Study Protocol (Version 2; dated 14/4/2011)

1. Title of project
Incorporation of omega-3 fatty acids from different chemical forms into blood lipid pools in healthy humans

Short title: Incorporation of omega-3 fatty acids

2. Principal investigator
Professor P.C. Calder, Institute of Human Nutrition, School of Medicine, University of Southampton

3. Funder of project
Vifor Pharma, Switzerland

4. Duration of research
36 months from start date

5. Places where research will be conducted
University of Southampton and Wellcome Trust Clinical Research Facility, Southampton University Hospitals NHS Trust

6. Researchers involved
Professor P.C. Calder, Dr G.C. Burdge, Miss A. West

7. Purpose of project/Background
The two long chain omega-3 (n-3) fatty acids of most importance to human health are eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). These fatty acids have been shown to lower risk of morbidity and mortality from cardiovascular disease [1-3]. They exert their protective effects by beneficially altering some of the recognised cardiovascular risk factors [1,2,4]. These fatty acids also exert benefit in inflammatory conditions [5] and perhaps in some cancers [6]. There is emerging evidence that they are important in development of learning and behaviour in childhood [7], in preventing psychiatric and psychological disorders in adults [8] and in slowing cognitive decline in the elderly [9]. As a result of these beneficial effects on human health, particularly the cardioprotective effects, there have been recommendations that individuals should increase their intake of long chain n-3 fatty acids [1,10-14]. The only naturally rich source of EPA and DHA is seafood, especially oily fish. Thus one strategy to increase intake of these fatty acids is to increase fish consumption, and there are recommendations to do so [13,14]. However, many consumers are resistant to taking this option despite the likely benefit. Also the n-3 fatty acid content is highly variable amongst fish species and even within species depending upon time of year, location at which caught etc. Thus, consumption of oily fish once or twice a week as recommended results in irregular intake of an unknown (to the consumer) amount of EPA plus DHA. In addition, some fish species are contaminated with heavy metals and other pollutants [13] and so their intake should be limited [13,14]. An alternative strategy to increase EPA plus DHA intake is to supplement with "fish oil" capsules. These present a useful strategy because capsules can provide a regular (daily) intake of a known amount of n-3 fatty acids. Furthermore, because of fish oil processing technologies, contaminants are removed, and so capsules represent a safe alternative to fish. There are many "fish oils" available and these present the n-3 fatty acids largely in one of two forms, as components of triglycerides (TAGs) or as ethyl esters (EEs). In addition to these forms of presentation, encapsulated n-3 fatty acids in the form of phospholipids (PLs) and of free fatty acids (FFAs) are also available commercially and have been used experimentally. An important question that is thus far not resolved is whether EPA and DHA are equally well incorporated into human blood, cells and tissues when presented in the form of TAGs, EEs, PLs

and FFAs. The literature is not clear about this, with better availability from TAGs being reported in some studies in rats [15,16], better availability from PLs being reported in humans [17], and similar availability from TAGs and EEs being reported in other studies in rats [18] and in humans [19,20]. Reasons for the differences between studies probably relate to differences in how studies were done and how data are reported and interpreted. The n-3 fatty acids can be interesterified between different positions in the parent TAG molecule and this is done in some supplements to increase n-3 fatty acid bioavailability; specifically it is believed that the fatty acid at the 2-position of the TAG has better bioavailability. Whether EPA and DHA are equally available, or not, when presented in different forms is an important scientific question. We believe that the answer to this question is very important to regulators, advisors and consumers, as well as to those involved in the fish oil industry. Therefore, we propose to conduct research in human volunteers that will address this question. We plan to investigate the appearance of EPA and DHA in the bloodstream over a period of weeks of consumption of those fatty acids in different chemical forms.

8. Objectives

The overall objective will be to follow the appearance of EPA and DHA in blood components when the fatty acids are consumed either as TAGs (two different formulations, one with the n-3 fatty acids interesterified and one without this modification), EEs, or FFAs.

The specific objectives are:

1. To follow the appearance over several weeks in plasma lipids and blood cells of EPA and DHA consumed as components of either TAGs or EEs or FFAs.
2. To follow the effect of the different omega-3 formulations on blood markers of inflammation (e.g. cytokines, adhesion molecules).

9. The study

General approach to be taken

Long term (several weeks to several months) studies are required to investigate and to compare the incorporation of n-3 fatty acids into transport pools (blood lipids) and functional pools (e.g. cells) which is a true measure of n-3 status. When included in the human diet EPA and DHA are incorporated into plasma lipid fractions, platelets, white cells, red cells and many other cell and tissue types. We have studied the detail of such fatty acid incorporation, particularly for plasma phospholipids and for white blood cells in at least eight studies in human volunteers and also in patients with advanced atherosclerosis and with Crohn's Disease. In these studies fish oil supplements of various types (but almost exclusively in the TAG form) have been used with blood being sampled at various time intervals over the course of several months. We have reported both the time- and dose-dependent nature of the incorporation into plasma phospholipids and white blood cells (e.g. see [21]). Incorporation of EPA and DHA is typically detectable within weeks. However the rate of incorporation and the time to reach a maximum varies according to turnover of the pool investigated. Thus incorporation into white cells is faster than into red cells. We will use the general approach used in our previous studies with human volunteers to investigate the appearance in plasma lipids (phospholipids, TAGs, cholesteryl esters, NEFAs, EEs), in white cells (mononuclear cells) and in red cells of EPA and DHA when supplemented in the TAG or EE or FFA form.

<u>Subjects and supplements</u>

Healthy males and females aged 18 to 45 years with a body mass index between 20 and 32 kg/m^2 (n = 100) will be recruited. Subjects will recruited via posters; email shots in the University of Southampton, Southampton General Hospital, and other organisations with which the researchers have contact; advertisements in local newspapers. Subjects who express an interest will be screened by telephone interview. 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, Southampton General Hospital.

Recruited subjects will be randomised to one of five groups (n = 20 per group):

- Placebo
- Omega-3 ethyl esters
- Omega-3 free fatty acids
- Omega-3 triglycerides (standard formulation)
- Omega-3 triglycerides (interesterified formulation).

The n-3 fatty acid preparations to be used are all commercially available as supplements and will be provided in capsules. Approximately 1.5 g EPA plus DHA (approx. 1.1 g EPA and 0.4 g DHA) will be given per day. Subjects, researchers and clinical staff will be blinded to group allocation.

Inclusion criteria

1. Aged 18 to 45 years
2. Body mass index 20 to 32 kg/m^2
3. Not consuming fish oil or other oil supplements
4. Not eating more than one oily fish meal per week
5. Willing to adhere to the study protocol
6. Being able to provide written informed consent

Exclusion criteria

1. Aged < 18 or > 45 years
2. Body mass index < 20 or > 32 kg/m^2
3. Being diabetic (type 1 or type 2)
4. Use of prescribed medicine to control inflammation
5. Chronic gastrointestinal problems (e.g. IBD, IBS, celiac disease, cancer)
6. Allergic to fish
7. Participation in another clinical trial
8. Use of fish oil or other oil supplements

Subject participation schedule

Subjects who express an interest will be screened by telephone interview. 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 and if they remain interested an appointment will be made for them to visit the Wellcome Trust Clinical Research Facility, Southampton General Hospital. At the first (and all subsequent) visit, subjects will attend in the fasted state (no food or drink except water after 9 pm the previous evening).

Subjects will visit the Wellcome Trust Clinical Research Facility, Southampton General Hospital on six occasions each between 8 and 10 am. On each occasion they will be in the fasted state. Visits will be at study entry and after approx. one, two, four, eight and twelve weeks of supplementation. On each occasion blood (20 ml) will be taken into heparin; at the visit made at study entry height and weight will be measured. At the end of each visit subjects will be offered toast and a hot drink. Subjects will receive a supply of supplements along with instructions on how to take them at the first study visit.

Sample analysis

Blood will be used to prepare plasma, mononuclear cells and red cells. Plasma will be aliquoted and frozen at minus 80^{o}C. Mononuclear cells will be frozen at minus 80^{o}C. Red cell membranes will be prepared using standard techniques currently in use in the PI's lab and then frozen at minus 80^{o}C. Lipid will be extracted from plasma, mononuclear cells and red cells using chloroform/methanol. Plasma TAGs, PLs, NEFAs and EEs will be isolated by solid phase extraction [21]. The fatty acid composition of plasma TAGs, PLs, NEFAs and EEs and of mononuclear cells and red blood cells

will be determined by gas chromatography. Inflammatory marker concentrations in blood will be measured by flow cytometry.

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 and the Institute of Human Nutrition 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:

- comparison of the change in EPA content of plasma phospholipids from study entry to week 12 between the different treatment groups (the primary outcome). This will be performed by one factor ANOVA.
- comparison of the change in EPA content of each of the other plasma lipid pools and of mononuclear cells and red blood cells from study entry to week 12 between the different treatment groups (the primary outcome). This will be performed by one factor ANOVA.
- comparison of the change in DHA content of each of the plasma lipid pools and of mononuclear cells and red blood cells from study entry to week 12 between the different treatment groups (the primary outcome). This will be performed by one factor ANOVA.
- comparison of the change of the EPA and DHA contents of each plasma lipid pool and of mononuclear cells and red blood cells over time following consumption of n-3 fatty acids in each chemical form. This analysis will be performed using two-factor repeated measures ANOVA, the two factors being time and group (i.e. "treatment").
- comparison of the change over time in blood concentrations of inflammatory markers according to treatment group. This analysis will be performed using two-factor repeated measures ANOVA, the two factors being time and group (i.e. "treatment").

All statistical comparisons will be performed at the end of the study using SPSS.

12. Sample size calculation

The study is powered according to the anticipated change in EPA content of plasma phospholipids (this is similar to the anticipated change in EPA in mononuclear cells and in red cells). Based upon previous studies of this sort, a supplement providing 1.1 g EPA/day is expected to increase the EPA content of plasma phospholipids by 100% from approximately 1 to approximately 2% of total fatty acids. It is considered that a 25% lower incremental increase in EPA content is meaningful. Typical standard deviations for the incremental increase in EPA content of plasma phospholipids from our previous studies are approximately 0.25%. Thus, sample size was calculated using mean values of 1 and 0.75 for the two groups, respectively, and standard deviations of 0.25 for each group. A sample size calculator revealed that a sample size of 17 per treatment group will give 90% power of

detecting this effect (i.e. a 25% increase) as statistically significant, by a pairwise comparison and setting $P < 0.05$, as is usual. In order to allow for a drop-out rate of 20% 20 subjects per group will be recruited (100 subjects in total).

13. Safety assessments

The supplements to be used are commercially available and are safe. The sole invasive procedure to be used is obtaining blood samples; this will be done by trained nursing staff limiting the likelihood of adverse events related to participation in the study. However 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 supplements. 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. In this case or any other instance in which a subject withdraws or is withdrawn from the study a volunteer withdrawal form will be completed. Where the reason is know to the investigator or is volunteered by the subject this will be recorded on the form. 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.

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. 12 weeks after the subject entered the study. 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 study will involve the participants consuming supplements over the course of several weeks and providing a series of blood samples. Participants will not be aware of which supplements they are taking at any given visit. 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 will most likely be familiar with having blood sampled. 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
This study is funded by Vifor Pharma, a producer of dietary supplements.

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 provided to the study funder and subject to approval subsequently presented at scientific conferences and published in relevant scientific journals. An agreement between the funder and the University of Southampton specifies the conditions that will govern such dissemination.

Study participants will be informed of the findings of the study, and the results of their samples if they so wish.

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