Bioavailability of Omega-3 Fatty Acid Formulations and Their Effect on the Intestinal Microbiota

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Published work from this thesis are detailed below:

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Abstract

Due to their proposed anti-cancer effects, omega-3 fatty acids (O3FA) may have a role to play in both chemoprevention and the adjuvant treatment of colorectal cancer (CRC). Novel O3FA drink formulations may provide a more effective method of delivering O3FA supplementation, although O3FA bioavailability in these preparations compared to traditional capsules has not been ascertained. There is also a lack of research exploring the effects of O3FAs on the colonic microbiota and whether this may have any protective effect on CRC carcinogenesis.

This thesis reports the findings of a randomised cross-over trial in healthy volunteers comparing the bioavailability of equivalent doses of O3FA supplementation (2g EPA and 2g DHA daily for 8 weeks) in capsule and drink carton formulations. The trial also explores the effects of O3FA on faecal microbiome profiles. In addition I report the analysis of red blood cell membrane (RBC) EPA levels from the previously reported EMT trial, a Phase II randomised, double-blind, placebo-controlled trial in which patients with colorectal cancer liver metastasis (CRCLM) received EPA (2g daily) prior to surgery.

O3FA supplementation provided in a drinks carton supplementation was non-inferior to an equivalent dose of EPA and DHA provided in capsule form. Faecal microbiome profile analysis revealed subtle changes to the colonic microbiota including reversible increases to *Lactobacillus* and *Bifidobacterium*. Analysis of RBC samples from the EMT study revealed a positive correlation between RBC membrane and CRCLM tissue EPA levels. Participants with EPA RBC membrane levels of >1.22 also exhibited improved overall survival.

This work provides evidence that an O3FA containing drink formulation is of equivalent bioavailability to traditional capsules. Due to their additional nutritional contents they may be of benefit in CRC patients. The effects of O3FAs on faecal microbiome profiles is of significant interest particularly their impact on bacteria associated with anti-CRC effects. Further work is required to elucidate whether O3FAs have a role in CRC chemoprevention or adjuvant treatment via their effects on the colonic microbiota.

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Abbreviations

AA - Arachidonic acid

AICR - American Institute for Cancer Research

ALA - Alpha linolenic acid

AOM – Azoxymethane

AOM-DSS - Azoxymethane Dextran-Sodium Sulfate

BMI - Body mass index

COX - Cyclo-oxygenase

CRC - Colorectal cancer

CRCLM - Colorectal cancer liver metastases

DAABD-AE - 4-[2-(N.N-dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole

DHA - Docosahexaenoic acid

DMAP - 4-(dimethylamino) pyridine

DPA - Docosapentaenoic acid

DSS - Dextran Sodium Sulfate

EDC - 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl

EDTA - Ethylenediaminetetraacetic acid

EGFR - Epidermal growth factor receptor

EMT - Eicosapentaenoic Acid for Treatment of Colorectal Cancer Liver Metastases

EPA - Eicosapentaenoic acid

FADS - Fatty acid desaturase

FAO - Food and Agriculture Organisation

FAP - Familial Adenomatous Polyposis

F:B - *Firmicutes:Bacteriodetes* ratio

FFA - Free fatty acid

GC-MS - Gas chromatography-mass spectrometry

GPCR - G-protein-coupled receptor

HARU - Human Appetite Research Unit

HPLC - High performance liquid chromatography

HNPCC - Hereditary non-polyposis colorectal cancer

HTA – Human Tissue Authority

IBS - Irritable bowel syndrome

IL – Interleukin

ISRCTN - International Standard Randomised Controlled Trial Number

LA - Linoleic acid

LC-MS/MS - Liquid chromatography tandem mass spectroscopy

LIMR - Leeds Institute of Medical Research

LOX - Lipoxygenase enzymes

LNA - Alpha-linolenic acid

LNA-d14 - Deuterated alpha-linolenic acid

MAPK - Mitogen-activated protein kinases

MS – Mass spectroscopy

m/z - mass to charge ratio

NAFLD - Non-alcoholic fatty liver disease ()

NF-κB - Nuclear transcription factor Kb

O3FA - Omega-3 fatty acid

O6FA - Omega-6 fatty acid

OTU - Operational taxonomic unit

OA – Oleic acid

PA – Palmitic acid

PCoA - Principal Coordinate Analysis

PCR - Polymerase chain reaction

PGE_2 - Prostaglandin E2

PGE_3 - Prostaglandin E3

PGE-M - Prostaglandin metabolite

PPAR - Peroxisome proliferator-activated receptor

PUFA - Polyunsaturated fatty acids

RBC - Red blood cells

RCF - Relative Centrifugal Force

RCT – Randomised Controlled Trial

REC - Research Ethics Committee

RNA

ROS – Reactive oxygen species

RPM - revolutions per minute

RvE - Resolvin

SCFA - Short chain fatty acids

SE – Standard error

SEAFOOD - Systematic Evaluation of Aspirin and Fish Oil

SMEDS - Self-micro-emulsifying-delivery systems

SPRI - Solid Phase Reversible Immobilisation

SA - Stearic acid

TE - Tris Ethylenediaminetetraacetic acid

TLR - Toll like receptor

TNBS - Trinitrobenzenesulfonic acid

TNFα - Tumour necrosis factor

TBE - Tris-Borate- Ethylenediaminetetraacetic acid

UV - Ultraviolet spectrum

VEGF - Vascular endothelial growth factor

WCRF - World Cancer Research Fund

WHO - World Health Organisation

Chapter 1: Introduction

1.1 Omega-3 fatty acids and colorectal cancer: potential clinical applications

The omega-3 fatty acids (O3FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential long chain polyunsaturated fatty acids (PUFA) obtained predominantly via dietary intake. They are of particular interest due to their potential anti-inflammatory and anti-colorectal cancer (CRC) activities. The ever-increasing burden of CRC and associations with various dietary risk factors has led to interest in the role of O3FAs in CRC carcinogenesis, particularly their potential applications in chemoprevention and adjuvant CRC treatment.

There is growing understanding of the mechanisms via which O3FAs modulate CRC risk and carcinogenesis. Inflammation is considered key in the stepwise progression from precursor benign adenomas to invasive adenocarcinomas. There is increasing interest in the role of O3FAs as a method of chemoprevention, particularly via modulation of inflammatory pathways (Schottenfeld & Beebe-Dimmer, 2006; Gu *et al.*, 2015; Park *et al.*, 2013). Chemoprevention is defined as the use of natural, synthetic, or biologic chemical agents to reverse, suppress, or prevent carcinogenic progression to invasive cancer (Tsao *et al.*, 2004; Benetou *et al.*, 2015).

Although there has been extensive work to elicit the various mechanisms via which O3FAs are understood to have anti-cancer effects, there has been limited research into the effect of O3FAs on the community of microorganisms (microbiota) within the colon and whether this affects CRC risk (Valdes *et al.*, 2018; Ursell *et al.*, 2012). The colonic microbiome is the collective genome of the

microorganisms residing within the colon and is of particular interest as there is growing evidence that the gut microbiota plays an important role in colorectal carcinogenesis (Valdes *et al.*, 2018; Ursell *et al.*, 2012). Dietary modification and exposure to O3FAs are therefore a potential avenue of chemoprevention and ameliorating CRC risk through both well recognised anti-inflammatory pathways and lesser understood effects on the colonic microbiome.

The anti-cancer properties associated with O3FAs have also led to supplementation being considered as a potential adjunct in the treatment of CRC. A recent review highlights that O3FAs may have synergistic effects in chemotherapy regimens, ameliorate cancer associated cachexia and potentially improve survival outcomes (Lee *et al.*, 2017). O3FA supplements are traditionally provided in capsule form. However recently developed novel O3FA liquid supplementation drinks are of particular interest as they contain additional calorific and nutritional value that would be beneficial in patients recovering from CRC treatment. As either a method of chemoprevention or adjuvant treatment in CRC, the most effective route of O3FA supplementation is unclear, particularly whether the formulations employed in O3FA supplement drinks are an effective method of O3FA supplementation.

1.2 Colorectal cancer

1.2.1 Epidemiology

The research into O3FAs as either a form of chemoprevention or adjuvant treatment in CRC is in part driven by the prevalence of the disease. CRC is the commonest gastrointestinal malignancy worldwide and the second most common cause of cancer deaths in the United Kingdom (Bray *et al.*, 2018; Cancer Research UK, 2014). There are an estimated 41,000 new cases

diagnosed each year, resulting in approximately 16,000 cancer deaths across the United Kingdom (UK) (Cancer Research UK, 2014). Epidemiological data suggests that across western society the incidence of CRC diagnoses is steadily increasing (Center *et al.*, 2009). Interestingly, this increase has also been mirrored in the developing world where the adoption of a western style diet has been implicated (Center *et al.*, 2009). In the UK, CRC incidence started to fall in the 1990s but has again increased since the advent of CRC screening where there is detection of earlier asymptomatic cancers (Jones *et al*., 2009; Logan *et al.*, 2012). Across both genders CRC is predominantly a disease of the over 50s with incidence rates rising steeply between the ages of 50 and 54 years and peak incidence between the ages of 80 and 84 years (Cancer Research UK, 2014). Therefore with an ever-increasing elderly population it is predicted that incidence rates will continue to rise. Both in the UK and across the world the incidence of CRC is higher in males with an age-specific incidence rate 1.7 times higher than females between the ages of 60 and 64 years (Cancer Research UK, 2014). Survival outcomes are related to the stage of disease at diagnosis, with overall survival rates at 1 year, 5 years and 10 years reported as 75.7%, 58.7% and 56.6% respectively (Cancer Research UK, 2014).

1.2.2 Risk factors

There are a number of recognised risk factors associated with the development of CRC. Hereditary conditions associated with CRC include familial adenomatous polyposis (FAP) and the autosomal dominant syndrome hereditary non-polyposis colorectal cancer (HNPCC). Inflammatory bowel disease, particularly Ulcerative Colitis, is also associated with an increased risk of developing CRC (Choi *et al.*, 2017).

Lifestyle factors contribute to an individual's risk of developing CRC. These include obesity, physical inactivity, smoking, excess alcohol consumption, diets high in red meat and inadequate dietary fruit and fibre intake (Giovannucci, 2002; Botteri *et al.*, 2008; Haggar & Boushey, 2009). The risk of developing CRC is associated with obesity as examined in a meta-analysis by Xue *et al.*, which reported an increased risk of developing CRC of 12% and 18% in obese women and men respectively, when compared to persons of normal body mass index (BMI) (Xue *et al.*, 2017). Specific dietary factors implicated as risk factors for the development of CRC include consumption of red meat and low intake of dietary fibre (Parkin, 2011). There are a number of meta-analyses that report CRC risk to be 17% to 30% higher per 100-120g/day of red meat consumption (Parkin, 2011; Aune *et al.*, 2011). Low intake of dietary fibre is a risk factor in approximately 12% of CRC cases (Parkin, 2011). With a range of dietary factors implicated in CRC carcinogenesis there is increasing interest in the potential protective effects of O3FAs on CRC carcinogenesis, the mechanisms by which these might act and whether there are any potential clinical applications.

1.2.3 Aetiology

Colorectal adenocarcinoma is the predominant histological type. The location of colorectal adenocarcinoma within the colon is variable. The rectum and sigmoid colon are the most common sites of primary colorectal tumours (approximately 50% and 30% respectively) (Cancer Research UK, 2014). The remainder of colorectal malignancies are evenly distributed throughout the ascending, transverse and descending colon. CRC is a disease of the colonic mucosa with benign colorectal adenomatous polyps the precursor in the 'adenoma-carcinoma' sequence (Fearon & Vogelstein, 1990; Cotton *et al.*, 1996).

Development of CRC is multi-factorial with the genetic events that lead to tumour development now widely understood. There are a number of genetic mutations that inactivate tumour suppressor genes or drive oncogene expression, subsequently leading to the transition from benign to malignant invasive disease (Goss & Groden, 2000; Morin *et. al*, 1997; Vazquez *et al.*, 2008; Baker *et al.*, 1990; Downward, 1998; Bos *et al.*, 1987).

1.3 Omega-3 fatty acids

1.3.1 O3FA structure and metabolism

O3FAs are long chain PUFAs, in which the first carbon to carbon double bond is positioned at the third carbon from the methyl (CH_3) end of the carbon chain. Eicosapentaenoic (c20:5ω-3, EPA, figure 1) and docosahexaenoic acid (c22:6ω-3, DHA, figure 1) are found naturally in oily fish as a result of the high levels of EPA and DHA contained within algae consumed by fish (Monroig *et al.*, 2013). Humans primarily acquire EPA and DHA through dietary intake and therefore they are referred to as essential fatty acids. EPA consists of a 20-carbon chain and five double bonds, whereas DHA is a 22-carbon chain with six double bonds. Both are classified as O3FAs due to the location of the first double bond at the third carbon from the omega end of the fatty acid structure. As shown in figure 2, EPA and DHA are also synthesised from the shorter chain precursor O3FA α-linolenic acid (ALA), which is found in plant sources such as flax seed (Arterburn *et al.*, 2006; Schuchardt & Hahn, 2013). ALA is converted to EPA and eventually DHA via a series of enzymatic reactions (Arterburn *et al.*, 2006). In humans, this conversion process is relatively inefficient due to limited Delta-6 desaturase activity. Therefore relatively low levels of ALA are converted to the biologically active EPA and DHA (Horrobin, 1993; Nakamura & Nara, 2003). The conversion

rate of ALA to EPA and DHA is greater in women at approximately 14% compared to 10% in men (Yang *et al.*, 2017; Falkiow, 2016). Diets high in linoleic acid (LA), for example from sunflower oil, further reduce the conversion of ALA to the longer chain O3FAs by substrate competition for the delta-6 desaturase enzyme (Emken *et al.*, 1994). Reducing consumption of LA may therefore induce modest increases in EPA and DHA levels (Wood *et al.*, 2015).

PUFA metabolism is affected by single nucleotide polymorphisms (SNP) in the FADS1, FADS2 and ELOVL genes which encode for the delta 5 desaturase, delta 6 desaturase and elongase enzymes (figure 2) (Marquardt *et al.*, 2000; Guillou *et al.*, 2010). SNP in the FADS1 and FADS2 genes may be associated with differences in PUFA metabolism exhibited across both sex and ethnicities. The rs174547 polymorphism in the FADS1 gene is reported to be associated with reduced activity of both delta 5 desaturase and delta 6 desaturase enzymes which results in lower DHA and ALA levels, with a higher proportion of LA (Guo *et al.*, 2017). A European population based study details a number of FADS1 and FADS2 haplotypes that exhibit differential serum DPA and EPA phospholipid levels (Schaeffer *et al.*, 2006). Specific SNP examined in the FADS2 gene are located in CpG islands within promoter regions of the gene which may therefore affect transcription of the FADS2 gene and subsequent expression of the delta 6 desaturase enzyme (Schaeffer *et al.*, 2006; Lattka *et al.*, 2010). The metabolism of AA appears to be affected most by a number of SNP in the FADS1 and FADS2 genes that affect both delta 5 and delta 6 desaturase enzyme activity (Bokor *et al.*, 2010; Schaeffer *et al.*, 2006). Much of the scientific literature examining various SNP in the FADS1 and FADS2 genes measure PUFA levels within serum phospholipids, which may not reflect PUFA metabolism in RBC membranes. However there is emerging evidence that the differential activity of

both delta 5 desaturase and delta 6 desaturase enzymes associated with various SNP is also demonstrated in RBC membranes (zehak *et al.*, 2008).

SNP may also influence an individual's response to O3FA supplementation. This was apparent in a parallel double-blind placebo controlled trial where carriers of minor alleles of the ELOVL2 gene exhibited a greater response to EPA/DHA supplementation when measuring plasma O3FA levels (Alsaleh *et al.*, 2014).

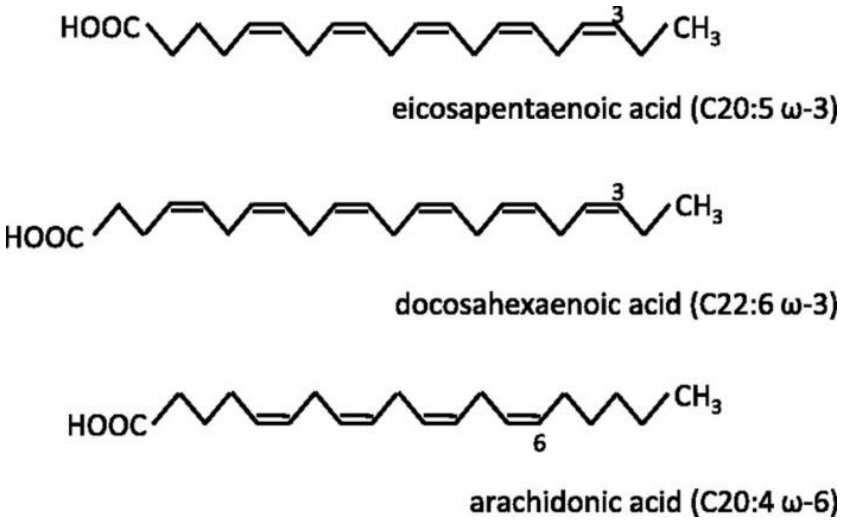


Figure 1. Chemical structure of EPA, DHA and AA.

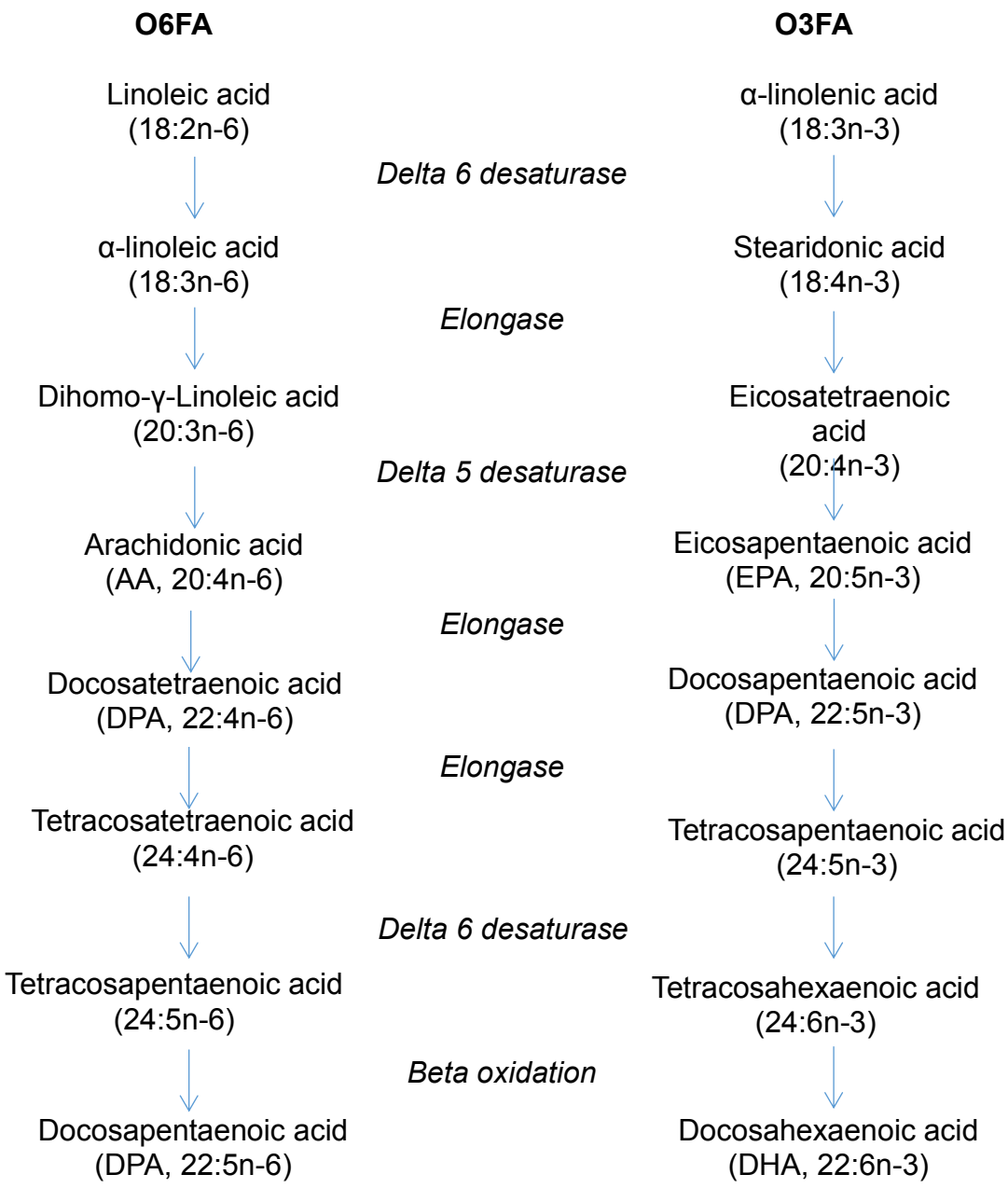


Figure 2. PUFA metabolism and enzymatic steps.

1.3.2 Sources of O3FAs

Although the modern western diet is plentiful in omega-6 fatty acids (O6FA) such as linoleic acid (c18:2ω-6, LA), which are primarily found in vegetable oils, the major dietary supply of O3FAs is from oily fish such as salmon, mackerel, sardine and herring (Arterburn *et al.*, 2006; Shirai *et al*., 2006; Bandarra *et al.*, 2018). A recent American study highlights that the general population consumes on average less than a third of the daily recommended amount of O3FAs (0.5g/day) and that in part was due to low levels of fish consumption (Richter *et al.*, 2017). O3FAs are widely available as over-the-counter nutritional supplements, due to the emerging apparent health benefits. O3FA 'nutraceuticals' are most commonly found in capsule form, although alternative juice drink cartons are now available. O3FAs are considered safe up to daily doses exceeding 4g, although doses exceeding 2g daily usually require split dosing (Wang *et al.* 2006). Side-effects observed in large cardiology trials with mixed O3FA capsule formulations are usually minor and predominantly relate to the gastrointestinal tract, with nausea, eructation, abdominal discomfort and diarrhoea in 0-10% of cases (Wang *et al.,* 2006; Abdelhamid *et al.*, 2018). A recent meta-analysis of 29 trials including over 65000 participants indicated that around three percent of individuals reported a gastrointestinal side-effect associated with O3FA supplementation (Abdelhamid *et al.*, 2018).

1.3.3 Chemical forms and absorption of O3FAs

O3FAs are found in various chemical forms. Marine O3FAs are usually in the triglyceride form, whereby long chain O3FAs are bound to a glycerol backbone. When consumed in triglyceride form, free fatty acids (FFA) are liberated in the small intestine by hydrolysis by pancreatic lipases, after which they are re-

esterified within enterocytes, subsequently incorporated into chylomicrons and then released into circulating lymph for systemic distribution (Schuchardt & Hahn, 2013). Adequate fat intake is required for absorption of triglyceride-bound O3FA supplements, in order to stimulate secretion of pancreatic lipases (Schuchardt & Hahn, 2013; Elvevoll *et al.*, 2006). It is therefore usually necessary to consume triglyceride bound O3FA supplement capsules with a meal, which also limits the gastrointestinal side-effects associated with O3FA supplementation (Abdelhamid *et al.*, 2018). To a lesser extent FFA O3FAs are also found in natural fish oil sources. There are also ethyl-ester O3FA preparations found in refined fish oil preparations (Breivik *et al.*, 1997). The chemical process of re-esterification enables production of re-esterified triglyceride-bound O3FAs that contain much higher concentrations of EPA and DHA (Dyerberg *et al.*, 2010). The importance of the chemical form of O3FA and the relationship to bioavailability will be discussed later.

1.4 Bioavailability of O3FAs

1.4.1 Measurement of O3FA bioavailability

The bioavailability of O3FAs is key when considering any potential clinical applications. Bioavailability is often cited as the amount of active substance that is available within the systemic circulation and is affected by a number of factors including absorption from the gastrointestinal tract. However, as discussed later, the O3FAs are physiologically active within tissues and may take a number of weeks to build up to steady state levels. It is therefore important to appreciate that measures of bioavailability from blood and plasma simply represent the amount of O3FAs available rather than the proportion of physiologically active O3FAs.

There are a number of studies exploring the most appropriate measure of O3FA bioavailability. O3FAs compete with O6FAs for incorporation into cellular membranes with subsequent activation of multiple cellular mediators (Calder, 2006). O3FAs are incorporated into the phospholipid membrane of tissues throughout the body including the cellular membranes of erythrocytes, which provide a convenient measure of O3FA content via collection of red blood cells (RBC) (Surette, 2008; Arterburn *et al.*, 2006; Brenna *et al.*, 2018).

Although plasma O3FA measurement provides a measure of short-term systemic O3FA levels, they are subject to significant short-term variability (von Schacky & Weber, 1985; Cao *et al.*, 2006; Arterburn *et al.*, 2006). Scaioli *et al*. highlighted a rapid increase in O3FA plasma content compared to RBC membrane content in a parallel study comparing 2g daily EPA supplementation in both patients diagnosed with inflammatory bowel disease and healthy volunteers (Scaioli *et al.*, 2015). Steady state O3FA plasma levels were reached at two weeks compared with four to eight weeks in RBC membranes, similar to other published results (Katan *et al.*, 1997; Cao *et al.*, 2006; Stonehouse *et al.*, 2011). There is significant variability of measured O3FA levels in plasma compared with RBC membranes, which appear to provide a more consistent measure of long-term O3FA incorporation as highlighted by Harris *et al*. who reported O3FA level intra-individual variability of 4.1% +/- 1.9% in RBC membranes compared to 15.9% +/- 6.4% in plasma (Harris & Thomas, 2010).

When used in short-term nutritional studies measuring relatively low daily intakes of O3FAs, RBC membrane O3FA levels do not exhibit as strong dose-related relationship to other measures of O3FA bioavailability, for example plasma (Browning *et al.*, 2012). However for intervention studies that utilise O3FA

dosages that far exceed recommended daily intakes, measurement of RBC membrane O3FA content is considered an accurate reflection of long-term O3FA content within multiple target tissues, in part due to the relatively long half-life of RBCs within the systemic circulation (Metcalf *et al.*, 2007; Harris *et al.*, 2004; Brenna *et al.*, 2018). This is of importance when assessing compliance and determining any correlation between O3FA supplementation and clinical outcomes. With specific reference to gastrointestinal tissues, Gurzell *et al.* report that individual EPA and DHA RBC levels, as well as combined EPA and DHA levels, the so-called O3FA index (discussed later) are strongly correlated with EPA and DHA levels measured within colonic tissue of mice, using a variety of O3FA doses up to an equivalent human dosage of 4g per day (Gurzell *et al.*, 2014).

There is limited data in the current scientific literature exploring the bioavailability of O3FAs in CRC patients. There are two randomised controlled trials (RCT) in CRC patients that report rapid incorporation of EPA into colonic mucosal tissue following seven to 12 days of O3FA supplementation (Sorensen *et al.*, 2014; Gee *et al.*, 1999). However neither study examined the bioavailability within colorectal tumour tissue or assessed whether there was any correlation with RBC membrane incorporation.

The O3FA index is defined as a measure of EPA and DHA content within RBC membranes. It is calculated as the sum of EPA and DHA as a percentage of total fatty acid content within RBC membranes and was initially widely used as a biomarker for determining the risk of cardiac death (Harris & von Schacky, 2004; Harris, 2008). An O3FA index greater than or equal to 8% is considered to have cardioprotective benefit using a daily EPA/DHA dose of 1g (Harris & von

Schacky, 2004). A number of observational studies highlight that higher intake of O3FAs, either via supplementation or increased dietary intake is associated with increases in the O3FA index (Block *et al.*, 2008; Sands *et al.*, 2005; Itomura *et al.*, 2008). A RCT examining the effect of varying O3FA supplementation dosages on the O3FA index reported a dose-dependent relationship, and that factors including age, gender and baseline O3FA index levels were predictors of greater increases in the O3FA index post-supplementation (Flock *et al.*, 2019). The O3FA index may be the most suitable measure of O3FA bioavailability in long-term intervention studies given the combined nature of many O3FA supplements and its dose-dependent properties.

1.4.2 Factors affecting O3FA absorption and bioavailability

A number of factors determine absorption and subsequent bioavailability of O3FAs, including chemical form. There are multiple studies examining the effect of chemical binding type on the bioavailability of O3FAs. However there is significant methodological variation, in particular with respect to the blood compartment measured, the duration of supplementation and the dosages of O3FAs administered, with doses up to 7.3g/day of EPA and DHA combined in one study (Krokan *et al.*, 1993). Much of the available literature examines the impact of the chemical form of O3FA supplementation on plasma or serum O3FA levels, rather than RBC O3FA membrane levels. These studies do not provide insight into long-term bioavailability, which is of particular relevance when considering the effects of O3FA supplementation and any role in chemoprevention or adjuvant cancer treatment (Dyerberg *et al.*, 2010; Reis *et al.*, 1990; Hansen *et al.*, 1993; Offman *et al.*, 2017).

FFA O3FA formulations exhibit enhanced EPA and DHA bioavailability in plasma compared with both triglyceride and ethyl-ester forms of O3FA supplementation, which is likely secondary to the bypassing of the pancreatic lipase breakdown pathway (El Boustani *et al.*, 1987; Lawson & Hughes, 1988; Davidson *et al.*, 2012). Re-esterified triglyceride formulations, which have undergone chemical modification to produce concentrations of EPA and DHA of up to 90%, provide the highest levels of O3FA bioavailability in studies examining both plasma and RBC membrane levels (Neubronner *et al.*, 2011; Dyerberg *et al.*, 2010). A RCT of 129 participants comparing re-esterified triglyceride-bound O3FA supplementation and ethyl-ester forms of O3FA supplementation, over a six month period, demonstrated enhanced RBC membrane incorporation in the triglyceride-bound group at three and six monthly intervals, compared with baseline (Neubronner *et al.*, 2011).

Other factors that may impact O3FA uptake and bioavailability include dosing with food. Consumption of triglyceride-bound O3FA supplements requires the concomitant intake of dietary fat to ensure adequate pancreatic lipase secretion for hydrolysis and subsequent uptake of O3FAs within the small intestine (Schuchardt & Hahn, 2013). It is therefore a requirement to consume many O3FA supplements with a meal. The aforementioned long-term studies examining bioavailability of O3FAs in various chemical formulations fail to provide any measure of dietary fat intake (Neubronner *et al.*, 2011; Dyerberg *et al.*, 2010). Short-term supplement studies of O3FA plasma levels report that concomitant consumption of higher amounts of dietary fat result in increased O3FA plasma levels of both triglyceride and ethyl-ester formulations (Lawson & Hughes, 1988; Davidson *et al.*, 2012). This is of significant importance when considering O3FA supplementation as either a chemoprevention or adjuvant treatment, as for both

purposes the requirement to consume supplements with sufficient dietary fat intake may impact acceptability to both healthy individuals and CRC patients. Dietary calcium intake may also affect O3FA bioavailability due to the interaction between calcium ions and FFAs (Scarsi *et al.*, 2015).

The chemical stability of various O3FA preparations is also likely to impact the bioavailability of O3FAs. Due to the presence of double bonds within the O3FA chain they are prone to oxidisation and are replaced by oxidation products including aldehydes and ketones (Shahidi & Zhong, 2010; Benzie, 1996). A study of 32 individual encapsulated O3FA supplements identified that only three (9%) contained equivalent amounts to labelled claims (Albert *et al.*, 2015). The addition of antioxidants which reduce the rate of the oxidation process, for example α-tocopherol, is now commonplace (Zuta *et al.*, 2007).

1.4.3 Novel methods of O3FA supplementation

The requirement to consume many O3FA supplements with dietary fat to facilitate absorption has led to the development of new technologies designed to limit the requirement to ingest O3FA supplements with high fat meals (Lopez-Toledano *et al.*, 2017; Maki *et al.*, 2018). These include self-micro-emulsifying-delivery systems (SMEDS), which enhance the absorption of ethyl-ester preparations of EPA and DHA in a fasting state (Maki *et al.*, 2018). An ethyl-ester preparation that is modified to form micelles in the absence of bile excretion has shown variable absorption of EPA and DHA in the absence of dietary fat (Lopez-Toledano *et al.*, 2017).

The development of novel O3FA supplement juice drinks such as Smartfish™ Remune has led to a potential role in both chemoprevention and as an adjuvant treatment in CRC patients. O3FA supplement drinks have been used in patients

with chronic obstructive pulmonary disease and shown to have clinical benefits in cachectic patients (Calder *et al.*, 2017). This is of relevance in CRC patients as the cartons may present a more acceptable method of O3FA supplementation in individuals whom also require calorific supplementation due to gastrointestinal disturbance, either as a result of surgery or chemotherapy. As a chemoprevention, supplementation drinks may be used as a method of delivering O3FAs in a healthy population, potentially as a substitute to normal dietary intake. However there is a lack of evidence exploring bioavailability of these preparations, specifically comparing RBC membrane incorporation over a prolonged period compared with traditional capsule supplementation. In a placebo-controlled trial Kohler *et al.* showed a mean increase of 2.43% in the O3FA index after daily supplementation for eight weeks with a convenience drink containing 500 mg EPA/DHA, although the chemical form of O3FA is not clear (Köhler *et al.*, 2010). The supplement drink was tolerated well with no severe adverse reactions, although the authors acknowledge that the sample size was insufficient to detect rare side-effects (Köhler *et al.*, 2010). Alternative O3FA preparations are available, including the supplement drink used in the present study which provides a combined EPA/DHA dosage of 2000 mg per carton. On review of the current literature this is equivalent to the O3FA dosages used in human CRC and polyp studies (Courtney *et al.*, 2007; West *et al.*, 2010; Sorensen *et al.*, 2014; Cockbain *et al.*, 2014). One of the key questions surrounding these drink supplements is whether they contain sufficient fat content to stimulate pancreatic lipase release, thus negating the requirement to take triglyceride-bound O3FA supplements with food.

1.5 Protective role of O3FAs in inflammation and CRC carcinogenesis

1.5.1 Epidemiological studies

Interest in the potential health benefits of O3FAs was first identified in population studies of the Greenland Inuits which reported low levels of cardiovascular disease in the presence of high dietary O3FA intake from fish (Bang *et al.*, 1971). A series of epidemiological studies followed, primarily focused on cardiovascular disease, which demonstrated an inverse relationship between fish consumption and cardiovascular mortality (Daviglus *et al.*, 1997; Hu *et al.*, 2002; Iso *et al.*, 2001). The potential cardio-protective benefits of O3FAs prompted research into possible interactions with other diseases including CRC. Initial studies suggested that as the diets of native Alaskans transitioned from one of relatively high fish consumption to one similar to that of non-native Americans there was an increase in the incidence of CRC (Byers, 1996).There are a number of epidemiological studies that suggest an inverse relationship between O3FA supplementation and CRC risk, some of which demonstrate differences across sex, ethnicity and tumour location (Kato *et al.*, 1997; Hall *et al.*, 2008; Kim *et al.*, 2010; Sasazuki *et al.*, 2011). The most tangible epidemiological evidence of a protective association between fish consumption, O3FAs and CRC is a comprehensive meta-analysis of 41 studies covering a 20 year period (total n=1,454,578 patients) which reported that fish consumption was inversely related to CRC risk (OR, 0.88; 95% CI, 0.80-0.95) (Wu *et al.*, 2012). The authors of the aforementioned study reported heterogeneity across retrospective case-controlled studies that was not evident in prospective cohort studies and which impacted on study outcomes. A meta-analysis of fourteen prospective cohort studies reported that the relative-risk of CRC incidence in those consuming the

highest amounts of fish compared to the lowest levels was 0.88 (Geelen *et al.*, 2007). It is apparent that direct comparison of studies is hampered by methodological heterogeneity, particularly quantification of dietary O3FA intake. This is highlighted by the latest consensus report from the 2017 World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR), which acknowledges an association between fish consumption and decreased CRC risk, but describes the evidence as limited (WCRF, 2017). Specific epidemiological evidence related to the consumption of O3FAs and reduced CRC risk is sparse. In a cohort study of 68109 individuals aged between 50 and 76 years, Kantor *et al.* quantified O3FA intake via a dietary questionnaire (Kantor *et al.*, 2014). They reported a 49% lower risk in the development of CRC in users of fish oil supplements compared to non-users.

1.5.2 Mechanisms of O3FA anti-neoplastic activity

In order to implement O3FA supplementation for the purposes of either CRC chemoprevention or adjuvant treatment, an understanding of the mechanisms via which O3FAs are proposed to have an anti-cancer effect is required. The process of inflammation is a driver of colorectal carcinogenesis, with emerging evidence to suggest PUFAs influence colorectal carcinogenesis via modulation of inflammatory pathways and production of eicosanoids such as prostaglandins, thromboxanes and leukotrienes (Gupta & Dubois, 2001; Marnett & DuBois, 2002). Pro-inflammatory eicosanoids promote carcinogenesis via a number of mechanisms including suppression of apoptosis, promotion of angiogenesis and stimulation of cell proliferation (Tsutsumi *et al.*, 2002; Leahy *et al.*, 2002; Cianchi *et al.*, 2001; Pai *et al.*, 2001). The effect of O3FAs on inflammation over prolonged periods is key to their potential application as a method of

chemoprevention in CRC. Both O3FAs and O6FAs are substrates for cyclo-oxygenase (COX) enzymes (Eisinger *et al.*, 2007). This is of significant interest in the context of CRC as eicosanoid generating COX enzymes, specifically COX-2 are overexpressed in 50-80% of tumours (Eberhart *et al.*, 1994; Sano *et al.*, 1995; Kargman *et al.*, 1995). O3FAs including EPA compete with AA for COX binding, thereby effecting eicosanoid production (Smith, 2005; Wang & Dubois, 2010). AA-derived eicosanoids are generally identified as pro-inflammatory, whereas their O3FA derived counterparts are anti-inflammatory. In the presence of EPA there is decreased production of the 'pro-tumorigenic' prostaglandin E2 (PGE_2) which is generated by AA interaction with COX enzymes (Hawcroft *et al.*, 2010). PGE_2 is identified at higher concentrations in CRC tissue compared to normal tissue (Chulada *et al.*, 2000). Reductions in other pro-inflammatory cytokines, including tumour necrosis factor (TNFα) and interleukin 1β (IL-1β) also occur following EPA supplementation (Caughey *et al.*, 1996). In CRC patients a RCT examining the effects of EPA in the treatment of colorectal cancer liver metastases (CRCLM) found a statistically significant reduction in prostaglandin metabolite levels, which may be considered a surrogate marker for PGE_2 production (Cockbain *et al.*, 2014). O3FA supplementation also results in displacement of AA from cell membranes (Crawford *et al.*, 2000). Additionally, EPA specifically exhibits a higher affinity for elongase enzymes than LA, leading to competitive inhibition and thus reducing the conversion of LA to AA (Rose & Connolly, 1999). Therefore supplementation with ALA, EPA or DHA results in a significant decrease in the concentration of AA as shown in a number of studies in healthy individuals (Yaqoob *et al.*, 2000; Thies *et al.*, 2001).

There is increasing evidence to suggest O3FAs influence the production of other inflammatory mediators including resolvins (Moro *et al.*, 2016). Biosynthesis of

these inflammatory mediators from O3FAs is modulated by lipoxygenase enzymes (LOX) (Serhan *et al.* 2015). The Resolvin RvE-1 is postulated to inhibit CRC carcinogenesis via activation of the ChemR23 protein coupled receptor and inhibition of nuclear transcription factor Kb (NF-κB) as demonstrated in mouse models (Kure *et al.*, 2010; Janakiram *et al.*, 2011). NF-κB is a transcription factor vital for propagating inflammatory pathways and tumour development (Clevers, 2004). There is also evidence to suggest that EPA acts as an alternative substrate for COX to generate the anti-inflammatory prostaglandin E3 (PGE_3) (Smith, 2005; Hawcroft *et al.*, 2010). Mouse models of colorectal carcinogenesis exhibit preferential production of anti-inflammatory PGE_3 in the presence of EPA (Vanamala *et al.*, 2008). Examples of the enzymatic generation of both eicosanoids and docosanoids from EPA, DHA and AA are briefly summarised in figure 3.

Incorporation of O3FAs into the cellular membrane may also affect cell membrane structure and integrity, thus influencing CRC development (Chapkin *et al.*, 2002). EPA and DHA are implicated in maintaining intestinal epithelial cell integrity via direct incorporation into the phospholipid membrane (Rees *et al.*, 2006; Nieto *et al.*, 2002). Of particular interest is the effect on 'lipid rafts' that are associated with cell membrane proteins identified in signalling pathways, which may subsequently promote tumour growth (Ibarguren *et al.*, 2014). Mouse models confirm that DHA supplementation results in decreased presence of the epidermal growth factor receptor (EGFR) protein within lipid rafts and a concomitant reduction in tumour weight compared to controls (Rogers *et al.*, 2010). O3FAs also interact with G-protein-coupled receptors (GPCR) located in cell membranes. There are multiple mechanisms of action via which O3FAs are reported to suppress inflammatory pathways, including activation of the GPR120

receptor in macrophages and dendritic cells, and also via production of inflammatory mediators such as resolvins (Arita *et al.*, 2005; Oh *et al.*, 2010). Oh *et al.* reported that DHA inhibits TNFα inflammatory signalling pathways in both *in-vitro* and *in-vivo* studies, where inhibition of the TNFα inflammatory gene is completely abrogated in GPR120 knockout mice (Oh *et al.*, 2010). The peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors are also activated by O3FAs and there is some evidence that this may modulate NF-κB inflammatory pathways (Krey *et al.*, 1997; He *et al.*, 2017). However, the mechanism by which this occurs is not fully understood as in the presence of PPAR inhibitors there continues to be O3FA-induced inhibition of NF-κB mediated inflammatory cytokine production (Draper *et al.*, 2011; Magee *et al.*, 2012).

The presence of a methylene group between two double bonds in the chemical structure of O3FAs makes them highly prone to attack by free radicals, even more so DHA which contains five methylene groups. The process of non-enzymatic lipid peroxidation by reactive oxygen species (ROS) results in the generation of highly toxic products such as aldehydes (Siddiquia *et al.*, 2008). In the context of CRC, lipid peroxidation increases the activation of apoptotic pathways by potentiation of caspase-3 activity in multiple CRC cell lines and presents a potential avenue for CRC treatment (Hossain *et al.*, 2008).

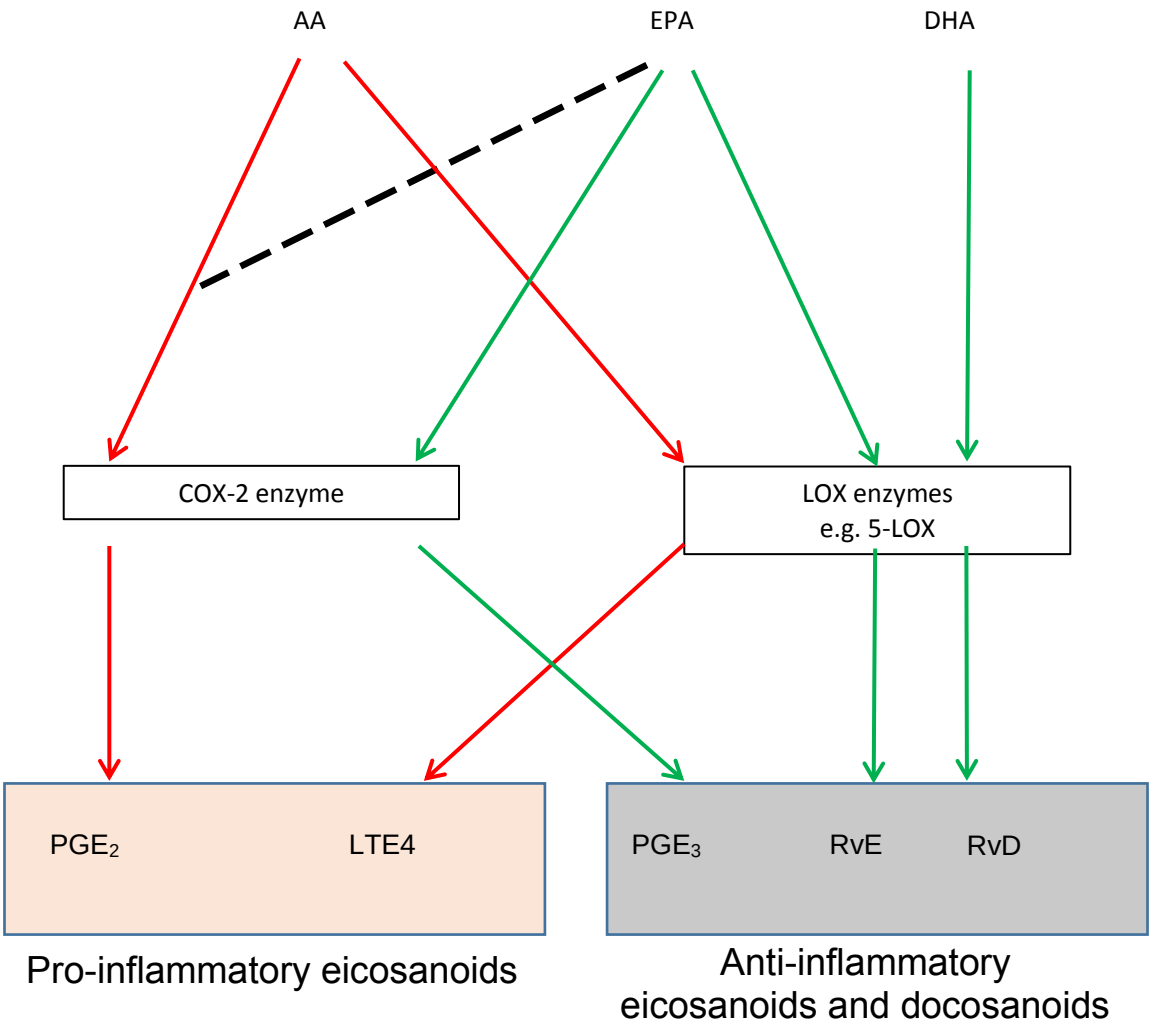


Figure 3. Diagrammatic representation of the enzymatic conversion of EPA, DHA and AA by COX-2 and LOX enzymes to generate pro-inflammatory and anti-inflammatory eicosanoids and docosanoids. Anti-inflammatory eicosanoids and docosanoids include PGE_3 and both D and E series resolvins (RvD and RvE). Pro-inflammatory eicosanoids include Leukotriene 4 (LTE4) and PGE_2

1.5.3 Animal studies exploring the effect of O3FAs on CRC carcinogenesis

The effect of O3FAs on CRC carcinogenesis in mouse models is well documented and provides evidence for the consideration of O3FA supplementation in both chemoprevention and as a CRC adjuvant treatment. There are a number of studies that report both decreased tumour multiplicity and

size following various O3FA containing diets (Petrik *et al.*, 2000; Boudreau *et al.*, 2001; Reddy *et al.*, 1991). The Apc(Min/+) mouse model data reported by Petrick *et al.* illustrated a reduction in both tumour number and size after supplementation with EPA and DHA (Petrik *et al.*, 2000). There was also concomitant reduction in mucosal PGE_2 tissue levels following O3FA supplementation. In the Apc(Min/+) mouse model Fini *et al*. reported that animals fed an EPA-containing diet had a 78% reduction in polyp numbers compared to animals fed a predominantly O6FA containing diet (Fini *et al.*, 2010). The animals fed an EPA rich diet also exhibited lower levels of mucosal AA and a reduction in COX-2 expression. These animal studies are of interest in the application of O3FAs as a method of chemoprevention as they provide evidence to suggest O3FAs impair the transition of normal colonic tissue to pre-malignant adenomas.

EPA and DHA are proposed to impair colorectal carcinogenesis via promotion of apoptosis (Fukunaga *et al.*, 2008). In an Azoxymethane (AOM) induced rat model of CRC, rats fed a combination of O3FAs and pectin exhibited a lower incidence of colorectal tumours compared to animals fed corn oil (Cho *et al.*, 2011). These animals also showed a concomitant increase in the apoptotic index within the colon, and there was also lower expression of genes associated with cell adhesion. Kansal *et al*. reported induction of apoptosis and suppression of cellular proliferation via inhibition of Ras-mediated cellular pathways in mice fed a combination of EPA and DHA (Kansal *et al.*, 2012). Ras controls the growth and differentiation of colonic cells and is of significant importance in colorectal carcinogenesis, in which mutations in the Ras oncogene are identified in a significant proportion of CRCs (Shirasawa *et al.*, 1993; Worthley *et al.*, 2007). The p53 tumour suppression gene is important in the regulation of apoptosis and maintenance of the cell cycle. Mutation of the p53 gene is accompanied by a loss

of function and is implicated in colorectal carcinogenesis (Hainaut *et al.*, 1998). A mouse study comparing the effects of EPA and DHA supplementation in two CRC cell lines, one possessing a p53 mutation (WiDr), the other with wild type p53 (COLO 205), showed decreased cell proliferation in both cell lines following O3FA treatment (Kato *et al.*, 2007). Interestingly, the degree to which tumour cell proliferation was inhibited was dependent on the type of O3FA supplementation, with DHA exhibiting a significantly increased inhibitory effect in wild type p53 cell lines compared to mutated p53 cell lines. This was not apparent in animals fed EPA, where the inhibitory effects of EPA were similar across both cell lines. The authors concluded that the anti-cancer effects of DHA are more profound than EPA and that the mechanism of DHA inhibition of colorectal carcinogenesis is p53 dependent, whereas EPA mechanisms are independent of p53 function. A GCPR mechanism of inhibiting colorectal carcinogenesis by EPA and DHA has been demonstrated in an AOM/DSS-induced CRC mouse model, in which there was significantly reduced tumour incidence and volume (Zhang *et al.*, 2016). There was also evidence of an O3FA dose-dependent reduction in cellular proliferation and an increase in apoptotic cells in an *in-vitro* study (Zhang *et al.*, 2016).

There is emerging evidence to suggest O3FAs ameliorate CRC carcinogenesis via epigenetic mechanisms, including methylation of DNA promoter regions, which subsequently inhibit gene transcription. A mouse study identified higher levels of 5-methylcytosine, a form of methylated DNA in mice treated with O3FAs, which corresponded with a statistically significant lower tumour incidence (Huang *et al.*, 2016). The Bcl-2 protein is an anti-apoptotic mediator which is upregulated in a variety of cancers including CRC (Kirkin *et al.*, 2004). Expression of the Bcl-2 protein is suppressed by methylation of the promoter

region of the BCL-2 gene, thus enhancing apoptosis (Cory & Adams, 2002). In an AOM-induced CRC rodent model, animals fed a combination of EPA and DHA exhibited decreased tumour incidence (Cho *et al.*, 2012). The study also identified a higher apoptotic index in O3FA fed animals, which corresponded with elevated Bcl-2 promoter methylation and lower levels of Bcl-2 transcripts. The downregulation of Bcl-2 expression and concurrent enhancement of apoptosis in the presence of O3FA supplementation has been replicated in other animal models (Hong *et al.*, 2003).

Angiogenesis, the development of new blood vessels, is stimulated in colorectal tumour tissue by the generation of vascular endothelial growth factor (VEGF) (Takahashi *et al.*, 1995). In a CRC mouse model, animals fed EPA or DHA exhibited a statistically significant reduction in tumour volume, tumour weight and microvessel density (Calviello *et al.*, 2004). These findings coincided with decreased expression of both VEGF and COX-2. The inhibition of angiogenesis by O3FA supplementation may be most relevant in the treatment of CRC or the adjuvant treatment of metastatic disease, since VEGF is produced by tumour tissue to stimulate neovascularisation and tumour growth (Guba *et al.*, 2004).

The effect of the ratio of O3FA to O6FA on colonic inflammation and CRC carcinogenesis has been demonstrated in mouse models where animals are fed a 2.5:1 ratio of O3FAs:O6FAs. There was a reduction in the number of N,N'-dimethylhydrazine-induced plaque lesions compared to mice fed a 1:1 O3FA:O6FA diet (Sarotra *et al.*, 2012). The role of the O3FA to O6FA ratio has also been demonstrated in *fat-1* transgenic mice. These mice contain a fat-1 gene that encodes for an O3FA desaturase which enables the conversion of O6FAs to O3FAs (Kang *et al.*, 2004). In *fat-1* transgenic mouse studies of DSS

induced chronic inflammation, the ratio of O6FAs to O3FAs was lower than wild-type mice, with concurrent significant reduction of a variety of inflammatory cytokines (Gravaghi *et al.*, 2011). Similar findings of a suppressive effect on colonic inflammation were identified in a trinitrobenzenesulfonic acid (TNBS)-induced colitis model of *fat-1* transgenic mice, with significant reductions in markers of oxidative stress, pro-inflammatory NF-κB activity and COX-2 expression (Yum *et al.*, 2017). Although the aforementioned studies were primarily focused on the interplay between PUFAs and inflammatory pathways, they are of importance in the consideration of O3FA supplementation as a method of chemoprevention, as inflammation is inextricably linked to colorectal carcinogenesis. In a *fat-1* transgenic mouse model of DSS/AOM induced colorectal carcinogenesis there were both a lower number and volume of colorectal tumours, with down regulation of NF-κB activity and COX-2 inhibition (Nowak *et al.*, 2007). The O6FA:O3FA ratio was also significantly lower in *fat-1* transgenic mice compared to wild type controls, suggesting the endogenously generated O3FAs garnered a protective effect. Similar findings have been reported in another study using *fat-1* transgenic mice where there were a reduced number of colorectal tumours and lower levels of PGE_2 (Jia *et al.*, 2008). There were also higher levels of the anti-inflammatory EPA-derived eicosanoid PGE_3.

1.5.4 The effect of O3FAs on CRC metastasis

Of relevance for consideration of O3FAs as a potential adjunct in the treatment of CRC, is any effect on metastatic disease. An *in-vivo* study exploring colorectal lung metastases identified a reduction in the number of lung metastases in mice treated with EPA and DHA, which the authors accounted for as being secondary

to alterations in cell membrane structure (Ligo *et al.*, 1997). The liver is the most common site of metastatic spread in CRC. In a mouse model of CRCLM Hawcroft *et al.* reported that EPA treatment was associated with a significant decrease in liver tumour burden (Hawcroft *et al.*, 2012). There was also evidence of a reduction in pro-inflammatory PGE_2 levels, with a reciprocal increase in the anti-inflammatory prostaglandin PGE_3. As previously described, PGE_2 is a cytokine associated with colorectal carcinogenesis (section 1.5.2). The effect of O3FA supplementation on CRCLM is also reported in other studies where 21% of mice fed an O3FA-rich diet exhibited tumours in the liver, compared to 70% in the control group that were fed coconut oil (Gutt *et al.*, 2007). It is therefore feasible that O3FAs may have a role in the adjuvant treatment of CRC metastasis. However there is a lack of evidence as to whether the effects on CRCLM exhibited in animal models would be reproducible in other anatomical sites of metastasis or circulating micrometastases, as it may be that the concentration of O3FAs is significantly higher in the liver due to absorption from the gastrointestinal tract directly into the portal circulation.

1.5.5 Clinical studies exploring the effect of O3FAs in CRC as a potential method of chemoprevention

Chemoprevention is defined as primary, secondary or tertiary. Primary and secondary prevention pertain to the implementation of treatments to disrupt the process of carcinogenesis in the general population and those deemed at increased risk of carcinogenesis secondary to the presence of pre-malignant lesions such as colonic polyps (Landis-Piwowar & Iyer, 2014). Tertiary chemoprevention refers to prevention of recurrent or secondary malignancy. There is limited scientific literature pertaining to O3FAs for the purposes of CRC

chemoprevention. Most study outcomes are focussed on biomarkers associated with inflammation and colorectal carcinogenesis, rather than clinical outcomes or the incidence of CRC as a primary endpoint.

In the context of chemoprevention, many of the studies exploring CRC risk are retrospective, do not implement an O3FA intervention and fail to provide details of O3FA bioavailability. Tokudome *et al.* undertook a randomised control trial over a two year period in Japanese participants who had undergone previous removal of colonic polyps and therefore most relevant to the purpose of secondary chemoprevention (Tokudome *et al.*, 2015). The O3FA intervention was achieved via both modification of dietary intake and O3FA supplementation. PUFA bioavailability was measured by analysing RBC membranes. Although there were multiple confounding factors, there were statistically significant elevations in EPA and DHA levels, with concomitant reductions in AA levels in RBC membranes. However this did not translate to a decrease in CRC incidence. There are a number of randomised placebo-controlled trials implementing various concentrations and formulations of EPA and DHA that show a reduction in the proliferation index within colonic mucosa, a surrogate marker of the likelihood of tumour development (Cockbain *et al.*, 2012). Given the adenoma-carcinoma sequence via which CRC develops, polyp prevention trials have been undertaken to ascertain the feasibility of O3FAs in chemoprevention (Fearon & Vogelstein, 1990; Cotton *et al.*, 1996). West *et al.* reported a reduction in both the number and size of colonic polyps in a RCT of 58 patients given 2g of EPA daily for 6 months (West *et al.*, 2010). However this trial was performed in patients with a diagnosis of FAP and therefore may not translate to the development of sporadic adenomas or CRC in the general population. A retrospective case-control study examining 363 cases with adenomas reported

an inverse relationship between the highest levels of serum O3FAs and colorectal adenoma risk (Pot *et al.*, 2008). There was also a statistically significant increased risk of developing colorectal adenomas with higher O6FA serum levels. The most recent data providing evidence for the implementation of O3FAs as a method of secondary chemoprevention is from the Systematic Evaluation of Aspirin and Fish Oil (SEAFOOD) polyp prevention trial. This was a multicentre, randomised, double-blind, placebo-controlled, 2x2 factorial trial in patients aged 55 to 73 years of age with previously identified colonic polyps (Hull *et al.*, 2018). Of the 709 patients recruited into the study, 153 were given 2g EPA per day over a 12 month period at which point a surveillance colonoscopy was undertaken. Although there appeared to be no overall reduction in the development of colonic adenomas after 12 months treatment with EPA, there was evidence of a risk-reduction in the development of left-sided colonic adenomas. The aforementioned studies are the most convincing evidence for the application of O3FA supplementation in CRC chemoprevention, although the potential protective benefits are most apparent in 'at risk' patients with previously identified colonic polyps.

1.5.6 Clinical studies exploring the effect of O3FAs in CRC as a potential method of treatment

Clinical studies examining the effects of O3FAs in CRC patients are limited and primarily focus on nutritional outcomes and measurement of inflammatory markers (Moldawer & Copeland, 1997; Gee *et al.*, 1999; Liang *et al.*, 2008; Sorensen *et al.*, 2014). A RCT in 148 patients awaiting surgical resection of colorectal tumours showed decreased levels of inflammatory markers compared to controls (Sorensen *et al.*, 2014). A smaller RCT in 42 patients undergoing

colorectal resections also exhibited decreased levels of inflammatory markers, but these findings did not translate into any clinical benefit related to mortality or morbidity (Liang *et al.*, 2008). It is of note that both studies were relatively small and undertook O3FA supplementation over a relatively short period (seven days), with the study by Liang *et al.* administering the O3FA intravenously. Due to the anti-inflammatory nature of O3FAs there has been some interest into whether short-term provision of O3FA supplementation would be of any clinical benefit in the post-operative period. A meta-analysis of eleven studies (n=694) identified statistically significant reductions in both length of stay and infectious complications in patients that had received either enteral or parenteral O3FA treatment in the peri-operative period (Xie & Chang, 2016).

With regard to metastatic disease Cockbain *et al.* examined the effect of EPA treatment in a RCT of patients awaiting resection of CRCLM (Cockbain *et al.,* 2014). Although there was no evidence of increased apoptotic activity or reduced cellular proliferation, the study did suggest impairment of vascularity in tumour tissue and even possible improved overall survival. Further examination of the RBC samples from the aforementioned study is undertaken in this thesis to ascertain whether RBC membrane incorporation of EPA has an association with incorporation of PUFAs into CRCLM tissue or patient survival outcomes.

One of the potential applications of O3FAs in the adjuvant treatment of CRC is in combination with standard chemotherapy agents. The mechanisms via which the synergistic effects of O3FA and chemotherapy drugs might take effect have not been fully elucidated. Both *in-vitro* and mouse studies, using a combination of O3FAs and 5-fluorouracil, indicate potentiation of pro-apoptotic effects via a number of mechanisms including downregulation of Bcl-2 expression (Calviello

et al., 2005; Rani *et al.*, 2017). Clinical outcomes including disease-free survival are reported in an adjuvant chemotherapy study of 1011 patients with lymph node positive CRC following surgical resection (Van Blarigan *et al.*, 2018). Dietary marine O3FA intake was measured using a food frequency questionnaire and participants followed up for seven years. There was an association between patients receiving adjuvant chemotherapy and intake of marine O3FAs, with patients in the highest quartile of marine O3FA consumption exhibiting both statistically significantly increased recurrence-free and disease-free survival. Of note was that the estimated median marine O3FA intake in the highest tertile (0.4g/day) was far lower than the dosages used in most O3FA intervention studies. O3FA intervention studies in combination with standard chemotherapy regimens are limited by relatively small numbers of patients (maximum, 23 patients), with treatment periods between 9 to 12 weeks. (Silva *et al.*, 2012; Mocellin *et al.*, 2013; Trabal *et al.*, 2010). These studies indicate a reduction in inflammatory marker levels and improved nutritional outcomes including gains in body weight, which in the setting of adjuvant chemotherapy may be of clinical benefit. These findings are of particular interest with respect to cancer-associated cachexia, which is mediated by a number of inflammatory cytokines and is associated with poor quality of life and increased mortality (Moldawer & Copeland, 1997; Tisdale, 2002).

1.6 O3FAs and the gut microbiome

1.6.1 The colonic microbiota

The microbiota is defined as the collection of microorganisms in a specified location, for instance the colonic microbiota (Claesson *et al.*, 2017). There is an important symbiotic relationship between the colonic microbiota and human host.

Microorganisms are involved in multiple biological processes that contribute to maintaining homeostatic conditions, including production of metabolites that aid healthy gut and immune function (Kau *et al.*, 2011; Clemente *et al.*, 2012). Bacteria residing within the large intestine are predominantly anaerobic, although there are variations throughout the colon according to various factors including pH and bile salt concentration (Neish, 2009). The most abundant phyla within the large intestine are gram-positive *Firmicutes* and gram-negative *Bacteroidetes* (Hollister *et al.*, 2014; Kurokawa *et al.*, 2007). There is evidence to suggest that the gut microbiota influences both mucosal barrier integrity and inflammatory processes within the colonic epithelium. Imbalance of the constituent bacteria within the colon, a so-called 'dysbiosis' is believed to influence multiple disease processes. There is emerging evidence implicating the gut microbiota in colonic inflammation, colorectal carcinogenesis and systemic metabolic dysfunction including insulin resistance and diet induced obesity (Ding *et al.*, 2010; Delzenne *et al.*, 2011; Irrazábal *et al.*, 2014; Sears & Garrett, 2014; Sherafat *et al.*, 2018). The generation of short chain fatty acids (SCFA) including butyrate, proprionate and acetate via bacterial fermentation of fibre is relevant to maintaining intestinal health and is proposed to be protective against colorectal carcinogenesis (Song & Chan, 2019; Wu *et al.*, 2018). Butyrate is also the primary energy source of the intestinal epithelium, again highlighting the synergistic role of the colonic microbiota in maintaining intestinal homeostasis (Wu *et al.*, 2018). Despite mounting evidence to show that dietary modification influences the colonic microbiota and subsequent CRC risk there is limited data to explore whether O3FAs have any effect (Yang & Yu, 2018). This is of particular relevance in relation to the application of O3FA supplementation as a method of chemoprevention.

1.6.2 Microbiome analysis

The advent of microbiome analysis has led to research examining the microbiota residing within the gastrointestinal tract, in particular the colon. The colonic microbiome is defined as the collective genome of the microorganisms residing within the colon (Zoetendal *et al.*, 2004). The technique of microbiome analysis utilises molecular sequencing to identify microorganisms within different environments, allowing identification of bacterial species previously not possible with traditional culture techniques.

Taxonomy and phylogeny are important concepts for classification of bacteria in the process of microbiome analysis. Taxonomic classification of bacteria is a hierarchical system that classifies bacteria according to common characteristics (figure 4) (Washburne *et al.*, 2018). Microbiome analysis identifies bacteria at various taxonomic levels including individual species. Phylogenetic classification is also implemented in microbiome analysis, particularly to measure the diversity of bacteria present within an environment. Phylogeny classifies bacteria according to their evolutionary relatedness and is therefore able to trace bacterial species to common bacterial ancestors (Washburne *et al.*, 2018).

Microbiome analysis utilises the presence of a highly preserved 16S ribosomal RNA subunit in bacterial genomic DNA, enabling polymerase chain reaction (PCR) amplification of bacterial DNA for subsequent genetic sequencing and identification of bacterial species (Zoetendal *et al.*, 1998; Vaughan *et al.*, 2000). The 16S rRNA gene is used for microbiome analysis as it contains both highly conserved regions surrounded by hyper-variable regions which provide optimal species identification (Neefs *et al.*, 1990; Claesson *et al.*, 2017). Ideally the entirety of the 16S rRNA gene would be sequenced, but due to the limited read

length of next generation sequencing a hyper-variable region is selected (Santamaria *et al.*, 2012). The 16S rRNA subunit gene contains conserved sites for binding of universal primers to allow PCR amplification and DNA sequencing. The primer sites are flanked by regions that contain variable sequences that can be exploited to identify bacteria at different taxonomic levels (Claesson *et al.*, 2017).

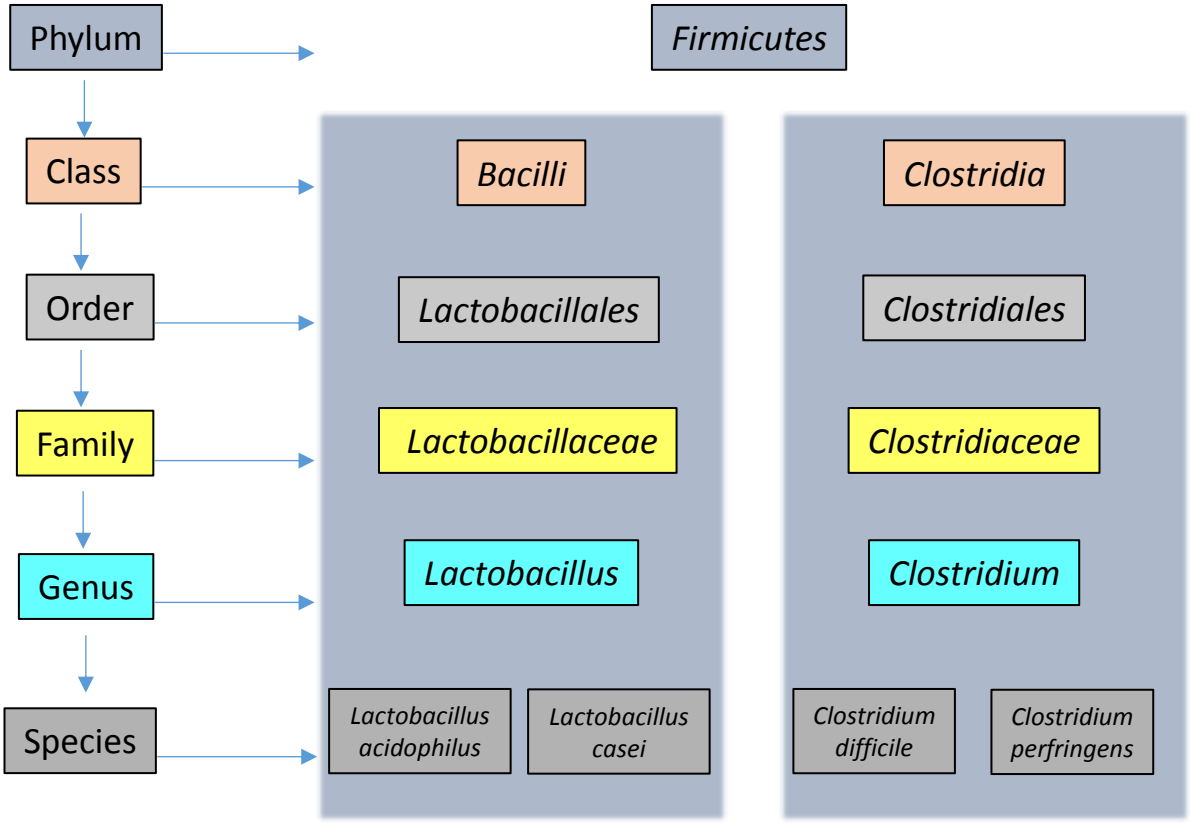


Figure 4. Taxonomic classification of bacteria with examples from the phylum *firmicutes*.

There are nine hyper-variable regions within the gene coding for the 16S rRNA subunit, each exhibiting various degrees of conservation (Vetrovsky & Baldrian, 2013). Each hyper-variable region is approximately 30 to 100 basepairs in length and therefore ideal for next generation sequencing. The degree of preservation across each hyper-variable region guides the taxonomic level to which microbiome analysis can be determined. Therefore hyper-variable regions displaying lower levels of sequence preservation can resolve bacteria at species

level, whereas more highly preserved regions identify bacteria at genus and phylum levels (Jovel *et al.*, 2016). The V4 hyper-variable region of the 16S rRNA subunit is often selected for PCR amplification and sequencing across reported microbiome studies (Caporaso *et al.*, 2011). This region appears to be optimum for resolving bacteria at the phylum level but may fail to identify closely related bacteria at species level that exhibit high levels of sequence similarity (Yang *et al.*, 2016; Jovel *et al.*, 2016). Sequences derived from the same genome, often exhibit a degree of diversity, either due to evolutionary mutations or sequencing errors. To avoid classifying near identical sequences as different organisms a process of binning is performed whereby sequences of typically greater than 95% similarity are clustered into a single Operational Taxonomic Unit (OTU) (Schloss, 2010; Matsen, 2014). This process is particularly important for assessing the bacterial diversity within an environment. α-diversity and β-diversity are commonly quoted measures in microbiome analyses (Wagner *et al.*, 2018). α-diversity is a measure of microbial diversity within a specific environment, for example a stool sample. β-diversity measures the degree to which taxa are shared between two different samples (Wagner *et al.*, 2018).

1.6.3 Gut microbiota and CRC

There is increasing evidence to implicate the intestinal microbiota in playing a role in colorectal carcinogenesis. The concept of dysbiosis is of particular importance in relation to the associations between microbial composition and colorectal carcinogenesis. In the context of CRC, dysbiosis is broadly referred to as an alteration in microbial composition between cancer patients and healthy individuals. It is characterised by both overrepresentation and depletion of a variety of bacteria species that will be discussed later. There are a number of

rodent models that provide evidence of a complex interplay between the microbiota, colonic inflammation and carcinogenesis. In Apc(Min/+) mouse models, mice grown in a germ free environment exhibited a significant reduction in the occurrence of intestinal tumours (Dove *et al.*, 1997; Li *et al.*, 2012).

The colonic bacteria indirectly influence colorectal carcinogenesis via alteration of mucosal integrity and production of inflammatory mediators (Arthur & Jobin, 2011). There are numerous pattern recognition receptors covering intestinal epithelial cells which interact with microbial proteins, resulting in both initiation and regulation of various apoptotic, inflammatory and cellular proliferation pathways (Arthur & Jobin, 2011). Chronic inflammation is associated with development of CRC as shown in individuals diagnosed with inflammatory conditions such as ulcerative colitis (Eaden *et al.*, 2001). Regulation of colonic inflammation is mediated by a single layer of intestinal epithelial cells that play a crucial role in the innate immune response. These cells possess the type I transmembrane glycoproteins, Toll-like receptors (TLR) (Li *et al.*, 2014). Dysregulation of TLR mediated immunity is key in the process of inflammatory-driven colorectal carcinogenesis. A number of TLR polymorphisms are implicated in both the onset and progression of CRC (Messaritakis *et al.*, 2018). TLRs are pattern recognition receptors that respond to a range of ligands, so-called pattern associated molecular patterns, including lipopolysaccharides derived from bacteria (Li *et al.*, 2014; Pradere *et al.*, 2014). Activation of TLRs by pathogenic bacteria activates inflammatory pathways, that if dysregulated may be lead to chronic inflammation and induction of carcinogenesis. TLRs promote carcinogenesis via MyD88 mediated activation of the NF-kB transcription factor, which inhibits apoptosis and initiates production of a variety of inflammatory cytokines including TNFα (Li *et al.*, 2014; Pradere *et al.*, 2014).

TLR4 has been shown in mouse models to be the key TLR implicated in inflammation-driven carcinogenesis, with TLR4 knockout mice showing decreased levels of inflammation and tumour burden in a (AOM)-DSS model (Fukata *et al.*, 2009). Conversely in transgenic mice in which TLR4 is overexpressed, inhibition of TLR4 resulted in fewer polyps in a (AOM)-DSS model of colorectal carcinogenesis (Fukata *et al.*, 2011). The same study also identified that expression of TLR4 was higher in ulcerative colitis patients with CRC compared with patients with no evidence of dysplasia. Modulation of TLR mediated colonic inflammation via changes in the colonic microbiota is therefore of significant interest in colorectal carcinogenesis.

A number of human studies highlight changes in the microbiome profiles of colorectal tumour tissue compared with colonic mucosa in healthy controls or non-tumour tissue from the same individual. These studies have sought to determine any association with CRC carcinogenesis (Chen *et al.*, 2012; Kostic *et al.*, 2012; Geng *et al.*, 2013; Geng *et al.*, 2014; Mira-Pascual *et al.*, 2015; Nakatsu *et al.*, 2015). The studies identify a variety of differences between normal colonic tissue and tumour tissue, thus inferring changes to the microbiome may be associated with colorectal carcinogenesis. However the results are varied and there is a lack of consensus across studies as to the specific changes to microbial composition that occur. The only commonality across any of the aforementioned studies are the results of diversity measures, which are representative of the dysbiosis that is purported to be associated with CRC. Three of the aforementioned studies report that there was an increase in measures of microbial diversity in CRC tissue compared to normal colorectal tissue in the same individual or adenomatous tissue (Mira-Pascual *et al.*, 2015; Geng *et al.*, 2013; Nakatsu *et al.*, 2015). However the largest microbiome

analysis of CRC tissue (n=95) and paired normal tissue did not reveal any differences in microbial diversity (Kostic *et al.*, 2012).

In an effort to determine whether changes in microbial composition and diversity in faeces are associated with CRC, there are a number of human studies examining the microbial composition of faecal samples in CRC patients compared to healthy controls (Sobhani *et al.*, 2011; Wu *et al.*, 2013; Weir *et al.*, 2013; Wang *et al.*, 2011). Again, similar to mucosal tissue studies there are differences between CRC patients and healthy volunteers, but there is significant variation as to the specific changes that occur. With regard to microbial diversity there are conflicting results, with Ahn *et al.* and Yu *et al.* suggesting reduced diversity in CRC patients, whilst other studies do not reveal any differences (Ahn *et al.*, 2013; Yu *et al.*, 2017; Wu *et al.*, 2013; Weir *et al.*, 2013; Wang *et al.*, 2011). Feng *et al.* report that there are no differences in α-diversity across CRC patients, patients with advanced adenomas and healthy controls (Feng *et al.,* 2015). However they did identify increased gene-richness in CRC stool samples compared to stool samples from patients with both advanced adenomas and healthy controls. The authors attribute this finding to the overgrowth of bacteria that may be associated with CRC.

One bacterium that appears to be over-represented across a number of human studies in either faecal samples or CRC tissue is *Fusobacterium nucleatum* (Ahn *et al.,* 2013; Wu *et al.,* 2013; Liang *et al.,* 2017; Yu *et al.,* 2017). The significance of this bacterium in colorectal carcinogenesis will be discussed later.

There is a question about whether the associations identified between the microbiome and CRC represent a causal relationship or if the changes in the microbiome reported are secondary to colorectal carcinogenesis and alterations

in the tumour microenvironment. Mouse studies have examined whether changes in the colonic microbiome result in the development of colorectal tumours or *vice versa*. One study exposed animals (Apc(Min/+)) to *Fusobacterium nucleatum* and reported both accelerated development and an increased number of colonic tumours (Kostic *et al.*, 2013). A second mouse study suggested the relationship between changes to the intestinal microbiome and CRC carcinogenesis is more complex (Zackular *et al.*, 2013). This study highlighted that not only did AOM-induced and DSS-induced inflammation and tumorigenesis induce changes to faecal microbiome profiles, but also conversely when the microbiota from tumour-affected mice was transferred to germ-free mice there was subsequent tumour development without carcinogenic exposure.

1.6.4 Limitations of studies exploring the relationship between the colonic microbiome and CRC carcinogenesis

The findings of studies analysing both faecal and tissue microbiome profiles at phylum, genus and species levels in CRC patients *versus* normal controls are varied, often without consensus. Differences across studies are in part likely due to variations in methodology. The bacterial changes identified in studies examining either cancerous or normal tissue cannot necessarily be interpreted as being representative of changes in the colonic faecal microbiome profiles. Microbiome analysis of luminal faecal samples compared to colonic tissue samples has shown significant differences in the composition of the three predominant phyla including *Firmicutes* and *Bacteroidetes* (Chen *et al.*, 2012). The lack of standardised hyper-variable region selection for PCR amplification and sequencing is likely to result in differences seen in microbiome analysis results, given that the choice of hyper-variable region impacts on the ability to

identify bacteria at phylum, genus and species levels (Jovel *et al.*, 2016). There are also methodological variations across studies with regard to sample storage and DNA extraction techniques which may account for some of the differences encountered in microbiome analysis in studies with otherwise similar designs. There are geographical variations across studies, with some evidence to suggest that the microbiome is influenced by ethnicity (Yazici *et al.*, 2017).

1.6.5 The effects of specific bacteria on CRC carcinogenesis

Studies have shown an increase in the presence of pathogenic bacteria including *Enterococcus* and *Escherichia coli* in CRC patients (Swidsinski *et al.*, 1998; Martin *et al.*, 2004; Grivennikov *et al.*, 2012; Bonnett *et al.*, 2014). *Escherichia coli* is reported to increase polyp burden in mouse models with specific bacterial strains proposed to produce toxic proteins that can induce double stranded DNA breaks (Cuevas-Ramos *et al.*, 2010). The gram-positive bacterium *Enterococcus faecalis* has been identified both *in-vivo* and *in-vitro* to promote CRC carcinogenesis via the generation of hydroxyl radicals that may induce point mutations and chromosomal instability (Huycke & Moore, 2002; Huycke *et al.*, 2001; Cooke *et al.*, 2003; Evans *et al.*, 2004).

A number of human studies have shown depletion of *Bacteroidetes* and *Clostridia* in both stool and tissue samples (Kostic *et al.*, 2012; Ahn *et al.*, 2013; Weir *et al.*, 2013; Wang *et al.*, 2012). Other studies have shown an increased presence of the genus *Bacteroides*, which may be linked to the release of endotoxins causing direct DNA damage and the induction of cellular proliferation pathways such as NF-κB (Qin *et al.*, 2010; Sobhani *et al.*, 2011; Wu *et al.*, 2009; Wu *et al.*, 2013; Karrasch *et al.*, 2007).

The presence of butyrate-producing bacteria such as *Clostridia* appears to be of significant importance (Van den Abbeele *et al.*, 2013). The SCFA butyrate is a breakdown product of carbohydrate and fibre metabolism. It is proposed to have various anti-inflammatory and anti-cancer properties, primarily the regulation of gene expression via inhibition of Histone deacetylase function (Donohoe *et al.*, 2012; McIntyre *et al.*, 1993; Davie, 2003). A large case-control study comparing 344 advanced CRC patients with a matched number of healthy controls illustrated an increased abundance of pathogenic bacteria including *Enterococcus*, whereas healthy controls were found to exhibit increased presence of butyrate-producing bacteria (Chen *et al.*, 2013). Other bacteria which are proposed to be protective against inflammation and CRC carcinogenesis include the butyrate-generating bacteria *Bifidobacteria* and *Lactobacillus* (Wu *et al.*, 2018). In a RCT of patients with a diagnosis of ulcerative colitis, patients treated with a *Bifidobacteria* probiotic exhibited reduced inflammation as measured by a reduction in the presence of inflammatory cytokines including TNF (Furrie *et al.*, 2005). *Lactobacillus and Bifidobacterium* species are protective against colonic inflammation via suppression of both NF-κB and IL-8 (Li *et al.*, 2019). Uronis *et al*. illustrated that pathogen free mice exhibited fewer tumours in AOM-initiated colitis associated CRC, when *Bacteroides vulgatus* was present (Uronis *et al.*, 2009).

The bacteria *Fusobacterium nucleatum* is widely reported to be associated with CRC both in mucosal and faecal samples (Kostic *et al.*, 2012; Ahn *et al.*, 2013). A case-control study identified the presence of *Fusobacterium nucleatum* in 32% of CRC subjects (n=47) compared with only 12% of healthy control samples (n=94) (Ahn *et al.*, 2013). The over-representation of *Fusobacterium* in faeces was also identified in a further study comparing CRC patients and healthy

controls (Feng *et al.*, 2015). A study examining the normal colonic mucosa of adenoma patients and healthy individuals, not only identified that the abundance of *Fusobacterium* was higher in adenoma patients, but also that the magnitude of the abundance of the bacteria was correlated with the likelihood of the presence of adenomas (McCoy *et al.*, 2013). Other studies have examined the bacterial content of colonic tumour tissue to ascertain any relationship between specific bacteria and CRC. One study of 99 CRC subjects reported that the abundance of *Fusobacterium* was 415 times greater in tumour tissue than in normal colonic tissue of the same individual, adding weight to the argument that the tumour micro-environment may confer some benefit to the bacterium (Castellarin *et al.*, 2012).

There is evidence to suggest the presence of *Fusobacterium* is associated with particular subtypes of CRC that have specific molecular traits including microsatellite instability (Tahara *et al.*, 2014). There is also a study suggesting that *Fusobacterium nucleatum* specifically, is enriched in CRC metastases, perhaps indicating that the bacterium has an important role in specific molecular mechanisms of CRC carcinogenesis (Kostic *et al.*, 2012).

The largest study exploring the relationship between *Fusobacterium* and CRC was from a follow-up of health professionals in the United States which included 1069 individuals with CRC (Mima *et al.*, 2015). This study identified that the presence and burden of DNA representative of *Fusobacterium nucleatum* in colonic tumour tissue had a negative impact on CRC specific mortality.

The molecular mechanisms of *Fusobacterium*-initiated CRC carcinogenesis are poorly understood. *Fusobacterium nucleatum* is proposed to generate proteins including Fap2 which inhibit natural killer cells and therefore contribute to

colorectal carcinogenesis (Gur *et al.*, 2015). Other proteins including the adhesion Fad A are implicated in activating various cell signalling pathways including β-catenin and driving the activation of various transcription factors and oncogenes that stimulate CRC cell growth (Rubinstein *et al.*, 2013). The bacteria may also have an effect on the ability of the immune system to inhibit carcinogenesis by increasing the presence of tumour permissive myeloid-derived suppressor cells as exhibited in mouse models (Kostic *et al.*, 2013).

1.6.6 The effect of O3FAs on colonic inflammation, gut microbiota and CRC

There is limited literature examining the effects of O3FAs on the gut microbiota. Most are studies examining the effects of O3FAs on mucosal integrity and intestinal inflammation rather than CRC carcinogenesis specifically (Rees *et al.*, 2006; Nieto *et al.*, 2002). There are a number of mouse studies reporting alterations in the microbiome profiles of mice fed O3FA-rich diets. Myles *et al.* report an increase in the *Firmicutes:Bacteroidetes* ratio and anti-inflammatory effects including increased levels of the cytokine IL-10 (Myles *et al.*, 2014). Interestingly, the same group report evidence of an increased inflammatory response when mice were fed high fat O6FA-rich diets. Ghosh *et al.* showed that mice fed an O6FA rich diet exhibited an increased presence of bacteria implicated in intestinal inflammation, namely *Clostridia* and *Enterobacteriaceae*, whereas mice fed an O3FA-rich diet showed an increased presence of *Lactobacillus* and *Bifidobacteria*, with a concomitant reduction in levels of bacteria associated with intestinal inflammation (Ghosh *et al.*, 2013). Similar findings are also reported in a study of rats with chronic intestinal inflammation, whereby O3FA supplementation reduced the proportion of the inflammatory associated bacteria *Esherichia coli* and *Bacteroides*, whilst there was an

increase in *Lactobacillales* (Li *et al.*, 2011). This corresponded with improved epithelial tight junction integrity in chronically inflamed intestinal mucosa. One of the limitations of studies examining the effects of O3FAs on the intestinal microbiome is that incorporation of O3FAs into tissues is rarely measured. This precludes any investigation of whether the effects of O3FAs on the intestinal microbiome might in fact be secondary to systemic incorporation of O3FAs into intestinal tissue rather than direct luminal exposure. A single study examining the effects of fish oil in mice identified that the microbiome was enriched with *Bifidobacteria* and importantly this corresponded with increased tissue levels of EPA and DHA (Patterson *et al.*, 2011). A limitation of many animal studies examining the effects of O3FAs on the intestinal microbiome is the confounding factors associated with dietary O3FA supplementation. In order to negate these potential dietary confounding factors researchers have employed animal models using the *fat-1* mouse that expresses the *Caenorhabditis elegans* fat-1 gene and therefore endogenously produces O3FAs from O6FAs (Kaliannan *et al.*, 2016; Kaliannan *et al.*, 2015). In one study elevated tissue levels of O3FAs resulted in attenuation of an antibiotic-induced increase in the *Firmicutes:Bacteroidetes* ratio seen in wild-type mice (Kaliannan *et al.*, 2016). In a separate study the same authors reported lower quantities of *Enterobacteriaceae, Escherichia coli*, *Fusobacterium* and *Clostridium* in *fat-1* mice than wild-type mice (Kaliannan *et al.*, 2015). These bacteria are associated with intestinal inflammation and CRC carcinogenesis.

A mouse study examining the caecal contents of mice fed a fish oil diet reported increases in *Lactobacillus*, *Bifidobacteria* and *Akkermansia muciniphilia* (Caesar *et al.*, 2015). As stated previously, both *Lactobacillus* and *Bifidobacteria* are proposed to have a protective effect against colonic inflammation. Any

protective role of *Akkermansia* associated with colorectal inflammation and carcinogenesis is unclear. Some studies suggest the presence of this bacteria is associated with colorectal inflammation via degradation of the protective mucus barrier within the colon and that O3FA supplementation reduces the presence of *Akkermansia* (Pusceddu *et al.*, 2015). A single human study in CRC patients which was limited by the small cohort size identified an increased presence of *Akkermansia muciniphilia* in the faecal samples of CRC patients (Weir *et al.*, 2013). There is a single comprehensive study exploring the effect of OF3As on the intestinal microbiota. A study of 867 individual female twins reported that serum O3FA levels correlated with microbiome diversity and that there was increased representation of the butyrate-producing bacteria *Lachnospiraceae* (Menni *et al.*, 2017).

There is very little evidence to identify any protective benefit of O3FA supplementation in relation to interaction between the intestinal microbiota and CRC carcinogenesis. Piazzi *et al*. reported that a diet containing EPA resulted in reduced number and size of colonic tumours in an AOM-DSS model of colitis-associated CRC (Piazzi *et al.*, 2014). These mice also showed increased presence of *lactobacillus* within microbiome profiles, although it is not possible to determine whether the altered microbiota was responsible for this protective effect as there was also increased activation of apoptotic pathways and inhibition of notch signalling pathways which have both been implicated in CRC carcinogenesis.

The bacterial profile within the gastrointestinal tract may also affect the availability of O3FAs within the colon. It is reported that *lactobacillus* alters the proportion of PUFAs *in-vitro*, including EPA via metabolic conversion

(Kankaanpää *et al.*, 2004). This is of interest when considering if colonic exposure to O3FAs is secondary to systemic incorporation or via direct luminal exposure.

The paucity of evidence regarding the effect of O3FAs on the gut microbiota highlights this as an area of interest, especially given there is increasing understanding of the role of the intestinal microbiota in inflammation and colorectal carcinogenesis. Although there are a number of animal studies that suggest beneficial effects of O3FAs on the intestinal microbiota it is unclear whether O3FAs result in changes to the gut microbiota that may be associated with reduced CRC risk. Amelioration of CRC risk via modulation of the colonic microbiota presents a potential avenue via which O3FA supplementation could be utilised as a form of chemoprevention.

Chapter 2: Aims and hypotheses

2.1 Aims and hypotheses

EPA and DHA are essential O3FAs required for multiple biological functions including cell signalling and gene expression. They are of particular interest due to their potential anti-inflammatory and anti-CRC activities. Therefore O3FA supplementation has a potential role in both CRC chemoprevention and adjuvant treatment as a so-called ‘nutraceutical’.

Despite much research on O3FAs there is a lack of evidence exploring the effect of O3FAs on the intestinal microbiota. This is of significance as epidemiological and clinical evidence suggests a complex interplay between diet, the intestinal microbiota and a potential role in CRC carcinogenesis. Therefore I conducted a randomised, open-label, cross-over trial in healthy volunteers to test the hypothesis that O3FA supplementation alters the colonic microbiota as examined in faecal microbiome profiles from stool samples.

Although naturally present in oily fish, O3FA capsules are usually administered in clinical studies. There are new preparations of O3FA supplements including juice drinks which are of potential clinical use in cancer patients who may also require calorific supplementation. There is a lack of scientific literature exploring bioavailability of these novel supplements. Therefore a secondary aim of the study was to test the hypothesis that an O3FA-containing drink supplement was not inferior to an equivalent O3FA daily dose given in soft-gel capsule form as measured by the change in relative RBC membrane levels of EPA and DHA following supplementation.

In summary the objectives of the study were as follows:

1. To conduct a randomised, cross-over trial in healthy volunteers aged >50 years randomised to O3FA supplementation for a period of eight weeks with either O3FA supplementation in a carton drink form or an equivalent dose in capsules.
2. To determine the effect of O3FAs on the colonic microbiome through PCR amplification of the V4 hyper-variable region of the 16S rRNA gene in faecal samples.
3. To compare the bioavailability of O3FAs in drink and capsule forms by measuring the change in RBC membrane percentage content of EPA and DHA at eight weeks compared with baseline using liquid chromatography tandem mass spectrometry (LC-MS/MS).
4. To measure the difference in RBC membrane percentage content of AA at eight weeks compared with baseline using LC-MS/MS.
5. To assess the acceptability, compliance, tolerability and adverse events related to each O3FA preparation.

The molecular gastroenterology group published the Eicosapentaenoic Acid for Treatment of Colorectal Cancer Liver Metastases (EMT) study (Cockbain *et al.*, 2014). This was a Phase II randomised, double-blind, placebo-controlled trial examining EPA treatment in patients awaiting surgical resection of CRCLM. As part of this study, RBC samples were collected to measure baseline, post-treatment and washout PUFA incorporation levels in RBC membranes, including EPA, DHA and AA. This was not performed at the initial analysis of the study due to financial constraints. As a further aim of his study I measured RBC membrane PUFA levels from the EMT study in order to determine the bioavailability and

PUFA profiles in patients with CRCLM and identify any relationship with clinical outcomes. The objectives were as follows:

1. To measure the change in RBC membrane percentage content of EPA after O3FA treatment and washout using LC-MS/MS.
2. To ascertain whether there is a correlation between RBC EPA membrane levels and CRCLM tumour tissue EPA levels.
3. To perform survival analysis in order to determine whether there is an association between RBC EPA membrane levels and overall survival in patients undergoing curative resection of CRCLM.

Chapter 3: A randomised cross-over trial examining the bioavailability of O3FAs in capsules versus an equivalent dose in a drinks formulation and the effects of O3FA supplementation on faecal microbiome profiles.

3.1 Introduction

The potential application of O3FAs as a method of CRC chemoprevention or adjuvant treatment requires examination of the bioavailability of O3FAs in various forms of supplementation. There is limited data comparing RBC membrane PUFA incorporation following supplementation with O3FAs in a 'nutritional drink' formulation and traditional capsule supplementation. Nutritional drinks are a potential alternative to capsule supplementation, and may be more acceptable to both healthy individuals and CRC patients with gastrointestinal disturbance. O3FA containing drinks may contain sufficient fat content to stimulate pancreatic lipase secretion which is required to facilitate absorption of triglyceride-bound O3FAs from the small intestine.

There is an increasing recognition and interest in the intestinal microbiota and its role in health and disease. However there is a lack of published evidence studying the effect of oral O3FA supplementation on the human intestinal microbiome, in particular with relation to CRC risk. This is of particular significance with regard to utilising O3FA as a potential chemoprevention.

3.2 Overview of study design

An open label, randomised, cross-over trial in healthy volunteers aged >50 years randomised to O3FA supplementation for a period of eight weeks with either (figure 5):

- Two Smartfish™ Remune drinks per day (2000mg EPA and 2000mg DHA per day; see Appendix 1 for full contents)
- Four O3FA capsules twice daily taken with food (2000mg EPA and 2000mg DHA per day)

A minimum 12-week 'washout' period followed each intervention period (figure 5). Participants over 50 years of age were selected to reflect the population in which CRC incidence is highest.

3.3 Primary outcome measure

- Change in RBC membrane percentage content of EPA and DHA at eight weeks compared with baseline

The cross-over trial design allowed intra-individual comparison of both changes to faecal microbiome profiles and O3FA RBC membrane levels after supplementation with capsules and O3FA-containing nutrition drinks. An eight week intervention period was chosen as this would allow steady-state RBC membrane O3FA levels to be reached (Scaioli *et al.*, 2015; Cao *et al.*, 2006; Stonehouse *et al.*, 2011). The intervention period would also be sufficient for changes in microbiome profiles to be apparent as highlighted in studies where rapid reversible changes to the intestinal microbiome have been noted following short-term dietary modifications of between 24 hours and two weeks (Wu *et al.*, 2011; Klimenko *et al.*, 2018). A 12-week 'wash-out' period was adequate for a return to baseline O3FA RBC membrane levels.

Figure 5. Timing and nature of study visits

3.4 Secondary outcome measures

- Change in faecal microbiome profiles after eight weeks O3FA supplementation
- Difference in RBC membrane percentage content of AA at 8 weeks compared with baseline
- Acceptability and compliance with each O3FA preparation
- Tolerability and adverse events related to each O3FA preparation

3.5 Ethical and regulatory approval

The Medicines and Health Products Regulatory Authority (MHRA) confirmed that the use of O3FA supplement capsules was not considered a Clinical Trial of an Investigational Medicinal Product (13/02/2015; Appendix 2). Ethical approval from the South Yorkshire Research Ethics Committee (REC) was received on April 29th 2015 (REC reference 15/YH/0142; Appendix 3). The trial was registered on the International Standard Randomised Controlled Trial Number (ISRCTN) database on 22nd June 2015 (ISRCTN: 18662143).

3.6 Trial design

3.6.1 Timing and nature of study visits (figure 5)

Following an initial screening visit to check eligibility for enrolment into the study, participants were required to attend five scheduled study visits over a 10-month study period. There were two eight week intervention periods followed by a 12-week washout period. Exact timing of the eight week visits during both intervention periods were adhered to whenever possible, although a 24 hour window either side of all planned visit dates was permitted. If attendance at visits 2 and 4 could not be guaranteed then the start of each intervention period (visits

1 and 3) was moved back to ensure the timing of the week eight visit, thus delaying the start of the study (intervention 1) or prolonging the first 'wash-out' period (prior to intervention 2). The washout period was a minimum of 12 weeks' duration. If the first washout period was prolonged, the duration of the second washout period was matched (within one week either side).

3.6.2 Screening visit assessments

At screening, participants completed a questionnaire to check eligibility. Volunteers underwent a taste-test of the study drink. A score of five or above on a 10-point acceptability scale was required in order for the participant to remain eligible. Potential participants were also required to swallow two study O3FA capsules with a glass of water to increase confidence they were able to tolerate swallowing eight capsules per day (four capsules twice a day with meals). Participants were asked to provide written informed consent if eligible to take part in the study. A baseline health assessment was also carried out.

3.6.3 Study visit assessments and sample collection

Blood, urine and stool samples were collected at each study visit. Any adverse events were assessed at visit 2 and all subsequent study visits. Assessment of capsule and drinks carton acceptability using a questionnaire was performed at the end of each intervention period (visits 2 and 4). Volunteer height and weight were collected at visit 3.

3.7 Inclusion & exclusion criteria

The inclusion and exclusion criteria for the trial are listed in table 1. In order to reflect a relevant risk group for CRC chemoprevention, only individuals over the age of 50 years were invited for recruitment into the study.

Inclusion Criteria	Exclusion Criteria
- Healthy male and female subjects ≥50 years	- Ongoing or planned use of other O3FA or cod-liver oil supplements
- Signed informed consent	- Seafood allergy
- Be able to understand and comply with the requirements of the study, as judged by the Investigator	- Unable to provide written informed consent
	- Unable to commit to study timetable
	- Concomitant use of non-steroidal anti - inflammatory medications including aspirin
	- Current treatment for any chronic inflammatory condition or malignancy
	- Previous colonic or small bowel resection
	- Current smoker (minimum of 6 months smoking cessation)
	- Pregnancy
	- Any other condition which according to the Investigator would interfere with the study or safety of the patient
	- Administration of any unlicensed or study product within 4 weeks of entry to the study or during the study
	- Involvement in the planning and conduct of the study

Table 1. Inclusion and exclusion criteria for participating in the randomised cross-over trial.

3.8 Participant Recruitment

Participants were identified from a database of potential volunteers available to the Human Appetite Research Unit (HARU) at the University of Leeds. Poster advertisement around the university campus and use of university approved electronic mailing lists were also employed to identify potential volunteers.

3.9 Randomisation

Participants were randomised to the order of which they received the O3FA intervention at visit one (capsules followed by cartons or *vice versa*). A randomisation schedule was produced by Leeds Teaching Hospitals Pharmacy. Allocation to either intervention order was concealed using envelopes.

3.10 Sample size calculation

There was no published data pertaining to the effects of O3FA supplementation on the human intestinal microbiota. Therefore the sample size calculation was based on the hypothesis that Smartfish™ Renume was not inferior to the equivalent O3FA daily dose given in soft-gel capsule form for the change in relative RBC membrane levels of EPA and DHA following O3FA supplementation.

There were no data available in relation to percentage RBC membrane EPA and DHA levels in subjects consuming Smartfish™ Remune. Based on data from a study in healthy individuals consuming O3FA capsules that reported intra-individual variability of four percent we set a non-inferiority margin of three percent (Harris & Thomas, 2010). Therefore, assuming a participant drop-out rate of 20%, we estimated a sample size of 20 patients was required to exclude a difference in levels of RBC DPA and EHA of three percent or greater with 90% power and at a significance level of 0.05.

3.11 Adverse events and data monitoring

Adverse events were classified as mild, moderate and severe. A mild adverse event was defined as a known side-effect associated with O3FA supplementation but which did not result in cessation of the trial supplementation

(e.g. diarrhoea, eructation, abdominal bloating or discomfort). A moderate adverse event was defined as requiring the participant to cease or reduce the dose of the trial supplementation, whereas a severe adverse event occurred if a participant required hospitalisation as a result of taking the trial O3FA supplements. A regular report was submitted to an independent Gastroenterologist to oversee any adverse events experienced during the study.

Chapter 4: Laboratory methods

4.1 Sample collection and processing for storage

4.1.1 Venous blood

Venous blood samples were obtained using the S-Monovette 7.5 ml, K2 Ethylenediaminetetraacetic acid (EDTA) Gel blood collection system (Sarsedt, Numbrecht, Germany). Blood samples underwent immediate centrifugation at 1000 Relative Centrifugal Force (RCF) for five minutes at 4ºC. Using a 1ml Pasteur pipette, the plasma was aliquoted into two separate 2ml cryovials. The buffy layer containing leukocytes and platelets was carefully discarded. The bottom layer of RBCs was divided equally into two separate 2ml cryovials. All samples were stored at -20ºC prior to transfer on dry ice for storage in the HTA-approved -80 ºC freezers at Leeds Institute of Medical Research (LIMR) at St. James University Hospital, Leeds.

4.1.2 Stool samples

Stool samples were collected using the Fe-Col™ faecal collection device (Alphalabs, Eastleigh, UK) and sent via post to LIMR using the Royal Mail Safebox™ system (Royal Mail, UK). Alternatively, patients were also able to provide a stool sample at the time of the scheduled study visit. A 250mg sample of participant faeces was weighed and aliquoted into a 1.5ml microcentrifuge tube. The microcentrifuge tubes were filled to 1.5ml with an appropriate volume of RNAlater stabilization solution (Thermo Fisher Scientific, MA, USA) and stored in a secure -20ºC freezer at LIMR. The remaining sample was stored in the original stool collection tube at -80ºC in the HTA-approved freezer in LIMR.

4.1.3 Urine samples

Urine samples were collected into a single 20ml universal container and transferred on ice to LIMR. Samples were centrifuged at 1000RCF for five minutes prior to aliquoting in equal volumes into two separate 5ml universal containers for storage in the -80ºC freezer at LIMR.

4.2 Microbiome analysis

4.2.1 Faecal microbial DNA isolation, extraction and recovery

Faecal microbial DNA extraction was adapted from Yu & Morrison (Yu & Morrison, 2004). Participant stool samples were removed from storage in -80°C freezers. A 250µg sample of participant faeces was weighed and aliquoted into a 1.5ml Pathogen Lysis tube to which 1ml of Buffer ASL (Qiagen, Hilden, Germany) was added. The samples were placed on a vortex shaker for 10 minutes at 22000 revolutions per minute (rpm) and transferred to a shaker incubator with parameters set at 95°C and 850rpm for 15 minutes. The samples were centrifuged for one minute at 14000rpm to pool the beads within the pathogen lysis tubes. The supernatant was then aliquoted into a 2ml microcentrifuge tube and placed on ice. The aforementioned process was repeated using 300µl of Buffer ASL. The supernatant was added to the initial sample stored on ice.

Two hundred and sixty microliters of 10M Ammonium Acetate was added to the supernatant contained in the microcentrifuge tube and vortexed to mix. The samples were placed on ice in a Biohazard Class 1 cabinet for five minutes after which samples were centrifuged for five minutes at 14000rpm. The supernatant was aliquoted in equal volumes into two separate 1.5ml microcentrifuge tubes. An equal volume of isopropanol was added to each microcentrifuge tube,

vortexed to mix and placed on ice in a Biohazard Class 1 cabinet for 30 minutes. The samples were centrifuged at 14000rpm for 10 minutes. The supernatant was discarded and 1ml of 70% (v/v) ethanol added to the remaining pellet within each of the 1.5ml microcentrifuge tubes. The samples were centrifuged at 14000rpm for three minutes and the ethanol then discarded. The process was repeated using 500µl of 70% (v/v) ethanol. The samples were left in the Biohazard Class 1 cabinet for 30 minutes to ensure complete evaporation of the ethanol. One hundred microliters of 1x Tris EDTA (TE) Buffer (Invitrogen, California, USA) was added to each sample and vortexed to mix to re-suspend the DNA pellet. Purification of the faecal DNA was performed using the QIAGEN QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). The two separate samples containing the re-suspended DNA pellet in 1xTE buffer were pooled into a single clean 1.5ml microcentrifuge tube to which 200µl of Buffer AL (Qiagen, Hilden, Germany) and 15µl of Proteinase K (Qiagen, Hilden, Germany) were added. The sample was then vortexed to mix and transferred to the shaker incubator for 10 minutes, with parameters set at 70°C and 650rpm. Two hundred microliters of 100% ethanol was added to each sample and vortexed to mix, followed by centrifugation at 3500rpm for five seconds. The supernatant was transferred to a QIAamp Mini Spin Column (Qiagen, Hilden, Germany) and centrifuged at 14000rpm for one minute. The column was then transferred to a clean 2ml collection tube to which 500µl of Buffer AW1 (Qiagen, Hilden, Germany) was added and then then centrifuged at 14000rpm for one minute. The column was then transferred to a clean 2ml collection tube to which 500µl of Buffer AW2 (Qiagen, Hilden, Germany) was added and then then centrifuged at 14000rpm for three minutes. The column membranes were dried by transferring to a clean collection tube and centrifuging at 14000 rpm for one minute. The DNA bound to the column

membrane was eluted into 100µl of molecular grade water (Invitrogen, California, USA) in a clean 1.5ml microcentrifuge tube by centrifuging at 14000rpm for one minute.

Quantification of faecal DNA was performed using the Nanodrop photospectrometer (Thermofisher Scientific, MA, USA). The ratio of sample absorbance at 260nm and 280nm is a measure of DNA purity. A 260/280 ratio of greater than 1.8 was accepted as representing adequate DNA purity with lower values indicating presence of contaminants from the DNA extraction process. The 260/230 ratio is a secondary measure of nucleic acid purity. 260/230 ratio values between 1.8 and 2.2 are accepted as representing pure DNA. Faecal DNA was stored at -80ºC.

4.2.2 PCR amplification of the 16S rRNA gene

16S rRNA V4 amplification and library preparation were adapted from Taylor *et al.* (Taylor *et al.*, 2017). Thawed faecal DNA samples were diluted into molecular grade water (20ng/µl) and 1µl of sample added to individual wells in a sterile 96 well plate. Two and a half microliters of both forward and reverse primers (10nM) specific to the V4 hyper-variable region of the 16S rRNA gene (V4F-5′-AYTGGGYDTAAAGNG, V4R- 5′— TACNVGGGTATCTAATCC) were prepared into a reaction volume (50µl) containing 1µL of 20ng/µL template DNA, 10µL of 5× QC Reaction Buffer, 0.5µL of Q5 Hot Start High Fidelity DNA Polymerase, 10µL of 5× Q5 High QC Enhancer, 1µL of 10mM deoxynucleotides (all New England Biolabs, MA, USA) and 22.5µl molecular grade water and added to each well. Degenerate base symbols N, V and Y represent alternative bases that occupy a position in the DNA primer sequence. Both a negative control sample containing 1µl of C2020 DNA from a B-lymphoblastoid cell line and a positive

control containing 1µl of *E. Coli* DNA were added to two separate wells. A well containing 1µl of molecular grade water was also prepared as an additional negative control. The 96 well plate was vortexed to mix at 2500rpm for five seconds.

The PCR reaction was undertaken in a Bio-rad C1000 thermocycler (Bio-rad, CA, USA). Initial denaturation was performed at 98ºC for a period of 30 seconds. This was followed by 30 cycles programmed to undergo denaturation at 98ºC for five seconds followed by a 10 second annealing step at 42ºC and a 20 second elongation step at 72ºC. A final elongation step was carried out at a temperature of 72ºC for a period of 120 seconds.

To ensure successful PCR amplification of faecal DNA samples, gel electrophoresis was performed using a 3% agarose gel and 1x Tris-Borate-EDTA (TBE) buffer (Thermo Fisher Scientific, MA, USA). The gel was run at 100 volts for 25 minutes. Gels were stained using 0.05% ethidium bromide and visualised under ultraviolet (UV) light. A 50bp DNA ladder (New England Biolabs, MA, USA) was also run on the gel for size comparison.

4.2.3 Purification of amplified 16S rRNA PCR product

Purification of the PCR amplification product was performed using the Minelute PCR purification kit (Qiagen, Hilden, Germany). Fourty five microlitres of amplified PCR product was added to 225µl of PB buffer in a clean spin column. Samples were centrifuged at 13000rpm for one minute. The collection tube was discarded and 750µl of buffer PE added to the spin column and centrifuged at 13000rpm for one minute. Spin column membranes were dried by further centrifugation at 13000rpm for one minute. Purified PCR product was eluted into 10µl of buffer EB after centrifuging at 13000rpm for one minute.

4.2.4 Quantification of PCR product

In order to determine the concentration of double stranded DNA following PCR amplification of the V4 hyper-variable region of the 16 rRNA gene the Quant-iT dsDNA Broad Range Assay Kit (Life Technologies, CA, USA) was employed. This requires Picogreen which is a cyanine dye that fluoresces upon binding to double stranded DNA despite the presence of various contaminants including RNA, single stranded DNA and free nucleotides.

Firstly a standard curve was prepared using known quantities of double stranded DNA (5ng, 10ng, 20ng, 40ng, 60ng, 80ng, 100ng). Two microliters of purified PCR product from each participant sample was added to a well of a clean 96 well plate. One microliter of 2x Picogreen (Thermo Fisher scientific, MA, USA) reagent buffer was added to 197µl of 10x Quant-it buffer and aliquoted into each individual sample well. Fluoresence was measured using a Fluoroskan Ascent 2.6 fluorometer (Thermofisher Scientific, MA, USA).

4.2.5 Library Preparation

In order to perform DNA sequencing a series of modifications to the purified PCR product from each participant sample were necessary. Library preparation was performed under the supervision of Dr. Morag Taylor at the University of Leeds using the NEBNext Ultra DNA Library Prep Kit for Illumina and NEBNext Singleplex Oligos for Illumina (New England Biolabs, MA, USA).

4.2.5.1 End prep of PCR product

Firstly the 3'-5' and 5'-3' cohesive overhangs of each DNA fragment were enzymatically removed to facilitate ligation of an adaptor. One hundred nanograms of purified PCR product from each participant sample was required

for library preparation. The amount of purified PCR product required for each participant was determined from the concentration of DNA calculated in the picogreen assay. The appropriate amount of purified PCR product for each participant sample was added to individual wells in a 96 well plate after vortexing to mix. Varying amounts of TE buffer (Invitrogen, CA, USA) were added to each well to make a total reaction volume of 55.5µl per well. The NEBNext End Prep Enzyme Mix (3µl) was added to the NEBNext End Repair Reaction Buffer (6.5µl) which was subsequently aliquoted into each sample well containing 100ng of participant DNA (Total volume 65µl). The 96 well plate was placed on to the Biorad C1000 thermocycler at 20ºC for 30 minutes followed by period of 30 minutes at 65ºC to denature the enzymes.

4.2.5.2 Adaptor Ligation

Oligonucleotide adapters were attached to the blunt ended DNA fragments to facilitate binding to the flow cell of the Illumina next generation sequencer. Firstly the NEBNext Adaptor for Illumina (New England Biolabs, MA, USA) was diluted in molecular grade water. Each blunt ended DNA sample (65µl) was added to an individual well of a clean 96 well plate. A mix of 15µl of Blunt/TA ligase master mix, 2.5µl of NEBNext adaptor for Illumina and 1µl ligation enhancer were aliquoted into each well to make a total volume of 83.5µl per sample. The plate was then vortexed to mix and centrifuged at 3700 rpm for 10 seconds followed by a period of incubation at 20°C for 15 minutes on a thermal cycler. The plate was subsequently centrifuged at 3700rpm for one minute before adding 3µl of USER enzyme (New England Biolabs, MA, USA) to each sample, vortexed to mix and centrifuged at 3700 rpm for 10 seconds. The plate was incubated for a period of 15 minutes at 37°C.

4.2.5.3 Purification of ligated PCR product

Following ligation of the adaptor to the blunt ended DNA, a purification process was required to remove various contaminants and ligation enzymes. Solid Phase Reversible Immobilisation (SPRI) beads are para-magnetic beads activated by a magnetic field and to which DNA reversibly binds in the presence of certain chemicals. AMPure XP Beads (Beckman Coulter, CA, USA) were added to the ligated DNA samples in the 96 well plate in a ratio of 0.8 of the volume of modified DNA mixture. The beads were mixed by gentle pipetting and incubated at room temperature for five minutes. The 96 well plate was placed on a magnetic rack to activate the magnetic beads and facilitate movement out of solution to the base of the plate. The supernatant was removed using a pipette and disposed of. The beads were washed with 200µl of 80% (v/v) ethanol, incubated at room temperature for 30 seconds and the ethanol then removed. The process of washing the beads with ethanol was then repeated. In order to remove the ligated DNA from the SPRI beads 17µl of Buffer EB (Qiagen, Hilden, Germany) was added to each well and mixed by pipetting. To separate the SPRI beads from the purified ligated DNA product the 96 well plate was placed on the magnetic rack for five minutes. Fifteen microliters of purified ligated DNA product was aliquoted from each well into a clean 96 well plate ensuring the SPRI beads were not disturbed.

4.2.5.4 Enrichment of ligated PCR product

PCR amplification of the ligated PCR product was performed in order to facilitate successful sequencing. A reaction mixture of 25µl NEBNext Q5 Hot Start (2X) plus 5µl Universal PCR Primer (10µM) was added to each well of the 96 well plate containing 15µl of purified ligated DNA product from each participant

sample. Each sample was mixed by gentle pipetting. Five microliters of unique in-house designed index primers (Integrated DNA Technologies, IA, USA) were added to enable amplification of the ligated adaptor sequence of the modified PCR product. Each unique index primer corresponded to an individual participant stool sample. The 96 well plate was sealed and vortexed to mix followed by centrifugation at 3000rpm for five seconds. The PCR reaction was undertaken in a Biorad C1000 thermocycler. Initial denaturation was performed at 98ºC for a period of 30 seconds. This was followed by 12 cycles programmed to undergo denaturation at 98ºC for 10 seconds followed by a 75 second annealing step at 65ºC. A final elongation step was carried out at a temperature of 65ºC for a period of five minutes.

The enriched PCR library preparation then underwent purification using SPRI beads. AMPure XP Beads (Beckman Coulter, CA, USA) were added to the ligated DNA samples in the 96 well plate in 1:1 ratio to the volume of enriched library preparation PCR product. The beads were mixed by gentle pipetting and incubated at room temperature for five minutes. The 96 well plate was placed on a magnetic rack to activate the magnetic beads and move out of solution to the base of the plate. The supernatant was removed using a pipette and disposed of. The beads were washed with 200µl of 80% (v/v) ethanol, incubated at room temperature for 30 seconds and the ethanol then removed. The process of washing the beads with ethanol was then repeated. In order to remove the ligated DNA from the SPRI beads, 33µl of Buffer EB (Qiagen, Hilden, Germany) was added to each well and mixed by pipetting. To separate the SPRI beads out of solution from the purified ligated DNA product the 96 well plate was placed on the magnetic rack for five minutes. Twenty eight microliters of purified ligated

DNA product was aliquoted from each well into a clean 96 well plate ensuring the SPRI beads were not disturbed.

4.2.5.5 Quantification of the library preparation product

Quantification of the library preparation product was performed using the Quant-iT dsDNA Broad Range Assay Kit as described previously. The Tapestation D1000 (Agilent technologies, CA, USA) was used to ensure the appropriate size of enriched library preparation product. Two microliters of D1000 sample buffer was added to an equal volume of enriched library preparation product from each sample.

4.2.6 Library sequencing

Next generation sequencing was performed on the Ilumina miseq sequencer (Illumina, CA, USA). Sample sequencing was undertaken by Carolina Lascelles and Sally Harrison at the University of Leeds. This sequencing technology utilises ‘sequencing by synthesis’ to both amplify and determine the sequence of DNA fragments of interest. The library preparation detailed earlier results in the addition of adaptor fragments to sample DNA fragments. During sequencing the adapted fragments bind to complementary sequences on a flow cell. A DNA polymerase reaction is initiated whereby sequences are amplified to generate a series of clusters, each with numerous copies of the same sequence within each cluster. Each cluster then undergoes simultaneous sequencing whereby a complementary fluorescently labelled base is added to the sequence of interest. The flow cell is excited by light to generate a specific signal corresponding to the fluorescently labelled base in each sequence cluster. This cycle is repeated to sequence the entirety of each cluster.

4.2.7 Bioinformatics microbiome analysis

Analysis of the stool microbial DNA sequences was undertaken with Dr. Henry Wood and Dr. Suparna Mitra at LIMR. FASTQ data files generated by the Illumina sequencing platform were separated according to the unique index primer associated with individual participant samples. The adapter sequences were removed using cutadapt. Phylogenetic classification was performed using the Ribosomal Database Project (RDP) Naive Bayesian Classifier. The RDP database contains data pertaining to all known bacterial 16S rRNA gene sequences, thus allowing alignment of sequencing data from microbiome analysis to known bacterial sequences within the database. Using MOTHUR computer software additional analyses were performed including assessment of α-diversity and β-diversity.

4.3 Measurement of RBC membrane PUFA content

Extraction of fatty acids from RBC membranes and subsequent LC-MS/MS were performed by Jade Spencer and Professor Paul Loadman at the Institute of Cancer Therapeutics at the University of Bradford (Volpato *et al.*, 2017; Rose & Oklander, 1965)**.**

4.3.1 PUFA extraction from RBC membranes

RBC samples were removed from storage in -80 ºC freezers and thawed. RBCs were washed using five volumes of 0.89% (w/v) sodium chloride by mixing by inversion and centrifuging at 5000RCF for five minutes. The supernatant was removed and the process of washing repeated twice. Fifty microliters of washed RBCs were transferred into a clean Eppendorf tube to which was added an equal volume of distilled water containing deuterated ALA (LNA-d14) as an internal

standard at a concentration of 2µg/ml. The samples were allowed to stand at room temperature for 15 minutes. A 550µl volume of isopropanol was added to the washed RBCs, mixed and incubated at room temperature for one hour, occasionally vortexing to mix. A volume of 350µl of chloroform was added to each sample, incubated at room temperature for one hour and centrifuged at 10000RCF for five minutes. The supernatant was then evaporated to dryness in a rotary evaporator (EZ-2 plus rotary evaporator, Genevac Ltd, Suffolk, UK). A proportion of PUFAs are found in triglyceride-bound form. In order to measure total PUFA levels, a process of saponification was required, whereby fatty acids were cleaved from the glycerol backbone by the addition of hydrochloric acid and neutralised with sodium hydroxide. Each sample was then reconstituted in 500µl of acetonitrile and 50µl of 5M Hydrochloric acid. The samples were incubated at 80ºC for one hour and then allowed to cool at room temperature for five minutes. A volume of 50µl of 5M sodium hydroxide was then added to each sample and mixed by vortexing, followed by the addition of 350µl of chloroform. The samples were left to stand for five minutes and the top 800µl of sample removed, leaving a layer of water behind. The sample was then evaporated to dryness in a rotary evaporator and subsequently reconstituted in 50µl of methanol.

4.3.2 LC-MS/MS

Identification and measurement of relative quantities of individual PUFAs was performed using LC-MS/MS. Although gas chromatography-mass spectrometry (GC-MS) has traditionally been the method of choice for quantification of PUFAs in biological samples, LC-MS/MS is a valid alternative (Volpato *et al*., 2017). Particular benefits of LC-MS/MS over GC-MS include high throughput and the ability to measure non-volatile and thermally fragile molecules, thus avoiding

thermal degradation of PUFAs (Perez *et al.*, 2016). LC-MS/MS is a two stage technique for identification and measurement of PUFA with the synergism of the two analytical techniques being both highly sensitive and specific.

In high performance liquid chromatography (HPLC) a sample mixture is dissolved in a solvent (mobile phase) that transits through an adsorbent column (stationary phase) at high pressure. Compounds are separated within the mobile phase at varying rates due to differential polarity and interactions with the stationary phase. Analytes are subsequently analysed based on absorption of light from the UV spectrum and time taken to transit through the column (retention time). When used in combination with tandem mass spectrometry analytes of interest can be diverted from the chromatography column for spectral analysis.

Tandem mass spectrometry identifies molecules according to their mass to charge ratio (m/z). Parent molecules injected into a mass spectrometer are ionised using various high energy sources, thus generating charged ions which transit through a magnetic field striking a detector to create an electrical current. Tandem mass spectrometry utilises a second ionisation process which further ionises parent molecule ions to create ‘daughter’ ions. This second ionisation process increases specificity of detection by enabling differentiation between molecules of similar m/z. The combination of tandem ionisation processes and the time taken to transit through the magnetic field and strike the detector produces specific spectra which correspond to specific molecular structures. The relative ion intensities generated during the mass spectrometry analysis correspond to the abundance of that molecule within a sample.

4.3.2.1 Derivatisation

Derivatisation is a process of chemically altering sample molecules to enhance the sensitivity of the LC-MS/MS analysis. The chemical alteration increases specificity and sensitivity of LC-MS/MS by optimising the ionisation process and may also increase limit of detection, where low amounts of analyte that may otherwise not be distinguished from zero can be detected (Thompson *et al.*, 2002). Derivatisation was performed using the derivatising agent 4-[2-(N.N-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). Fifty microliters of derivatising agent was added to 50µl of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC) and 50µl of 4-(dimethylamino) pyridine (DMAP). This was then mixed with the fatty acid extracted from each participant RBC sample (50µl) and incubated in amber glass HPLC vials for 24 hours.

4.3.2.2 LC-MS/MS protocol

LC-MS/MS was performed using a Waters Alliance 2695 High Pressure LC separations module in combination with a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters UK, Hertfordshire). A two microliter derivatised sample from each participant was separated on a HiChrom RPB column (2.1mm× 250mm, 5µm) (HiChrom Ltd, Berkshire, UK). Mobile phase (MP) A consisted of 90% dH2O, 10% MeOH, 0.1% formic acid and MPB consisted of 90% MeOH, 10% dH2O and 0.1% formic acid. Gradient conditions were as follows: Starting at 80% MPB changing to 83% MPB over 8 min, then increasing to 95% MPB at 15 min, remaining at 95% MPB until 17 min before returning to starting conditions at 18 min. The overall run time was 25 min. The flow rate was set at 0.5ml/min and split post-column with 0.3ml/min delivered to

the mass spectrometer. Samples were analysed in multiple reaction monitoring (MRM) mode for the following fatty acids: EPA, DPA, DHA, ALA, AA, LA, SA, OA, PA and internal standard LNA-d14. The instrument settings were as follows: Capillary voltage, 3kV; Cone energy, 15eV; collision energy, 25eV; source temperature, 120°C and desolvation temperature, 300°C.

Results for each of the aforementioned PUFAs were reported as the percentage of the total analysed fatty acid content for that sample. This was calculated as the sum of the ratio of each fatty acid peak area to the internal standard LNA-d14 peak area (%FA).

4.4 Statistical analysis

Statistical analysis was undertaken with Graphpad version 7 (Graphpad, CA, USA) and SPSS software (IBM, NY, USA). Normality of data was tested using the D'Agostino & Pearson normality test. Correlations were assessed using the Pearson's correlation co-efficient. The one sample t-test was used to assess changes to RBC PUFA membrane levels from baseline. For paired data the paired t-test or Wilcoxian test were used to compare interventions. For unpaired data the Student's un-paired t-test or Mann Whitney U test were employed. Multivariate analysis was carried out using multivariate linear regression. Survival analysis was performed with Kaplan-Meier curves and the log rank test.

Chapter 5: Results of the randomised cross-over trial exploring PUFA RBC membrane levels and tolerability of equivalent doses of EPA and DHA in a drink versus capsule formulation

5.1 Introduction

The following chapter details the results of the randomised cross-over trial in healthy volunteers. There is specific focus on comparing the bioavailability of EPA and DHA, as measured by RBC membrane levels, following capsule and drink supplementation. This was performed in order to determine whether O3FA-containing drinks are a valid alternative to traditional capsule supplementation, which therefore may have a clinical application as either an adjunct in CRC treatment or chemoprevention.

5.2 Patient recruitment

Twenty two individuals expressed an interest to take part in the study and subsequently attended the HARU to undertake a screening visit. All 22 individuals fulfilled the inclusion criteria and were therefore eligible to consent to enter the study. The first study participant attended visit 1 on 15th July 2015. There was a period of approximately three months between the first and final participant entering the study, with the final participant attending visit 1 on 21st October 2015. The study finished on 16th December 2015 after the final study 5 visit.

The consort diagram outlined in figure 6 details recruitment through the study. At visit 1, 12 participants were randomised to the capsule followed by drinks intervention group (C/D). Ten participants were randomised to consume drinks

followed by capsules (D/C). Withdrawals throughout the study period are discussed later.

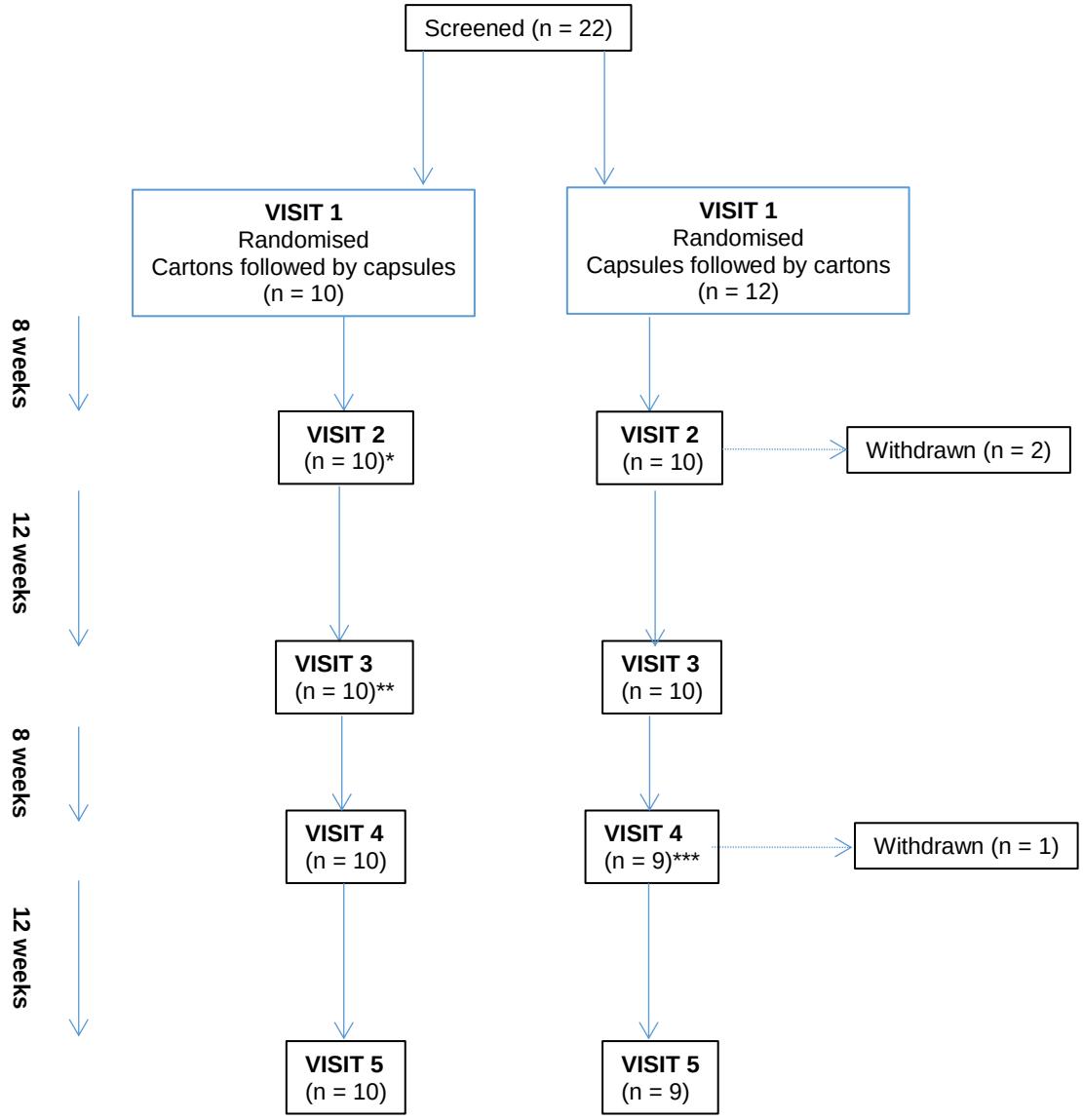


Figure 6. Consort diagram of recruitment, participant flow and withdrawals throughout the study period. *Participant ceased carton intervention early (remained in study), **Participant that ceased carton intervention early recommenced intervention period 2 (capsules), *** Participant ceased carton intervention early (remained in study).

5.3 Baseline characteristics

Twelve of the 22 (55%) participants recruited into the study were female. Following withdrawal of two female participants early in the first intervention period, the sex distribution within the study was balanced (10 male; 10 female).

The median age across all 22 participants in the study was 57 years (range; 51 to 65 years). The median BMI was 27kg/m^2 (range; 22.0 kg/m^2 to 33.8kg/m^2).

Prior to starting the first intervention period, participants were assessed with regard to any symptoms that might be associated with O3FA supplementation (table 2). Of the three participants in the first capsule intervention group that experienced loose motions prior to entering the study, two had been formally diagnosed with irritable bowel syndrome (IBS). The single participant in the D/C intervention group that experienced loose motions prior to entering the study had a previous cholecystectomy which may be associated with chronic diarrhoea, particularly in the presence of bile salt malabsorption. Only one individual in the C/D intervention group experienced any other recognised O3FA-related symptoms, specifically abdominal discomfort in an individual with a known diagnosis of IBS.

	CAPSULES (n = 12)	**DRINKS (n = 10)**
Loose stools	3 (IBS - 2)	1 (cholecystectomy)
Abdominal discomfort	1 (IBS)	0
Burping	0	0
Indigestion	0	0
Nausea/Vomiting	0	0

Table 2. Symptoms associated with O3FA supplementation reported by study participants prior to commencing O3FA supplementation at visit 1.

5.4 Withdrawals

Of the 22 patients that commenced the study, two female participants withdrew from the first intervention group randomised to initially consume capsules (C/D) after 49 days and 7 days respectively. These individuals withdrew due to experiencing gastrointestinal symptoms associated with O3FA supplementation. A third patient (male) withdrew after visit 3, completing both the initial intervention and washout periods (randomised to C/D). He withdrew due to concerns regarding the calorific content of the O3FA drinks.

Seventeen of the 22 participants that enrolled in the study completed both eight week intervention and 12-week washout periods. Nineteen participants commenced both intervention periods with 20 participants completing at least one intervention and corresponding washout period. One participant attended all planned study visits providing both venous blood and stool samples, but ceased O3FA drink supplementation 14 days prior to the planned intervention study visit (visit 4). Therefore, 16 participants took O3FA supplements as per protocol during both intervention periods up until the relevant study visits (2 and 4).

5.5 Duration of supplementation and compliance

The median duration of capsule supplementation across the 22 participants that commenced the capsule intervention period was 57 days (range 7 to 63 days). When adjusting for the two participants that withdrew from the study in the first intervention period (at 7 and 49 days) the median duration of capsule supplementation was 57 days (range 55 to 63 days). Nineteen participants commenced the drinks intervention period with a median duration of supplementation of 57 days (range 7 to 59 days). Adjusting for participants that

provided RBC samples at the planned end of intervention study date, median duration of supplementation was 57 days (n=20, range 55 to 63 days) and 57 days (n=16, range 54 to 57 days) for capsules and drinks respectively.

Compliance was measured by counting the number of capsules or drinks returned by a participant at the end of each intervention period. Median compliance across the 20 participants that completed the capsule intervention period was 100% (range 93% to 108%) versus 98% in the 16 participants that completed the drinks intervention period (range 85% to 106%). Compliance greater than 100% was due to participants returning fewer capsules or drinks than expected from the excess number given at the initial study visit.

5.6 Acceptability and adverse events

Adverse events were examined across the 22 and 19 participants that commenced the capsule and drink intervention periods respectively. Overall there were 29 adverse events recorded in 14 participants during the capsule intervention period versus 17 adverse events in 10 participants consuming drinks. The specific symptoms associated with O3FA supplementation are detailed in table 3. Although there were a greater number of adverse events experienced in the capsule intervention group there was no statistically significant difference identified between each intervention as measured by Fisher's exact test. All 14 participants that experienced symptoms during the capsule intervention period reported resolution of the symptoms during the corresponding washout period, compared with nine of 10 participants in the drinks intervention.

With regard to classification of severity of O3FA related symptoms, three participants in both capsule and drink interventions developed symptoms defined

as ‘moderate’, thus requiring cessation of supplementation either temporarily or permanently. Two participants (2/22) experienced symptoms that required cessation of capsule supplementation and subsequent withdrawal from the study after seven and 49 days. The third participant ceased capsule supplementation for a period of two days after experiencing flu-like symptoms which were deemed unrelated to the capsules. All three participants that experienced O3FA related symptoms defined as moderate during the drink intervention period ceased supplementation after seven, 10 and 43 days.

Of the 10 participants that were randomised to commence capsules during the first intervention period (C/D), four participants also experienced symptoms associated with O3FA supplementation during the second intervention period when consuming drinks. Four of nine participants that were initially randomised to the drink intervention (D/C) also experienced O3FA-related symptoms during the second capsule intervention period.

	CAPSULES (n = 22)	**DRINKS (n = 19)**	**p value****
Total number of adverse events during intervention periods	29 (14*)	17 (10*)	0.54
Diarrhoea	5	5	1.00
Abdominal discomfort	6	3	0.47
Burping	10	4	0.19
Indigestion	5	1	0.19
Nausea/vomiting	3	4	0.68

Table 3. Number of adverse events associated with O3FA supplementation reported by study participants at the end of either capsule or drink supplementation irrespective of intervention order. * Total number of participants reporting adverse events. * * Fisher’s exact test, $p < 0.05$ statistically significant.

Acceptability was measured for both capsule and drink interventions. An adapted questionnaire provided by the drinks manufacturer Smartfish^{TM} was used to assess carton acceptability. The questionnaire measured four domains, specifically, smell, taste, texture and tolerability. Each domain was measured using a visual analogue scale with a maximum score of seven points for each domain (1; I dislike it very much, 7; I like it very much) and maximum total score of 28 points across all four domains. The median drinks acceptability score across the 17 participants that completed the questionnaire was 21/28 (range 13 to 28). For a direct comparison of drinks and capsule O3FA supplementation, study participants were asked whether they would consider continuing the relevant supplementation following completion of the study. Of the 19 participants that commenced drinks supplementation, 11 (58%) stated they would consider continuing the drinks supplementation at the end of the study, compared to 15 of 22 (68%) participants that commenced the capsule supplementation. There was no statistically significant difference between whether participants would consider continuing either type of O3FA supplementation (p=0.53).

5.7 Measurement of PUFA RBC membrane levels

Ninety seven RBC samples from 20 of the 22 participants that enrolled into the study successfully underwent LC-MS/MS PUFA analysis. Two participants that withdrew from the study during the first intervention period and therefore only supplied an initial visit 1 pre-supplementation venous blood sample were excluded from analysis. A participant that ceased the drink intervention early during the first intervention period did not supply a post-intervention period venous blood sample (visit 2). A second individual completed the initial capsule

intervention and washout period, but elected not to start the drinks intervention period and therefore did not provide blood samples from visits 4 and 5.

Levels of individual PUFAs in RBC membranes were analysed and expressed as a percentage of the total RBC membrane fatty acid content. The absolute change in percentage values between baseline and post-supplementation or washout was calculated for individual PUFAs. The one sample t-test was employed to assess absolute percentage RBC membrane PUFA change in individuals at post-supplementation and washout. The paired t-test was used to compare absolute change post-supplementation and at the end of the washout period across both interventions. The Wilcoxon matched-pairs signed rank test was used when data were not normally distributed.

5.7.1 Treatment intervention order

Analysis of PUFA RBC levels based on the intervention order to which participants were randomised was performed to determine whether the 12-week washout period ensured a return to baseline RBC membrane levels (Figure 7).

The 12-week washout period was of satisfactory length to return EPA RBC membrane levels to near baseline measurements irrespective of capsule intervention order. Baseline EPA levels in participants randomised to receive the capsule intervention first (C/D, n=10) were similar to EPA levels in patients randomised to receive capsule supplementation during the second intervention period (D/C, n=10) with mean EPA RBC membrane levels of 0.72% and 0.83% respectively (p=0.52). Although DHA levels in participants randomised to consume capsules during the first intervention period (C/D) remained elevated after the 12-week washout period, this had no effect on baseline levels with regard to intervention order (C/D: 2.73% versus D/C: 3.32%, p=0.21). There was

no difference between the absolute change in eight week post-supplementation EPA RBC membrane levels based on the order to which the capsule intervention was received (C/D: 1.52% points, D/C: 1.54% points, p=0.52), which was also similar when examining DHA RBC membrane levels (C/D: 1.51% points, D/C: 1.31% points, p=0.63).

Nine individuals (9/10) randomised to consume drinks during the first intervention period (D/C) completed eight weeks of supplementation compared to seven participants taking the drink supplements during the second intervention period (C/D). EPA RBC membrane levels were higher at baseline in patients randomised to receive drinks during the second intervention period (D/C: 0.67%, C/D: 1.13%, p = 0.05). There were similar findings exhibited in DHA RBC membrane levels with mean levels at baseline of 3.36% and 4.15% in participants randomised to taking drinks during the first and second intervention periods respectively, although this difference was not statistically significant. The adequacy of the 12-week washout period was confirmed by examination of the absolute change in EPA and DHA levels after 12 weeks' washout, neither of which were statistically significant regardless of intervention order.

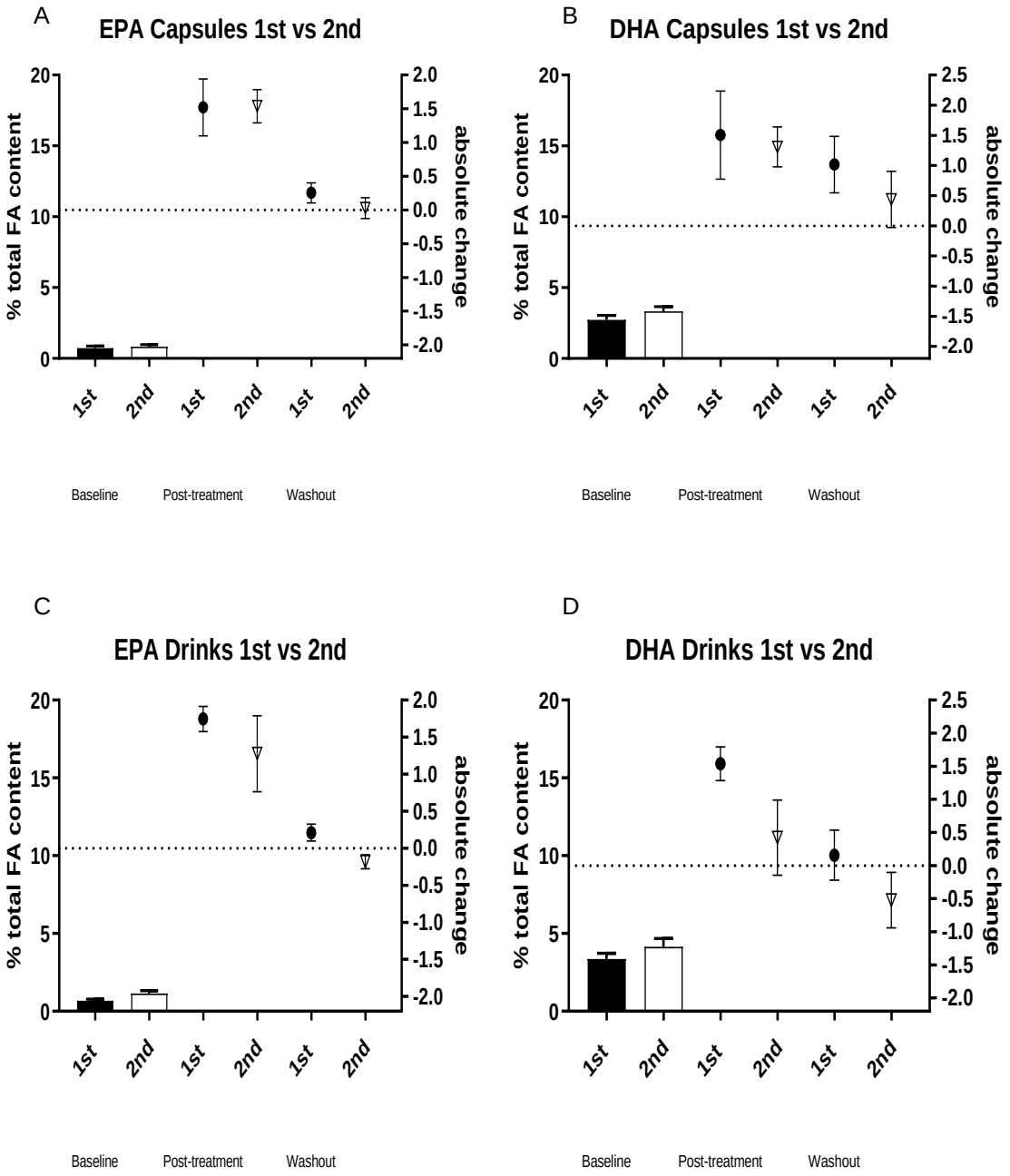


Figure 7. Comparison of RBC EPA and DHA levels depending on the intervention order to which either the drinks or capsules were received (first or second). A,B: EPA and DHA RBC membrane levels in participants randomised to receive capsules during the first intervention period (n=10) versus the second intervention period (n=10). C,D: EPA and DHA RBC membrane levels in participants randomised to receive drinks during the first intervention period (n=9) versus the second intervention period (n=7). The left y-axis is the absolute mean percentage PUFA content measured for each supplementation type prior to either intervention period (visits 1 or 3). The right y-axis is the absolute mean change from baseline following the intervention period and washout dependant on whether the supplementation was received during the first or second intervention period. Error bars denote the standard error (SE) of the mean

5.7.2 Primary outcome measure

To test the hypothesis that O3FA supplement drinks were non-inferior to an equivalent dose of O3FAs in capsule form the difference between absolute eight week post-supplementation O3FA RBC membrane levels (EPA + DHA) in both interventions were examined. Across the 16 participants that completed and provided a venous blood sample at the planned eight week study visit, seven participants exhibited lower O3FA RBC membrane levels following drinks compared to capsules. Measured absolute O3FA RBC membrane levels were 0.12% points lower in drinks than capsules, although the 95% confidence interval of -1.84% points to 1.61% points revealed that this difference was not significant and therefore O3FA supplementation with Smartfish^{TM} drinks was non-inferior to an equivalent dose of O3FAs in capsule form.

5.7.3 EPA (figure 8a)

Comparison of baseline EPA RBC membrane levels revealed no difference between participants randomised to either intervention (drinks; 0.88%, caspules; 0.77%, p=0.47). At the end of the eight week intervention period, both drink and capsule interventions exhibited similar statistically significant elevations in EPA RBC membrane levels from baseline of 1.54% points and 1.53% points respectively (both $p<0.0001$). However, there was no difference with regard to absolute change in EPA RBC membrane levels between capsule and drink interventions (p=0.93). After the 12-week washout period EPA RBC membrane levels in both interventions returned to baseline levels (absolute change from baseline; drinks, 0.04% points; capsules, 0.14% points, p=0.63).

5.7.4 DPA (figure 8b)

Mean DPA RBC membrane levels were similar across both interventions at baseline (drinks; 1.95%, capsules; 1.65%, p=0.20). Post-supplementation DPA RBC membrane levels were higher following the capsule intervention period, although this did not reach statistical significance when compared to the drinks intervention period (absolute change from baseline; capsules, 0.35% points; drinks, -0.01% points, p=0.16). After the 12-week washout period mean DPA RBC membrane levels in the drinks intervention were 0.33 percentage points lower than those measured at baseline (one sample t-test; p=0.04). However a difference between capsule and drink intervention DPA RBC membrane levels, as measured by absolute change failed to reach statistical significance in a paired analysis (drinks; -0.33% points, capsules; 0.26% points, p=0.07).

5.7.5 DHA (figure 8c)

Mean DHA RBC membrane levels were similar across both interventions at baseline (drinks; 3.70%, capsules; 3.03%, p=0.32). At the end of the eight week intervention period, both drink and capsule intervention groups exhibited elevations in DHA RBC membrane levels from baseline of 1.05% points (p=0.02) and 1.41% points (p<0.0001) respectively. However the difference between interventions failed to reach statistical significance (p=0.86). Following the 12-week capsule washout period, DHA RBC membrane levels remained elevated from baseline (absolute change from baseline 0.73% points, p=0.04). The same elevation was not exhibited following the drink washout period where DHA RBC membrane levels returned to near baseline levels (absolute change from baseline -0.14% points, p=0.63). However the difference between capsule and drink interventions was not statistically significant (p=0.38).

5.7.6 AA (figure 8d)

Baseline AA RBC membrane levels were similar across both interventions (drinks; 7.42%, capsules; 6.61%, p=0.42). There was a significant decrease from baseline in AA RBC membrane levels post-supplementation in the drinks intervention (-1.58% points, p<0.01), which was not apparent following the capsule intervention (-0.31% points, p=0.68). However this difference failed to reach statistical significance (p=0.23). Following the washout period AA RBC membrane levels remained lower than baseline levels in the drinks intervention (-1.27% points, p=0.05). However AA RBC membrane levels returned to near baseline levels in the capsule intervention (0.19% points, p=0.80).The difference between capsule and drinks interventions was not statistically significant.

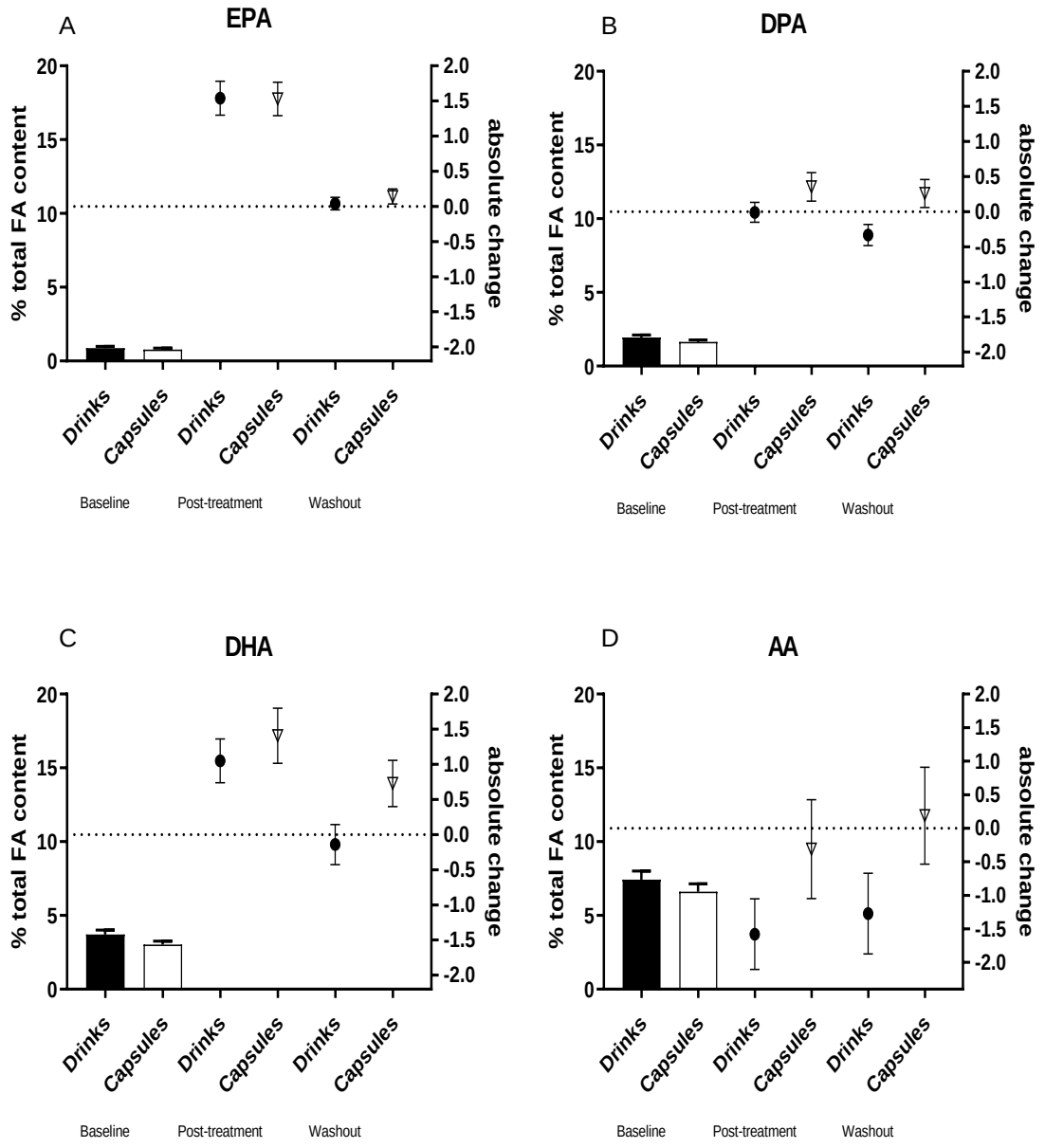


Figure 8. Individual RBC PUFA levels comparing pooled data from the drink *versus* capsule intervention independent of intervention order (A: EPA, B: DPA, C: DHA, D: AA). Number of participants completing drinks intervention (n=16), number of participants completing capsule intervention period (n=20). The left y-axis is the absolute percentage PUFA content measured for capsule and drink interventions at baseline (visits 1 or 3). The right y-axis is the absolute change from baseline following the intervention period and washout dependent on the intervention type. Error bars denote the SE of the mean.

5.7.7 O3FA index (figure 9)

The total EPA and DHA RBC membrane levels were analysed to reflect the combined O3FA composition of both the capsule and drink interventions. There was no significant difference between baseline EPA+DHA RBC membrane levels (drinks, 4.58%; capsules, 3.81%, p=0.43). Both capsule and drink interventions exhibited a statistically significant increase in EPA+DHA RBC membrane levels from baseline of 2.94% points (range: 0.33% points to 12.38% points, p=0.0001) and 2.60% points (range: -1.94% points to 5.68% points, p=0.0001) respectively. Post-supplementation there was no statistically significant difference between intervention types (p=0.90). Following the 12-week washout period EPA+DHA RBC membrane levels remained elevated in the capsule intervention (0.87% points, p = 0.06) compared to the drinks intervention (-0.10% points, p=0.78), however this difference failed to reach statistical significance (p=0.40).

5.7.8 O3FA:O6FA ratio (figure 10)

The ratio of O3FAs to O6FAs is often quoted to reflect the proposed anti-inflammatory role of EPA and DHA in comparison to pro-tumorigenic AA (DiNicolantonio & O'Keefe, 2018; Simopoulos, 2008). The ratio was defined as (EPA+DHA)/AA. At baseline, the mean O3FA:O6FA ratio was similar for both capsule and drink interventions (0.61 versus 0.63). Following eight weeks supplementation, the O3FA:O6FA ratio was higher for both capsule and drink interventions compared to baseline. Comparison of intervention type revealed that the increase in the O3FA:O6FA ratio from baseline was significantly higher following drinks (0.64) compared with capsules (0.48) (p=0.02). Following the 12-week washout period the O3FA:O6FA ratio fell but remained elevated

compared to baseline for both capsule and drink interventions with mean absolute change from baseline of 0.13 and 0.09 respectively ($p<0.0001$ in both capsule and drink interventions).

5.7.9 Individual participant analysis

All participants (n=20) exhibited elevated EPA RBC membrane levels following eight weeks capsule supplementation. With the exception of one individual that ceased drink intervention two weeks prior to the planned eight week study visit (participant 007), the remaining participants that provided a post-intervention venous blood sample exhibited a rise in absolute EPA RBC membrane levels following drinks supplementation.

Nineteen of the 20 participants showed increased DHA RBC membrane levels post-O3FA capsule supplementation (participant 005 – decreased RBC DHA membrane levels). DHA RBC membrane levels fell in three individuals (participants 006, 007 and 018) following eight weeks supplementation with O3FA containing drinks. Participant 007 ceased O3FA drinks supplementation two weeks prior to providing a venous blood sample. Irrespective of intervention type, the remaining three participants (capsules n=1, drinks n=2) with a decrease in measured post-supplementation RBC DHA membrane levels were all apparent following the second intervention period.

Examination of individual PUFA profiles throughout the course of the trial revealed significant inter-individual variation. Absolute change in EPA RBC membrane levels post-capsule supplementation ranged from 0.56% points to 3.28% points. This variation was also apparent following drinks supplementation (0.37% points to 3.57% points). Absolute change in DHA capsule RBC membrane levels ranged from -0.23% points to 7.86% points. There was also

variation following drinks supplementation although to a lesser degree (-2.32% points to 2.70% points).

Measured AA RBC membrane levels were lower following O3FA drinks supplementation in 14 of 17 individuals (82%) that provided venous blood samples. Across the 17 participants, absolute change in AA RBC membrane levels post-supplementation ranged from -6.27% points to 0.75% points. Comparatively only 13 of 20 individuals (65%) showed a decrease in AA RBC membrane levels following capsule supplementation. Absolute change post-supplementation in capsules ranged from -5.85% points to 10.47% points across the 20 participants. When taking into account intervention order, 9 of 10 individuals randomised to capsules first exhibited a reduction in RBC AA membrane levels compared to 4 of 10 randomised to the capsules during the second intervention period. This difference is likely due to the previously described finding that following washout in the drinks intervention, AA RBC membrane levels persisted to be lower than baseline.

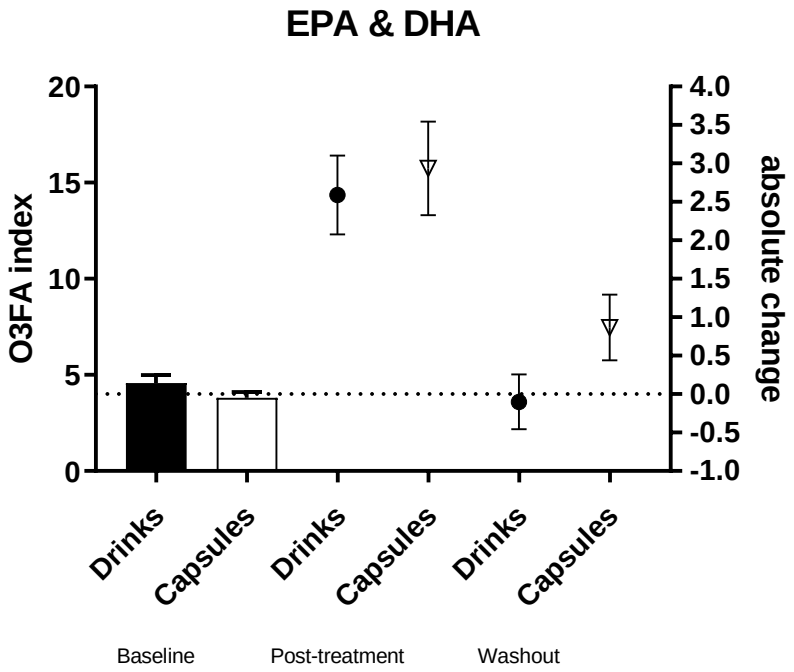


Figure 9. Measurement of the O3FA index (EPA + DHA) in drinks *versus* capsules independent of intervention order. Number of participants completing drinks intervention (n=16), number of participants completing capsule intervention period (n=20). The left y-axis is the absolute O3FA index measured for capsule and drink interventions at baseline (visits 1 or 3). The right y-axis is the absolute change from baseline following the intervention period and washout dependant on intervention type. Error bar denote the SE of the mean.

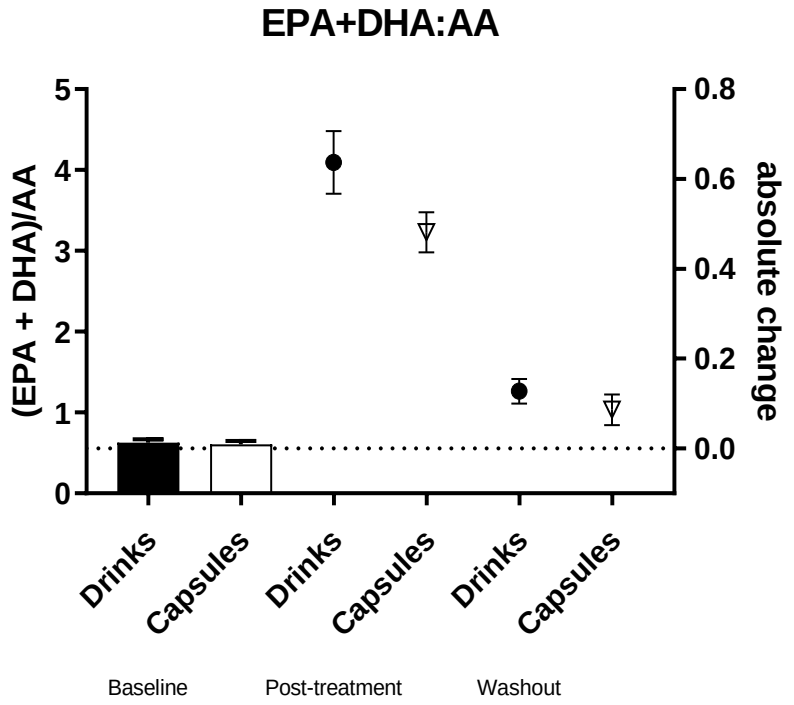


Figure 10. Measurement of the O3FA:O6FA ratio ([EPA + DHA]/AA) in drinks *versus* capsules independent of intervention order. Number of participants completing drinks intervention (n=16), number of participants completing capsule intervention period (n=20). The left y-axis is the absolute O3FA index measured for capsule and drink interventions at baseline (visits 1 or 3). The right y-axis is the absolute change from baseline following the intervention period and washout dependant on intervention type. Error bars denote the SE of the mean.

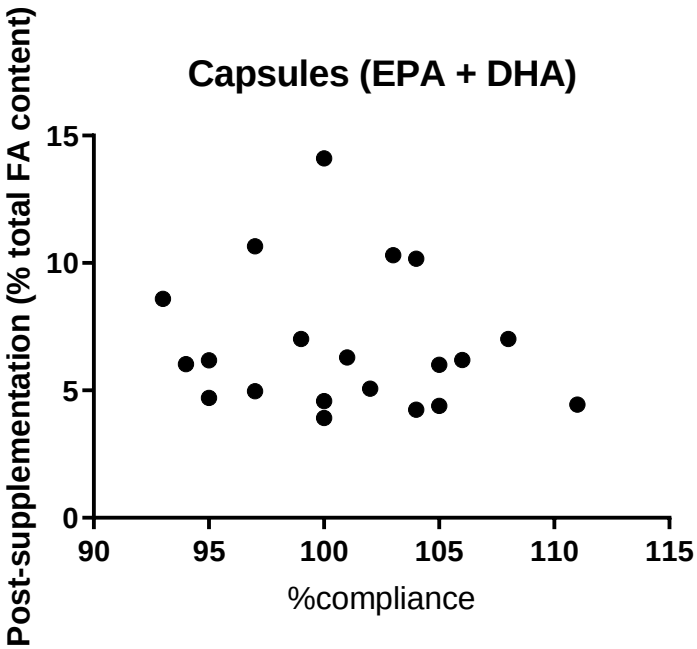
5.7.10 Relationship between compliance and O3FA RBC membrane levels (Figure 11)

In order to ascertain whether there was a relationship between O3FA RBC membrane levels and compliance with taking either supplementation, the Pearson correlation coefficient was calculated. The O3FA index was used for analysis as both capsule and drink preparations contained a combination of EPA and DHA. Compliance was based on the number of drinks cartons or capsules returned as described previously. There was no correlation between percentage compliance and measured post-supplementation O3FA RBC membrane levels (EPA + DHA) for either drinks (r=-0.13, 95% CI -0.54 to 0.33) or capsules (r=-0.10, 95% CI -0.57 to 0.42) interventions. This analysis is limited by the inability to verify whether the capsule and drinks carton counts were accurate.

5.7.11 Relationship between baseline O3FA RBC membrane levels and post-supplementation O3FA RBC membrane levels (Figure 12)

To determine whether there was a relationship between baseline O3FA RBC membrane levels and post-supplementation EPA or DHA RBC membrane levels following either capsule or drink interventions the Pearson correlation coefficient was calculated. There was no correlation between baseline and post-supplementation RBC membrane levels for either EPA or DHA following capsule supplementation. However there was a statistically significant correlation between baseline and post-supplementation DHA RBC membrane levels following drinks supplementation (r=0.52, 95% CI 0.03 to 0.81, p=0.04).

A



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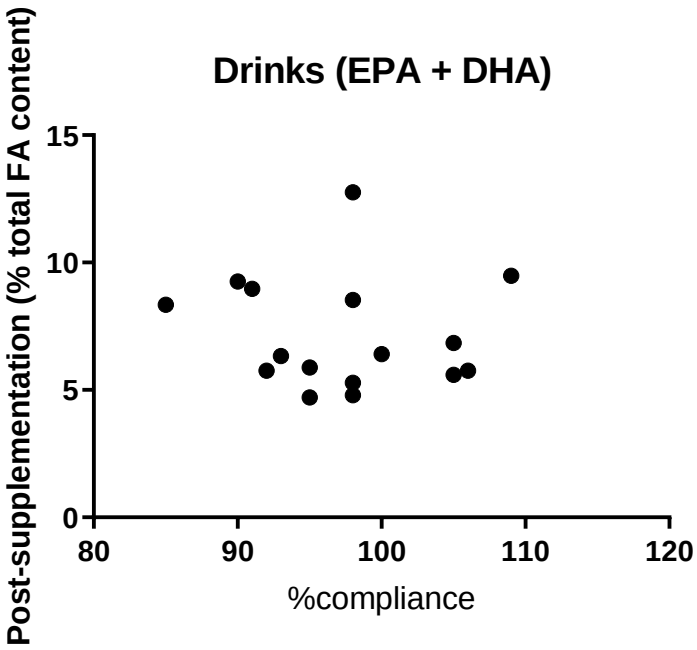


Figure 11. Correlation between post-treatment O3FA (EPA + DHA) RBC membrane content (%) and compliance in each intervention type. A: capsules (n=20, r=-0.13), B: drinks (n=16, r=-0.10).

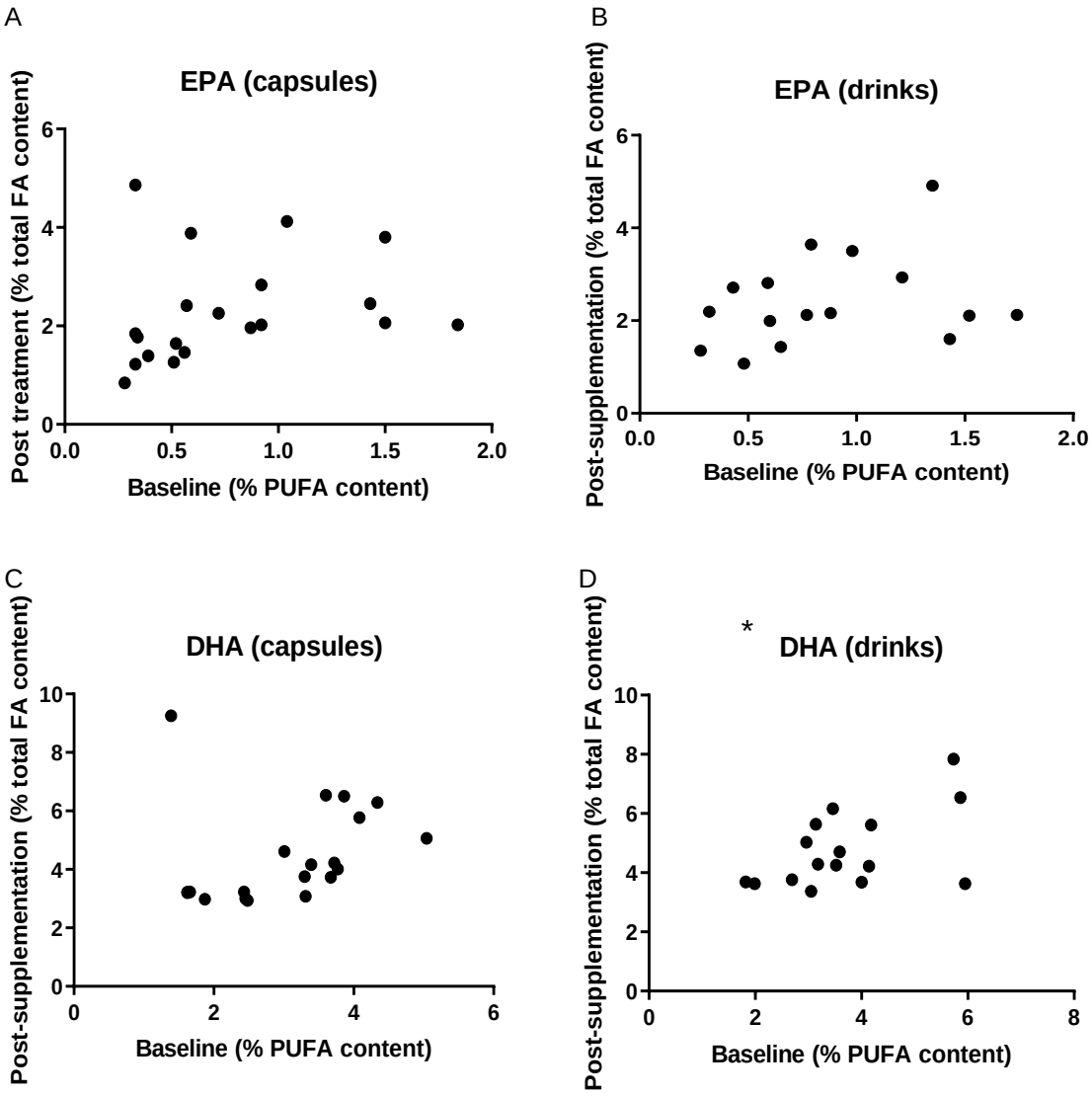


Figure 12. Correlation between baseline O3FA RBC membrane content (%) and post treatment O3FA RBC membrane levels (%). A: EPA-capsules (n =20, r=0.25), B: EPA-drinks (n=16, r=0.28), C: DHA-capsules (n=20, r=0.23), D: DHA-drinks (n=16, r=0.52, p=0.04). *denotes statistically significant result (p<0.05)

5.8 Discussion

The randomised cross-over trial confirmed the hypothesis that EPA and DHA supplementation with drinks was non-inferior to an equivalent dose in capsule form as measured by RBC PUFA membrane levels. The cross-over trial design and adequacy of the 12-week washout period were validated by the finding that, irrespective of intervention order, baseline RBC membrane levels of both EPA

and DHA were similar. Baseline EPA and DHA levels were comparable to published healthy volunteer studies (Kohler *et al.*, 2010; Scaioli *et al.*, 2015).

Both capsule and drinks O3FA preparations were in triglyceride form, thus requiring hydrolysis by pancreatic lipases in the small intestine (see section 1.3.3). The drinks cartons contained adequate fat content to stimulate secretion of pancreatic lipases and subsequent absorption of triglyceride-bound EPA and DHA. Due to the lack of dietary assessment over the course of the study it could not be ascertained whether participants took the drinks with or without food and if they consumed additional dietary fat. This would potentially affect both absorption and subsequent incorporation of EPA and DHA into RBC membranes.

The findings of the present study are in agreement with an eight week placebo-controlled trial of another 'convenience' drink containing a total of 500mg combined EPA and DHA developed by the same manufacturer used in the present study. Interestingly, despite the aforementioned study employing a far lower daily dose of EPA and DHA (500mg versus 4000mg) the authors reported similar increases to the O3FA index of 2.43% compared to the 2.60% increase reported here (Kohler *et al.*, 2010). This would suggest that a daily dose of 4000mg of EPA and DHA is far in excess of that required to achieve maximal RBC membrane incorporation levels. Given the timing of RBC membrane measurements it is not possible to determine whether the rate at which maximal RBC membrane levels were achieved was more rapid with this higher dose of EPA and DHA. Of note was that following drinks supplementation and washout there was a decrease in both RBC DPA and DHA levels which was not apparent following capsule supplementation. The reason behind this difference is unclear

but there may be additional ingredients within the drinks that effect enzymatic conversion of EPA to DPA and subsequent conversion to DHA.

There was significant inter-individual variation with respect to individual healthy participant PUFA profiles across both capsule and drinks interventions. This was also evident in the individual RBC membrane PUFA profiles of patients in the study of CRCLM (chapter 7). These findings correspond with the previously described O3FA drinks formulation study, where there was significant post-supplementation variability to the O3FA index ranging from -0.03% to 7.16% across 40 participants (Kohler *et al.*, 2010). There are a number of potential explanations for the variation exhibited across individual PUFA profiles in the present study. There is potential widespread variation across individuals in relation to the rate at which PUFAs entered various metabolic pathways, including β-oxidation and generation of ATP, fatty acid conversion, or entering into various lipid signalling pathways (Kanamori *et al.*, 2018; Camões *et al.*, 2009). The metabolic fate of PUFAs is likely to affect the measurement of RBC membrane PUFA levels at any given time point. One might also expect compliance to have a significant influence on EPA and DHA RBC membrane levels, although this was not apparent in the present study. A possible explanation was that with compliance across both interventions of greater than 98% there was insufficient variation to detect a relationship. There are also likely differences across participants with respect to their ability to absorb EPA and DHA from the gastrointestinal tract, specifically variations in pancreatic lipase excretion and enzyme activity, which may also be influenced by unmeasured concomitant dietary fat intake (Schuchardt & Hahn, 2013).

The significant inter-individual variation of response to O3FA supplementation is of relevance in the potential clinical applications of O3FA supplementation. RBC membrane O3FA levels are accepted as a reflection of O3FA levels within multiple tissues, and as I report later there was a correlation between RBC EPA membrane levels and CRCLM tissue levels (Metcalf *et al.*, 2007; Harris *et al.*, 2004; Tu *et al.*, 2013; Brenna *et al.*, 2018). If there is a potential clinical benefit, particularly in the context of treating CRC micrometastases and chemoprevention in healthy individuals, the efficacy of any anti-cancer effect may also vary according to an individual's ability to incorporate EPA and DHA into target tissues. A major limitation of this trial was the inability to gather colonic tissue to confirm whether the changes in RBC membrane PUFA levels correspond to colonic mucosal PUFA levels. This is of significant importance as there is evidence that incorporation of O3FAs into cell membranes may influence CRC development via influencing cell membrane structure and integrity (Chapkin *et al.*, 2002). It is also evident from the literature that many *in-vivo* and *in-vitro* studies examine the effect of O3FAs on colorectal carcinogenesis in both colonic and CRC tissue (Cockbain *et al.*, 2012). Therefore changes to colonic PUFA tissue profiles would be of significant interest. Participants in this study were healthy volunteers and therefore it would have been deemed to pose an unacceptable risk to subject individuals to endoscopic biopsy to obtain colonic tissue.

When examining individual PUFA levels, the relationship between EPA, DHA and AA was of significant interest. The ratio of O3FAs to O6FAs is quoted to reflect the proposed anti-inflammatory role of EPA and DHA in comparison to pro-inflammatory AA, via their competitive interactions with the COX family of enzymes (Smith, 2005). In this study the O3FA:O6FA was defined as

(EPA+DHA)/AA. Irrespective of intervention type, there were increases in the O3FA:O6FA following the eight week supplementation period. Similar findings are reported elsewhere (Young *et al.*, 2005; Burns *et al.*, 2007; Gibney *et al.*, 1993). Cao *et al.* do not state the RBC membrane O3FA:O6FA ratio following EPA/DHA supplementation (2000mg) but do report both an increase in O3FA RBC membrane levels with a reciprocal decrease in AA RBC membrane levels over a period of eight weeks supplementation (Cao *et al.*, 2006). It is unclear whether O3FA supplementation has a dose-dependent effect on the O3FA:O6FA given that the present study reports higher O3FA:O6FA ratios than a study using a combined EPA/DHA daily dose of approximately 35g (Young *et al.*, 2005). However, it is noted that the aforementioned study measures plasma PUFA levels rather than RBC membrane levels. The magnitude of the increase in the O3FA:O6FA ratio was statistically significantly higher in drinks compared to capsules, a likely reflection of the greater drop in AA RBC membrane levels during the drinks intervention period. The reason behind this difference is unclear given that EPA and DHA RBC membrane levels were similar across both interventions and therefore one would expect any potential membrane displacement of AA by EPA and DHA to follow suit. One possible explanation is that during the drinks intervention period participants may have substituted their regular dietary routine with the drinks carton, which they did not subsequently follow during the capsule intervention period. Therefore their dietary intake of AA from for example eggs may have been lower during the drinks intervention period. If an individual also substituted LA containing foods during the drinks intervention period this may have resulted in reduced amounts available for conversion to AA (Rose & Connolly, 1999). The drinks cartons may also have ingredients that affected AA metabolism, particularly given that the drinks

contained approximately 600mg to 1100mg of unspecified PUFAs in addition to the EPA and DHA contents.

The O3FA:O6FA ratio is pertinent to the interaction of PUFAs with the COX family of enzymes. This is one of the key mechanisms via which O3FAs are proposed to have anti-cancer properties and therefore potential clinical applications (see section 1.5). COX-2 is overexpressed in 50-80% of colorectal tumours (Eberhart *et al.*, 1994). In the presence of EPA, there is decreased production of PGE_2 which is generated by AA interaction with COX enzymes (Hawcroft *et al.*, 2010). PGE_2 is identified at higher concentrations in CRC tissue compared to normal tissue (Chulada *et al.*, 2000). It was also reported that PGE_2 levels were lower in CRCLM tissue following EPA supplementation (Cockbain *et al.*, 2014). The production of pro-inflammatory eicosanoids such as PGE_2 promotes carcinogenesis via a number of mechanisms including suppression of apoptosis, promotion of angiogenesis and stimulation of cell proliferation (Cheuk *et al.*, 2002; Tsutsumi *et al.*, 2002; Leahy *et al.*, 2002; Cianchi *et al.*, 2001; Pai *et al.*, 2001). It would be of interest to measure whether levels of the urinary prostaglandin metabolite (PGE-M) decreased following O3FA supplementation with both drinks and capsules (Cockbain *et al.*, 2014). Although there was insufficient funding there is still scope to perform this analysis in the future, as urine samples were collected at each time point over the course of the study.

Both drinks and capsule O3FA formulations were well tolerated as highlighted by the limited number of individuals that ceased supplementation during either intervention period (two individuals and three individuals from capsule and drinks respectively). There was no appreciable difference with regard to the adverse event and acceptability profiles of both the drinks and the capsules, with results

similar to data reported previously (Wang *et al.*, 2006). It is not possible to determine whether there was a dose-related response with regard to O3FA supplementation in either capsule or drink preparations as no individual reduced the dose of O3FA intervention over the course of either intervention period. Of interest is that less than 50% of individuals that experienced adverse events during the first intervention period taking either capsules or drinks, reported any adverse events when taking the alternative intervention during the second intervention period. Given there was O3FA dose equivalence across both capsule and drink interventions, there may have been additional factors associated with both interventions responsible for adverse events. One measure that suggested participants preferred capsules to the drinks, was whether an individual would consider taking either supplement in the future. A greater number of participants answered they would consider continuing the capsules (68%) than the drinks (58%), although this was not statistically significant. It is clear that although the adverse event profile and acceptability of the drinks preparation was comparable to capsules, there continued to be hesitancy from this healthy study population as to whether long-term or indefinite supplementation would be tolerated.

The strengths of the present study included a relatively low participant withdrawal rate and high concordance with both provision of RBC samples and consumption of O3FA supplements. The cross-over trial design allowed direct intra-individual comparison of O3FA supplement types in an effort to limit the effect of inter-individual variability.

The main limitation of the study was the lack of dietary assessment which precluded measuring the effect of dietary fat intake on the absorption of O3FAs

in either intervention type. This relatively small study was also insufficient to properly assess the difference in tolerability and acceptability between both forms of supplementation. The inability to assess for any correlation between PUFA incorporation into RBC membranes and colonic mucosal tissue is a significant limitation. Given that the colonic mucosa is the site at which colorectal carcinogenesis occurs, it is an important target for O3FA incorporation when considering O3FA supplementation as either a form of CRC chemoprevention or CRC treatment adjunct.

5.9 Conclusion

The findings of the present study provide evidence that O3FA containing drinks exhibit a similar O3FA RBC membrane incorporation profile to traditional O3FA containing capsules. Given the previously described anti-inflammatory and anti-neoplastic properties associated with O3FAs, supplement drinks have a potential clinical application in chemoprevention or as an adjunct in CRC treatment. The non-inferiority of drinks compared to capsules with regard to bioavailability of O3FAs is of key significance in CRC patients. Many patients recovering from surgery have an impaired appetite and are unable to consume conventional dietary fats required for absorption of traditional O3FA capsule supplementation. In addition, fortified O3FA nutrition drinks may be of nutritional value in providing additional calories to patients who are unable to achieve adequate calorific input, as is already commonplace with the use of various calorific supplements. Further research in a population of CRC patients is required to ascertain whether there are similar bioavailability, acceptability and tolerability profiles to those exhibited in this study of healthy volunteers.

Chapter 6: Results of faecal microbiome analysis from the randomised cross-over trial following O3FA supplementation

6.1 Introduction

The following chapter describes the results of faecal microbiome profile analysis of healthy volunteer stool samples following O3FA supplementation in the randomised cross-over trial. The effect of O3FA supplementation on bacteria at various taxonomic levels was examined, with particular attention to faecal microbiome profile changes associated with CRC carcinogenesis.

6.2 Sampling (see section 4.1)

When taking into account participant withdrawals during the course of the study, 97 of an expected 98 stool samples (99%) were obtained for faecal microbiome analysis from 20 study participants.

6.3 Stool DNA extraction and purification

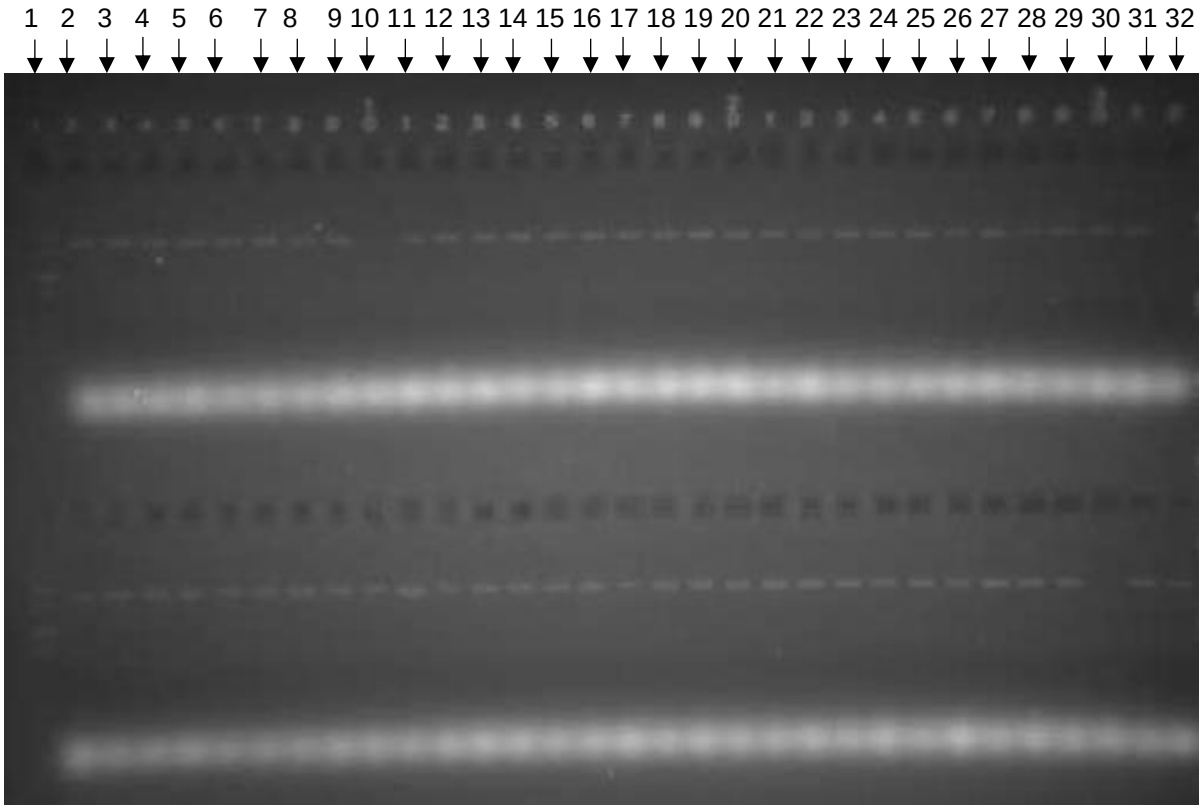
Ninety seven stool samples stored at -80ºC underwent DNA extraction. The median 260/280 and 260/230 ratios of DNA samples were 2.05 (range 1.66 to 2.22) and 2.00 (range 1.17 to 2.28) respectively. When analysing both 260/230 and 260/280 ratios, eight samples had markedly low ratio values (<10% of recommended ratio values). Therefore the relevant frozen stool samples underwent repeat DNA extraction and purification. Two of the eight repeated stool samples achieved higher 260/230 ratios but neither were within the desired range (greater than 1.8). The eight stool samples that reported 260/230 and 260/280 ratios below the desired range were still taken forward for faecal microbiome analysis. A repeat sample was only taken forward if both the purity of the sample and DNA concentration were higher than the original sample

extraction. This was exhibited in only one of the eight repeat extractions. The median DNA concentration across all 97 samples was 1025ng/µl (range 140ng/µl to 3162ng/µl).

6.4 PCR amplification of the 16S rRNA gene

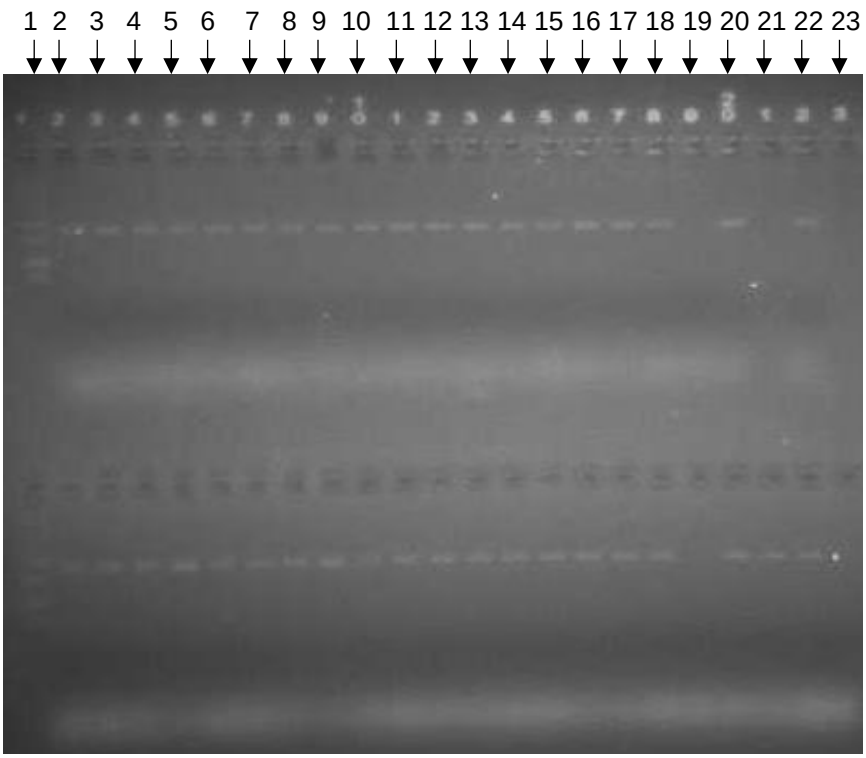
Gel electrophoresis of the product from PCR amplification of the V4 hyper-variable region of the 16S rRNA gene illustrated that 96 of 97 samples underwent successful PCR amplification as confirmed by a PCR product of expected size (approximately 250bp; Figures 13A & 13B). This was further highlighted by the presence of PCR product in the positive control *E. coli* sample (lane 32, row 2 of figure 13A; lane 21, row 2 of figure 13B) and the absence of PCR product in both the negative PCR sample and C2020 human cell line sample (lane 32, row 1 and lane 30, row 2 of figure 13A; lanes 19 and 23, row 2 of figure 13B) which did not contain any bacterial DNA. PCR amplification was unsuccessful in both the initial and repeated DNA extraction of the visit 1 stool sample from participant 005. This was evident in lanes 10 (row 1) and 19 (row 1) of figures 13A and 13B respectively where there was no evidence of PCR product. The purity of this DNA sample was below the desired range.

A



Row 1		Row 1		Row 2		Row 2	
1	50bp ladder	17	019V1	1	50bp ladder	17	020V2
2	001v1	18	009V1	2	011V2	18	008V3
3	012V1	19	020V1	3	012V2	19	012V3
4	002V1	20	010V1	4	001V3	20	009V3
5	013V1	21	001V2	5	013V2	21	013V3
6	003V1	22	011V1	6	002V3	22	010V3
7	014V1	23	002V2	7	014V2	23	014V3
8	004V1	24	005V2	8	003V3	24	011V3
9	015V1	25	003V2	9	015V2	25	015V3
10	005V1	26	006V2	10	004V3	26	019V3
11	016V1	27	004V2	11	016V2	27	016V3
12	006V1	28	007V2	12	005V3	28	020V3
13	017V1	29	009V2	13	017V2	29	017V3
14	007V1	30	008V2	14	006V3	30	C2020
15	018V1	31	010V2	15	018V2	31	018V3
16	008V1	32	NEG PCR	16	007V3	32	E.COLI

B



Row 1		Row 1		Row 2		Row 2	
1	50bp ladder	13	018_V4	1	50bp ladder	13	018_V5
2	001v4	14	007v4	2	001_V5	14	007_V5
3	012v4	15	019_V4	3	012_V5	15	019_V5
4	002v4	16	008_V4	4	002_V5	16	008_V5
5	013_V4	17	020_V4	5	013_V5	17	020_V5
6	003v4	18	009_V4	6	003_V5	18	009_V5
7	014_V4	19	005_V1	7	014_V5	19	C2020
8	004v4	20	010_V4	8	004_V5	20	010_V5
9	016_V4	21	empty	9	016_V5	21	E.Coli
10	005_V4	22	011_V4	10	005_V5	22	011_V5
11	017_V4	23	empty	11	017_V5	23	Neg PCR
12	006_V4			12	006_V5		

Figure 13A&B: Gel electrophoresis of PCR product following PCR amplification of the V4 hypervariable region of the 16S rRNA gene. Individual numbered lanes represent individual participant stool samples provided at each study visit (v1, v2, v3, v4, v5). Reference ladder (50bp) and control samples also individually labelled.

6.5 Quant-it assay of PCR product

The concentration of DNA following PCR amplification of the V4 hyper-variable region of the 16S rRNA gene was determined for each participant stool sample. The median concentration of DNA post PCR amplification was 23.1ng/µl (range 7.0ng/µl to 34.6ng/µl). The concentration of PCR product from the participant stool sample that showed no evidence of successful PCR amplification at gel electrophoresis was also quantified using the Quant-it assay (participant 005, visit 1). The concentration of the initial sample was below the reference range of the know standard. The re-run sample concentration measured 0.4ng/µl which was insufficient for sequencing library preparation. The Quant-it assay measure of DNA concentration was validated by the control samples. The negative control, the C2020 human cell line and sample containing no PCR product showed no recordable DNA concentrations. The positive *E. Coli* control showed adequate PCR amplification of the V4 hyper-variable region of the 16S rRNA gene (19.3ng/µl).

6.6 Tapestation assay of library preparation PCR product

Ninety seven stool samples were taken forward for library preparation resulting in the addition of an adaptor sequence to enable DNA sequencing on the Illumina platform. The adaptor sequence was 120bp in length with an expected overall library preparation product of approximately 360bp to 390bp in length. To confirm that successful library preparation had taken place, samples were analysed on the Tapestation analyser. The Tapestation system identifies the DNA fragment by size and also determines the presence of any isolated adaptor sequences or contaminant DNA from the purification process. Isolated adaptor sequences

following library preparation may interfere with sequencing as they preferentially bind to the flow cell of the sequencer.

Ninety seven samples analysed contained a predominant DNA fragment of between 360bp to 390bp in length, corresponding to the modified library preparation product with adaptor sequence. The median percentage integrated area, which is a measure of the presence of the desired PCR product with an adaptor sequence was 72%. Therefore samples contained a greater amount of adapted PCR product than other background or contaminating sequences and were suitable for sequencing. One sample, (participant 005, visit 1) did not exhibit a DNA fragment of the expected size. This corresponded to the same sample for which there was no PCR product post PCR amplification at gel electrophoresis or Quant-it analysis. Ten samples exhibited an adaptor peak of approximately 120bp in size, however the magnitude of isolated adaptor sequences present was below the threshold to inhibit DNA sequencing.

6.7 Quant-it assay of library preparation product

The concentration of library preparation DNA for each of the 97 stool samples was measured. Median concentration was 69.1ng/µl (range 21.2ng/µl to 99.1ng/µl). The library preparation sample from participant 005 visit 1 contained no library preparation product, which corresponded to the absence of a corresponding sized DNA fragment in the Tapestation analysis. Six library preparation samples were below the required concentration for sequencing on the Illumina next generation sequencer (range 21.2ng/µl to 42.2ng/µl) and therefore the process of library preparation was repeated. The six repeat samples that underwent library preparation were of adequate concentration as measured by the Quant-it assay (range 58.3ng/µl to 89.7ng/µl).

6.8 Illumina next generation DNA sequencing

Ninety six stool samples were taken forward for DNA sequencing. A single sample (participant 005, visit 1) was excluded from further faecal microbiome analysis as there was no evidence of library preparation product in either Tapestation or Quant-it assays. The number of reads per sample is a measure of sequence coverage. An increased number of reads provides improved accuracy of genomic sequencing as sequencing errors in any individual sequence may be indistinguishable from a sequence variant (Sims *et al.*, 2014). Across the 96 samples analysed the median number of reads was 46603 (range 26387 reads to 114130 reads).

6.9 Bray-Curtis principal co-ordinate analysis (PCoA)

The 96 stool samples were analysed to measure both the degree of inter-individual and intra-individual variation between participant stool samples, so-called β-diversity. The samples were analysed on the basis of OTUs, whereby nearly identical sequences, typically greater than 95% similarity are classified as identical genomes (Schloss, 2010; Matsen, 2014). The Bray-Curtis dissimilarity calculation quantifies β-diversity and is graphically represented in figure 14 using a PCoA. Each point is colour coded by participant and represents a stool sample from each of the five study visits irrespective of intervention order. Each data point is plotted to create a visual representation of variability and therefore microbial compositional differences between samples (Goodrich *et al.*, 2014). Assessment of the PCoA illustrates consumption of either O3FA containing drinks or capsules had no significant effect on faecal microbiome profiles, although inter-individual variation across samples precluded assessment of the effect of O3FA supplementation on the microbiome within individual participants.

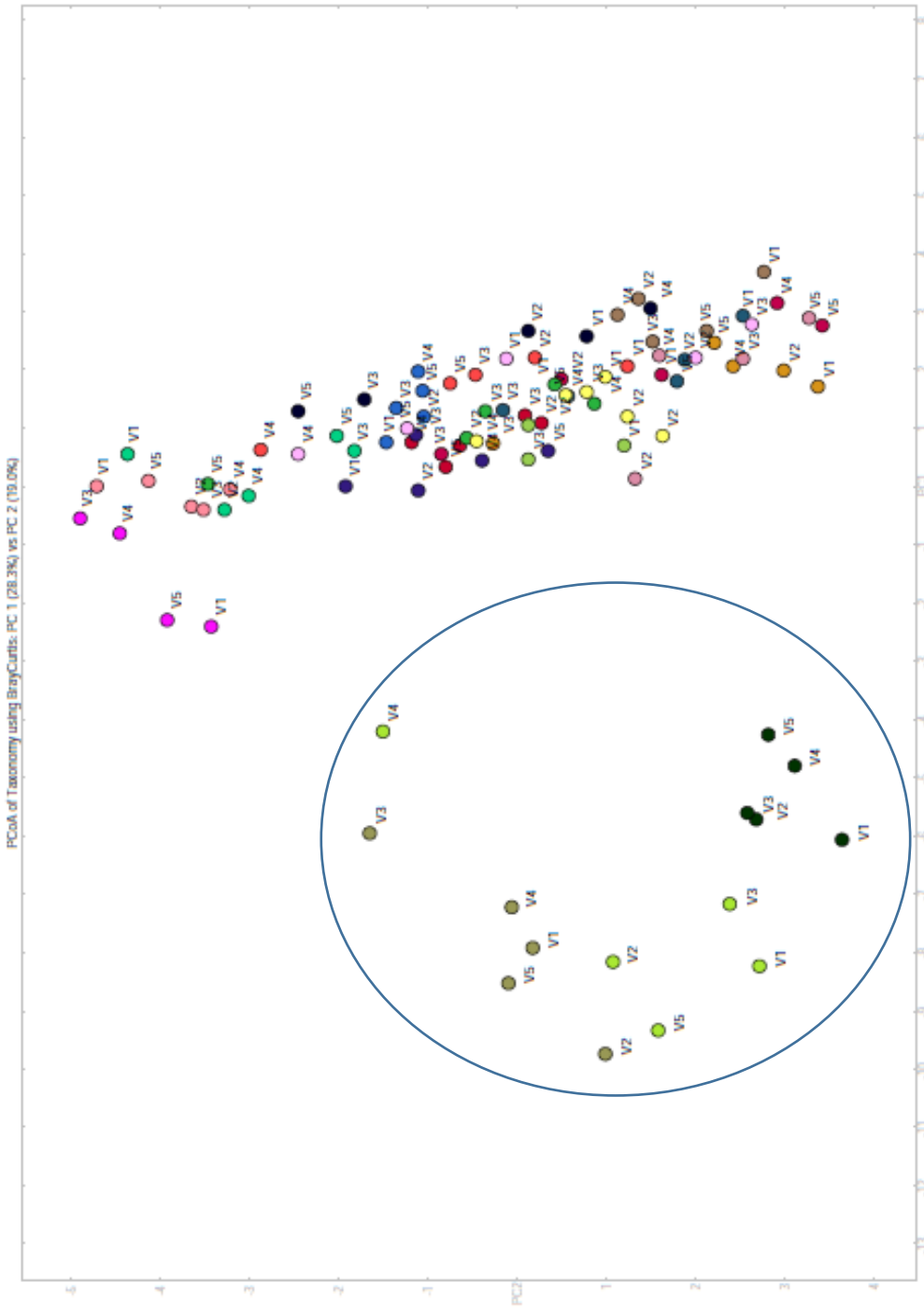
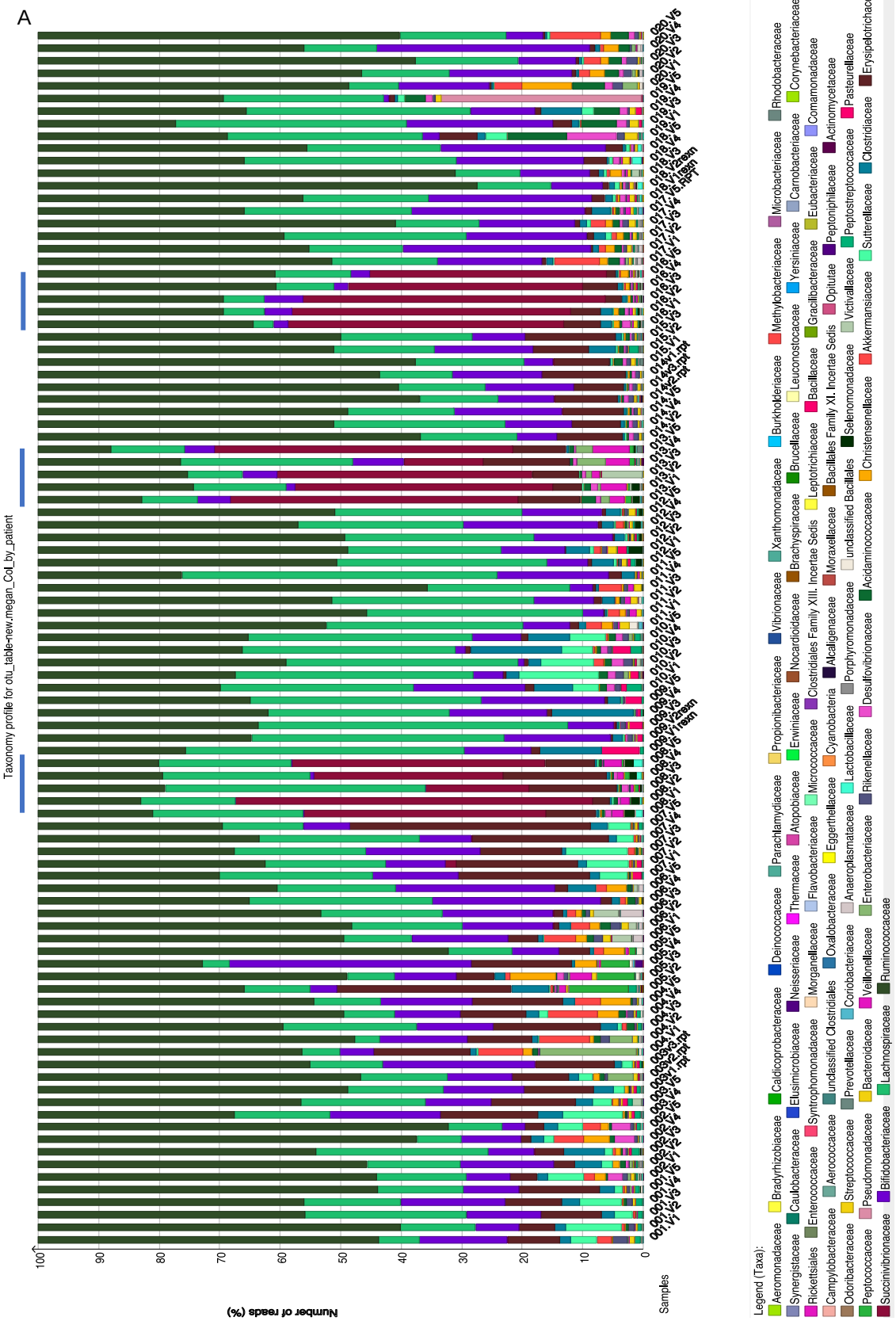


Figure 14: PCoA of β-diversity across 96 stool samples (20 participants) as measured by Bray-Curtis Dissimilarity calculation. Individual participants (001 to 020) are uniquely colour coded across each of the five planned study visits (V1, V2, V3, V4, V5) irrespective of intervention order. Participants 008, 013 and 016 are highlighted (blue circle).

Three individuals displayed markedly different faecal microbiome profiles (participants 008, 013 and 016). Participants 008 and 013 differed from the remaining participants with a reduced proportion of the class *Clostridia* and a larger proportion of the class *Gammaproteobacteria* with high abundance of the family *Succinivibrionaceae* (figure 15a). Analysis of the microbiome at family level excluding *Succinivibrionaceae* revealed that these microbiome profiles were actually similar to the remaining study participants (figure 15b). Examination of the baseline characteristics of participants 008 and 013 did not reveal differences from the other participants in the study, specifically the use of medications, vitamin supplements or underlying medical conditions. Participant 016 displayed a very high abundance of the family *Succinivibrionaceae*, specifically the genus *Succinivibrio* but otherwise the faecal microbiome profile was similar to the remaining volunteers in the study as detailed in figure 16. Of specific note was this participant was the only individual within the study prescribed a proton pump inhibitor (omeprazole) prior to entering the study. As participants 008 and 013 exhibited markedly different faecal microbiome profiles with respect to the classes *Clostridia* and *Gammaproteobacteria* they were excluded from subsequent pooled analyses.


A
Taxonomy profile for otu_table-new.megan_CoI_by_patient
Number of reads (%)
Samples
Legend (Taxa):
Aeromonadaceae
Bradyrhizobiaceae
Caldicoprobacteraceae
Deinococcaceae
Parachlamydiaceae
Propionibacteriaceae
Vibrionaceae
Xanthomonadaceae
Burkholderiaceae
Methylobacteriaceae
Microbacteriaceae
Rhodobacteraceae
Synergistaceae
Caulobacteraceae
Elusimicrobiaceae
Neisseriaceae
Thermaceae
Atopobiaceae
Erwiniaceae
Nocardioidaceae
Brachyspiraceae
Brucellaceae
Leuconostocaceae
Yersiniaceae
Carnobacteriaceae
Corynebacteriaceae
Rickettsiales
Enterococcaceae
Syntrophomonadaceae
Morganellaceae
Flavobacteriaceae
Micrococcaceae
Clostridiales Family XIII. Incertae Sedis
Leptotrichiaceae
Bacillaceae
Gracilibacteraceae
Eubacteriaceae
Comamonadaceae
Campylobacteraceae
Aerococcaceae
unclassified Clostridiales
Oxalobacteraceae
Eggerthellaceae
Alcaligenaceae
Moraxellaceae
Bacillales Family XI. Incertae Sedis
Opitutae
Peptoniphilaceae
Actinomycetaceae
Odoribacteraceae
Streptococcaceae
Prevotellaceae
Coriobacteriaceae
Anaeroplasmataceae
Cyanobacteria
Lactobacillaceae
Porphyromonadaceae
unclassified Bacillales
Selenomonadaceae
Victivallaceae
Peptostreptococcaceae
Pasteurellaceae
Peptococcaceae
Pseudomonadaceae
Bacteroidaceae
Veillonellaceae
Enterobacteriaceae
Rikenellaceae
Desulfovibrionaceae
Acidaminococcaceae
Christensenellaceae
Akkermansiaceae
Sutterellaceae
Clostridiaceae
Erysipelotrichaceae
Succinivibrionaceae
Bifidobacteriaceae
Lachnospiraceae
Ruminococcaceae

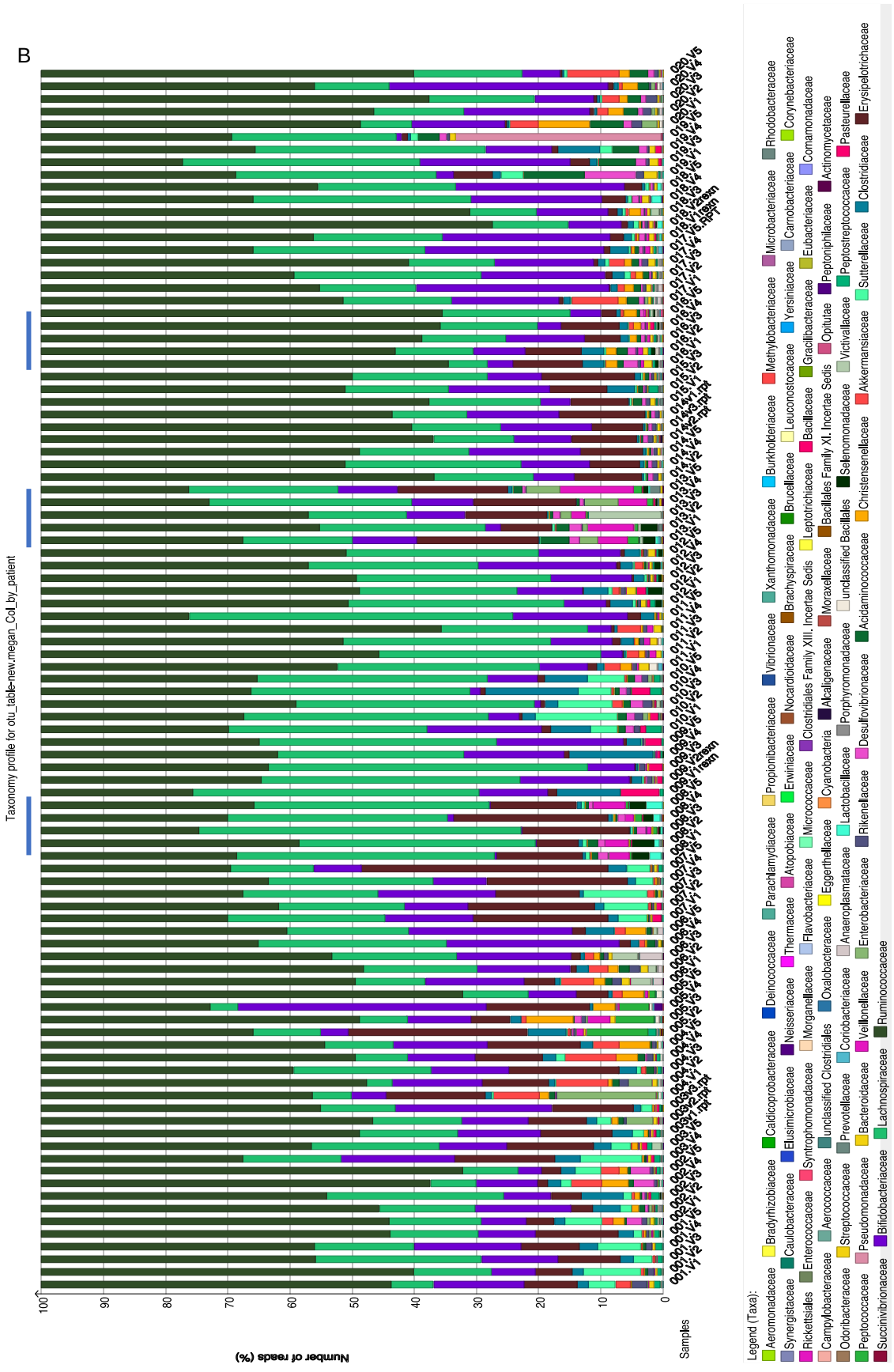


Figure 15A&B. Microbiome analysis across all 20 participants at individual study visits (V1 to V5) at family taxonomic level as measured by number of reads. Figure A includes all families identified. Figure B excludes the family *Succinivibrionaceae.* Participants 008, 013 and 016 are highlighted by a blue line above relevant study visits.

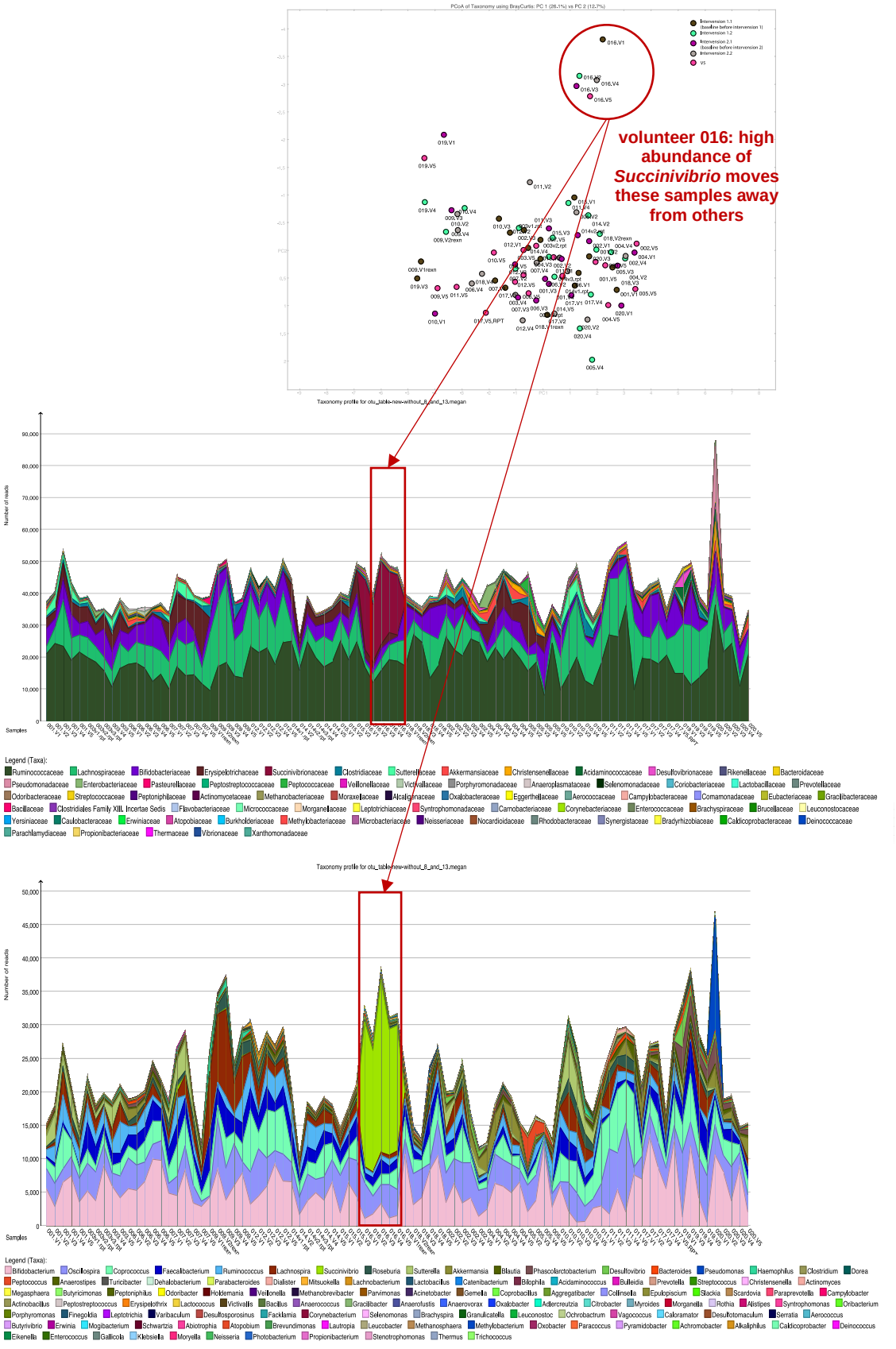


Figure 16. Family- and genus-level analysis of all stool samples as measured by number of reads (excluding volunteers 008 and 013). PCoA included to demonstrate the presence of a high proportion of *Succinivibrio (f*amily, *Succinivibrionaceae*) in participant 016 results in differences exhibited in β-diversity analysis (highlighted in red).

The following analyses measure changes to faecal microbiome profiles based on the order to which study participants received the carton and drink supplements. Therefore figures 17 and 18 label participants randomised to capsules followed by drinks as Int1_Int2. Participants randomised to take drinks followed by capsules are labelled as Int2_Int1. The PCoA in figure 17 illustrates that irrespective of intervention order both the baseline and washout faecal microbiome profiles of all participants within the study (excluding participants 008 and 013) were similar. It also appears that following O3FA supplementation there was a distinct change to faecal microbiome profiles in participants randomised to both intervention orders, although the change was less marked in participants randomised to consume capsules during the second intervention period. On the basis that following both washout periods, regardless of intervention order the pooled faecal microbiome profiles of all participants were similar to baseline, subsequent analyses were performed with capsule and carton interventions merged. Review of faecal microbiome profiles dependent on intervention order did not exhibit any significant differences as measured by the percentage number of reads at family level (figure 18). Microbiome analysis irrespective of intervention order was further justified by the previously described finding that EPA and DHA RBC membrane levels returned to near baseline following the washout period across both intervention orders. Subsequent analyses presented are performed irrespective of intervention order and are labelled as pre (1.1) and post (1.2) capsule or pre (2.1) and post (2.2) drinks supplementation.

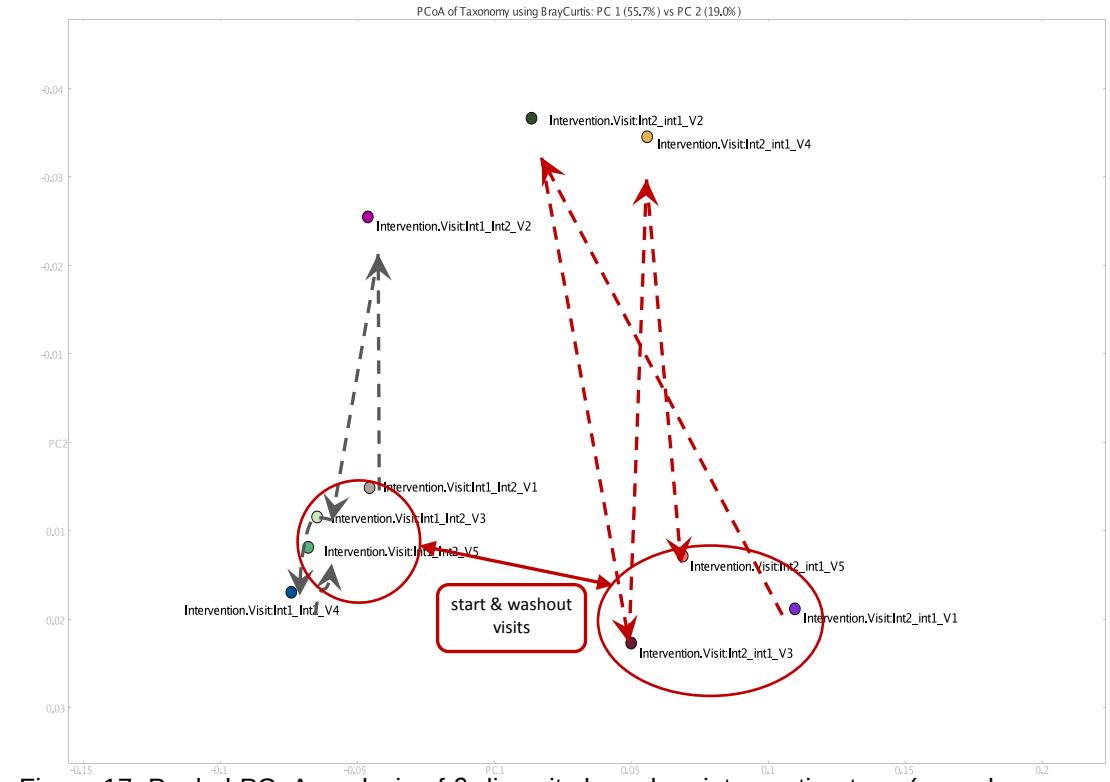


Figure 17. Pooled PCoA analysis of β-diversity based on intervention type (capsules versus drinks) at individual study visits (V1 to V5). Consumption of capsules are labelled as Int1. Consumption of drinks are labelled as Int2. Arrows highlight the flow of participants through the study from visit 1 to visit 5.

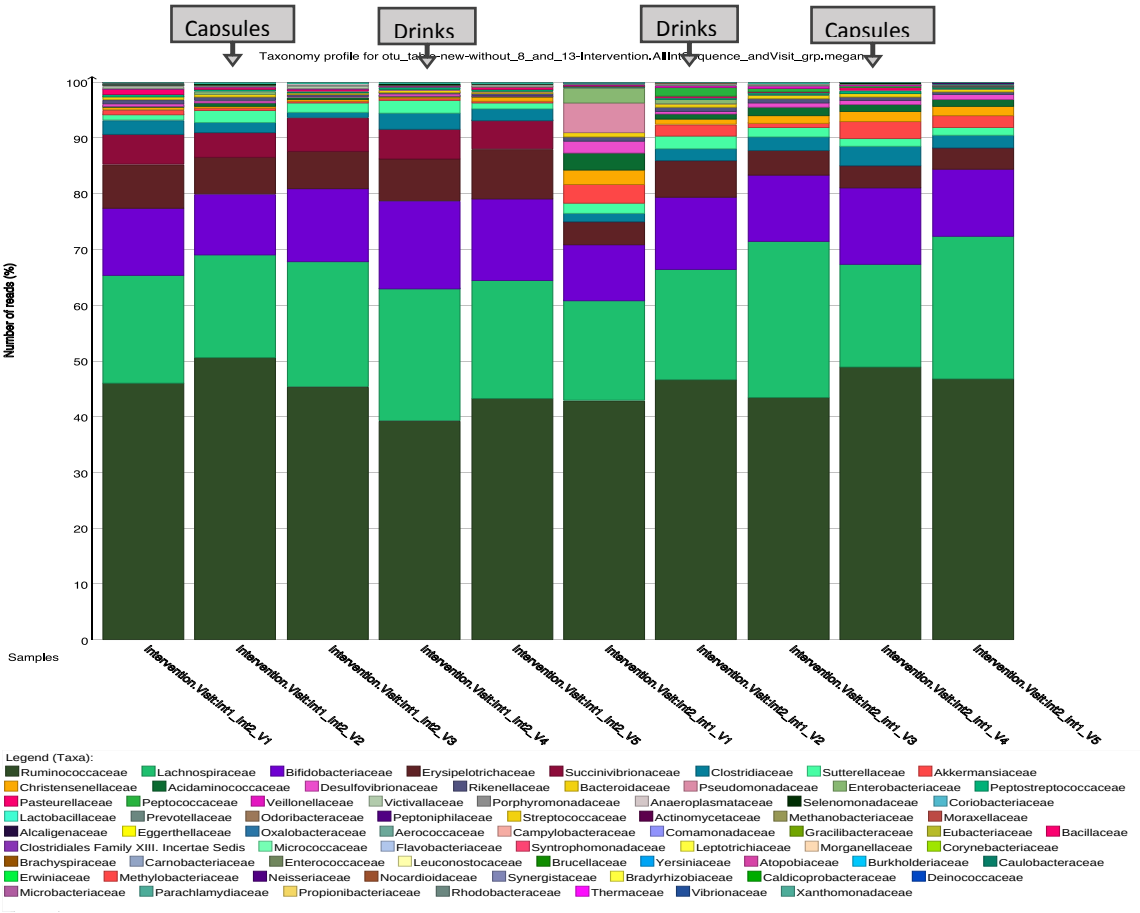


Figure 18. Analysis of effect of intervention order on microbiome profile analysis as measured by percentage number of reads at family taxonomic level at each study visit. Capsule and drink intervention periods labelled.

6.10 β-diversity as measured by Unifrac pooled analysis

To further assess changes to faecal microbiome profiles in a pooled analysis across the five study visits, β-diversity was measured. β-diversity measures the degree to which taxa are shared between the overall study population at each study visit. Unifrac analysis differs from previously described Bray-Curtis dissimilarity measures by incorporating phylogenetic distances which assess not only the abundance, but also the phylogenetic relatedness of organisms within a study population. Figure 19 illustrates both weighted and unweighted Unifrac analyses. The weighted analysis is a quantitative measure of β-diversity, whereas the unweighted analysis is qualitative and only determines the presence or absence of organisms (Lozupone *et al.*, 2007). It is apparent that neither weighted nor unweighted Unifrac measures identify any significant changes in β-diversity following either capsule or drink O3FA supplementation. This is at odds with the Bray-Curtis PCoA analysis previously described (figure 17) where there appeared to be marked changes post O3FA supplementation irrespective of intervention order. This apparent difference between β-diversity analyses may in part be due to the method of Unifrac analysis which accounts for phylogenetic similarities between separate OTUs. Individual OTUs across the five separate study visits may be phylogenetically closely related and therefore any differences illustrated in the Bray-Curtis PCoA analysis are no longer apparent.

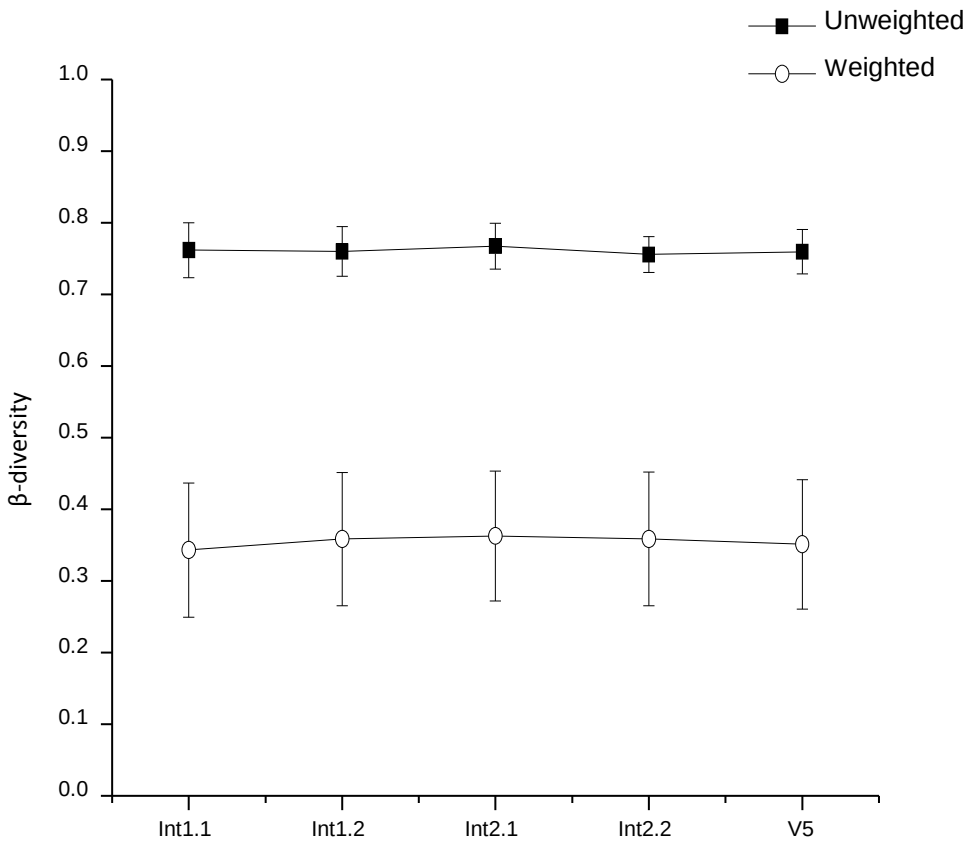


Figure 19. Weighted and unweighted unifrac analysis of β-diversity based on intervention type (capsule versus drinks) irrespective of intervention order. Int 1.1 and 1.2 consumed are pre and post-supplementation results in the capsule intervention. Int2.1 and 2.2 are pre and post supplementation results in the drinks intervention. Weighted (open circles), unweighted (solid squares). Results shown are mean values and standard deviation.

6.11 α-diversity

α-diversity is a measure of microbial diversity within a specific environment as measured by the Shannon diversity index. For the purpose of this study the environment is the participant faecal sample at each study visit. The index is calculated as the proportion of each species relative to the total number of species in the participant environment under analysis. The index is a measure of both the richness and distribution of taxa within a single population. Figure 20 is a pooled analysis of participant faecal samples at each visit in the study irrespective of intervention order. The microbial diversity within participant stool samples as measured by the Shannon diversity index was unchanged following

O3FA supplementation in both capsule and drink forms. There was no change to faecal microbial diversity following either of the eight week intervention periods or 12-week washout periods.

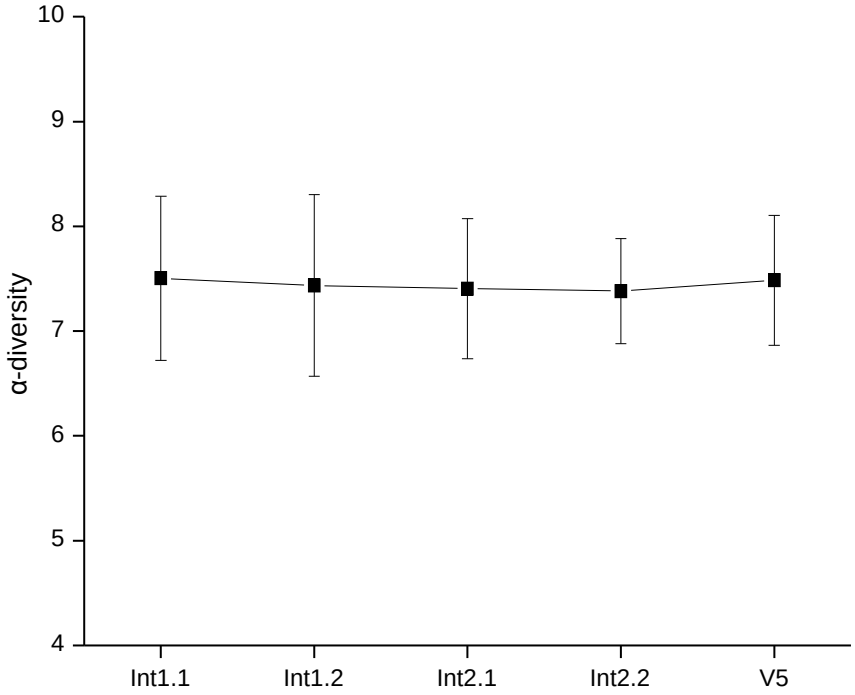


Figure 20. Shannon α-diversity index of effect of intervention type on microbiome profiles. Int 1.1 and 1.2 consumed are pre and post supplementation results in the capsule intervention. Int2.1 and 2.2 are pre and post supplementation results in the drinks intervention. Results shown are mean values and standard deviation.

6.12 The *Firmicutes:Bacteriodetes* (F:B) ratio

Two of the major phyla colonising the human colon are *Firmicutes* and *Bacteroidetes*. The ratio of *Firmicutes* to *Bacteroidetes* is often cited in studies relating to microbial dysbiosis and CRC risk (Sun & Kato, 2016; Raskov *et al.*, 2017). Paired data were available for 20 and 17 participants in the capsule and drink groups respectively as shown in figure 21. Thirteen of 20 participants and 10 of 17 participants in the capsule and drink interventions groups respectively reported an elevation in F:B ratio following the intervention period. However there was no statistically significant change in the F:B ratio following either drink or capsule O3FA interventions.

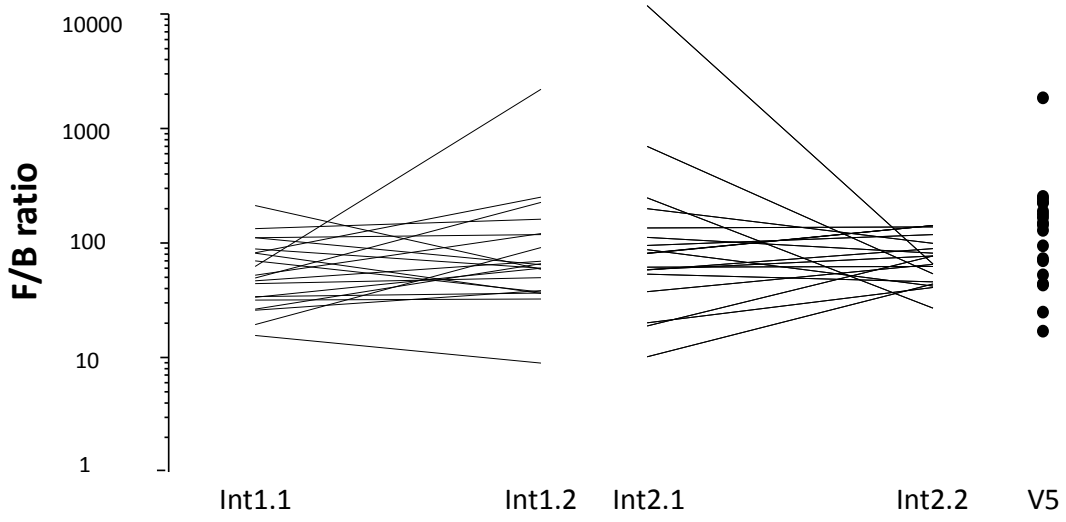


Figure 21. Paired *Firmicutes*/*Bacterioidetes* (F:B) ratio values before and after capsules (Int1) and drinks (Int2) for both orders of intervention. V5 are the final visit (second washout) values.

6.13 Analysis of faecal microbiome profiles at family and genus levels

Faecal microbiome profiles were examined at both genus and family levels to determine changes not apparent in the global measures of α-diversity and β-diversity.

Results were merged according to O3FA intervention type (capsule or drink) irrespective of intervention order (Figure 22). The bacteria were quantified at both family and genus levels according to the measured number of reads for individual OTUs. At family level, the abundance of both *Clostridiaceae* and *Akkermansiaceae* of the phyla *Firmicutes* and *Verrucomicrobia* respectively, increased after both capsule and carton intervention periods with subsequent reversibility following the corresponding washout periods.

There was an increase in the abundance of *Sutterellaceae* of the phylum *Proteobacteria*. This family of bacteria also exhibited a return to near baseline levels following the 12-week washout periods in both drink and capsule interventions.

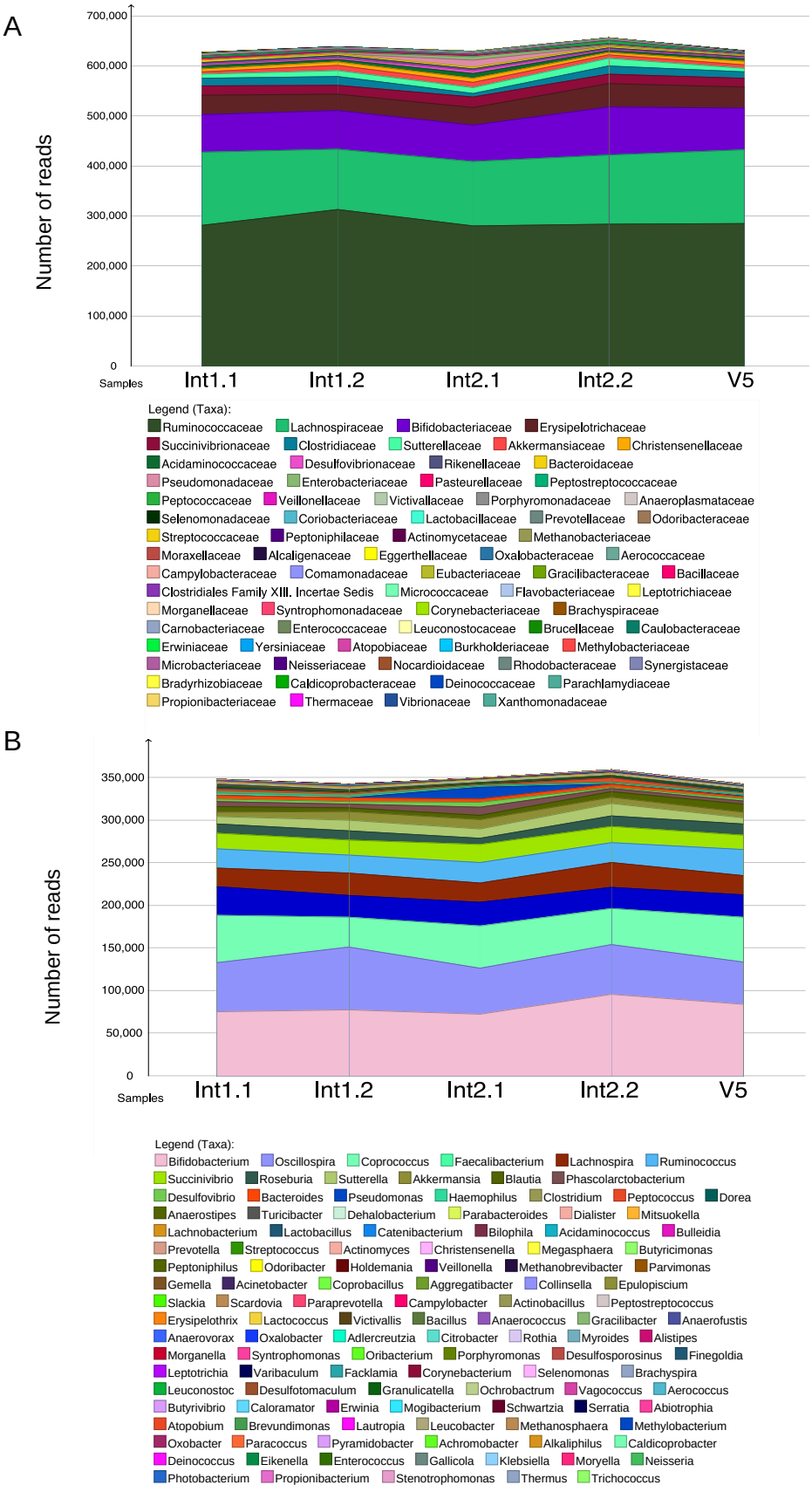


Figure 22. Family (A) and Genus (B) taxonomic level microbiomes profiles based on intervention type pre-and post-supplementation as measured by number of reads. Int 1.1 and 1.2 consumed are pre and post supplementation results in the capsule intervention. Int2.1 and 2.2 are pre and post supplementation results in the drinks intervention.

To identify changes to faecal microbiome profiles not apparent at family level, the genus level was examined in an unpaired analysis, with specific attention to the top 5 abundant genera across study participants (figure 23).

There was an increased abundance of *Bifidobacterium* of the family *Bifidobacteriaceae* (Phylum, *Actinobacteria*) following both capsule and drink interventions. The increase was most notable following the drinks intervention period. The abundance of *Bifidobacterium* appeared to remain elevated following the second 12-week washout period compared to pre-intervention measurements for either intervention type.

The remaining four most abundant genera identified within the stool samples of study participants were from the phylum *Firmicutes*. Following both capsule and drink intervention periods there was an increase in abundance of both *Oscillospira* of the family *Ruminococcaceae* and *Lachnospira* of the family *Lachnospiraceae*. The magnitude of increase in abundance of *Lachnospira* was greatest following the O3FA drinks intervention period, whereas the increase in abundance of the genus *Oscillospira* was greater following the O3FA capsule intervention. At the end of the final 12-week washout period (visit 5) the abundance of both aforementioned genera returned to pre-intervention levels.

The abundance of *Coprococcus* of the family *Lachnospiraceae* and *Faecalibacterium* of the family *Clostridiaceae* were reduced after both capsule and drink interventions. The reduction to both genera was most pronounced following the O3FA capsule intervention period. However it was noted that the mean number of reads for both *Coprococcus and Faecalibacterium* were higher prior to the capsule intervention period when compared to the O3FA drinks period.

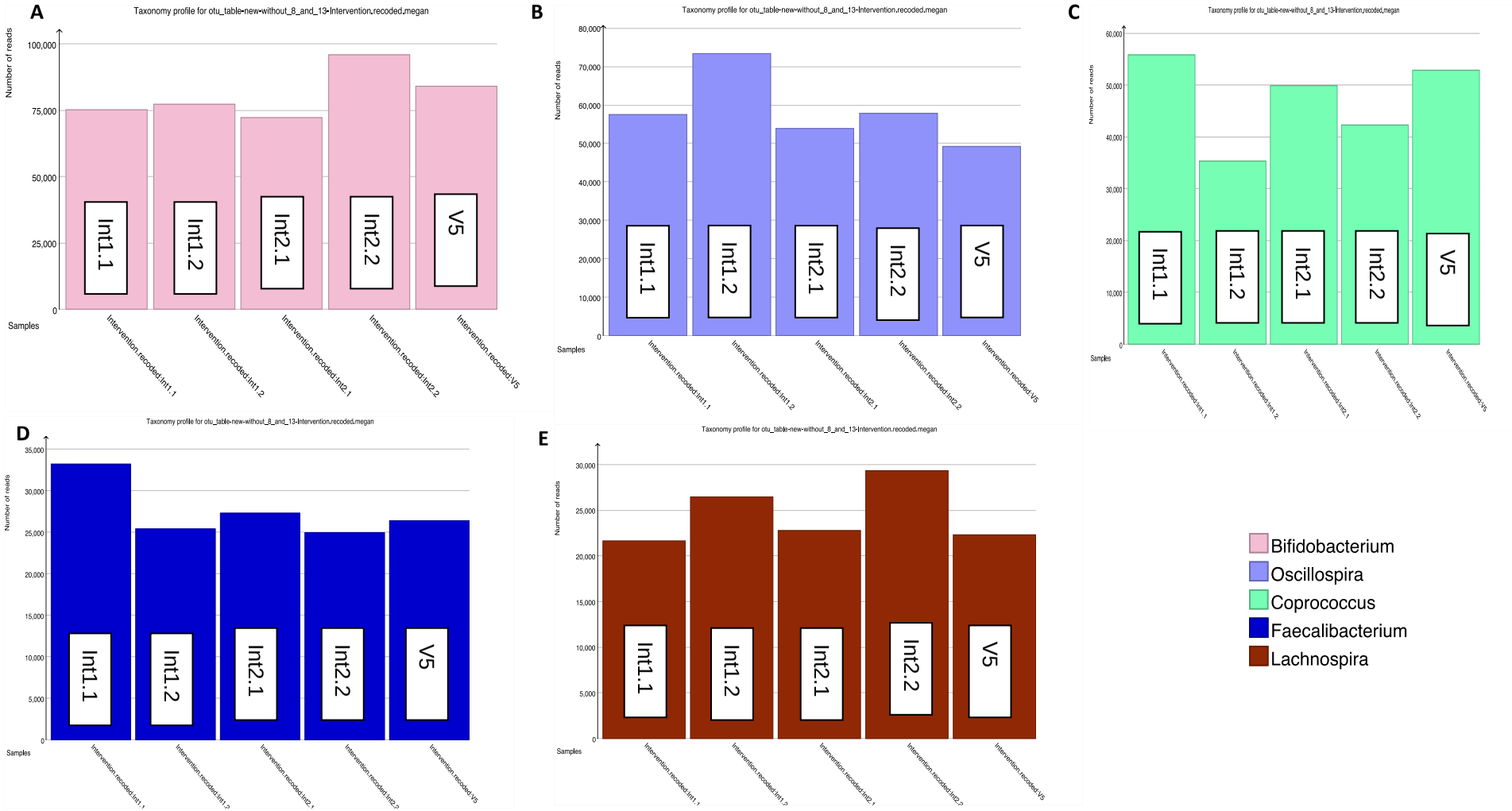


Figure 23. Analysis of the 5 most abundant genera following O3FA supplementation (capsule *versus* drinks) as measured by number of reads. Int 1.1 and 1.2 consumed are pre and post supplementation results in the capsule intervention. Int2.1 and 2.2 are pre and post supplementation results in the drinks intervention.

Alterations to faecal microbiome profiles in relation to the genus *lactobacillus* of the family *Lactobacillaceae* (Phylum, *Firmicutes*) following O3FA supplementation were also examined. Figure 24 illustrates an increase in *lactobacillus* following both O3FA capsule and drink interventions. The rise was most prominent following the O3FA drinks intervention period. The increase in *Lactobacillus* associated with either intervention type was not statistically significant (capsules, p=0.11; drinks, p=0.9). Following the final 12 week washout period (visit 5), the abundance of *Lactobacillus* fell but remained elevated compared to baseline levels.

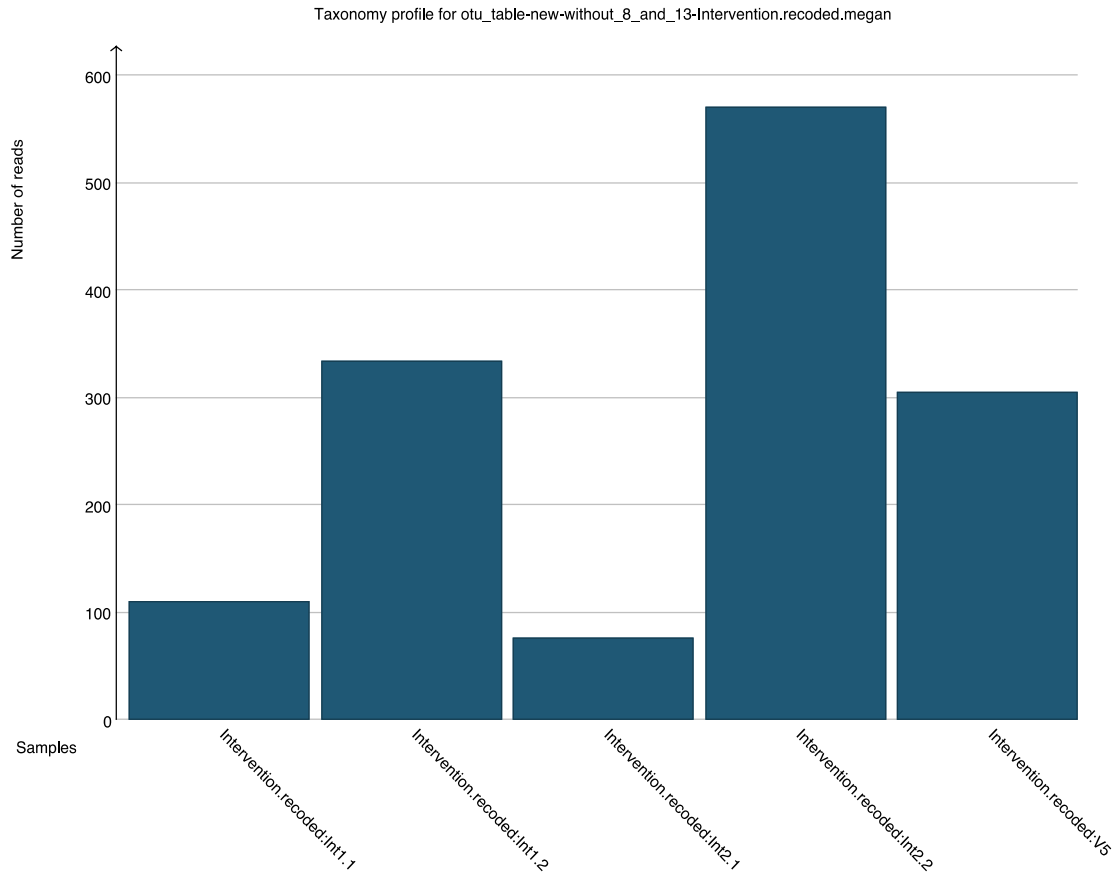


Figure 24. Analysis of the effect of O3FA supplementation on *lactobacillus* (capsule versus drinks) as measured by number of reads. Int 1.1 and 1.2 consumed are pre and post supplementation results in the capsule intervention. Int2.1 and 2.2 are pre and post supplementation results in the drinks intervention.

6.14 Paired analysis of changes to faecal microbiome profiles

Paired analysis of the 20 most abundant genera was performed to assess changes to faecal microbiome profiles following each intervention type (figure 25). The mean change in the observed number of OTUs was calculated by subtracting the number of OTUs measured post-intervention from the pre-intervention value. Seventeen study participants were included within the analysis that completed both capsule and drinks O3FA supplementation periods. The paired analysis revealed similar findings to the unpaired analysis previously described. It confirmed *Bifidobacterium, Oscillospira* and *Lachnospira* increased following O3FA supplementation. Across all three of the aforementioned genera the increase in the mean number of OTU reads was highest following the O3FA drink intervention period. Both *Bifidobacterium* and *Oscillospira* also exhibited notable positive changes in the observed number of OTUs post O3FA capsule supplementation, whereas the increase in abundance of *Lachnospira* following capsule O3FA supplementation was negligible.

There was a reduction in the number of OTUs observed between pre and post-intervention periods in both capsule and drink interventions for *Coprococcus and Faecalibacterium*. However only the negative change identified in *Coprococcus* during the capsule supplementation period reached statistical significance ($p=0.01$, one sample t-test). When adjusting for multiple testing the aformentioned change to Coprococcus was not statistically significant ((False discovery rate, Benjamini, Krieger and Yekutieli method)

A number of genera, within the microbiome including *Akkermansia*, *Roseburia* and *Rumminococcus* illustrated opposing changes dependent on O3FA intervention type, although this only reached statistical significance when

examining *Roseburia* (p=0.03, one sample t-test) where there was an increase post O3FA drink intervention. However with adjustment for multiple testing this was not significant (False discovery rate, Benjamini, Krieger and Yekutieli method).

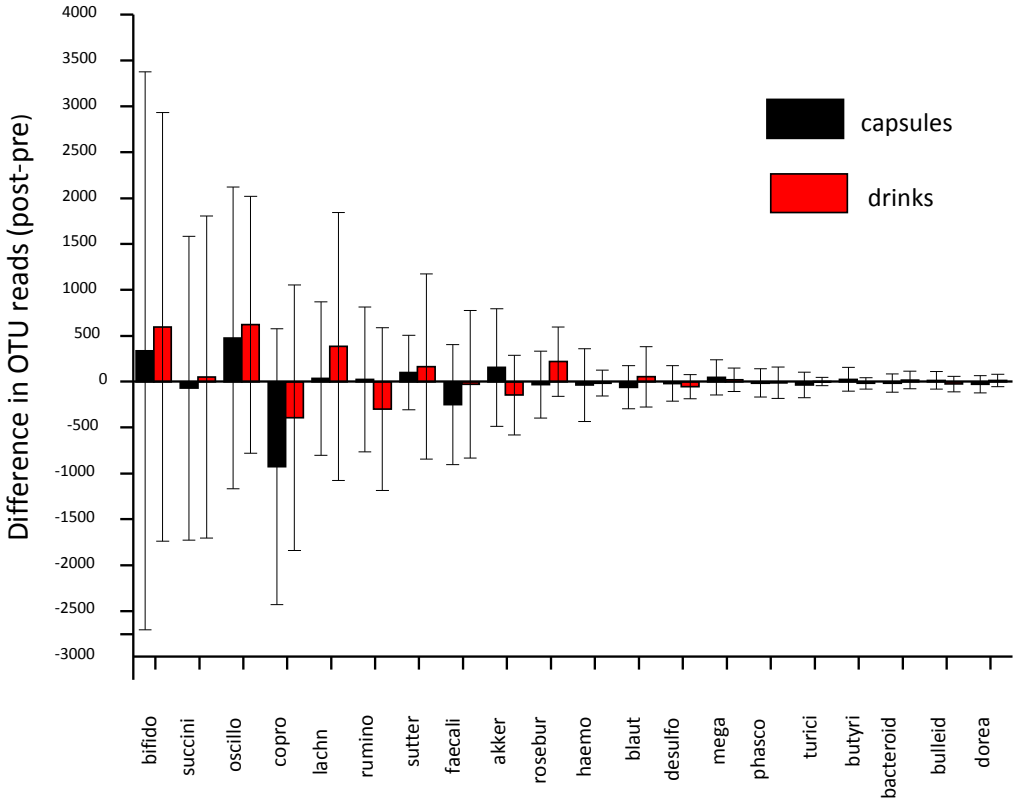


Figure 25. Changes in the abundance of specific genera following 8 weeks O3FA supplementation as measured in the difference in number of reads of OTUs. Red bars are drinks. Black bars are capsules.

6.15 Bacteria of interest

Faecal microbiome profiles from this cohort of healthy volunteers were examined for specific bacteria associated with CRC carcinogenesis.

Fusobacterium of the family *Fusobacteriaceae* is associated with CRC carcinogenesis (Ahn *et al.,* 2013; Wu *et al.,* 2013; Liang *et al.,* 2017; Yu *et al.,* 2017). *Fusobacterium nucleatum* has previously been identified in studies as a species which is overrepresented in CRC patients compared to controls. Analysis of the sequenced data from faecal microbiome analysis did not identify any OTUs representative of the genus *Fusobacterium* within this cohort of healthy volunteers.

Bacteroides fragilis of the genus *Bacteroides* (family, *Bacteroidaceae*) is associated with CRC carcinogenesis (Qin *et al.*, 2010; Sobhani *et al.*, 2011; Wu *et al.*, 2009; Wu *et al.*, 2013; Karrasch *et al.*, 2007). At genus level *Bacteroides* was present in the stool samples of healthy volunteers. At a species level, *Bacteroides fragilis* and specifically an enterogenic toxin producing strain which has been implicated in CRC carcinogenesis was not identified (Toprak *et al.*, 2006).

Escherichia coli of the genus *Escherichia* (family, *Enterobacteriaceae*) is cited in studies as being overrepresented in the faecal samples of CRC patients (Swidsinski *et al.*, 1998; Martin *et al.*, 2004; Grivennikov *et al.*, 2012; Bonnett *et al.*, 2014). The present study did not identify the genus *Escherichia* in the faecal samples analysed from healthy volunteers. At higher taxonomic levels the family *Enterobacteriaceae* was identified but there was no significant difference across intervention and washout periods for either intervention type.

6.16 Discussion

The present study highlights subtle changes to faecal microbiome profiles following O3FA supplementation in both drink and capsule forms. The significance of these changes is unclear given the significant inter-individual

variation demonstrated in the faecal microbiome profiles of healthy volunteers. The variation across individual participant faecal microbiome profiles both within and between individuals is likely to mask any changes induced by O3FA supplementation over a relatively short intervention period. Long-term, rather than short-term dietary alterations are more likely to induce changes to individual faecal microbiome profiles (Flint *et al.*, 2017; Wu *et al.*, 2011). When examining the effects of O3FA supplementation across the entire study population, O3FA supplementation had little or no effect on microbiome diversity measures irrespective of intervention order. This would appear to correspond with rodent studies that also show O3FA supplementation has no measurable effect on microbiome diversity (Caesar *et al*., 2015; Robertson *et al.*, 2017).

Measures of β-diversity varied according to the method of analysis performed. Pooled analysis of participant samples taking into account intervention type revealed changes to the microbiome following O3FA supplementation that returned to baseline profiles after the 12-week washout period. However, the Unifrac measure of β-diversity which takes into account the phylogenetic distances between OTUs did not show any change to microbiome profiles during the course of the study (Lozupone *et al.*, 2007). The likely explanation for this difference is that individual OTUs across the five separate study visits were phylogenetically closely related and therefore any differences illustrated in the Bray-Curtis PCoA analysis were no longer apparent.

The lack of significant change to faecal microbiome profiles was further confirmed by the analysis of α-diversity, a measure of overall bacterial diversity within a specified environment (Morgan & Huttenhower, 2012). There was no change to microbiome profiles following O3FA supplementation, irrespective of

the order in which participants received the capsule and drink supplementation. Although the present intervention study does not suggest any changes to diversity associated with O3FA supplementation, a recent study by Menni *et al.* of over 800 individuals enrolled in the TwinsUK registry highlighted a correlation between DHA serum levels and microbial α-diversity as measured by the Shannon index (Menni *et al.*, 2017). However, this apparent linkage is likely to reflect longer term exposure to O3FAs compared to the present study.

The aforementioned measures of diversity give an insight into changes to faecal microbiome profiles but overlook subtle alterations at various taxonomic levels which may be influenced by O3FA supplementation. When microbiome profiles were analysed on a genus level, more nuanced changes to microbiome profiles of participants within the study were identified. There were increases to varying degrees at genus level for *Bifidobacterium*, *Lachnospira*, *Roseburia* and *Lactobacillus*. Interestingly, the magnitude of change to the abundance of the aforementioned bacteria differed between capsule and drinks interventions. An increased abundance of *Roseburia* and *Lachnospira* was only observed during the drink intervention. This again suggests that differences between the contents of the drinks and capsules may be responsible for alterations to faecal microbiome profiles, including the presence of additional proteins, carbohydrates and fibre (see appendix 1).

The genera *Bifidobacterium*, *Lachnospira*, *Roseburia* and *Lactobacillus* are of particular interest in the context of CRC carcinogenesis as they are butyrate-producing bacteria that generate SCFAs from the fermentation of fibre. These SCFAs are proposed to have a number of beneficial effects including modulating the pH of the colon, regulating apoptosis of CRC cells, modulating inflammatory

pathways within the colon, reducing oxidative stress and improving gut barrier function by promoting mucin formation (Hamer *et al.*, 2008; Havenaar, 2011; Macfarlane & Macfarlane, 2012; Louis & Flint, 2009; Wu *et al.*, 2018; Zeng *et al.*, 2019). Butyrate also plays a role in the epigenetic regulation of gene expression via inhibition of histone deacetylase function (Donohoe *et al.*, 2012; McIntyre *et al.*, 1993; Davie, 2003; Chang *et al.*, 2014; Han *et al.*, 2018). Large scale case-control studies have identified differences in the abundance of butyrate-producing bacteria across CRC patients and healthy individuals (Chen *et al.*, 2013). Although not universal, there was a degree of reversibility following the washout period to changes exhibited in the abundance of butyrate-producing bacteria. This may suggest the presence of high doses of EPA and DHA were responsible for any increase. There is limited evidence from human studies examining the effect of O3FAs on colonic microbiome profiles. Menni *et al*. report that serum and dietary levels of DHA correlate with an increased presence of bacteria from the *Lachnospiraceae* family which include *Lachnospira* and *Roseburia* (Menni *et al.*, 2017). Further evidence that O3FA-rich diets result in an increased presence of butyrate-producing bacteria is provided by an O3FA supplementation study implementing high dietary fish intake (Noriega *et al*., 2016). They report reversible increases to *Roseburia* and *Coprococcus* both of the *Lachnospiraceae* family. However the study by Noriega *et al.* was limited by examining the faecal microbiome profile of a single male individual. An increased abundance of bacteria from the *Lachnospiraceae* family is also reported in a study of 30 individuals at risk of metabolic syndrome following supplementation with DHA-enriched canola oil over a 30 day period (Pu *et al.*, 2016). However, it is necessary to consider that individuals prone to developing metabolic syndrome may have multiple underlying metabolic factors that influence changes

to microbiome profiles. Given that butyrate is generated by the fermentation of fibre it would be of interest to assess whether the intake of fibre in conjunction with O3FA supplementation has any synergistic effect on CRC risk, as already reported in rats (Crim *et al.*, 2008).

Although there is limited evidence pertaining to human studies exploring the influence of O3FAs on microbiome profiles, there are animal studies that report similar changes to the abundance of various genera exhibited in the present study. In the context of CRC, Piazzi *et al.* reported that EPA supplementation not only impaired tumour development in the AOM-DSS mouse model of colitis-associated CRC, but also showed an increase in the abundance of *Lactobacillus* (Piazzi *et al.*, 2014). Lactic acid-producing bacteria including *Lactobacillus* are proposed to modulate a number of CRC pathways including enhancement of mitogen-activated protein kinase (MAPK) enhanced apoptosis and expression of tumour suppressor genes (Iyer *et al.*, 2008; Wu *et al.*, 2018). There is evidence to suggest these bacteria generate an anti-tumour immune response via generation of cytokines including TNFα and also by inhibition of ROS induced DNA damage (Zhong *et al.*, 2014).

The balance between the two predominant phyla *Firmicutes* and *Bacteroidetes* is proposed to be significant in colorectal carcinogenesis (Sun & Kato, 2016; Raskov *et al.*, 2017). A reduction to the *F:B* ratio is associated with CRC in animal studies (Myles *et al.*, 2014). However, O3FA induced changes to the *F:B* ratio were not apparent in the present study. There was evidence of an increased abundance of individual bacteria from the phylum *Firmicutes*, including various SCFA producing bacteria, but there was no corresponding alteration to the *F:B* ratio. The association of the *F:B* ratio with mechanisms of CRC carcinogenesis

is unclear. It may be that the *F:B* ratio *per se* is not of significance in CRC carcinogenesis, but instead it is individual changes to bacteria from the *Firmicutes* phylum. A recent study comparing healthy individuals and CRC patients reported that contrary to current thinking, there was in fact a higher *F:B* ratio in CRC patients compared to healthy individuals (Bamolaa *et al.*, 2017). This study also reported that the *F:B* ratio was significantly higher in vegetarians compared to non-vegetarians, suggesting that there are multiple other dietary factors that may outweigh changes induced by O3FA supplementation (Bamolaa *et al.*, 2017).

There are a number of bacterial species associated with CRC carcinogenesis including *Escherichia coli*, *Bacteroides fragilis* and most notably *Fusobacterium nucleatum,* which were not identified at any point in the current study (Kostic *et al.*, 2012; Ahn *et al.*, 2013; Wu *et al.*, 2013; Liang *et al.,* 2017). Many studies identifying an association between *Fusobacterium nucleatum* and colorectal carcinogenesis were noted to identify pathogenic strains within colonic tumour tissue samples rather than faecal samples of individuals diagnosed with CRC when compared to controls (Castellarin *et al.*, 2012; Mima *et al.*, 2015)

Escherichia coli was not identified in the present study. A potential explanation is that some strains of the bacterium are pathogenic and therefore unlikely to be present in this cohort of 'healthy' study participants. Equally it may be a technical limitation of the microbiome analysis methodology that was unable to resolve these species in stool samples.

It is unclear whether changes to faecal microbiome profiles of healthy volunteers reflect changes to microbiome profiles within colonic mucosa or tumour tissue, and whether this governs any protective effect in relation to CRC risk. Recent

evidence indicates that faecal microbiome profiles differ to those found at mucosal level both in healthy individuals and CRC patients (Flemer *et al.*, 2017). Furthermore, the aforementioned study reported that mucosal microbiome profiles varied according to colonic tumour location, suggesting that the effects of alterations to the microbiome are complex and may vary according to the specific environment at different points within the colon.

An inherent limitation of all 'healthy' volunteer studies is the inability to describe a single profile that constitutes a healthy microbome. Approximately 60% of the microbiome is influenced by host factors and only 40% of humans share any form of consistent profile (Raskov *et al.*, 2017). The individuals examined in this study were by definition 'healthy' and therefore it was not possible to determine whether any alterations to the microbiota induced by O3FAs are able to ameliorate other factors including obesity and consumption of red meat, both of which may increase the risk of CRC development. It is also unclear whether the potential beneficial effects of O3FAs on faecal microbiome profiles are of a magnitude needed to overcome dietary factors that may alter the faecal microbiome profile to one that increases CRC risk.

There are also multiple uncontrolled factors in this study that may have influenced changes to faecal microbiome profiles. The O3FA drinks contained multiple ingredients which over an eight week supplementation period may have influenced changes to microbiome profiles or conversely masked any subtle changes induced by O3FA supplementation. The present study reported variations in bacterial changes following O3FA supplementation dependent on intervention type, for example opposing changes exhibited by the genera *Akkermansia*, *Roseburia* and *Rumminococcus*. This suggests that despite the

dose equivalence of O3FAs in both capsule and drinks interventions, there were additional unmeasured properties unique to each intervention that outweighed EPA and DHA induced changes to faecal microbiome profiles. Over the course of the study there were multiple unmeasured dietary factors that may have affected faecal microbiome profiles.

It was not possible to determine whether the absorption of O3FAs from the small intestine and therefore the amount of O3FAs transiting into the colon had any influence on changes to faecal microbiome profiles. It is unclear at present whether alterations to faecal microbiome profiles were influenced by direct luminal exposure to O3FAs or via systemic incorporation into colonic mucosal tissue. There is limited evidence from a study measuring O3FA levels in patients with an ileostomy fashioned from terminal ileum, that approximately 99% of orally supplemented O3FAs are absorbed within the small bowel (Sanguansri *et al.*, 2013). In the context of the present study, it is feasible that the subtle changes to faecal microbiome profiles potentially induced by O3FA supplementation, were in fact secondary to colonic mucosal incorporation following systemic EPA and DHA exposure. Alternatively it may be that only subtle changes to faecal microbiome profiles were evident because of limited direct exposure to O3FAs in the colonic lumen.

There are a number of technical factors that require consideration when drawing conclusions form the present study. There remains significant methodological variation across studies performing microbiome analysis, particularly with respect to the 16S rRNA gene hyper-variable region selected for primer design. There is data to suggest primer selection influences both the number of OTUs generated and various measures of microbial diversity (Rintala *et al.*, 2017). Due

to the nature of the present study there was inevitable variation in the duration of time between collection, processing and storage of stool samples which may have affected the extraction process and overall microbiome profile. Published recommendations from the International Human Microbiome Standards describe a similar protocol for stool sample collection to that used in the present study for a period of up to seven days prior to processing (Dore *et al.*, 2015). However a key variant that may affect the DNA extraction process was the inability to perform immediate freezing of volunteer samples post-collection. In the present study stool samples took up to 48 hours to arrive in the laboratory prior to processing for freezing. It is unclear whether the duration of time between initial storage of stool samples at -80ºC and subsequent DNA extraction had any detrimental effect on the results of faecal microbiome analysis. A previous study of stool samples from a limited number of individuals detailed variations in microbial diversity dependent on the length of time between sample collection, freezing and subsequent DNA extraction (Cardona *et al.*, 2012). The main determinant of variation across microbiome analysis of stool samples in the aforementioned study was the process of repeated freezing and thawing of samples which resulted in fragmentation of DNA and thus affected analysis at both species and genus levels (Cardona *et al.*, 2012).

6.17 Conclusion

In conclusion, O3FA supplementation appeared to alter the abundance of butyrate-producing bacteria within faecal microbiome profiles, which in the context of CRC may confer a protective effect. The results of the healthy volunteer study should be interpreted with caution given some of the technical limitations and the inability to control for all the potential confounding factors. It

is unclear whether changes to faecal microbiome profiles induced by O3FA supplementation have a direct influence on long-term CRC risk and whether there is any interaction with other known risk factors. At present there is a lack of clinical evidence in humans to ascertain a microbiome profile associated with CRC and any causal relationship with the various mechanisms of colorectal carcinogenesis. In order to determine whether O3FA supplementation has a potential application as a dietary chemoprevention, further research is required to elucidate both the effects of O3FAs on colonic microbiome profiles and the mechanisms via which both the microbiota and O3FAs influence CRC carcinogenesis.

Chapter 7: Analysis of RBC membrane PUFA levels from the EMT trial

7.1 Introduction

The molecular gastroenterology group at LIMM previously published the results of the EMT study (Cockbain *et al.*, 2014). EMT was a Phase II randomised, double-blind, placebo-controlled trial examining EPA treatment in patients awaiting surgical resection of CRCLM. As part of this study venous blood samples were collected to measure baseline, post treatment and washout RBC membrane PUFA levels including EPA, DHA and AA. This was not performed at the initial analysis of the study due to financial constraints. Therefore I measured RBC membrane PUFA levels from the EMT study in order to determine PUFA incorporation profiles in patients with CRCLM. Measurement of individual RBC membrane PUFA profiles allowed assessment of compliance, variability and correlation with tumour tissue PUFA content. There was also the opportunity to explore any association between RBC EPA membrane levels and clinical outcomes in patients following resection of CRCLM.

I tested the hypothesis that patients undergoing surgical resection of CRCLM exhibited elevated RBC membrane EPA levels following EPA treatment compared to patients randomised to placebo.

7.2 Analyses

Primary analysis

- Change in percentage RBC membrane EPA content compared with baseline following EPA treatment.

Secondary analyses

- Change in RBC EPA membrane levels after washout compared with post-treatment
- Change in RBC membrane levels of DHA, DPA and AA after EPA treatment compared with baseline.
- Change in RBC membrane levels of DHA, DPA and AA after washout
- Effect of sex, duration of treatment and compliance on EPA RBC membrane levels
- Correlation between EPA RBC membrane levels and CRCLM tumour tissue EPA levels
- Correlation between EPA RBC membrane levels and tumour vascularity as measured by CD31 immunohistochemistry
- Survival analysis based on EPA RBC membrane levels

7.3 Overview of study

The EMT study design is detailed by Cockbain *et al*. (Cockbain *et al.* 2014). In summary, 88 patients awaiting surgical resection of CRCLM were enrolled into the EMT study. Patients were randomised to either 2g EPA (FFA) orally per day (n=43) or placebo (n=45). The duration of pre-operative EPA supplementation was variable across all participants randomised into the study with a median duration of 26 days (15 to 73 days) and 30 days (12 to 65 days) in the intervention and placebo groups respectively. This is of particular relevance in the context of measuring RBC PUFA membrane levels. Venous blood samples were collected

at randomisation (pre-treatment n=87) and on the day prior to liver surgery (post-treatment n=80). There was a 'washout' period (n=71) of approximately 6 weeks after cessation of intervention following surgery (EPA, median number of days 47, range 16–110 days; placebo, median number of days 44, range 21–68 days). Baseline characterisitics of patients in placebo and intervention groups are detailed in table 4.

The main finding of the EMT study was a statistically significantly higher percentage EPA content in CRCLM tissue in the EPA treatment group compared to patients receiving the placebo (1.82% versus 1.30%, p<0.001). Although not statistically significant, there was reduced tumour vascularity as measured by CD31 immunohistochemistry, in patients receiving EPA supplementation compared with placebo. Kaplan-Meier survival analysis also indicated a possible overall survival benefit in patients receiving EPA supplementation, although the study was not powered to answer this question formally.

	Placebo (n=45)	**EPA (n=43)**
Age (years)	71 (35-87)	68 (44-82)
Sex (male:female, % male)	35:10 (78%)	26:17 (61%)
Previous fish oil supplement use (%)	9 (20%)	5 (12%)
Primary CRC Dukes stage (%)	A 2 (4%) B 13 (29%) C 10 (22%) D 20 (44%)	A 2 (5%) B 11 (26%) C 17 (40%) D 13 (30%)
Lymph node positive (%)	25 (56%)	26 (61%)
Interval between primary CRC surgery and presentation with CRCLM (months)*	19 (3-80) (n=30)	24 (6-91) (n=32)

Table 4. Baseline characteristics of patients enrolled into the EMT study.*15 patients in the placebo group and 11 patients in the EPA group underwent first resection of synchronous CRCLM

7.4 PUFA analysis

Although this was a purely EPA intervention study, it was necessary to analyse the effect of EPA treatment on DPA, DHA and AA RBC membrane levels. As previously highlighted in Figure 2 DPA and DHA are downstream products of EPA metabolism. EPA, DHA and AA are proposed to affect both inflammatory pathways and mechanisms of colorectal carcinogenesis (See section 1.5).

7.4.1 EPA (Figure 26a)

Baseline mean EPA RBC membrane levels were similar in both EPA intervention and placebo groups (EPA, 1.06%; Placebo, 1.11%). Following EPA treatment there was an increase from baseline in measured mean RBC EPA membrane levels, which was not apparent in the placebo group (absolute change from baseline: EPA, 1.26% points, one sample t-test $p < 0.0001$; placebo, -0.03% points, one sample t-test $p=0.57$). The increase in post-treatment RBC EPA membrane levels in the intervention group amounted to a 2.40 fold increase from baseline (95% CI, 2.07 to 2.73), whereas EPA RBC membrane levels were unchanged in patients randomised to placebo (fold change from baseline, 1.02; 95% CI, 0.94 to 1.10) Following the washout period there was a decrease in RBC EPA membrane levels, although there appeared to be incomplete washout from RBCs, with a mean change of absolute EPA RBC membrane levels from baseline of 0.20% points (95% CI, 0.04% points to 0.35% points; one sample t-test, $p=0.02$). As shown in figure 27 the magnitude of EPA RBC membrane incorporation post-treatment in the intervention group varied between individual patients. However 92% (33/36) of patients that provided the full complement of venous blood samples exhibited an EPA RBC membrane rise post-treatment followed by a reduction after washout.

7.4.2 DPA (figure 26b)

Mean DPA RBC membrane levels were similar across both EPA intervention and placebo interventions at baseline (EPA, 2.43%; capsules, 2.33%). Post-treatment DPA RBC membrane levels as measured by mean absolute change were higher in the EPA intervention group than in patients that received placebo (EPA, 0.60% points, one sample t-test p=0.002; placebo, 0.07% points, one sample t-test p=0.39). After the washout period mean DPA RBC membrane levels in patients receiving EPA treatment were 0.26% points higher than EPA RBC membrane levels measured at baseline (one sample t-test, p=0.03), which was not apparent in the patients receiving placebo (mean absolute change, 0.00% points)

7.4.3 DHA (figure 26c)

Mean baseline DHA RBC membrane levels were 4.65% and 4.24% in EPA intervention and placebo groups respectively. Post-treatment there was a 0.31% points decrease from baseline in DHA RBC membrane levels which was not statistically significant (one sample t-test, p=0.20). The lower than baseline DHA membrane levels persisted following the washout period (mean absolute change; -0.40% points; 95% CI, -0.78% points to -0.02% points; one sample t-test, p=0.04) and was not apparent in the placebo group.

7.4.4 AA (figure 26d)

The AA RBC membrane levels in EPA intervention and placebo groups were similar at baseline (12.56% and 11.41% respectively). Following EPA treatment there was a statistically significant decrease in absolute AA RBC membrane levels of -1.31% points (95% CI, -2.46% points to -0.16% points; one sample t-

test, p = 0.03) which was not seen in the placebo group. This decrease appeared to persist following the washout period but was not statistically significant (mean absolute change, -0.90% points; one sample t-test, p=0.07).

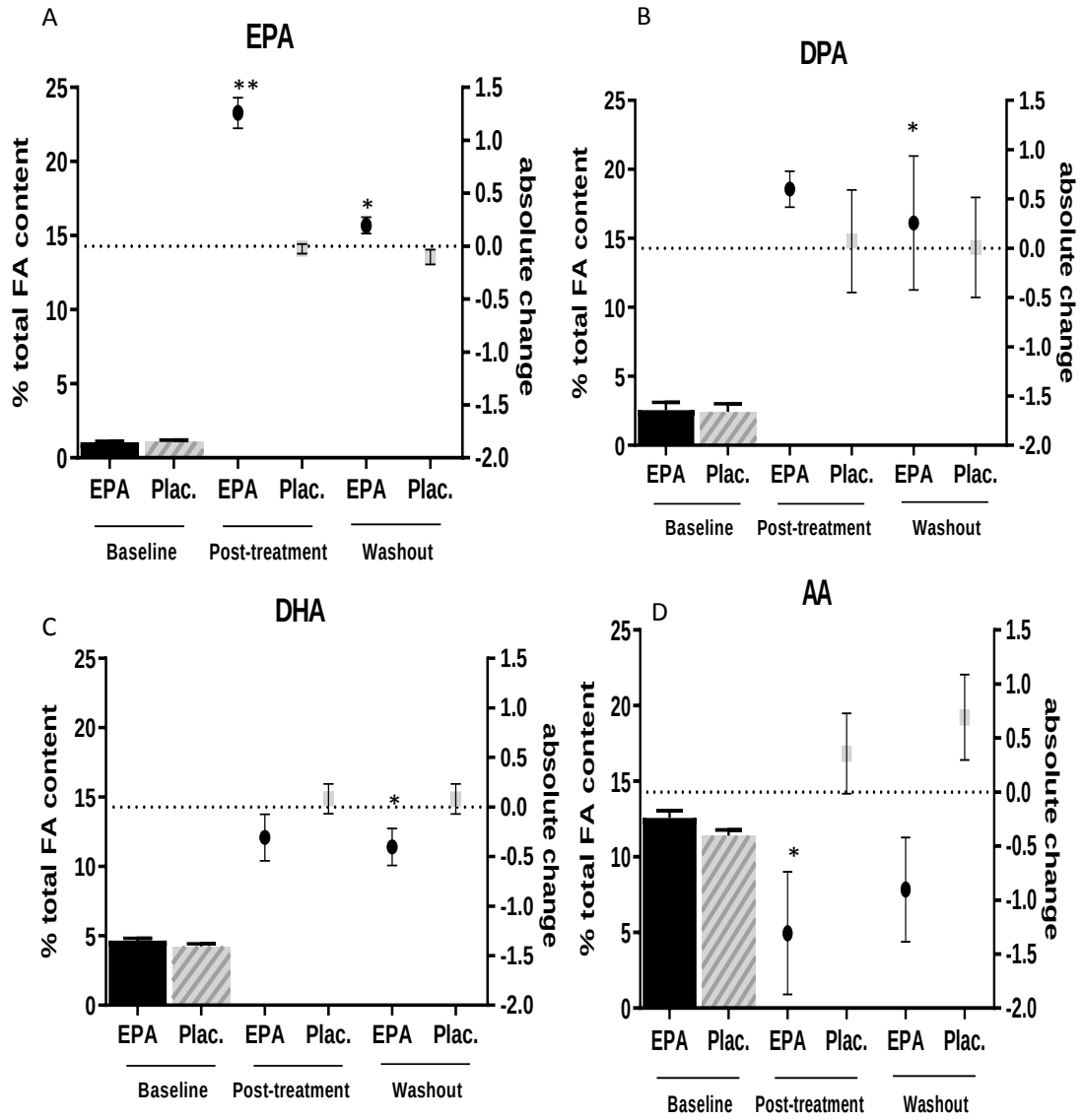


Figure 26. Baseline % RBC PUFA level and absolute difference in % RBC PUFA levels between baseline and post-treatment or after surgery (washout). The left y-axis is the baseline % RBC PUFA value and the right y-axis is the absolute difference between the post-treatment value or 'washout' post-operative value and baseline % level. Columns (baseline % values) and symbols (absolute difference in % value from baseline) denote the mean for EPA and placebo (Plac.) groups. Error bars denote the SE of the mean. * $p < 0.05$, ** $p < 0.001$; one sample t-test.

7.4.5 Individual PUFA analysis

Figure 27 illustrates individual PUFA profiles of trial participants in both EPA intervention and placebo arms of the study. There was marked inter-individual variation across study participants particularly at baseline in both the intervention and placebo arms. Twenty three participants were assessed prior to entering the study as either having regular intake of O3FA rich fish or being regular users of O3FA containing nutritional supplements. These individuals demonstrated statistically significant higher EPA RBC membrane levels at baseline (1.39 ± 0.15%) compared to naïve O3FA participants (0.97 ± 0.04%; n=64; p=0.003) irrespective of intervention group. Individuals identified as being regular users of O3FA supplements were required to cease supplementation prior to entering the study.

Closer examination of the profiles of individuals within the placebo group identified four participants with notably higher EPA RBC membrane levels (greater than two percent) at baseline. These individuals had been identified as prior users of O3FA supplements with three of these individuals demonstrating a decrease in RBC EPA membrane levels following commencement of the study and therefore presumed cessation of additional O3FA supplements (Figure 27: EPA placebo).

As previously stated 92% (33/36) of patients randomised to the EPA treatment group that provided a full complement of venous blood samples exhibited an EPA RBC membrane rise post-treatment followed by a reduction after washout. A single individual demonstrated notably higher EPA, DPA, DHA and AA RBC membrane levels following EPA treatment.

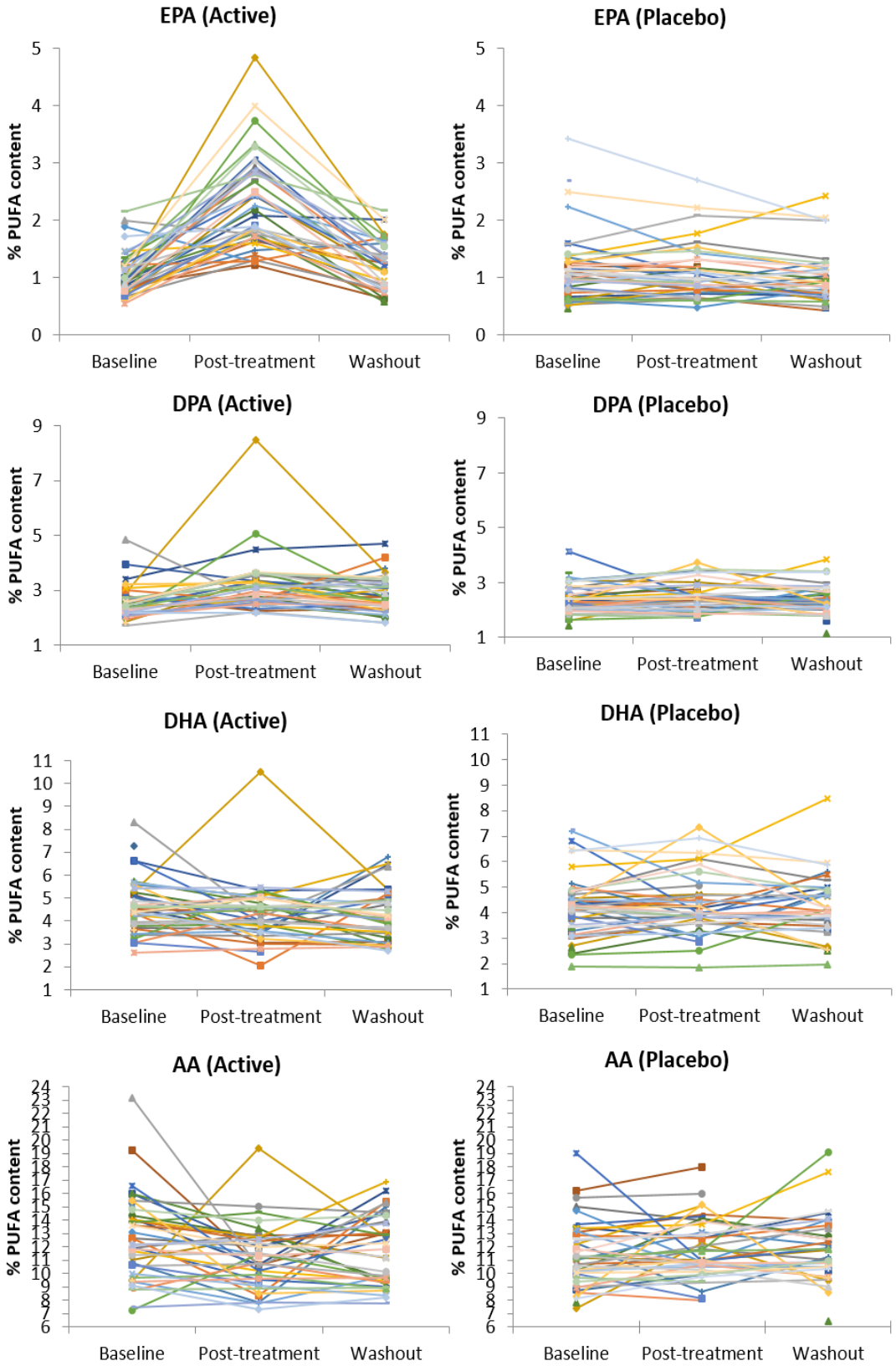


Figure. 27. Individual % PUFA profiles in active and placebo groups. Each coloured line and symbol denotes an individual participant %RBC membrane level at baseline, post-treatment and washout.

7.4.6 EPA:AA (figure 28)

As discussed in chapter 1, the ratio of O3FAs to AA is reported in studies exploring the relationship and mechanisms by which O3FAs are thought to derive anti-inflammatory benefits. In the context of an EPA treatment study the EPA:AA ratio was calculated. Baseline EPA:AA RBC membrane levels were 0.09 and 0.10 in the EPA intervention and placebo groups respectively. In line with the changes exhibited in individual EPA and AA RBC membrane levels, there was a statistically significant increase of 0.12 to the EPA:AA ratio in the EPA intervention group post-treatment (95% CI, 0.10 to 0.14; one sample t-test, $p<0.0001$) which was not apparent in the placebo group. Following the washout period there was a fall in the EPA:AA ratio in the EPA intervention group, although it remained elevated compared to baseline (95% CI, 0.01 to 0.03; one sample t-test, $p=0.0006$).

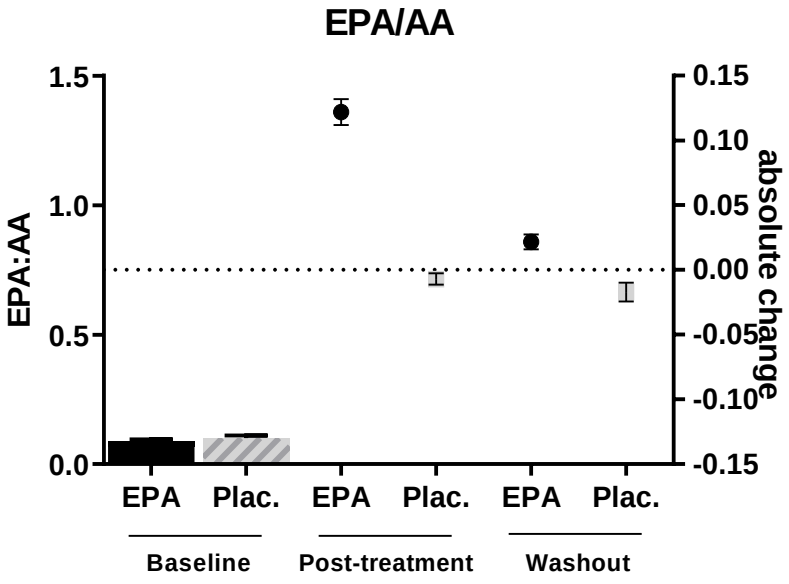


Figure. 28. Baseline RBC membrane EPA:AA ratio and absolute difference in RBC membrane EPA:AA ratio between baseline and post-treatment or after surgery (washout). The left y-axis is the baseline EPA:AA ratio and the right y-axis is the absolute difference between the post-treatment value or 'washout' post-operative value and baseline EPA:AA ratio. Columns and symbols denote the mean for EPA and placebo (Plac.) groups. Error bars denote the SE of the mean.

7.5 Univariate analyses

A number of variables were examined to explore any relationship with RBC EPA membrane levels. Analysis was performed using the Pearson correlation coefficient for continuous data. The unpaired t-test was employed when analysing categorical variables and the Mann-Whitney test for non-normally distributed data.

7.5.1 Duration (figure 29)

There was no relationship between the duration of EPA supplementation and post-treatment EPA RBC membrane levels (n=38, range; 12 to 65 days, r =0.25, 95% CI; -0.07 to 0.53, p=0.12). Analysis was also undertaken based on the absolute change in EPA RBC membrane levels between baseline and post-treatment. Again, there was no statistically significant correlation (r=0.29, 95% CI; -0.03 to 0.56, p=0.07).

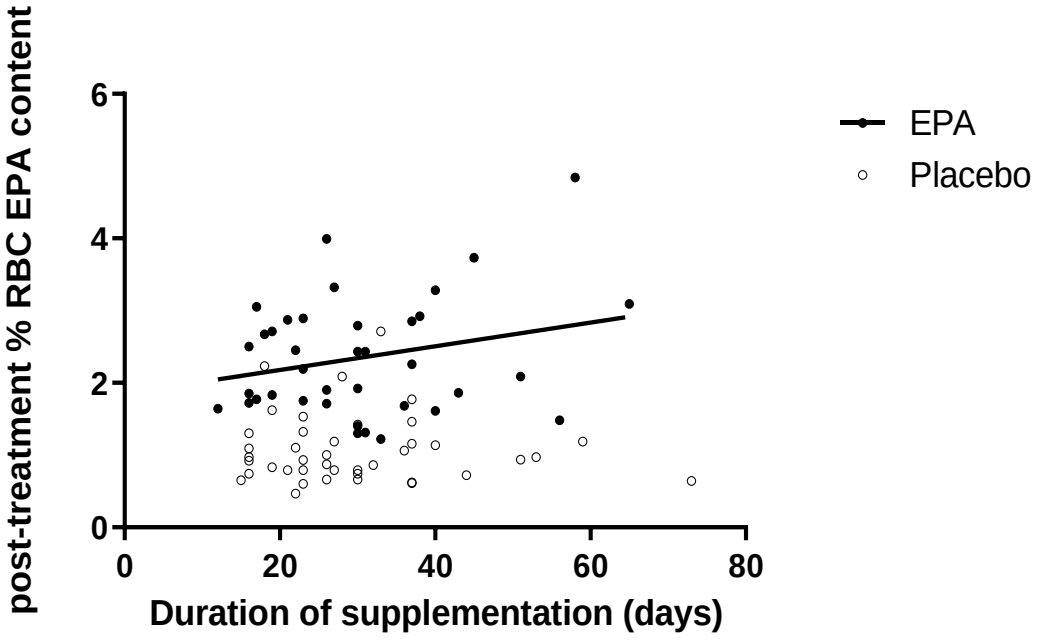


Figure 29. Relationship between the % RBC EPA level at the end of the trial intervention and treatment duration. Open symbols denote individual data from the placebo group and filled symbols denote EPA group data. Line of best fit plotted for EPA treatment group.

7.5.2 Compliance (figure 30)

Compliance was calculated based on a capsule count undertaken following the intervention period. It was calculated as the following, (expected–returned)/expected and expressed as a percentage. A number of patients reported compliance of over 100%, whereby a participant had returned fewer capsules than expected. Post-supplementation capsule count data was available for 34/43 patients (79%) in the EPA intervention group. Median compliance in the active intervention group was 95%, ranging from 41% to 117%. There was no correlation between percentage compliance in patients receiving EPA treatment and post-treatment RBC membrane EPA levels (Spearman's correlation; r=0.15, 95% CI; -0.21 to 0.47, p=0.40). Examination of absolute change between baseline and post-treatment RBC EPA membrane levels also revealed no correlation with compliance (r=0.22, 95% CI; -0.13 to 0.52, p=0.21).

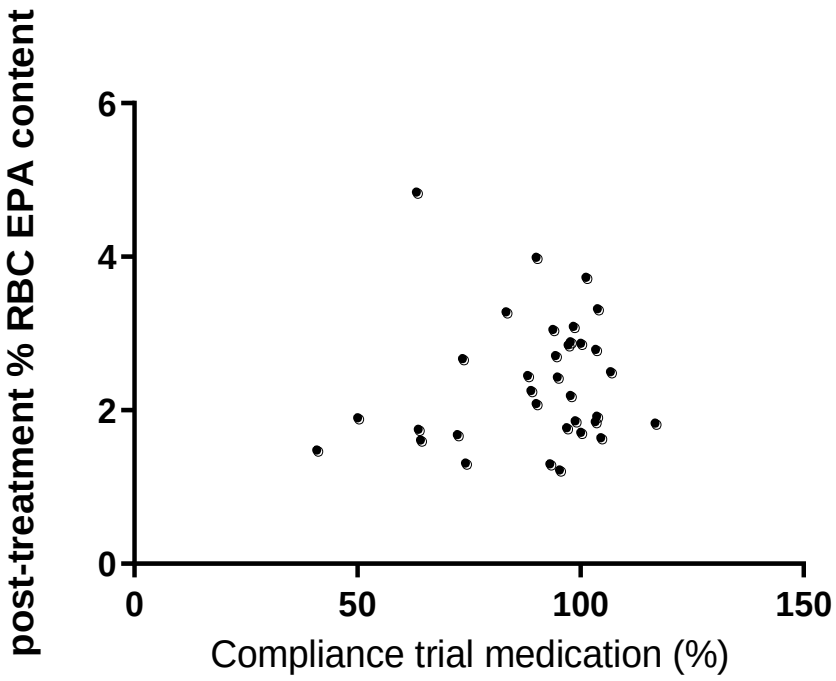


Figure 30. Relationship between the % RBC EPA level at the end of the trial intervention and %compliance in the active EPA intervention group.

7.5.3 Sex

The effect of sex on EPA RBC membrane levels in the EPA intervention group was examined. Median RBC membrane EPA levels were similar in both sexes at baseline, with measured levels of 0.99% and 0.95% in female and male participants respectively. Post-treatment RBC EPA membrane levels were measured in 23 of 25 males and 16 of 17 females randomised to the EPA intervention group. Median post-treatment EPA membrane levels were higher in female patients compared to their male counterparts, at 2.69% (range 1.31% to 4.84%) and 1.83% (range 1.22% to 3.32%) respectively. Analysis revealed the difference between male and female EPA RBC membrane levels post-treatment was statistically significant (p=0.02).

7.6 Multivariate analysis

In order to verify that sex was a statistically significant predictor of EPA RBC membrane incorporation, linear regression analysis was undertaken taking into account gender, compliance and duration of treatment. This confirmed that gender was the only statistically significant predictor of RBC EPA membrane levels in the EPA intervention group (beta −0.35, p=0.039).

7.7 Relationship between EPA measurements in RBC membranes and CRCLM tumour tissue EPA and AA levels

The relationship between EPA RBC membrane levels and corresponding CRLCM tissue levels from resected liver metastases was explored (figure 31). RBC EPA membrane levels are a surrogate marker of EPA levels in a variety of tissues, but there is a lack of evidence pertaining to EPA incorporation into tumour tissue (Metcalf *et al.*, 2007; Harris *et al.*, 2004). In the context of CRC

treatment it is important to determine whether EPA RBC membrane levels are related to tumour tissue levels as the proposed anti-CRC mechanisms of O3FAs are likely to take place in tumour tissue (see section 1.5). The RBC samples were collected on the day prior to surgery. There were 37 patients in each of the EPA intervention and placebo groups with measurements of EPA levels for both RBC membrane and corresponding CRCLM tumour tissue. Irrespective of intervention group there was a correlation between post-treatment RBC membrane EPA levels and CRCLM tumour levels. The correlation appeared to be stronger in the placebo group of patients ($r=0.72$, $p<0.0001$) compared to the EPA intervention group ($r=0.36$, $p=0.03$). Analysis of the correlation between RBC membrane EPA content and tumour tissue EPA content was also undertaken irrespective of intervention group where there was a statistically significant correlation (figure 32; $r=0.65$, $p<0.0001$). There was no correlation between CRLCM tumour levels of either DPA or DHA and EPA RBC membrane levels in the EPA intervention group. There was a weak inverse relationship between post-treatment RBC membrane EPA levels and CRCLM tumour tissue AA levels in both placebo ($r=-0.18$, $p=0.29$) and EPA ($r=-0.27$, $p=0.10$) intervention groups, although neither reached statistical significance. Merging of intervention intervention groups revealed a statistically significant inverse correlation between post-treatment RBC EPA membrane content and CRLCM tumour tissue AA content (Figure 33; $r=-0.25$, $p=0.03$)

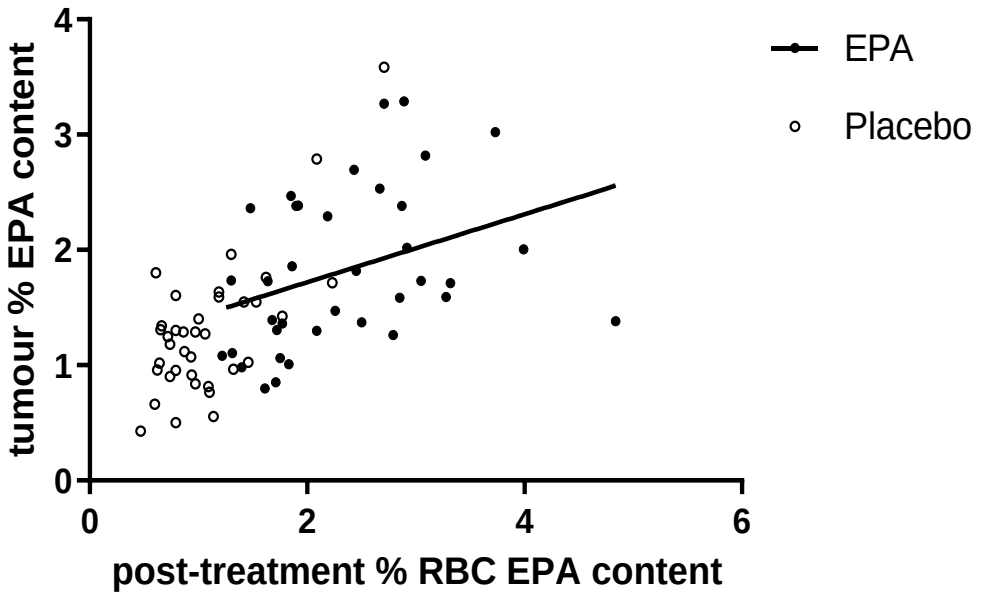


Figure 31. Relationship between the % RBC EPA level and % EPA CRCLM tumour tissue content at the end of the trial intervention. Open symbols denote individual data from the placebo group and filled symbols denote EPA group data. Line of best fit plotted for EPA treatment group.

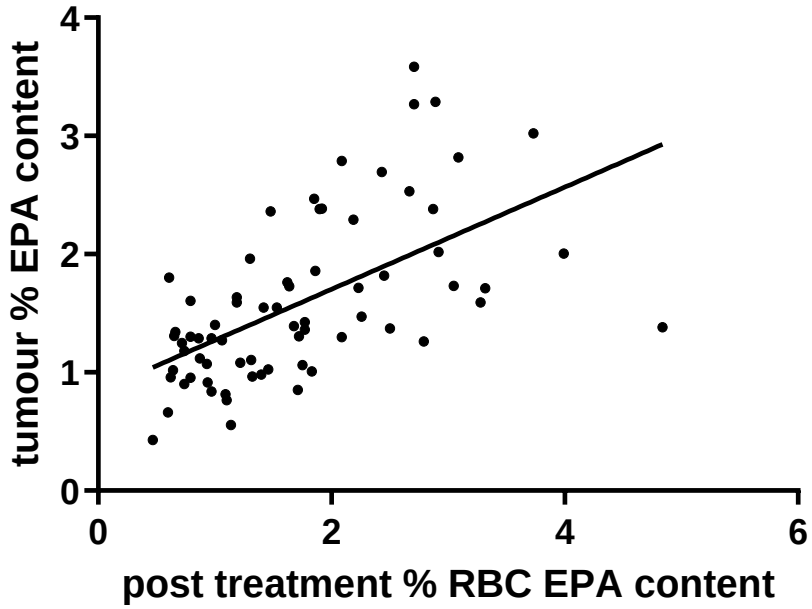


Figure 32. Relationship between the % RBC EPA level and % EPA CRCLM tumour tissue content irrespective of intervention.

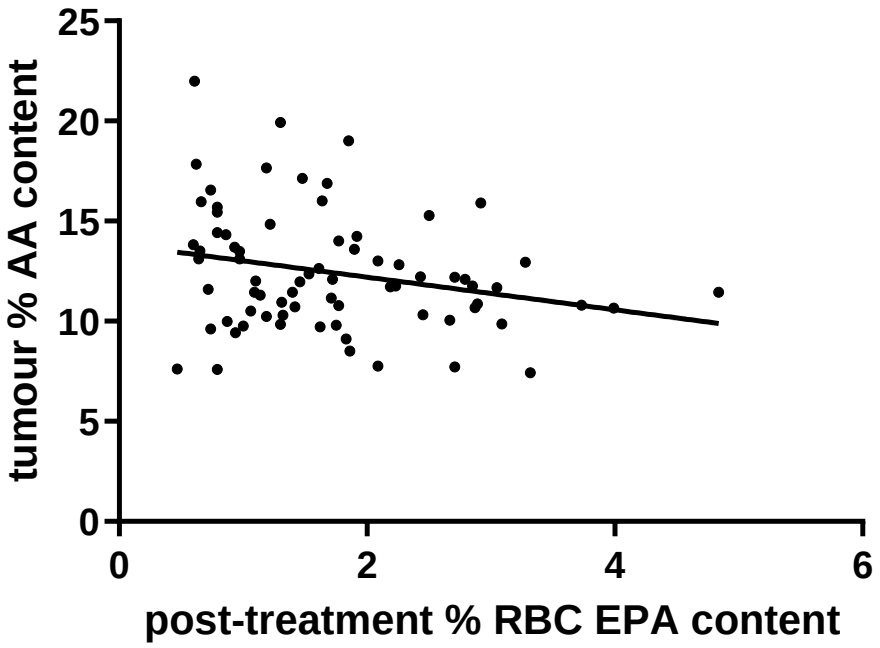


Figure 33. Relationship between the % RBC EPA level and % AA CRCLM tumour tissue content irrespective of intervention.

7.8 Relationship between EPA RBC membrane levels and CRCLM tumour tissue vascularity

A finding of the EMT study was a reduction in tumour vascularity as measured by CD31-positive microvessel density in patients treated with EPA. Therefore analysis of the relationship between RBC EPA membrane levels and CRCLM tumour CD31-positive microvessel density was performed (figure 34). There were 33 and 36 patients in the EPA intervention group and placebo group respectively with both RBC membrane EPA levels and CD31-positive microvessel density measurements. There was no correlation in the EPA intervention group. However in the placebo group it appeared that a higher RBC EPA membrane level was associated with a reduction in tumour vascularity as measured by CD31-positive microvessel density measurements (r=-0.37, p=0.03). The association was again demonstrated when analysis of tumour

vascularity was performed irrespective of intervention group (r=-0.14), although this was not statistically significant (p=0.26).

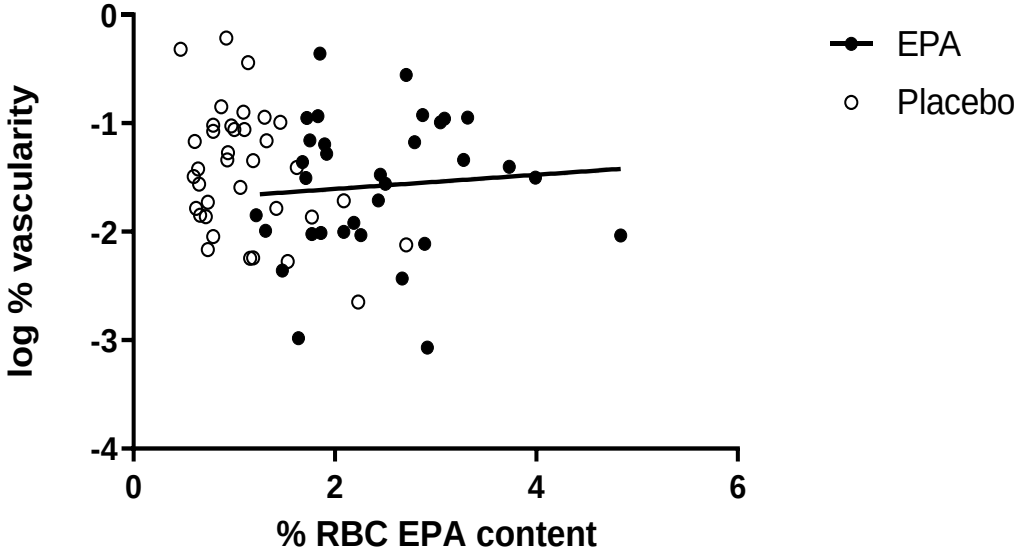


Figure 34. Relationship between log % vascularity in CRCLM tumour tissue and % RBC EPA membrane levels. Open symbols denote individual data from the placebo group and filled symbols denote EPA group data. Line of best fit plotted for EPA treatment group.

7.9 RBC EPA membrane levels and overall survival

On the basis that there was a correlation between RBC membrane and CRCLM tissue EPA levels in both EPA intervention and placebo groups, Kaplan-Meier survival analysis was performed to assess whether there was any association with overall survival outcomes (figure 35). An intervention independent survival analysis was performed. RBC EPA membrane levels were dichotomised based on the lowest post-treatment RBC EPA membrane level measured in the EPA intervention group following treatment (1.22%). Fourty nine patients exhibited measured RBC EPA membrane levels of 1.22% or greater post-treatment, versus 29 patients with measured RBC membrane EPA levels of less than 1.22%. Ten patients randomised to the placebo treatment group exhibited post-treatment RBC EPA membrane levels of 1.22% or greater and therefore for the

purpose of survival analysis were pooled with patients that had been randomised to the EPA treatment group. The survival analysis illustrated that a RBC EPA membrane level of greater than or equal to 1.22% was associated with overall survival benefit. The associated hazard ratio was 0.4 (Mantel-Haenszel) with a 95% confidence interval of 0.16 to 0.96, which was statistically significant (log rank, p =0.04).

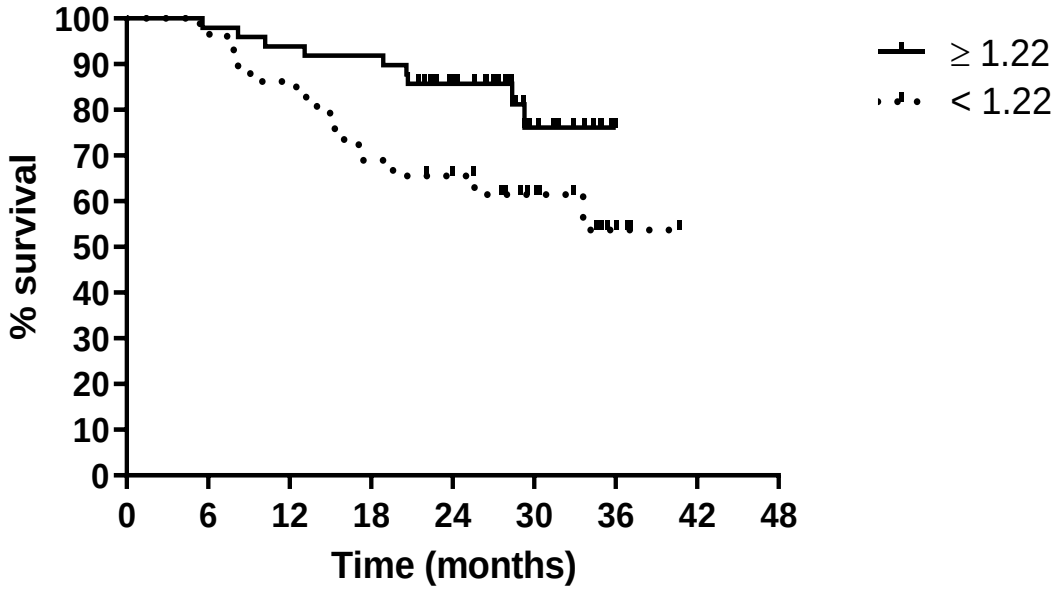


Figure 35. Overall survival analysis of CRCLM patients stratified on the basis of post-treatment % RBC EPA level, irrespective of treatment allocation with a cut off % RBC EPA membrane level of 1.22% corresponding with the lowest post-treatment % EPA RBC membrane value in the intervention group. Solid line denotes events in individuals with post-treatment RBC % EPA level ≥1.22%. Dashed line denotes events in individuals with post-treatment RBC % EPA level < 1.22%. Log rank P=0.04.

7.10 Discussion

The present study clearly demonstrates incorporation of EPA into the RBC membrane of CRC patients with CRCLM following treatment with EPA prior to surgical resection of CRCLM. The RBC PUFA membrane profiles in CRC patients are similar to other studies in healthy volunteers and women with breast hyperplasia (Cao *et al.*, 2006; Ohnishi & Saito, 2013; Fabian *et al.*, 2015). In the

context of CRC, Coviello *et al.* reported baseline EPA RBC levels of 0.46% compared to approximately 1% in the present study (Coviello *et al.*, 2014). Mikirova *et al.* have previously reported that RBC EPA and DHA membrane levels are lower in CRC patients, which may in part be due to differential activity of the delta-6 desaturase enzyme (Mikirova *et al.*, 2004). However comparison of baseline O3FA RBC membrane levels in the present study to healthy volunteers does not support this finding (Cao *et al*., 2006).

The consensus across studies examining the bioavailability of O3FAs is that maximal RBC membrane levels are reached within four to eight weeks of starting supplementation (Katan *et al.*, 1997; Cao *et al.*, 2006; Stonehouse *et al.*, 2011). It is unclear whether maximal RBC levels were achieved within the median 30 day intervention period of this study. There are few studies for direct comparison of RBC PUFA levels following EPA treatment in CRC patients. A double-blind RCT in lung cancer patients treated with a preparation containing a mix of EPA and DHA, but providing an equivalent daily dose of EPA to the present study showed similar levels of EPA RBC membrane incorporation at around 2% following a 22 day treatment period (Finocchiari *et al.*, 2012). Scaioli *et al*. examined an identical EPA preparation to that used in the present study in both healthy volunteers and patients with inflammatory bowel disease (Scaioli *et al.*, 2015). They report a greater increase in O3FA RBC membrane levels following an eight week treatment period with an absolute post-treatment increase from baseline of approximately of 4.3% compared to 1.3% in the present study. In part this may be explained by the longer treatment period of eight weeks compared to a median treatment period of 26 days in this study. In the aforementioned study, EPA RBC membrane levels continued to rise at measurement intervals of four and eight weeks, suggesting that the 30 day treatment period may have

been insufficient to reach maximal RBC membrane EPA incorporation. This possible explanation is also supported by a study in lung cancer patients in which both EPA and DHA RBC membrane levels continued to rise between RBC measurements intervals of 22 and 66 days (Finocchiari *et al.*, 2012). Both inflammatory bowel disease patients and healthy volunteers from the study by Scaioli *et al.* exhibited significantly lower baseline RBC EPA, DPA and DHA levels compared to the present study, which may also explain the apparent differential in EPA RBC membrane incorporation.

There was no correlation between either the compliance or duration of treatment and post-treatment EPA RBC membrane levels. This may in part be due to a relatively small sample size in the EPA treatment group which was not powered to detect changes on the basis of RBC PUFA analyses. There is also unreliability associated with a capsule count reliant on study participants returning unused trial medication. This may mask any underlying relationship between compliance and RBC membrane PUFA levels (Lam & Fresco, 2015).

The finding that there was an increase in RBC membrane DPA levels following EPA treatment is expected secondary to enzymatic elongation of EPA by the ELOVL2 elongase enzyme (Gregory *et al.*, 2011). Increases in DPA have been reported elsewhere in studies of healthy individuals following O3FA supplementation (Scaioli *et al.*, 2015; Gregory *et al.*, 2011). The present study suggests that elongase conversion of EPA to DPA also occurs in patients with CRCLM. However it is unclear whether there is differential elongase activity in tumour tissue or whether the magnitude of elongase activity in cancer patients differs to that of healthy individuals. There may also be specific PUFA metabolism profiles in patients with metastatic CRC, as highlighted in a recent

study where metastatic colorectal tissue exhibited a different PUFA profile to primary tumour tissue in patients with localised disease, although RBC PUFA membrane profiles were similar (Notarnicola *et al.*, 2018).

Despite an increase in both EPA and DPA RBC membrane levels, there was no evidence of increase in DHA RBC membrane levels. Conversely there was a statistically significant decrease in DHA RBC membrane levels that persisted following cessation of EPA treatment. One might expect a stepwise conversion of EPA to DPA and DHA by a series of enzymatic reactions involving elongase and desaturase enzymes (figure 2). There are a number of possible explanations for this finding including displacement of DHA from RBC membranes by the presence of excess EPA. It is also possible that various stages of the enzymatic conversion of EPA to DHA were saturated by the presence of high levels of EPA thus limiting conversion to DHA. Gregory *et al.* reported saturation of the elongase (Elovl2) step in the conversion of DPA to 24:5n-3 DPA as a potential rate-limiting step in the presence of excess EPA, as demonstrated in rodent models (Gregory *et al.*, 2011).

The relationship between O3FAs and O6FAs is of significant interest with respect to the role of PUFAs in inflammatory pathways and CRC carcinogenesis. O3FAs including EPA compete with AA for COX binding, thereby effecting the dynamics of eicosanoid production (Smith, 2005). The present study demonstrates a statistically significant decrease in AA RBC membrane levels after EPA treatment which persisted following the washout period. Baseline RBC membrane levels of AA prior to EPA treatment were similar to those reported elsewhere (Scaioli *et al.*, 2015). The ratio of O3FA to O6FA is a measure of the dynamic relationship between O3FAs and O6FAs. As one might expect, when

patients receiving EPA treatment demonstrated an increase in RBC EPA membrane levels and reciprocal decrease in AA levels there was also a statistically significant increase in the EPA:AA ratio that persisted following the washout period. This has been widely reported in the scientific literature and suggests that there is displacement of AA from the RBC membrane by EPA (Cao *et al.*, 2006; Gibney *et al.*, 1993).Whether this is of any clinical benefit in CRCLM patients is unclear, although animal studies have suggested that there may be an anti-cancer effect primarily via interaction with COX enzymes and reduction in generation of the pro-inflammatory PGE_2 (Sarotra *et al.*, 2012). The presence of excess EPA may also induce a reduction in AA by acting as a competitive substrate for desaturase and elongase enzymes which are required for conversion of LA to AA (Rose & Connolly, 1999).

The individual PUFA profiles generated by this study reveal widespread inter-individual variation in EPA RBC membrane levels following EPA treatment. This has also been reported by Kohler *et al.* when examining the O3FA index following EPA and DHA supplementation (Kohler *et al.*, 2010). The inter-individual variation exhibited was not dependent on compliance, although there are clear limitations with the validity of a 'capsule count' performed at the end of a study period. The lack of correlation between duration of EPA supplementation and EPA RBC membrane levels also fails to explain why there was significant variation across participants in the intervention group.

There is variation in the metabolism and bioconversion of PUFAs across humans with the conversion of ALA to EPA inefficient due to limited delta-6 desaturase activity (Horrobin, 1993). There is inter-individual differential expression of the fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) genes that

encode for the delta-5 and delta-6 desaturase enzymes required for enzymatic conversion of ALA to EPA, with a significant proportion of individuals unable to perform enzymatic conversion (Rzehak *et al.*, 2009; Xie & Innis, 2008; Plourde & Cunnane, 2007). A potential explanation for the inter-individual variation exhibited in the present study is the variation in conversion of dietary ALA to EPA and DHA due to differential enzyme activity (Rzehak et al., 2009; Xie & Innis, 2008). However it is unlikely that the relatively small contribution of EPA to RBC membrane EPA levels from dietary ALA would make a significant difference to overall membrane levels generated by EPA incorporation from capsule treatment, unless an individual was a highly efficient converter of ALA. It was not possible to determine whether dietary intake of ALA during the intervention period influenced O3FA RBC membrane levels as it was not measured. A proportion of PUFAs undergo mitochondrial and peroxisomal degradation via β-oxidation to generate energy (Kanamori *et al.*, 2018, Camões *et al.*, 2009). It is feasible that variation exists across individuals as to both the rate and quantity to which β-oxidation take place, which may subsequently impact on RBC membrane EPA levels.

The individual PUFA profiles provide an insight into possible 'contamination' by the use of additional PUFA supplements outside the study protocol. In the placebo group, individuals exhibiting the highest baseline EPA levels were identified as either prior O3FA supplement users or having had a diet containing high amounts of oily fish. These individuals showed decreased RBC membrane levels at subsequent visits in the study, suggesting that they had complied with the study protocol and ceased any prior O3FA supplementation. With respect to both the treatment and placebo groups there was evidence that individuals had taken O3FA supplementation outside the prescribed study protocol. This was

evident in the placebo group where following the intervention period a number of individuals exhibited elevated RBC EPA and DHA membrane levels and on occasion elevated AA levels. A single individual in the EPA treatment group exhibited significant increases in EPA, DPA, DHA and AA RBC membrane levels over the treatment period of the study. This rise in both O3FAs and O6FAs suggests the use of a supplement that was not disclosed to researchers over the course of the treatment period. An alternative explanation is that this individual was able to convert EPA to DHA highly effectively.

This is the first published data from a RCT that illustrates a correlation between EPA RBC membrane levels and CRCLM tissue, providing evidence that RBC EPA levels may be used as a surrogate marker of tumour tissue incorporation. It is already widely accepted that RBC membrane O3FA levels are an accurate reflection of multiple target tissues (Metcalf *et al.*, 2007; Harris *et al.*, 2004; Tu *et al.*, 2013; Brenna *et al.*, 2018). However from the present study it is not possible to ascertain whether colorectal tumour tissue demonstrates differential incorporation of O3FAs compared to healthy colonic tissue which may be relevant in the context of the proposed anti-inflammatory and anti-neoplastic mechanisms of O3FAs.

Based on the assumption that there appeared to be O3FA contamination within both the placebo and treatment arms of the study survival analysis irrespective of treatment allocation was undertaken. An absolute RBC EPA membrane level of 1.22% was selected on the basis of the minimum post-treatment rise in the EPA treatment group, thus including individuals from the placebo group that exhibited higher or equivalent RBC membrane levels to those in the EPA treatment group. Individuals from the placebo group that were effectively moved

into the EPA treatment group for the purpose of survival analysis, invariably corresponded with individuals previously identified as consumers of high amounts of oily fish or prior users of O3FA supplements. Although seemingly arbitrary, the dichotomisation of participants within the study at a cut off of 1.22% was similar to a study that explored the risk of developing colorectal adenomas where EPA RBC membrane levels above 0.89% were associated with a decreased risk of adenoma development (Cottet *et al.*, 2013). The present study provides the first evidence that RBC EPA membrane levels are associated with improved overall survival in patients with CRCLM. Whether this finding is indicative of a direct mechanistic effect on colorectal carcinogenesis cannot be determined. There was no indication of any relationship between O3FA RBC membrane levels and CRCLM tumour tissue vascularity, with anti-angiogenesis within tumour tissue one of the mechanisms by which O3FAs are thought to exert anti-cancer effects (Calviello *et al.*, 2004). It may be that higher EPA levels have more global beneficial health effects given that there is recent evidence to suggest that higher RBC membrane O3FA levels are associated with reduced risk for all-cause mortality (Harris *et al.*, 2017).

Following multi-variate analysis it was apparent that there were statistically significantly higher post-treatment EPA RBC membrane levels in females compared to males. Much of the published literature on sex and PUFA levels is focused on the measurement of plasma PUFA levels as highlighted in two comprehensive reviews (Childs *et al.*, 2008, Lohner *et al.*, 2013). A systematic review of twelve studies examining the effect of sex on PUFA levels identified higher levels of DHA in the RBC membrane of females compared to males, but no difference in relation to EPA (Lohner *et al.*, 2013). The higher post-treatment EPA RBC membrane in females compared to males suggests that hydrolysis of

high doses of triglyceride-bound EPA by pancreatic lipases and subsequent gastrointestinal absorption of EPA differs between sexes. Alternatively EPA may be metabolised at a more rapid rate in males thus reducing the amount present in the RBC membrane. The difference between sexes in DHA RBC membrane levels has been proposed to be secondary to differential β-oxidation and variations across the activity of desatuarase and elongase enzymes as influenced by circulating hormones (Childs *et al.*, 2008). A study of transsexuals identified that circulating plasma levels of DHA were higher in males that had transitioned to female within four months of taking oral ethyinyl oestradiol and cyproterone acetate hormones, whereas females who had transitioned to males exhibited lower circulating DHA levels when taking testosterone after four months (Giltay *et al.*, 2004).

Given the concerns of placebo or treatment arm contamination in any human trials by readily available 'over the counter' PUFA supplements the present findings suggest it would be appropriate to perform additional analyses irrespective of treatment allocation when exploring the effects of RBC PUFA membrane levels, an approach already undertaken in a study on the effects of O3FAs on non-alcoholic fatty liver disease (NAFLD) (Scorletti *et al.*, 2014). In addition the individual PUFA profiles reported in this study suggest that RBC membrane PUFA levels may be a potential method of monitoring compliance to O3FA treatments in clinical studies and also allow identification of contamination by O3FA supplement consumed outside a study protocol. A limitation to this method of monitoring compliance is that as exhibited in the present study there is substantial variability in the RBC EPA membrane incorporation following EPA treatment which may make it particularly difficult to differentiate whether an

individual is not compliant or whether the magnitude at which they incorporate EPA into the RBC membrane is low.

7.11 Conclusion

In summary there was incorporation of EPA into the RBC membrane following pre-operative EPA treatment in CRCLM patients, corresponding with a reciprocal decrease in RBC membrane AA levels. There was also evidence of a correlation between RBC EPA membrane levels and CRCLM tumour tissue EPA levels. This study also suggests that RBC EPA membrane levels of 1.22% and above are associated with a potential survival benefit. Therefore O3FA supplementation as an adjunct in the treatment of CRC is of potential future clinical significance, particularly in the context of treatment of micrometastases. However further research is required to elucidate the mechanisms of action by which any survival benefit is conferred.

Chapter 8: Discussion

8.1 Suitability of O3FA drinks cartons for chemoprevention or adjuvant treatment of CRC

The present study provides evidence that O3FA supplement drinks exhibit a similar O3FA RBC membrane incorporation profile to traditional triglyceride-bound O3FA capsules. Given the previously described anti-inflammatory and anti-neoplastic properties they have a potential clinical application as both a CRC chemoprevention and as an adjunct in CRC treatment (see section 1.5). The widespread inter-individual variation of O3FA bioavailability across both intervention types requires further investigation to determine whether there is any influence on the efficacy of mechanisms via which O3FAs have both anti-inflammatory and anti-cancer effects.

With respect to O3FA bioavailability, the non-inferiority of drinks compared to capsules is of key significance when considering O3FA supplementation as a treatment adjunct in CRC patients. A significant proportion of patients recovering from surgery or other treatment modalities have an impaired appetite and may be unable to consume conventional dietary fats required for O3FA capsule supplementation. In addition fortified O3FA nutrition drinks may provide additional calories in patients who are unable to achieve adequate calorific intake, as is already commonplace with the use of various calorific supplements. There is also a potential application for O3FA supplementation in combination with traditional chemotherapy regimens, with evidence to suggest synergistic effects and the amelioration of cancer cachexia (Lee *et al.*, 2017). Further research in a population of CRC patients is required to ascertain whether there would be similar acceptability and tolerability in this cohort of patients.

Both preparations of O3FA supplementation demonstrated similar acceptability and tolerability profiles, although only 58% and 68% of participants would consider continuing drinks cartons and capsules respectively for an indefinite period. Long-term acceptability and tolerability are key properties for any chemoprevention and therefore traditional O3FA containing capsules may be more suited to this purpose than drinks. As evidenced in the present study there were concerns regarding the calorific content of the O3FA supplement drinks which led to a participant withdrawing from the study. Although in the US there is a relatively high proportion of the population (approximately 8%) taking O3FA supplements, it is important to recognise that the general population do not yet have irrefutable evidence to support the use of O3FA supplementation as a chemoprevention for CRC (Clarke *et al.*, 2015; Black *et al.*, 2015). Indeed, if the mechanisms and body of evidence to support the use of O3FAs as a chemoprevention in CRC were more robust, then the adoption of long-term supplementation may increase.

Although there are a number animal models and human polyp prevention studies, there is at present a lack of clinical data to justify recommendation to implement O3FA supplementation as a form of chemoprevention or adjuvant treatment in CRC (See section 1.5). A long-term study is required to explore implementing O3FA supplementation as a method of CRC chemoprevention, rather than retrospective estimation of exposure to O3FAs via dietary assessment. Any such study is likely to be of complex design and it remains to be seen how investigators would control for a plethora of known risk factors associated with CRC.

8.2 The effect of O3FA supplementation on faecal microbiome profiles and association with colorectal carcinogenesis

O3FA supplementation was associated with an increased abundance of the genera *Bifidobacteria, Roseburia, Lachnospira* and *Lactobacillus*, with some evidence of reversibility during the washout periods. The present study contributes to mounting evidence in both animal models and humans that EPA and DHA induce subtle changes to the abundance and composition of the faecal microbiota (Costantini *et al.*, 2017). The enrichment of SCFA-producing bacteria including *Lactobacillus* in the faecal microbiome profiles of healthy participants following O3FA supplementation is of significant interest with respect to colorectal carcinogenesis. SCFAs including butyrate and acetate are generated by bacterial fermentation of fibre in the colon (Dahl *et al.*, 2017). Butyrate is proposed to have anti-CRC effects via both regulation of inflammatory pathways and maintenance of the intestinal epithelium (Peng *et al.*, 2009; Kim *et al.*, 2013; Chang *et al.*, 2014; Chen *et al.*, 2017). Although butyrate is an energy source for normal colonocytes, in colon cancer cells it has a pro-apoptotic effect via inhibition of histone deacetylase (Bergman, 1990; Encarnacao *et al.*, 2015; Bultman, 2016; Han *et al.*, 2018). It is unclear whether any anti-CRC effects associated with O3FAs and alterations in the abundance of SCFA-producing bacteria are also dependant on fibre intake.

There are unanswered questions regarding the effects of O3FAs on the colonic microbiota as measured by changes to faecal microbiome profiles. The present study did not investigate whether compositional changes to faecal microbiome profiles induced by O3FA supplementation are drivers of any beneficial effects on the human colon and amelioration of CRC risk. There is also a lack of data

with respect to the interaction between O3FAs and bacteria, particularly whether O3FAs modulate bacterial metabolic function. It remains to be seen whether O3FAs are directly metabolised by bacteria and if the products of bacterial O3FA metabolism affect the colonic environment. The lack of consensus as to the specific microbiome profile associated with colorectal carcinogenesis is also a key limitation when assessing the effect of O3FAs on CRC risk. The mechanisms via which changes to microbiome profiles influence colorectal carcinogenesis and the complex interplay with genetic, environmental and dietary risk factors are not completely understood. It is unclear whether changes to the microbiome profiles of CRC patients are a causative factor in colorectal carcinogenesis or merely an association.

Differences between how EPA and DHA individually alter faecal microbiome profiles are not explored in the present study and there is limited data in the wider published literature. There is a single human study utilising a DHA containing intervention, but no EPA study for comparison (Pu *et al.*, 2016). A mouse study exploring the effect of DHA on faecal microbiome profiles showed an increase in the presence of SCFA-producing bacteria (Davis *et al.*, 2017). At present it is not possible to determine whether EPA and DHA have differing contributions to alterations exhibited in faecal microbiome profiles.

The present study examined faecal microbiome profiles. The bacterial composition and microbiome profile of the colon varies according to anatomical location and colonic environmental conditions (Donaldson *et al.*, 2016). In both CRC patients and healthy individuals luminal faecal microbiome profiles vary from those found within the colonic mucosa (Chen *et al.*, 2012; Flemer *et al.*, 2017). Given that the molecular mechanisms of colorectal carcinogenesis take

place within the colonic mucosa it is unclear whether changes to the faecal microbiome profile observed in this study bear any relationship to ameliorating CRC risk.

The results of the present study should be interpreted with caution given some of the technical limitations of the study and the inability to control for all the potential confounding factors which are inherent to a healthy volunteer study. There were additional ingredients in the drink cartons that may have influenced alterations to faecal microbiome profiles. It is unclear whether changes to faecal microbiome profiles induced by O3FA supplementation have a direct effect on long-term CRC risk, especially given that the present study was performed in a 'healthy' population. There is also lack of understanding as to any interaction with other known risk factors, particularly whether the potential beneficial changes induced by O3FA supplementation are sufficient to ameliorate other risk factors.

The current evidence base exploring the effect of O3FAs on the colonic microbiota and specifically CRC risk is limited. In part, advancing the understanding of how changes to the microbiota affects CRC risk is hampered by a lack of standardisation across the field of microbiome analysis. It is clear that further research is required to determine whether O3FA supplementation induced changes to microbiome profiles exhibited in healthy individuals translate to a potential application as a dietary CRC chemoprevention via modulation of the colonic microbiota.

8.3 RBC cell PUFA analysis in CRCLM patients receiving pre-operative O3FA supplementation

In summary, analysis of CRCLM patient PUFA profiles following pre-operative EPA treatment highlighted incorporation of EPA into RBC membranes. This corresponded with a reciprocal decrease in RBC membrane AA levels. This data provides evidence of EPA bioavailability profiles in CRC patients. Of potential application in future clinical trials is the interpretation of RBC membrane PUFA profiles to assess compliance with trial design, as on a number of occasions PUFA profiles highlighted potential contamination by 'own use' O3FA supplements.

There was a correlation between RBC EPA membrane levels and CRCLM tumour tissue EPA levels, which suggests there is no difference in the ability of CRCLM tissue to incorporate O3FAs compared to other tissues (Metcalf *et al.*, 2007; Harris *et al.*, 2004). However, a significant limitation of this work is the inability to compare RBC membrane PUFA profiles with thiose in normal liver tissue or colonic mucosal tissue. The finding that there was an association between RBC membrane EPA levels and overall survival in patients undergoing resection of CRCLM is of clinical significance, specifically the use of O3FA supplementation as a potential adjunct in the treatment of CRC. Song *et al.* identified that a higher intake of marine O3FAs (>0.3g/day) is associated with a lower risk of CRC specific mortality in a study of 1659 individuals diagnosed with CRC over a median follow-up period of 10.4 years (Song *et al.*, 2017). Whether there is a causal link between RBC EPA membrane incorporation and survival remains to be seen. There are a number of potential avenues via which EPA supplementation may confer a survival advantage including both anti-

inflammatory and anti-neoplastic mechanisms. In addition there is evidence to suggest O3FAs may have more general health benefits including the treatment and prevention of cachexia associated with cancer (Harris *et al.*, 2017; Lee *et al.*, 2017). As an adjunct in CRC treatment there is also a potential clinical application in patients undergoing curative resection of CRC to treat micrometastases. However, before O3FA supplementation can be recommended as a treatment adjunct in CRC further research is required to confirm whether O3FAs provide a definite survival advantage and to elucidate the mechanisms via which this is conferred. Currently there is a phase III multi-centre randomised, double-blind, placebo-controlled trial of EPA supplementation in patients undergoing surgical resection of CRCLM with curative intent (EMT2 study; ClinicalTrials.gov NCT: 03428477).

8.4 Future work

At present a study is being undertaken to determine the amount of O3FAs in ileal fluid (REC reference: 15/YH/0547; ISRCTN: 14530452, see appendix 4 & 5). This study is being performed to determine the proportion of O3FAs, given in doses similar to those employed in clinical studies, absorbed in the small intestine. The study is of interest as it will develop our understanding of O3FA bioavailability, specifically whether colonic exposure to O3FAs is secondary to direct luminal exposure or systemic distribution and subsequent incorporation into the colonic mucosa. Participants with a loop ileostomy are required to take O3FA containing gelatin capsules twice a day for four weeks, providing a combined daily total of 1000mg EPA and 1000mg DHA. Stoma fluid samples are to be collected prior to starting supplementation, within two hours of taking the first O3FA capsules and at the end of the study. RBC membrane PUFA levels

will be measured prior to starting supplementation and at the end of the four week intervention period. Subjects with a loop ileostomy have been selected as these individuals have maximal length of disease free small intestine, which near replicates the small intestine of healthy individuals prior to ejection of terminal ileal fluid into the colon. It is hoped this work will inform the design, and specifically the dosing of O3FAs to be employed in future studies using *in-vitro* modelling of the colonic microbiome. The *in-vitro* gut model has been reported in *Clostridium difficile* research and will allow assessment of the effect of O3FA supplementation on the colonic microbiota, with the ability to control for multiple confounding factors that to date have significantly limited explorative studies in both human and animal models (Baines *et al.*, 2005; Freeman *et al.*, 2007; Baines *et al.*, 2009).

The present study highlights subtle changes to the composition of faecal microbiota induced by O3FA supplementation. Further research is required to determine whether changes to microbiome profiles also take place within the colonic mucosa. The most appropriate application for O3FA supplementation as a potential method of beneficially modulating the colonic microbiota is yet to be determined. Many studies examining the effect of O3FAs on colonic mucosal or faecal microbiome profiles employ O3FAs as a prebiotic (Pu *et al.*, 2016; Rajkumar *et al.*, 2014). A prebiotic is defined by the World Health Organisation (WHO) and Food and Agriculture Organisation (FAO) as a non-viable food component that confers a health benefit via modulation of the microbiota (Pineiro, 2008). The present study in healthy volunteers provides further evidence supporting the application of EPA and DHA as a prebiotic, particularly relating to an increased abundance of beneficial butyrate-producing bacteria. There is a lack of research into the effects of O3FAs on bacterial metabolic

function, where it is unclear if O3FAs are metabolised by bacteria and if any products of bacterial O3FA metabolism have an effect on the colonic mucosa. There is emerging evidence to employ O3FAs in conjunction with probiotic bacteria to provide synergistic benefits on the colonic microbiota. A placebo controlled RCT in 140 CRC patients undergoing chemotherapy, tested a combination of probiotic bacteria (*Lactobacillus* and *Bifidobacterium*) with a 2g daily dose of O3FAs (Golkhalkhali *et al.,* 2018). The study reported improved quality of life and a reduction in measured inflammatory markers. Further research into how O3FAs affect the colonic microbiota and any impact on long-term CRC risk is required. To account for the plethora of confounding factors associated with O3FA supplementation and microbiome analysis a RCT would be desirable, although a cohort study would be a more viable study design.

There is now extensive data pertaining to the bioavailability of O3FAs following O3FA supplementation. The present study suggests O3FA containing drink supplements exhibit equivalent bioavailability profiles to traditional capsule supplementation. There is still debate as to whether O3FA supplementation is required to deliver the proposed health benefits associated with O3FAs, or if this could be achieved via dietary intake (Song *et al.*, 2017). In order to justify the clinical application of O3FA supplementation as either a method of chemoprevention or adjuvant treatment in CRC, further evidence is required to elicit their anti-CRC mechanisms. There is significant scope for research into the fate of O3FAs following RBC membrane incorporation. There are a number of potential pathways that O3FAs enter including lipid signalling pathways, β-oxidation and PUFA conversion (Kanamori *et al.*, 2018; Camões *et al.*, 2009). Of current interest is the field of lipidomics with research focused on the metabolic

fate and function of various lipids including EPA and DHA (Zárate *et al.*, 2017; Schmöcker *et al.*, 2018).

In summary, future work to justify the implementation of O3FA supplementation for chemoprevention or adjuvant treatment of CRC requires focus on long-term effects and the mechanisms via which these take place. There is a particular paucity of data examining the effects of O3FA on the colonic microbiota, for which the present study has given a key insight.

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Appendix 1.

Content of Smartfish® Remune (per 200 ml)

Energy 233 kcal

Fat (53% total energy) 13 g

Saturates 2.8 g

Monounsaturates 5.3 g

Polyunsaturates 3.1 g

Omega-3 PUFAs 2.0-2.5 g

EPA 850-1100 mg

DHA 1100-1300 mg

Carbohydrates (33% total energy) 19 g

Fibre (2% total energy) 1 g

Protein (12% total energy) 10 g

Calcium 56 mg

Vitamin D3 10 µg

Vitamin E 4.1 µg

Osmolarity 698 mOsmol/l

Appendix 2.

From: Clinical Trial Helpline <ctdhelpline@mhra.gsi.gov.uk>
Sent: 13 February 2015 15:06
To: Henry Watson [ugm3hsw]
Subject: RE: Omega-3 nutritional supplement study

Notification that a Clinical Trial Authorisation (CTA) is not required

Dear Mr Watson

Thank you for your email dated 25th January 2015, and please accept our sincere apologies for the delay in responding to you.

Following a review of your protocol, I can confirm that your proposal is not a Clinical Trial of an Investigational Medicinal Product (IMP) as defined by the EU Directive 2001/20/EC and no submission to the Clinical Trials Unit at the MHRA is required.

Kind regards

Clinical Trial Helpline
MHRA

Your views matter. Please tell us what you think of the service you have received from us by following the link below:
https://www.surveymonkey.com/s/ClinicalTrialHelplineFeedback

Appendix 3.

NRES Committee Yorkshire & The Humber - South Yorkshire
Unit 001
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Rolling Mill Road
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NE32 3DT
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29 April 2015
Professor M A Hull
University of Leeds
Level 9
Leeds Institute of Biomedical and Clinical Sciences
St James' University Hospital
Leeds LS9 7TF
Dear Professor Hull

Study title:	**A randomised cross-over trial to compare the bioavailability, acceptability and tolerability of omega-3 fatty acids in a drink carton formulation with an equivalent dose of omega-3 fatty acids in soft gelatin capsule form.**
REC reference:	**15/YH/0142**
IRAS project ID:	**166337**

Thank you for your letter of 23 April 2015, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Mrs Helen Wilson, nrescommittee.yorkandhumber-southyorks@nhs.net .Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **favourable ethical opinion** for the above research on the basis described in the application form, protocol and supporting documentation [as revised], subject to the conditions specified below.

With the Committee's best wishes for the success of this project.
Yours sincerely
pp
Dr Ian Woollands (Chair)

Appendix 4.

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 a temporary 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 a temporary ileostomy following anterior colonic resection for colorectal cancer.

Individuals with a temporary loop 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 also have an intact small bowel in continuity and therefore is likely to 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 50 years or over
- Either gender
- Temporary ileostomy fashioned at least 2 months prior to commencing study
- Able to self medicate
- 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
- Metastatic colorectal cancer
- Less than 4 weeks since any chemotherapy or radiotherapy
- Inflammatory bowel disease or other intestinal disease (e.g. coeliac disease)

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 ileostomy following anterior resection for colorectal cancer will be identified from electronic colorectal surgery department records at St James' University Hospital. Currently, reversal of the ileostomy is undertaken at between 6 to 12 months following primary 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.

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Appendix 5.

NRES Committee Yorkshire & The Humber - South Yorkshire
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05 January 2016
Professor MA Hull
Level 9, Leeds Institute of Biomedical and Clinical Sciences
St James' University Hospital
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Beckett Street
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Dear Professor Hull

Study title:	**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 a temporary ileostomy.**
REC reference:	**15/YH/0547**
IRAS project ID:	**182428**

Thank you for your letter of 23rd December 2015, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair. We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Miss Christie Ord, nrescommittee.yorkandhumber-leedswest@nhs.net.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

pp
Dr Vera Neumann
Vice Chair

Gut Online First, published on September 26, 2017 as 10.1136/gutjnl-2017-314968

ORIGINAL ARTICLE

A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota

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ABSTRACT

Objective Omega-3 polyunsaturated fatty acids (PUFAs) have anticolorectal cancer (CRC) activity. The intestinal microbiota has been implicated in colorectal carcinogenesis. Dietary omega-3 PUFAs alter the mouse intestinal microbiome compatible with antineoplastic activity. Therefore, we investigated the effect of omega-3 PUFA supplements on the faecal microbiome in middle-aged, healthy volunteers (n=22).

Design A randomised, open-label, cross-over trial of 8 weeks' treatment with 4 g mixed eicosapentaenoic acid/docosahexaenoic acid in two formulations (soft-gel capsules and Smartfish drinks), separated by a 12-week 'washout' period. Faecal samples were collected at five time-points for microbiome analysis by 16S ribosomal RNA PCR and Illumina MiSeq sequencing. Red blood cell (RBC) fatty acid analysis was performed by liquid chromatography tandem mass spectrometry.

Results Both omega-3 PUFA formulations induced similar changes in RBC fatty acid content, except that drinks were associated with a larger, and more prolonged, decrease in omega-6 PUFA arachidonic acid than the capsule intervention (p=0.02). There were no significant changes in α or β diversity, or phyla composition, associated with omega-3 PUFA supplementation. However, a reversible increased abundance of several genera, including *Bifidobacterium*, *Roseburia* and *Lactobacillus* was observed with one or both omega-3 PUFA interventions. Microbiome changes did not correlate with RBC omega-3 PUFA incorporation or development of omega-3 PUFA-induced diarrhoea. There were no treatment order effects.

Conclusion Omega-3 PUFA supplementation induces a reversible increase in several short-chain fatty acid-producing bacteria, independently of the method of administration. There is no simple relationship between the intestinal microbiome and systemic omega-3 PUFA exposure.

Trial registration number ISRCTN18662143.

Significance of this study

What is already known on this subject?

- The naturally occurring omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have anticolorectal cancer (CRC) activity.
- High-purity EPA and DHA can be provided in soft-gel capsule form or as a 'nutrition' drink providing greater than 2 g omega-3 PUFAs daily.
- The intestinal microbiota are implicated in colorectal carcinogenesis, as well as modulation of chemotherapy and immunotherapy of CRC.

What are the new findings?

- Oral high-dose omega-3 PUFAs do not produce marked changes in the intestinal microbiome in healthy volunteers, even in individuals with treatment-emergent diarrhoea.
- Intake of 4 g daily mixed EPA/DHA for 8 weeks was associated with a reversible increase in Bifidobacterium, Oscillospira, Roseburia and Lachnospira species, but decreased Coprococcus and Faecalibacterium.
- Similar effects of omega-3 PUFAs on the faecal microbiome were observed for both capsule and drink formulations.
- Capsule and drink formulations provide equivalent tissue omega-3 PUFA incorporation, as measured by red blood cell levels, but only drinks were associated with prolonged suppression of proinflammatory arachidonic acid levels.

How might it impact on clinical practice in the foreseeable future?

- An increase in short-chain fatty acid-producing bacteria may be relevant to the beneficial anti-CRC effects of EPA in both prevention and adjuvant treatment settings.
- Clinical evaluation of the anticancer properties of omega-3 PUFAs needs to consider the intestinal microbiota and its role in carcinogenesis and immune regulation.

INTRODUCTION

The two main omega-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA; C20:5 ω3) and docosahexaenoic acid (DHA; C22:6 ω3) are widely used as nutritional supplements, as fish oil or in more concentrated 'nutraceutical' form.[1] Multiple health benefits have been claimed for these long-chain omega-3 PUFAs, including secondary prevention of ischaemic heart disease,[2] treatment of

rheumatoid arthritis[3] and anticancer activity,[4] some of which are supported by evidence from randomised trials.[5 6]

The mechanism(s) underlying the colorectal cancer (CRC) chemopreventative activity of EPA reported by West *et al* is unclear.[5] It has been proposed that the intestinal microbiota may play a role in colorectal carcinogenesis based on the association of CRC with a specific intestinal microbiome profile, or so-called dysbiosis, characterised by low phylogenetic diversity, altered *Firmicutes*/*Bacteriodetes* ratio, under-representation of short-chain fatty acid (SCFA)-producing genera such as *Roseburia* and *Eubacterium* as well as presence of putative pathobionts such as *Fusobacterium nucleatum*.[7 8] One possibility is that modulation of the intestinal microbiota may contribute to the cancer-preventative properties of omega-3 PUFAs.

Data from mouse models suggest that dietary omega-3 PUFA intake or high tissue levels of omega-3 PUFAs are associated with differences in intestinal microbiota, including increased quantities of certain genera, including *Bifidobacterium* and *Lactobacillus*.[9 10] There has been only one case report of the effect of an omega-3 PUFA-rich diet on human intestinal microbiota.[11] In this case, there was a notable increase in several SCFA (butyrate)-producing genera including *Blautia*, *Bacterioides*, *Roseburia* and *Coprococcus*.[11]

Therefore, a plausible hypothesis is that omega-3 PUFA intake alters the composition of human intestinal microbiota, thereby attenuating the intestinal dysbiosis associated with colorectal carcinogenesis.

Nutritional supplementation with omega-3 PUFAs can occur in several ways, either as unrefined fish oil, in 'nutraceutical' form, usually as the triglyceride or ethyl ester conjugate in soft-gel capsules, taken with food or more recently as an emulsion in drink form.[1]

To address the above hypothesis and, at the same time, compare two different formulations of omega-3 PUFAs, we performed a randomised, open-label, cross-over study of the effect of omega-3 PUFAs on the intestinal microbiome of healthy volunteers aged over 50 years (a population relevant to CRC screening and chemoprevention), thus comparing equivalent doses of equimolar amounts of EPA and DHA in capsule or drink form with an integrated 'washout' period, with which to determine reversibility.

METHODS

Study design and interventions

The randomised, cross-over trial was carried out in the Human Appetite Research Unit (HARU), University of Leeds. Approval was obtained from the South Yorkshire Research Ethics Committee (15/YH/0142). Interventions were not classified as Investigational Medicinal Product by the Medicines and Healthcare Products Regulatory Agency. The study was registered with International Standard Randomised Controlled Trials Number (18662143).

Healthy volunteers aged ≥50 years of both sexes were sought using a HARU volunteer database and advertising across the university. Participants received £20 per study visit. At a screening visit, the following exclusion criteria were considered: ongoing or planned regular use of other omega-3 PUFA or cod liver oil supplements; seafood allergy; concomitant use of non-steroidal anti-inflammatory medications, including aspirin; current treatment for any chronic inflammatory condition or malignancy; previous colonic or small bowel resection; current smoker (minimum 6 months smoking cessation) and pregnancy. If an individual was eligible, he/she underwent a 'lead-in' taste test of the peach-flavoured drink and also swallowed two study capsules with water, after providing written informed consent to confirm likely compliance and minimise dropout.

Visit 1 occurred within 2 weeks of the screening visit, at which participants were randomised to take either two 200 mL Smartfish Remune drinks (see online supplementary methods for content) per day (providing approximately 2000 mg EPA and 2000 mg DHA, as the triglyceride) at any suitable time of day or four soft-gel capsules (each containing 250 mg EPA and 250 mg DHA as the ethyl ester) twice daily with meals (providing 2000 mg EPA and 2000 mg DHA per day), both for 8 weeks (intervention A; figure 1). After a 12-week 'washout' period, participants took the second intervention for 8 weeks (Intervention B; figure 1). We also included a final study visit after a second 12-week 'washout' period (V5; figure 1). Randomisation was performed by Leeds Teaching Hospitals Pharmacy using random permuted block allocation in concealed envelopes. Neither participants nor researchers were blinded to the interventions and hence allocation order.

At each visit, adverse event (AE) monitoring was undertaken by a brief interview based on questioning for recognised AEs of omega-3 PUFA supplements, including loss of appetite, eructation ('fishy' burping), nausea, vomiting, dyspepsia, abdominal pain and diarrhoea, as well as bleeding events. Review of AEs was performed by an independent data monitoring committee every 3 months. Tolerability of both drinks and capsules was assessed with a palatability questionnaire and capsule acceptability questionnaire at the end of each 8-week intervention period (visit 2 or 4). Participants' height and weight were measured at the start of the study.

Blood and urine were collected at each visit and a faecal sample (obtained with a Fe-Col faecal collection device) was returned by hand or by Royal Mail Safebox within 2 days of each visit. Participants did not start either intervention until the baseline (visit 1 or 3) faecal sample had been collected. Faecal samples were stored at −20°C in RNA later (Thermofisher Scientific) until DNA extraction, which occurred within 2 weeks of collection in the majority of cases.

Omega-3 PUFA measurement

Red blood cells (RBCs) were obtained from whole blood EDTA samples as described.[12] Samples were stored at −80°C until lipid extraction and measurement of fatty acids by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described.[13] Data are presented as the % (w/w) content of the total fatty acid pool measured.[13]

Intestinal microbiome analysis

Microbial DNA extraction, PCR and Illumina sequencing were performed as described (see online supplementary methods).[14]

Demultiplexed FASTQ files were trimmed of adapter sequences using cutadapt.[15] Paired reads were merged using fastq-join[16] under default settings and then converted to FASTA format. Consensus sequences were removed if they contained any ambiguous base calls, two contiguous bases with a Phred quality score lower than 33 or a length more than 2 bp different from the expected length of 240 bp. Further analysis was performed using QIIME.[17]Operational taxonomy units (OTUs) were picked using Usearch[18] and aligned to the Greengenes reference database using PyNAST.[19] Taxonomy was assigned using the RDP 2.2 classifier.[20] The resulting OTU biom files from the above analyses were imported in MEGAN for detailed group-specific analyses, annotations and plots.[21]

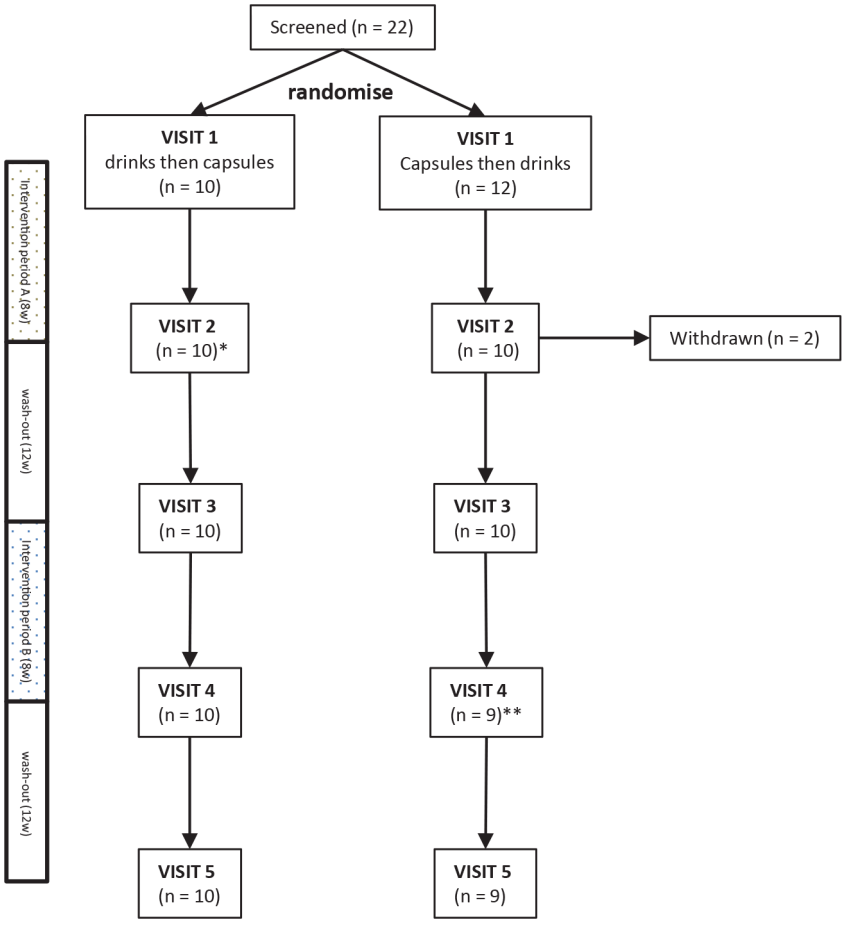

Figure 1 Schedule of study visits and participant flow through the study. The duration of the intervention and washout periods in weeks (**w**) is noted in the left column. *One participant ceased the drink intervention early and completed intervention period B (capsules). **Two participants ceased drink intervention early (remained in study). n, number of individuals in study as per protocol.

As well as comparisons of observed taxa, rarefication was performed to various levels to compare α diversity. All groups were rarefied to the lowest read number, and β diversity calculated using weighted and unweighted UniFrac as well as the non-phylogenetic Bray-Curtis dissimilarity measure.[14] β diversity was compared using principal coordinate analysis (PCoA) on all samples. Correlations between the microbiome and RBC fatty acid data were computed using package 'Hmisc'[22] in R.[23]

End points and sample size calculation

In the absence of any data on the effect of omega-3 PUFAs on human intestinal microbiota, we determined the sample size based on testing non-inferiority of the drink formulation compared with capsules for the RBC level attained after 8 weeks' dosing. There are no prior RBC omega-3 PUFA data available in subjects consuming Smartfish Remune. However, based on data from healthy individuals consuming omega-3 PUFA capsules that reported intraindividual variability of 4%,[24] we set a non-inferiority margin of 3%. Assuming participant dropout of 20%, we estimated that a sample size of 20 participants would be required to exclude a difference in combined levels of RBC, EPA and DHA between the intervention groups of greater or equal to 0.3 absolute per cent points, with 90% power at a significance level of 0.05.

Data were analysed on an intention-to-treat basis. The paired Student's t-test was used to evaluate the difference between RBC omega-3 PUFA levels for participants who completed both intervention periods. Unpaired data from participants, who did not complete both intervention periods, were included in unpaired data analyses. Comparison of absolute presupplementation and postsupplementation levels of RBC omega-3 PUFA levels within intervention groups was by paired Student's t-test. AEs in each group were compared by χ^2 analysis.

For intestinal microbiota analysis, OTU read numbers at different time-points for the two interventions were compared by unpaired and paired analysis. Having excluded a significant treatment order effect on both RBC omega-3 PUFA incorporation and the intestinal microbiome profile, data for the capsule intervention (termed Int1) and the drink intervention (termed Int2) were combined regardless of the intervention order (either A or B).

RESULTS

Twenty-two participants were randomised between July 2015 and April 2016 (figure 1). There were 12 women and 10 men with a median age of 57 years (range 51–65 years) and a median body mass index of 27.1 kg/m^2 (22.0–33.8 kg/m^2). Both interventions were well tolerated with good acceptability scores (see online supplementary results). Two individuals withdrew from

Table 1 AEs during capsule and drink intervention periods

	Capsules (n=22)	Drinks (n=19)
AE during intervention period (no of participants)	29 (14)	17 (10)
Diarrhoea	5	5
Abdominal discomfort	6	3
Eructation (burping)	10	4
Dyspepsia	5	1
Nausea/vomiting	3	4
One or more symptoms defined as moderate	3 (2 ceased intervention)	3 (3 ceased intervention early)
Resolution of symptoms after washout (no of participants)	14	9
Symptoms experienced during both intervention periods (proportion occurring during second intervention that were present during the first intervention)	4/10 (40%)	4/9 (44%)

AE, adverse event.

the study during intervention period A (both capsules) because of an AE. One individual did not take drinks because of concern about calorific intake but remained in the study. Of those individuals completing intervention periods for drinks (n=16) and capsules (n=20), the median duration of the intervention period was 57 (54–59) and 57 (55-63) days, respectively.

Symptoms reported during all capsule interventions (n=22) and all drink interventions (n=19) are detailed in table 1. In general, there was an excess of treatment-emergent AEs during the capsule intervention, particularly for dyspeptic symptoms (heartburn, acid regurgitation). All AEs were defined as minor (any symptom experienced during intervention but did not require cessation of intervention or dose reduction) except for three AEs in each group that were classified as moderate (any symptom experienced that required subsequent dose reduction or cessation of intervention) and led to cessation of the intervention. All the AEs during the capsule intervention and the AEs in 9/10 individuals during the drink intervention had resolution of the AE during washout, suggesting that these were definite adverse reactions.

Changes in RBC omega-3 PUFA content

Fatty acid analysis was performed on those samples from participants who completed one or both intervention periods and washout (n=20). Only three individuals did not complete a second intervention washout cycle with full blood sampling. Overall, 98 RBC samples underwent lipid extraction for omega-3 PUFA analysis. Data were available for 97 samples as one sample did not extract well and was uninterpretable.

Individual PUFA data are represented as the absolute per cent level, change between baseline and the post-treatment level and difference between baseline and 'washout' value in figure 2. Data for the order of intervention (drinks then capsules or capsules then drinks) are shown only for EPA and DHA (figure 2A and B). The 'washout' period (12 weeks) was effective with return to baseline RBC levels of EPA and DHA for both drinks and capsules for either order of intervention. There was a lower post-treatment % RBC content of EPA or DHA (figure 2A and B) if drinks were consumed after capsules compared with the opposite intervention order, but this difference was not statistically significant. There were no intervention order effects on content of the other fatty acids measured (data not shown).

As there was no significant difference in RBC PUFA levels related to the order of intervention, data for individual PUFAs were pooled, independently of the intervention order, for the further comparison of drinks and capsules (figure 2C-F). Baseline and post-treatment levels of EPA, docosapentaenoic acid (DPA) and DHA were well matched with no significant difference between the post-treatment level of EPA and DHA attained following consumption of drinks or capsules (figure 2C and E). The mean individual difference between the combined RBC EPA and DHA level attained after the drink intervention and that attained after capsule use was −0.12 (95% CI −1.84 to 1.61; n=16 paired data), thereby confirming non-inferiority of the drink formulation compared with capsules for omega-3 PUFA incorporation in RBCs. There was little conversion to C22:5 ω-3 DPA from EPA (figure 3D). Incomplete washout of DHA from RBC membranes after the capsule intervention was observed unlike that after the drink intervention (figure 2E). Drink intake was associated with a larger decrease in relative C20:4 ω-6 arachidonic acid (AA) content compared with capsules, which remained evident at the end of washout (figure 2F). However, there was no statistical post-treatment difference in the per cent AA content between drink and capsule use (figure 2F). The omega-3:omega-6 ratio is commonly used as a biomarker of omega-3 PUFA bioactivity.[12] Therefore, we examined the effect of drink consumption and capsule intake on the EPA+DHA:AA ratio (figure 2G). The larger decrease in AA content contributed to the higher EPA+DHA/AA ratio gained after use of drinks compared with capsules (figure 2G), a difference which was statistically significant (p=0.02).

Online supplementary figures 1 and 2 demonstrate individual EPA and DHA profiles based on intervention order. As expected, there was wide variability in omega-3 PUFA incorporation between individuals. In general, EPA profiles were less variable, for both capsule and drink interventions (see online supplementary figure 1) than for RBC DHA levels, both within and between individuals (see online supplementary figure 2). Incorporation of either EPA or DHA with one intervention did not predict the individual response to the other intervention.

Changes in the intestinal microbiome

A faecal sample was collected at all five time-points from all 20 volunteers. Three samples were excluded from PCR amplification because of repeated poor quality DNA extraction, leaving 97 samples that were analysed (median 46 603 reads; minimum 26 387, maximum 114 130). Bray-Curtis PCoA of all samples considering all taxonomic levels is shown in figure 3A. Interindividual differences in the intestinal microbiome exceeded any treatment effect of omega-3 PUFA in either capsule or drink form. In particular, volunteers 8 and 13 were markedly different from the others related to a reduced proportion of *Clostridia* and a larger proportion of the class *Gammaproteobacteria* with high abundance of *Succinivibrionaceae* (see online supplementary figure 3). For unpaired analysis of microbiome profiles across time-points, data from these volunteers were excluded. Volunteer 16 also had a different microbiome with very high abundance of *Succinivibrionaceae*, but the rest of the taxonomic groups in this case were consistent with the other volunteers (see online supplementary figure 4). Therefore, we did not exclude this (volunteer 16) individual's data from further analysis. For the subsequent analyses, all capsule (Int1) and drink (Int2) intervention data were combined, regardless of the order of the interventions, on the basis of complete tissue (RBC) omega-3 PUFA washout

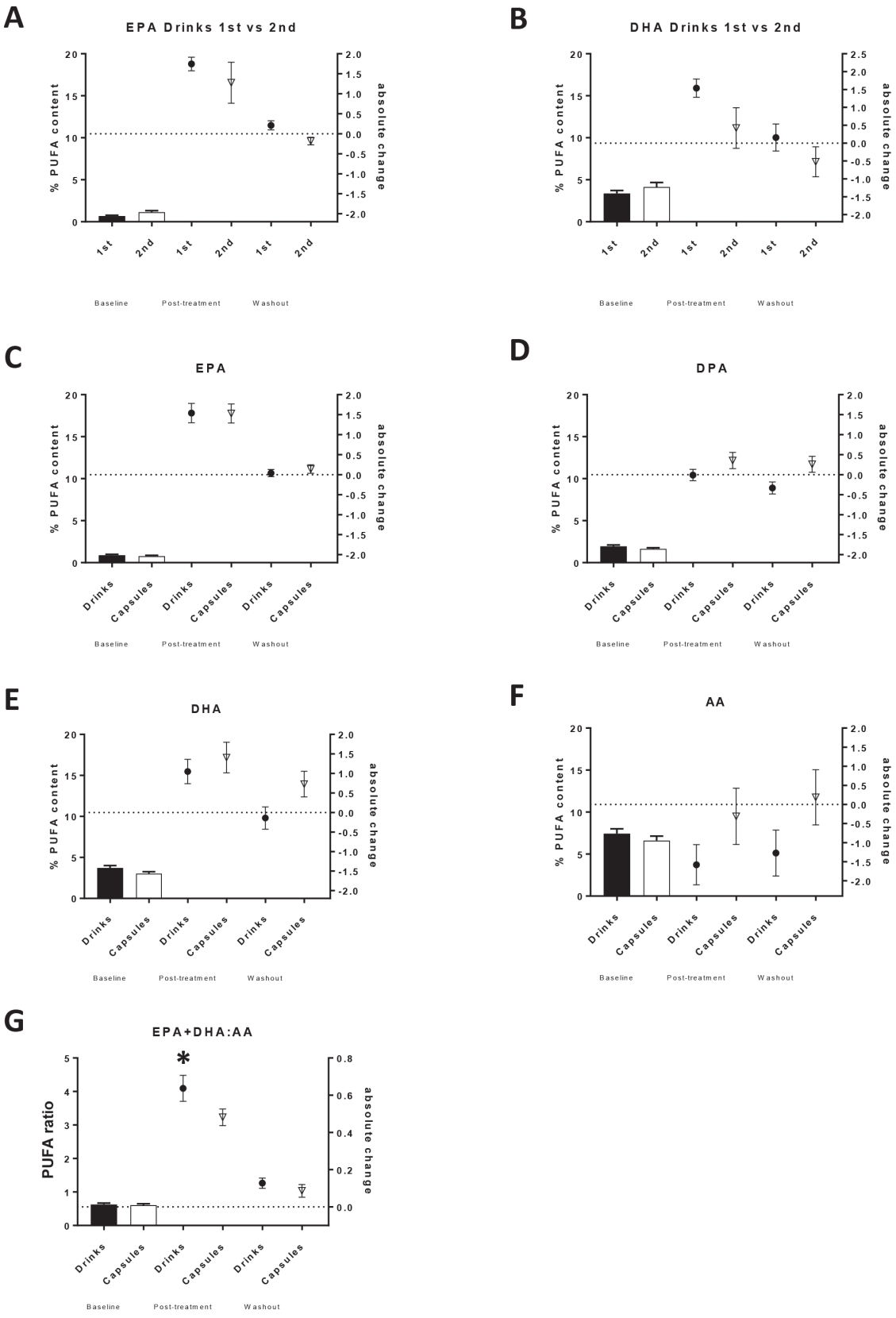

Figure 2 Red blood cell PUFA levels during the study. In each case (A–G), the left y axis is the baseline % RBC PUFA value or ratio, and the right y axis is the absolute difference between the post-treatment value or 'washout' value and the baseline % level. Columns (baseline per cent values) and symbols (absolute difference in per cent value from baseline) denote the mean. Error bars denote the SE of the mean. (A–B) Comparison of RBC EPA and DHA levels depending on whether the drink intervention was first or second. (C–F) Individual RBC PUFA levels comparing pooled data from the drink versus capsule intervention, independently of the intervention order. (G) Comparison of the EPA+DHA/AA ratio at baseline, post-treatment and after washout for the drink and capsule intervention. *p<0.05 for the difference between drinks and capsules; paired t-test. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; RBC, red blood cell.

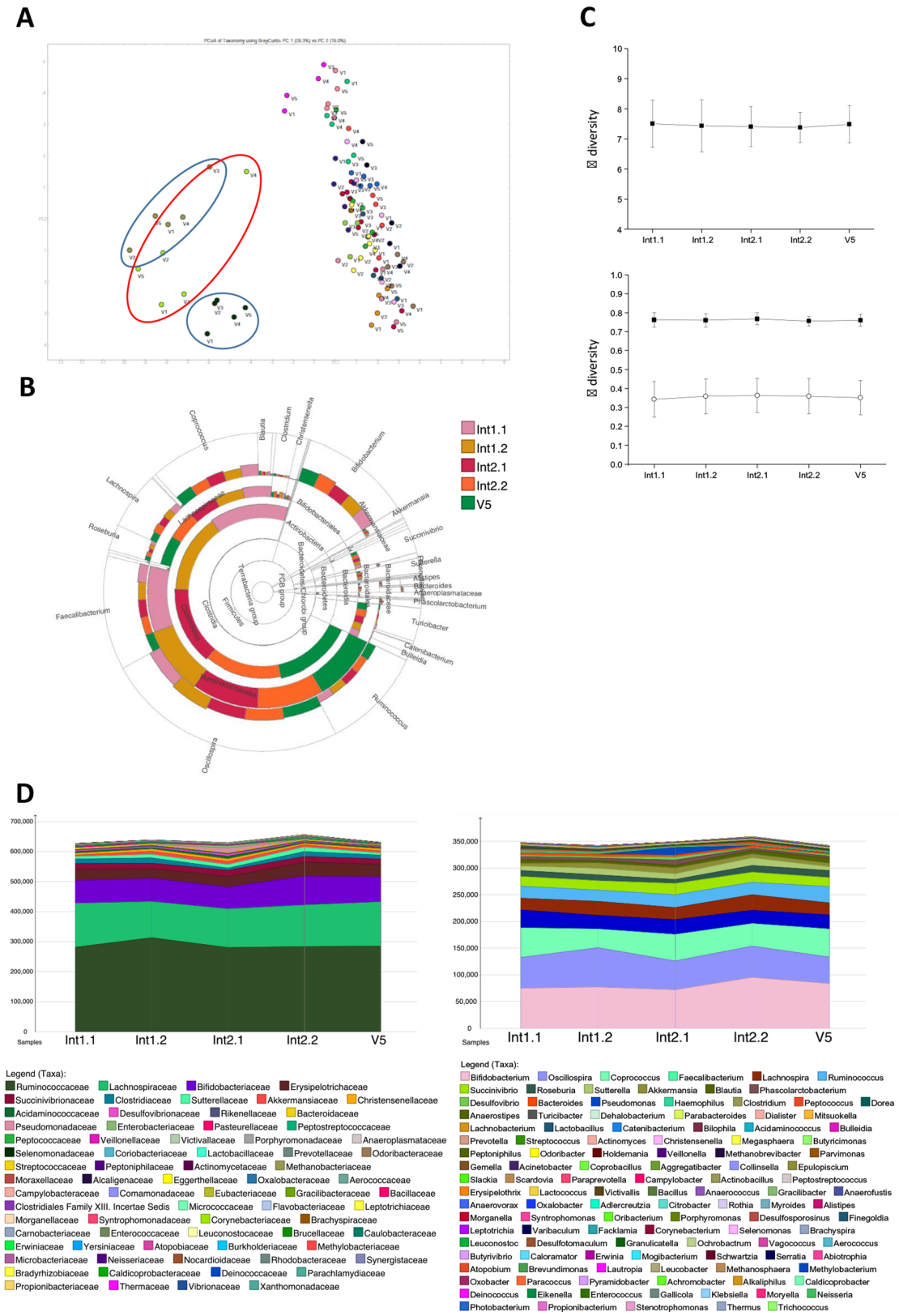

Figure 3 Changes in the intestinal microbiome associated with omega-3 PUFA supplementation. (A) Principal coordinate analysis of all samples (V1–V5) for all participants. Each participant is denoted by a different colour. Clustering of data from individuals is prominent with relatively small differences between different time-points per individual. Circles highlight participants with high *Gammaproteobacteria* (blue) or *Succinivibrioacceae* alone (red). (B) Cladogram. Int1.1 and Int1.2 denote visits/samples before and after the capsule intervention regardless of the intervention order (A then B, and B then A). Int2.1 and Int2.2 are corresponding time-points for the drinks intervention regardless of intervention order. (C) Shannon α diversity index (23 305 sequences per sample) and weighted (open circles) and unweighted (solid squares) unifrac β diversity scores for all participant samples from each visit (Int1.1–1.2 and Int2.1–2.2 and V5). Symbols denote the mean and bars represent the SD. (D) Family and genus-level profiles before and after capsule and drink interventions and at final 'washout'. PUFA, polyunsaturated fatty acid.

and similarity of the microbiome profile at the two baseline assessments at visit 1 and visit 3 for either order of interventions (see online supplementary figure 5). The cladogram highlights that the omega-3 PUFA intervention, either as capsules or as drinks, was not associated with any significant overall taxonomic shift (figure 3B). There was no significant change in α (Shannon diversity index) or β (unweighted and weighted unifrac distance) diversity at the end of each intervention period (Int1.2 and Int2.2) compared with baseline (Int1.1 and Int2.1) or the final washout time-point (V5) (figure 3C).

The *Firmicutes:Bacteriodetes* (*F:B*) ratio is frequently reported in cohort studies investigating the relationship between intestinal dysbiosis and CRC.[7] However, neither capsule nor drink intake was associated with statistically significant changes in the *F:B* ratio (see online supplementary figure 6).

However, presentation of data at individual family and genus level revealed consistent differences associated with both capsule omega-3 PUFA and drink omega-3 PUFA interventions that returned towards baseline on cessation (figure 3D). For example, some relatively low abundance families including *Clostridiaceae*, *Sutterellaceae* and *Akkermansiaceae* were each increased at the end of both interventions with reversibility after the washout period (figure 3D). A similar phenomenon was observed at genus level, for example *Oscillospira* and *Lachnospira* (figure 3D). Individual histograms are shown for the top five abundant genera in figure 4A-E, which highlight changes associated with 8 weeks' intervention with capsules or drinks, each time with reversibility after 12 weeks. Supplementation with omega-3 PUFAs was associated with increased abundance of *Bifidobacterium*, *Oscillospira*, *Lachnospira* and a reduction in abundance of *Coprococcus* and *Faecalibacterium* (figure 4A-E).

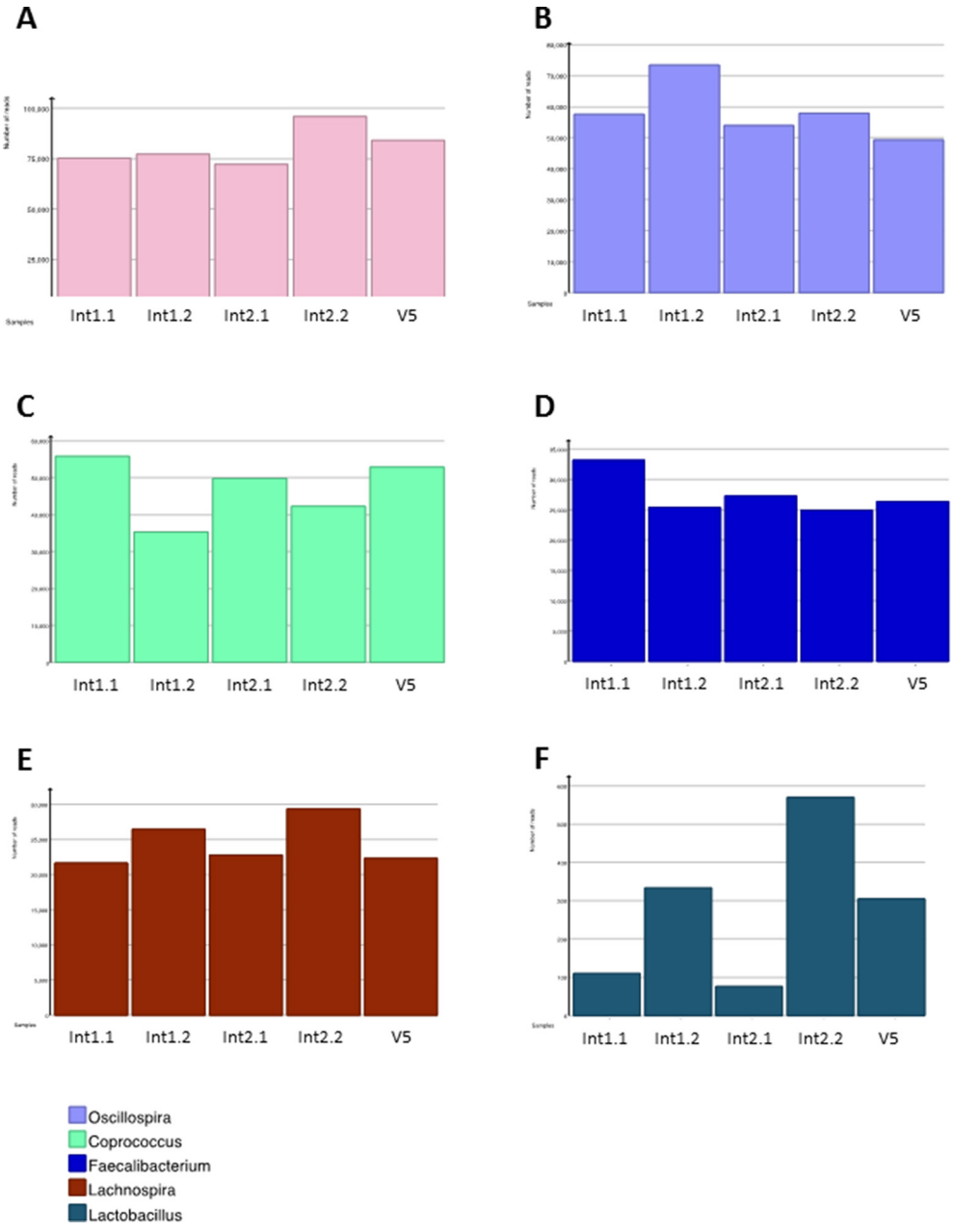

Figure 4 Abundance of top five genera and *Lactobacillus* at each visit. Columns represent the mean value for each time-point for each intervention (Int1 and Int2) irrespective of the order of the interventions (see figure 3 legend).

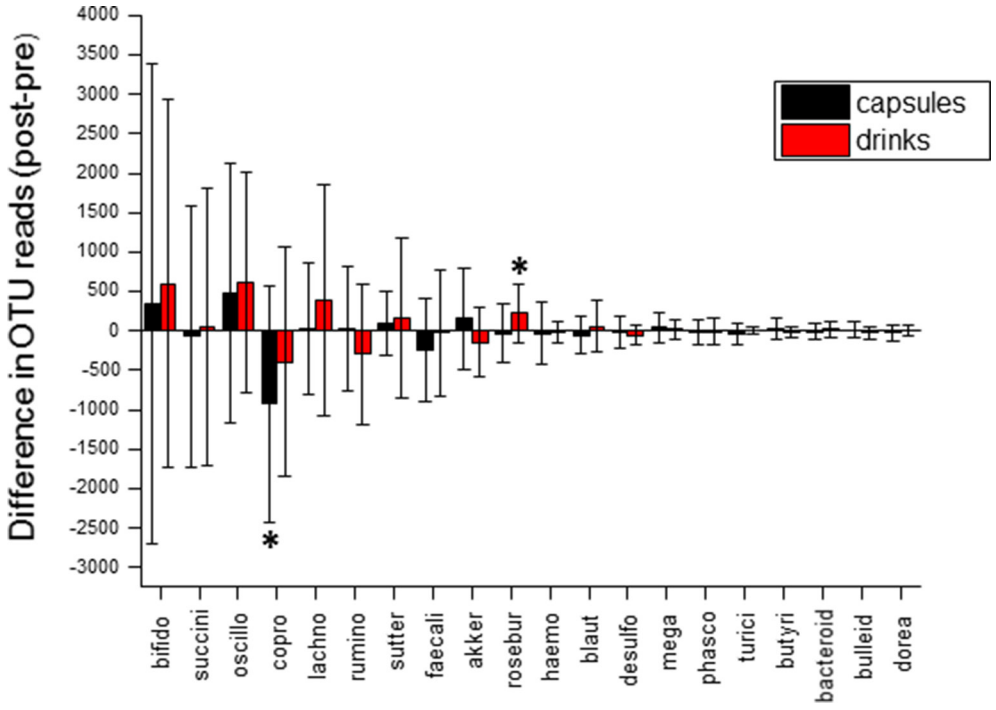

Figure 5 Changes in abundance of genera at the end of the 8-week omega-3 PUFA intervention period. Differences in OTU read number are calculated as the value at the end of the treatment period minus the value at the start of the intervention. Bars denote the mean value and bars denote the SD Black and red bars distinguish capsule and drink intervention data, respectively. Positive values represent an increase associated with intervention and negative values represent a decrease in abundance at the end of the intervention period. *p<0.05 (one sample t-test). OTU, operational taxonomical unit; PUFA, polyunsaturated fatty acid.

We also performed a paired analysis, in which OTU read numbers for the top 20 genera were compared before and after capsule or drink interventions within individuals. This confirmed that *Bifidobacterium* and *Oscillospira* (increase), as well as *Coprococcus* (decrease), changed most for both interventions, whereas an increase in *Roseburia* and *Lachnospira* abundance was prominent only after the drink omega-3 PUFA intervention (figure 5). A paired t-test comparing the treatment effect (in OTU reads) between the capsule and drink intervention for the 17 individuals who had complete paired (before and after intervention) data revealed that the drink intervention was associated with increased abundance compared with capsules for *Roseburia* only ($p<0.05$). Mouse studies have consistently demonstrated an increase in *Lactobacillus* abundance after dietary fish oil supplementation.[9 10 25 26] Therefore, we analysed changes in this lower abundance genus following both omega-3 PUFA interventions. There was an increase in *Lactobacillus* OTU number after both interventions that returned towards baseline values after washout (figure 4F). However, these differences were not statistically significant between baseline and postintervention timepoints ($p=0.11$ capsules; $p=0.9$ drinks) or comparing drinks versus capsules in a pair-wise manner ($p=0.59$). *Fusobacterium* spp. (including *nucleatum*) were not detected in faeces from this cohort of non-cancer individuals.

There was no effect of gender on baseline microbiome profile or treatment effect of either omega-3 PUFA intervention (data not shown). We also tested whether development of diarrhoea on omega-3 PUFA supplementation was associated with a shift in the intestinal microbiome. There was no consistent profile observed in stool samples collected at the visit during/after which diarrhoea was reported (see online supplementary figure 7). Moreover, we investigated whether the RBC EPA and DHA levels generated by each of the interventions correlated with changes in the intestinal microbiome. Correspondence analysis and correlation testing did not demonstrate any consistent relationship between RBC EPA/DHA levels and microbiome profile, with the relationship for EPA and DHA being weak in comparison with the other FAs tested (see online supplementary figure 8).

DISCUSSION

We report small, consistent and reversible changes in the human intestinal microbiome associated with omega-3 PUFA supplementation for 8 weeks. The lack of significant change in microbial diversity associated with omega-3 PUFA intervention is consistent with mouse studies, in which there was either no change,[10] or a small change,[9] in α diversity. Our data are also consistent with the concept that short-term dietary interventions do not overcome the dominant interindividual variation in the intestinal microbiome.[27]

The increased abundance of butyrate-producing, so-called 'beneficial', bacterial genera such as *Bifidobacterium*, *Lachnospira*, *Roseburia* and *Lactobacillus* during one or both types of omega-3 PUFA intervention is consistent with the mouse literature.[9 10 25 26 28–30] Whether the relatively small changes in intestinal microbiome that we observed at the end of the 8-week intervention period have functional consequences, including an increase in luminal SCFA levels, is a question that will require a metabolomic approach, including lipidomic profiling. The increase in *Roseburia* and *Lachnospira* was only observed during the drink intervention. Further studies are required to determine what aspect of the drink formulation may explain this difference.

Studies of CRC and colorectal adenoma patients have reported a reduction in OTUs of SCFA-producing bacteria compared with healthy controls.[7 31–33] An increase in

SCFA-producing bacteria such as *Bifidobacterium*, leading to enhanced mucosal SCFA exposure has been suggested to reduce mucosal inflammatory tone.[34] Therefore, our findings are compatible with a hypothesis that omega-3 PUFA intake is associated with intestinal microbiota changes driving increased luminal SCFA exposure. In future studies, it will be key to investigate the effect of omega-3 PUFAs on the colonic metabolome in concert with dietary fibre assessment. Consistent with an interaction between omega-3 PUFAs and fibre, Crim *et al* have reported that fish oil and dietary pectin have synergistic antineoplastic activity in rats.[35]

We did not observe any relationship between intestinal microbiome changes and RBC omega-3 PUFA levels (a measure of systemic omega-3 PUFA exposure). Therefore, we do not provide any evidence that systemic 'bioavailability' of omega-3 PUFAs was modulated by the intestinal microbiota in our study. However, mouse studies have demonstrated that supplementation with *Bifidobacterium breve* was associated with increased tissue EPA and DHA content, suggesting an influence of intestinal microbiota on tissue fatty acid levels.[36] It remains unclear whether RBC omega-3 PUFA levels after oral omega-3 PUFA supplementation predict omega-3 PUFA exposure in the gut lumen. In a study of patients (n=6) with a permanent ileostomy and minimal terminal ileum removed, less than 1% of the total oral omega-3 PUFA dose was recovered in ileal effluent suggesting efficient proximal small intestinal absorption.[37] However, the daily dose of EPA and DHA was less than 300 mg for 4 days only.[37] It remains unclear what the small intestinal and colonic bioavailability of omega-3 PUFAs is at oral dosing ≥2000 mg daily.[5 6]

A randomised trial of control versus sardine diet (100 g sardines for 5 days per week for 6 months providing approximately 3 g daily of EPA and DHA combined) in type II diabetes patients reported a significant decrease in *F:B* ratio in the sardine diet arm compared with controls.[38] We did not observe a significant change in the *F:B* ratio in our study, which may be explained by differences in omega-3 PUFA delivery and duration of the intervention.

RBC fatty acid changes were similar during either the drink or capsule intervention with a significant increase in RBC EPA and DHA (and a parallel decrease in AA content) consistent with dosing at 4 g per day for 8 weeks.[39] It is not clear why the drink intervention was associated with a larger decrease in relative AA content, which led to a significantly higher EPA+DHA:AA ratio. We acknowledge that despite daily dose equivalence of EPA and DHA between drink and capsule interventions, the omega-3 PUFAs were triglyceride and ethyl ester conjugates, respectively. In the longest comparative study of capsules providing 1.68 g EPA/DHA per day (6 months), the RBC omega-3 index was higher after consumption of omega-3 PUFA triglycerides compared with ethyl esters.[40] Our study suggests that, at high doses (4 g) over several weeks, possible small differences in intestinal bioavailability related to PUFA delivery are not relevant to omega-3 PUFA tissue incorporation and subsequent changes in the intestinal microbiota. Further investigation of the effect of the other macronutrients (eg, fibre) and micronutrients (eg, vitamin D) in the Smartfish intervention on tissue PUFA incorporation and metabolism is required.

Smartfish Remune drinks providing 4 g per day of EPA and DHA combined were well tolerated by healthy middle-aged individuals. Treatment-emergent AEs were similar between drinks and capsules with a suggestion that upper GI symptoms were more common during the capsule intervention. An AE occurring during one intervention did not consistently predict occurrence during the other intervention. The excellent acceptability and tolerability, aligned with good omega-3 PUFA absorption characteristics, mirrors previous experience with an earlier version of the Smartfish drink.[41] The aetiology of the dose-dependent diarrhoea caused by omega-3 PUFA intake in up to 5%–10% of individuals remains unclear. We describe, for the first time, that diarrhoea associated with omega-3 PUFA intake is not associated with a significant change in intestinal microbiota or predicted by a particular baseline intestinal microbiome profile.

The strengths of this study include the cross-over design that allowed a direct comparison between two omega-3 PUFA formulations, thus minimising the effect of interindividual variability in omega-3 PUFA incorporation[42] and intestinal microbiome profile,[43] as well as the middle-aged demographic of the study cohort relevant to CRC prevention. A methodological weakness was the lack of colorectal tissue to study the effect of omega-3 PUFA treatment on the mucosa-associated microbiome. This would be particularly relevant if luminal bioavailability of oral omega-3 PUFAs is confirmed to be low and omega-3 PUFA 'exposure' is predominantly via membrane fatty acids from surface and/or shed enterocytes. We also did not obtain data on the background diet and dietary omega-3 PUFA intake.

In summary, we report that a high dose (4 g daily) of mixed omega-3 PUFAs (EPA and DHA) given for 8 weeks is associated with small changes in the intestinal microbiota that are consistent across two different omega-3 PUFA interventions.[9 10 25–28] The increase in density of bacteria known to be butyrate producers concurs with the existing preclinical literature and is compatible with the known anti-inflammatory and antineoplastic properties of omega-3 PUFAs.

Contributors HW and FCC performed the randomised study and collected samples. HW, SM, MT, HMW, SP, JAS and PL analysed samples and contributed to data analysis. MH, LD, CLL, GJT and PQ designed the study and obtained funding. MH and SM wrote the paper, which was reviewed and approved by all authors.

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Competing interests MH acts as a consultant advisor for Thetis Pharmaceuticals.

Ethics approval South Yorkshire research ethics committee.

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GUT

A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota

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Measurement of red blood cell eicosapentaenoic acid (EPA) levels in a randomised trial of EPA in patients with colorectal cancer liver metastases☆

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ABSTRACT

We investigated red blood cell (RBC) PUFA profiles, and the predictive value of RBC EPA content for tumour EPA exposure and clinical outcomes, in the EMT study, a randomised trial of EPA in patients awaiting colorectal cancer (CRC) liver metastasis surgery (Cockbain et al., 2014) [8]. There was a significant increase in RBC EPA in the EPA group (n=43; median intervention 30 days; mean absolute 1.26[± 0.14]% increase; $P < 0.001$), but not in the placebo arm (n=45). EPA incorporation varied widely in EPA users and was not explained by treatment duration or compliance. There was little evidence of 'contamination' in the placebo group. The EPA level predicted tumour EPA content (r=0.36; P=0.03). Participants with post-treatment EPA≥1.22% (n=49) had improved OS compared with EPA < 1.22% (n=29; HR 0.42[95%CI 0.16–0.95]). RBC EPA content should be evaluated as a biomarker of tumour exposure and clinical outcomes in future EPA trials in CRC patients.

1. Introduction

The clinical efficacy of omega-3 polyunsaturated fatty acids (PUFAs) has been tested in randomised, double-blind, placebo-controlled trials in multiple adult healthcare settings including studies of cardiovascular disease [1], inflammatory bowel disease [2], non-alcoholic fatty liver disease [3] and cancer [4,5]. However, despite the widespread availability of mass spectrometric techniques for measuring PUFAs, only a minority of clinical omega-3 PUFA studies with a primary or secondary clinical endpoint have reported target organ PUFA incorporation and/or individual blood PUFA profiles [3,5,6], with which to interpret PUFA 'bioavailability' (a term usually used in omega-3 PUFA studies to indicate the tissue or blood level of omega-3 PUFAs), compliance and 'contamination' by excess 'own use' of the intervention or dietary omega-3 PUFA intake, the latter being a particular threat to correct interpretation of trial data and the statistical power of a trial for any substance that is easily available 'over the counter' or as a dietary constituent [7].

We have previously reported the results of a Phase II randomised, double-blind, placebo-controlled trial of 99% pure eicosapentaenoic acid (EPA) in the free fatty acid (FFA) form in patients undergoing liver resection surgery for colorectal cancer liver metastasis (CRCLM), called the EMT study [8]. In this 'window of opportunity' trial, gastro-resistant EPA-FFA or identical placebo capsules were taken for a variable amount of time between the decision to undergo surgery and liver resection, at which time the trial intervention was stopped. We demonstrated that EPA-FFA 2 g daily before liver surgery was safe and well-tolerated. Analysis of resected CRCLM tissue confirmed that orally administered EPA was incorporated into target tumour tissue and was associated with a reduction in tumour vascularity as measured by CD31-positive microvessel density [8]. An intriguing preliminary observation was that EPA may provide prolonged overall survival (OS) and disease-free survival (DFS) benefit after treatment cessation following liver surgery [8].

A methodological limitation of the trial was that we were only able to obtain CRCLM tissue at surgery after EPA treatment, thus excluding any longitudinal analysis of PUFA incorporation and 'washout' in target CRCLM tissue. However, we obtained blood from participants at randomisation (baseline), the day prior to liver surgery (post-treatment), and several weeks after surgery ('washout'). Red blood cell

Abbreviations: AA, arachidonic acid; CRC, colorectal cancer; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LM, liver metastasis; OS, overall survival; PUFA, polyunsaturated fatty acid; RBC, red blood cell

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(RBC) membrane omega-3 PUFA content is widely accepted as the best surrogate biomarker of omega-3 PUFA content in other tissues such as the heart and liver [9,10].

Therefore, we analysed the RBC membrane omega-3 PUFA content at baseline, post-treatment and at follow-up after surgery in the EMT study, allowing us to interpret individual PUFA content profiles in both active (EPA) and placebo groups, correlate RBC PUFA content with CRCLM levels, and explore the use of RBC EPA content as a predictor of survival in patients who have undergone liver surgery for CRCLM.

2. Methods

2.1. The EMT study

This Phase II randomised, double-blind, placebo-controlled trial (ClinicalTrials.gov NCT01070355) has been described in detail previously [8]. Participants were randomised to EPA-FFA 2 g taken as two gastro-resistant capsules twice daily with food for a median (range) duration of 30 (12–65) days (n=43) or placebo (capric and caprylic acid triglycerides) capsules for a median 26 (15–73) days (n=45). Blood samples were obtained at randomisation (baseline; n=87), the day prior to liver surgery (post-treatment; n=79), and approximately six weeks (EPA median [range] 47 [16–110] days; placebo 44 [21–68]) after surgery (washout; n=70). Participants were stratified by prior fish oil supplement use and/or high dietary (>2 oily fish portions per week) omega-3 PUFA intake. We have previously reported that dietary omega-3 PUFA intake did not change significantly in either treatment group during the trial as measured by a modified validated food frequency questionnaire [8]. Percentage compliance with capsules was calculated from the difference in actual and expected capsules use based on a capsule count at the trial visit immediately prior to surgery. Participants were all given capsules in slight excess of requirements and were admitted for surgery at different times after randomisation. A lower than expected capsule count before surgery is expressed as 'compliance' >100%.

2.2. PUFA measurement

Blood was collected in two EDTA-coated VACUETTE® tubes on ice and the RBC slurry was obtained after centrifugation at 700*g* for 10 min at 4 °C within three hours of venipuncture. Isolated RBCs were stored at −80 °C until fatty acid extraction.

Fatty acids were extracted from erythrocytes using the following method adapted from the protocol first published by Rose et al. [11]. Isolated erythrocytes were washed three times with 5 volumes of 0.89% Sodium chloride (NaCl), before transferring 50 μL into a 1.7 mL Eppendorf® tube. Erythrocytes were mixed with 50 μL distilled water and allowed to stand for 15 min. 550 μL isopropanol was added slowly with mixing, and following incubation for 1 h at room temperature, 350 μL chloroform was added. After a further 1 h incubation, samples were centrifuged at 10 000*g* for 5 min. The supernatant was then evaporated in a rotary evaporator and reconstituted in 500 μL acetonitrile. Hydrolysis was performed with the addition of 50 μL 5 M HCl followed by incubation at 80 °C for 1 h. 50 μL of 5 M NaOH was then added, and the sample mixed, before addition of 350 μL chloroform. The sample was left to stand for 5 min to allow the solvent layers to separate, before taking the top 800 μL and evaporating in a rotary evaporator. Extracted fatty acids were then reconstituted in 50 μL methanol prior to derivatization.

We measured FAs by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [12]. The following FAs were measured with the relative level of each PUFA expressed as the % of the total FA content: alpha-linolenic acid (C18:3ω3), EPA (C20:5ω3), docosapentaenoic acid (DPA; C22:5ω3), docosahexaenoic acid (DHA; C22:6ω3), linoleic acid (C18:2ω6), arachidonic acid (AA;C20:4ω6), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1ω9). Tumour tissue PUFA content was measured previously and has been reported in the EMT study publication [8].

2.3. Statistical analysis

PUFA data are quoted as the mean (±standard error of the mean [SEM]) relative (%) amount of each PUFA compared with the total fatty acid content measured by LC-MS/MS. Individual absolute and fold changes in PUFA content over time were compared using the one-sample *T* test. Pairwise differences in relative PUFA content over time were analysed by the paired-sample T test. Data that were not normally distributed were analysed by the Mann Whitney U test. Pearson's correlation test was used to explore the relationship between continuous variables including RBC and tumour % PUFA content. Comparison of OS in trial participants with a % RBC EPA level or 1.22 (the lowest post-treatment % EPA level in the EPA group) was performed using Kaplan-Meier survival analysis and log rank test.

3. Results

3.1. Time course of RBC EPA incorporation and 'washout' in CRCLM patients

Baseline % RBC PUFA levels in patients with CRCLM are displayed in Fig. 1. There was a rise in RBC EPA content in patients randomised to EPA-FFA treatment with a mean absolute 1.26 (±0.14 [SEM])% increase during treatment ($P < 0.001$, one-sample T test; Figs. 1A and 2A), which was not observed in the placebo group (−0.03 ± 0.04%; Figs. 1A and 2B). This equated to a mean 2.40 (±0.16)-fold increase in RBC EPA content during EPA treatment (placebo 1.02 ± 0.04 fold change). There was also a smaller increase in RBC DPA content (mean 0.60 ± 0.18[SEM] absolute increase; $P < 0.05$; Figs. 1B and 2C). A small, but statistically significant, reduction in relative DHA content in RBC membranes (mean absolute 0.31 ± 0.24% reduction) was also seen following EPA treatment (Fig. 1C). There was a concurrent, statistically significant decrease in omega-6 PUFA AA content (mean absolute 1.31 ± 0.57% reduction) in those individuals receiving EPA ($P < 0.05$; Figs. 1D and 2G). The EPA/AA ratio is widely quoted as a predictive biomarker of omega-3 PUFA activity, particularly in cardiology studies [13]. There was a mean absolute increase in EPA/AA ratio of 0.12 (SEM 0.01) at the end of the intervention period in those allocated to EPA with RBC EPA/AA ratio values at the end of the intervention period ranging between 0.09 and 0.37 (mean 0.21).

There was no overall change in % RBC content of any PUFA in the placebo group (Figs. 1 and 2B, D, F and H). For each PUFA, there was significant, but incomplete, reversal of the changes in PUFA content related to EPA treatment during the post-intervention 'washout' period (Figs. 1 and 2A, C, E and G). A persistent, small elevation in RBC EPA and DPA content was observed at six weeks after cessation of EPA treatment (Fig. 1A and B), along with reduced AA content (Fig. 1D).

Individual RBC PUFA content profiles varied significantly between individuals in both EPA and placebo groups (Fig. 2A–H). Previous fish oil supplement users (who were required to stop this for the duration of the trial) and those with high dietary omega-3 PUFA intake (n=23) had a significantly higher baseline % RBC EPA content (1.39 ± 0.15%) than participants 'naïve' to both (0.97 ± 0.04%; n=64; P=0.003). The four participants in the placebo arm with the highest baseline % EPA content (all >2%) were all fish oil supplement users or those classified as having a high dietary omega-3 PUFA intake (Fig. 2B). All these individuals that supplied blood samples after baseline displayed a reduction in RBC EPA content during the intervention period consistent with adherence to the protocol, which demanded cessation of existing omega-3 PUFA supplement use (Fig. 2B). Thirty-three out of 36 (92%) participants, who received EPA and for whom data from all three time-points were available, demonstrated an increase in RBC EPA content during the intervention period, which then reduced after

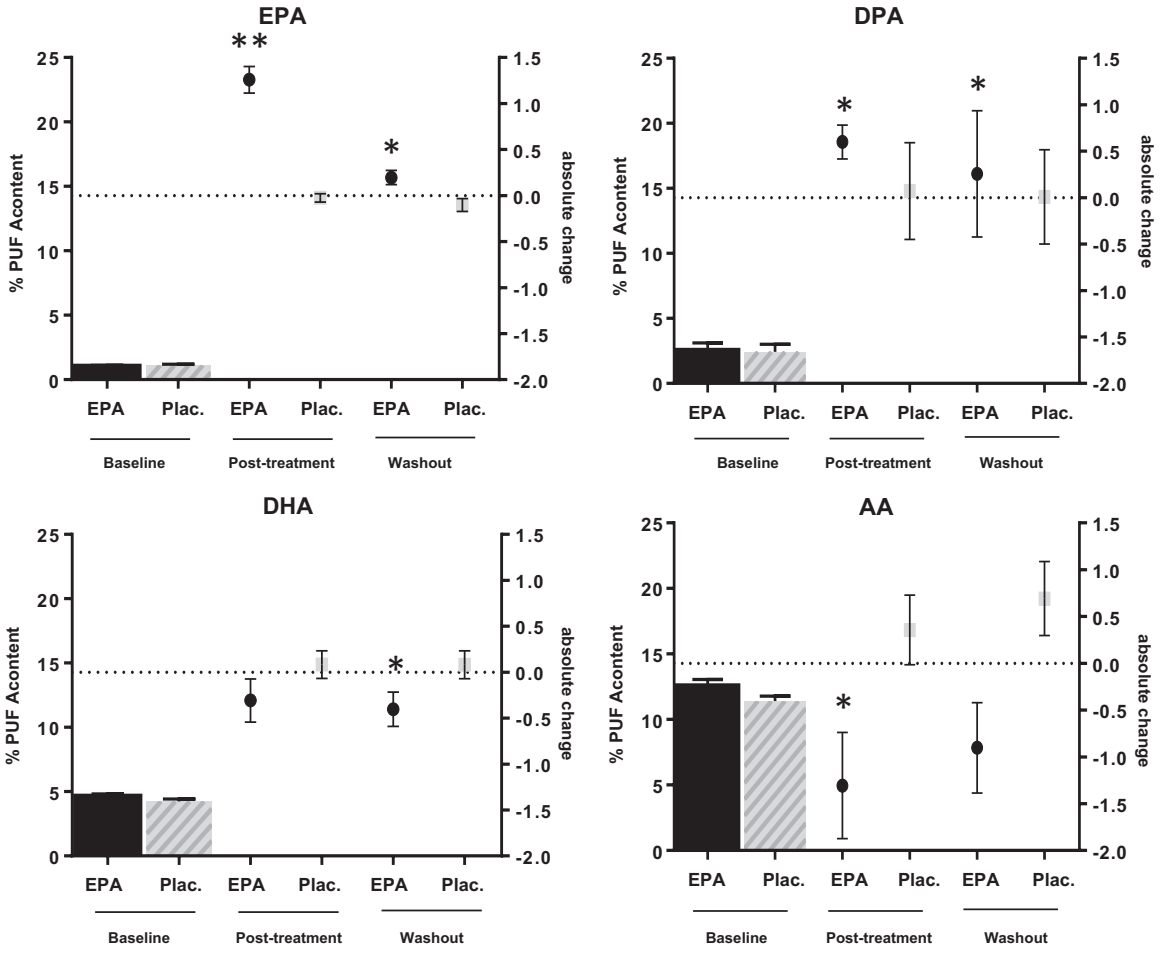

Fig. 1. Baseline % RBC PUFA level and absolute difference in % RBC PUFA level between baseline and post-treatment or after surgery (washout). In each case (A–D), the left y axis is the baseline % RBC PUFA value and the right y axis is the absolute difference between the post-treatment value or 'washout' post-operative value and baseline % level. Columns (baseline % values) and symbols (absolute difference in % value from baseline) denote the mean for EPA and placebo (Plac.) groups. Error bars denote the standard error of the mean.*P < 0.05, **P < 0.001; one-sample *t* test.

treatment cessation (Fig. 2A). This suggests excellent compliance with trial treatment. However, the magnitude of the EPA 'response' varied widely with only 20 individuals displaying a %EPA RBC content of greater than 2% (Fig. 2A).

There was no statistically significant correlation between the % RBC EPA content at the end of the intervention period and the duration of EPA treatment (r=0.25; P=0.12; Fig. 3A). The post-treatment absolute EPA level attained in females (mean 2.68 ± 0.23%; n=16) was greater than in male participants (mean absolute 2.07 ± 0.0.14%; n=23) in the EPA group (P=0.02). There was no difference in baseline or post-intervention RBC PUFA levels in concurrent aspirin users (n=9) compared with non-users in the EPA group (data not shown). 'Compliance' with capsules measured by 'pill counting' in both active and placebo groups was good and has been reported previously [8]. Mean compliance in the EPA group was 89.5 ± 2.9% (range 40.9–116.7). There was no significant correlation between % compliance and post-treatment % RBC EPA level (r=0.09; P=0.59) in EPA users. Multivariate linear regression inputting gender, treatment duration and compliance confirmed that gender alone (beta −0.35, P=0.039) remained the only significant predictor of post-treatment RBC EPA level in the EPA treatment group.

Of note, the individual with the largest increase in RBC EPA content (gold symbols and line) also demonstrated a marked increase in DPA and DHA, but also AA (Fig. 2A, C, E and G). The most likely explanation is 'contamination' by concurrent use of a mixed omega-3 and omega-6 PUFA supplement, many of which are available commercially. Only one individual allocated to placebo demonstrated an increase in RBC EPA content over time (yellow crosses and line), also suggesting possible 'contamination' by 'own' omega-3 PUFA use.

3.2. Relative RBC EPA content predicts the CRCLM tissue EPA level

Measurement of the % RBC EPA content the day before surgery allowed a pairwise comparison of RBC EPA content and the corresponding tumour EPA level at CRCLM resection (Fig. 3B). There was a significant correlation between the % RBC EPA level just prior to surgery and the CRCLM EPA content in those participants who received EPA (r=0.35, P=0.03) and placebo (r=0.72; P < 0.001: Fig. 3B). Interestingly, those individuals in the placebo group with relatively high % RBC EPA content, as a consequence of prior omega-3 PUFA use or high dietary intake, had a correspondingly high tumour EPA content (Fig. 3B).

3.3. RBC EPA incorporation and long-term clinical outcomes

Long-term clinical outcomes were an exploratory end-point in the Phase II EMT study [8]. A preliminary finding was that those patients who were randomised to EPA treatment had OS benefit compared with placebo, although the difference was not statistically significant in this small randomised trial [8]. On the basis that the lowest post-treatment % RBC EPA level attained in the EPA group was 1.22%, which is higher than all the respective placebo group values except for 10 individuals (6 of whom had high baseline levels due to significant prior dietary or supplement omega-3 PUFA exposure), we compared OS in individuals

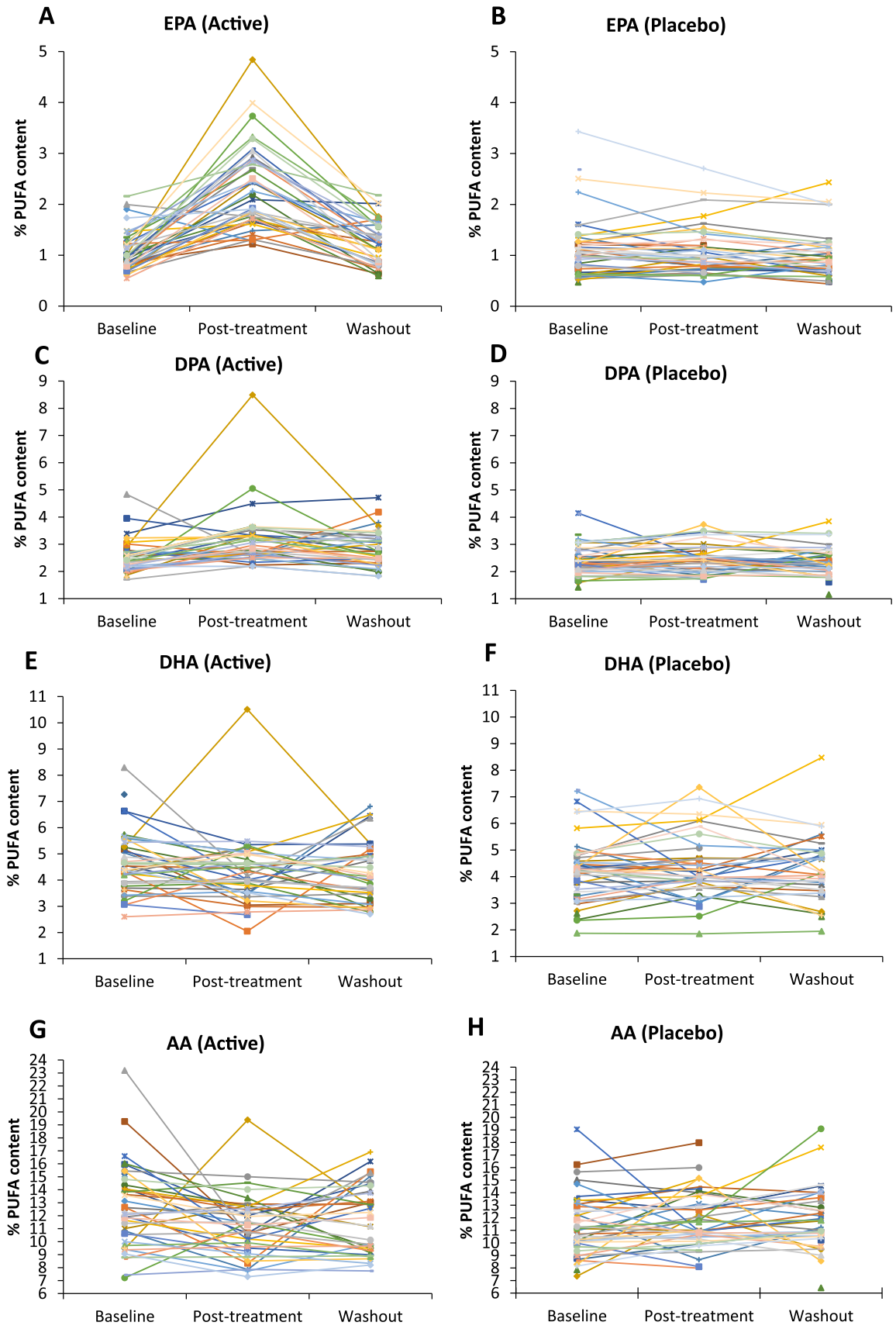

Fig. 2. Individual % RBC PUFA profiles for individuals randomised to either EPA (A, C, E, G) or placebo (B, D, F, H). Different coloured lines join data points for all individuals.

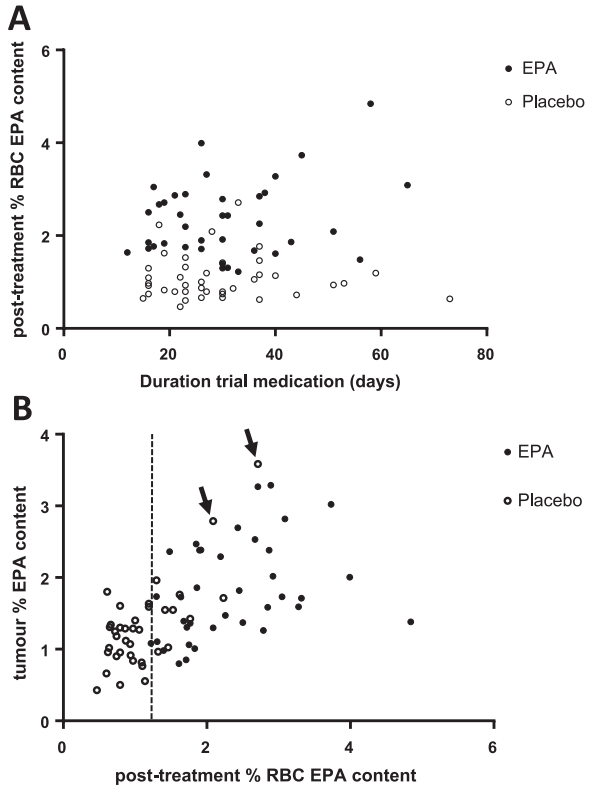

Fig. 3. Relationship between the % RBC EPA level at the end of the trial intervention and either treatment duration (A) or tumour EPA content at surgery (B). Open symbols denote individual data from the placebo group and filled symbols denote EPA group data. B) The dashed line denotes the RBC % EPA 'cut-off' (1.22 for survival analysis). Arrows denote examples of individual patient data in prior omega-3 PUFA supplement users allocated to placebo, who had high tumour % EPA content.

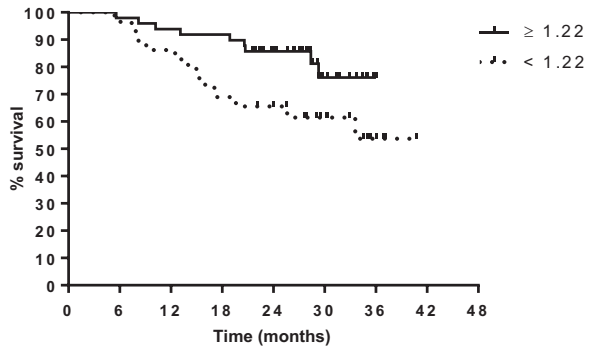

Fig. 4. Overall survival analysis of CRCLM patients stratified on the basis of post-treatment % RBC EPA level, not treatment allocation. The cut-off of 1.22% was based on the lowest value indicative of an EPA 'response' (evidenced by an increase in EPA level from baseline to post-treatment measurement and then 'washout') in the EPA group. Vertical ticks denote censored cases. Solid line denotes events in EPA 'incorporators' (post-treatment RBC % EPA level≥1.22%. Dashed line denotes events in EPA 'non-incorporators' (post-treatment RBC % EPA level < 1.22%. Log rank P=0.04.

with a post-treatment % RBC EPA content of ≥1.22% with those with a post-treatment RBC EPA content of < 1.22%, regardless of treatment allocation in the trial. Participants with a post-treatment % RBC EPA content of ≥1.22% (n=49) demonstrated improved OS compared with those with a RBC EPA level < 1.22% (n=29; Fig. 4). The hazard ratio was 0.42 (95% confidence interval 0.16–0.95) with a log rank P value of 0.04.

4. Discussion

This is the first time that individual RBC and tumour PUFA content values have been analysed together in a randomised, placebo-controlled trial of an omega-3 PUFA in cancer patients. We measured % RBC omega-3 PUFA content as the biomarker of choice, with least within-subject variability, for assessment of omega-3 PUFA 'bioavailability' in a clinical study lasting several weeks [9,10].

We report that RBC EPA incorporation predicts CRCLM EPA content and therefore has potential as a surrogate biomarker of short-term target organ EPA exposure, at least in CRCLM. Consistent with this finding, RBC and cardiac/breast tissue EPA content correlate strongly after omega-3 PUFA supplementation in humans [14–16], and RBC omega-3 PUFA levels correlate with omega-3 PUFA content in multiple mouse and rat tissues after dietary supplementation [17,18].

Baseline and post-treatment RBC PUFA levels in EMT study participants were similar to those reported previously in studies of omega-3 PUFA supplementation in healthy volunteers [13,19], women with breast hyperplasia [20,21] and in patients with lung cancer [22]. In particular, post-treatment % RBC EPA values were similar to those observed in an eight-week study of exactly the same EPA-FFA formulation and dose in healthy volunteers and inflammatory bowel disease patients [23]. In keeping with previous studies of pure EPA treatment, there was some evidence of elongation of EPA to DPA leading to a small, statistically insignificant increase in RBC DPA incorporation [8,23,24]. However, there was no evidence of DHA incorporation in RBC membranes, with an actual small reduction in % DHA content in EPA users, unlike the previous study with EPA-FFA [23]. This may be explained by the shorter duration of EPA supplementation in the EMT study, which may lead to displacement of membrane DHA by excess EPA-FFA, prior to *de novo* synthesis of DHA from EPA. Alternatively, it is understood that excess EPA may saturate the second *ELOVL2* elongase reaction necessary for DPA to DHA conversion, which could lead to a reduced relative DHA content [24].

The literature on omega-3 PUFA supplementation in healthy volunteers and patient groups would suggest that omega-3 PUFA incorporation is associated with a reciprocal reduction in membrane content of the omega-6 PUFA counterpart to EPA, AA [19–21,23,25,26]. We also observed this phenomenon in the EMT study, despite the relatively short duration of EPA supplementation.

Overall, there was consistent and uniform, but incomplete, EPA 'washout' over the relatively short time period between surgery and post-operative follow-up mandated in the trial. Longer-term follow-up would have been required in order to confirm the return of the PUFA profile in RBCs to that observed pre-intervention. 'Washout' kinetics were not dependent on the prior degree of RBC incorporation or duration of EPA treatment and are similar (in these CRC patients) to the decay in RBC EPA content observed in at least three healthy volunteer studies, in which peak RBC EPA content was similar to that seen in the EMT study [13,19,27], as well as two more recent studies of pre- and post-menopausal women [20,21].

Individual omega-3 PUFA profiles allowed us to investigate inter-patient variability in EPA incorporation in CRC patients. There was significant heterogeneity in the increase in EPA content of RBC membranes in response to EPA treatment, which has previously been noted in intervention studies of daily supplementation with combined EPA and DHA formulations (measured as the omega-3 index) in atherosclerotic patients [28,29], but not cancer patients. This is in contrast to the more uniform incorporation of EPA into RBC membranes observed in the healthy volunteer study of EPA-FFA by Scaioli et al. [23]. A similar degree of inter-individual variability in plasma phospholipid DHA level was observed in a DHA intervention study in breast cancer patients [30].

There was a small but statistically significant difference in RBC EPA incorporation in women compared with men in the EPA group, which remained even after adjustment for treatment duration and compliance. A meta-analysis of gender differences in omega-3 PUFA levels in RBC membranes has previously reported a significantly higher DHA,

but not EPA, content in RBCs in women compared with men [29]. However, a previous supplementation study, which undertook a gender-specific analysis, reported on gender difference in omega-3 index [31].

Individual omega-3 PUFA profiles also highlighted possible cases of 'contamination' by prior or concurrent omega-3 PUFA intake separate from the Investigational Medicinal Product. This was most easily observed in several instances in the placebo arm, in which a concurrent increase in DHA (but not DPA) suggested use of a combined EPA/DHA omega-3 PUFA formulation in addition to placebo. 'Contamination' by extra omega-3 PUFA use may also explain the high EPA and DHA 'outlier' in the EPA arm of the trial although the concurrent high RBC DPA content may signal that this individual was, in fact, capable of extremely efficient EPA-DPA-DHA conversion. There is increasing understanding of the genetic basis of inter-individual variability in omega-3 PUFA interconversion, underlying which genetic polymorphisms in the *FADS1-FADS2* cluster and *ELOVL2/5* genes modulate desaturase and elongase activities [32].

Placebo group contamination is an ever-present risk in a randomised trial of any intervention available 'over the counter' [33], particularly when fish oil use is so widespread (7.8% of US adults in 2012 [34]). However, contamination by omega-3 PUFA use in the placebo arm (suggested by an increase in omega-3 PUFA RBC content during the intervention period) appeared relatively low, occurring in perhaps 4 (9%) of 45 individuals allocated to placebo, and possibly in a single case (2%) in the EPA group. Therefore, contamination by additional omega-3 PUFA use is unlikely to have affected significantly the laboratory and clinical endpoints of the EMT study.

There has been only one previous randomised omega-3 PUFA intervention trial in cancer patients that has reported individual omega-3 PUFA levels during treatment [6] and none that have included an analysis of omega-3 PUFA 'washout' after cessation of omega-3 PUFA supplementation. Van der Meij et al. described variability in plasma phospholipid EPA levels after double-blind intervention for 5 weeks with a daily supplement containing 2 g EPA and 0.9 g DHA or isocaloric control in patients with non-small cell lung cancer [6]. These authors obtained similar individual EPA profiles to the EMT study with a number of 'non-responders' in the intervention arm and, conversely, some individuals with high baseline EPA values or an 'EPA response' in the control arm of the study [6]. We suggest that contamination is likely to occur in all trials of omega-3 PUFA supplements with individual randomisation and can be, at least partly, adjusted for by analysis by absolute or relative change in RBC omega-3 PUFA levels.

To the best of our knowledge, survival analysis based on EPA incorporation in RBCs, as a surrogate biomarker of tumour EPA exposure, has not been reported previously. Despite the relatively small sample size of the EMT trial, there was a statistically significant difference between individuals dichotomised on the basis of the RBC EPA content at the end of the intervention period (irrespective of the trial treatment allocation). Effectively, this moved those individuals in the placebo group with relatively high RBC EPA levels (explained mainly by high dietary marine omega-3 PUFA intake or prior supplement use) into the active (EPA) group. A methodological weakness of this approach is the rather arbitrary cut-off value, which, in this case, was selected as the lowest relative RBC EPA level in an 'EPA-responder' (increase in EPA content during intervention and then reduction during 'washout'). However, the EPA value used (1.22%) was similar to equivalent values used in previous cohort and case-control studies of the association between omega-3 PUFA levels and CRC risk [35], in particular the study of Cottet et al. which measured RBC EPA content [36]. Moreover, in the absence of a widely recognised, standardized method for measurement of RBC fatty acid content, we used a LC-MS/MS method, thus restricting future comparison with other studies.

We suggest that a so-called 'contamination-adjusted' secondary analysis [7] should be undertaken in future randomised, double-blind trials of omega-3 PUFAs in which PUFA levels are measured. Such an approach was recently taken in the WELCOME study of non-alcoholic fatty liver disease patients, in which placebo- and active-group contamination occurred to a similar extent to the EMT study [3].

In conclusion, we report significant variability in RBC omega-3 PUFA profiles during a randomised trial of EPA in patients with CRCLM awaiting liver resection. There was evidence of contamination by 'own use' omega-3 PUFA in only a small proportion of participants. There was a significant correlation between the RBC EPA content and CRC liver metastasis EPA level measured within 24 h of each other in this EPA intervention trial, implying that the RBC omega-3 PUFA content may reflect tumour omega-3 PUFA exposure, at least in CRCLMs.

A fascinating preliminary observation is that OS analysis based on post-intervention RBC EPA content, rather than treatment allocation, suggested OS benefit in those individuals with a high relative EPA level. This finding adds further to the rationale for a phase III omega-3 PUFA intervention trial in CRCLM patients. Consistent with the hypothesis that EPA therapy may provide survival benefit in this group of patients, Song et al. have recently reported that higher marine omega-3 PUFA intake is associated with reduced post-diagnosis CRC mortality in two US cohort studies [37].

Conflict of interest statement

Mark Hull has received a travel grant and unrestricted scientific grant from SLA Pharma AG. Mark Hull has received an unrestricted scientific grant from Smartfish®. Mark Hull is a member of the Scientific Advisory Board for Thetis Pharmaceuticals LLC. None of the other Authors declares a potential Conflict of Interest.

Author contributions

AJC, GJT and MAH designed the EMT study and PUFA analysis; HW, JS, AR, MV and PML conducted the research; HW and MAH analysed data; HW and MAH wrote the manuscript; MAH has primary responsibility for final content. All Authors read and approved the final manuscript.

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