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Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chain omega-3 fatty treatment in non-alcoholic fatty liver disease

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) represents a range of liver conditions from simple fatty liver to progressive end stage liver disease requiring liver transplantation. NAFLD is common in the population and in certain sub groups (e.g. type 2 diabetes) up to 70% of patients may be affected. NAFLD is not only a cause of end stage liver disease and hepatocellular carcinoma, but is also an independent risk factor for type 2 diabetes and cardiovascular disease. Consequently, effective treatments for NAFLD are urgently needed.
Objectives: The WELCOME study is testing the hypothesis that treatment with high dose purified long chain omega-3 fatty acids will have a beneficial effect on a) liver fat percentage and b) two histologically validated algorithmically-derived biomarker scores for liver fibrosis.
Design: In a randomised double blind placebo controlled trial, 103 participants with NAFLD were randomised to 15–18 months treatment with either 4 g/day purified long chain omega-3 fatty acids (Omacor) or 4 g/day olive oil as placebo. Erythrocyte percentage DHA and EPA enrichment (a validated proxy for hepatic enrichment) was determined by gas chromatography. Liver fat percentage was measured in three discrete liver zones by magnetic resonance spectroscopy (MRS). We also measured body fat distribution, physical activity and a range of cardiometabolic risk factors.
Methods: Recruitment started in January 2010 and ended in June 2011. We identified 178 potential participants, and randomised 103 participants who met the inclusion criteria. The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165; www.clinicalTrials.gov registration number NCT00760513).

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is defined by the presence of steatosis (characterised by lipid droplets) in more than 5% of hepatocytes [1]. NAFLD may progress over time to non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma (HCC) and end stage liver disease

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[1]. Since the prevalence of major risk factors for NAFLD (obesity, insulin resistance and type 2 diabetes) has increased worldwide, NAFLD is rapidly becoming an important problem for patients and health care professionals. Progression of liver disease is often silent and NAFLD may be overlooked as it presents with other serious conditions such as type 2 diabetes and cardiovascular disease (CVD).

NAFLD is often associated with obesity and other features of the metabolic syndrome (MetS) and strategies that produce weight loss can be very effective in lowering liver fat content, although whether such strategies are effective in improving other components of NAFLD is uncertain [2,3]. Although treatments are available for individual components of the MetS (e.g. glucose intolerance, increased blood pressure, dyslipidaemia, and obesity) [4], whether these treatments are effective in NAFLD is unproven. Studies that have attempted to treat NAFLD by targeting specific pathways in the pathogenesis of NAFLD have to date met with limited success. For example, the results of trials testing treatment with thiazolidinediones [5] and anti-oxidants such as vitamin E [6], that have focussed primarily on modifying pathways affecting insulin resistance [7], oxidative stress [6] and lipid metabolism [8] have produced variable results. These relatively small trials have also generated controversy, not least because any positive effects of treatment are limited by side-effects and concerns about long term safety of glitazones [9,10] and the high dose of vitamin E required [11].

Typically, within a Westernized diet, omega-6 fatty acid consumption is markedly greater than omega-3 fatty acid consumption [12]. The potential consequences of an increased ratio of omega-6 to omega-3 fatty acid consumption are increased production of pro-inflammatory arachidonic acid-derived eicosanoids and impaired regulation of hepatic and adipose function, predisposing to NAFLD. Recently, several studies have shown that a diet with an inadequate intake of “omega-3 essential fatty acids” is associated with metabolic syndrome [13], cardiovascular disease [14], dyslipidaemia and fatty liver disease [12,15].

To assess whether high dose purified long chain omega-3 fatty acids have a beneficial effect on liver fat and biomarkers for liver fibrosis in people with NAFLD, we designed a randomised, double blind placebo controlled trial: the WELCOME study (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy). The WELCOME study is testing the hypothesis that treatment with high dose purified long chain omega-3 fatty acids will have a beneficial effect on a) liver fat percentage and b) two histologically validated algorithmically-derived biomarker scores for liver fibrosis.

2. Methods

2.1. Study design

The WELCOME study (Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy; www.clinicalTrials.gov registration number NCT00760513) is a randomized double blind placebo controlled trial testing the effects of high dose omega-3 fatty acid ethyl esters in participants with NAFLD recruited from six hospitals in the South of England. The WELCOME study was approved by the local ethics committee (REC: 08/H0502/165) and the primary end points of the study were to test whether treatment with purified long chain omega-3 fatty acids ethyl esters over 15–18 months decreases mean liver fat percentage from three discrete liver zones, measured by a magnetic resonance spectroscopy (MRS) scan; and improves two validated algorithmically-derived liver fibrosis scores. The primary end points of the study will be tested using intention-to-treat (ITT) analysis and per protocol analysis.

2.2. Treatments groups

We identified 178 potential participants, and randomised 103 participants who met the inclusion criteria. Participants were block randomised to either omega-3 fatty acid ethyl esters 4 g/day or placebo (olive oil) 4 g/day for a minimum of 15 months and a maximum of 18 months of treatment. To date, the few trials that have tested the effects of omega-3 long chain fatty acids in adults [16–22] and in children with NAFLD [23] have used doses of omega-3 fatty acids between 250 mg DHA and 9 g of fish oils. However, since our trial was a proof of concept study to test the highest licensed (and safe) dose of Omacor/Lovaza that is used in the treatment of hypertriglyceridemia, we chose to test that dose (i.e. 4 g/day) in NAFLD. Olive oil that contained ~67% oleic acid, ~15% linoleic acid, ~15% palmitic acid, ~2% stearic acid and ~1% alpha linolenic acid was chosen as the placebo. We chose 4 g/day olive oil because oleic acid is common within the diet, and this dose of olive oil was isocaloric with the intervention. This dose and choice of placebo is the same as has been used by others, testing the effects of 8 weeks treatment with 4 g/day omega-3 oil (4 × 1000-mg capsules of 56% docosahexaenoic acid and 27% eicosapentaenoic acid) on NAFLD in women with polycystic ovarian syndrome [22]. In many of the trials testing the effects of omega-3 fatty acids in NAFLD to date, the exact nature of the placebo is unclear.

2.2.1. Active group

51 participants were randomised to receive 4 g per day of omega-3 fatty acid ethyl esters: Omacor 1 g contains eicosapentaenoic acid (EPA) 460 mg and docosahexaenoic acid (DHA) 380 mg as ethyl esters (Omacor/Lovaza, Pronova, Sandefjord, Norway; approved by the Food and Drug Administration and the European Medicines Agency for the treatment of hypertriglyceridemia).

2.2.2. Placebo group

52 participants were randomised to receive 4 g per day of olive oil.

Patients were randomised according to standardized procedures (computerized block randomisation) by a research pharmacist at University Hospital Southampton NHS Foundation Trust. Simple randomisation in blocks of four, either to trial medication or placebo was used. During the study period there was no specific intervention to advocate change in lifestyle that might influence NAFLD. As part of usual patient care in the region, all patients with NAFLD attending clinic are routinely given general healthy lifestyle advice [24].

2.3. Outcome measures

The primary outcomes are to assess: a) decrease in percentage liver fat measured by MRS scan and b) improvement in two histologically validated algorithmically-derived biomarker scores for liver fibrosis [25,26]. The sensitivity and specificity of these two biomarker scores for the diagnosis of fibrosis in NAFLD has recently been summarised [27]. The fibrosis score using TIMP-1, HA and PIIINP [28] has excellent performance (AUROC 0.9) for the diagnosis of severe fibrosis, good performance (AUROC 0.82) for moderate fibrosis, and fair performance (AUROC 0.76) for no fibrosis. Sensitivity and specificity were 89% and 96% respectively for diagnosing and excluding advanced fibrosis. In contrast, the NAFLD fibrosis score [26] has good sensitivity (82%) for diagnosing no fibrosis, but poorer sensitivity for diagnosing advanced fibrosis (51%). This test has excellent specificity for excluding advanced fibrosis (98%) and moderate to good specificity for excluding no fibrosis (77%). Consequently, because these two scores provide different sensitivities and specificities according to the amount of fibrosis that is present, we will report the effects of the intervention on both fibrosis scores.

Additionally there are several hypothesis-generating secondary outcomes. These are to test the effect of the intervention on changes in a) measures of insulin sensitivity, b) microvascular function, c) neurological function, d) carotid intima-media thickness, e) echocardiographic parameters, f) plasma cardiovascular risk markers, g) ankle brachial pressure index and h) pulse wave velocity.

2.4. Sample size calculations

Based on the small amount of inconclusive published literature at the time of the design of the protocol in 2007/2008, we estimated that a 15% decrease in liver fat may result from omega-3 fatty acid treatment. Subsequently, in 2012 a systematic review and meta-analysis of omega-3 fatty acid supplementation in the treatment of NAFLD produced a similar estimate of the effect size of treatment to decrease liver fat [8]. With an estimated value for sigma of 0.3, and an alpha of 0.05, a sample size of 50 participants in each arm would give 94% power to detect this difference in liver fat. Allowing for 15% drop out, there would be 90% power to detect this effect. The exact change in liver fibrosis score [25] that equates to a clinically meaningful change is uncertain, but we assumed that a 0.6–1.0 unit change in fibrosis score might be clinically significant [25]. To detect a 1.0 unit change, a total of 32 patients would be required, with 80% power at the 5% significance level. To detect a 0.6 change in score a total of 100 patients would be required, with 80% power at the 5% significance level. Since we speculated that an ideal change in fibrosis score [25] would likely be between 0.6 and 1.0, we estimated that 100 patients should be recruited for the study, allowing for a 15% drop out of participants from the study. A sample size calculation was not undertaken for the NAFLD fibrosis score [29].

2.5. Statistical analysis

Statistical analyses will be performed using SPSS for Windows. The main outcomes that will be tested are: a decrease in liver fat percentage, and improvement in two histologically-validated, algorithmically derived liver fibrosis biomarker scores [25,26], with omega-3 tissue enrichment due to Omacor treatment. The normal distribution of the data will be tested by the Shapiro–Wilk and Kolmogorov–Smirnov tests. We will adjust for potential confounders (e.g. age, sex, alcohol consumption, and weight loss, increased physical activity and change in diet during the study).

Data will be reported as mean and standard deviation for normally distributed variables, or as median and interquartile range for non-normally distributed variables. Comparison of mean values of continuous variables from the two groups will be undertaken using paired *t*-test and when variables are not normally distributed, the Wilcoxon Signed rank test will be undertaken. The Pearson and Spearman rank correlation coefficients will be used to investigate factors associated with liver fat percentage at baseline. Multivariable linear regression will be undertaken with liver fat percentage and each of the algorithmically-derived liver fibrosis scores, as the respective outcomes to test the effects of tissue omega-3 fatty acid enrichment due to the intervention. To test the effects of omega 3 fatty acid enrichment due to Omacor, we will include in the regression model as an explanatory variable: 1) Percentage enrichment of EPA or DHA, measured in erythrocytes as the difference between baseline and end of study percentages; and separately 2) allocation to Omacor or to placebo, as a binary indicator variable.

Where variables are not normally distributed, logarithmic transformation will be undertaken to normalise the distribution. In order to adjust the regression models for potential confounding factors such as change in weight, change in diet, or change in physical activity during the period of the intervention, we will calculate the mathematical difference between baseline and end of study data for weight, prudent diet score [24] (see below) and metabolic equivalent of tasks (METs) (as a measure of energy expenditure) (see below) [30]. Analyses will also include adjustment for NAFLD severity (as indicated by the measurement of liver fat percentage, NAFLD fibrosis biomarker scores and Cytokeratin-18) and BMI.

We will undertake both intention-to-treat (ITT) analysis and per protocol analysis. For ITT analyses, complete case analysis will be undertaken with exclusion of cases with missing data. Analytic comparisons of baseline data in participants with missing end of study data and participants with complete data will be undertaken. Per protocol analysis will include all participants who consumed $\geq$50% of their supplement in the time period from randomization to final visit and had a baseline average liver fat percentage $\geq$5% for the mean of the three liver zones. A p-value of <0.05 will be considered statistically significant for all analyses.

No interim analyses during the trial were planned.

3. Conduct of the trial

3.1. Patient selection

We identified a cohort of people with fatty liver disease from secondary care clinics, diagnosed on either radiological or biopsy criteria for NAFLD.

3.2. Inclusion and exclusion criteria

Briefly, the inclusion criteria for participation in the study were age > 18 years and: 1) a recent (<3 years) histological diagnosis of non-alcoholic steatosis or steatohepatitis in keeping with NAFLD [31]; or 2) steatosis diagnosed by ultrasound, CT or magnetic resonance imaging in a patient who also had either diabetes and/or features of the metabolic syndrome. All participants underwent an assessment of liver fat percentage by MRS examination at recruitment, to establish the baseline liver fat percentage at entry into the trial. Exclusion criteria included known other causes of liver disease (e.g. hepatitis A, B or C, primary biliary cirrhosis, Wilson's disease, autoimmune hepatitis and haemochromatosis). These conditions were excluded with blood tests. Subjects were also excluded if alcohol consumption was >35 units per week for women and >50 units per week for men. These thresholds of alcohol consumption were chosen because at the time of the study design alcohol intake above these thresholds was considered harmful to the liver [32]. Nonetheless, at recruitment, only one man was consuming >21 units of alcohol per week and one woman was consuming >14 units per week.

Additional exclusion criteria were: decompensated acute or chronic liver disease; cirrhosis; pregnancy or breast feeding; and hypersensitivity to Omacor, soya or any of the excipients.

3.3. Recruitment

At University Hospital Southampton NHS Foundation Trust, potential participants with a diagnosis of NAFLD established as part of their attendance at hospital clinic were contacted by means of personal contact from the research team. Contact occurred at their Hospital Clinic attendance or by letter of invitation from the research team.

Outside Southampton, at Poole Hospital NHS Trust, Portsmouth Hospitals NHS Trust, Royal Bournemouth and Christchurch Hospitals NHS Trust, Basingstoke and North Hampshire NHS Trust, Winchester and Eastleigh Healthcare NHS Trust, and the Isle of Wight NHS Trust, our collaborators (medical doctors responsible for the care of people with NAFLD) acted as 'post boxes' and informed potential participants about the study providing them with a patient information sheet and asking them if interested to get in touch with the research team members in Southampton listed on the patient information sheet.

3.4. Randomisation

All participants gave written, informed consent. After completion of baseline study tests, participants were block randomised in groups of four to either omega-3 fatty acids 4 g/day or placebo 4 g/day for 15–18 months treatment. Participants were advised to take 2 g b.d.; but if participants preferred to take 4 g o.d., that was considered acceptable.

3.5. Study visit overview and follow-up

During the trial, participants attended the clinical research facility (Wellcome Trust Clinical Research Facility at University Hospital Southampton NHS Foundation Trust) at 6 months and at 12 months for simple haematological and biochemical blood tests that were undertaken as a safety check (Fig. 1). Omacor and placebo capsules were of similar appearance and taste; capsules were stored at the hospital pharmacy and dispensed at baseline visit and at 6 and 12 months follow-up. We evaluated the compliance to the treatment by capsule count at every visit, and serious adverse events were recorded. At each visit, participants were specifically asked about any adverse events. We also reviewed medication records during the study. After completing 15–18 months randomisation to either Omacor or placebo, subjects returned for end of study investigations that included a repeat measurement of liver fat by MRS

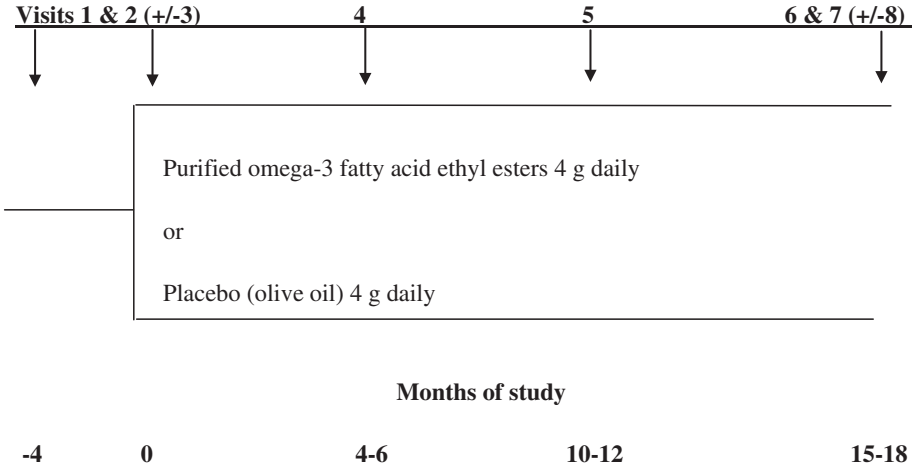

Fig. 1. Schedule, visits and timescale of the randomized controlled trial. Schematic figure of study design. The period of time from −4 to 0 months of study was dedicated to recruitment of potential participants, screening and baseline visits (visit 1 and visit 2). At time point 0 we block-randomised participants to purified omega-3 fatty acid ethyl esters 4 g daily or placebo olive oil 4 g daily. During the trial, participants attended the clinical research facility at 6 months and at 12 months for simple blood tests undertaken as a safety checklist (visit 4 and visit 5). The end of study visits were performed at 15–18 months (visit 6 and visit 7). Visit 3 and visit 8 were extra visits to allow participants with restricted time to complete at their convenience the clinical tests.

examination. 95/103 randomised participants completed the study. Details of reasons for participant withdrawal from the study are presented in Table 1.

The progress of the trial was divided into 6 steps:

Months 0–3 Staff recruitment, Ethics application
Months 4–15 Recruitment of retrospective cohort & baseline phenotyping
Months 4–15 Randomisation to 15–18 month placebo controlled trial
Months 4–15 Recruitment of prospective new referrals
Months 4–41 Placebo controlled trial and analyses of baseline data
Months 41–48 Follow-up phenotyping and analyses of results.

3.6. Baseline and end of study measurements

3.6.1. Biochemical and anthropometric measurements

Glucose, insulin, total cholesterol, HDL-cholesterol, triglycerides, platelets, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyl transferase were measured in fasting serum using commercially available kits according to the manufacturers' instructions. Tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) and hyaluronic acid (HA) were analysed using an ELISA kit Dynex DS2 platform. The Procollagen-III N-terminal Propeptide (PIIINP) assay was performed with a UniQ radioimmunoassay kit supplied by Orion Diagnostica (Product no.68570). Plasma 3-hydroxybutyrate (3-OHB) was measured as a proxy for β-oxidation [33] using an ILAB650 clinical analyser.

We generated two different histologically-validated liver fibrosis scores [25,26]. The first of these scores comprised measurement of HA, PIIINP and TIMP-1 [25] and the second validated algorithmically-derived score (NAFLD fibrosis score) used age (years), BMI, impaired fasting glucose/diabetes (yes/no), ALT/AST ratio, platelet count and albumin concentration [26].

Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) on the non-dominant arm after subjects had become acclimatised and had rested for at least 60 min; the mean of three measurements was calculated. Waist circumference was measured over bare skin midway between the costal margin and the iliac crest. Hip circumference was measured at the widest part between the greater trochanter and lower buttock level. BMI was calculated as height (m) squared divided by weight (kg). Bioelectrical impedance (Bodystat 1500; Bodystat, Isle of Man, UK) was used to determine body composition. Metabolic syndrome was defined using the International Diabetes Federation criteria [34] (Table 2).

Table 1
Reasons for withdrawal.

Reasons for withdrawal
· Pre-existing difficulties with venesection/priority of other on-going clinical treatment
· Death from bronchopneumonia diagnosed following randomisation
· Allergy to salmon discovered following recruitment
· Insufficient time to continue with study
· Reliant on friends and family to travel long distance for visits and felt that health was also not up to the commitment. Offers of travel assistance and reduced visit schedule declined. Also could not tolerate MRI and MRS examination
· Serious health concerns that, although successfully treated, meant the volunteer felt unable to continue frequent hospital visits and tests
· Withdrawal for personal/family reasons that volunteer was unwilling to discuss with study team
· No longer able to fulfil time commitment due to family problems
· Relocation of work and no longer able to fulfil time commitment
· Unwilling to continue. Research team unable to contact participant

N.B. Some participants had more than one reason for withdrawal.

3.7. Assessment of compliance and measurement of erythrocyte DHA and EPA enrichment

3.7.1. DHA and EPA enrichment of erythrocytes

We measured DHA and EPA concentrations in erythrocytes at the beginning and at the end of the study to evaluate erythrocyte enrichment during the study in all participants. Enrichment was defined as the difference between end of study and baseline measurements. We were specifically interested in measuring enrichment in participants randomised to Omacor but we were also interested in checking that no enrichment occurred in the placebo group. Measurement of omega-3 fatty acids in erythrocytes is a validated proxy for liver tissue concentrations of omega-3 fatty acids [35–37]. Measurement of omega-3 fatty acids in erythrocytes at baseline and end of study was also used as a measure to assess compliance with study allocation to Omacor in that arm of the trial.

To quantify the magnitude of tissue enrichment with omega-3 fatty acids due to the effects of Omacor treatment, erythrocyte fatty acids were analysed by gas chromatography at both baseline and upon completion of the trial period of intervention. Thawed packed red cells (1 ml) were mixed vigorously with 5 ml chloroform:methanol (2:1 vol/vol); butylated hydroxytoluene (50 mg/L) was included in the chloroform:methanol as an antioxidant. After centrifugation the organic phase that includes the extracted total lipid was collected. This was dried down under nitrogen at 40 °C and redissolved in 0.5 ml toluene. Fatty acid methyl esters (FAMEs) were formed by incubation of the entire lipid extract with 1 ml methanol containing 2% (vol/vol) H_2SO_4 at 50 °C for 2 h. After cooling, samples were neutralized by addition of 1 ml of a solution of 0.25 M $KHCO_3$ and 0.5 M K_2CO_3. Then FAMEs were extracted into 1 ml hexane, dried down, redissolved in a small volume (150 μl) of hexane, and separated by gas chromatography. Gas chromatography was performed on a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 column (30 m × 0.22 mm × 0.25 μm). Inlet temperature was 300 °C. Oven temperature was initially 115 °C and this was maintained for 2 min post-injection. Then the oven temperature was programmed to increase to 200 °C at the rate of 10 °C/min, to hold at 200 °C for 16 min, and then to increase to 240 °C at the rate of 60 °C/min and then to hold at 240 °C for 2 min. The total run time was 37 min. Helium was used as the carrier gas. FAMEs were detected by a flame ionization detector held at a temperature of 300 °C. The instrument was controlled by, and data were collected using, HPChemStation (Hewlett Packard). FAMEs were identified by comparison of retention times with those of authentic standards run previously. Intra-assay coefficient of variance CVs for EPA, DPA and DHA were 3.0%, 1.0% and

Table 2
Individual study visit plan.

Action	Visit numbers							
	V.1	V.2	V.3	V.4	V.5	V.6	V.7	V.8
Informed consent	X							
Adverse events		X		X	X	X		
Ankle-brachial Index	X					X		
Anthropometry	X			X	X	X		
Bioimpedance	X					X		
Blood pressure	X			X	X	X		
Blood samples	X			X	X	X		
Blood sample for DNA				X				
Calorimetry	X					X		
Cardiorespiratory fitness		X					X	
Concomitant medications	X	X		X	X	X	X	
DEXA	X					X		
Fasting bloods, including lipids, glucose kidney and thyroid function	X					X		
Hand grip strength	X					X		
Height	X					X		
Blood samples for inflammatory markers		X					X	
Laser-doppler flowmetry		X					X	
Liver Biopsy	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OGTT	X					X		
Physical activity energy expenditure — SenseWear Pro	X					X		
Pulse wave/analysis	X					X		
Questionnaires: QOL & physical activity	X					X		
Randomisation & receive drug		X						
Urinalysis	X					X		
Venous occlusion plethysmography	X					X		
Vibrotactile perception & temperature sensation		X					X	
Weight	X			X	X	X		
MRI and MRS scanning			X					X
Carotid Doppler		X					X	
Echo		X					X	

OGTT: oral glucose tolerance test.
QOL: quality of life.
DEXA: dual-energy X-ray absorptiometry.
MRI: magnetic resonance imaging.

2.0% respectively. Inter-assay CVs for EPA, DPA and DHA were 5.0%, 6.1% and 2.2% respectively.

3.7.2. Body fat (total body fat, regional body fat and visceral fat)

Radiological assessments of body fat i.e. dual-energy X-ray absorptiometry (DEXA), and magnetic resonance imaging (MRI) and liver fat magnetic resonance spectroscopy (MRS) were undertaken at both baseline and end of study.

We used dual-energy X-ray absorptiometry (DEXA) and horizontal five-slice cross-sectional magnetic resonance imaging (MRI) to evaluate in detail absolute amount and relative percentages of body fat, truncal fat and visceral fat. DEXA scanning was undertaken with a Delphi W instrument (Hologic, Bedford, MA, USA) using a standard visual method to divide images into trunk, limb and head. MRI images were acquired from five non-contiguous slices of the abdomen, extending from 5 cm below to 15 cm above L4–L5, to obtain a more accurate estimation of visceral fat than from a single slice. Axial scans were acquired with participants in the supine position. Participants were scanned on a 1.5 T MR scanner (Siemens Avanto, Syngo software release B17; Siemens AG, Munich, Germany) using a 32-channel body coil. A gradient echo 2D FLASH (fast low angle shot) sequence (TR = 111 ms, TE = 4.18 ms, flip angle = 70°, slice width = 10 mm, slice spacing = 50 mm) was used to obtain T1-weighted images. In order to accommodate the circumference of the individual being scanned within the image, the field of view was varied. The MR images were analysed using a proprietary software package (Mimics 14.0; Materialise NV, Leuven, Belgium) to identify regions of subcutaneous and visceral fat within the cross-sectional abdominal MR images. This package enabled identification of subcutaneous and visceral fat. By examining the histogram of pixel values present in the image, threshold levels could be set. Since fat pixels were the highest value pixels in the image, fat tissue could be identified from other tissue in the images. Some manual intervention was required when using this technique, as there was some variation in signal intensity across the image, which is often the case in large field-of-view MR images. Three different masks were created; one comprising the whole cross-section of the body, one containing the visceral fat region and one containing the subcutaneous fat region. It was possible to determine the number of pixels contained within each of these masks, and hence calculate the areas of subcutaneous fat and visceral fat, and compare them with the total cross-sectional area. Adipose tissue volume was converted to mass in kg using a density of 0.92 kg/l for adipose tissue.

3.7.3. Mean liver fat percentage

Participants underwent MR spectroscopy (MRS) of the liver to measure the quantity of liver fat accumulated in three discrete liver zones, at baseline and follow-up. Three

$20 \times 20 \times 20$ mm^3 spectroscopic volumes of interest (VOI) were positioned within segments 3 (inferior sub-segment of the lateral segment), 5 (inferior sub-segment of the anterior segment) and 8 (superior sub-segment of the anterior segment) of the liver, avoiding major blood vessels, intra-hepatic bile ducts, and the lateral margin of the liver. For the second visit scan, these VOI positions were copied from the first scan, to ensure consistency. A PRESS (point resolved spectroscopy) spin echo pulse sequence was used to acquire the spectroscopic data. The pulse sequence used a TR = 1500 ms, TE = 3 ms, flip angle = 90°, bandwidth = 1000 Hz, 8 averages and acquisition duration of 1024 data points, with no water suppression. The acquisition was obtained in a breath hold examination of 18 s. Spectra were post-processed using Siemens scanner software. This was a fully automated process and involved several steps, starting with filtering the data using a Hanning filter, zero-filling the data, baseline correction, phase correction and finally curve fitting was performed (with 4 iterations) to identify the water and lipid peaks. Values for the lipid and water peak integrals were produced for each VOI and recorded for each subject.

3.7.4. Diet

We assessed any change in diet during the study period by completion of a food frequency questionnaire (FFQ) at baseline and at the end of the study. This questionnaire comprises more than twenty groups of food and the frequency options where based on weekly consumption. From these reported weekly frequencies of foods consumption we generated a 'prudent diet score' using principal component analyses [24]. These components are independent linear combinations of dietary variables that account for maximum variance and each component identifies a pattern of consumption of certain foods. We identified a "prudent diet" as being a healthy diet specifically rich in fruit and vegetables and that contained fewer dietary calories from simple carbohydrates and saturated or trans-fatty acids (e.g. prudent diet: wholemeal bread, yoghurt, and fish). The prudent diet score is a continuous variable and changes in this measurement during the trial will be analysed by comparing baseline measurement with end of study measurement, in order to assess whether participation in the trial results in a change in the participant's diet.

3.7.5. Cardiorespiratory fitness

Cardiorespiratory fitness measured in terms of maximal oxygen uptake (peak VO_2) was determined from breath-by-breath analysis of oxygen consumption and CO_2 production using a Cortex metalyser 3B instrument (Cortex Biophysik, Germany) during maximal treadmill exercise (Woodway P55 treadmill) utilising a modified Bruce protocol, with 12-lead ECG monitoring throughout the test. Volunteers were advised to avoid strenuous exercise and alcohol for 24 h prior to testing. Volunteers were encouraged to continue until the respiratory exchange ratio was >1.1 and they reached at least 90% of their predicted maximum heart rate (as determined by 220 − age), unless they experienced chest pain or felt unwell. Cardiorespiratory fitness was measured by peak VO_2 which was corrected for total body weight.

3.7.6. Physical activity energy expenditure

Physical activity levels were assessed at baseline and at the end of the study by measuring total energy expenditure using a SenseWear Pro3 armband (Bodymedia, Pittsburgh, USA) [38] for approximately 4 days on each occasion. The SenseWear armband is a compact and lightweight ~82 g device worn around the upper arm that is well tolerated and contains sensors for 2 plane accelerometry, near body temperature, skin temperature and the galvanic skin response. The SenseWear Pro3 armband allows reliable measurement of physical activity energy expenditure levels and calculation of total energy expenditure recordings. Assessment of any change in physical activity at the end of the trial will be undertaken by comparing baseline and end of study data to assess whether participation in the trial resulted in any change in this behaviour.

3.7.7. Cardiac function

Transthoracic echocardiography was performed using the Philips iE33 ultrasound system and 2.5 MHz transducers. Standard parasternal and apical views are acquired. Complete 2D and M-mode echocardiogram, conventional Doppler, and tissue Doppler imaging (TDI) were obtained for all study participants. Standard 2D measurements [(LV end-diastolic and end-systolic dimensions (mm), septal and posterior wall thickness at end-diastole (mm)] were determined. LV ejection fraction (%) was calculated using the biplane modified Simpson's method [39]. LV mass was calculated using the formula proposed by Levy et al. [40] and normalized for body surface area (LV mass index, g/m^2). LA dimension (mm) was measured in the 2D parasternal view, and LA volume (ml/m^2) was measured using the area–length method in the apical view and normalized for body surface area. Transmitral flow velocities were obtained by pulsed-wave Doppler echocardiography, positioning a sample volume at the level of a mitral tip in an apical four-chamber view. Mitral flow parameters, including peak velocities at early diastole (E) and late diastole (A) and E-wave deceleration time, were measured and E/A ratio was calculated.

Grey-scale and colour tissue Doppler images from apical four-, three-, and two-chamber views were recorded at a high frame rate (>120 frames/s) that was automatically changed by narrowing the sector width. Tissue velocity curves were obtained from colour tissue Doppler imaging. A sample volume was placed separately at the septal and lateral annulus in the apical four-chamber view, and peak myocardial systolic, early diastolic and late diastolic velocities (Sm, Em, and Am, respectively) were measured and the ratio of mitral to myocardial early diastolic peak velocity (E/Em) was calculated [41].

To obtain longitudinal myocardial velocity, strain rate, and strain images with high quality, a narrow sector angle was used, and image depth was adjusted to allow for a high frame rate (>120 frames/s) with care taken to avoid angulations. The myocardial time–velocity and time–deformation curves were reconstructed off-line from colour-coded 2D tissue Doppler image loops (average of 3 consecutive heart cycles) using Philips QLab version 8 software. The peak systolic (Sm), early diastolic (Em), and late diastolic longitudinal myocardial velocities were measured at basal and

mid-segments of the septal, lateral, inferior, anterior, posterior and anteroseptal walls from apical views. Likewise, peak systolic, early diastolic and late diastolic strain rates, and peak systolic strain were also measured at the same segments and expressed as absolute values. A 10 mm sample volume was positioned at each wall of the LV in four-, three-, and two-chamber views and manually tracked frame-by-frame to maintain its position within LV walls. The average values were used to compare global LV function of subjects before and after intervention [42]. Data will be excluded if a smooth SR curve is not obtained or when the angle between the scan line and LV wall is more than 30° in order to preclude angle-dependency of these parameters. Inter- as well as intra-observer variability coefficients were measured to assess reproducibility of results.

3.7.8. DNA analyses

Blood was collected from participants at baseline for DNA analyses for assessment of genetic markers and polymorphisms (e.g. Patatin-like phospholipase domain-containing protein 3 (PNPLA3) that have been shown to be associated with NAFLD).

3.7.9. Vibrotactile perception thresholds

Vibration stimuli were generated by an *HVLab* Vibrotactile Perception Meter [43], fitted with a thermocouple to measure skin temperature. The device is computer-controlled using *HVLab* Diagnostics Instruments software. The Vibrotactile Perception Meter is CE-marked. Vibrotactile perception thresholds were measured at two locations on both feet of each participant (pulp of the great toe and heel) and at the index finger. All measurements were taken at baseline and end of study. At all locations the thresholds were measured at 20 and 125 Hz.

3.7.10. Temperature perception thresholds

Temperature stimuli were generated by an *HVLab* Thermal Aesthesiometer [44], which is computer-controlled using *HVLab* Diagnostics Instruments software. The *HVLab* Thermal Aesthesiometer is CE-marked. Subjects were exposed to a metal surface, which is heated and cooled via a Peltier element. The *HVLab* Thermal Aesthesiometer has two safety cutoff features: the first is software cutoff set at 5°C and 55°C and the second is a hardware cutoff set at 3°C and 57°C. Warm thresholds and cold thresholds were determined at two locations on both feet (pulp of the great toe and heel) and at the index finger. All measurements were taken using the method of limits at baseline and end of study.

3.7.11. Microvascular function

Microvascular function was assessed by venous congestion plethysmography and laser Doppler fluximetry at baseline and end of study. For venous congestion plethysmography, an elastic gauge was placed around the calf and a congestion cuff was placed around the thigh. Small (~10 mm Hg) increases in pressure, from zero to a pressure not exceeding subjects' diastolic blood pressure were applied and maintained. The changes in limb volume measured by the gauge were used to estimate the rate of fluid exchange across the microvasculature. For laser Doppler fluximetry, two small laser Doppler probes were placed on the volar surface of the forearm and finger to detect blood flow in the superficial dermal vasculature. A blood pressure cuff was placed around the upper arm and blood flow was measured before, during and after inflation of the cuff to supra-systolic pressure and maintained for up to 3 min. The reactive hyperaemic response was used to assess the capacity of the vasculature to dilate under rested conditions.

3.7.12. Carotid-intima media thickness

The carotid arteries were studied with a duplex scanner using a 7.5 MHz linear array transducer (Philips IE33 4–8 MHz) with ECG monitoring. Carotid intima-media thickness (IMT) is a well-validated screening tool for the prediction of CV disease in asymptomatic subjects [45,46]. Ultrasound parameters (dynamic range, depth range, power output and greyscale) for B-Mode carotid imaging were adjusted during image acquisition to optimize image quality. All scans were carried out according to a standardised protocol [47]. Briefly, subjects lie supine with the neck slightly rotated and a transverse scan is first performed as a screening measure and also to identify the carotid bifurcation. Longitudinal images of the near and far walls of the common, proximal portion of the internal and external carotid arteries and the carotid bifurcation are examined and multiple images of 4 cine-loop cycles of the carotid artery are recorded and stored digitally for subsequent off-line analysis using Philips Q-Lab version 8 software. For each subject, a 10 mm plaque-free segment of IMT at the far wall of the common carotid artery immediately proximal to the carotid bulb was measured using QLAB automated software. An average of three different cardiac cycle measurements of IMT from each of the left and right common carotid arteries was calculated. The presence of carotid plaque at the distal common carotid, carotid bulb and proximal internal carotid arteries was also recorded [47].

3.7.13. Ankle/brachial pressure index (ABPI)

ABPI was measured at baseline and at the end of the study. ABPI is a simple measurement for assessing peripheral macrovascular function. Low ABPI (<0.9) is an independent predictor of increased cardiovascular disease risk [48]. With the patient rested and lying supine blood pressure cuffs were placed bilaterally on the upper arm (brachial pressure) and ankle, and inflated to 20 to 30 mm Hg above systolic pressure. An ultrasound Doppler probe was placed over the brachial, dorsalis pedis and posterior tibialis arteries and was used to detect the return of the arterial signal at the highest systolic pressure. The ABPI was calculated by dividing the ankle pressure by the brachial systolic pressure.

3.7.14. Pulse wave analysis

This was undertaken by applanation tonometry using SphygmoCor software to derive non-invasively central aortic pressure and haemodynamic indexes [49].

4. Results

The recruitment started in January 2010 and ended in June 2011. We identified 178 potential participants, and randomised 103 participants who met the inclusion criteria. We will test the primary outcomes of the study in complete

case analysis of the 95 participants who completed the study. We will comment on any differences in subjects withdrawing and those completing the trial.

5. Challenges and limitations

5.1. Assessment of histological improvement in NAFLD with treatment

In 2009, we had local ethics committee approval to undertake an end of trial liver biopsy performed by an interventional radiologist under ultrasound guided control. In 2011/2012 when the first participants were completing the trial, there were two deaths in our hospital amongst routine NHS patients undergoing a diagnostic liver biopsy within our Hepatology service. Our study team collectively and unanimously decided to withdraw liver biopsy from the protocol as an end of study test and the local ethics committee were notified and accepted this decision and change to the protocol. We considered that the risks of liver biopsy in research participants had become unacceptable, and moreover, many of our unpaid participants, who were altruistically giving up their time, had also expressed reservations at recruitment about having an end of study liver biopsy (if it was not clinically indicated). Since NAFLD is also well known to be a patchy liver disease, we had also had reservations at the outset in designing the protocol about inclusion of a liver histological end point.

However, it is acknowledged that a limitation of the trial is that it does not also have a histological end point. Ideally, the combination of non-invasive (MRS, biomarkers for NASH) and invasive (histological) assessment would have provided an even better test of the efficacy of omega-3 treatment in NAFLD.

5.2. Assessment of the amount of DHA and EPA tissue enrichment in liver that is required to produce an effect in NAFLD

A challenge that we faced, was that at completion of the trial, we wished to show (with an objective measurement) that participants who had been randomised to Omacor were compliant with the medication (and therefore showed an increase in tissue DHA and EPA concentrations). Furthermore, we also wished to show that participants who had been randomised to placebo did not show an increase in tissue DHA and EPA concentrations. We reasoned that if subjects in the placebo group were able to detect (by crushing capsules) that they had been randomised to placebo, they might increase their fish consumption or take over the counter fish oil preparations. For these reasons we measured baseline and end of study DHA and EPA concentrations in erythrocyte. A further challenge that we faced was that it was uncertain precisely how much tissue enrichment with omega-3 fatty acid would be required to produce a beneficial effect in NAFLD. Based on our previous studies and recent literature [36,37,50,51], we hypothesised a priori, that the intervention should produce a minimum two fold increase in baseline percentage erythrocyte DHA and EPA. Thus, after considering the baseline percentage DHA and EPA concentrations, we considered a threshold of 2% for DHA erythrocyte enrichment and a threshold of 0.7% for EPA enrichment should be achieved with the Omacor intervention if participants were compliant with the intervention.

6. Discussion

To date there is no licensed treatment for NAFLD. Because of the burgeoning health care problem caused by a marked burden of disease affecting many patients worldwide, new, safe treatments for NAFLD are urgently needed. Although the results of trials testing treatment with thiazolidinediones [5] and anti-oxidants such as vitamin E [6], have looked promising, as stated above, long safety concerns of both classes of drugs will inevitably limit their usefulness in practice. Furthermore other agents that include specific lipid modifying treatments have also been tested recently using various proxy measures for NAFLD. These data have been obtained utilising data obtained from trials testing the effects of statin treatment in cardiovascular end point trials [16,52–54] and in safety and efficacy studies [53,55]. The results from these studies were summarised in December 2012 [56] and show that statins, in particular, are safe in NAFLD, and may provide some benefit. However these trials were not usually designed a priori as randomised placebo double blind trials to test the effects of lipid lowering therapy on NAFLD. Moreover, statins and other lipid lowering therapy (e.g. fibrates) are not licensed for the treatment of NAFLD and further specific randomised, placebo control trial evidence to test the efficacy of lipid modifying therapy on the different stages of NAFLD is urgently needed. Such evidence is required before they can be advocated specifically as a treatment for NAFLD.

Over the last decade there has been growing interest in a potential role for long chain omega-3 fatty acid treatment in NAFLD and several biological mechanisms have been suggested, proposing a benefit of this treatment. For example biological actions that may be relevant to NAFLD include beneficial effects of these fatty acids on bioactive metabolites (protectins, resolvins and maresins), cell surface receptors (e.g. G-protein coupled receptor (GPR120)), alteration of transcription factor activity, peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) [57]. To date there have been a few trials testing the effects of omega-3 long chain fatty acids in adults [16–22] and in children with NAFLD [23]. Most of these trials have used ultrasound or liver enzymes as a semi-quantitative or non-specific measure of NAFLD severity. Trials were mainly of short duration (8–26 weeks), all used different doses of omega-3 fatty acids of differing degrees of purity for different periods of time; and furthermore the findings were not consistent across the different studies. This confusing evidence led to the authors of a recent systematic review and meta-analysis in 2012 to conclude that “well designed RCTs which quantify the magnitude of effect of omega-3 PUFA supplementation on liver fat are needed” [8].

7. Summary

The WELCOME study is a single centre, randomised, double-blind placebo controlled trial in participants with

NAFLD. The aim of the WELCOME study is to test the effect of 15–18 months treatment with Omacor (Lovaza) that contains purified long chain omega-3 fatty acids in the early stages of NAFLD. Because of the risk of 'contamination' in the placebo group, and the risk of increased fish consumption or over the counter purchase of fish oil capsules and to monitor compliance, we will also assess enrichment of tissue fatty acids, to test whether enrichment with EPA and DHA improves the pre-specified outcomes.

Disclosures/Conflicts of interest

PCC serves on the Clinical Advisory Board of Pronova Biopharma and has acted as a consultant to Amarin. None of the other authors has any disclosures.

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