Study Protocol (Version 4; dated 18May17)

1. Title of project

Can oils derived from genetically-modified plants replace fish oil as a source of long chain n-3 polyunsaturated fatty acids in the human diet

Short title: **Comparison of fish-oil vs modified seed oil (containing fish-oil fatty acids)**

Lay title: **Does consuming modified plant seed oil containing fish oil fatty acids act in the same way as consuming fish oil?**

2. Chief investigator

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3. Funder of project

Biotechnology and Biological Sciences Research Council

4. Duration of research

42 months from start date

5. Places where research will be conducted

University of Southampton and NIHR Wellcome Trust Clinical Research Facility, Southampton University Hospitals NHS Trust

6. Researchers involved

Professor G.C. Burdge, Professor P.C. Calder, Professor J. Napier, Professor K.A. Lillycrop, Dr E.A. Miles.

7. Purpose of project/Background

There is a robust literature that demonstrates that adequate consumption of omega 3 (or n-) polyunsaturated fatty acids (PUFA) derived from marine fish, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is important for maintaining health across the life course. For example, EPA+DHA status[1] and dietary intake[2] have been shown consistently to improve cardiovascular health and pharmaceutical preparations of these fatty acids have been demonstrated in meta-analyses to be as effective as statins in preventing myocardial infarction[3]. Anti-inflammatory effects of increased EPA+DHA intake include consistent improvement across studies in the severity of rheumatoid arthritis[4] and there are emerging findings that support beneficial effects in asthma and inflammatory bowel disease[5]. The recommended daily intakes vary between 250 mg/day (European Food Safety Authority[6] to 1000 mg/day (Japanese Ministry of Health[7]) of EPA+DHA. In the UK, the Scientific Advisory Committee on Nutrition (SACN 2004) has recommended that adults should consume at least 450 mg/day of EPA+DHA[8]. UK adults consume approximately 50% of the amount of EPA+DHA that is recommended to promote health, while intakes in children are about 10% of that in adults[9]. This is consumed in oily fish or fish oil containing supplements. The low intake of EPA+DHA may be as a result of issues such as the palatability or the cost of consuming oily fish and fish oil. To increase the intake of oily fish and fish oil in the population would be challenging and would also increase the pressure on fish stocks. Algal oils that contain DHA or EPA+DHA have also been proposed as an alternative source of these fatty acids[10] and these currently account for 2% of human EPA+DHA consumption[11]. However, the current costs of fermentation and refining processes are substantially greater than for FO production and are unlikely to decrease in the medium term[12]. Therefore there is a need for an alternative cost effective sustainable source of EPA+DHA.

Vegetable oils derived from genetically modified plants represent a potentially attractive alternative means of providing EPA+DHA for the human population. Such oils potentially overcome issues of the palatability, sustainability and also concerns about contamination of FO with marine pollutants[13].
Insertion of desaturase and elongase enzymes from yeast and algae into the model plant Arabidopsis thaliana produced an oil containing 12-15% DHA (but little EPA), which could produce per hectare sufficient DHA to replace 10,000 oily fish[14]. It is important to note that most standard FO preparations contain ~18% EPA and ~12% DHA. The health benefits derived from FO are due to both EPA and DHA, and thus plant oils containing only one or the other of these PUFA may have limited effectiveness in supporting optimal health. *Camelina sativa* is a seed oil plant which was the most important seed oil crop in Europe up until the last century. Camelina sativa plants have been modified by the addition of genes from other plant sources to be able to provide seed oil with around 10% EPA and 10% DHA. This oil from genetically modified *Camelina sativa* (high omega 3 Camelina oil) is an alternative dietary source of these fatty acids usually found in fish oil and oily fish. The high omega 3 Camelina oil does not represent the synthesis of an entirely new compound, but is a "blend" of the fatty acids found in either vegetable or fish oil. This high omega 3 Camelina oil offers a final possible advantage over conventional fish oil. Fatty acids can be attached to the carrier molecule (triglyceride) in one of three positions (named sn-1, sn-2 and sn-3). The fatty acids are removed from the carrier molecule by enzymes, which release them to be used within the body. These enzymes are better able to remove fatty acids at the sn-1 and sn-3 position and less efficient at removing them from the sn-2 position. In fish oil almost all of the DHA and nearly half of the EPA is attached at the sn-2 position[15]. In the high omega 3 Camelina oil, EPA and DHA are attached predominately at the sn-1 and sn-3 positions suggesting the possibility that EPA+DHA in the high omega 3 Camelina oil is more easily released for use by the body.
Therefore, high omega 3 Camelina oil offers a sustainable, cost-efficient source of EPA+DHA. Compared with the current major source of EPA+DHA (fish oil), it avoids issues of contamination with marine pollutants and pressure on fish stocks. Finally it offers the possibility of being more efficacious compared with fish oils due to the way it carries the EPA+DHA.

This study will measure the effectiveness of high omega 3 Camelina oil compared with equivalent fish oil (similar EPA+DHA content) to:
a) Increase the body EPA+DHA status in the short and longer term.
b) Exert anti-inflammatory and immunomodulatory influences.

8. Aims & Objectives
The overall aim is to test whether the efficacy of dietary high omega 3 Camelina oil is comparable with fish oil for the following:
1 The magnitude of the increase and turnover of EPA+DHA in plasma lipids following acute consumption (postprandial period).
2 The magnitude of the increase in EPA+DHA derived in plasma lipids and blood cells following consumption over a longer period (provided as a dietary supplement for 8 weeks).
3 In vivo and ex vivo anti-inflammatory effects following acute and longer duration consumption.

Objectives
1. To measure the appearance of EPA and DHA in individual blood lipid classes (phospholipids, triacylglycerol (TAG), non-esterified fatty acids (NEFA) and cholesteryl esters (CE)) and specific lipoprotein fractions (chylomicrons and very low density lipoprotein) and blood cells (erythrocytes and peripheral blood mononuclear cells) in the blood of healthy subjects over 8 hours following consumption of a standard high fat meal containing high omega 3 Camelina oil compared with consuming the same meal containing an equivalent fish oil (similar EPA+DHA content). (Study A)
2. To measure the appearance of inflammatory mediators (including CCL2/MCP-1, CCL4/MIP-1 beta, CRP, IL-1beta, IL-6, IL-8, IL-10, IL-12p70, sICAM-1, Serpin E1/PAI-1, sVCAM-1, TNF-alpha and VEGF and CRP) in the blood of healthy subjects over 8 hours following consumption of a standard high fat meal containing high omega 3 Camelina oil compared with consuming the same meal containing an equivalent fish oil (similar EPA+DHA content). (Study A).

3. To measure the concentration of EPA and DHA in individual blood lipid classes (phospholipids, triacylglycerol (TAG), non-esterified fatty acids (NEFA) and cholesteryl esters (CE)) and blood cells (erythrocytes and peripheral blood mononuclear cells) in the blood of fasted healthy subjects following consumption of high omega 3 Camelina oil for 8 weeks compared with consuming an equivalent fish oil (similar EPA+DHA content) for the same time period. (Study B).
4. To measure in vitro immune responses (mononuclear cell mediator production, mononuclear cell and neutrophil phagocytosis and respiratory burst response, T cell signalling) by cells from the blood of fasted healthy subjects following consumption of high omega 3 Camelina oil for 8 weeks compared with consuming an equivalent fish oil (similar EPA+DHA content) for the same time period. (Study B).

9. The study

Subjects

For each study (A and B) we will recruit to enrol 10 healthy men and 10 healthy women each in two age ranges (18 to 30 years or 50 to 65 years). We estimate (based on past experience) that this will require screening of approximately 30 men and 30 women in each age group for each study[55].
For the purpose of this study healthy will be defined as having health screening results that are within normal ranges, a body mass index of 18.5-30.0 kg/m^2 and without diagnosis of chronic illness.

Inclusion criteria

1. Male or female
2. 18 to 30 years or 50 to 65 years
3. Body mass index 18.5-30.0 kg/m^2
4. Health screening results that are within normal clinical ranges (systolic blood pressure ≤ 140 mm/Hg, diastolic blood pressure ≤ 90 mm/Hg, total cholesterol concentration < 7.5 mmol/L, random glucose concentration < 11 mmol/L)
5. Not consuming fish oil or other oil supplements
6. Not eating more than one oily fish meal per week
7. Willing to adhere to the study protocol
8. Being able to provide written informed consent

Exclusion criteria

1. Aged < 18 years; 31-49 years or > 65 years
2. Body mass index < 18.5 or > 30 kg/m^2
3. Current smoker
4. Random sample blood cholesterol or glucose concentrations outside the normal concentration range
5. Diagnosed chronic illness (for example diagnosed cardiovascular disease, diabetes, cancer, rheumatoid arthritis)
6. Diagnosed food allergy.
7. Regular use of anti-inflammatory medication (e.g. aspirin, ibuprofen, diclofenac) or anti-coagulant therapy.
8. Use of prescribed medication to control blood lipids (e.g. statins, fibrates (fenofibrate), Omacor, bile acid resins) or fat absorption (e.g. orlistat)
9. Use of prescribed medication to control blood pressure (ACE inhibitors, angiotensin 2 receptor blockers, calcium channel blockers, α-inhibitors, thiazide diuretics)Use of fish oil or other oil supplements
10. Chronic gastrointestinal problems (e.g. IBD, celiac disease, cancer)
11. Pregnant or planning to become pregnant within the study period
12. Participation in another clinical trial

Medications which are not incompatible with the study and are thus acceptable include contraceptives, hormone replacement therapy, antacids, anti-depressants, inhaled steroids or beta-agonists for

asthma, topical steroids or other nasal sprays for seasonal or perennial rhinitis, thyroxine. Certain stable, managed, chronic conditions do not constitute an exclusion (e.g. asthma, seasonal or perennial rhinitis, hypothyroidism).

Subjects will recruited via posters, intranet and email shots in the University of Southampton, via posters, and email shots in Southampton General Hospital, and other organisations with which the researchers have contact; and via advertisements in local media including newspapers and radio. Subjects who express an interest will be contacted by telephone or email. If they fit the inclusion and exclusion criteria (see below) they will be sent the information sheet. They will be contacted about 7 days later to confirm their interest or not, and if they remain interested an appointment will be made for them to visit the Wellcome Trust Clinical Research Facility at Southampton General Hospital. At this first visit the study will be explained, any questions answered and written consent obtained.

Supplements

The fish oil n-3 fatty acid preparation to be used is commercially available as a supplement and will be provided incorporated into the high fat meal for study A and as a dietary supplement oil for study B. The Camelina oil is an edible plant seed oil but additionally will contain the fish oil-type fatty acids EPA plus DHA. Approximately 450 mg EPA plus DHA will be provided by the oils (fish oil or high omega 3 Camelina oil) for the meal challenge (study A). For study B the oils (fish oil or high omega 3 Camelina oil) will provide 450 mg EPA plus DHA per day for the 8 weeks of the intervention period (providing the recommended UK daily intake)[17].

The high omega 3 Camelina oil contains around 10% of fatty acids as EPA and 10% as DHA. It will be given incorporated into the high fat meal for study A and as dietary supplement oil for study B. Subjects will be randomised to allocate order of supplement. Subjects, researchers and clinical staff will be blinded to group allocation.

Subject participation schedule

Study A

Subjects who express an interest will be contacted by telephone or email. If they fit the inclusion and exclusion criteria they will be sent the information sheet. They will be contacted about 7 days later to confirm their interest or not. If they remain interested an appointment will be made for them to visit the NIHR Wellcome Trust Clinical Research Facility, Southampton General Hospital. At this first visit the study will be explained, any questions answered and written consent obtained; subjects will then have height, weight, and blood pressure measured and a screening blood sample collected (10 ml) to confirm that they meet the inclusion criteria. An appointment will be made for the subsequent clinic visit; one further clinic visit will be made (i.e. three clinic visits in total). Subjects will attend the 2 latter clinic visits in the fasted state (no food or drink except water after 8 pm the previous evening).

Subjects will visit the Wellcome Trust Clinical Research Facility, Southampton General Hospital on three occasions.

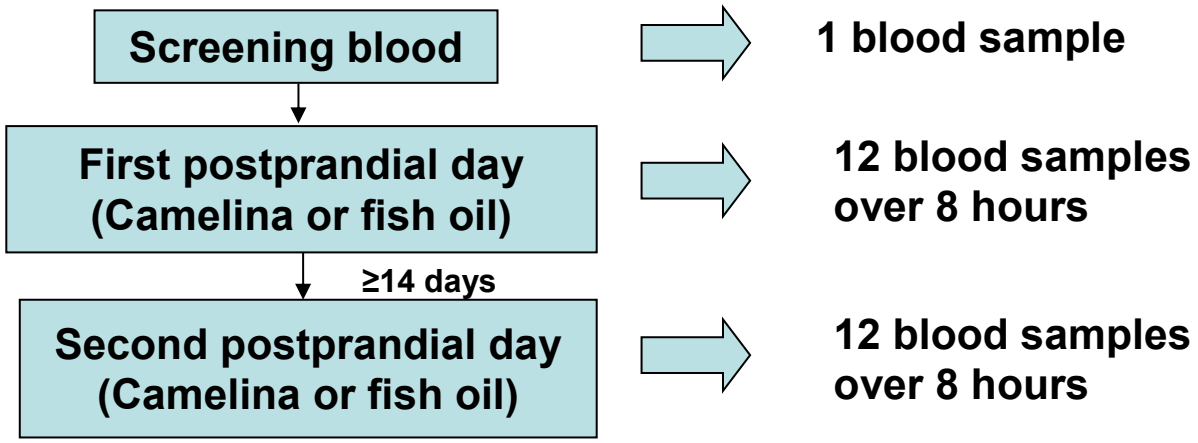
At the second and third visits the subjects will attend the clinic between 7 and 9 am and will have a cannula inserted into a forearm vein and a baseline blood sample (10 ml) collected. Subjects will then consume one of the test meals on each of two study visits in random order.

The composition of the test meals will be based upon typical UK nutrient intakes as we have used in previous post-prandial studies. The lipid component will be derived from a blend of fat sources (fish oil or high omega 3 Camelina oil, safflower oil, double cream, linseed oil and olive oil) to model the fatty acid profile of the UK diet. Approximately 450 mg EPA plus DHA will be provided by the oils (fish oil or high omega 3 Camelina oil) for the meal challenge (providing the recommended UK daily intake)[17].

Blood samples (10 ml) will then be collected for 8 hours (at 30 minute intervals up to 3 hours and then hourly; total volume sampled 120 ml). A light meal (e.g. sandwiches) will be provided at the end of

the visit. Subjects will then consume the other test meal at the next study visit which will be at least 14 days after the first. This will complete study A.

Study A



Study B

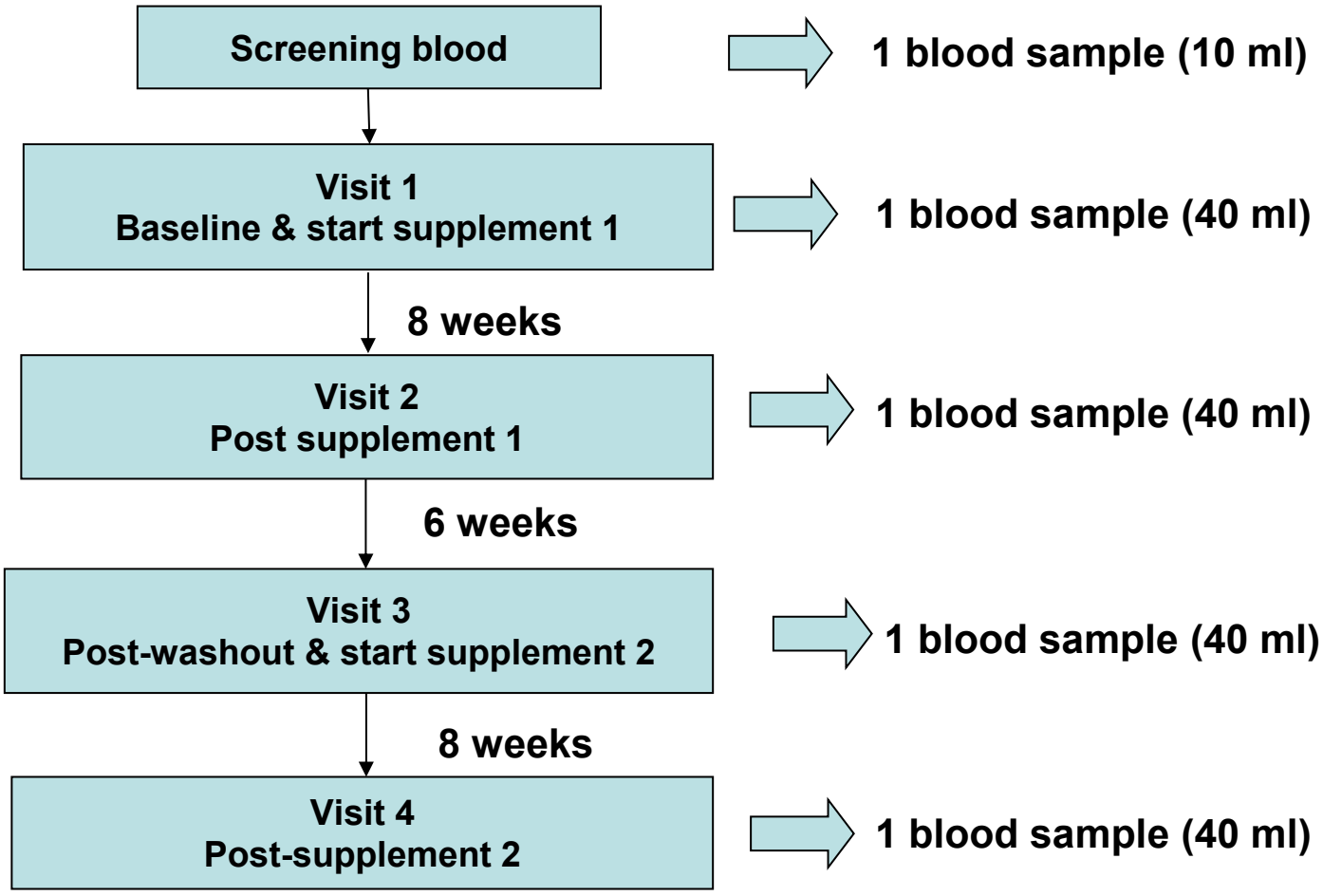
Subjects who express an interest will be contacted by telephone or email. If they fit the inclusion and exclusion criteria they will be sent the information sheet. They will be contacted about 7 days later to confirm their interest or not. If they remain interested an appointment will be made for them to visit the NIHR Wellcome Trust Clinical Research Facility, Southampton General Hospital. At this first visit the study will be explained, any questions answered and written consent obtained; subjects will then have height, weight, and blood pressure measured and a screening blood sample (10 ml) to confirm that they meet the inclusion criteria.

Subsequently four further clinic visits will be made. Subjects will attend each of these four clinic visits in the fasted state (no food or drink except water after 9 pm the previous evening).

Subjects will visit the NIHR Wellcome Trust Clinical Research Facility, Southampton General Hospital on five occasions the first to obtain consent and collect a screening blood sample, then to attend four study appointments, each between 8 and 11 am. On each occasion following the first visit they will be in the fasted state.

At the four subsequent study visits the subjects a blood sample (40 ml) will be collected. The first of these visits will be a baseline visit and the subject will receive either the fish oil or high omega 3 Camelina oil supplement (allocated by randomisation). The subject will return for the second study visit after consuming the supplement for 8 weeks. At study visit 2 a blood sample will be collected and the subject will commence the 6 week wash out period. After this period they will return for study visit 3 where a blood sample will be collected (as verification that the subject's blood fatty acid composition has returned to the values seen pre-supplementation) and the subject given the second supplement. The subject will consume this daily for 8 weeks and then return for study visit 4. This will complete study B.

Study B



Schedule of subject visits and activities performed at each visit

X indicates the activity will take place.

Visits 1 and 2 and 3 to 4 will be around 8 weeks apart and subjects will consume fish oil or high omega 3 Camelina oil daily during that period. Visits 2 to 3 will be a 6 week wash-out period.

Activity	Screening Visit	Study Visit 1	Study Visit 2	Study Visit 3	Study Visit 4
Subject fasted		X	X	X	X
Informed consent	X				
Height	X				
Weight	X	X	X	X	X
Blood for screening	X				
Blood for fatty acids, and in vitro immune and inflammatory responses		X	X	X	X

Sample analysis

Blood collected at study entry will be used for screening. This will be analysed by the University Hospital Southampton NHS Foundation Trust Chemical Pathology Laboratory for blood cholesterol and blood glucose.

Study A. At the 2 study visits blood will be used to prepare plasma, mononuclear cells and red cells. Plasma will be aliquoted and frozen at minus 80°C. Mononuclear cells and red cells will be frozen at minus 80°C. Lipid will be extracted from plasma and red cells using chloroform/methanol and fatty

acid composition determined using standard techniques. Size distribution and concentration of chylomicron, VLDL, LDL and HDL lipoprotein particles will be analysed by NMR in samples collected at baseline, 3 and 8 hours. Immune and inflammatory mediators will be measured in plasma using multiplex immunoassays.

Study B. At the 4 study visits blood will be used to prepare plasma, mononuclear cells and red cells. Plasma will be aliquoted and frozen at minus 80°C. Mononuclear cells and red cells will be frozen at minus 80°C. Lipid will be extracted from plasma and red cells using chloroform/methanol and fatty acid composition determined using standard techniques.

Plasma will be used to evaluate blood lipid concentration and particle sizes

Mononuclear cells will be cultured for 24-48 hours and immune mediators in response to stimulation measured in plasma will be measured using multiplex immunoassays and ELISA.

Whole blood will be used to investigate neutrophil phagocytosis and respiratory burst response to bacterial challenge. Whole blood will be used to measure mononuclear cell phagocytosis.

T cells from the mononuclear cells will be mitogen stimulated and the RNA harvested in prepared for microarray analysis of the T cell transcription.

Data will be compared across time and between groups by ANOVA with repeated measures.

10. Data handling and record keeping

- All data will be entered onto a spreadsheet (Microsoft Excel) by the researchers involved.
- All data will be entered on a password-protected computer. This data will be accessed only by the PI and the researchers involved.
- All data will only be linked to study codes and thus not identifiable with the source volunteer. However, the caveat to this will be a data set recording the volunteer name and study code without any other volunteer details.
- All data recorded on paper will be kept in a locked filing cabinet in the researchers' office and/or in a dedicated, restricted access, clinical data storage area on Level D of the IDS Building, University of Southampton.
- Data of an identifiable nature (i.e. volunteer names, contact details, addresses) will be destroyed 12 months after the end of the study. All other data will be kept securely for 15 years and then destroyed.
- Data will be obtained, handled and stored in adherence to the principle set out in the Data Protection Act 1998.
- The investigators will permit monitoring, audits, REC and MHRA review (as applicable) and provide direct access to source data and documents.

11. Statistical Analysis

The statistical analysis will involve the comparison of inflammatory markers (proteins, lipids etc) and of fatty acids between fish oil and High omega 3 Camelina oil supplements. All statistical comparisons will be performed at the end of the study using SPSS version 21 (or later).

12. Sample size calculation

Study A. Based on our published findings[16], ten subjects per group will provide 85% power for detecting a 10% difference in peak postprandial concentrations of EPA or DHA in plasma TAG at $\alpha = 5\%$ and $\beta = 7.5\%$ in a two tailed analysis. Based on our previous findings of the effect of consuming a FO-enriched meal on lipoprotein size and concentration[17], Ten subjects per group will provide 85% power for detecting 13 nmol/l difference in peak postprandial VLDL concentration and 11 nm difference in VLDL particle size ($\alpha = 5\%$ and $\beta = 7.5\%$) in a two tailed analysis. Based on our preliminary data, ten subjects per group will provide 80% power for detecting a maximum change of 0.8pg/ml IL-6 (30% difference) in a two-tailed test ($\alpha = 5\%$ and $\beta = 20\%$).

Study B. Based upon our previous work[18], ten subjects per group will provide 80% power for detecting a 40% increase in EPA or DHA in plasma phospholipids (α = 5% and β = 7.5%) in a two tailed test based upon after 30 days supplementation. Based upon our previous work[19], ten subjects per group will provide 85% power for detecting a 10% increase in concanavalin A stimulated IL-4 and IL-2 secretion in vitro (α = 5% and β = 7.5%) in a two tailed test based upon after 30 days supplementation. In the first instance, data from the two phases of the cross over studies will be compared for time effects and if no significant time effects are detected, the data sets for each treatment will be combined[18]. Non-parametric data will be normalised by log transformation. The postprandial changes in plasma metabolites will be analysed by ANOVA with treatment, age and sex as fixed factors and time as a repeated measure. The effect of the different oils on plasma metabolites and immune function in the dietary supplementation study will be assessed by ANCOVA with baseline as a covariate.

13. Safety assessments

The supplements are edible oils (fish oil and Camelina oil) which are already consumed in the human diet and have been for many hundreds of years. The Camelina oil will be produced from the seed of the seed oil crop plant *Camelina sativa* which has been genetically modified by inclusion of genes from other plant sources which enable this seed oil plant to make fish oil-type fatty acids (long-chain omega 3 fatty acids). Oils produced from GM plants have been produced and are used for human consumption in a number of countries including the United States and Canada. The use of genetic modification to improve the nutritional characteristics of the types of fatty acids within the oil seed crops has been achieved successfully in other seed crops and has been approved for food use by Health Canada and the European Food Safety Authority[1,2,3] . Genetically modified soybean which produces oil with a much higher oleic acid content (considered to be a healthier fatty acid) has been approved for import and processing for food use in the UK[4].
Camelina sativa, a member of the cabbage family (*Brassicaceae*) and related to mustard, is a traditional food plant which has been used in the human diet for thousands of years[5]. It was the most important seed oil crop in Europe up until the nineteen hundreds[3]. In the USA and Canada it has been cultivated and used as a biofuel[6,7,8]. More recently, cold-pressed *Camelina sativa* oil has been approved as a food ingredient in Canada[9]. It is naturally rich in the shorter chain omega 3 fatty acid alpha-linolenic acid, and has been used in human intervention studies and reported to reduce LDL cholesterol in hypercholesteraemic subjects[10]. The oil produced from the genetically modified Camelina sativa will contain more of the long-chain omega 3 fatty acids which are normally consumed in fish oils or oily fish.

Two invasive procedures will be used to collect blood samples, siting of a venous cannula and venipuncture. The siting of the cannula or venipuncture and collection of blood samples will be undertaken by trained nursing staff in a clinical setting (NIHR Welcome Trust Clinical Research Facility, Southampton General Hospital) limiting the likelihood of adverse events and ensuring that any such events are dealt with rapidly by the correct method. If any volunteer reports any untoward medical occurrence this will be recorded on an adverse event or serious adverse event form and the PI informed immediately. If the investigator suspects that a serious adverse event is either a) related to the intervention or b) unexpected, the PI will report the event to the main REC and to a representative of the supplier of the supplement. An adverse or serious adverse event may result in the volunteer wishing to withdraw from the study or being unable to continue with the study schedule. The subject will not be required to give any reason for withdrawing themselves from the study and will not be asked to do so by the investigator.

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and feed uses, import and processing under Regulation (EC) No 1829/2003 from Monsanto EFSA Journal 2014;12(5):3644 [41 pp.].
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14. Stopping/Discontinuation of intervention

Completion of each subject's involvement in the study will be when the last blood sample is taken, which will be approx. 3 weeks after the subject entered the study for study A and 24 weeks for study B. If there is any reason for discontinuing the intervention prior to its completion the PI will arrange for the research team to inform all volunteers immediately. The PI will also inform the sponsor and the main REC.

15. Monitoring

The project will be overseen and monitored by the Southampton University Hospitals Trust R&D Office.

Steps taken to ensure quality of research

Standard operating procedures will be developed for all aspects of the study. Staff will be fully trained in all procedures in which they are involved. All activities will conform to local health and safety regulations and staff will be adequately trained in these. Good clinical practice and good laboratory practice will be used throughout the study. Staff involved in blood sampling will be properly trained for this. All study samples will be labelled clearly, uniquely, accurately and durably using distinctive water resistant labels printed via computer. All samples will be tracked. The temperatures of fridges and freezers in which samples are stored will be monitored to ensure proper functioning. All analyses will be conducted to the highest standards. All equipment to be used is modern, in good working order and maintained on service contracts. All pipettes to be used are serviced regularly. All data will be recorded in laboratory notebooks that will be signed off by the PI at regular intervals. Data entry into spreadsheets will be carefully monitored. All data will be stored securely.

16. Ethical considerations

The studies will involve the participants consuming a supplement as part of a meal or over the course of several weeks and providing a series of blood samples. Participants will not be aware of whether

they are consuming the fish oil or high omega 3 Camelina oil supplement. Participants will be given an information sheet outlining the nature of the study and they will have the opportunity to discuss any issues they may have with the research staff. Participants may be familiar with having blood sampled, but may not have had a cannula sited. This will be explained fully. Trained researchers will address any concerns that the participants may have. If they remain concerned they will be reminded that they can opt out of any procedure at any time.

17. This study will be conducted in accordance with approvals from the LREC and the Southampton University Hospitals Trust R&D Office.

18. This study will be conducted in compliance with the Research Governance Framework for Health and Social Care, the Medicine for Human Use (Clinical Trials) Regulation 2004 and ICH GCP.

19. Financial arrangements
Funding from the Biotechnology and Biological Sciences Research Council

20. Indemnity
University of Southampton insurance will apply; since an NHS Trust will act as study sponsor, CNST may also apply. University of Southampton insurance may also apply where the cause of harm was not due to clinical negligence as covered by CNST.

21. Reporting and dissemination
Results will be presented at scientific conferences and published in relevant scientific journals. Study participants will be informed of the findings of the study, and the results of their samples if they so wish.

References
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