs, involving a total of 64,000 participants (ASCEND: n=15,480; VITAL: n=25,874; STRENGTH: n=13,000 and REDUCE-IT: n=8000), should provide additional knowledge about the effects of omega-3 FA supplements on major vascular events, CHD and subtypes of CHD (124, 190, 220, 239). Importantly, the STRENGTH and REDUCE-IT trials will test the effects of major vascular events of much higher doses of omega-3 FA (3-4g/day) which will lower plasma levels of triglycerides without risk of bleeding or effect on blood glucose (detail discussion in section 2.5.3). These latter trials will also test the triglyceride-lowering hypothesis (with relatively higher dose compared to previous large RCTs in OTTC) in high-risk statin-treated patients with persistently elevated triglyceride levels.

The 2016 ESC/EAS Guidelines for the Management of Dyslipidaemia state that more data on the efficacy of omega-3 FA supplements for prevention of clinical outcomes are needed to justify their prescription (124). The results of the present meta-analysis provide no support for the recommendations to use of about 1g/day of omega-3 FA supplements in high-risk individuals for prevention of vascular events, cancer or all-cause mortality. The results of the ongoing trials are required to assess if higher doses of omega-3 FA (3-4g/day) have any beneficial effects on risk of MVEs.

In conclusion, this meta-analysis of large RCTs is the largest such meta-analysis carried out to date and has shown no significant effect of omega-3

FA supplementation on non-fatal MI, sudden death or on any major vascular events. No beneficial effect was also seen for stroke or revascularisation. The results of on-going large RCTs (ASCEND and VITAL) will provide additional evidence in a further 35,000 participants for the effects of omega-3 FA for prevention of CHD. The present results do not support recommendations to use omega-3 FA supplements in high-risk people for prevention of CHD (221). (OTTC paper is now published in JAMA Cardiology)

5.2 Discussion of biochemical results

In the ASCEND sub-study of the biochemical effectiveness of study medications, blood and urine samples were requested to 1500 participants at mid randomization phase, (10% of the total study population of randomly selected participants who returned samples at the baseline sample collection). In total 1288 samples were returned (1265 returned blood and urine samples, 6 blood only, 17 urine only, 512 did not return any samples). This selective randomisation method gave the opportunity to compare the analysis of biochemical measurement at two time-points for each individual, at baseline and post-randomisation. However, this approach may have a possible implication that those participants may tend to compliance more with study medication compared with non-responders to sample request in the first place. The percentage response rate of blood and urine sample collection for ASCEND on both time points was similar (around 78%-84%). About 98% of returned samples arrived to the central laboratory within the calculated time frame of 10 days from blood and urine collection at their local

surgery. Urine samples were frozen on arrival until measured. The impact of this was evaluated as part of the assays validation process (detail in section 3.4.2) and showed no significant variation in the measurement of UTxB2. The methodology of adopting randomization between treatment group and placebo group for this sub-study should have given the reliable and meaningful results between groups. There was a plasma sparing effect by choosing red-cell membrane omega-3 index measurement by dried blood spots which allow stored plasma samples for future biochemical measurement in ASCEND.

5.2.1 **Biochemical results of Omega-3 index**

Follow-up omega-3 index was increased by 33% (95% CI 26%-39%) from baseline in those allocated omega-3 FA compared to placebo. Our finding suggested that the increment was higher in patients with low omega-3 Index at baseline compared to a group of patients with high omega-3 index (>8%). Our findings are in agreement with a previously reported range of increase in measurement with omega-3 FA supplement. In a sub-study of the Framingham offspring cohort study for fish oil supplement, it was reported that omega-3 index increased by 41% (95% CI- 31-52%) in the active treatment group (200). The previously reported dose-response study reported that omega-3 index (mean +/-CI) increased from 4.7% (+/-0.9) to 7.9% (+/-1.7) with 0.5 g/day omega-3 FA supplement, to 9.9% (+/-2.9) with

1g/day and to 11.6% (+/-2.4) with 2g/day. Supplementation of 1 g Omega-3 FA in the ASCEND trial increased omega-3 FA index to 9.1% (8.79-9.41) in the active treatment group compared with placebo consistent with previously reported response of the omega-3 index after supplementation.

The omega-3 index has been used in various trials in different populations. In a diabetic population, the mean level was reported as 3.47% (CI +/-1.20) in line with the baseline estimates in ASCEND. One US study reported results in patients with major depression in the USA showing the omega-3 index was very low at 2.9% (+/-1.5) (240). Trials of patients with end-stage renal failure having haemodialysis, peritoneal dialysis or renal transplant reported high omega-3 index level which was thought to be due to significant alterations in erythrocyte membrane FA by the dialysis method and the omega-3 FA supplementation (241). Fish consumption had a major effect on omega-3 Index by 13% ($p< 0.0001$) increase for each serving level increase in oily fish intake (198). Diet was not included in the follow-up questionnaires in ASCEND, however, in this sub-study which measured omega-3 FA index at two time points (baseline and on-trial), the results indicate the desired increase in treatment group (follow-up omega-3 index was increased by 33% (95% CI 26%-39%) in those allocated omega-3 FA compared to placebo ($p<0.0001$, geometric mean 9.10% versus 6.54%).

In the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Heart Failure (GISSI-HF) study, the omega-3 index was measured in a subset of patients (n=461 out of 6975 randomised), at

baseline and after 3 months of omega-3 FA supplementation (1 g daily). After treatment, the omega-3 index increased from 4.8±1.7% to 6.7±1.9% in the treatment group but was unchanged in the placebo group (4.7±1.7 to 4.8±1.5%) (P<.0001) (242). Previously omega-3 index has been suggested as a risk factor for cardiac death and was inversely associated with risk for CHD mortality coding >8% of the omega-3 index with the greatest cardioprotection (243). Based on this data the proposed cutoffs for healthy levels of the omega-3 index were thought to be in the range of desirable >8%, intermediate level 4%-8% and undesirable high-risk level of <4% for cardioprotective effect (243). Overall, in the ASCEND trial, omega-3 Index (mean 95% CI) in the treatment group was 9.10% (8.79 to 9.41) compared to 6.54% (6.20 to 6.91) in the placebo group in the trial. Biochemically the finding has confirmed that supplementation with 1g daily EPA+DHA increased the omega-3 index to a level believed to be cardioprotective. Previous omega-3 FA trials with null results were criticized for low bioavailability, and issues in study design (small sample size and insufficient duration). It was also assumed that with no difference in levels of omega-3 FA between treatment and placebo groups, a difference in study outcome may not be possible (240). However, the large randomised ASCEND trial (N>15,000) with a mean duration of >7 years follow-up and biochemical confirmation of biochemical effectiveness measured by the omega-3 index, should provide reliable evidence for the effects of omega-3 FA supplementation on cardiovascular outcomes.

5.2.2 Discussion of the results of the Urinary Thromboxane B2 measures

Several groups have reported that the antiplatelet activity of aspirin in people with diabetes may be reduced or variable leading to aspirin resistance due to hyperglycaemia or rapid platelet turnover (138, 139, 143, 144, 244). In one study the prevalence of aspirin resistance was reported as being up to 18% in diabetic patients suggesting that such patients were at risk of arterial thrombotic events despite aspirin therapy (244). However, our results show a significant reduction in UTxB2 level (67% (95% CI 61%-74%)) reflecting suppression of TXA production due to COX 1 inhibition in diabetic patients with aspirin allocation compared to placebo group ($p<0.0001$) in the ASCEND trial. Our findings do not support the previously reported high prevalence of aspirin resistance in a diabetic population but show that aspirin has a similar biochemical effect in diabetes as seen in other populations. However, the limitation of this pilot study to add to consideration included a small number (n=337), randomly selected from those 1260 participants who sent their blood and urine samples at both time points (baseline and 3.5 years follow-up) of sample collection. In addition, by being a mailed-based sample, aspirin ingestion was not witnessed and therefore the exact time difference between aspirin ingestion and sample collection was self-reported.

It has also been suggested that diabetic patients require higher doses or more frequent dosing of aspirin to have the desired biochemical effect and

preventive effect on atherosclerotic events (136, 144, 245). In our study, in compliant participants, there was no evidence of a decrease in effect by time since ingestion of the active/placebo aspirin tablet (reductions 67% [95% CI 63%-72%] at 0-12 hours and 70% [64%-74%] at 12-24 hours). Our results do not suggest rapid recovery of TxB2 generation with once a day dosing nor higher UTxB2 measurement after 12-hour post-aspirin intake.

Measurement of serum thromboxane is regarded as the preferred method of assessment for COX inhibition by aspirin rather than UTxB2. However, we have shown that UTxB2 can also be used to reliably assess the biochemical effect of aspirin. Using measures of UTxB2 will allow trials to collect samples by mail or without any requirement for on-site sample preparation as it is a stable metabolite. One study has shown discrepancies between serum and urine thromboxane results and this may be related to a variety of factors (*e.g.* BMI) but in the context of this randomised trial, it appears that UTxB2 levels were suppressed as expected (136). Although it was well recognized that UTxB2 level can be influenced by various factors, it has shown the percentage difference on a comparison between treatment group and placebo group in our pilot study.

Another concern is that the preparation of aspirin with an enteric coating may lead to a failure to achieve complete inhibition of TxB2 generation due to incomplete absorption leading to possible aspirin resistance in diabetic patients (246). In ASCEND, the preparation was enteric coated aspirin and our results do not suggest less bioavailability as shown by the desired

significant reduction of UTxB2 after aspirin ingestion. Medication adherence might play a large role in aspirin resistance, and may, in fact, be the largest contributory factor and this should be fully investigated before “aspirin resistance” is invoked.

Considering possible side effects of aspirin with bleeding risk, using higher or frequent dosing of aspirin should not be taken lightly especially with a recent report from The Japanese Primary Prevention Project (JPPP) for elderly people with multiple cardiovascular risk factors (N = 14,464, aged 60 to 85 years) (247). The study was terminated early by the Data Monitoring Committee after a median follow-up of 5.02 years based on likely futility. The 5-year cumulative primary outcome event rate was not significantly different between the groups (2.77% [95% CI, 2.40%-3.20%] for aspirin vs 2.96% [95% CI, 2.58%-3.40%] for no aspirin; hazard ratio [HR], 0.94 [95% CI, 0.77-1.15]; P = 0.54) (247). However, it showed significantly increased the risk of extracranial hemorrhage requiring transfusion or hospitalization (HR, 1.85 [95% CI, 1.22-2.81]; P = 0.004). The ongoing, ASPREE (a double-blind, randomised, placebo-controlled trial of oral 100mg enteric-coated acetylsalicylic acid (ASA) or matching placebo being conducted in Australian and US populations), will examine whether the potential primary prevention benefits of low-dose aspirin outweigh the risks in healthy older individuals (248). ASCEND will complete and results will be available in 2018 to answer whether aspirin reduces the risk of cardiovascular events in individuals with diabetes who do not already have diagnosed occlusive arterial disease and whether such benefits outweigh any potential hazards from bleeding. A study

with n=>15,000 should have excellent power (95% Power 2p<0.01) to detect a 12-15% proportional reduction in the cardiovascular event rate among such patients with biochemical confirmation that aspirin inhibiting COX1 as shown by reduction in UTxB2.

The work described in the present thesis has demonstrated the reliability of questionnaire-based, self-reported compliance with study medication for this large mail based trial. Whether the biochemical effects will translate into beneficial effects on clinical outcomes will become clear on completion of ASCEND in mid-2018.

5.2.3 **Aspirin: continuing clinical uncertainty**

Substantial uncertainty still persists about whether or not aspirin should be recommended for the primary prevention of cardiovascular disease in people with diabetes. This uncertainty is reflected by the discrepant recommendations for use of aspirin in cardiovascular prevention guidelines. When ASCEND was designed, the American Diabetes Association recommended aspirin use for primary prevention in people with diabetes with one additional risk factor (249) but, at that time, the UK and European guidelines were more circumspect (250, 251). More recently, the UK National Institute for Health and Care Excellence (NICE) 2015 Guideline for Type 2 Diabetes has included recommendations not to offer antiplatelet therapy for adults with type 2 diabetes without cardiovascular disease (252) and similarly the most recent European Guidelines (124). By contrast the U.S. Preventive

Services Task Force now recommends initiating low-dose aspirin use for the primary prevention of cardiovascular disease and colorectal cancer in adults aged 50 to 59 years who have a predicted risk for MI or stroke of at least 10% over 10 years, are not at increased risk for bleeding, and are willing to take low-dose aspirin daily for at least 10 years, irrespective of their diabetes status (253) For the analogous group aged 60-69 the recommendation is “optional”.

The ATTC meta-analyses have shown that the absolute risks for cardiovascular disease and for bleeding events are positively correlated. A diagnosis of diabetes increases an individuals’ risk both of occlusive vascular disease and of bleeding. Without direct evidence of the balance of benefits and harm of antiplatelet therapy in this population, it is difficult to make clear recommendations. Furthermore, since the ASCEND trial started, the potential role of aspirin for the prevention of cancer has emerged as an important additional factor to be considered.

In addition to ASCEND, there are other ongoing trials in intermediate-risk populations which will provide further data. The Aspirin and Simvastatin Combination for Cardiovascular Events Prevention Trial in Diabetes (ACCEPT-D) study in Italy originally aimed to randomize 5170 diabetic patients aged over 50 years to aspirin 100mg daily versus open control, with follow-up until there have been 515 primary endpoints (CV death, MI, stroke or hospitalisation for vascular disease) (254) ACCEPT-D represented the only other major trial in diabetes but recruitment stopped at 2100 participants,

significantly reducing its ability to produce a reliable answer in the next few years. The ASPREE (ASPirin in Reducing Events in the Elderly) trial has randomized 19,000 healthy people over the age of 70 years from Australia and the USA to aspirin 100mg daily versus placebo but the proportion of patients with diabetes is likely to be under 10% (~1500); results are expected in 2018 (248). Finally, the ARRIVE study (Aspirin to Reduce the Risk of Initial Vascular Events) Study has recruited over 12,000 participants at moderate risk of major CHD events (estimated 10-20% 10 year CHD risk), but patients with diabetes at an estimated risk >20% over 10-year risk are excluded; the expected completion date is 2018.
(https://clinicaltrials.gov/ct2/show/NCT00501059).

Therefore, in the next 2-3 years, there should be substantially more data about the relevance of aspirin for primary prevention of cardiovascular disease. ASCEND will provide almost half of the available data in diabetes. In addition, there will be considerably more evidence in other intermediate-risk groups including healthy elderly people. It is likely that a meta-analysis of all of these trials will be needed to provide reliable evidence about the effects of aspirin in people with diabetes. It is, therefore, data from ASCEND will be combined with data from the other relevant trials in a further meta-analysis coordinated by the ATTC.

5.2.4 **Omega 3 fatty acids and OTTC results on ASCEND**

Since ASCEND started, several large trials have reported their results with no clear evidence to support the use of omega-3 fatty acid supplementation

for the prevention of cardiovascular disease. Given OTTC data, it is unlikely that ASCEND will show benefits from allocation to omega-3 FA on risk. Nevertheless, it will provide additional randomised evidence on both efficacy and safety which may help elucidate whether there are particular groups who might benefit from omega-3 FA supplementation.

Impact of this thesis on current literature

1. Trial methodology describing large randomised trial by mail (paper published in Trial in 2016 (190)).
2. Design and baseline characteristics of the ASCEND trial (Published in AHJ (182)).
3. Meta-analysis of omega 3 FA: no significant effects on the cardiovascular outcome (Paper published JAMA Cardiology (221)).
4. Biochemical measurement supporting the compliance of trial medication in a mail-based trial (abstract submitted to ESC Congress 2017 and presented in August 2017 at Barcelona).

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7 Appendices

Appendix 1: Ethic approved documents for blood and urine collection

Appendix 2: ASCEND study protocol

Appendix 3: Published papers from this thesis

Appendix 1: Ethic approved documents for blood and urine collection

ASCEND

ASCEND
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[Date]

Dear Colleague

Request for blood sample for the ASCEND trial

Your patient is participating in the ASCEND (A Study of Cardiovascular Events iN Diabetes), which is a nationwide study with over 15,000 men and women with diabetes taking part. It is a randomised trial assessing the benefits and risks of aspirin and/or omega-3 fatty acids in patients with diabetes with no previous history of cardiovascular disease. Further information about this study is available on www.ctsu.ox.ac.uk/ascend.

An optional, but we believe valuable, part of the study involves obtaining blood and urine samples to measure glycaemic control, lipids, renal function and biochemical measurement of the effect of the study medications.

We should be very grateful, therefore, if you would help us by collecting a blood sample and transferring the urine specimen brought by the participant to the two different containers. In addition, it would be most helpful if you could record both the time of the last dose of study aspirin/placebo and their current medication on the enclosed consent form. Detailed instructions are printed on the back of this letter.

We apologise for troubling you with this request, but your collaboration would be extremely helpful. Many thanks.

Yours sincerely

Professor Jane Armitage

Dr Louise Bowman

Study Coordinators

Instructions for collecting and mailing of blood and urine samples

1. Please check that the name of the study participant corresponds to that on the enclosed barcode labels and consent form.

2. Ensure that the consent form has been completely filled-in, signed and dated. (N.B. If the patient has not brought the consent form, please call the Freefone number below and ask for a copy to be faxed to you.)

3. Please measure the patient's weight and record this on the consent form as indicated. Please fill in the list of medication the patient is currently taking.

4. Collect 9ml of blood into the purple-topped vacuette [EDTA] and label this using one of the blood sample labels from the enclosed sheet. **Please attach the label as straight as possible lengthways, and as close to the bung as possible.** Then place the labelled vacuette (top upright) in one lined, green-topped, opaque white plastic tube, ensuring that the cotton-wool bung provided is inserted into the green lid, before securing it tightly.

   (N.B. If the vacuette fails or is broken we should be most grateful if you would use one of your own ETDA-containing vacutainers (9ml if possible, otherwise 5ml). A spare blood sample barcode label is enclosed should you need it.

5. Using the pipette, transfer 5ml of urine from the universal container brought by the participant to the small white topped tube and 10 ml of urine to the white top tube which contains small white tablet (a preservative), and label those using the urine sample labels from the enclosed sheet. Then place the labelled tubes (top upright) in the green-topped plastic tubes provided, again ensuring that the cotton-wool bungs are inserted into the green lids before securing those tightly. Then discard the universal container with remaining urine.

6. Please indicate on the consent form that a blood and/or urine sample has been obtained and date the form.

7. Put three green-topped mailing tubes in the cardboard box and secure the lid. (Please do not write on the box.) Put this, **together with the completed**

consent form, in the pre-addressed reinforced white envelope (no stamp required).

8. **Please mail the envelope today,** as any delay will result in sample degradation.

Please call the ASCEND study office on Freefone 0800 585323 if you have

any queries regarding this.

Many thanks for your collaboration.

ASCEND

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[Date]

[Our GP ref]

[GP address]

Dear Dr [GP name],

[Patient name], [DOB], [address]
ASCEND Study Ref: [100-1234]
[Blood] [and] [urine] results from the ASCEND trial

As you know [Mr Patient] [is participating in the ASCEND study. He recently provided [a non-fasting blood sample] [and] [a urine specimen] which was mailed to our central laboratory. Routine analysis of [this sample] [these samples] revealed the following results:

Total cholesterol:	mmol/L
$HbA1_C$ DCCT:	%
$HbA1_C$ IFCC :	mmol/mol
HDL-cholesterol:	mmol/L

Urinary microalbumin/creatinine ratio*: mg/mmol creatinine

* NICE guidelines: microalbuminuria defined as ratio ≥ 2.5 mg/mmol [men] or 3.5 mg/mmol [women]

Please feel free to telephone Freefone 0800 585323 if you wish to discuss any aspect of the study. Many thanks for your help.

Yours sincerely

Dr Jane Armitage

Dr Louise Bowman

Study Coordinators

Consent for additional blood and urine collection, storage, and analysis

Please cross (X) each of the following statements to which you agree:	
Yes ☐ No ☐	I confirm that I have read and understood the information about blood and urine sampling. I understand that providing a blood and urine sample is optional, and I am free to continue taking part in the trial without agreeing to my blood or urine being taken.
Yes ☐ No ☐	I agree to my samples being used for immediate measurements of glucose control, lipids (cholesterol), kidney function and biochemical tests to assess the study treatment effects, and for relevant results to be provided to my general practitioner.
Yes ☐ No ☐	I agree that samples of my blood and urine may be stored for future biochemical tests to help understand the effects of the study treatment and the causes of diabetes and circulatory disease. This is the understanding that the investigations will be for medical research only and my results will be kept confidential.

PRINTED name of participant	Signature	Date (Day / Month / Year)
		/ / 2 0 1

THANK YOU FOR YOUR HELP

To be completed by the practice nurse (in blue or black ink). If possible, please

Current drug list	
Are you currently taking the study white tablets?	Yes ☐ No ☐ Are you currently taking the study brown capsule? Yes ☐ No ☐
Please give date and time last dose of white study tablet was taken	Day / Month / Year 2 0 0 4 1 2 3 0 Time 3 0
Weight:	Kgs or Stones lbs
Has a blood sample been obtained?	Yes ☐ No ☐ Is a urine sample provided? Yes ☐ No ☐

Date and time blood sample was taken:	Day Month Year Time
Name of person taking blood sample	
Signature	
Contact Telephone No.	

Please ensure that the participant has read and signed the consent above and return the completed form with the blood and/or urine sample to the ASCEND coordinating centre in the Freepost envelope provided. ***Please mail it today*** *as delays in the post can affect the measurements. If you require*

«Title» «Forename» «Surname»

«Address1»

«Address2»

«Address3»

«Address4»

Dear «Title» «Surname»,

Optional blood and urine tests for the ASCEND study

Thank you for continuing to take part in ASCEND (A Study of Cardiovascular Events iN Diabetes).

A worthwhile, but optional, part of this research study, involves the analysis of blood and urine samples from study participants. More information about this aspect of the study is included in the enclosed leaflet. Please read the leaflet carefully. If you are prepared to provide a sample of blood and urine for the ASCEND study, please do the following:

1. Read, sign and date the consent form overleaf, remembering to cross (**X**) a "Yes" or "No" box for each statement.
2. Make an appointment for a blood test either at your GP surgery, diabetes clinic or local hospital. (Some GP practices are unable to offer a blood test service. We would advise you to phone to check first.) Ideally, your appointment should be early in the week, i.e. on a Monday, Tuesday or Wednesday. N.B. This does not need to be a fasting blood sample.
3. On the day of this appointment, fill the large clear plastic container (white screw-top) with a specimen of urine.
4. Hand this specimen to the nurse, along with the blood sampling kit, labels, letter with instruction sheet and completed consent form.

If you are willing, we would also like to obtain a measurement of your weight and a list of your current medications. In most cases, this can be done when you go to have your blood sample taken.

The results of the blood and urine tests will be sent to your GP. We will also send you a copy of the signed consent form for you to keep. If you feel unable to provide either one or both samples you are still a valuable member of the study and are strongly encouraged to continue in ASCEND.

Should you have any questions about this, or any other part of the study please call the ASCEND study office (Freefone 0800 585323) and speak to a study nurse or doctor.
Thank you for your help.

Yours sincerely

Professor Jane Armitage Dr Louise Bowman

Study Coordinators

Enc: Sample kit, labels, instructions letter, sampling information leaflet

ASCEND

ASCEND
Clinical Trial Service Unit
(CTSU)
Richard Doll Building
University of Oxford
Old Road Campus
Headington
Oxford
OX3 7LF

Office telephone: 01865 743888
Office fax: 01865 743981
Freefone: 0800 585323
e-mail: ascend@ctsu.ox.ac.uk

[Date]

[Our GP ref]

[GP address]

Dear Dr [GP name],

[Patient name], [DOB], [address]
ASCEND Study Ref: [100-1234]
[Blood] [and] [urine] results from the ASCEND trial

As you know [Mr Patient] [is participating in the ASCEND study. He recently provided [a non-fasting blood sample] [and] [a urine specimen] which was mailed to our central laboratory. Routine analysis of [this sample] [these samples] revealed the following results:

Total cholesterol:	mmol/L
$HbA1_C$ DCCT:	%
$HbA1_C$ IFCC :	mmol/mol
HDL-cholesterol:	mmol/L
Urinary microalbumin/creatinine ratio*:	mg/mmol creatinine

Please feel free to telephone Freefone 0800 585323 if you wish to discuss any aspect of the study. Many thanks for your help.

Yours sincerely

Dr Jane Armitage

Dr Louise Bowman

Study Coordinators

ASCEND (A Study of Cardiovascular Events iN Diabetes):

A randomised 2x2 factorial design study of aspirin versus placebo, and of omega-3 fatty acid supplementation versus placebo, for the primary prevention of cardiovascular events in people with diabetes

Should aspirin be used routinely in people with diabetes but no vascular disease?

The role of antiplatelet therapy (chiefly aspirin) for the *secondary* prevention of cardiovascular disease is firmly established for many high-risk groups with diagnosed occlusive arterial disease, and the proportional reductions in heart attacks and strokes appear to be similar whether or not these patients have diabetes. But, most younger and middle-aged people with diabetes do not have manifest arterial disease – although they are still at significant cardiovascular risk – and yet the available randomised evidence for the use of antiplatelet therapy in such individuals is sparse. As a result, there is major uncertainty about the role of antiplatelet therapy for the *primary* prevention of cardiovascular events among people with diabetes, and only a small minority receives it.

ASCEND aims to demonstrate whether aspirin reduces the risk of cardiovascular events in individuals with diabetes who do not already have diagnosed occlusive arterial disease and whether such benefits outweigh any potential hazards from bleeding. In order to do this reliably, at least 15,000 patients with diabetes and no clinical evidence of occlusive arterial disease will be randomly allocated to receive 100mg aspirin daily or matching placebo tablets for at least 7 years. A study of this size should have excellent power to detect a 12-15% proportional reduction in the cardiovascular event rate among such patients.

Do omega-3 fatty acids (fish oils) reduce cardiovascular risk in people with diabetes?

There is consistent evidence from observational studies of lower rates of cardiovascular disease (particularly cardiac and sudden death) in people with higher intakes, or higher blood levels, of omega-3 fatty acids (FA). Randomised evidence among people who have survived a heart attack suggests modest, but potentially worthwhile, reductions in coronary events of 15-20%. There is, however, no large-scale randomised evidence for the use of omega-3 fatty acids in the primary prevention of vascular events. People with diabetes are at increased cardiovascular risk, and may gain particular benefit from the effects of omega-3 fatty acid supplementation on platelet aggregation and dyslipidaemia. Hence, participants in ASCEND will also be randomly allocated in a 2x2 factorial design to receive 1g omega-3 FA daily or matching placebo capsules for at least 7 years. Such a study

design allows all randomised patients to contribute fully to the assessment of the separate effects of aspirin therapy and of omega-3 fatty acids.

ASCEND: A streamlined, mail-based trial collecting only essential data

The reliable assessment of the important questions that ASCEND is addressing requires the randomisation of a very large number of people with diabetes, and their long-term treatment and follow-up. In order to be able to study 15,000 people with diabetes for at least 7 years at low cost, ASCEND is streamlined and being undertaken predominantly by mail (supplemented by central records). If it can reliably demonstrate that aspirin and/or omega-3 fatty acids safely reduces the risk of cardiovascular events and deaths in patients with diabetes who do not have pre-existing occlusive arterial disease, then this would be relevant to some tens of millions of people world-wide (who are currently not receiving such therapy) and could save tens of thousands of lives each year. Consequently the British Heart Foundation is supporting this large streamlined trial.

CONTENTS

1. BACKGROUND AND RATIONALE

1.1 Reliable assessment of the effects of aspirin for primary prevention of cardiovascular events in diabetes mellitus

1.1.1 Diabetes mellitus: An increasingly common cause of cardiovascular disease

Diabetes mellitus affects about 150 million individuals worldwide, with at least 40 million cases in the Established Market Economies and over one million diagnosed cases in the UK.[1] Moreover, the prevalence is increasing rapidly, and it is estimated that there will be 300 million people worldwide with type 2 diabetes mellitus by 2025, and a further 30 million with type 1 disease.[2] Patients with diabetes of either type are at substantially increased risk of cardiovascular events and death, and the majority (60-70%) of deaths in both types of diabetes are attributed to vascular causes.[3,4] However, among prevalent cases of diabetes in younger and middle age, the majority will not have a history of vascular disease.[5,6]

1.1.2 Lack of reliable evidence for benefit with antiplatelet therapy in patients with diabetes

In the "secondary" prevention of cardiovascular disease, there is reliable randomised evidence that antiplatelet therapy (chiefly aspirin) reduces the risk of further cardiovascular events by about one-quarter among a wide range of different high-risk groups with occlusive arterial disease,[7,8] and the benefits appear to be similar whether or not such patients also had diabetes (Figure 1). As a consequence, most patients with diabetes who have diagnosed vascular disease are currently receiving antiplatelet therapy[9,10] and its use is widely included in guidelines for secondary prevention.[11,12]

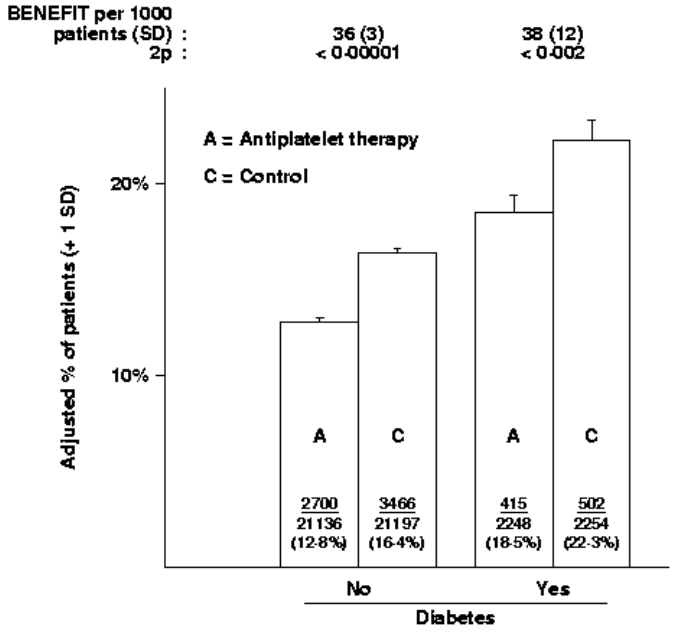

***Figure 1: Absolute effects of antiplatelet therapy on vascular events among patients with occlusive arterial disease in the absence and presence of diabetes*[7]**

However, the majority of people with diabetes do **not** have manifest occlusive arterial disease[5,6] (at least 0.5 million in Britain and several tens of million worldwide), and for them there is no

direct evidence of benefit with aspirin or any other antiplatelet agent. The main randomised evidence currently available on the effects of antiplatelet therapy in such patients with diabetes comes from 9 trials involving a total of about 5000 patients, and a meta-analysis of their results indicates a much smaller proportional reduction in cardiovascular events than has been found in the secondary prevention setting (just 7% compared with about 20-25%: Figure 2).[8] Even in aggregate, however, those studies in people with diabetes involved relatively few events, and the confidence interval for the estimated effect is wide, ranging from a 23% risk reduction to an 8% hazard.

Given the consistency of the beneficial effect in other high-risk settings (including patients with diabetes with arterial disease: Figure 1), it seems likely that the true effect of antiplatelet therapy in people with diabetes alone is similar to the reduction of about one-quarter seen overall in high-risk patients as, for example, has been shown with cholesterol-lowering[13] and anti-hypertensive therapies[14]).

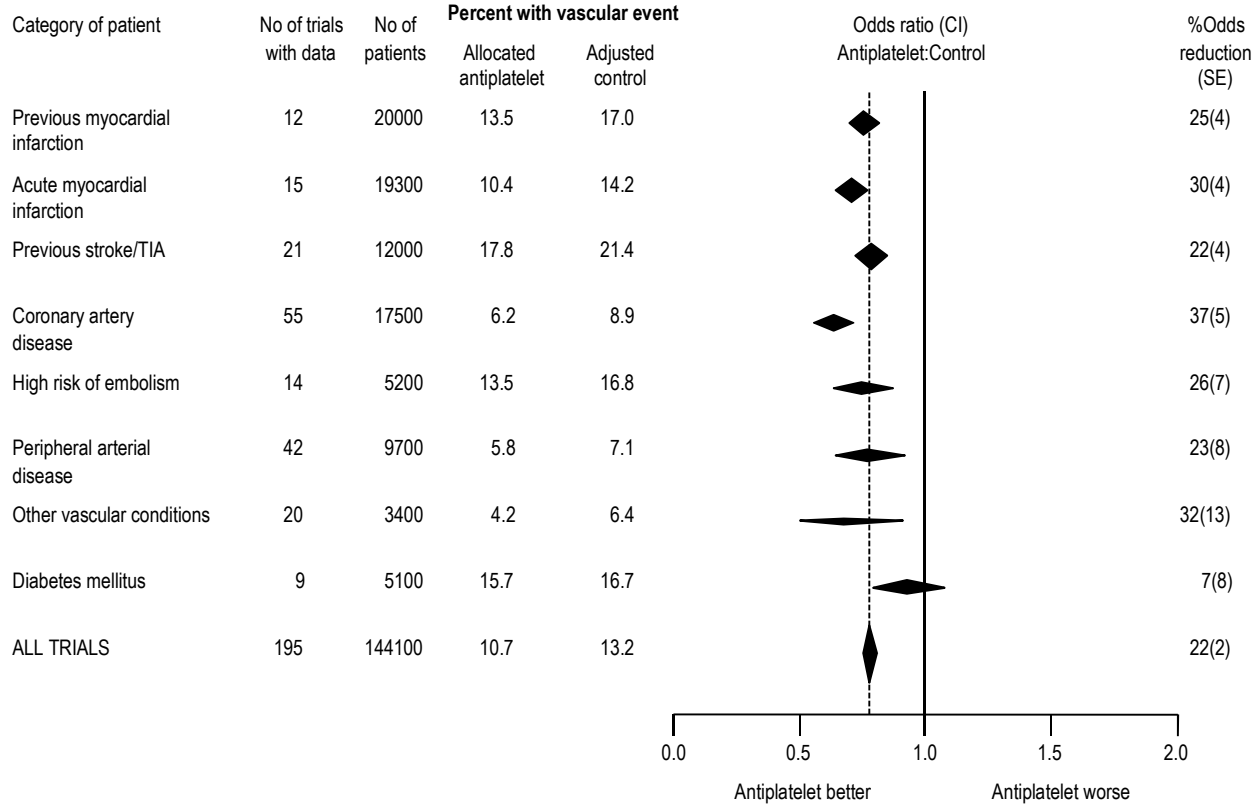

Figure 2: Proportional effects of antiplatelet therapy on vascular events in 195 trials among high-risk patients subdivided by disease category[8]

1.1.3 Aspirin increases the risk of major bleeding (but appears to be relatively safe in diabetes)

In the meta-analysis of previous trials among people with occlusive arterial disease, antiplatelet therapy was found to increase the risk of cerebral haemorrhage by about 25% and the risk of major extracranial bleeds by about 60%, with similar proportional increases in the different types of patient studied.[8] Among such high-risk patients, the absolute reductions in heart attacks and ischaemic strokes with antiplatelet therapy substantially outweighed the relatively small absolute risks of cerebral haemorrhage and major extracranial bleeds. There is also good evidence from the previous trials that antiplatelet therapy is not associated with any special

risks in patients with diabetes. In particular, the Early Treatment Diabetic Retinopathy Study (ETDRS) of 650 mg aspirin daily versus placebo among 3700 people with diabetes indicated that aspirin did not increase the risk of retinal or vitreous haemorrhage.[15] Nevertheless, there is a lack of reliable direct evidence that the balance of benefits and risks of antiplatelet therapy among patients with diabetes alone is favourable.

1.1.4 Large-scale randomised evidence is required to demonstrate directly that the benefits of aspirin outweigh any risks in people with diabetes

The emergence of reliable evidence about the substantial net benefits produced by aspirin in people with occlusive arterial disease has rapidly lead to its widespread use in such patients (with, for example, over 80% of those with a history of previous heart attacks or strokes receiving some form of antiplatelet therapy).[10] Based on extrapolation from the evidence in these other high-risk settings, the American Diabetes Association (ADA) has recommended the use of aspirin in people with type 2 diabetes and at least one additional risk factor (e.g. hypertension or hypercholesterolaemia).[16] By contrast, UK and European guidelines are more circumspect in their recommendations about aspirin use for people with diabetes alone.[11,12] Presumably as a result of the current uncertainties about the net benefit of antiplatelet therapy in this setting, surveys in the US and UK indicate that only about 10-20% of patients with diabetes without diagnosed occlusive arterial disease are taking antiplatelet therapy regularly.[17,18] Similarly, less than 20% of diabetic patients without vascular disease were taking aspirin regularly in the United Kingdom Prospective Diabetes Study (UKPDS) and the MRC/BHF Heart Protection Study[19] (HPS) conducted in Britain, as well as in the ongoing FIELD trial conducted in Australia, New Zealand and continental Europe. Data from the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) also indicate that less than 20% of the hypertensive patients with diabetes and no occlusive vascular disease were taking aspirin in the last 6 months of the study in Sweden, Denmark and Norway (personal communication).

Currently, the only ongoing comparison of antiplatelet therapy versus no antiplatelet therapy in patients with diabetes without pre-existing occlusive arterial disease involves 2000 of the participants in the Women's Health Study (WHS), which is too few to assess the effects of treatment in such individuals reliably (see below). The Prevention of Progression of Asymptomatic Diabetic Arterial Disease (POPADAD)[20] study involves the assessment of aspirin among a further 1600 patients with diabetes, but all of the participants in that trial have diagnosed peripheral arterial disease. Further information about the effects of antiplatelet therapy among diabetic patients without pre-existing arterial disease will emerge from a collaborative meta-analysis of individual participant data from all of the previous "primary" prevention aspirin trials. But, preliminary results among the 3000 low-risk diabetic participants involved in that analysis indicate only a non-significant 25% (SD 16) reduction in coronary events (59 [3.9%] aspirin-allocated versus 71 [4.9%] placebo-allocated events; 2P=0.1) during median follow-up of 5 years (personal communication). Hence, there is a real need to initiate a much larger randomised trial of antiplatelet therapy in people with diabetes without occlusive arterial disease for whom there is not considered to be any clear indication for such treatment.

1.1.5 Aspirin 100mg (enteric coated) daily: an effective and well-tolerated antiplatelet regimen

The Anti-Thrombotic Trialists' (ATT) collaborative meta-analysis of previous trials found that high doses of 500-1500mg aspirin daily (which are more gastrotoxic[21]) are no more effective than lower doses of 75-100mg/day either in direct comparisons or in indirect comparisons (Figure 3).[7] As a consequence, daily doses of 75-150mg are generally preferred for long-term treatment as protection against serious vascular events in high-risk patients. The use of enteric-

coating delays the dissolution of the contents of the tablet until the higher pH of the duodenum is reached, and so may reduce gastric injury and symptoms.[22] Hence, a regimen of 100mg daily enteric-coated aspirin is to be used in this study.

Aspirin dose mg/day	No of trials with data	No of patients	Percent with vascular event: Allocated aspirin	Percent with vascular event: Adjusted control	%Odds reduction (SE)
500-1500	34	22500	14.5	17.2	19(3)
160-325	19	26500	11.5	14.8	26(3)
75-150	12	6800	10.9	15.2	32(6)
<75	3	3700	17.3	19.4	13(8)
Any aspirin	65	59400	12.9	16.0	23(2)

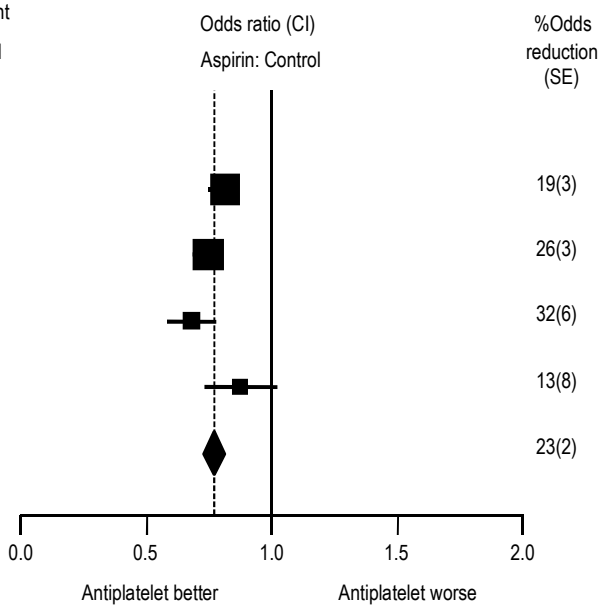

Figure 3: Proportional effects of different doses of aspirin on vascular events in

1.2 Reliable assessment of the effects of dietary supplementation with omega-3 fatty acids

1.2.1 Higher intake of omega-3 fatty acids is associated with less coronary heart disease

Omega-3 fatty acids are long-chained polyunsaturated fatty acids (PUFA) with their first double-bond found at the third carbon atom from the methyl group (which is why they are referred to as n-3 or omega-3 fatty acids). Man is unable to manufacture these omega-3 fatty acids (FA) and is reliant upon intake from plants and animals. The richest dietary sources of the two principal omega-3 fatty acids, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), are marine animals.[23] Consumption of oily fish 2-3 times per week provides about 500mg daily of EPA and DHA combined, but consumption is less than about 50mg per day in people who do not eat fish regularly.[24]

The possible link between high intake of omega-3 FA and prevention of coronary heart disease was first noted in the 1940s when the diets of Greenland Eskimos, among whom coronary disease was rare, were compared with those of Danes living in Denmark where coronary heart disease (CHD) rates were about 10 times higher.[25] Despite similar total fat intake (about 40% of total calories), eskimo diets contained significantly greater proportions of omega-3 FA (>4%) compared with the Danes (<0.1%). These observations stimulated a large number of observational studies of omega-3 FA intake and heart disease risk in different populations. A 1999 systematic review of all of the observational data concluded that in high-risk populations consumption of the equivalent of 40-60 grams of fish per day (providing about 0.2-1g daily of omega-3 FA depending on the type of fish) is associated with 40-60% lower rate of cardiac death.[26] More recently, other observational studies have found similar protective associations of

fish consumption and incidence of CHD,[27-29] (including among 5000 women with diabetes followed for about 9 years[30]) and stroke.[24]

1.2.2 Randomised trials of omega-3 FA supplementation in post-MI patients suggest 15-20% reductions in cardiovascular events but there is no information in diabetes

In the only large randomised trial of omega-3 FA supplementation that has been conducted to date, 11,000 heart attack survivors in Italy were allocated to receive 1g daily of n-3 PUFA (containing 0.4g of EPA and 0.3g of DHA) versus no PUFA treatment for 3.5 years.[31] Marginally significant reductions of 13% (95% CI 1-24%, p=0.04) in coronary events (i.e. non-fatal myocardial infarction [MI] or coronary death) and of 17% (95% CI 3-29%, p=0.02) in cardiovascular deaths, were observed among those allocated PUFA capsules in this GISSI-Prevenzione trial. This was despite 80-90% of patients in both groups eating fish at least once a week, and high use of cardioprotective drugs (including aspirin). In another randomised trial, 2000 men with a history of myocardial infarction in Wales were allocated to a recommended intake of at least 2 portions of fatty fish per week (or 1.5g Maxepa capsules daily, which contain about 0.5g EPA) versus no change in fish intake for 2 years.[32] There was a non-significant trend towards 17% fewer (95% CI 35% reduction to 8% excess) coronary events among patients allocated increased fish intake, and cardiac deaths were by 35% (95% CI 13-52%, p=0.004). Background intake of fish in that Welsh population was low, and only about 10% of the patients were taking aspirin. In a meta-analysis of all of the available unconfounded randomised evidence for increased omega-3 FA intake from these two trials and 9 much smaller trials[33,34] (which tested doses of EPA and DHA in the range 1-6g per day among a total of about 2000 patients), there was a highly significant reduction in coronary events of 18% (95% CI 8-27%, p=0.0008). Based on these studies – which were conducted chiefly among people with vascular disease – it would seem plausible that omega-3 FA supplementation might produce a 15-20% reduction in coronary and other occlusive vascular events among high or intermediate risk populations, including people with diabetes.

1.2.3 Cardioprotective effects of omega-3 fatty acids may be additional to those of aspirin

Aspirin irreversibly inhibits platelet cyclo-oxygenase, the enzyme that controls the conversion of arachidonic acid to prostaglandins and thromboxanes, which reduces the formation of thromboxane A2 in platelets and produces a potent anti-aggregatory effect.[21] But, aspirin also reduces the formation of prostacyclin, which is a potent vasodilator, and so may lead to vasoconstriction. Omega-3 FA (particularly EPA) compete with arachidonic acid for cyclo-oxygenase,[23] and so reduce thromboxane A2 production in platelets (albeit to a lesser extent than aspirin). Unlike aspirin, however, omega-3 FA enhance prostacyclin production in endothelial cells. Moreover, when aspirin and omega-3 FA are given together, there is a shift towards increased prostacyclin formation in endothelial cells and vasodilatation.[35] Consequently, any beneficial effects of aspirin and omega-3 FA on vascular disease that are mediated through these effects on prostaglandins and thromboxanes should be complementary.[35] Omega-3 FA might also have other cardioprotective effects, including: reducing myocardial susceptibility to ventricular arrhythmias;[36] increasing the stability of atherosclerotic plaques through anti-inflammatory effects that are mediated by prostaglandins and leukotrienes;[37] reducing blood pressure;[38] and reducing plasma concentrations of triglycerides (TG) and very-low-density lipoproteins, and inhibiting post-prandial lipaemia.[39-41] These effects of omega-3 FA on lipoproteins are seen both in the presence, and in the absence, of statin therapy.[34] As cardiovascular disease in diabetes derives both from platelet activation[42] and from disordered triglyceride metabolism,[3] omega-3 FA may be particularly worthwhile for people with diabetes.

1.2.4 Omega-3 fatty acids are considered safe and well tolerated

The Food and Drug Administration (FDA) consider omega-3 FA doses of up to at least 3g daily to be safe,[23] with no significant risk of bleeding. In the large GISSI Prevenzione trial,[31] 90% of participants were taking aspirin, but no excess of bleeding was observed with the addition of 1g omega-3 FA daily. The only side-effects reported in that open-label study were a slight fishy after-taste and some gastrointestinal disturbances, but only 3.8% of participants stopped their omega-3 FA supplements because of these side effects. Omega-3 FA have no effect on glycaemic control in diabetes[40,43] and their small, potentially adverse, effects on plasma concentrations of LDL-cholesterol may be offset by beneficial changes in lipoprotein particle size.[39,41] For the present trial, a daily dose of approximately 1g of omega-3 FA (0.4g EPA and 0.3g DHA is to be used (as in GISSI), which can be conveniently provided in 1 capsule of the concentrated preparation (with matching placebo capsules containing olive oil).

1.2.5 Need for a large-scale study of omega-3 FA supplementation in people with diabetes

As discussed above, diabetes is associated with a 2-4 fold increase in the risk of cardiovascular disease and the incidence of diabetes worldwide is increasing rapidly. Consequently, the demonstration that an inexpensive and readily available food supplement – such as omega-3 FA – reduces cardiovascular risk in patients with diabetes would have important public health consequences. By adopting a 2x2 factorial design within this large streamlined study, it will be possible to assess the separate and combined effects of both aspirin and omega-3 FA supplementation in a particularly cost-effective manner.

1.3 Mail-based studies for efficiency and cost-effectiveness

1.3.1 Previous successful experience of conducting cost-effective randomised trials by mail

Both aspirin and omega-3 FA are widely available and used, the hazards are low and well characterised, and neither requires biochemical monitoring. Several large randomised trials have been conducted using mailed drug supply and follow-up, including the CTSU-coordinated British Doctors' Study[44] and the (first) US Physicians Health Study[45] of aspirin for the prevention of myocardial infarction. Currently, there are 3 large studies[46-48] of either aspirin or various supplements being conducted entirely by mail in the US: the (second) US Physicians' Health Study II, the Women's Antioxidant Study (WACS) and the Women's Health Study (WHS). The latter study includes 40,000 American women from a wide range of educational and social backgrounds randomised to aspirin or matching placebo, and in a factorial design to different vitamin and mineral combinations. Experience from these studies shows that - with appropriate attention to the wording of information leaflets, consent forms and questionnaires, - good response rates and compliance can be achieved and reliable information about medical events gathered.[49] In addition, the 24-hour Freefone service established by CTSU for other large heart disease trials will allow study participants to discuss any aspects of the study with experienced clinical staff, and so help ensure good compliance and the early identification of serious problems.

2. PLAN OF INVESTIGATION

2.1 Study aims: assessment of outcomes

The aim of ASCEND is to determine whether 100mg daily aspirin and/or supplementation with 1 gram capsules containing 90% omega-3 fatty acids (0.41g EPA, 0.34g DHA) daily prevents cardiovascular events in patients with diabetes who do not already have clinically manifest arterial disease (without leading to significant bleeding or other adverse events).

2.1.1 Primary assessments

Aspirin therapy: The primary comparison will involve "logrank" analyses[54] of "serious vascular events" (defined as the combination of non-fatal myocardial infarction, non-fatal stroke or transient ischaemic attack, or vascular death excluding confirmed cerebral haemorrhage during the scheduled treatment period among all those allocated aspirin tablets versus all those allocated placebo tablets (i.e. "intention-to-treat" comparisons). (Vascular death includes ICD I00-I52 and I63-99 in the 10th International Classification of Diseases.)

Omega-3 fatty acid supplementation: The primary comparison will involve "logrank" analyses of "serious vascular events" during the scheduled treatment period among all those allocated omega-3 fatty acid capsules versus all those allocated placebo capsules.

2.1.2 Secondary assessments

The principal subsidiary comparisons will be of the effect of allocation to aspirin versus allocation to placebo tablets and, separately, of allocation to omega-3 FA versus allocation to placebo capsules on:

(i) The incidence of serious vascular events, and the combined endpoint of "serious vascular events or revascularisations" (i.e. serious vascular event, or coronary or non-coronary revascularisation) in various prognostic subgroups (e.g. older versus younger, men versus women, longer versus shorter duration diabetes); and in the presence and absence of the other study treatments

(ii) The incidence of confirmed cerebral haemorrhage and, separately of other "major haemorrhage" (defined as any other bleeding episode that requires hospitalisation or transfusion, or is fatal or disabling).

2.1.3 Tertiary assessments

In addition, comparisons will be made of the effects during the scheduled treatment period of each of the study treatment allocations on: total and cause-specific mortality (coronary, other vascular and non-vascular death separately); total coronary events (ie. non-fatal myocardial infarction, coronary death or coronary revascularisations [i.e. CABG and PTCA]); non-haemorrhagic strokes or transient ischaemic attacks; venous thromboembolism; total and site-specific cancers; and hospitalisations for various other causes. Allowance for multiple hypothesis testing in these analyses will be made using the "Bonferroni" correction.

2.2 Sample size and predicted number of events

- **2.2.1 Random allocation of at least 15,000 patients with diabetes without arterial disease should provide good statistical power to detect plausible effects**

One particular cohort of people with diabetes and no evident cardiovascular disease had a coronary event rate of around 3% per annum.[55] However, although that study is widely quoted, event rates may not be as high in diabetic populations with lower levels of other risk factors. For example, among the 5000 men and women with newly diagnosed type 2 diabetes (mean age 53 years) randomised in the UKPDS,[56] annual rates were 1.6% for coronary events and 1.1% for death due to macrovascular disease (i.e. fatal MI, stroke or sudden death). Similarly, among about 3000 people with diabetes (mean age 63) without diagnosed occlusive arterial disease

randomised in HPS,[13,57] the annual overall rate of cardiovascular events (fatal or non-fatal myocardial infarction or stroke) was 2.2%. Hence, it would seem prudent to base sample size calculations for any randomised trials in patients with diabetes and no arterial disease on serious vascular event rates of no more than about 2% per annum.

Aspirin has been shown to reduce cardiovascular event rates by about one quarter in a wide range of high-risk groups with arterial disease, with similar proportional reductions irrespective of whether or not diabetes is present (see Figure 1).[7] Hence, as in other high-risk populations, it seems plausible that aspirin might reduce the risk of serious vascular events by around one-quarter in patients with diabetes who do not have clinical evidence of arterial disease. Similarly omega-3 FA have reduced risk of cardiovascular events by 15-20% in high-risk populations. Proportional reductions of 15-20% among people with diabetes without diagnosed arterial disease would still correspond to substantial absolute benefits (see Table 1). But, even if such benefits do exist, at least 10,000 patients with diabetes would need to be randomised and followed for 5 years to detect these effects reliably. During the trial it is intended that blinded event rates (i.e. active and placebo groups combined) will be monitored and, if they are substantially lower than anticipated, the Steering Committee will have the option of increasing the sample size or prolonging the scheduled treatment period (see below)

Table 1: Statistical power to detect 15-20% proportional reductions in serious vascular events among 10,000 randomised patients (based on 10% 5 year control group event rate)

Proportional reduction	Control group 5000	Active group 5000	Power at 2P<0.01	Power at 2P<0.05	Events avoided/ 1000 over 5 years
25%	500	375	>95%	>95%	25
20%	500	400	80%	>90%	20
15%	500	425	60%	70%	15

Protocol addition January 2011: Accumulating evidence from within ASCEND, suggests that the overall (i.e. blinded) annual event rate (including transient ischaemic attacks) is likely to be about 1.2%, i.e. somewhat lower than the initial estimate of 2% pa in the control group. In addition, a recent meta-analysis of primary prevention trials of aspirin suggests that reductions in serious vascular events of 12-15% may be more likely than reductions of 20-25%.[58] Table 2 indicates that, with an annual event rate of 1.2% in the control group, randomisation of 15,000 patients with follow-up of 7.5 years would provide robust statistical power to detect plausible risk reductions of 12-15%. Hence, the Steering Committee agreed during 2010 to increase the sample size to 15,000 and the duration of follow-up to at least 7 years.

Table 2: Statistical power to detect plausible effect sizes with 15,000 participants followed for up to 7.5 years at 1.2% per annum serious vascular event rate in the control group.

15,000 patients randomised

Proportional reduction	Median duration of	Number of events (%)		Power		
		Control (7500)	Active (7500)	2p<0.05	2p<0.01	Events avoided/1000

	follow-up (years)					over 7.5 years
10%	5	450 (6%)	405 (5.4%)	60%	36%	
	7.5	675 (9%)	608 (8.1%)	79%	58%	9
12%	5	450 (6%)	398 (5.3%)	76%	54%	
	7.5	675 (9%)	593 (7.9%)	92%	78%	11
15%	5	450 (6%)	383 (5.1%)	92	79%	
	7.5	675 (9%)	570 (7.6%)	99	95%	14

2.2.2 Full efficiency of a 2 x 2 factorial design: separate assessment of both study questions without any material effect on non-drug cost or sample size requirements.

A factorial design will be used, with at least 7500 patients being randomly allocated to receive aspirin tablets versus 7500 patients allocated to receive matching placebo tablets (see Figure 4). Similarly, at least 7500 patients will be separately randomised to receive omega-3 FA capsules versus 7500 patients allocated to receive placebo capsules. The primary analyses will involve two-way comparisons of all those allocated aspirin versus all those allocated matching placebo tablets, irrespective of the omega-3 FA allocation (Figure 4: subtotal A versus subtotal B), and of all those allocated omega-3 FA versus all those allocated matching placebo capsules irrespective of the aspirin allocation (i.e. subtotal 1 versus subtotal 2). Hence, reliable assessment of the effects of aspirin will not interfere with reliable assessment of omega-3 FA (or vice versa), as outcomes among all those allocated active aspirin can still be compared with those among all those allocated placebo aspirin (even though half of each group will have received omega-3 FA). Use of such a factorial design instead of a simple 2-way design has little or no effect on the statistical sensitivity with which the overall effects can be assessed, or on the total number of patients required in the study.[53]

Figure 4: Factorial design of trial

	Aspirin Tablets	**Placebo Tablets**	
Omega-3 FA capsules	3750 Aspirin + Omega-3 FA	3750 Omega-3 FA	Subtotal 1: 7500 **Omega-3 FA**
Placebo capsules	3750 Aspirin	3750 Neither	Subtotal 2: 7500 **Placebo**
	Subtotal A: 7500 **Aspirin**	Subtotal B: 7500 **Placebo**	

2.3 Data and safety monitoring

2.3.1 Interim analyses: role of the Data Monitoring Committee and Steering Committee

During the study, the independent Data Monitoring Committee will review unblinded interim analyses, at least annually, of mortality, of cardiovascular events and of other serious adverse events, along with any other analyses requested. In the light of these analyses and the results of any other relevant trials or meta-analyses of trials, the Data Monitoring Committee will advise the Steering Committee if, in their view, the randomised comparisons in the study have provided **both** (a) "proof beyond reasonable doubt"* that for all patients, or for some specific types, aspirin therapy is clearly indicated or clearly contraindicated in terms of a net difference in mortality, **and** (b) evidence that might reasonably be expected to influence materially the patient management of many clinicians who are already aware of any other main trial results. The Steering Committee can then decide whether to end or modify the study (or to seek extra data). Unless this happens, the Steering Committee, the collaborators and the coordinating centre staff (except those who supply the confidential analyses) will remain ignorant of the interim results on mortality and morbidity until the study is terminated. Collaborators, and all others associated with the study, may write (preferably through the Oxford coordinating centre) to the chairman of the Data Monitoring Committee, drawing attention to any worries they may have about the possibility of particular side-effects, or about particular categories of patient requiring special consideration, or about any other matters that may be relevant.

2.3.2 Monitoring of any serious adverse events believed to be due to the study treatment

Throughout the trial, all serious adverse events believed with a reasonable probability to be due to study treatment are to be reported immediately by telephoning the 24-hour telephone service (see Section 3.6). A “serious” adverse event is defined as any untoward medical occurrence which results in death, is life-threatening, requires hospitalisation or the prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, or is a congenital anomaly or birth defect. During this telephone call, standard information (i.e. identity of the patient and of the person reporting the event, nature and date of event, and reasons for attribution to study treatment) will be recorded directly on the coordinating centre computer. These reports will be reviewed immediately, blind to treatment allocation, by the clinical coordinators, and any further information required sought urgently. Confirmed reports will then be promptly forwarded “unblinded” to the chairman of the Data Monitoring Committee. and to Bayer Healthcare AG or to Abbott Products Operations AG, as appropriate and included in the Annual Safety Report sent to the Research Ethics Committee (REC). Any such serious adverse events that are also unexpected will be reported in an expedited fashion to the REC and relevant drug regulatory agencies.

2.4 Central Coordination

2.4.1 Central coordination and local collaboration

The Clinical Trial Service Unit (CTSU) at Oxford University is coordinating this study and will have overall responsibility for the administration and coordination of the study. There will be a

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* Appropriate criteria of proof beyond reasonable doubt cannot be specified precisely, but in general a difference of **at least** 3 standard deviations in an interim analysis of a major endpoint would be needed to justify halting, or modifying, such a study prematurely, especially if the comparison was based on relatively few events (e.g. less than 100). If this criterion were to be adopted, it would have the practical advantage that the exact number of interim analyses would be of little importance, and so no fixed schedule is proposed [53].

Steering Committee to oversee the trial conduct (back page). CTSU is responsible for obtaining Multicentre Research Ethics Committee approval; for the training and monitoring of all staff directly involved in the study; for the supply of conveniently packaged study drugs and other study materials; for the identification, with the assistance of the local medical collaborators, of potentially eligible participants; for obtaining any relevant permissions to invite suitable patients to participate; for the initial invitation of participants and subsequent randomisation and follow-up by mail; for the provision of a 24-hour Freefone telephone service (for queries from participants or medical staff, for unblinding when medically necessary, and reporting of any serious adverse events believed to be due to study treatment); and for the collection and analysis of data, and blood samples. The medical collaborators around the UK are responsible, with the help of the Oxford coordinating centre, for obtaining local ethics committee approval and for assisting in the identification of potentially eligible individuals with diabetes (including liaison with hospital medical records staff). (This is summarised below, and is described in detail in the coordinating centre standard operating procedures [SOP].)

2.4.2 Training and monitoring

The administrative and nursing staff in the Oxford coordinating centre will be trained in correct study procedures (as summarised in Section 3 of the protocol and described in detail in the SOPs). The coordinating centre staff will also arrange regular meetings of all the collaborators to discuss the progress of the study and other general issues, and to provide an update on the results of any other relevant studies. Collaborators will be encouraged to contact the coordinating centre office (or 24-hour telephone service for urgent queries) if they wish to discuss some problem or other issue related to the study.

2.4.3 Supply of study materials

Aspirin and matching placebo tablets are to be manufactured and provided by Bayer Healthcare AG. Omega-3 FA capsules and matching placebo capsules are to be provided by Abbott Products Operations AG (formerly Solvay Pharmaceuticals). Both treatments are to be delivered in bulk to Brecon Pharmaceuticals for packaging and labeling prior to dispatch to participants. All study medication will be supplied in convenient treatment packs appropriate for mailing which contain the appropriate number of blister-strips for each period of the study. An inventory of study drug supply will be maintained on the coordinating centre computer, and any study drug not required by participants is to be returned to the coordinating centre for disposal.

2.4.4 Data handling

Lists of potentially eligible people with diabetes will be sought, preferably in computerized format, by the Oxford coordinating centre from medical collaborators who have access to diabetes registers, from trial databases and from general practitioners (GPs). This information will be used by the coordinating centre to generate invitations, in the name of the local medical collaborator, for patients to join the study (see Section 3.3). Hospital collaborators, general practitioners or practice nurses will also be able to offer a standard “invitation pack” (containing patient information leaflet, screening questionnaire and freepost envelope) to potentially eligible participants when they are seen for routine care in their clinic. In addition, randomised participants will have the option to recommend any friend or relative they think may be eligible and interested in participating in the study and potential participants can volunteer themselves if they hear about the study from any source. Responses from participants will be collected on questionnaires which are to be returned to the coordinating centre either on paper or electronically. Any coding that is required will be undertaken and data will then be entered into the coordinating centre computer (following an operating procedure for data handling). Any failure by participants to return Follow-up questionnaires will result in two mailed reminders

being sent. Subsequently, when necessary, study coordinating staff will undertake a telephone follow-up.

Errors or omissions in the completion of study forms will result, if appropriate, in computer-generated correction requests being sent to participants for completion. All such corrections to the data will be entered on the central computer with an appropriate audit trail. The coordinating centre is also responsible for seeking confirmation and additional information about any relevant clinical events reported during follow-up, and for obtaining details from national registries of any deaths, non-fatal cancers or other relevant events available among study participants (see Section 3.7).

2.4.5 Laboratory measurements and sample storage

Blood and urine samples taken at GP practices from those patients who agree to start Run-in treatment (Section 3.3.2) and samples taken from a randomly selected group of patients during follow up (Section 3.3.1) will be mailed to the coordinating centre laboratory in Oxford. The central laboratory will use part of each blood sample for immediate assays, with the remainder being frozen for subsequent assays. The laboratory uses a number of internal and external quality control procedures and follows a standard operating procedure (in accordance with Good Laboratory Practice guidelines). Checked assay results will be transferred to the central computer and linked to the patients' other data.

2.4.6 Source documents and archiving

The lists of potentially eligible patients provided to the Oxford coordinating centre by collaborators, the returned questionnaires from these patients, the additional information obtained on reported outcome measures and other relevant events, the death certificates, the blood assay results and the drug supply records constitute the "source documents" for the study. The coordinating centre will retain these data and records for at least 15 years. Regulatory agencies and the companies providing the study medications will have the right, in accordance with Good Clinical Practice guidelines, to commission a confidential audit of such records kept in the coordinating centre, as long as this does not result in unblinding of the interim results while the study is still in progress.

2.4.7 Source of support and non-negligent liability cover

Funding has been obtained from the British Heart Foundation to cover the administrative and coordination costs of the trial. A supply of aspirin and matching placebo is to be provided by Bayer Healthcare AG, and a supply of omega-3 FA and matching placebo capsules by Abbott Products Operations AG (formerly Solvay Pharmaceuticals), with some funding from each company to cover drug packaging.

The trial is to be conducted, analysed and interpreted by CTSU entirely independently of the funding sources, which have no representation in its organisation and will, like the Steering Committee, remain blind to the main results as they accumulate. This arrangement is intended to ensure that no suggestions of lack of objectivity of the findings can be justified.

2.4.8 Publication in the names of all the collaborators

The success of this study depends on the wholehearted collaboration of a large number of doctors, nurses and patients. For this reason, chief credit for the main results will be given not to the central organizers, but to all those who have collaborated in the study. Draft copies of any manuscripts will be provided to all collaborators for review prior to their publication and will be published in the name of the collaboration.

3. SUMMARY OF PRACTICAL PROCEDURES

POTENTIALLY ELIGIBLE

- Diabetes mellitus (type 1 or 2)
- Male or female
- No diagnosed occlusive arterial disease
- Aged ≥ 40 years

IDENTIFICATION AND INVITATION

- Potentially eligible patients identified from existing diabetes registers or databases and other sources
- Invited by GP, diabetologist or study coordinators, either in person or by mail. Invitation includes Information Leaflet, Consent Form and brief Screening Questionnaire
- Central Freefone number for any questions

SCREENING PROCESS (-2 months)

- Screening Questionnaire returned, which identifies eligible and consenting patients
- Run-in pack with 2-month supply of placebo treatment mailed to patient
- GP informed of patient's possible participation, and asked to return form if patient **not** to be randomised
- Blood and urine samples (optional) collected locally and mailed to central laboratory
- Freefone number (0800 585323) for medical advice and any questions

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RANDOMISATION (0 months)

- Randomisation Questionnaire sent to re-confirm eligibility, and to characterize the patient more fully
- Randomisation Questionnaire returned, and eligible patient randomised by central computer
- Allocated treatment pack mailed to patient: 100 mg aspirin daily or matching placebo tablet, and 1g omega-3 FA daily or matching placebo capsule
- GP informed of patient's randomisation

FOLLOW-UP QUESTIONNAIRES (6-monthly)

- Follow-up Questionnaires and treatment packs sent 6-monthly
- Freefone number (0800 585323) for medical advice and any questions
- Further details sought from responsible clinicians about any relevant events reported on Follow-up questionnaires

- Flagging for mortality and cancer at central registries

3.1 Eligibility for ASCEND

Men or women aged at least 40 years at the time of invitation for Screening are eligible for the study, provided they fulfil **all** of the following criteria:

- **Clinical diagnosis of diabetes mellitus:** The participant's own doctor considers them to have type 1 or type 2 diabetes (based on standard WHO or ADA diagnostic criteria[50,51]);

- **No clear indication for aspirin:** The participant has no diagnosed occlusive arterial disease (i.e. a history of myocardial infarction, angina pectoris, coronary or non-coronary revascularisation procedure [i.e. peripheral arterial bypass surgery or angioplasty], stroke or transient ischaemic attack);

- **No clear contra-indication to aspirin:** The participant is not at high risk of bleeding due to: gastrointestinal haemorrhage or peptic ulcer within the previous 6 months; active hepatic disease such as cirrhosis or active hepatitis; use of warfarin, or other anti-coagulant therapy; or has a history of aspirin allergy;

- **Substantial uncertainty about whether antiplatelet or omega-3 FA therapy confers worthwhile benefit:** Neither the participant nor the participant's own doctor considers there to be a definite need for the patient to take aspirin or omega-3 FA supplements regularly (or a definite need not to do so);

- **No other predominant life-threatening medical problem:** The participant does not have some condition (other than diabetes) that might limit compliance with 5 years of study treatment, such as cancer (other than non-melanoma skin cancer).

3.2 Identification of participants

3.2.1 Large numbers of potentially eligible patients can be identified through diabetes registers, trial databases and general practice

Based on our previous experience, large numbers of potentially suitable individuals may need to be approached to randomise at least 15,000 eligible patients into this long-term trial. People with diabetes will be sought from 3 main sources: diabetes registers, trial databases and general practice. Diabetologists from around the UK will be invited to collaborate and allow invitation of potentially suitable individuals from locally held diabetes registers (such as those held for retinopathy screening or for service provision). Such registers vary in size from a few thousand to many thousands and at least one third of participants are expected to be recruited from these sources. Other people with diabetes will be identified from among the populations taking part in HPS and other diabetes trials. In order to streamline the invitation process, the contact details of potentially eligible people will be sought electronically whenever possible to allow central mailings in the name of the local doctor. This approach has allowed large numbers to be recruited by CTSU into the HPS and SEARCH trials, and is more efficient and cost-effective than mailings sent from individual centres or practices. It also allows over-selection of certain groups (e.g. older individuals) to ensure an appropriate balance of different types of participant. The third source will be directly from general practice. Diabetologists and other collaborators will be asked to identify 20-30 local general practices with computerized diabetes registers, and to seek their agreement to mailing a single batch of letters to potentially eligible individuals. Experience of screening notes in general practice indicates that ~3.5% of patients

aged 50-65 have diabetes without diagnosed arterial disease. Hence, a typical group practice of about 10,000 registered patients may have 100-150 potentially eligible individuals. To complement these 3 main methods of recruitment, hospital collaborators, general practitioners or practice nurses will also be able to offer a standard "invitation pack" (containing patient information leaflet, screening questionnaire and freepost envelope) to potentially eligible participants when they are seen for routine care in their clinic, or directly by mail if they have previously agreed to be approached for research. In addition, randomised participants will have the option to recommend any friend or relative they think may be eligible and interested in participating in the study and potential participants may volunteer themselves if they hear about the study from any source.

3.3 Screening (- 2 months)

3.3.1 Establishing eligibility

Patients with diabetes that are identified from any source as being possibly suitable will be invited by letter to take part. An invitation letter will be sent enclosing an information leaflet (Appendix 1) and a brief one-page Screening questionnaire to determine eligibility and to seek consent (Appendix 2), along with a Freepost envelope. Preliminary eligibility for the pre-randomisation Run-in phase will be based on information provided on the completed Screening questionnaire (i.e. diagnosis of diabetes, no history of diagnosed occlusive arterial disease, no contraindication to regular aspirin and signed consent to participate).

3.3.2 Pre-randomisation Run-in treatment and optional blood and urine sampling

Eligible patients will be sent a Run-in pack of medication (containing placebo tablets and placebo capsules) and asked to take one tablet and one capsule daily for 2 months. An information sheet about the medication will be provided and a copy of their signed agreement to participate will also be sent to them. About 2-4 weeks later, participants will be sent an optional blood and urine sampling kit, and asked to take this kit to their general practice for sample collection (and for measurement of blood pressure, height and weight), with this sample then mailed to the central laboratory in the containers provided. A supplementary information leaflet is to be provided and separate consent sought for this 5-10ml blood and urine collection which will allow baseline stratification by important biochemical prognostic variables (such as blood HbA_1C, lipids and markers of renal function, and urinary albumin/creatinine ratio).

During the Run-in period, the participant's general practitioner will be informed by letter of their patient's possible involvement in the study and asked to return a form if they consider there to be any reason **not** to randomise their patient (in which case the patient would be informed of their GP's decision and withdrawn before randomisation). Patients are to be randomised only if, at the end of the Run-in period, they seem likely to comply with the study protocol for several more years. By this process, many potential drop-outs should be excluded before becoming part of the randomised comparison, with a consequent improvement in statistical sensitivity of the "intention-to-treat" analyses.[52] Patients who are not eligible will be thanked for completing the questionnaire, but will not proceed further.

3.4 Randomisation (0 months)

3.4.1 Final check of eligibility and compliance before randomisation

About 2 months after they have been sent their Run-in pack, participants will be sent a further more detailed Randomisation questionnaire asking about any significant problems (including

any cardiovascular events) and their compliance with the study treatments during the Run-in period. Details of their diabetes history (in particular to allow classification as type 1 or 2), current medication, ethnic group, and smoking history will be sought to allow baseline risk stratification.[59] Participants will be asked to reiterate their commitment to a 7-year study and also, if willing, to provide details of a friend or relative living at a different address who may be contacted in the event of loss of contact with the participant.

3.4.2 Random allocation of aspirin 100mg daily versus placebo, and of 1g daily capsules containing omega-3 fatty acids versus placebo

Participants who indicate on the randomisation questionnaire that they remain eligible and willing to continue into the long-term part if the study will be randomised by the central computer in CTSU, using a minimisation algorithm to ensure balance by important baseline variables.[60] Eligible patients will be randomised in a 2 x 2 factorial blinded design between:

- Aspirin 100mg daily versus matching placebo
- Omega-3 fatty acid capsules 1 daily versus placebo

One aspirin tablet and one capsule are to be taken each day for about 7 years unless some clear reason to stop develops.

They will then be mailed a pack containing a 24-week supply of their allocated study treatment, along with relevant information about the medication and the CTSU Freefone number for any trial-related queries. The patient's general practitioner will be informed by letter of their patient's randomisation into the trial and the results of any relevant blood tests taken during Run-in (e.g. lipid profile and HbA_1C).

3.5 Post-randomisation Follow-up

- **3.5.1 6-Monthly follow-up questionnaires sent by mail (with telephone back-up)**

Follow-up questionnaires asking about cardiovascular events, other serious adverse events (including bleeding episodes), compliance with study treatment and use of relevant non-study treatments will be sent 6-monthly with a further supply of the participant's allocated study treatment. All randomised patients - irrespective of whether or not they continue to take study treatments - are to be encouraged to return their questionnaire with up to 2 mailed reminders sent routinely. Failure to return a questionnaire will result in a study administrator telephoning the patient in order to complete the Follow-up questionnaire. Those who do not agree to being contacted in this way will be followed via their GPs and central registries.

3.5.2 Modifying study treatment

The aspirin component of the study treatment will be discontinued if a patient starts to use regular non-study aspirin or warfarin or is considered to have developed some other clear contraindication to the study aspirin (e.g. high risk of bleeding or aspirin allergy).
(N.B. Patients who stop the aspirin component of the study will be encouraged to continue the omega-3 FA component, unless this is thought to be clearly contraindicated.)

The study treatments will also be stopped if a serious adverse experience believed with a reasonable probability to be due to study treatment is reported (see Section 3.6). Patients may also stop either study treatment at their own request, or at the request of their own doctors. But, any patient who stops the study medications would still be encouraged to continue returning their Follow-up questionnaires and, if appropriate, to continue taking either study treatment alone if the other is to be stopped.

- **3.5.3 Follow-up of deaths and of non-fatal cancers through central registries**

All randomised patients will be flagged through the Office for National Statistics and other central registries for death, cancer and other relevant events. Consequently, unbiased cause-specific mortality and site-specific cancer incidence data for all patients can be obtained, independent of whether they are still complying with study medication or responding to questionnaires.

3.6 Reporting serious adverse events

3.6.1 Immediate reporting of expected and unexpected serious adverse events believed with a reasonable probability to be due to study treatment

To fulfil regulatory authority requirements, serious adverse events believed with a reasonable probability to be due to study treatment are to be reported immediately by telephoning the 24-hour Freefone service, where a few brief details will be recorded. For the purposes of this study, the only adverse events that need to be reported in this way are those that are **both**:

- (i) serious (defined as any untoward medical occurrence which results in death is life-threatening, requires hospitalisation or the prolongation of existing hospitalisation, results in persistent or significant disability or incapacity, congenital abnormality, or the result of an overdose); **and**
- (ii) believed with a reasonable probability to be due to study treatment.

All such serious drug related adverse events (whether expected or not) will be reported (unblinded) to the Chairman of the independent Data Monitoring Committee, and included in the Annual Safety Report for the Research Ethics Committee, to Bayer Healthcare AG and to Abbott Products Operations AG (formerly Solvay Pharmaceuticals). Any such serious drug related adverse events which are unexpected (SUSARs) will be reported, unblinded in an expedited fashion to the Medicines and Healthcare products Regulatory Agency (MHRA) and to the companies. Expected aspirin related serious adverse events might include those due to bleeding, gastro-intestinal perforation, broncho-spasm or other recognised side-effects of aspirin; there are no expected omega-3 fatty acid related serious adverse events.

3.6.2 Reporting of other serious adverse events on routine follow-up questionnaires

Any serious adverse events that are not thought to be due to study treatment, including study endpoints, should not be reported in this way. Such events are, however, to be routinely recorded on the Follow-up questionnaires (see Section 3.5) for central analysis and regular review by the Data Monitoring Committee (see Section 2.3).

3.6.3 Unblinding of study treatment allocation

Unblinding of study treatment allocation is available via the 24-hour Freefone service, where all such unblindings are logged. In general, unblinding of patients is only likely to be necessary if knowledge of treatment allocation will influence immediate patient management or for onward reporting of serious drug related adverse events (see Section 3.6.1).

3.7 Central ascertainment of biochemical effects and confirmation of reported vascular events, cancers and death

3.7.1 Assessing biochemical efficacy of study treatments by random sampling

As well as asking all participants routinely about their compliance with allocated study treatments, compliance will be assessed in a random sample of participants at intervals during the study. A randomly selected sub-set of randomised participants (5-10%) will be sent a kit for blood collection by their GP and mailing to the coordinating centre. Assays of serum or urine thromboxane levels to assess aspirin effects[61] and blood markers of omega-3 FA intake,[62] will be measured to estimate compliance with study treatments. At least once during follow-up assessments will be made in a random sample of participants of the effects of study treatments on blood HbA1c, lipids and markers of renal function, and on urinary albumin/creatinine ratio.

3.7.2 Confirmation of patient reported cardiovascular and other significant serious adverse events using mail-based systems

The coordinating centre will seek confirmation and additional information (including, if necessary, any relevant hospital discharge records) from the participant's GPs about each suspected myocardial infarction, stroke, coronary or non-coronary angioplasty, arterial surgery, cancer, or other relevant hospitalisation or serious adverse event recorded on Follow-up questionnaires or reported by participants during telephone calls or other contact. Similarly, further information will be sought from participant's GPs and other relevant sources about all cancers and deaths identified from national registries. All such information will then be reviewed, blind to treatment allocation, by coordinating centre clinical staff and coded in accordance with pre-specified criteria. The diagnosis of myocardial infarction (MI) requires information about either: (i) the presence of two or more of: (a) typical ischaemic chest pain, pulmonary oedema, syncope or shock; (b) development of pathological Q-waves and/or appearance or disappearance of localised ST-elevation followed by T-wave inversion in two or more of twelve standard electrocardiograph leads; and (c) increase in concentration of biochemical markers consistent with MI (e.g. CK >2xULN, or elevated troponins); or (ii) necropsy findings of MI of an age corresponding to time of onset of symptoms. (Silent myocardial infarctions are not to be included.) Stroke is defined as rapid (or uncertain) onset of focal or global neurological deficit lasting >24 hours or leading to death and transient ischaemic attack is defined by the same symptoms lasting <24 hours. Information (e.g. CT/MRI scan results) will be sought to ascertain the likely aetiology of the stroke (i.e. haemorrhagic or not). These procedures for reviewing reports from patients and other sources of possible study outcomes was developed by CTSU for the MRC/BHF Heart Protection Study, and allowed over 98% of such reports to be successfully confirmed or refuted.

Appendix 1: Information leaflet for potentially eligible patients

- # ASCEND: Invitation to join a large medical research project

A randomised study of aspirin and of natural oils for the primary prevention of cardiovascular events in diabetes

You are being invited to take part in a research study. Before you decide whether to participate, it is important for you to understand why the research is being done and what is involved. Please take time to read the following information carefully and discuss it with friends or relatives if you wish. You are entirely free to decide whether or not to take part in this trial. If you choose not to take part, the standard of care you are given by your own doctors will not be affected. If there is anything that is not clear, or if you would like more information, please telephone the ASCEND Freefone number (0800 585323) and speak to a study nurse or doctor. The study is to be conducted mainly by mail, so no extra clinic visits will be required.

- **Aspirin, heart disease and strokes**

Patients with diabetes may be at increased risk of developing heart disease or suffering a stroke. Aspirin prevents heart attacks and strokes in people who have existing problems with their heart or blood circulation. But it is not known whether aspirin would be helpful in people with diabetes who have not yet been diagnosed with heart or circulatory problems. Serious (but uncommon) complications from the regular use of aspirin are bleeding in the stomach or intestinal tract. Typically this might happen in only about 1 per 1000 people taking aspirin regularly each year. Extremely rarely, aspirin may cause bleeding in the brain (about 1-2 per 10,000 people taking aspirin each year). Previous studies in people with known circulatory problems have shown that about 10 times as many people given aspirin have avoided a heart attack or stroke as have experienced a serious complication. However, in people with diabetes and no circulatory problems, it is not known whether the benefits of aspirin will outweigh the possible risks.

Omega-3 fatty acids and diabetes

Naturally occurring oils that are rich in omega-3 fatty acids (such as fish oils) may reduce the chances of a recurrent heart attack among people who have survived at least one heart attack. These oils have not been widely tested in people with diabetes, but there are reasons to hope that they may be helpful (although this is unproven). Taking regular supplements of such oils may have little or no beneficial effect among people living in a country (such as Britain) where most people eat a balanced diet. It is also possible that the long term use of these oils could, on balance, be slightly harmful – but this too is unknown.

What the study hopes to answer

The main purpose of the ASCEND study, is to find out whether long-term treatment with aspirin in people with diabetes who are not already known to have heart or circulatory problems, produces benefits by preventing heart attacks and strokes which outweigh the possible risks of bleeding. ASCEND will also help establish whether oils rich in omega-3 fatty acids are of any importance in reducing the chances of having a heart attack in people with diabetes who have not yet got circulatory problems.

Why have I been chosen?

ASCEND will involve at least ten thousand men and women from around Britain, who like you, are being invited to take part because they have diabetes. This invitation has come from either your own GP or a local Specialist because they think you might be suitable for the study. Alternatively you may

have been recommended by a friend or relative who is already taking part in the study or volunteered yourself having read about the study. It is up to you to decide whether or not to take part in this study. If you do decide to take part, you would, of course, be free to withdraw from the study treatment at any time without necessarily giving any reason (and without adversely affecting the medical care you can expect from your own doctors). In particular, at the end of the first 2 months, when you finish your first box of treatment, you will have the chance to withdraw if you have any second thoughts or problems with study treatment.

What taking part in ASCEND involves

Everyone taking part will have agreed to do so voluntarily, knowing that it may involve them in taking study treatment for at least 7 years. The daily study treatments (which would be sent to you by mail) will be a single white tablet and a single brown capsule taken from a blister pack. The white tablets will contain either active aspirin (100mg) or a similar looking inactive substance called a "placebo". Whether or not a participant receives active or placebo tablets will be determined randomly (like tossing a coin). Each participant will have a 50% chance of receiving active aspirin and a 50% chance of receiving placebo ("dummy") tablets. The brown capsules will each contain 1 gram of a naturally occurring oil, either mainly omega-3 fatty acids or mainly olive oil. Each participant will have a 50% chance of receiving the omega-3 containing capsules and a 50% chance of receiving olive oil capsules. The type of study treatment being taken will not generally be known by you or your doctor. This information will be known only by certain staff at the coordinating centre in Oxford, but it would be made available to your doctor if this were ever medically necessary. This design helps ensure that reliable information will be obtained about the effects of these potentially important treatments.

- **What you have to do to join the study**

If you might like to participate in this study you should complete the brief Screening Questionnaire on the inside of the letter, sign the Agreement to Participate and return them both in the enclosed Freepost envelope. We will use your answers on the questionnaire to check that you are suitable for the study. If you are suitable, then we will send a box of conveniently packaged study treatments, and ask you to start taking one tablet and one capsule each day by mouth for the next 2 months. We shall also inform your general practitioner of your involvement in the study and check that they are happy for you to continue in the study.

Within a few weeks of receiving this first pack of study treatment, you will also be sent an **optional** blood and urine sampling kit. If it is convenient for you to do so, you would be asked to attend your local surgery to have a small blood sample taken (about 2 teaspoons full) and to provide a urine specimen. Measurements of your height, weight and blood pressure would also be recorded at the surgery and this information, along with the sample, would then be mailed to the ASCEND coordinating centre.

Long-term commitment to the study

Towards the end of the 2 months you will be sent a second study questionnaire. This will allow you to indicate whether or not you would be willing to continue taking the study treatments long-term. Participation in the study does require a commitment to take the study treatments regularly for at least 7 years and to complete questionnaires regularly. **If you do not think that you would be willing or able to do this then it would be better not to join in the first place.**

If you decide to continue you would then be sent further supplies of the study treatments and asked to take one tablet (which would be active or dummy aspirin) and one capsule (containing one or other naturally-occurring oil) every day for the next several years. Further questionnaires would be sent out at 6-monthly intervals. We would ask you to tell us about your current medication and any changes to

your health since your last questionnaire. Additional supplies of study treatment would be sent to you 6-monthly if you were willing to continue taking it. If you do stop during the first 2 months then no further enquiries will be made of you. But, if you decide to continue, we would like to remain in contact with you for the next several years – even if you stop taking the study treatment during this period. **Throughout the study, your own doctors would remain fully responsible for all your other medical care as usual.** However, if you develop any unexpected symptoms which you believe may be due to study treatment you should contact a study doctor on the 24-hour Freefone service: 0800 585323.

What are the side-effects and risks of taking part?

A low dose of aspirin is being used in this study in order to minimise any stomach upset or other gastrointestinal problems. Some minor bleeding (e.g. after having blood taken) and bruising may be experienced by some people, but serious bleeding is likely to be rare. We shall monitor whether aspirin causes an unacceptable level of bleeding during the study. Bleeding risks with aspirin may be somewhat greater among those who are taking warfarin (Marevan) or other blood thinning drugs (e.g. Acenocoumarol (Nicoumalone, Sinthrome) or Phenindione). So, if you are taking any of these blood thinning drugs you would not be suitable to join the study, and if you are prescribed them later we recommend stopping the study aspirin/placebo tablets. People who join the study would be asked to avoid taking aspirin-containing painkillers, and to take an alternative, (such as paracetamol), whenever pain relief is necessary. All other prescribed treatments can be taken as usual. There are no other lifestyle or dietary restrictions required. The doses of the naturally occurring oils being tested in ASCEND are not known to cause any particular problems, although some people may experience gastro-intestinal ("tummy") disturbances. If you did experience any symptoms that you thought were related to either of the study treatments, medical advice is available at all times through the 24-hour Freefone service: 0800 585323.

What are the possible benefits of taking part?

We hope that both the study treatments may help you. However, this cannot be guaranteed. The information we get from this study may help us to treat future patients with diabetes better and may help to prevent many thousands of heart attacks and strokes.

What if new information becomes available?

Sometimes during the course of a research project, relevant new information becomes available about the treatment that is being studied. If this happens we will tell you and your general practitioner about it and you can discuss whether you want to continue in the study. A study doctor is available through the 24-hour Freefone service if either you or your GP need to discuss any new information.

What happens at the end of the study?

When the research study finishes, we will inform you and your GP of the study results. You will then be able to decide whether or not you should take aspirin and/or omega-3 fatty acids. After the study finishes we will no longer continue to provide study medication for you. But, if the study results suggest possible benefit, you could discuss with your GP whether you should take either of these treatments routinely. We will also publish the study results in a professional medical journal as soon as possible after the study finishes. You would not be identified individually in any published report.

What if something goes wrong?

In the unlikely event of you being harmed as a result of taking part in the ASCEND study, the University of Oxford provides insurance cover and you would retain the same rights of care as any other patient treated in the National Health Service.

Will my taking part in this study be kept confidential?

The coordinating centre would seek information from participants' own doctors and from NHS and other central registries about any serious illnesses (such as heart attacks, strokes, cancers etc) that occur. All such information would be used, in confidence, only for medical research purposes and for routine regulatory and audit purposes.

Study organisation

The ASCEND study has been designed, and is coordinated, by Oxford University's Clinical Trial Service Unit. It involves the collaboration of many doctors and nurses around the country. The study design has been reviewed and agreed by independent Research Ethics Committees, which include people from outside the medical profession. The British Heart Foundation has provided a grant to conduct this research study, and packaged study treatment has been provided free by Bayer (makers of the aspirin/placebo) and Abbott (who are providing the natural oils). An independent Data Monitoring Committee will review various outcomes among participants during the study, and will inform the organisers if any important new information has emerged that needs to be provided to participants and their doctors. Any questions about the study should be directed to the coordinating centre in Oxford either by telephone (24-hour Freefone service: 0800 585323) or by mail to: ASCEND Study, CTSU, Richard Doll Building, University of Oxford, Old Road Campus, Oxford, OX3 7LF. Alternatively you can e-mail us on ascend@ctsu.ox.ac.uk.

- **ASCEND: Summary of invitation to join a large medical research project**

- Having diabetes may increase the risk of heart attacks and strokes

Aspirin and omega-3 fatty acids benefit people who have survived a heart attack

- It is not clear whether people with diabetes who have not shown signs of circulatory problems should take aspirin or omega-3 supplements regularly

- Most people with diabetes and no circulatory problems do not take aspirin or omega-3 supplements regularly

- Low-dose aspirin is generally very safe, but does increase the risk of bleeding

- Omega-3 fatty acids at the doses being taken in ASCEND are also considered safe

- The purpose of ASCEND is to find out whether aspirin and/or omega-3 fatty acid supplementation prevents heart attacks and strokes in people with diabetes who have not shown signs of circulatory problems

- If these treatments are shown to be safe and effective for people with diabetes, then their widespread use could lead to the prevention of many thousands of heart attacks and strokes and the saving of many lives

- With your help we can answer these questions reliably with the ASCEND study

If you have any questions about the study then please feel free to contact the coordinating centre on Freefone: 0800 585323

If you think you might be interested in joining this research study please complete and return the attached questionnaire and agreement to participate.

A copy of your signed agreement to participate will be returned to you when your first pack of study treatment is sent out.

Please keep this information sheet for your own records.

THANK YOU FOR YOUR HELP

Appendix 2: Consent form

Need help completing this form? Please call Freefone 0800 585323

Please read this **Agreement to Participate**, and if you are willing then please CROSS the boxes, SIGN and DATE the form using blue or black ink, and return it in the FREEPOST envelope provided.

7. Agreement to Participate

Please cross (☒) **EVERY box** to confirm that you have read and understood the following:

☐	I have read and understood the leaflet **"ASCEND: Invitation to join a large medical research project"** *[Version number of accompanying Patient Information Leaflet will be inserted here]*
☐	I have had an opportunity to telephone the Freefone number 0800 585323 and ask any relevant questions. All my questions have been answered to my satisfaction **OR** I decided that I did not need to ask any questions
☐	I understand that my participation in the ASCEND study is voluntary and that I am free to withdraw from the study at any time without my medical care or rights being affected
☐	I understand that information about my progress in the ASCEND study will be recorded on a computer database, and that these data will be stored securely and confidentially on a computer at Oxford University
☐	I agree that information about any serious illnesses (such as heart attacks, strokes or cancers) may be supplied in confidence to the study coordinators by my own doctors and by NHS and other central registries for use in the ASCEND study
☐	I agree that my hospital and other health records may be looked at in confidence by authorised individuals from the ASCEND study and by regulatory authorities to check the study is being carried out correctly
☐	I understand that my GP will be informed about this provisional agreement to participate in the ASCEND study, and that in about 2 months time I will have another opportunity to decide whether or not I want to join the long-term part of the study

I am happy to take part in ASCEND:

ASCEND Screening Questionnaire [V3.4_240407]

Signature: _____

& **PRINTED** name: _____

(Please use blue or black ink)

Today's date: ☐☐ / ☐☐ / 2 0 ☐☐
Day / Month / Year

Please check that you have answered every question, and signed and dated the form. Return the completed form in the Freepost envelope provided (no stamps needed) to:-

Freepost RLUJ-TKES-SURB, ASCEND, Richard Doll Building, University of Oxford, Old Road Campus, Headington, Oxford, OX3 7LF

If you have any questions about the study, please contact the coordinating centre in Oxford on FREEFONE: 0800 585323 (preferably during office hours 9 am - 5 pm, Monday to Friday)

If this questionnaire indicates that you are suitable to enter the preliminary part of ASCEND, a box containing ASCEND tablets (aspirin or placebo) and capsules (one or other natural oil) will be mailed to you. A copy of this Agreement to Participate, for you to keep, will also be mailed.

If the questionnaire suggests that the study medications may not be suitable for you, then we shall write and tell you.

Thank you very much

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Appendix 3: Published papers

Aung et al. Trials (2016) 17:286
DOI 10.1186/s13063-016-1354-9

Trials

METHODOLOGY Open Access

Cost-effective recruitment methods for a large randomised trial in people with diabetes: A Study of Cardiovascular Events iN Diabetes (ASCEND)

Theingi Aung[2], Richard Haynes[1], Jill Barton[1], Jolyon Cox[1], Aleksandra Murawska[1], Kevin Murphy[1], Michael Lay[1], Jane Armitage[1], Louise Bowman[1*] and ASCEND Study Collaborative Group

Abstract

Background: Clinical trials require cost-effective methods for identifying, randomising, and following large numbers of people in order to generate reliable evidence. ASCEND (A Study of Cardiovascular Events iN Diabetes) is a randomised '2 × 2 factorial design' study of aspirin and omega-3 fatty acid supplements for the primary prevention of cardiovascular events in people with diabetes; this study used central disease registers and a mail-based approach to identify, randomise, and follow 15,000 people. In collaboration with UK consultants and general practitioners (GPs), researchers identified potentially eligible people with diabetes from centrally held registers (e.g. for retinopathy screening) and GP-held disease registers. Permission was obtained under section 251 of the National Health Service Act 2006 (previously section 60 of the NHS act 2001) to allow invitation letters to be generated centrally in the name of the holder of the register. In addition, with the collaboration of the National Institutes for Health Research (NIHR) Diabetes and Primary Care Research Networks (DRN and PCRN), general practices sent pre-assembled invitation packs to people with a diagnosis of diabetes. Invitation packs included a cover letter, screening questionnaire (with consent form), information leaflet, and a Freepost envelope. Eligible patients entered a 2-month, pre-randomisation, run-in phase on placebo tablets and were only randomised if they completed a randomisation form and remained willing and eligible at the end of the run-in. Follow-up is ongoing, using mail-based approaches that are being supplemented by central registry data.

Results: Information on approximately 600,000 people listed on 58 centrally held diabetes registers was obtained, and 300,188 potentially eligible patients were invited to join the study. In addition, 785 GP practices mailed invitations to 120,875 patients. A further 2,340 potential study participants were identified via other routes. In total, 423,403 people with diabetes were invited to take part; 26,462 entered the 2-month, pre-randomisation, run-in phase; and 15,480 were randomised.

Conclusion: If sufficient numbers of potentially eligible patients can be identified centrally and the trial treatments do not require participants to attend clinics, the recruitment and follow-up of patients by mail is feasible and cost-effective. Wider use of these methods could allow more, large, randomised trials to be undertaken successfully and cost-effectively.
(Continued on next page)

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(Continued from previous page)

Trial registration: Current Controlled Trials, ISRCTN60635500, registered on 14 July 2005

Keywords: Aspirin, Omega-3 fatty acids, Diabetes, Cardiovascular disease, Randomised controlled trial, Recruitment methodology

Background

Randomised controlled trials are the cornerstone for reliably evaluating the safety and efficacy of therapeutic strategies [1]. For chronic conditions, where many treatments are expected to have only moderate effects, trials need to be large in size and long in duration to achieve sufficient statistical power and ensure a robust result. The regulations surrounding clinical trials are becoming increasingly burdensome [2, 3], and as a result, the cost and complexity of a standard approach to evaluating therapies is prohibitive (typically at least £3–400 M for large clinical outcome trials), and the model is unsustainable [4]. The development of potentially effective drugs is often stopped prematurely on financial, rather than scientific grounds, and it has become more difficult to do academic trials of important scientific questions; this difficulty has resulted in the distortion of the scientific agenda.

Clinical trials are typically undertaken in a clinic-based setting either in primary or secondary care, and the recruitment of large numbers of participants may require many sites, resulting in organisational complexity and very substantial costs [4]. However, for interventions that require no ongoing physical or laboratory safety monitoring, conducting the trial by mail offers a cost-effective alternative. Several large, successful, randomised trials have been conducted using both a mailed drug supply and follow-up [5–8]. Experience from these studies shows that, with appropriate attention to the wording of the information leaflets, consent forms and questionnaires, good response rates and compliance can be achieved, and reliable information about medical events, gathered. However, previous trials had been conducted among healthcare professionals (i.e. doctors or nurses), and it was not known if such mail-based approaches to clinical trials would be feasible and acceptable in people without such a background.

ASCEND (A Study of Cardiovascular Events iN Diabetes) is a 2 × 2 factorial design randomised study to assess whether aspirin 100 mg daily versus placebo and separately, omega-3 fatty acids 1 g daily versus placebo, reduce the risk of cardiovascular events in individuals with diabetes who do not already have diagnosed occlusive arterial disease, and whether any such benefits outweigh any hazards from bleeding. To minimise costs sufficiently to allow ASCEND to be funded by non-commercial sources, the study was designed to be run mainly by mail with back-up from a 24-hour Freefone service. The rationale and design are available on the study website (http://www.ctsu.ox.ac.uk/ascend/further_pro.htm). This report describes the highly cost-effective mail-based-recruitment methods, which allowed the randomisation of 15,480 people with diabetes from around the UK into ASCEND, making it one of the largest ever trials in this patient group.

Methods

Trial coordination and approvals

The University of Oxford's Clinical Trial Service Unit & Epidemiological Studies Unit (CTSU) is coordinating the study and has overall responsibility for the administration and management of the study under the guidance of a Trial Steering Committee. The University of Oxford is the regulatory sponsor of the trial. After the study had secured initial funding from the British Heart Foundation and a commitment to provide packaged aspirin and matching placebo tablets from Bayer Pharmaceuticals and omega-3 fatty acid capsules and matching placebo capsules from Solvay Pharmaceuticals (subsequently Abbott and now Mylan), Multi-centre Research Ethics Committee (MREC) approval was obtained in 2003 (North West REC, ref 03/8/087) for the study protocol and, in particular, to use centrally held diabetes registers to identify potential participants. Since local doctors were not directly involved in recruitment, the MREC approval indicated that local ethics committees need only be informed of the study, and site-specific approval was not required. Regulatory approval was obtained from the Medicines and Healthcare Products Regulatory Agency (MHRA), and permission to obtain identifiable details of people with diabetes without their explicit consent (in order to invite them to participate in the trial) was obtained from the Patient Information Advisory Group (PIAG), constituted under Section 60 of the NHS Act 2001 (subsequently the National Information Governance Board under section 251 of the National Health Service Act 2006, and more recently the Confidentiality Advisory Group). The coordinating centre ensured that the necessary Research Governance approvals were also in place for the invitations to be sent from general practices.

Identification of participants

People with diabetes were identified from two main sources: (1) centrally held diabetes registers and (2) general practice diabetes registers. Once potentially eligible

individuals had responded to their invitation, subsequent processes were identical for each route of identification (Fig. 1).

Centrally held registers

Consultant diabetologist physicians and other relevant doctors from around the UK were invited to collaborate with the investigators in Oxford in order to allow invitation of potentially suitable individuals with diabetes from their locally held diabetes registers (such as those held for retinopathy screening). To streamline the invitation process, and in accordance with the PIAG approval, the individuals' contact details, date of birth, and GP details were sought electronically, and lists were sent to the coordinating centre. Prior to contacting anyone, lists of potential invitees were sent to the relevant GP asking that they inform the coordinating centre if they did not wish their patients (either specific individuals or all potentially eligible patients) to be contacted about the study. No response from the GP after a reminder letter was taken as agreement to contact the patients. Immediately prior to the invitation being sent, the vital status of the person was checked with the Office for National Statistics (subsequently Health and Social Care Information Centre), to help avoid inadvertent invitation of people who had died (although delays in the availability of up-to-date information could not prevent this entirely). Large-scale, automated, mailing systems were used to generate individualised invitation letters, which

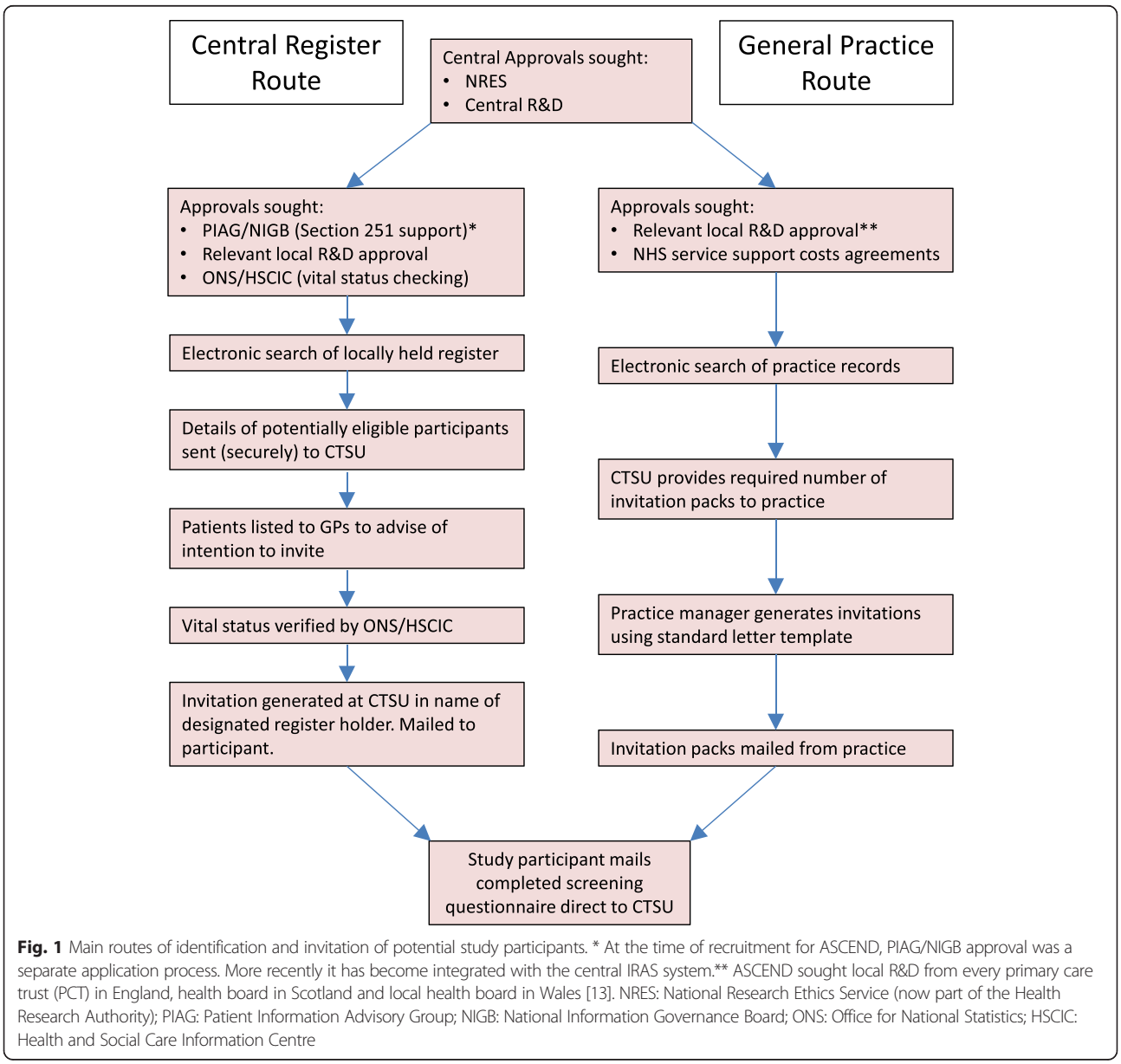

Fig. 1 Main routes of identification and invitation of potential study participants. * At the time of recruitment for ASCEND, PIAG/NIGB approval was a separate application process. More recently it has become integrated with the central IRAS system.** ASCEND sought local R&D from every primary care trust (PCT) in England, health board in Scotland and local health board in Wales [13]. NRES: National Research Ethics Service (now part of the Health Research Authority); PIAG: Patient Information Advisory Group; NIGB: National Information Governance Board; ONS: Office for National Statistics; HSCIC: Health and Social Care Information Centre

were sent with the computer-generated signature of the designated holder of the diabetes register. The invitation pack included the signed cover letter, a screening questionnaire (including the consent form), the study patient information leaflet and a Freepost return envelope addressed to the coordinating centre (see Additional files 1 and 2). A 24-hour Freefone telephone service was available for trial-related enquiries from both potential participants and medical staff.

General practice registers

Consultants and other collaborators were also asked to identify 20–30 local GPs with computerised disease registers and to seek their agreement to mail invitations to potentially eligible individuals. In addition, the National Institutes for Health Research (NIHR) Diabetes Research Network (DRN) and the NIHR Primary Care Research Network (PCRN) identified other interested general practices and provided support for practice staff in the recruitment process. Staff in collaborating practices performed an electronic search on their practice database for potentially eligible patients. Having reviewed the list generated by this search to remove anyone considered unsuitable for the trial, they informed the coordinating centre of the number of invitation packs required, and these were sent to the practice. At the practice, an approved invitation letter was mail-merged with the patient's name and address onto practice headed paper, and these invitation letters were added to the invitation packs provided by the coordinating centre and mailed. For practices identified via the DRN and PCRN, local network support funding was available to help with these administrative tasks.

Other identification routes

Other potentially eligible patients with diabetes were identified from among participants in the Medical Research Council/British Heart Foundation (MRC/BHF) Heart Protection Study (HPS) [9], and they were sent similar invitation packs, with the cover letter adapted accordingly. In addition hospital-based collaborators were sent pre-assembled invitation packs that they could hand to potentially eligible patients seen in their outpatient clinics, and randomised participants had the option of recommending a friend or relative they thought might be eligible and interested in participating in the study. With their friend's or relative's permission, their contact details were sent to the coordinating centre, and an invitation pack was mailed directly to the individual. The study website also facilitated the registration of potential volunteer participants. Diabetes UK, the UK patient, healthcare professional, and research charity, published a brief article about the study in their patient magazine 'Balance', which resulted in a number of 'self-referrals'.

Method of recruitment

People were potentially eligible if aged over 40 years, had type 1 or 2 diabetes, and were not thought to have occlusive vascular disease. Preliminary eligibility was based on information provided on the completed screening questionnaire (i.e. confirmation of diabetes diagnosis, no reported history of diagnosed occlusive arterial disease, no contraindication to regular aspirin, and signed consent to participate – see Additional file 1). Completed screening questionnaires were returned (Freepost) to the coordinating centre where they were logged and then scanned using optical character recognition software to facilitate the efficient transfer of information into the study databases. Bespoke computer programs were used to validate the data, with study administrators, nurses, or clinicians performing additional checks where needed or contacting participants for clarification of responses if necessary.

Consent and pre-randomisation run-in

The screening questionnaire included specific questions related to consent (Additional file 1), which the participants had to sign to confirm that they had understood, and that if they had any questions, these had been addressed by study staff. The 24-hour Freefone service was available if they had any questions about the trial or wished to speak to a doctor about their involvement. During the day, this was manned by the study team, and outside working hours, a clinician was available via a radio pager. Based on the screening questionnaire responses, willing and eligible patients, all of whom had provided signed informed consent, entered a 2-month, placebo, run-in phase (single-blind) and were mailed a pre-randomisation 'run-in' pack of medication, which contained 8-weeks of placebo aspirin and placebo omega-3 fatty acids (Fig. 2). An information sheet about the medication was provided (including a list of contra-indicated medications – see Additional file 3), along with a copy of the scanned image of their signed agreement to participate.

The purpose of the run-in was to check that patients would take the study medication and return the questionnaires regularly, thereby aiming to increase the chance that, if randomised, they would remain compliant and complete follow-up [10]. The run-in also provided the time and opportunity for the coordinating centre to inform the GP of their patient's provisional agreement to enter the study, with an option for the GP to advise against it if they wished, and for the coordinating centre to send a blood and urine kit (see below) to the participant to obtain baseline biological samples.

Fig. 2 Packaged study drugs for mail-based trial

During the run-in phase, a blood and urine sampling kit was sent with a supplementary information leaflet and consent form (Fig. 3 and Additional file 4). Participants were asked to take this kit to their general practice for sample collection and the samples were then mailed to the central laboratory in the containers provided. With the exception of those practices in which phlebotomy services were very limited, this approach was widely accepted, and most practices agreed to provide this service without requesting additional payment. The practice nurse was also asked to record the patient's blood pressure and height and weight on the form provided. This allowed minimised

Fig. 3 Blood and urine sampling kit for mail-based trial

randomisation by relevant biochemical prognostic variables (e.g. lipids, HbA1c) as well as the collection of samples for long-term storage and future analyses (including DNA).

Approximately 2 months after entering the run-in, the participants were sent a randomisation questionnaire to confirm their eligibility, collect more detail about their diabetes and current medications, and check their compliance with the study treatments during the run-in period (Additional file 5). Participants were randomised if they completed and returned a randomisation form and remained willing and eligible to participate.

Results

A total of 423,403 potentially eligible individuals were invited via the different routes, of which, 29 % (121,254 people) returned the screening questionnaires to the coordinating centre (Table 1). Approximately one-third of those returning the questionnaire agreed to join the trial. After review of the questionnaire data, 26,462 participants (6 % of those originally invited) were willing and eligible to join ASCEND and entered the 2-month run-in period.

Randomisation questionnaires were sent to 22,579 patients. Of these, 15,480 people returned a completed questionnaire, remained willing and eligible to participate, and were randomised into ASCEND using a computer-based minimisation algorithm. Approximately 40 % of all patients who entered the run-in dropped out before randomisation, and half of these (approximately 5500 participants) had no clinical reason to stop the trial but simply declined to continue. Overall, 4 % of those invited were randomised: 3 % from centrally held registers (9013 patients) and 5 % from GP registers (6037 patients).

The recruitment process took longer than expected (Fig. 4) but accelerated after mid-2009 due to both the increased availability of the large central registry data (regional retinopathy registers) and to the support from the DRN and PCRN. More than 700 general practices helped with recruitment for the study, from which approximately 6000 of the randomised patients were identified. The majority of practices were identified with the help of the networks, whose support of ASCEND resulted in more than 5000 participants being recruited into the study (Table 2).

If a completed screening questionnaire was not received within approximately 2 weeks of the initial invitation from a centrally held register, a reminder questionnaire was sent. Approximately one-fifth (38,785 of 203,083) of those who received a reminder returned either the original or the reminder screening questionnaire. Similarly, a reminder was sent if a randomisation questionnaire was not returned within 2 weeks. Approximately two-thirds (3110 of 5101) of those to whom randomisation questionnaire reminders were sent, replied, and this led to 2183 patients being randomised. Overall, nearly half (4111 of 9013) of all randomised patients recruited via the centrally held register route were sent a reminder for at least one of the questionnaires.

The availability of information (e.g. sex, date of birth, and post code) from the centrally held registers allows the response rate to the invitation to be compared among the different types of people (Table 3). Younger invitees were more likely to express an interest in participating in the study, even if they were not eligible based on their returned screening form (14 % of those < 50 years old vs 7 % of those ≥ 70 years old, trend p value < 0.0001). However, amongst people who were eligible for the trial and entered the run-in phase, the percent randomised did not vary substantially according to age. There was also a slightly better initial response from men than women (11 % vs 8 %, chi-square $p < 0.0001$), but when those who were ineligible at screening are taken into account, the proportion randomised of those entering run-in was similar by sex (56.2 % men vs 55.5 % women, chi-square $p = 0.4$) (Table 3).

A particular advantage of the mail-based trial methodology used in ASCEND is that with no requirement to attend study clinics, participation is not limited by geographical proximity to a study centre. Figure 5 shows the location of the home addresses of the randomised participants in ASCEND, with recruitment covering both rural and urban areas across the UK. The response to invitation was slightly greater from those living in rural areas compared with those in cities (10.9 % vs 9.7 %, $p < 0.0001$, Table 3). This is likely to be in part due to differences in the Townsend index (a measure of material deprivation based on the subject's home post code), in which a

Table 1 Recruitment by route of identification

	Central registers	General practitioner (GP) registers	Others[a]	Total (% of those invited)
Invited	300,188	120,875[b]	2340	423,403
Returned valid screening form	100,563	19,478	1213	121,254 (29 %)
Entered run-in	16,091	9739	632	26,462 (6 %)
Sent randomisation form	13,481	8541	557	22,579 (5 %)
Randomised (% of those invited)	9013 (3 %)	6037 (5 %)	430 (18 %)	15,480 (4 %)

[a]MRC/BHF Heart Protection Study/self-referral/Friends & Family referral/consultant clinic invitations
[b]Based on number of screening forms sent by coordinating centre to GP practices to be mailed to participants

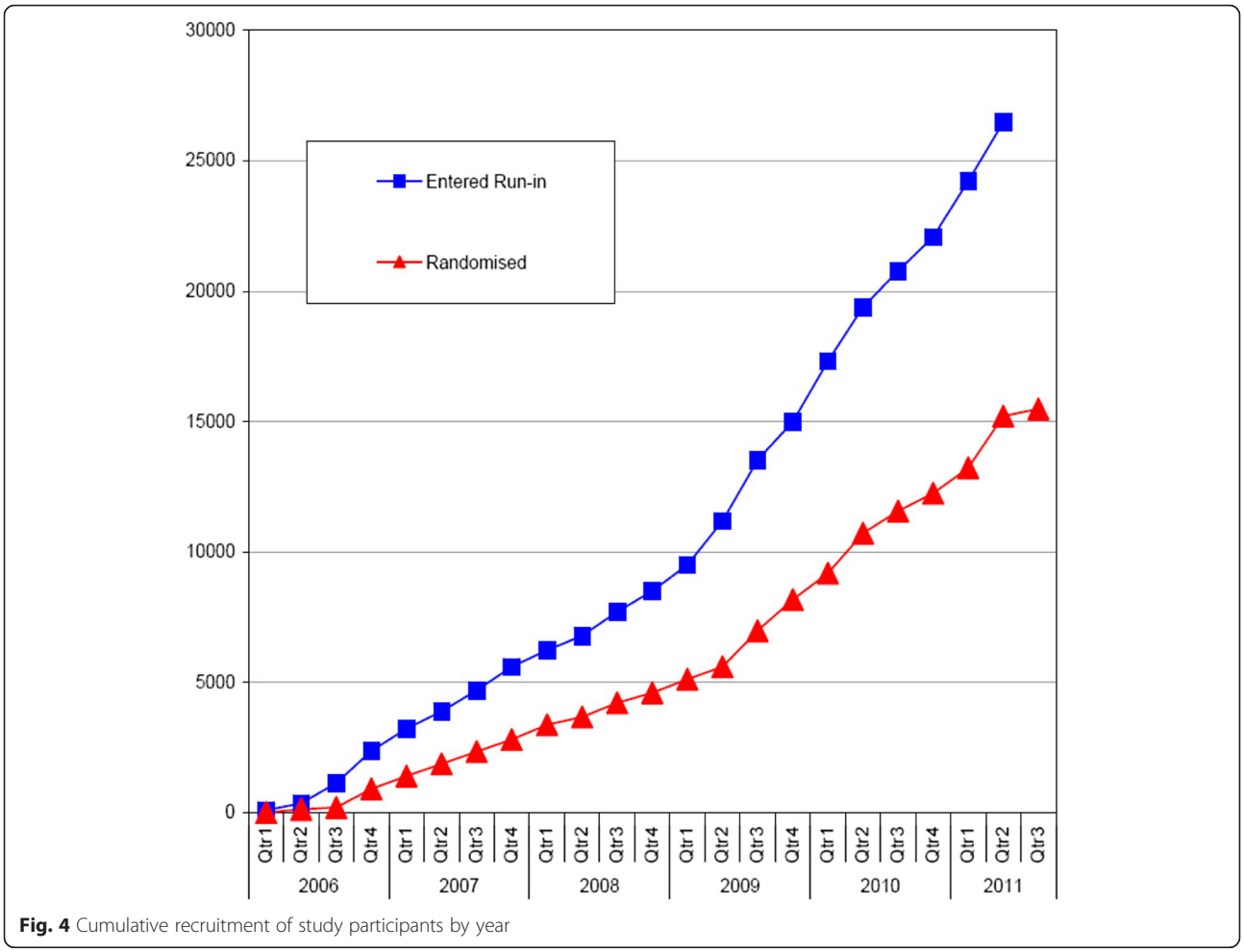

Fig. 4 Cumulative recruitment of study participants by year

substantial variation is observed from 12 % among those in the least deprived areas to 8 % from the most deprived areas (trend p value < 0.0001, Table 3). However, a highly significant rural vs urban effect still persists after allowing for Townsend index ($p < 0.0001$).

The number of days between mailing a study invitation and receipt of the response could be recorded for screening questionnaires sent by the coordinating centre and for all randomisation questionnaires. The mean (SD) time from original invitation to response was 24 (27) days and 14 (15) days for the screening and randomisation questionnaires, respectively. To keep study costs down, second class postage was used for all routine mailings, so the minimum achievable response time was, therefore, 4 days. Ninety-five percent of responses to the screening and randomisation questionnaires were received within approximately 2 months and 1 month, respectively.

A 24-hour Freefone telephone service for queries from participants or their doctors was available to support the recruitment process. Over the 6-year recruitment phase, 8800 telephone calls were logged to this service: 3500 were incoming calls with enquiries from participants or

Table 2 Number of GP practices identified and participant recruitment via the Primary Care Research Network (PCRN), Diabetes Research Network (DRN) and other routes

	Number of practices recruited	Number of patients invited	Number of patients entered run-in	Number of patients randomised (% of those invited)
PCRN	512	79,471	6733	4207 (5 %)
DRN	156	24,420	1772	1065 (4 %)
Other	117	16,984	1234	765 (5 %)
Total	785	120,875	9739	6037 (5 %)

Table 3 Response to invitation; entering pre-randomisation run-in phase; and randomised by age, sex, and Townsend Index (central register route only)

	No. invited	Responded and willing to participate[a] (% of invited)	Entered run-in (% of invited)	Randomised (% of invited)	Percent of those entering run-in who are subsequently randomised
Age (years)[b]					
<50	12,753	1,729 (14 %)	1,262 (10 %)	694 (5 %)	55 %
≥50, < 60	59,635	7,580 (13 %)	4,914 (8 %)	2,801 (5 %)	57 %
≥60, < 70	93,526	11,040 (12 %)	6,103 (7 %)	3,543 (4 %)	58 %
≥70	134,274	9,508 (7 %)	3,812 (3 %)	1,975 (1 %)	52 %
Sex					
F	130,889	10,642 (8 %)	5,931 (5 %)	3,297 (3 %)	56 %
M	169,299	19,215 (11 %)	10,160 (6 %)	5,716 (3 %)	56 %
Townsend Index[c]					
< -3	64,054	7,635 (12 %)	4,649 (7 %)	2,781 (4 %)	60 %
≥ -3 < 0	100,057	10,544 (11 %)	6,022 (6 %)	3,467 (3 %)	58 %
≥0 < 2	47,597	4,207 (9 %)	2,179 (5 %)	1,201 (3 %)	55 %
≥2 < 4	41,932	3,576 (9 %)	1,697 (4 %)	838 (2 %)	49 %
≥4 < 6	30,637	2,466 (8 %)	1,009 (3 %)	496 (2 %)	49 %
≥6	15,354	1,287 (8 %)	458 (3 %)	195 (1 %)	43 %
Urban/rural location[d]					
Urban	244,718	23,729 (10 %)	12,590 (5 %)	6,960 (3 %)	55 %
Rural	54,116	5,923 (11 %)	3,397 (6 %)	2,004 (4 %)	59 %
Unknown	1,354	205 (15 %)	104 (8 %)	49 (4 %)	47 %
Total					
	300,188	29,857(10 %)	16,091 (5 %)	9,013 (3 %)	56 %

[a]Includes willing but ineligible responses. Eligibility likely to vary in subgroups due to differing incidence of prior vascular disease
[b]Based on age on the date screening invitation generated
[c]Based on postcode at screening (lower values indicate least deprived). Score unknown for 557 of those invited
[d]Based on postcode at screening (using ONS 2011 Rural-Urban Classification for Small Area Geographies)

their carers/relatives or doctors, and 5300 were outgoing calls made by study staff, typically to clarify information that had been written on the screening and randomisation questionnaires. Approximately half of all telephone calls (both outgoing and incoming) related to participants who were not subsequently randomised.

Blood and urine kits were sent to 22,858 patients who entered the pre-randomisation phase and who had not informed the coordinating centre that they wished to withdraw before the kits were due to be sent. Samples (either blood or urine or both) were received by the laboratory from 13,270 individuals, among whom 11,685 were subsequently randomised. The average delay between sample collection and receipt at the coordinating centre laboratory (sent by first class post to limit sample delays) was 2 days.

Discussion

ASCEND is one of the largest ever randomised trials among people with diabetes. It achieved its recruitment target of 15,000 participants by means of central and local diabetes registers to identify patients who might be suitable and by using highly cost-effective mail-based systems to send screening and randomisation questionnaires, provide study drugs, and collect biological samples. The trial is funded by a grant to the University of Oxford from the British Heart Foundation (£3.7 million), which covers the costs of running the study over a 15-year period (to include planning, recruitment, follow-up, and study close-out and reporting activities). Within this budget, the costs of printing and postage for the mail-based recruitment process were less than £0.5 million, which is substantially less than the clinic staff costs that would be required for a standard clinic-based approach at this large scale. Study drug and additional funding for drug packaging (£3.6 million) was provided by Bayer and Solvay Pharmaceuticals (subsequently Abbott, and now Mylan). In addition, the 668 practices identified via the DRN and PCRN were eligible for local network support funding (typically around £500 per practice) to help with recruitment activities. Overall, the total costs of this major trial are therefore an order of magnitude lower than those of a typical commercial clinic-based study (generally at least £3–400 M for large clinical outcome trials [4]).

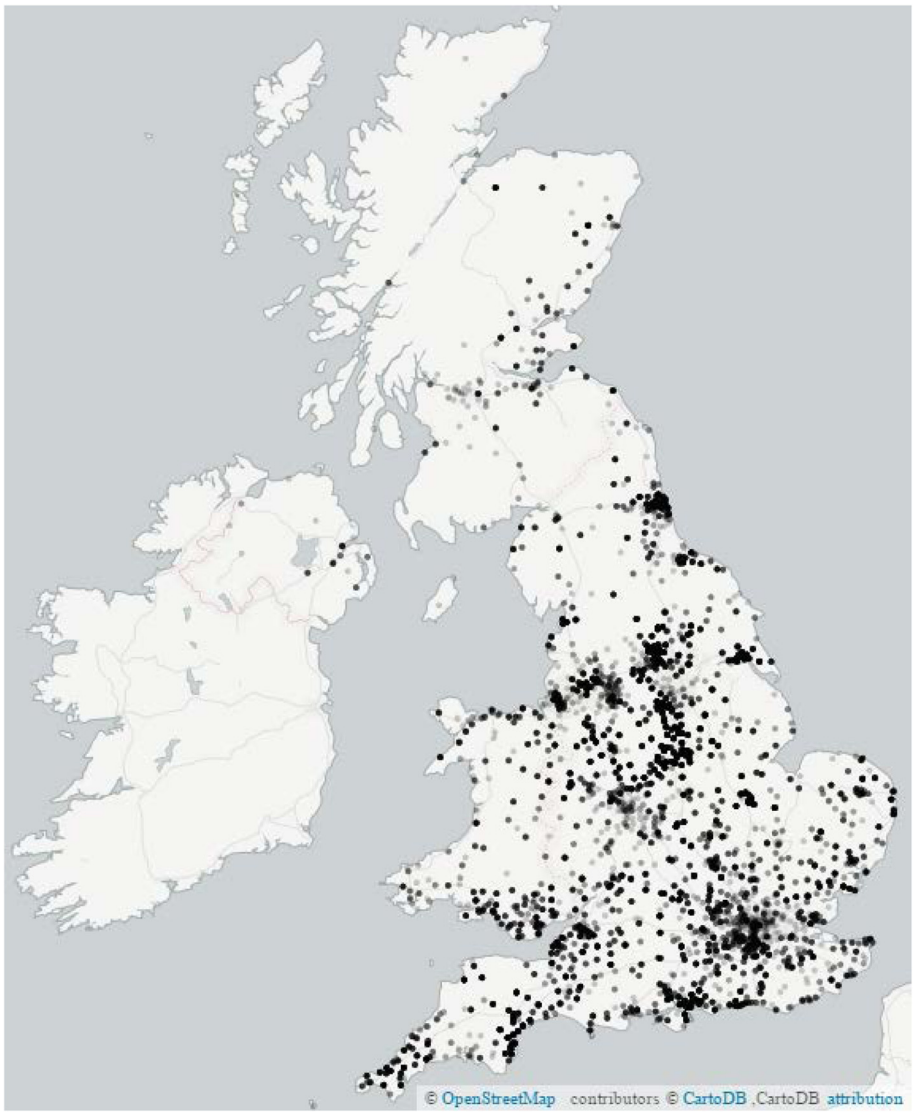

Fig. 5 Location of randomised participants in the UK (postcode of home address)

Detailed baseline characterisation of the randomised participants has been possible from the information collected on the mailed screening and randomisation questionnaires and from mailed blood and urine samples. ASCEND illustrates that if large enough numbers of potentially eligible patients can be identified, and automated methods can be adopted, it is possible to recruit a large study population successfully by mail.

Access to centrally held registers of potentially eligible patients was crucial to the success of recruitment into ASCEND. Although this required the transfer of patient-identifiable information from the register holder to the coordinating centre without the patient's prior consent, existing legislation allows this to be done within a strict legal framework. Without access to these registries, more than 2000 GP practices would need to have been identified and, even if this had been possible, such an approach would have increased the costs and time to recruit significantly. Previous patient volunteer focus group work conducted by CTSU for other studies has confirmed that most patients find this approach acceptable, as long as robust information governance standards are adhered to.

In ASCEND, a small number of complaints were received about the transfer of data without consent. In the majority of cases, the complainant was unaware of the relevant legislation, and a simple verbal explanation of the process was sufficient to clarify any concerns, with many such individuals subsequently agreeing to join the trial. The complainant remained dissatisfied in only 28 cases (0.01 % of 300,188 people invited from centrally held registers) and requested removal of personal data from the study database. The coordinating

centre had a standard operating procedure for such requests, which were acted on promptly. Whereas previous mail-based trials had successfully recruited from populations of healthcare professionals [5–8], ASCEND has demonstrated the acceptability of this approach in a general diabetic population.

Overall, less than one-third of those invited replied to the invitation to take part, and the majority of those who did reply declined to join the study. Other groups have reviewed possible methods to improve the response to postal and electronic questionnaires in order to identify effective strategies to improve recruitment to trials and epidemiological studies. A Cochrane review reported substantial heterogeneity among trials, evaluating more than 100 different approaches to increasing the response to postal questionnaires (typically for epidemiological studies) [11]. Strategies involving monetary incentives and the use of recorded delivery of the questionnaire appeared to be successful, approximately doubling the response rate to postal questionnaires. However, such approaches would add substantially to the cost of a large trial such as ASCEND. Furthermore, those recruited by means of financial incentives might not remain adequately compliant with follow-up and study treatment in longer-term studies. Sending reminders in ASCEND was an effective strategy, which substantially improved the response rate, without which, an additional 140,000 potentially eligible people would have needed to be invited.

Follow-up in ASCEND is ongoing, using mail-based approaches supplemented by central registry data. Study participants receive requests every 6 months for follow-up information. This can be provided either by means of a paper questionnaire, by telephone to the coordinating centre, or using a web-based interface via an internet browser, according to the individual's preference. For participants who are no longer able or willing to complete questionnaires, follow-up information is obtained from their GP. Overall, good rates of follow-up are being achieved using these cost-effective methods. At present, approximately 95 % of all live study participants have follow-up information available from within the last 12 months, and efforts are ongoing to contact those participants for whom follow-up is due.

The currently observed compliance (blinded) with the aspirin/placebo study treatment at the end of the first year post-randomisation is approximately 85 %, with a further 5–7 % decline in compliance annually thereafter. Despite participants having no routine direct contact with the study team, this is comparable to clinic-based trials in similar populations [12]. However, notably, the compliance with study treatments is somewhat lower among those who were sent a reminder randomisation form compared with those who replied to the initial mailing. For example, at the study mid-point (45 months after randomisation), 61 % of those sent a randomisation reminder were compliant with their aspirin/placebo study tablets versus 68 % of those where no reminder was sent ($p < 0.0001$). This reduction in compliance became apparent within the first 6 months after randomisation and, although reminders were essential for the completion of recruitment, the implications for reduced compliance in those who do not readily respond to questionnaire mailings is an important consideration.

On the other hand, the use of a pre-randomisation run-in is a valuable methodological tool to enhance compliance, especially in the early phase of a long-term study [10]. Of those who entered the single-blind placebo run-in period in ASCEND, approximately 40 % dropped out of the study before randomisation. Had there been no run-in, these withdrawals would probably have occurred after randomisation (most likely in the first 6–12 months), thereby substantially reducing the statistical power of the study.

Recruitment into ASCEND took longer than initially hoped as a result of a variety of factors, including research governance delays [13], the time taken to obtain the electronic records from the diabetes registers, establishing robust IT systems to monitor the study, and an increase in the original recruitment target. However the involvement of the former local NIHR Diabetes and Primary Care Research Networks across England and Scotland provided a valuable extra resource, which boosted recruitment and, had they been established sooner, might have shortened the recruitment period. A substantial infrastructure for patient recruitment to research studies continues to be available through the NIHR Clinical Research Network. The response to invitation was higher among those identified from general practice compared with those in central registries (5 % vs 3 % of those invited were eventually randomised). This may have been partly due to the ability to pre-screen potential participants to exclude those with established vascular disease but also because participants were more likely to respond positively to a GP whom they knew.

The design of ASCEND included an optional baseline blood and urine sample collection during the pre-randomisation run-in phase. This exercise was funded by a separate project grant from the British Heart Foundation (£140 k). Previous transport studies have demonstrated that a wide range of analytes (including HbA1c lipids and cystatin C as a measure of renal function) and genetic polymorphisms can be reliably measured in whole blood samples with delayed separation [14, 15]. During the past few years, extensive experience has been gained with obtaining cardiovascular risk factor measurements from mailed blood samples [16]. In ASCEND, this approach has allowed measured baseline risk factor information to be obtained from 75 % of those

randomised at very low cost, which will allow the effects of the study treatments to be assessed within subgroups defined by biological measures.

Conclusions

ASCEND is designed to be streamlined and highly cost-effective. When completed, the trial will have cost < £10 million overall. This includes both the NHS service support costs provided to general practices and the substantial costs of drug packaging and distribution (which are typically covered by the pharmaceutical industry and not usually included in the budget quoted for many large-scale outcome studies). Using the methods described, ASCEND has randomised nearly 15,500 people with diabetes, making it one of the largest ever randomised trials in this patient group. The questions it aims to address are clinically relevant for the hundreds of millions of people worldwide with diabetes and, with good follow-up and compliance, will add valuable information on the balance of benefits and risks of these treatments, including important data on the use of aspirin for cancer prevention. The strategies which helped make recruitment successful include (1) simple inclusion and exclusion criteria, (2) the central coordination of recruitment, (3) the ability to identify a large pool of potentially eligible people, and (4) the involvement of local research networks. The success of these methods in ASCEND show that, with good planning, mail-based methodology is cost-effective and could be more widely adopted for the assessment of interventions that require little monitoring.

Additional files

Additional file 1: Example of screening questionnaire, including consent form. (PDF 167 kb)

Additional file 2: Patient information leaflet. (PDF 367 kb)

Additional file 3: Study treatment information leaflet. (PDF 113 kb)

Additional file 4: Blood and urine sampling information leaflet. (PDF 521 kb)

Additional file 5: Randomization questionnaire. (PDF 198 kb)

Additional file 6: ASCEND Study Collaborative Group. (PDF 19 kb)

Abbreviations

ASCEND: A Study of Cardiovascular Events iN Diabetes; BHF: British Heart Foundation; CTSU: Clinical Trial Service Unit; DRN: Diabetes Research Network; GP: general practitioner; MRC: Medical Research Council; PCRN: Primary Care Research Network.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TA provided clinical oversight of the day-to-day running of the study and drafted the manuscript. RH provided clinical oversight of the day-to-day running of the study. JB contributed to the study coordination and data collection. JC developed the computer programmes for participant invitation and data collection. AM developed the computer programmes for participant invitation and data collection. KM contributed to the study coordination and data collection. ML developed the computer programs for participant invitation and data collection JA designed and coordinated the study and drafted the manuscript. LB designed and coordinated the study and drafted the manuscript. All authors contributed to running the study, data collection, analysis, and interpretation. All members of the Writing Committee and trial Steering Committee read and approved the final manuscript. The ASCEND Study Collaborative Group members participated in the identification and recruitment of the study participants. All authors read and approved the final manuscript.

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ASCEND: A Study of Cardiovascular Events iN Diabetes: Characteristics of a randomized trial of aspirin and of omega-3 fatty acid supplementation in 15,480 people with diabetes

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Objectives The use of aspirin for the secondary prevention of cardiovascular disease (CVD) is firmly established, and the proportional reductions in heart attacks and strokes appear to be similar in people with and without diabetes. Uncertainty remains about the role of antiplatelet treatments for primary prevention of CVD, and guidelines vary in their recommendations. It has also been hypothesized that long-term aspirin can prevent gastro-intestinal and other cancers.

Observational studies suggest associations between higher intakes of omega-3 fatty acids (FA) and lower rates of CVD, but there is no large-scale randomized evidence to support using prophylactic omega-3 FA supplementation in primary prevention. ASCEND is a randomized trial assessing whether 100 mg daily aspirin safely prevents CVD and cancer in patients with diabetes without known arterial disease. It is also assessing whether supplementation with 1 g omega-3 FA daily prevents CVD. This paper describes the methods and baseline characteristics of the randomized participants.

Methods and results Between 2005 and 2011, using mail-based methods, 15,480 people with diabetes were randomized to aspirin versus placebo and, in a factorial design, to omega-3 FA supplementation versus placebo. Blood and urine samples were collected to allow baseline stratification by biochemical prognostic variables (e.g. HbA1c, blood lipids). Follow-up is for a median of at least 7 years.

Conclusions Demonstrating that prophylactic aspirin safely reduces the risk of CVD or cancer in the primary prevention setting, or that omega-3 FA supplementation prevents CVD, would be relevant to hundreds of millions of people worldwide who are currently not receiving such therapies. The results of ASCEND will be reported in 2018. (Am Heart J 2018;198:135-144.)

ASCEND is a randomized placebo-controlled trial aiming to determine whether 100 mg daily aspirin prevents cardiovascular events or cancer in 15,480 UK patients with diabetes who are not already known to have occlusive arterial disease, without leading to significant bleeding or other adverse events that outweigh any benefits. It is also assessing whether supplementation with 1 g omega-3 fatty acids (FA) daily prevents CVD.

Aspirin in primary prevention

The Anti-Thrombotic Trialists' Collaboration (ATTC) demonstrated conclusively that antiplatelet therapy (chiefly aspirin) reduces the risk of myocardial infarction (MI), stroke or cardiovascular death by about one-quarter in people with occlusive vascular disease, including among those who have diabetes.[1] However, most of the 3 million people with diabetes in the UK, and the estimated 400 million worldwide,[2] do not have manifest vascular disease. The 2009 ATTC individual-patient-data meta-analysis of 95,000 patients in 6 primary prevention trials found that allocation to aspirin yielded a 12% (95% CI, 6%-

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18%) proportional reduction in occlusive vascular events, mainly due to a reduction in non-fatal MI of about one fifth.[3] However, given the approximate 50% proportional increase in the risk of bleeding with aspirin, on average the bleeding hazard counterbalanced much of the benefit in these low-risk primary prevention patients. Among the participants in these primary prevention trials, only about 4% had diabetes and the relative risk reduction among them was similar to that observed in those without diabetes. Consequently, since people with diabetes are generally at 2- to 3-fold higher risk of vascular events than those without it,[4] the absolute risk reduction with aspirin is likely to be greater than for healthy volunteers. However, the ATTC analyses also found that people with diabetes had a higher risk of both major extra-cranial bleeds (rate ratio [RR], 1.55; 95% CI 1.13–2.14) and of hemorrhagic stroke (RR, 1.74; 95% CI, 0.95-3.17, respectively) compared to those who did not have diabetes irrespective of aspirin allocation.[3]

A further four trials of aspirin for primary prevention of cardiovascular events have reported results since the ATTC analyses were published in 2009: two specifically in diabetes,[5,6] and two in wider populations that included people with diabetes.[7,8] The Prevention of Progression of Arterial Disease and Diabetes (POPADAD) trial in 1276 patients with diabetes and reduced ankle-brachial index observed no effect on vascular events over 6.7 years (18.2% vs 18.3%; HR, 0.98; 95% CI, 0.76-1.26), while the Japanese Primary Prevention of Atherosclerosis With Aspirin for Diabetes Trial (JPAD) in 2539 patients with type 2 diabetes and no history of atherosclerotic disease followed for 4.4 years observed a non-significant reduction in vascular events based on a very low event rate (1.36% vs 1.70%; HR, 0.80; 95% CI, 0.58-1.10). Neither of these trials reported detailed information about bleeding, so the balance of benefits and risks with aspirin use for primary prevention in diabetes remains uncertain.

Aspirin in primary prevention of cancer

Recent retrospective meta-analyses of randomized trials have suggested that aspirin may produce 15% to 20% proportional reductions in cancer incidence or death, with 30% to 40% reductions in gastrointestinal cancers (particularly colorectal cancer), and that these effects increase with more prolonged exposure.[9-13] If such effects on cancer are confirmed prospectively in randomized trials of sufficient duration, they could have significant implications for the balance of benefits and hazards of using aspirin for primary prevention. ASCEND provides the opportunity to test this hypothesis prospectively with good statistical power since there about as many incident cancers (approximately 1500 during the scheduled treatment period) as in the meta-analyses that generated the hypothesis of protection against cancer and it involves prolonged exposure to aspirin (a median of at least 7 years), with longer-term follow-up available from central registers.

Omega-3 fatty acids in diabetes: adding to the randomized evidence

A possible link between intake of omega-3 fatty acids (FA) and prevention of coronary heart disease (CHD) was first noted in the 1940s when the diets of Greenland Eskimos, among whom CHD was rare despite a high fat intake largely due to sea food, were compared with those of Danes in Denmark who had similarly high fat intake from more mixed diets but CHD rates that were about 10 times higher.[14] A large number of observational studies of omega-3 FA intake and heart disease risk were subsequently conducted in different populations. A systematic review of these observational data concluded that consumption of the equivalent of 40–60 grams of fish per day (providing about 0.2–1 g daily of omega-3 FA, depending on the type of fish) is associated with about a halving in rates of cardiac death.[15] However, there were only limited data available from randomized controlled trials of the effects of increasing omega-3 FA intake on cardiovascular disease outcomes. The results in one small randomized trial involving 2000 male heart attack survivors were consistent with the observational studies, with a 29% (95% CI, 7–46%) significant reduction in total mortality and a 16% (95% CI, +7 to 24%) non-significant reduction in ischemic heart disease events.[16] Similarly, in a trial of omega-3 FA (1 g daily) among 11,000 patients who had survived a myocardial infarction, there was a 13% (95% CI, 1–24%) proportional reduction in coronary events but, both this and the 10% (95% CI, 1–18%) reduction in the primary outcome of cardiovascular events, were only marginally significant.[17] As a consequence, several large randomized trials (including the present ASCEND trial) were started in order to generate more reliable evidence about the effects of omega-3 FA supplementation. Some of those trials have now reported their results, and combined in a tabular data meta-analysis of 10 such trials that each included at least 500 participants treated for at least 1 year involved over 11,000 vascular events in 78,000 participants.[18] Allocation to omega-3 FA supplements (weighted mean daily dose of 1.1 g) for an average duration of 4.4 years appeared to have no significant effect on major vascular events, either overall (RR 0.97; 95% CI 0.93-1.01) or in any particular subgroup. ASCEND will contribute important additional data on both efficacy and safety of such supplementation and, given its large size and longer duration than any previous trial, may be able to detect any modest effects of omega-3 FA supplementation.

Methods

Objectives

The aim of ASCEND is to determine whether daily 100 mg aspirin prevents cardiovascular events or cancer in patients with diabetes who are not known to have occlusive arterial disease, as well as to assess the

magnitude of any effects on significant bleeding or other serious adverse events. It is also assessing whether supplementation with daily 1 g capsules containing 90% omega-3 fatty acids (0.41 g eicosapentaenoic acid, 0.34 g docosahexaenoic acid) prevents CVD.

Eligibility

Men or women aged at least 40 years at the time of invitation for screening were eligible for the study, provided they fulfilled all of the following criteria:

i) *Clinical diagnosis of diabetes mellitus:* the participant's own doctor considered them to have type 1 or type 2 diabetes (based on standard WHO or ADA diagnostic criteria[19,20]);
ii) *No clear indication for aspirin:* the participant had no diagnosed occlusive arterial disease (i.e. a history of MI, angina pectoris, coronary or non-coronary revascularization procedure [ie, peripheral arterial bypass surgery or angioplasty], stroke or transient ischemic attack);
iii) *No clear contra-indication to aspirin:* The participant was not at high risk of bleeding due to gastrointestinal hemorrhage or peptic ulcer within the previous 6 months, active hepatic disease (such as cirrhosis or active hepatitis), or use of warfarin or other anti-coagulant therapy; and had no history of aspirin allergy;
iv) *Substantial uncertainty about whether anti-platelet or omega-3 FA therapy confers worthwhile benefit:* the participant and their own general practitioner (GP) did not consider there to be a definite need to use aspirin or omega-3 FA supplements regularly (or a definite need not to do so);
v) *No other predominant life-threatening medical problem:* the participant did not have some condition (other than diabetes) that might be expected to prevent them from taking at least 5 years of study treatment.

Participant recruitment and follow-up

In order to be cost-effective, UK-wide recruitment into ASCEND was conducted by mail. The highly streamlined recruitment methods have been described previously.[21] The coordinating center provided a 24-hour Freefone service to answer questions about the trial from participants and their GPs.

Invitation and screening. In collaboration with medical consultants and GPs around the UK, and supported by the National Institutes for Health Research (NIHR) Diabetes and Primary Care Research Networks (DRN and PCRN), potentially eligible patients with diabetes were identified from centrally-held registers (e.g. for retinopathy screening) and GP-held disease registers. Potential study participants were mailed an invitation pack, including a cover letter, screening questionnaire (to determine eligibility and to seek consent), Freepost envelope and Information Leaflet.

Pre-randomization run-in period. Willing and eligible patients entered a pre-randomization run-in phase and were sent a run-in pack of medication (single blind: containing placebo aspirin tablets and placebo omega-3 FA capsules) and asked to take one tablet and one capsule daily for 2 months. During the run-in period, the participant's GP was informed by letter of their patient's possible involvement in the study and asked to return a form if they considered there to be any reason not to randomize their patient. Patients were randomized only if, at the end of the run-in period, they seemed likely to comply with the study protocol for several more years. By this process, many potential dropouts could be excluded before becoming part of the randomized comparison, with a consequent improvement in statistical sensitivity of the "intention-to-treat" analyses.[22]

Randomization. About 2 months after starting the run-in, participants were sent a more detailed randomization questionnaire asking about any significant problems (including any cardiovascular events), their compliance with the study treatments during the run-in period, details of their diabetes history (to allow classification as type 1 or 2),[23] current medication, ethnic group and smoking history.

Participants who remained eligible based on the randomization questionnaire and were willing to continue on the study were randomized centrally at the Clinical Trial Service Unit (CTSU), University of Oxford, using a minimization algorithm to ensure balance by prognostic variables (age, sex, duration of diabetes, history of treated hypertension, smoking status, ethnic origin, and, if available from centrally measured blood and urine samples [see below], total cholesterol, HbA1c, and urinary albumin/creatinine ratio). Eligible patients were randomized in a 2 × 2 factorial blinded design between aspirin 100 mg daily and matching placebo, and, separately, between omega-3 fatty acid capsules 1 g daily and placebo.

Post-randomization follow-up. Follow-up was also conducted largely by mail, supplemented by information from central registries. Randomized participants received a follow-up questionnaire 6-monthly (either paper or via a weblink to a secure online version[24]) asking about the occurrence of any cardiovascular events, bleeding events, cancer diagnoses, compliance with study medication and use of other relevant medications (such as anti-platelet agents or anti-coagulants). Confirmation and further information was then sought from GPs about reports of possible cardiovascular events and serious bleeds. All such information is reviewed by coordinating center clinicians, blind to treatment allocation, and events are

adjudicated according to pre-specified criteria. Additional follow-up for death, cancer and hospitalizations is being obtained from NHS Digital (formerly the Health and Social Care Information Centre) in England and Wales and the Information Services Division of NHS Scotland. Ethics approval has been obtained for additional follow-up after the scheduled treatment period via these central registries to assess the longer-term effects on cancer and on other outcomes.

Central biological sample assays

About 2 to 4 weeks after entering the pre-randomization run-in period, participants were sent an optional blood and urine sampling kit, and asked to take it to their general practice or other usual phlebotomy service for sample collection. The kit was sent with an information leaflet explaining the reasons for sample collection, a consent form for sample storage and assay (which included a section for recording blood pressure, pulse, height and weight measured by the practice nurse), a letter for the practice nurse with instructions for sample collection, and barcoded labels for the sample tubes. The completed consent form and blood (EDTA whole blood) and urine samples were to be mailed to the central laboratory at CTSU. Previous transport studies have demonstrated that a wide range of analytes (including HbA1c, lipids and cystatin C as a measure of renal function) and genetic polymorphisms can be reliably measured in whole blood samples despite delayed separation.[25,26]

Blood levels of total cholesterol, HDL-cholesterol, apolipoprotein-B, apolipoprotein-A1, HbA1c and cystatin C, and urinary creatinine and albumin were assayed (see Supplementary Appendix 1 for methods) in the central CTSU laboratory, which is a UKAS accredited testing laboratory. Aliquots of plasma, urine, red cells and DNA-containing buffy coat from all participants who provided samples have been stored in liquid nitrogen for future analyses (consistent with consent provided by participants).

Sample size and predicted number of events

When ASCEND was designed in 2003, it was anticipated that 10,000 participants followed for 5 years with an expected 2% annual rate (based on previous trials in similar populations) of the composite primary efficacy outcome of non-fatal MI, non-fatal stroke or vascular death (excluding confirmed intracranial hemorrhage) would provide sufficient power to detect a relative reduction in vascular risk of 20–25%. However, as specified in the protocol, the Steering Committee monitored the overall (i.e. blinded) vascular event rate among the randomized participants in order to decide whether the original sample size assumptions remained valid or whether changes might be needed.

Based on information that subsequently became available from the ATTC meta-analysis of primary prevention trials,[3] a relative reduction in the risk of occlusive vascular events with aspirin therapy seemed more likely to be only about 12% to 15%. In addition, the rate of the composite primary outcome observed among the first few thousand randomized participants during the first few years of the study was significantly lower than anticipated, at around 0.6% per annum. Consequently, the Trial Steering Committee decided to modify the trial design blind to any treatment related results in the following ways:

Inclusion of transient ischemic attacks (TIA) in the primary efficacy outcome. Patients are now routinely started on aspirin after a TIA,[27] so its inclusion in the primary efficacy outcome increases the chances of detecting any effects of aspirin on cerebrovascular events (rather than having them diluted by post-TIA treatment).

Increase in sample size. The availability of large regional retinopathy registers from which potential participants could be invited provided an opportunity to increase the study population to 15,000 in a cost-effective manner.

Increase in study duration. It was possible to secure additional funding and drug supplies to extend median duration from 5 to at least 7 years.

Revised power calculations based on a 1.3% per annum rate of the revised primary efficacy outcome of serious vascular events (SVE: defined as non-fatal MI, non-fatal stroke, or vascular death, excluding confirmed intracranial hemorrhage but including TIA) indicate that ASCEND has good statistical power (ie, >90% at $2P < .05$) to detect a 15% proportional reduction during extended follow-up of 7.5 years. The expected 1300 incident vascular events will approximately double the information currently available about the effects of using aspirin for primary prevention in people with diabetes. Consequently, inclusion of these data in an updated meta-analysis should help determine whether there are particular types of diabetic patient (eg, those at higher vascular risk) who would benefit.

Planned comparisons of outcome

For aspirin therapy, the primary efficacy comparison will involve log-rank analyses of SVEs during the scheduled treatment period among all those allocated aspirin tablets versus all those allocated placebo tablets (ie, "intention-to-treat" comparisons). Similarly, for the omega-3 fatty acid supplementation, the primary efficacy comparison will involve log-rank analyses of SVEs during the scheduled treatment period among all those allocated omega-3 fatty acid capsules versus all those allocated placebo capsules. No allowance will be made for multiple hypothesis testing in these 2 separate primary comparisons (see Data Analysis Plan).

A key secondary outcome for aspirin will be the incidence of gastrointestinal tract cancers during the scheduled treatment period. However, little or no

treatment effect is expected before about 3 years,[9] limiting the statistical power to detect plausible effects of aspirin during the scheduled treatment period. There are expected to be ~430 GI tract cancers during the 7.5 years of follow-up. These numbers provide ~86% power at $2P < .05$ to detect a 40% reduction in risk and 60% power at $2P < .05$ for a 30% reduction in risk. Analyses excluding the first 3 years of follow-up are prespecified to assess whether effects are increasing with time from randomization. However, the main focus of the cancer analyses will be during longer term follow-up, when there will be much better power to detect plausible differences between the arms due to larger numbers of events. At about 5 years after the scheduled treatment period, there will be >90% at $2P < .01$ to detect a 30% or greater risk reduction and >90% at $2P < .05$ to detect a 25% reduction in any GI tract cancer risk.

The key safety outcome for aspirin will be any major bleed, defined as any confirmed intracranial hemorrhage (including intracerebral, subarachnoid, subdural or any other intracranial hemorrhage), sight-threatening eye bleeding, or any other serious bleeding episode (i.e. requiring hospitalization or transfusion, or fatal or disabling). Further details regarding pre-specified comparisons and statistical methods are provided in the Data Analysis Plan (see Supplementary Appendix 3).

Organization and funding

The University of Oxford is the academic sponsor of ASCEND. The study is funded by a grant to the University of Oxford (a Special Project Grant from 2003 to 2008 (SP/03/002), followed by two renewals of the grant in 2009-2013 (SP/08/010/259) and 2015-2019 (SP/14/3/31114) from the British Heart Foundation (BHF) to cover the administrative and coordination costs of the trial. A separate BHF project grant (PG/05/013/18296) was obtained for the addition of blood and urine sampling to the study protocol. Aspirin and matching placebo are being provided by Bayer AG, and omega-3 fatty acid and matching placebo capsules by Mylan EPD (formerly by Abbott Product Operations AG and Solvay Pharmaceuticals GmbH), with funding from each company to cover drug packaging. The MRC Population Health Research Unit within the Clinical Trial Service Unit (CTSU) at Oxford University supports some study staff and receives additional funding from the British Heart Foundation and Cancer Research UK. Staff from the National Institute for Health Research Clinical Research Network, and the Scottish Primary Care Research Network, assisted with recruitment activities. The study was designed, and has been conducted, analyzed, interpreted and reported, independently by CTSU. The study is overseen by an independent Steering Committee, including UK diabetologists, clinical trialists, statisticians and representatives from the BHF. Representatives from Bayer AG and Mylan attend Steering Committee meetings as non-voting observers. The authors are solely responsible for the design and conduct of this study, all study analyses, the drafting and editing of the paper and its final contents. The first and last authors act as guarantors for this work.

Results

Ethics, regulatory, and research governance approvals

Multi-centre Research Ethics Committee (MREC) approval was granted for ASCEND by the North West MREC. Doctors and Dentists Exemptions (DDXs) for the use of aspirin and omega-3 fatty acids in ASCEND were obtained from the Medicines and Healthcare products Regulatory Agency (MHRA) prior to 1 May 2004. These DDXs were automatically converted to clinical trial authorizations following the implementation of the Medicines for Human Use (Clinical Trials) Regulations 2004. A separate application for research governance approval was made to all relevant NHS Trusts (both Hospital Trusts and Primary Care Trusts (PCTs), including Local Health Boards (LHBs) in Wales and Scotland).

Participant recruitment

A total of 423,403 potentially eligible individuals were invited via the different routes of recruitment,[21] of which 29% (121,254 people) returned a screening questionnaire. About two-thirds of those who responded declined to join the trial and a further 14,000 did not meet the eligibility criteria (Table I). After review of the questionnaire data, 26,462 participants (6% of those originally invited) were willing and eligible to join ASCEND and entered the 2-month run-in period (Figure).

About 40% of all patients who entered the run-in dropped out before randomization. Supplementary Table I gives the reasons for withdrawal: about half had no clinical reason but simply declined to continue. Without this pre-randomization phase, many such withdrawals might instead have occurred early after randomization, resulting in a substantial reduction in statistical power. Towards the end of the 2-month run-in, randomization questionnaires were sent to 22,579 individuals, of whom 15,480 responded that they remained willing and eligible, and were randomized into ASCEND (Figure). Overall 3.7% of those invited were randomized.

Baseline characteristics of randomized participants

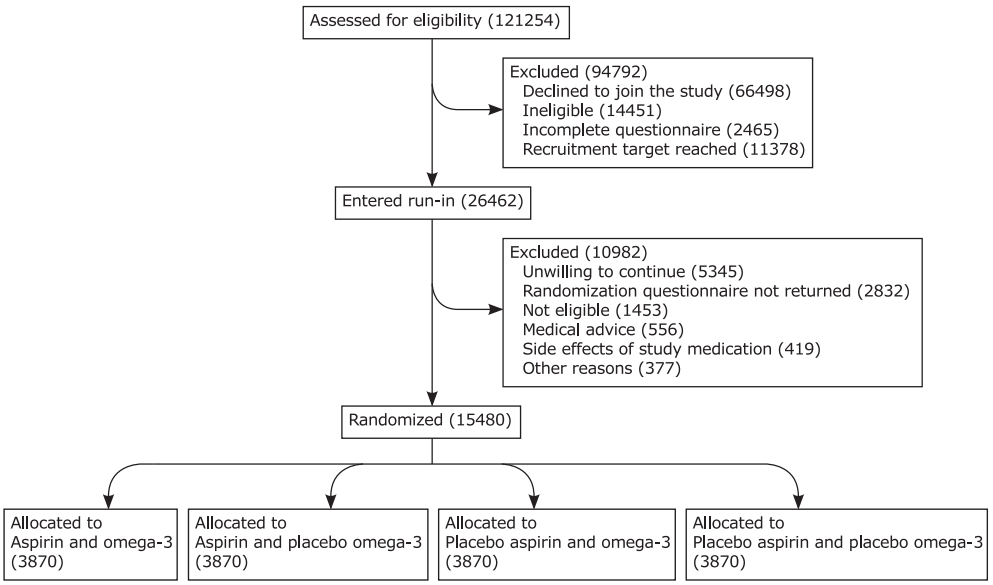
In total 15,480 people (9684 men and 5796 women), average age 63.3 years (SD 9.2) were randomized between June 2005 and July 2011 (Table II). Participants had diabetes (94% type 2) diagnosed a median of about 7 years before randomization. For 16% of participants, their diabetes was managed by diet alone; 25% were using insulin at baseline (with or without other agents); and 58% were using hypoglycemic agents but not insulin. The majority (85%) were overweight or obese at baseline (BMI $\geq$25 kg/m^2), and 62% reported taking treatment for hypertension. In those participants for whom baseline blood

Table I. Reasons for not entering run-in at screening.

Reason for not entering run-in	Number of patients
Declined to join the study	66,498 (70%)
Ineligible at screening*	14,004 (15%)
Prior coronary artery disease	6406
Declined to stop pre-study aspirin	3928
Declined to avoid non-study aspirin	3657
Prior stroke or transient ischemic attack	2374
On warfarin/acenocoumarol/phenindione	1312
Allergic to aspirin or omega-3	1224
Cancer in the last 5 years	871
Gastrointestinal bleeding in the last 6 months	780
Active peptic ulcer in the last 6 months	605
Did not have diabetes	501
Other serious illness	408
Prior non-coronary revascularization	230
Liver disease	158
Too young†	22
Incomplete questionnaire - unable to process	2465 (3%)
Potentially eligible at screening but subsequently ineligible‡	447 (<1%)
Screening form not processed as recruitment target reached	11,378 (12%)
Total (completed a screening form but did not enter run-in)	94,792 (100%)

* More than one reason may apply per patient.
† This includes some individuals who recorded their date of birth incorrectly.
‡ This includes individuals who were potentially eligible on the basis of their screening form but who did not enter run-in for a variety of reasons including technical difficulties in processing the form or further information from the patient indicating that they were ineligible or unwilling to take part.

Figure



Trial profile: Flow of participants through the ASCEND trial.

pressure measurements were available (at the time of blood sampling), the mean systolic blood pressure was 136 mmHg.

Blood and urine kits were sent to 22,858 patients who entered the pre-randomization phase and who had not informed the coordinating center that they wished to withdraw before the kits were due to be sent. Samples (either blood or urine or both) were received by the laboratory from 13,270 individuals, among whom 11,685 were subsequently randomized. Samples received from about 1800 participants were not deemed usable as a result of inadequate sample volume, incomplete consent or delays in sample receipt at the central laboratory.

Table II. Baseline characteristics of study population.

	Male	Female	Total
Total randomized	9684 (63%)	5796 (37%)	15,480 (100%)
Age at randomization (years)			
<50	582 (6%)	507 (9%)	1089 (7%)
≥50, <60	2888 (30%)	1613 (28%)	4501 (29%)
≥60, <70	3944 (41%)	2303 (40%)	6247 (40%)
≥70	2270 (23%)	1373 (24%)	3643 (24%)
Mean age (SD)	63.3 (9.1)	63.1 (9.4)	63.3 (9.2)
Body mass index (kg/m^2)*			
<25	1385 (14%)	864 (15%)	2249 (15%)
≥25, <30	3883 (40%)	1646 (28%)	5529 (36%)
≥30, <35	2666 (28%)	1574 (27%)	4240 (27%)
≥35	1449 (15%)	1512 (26%)	2961 (19%)
Unknown	301 (3%)	200 (3%)	501 (3%)
Mean body mass index (SD)	30.1 (5.6)	31.7 (7.1)	30.7 (6.3)
Type of diabetes†			
Type 1	518 (5%)	393 (7%)	911 (6%)
Type 2	9166 (95%)	5403 (93%)	14,569 (94%)
Diabetes management			
Diet only	1502 (16%)	1027 (18%)	2529 (16%)
Any hypoglycemic agent but not insulin	5816 (60%)	3204 (55%)	9020 (58%)
Insulin +/− other hypoglycemic agent	2366 (24%)	1565 (27%)	3931 (25%)
Duration of diabetes (years)			
<5	2991 (31%)	1900 (33%)	4891 (32%)
≥5, <10	2728 (28%)	1606 (28%)	4334 (28%)
≥10, <20	2287 (24%)	1250 (22%)	3537 (23%)
≥ 20	1150 (12%)	712 (12%)	1862 (12%)
Unknown	528 (5%)	328 (6%)	856 (6%)
Median duration of diabetes (IQR)	7 (3–13)	7 (3–12)	7 (3–13)
Systolic blood pressure (mmHg)‡			
<130	1961 (20%)	1433 (25%)	3394 (22%)
≥130, <140	1931 (20%)	1160 (20%)	3091 (20%)
≥140	2965 (31%)	1590 (27%)	4555 (29%)
Unknown	2827 (29%)	1613 (28%)	4440 (29%)
Mean systolic blood pressure (SD)	136.9 (15.2)	134.9 (15.3)	136.2 (15.3)
Other Factors§			
Reported treated hypertension (n = 15,368)	5854 (60%)	3679 (63%)	9533 (62%)
Current smoker (n = 15,307)	778 (8%)	501 (9%)	1279 (8%)
Diabetic retinopathy (n = 15,336)	1875 (19%)	1148 (20%)	3023 (20%)
Ethnic origin			
White	9331 (96%)	5604 (97%)	14,935 (96%)
Indian/Pakistani/Bangladeshi	141 (1%)	43 (<1%)	184 (1%)
African/Caribbean	79 (<1%)	61 (1%)	140 (<1%)
Other/unknown	133 (1%)	88 (2%)	221 (1%)

* Based on self-reported height and weight
† Based on a broad clinical definition involving age at diagnosis of diabetes, use of insulin within one year of diagnosis and BMI
‡ From blood and urine consent form, generally before randomization
§ Reported by participant on randomization questionnaire.

Baseline biochemical measures are shown in Table III. Among the 9813 participants with baseline HbA1c available, only 31% achieved target levels for glucose control of <6.5% (48 mmol/mol). By contrast, 4554 participants (46% of those with measures available) had a total cholesterol <4.0 mmol/L. Supplementary Table II provides the baseline characteristics of those participants with a usable baseline blood sample and indicates that they were generally representative of the full study population.

Non-study medication use was reported on the randomization form and is shown in Supplementary Table III. Three quarters of participants reported taking a statin, and over a third were previously on aspirin but had no clear clinical indication for it (and they and their GP were agreeable to stopping this in order to take part in ASCEND).

Post-randomization follow-up

Follow-up of ASCEND participants is scheduled to finish in 2017, by which time there will be a median duration of follow-up of at least 7 years. Follow-up questionnaires are sent 6-monthly. In order to ensure completeness of follow-up, if no reply is received to the initial follow-up questionnaire (paper or emailed request), two reminders are sent to non-responders,

Table III. Biochemical measures assessed during pre-randomization run-in phase.

	Male	Female	Total
Total cholesterol (mmol/L) (n = 9819)			
<4.0	3175 (52%)	1379 (37%)	4554 (46%)
≥4.0, < 5.0	2193 (36%)	1602 (43%)	3795 (39%)
≥5.0	716 (12%)	754 (20%)	1470 (15%)
Mean (SD)	4.0 (0.8)	4.4 (0.9)	4.2 (0.9)
HDL cholesterol (mmol/L) (n = 9800)			
<1.0	1751 (29%)	419 (11%)	2170 (22%)
≥1.0, < 1.5	3466 (57%)	2057 (55%)	5523 (56%)
≥1.5	855 (14%)	1252 (34%)	2107 (22%)
Mean (SD)	1.2 (0.3)	1.4 (0.4)	1.3 (0.4)
Non-HDL cholesterol (mmol/L) (n = 9800)			
<2.5	2187 (36%)	1203 (32%)	3390 (35%)
≥2.5, < 3.5	2693 (44%)	1700 (46%)	4393 (45%)
≥3.5	1192 (20%)	825 (22%)	2017 (21%)
Mean (SD)	2.9 (0.8)	3.0 (0.9)	2.9 (0.8)
Apolipoprotein B (mg/dL) (n = 9779)			
<70	1900 (31%)	947 (25%)	2847 (29%)
≥70, < 90	2386 (39%)	1516 (41%)	3902 (40%)
≥90	1766 (29%)	1264 (34%)	3030 (31%)
Mean (SD)	80.8 (20)	84.3 (21)	82.1 (21)
Apolipoprotein A1 (mg/dL) (n = 9799)			
<130	1781 (29%)	403 (11%)	2184 (22%)
≥130, < 160	3030 (50%)	1669 (45%)	4699 (48%)
≥160	1259 (21%)	1657 (44%)	2916 (30%)
Mean (SD)	143.5 (23)	159.3 (26)	149.5 (25)
HbA1c DCCT % (IFCC mmol/mol) (n = 9813)			
<6 (42)	734 (12%)	454 (12%)	1188 (12%)
≥6 (42), < 6.5 (48)	1077 (18%)	744 (20%)	1821 (19%)
≥6.5 (48), < 7 (53)	1317 (22%)	790 (21%)	2107 (21%)
≥7 (53), < 7.5 (58)	1057 (17%)	651 (17%)	1708 (17%)
≥7.5 (58)	1895 (31%)	1094 (29%)	2989 (30%)
Mean (SD)	7.2 (55) (1.2 (13))	7.1 (55) (1.2 (13))	7.2 (55) (1.2 (13))
eGFR (ml/min/1.73m^2) (n = 9815)*			
≥90	2966 (49%)	1557 (42%)	4523 (46%)
≥60, < 90	2413 (40%)	1603 (43%)	4016 (41%)
≥45, < 60	490 (8%)	379 (10%)	869 (9%)
≥30, < 45	167 (3%)	155 (4%)	322 (3%)
<30	46 (<1%)	39 (1%)	85 (<1%)
Mean (SD)	86.9 (21)	82.3 (21)	85.2 (21)
Urinary albumin/creatinine ratio (mg/mmol) (n = 9774)			
<3	5176 (85%)	3350 (90%)	8526 (87%)
≥3, < 30	764 (13%)	324 (9%)	1088 (11%)
≥30	123 (2%)	37 (<1%)	160 (2%)
Median	0.59	0.51	0.55

HDL, High-density lipoprotein; IFCC, International Federation of Clinical Chemistry
*Calculated from blood cystatin c concentration using the CKD-EPI formula.

followed by a telephone call by coordinating centre staff. In late 2014, the option of completing a follow-up form via the internet was introduced,[24] and around 15% to 20% of responses are now received online. In addition, electronic information about all deaths and cancers is received periodically from central NHS registries.

Discussion

There remains continuing clinical uncertainty about whether or not aspirin should be recommended for the primary prevention of cardiovascular events in people with diabetes.[13] This is reflected in the differing and changing recommendations in cardiovascular prevention guidelines. When ASCEND was designed, the American Diabetes Association recommended aspirin use for primary prevention in people with diabetes with one additional risk factor[28] but, at that time, the UK and European guidelines were more circumspect.[29,30] More recently, both the 2015 UK National Institute for Health and Care Excellence (NICE) guideline for type 2 diabetes[31] and the 2016 European Guidelines[32] have advised not offering antiplatelet therapy for adults with type 2 diabetes

but without cardiovascular disease. By contrast, the US Preventive Services Task Force now recommends low-dose aspirin for the primary prevention of cardiovascular disease and colorectal cancer in adults aged 50–59 years who have a 10-year cardiovascular risk of at least 10% and are not considered at increased risk for bleeding, irrespective of their diabetes status.[33]

In addition to ASCEND, three other randomized trials of aspirin therapy in similar intermediate-risk populations are anticipated to announce their results during the next 2–3 years,[34-36] providing substantially more data than is currently available about the value of using aspirin in the primary prevention setting. ASCEND will be responsible for almost half of the available data in diabetes and, in addition, will provide one of the first large-scale prospective tests of aspirin for the prevention of cancer. If aspirin is shown to be effective for cancer chemoprevention then this could significantly alter the balance of benefits and risks for its use in primary prevention.

Given recent data, it is unlikely that ASCEND will show benefits from the omega-3 fatty acid allocation of the magnitude that had been anticipated. Nevertheless, the increase in size and duration of exposure beyond that originally planned means that its ability to detect more modest effects of omega-3 fatty acids has increased. In addition, it should be large enough to determine whether (or not) any particular types of patient benefit to a worthwhile extent from such supplementation.

Conclusion

The current global epidemic of diabetes makes robust evidence about the effects of low-cost prophylactic interventions especially important. For example, demonstrating that primary prevention with aspirin prevents cardiovascular events or cancers, and that the benefits outweigh the risks of bleeding, would be relevant to some hundreds of millions of people worldwide who are at risk of such events but are currently not taking low-dose aspirin. On the other hand, if the risks of serious bleeding outweigh any benefits then these risks could be avoided by the very large numbers of people who are currently using aspirin for primary prevention.

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Author contributions

JA and LB designed and coordinated the study and drafted the manuscript. MM provided clinical oversight of the day-to-day running of the study and drafted the manuscript. WS performed the data analysis and prepared the tables and figure. RH, TA and FC provided clinical oversight of the day-to-day running of the study. GB performed the statistical analyses. RC designed the study and helped revise the manuscript. All authors contributed to running the study, data collection, analysis, and interpretation. All members of the Writing Committee and trial Steering Committee read and approved the final manuscript. The ASCEND Study Collaborative Group members participated in the identification and recruitment of the study participants.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ahj.2017.12.006.

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Associations of Omega-3 Fatty Acid Supplement Use With Cardiovascular Disease Risks

Meta-analysis of 10 Trials Involving 77 917 Individuals

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IMPORTANCE Current guidelines advocate the use of marine-derived omega-3 fatty acids supplements for the prevention of coronary heart disease and major vascular events in people with prior coronary heart disease, but large trials of omega-3 fatty acids have produced conflicting results.

OBJECTIVE To conduct a meta-analysis of all large trials assessing the associations of omega-3 fatty acid supplements with the risk of fatal and nonfatal coronary heart disease and major vascular events in the full study population and prespecified subgroups.

DATA SOURCES AND STUDY SELECTION This meta-analysis included randomized trials that involved at least 500 participants and a treatment duration of at least 1 year and that assessed associations of omega-3 fatty acids with the risk of vascular events.

DATA EXTRACTION AND SYNTHESIS Aggregated study-level data were obtained from 10 large randomized clinical trials. Rate ratios for each trial were synthesized using observed minus expected statistics and variances. Summary rate ratios were estimated by a fixed-effects meta-analysis using 95% confidence intervals for major diseases and 99% confidence intervals for all subgroups.

MAIN OUTCOMES AND MEASURES The main outcomes included fatal coronary heart disease, nonfatal myocardial infarction, stroke, major vascular events, and all-cause mortality, as well as major vascular events in study population subgroups.

RESULTS Of the 77 917 high-risk individuals participating in the 10 trials, 47 803 (61.4%) were men, and the mean age at entry was 64.0 years; the trials lasted a mean of 4.4 years. The associations of treatment with outcomes were assessed on 6273 coronary heart disease events (2695 coronary heart disease deaths and 2276 nonfatal myocardial infarctions) and 12 001 major vascular events. Randomization to omega-3 fatty acid supplementation (eicosapentaenoic acid dose range, 226-1800 mg/d) had no significant associations with coronary heart disease death (rate ratio [RR], 0.93; 99% CI, 0.83-1.03; $P = .05$), nonfatal myocardial infarction (RR, 0.97; 99% CI, 0.87-1.08; $P = .43$) or any coronary heart disease events (RR, 0.96; 95% CI, 0.90-1.01; $P = .12$). Neither did randomization to omega-3 fatty acid supplementation have any significant associations with major vascular events (RR, 0.97; 95% CI, 0.93-1.01; $P = .10$), overall or in any subgroups, including subgroups composed of persons with prior coronary heart disease, diabetes, lipid levels greater than a given cutoff level, or statin use.

CONCLUSIONS AND RELEVANCE This meta-analysis demonstrated that omega-3 fatty acids had no significant association with fatal or nonfatal coronary heart disease or any major vascular events. It provides no support for current recommendations for the use of such supplements in people with a history of coronary heart disease.

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Supplemental content

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Observational studies in Western and Asian populations have reported that regular consumption of fish once or twice a week is associated with lower risks of death from coronary heart disease (CHD).[1,2] These observations, together with the lower rates of CHD in populations that consumed large amount of foods rich in very-long-chain polyunsaturated fatty acids containing omega-3 fatty acids have prompted interest in assessing whether consumption of marine-derived very-long-chain omega-3 fatty acids (abbreviated "omega-3 FA" in this article) may be protective for CHD.[3] These marine-derived omega-3 FAs include eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) found in fish and other seafood, but not alpha-linolenic acid, which is plant-derived.

The initial Diet and Reinfarction Trial-1 study[4] examined the associations of consumption of oily fish twice or more per week with CHD risk in men who had had a myocardial infarction and reported that fish consumption was associated with a significant reduction in fatal CHD and all-cause mortality but had no association with nonfatal myocardial infarction (MI) recurrence.[4] However, the subsequent Diet and Reinfarction Trial-2 study in men with angina reported that consumption of fish or fish oil supplements increased the risk of CHD death.[5] Subsequently, several large trials have reported conflicting results of the associations of supplementation with omega-3 FA supplements vs placebo or untreated controls on fatal and nonfatal vascular events.[6-16]

Ten large randomized trials[6-15] have been conducted comparing the associations of treatment with omega-3 FA supplementation vs placebo or no treatment for at least 12 months in populations with prior CHD, stroke, or high risk of cardiovascular disease (CVD). These trials have reported conflicting results for the associations of treatment with fatal CHD, nonfatal CHD, or other subtypes of CVD. The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione trial,[6] an open-label trial involving 11 323 recent survivors of MI, reported that patients who received supplementation with omega-3 FAs experienced a 10% reduced risk of major cardiovascular events compared with untreated controls. The Japan EPA Lipid Intervention Study (JELIS) trial, an open-label trial involving 18 645 participants with total cholesterol of 243.24 mg/dL (to convert to mmol/L, multiply by 0.0259) or greater, of whom only 20% with prior CHD, also reported[14] that supplementation with fish oil was associated with a 19% reduction in major CHD events (95% CI, 5%-31%). None of the other large placebo-controlled trials reported any significant association with CHD or mortality. Hence, it is unclear whether the discrepant results reflect different associations of omega-3 FAs with CHD subtypes, different outcomes in primary vs secondary prevention of CHD, increasing use of statins with better control of lipid levels, or an artifact of chance or bias in open-label trials. Previous meta-analyses of these trials of omega-3 FA supplements[16-18] appeared to suggest a significant beneficial association of omega-3 FAs with fatal CHD but not nonfatal CHD. However, these meta-analyses were constrained because they included trials of dietary advice to eat fish[17] or excluded trials that did not include a placebo control.[18]

The Omega-3 Treatment Trialists' Collaboration was established to conduct a collaborative meta-analysis based on aggregated study-level data obtained from the principal investigators of all large randomized clinical trials of omega-3 FA supplements for the prevention of cardiovascular disease, using a prespecified protocol and analysis plan. The aims of this meta-analysis were to assess the associations of supplementation with omega-3 FAs on (1) fatal CHD, nonfatal MI, stroke, major vascular events, and all-cause mortality and (2) major vascular events in prespecified subgroups.

Key Points

Question Does supplementation with marine-derived omega-3 fatty acids have any associations with reductions in fatal or nonfatal coronary heart disease in people at high risk of cardiovascular disease?

Findings This meta-analysis of 10 trials involving 77 917 participants demonstrated that supplementation with marine-derived omega-3 fatty acids for a mean of 4.4 years had no significant association with reductions in fatal or nonfatal coronary heart disease or any major vascular events.

Meaning The results provide no support for current recommendations to use omega-3 fatty acid supplements for the prevention of fatal coronary heart disease or any cardiovascular disease in people who have or at high risk of developing cardiovascular disease.

Methods

We performed a systematic search of randomized clinical trials in PubMed and Medline data sets, supplemented by manual hand-searching of reference lists from individual trials, review articles, or previous meta-analyses of omega-3 FAs and CVD (eFigure 1 in the Supplement). Search terms included "omega-3 FA," "omega-3 polyunsaturated fat," "fish oils," and "ω-3 FA" and "cardiovascular disease" or "coronary heart disease" or "stroke" (eFigure 1 in the Supplement). The prespecified eligibility criteria were randomized clinical trials of marine-derived very-long-chain omega-3 FA supplements vs placebo or open-label control, with a sample size of at least 500 participants and a scheduled duration of treatment of at least 1 year. All eligible trials required use of supplements, but no minimum daily dose of EPA or DHA was specified. The prespecified end points included nonfatal MI; death caused by CHD; ischemic, hemorrhagic, and unclassified stroke; coronary or noncoronary arterial revascularization events; major vascular events (a composite of first occurrence of nonfatal MI or death caused by CHD; nonfatal or fatal stroke; or any revascularization procedure); and all-cause mortality. Deaths caused by CHD included sudden cardiac deaths, deaths due to ventricular arrhythmias, and heart failure in patients with CHD, MI, or deaths occurring after coronary revascularization or heart transplant.

All included trials were also assessed for risk of bias. Individual trials had approval from their respective institutional review boards, and all participants provided written informed consent. No additional ethical approval was required for this meta-analysis.

Table. Characteristics of Included Trials

Study (Year)	Patients, No.	Dose of EPA/ DHA (mg/d)	Male, No (%)	Mean Trial Duration, y	Mean (SD) Age, y	No (%)			
						Prior CHD	Prior Stroke	Prior Diabetes	Statin Use
DOIT (2010)	563	1150/800	563 (100)	3	70 (3)	133 (23.6)	37 (6.6)	46 (8.2)	NA
AREDS-2 (2014)	4203	650/350	1816 (43.2)	4.5	74 (NA)	405 (9.7)	211 (5.0)	546 (13.0)	1866 (44.4)
SU.FOL.OM3 (2010)	2501	400/200	1987 (79.4)	4.7	61 (NA)	1863 (74.5)	638 (25.5)	440 (17.9)	2079 (83.1)
JELIS (2007)[a,b]	18 645	1800/NA	5859 (31.4)	4.6	61 (8)	NA	NA	3040 (16.3)	18 645 (100.0)
Alpha Omega (2010)	4837	226/150	3783 (78.2)	3.3	69 (6)	4837 (100.0)	345 (7.2)	1014 (21.0)	4122 (85.2)
OMEGA (2010)	3818	460/380	2841 (74.4)	1	64 (NA)	796 (22.5)	192 (5.5)	948 (27.0)	3566 (94.2)
R&P (2013)	12 505	500/500	7687 (61.5)	5	64 (NA)	Not stated (30)	594 (4.8)	7494 (59.9)	12 505 (100.0)
GISSI-HF (2008)	6975	850/950	5459 (78.3)	3.9	67 (11)	3614 (51.8)	346 (5.0)	1974 (28.3)	NA
ORIGIN (2012)	12 536	465/375	8150 (65.0)	6.2	64 (8)	8094 (64.6)	10 877 (86.8)	11 081 (88.4)	6739 (53.8)
GISSI-P[b] (1999)	11 334	850/1700	9658 (85.2)	3.5	59 (11)	11 334 (100.0)	NA	2139 (18.9)	NA
Total	77 917	NA	47 803 (61.4)	4.4	64	31 076/46 767 (66.4)	13 240/47 938 (27.6)	28 722 (36.9)	49 522 (83.4)

Abbreviations: AREDS-2, Age-Related Eye Disease Study 2; DOIT, Diet and Omega-3 Intervention Trial; GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Heart Failure; GISSI-P, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione; JELIS, Japan Eicosapentaenoic Acid (EPA) Lipid Intervention Study; NA, not available; OMEGA, Effect of Omega 3-Fatty Acids on the Reduction of Sudden Cardiac Death After Myocardial Infarction; ORIGIN, Outcome Reduction With Initial Glargine Intervention; SU.FOL.OM3, Supplémentation en Folates et Omega-3; R&P, Risk and Prevention Study.

[a] All trials used eicosapentaenoic acid and docosahexanoic acid supplements, with the exception of the JELIS trial (eicosapentaenoic acid only).

[b] All trials were blind, placebo-controlled randomized clinical trials with the exception of JELIS and GISSI-P, which were open-label without placebo.

A protocol outlining the eligibility criteria, prespecified analyses, and plans for publication together with standardized data request forms were sent to the principal investigators of all eligible trials. The study used the PRISMA guidelines for the conduct of meta-analysis of randomized trials.[19] Aggregated study-level (tabular) data were successfully obtained from 9 of the 10 trials (**Table**; eTable in the Supplement).[6-13,15] The JELIS trial[14] declined to participate in this collaboration, but the published results of the trial were sufficiently detailed to allow its inclusion in this study. Any discrepancies between data supplied and the published reports were clarified by contacting trial investigators.

Statistical Analysis

The association of treatment with outcomes in each trial was analyzed separately, and summary statistics were calculated for each trial. For each trial, we calculated the observed minus expected statistic (O–E) and its variance (V) from the number of patients who developed the relevant end point and the total number of patients in each treatment group, using standard formulas for 2 × 2 contingency tables. One O–E value from each trial was summed to produce a grand total (G), with variance (V) equal to the sum of their separate variances. The value exp(G/V) is Peto 1-step estimate of the rate ratio (RR), and its continuity-corrected 95% confidence interval is given by $\exp(G/V \pm [0.5/V + (1.96/\sqrt{V})])$.[20] Rate ratios are given with 95% CI for the overall results for major diseases and with 99% CI (which is calculated by replacing 1.96 in the formula above by 2.58) for the results of individual trials or subgroups of trials or subgroups of such major diseases. Heterogeneity between the different subgroups is assessed by first calculating $S–(G^2/V)$, where S is the sum of $(O–E)^2/V$ for each trial (or subgrouping), and then testing this statistic against a χ^2 distribution with the degrees of freedom equal to 1 fewer than the number of subgroups. The meta-analysis was repeated after excluding the JELIS trial,[14] since it tested EPA alone rather than the combination of EPA and DHA used in all other trials.[6-13,15]

Additional analyses of the primary outcomes assessed the associations of treatment with major vascular events in predefined subgroups, including age, sex, prior CHD, prior stroke, prior diabetes, blood lipids (total cholesterol, triglyceride, high-density lipoprotein, and calculated or measured low-density lipoprotein), prior use of statins, and trial design (open-label or blinded). In interpreting subgroup results, the chief emphasis was placed on the overall results, unless there was strong evidence of heterogeneity ($P < .001$). Sensitivity analyses compared the results of the Peto method with log-rank method in the 1 trial that had also provided individual participant data on all events.

Results

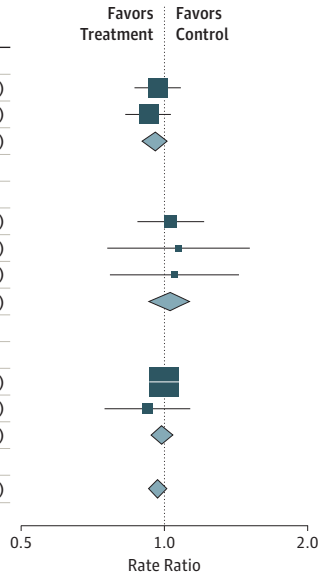
Characteristics of Individual Trials

Study level data were obtained on a total of the 10 trials[6-15] that met the inclusion criteria. A total of 77 917 participants were involved, and trials ranged in size from 563 to 18 645 participants (Table; eTable in the Supplement). Of the 10 trials, 8 had a double-blind design and used a placebo control, and 2 trials had an open-label design.[6,14] The risk of bias of the included trials was low, with exception of the 2 trials that did not use a placebo-treated control group[6,14] (eFigure 2 in the Supplement).

Combinations of polyunsaturated fatty acid ethyl esters of EPA and DHA were used in all but 1 trial,[14] which tested daily dose of 1800 mg EPA alone. The daily doses of EPA varied from 226 to 1800 mg/day, and DHA varied from 0 to 1700 mg/day. The mean duration of treatment in individual trials varied from 1.0 year to 6.2 years (weighted mean, 4.4 years).

Figure 1. Associations of Omega-3 Fatty Acids With Major Vascular Events

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Coronary Heart Disease			
Nonfatal myocardial infarction	1121 (2.9)	1155 (3.0)	0.97 (0.87-1.08)
Coronary heart disease death	1301 (3.3)	1394 (3.6)	0.93 (0.83-1.03)
Any	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12
Stroke			
Ischemic	574 (1.9)	554 (1.8)	1.03 (0.88-1.21)
Hemorrhagic	117 (0.4)	109 (0.4)	1.07 (0.76-1.51)
Unclassified/Other	142 (0.4)	135 (0.3)	1.05 (0.77-1.43)
Any	870 (2.2)	843 (2.2)	1.03 (0.93-1.13)
			P = .60
Revascularization			
Coronary	3044 (9.3)	3044 (9.3)	1.00 (0.93-1.07)
Noncoronary	305 (2.7)	330 (2.9)	0.92 (0.75-1.13)
Any	3290 (10.0)	3313 (10.2)	0.99 (0.94-1.04)
			P = .60
Any major vascular event	3230 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10



The number of events by allocated treatment are presented for individual trials and subgroups of trials; participants can contribute only once to subtotals and totals of major vascular events. Rate ratios for individual trials or subgroups of trials are indicated by squares and 99% CIs by horizontal lines. Overall totals and their 95% confidence intervals are represented by diamonds. The size of the squares and the diamonds are proportional to the statistical information conveyed.

Of the 77 917 participants, 47 803 (61.4%) were men, and the mean age at entry was 64 years. After accounting for missing data, about two-thirds of participants had a prior history of CHD (31 076/46 767; 66.4%), 13 240 of 47 938 (28%) had prior stroke, and 28 722 of the total 77 917 participants (37%) had prior diabetes. Among the 77 917 participants, there were a total of 12 001 major vascular events (15.4% of 77 917 participants), including 2276 incidents of nonfatal MI (2.9%), 2695 CHD deaths (3.5%), 1713 strokes (2.2%), and 6603 revascularization events (8.5%) during the study duration (eTable in the Supplement). Data were available on the association of treatment by prior use of statin therapy in 7 trials involving 49 522 participants.[8,10-12,14,15]

Associations of Omega-3 Fatty Acid Use With CHD and Major Vascular Events

Figure 1 shows that randomization to receive omega-3 FA supplementation had no significant association with the rate ratios (RRs) for any CHD event (RR, 0.96; 95% CI, 0.90-1.01; *P* = .12) and no significant association with RRs in subgroups of CHD events, including CHD death (RR, 0.93; 99% CI, 0.83-1.03; *P* = .05) and nonfatal myocardial infarction (RR, 0.97; 99% CI, 0.87-1.08; *P* = .40). Likewise, randomization of patients to an omega-3 FA supplementation regimen had no associations with the RRs for major vascular events (RR, 0.97; 95% CI, 0.93-1.01; *P* = .10), stroke (RR, 1.03; 95% CI, 0.93-1.13; *P* = .56), or revascularization events (RR, 0.99; 95% CI, 0.94-1.04; *P* = .61). This meta-analysis also showed no significant heterogeneity between the results of individual trials for nonfatal MI, CHD death, any CHD events, or all major vascular events (**Figure 2**). The association of omega-3 FA supplementation with major vascular events were unaltered after excluding the JELIS trial[14] (odds ratio [OR], 0.98; 95% CI, 0.94-1.02; *P* = .30) (eFigure 3 in the Supplement). Additional sensitivity analyses in 1 trial[12] that compared the results of the Peto method (O–E statistic) with the log-rank method demonstrated that analysis of individual participant and study-level data yielded identical results for association of omega-3 FA supplementation with major vascular events (eFigure 4 in the Supplement).

Associations of Omega-3 Fatty Acid Use With Major Vascular Events in Prespecified Subgroups

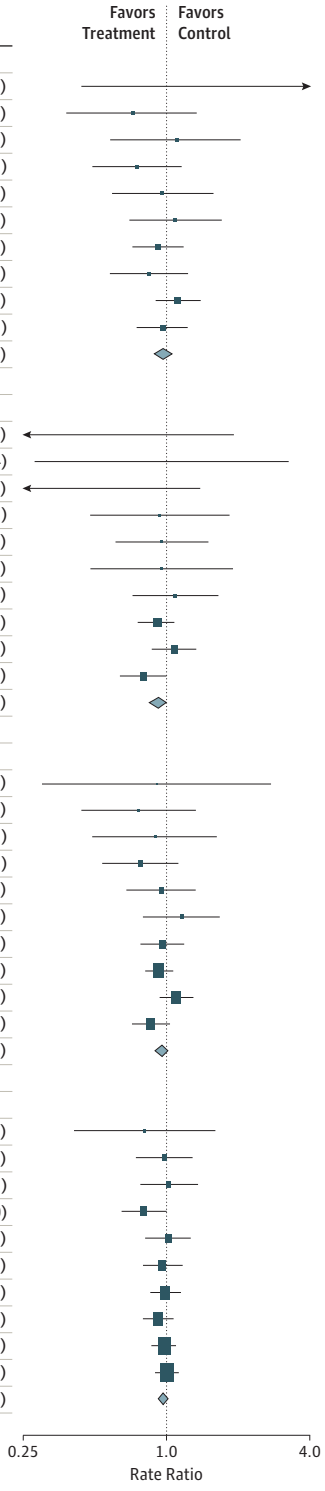
Figure 3 shows that after adjustment for multiple testing, randomization of patients to study arms involving supplementation by omega-3 FAs had no significant association with major vascular events in any of the prespecified subgroups, including those defined by sex, history of CHD, history of diabetes, pretreatment levels of total cholesterol, high-density lipoprotein levels, low-density lipoprotein levels, triglyceride levels, or prior use of statin therapy. However, there was some evidence of heterogeneity in the associations of omega-3 FAs with major vascular events by age (unadjusted *P* = .02) and by history of stroke (*P* = .06), respectively. While it was not possible to assess the associations of treatment with race, the results were unaltered after exclusion of the JELIS trial,[14] which was conducted in a Japanese population only (eFigure 3 in the Supplement).

Associations of Omega-3 Fatty Acid Use With CHD Events by Study Design

Figure 4 demonstrates that randomization of patients to receive omega-3 FAs had no significant association with their experience of nonfatal MI, CHD death, or overall CHD in trials that used either an open-label and blind design. However, there was some evidence of heterogeneity between the results of open-label trials vs blind trials for all participants with CHD (open-label trials: RR, 0.85; 99% CI, 0.72-0.99; *P* = .01; blinded trials: RR, 0.99; 99% CI, 0.91-1.07; *P* = .69; heterogeneity *P* = 0.03), but not for either fatal CHD or nonfatal MI, respectively. Overall, the results of this meta-analysis demonstrated no significant association of supplementation with omega-3 FAs for a mean duration of 4.4 years with the risk of fatal CHD, nonfatal MI, any CHD, or any major vascular events in the full study population and in all relevant subgroups.

Figure 2. Associations of Omega-3 Fatty Acids With Subtypes of Coronary Heart Disease and Major Vascular Events, by Trial

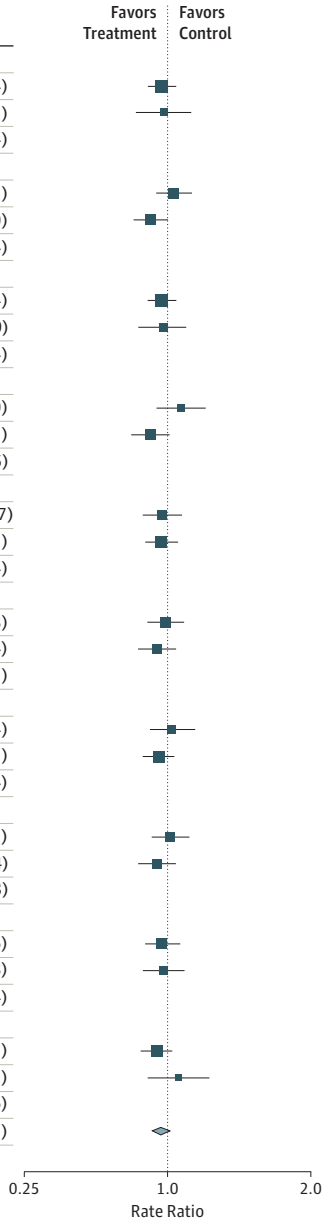
Source	No. of Events (%)		Rate Ratios (CI)
	Treatment	Control	
Nonfatal myocardial infarction			
DOIT	8 (2.8)	4 (1.4)	1.97 (0.44-8.85)
AREDS-2	30 (1.4)	40 (1.9)	0.72 (0.38-1.33)
Su.Fol.OM3	32 (2.6)	29 (2.3)	1.10 (0.56-2.15)
JELIS	62 (0.7)	83 (0.9)	0.75 (0.49-1.05)
Alpha Omega	56 (2.3)	59 (2.4)	0.96 (0.59-1.56)
Omega	74 (3.8)	67 (3.6)	1.08 (0.70-1.69)
R&P	228 (3.7)	248 (4.0)	0.92 (0.72-1.17)
GISSI-HF	92 (2.6)	108 (1.3)	0.82 (0.58-1.22)
ORIGIN	316 (5.0)	285 (4.5)	1.11 (0.90-1.38)
GISSI-P	223 (3.9)	233 (4.1)	0.96 (0.75-1.22)
All	1121 (2.9)	1155 (3.0)	0.97 (0.89-1.05)
			P = .43
Coronary heart disease death			
DOIT	3 (1.1)	8 (2.8)	0.39 (0.08-1.90)
ALREDS-2	9 (0.4)	9 (0.4)	0.96 (0.28-3.24)
Su.Fol.OM3	5 (0.4)	13 (1.0)	0.41 (0.12-1.38)
JELIS	29 (0.3)	31 (0.3)	0.93 (0.48-1.82)
Alpha Omega	67 (2.8)	71 (2.9)	0.95 (0.61-1.49)
OMEGA	28 (1.5)	29 (1.5)	0.95 (0.48-1.89)
R&P	82 (1.3)	76 (1.2)	1.08 (0.72-1.64)
GISSI-HF	512 (14.7)	555 (15.9)	0.91 (0.76-1.07)
ORIGIN	319 (5.1)	296 (4.7)	1.08 (0.87-1.33)
GISSI-P	247 (4.4)	306 (5.4)	0.80 (0.64-1.00)
All	1301 (3.3)	1394 (3.6)	0.93 (0.85-1.00)
			P = .05
Any Coronary heart disease			
DOIT	11 (3.9)	12 (4.3)	0.91 (0.30-2.73)
AREDS-2	39 (1.8)	49 (2.4)	0.76 (0.44-1.32)
Su.Fol.OM3	37 (3.0)	41 (3.3)	0.90 (0.49-1.62)
JELIS	88 (0.9)	113 (1.2)	0.78 (0.54-1.12)
Alpha Omega	120 (5.0)	128 (5.3)	0.95 (0.68-1.32)
OMEGA	112 (5.8)	96 (5.1)	1.16 (0.80-1.67)
R&P	130 (5.0)	324 (5.2)	0.96 (0.78-1.18)
GISSI-HF	1309 (37.5)	1360 (39.1)	0.93 (0.82-1.06)
ORIGIN	635 (10.1)	580 (9.3)	1.10 (0.94-1.29)
GISSI-P	424 (7.5)	485 (8.6)	0.86 (0.72-1.03)
All	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12
Major vascular events			
DOIT	29 (10.3)	35 (12.5)	0.81 (0.41-1.60)
AREDS-2	213 (9.9)	208 (10.1)	0.98 (0.75-1.28)
Su.Fol.OM3	216 (17.2)	211 (16.9)	1.02 (0.78-1.35)
JELIS	262 (2.8)	324 (3.5)	0.80 (0.65-1.00)
Alpha Omega	332 (13.8)	331 (13.6)	1.02 (0.82-1.26)
OMEGA	534 (27.7)	541 (28.6)	0.96 (0.80-1.16)
R&P	733 (11.7)	745 (11.9)	0.99 (0.86-1.14)
GISSI-HF	783 (22.4)	831 (23.9)	0.92 (0.80-1.07)
ORIGIN	1276 (20.3)	1295 (20.7)	0.98 (0.87-1.09)
GISSI-P	1552 (27.4)	1550 (27.3)	1.00 (0.90-1.12)
All	5930 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10



Symbols and conventions as in Figure 1. Study names are AREDS-2, Age-Related Eye Disease Study 2; DOIT, Diet and Omega-3 Intervention Trial; GISSI-HF, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Heart Failure; GISSI-P, Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico-Prevenzione; JELIS, Japan Eicosapentaenoic Acid (EPA) Lipid Intervention Study; OMEGA, Effect of Omega 3-Fatty Acids on the Reduction of Sudden Cardiac Death After Myocardial Infarction; ORIGIN, Outcome Reduction With Initial Glargine Intervention; Su.Fol.Om3, Supplémentation en Folates et Omega-3; R&P, Risk and Prevention Study. Rate ratios for individual trials or subgroups of trials are indicated by squares and the 99% CIs by the horizontal lines. Overall totals and their 95% confidence intervals are represented by diamonds. Arrowheads indicate error bars that extend beyond the area shown. Heterogeneity between all trials (χ^2_9 in all cases) for nonfatal myocardial infarction, coronary heart disease death, any coronary heart disease, and major vascular events were 10.18 (*P* = .34), 12.3 (*P* = .20), 12.92 (*P* = .17), and 7.68 (*P* = .57), respectively.

Figure 3. Associations of Omega-3 Fatty Acids With Major Vascular Events, in Prespecified Subgroups

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Sex			
Male	3989 (23.2)	4040 (23.7)	0.97 (0.91-1.04)
Female	946 (15.1)	962 (15.5)	0.98 (0.86-1.12)
Missing	995 (6.4)	1069 (6.9)	0.93 (0.82-1.04)
Age, y			
<65	2528 (21.8)	2432 (21.3)	1.03 (0.95-1.12)
≥65	2407 (20.5)	2570 (22.0)	0.92 (0.85-1.00)
Missing	955 (6.4)	1064 (6.9)	0.93 (0.82-1.04)
Prior coronary heart disease			
Yes	3499 (22.9)	3550 (22.9)	0.97 (0.91-1.04)
No	1435 (18.5)	1448 (19.0)	0.98 (0.87-1.09)
Missing	996 (6.4)	1073 (6.9)	0.92 (0.82-1.04)
Prior stroke			
Yes	1332 (21.1)	1262 (20.0)	1.07 (0.95-1.20)
No	2046 (18.3)	2185 (19.6)	0.92 (0.84-1.01)
Missing	2552 (12.0)	2624 (12.3)	0.97 (0.89-1.05)
Prior diabetes			
Yes	2087 (22.8)	2111 (23.3)	0.97 (0.89-1..07)
No	2846 (20.1)	2889 (20.5)	0.97 (0.90-1.05)
Missing	997 (6.4)	1071 (6.9)	0.93 (0.82-1.04)
Total cholesterol			
<193.1	2326 (21.5)	2315 (21.5)	0.99 (0.91-1.08)
≥193.1	2346 (22.8)	2441 (23.7)	0.95 (0.87-1.04)
Missing	1258 (7.1)	1315 (7.4)	0.94 (0.84-1.05)
High-density lipoprotein			
<38.6	1532 (25.4)	1512 (24.8)	1.02 (0.92-1.14)
≥38.6	2961 (20.7)	3041 (21.5)	0.96 (0.89-1.03)
Missing	1437 (7.8)	1518 (8.2)	0.94 (0.85-1.04)
Low-density lipoprotein			
<115.8	2096 (21.0)	2051 (20.6)	1.01 (0.93-1.11)
≥115.8	2295 (23.2)	2385 (24.2)	0.95 (0.87-1.04)
Missing	1539 (8.1)	1635 (8.6)	0.93 (0.84-1.03)
Triglycerides			
≤150.4	2626 (21.3)	2638 (21.7)	0.97 (0.90-1.06)
>150.4	1998 (23.4)	2061 (23.7)	0.98 (0.89-1.08)
Missing	1306 (7.3)	1372 (7.7)	0.94 (0.84-1.04)
Prior statin use			
Yes	2824 (11.4)	2959 (11.9)	0.95 (0.88-1.02)
No	742 (15.4)	696 (14.9)	1.05 (0.91-1.22)
Missing	2364 (25.0)	2416 (25.6)	0.97 (0.89-1.06)
All	5930 (15.2)	6071 (15.6)	0.97 (0.93-1.01)
			P = .10



Symbols and conventions as in Figure 1. Total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides were measured in mg/dL (to convert cholesterol to mmol/L, multiply by 0.0259; triglycerides, multiply by 0.0113). Heterogeneity between all trials (χ^2_1 in all cases) was 0.04 (P = .84) for sex, 5.59 (P = .02) for age, 0.0 (P = .96) for prior coronary heart disease, 7.03 (P = .01) for prior stroke, 0.0 (P > .99) for prior diabetes, 0.87 (P = .35) for total cholesterol, 1.56 (P = .21) for high-density lipoprotein, 1.8 (P = .18) for low-density lipoprotein, 0.02 (P = .89) for triglycerides, and 2.55 (P = .11) for prior statin use.

Associations of Omega-3 Fatty Acid Use With All-Cause Mortality

Randomization to omega-3 FA intervention had no significant association with RRs of all-cause mortality (RR, 0.96; 95% CI, 0.92-1.01; P = .16). Further information is presented in eFigure 5 in the Supplement.

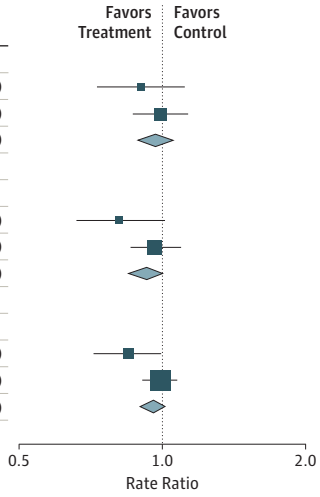
Discussion

This meta-analysis of 10 randomized clinical trials, involving 77 917 participants, demonstrated that randomization to trial arms with omega-3 FA supplementation for a mean of 4.4 years had no significant effect on either of fatal CHD, nonfatal MI, stroke, revascularization events, or any major vascular events. Importantly, this meta-analysis also demonstrated no significant effect on major vascular events in any particular subgroups, including prior vascular disease, diabetes, lipid levels, or statin use. Likewise, the present meta-analysis showed no significant association of omega-3 FA supplementation with all-cause mortality or cancer (data not shown). Moreover, the overall results were unaltered after exclusion of the JELIS trial,[14] which tested the effects of EPA alone rather than EPA and DHA combined.

The chief strength of this study was the availability of study-level data extracted by the trial principal investigators for all prespecified outcomes in this meta-analysis (with the exception of the JELIS trial,[14] in which the published data

Figure 4. Associations of Omega-3 Fatty Acids With Fatal and Nonfatal Vascular Events, by Trial Design

Source	No. of Events (%) Treatment	Control	Rate Ratios (CI)
Nonfatal myocardial infarction			
Open	285 (1.9)	316 (2.1)	0.90 (0.73-1.11)
Blind	836 (3.5)	839 (3.5)	0.99 (0.87-1.13)
All	1121 (2.9)	1155 (3.0)	0.97 (0.89-1.05)
			P = .40
Coronary heart disease death			
Open	276 (1.8)	337 (2.2)	0.81 (0.66-1.01)
Blind	1025 (4.3)	1057 (4.4)	0.96 (0.86-1.09)
All	1301 (3.3)	1394 (3.6)	0.93 (0.85-1.00)
			P = .05
Any coronary heart disease			
Open	512 (3.4)	598 (4.0)	0.85 (0.72-0.99)
Blind	2573 (10.7)	2590 (10.8)	0.99 (0.91-1.07)
All	3085 (7.9)	3188 (8.2)	0.96 (0.90-1.01)
			P = .12



Symbols and conventions as in Figure 1. Heterogeneity between trial designs (χ^2_1 in all cases) was 1.05 (*P* = .31) for nonfatal myocardial infarction, 3.26 (*P* = .07) for coronary heart disease death, and 4.81 (*P* = .03) for any coronary heart disease.

were used). The inclusion criteria and vascular disease outcomes differed from previous meta-analyses of the published results.[16-18] The present meta-analysis had a low risk of selection bias or confounding because it did not include trials testing the effects of dietary advice to eat fish nor trials that were either too small or insufficient in treatment duration. In contrast with previous meta-analyses, the present meta-analysis also examined effects of supplementation with omega-3 FA supplementation in prespecified subgroups of major vascular events by history of disease, history of diabetes, lipid levels, or statin use.

The reasons for the discrepant results of the previous trials of omega-3 FA supplementation on fatal and nonfatal CHD events are unclear. In contrast with the null findings for most trials, the GISSI-Prevenzione trial[6] reported a 14% reduction in major vascular events, chiefly owing to an 11% reduction in cardiac deaths. But the JELIS trial reported a 19% (95% CI, 5%-31%) reduction in major CHD events (albeit based on only 586 events), chiefly owing to a reduction in nonfatal CHD events.[14] It is unclear whether differences in inclusion criteria for prior diseases, concomitant use of statins, or other secondary prevention treatments may explain some of the conflicting results of individual trials.

For example, previous reports had suggested that the effects of omega-3 FA use may vary by patients' prior use of statin medications.[21,22] The Alpha Omega trial reported that use of low-dose omega-3 FAs reduced the risk of major vascular events in patients with prior MI who were not treated with statin medications.[22] However, the present meta-analysis demonstrated no heterogeneity in the effects of omega-3 FA supplementation on CHD death or nonfatal MI between the individual trials and reported no differences in the effects of omega-3 FAs on major vascular events by subgroups of those with or without prior cardiovascular disease or diabetes; those with lipid levels less than or greater than specified cutoff points; or those who had histories of statin therapy. The results of the present meta-analysis were also unaltered by the exclusion of the JELIS trial,[14] in which all participants were also treated with statin medications.

The present meta-analysis reported weak evidence of heterogeneity between the results of open-label vs blind trials for any CHD. This may reflect reporting bias, chance, or greater compliance in the open-label trials than in the blinded trials.

Previous meta-analyses of omega-3 FA trials,[16-18] which were limited by being incomplete, including trials of dietary advice to increase fish consumption,[16,17] or failure to distinguish the effects on a wide range of subtypes of CVD.[16-18,23,24] In contrast, the present meta-analysis demonstrated that omega-3 FA supplementation had no significant effect on fatal CHD or any other CVD subtypes. Moreover, the conclusions of the present meta-analysis are consistent with those of a 2016 report[24] for the US Agency for Healthcare Research and Quality that also involved study-level data from the same 10 large trials for prevention of major vascular events, and concluded that omega-3 FA supplementation had no association with the risk of major vascular events, all-cause mortality, sudden cardiac death, or revascularization. In contrast with this report, the present article was able to assess effects on a wide range of subtypes of CVD and on major vascular events in all relevant subgroups.[24]

Limitations

This meta-analysis had several limitations. The protocol did not prespecify assessment of the effects of treatment by smoking status or by site-specific cancer incidence. An additional limitation of this meta-analysis involved the use of aggregated study-level data rather than individual-level data. A meta-analysis of individual participant data may have a greater chance of detecting effects of omega-3 FA supplements on subtypes of fatal CHD events (ie, sudden death or ventricular arrhythmias) in a wider range of subgroups. However, the overall null results of the present meta-analysis, which assesses effects on a wide range of prespecified CVD subtypes, provides little encouragement for such an approach. In addition, sensitivity analyses

using data from 1 trial[12] that also provided data on all individual participants indicated identical effect estimates and 99% CI for analyses using both O-E and log-rank methods.

The 95% CI in the present meta-analysis of 10 trials, involving 77 917 high-risk individuals, 12 001 major vascular events, and 6273 CHD events, cannot exclude a 7% lower risk of major vascular events and a 10% lower risk of CHD associated with omega-3 FA supplements. Several ongoing large randomized trials involving a total of 54 354 additional participants (A Study of Cardiovascular Events in Diabetes [ASCEND],[25] n = 15 480; VITamin D and OmegA-3 TriaL [VITAL],[26] n = 25 874; STatin Residual risk reduction with EpaNova in hiGh CV risk patienTs with Hypertriglyceridemia [STRENGTH],[27] n = 13 000 and Reduction of Cardiovascular Events With EPA-Intervention Trial [REDUCE-IT], n = 8000) will provide additional evidence about the associations of omega-3 FA supplementation with the risk of major vascular events, any CHD, and subtypes of fatal and nonfatal CHD. Importantly, the STRENGTH[27] and REDUCE-IT trials will test the effects on major vascular events of much higher doses of omega-3 FAs (3-4 g/d), which will lower plasma levels of triglycerides.

Conclusions

The 2016 European Society of Cardiology and European Atherosclerosis Society guidelines for prevention of cardiovascular disease[28] indicated that it is debatable whether omega-3 FAs may exert a protective effect, and the 2016 guidelines on the management of dyslipidaemia[29] indicated that more evidence on the efficacy of omega-3 FA supplements for prevention of clinical outcomes is needed to justify their prescription. In contrast, the American Heart Association recommended[30] that the use of omega-3 FAs for prevention of CHD is probably justified in individuals with prior CHD and those with heart failure and reduced ejection fractions. However, the results of the present meta-analysis provide no support for the recommendations to use approximately 1 g/d of omega-3 FAs in individuals with a history of CHD for the prevention of fatal CHD, nonfatal MI, or any other vascular events. The results of the ongoing trials are needed to assess if higher doses of omega-3 FAs (3-4 g/d) may have significant effects on risk of major vascular events.

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