A pilot study to measure omega-3 fatty acid levels in terminal ileal content following four weeks of omega-3 fatty acid supplementation in patients with an ileostomy.

The omega-3 fatty acids (O3FAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential fatty acids predominantly found in oily fish. O3FAs are commonly used as nutritional supplements. O3FAs are considered safe up to, and exceeding, daily doses of 4 g, although doses exceeding 2 g daily usually require split dosing[1]. Side effects that have been observed in large cardiology trials with O3FA capsule formulations are usually minor and predominantly relate to the gastrointestinal tract with nausea, eructation, abdominal discomfort and diarrhoea in up to 10% of cases[2].

Marine O3FAs are naturally present predominantly as the triglyceride (TG) conjugate[1]. 'Nutraceutical' O3FA TGs, as well as free fatty acid and ethyl ester forms, are also available for use in capsule form[1]. O3FAs in the triglyceride form undergo hydrolysis by pancreatic lipase, after which the free fatty acids are re-esterified to a TG conjugate within enterocytes in the small intestine. O3FAs are then incorporated into chylomicrons and subsequently released into circulating lymph for systemic distribution[1]. Absorption is believed to take place predominantly in the small intestine, but a proportion of O3FAs may be directly bioavailable in the large intestine. There is a single study which examined O3FA levels in ileostomy fluid in order to determine absorption of different forms of O3FA formulation[3]. The authors reported that approximately 99 % of O3FAs were absorbed in the small intestine within 24 hours of administering a single O3FA capsule. However, this study was limited by the relatively small dose of O3FA supplementation used (266 mg) and the failure to measure both stoma fluid and plasma O3FA concentration. It has not been studied whether there is colonic luminal exposure to O3FAs and whether it is explained by direct transition of O3FAs through the gastrointestinal tract into the colon, or whether it is secondary to shedding of enterocytes that have incorporated O3FAs following systemic distribution of O3FA after small bowel absorption. This question is of increasing relevance when considering the role of O3FAs in colorectal cancer chemoprevention and potential effects of O3FAs on the intestinal microbiota.

There is increasing recognition and interest in the intestinal microbiota and its role in health and disease[4]. Change in diet alters the intestinal microbiome significantly[5]. However there has been no study of the effect of oral O3FA supplementation on the intestinal microbiome. Moreover, the intestinal microbiota may incorporate and metabolise O3FAs altering host O3FA bioavailability and the overall host O3FA 'lipidome'.

Study synopsis

A pilot study to measure fatty acid levels in terminal ileal luminal fluid content at the beginning of and after 4 weeks O3FA supplementation with two O3FA capsules twice daily (total daily dose 1000 mg EPA and 1000 mg DHA) in patients who have an ileostomy. Individuals with an ileostomy have been identified as an appropriate group of patients to study as terminal ileal content is accessible non-invasively from a stoma and is directly relevant to O3FA exposure in the proximal colon. These individuals have had minimal small bowel resected or have an intact small bowel in continuity and are therefore likely to best reflect small bowel physiology (particularly transit time) in healthy individuals.

The primary endpoint of the study is the level of EPA and DHA in terminal ileal luminal content after four weeks of oral O3FA supplementation compared with O3FA levels detected at the start of supplementation. Secondary endpoints include change in erythrocyte membrane EPA and DHA levels after four weeks O3FA supplementation and levels of other FAs.

The data will be used to inform the design of a future study to determine the contribution of either direct local O3FA delivery to the colon and/or colonic exposure to O3FAs secondary to systemic incorporation, to O3FA concentrations in the proximal colon.

Hypothesis

After 4 weeks of O3FA supplementation there is an increase in terminal ileal EPA and DHA levels compared with basal levels.

Primary endpoint

Change in terminal ileal fluid EPA and DHA concentration at four weeks compared with baseline.

Secondary endpoints

- Change in percentage erythrocyte membrane content of EPA and DHA at four weeks compared with baseline
- Change in terminal ileal fluid EPA and DHA concentration within 24 hours of taking the first O3FA dose.

- Tolerability and adverse events related to O3FA supplementation

Exploratory endpoints

Changes in the intestinal microbiome after O3FA intake for 4 weeks

Inclusion criteria

- Aged 16 years or over
- Either gender
- Temporary or permanent ileostomy fashioned at least 2 months prior to commencing study
- Able to self-medicate and give informed consent
- A minimum period of 2 months availability for the study prior to planned ileostomy reversal

Exclusion criteria

- Seafood allergy
- Ongoing and/or previous use (within 4 weeks of commencing the study) of other O3FA or cod-liver oil supplements
- Previous small bowel resection more than 10 cm
- Metastatic colorectal cancer
- Less than 4 weeks since any chemotherapy or radiotherapy
- Inflammatory bowel disease or other intestinal disease (e.g. coeliac disease) affecting the small intestine

Intervention

A four week intervention period in which participants are required to take two O3FA containing soft gel capsules twice daily with meals (total 1000 mg EPA and 1000 mg DHA daily).

As confirmed for a previous study (ISRCTN 18662143), the Medicines and Healthcare Products Regulatory Authority (MHRA) considers O3FA capsules to be a nutritional supplement and therefore the study does not require Clinical Trials Authorisation.

Recruitment

Suitable patients with a temporary loop or permanent ileostomy will be identified from electronic colorectal surgery department records at St James' University Hospital. For those individuals with a temporary ileostomy, the function and management of the stoma will be assessed in conjunction with the colorectal stoma nurse team. Currently, restoration of intestinal continuity by reversal of the ileostomy is undertaken at between 6 to 12 months following primary colorectal cancer resection. Potential participants will be sent a covering letter and participant information leaflet by post or e-mail. This will include a phone number and e-mail address to contact if an individual wishes to be considered for recruitment into the study. Individuals that express an interest in participating in the study will be contacted by phone at least 24 hours later to assess eligibility for inclusion in the study and, if appropriate, will be invited to an appointment at SJUH. At the hospital visit, a researcher will confirm eligibility for inclusion into the study, answer any questions and complete the consent process, if appropriate.

When any potentially suitable patients are already scheduled for a out-patient/stoma-related hospital visit, a covering letter and participation information leaflet will be sent at least one week prior to the clinic visit. A researcher will be present at the clinic to check eligibility for inclusion into the study and answer any questions. If an individual agrees to take part in the study a consent form will be completed.

O3FA capsules will be issued to patients after completing the consent process. A planned start date for commencing the capsules will be agreed based on the timing of the most convenient next hospital visit.

Timing of intervention and study visits

To ensure participants are able to complete the 4 week intervention period, patients will be asked to commence taking O3FA capsules 4 weeks prior to a planned hospital study visit. Participants will be expected to self-administer capsules with meals for 28 days up until and including the day of the scheduled study visit. Participants will be asked to provide a pre-supplementation baseline stoma sample at the first hospital visit. Current evidence indicates that maximal O3FA content in stoma fluid occurs at between 2 to 8 hours after low dose administration[3]. Therefore participants will be required to provide a stoma fluid sample between 2 and 8 hours after the first capsule dose to identify any immediate change in terminal ileal O3FA concentration. After 4 weeks, participants will be required to provide a post intervention stoma sample after their final O3FA capsule dose at the same time after dosing, at which the first sample was obtained (plus or minus 1 hour). An extra two weeks of O3FA supplements will be supplied in the eventuality that a participant cannot attend the

scheduled study visit. Dietary intake in the past 24 hours will be recorded in order to determine the fat and calcium intake in the 24 hour period prior to obtaining each stoma fluid sample[6]. If an individual experiences side-effects, which are believed to be related to O3FA use during the supplementation period, he/she will be advised to reduce the number of capsules consumed per day e.g. two capsules per day with a subsequent attempt to increase the dose again, symptoms permitting. Any adverse events occurring over the four week supplementation period will be recorded by a researcher. Participants will also be provided with the contact details of the research fellow overseeing the study.

Study visit one

At the initial study visit, a researcher will be present to answer any questions and administer a baseline questionnaire to ensure eligibility for inclusion into the study. If eligible the participant will be asked to complete a consent form.

Those agreeing to take part in the study will be asked to provide a venous blood sample for measurement of erythrocyte membrane EPA/DHA levels. Participants will be asked to provide a stoma fluid sample from their ileostomy bag for measurement of baseline O3FA levels. Dietary intake in the 24 hours prior to obtaining the first stoma fluid will be assessed. In order to assess individual stoma output, participants will be asked to report the number of stoma bag changes, both on the visit day and the day prior to the hospital visit. A convenient date will be arranged to undertake visit 2 and commence taking the O3FA capsules (28 days earlier).

Study visit two

Participants will be required to provide a stoma fluid sample within 2 to 10 hours of the first capsule dose to identify any immediate change in terminal ileal O3FA concentration. If this cannot be done at St James's University Hospital, a researcher will arrange to visit the participant at their home to collect a stoma fluid sample after the first O3FA capsule dose. The time of the first capsule dose and the time at which the stoma sample is collected will be recorded. The researcher will also check dietary intake in the few hours since the first capsule dose was taken.

Study visit three

At the final hospital visit, a venous blood sample will be taken for measurement of erythrocyte membrane EPA/DHA levels. A pill count will be performed and an adverse events questionnaire

administered. The patient will be asked to provide a stoma fluid sample for measurement of O3FA levels. The stoma fluid sample should be collected within 2 to 8 hours of taking the last capsule dose, at a time point matched (+/- 1 hour) to the time the visit 2 stoma fluid sample was taken after capsule dosing. The time of the final capsule dose and the time at which the stoma fluid sample is collected will be recorded. In order to assess individual stoma output, participants will be asked to report the number of stoma bag changes, both on the day and the day prior to the hospital visit. The dietary intake in the 24 hours prior to the last capsule dose will be recorded. If an individual stops taking capsules during the four week supplementation period they will be still asked to attend to provide a stoma fluid and venous blood sample.

Laboratory analyses

Venous blood will be collected into two EDTA sample tubes and transferred to the Leeds Institute of Biomedical & Clinical Sciences (LIBACS) in the Wellcome Trust Brenner Building, where they will undergo immediate centrifugation (800 g for 5') for preparation of plasma and erythrocytes. Plasma samples from each participant will be split between four individual vials for subsequent storage at -80^{o}C across two separate freezers at the LIBACS. For the measurement of erythrocyte membrane EPA and DHA levels, the blood samples will be transported to the Institute of Cancer Therapeutics at the University of Bradford to undergo liquid-chromatography tandem mass-spectrometry analysis.

Stoma contents will be collected into 20ml sterile plastic containers and transferred to the Leeds Institute of Biomedical & Clinical Sciences (LIBACS) in the Wellcome Trust Brenner Building on the St James's site. Samples will be stored at -80^{o}C across two separate freezers at the LIBACS. For the measurement of stoma content EPA and DHA levels, the samples will be transported to the Institute of Cancer Therapeutics at the University of Bradford to undergo liquid-chromatography tandem mass-spectrometry analysis.

Stool samples will be stored at -80^{o}C for a period of less than 2 weeks prior to total DNA extraction by bead-beating followed by the Qiagen© Stool DNA extraction method. Stool DNA will be stored at -80 ^{o}C in the LIBACS Human Tissue Authority-approved freezer. If an increase in EPA and/or DHA level in ileal fluid is observed, we have the opportunity to investigate whether this is associated with changes in intestinal microbiome. PCR amplification of hypervariable regions of bacterial 16S rRNA genes will be performed using barcoded universal primers. In order to identify the variety of bacterial species present, the PCR amplified samples will be sequenced on the Illumina MiSeq platform, followed by classification at a genus level using the Ribosomal Database Project (RDP)

Naive Bayesian Classifier in Mothur. Analysis of the relative proportions of individual bacterial taxa will be performed using quantitative PCR of the total 16S rRNA gene content.

Sample size calculation and statistical analysis

There are no data available in relation to terminal ileal or faecal EPA and DHA levels in subjects consuming O3FA supplements and so we are therefore unable to perform a formal sample size calculation. This is a pilot study in order to inform an appropriate sample size calculation for a future study. In view of previous studies examining O3FA erythrocyte membrane incorporation there is likely to be both significant inter-individual (between 4.4 and 11.8 %) and intra-individual variability (4.1 % +/- 1.9 %) in terminal ileal content of O3FAs[7]. Therefore, we estimate eight participants will be sufficient to produce an appropriate 95 % confidence interval for EPA/DHA concentration in terminal ileal fluid.

According to St James' University Hospital Colorectal stoma department records approximately 102 patients underwent formation of a temporary ileostomy between June 2014 and June 2015. Therefore it is feasible to recruit eight participants into the study over a 10 week period assuming a 50 % recruitment rate.

For the purpose of comparing absolute pre- and post-supplementation levels of stoma content and erythrocyte O3FA levels, a paired Student's t-test will be employed. Adverse events in each group will be compared by Fisher's exact test.

Confidentiality

The participant information sheet (PIS) and participant consent form will specify clearly that any records identifying the participants (e.g. the recruitment questionnaires) and all the information collected from participants during the course of the research will be kept strictly confidential. Participants will be given a unique ID number on entry to the study and all study paperwork (e.g. questionnaires) will coded by ID number rather than participant name or other participant identifier. All electronic data will be kept on a password-protected computer in LIBACS. Hard copy data will be kept securely in a locked cabinet in a locked office in LIBACS.

References

1. Wang C, Harris WS, Chung M et al. 2006. n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary and secondary-prevention studies: a systematic review. *American Journal of Clinical Nutrition*. **84**, pp. 5-17.

2. Schuhardt JP and Hahn J. 2013. Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*. **89**, pp. 1-8.

3. Sanguansri L, Shen Z, Weerakkody et al. 2013. Omega-3 fatty acids in ileal effluent after consuming different foods containing microencapsulated fish oil powder – an ileostomy study. *Food & Function*. **4**, pp. 74-82.

4. Tlaskalová-Hogenová H, Štěpánková R, Kozáková H et al. 2011. The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases. *Cellular & Molecular Immunology*. **8**, pp. 110–120.

5. David L, Maurice CF, Carmody RN et al. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature. 505, pp559-563.

6. Bingham SA, Gill C, Welch A et al. 1997. Validation of dietary assessment methods in the UK arm of EPIC using weighed records, and 24-hour urinary nitrogen and potassium and serum vitamin C and carotenoids as biomarkers. *International Journal of Epidemiology* .**26**(Suppl 1):S137-S151

7. Harris WS and Thomas RM. 2010. Biological variability of blood omega-3 biomarkers. *Clinical Biochemistry*. **43**, pp. 338-340.