Study Protocol (Version 2; dated 1/3/2010)

1. Title of project
Bioavailability and disposition of omega-3 fatty acids from different chemical forms

Short title: Bioavailability 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 available to the human body 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] and

similar availability from TAGs and EEs being reported in other studies in rats [17] and in humans [18,19]. Reasons for the differences between studies probably relate to differences in how studies were done and how data are reported and interpreted. 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 initially investigate the acute appearance of EPA and DHA in the bloodstream in the hours after 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, as EEs, or as FFAs.

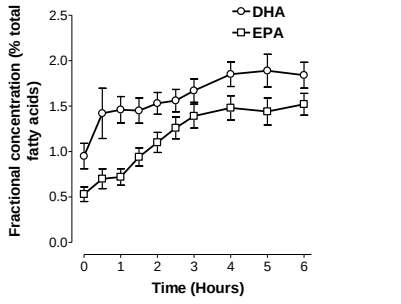
The specific objectives are:

1. To follow the acute appearance (i.e. over the first 6 hours) in plasma lipids and blood cells of EPA and DHA consumed as components of either TAGs or EEs or FFAs.
2. To follow the acute appearance (i.e. over the first 6 hours) in blood of markers of inflammation (e.g. cytokines, adhesion molecules).

9. The study

General approach to be taken

Most fat in the diet is in the form of TAGs. The TAG fatty acids are released in the small intestine as a result of the action of pancreatic lipase. The released fatty acids and unhydrolysed monoacylglycerol are absorbed into enterocytes where TAGs are reformed. These are then packaged into chylomicrons and released first into the lymphatics and then into the bloodstream. Hence there is net appearance of TAG in the bloodstream after consumption of a meal. The fatty acid composition of plasma TAGs over the post-prandial period closely resembles that of the diet just consumed. Thus it is possible to use the fatty acid composition of plasma TAGs over the few hours following consumption of a meal as an indicator of absorption of fatty acids from that meal (although strictly speaking the fatty acid composition will reflect the balance between input from the gut and loss to peripheral tissues). In the experimental setting a standardized meal can be given to human volunteers and this results in a predictable plasma TAG response (see Figure 2 of Ref [20]). We have shown that inclusion of EPA plus DHA (2.3 g in TAG form) in the standard meal results in significant time-dependent appearance of those fatty acids in plasma TAG over a period of 6 hours (see figure).



Concentrations of EPA and DHA in plasma triglyceride following a meal containing EPA+DHA as triglyceride

This approach will be used to investigate the appearance in plasma TAG (and in other plasma lipids and in blood cells) of EPA and DHA when included in the standard meal in TAG or EE or FFA form. In addition, since EEs may escape intestinal hydrolysis and be absorbed directly we will measure appearance of EPA and DHA in plasma EEs.

Subjects and supplements

Healthy male volunteers aged 18 to 45 years with a body mass index between 20 and 32 kg/m^2 (n = 10) will be recruited. They will consume a test meal on five occasions each four weeks apart; the composition of the test meal consumed on each occasion will be identical and will be based upon the control meal used in Ref. [20] (see below). Subjects will consume EPA plus DHA in TAG (three forms), EE, or FFA form provided in capsules with the test meal; this will be done in random order. EPA plus DHA in the TAG (three different TAG formulations will be tested), EE, or FFA form will be sourced by Vifor Pharma; all forms will contain the same (or a very similar) ratio of EPA to DHA

(approx. 3:1) and overall approx. 1.5 g EPA plus DHA (approx. 1.1 g EPA and 0.4 g DHA) will be given per meal.

Inclusion criteria

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

Subject participation schedule

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 and interest will be screened by telephone interview. If they fit the inclusion and exclusion criteria they will be 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 subsequent) visit subjects will attend in the fasted state (no food or drink except water after 9 pm the previous evening).

Subjects will make clinic visits on five occasions; each visit will be at least two weeks apart.

Visit 1

Subjects will arrive at about 7 am and will be in the fasted state. Inclusion and exclusion criteria will be verified (e.g. height and weight will be measured and BMI calculated). Subjects will have the opportunity to discuss the study and have any questions answered. If they are satisfied the consent form will be signed. They will be cannulated and a fasting blood sample (5 ml) taken. They will then consume the test meal (see [20]). The test meal will comprise toast with jam or marmalade (to provide the carbohydrate) and a milkshake made from milkshake powder, double cream, oils and water to provide fat and protein and it will have the following energy and nutrient composition:

Test meal composition

Total fat (g)	55.1
*Total carbohydrate (g)	130.0
*Total protein (g)	12.0
Total energy (kJ)	4.3
	Major sources of fatty acids
Safflower oil (ml)	8.8
Double cream (ml)	47.6
Linseed oil (ml)	1.8
Olive oil (ml)	6.9
	Fatty acid composition (%)
Lauric acid	1.8
Myristic acid	6.3
Palmitic acid	21.5
Stearic acid	8.4
Palmitoleic acid	1.5
Oleic acid	34.0
Linoleic acid	22.1
α-linolenic acid	3.7

* Provided by Nesquik milkshake power, casein food supplement, and sucrose.

During the meal subjects will consume one of five supplements provided in capsule form. These will be consumed in random order and will provide 1.1 g EPA plus 0.4 g DHA.
Blood will be collected again 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 6 h after consuming the meal. Subjects will have nothing further to eat or drink (except water) until the last blood sample is collected.
Blood will be taken into heparin. At each time point 5 ml blood will be collected; total blood volume collected will be 50 ml.

Visits 2, 3 4 and 5
These will be identical to visit 1 except that a different form of supplement will be consumed with the breakfast.

Sample analysis
Blood will be used to prepare plasma, leukocytes and red cells. Plasma will be aliquoted and frozen at minus 80^{o}C. Plasma TAG and NEFA concentrations will be measured. Leukocytes 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, leukocytes and red cells using chloroform/methanol. Plasma TAGs, PLs, NEFAs and EEs will be isolated by solid phase extraction. The fatty acid composition of plasma TAGs, PLs, NEFAs and EEs and of leukocytes and red blood cells will be determined by gas chromatography. Inflammatory marker concentrations in plasma will be measured by ELISA.

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 the comparison of the EPA and DHA content of each plasma lipid pool and white blood cells over time following consumption of n-3 fatty acids in each chemical form. This analysis will be conducted using two-factor repeated measures ANOVA, the two factors being time and chemical form (i.e. "treatment").

In addition, the change in blood concentrations of inflammatory markers will be assessed using the same statistical approach.

All statistical comparisons will be performed at the end of the study using SPSS version 14.

12. Sample size calculation

The study is powered according to the anticipated change in EPA content of plasma TAG. Based upon our recent study [20] providing 1.1 g EPA is expected to increase the EPA content of plasma TAG at 4 h by 180% from approximately 0.5% to approximately 1.4% of total fatty acids. Using standard deviations for EPA content of plasma TAG from our previous study, it is evident that a sample size of 10 will give 80% power of detecting this effect as statistically significant.

13. Safety assessments

The supplements used are commercially available and are safe. All invasive procedures will be conducted 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 the subject involvement in the study will be when the last blood sample is taken, which will be 10 to 20 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 at the same time as consuming a meal (breakfast) on five different occasions and then providing a series of blood samples over the following six hours. 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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