Intestinal bioavailability of marine omega-3 polyunsaturated fatty acids and associated changes to the gut microbiota.

Gaël Romuald Nana

Submitted in accordance with the requirements for the degree of

Doctor of Medicine

The University of Leeds

Leeds Institute of Medicine and Research

August 2025

The candidate confirms that the work submitted is his/her own and that appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

This research has been carried out by a team that included Mitra S, Watson H, Young C, Wood HM, Perry SL, Race AD, Quirke P, Toogood GJ, Loadman PM and Hull MA. My own contributions, fully and explicitly indicated in the thesis, have been as follows: designing the studies, obtaining ethics approval, Participant recruitment, sample collection and laboratory methods, result analysis, writing and publishing the paper and thesis.

The other members of the group and their contributions have been as follows:

S. Mitra, H Wood, C Young – microbiome analysis, data handling and interpretation

H Watson – designed, obtained ethics approval and carried out the study to obtain faecal samples from volunteers.

AD Race, PM Loadman – Fatty acid measurements and analysis

P Quirke, G Toogood – obtained funding, designed the study

MA Hull – formulated the research question, supervised the study design, data collection, analysis and interpretation and revised the published manuscripts and thesis.

Published work from this thesis:

Nana G, Mitra S, Watson H, Young C, Wood HM, Perry SL, Race AD, Quirke P, Toogood GJ, Loadman PM, Hull MA. *Luminal Bioavailability of Orally Administered ω-3 PUFAs in the Distal Small Intestine, and Associated Changes to the Ileal Microbiome, in Humans with a Temporary Ileostomy.* J Nutr. 2021 Aug 7;151(8):2142-2152. doi: 10.1093/jn/nxab113. PMID: 34036331

Oral Presentation - United European Gastroenterology Conference in Barcelona, Spain - October 2019

Abstract

Omega-3 polyunsaturated fatty acids (omega-3 PUFAs) have anti-colorectal cancer properties. The mechanistic pathways by which oral omega-3 PUFA supplementation influences bioavailability along the gut-blood axis remain poorly understood. This thesis aimed to quantify the concentrations/abundance of omega-3 PUFAs in the small intestine and colonic lumina, assess their association with tissue incorporation and evaluate changes in the distal ileal microbiome.

In a clinical study, ileostomy and blood samples were collected in 11 human volunteers with an ileostomy at baseline, after the first oral dose and after the final oral dose of 4g of mixed EPA/DHA 1:1 capsules for a minimum of 28 days. Ileal fluid omega-3 PUFAs concentrations and microbiome changes were measured.

A secondary analysis was conducted on a published cross-over trial in which participants were randomised to 8 weeks' supplementation with 4g mixed EPA/DHA in capsule or drink formulation, separated by a 12 week 'washout' period. Faecal EPA and DHA abundance and its correlation with red cell membrane (rbc) omega-3 PUFA were explored.

Oral supplementation with omega-3 PUFAs for ≥28 days results in an increase in Ileostomy fluid (IF) EPA/DHA, returning to baseline on cessation. There was a statistically significant association with increased rbc EPA/DHA($p<0.05$). There was a positive correlation with increased abundance of *Bacteroides* in the ileal lumen. Secondary analysis of the cross-over trial showed a rise in faecal EPA /DHA with supplementation irrespective of formulation, although capsules reached statistical significance. There was a weak negative correlation between the rise in faecal and rbc EPA/DHA. There was greater individual variability with capsules compared to drinks, but no statistically significant difference between the absolute change from baseline with either formulation.

This integration of luminal, systemic biomarkers and microbiome shifts, offers novel insights on mechanistic pathways of omega-3 PUFA activity. EPA/DHA concentrations could inform future studies with gut fermentation models.

Table of contents

List of Figures

List of Tables

List of abbreviations

Abbreviation	Full term
°C	Degrees Celsius
AA	Arachidonic acid
ALA	Alpha linoleic acid
Bft	*Bacteroides fragilis* toxin
CIMP	CpG island methylator phenotype
CIN	Chromosomal Instability
CRC	Colorectal cancer
CTIMP	Clinical Trial of an Investigational Medicinal Product
DAABD-AE	4-[2-(N,N-Dimethylamino) ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole
dH_2O	Distilled water
DHA	Docosahexaenoic acid
DMAP	4-(Dimethylamino) pyridine
DPA	Docosapentaenoic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl
EE	Ethyl ester
EGFR	Epidermal Growth Factor Receptor
EPA	Eicosapentaenoic acid
EPIC	European Prospective Investigation into Cancer and Nutrition
ETBF	Enterotoxin *Bacteroides fragilis*
ETEs	Epoxyeicosatrienoic acids
FA	Fatty acid
FAP	Familial Adenomatous Polyposis
FATP4	fatty-acid transport protein 4
FDA	Food & Drug Administration
FFA	Free fatty acid

GC-MS	gas chromatography mass-spectrometry
HDAC	histone deacetylase
HETEs	Hydroxyeicosatetraenoic acids
HPETEs	Hydroxyperoxyeicosatetraenoic acids
HPFS	Health Professionals Follow-Up Study
ICT	Institute of Cancer Therapeutics
IF	Ileostomy fluid
IFABP	Intestinal fatty acid binding protein
IL-1	Interleukin – 1
ISRCTN	International Standard Register Clinical/social sTudy Number
ISRCTN	International Standard Register Clinical/social sTudy Number
LA	Linoleic acid
LCMS	Liquid chromatography tandem-mass spectrometry
LIMR	Leeds Institute of Medical Research
MeOH	Methanol
MHRA	Medicines and Healthcare Products Regulatory Authority
MMR	Mismatch Repair
MPA	Mobile Phase A
MPB	Mobile Phase B
MRM	Multiple Reaction Monitoring
MSI	Microsatellite Instability
NHS	Nurses' Health Study
NSAID	Non-steroidal anti-inflammatory drug
O3I	Omega-3 index
OA	Oleic acid
OTUs	Operational taxonomic units
PA	Palmitic acid

PCR	Polymerase Chain Reaction
PGE2	prostaglandin E2
PGE3	prostaglandin E3
pks	Polyketide synthase
PL	Phospholipid
PUFAs	Polyunsaturated fatty acids
Rbc	Red blood cell
RCT	Randomized controlled trial
ROS	Reactive Oxygen Species
RPM	Reps per minute
SA	Stearic acid
SCFA	short-chain fatty acid
TAG	Triacylglycerol
TGF β	Transforming Growth Factor Beta
TNF	Tumour Necrosis Factor
uHPLC	Ultra-high-performance liquid chromatography
VITAL	Vitamin D and Omega-3 Trial
α	Alpha
β	Beta

Chapter 1 Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related death worldwide (Cancer Research UK, 2023). Although screening and endoscopic surveillance form the mainstays of CRC prevention strategies, there is growing evidence that pharmacological interventions may prevent, arrest or reverse carcinogenesis – this is termed chemoprevention or therapeutic prevention (Chapelle et al., 2020). Such chemopreventive agents are usually taken for a long time, thus they need to be easy to administer, with a low side-effect profile, and be cost-effective in the long term.

The inhibition of cyclo-oxygenase-2 and reduction of pro-inflammatory prostaglandin release has been postulated as a mechanism of action of chemopreventive compounds such as non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin, in particular has demonstrated long-term efficacy in CRC prevention (Ghaddaf et al., 2021), and a lower gastrointestinal and cardiovascular side-effect profile, compared to other NSAIDs like rofecoxib (Bresalier et al., 2005) and celecoxib (Bertagnolli et al., 2006).

Other chemopreventive compounds have been studied, either alone or in combination with aspirin. These include calcium and vitamin D (Baron et al., 2015), dietary fibre (Pan et al., 2018), metformin (Ng et al., 2020), and omega-3 polyunsaturated fatty acids (PUFAs) (Hull et al., 2018; West et al., 2010). The latter are of particular interest as observational studies suggest that high dietary intake of fish oils is not only associated with lower incidence of CRC, but may also have a role in the treatment of CRC (Aldoori et al., 2022). The mechanisms for this remain poorly understood but one hypothesis is that oral consumption of high dose omega-3 PUFA alters the abundance of certain bacterial taxa that inhabit the bowel – the gut microbiota – in favour of protective, short-chain fatty acid-producing species (M. Song, Chan, et al., 2020). It is unclear whether this action is either via direct luminal exposure to omega-3 PUFAs in the small and large bowel or systemic incorporation. This thesis will seek to explore this relationship.

1.1 Fatty acids

Fatty acids (FA) are the simplest lipids, building blocks of cell membranes and key substrates for intracellular signal transduction (Berg et al., 2002) These organic compounds consist of a long hydrocarbon (C-C) chain attached to a

hydrophilic carboxyl group (-COOH) at one end and a terminal, hydrophobic, methyl group (-CH_3). Hydrocarbon chains vary in length of carbon atoms linked by single (C-C) or double (C=C) covalent bonds. FAs linked by single bonds are termed saturated e.g. palmitic acid (PA, C16:0) and stearic acid (SA, C18:0) have 16 and 18 carbon atoms respectively, linked by single bonds. Those linked by at least one double bond are termed unsaturated. Unsaturated FAs can have a single double bond in the hydrocarbon chain, e.g. Oleic acid with a double bond at position 9 (OA, C18:1 n-9). Alternatively, when multiple sites of double-bonded carbon atoms exist within a long carbon chain, the FA is called a polyunsaturated fatty acid (PUFA) e.g. docosapentaenoic acid (DPA, 22: 5n-3) has a twenty-two hydrocarbon chain with 5 double bonds, the first occurring at the 3rd carbon atom from the methyl end. The hydrogen atoms attached to the carbon atoms linked by double covalent bonds may be oriented in the same direction (cis-configuration) causing a bend in the FA chain. Alternatively, they may be attached in opposite directions (trans-configuration). Trans fatty acids are commonly associated with an increased risk of atherosclerosis and cardiovascular disease (Kiralan et al., 2021; Russo, 2009).

1.1.1 Chemical structure of omega-3 polyunsaturated fatty acids

The term omega refers to the carbon atom at the methyl end. Therefore an omega-3 PUFA is a long chain FA with cis-double bonds numbered from the 3rd carbon atom from the methyl end (Figure 1). (Khan et al., 2015; Nabavi et al., 2015)

These include:

- Alpha-linolenic acid (ALA, 18:3n-3) - eighteen hydrocarbon chain with 3 double bonds
- Eicosapentaenoic acid (EPA, 20:5n-3) – twenty hydrocarbon chain with 5 double bonds
- Docosapentaenoic acid (DPA, 22: 5n-3) – twenty-two hydrocarbon chain with 5 double bonds
- Docosahexaenoic acid (DHA, 22:6n-3) – twenty-two hydrocarbon chain with 6 double bonds

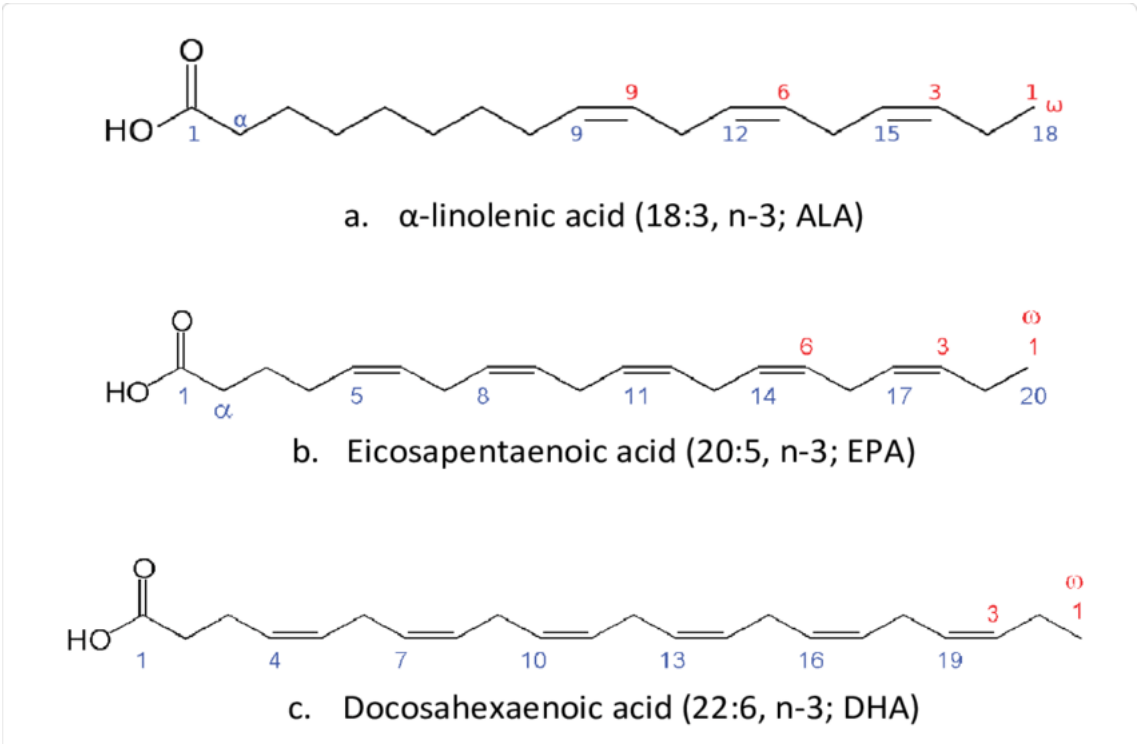


Figure 1: The chemical structure of the omega-3 PUFAs (Khan et al., 2015)

This work focused on those FAs that are of limited production by the human body; thus have to be supplemented through dietary consumption; the so-called essential fatty acids, EPA and DHA (Das, 2006). The other widely studied and biologically relevant group of PUFAs are the omega-6 (n-6) PUFAs including Linoleic acid (LA, 18:2n-6) and Arachidonic acid (AA, 20:4n-6) (Das, 2006; Russo, 2009)

1.1.2 Sources of omega-3 polyunsaturated fatty acids

Different pathways exist for synthesis of essential fatty acids from the parent precursors, ALA and LA.

ALA is abundant in plant oils – canola (9.2%), soy bean (7.8%), hemp oil (20%). Seed oils including perilla, flax and chia seed are also a major source. (Nabavi et al., 2015; Saini & Keum, 2018; Schuchardt & Hahn, 2013).

EPA and DHA are more abundant in wild or cold water fish, such as Antarctic salmon, mackerel, tuna or krill. Hence they are also referred to as marine omega-3 PUFA. Their abundance is a result of the phytoplankton consumed by fish in this environment being rich in EPA and DHA. The higher number of double bonds in the hydrocarbon chains of omega-3 PUFA, lower the melting point and improve

fluidity of the cell membrane in a cold environment. (Pacetti et al., 2013; Saini & Keum, 2018)

Dietary seafood intake is geographically variable: highest in Pacific island nations, Japan, South Korea, the Nordic countries - Denmark, Norway, Greenland and Iceland - while the rest of the world including the UK and North America consumes below 250mg/day (Micha et al., 2014; Stark et al., 2016).

1.2 Oral Supplementation of omega-3 polyunsaturated fatty acids

The UK government recommends that a healthy balanced diet should include at least 2 portions of fish per week, including one of oily fish. A portion of oily fish should be equivalent to at least 140g (NHS, 2022). Despite this, secondary analysis of the 2019 National Diet and Nutrition Survey data revealed PUFA intake was consistently lower than guideline recommendation, with only 7.3% of children, 12.8% of teenagers and 15.6% of young adults achieving the recommended oily fish intake (Derbyshire, 2019). Pregnant women in particular presented some of the largest dietary gaps. Risk of mercury poisoning, availability, cost, smell/taste, veganism all present barriers to consumption of oily fish. Furthermore, concerns regarding sustainable fish farming, oceanic pollutants and microplastics such as methyl mercury or bisphenol A have further reduced consumption of oily fish. (Ragusa et al., 2021; Ramírez et al., 2022). Research has therefore grown into possible alternative sources or delivery of omega-3 PUFA such as microalgae (Derbyshire et al., 2024; Karageorgou et al., 2023). Algal oil, derived from single-celled microalgae species e.g. *Crypthecodinium* contains unmodified triacylglycerol molecules of EPA and DHA. These natural oils must be refined prior to fortification of natural diets. Supplementation has come in the form of oral capsules or emulsions.

1.2.1 Forms and safe dosage of omega-3 polyunsaturated fatty acids

Encapsulation in soft gel capsules is widely used in the pharmacological sector for delivery of medication, protecting them from degradation by digestive enzymes in the gastrointestinal tract. In the case of omega-3 PUFAs, this has helped increase the shelf-life, bioavailability, targeted/controlled delivery and release given that omega-3 PUFAs are prone to oxidation. Interest in the health benefits of these ‘nutraceuticals’ has expanded, and they are now widely

available as over-the-counter supplements. A recent European study examining the health claims of 97 omega-3 PUFA supplements, from 63 producers across 23 countries, reported that 83.5% were in capsule form, 11.3% in liquid form, and only a few available as gummies, candies and powder. The omega-3 PUFA content of these supplements vary by composition, purity, concentration and claimed health benefit. (Banović Fuentes et al., 2024).

1.2.2 Consumption and Regulation of omega-3 PUFAs

Omega-3 PUFAs may be present in triacylglycerol (TAG), free fatty acid (FFA), phospholipid (PL) and ethyl ester (EE) form. The chemical form has an impact on digestion and intestinal absorption. This will be covered later in the bioavailability section of this introduction.

The US Food and Drug Administration (FDA) has approved two prescription omega-3 PUFA products, icosapent ethyl (Vascepa) and omega-3 ethyl acid esters (Lovaza), for the reduction of triglyceride levels and secondary prevention of cardiovascular disease (Fialkow, 2016). In 2019, the American Heart Association recommended dosages of 4g per day (>3g total EPA and DHA) were safe as monotherapy or adjuncts to other lipid lowering drugs (Skulas-Ray et al., 2019). The carboxylic acid (Epanova) and ethyl ester A preparations were later discontinued due to disappointing trial data however generic versions may remain in circulation (Astrazeneca, 2020).

Omega-3 PUFA dietary supplements, on the other hand, are not classed as prescription medication thus are not subject to strict medicines regulation (Fialkow, 2016). Therefore dosages, absolute amount of EPA and DHA, chemical form and additives vary between brands. This makes interpretation and generalisability of results from trials of omega-3 PUFA difficult, unless the specific preparations are described.

1.2.3 Absorption and metabolism of omega-3 polyunsaturated fatty acids

Fat absorption begins in the stomach where lingual and gastric lipase break down triacylglycerols to free fatty acids and diacyl glycerol. Hydrolysis continues in the small intestine catalysed by the pancreatic and carboxyl ester lipase enzymes to produce monoacyl glycerol and FFAs. Pancreatic lipase breaks down TAG, PL and FFA, but are much less effective at the hydrolysis of EE forms of EPA and DHA. Bile salts - concentrated in the gallbladder and released through the

presence of fatty food in the duodenum and the hormone, cholecystokinin - emulsify the fat mixture into an aqueous suspension of fatty droplets to maximise the surface area for hydrolysis. Bile salt secretion further enhances the activity of carboxy ester lipase – that is more effective at hydrolysing the EE form of EPA and DHA.

This mixture is combined with other constituents of bile (salts, cholesterol, lysophosphatidic acid and fat-soluble vitamins) to form mixed micelles that are absorbed via passive diffusion and active transport across the membranes of the enterocytes lining the brush border of the small intestinal villi. (Shi & Burn, 2004). In vitro studies have identified distinct membrane proteins that play a role in active transport across the enterocyte membrane: CD36, intestinal fatty acid binding protein (IFABP) and fatty-acid transport protein 4 (FATP4) (Stahl, 2001).

Once in the cell, FFA are re-esterified and incorporated into chylomicrons as triglycerides. Chylomicrons are then released into the lymphatic system via exocytosis and via the thoracic duct, gain entry into the blood stream. Efficient absorption results in less than 5% of lipids being excreted in faeces. (Feeney et al., 2023)

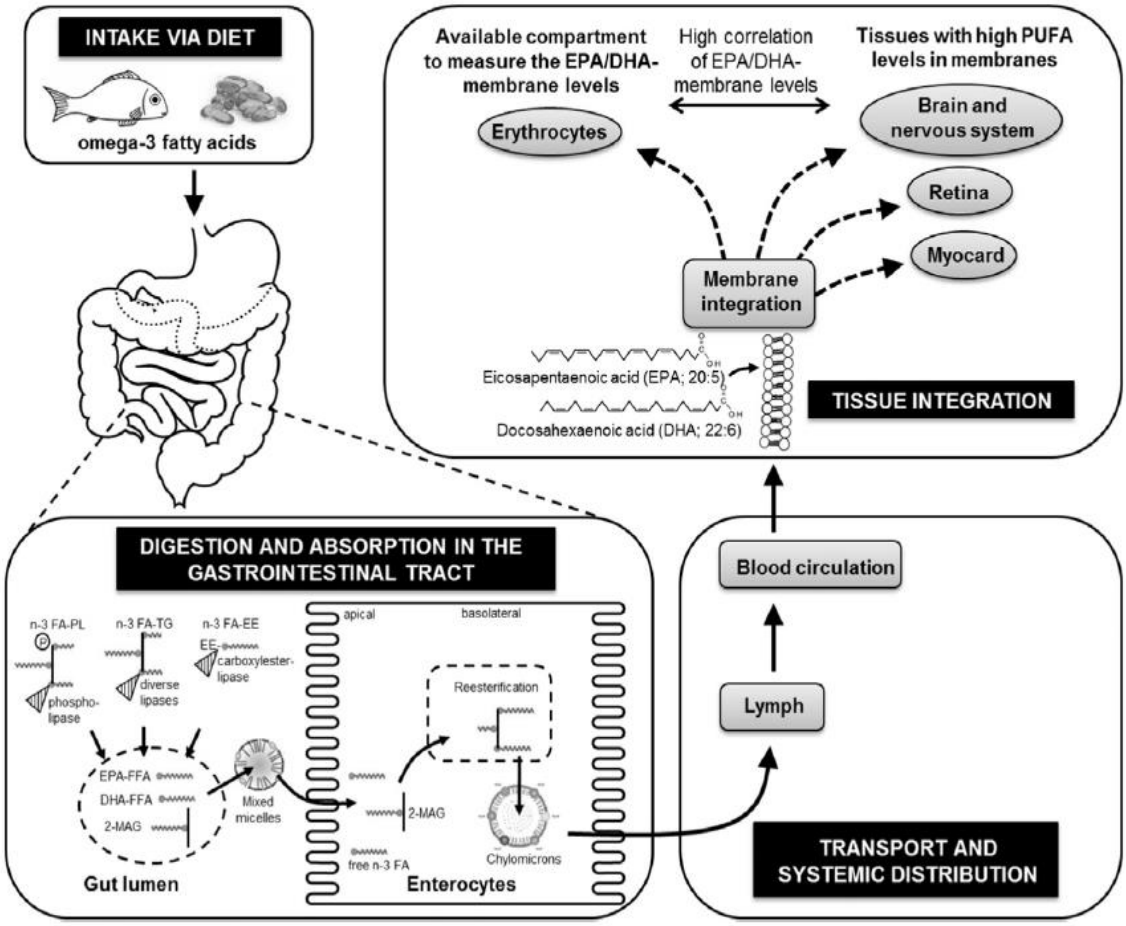


Figure 2 Route of digestion, absorption and tissue incorporation of omega-3 polyunsaturated fatty acids (n3 PUFA). n3FA-PL = omega-3 fatty acid phospholipid, n3FA-TG = omega-3 fatty acid triglyceride, n3FA-EE = omega-3 fatty acid ethyl ester; EPA = Eicosapentaenoic acid; DHA = Docosahexaenoic acid; FFA = free fatty acid; 2-MAG = monoacylglycerol. Adapted from Schuchardt & Hahn, 2013.

As the dietary content of ingested omega-3 PUFA may vary, it is an advantage that mammals can metabolise EPA/DHA from either omega-3 or omega-6 precursors (Saini & Keum, 2018). In the aerobic 'metabolic pathway 6', both substrates undergo a series of double-bond insertions catalysed by delta (Δ6 and Δ5) desaturases. Further carbon chain elongation is catalysed by Elongation of Very Long FAs (ELOVL) enzymes resulting in the formation of EPA and AA from ALA and LA respectively (Zarate et al., 2017). The Sprecher pathway is another mechanism in mammals through which EPA can be converted to DHA by peroxisomal β-oxidation (Sprecher, 2000). The relative abundance of these omega-3 PUFAs and their metabolites depend on dietary intake, genetic factors, gender differences and enzymatic factors. (L. Chen et al., 2025; Panda et al., 2022)

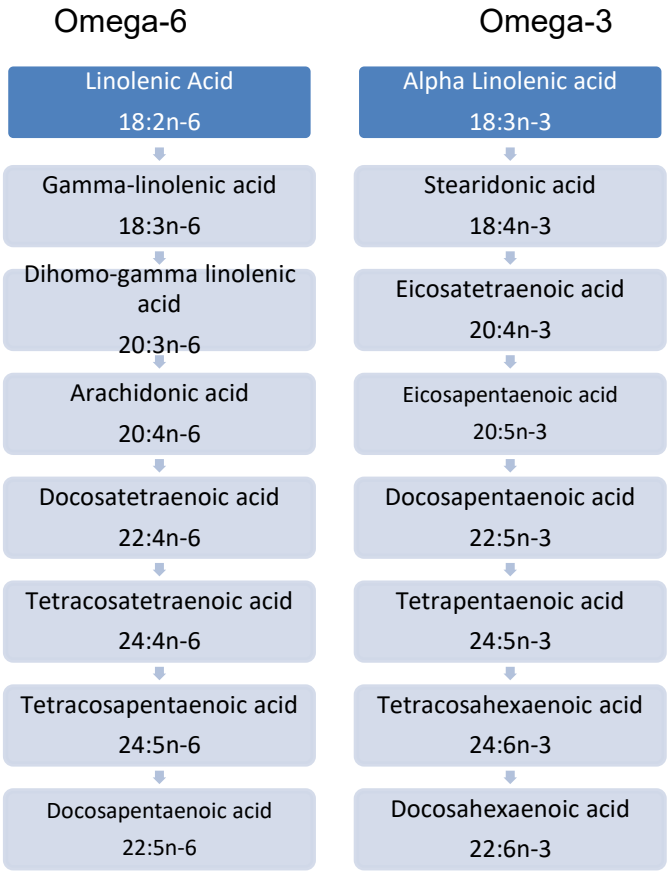


Figure 3: Metabolic pathway of EPA and DHA formation from omega-3 and omega-6 polyunsaturated fatty acids respectively.

The production of DHA from ALA is rate-limited by low levels of endogenous Δ6 desaturase activity in mammals (Burdge et al., 2002; Harauma et al., 2017). This is even lower in plant-based diets, as humans lack the Δ12 and Δ15 desaturases required to convert oleic acid in plants to LA and ALA respectively (Ruiz-Lopez et al., 2015). Furthermore single nucleotide polymorphisms (SNPs) in the fatty acid desaturase (FADS) 1 and 2 genes encoding for Δ5 and Δ6 desaturase enzymes respectively, have an effect in the EPA and DHA composition of serum and red-cell membrane phospholipid levels. (Rzehak et al., 2009; Schaeffer et al., 2006) A Dutch study of 175 volunteers (72 men and 103 women) confirmed gender differences, with higher DHA levels in women compared to men, due to oestrogenic upregulation of synthesis from ALA precursors (Giltay et al., 2004; Yang et al., 2017).

1.2.4 Safety of supplementation with omega-3 PUFAs

In a systematic review published in 2023, Chang analysed 90 multicentre placebo-controlled randomised controlled trials (RCTs) including over 100,000 patients who took either prescription or generic (non-prescription) PUFA formulations or placebo. (Chang et al., 2023) They were generally well tolerated with no serious adverse events related to taking omega-3 PUFAs. Although gastrointestinal, musculoskeletal, respiratory and skin disorders were described, only an altered 'fishy' taste (dysgeusia), diarrhoea and bleeding were statistically significant, compared to placebo (Chang et al., 2023). Prescription omega-3 PUFAs had a higher association with bleeding events, although no dose-dependent relationship was established. The adverse event of bleeding was specifically investigated in another meta-analysis of large cardiovascular outcomes trials (Javaid et al., 2024). This study also included patients with background use of antiplatelets such as aspirin. It concluded that there was no association between omega-3 PUFA use and increased bleeding risk. Generic omega-3 PUFA on the other hand were more associated with dysgeusia and diarrhoea (Chang et al., 2023). This could be explained by the variability in the additives as a result of the less rigorous regulation of these dietary supplements.

1.3 Precision nutrition with omega-3 PUFA

EPA and DHA are important for health. Their cardioprotective effects were long recognised more than 50 years ago when Hugh Sinclair first observed a lower incidence of mortality from myocardial infarction in Greenland Inuit people (Sinclair, 1956). Dyerberg later hypothesised that it was the higher dietary intake of omega-3 PUFAs by Greenland Inuits, that resulted in higher platelet-omega-3 PUFA levels, reduced platelet aggregation, reduced thrombotic events and contributed to lowering the mortality from acute myocardial infarction, compared to an age- and sex-matched Danish control cohort (Bang et al., 1971; Dyerberg & Bang, 1979). Since then several studies have demonstrated the benefits of omega-3 PUFAs in the management of cardiovascular disease (Javaid et al., 2024). As the control of local and systemic inflammation was established as a potential therapeutic avenue for management of cardiovascular disease, interest turned to investigating whether omega-3 PUFA could be effective in other inflammatory conditions. The evidence of a beneficial effect of EPA and/or DHA was demonstrated in the management of diabetes and insulin resistance (Brown et al., 2019), neurodegenerative disorders such as depression and Alzheimer's, autoimmune conditions such as inflammatory bowel disease or rheumatoid arthritis, and cancer (Zarate et al., 2017).

Building on early epidemiological (Calviello et al., 2007) and preclinical evidence of omega-3 PUFA efficacy against carcinogenesis (Fini et al., 2010; Minoura et al., 1988), West published a clinical proof-of-concept study that demonstrated that administration of 2g of EPA, in free fatty acid form, for 6 months, significantly reduced the number and size of colonic adenomas (West et al., 2010). More recent studies strengthen the evidence for the anticancer potential of omega-3 PUFAs. Clinical and cohort data, elegantly summarised by Aldoori et al indicate that the protective capabilities of EPA and DHA vary according to host factors including as baseline omega-3 PUFA status, ethnicity, systemic inflammatory state or tumour characteristics (anatomical location, histopathological subtype, microsatellite instability) (Aldoori et al., 2022). These observations have translated into clinically relevant health outcomes in large-scale cohort studies. For example, a recent UK Biobank analysis of 234598 participants, with a mean follow-up 13.4 years reported that elevated levels of plasma omega-3 PUFAs were associated with reduced colorectal cancer risk especially in men, and for proximal colon cancers. (Aldoori et al., 2025)

These findings support the study of omega-3 PUFA as a tool for precision nutrition whereby personalised nutritional recommendations and interventions to prevent disease are based on an individual's genetic risk or type and location of cancer (Chilton et al., 2017; Surh, 2003). To optimise the chemopreventive potential of omega-3 PUFAs, it is important to understand the pathogenesis of cancer, and potential mechanisms of omega-3 PUFA anticancer and anti-inflammatory efficacy.

1.4 Colorectal cancer and the gut microbiome

1.4.1 Colorectal cancer pathogenesis

Colorectal cancer remains an important public health concern with rising global incidence of 2-4% per year in younger adults i.e. age 50 and under at diagnosis. This contrasts with plateauing trends among those over 50, likely attributed to screening and lifestyle alteration e.g. smoking cessation (Bray et al., 2024; Sung et al., 2025). It develops in the normal glandular epithelium of the colon and follows the adenoma to carcinoma progression sequence first hypothesised by Fearon and Vogelstein (Vogelstein et al., 1988). Better understanding of genomic research has led to the understanding that the majority of cancers (80-85%) arise from chromosomal instability (CIN), while the remainder (10-15%) are due to deficient DNA mismatch repair (dMMR) causing microsatellite instability (MSI) (Grady & Markowitz, 2015). Genetic, environmental and carcinogenic risk factors cause mutations in oncogenes and tumour suppressor genes that result in activation of pathways including CIN, MSI, MMR gene (MLH1, MSH2, MSH6, PMS2) mutations, and CpG island methylator phenotype (CIMP) (Grady & Markowitz, 2015). These transform the epithelial cells of the colon, giving rise to unregulated cellular growth, proliferation, and metastasis if *KRAS* mutation is present. Other deregulated signalling pathways include the Wnt-beta-catenin, the transforming growth factor Beta (TGFB), Notch and Epidermal Growth Factor Receptor (EGFR) contributing to carcinogenesis and thus create opportunities for selective therapies against these molecular subclasses (Lee et al., 2017).

1.4.2 The gut microbiome

The gut microbiome refers to the ecosystem of commensal micro-organisms, including bacteria, fungi, viruses, fungi, that inhabit the mucosal surfaces of the human gastrointestinal tract (Sender et al., 2016). The micro-organisms are established from birth , and grow to the trillions, with the composition, diversity and function varying throughout the human host's life cycle (Gill et al., 2006). As such the composition of the gut microbiome is unique to each individual and stability of this environment is beneficial for health. Gut dysbiosis refers to an alteration in the composition and function of the microbiome, associated with pathogenesis (Alagiakrishnan et al., 2024; Petersen & Round, 2014). Numerous studies have shown that genetic factors, geographical location, dietary life style

choices including ultra-processed foods and additives influence gut dysbiosis and affect the risk of colorectal cancer (Bolyen et al., 2019; Kwao-Zigah et al., 2024; Wong & Yu, 2023).

Direct sampling methods, including mucosal aspirates and ingestible capsules have revealed that the human ileal microbiome is distinct from the colonic communities with high temporal diversity, lower diversity, and dominated by oral-mucosal taxa (Booijink et al., 2010; Yilmaz et al., 2022). Across studies, the dominant genera commonly identified in the ileum include *Streptococcus*, *Veillonella*, *Granulicatella* and facultative proteobacteria such as *Haemophilus* and *Neisseria*. (Shalon et al., 2023; Villmones et al., 2022; G. Wang et al., 2024)

The human colonic mucosal microbiome is on the other hand is compositionally distinct from faeces and enriched with consistent genera. Across healthy subjects, dominant colonic taxa include *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Blautia*, *Ruminococcus*, *Alistipes*, *Prevotella*, and mucus-associated *Akkermansia*, with lower diversity compared to stool and site-specific variation along the colon (Shalon et al., 2023; Yin et al., 2024).

1.4.3 Microbiome analysis

Identification of bacterial species involves sequencing technologies, dysbiosis indices and assessments of microbial metabolites (Alagiakrishnan et al., 2024; Terrón-Camero et al., 2022).

Due to ease of access, faecal specimens were initially used, however colonic mucosal biopsies or luminal brushings obtained through invasive methods (colonoscopy) are also used to perform bacterial DNA sequencing (Tang et al., 2020). Using PCR-amplified 16S ribosomal RNA sequencing or shotgun metagenome sequencing, the bacterial taxa and phyla composition of a sample can be identified (Hayashi et al., 2005). Dysbiosis indices include alpha (α) diversity indicating the relative abundance of a microbial specie in a biological sample. Beta (β) diversity on the other hand measures species diversity over time.

Bacteria produce metabolites such as lipopolysaccharides (LPS) , secondary bile acids and short chain fatty acids. Using gas chromatography or mass spectrometry, the various proportions of these metabolites can be measured to identify specific bacteria (Alagiakrishnan et al., 2024)

1.4.4 The gut microbiome in colorectal carcinogenesis

1.4.4.1 Overgrowth of pathogenic bacteria

A recent review of small-scale studies of CRC patients identified common bacterial pathobionts contributing to gut dysbiosis and increased CRC risk. Using next generation genomic sequencing, *Fusobacterium* genus (in particular *Fusobacterium nucleatum*), *Bacteroides fragilis, Escherichia coli* and *Enterococcus faecalis* were consistently more abundant in the tumour microbiome of CRC patients compared to non-tumour microbiome (M. Song, Chan, et al., 2020).

F. nucleatum, a bacterial specie present in the oral cavity, was most prevalent within colonic tumour cells of 116 CRC patients (Zepeda-Rivera et al., 2024) and associated with recurrence (Serna et al., 2020), metastasis (Bullman et al., 2017) and poor prognosis (LaCourse et al., 2021)

A review of 22 observational studies using human tissue biopsy, stool, mucosal samples and colonic washings found higher levels of Enterotoxin *Bacteroides fragilis* (ETBF) and *bacterium fragilis* toxin (bft) gene expression. Due to heterogeneity in study methods, a significant association between ETBF abundance and CRC could not be concluded (Scott et al., 2022)

E.coli strains with a polyketide synthase (*pks*) island gene encoding for colibactin were associated with CRC (Dziubańska-Kusibab et al., 2020). Colibactin, a genotoxin that alkylates DNA, caused double-stranded breaks at locations corresponding to somatic mutation hotspots in CRC genomes. A study involving 413 CRC patients found that *pks*-positive E.coli was more abundant in the tumour tissue from early CRC than normal or late stage CRC tissue (Miyasaka et al., 2024).

The presence and abundance of these oncogenic bacteria resulted in the depletion of gut bacteria with putative beneficial effects such as *Streptococcus thermophilus* or *Lactobacillus gallinarum* (Wong & Yu, 2023).

1.4.4.2 Alteration of gut metabolites

The intestinal flora carry out digestive and metabolic activities in the gut, thus dietary consumption may alter the gut flora resulting in an abundance of harmful metabolites that can induce tumorigenesis (Coker et al., 2022).

Increased dietary consumption of red and ultra-processed meat in the Western diet raises the proportion of bacteria that metabolize sulphur such as,

Fusobacterium nucleatum, Porphyromonas, and *Prevotella*. This in turn increases the colonic concentration of hydrogen sulphide, a carcinogen associated with DNA damage, impaired colonocyte nutrition, inflammation and upregulation of regulatory T-cell activity resulting in impaired anti-tumor immunity. These have been shown to be abundant in the luminal and stool microbiota of CRC patients (M. Song & Chan, 2017; Wong & Yu, 2023).

High fat consumption results in the production of secondary bile acids such as deoxycholic acid by *Clostridium* species. In individuals with inflammatory bowel disease, high deoxycholic acid levels have been associated with gut dysbiosis and increased CRC risk (Lavelle et al., 2022; Wirbel et al., 2019).

Bacteroides fragilis has been demonstrated to increase reactive oxygen species (ROS) that promote chronic inflammation resulting in cancer (Dejea et al., 2014).

1.4.4.3 The protective role of short chain fatty acids

Some beneficial bacterial metabolites have a potent anti-tumorigenic function. Following the observation that Africans had a markedly lower rate of CRC compared to other ethnicities possibly due to their higher fibre intake (Burkitt, 1971), studies have gone on to demonstrate a 17% reduction in CRC risk with ≥90g daily intake of whole grains (M. Song, Chan, et al., 2020). Soluble dietary fibre can be fermented by bacteria in the colonic lumen releasing short chain fatty acids (SCFAs) including acetate (60%), propionate (25%) and butyrate (15%). Butyrate, in particular is an important energy substrate for colonocytes, maintaining intestinal barrier immunity and immune function and can reduce the risk of CRC (Smith et al., 2013; Wong & Yu, 2023). Dietary fibre supplementation enriched the microbiome in favour of butyrate-producing bacteria such as *Bifidobacterium, Firmicutes, Clostridium, Anaerostipes, Eubacterium* and *Roseburia* species (O'Keefe et al., 2015; C.-H. Song et al., 2024). The anticancer effect of butyrate can be explained via its function as a histone deacetylase (HDAC) inhibitor, promoting the expression of tumour suppressor proteins FAS, p21 and p27; binding with tumour suppressive Free fatty acid receptor 2 (FFAR2) and FFAR3 to induce apoptosis (H.-M. Chen et al., 2013; Wong & Yu, 2023). In cancerous colonocytes, butyrate metabolism slows down. Cancer cells undergo aerobic glycolysis rather than using butyrate as the main substrate, the Warburg effect. This accumulation of butyrate in the cell nucleus results in downregulation of Acetyl-Coenzyme A- dependent genes responsible for tumour growth and angiogenesis (Donohoe et al., 2012; Zhang et al., 2025).

1.5 Mechanistic models of anti-colorectal cancer action of omega-3 PUFA

The anticancer activity of omega-3 PUFA is complex and mediated by multiple mechanisms. There is a robust evidence base focused on the anti-inflammatory actions, immune-modulating response and alteration of the gut microbiome to minimise carcinogenesis (M. Song & Chan, 2017; Tu et al., 2020).

1.5.1 Anti-inflammatory action of omega-3 PUFA

Inflammation is promoted by eicosanoids, a term that encompasses multiple signalling molecules generated from the conversion of AA and omega-3 PUFAs. These include:

- Prostaglandins, prostacyclins and thromboxanes - mediated by cyclooxygenase (COX) enzymes,
- Leukotrienes and lipoxins - mediated by lipoxygenase (LOX) enzymes,
- Hydroxyeicosatetraenoic (HETEs), Hydroxyperoxyeicosatetraenoic (HPETEs), Epoxyeicosatrienoic (EETs) acids - mediated by the cytochrome CYP 450)

The control of chronic inflammation is integral for the anticancer effects of omega-3 PUFAs (Tu et al., 2020). EPA effectively competes with AA for metabolism by COX-2, leading to reduced tissue concentrations of AA-derived pro-inflammatory prostaglandin E_2 (PGE_2) in favour of EPA-derived anti-inflammatory prostaglandin E_3 (PGE_3) (Hudert et al., 2006). This mechanism was demonstrated to reduce the CRC liver tumour burden in mice (Hawcroft et al., 2012).

Similarly, the 5-LOX metabolite of DHA, 4-hydroxy-docosahexaenoic acid (4-HDHA), has a potent antiangiogenic effect while the corresponding metabolite from AA stimulates angiogenesis (Sapieha et al., 2011).

EPA and DHA act as substrates for the CYP mono-oxygenase enzymes, generating 19,20-epoxydocosapentaenoic acid (19,20 EDP). A study showed that 19,20-EDP (dose = 0.5 mg/kg/day) potently inhibited VEGF-induced angiogenesis, reducing primary tumour growth in a MC38 xenograft CRC model in mice (Arnold et al., 2010; W. Wang et al., 2017).

EPA and DHA inhibit pro-inflammatory cytokines such as Interleukin (IL) -1, IL-2, IL-6 and Tumour Necrosis factor Alpha (TNF-α) via Specialized Proresolving

mediators (SPMs) consisting of resolvins, protectins and maresins that collectively reduce inflammation reduction and improve tissue regeneration (Bodur et al., 2025). Resolvins inhibit neutrophil chemotaxis and transmigration, thus reducing tissue infiltration and potential damage (Serhan & Levy, 2018). Protectin D1 (PD1) reduces leukocyte recruitment to sites of inflammation (Serhan, 2014). Maresin 1 promotes macrophage-mediated phagocytosis of apoptotic cells and debris (Saito-Sasaki et al., 2022).

1.5.2 Induction of oxidative stress

Omega-3 PUFAs accumulation within tumour cells induces apoptosis. Omega-3 PUFAs are highly susceptible to oxidation generating the superoxide radical. This depletes glutathione, the major intracellular antioxidant, inducing oxidative stress and resulting in apoptosis (Simon et al., 2000).

1.5.3 Increase cell membrane fluidity

Omega-3 PUFA impact the fluidity and flexibility of cell membranes, enhancing cellular communication, signal reception, and response to infections and inflammation in immune cells such as lymphocytes, macrophages, and dendritic cells. Increased membrane fluidity may disrupt cellular integrity. Furthermore, multiple signalling cascades may be altered via transcription and messenger molecules such as vascular endothelial cell growth factor, b-catenin, peroxisome proliferator-activated receptor gamma (PPAR-γ) to name a few. Reduced extracellular signal-related kinase-1/2 signalling, have been demonstrated in human CRC cell lines treated with omega-3 PUFAs (Bodur et al., 2025).

1.5.4 Disruption of cell signalling

Fatty acids exert significant indirect influence on the functionality of lipid rafts through nuclear receptor activation, transcription factor modulation and microRNA regulation. This impairs the signalling capabilities of key immune cells, including T-cells and macrophages. One example is the activation of PPAR-y also inhibits the activation of NF-*κ*B, a key transcription factor in the inflammatory cascade which results in decreased expression of proinflammatory genes (Bodur et al., 2025).

1.5.5 Cytokine production and immune modulation

EPA and DHA, both function as natural ligands of PPAR-*γ*. When activated, PPAR-*γ* physically binds specific DNA sequences cells known as peroxisome proliferator response elements (PPREs). These upregulate lipogenic genes, such as perilipin 2 (PLIN2), elongation of very long chain fatty acid 4 (ELOVL4), and sterol O-acyltransferase 1 (SOAT1) that are associated with lipid droplet formation, lipid biosynthesis and autophagy (Kim et al., 2020).

Fish oil rich in EPA led to a reduced release of IL-2 in T and B cells. This reduction in IL-2, alongside decreased serum immunoglobulin levels, indicates a mild immunosuppressive effect of omega-3 fatty acids on both T and B cell functions [*PMID: 1833105]*

1.6 Omega-3 PUFAs alter the gut microbiome in colorectal cancer

As the mechanistic models of CRC risk modulation by environmental and genetic factors became better understood, omega-3 PUFAs were demonstrated to have an effect on gut microbiota.

A study of 20 healthy volunteers taking 4g of EPA+DHA supplements daily in drinks or capsules form for 8 weeks. Blood and faecal samples were analysed. Irrespective of formulation, dietary supplementation increased the proportion of SCFA-producing bacteria including *Clostridiaceae, Sutterellaceae, Akkermansiaceae, Bifidobacterium, Oscillospira, Lachnospira, and Roseburia*, while decreasing *Faecalibacterium* (Watson et al., 2018).

In another study, 88 HIV infected individuals taking 3150mg DHA algae oil for 24 weeks, had increased diversity and abundance of *Bifidobacterium*, *Lactobacillus, Fusobacterium and Faecalibacterium,* while the abundance of *Bacteroides and Prevotella* were decreased (Dong et al., 2021). Blood and faecal samples were analysed.

In a third study, 876 middle-aged female twins took ~350mg DHA daily, measured via food frequency questionnaires. Increased total omega-3 PUFA and DHA serum levels correlated with an abundance of *Lachnospiraceaes*, *Ruminococcacae*, and *Bacteroidetes* and a decrease in *Enterobacteriaceae* (Menni et al., 2017).

Preclinical studies had demonstrated the beneficial effects of omega-3 PUFA on polyp/adenoma formation and combatting progression to cancer. $APC^{Min/+}$ mice fed a diet enriched with EPA-FFA for 12 weeks had reduced polyp formation (Fini et al., 2010). In another study, after 36 weeks of an EPA-enriched dietary supplementation , aberrant crypt formation and adenoma formation in the colon was supressed in 1,2-dimethylhydrazine- induced CRC models in rodents (Moreira et al., 2009). Omega-3 PUFA-enriched diets demonstrated a decrease in tumour multiplicity, incidence and maximum tumour size in colitis-associated CRC in mice. This was via alteration of the afore-mentioned Notch signalling pathway and alteration of gut microbiota in favour of Lactobacillus enrichment (Piazzi et al., 2014). Small intestinal polyposis was attenuated by high tissue levels of EPA, through inhibition of the Wnt-β catenin signalling pathway in fat-1 and $APC^{Min/+}$ transgenic mice (Han et al., 2016)

Furthermore, combined intake of omega-3 PUFA and dietary fibre was observed to play a role in reducing CRC risk through modulation of the microbiome in favour of SCFA-producing bacteria. Kraja et al analysed 4967 participants (222 CRC cases) and reported an increased risk of CRC associated with O3FA intake (including non-marine sources) in those with a less than median dietary fibre intake. There was a statistically significant association between dietary fibre and O3FA intake on CRC risk (p=0.02) (Kraja et al., 2015)

The Women's Health Initiative Dietary Modification Trial, a larger prospective study of 134 017 women (1952 CRC cases) with a mean follow-up of 11.7 (±3.5) years, reported that higher levels of O3FA intake were associated with a reduced risk of CRC (HR 0.90), with an association between EPA and DHA intakes and CRC risk only in those with lower levels of soluble fibre intake (HR 0.59, p=0.02) (Navarro et al., 2016).

Other clinical and epidemiological studies followed to further investigate the relationship between omega-3 PUFAs and CRC risk. One of the original double blind, placebo-controlled RCTs was discussed earlier (West et al., 2010). In this study 2g EPA-FFA intake was associated with reduced polyp number and size in Familial Adenomatous Polyposis (FAP) patients. The EPA for Metastasis Trial (EMT) was another small phase II double-blind, randomized, placebo-controlled trial. It concluded that 2g daily EPA intake for 12-65 days was well tolerated and increased overall survival, with reduced tumour vascularity in advanced CRC patients undergoing liver resection due to liver metastases (Cockbain et al., 2014). Aldoori elegantly reviewed the literature, highlighting potential benefit of omega-3 PUFA activity at various stages of CRC management – from primary prevention or post-screening reduction of polyps and adenomas to surgical

management of primary or adjuvant treatment of metastatic disease (Aldoori et al., 2022).

Large scale studies were designed to provide definitive data to support those preliminary conclusions. The Vitamin D and Omega-3 Trial (VITAL) was a double-blind, 2x2 factorial, placebo-controlled trial set up to primarily study cardiovascular outcomes (Manson et al., 2019), but it also contained a predefined ancillary study to analyse CRC risk. The investigators randomised 25871 adults in the US general population, free of cancer and cardiovascular disease to either 1g daily of mixed EPA and DHA (460mg and 380mg respectively) and vitamin D3 (2000 IU) daily versus placebo. Although secondary analyses did not show any significant association between omega-3 PUFA intake and reduced polyp size, omega-3 PUFA supplementation was associated with lower risk of conventional adenomas among participants who were either African-American or had low plasma baseline levels of EPA and DHA (M. Song, Lee, et al., 2020). Most recently, a UK Biobank analysis demonstrated inverse correlation between elevated plasma omega-3 PUFA levels and CRC risk. The study focused on circulatory plasma omega-3 PUFA levels but had no assessment of microbiome endpoints (Aldoori et al., 2025). Other large-scale cohort studies with long follow-up periods include the European Prospective Investigation into Cancer and Nutrition (EPIC) (n=521324) showed an inverse relationship between CRC incidence and red blood cell omega-3 PUFA (Linseisen et al., 2021). However these results were inconsistent with the Health Professionals Follow-Up Study (HPFS) and Nurses' Health Study (NHS) nested cohort studies that did not show any overall CRC risk reduction, and even increased distal colon cancer risk in certain individuals (M. Song et al., 2014; L. Wang et al., 2021). Another trial of omega-3 PUFA usage in adjuvant treatment of CRC is due to report in the near future (Hull et al., 2023).

1.7 Bioavailability

Studies of Omega-3 PUFA chemoprevention have shown variability in population groups, dosages, periods of supplementation and overall effect at the molecular level (Cockbain et al., 2014; Manson et al., 2019). Another challenge in the significant differences observed in studies of acute availability of EPA in tissues, is often inconsistent in studies of long-term supplementation. The effect from mouse studies and small scale observational studies is not always replicated in large RCTs e.g. After A Study of Cardiovascular Events iN Diabetes (ASCEND trial) randomised 15480 diabetic patients to 1g omega-3 PUFA supplementation

or olive oil placebo, there was no significant difference in the incidence of fatal cancer (The ASCEND Study Collaborative Group, 2018). Chemoprevention may require supplementation over a prolonged time frame thus understanding of kinetics, tissue distribution and response and tolerability are required.

Omega-3 PUFA effects depend on their bioavailability. According to the US FDA, bioavailability refers to the rate and extent to which the active moiety or ingredient is absorbed from a drug product and becomes available at the site of action (Center for Drug Evaluation and Research, 2020). In pharmacological terms, this would also refer to the pharmacokinetics. This is difficult to establish for omega-3 PUFA supplements as they are not pharmacological drugs, but nutrients. Another difficulty in measuring their bioavailability would be the exclusion of the endogenous presence of fatty acids in cell membranes, or its exogenous supplementation via dietary fat intake. As such reported biomarkers of bioavailability quoted in the literature on the subject vary depending on sampling time, tissue distribution, length of supplementation, and biological half-life of these biomarkers (Alijani et al., 2025).

1.7.1 Biomarkers of EPA/DHA bioavailability

Various biomarkers describe the bioavailability of omega-3 PUFAs (Alijani et al., 2025). The half-life of circulating FA in plasma is about 4 -14 days (Plourde et al., 2014; Zuijdgeest-van Leeuwen et al., 1999), reaching steady state, the point at which the absorption and elimination of a molecule are balanced, within 14-30 days for EPA and 14-60 days in DHA (Browning et al., 2012). Thus, plasma or blood EPA/DHA levels, collected ideally within 72 hours of supplementation, irrespective of formulation (EE, PL, FFA) are used as measures of the acute bioavailability of EPA/DHA. Platelet EPA/DHA levels have a biological half-life similar to the lifespan of platelets, 7-10 days (Gardner & Cohen, 1966), and may also be used for acute bioavailability but this is less common in the quoted literature. Due to this rapid cell turnover, and the sensitivity of these biomarkers to dietary fatty meal ingestion, meaningful conclusions about the associated health effects of omega-3 PUFAs should be drawn from chronic bioavailability studies (Alijani et al., 2025).

Compared to platelet and plasma FA levels, circulating FA on red blood cell (rbc) membrane levels persist for weeks to months, achieving steady state within 90 days (Browning et al., 2012; Shrestha et al., 2016). Rbc membranes are less likely to be affected by fluctuating dietary fat intake and strongly correlate with EPA/DHA in adipose tissues (Fenton et al., 2016). Therefore rbc FA levels are a better indicator of tissue distribution of fatty acids, a surrogate biomarker for

'chronic' supplementation - described in the literature as supplementation for >1 week (Alijani et al., 2025). Rbc levels of a specific FA may be described as the concentration of the FA or the % of the FA relative to the total fatty acid within the measured sample. Therefore, the omega-3 index (O3I), calculated as the relative sum of EPA and DHA on total FA within the measured red cell membrane sample has become a widely used biomarker of omega-3 PUFA supplementation (Schuchardt, Beinhorn, et al., 2024; Watson et al., 2018)

1.7.2 Laboratory methods for measuring omega-3 PUFAs

Gas chromatography techniques were traditionally employed for relative quantification of FA content of biological samples both in vivo and in vitro due to their high chromatographic resolution. However Liquid chromatography tandem-mass spectrometry has been increasingly favoured for its superior sensitivity to long chain fatty acids, with reduced thermal degradation. (Koch et al., 2021; Volpato et al., 2017) This will be further developed in the methods section 3.5.5.4.

1.7.3 Rationale for a study on bioavailability

In vivo and in vitro studies support the possibility of omega-3 PUFAs reducing the risk of colorectal cancer development, potentially through alteration of the ecosystem of commensal bacteria lining the human GI tract, the gut microbiome (Aldoori et al., 2022). Oral supplementation with omega-3 PUFAs favoured a reversible increase in SCFA-producing bacteria, *Bifidobacterium* and *Lactobacillus,* over other colonic, potentially oncogenic, bacteria (Watson et al., 2018). These bacteria stabilise the defective DNA-mismatch repair gene associated with microsatellite instability (in mouse models) of hereditary non-polyposis colorectal cancer (van der Beek et al., 2017). In the colon in particular, the abundance of these bacteria works synergistically with omega-3 PUFAs to increase butyrate and acetate production. In turn, this reduces inflammation, preserves intestinal barrier integrity, and prevents tumour development. It is unclear whether this anti-tumorigenic activity is mediated by the luminal or intracellular proportion of omega-3 polyunsaturated fatty acids after oral supplementation. Analysis of biomarkers of omega-3 PUFA activity also highlights differential CRC risk profile across different regions of the colon. For example, proximal colorectal cancers with a higher percentage of microsatellite

instability, were inversely associated with higher circulating plasma EPA levels after dietary omega-3 polyunsaturated fatty acids intake. (Aglago et al., 2020).

A review of 61 omega-3 PUFA bioavailability studies concluded that the mechanistic effects of EPA/DHA observed in acute bioavailability studies, as measured by plasma or platelet omega-3 PUFA levels, was inconsistent in chronic bioavailability studies (Alijani et al., 2025). Further research in human studies was therefore necessary to confirm the beneficial anticancer effects of omega-3 PUFAs observed in vivo and *in vitro* studies.

While EPA/DHA rbc membrane levels and O3I are the commonly used biomarkers of chronic supplementation in the literature, their accuracy can be questioned as they do not readily distinguish between gut luminal EPA and DHA absorption of the supplement and the contribution of endogenous metabolism. A few studies attempt to measure true bioavailability by using carbon-13 labelled EPA and DHA but they do not measure faecal excretion of EPA and DHA (Hachem et al., 2020; Léveillé et al., 2017; Metherel et al., 2019).

In addition to this, data on intestinal luminal concentrations of EPA and DHA are limited. A single study examined the small intestinal concentrations of EPA and DHA after oral supplementation with various microencapsulated forms of omega-3 polyunsaturated fatty acids in human volunteers with a permanent ileostomy (Sanguansri et al., 2013). The authors concluded that over 98% of administered omega-3 PUFA was absorbed in the small bowel, such that less than 1% of the total EPA and DHA consumed was present in the small bowel effluent, irrespective of the mode of delivery. That study also had a few limitations: firstly, a relatively small dose of omega-3 polyunsaturated fatty acids was taken with water (266mg of mixed EPA and DHA) compared to the higher doses (1- 4 g of omega-3 polyunsaturated fatty acids) administered in RCTs of omega-3 PUFAs on intestinal microbiota and colorectal cancer (Aung et al., 2018). Secondly, the study was conducted over a short time frame of two weeks, thus biomarker data related to acute bioavailability. Finally, the study did not report actual EPA/DHA concentrations nor attempt to study the effect on the ileal microbiota.

1.7.4 Methodological criteria for optimal design of omega-3 PUFA bioavailability studies

In addition to omega-3 PUFA biomarkers use and heterogeneity in the methodology across bioavailability studies, a review of 61 human intervention trials - 35 acute and 26 chronic bioavailability studies – made recommendations

on methodological criteria to guide the optimal design of future bioavailability studies (Alijani et al., 2025).

1.7.4.1 Information on the source, chemical form and distribution of EPA and DHA.

Marine omega-3 PUFA pharmacokinetics are controlled by lipid digestion in the GI tract. Thus, bioavailability is reduced subsequently from non-esterified Free fatty acid, phospholipid, triacylglycerol to ethyl ester forms of EPA/DHA supplements. As explained earlier, this is due to the enzyme driven ease of hydrolysis and absorption, thus EE absorption is aided with concomitant ingestion of a fatty meal. Ideally the fat content of meals or background omega-3 PUFA intake should be standardised to minimise confounding. Supplements should state the exact concentrations of EPA and DHA, together with the relative proportions of the various forms.

1.7.4.2 Information on delivery systems

Marketed supplements undergo a process of purification, concentration and deodorisation to increase palatability for human consumption. To further protect them from oxidation, EPA and DHA are enclosed in bovine gelatine soft gel capsules. Enteric coating may also offer some protection for targeted delivery beyond the stomach. Bioavailability studies did not show any significant difference between formulations with chronic supplementation (Alijani et al., 2025). Self-emulsifying formulations consisting of lipids, surfactants and co-solvents form a dispersible oil-water medium that increases the surface area for absorption. They can increase acute bioavailability but the data on chronic bioavailability is inconclusive. Other commercial delivery systems including nano-sized emulsions, gelled emulsions, microencapsulation with biopolymers, micellar matrices have been described (Abdelhafez et al., 2025).

1.7.4.3 Dosing

Dosage of the administered supplement should be consistent throughout the study. For acute bioavailability studies, a defined washout period of 7 to 10 days should allow for the FA levels to return to baseline. In contrast, chronic

supplementation studies vary in the amount and duration of supplementation thus no recommendation can be made on washout periods but at least 3 months from prior exogenous background omega-3 PUFA intake should be followed prior to enrolment (Alijani et al., 2025). A parallel controlled study design would be more suitable to test chronic bioavailability while cross-over design is more prevalent in acute bioavailability studies.

1.7.4.4 Controlling bias

Randomised allocation of the supplement reduces bias in parallel studies of chronic bioavailability. To maintain a homogenous population, screening of the participants for EPA/DHA intake, dietary fish consumption, and rbc EPA/DHA levels prior to enrolment in chronic availability studies is preferable.

Putting all this together, robustly designed small- and large-scale studies of targeted omega-3 PUFA use for CRC chemoprevention are necessary. This would aid our understanding of the anticancer effects of O3FA on tumour microenvironment, biomarker-driven assessment of response to supplementation, gastrointestinal bioavailability and the impact of omega-3 PUFA on the gut microbiome.

Chapter 2 Aims and hypotheses

Inflammation, at the molecular level, remains a feature of many disease processes, ranging from coronary artery disease to colorectal cancer, to name a few. Within the last two decades, there has been a growing evidence base exploring the anti-inflammatory and chemopreventive properties of the marine omega-3 PUFAs, EPA and DHA. Potential pathways include their incorporation into the cell membrane resulting in the formation of biologically active SPMs such as maresins, resolvins and protectins. These down-regulate inflammatory cytokines e.g. PGE2, IL-2, TNF-α, leading to a resolution of inflammatory processes. This property has led both to a recommendation for the supplementation of omega-3 PUFAs for cardiovascular disease prevention, and precision nutrition for cancer prevention. This is either through the diet or in the form of high-strength oral supplements. The absolute amount and duration of consumption remains under debate. By measuring biomarkers of bioavailability after oral supplementation, this thesis aims to quantify omega-3 PUFA concentrations in biological samples and explore relationships between luminal and systemic incorporation of orally consumed omega-3 PUFAs.

2.1 Primary Aim

To perform a clinical experimental study to measure the concentration of omega-3 PUFAs in the ileum immediately after initial dosing and after prolonged oral consumption of high dose omega-3 polyunsaturated fatty acid supplements. The primary hypothesis to be tested was:

- The intestinal luminal concentration of EPA and DHA increases after 4 weeks dosing with 4g mixed EPA/DHA oral supplements.

Collecting ileostomy effluent and blood from participants after consumption of oral omega-3 polyunsaturated fatty acids supplements to measure the fatty acid content within it would provide novel data on the concentration of EPA and DHA in the distal small bowel lumen after digestion and absorption. It would also permit investigation of any relationship between the intestinal omega-3 polyunsaturated fatty acids concentration and variation in the relative abundance of the microbiota in the ileal effluent.

2.2 Secondary aim

Watson and his group (Watson et al., 2018) published their findings that oral supplementation with 4g omega-3 polyunsaturated fatty acids over 8 weeks favoured a reversible increase in short-chain fatty acid (SCFA) producing bacteria, *Bifidobacterium* and *Lactobacillus*. Given that study used the same high dosage omega-3 polyunsaturated fatty acids capsules as the ileostomy effluent study described above, the aim of this secondary analysis was to use the faecal samples from the twenty healthy volunteers involved in Watson's trial to test the hypothesis that:

- omega-3 PUFA levels in faecal samples increase after oral EPA/DHA dosing.

The combination of these two streams of work will provide novel data on the bioavailability of intestinal EPA and DHA after high dose oral supplementation. It is hoped that this will provide a basis for future laboratory studies of how omega-3 polyunsaturated fatty acids interact with the gut microbiota

Chapter 3 Design and methodology of a clinical study to measure the ileal luminal bioavailability of oral omega-3 polyunsaturated fatty acids, and associated changes on the ileal microbiota in humans with a temporary ileostomy

3.1 Background

It is not known whether the microbiome is affected by direct colonic luminal exposure to omega-3 PUFAs or whether it is secondary to shedding of enterocytes that have incorporated omega-3 PUFAs following efficient small bowel absorption and systemic incorporation.

Therefore, a prospective clinical study to investigate the luminal concentration and 'bioavailability' of omega-3 PUFAs after oral administration of 4g EPA/DHA supplements would attempt to answer this. In pharmacology, bioavailability refers to the amount of an ingested drug/substance that reaches the systemic circulation or physiological site of action (Schuchardt & Hahn, 2013). In this context, it was used to describe the luminal and systemic amounts of EPA and DHA as the optimal physiological site of action remains under investigation.

The primary aim was to measure the small intestinal content of omega-3 PUFAs, after taking 4g omega-3 PUFAs in soft gel capsules orally (EPA to DHA ratio 1:1) for at least 28 days, in patients with a temporary ileostomy.

Sanguansri et al concluded that the omega-3 PUFA content of the ileostomy fluid (IF) in their study peaked at 2-8 hours post ingestion and declined 10hours after consumption (Sanguansri et al., 2013). Thus, sampling within a few hours and after longer term capsule consumption would help distinguish between immediate and longer-term enterocyte exposure to omega-3 PUFAs. This would provide an indication of the efficiency of intestinal absorption and inform the contribution of either direct local delivery to the colon or colonic exposure secondary to systemic incorporation of omega-3 PUFAs.

3.2 Primary endpoint

The primary endpoint was the change in terminal ileal fluid (IF) EPA and DHA concentrations at four weeks (28 days) compared to baseline.

3.3 Secondary endpoints

1. Change in terminal ileal fluid and rbc EPA and DHA concentration within 24 hours of taking the first omega-3 PUFA dose.
2. Change in omega-6 and other fatty acids after 24 hours, and four weeks of oral supplementation with EPA/DHA capsules.
3. Change in percentage rbc membrane content of EPA and DHA at four weeks compared to baseline
4. Tolerability and adverse events related to omega-3 PUFA supplementation
5. Relationship between ileal EPA and DHA and changes to the luminal microbiome in the ileum.

3.4 Registration and ethics

The Medicines and Healthcare Products Regulatory Authority (MHRA) considers omega-3 PUFA capsules to be a nutritional supplement and given there were no clinical endpoints in this experimental medicine study, the study did not require Clinical Trials Authorisation. However, the trial was registered with the International Standard Register Clinical/social sTudy Number (ISRCTN) registry (ISRCTN14530452). All other approvals for human subjects and tissue samples were obtained from the Yorkshire & Humber Leeds West Research Ethics Committee (15/YH/0547) .

3.5 Methods

3.5.1 Recruitment of participants

Adult patients with a temporary loop ileostomy following colonic resection were identified from electronic colorectal surgery department records at St James's University Hospital, Leeds, UK. All eligible patients were screened with the colorectal stoma nurses: identifying frail individuals, long waiters for reversal of ileostomy or upcoming clinic appointments. Potential participants were sent an invitation letter and participant information leaflet by post with a contact phone number if they wished to be considered for the study. A follow-up phone call was made to all participants within 5-7 days of sending out the invitation. Individuals that expressed an interest in participating in the study were contacted by the researcher by phone, and the consent process was initiated, with an appointment

to SJUH. At the hospital visit, eligibility for inclusion was assessed, questions answered, and a consent form signed, if appropriate.

3.5.1.1 Inclusion criteria

- Aged 18 years or over
- Either sex
- Temporary or permanent ileostomy fashioned at least 2 months prior to commencing study.
- Able to self-medicate and give informed consent
- A minimum period of 2 months availability for the study prior to planned ileostomy reversal

3.5.1.2 Exclusion criteria

- Seafood allergy
- Ongoing and/or previous use (within 4 weeks of commencing the study) of other omega-3 polyunsaturated fatty acids or cod-liver oil supplements
- Previous small bowel resection more than 10 cm
- Metastatic colorectal cancer
- Less than 4 weeks since any chemotherapy or radiotherapy
- Inflammatory bowel disease or other intestinal disease (e.g. coeliac disease) affecting the small intestine

3.5.1.3 Sample size and power calculation

The literature on absolute terminal ileal EPA and DHA levels in subjects consuming oral omega-3 polyunsaturated fatty acids supplements is limited. At the time of study design, the only research article in a similar subject area, was based on data from six volunteers to provide statistically significant results. Therefore, a formal sample size power calculation based on absolute omega-3 polyunsaturated fatty acids levels was not possible. In view of previous studies examining omega-3 polyunsaturated fatty acids rbc 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 omega-3 PUFAs (Harris & Thomas, 2010). Therefore, it was predicted that at least eight participants will be adequate to produce a measurable difference in terminal ileal EPA/DHA.

According to St James' University Hospital (SJUH) colorectal stoma department records, approximately 102 patients underwent formation of a temporary ileostomy between June 2014 and June 2015. Therefore, it was deemed realistic to recruit eight participants into the study over a 10-week period assuming a 50% recruitment rate.

This proved challenging in practice and the recruitment to the study was prolonged over at least 12 months, but the predicted sample size was reached and exceeded.

3.5.2 Active intervention

Omega-3 PUFA capsules were then issued to participants. The intervention was Incromega EPA/DHA soft gel omega-3 PUFA capsules (provided by EuroCaps Limited). Each 1g capsule contained 500mg EPA and 500mg DHA in triglyceride form, encapsulated in a clear bovine gelatine shell. Participants were instructed to take four capsules in total, daily.

A planned start date for commencing the capsules was agreed based on the timing of the most convenient next hospital visit. In total, three hospital or home visits were arranged for sample collection during the 4-week duration of the study.

3.5.3 Study visit schedule

3.5.3.1 Study visit one (V1)

Participants were expected to self-administer four capsules with meals daily for at least 28 days up until and including the day of the final scheduled study visit. Participants were asked to provide a pre-supplementation baseline stoma sample at the first hospital visit. Current evidence suggests that maximal omega-3 PUFA content in stoma fluid occurs between 2 to 8 hours after low dose administration. (Sanguansri et al., 2013) Therefore, participants were 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 omega-3 PUFA concentration.

A baseline questionnaire about their general health, drug history and dietary intake in previous 24hours was recorded. To assess individual stoma output, participants were asked to report the number of stoma bag changes, the day before and on the day of the hospital visit.

A venous blood and stoma sample were collected for baseline rbc membrane and ileostomy fluid (IF) EPA/DHA levels respectively. The participants were provided with six weeks' worth of omega-3 PUFA capsules. A convenient date was arranged to undertake visit 2 and start taking the omega-3 PUFA capsules.

3.5.3.2 Study visit two (V2)

The second visit was scheduled for the day the participant took the first capsules. All four capsules (4g) were taken orally with food and then participants provided a venous blood and stoma fluid sample within 2-10 hours of the first capsule dose to identify any immediate change in rbc membrane and ileostomy fluid EPA/DHA levels respectively. If this could not be done at SJUH, the researcher arranged to visit the participant at their home to collect the samples after the first omega-3 PUFA capsule dose. The time of ingestion of the first capsules and sample collection times were recorded. Dietary intake in the few hours after ingesting the first capsule dose was recorded. It was noted that visit one and two sometimes occurred on the same day as per the participants' convenience. However, visit two samples were always collected within 2-10 hours of first oral administration of the omega-3 PUFA capsules. Participants were advised to take the omega-3 PUFA capsules twice daily with meals for 4 weeks

3.5.3.3 Study visit three (V3)

The final hospital visit was scheduled 28 days after starting oral supplementation with the capsules. At the final hospital visit, a venous blood and stoma sample were collected for rbc membrane and ileostomy fluid EPA/DHA levels respectively. The samples were 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 ileostomy fluid sample was taken after capsule dosing. The time of the final capsule dose and the time the samples were collected was recorded. Dietary intake in the 24 hours prior to the last capsule dose and adverse events were recorded. A pill count was performed as a surrogate indicator of compliance. If an individual stopped taking capsules during the four-week supplementation period, they were asked to attend to provide a stoma fluid and venous blood sample. If they could not meet with the researcher on day 28, they were advised to continue taking the

omega-3 PUFA capsules until an appointment could be arranged. After 4 weeks, participants were required to provide a post-intervention stoma sample after their final omega-3 PUFA capsule dose at the same time after dosing, at which the first sample was obtained (plus or minus 1 hour). This was to minimise possible sample degradation by oxidation of omega-3 PUFAs or bacterial action in the ileostomy bag.

An extra two weeks' worth of omega-3 PUFA supplements was supplied in the eventuality that a participant could not attend the scheduled final study visit, and a delay was required. Dietary intake in the 24 hours prior to a hospital visit and sample collection was recorded in order to identify any substantial fish intake that may lead to misleading results. All adverse events were recorded. For any adverse event experienced, the participant was given a choice to stop or halve the daily number of capsules consumed (four to two capsules daily). All data was collated on a clinical research form.

3.5.4 Sample collection and preparation

3.5.4.1 Venous blood

At each visit, venepuncture was performed aseptically using a 21G needle and Vacuette® device. Venous blood was collected in 2 x 4ml EDTA-containing sample tubes, then transferred within 30minutes to the Leeds Institute of Medical Research (LIMR) at SJUH. The blood sample tubes were centrifuged at 800 x g for 5 minutes at 4°C within 30 minutes of venepuncture. A Pasteur pipette was used to remove the plasma layer and aliquot this into four individual cryogenic vials. The buffy coat layer was discarded, and the red blood cells were similarly aliquoted into four individual cryogenic vials. 2 vials of red blood cells and plasma were each stored in two separate freezers at -80°C for future analysis of the rbc membrane EPA and DHA content by liquid-chromatography tandem mass-spectrometry (LC-MS/MS) and plasma osmolality testing. This analysis was performed at the Institute of Cancer Therapeutics (ICT) at the University of Bradford. Samples were kept frozen until such a time, when they were transported across in dry ice.

3.5.4.2 Ileostomy fluid samples

The participants provided a sample of their ileostomy bag effluent in 2 sterile 20ml plastic containers. These were transferred on ice, within 30 minutes to the LIMR and stored in two separate freezers at -80°C. Aliquots for further analyses were prepared from the original samples by combining 150mg of the thawed ileostomy effluent with 3ml of water and vortexing for 1 minute to form a slurry. These aliquots were then transported on dry ice to the ICT at the University of Bradford to undergo LC-MS/MS

3.5.5 Omega-3 polyunsaturated fatty acids analysis

The preparation of samples for analysis, described below, was performed using a modification of a published protocol. (Volpato et al., 2017)

3.5.5.1 Fatty acid extraction

After thawing, rbcs were prepped for FA extraction by washing 500µl rbc in serum into Eppendorf cryogenic vial and 500µl sodium chloride (NaCl) added. The mixture was centrifuged at 1000 x g for 8 mins, the supernatant removed and discarded. The process was repeated twice to prepare homogenised rbc samples.

For IF samples, a 100µl aliquot was taken from the homogenised samples and underwent extraction, hydrolysis and derivatisation as subsequently described.

A 50µl rbc sample was mixed with 50µl of distilled water (dH_2O), containing internal standard (2µg/ml deuterated Arachidonic Acid – d8) and allowed to stand for 15 min. 550µl Isopropanol was slowly added to the mixture, vortexing twice during a 1-hour incubation period at room temperature. Following incubation, 350µl of chloroform was added. After a further 1h incubation, samples were centrifuged at 10,000 × g for 5 min. The supernatant was then evaporated to dryness in a rotary evaporator (EZ-2 plus rotary evaporator, Genevac Ltd, Suffolk, UK).

3.5.5.2 Fatty acid hydrolysis

The biological sample was reconstituted with 500 µl acetonitrile as a solvent. Saponification by acid hydrolysis was performed with the addition of 50 µl 5 M hydrochloric acid (HCl) followed by incubation at 80 °C for 1 h. Sequential additions of 50 µl of 5 M sodium hydroxide (NaOH) to neutralise, before the

addition of 350 µl chloroform. The samples were centrifuged to separate the solvent layers then left to stand for 5 min, before isolating the top 800 µl for evaporation to dryness.

3.5.5.3 Fatty acid derivatisation

Extracted FAs from hydrolysed dry samples (50 µl) were reconstituted in a mixture of 50 µl methanol (MeOH) and 150 µl derivatisation agents (50 µl of EDC, 50 µl of DMAP and 50 µl DAABD-AE all made to 10 mg/ml in methanol). The mixture was then incubated for 24h at room temperature in amber glass vials. The resulting FA derivatives were injected into the Liquid chromatography - electrospray ionisation - tandem mass-spectrometer (LC–ESI-MS/MS).

3.5.5.4 Liquid chromatography - electrospray ionisation - tandem mass-spectrometry (LC-ESI-MS/MS) measurement of fatty acids

Traditionally, gas chromatography mass-spectrometry (GC-MS) had been used to measure relative fatty acid content in plasma and rbc membranes both *in vivo* or *in vitro*. (Blanksby & Mitchell, 2010) However, the high energies for electron ionisation and the high temperatures required for GC-MS resulted in structural or thermal degradation of the various polyunsaturated fatty acids, rendering interpretation inaccurate (S.-H. Chen & Chuang, 2002). As LC-MS/MS became more widely available, it provided a robust and more sensitive technique without the drawbacks of GC-MS. Several liquid chromatography electrospray ionisation triple quadrupole tandem mass spectrometry techniques have been used in various interventional trials for FA content quantification. The LC-MS/MS method described by Volpato et al (Volpato et al., 2017) was adopted for this experiment. It was the first to compare directly to a ‘gold-standard’ GC-MS method; producing consistently reliable results across a range of long chain fatty acids. Its reliability and reproducibility in measuring omega-3 PUFA (in FFA or EE form) has been confirmed in animal and human interventional trials of EPA supplementation (Volpato et al., 2017; Watson et al., 2017). Specimen preparation via the steps listed above was required prior to injection into the mass spectrometer.

Red cell, plasma and IF samples were analysed 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). Two µl of derivatised sample was separated on a HiChrom RPB column (2.1 mm× 250 mm, 5 µm) (HiChrom Ltd, Berkshire UK). Mobile phase A (MPA) consisted

of 90% dH_2O, 10% MeOH, 0.1% formic acid and Mobile Phase B (MPB) consisted of 90% MeOH, 10% $dH_2O$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 0.5 ml/min and split post-column with 0.3 ml/min delivered to the MS. Samples were analysed in multiple reaction monitoring (MRM) mode for the following fatty acids: PA, LNA, LA, OA, SA, EPA, AA, DHA, DPA and deuterium-labelled internal standard (LNA-d8). The instrument settings were: capillary voltage, 3 kV; cone energy, 15 eV; collision energy, 25 eV; source temperature, 120 °C and desolvation temperature, 300°C. Results were expressed for each FA as the percentage and concentrations of the total analysed FA content for that sample.

3.5.6 Ileostomy fluid and plasma osmolality analysis

After thawing to room temperature and 2 cycles of centrifugation at 3000 x g for 10 minutes, 0.5ml of homogenised IF and plasma supernatant samples were obtained. Due to multiple analyses, the stock of IF provided by each participant was diminishing, thus some samples were reconstituted to 0.5ml with dH2O. Osmolality, the total solute concentration of an aqueous solution, was measured in duplicates for a volume of 20µl of supernatant using the Advanced 2020 Multi-Sample Osmometer (Advanced Instruments, Norwood, MA, USA) located at the pathology lab in SJUH. The machine used the principle of freezing point by inducing a mechanical freeze of the sample then raising its temperature to rise until the ice/water equilibrium was maintained, providing a readout of the osmolality of the sample within a working range of 0-2000 mOsm/kg H2O.

3.5.7 Measurement of ileal microbiota abundance

The Earth Microbiome Project (EMP) is a global collaborative, open science initiative to characterise all microbial species and patterns across the biomes and habitats of the planet. (The Earth Microbiome Project Consortium et al., 2017). The EMP 16S Illumina Amplicon methodology was designed to amplify bacteria and archaea for characterisation using primers aimed at the v4 region of the 16S SSU ribosomal RNA (rRNA). Expertise in this technique was validated at the

LIMR (Taylor et al., 2017), used for quantifying the faecal microbiome (Watson et al., 2018; Young et al., 2021); thus it was plausible to attempt a novel application for the ileal microbiome. This followed a three step process: extraction and purification of the bacterial DNA within the ileostomy fluid supernatant; Polymerase Chain Reaction (PCR) amplification of the V4 region of the 16S RNA gene, and sequencing according to the Earth Microbiome Project 16S Illumina Amplicon methodology.

3.5.7.1 Bacterial DNA extraction

A modified version of the QIAamp DNA Mini Kit (Qiagen) protocol was used. Two hundred mg of frozen IF sample was weighed into an Eppendorf tube and 1800µl of Buffer ASL (Qiagen) was added. This mixture was placed onto a 'Thermomixer Comfort' (Eppendorf UK) set at 23°C, 850 reps per minute (rpm) for 1 hour. One ml was transferred to pathogen lysis tubes (S) (Qiagen) and mixed on a shaker (Vibrax VXR, IKA, UK) at a motor setting of 1800 – 2200rpm for 10 minutes. This was returned to the Thermomixer set at 95°C, 850rpm for 15 minutes. After centrifuging at 14000rpm for 1 minute, the supernatant was transferred into a new Eppendorf tube.

To the supernatant, 173 µl of 10M ammonium acetate was added, vortexed and cooled on ice for 5 minutes. It was then centrifuged at 14000rpm for 5mins, and the supernatant added to 725 µl of propan-2-ol (Fisher Scientific, UK). Samples were then vortexed, cooled on ice for 30minutes, centrifuged at 14000rpm for 10mins, followed by removal of the supernatant. The precipitated bacterial DNA pellet was combined with 1ml of 70% ethanol and centrifuged at 14000rpm for 5 minutes. The supernatant was removed and discarded. Another 500µl of 70% ethanol was added to the pellet and centrifuged at 14000rpm for 3 minutes. The supernatant was again removed and discarded. The DNA pellet in the Eppendorf tube was left open to air dry for approximately 10minutes.

Samples were then processed as per the QIAamp DNA Mini Kit protocol. Sequential addition of 200 µl of 1x Tris EDTA buffer with the lids closed for 10minutes and 200 µl of Buffer AL, resulted in re-suspension of the DNA pellet. Fifteen µl of proteinase K was added, vortexed and left on the Thermomixer at 70°C, 650rpm for 10minutes. Another 200 µl of 100% ethanol was added and the mixture transferred to a QIAamp DNA Mini Spin Column (QIAamp DNA Mini Kit) and centrifuged at 14000rpm for 1 minute. The DNA column was then transferred to a clean tube.

DNA washing and elution consisted of addition of 500µl of Buffer AW1, centrifuging at 14000rpm for 1 minute, discarding the flow through and transfer

of the column to a clean 2ml collection tube. Following this, 500µl of buffer AW2 was added, centrifuged at 14000rpm for 3 minutes, the flow through discarded and the column transferred to a clean 2ml collection tube. The membranes were dried by centrifuging at 14000rpm for 1 minute. One hundred µL of UV-treated molecular grade water was added to the columns. This was incubated at room temperature for 5 minutes and centrifuged at 14000rpm for 1 minute, discarding the column at the end.

The purified DNA was quantified using the NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific Incorporated, UK). Following selection of nucleic acid tab, performing a wavelength verification check, and a blank tester (dH_2O), 2µl of the extracted DNA sample was pipetted into the lower pedestal and spectral analysis performed. Assessment of DNA purity via 260/280 ratio, 260/230 ratio and DNA concentrations are described in the results section 3.7.7

3.5.7.2 Bacterial DNA PCR reaction

3.5.7.2.1 Sample dilution

Pre-PCR DNA sample dilution was performed by adding molecular grade water to dilute 3µl DNA sample to a concentration of 20ng/ul in UV-treated 96-well plates. Reagents for PCR were as per the Earth Microbiome Project 16S Amplicon protocol. (Apprill et al., 2015; Parada et al., 2016; The Earth Microbiome Project Consortium et al., 2017)

3.5.7.2.2 Amplification

A PCR mastermix was created by mixing 12.5 µl of PCR grade water (Sigma), 10µl of Platinum Hot Start PCR Master Mix (2x) (ThermoFisher), 0.5µl of 10 µM reverse primer. The mastermix (23µL) was added to each well of a plate. To this, 1µl 5uM forward primer and 1µl bacterial DNA was added. Three wells were reserved for controls: 1µl each of E. coli DNA (Sigma-Aldrich) as the positive control, PCR grade water and human C2020 cell line DNA (Sigma-Aldrich) as the negative controls respectively. The plate was sealed and centrifuged briefly then placed on a 96-well Thermocycler (C1000 Touch, Bio-Rad, UK) set at 94°C for 3 minutes, 35 cycles at 94°C for 45s, 50°C for 60s, 72°C for 90s, an extension of 10 minutes at 72°C and 4°C hold. Once complete and plate integrity satisfactorily maintained, they were stored on ice until gel loading.

3.5.7.2.3 Post-amplification agarose gel electrophoresis

The gel was reconstituted using 1.5g of agarose powder, 100ml of TBE and 5µl of ethidium bromide in a conical flask, warming until dissolved, then cooled onto a tray with combs.

The PCR products (5µL) were vortexed with 1.5µl of orange loading dye. After addition of 3µl blue ladder dye into the first well of the gel, 5µl of DNA samples were loaded into subsequent wells. The gel was run at 100V for 25minutes (Bio Rad pack).

3.5.7.2.4 Post amplification quantification

The Quant-iT 'dsDNA Assay Kit, broad range' (Thermo Fisher Scientific, USA) was used for DNA quantitation. Individual master mixture consisting of 197µl Quant-iT buffer and 1µl Quant-iT dsDNA BR Reagent was combined with 2µl of PCR product. Quant-iT DNA standards were used to construct a standard curve. Fluorescence was read using a microplate fluorometer (Fluoroskan Ascent, ThermoFisher Scientific, USA) and DNA concentration in PCR products calculated.

3.5.7.2.5 Post amplification pooling and purification

Following amplification, the PCR products were purified using the MinElute PCR Purification kit (Qiagen, Germany). From the fluorometer concentration readings, the volume of PCR product to yield 240ng of DNA was calculated. From each sample, 240ng was obtained and pooled (total of three pools), and 5 times this volume of Buffer PB was added. Each pool was added to a MinElute column and centrifuged at 13000rpm for 1 minute. The flow through was discarded. 750 µl of Buffer PE was added and centrifuged at 13000rpm for 1 minute. The flow through was discarded. The empty column was centrifuged at 13000rpm for 1 minute. To elute the sample, 10 µl of Buffer EB, was added and centrifuged after 1 minute at 13000rpm for 1minute, and this was repeated to give a final elute of 20ul. The three cleaned pools were analysed using the 2200 TapeStation (Agilent, USA). The samples were stored at -20°C to be analysed as a batch with samples from other studies in one Illumina sequencing run.

3.5.8 Illumina sequencing

Samples were sequenced on a HiSeq 3000 2x150 base pair paired end lane, as part of a pool of 996 samples from various studies, all using the same library protocols (Caporaso et al., 2012). Reads were stripped of adapter sequences using Cutadapt (v1.18) (Martin, 2011).

Further initial processing was performed in the QIIME2 environment (v2019.10) (Bolyen et al., 2019). Reads were shortened to a maximum of 145bp based on base quality, before being merged, denoised and representative sequences picked using DADA2 (Callahan et al., 2016). Representative reads were aligned to the SILVA database (release 132) using the BLAST+ algorithm ((Camacho et al., 2009; Quast et al., 2012). All taxonomy assignments were exported to MEGAN 6 for further analyses and descriptive plots including Principal Coordinate Analysis (PCA), heatmap and taxonomy barplots (Huson et al., 2016).

3.5.9 Statistical analysis

Given the paucity of data on absolute ileal or faecal EPA and DHA concentrations generated following oral omega-3 PUFA supplement use, a formal sample size calculation could not be calculated. As explained in chapter 3.5.1.3, the predicted sample size of 8 participants would be deemed satisfactory. This would also be a larger sample size than the previous clinical study of ileal omega-3 PUFA content.

Given such a small sample size, hypotheses were tested with significance differences achieved with a p-value <0.05

Relative concentrations of each FA were expressed as a percentage of the total analysed FA content for that biological sample (%FA). Absolute FA concentrations in ileostomy fluid, rbc membrane and plasma were quoted in µg/ml. Scatter dot and bar plots were used for individual and group level data for EPA and DHA. Pre- and post- supplementation data were compared using two-sided Students' t-tests and ANOVA where appropriate. Non-parametric tests (Mann-Whitney U and Wilcoxon signed-rank) were used for group analyses of ileostomy fluid, RBC and plasma omega-3 PUFA profiles with a non-normal distribution. Differences were expressed with their 95% confidence intervals, accepting a p value <0.05 as significant. Graphs and statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, Inc.). Adverse

events in each group were compared by Fisher's exact test. Spearman correlation tests were used to assess the relationship between timing of sampling and concentrations of omega-3 PUFA.

Due to the multivariate nature of microbiome data, (Mallick et al., 2017) taxonomy assignments were exported to MEGAN. Analyses focused on level 6 (genus) assignments. Datasets were grouped for the three different visits and compared using barplots for the top 35 selected genera ranked by abundance. Ileal microbiome shifts across visits (V2-V1 and V3-V1) and PCA plots were used to assess and plot intra-individual differences in microbiome abundance across the three visits. Beta (β) diversity between visit samples and across participants was tested using the Adonis permutational multivariate ANOVA (PERMANOVA) test. Pearson correlation between ileal microbiome abundance and rbc and IF data were calculated using the R package (R Core Team (2020)., n.d.) using the Benjamini-Hochberg corrections to correct the false-discovery rate (Benjamini & Hochberg, 1995).

Chapter 4 Results of a clinical study to measure the ileal luminal bioavailability of oral omega-3 polyunsaturated fatty acids, and associated changes on the ileal microbiota in humans with a temporary ileostomy

The following section details the results of a clinical experiment to establish the luminal bioavailability of omega-3 PUFA in the distal small intestine specifically in participants with an end ileostomy following colorectal cancer surgery. A cohort of volunteers were administered 4g of 1:1 EPA/DHA oral capsules daily for a minimum of 28 days. The ileostomy fluid was collected to quantify the actual concentrations of fatty acids, with a particular focus on the omega-3 PUFAs EPA and DHA as the primary endpoint. The changes in the gut microbiome were also analysed to provide insight on the effects of long-term omega-3 PUFA supplementation on microbial composition. This data was presented at an international conference and published in the Journal of Nutrition in August 2021.

In addition to the data published, this thesis will present novel data on the total fatty acid presence within the ileostomy fluid. As elucidated earlier in the section 1.2.3, the omega-3 and omega-6 relationship are linked and this relationship will also be investigated, together with alterations in FA balance in the gut lumen after dietary supplementation.

4.1.1 Enrolment of participants

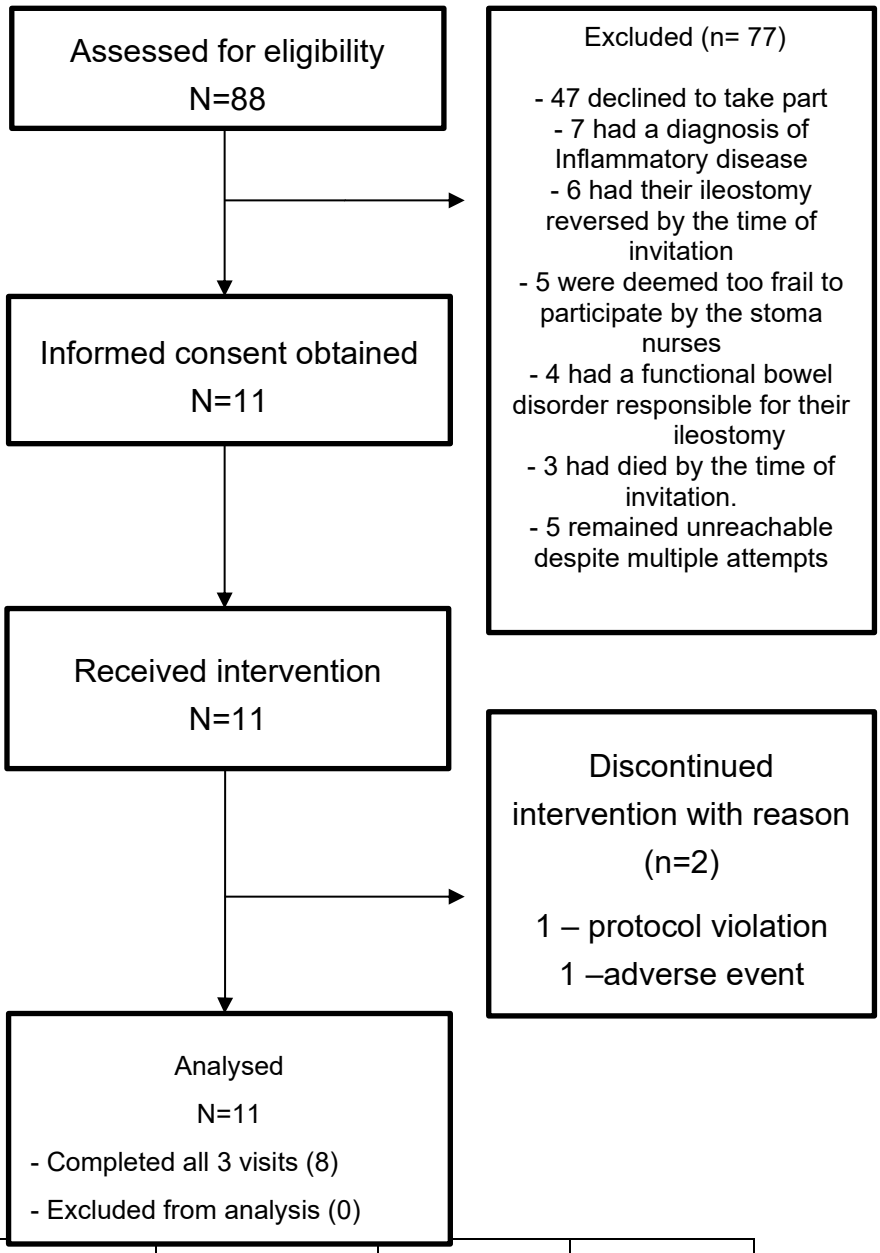
Participant recruitment occurred between April 2016 and December 2018. Eighty-eight CRC patients were screened for eligibility via the CRC stoma database. Seventy-seven participants were excluded – 13 were deemed ineligible while 64 did not consent. This is illustrated in the Figure 3 below.

The sample size consisted of 11 participants who volunteered to take part. All 11 participants provided a baseline sample of IF and venous blood at their first visit (V1).

They started oral supplementation either straight away (2 participants) or at a later date (9 participants), attending for a second visit to provide IF and venous blood samples (V2), except for one participant in whom venepuncture was unsuccessful. At this second visit, one participant was withdrawn because his age fell outside the inclusion criteria at the time (42 years of age, rather than over 50). This protocol violation led to a revision of ethical approval to include anyone

over the age of 18. Despite the participant's inability to continue with the study, they granted consent for their data to be included in the analysis.

The remaining 10 participants continued with the capsules; one patient did not provide enough for a IF sample at visit 3; another stopped taking capsules on day 24 after reporting severe gastrointestinal adverse events despite reducing the dose of active intervention. Only 8 participants completed the study per protocol. The results are presented in the flow diagram below:



Specimens provided	Visit 1	Visit 2	Visit 3	Total
Blood samples	11	10	9	30
Ileostomy fluid samples	11	11	9	31

Figure 4 Participant flow diagram

4.1.2 Participant characteristics, compliance, and tolerability

All 11 (8 male, 3 female) participants were colorectal cancer patients who had undergone an anterior resection with formation of a temporary loop ileostomy with a median time of 9 (2 – 18) months prior to recruitment. The median age was 63 (42-81) years. As earlier stated, one patient was suspended due to a protocol violation, noticed after 4 capsules had been ingested. The median length of time on the active intervention was 28 (1- 33) days. Each patient was given 168 capsules to last the study period. A median of 53 (32-164) capsules were returned, with individual compliance of 100 (50 – 107)% with intervention. Compliance was calculated using the formula below:

$$\frac{Observed\ number\ of\ capsules\ taken\ [total\ given\ to\ participant\ (168) - number\ returned]}{expected\ number\ of\ capsules\ taken\ (4\ x\ number\ of\ days\ taking\ capsules)}\ \text{x } 100\%$$

The frequency of ileostomy bag emptying was recorded as a proxy for diarrhoea/ loose stool secondary to supplementation with omega-3 PUFA Samples were collected on average within 2 hours of taking the capsules on visit 2 and 3. These results are summarised in Table 1.

One participant experienced abdominal discomfort, burping and nausea so discontinued supplements. The others tolerated the intervention without adverse events.

Participant characteristics	Results - median (range)
Age (years)	63 (42 – 81)
Sex (Male : Female)	8:3
Interval from stoma formation to enrolment onto the study (months)	9 (2- 18)
Duration of intervention (days)	28 (1-33)
Frequency of ileostomy bag emptying in last 24h Visit 1 Visit 3	 3 (1-7) 4 (1-7)
Time between last oral dose of omega-3 PUFA and sample collection (hours) Visit 2 Visit 3	 2 (1-4) 2 (1-4)
Capsules returned	53 (32-164)
Individual compliance (%)	100 (50 -107) %

Table 1 Participant characteristics.

4.1.3 Concentration of omega-3 PUFAs in ileostomy fluid

4.1.3.1 EPA concentration rises increases with dietary supplementation

At baseline prior to starting the capsules (V1), the mean (±standard deviation – SD) IF EPA was 0.99 (±0.52) µg/ml. There was an approximately 30-fold overall increase in mean EPA concentration to 29.7 ± 61.3 µg/ml after the first oral dose of omega-3 PUFA capsules (V2). By the end of treatment after 28 days of oral dosing (V3), there was a statistically significant rise in IF EPA to 6.88 ± 14.3 µg/ml (p= 0.027, Wilcoxon signed rank test). This is illustrated in Table 2 below:

FA	Baseline (V1)	Post 1st oral dose (V2)	Post final oral dose (V3)
LNA	1.7 (2.6)	0.7 (0.63)	0.98 (1.01)
EPA	0.99 (0.52)	29.7 (61.3)	6.88 (14.3)
DPA	0.03 (0.05)	0.13 (0.23)	0.07 (0.11)
DHA	0.98 (0.71)	28.6 (59.4)	7.37 (15.7)
LA	12 (12)	5.23 (3.51)	9.4 (12.4)
AA	5.6 (5.5)	8.29 (10.3)	4.42 (3.76)
OA	14 (16)	10.1 (6.92)	18.4 (16.6)
SA	363 (245)	308 (142)	557 (400)
PA	112 (81)	79.9 (34.7)	168 (134)

Table 2 Mean (±SD) FA concentrations (µg/ml) in Ileostomy fluid samples collected before (V1), after the first dose (V2) and after the final dose 28 days later (V3) of omega-3 PUFA capsules containing 4g of mixed 1:1 EPA and DHA. LNA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; SA, stearic acid; PA, palmitic acid

4.1.3.2 DHA concentration also increases after supplementation

Like EPA as illustrated in Table 2, mean baseline DHA levels of 0.98 ± 0.71 µg/ml rose 29-fold to 28.6 ± 59.4 µg/ml after initial oral supplementation (V2). This initial spike eventually fell with consistent intake of capsules such that after 4 weeks,

there was a seven-fold overall increase from baseline to final (V3) mean DHA levels of 7.37 ± 15.7 µg/ml. This mean DHA increase was statistically significant (p=0.01, Wilcoxon signed rank test) and similar to the increase in EPA. This therefore agrees with our primary hypothesis that EPA and DHA increase after 4 weeks of oral supplementation.

4.1.3.3 Subgroup analysis and inter-individual variation of EPA and DHA concentrations

In addition to baseline readings, individual IF measurements after initial consumption of oral supplements (V2) were investigated (Table 3). It was noticeable that a subgroup of 4 participants (participants #2, #3, #5 and #6) were responsible for this significant rise, while the other participants (participants #1, #4 and #7 - #11) did not display a clear increase in the IF EPA/DHA concentration at V2 (Figure 5). A similar occurrence was observed with DHA (compare Figures 5 and 6).

Participant	LNA	EPA	DPA	DHA	LA	AA	OA	SA	PA
P1 V1	0.1	1.5	0.01	0.8	1.3	2.4	3.0	240.5	62.2
P1 V2	0.3	1.5	0.08	1.8	3.1	4.8	5.4	273.6	67.2
P1 V3	0.1	1.6	0.02	1.6	1.9	2.7	3.2	325.4	74.6
P2 V1	**0.8**	**0.6**	**0.01**	**0.6**	**2.2**	**1.7**	**6.5**	**226.2**	**96.6**
P2 V2	**0.3**	**52.5**	**0.18**	**52.0**	**2.0**	**9.5**	**2.4**	**109.9**	**44.6**
P2 V3	**1.0**	**1.1**	**0.04**	**2.7**	**9.8**	**6.1**	**8.0**	**190.9**	**66.9**
P3* V1	**3.0**	**1.2**	**0.02**	**1.9**	**28.1**	**15.3**	**60.7**	**323.2**	**121.0**
P3* V2	**0.2**	**20.6**	**0.08**	**22.4**	**3.1**	**4.9**	**15.7**	**197.2**	**55.1**
P4 V1	0.3	0.6	0.01	0.2	2.9	1.6	6.0	367.7	93.7
P4 V2	0.6	0.7	0.01	0.1	2.0	0.9	10.5	573.3	95.5
P4 V3	0.2	1.1	0.01	1.0	0.8	1.0	5.3	634.4	118.0
P5 V1	**0.4**	**0.4**	**0.01**	**0.3**	**2.9**	**3.1**	**12.9**	**1067.1**	**343.9**
P5 V2	**2.3**	**207.1**	**0.79**	**200.5**	**9.7**	**37.2**	**25.7**	**273.1**	**74.2**
P5 V3	**0.3**	**0.8**	**0.00**	**0.3**	**1.7**	**0.9**	**7.6**	**461.3**	**76.2**
P6 V1	**9.2**	**1.1**	**0.10**	**2.2**	**15.8**	**13.5**	**17.7**	**417.0**	**114.1**
P6 V2	**0.2**	**34.4**	**0.11**	**28.0**	**4.2**	**10.8**	**6.3**	**208.8**	**53.8**
P6 V3	**3.0**	**3.6**	**0.07**	**3.9**	**12.9**	**4.5**	**38.6**	**663.0**	**212.6**
P7 V1	0.7	0.3	0.00	0.1	36.8	0.6	9.8	187.3	49.7
P7 V2	0.7	0.5	0.00	0.1	9.0	0.8	3.8	171.7	37.6
P7 V3	0.4	1.1	0.01	1.1	2.7	1.0	3.9	198.3	43.9
P8 V1	0.5	2.0	0.08	1.0	6.0	12.7	6.4	217.6	63.1
P8 V2	0.7	1.3	0.03	0.3	2.4	5.4	7.3	352.3	86.3
P8 V3	0.4	0.9	0.04	0.7	3.9	2.3	6.5	432.1	187.4
P9 V1	1.3	1.4	0.03	1.6	15.1	4.1	12.7	344.2	105.1
P9 V2	0.6	1.4	0.05	2.6	2.6	1.7	5.2	338.8	85.0
P9 V3	1.0	47.1	0.37	51.7	7.2	11.5	37.8	1472.4	441.5
P10 V1	0.2	0.8	0.04	1.0	3.5	5.4	6.6	251.5	70.3
P10 V2	0.4	1.0	0.13	1.0	8.4	12.0	15.5	510.4	143.0
P10 V3	0.8	4.2	0.09	5.3	10.7	10.0	30.3	940.6	355.4
P11 V1	2.1	1.0	0.11	1.5	12.6	1.4	9.9	347.1	115.6
P11 V2	1.4	5.7	0.03	5.9	11.0	3.2	12.8	375.8	136.6
P11 V3	2.6	7.2	0.03	5.4	42.5	4.1	42.6	249.0	103.0

Table 3 Absolute fatty acid concentrations in ileostomy fluid in µg/ml. P = individual participant number; V = Visit number; LNA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; SA, stearic acid; PA, palmitic acid. Participants with V2 spikes in EPA and DHA (#2,3,5,6) are in bold. P3* - no V3 value as patient did not provide a sample.

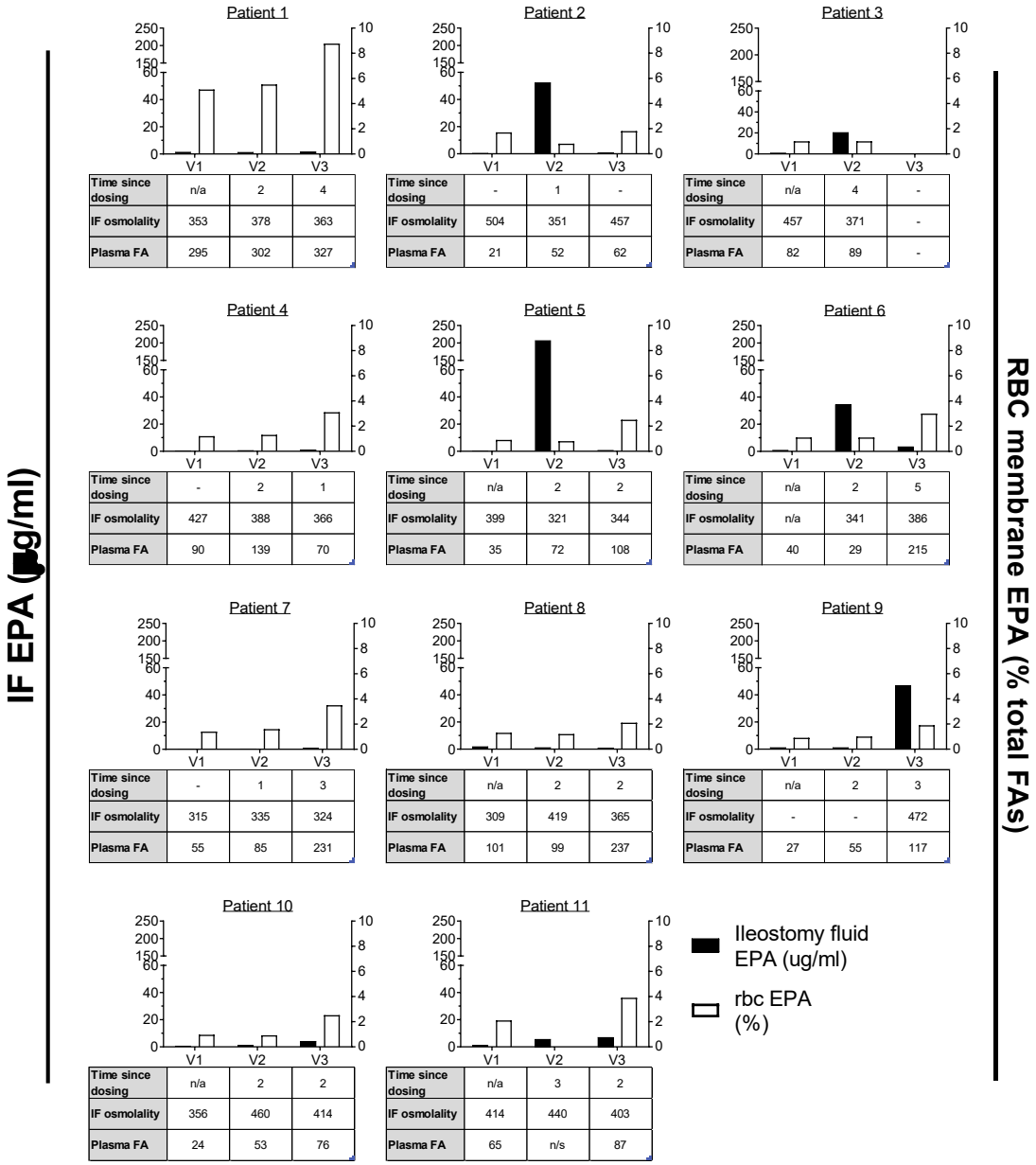


Figure 5 Absolute concentration of EPA in ileostomy fluid (IF) in µg/ml (left y-axis); red cell membrane (rbc) as % of total fatty acids (right y-axis); and plasma (µg/ml) for individual participants in the study.

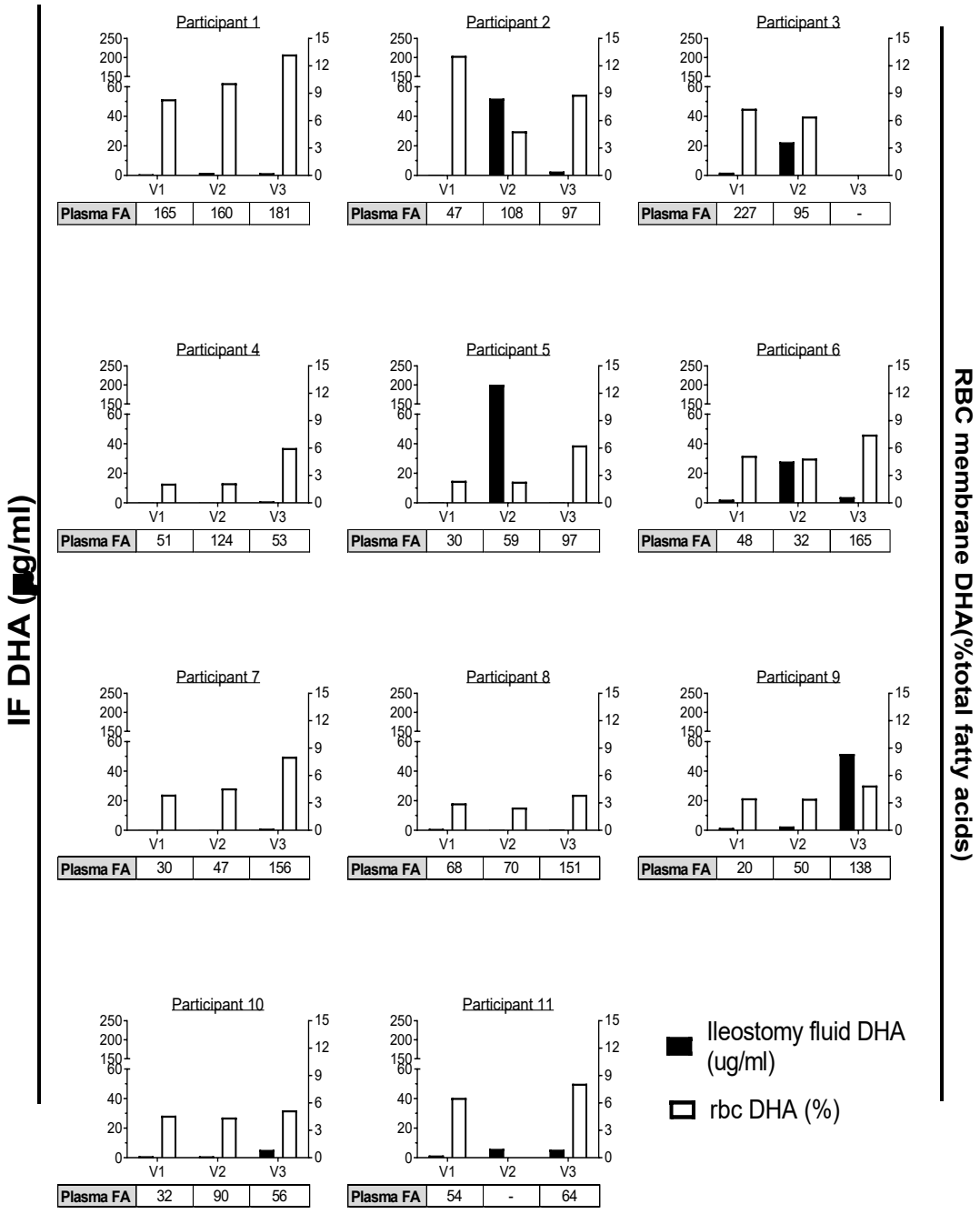


Figure 6 Absolute concentration of DHA in ileostomy fluid (IF) in µg/ml (left y-axis); red cell membrane (rbc) as % of total fatty acids (right y-axis); and plasma (µg/ml) for individual participants in the study.

Potential reasons for this observed disparity between V2 concentrations in this study cohort were explored.

The mean time interval from omega-3 PUFA supplement dosing to IF sample collection was 2 hours (range 1-4 hours) in the 36% of participants (participants

#2, #3, #5 and #6) who had a V2 spike in EPA/DHA concentration. There was no significant difference in mean time interval (2 hours, range 1-3 hours; p=1.0, Mann-Whitney U test) to sample collection between them and the other subgroup of participants (#1, 4, 7-11). See Table 4.

Overall, there was no relation between the ileal EPA/DHA concentration and time interval between oral dosing and IF sample collection at visit 2. [ρ=0.20, P=0.57 and ρ=-0.17, P=0.61 respectively, Spearman rank correlation]. It is worth noting that there were no significant differences between the sample collection interval after oral dosing, either after 1st (V2) or final (V3) dose. (p=0.22, Wilcoxon signed rank test)

Participant	Post 1st oral dose (V2)	Post final oral dose (V3)
P1	2	4
P2	1	-
P3	4	-
P4	2	1
P5	2	2
P6	2	5
P7	1	3
P8	2	2
P9	2	3
P10	2	2
P11	3	2

Table 4 Timing interval (hours) from oral capsule supplementation to sample collection. P2 and P3 had missing V3 values

4.1.3.4 Relationship between IF EPA/DHA concentration and osmolality

Another potential reason could be the solute concentration in the ileostomy effluent volume collected for each individual. I calculated the osmolality per sample in an attempt to reduce confounding due to IF volume disparities (Table 5)

Participant	Baseline (V1)	Post 1st oral dose (V2)	Post final oral dose (V3)
P1	353	378	362.5
P2	503.5	350.5	457
P3	456.5	371	-
P4	427	388	365.5
P5	399	320.5	343.5
P6	-	340.5	385.5
P7	314.5	335	323.5
P8	308.5	419	365
P9	-	-	471.5
P10	355.5	459.5	414
P11	414	439.5	402.5

Table 5 Ileostomy Fluid (IF) Osmolality (mOsmol /kg) by participant for each sample collection visit. (-) represent those samples with insufficient IF volumes for analysis to be performed.

The increase in IF EPA/DHA content was also not due to a highly concentrated ileostomy effluent as measured by IF osmolality for V2 samples (see mini tables in Figure 5 and 6). The mean IF osmolality for the 4 participants (P2,3,5,6) was 345 ± 21mOsm/kg compared to 403 ± 45 mOsm/kg (p=0.067, Mann-Whitney U).

In trying to understand the significance of this high EPA and DHA content in IF after initial oral dosing, I tested the hypothesis that this could be due to lower proximal intestinal absorption. This would be associated with an absence of a peak in plasma omega-3 PUFA concentrations after initial oral dosing. However there was no statistically significant difference between the two subgroups in

plasma EPA (mean 61 ±26 compared to 122 ±94 µg/ml, p=0.17, Mann-Whitney U test) or plasma DHA (mean 74 ±35 compared to 90 ±45 µg/ml, p=0.76, Mann-Whitney U test).

However, the lack of a noticeable acute peak in plasma omega-3 PUFA response to oral dosing was likely due to the insufficient time (2 to 4 hours) from oral dosing to sample collection for plasma analysis in this study. This was shorter than the 5-6 hours for maximum plasma FA concentration post oral dosing quoted in other studies (Braeckman et al., 2014).

4.1.3.5 Initial rise in EPA and DHA subsides to remain at an elevated concentration after oral dosing.

At the end of the 4-week supplementation period (V3), mean IF EPA concentration rose six-fold from 0.99 (±0.52) µg/ml to 6.88 ± 14.3 µg/ml. This increase was statistically significant (p= 0.027, Wilcoxon signed rank test). Two participants stood out in particular for higher than average increases after supplementation, P9 and P11 (Figure 5 and Table 3). Participant 9 demonstrated the highest increase in V3 IF EPA (a 33-fold increase from 1.41 to 47.08 µg/ml). Participant 11 had a more modest seven-fold increase. There was no strong correlation between baseline and final IF EPA concentrations (Spearman r=0.347, P= 0.32). Excluding the subgroup of individuals with an exponential rise in EPA and DHA in V2, oral dosing results in an increase in IF EPA and DHA in V3 compared to the corresponding concentration at V2, from baseline. This difference however was not statistically significant (p=0.06 for EPA, and 0.11 for DHA, respectively, Wilcoxon signed-rank test). This suggests that oral supplementation does indeed increase bioavailability in the small bowel lumen, within 2 hours (range 1-5 hours). Consistent with visit 2 data, there was no correlation between oral dosing and duration to sample collection for both EPA and DHA (ρ=0.25, P=0.53 and ρ=0.34, P=0.36 respectively, Spearman rank correlation)

4.1.3.6 Alpha-Linolenic acid (LNA) concentration

Mean IF LNA levels fell from baseline (1.70 ±2.6 µg/ml) to 0.7 ± 0.63µg/ml after the first oral dose (V2). After 4 weeks of continuous supplementation with omega-3 PUFAs, there was some recovery in final LNA concentration to 0.98 ± 1.01 µg/ml however this remained lower than the baseline level. There was no statistically significant difference between measured concentrations at V2 and V3

(p=0.35 and p=0.72 respectively, Wilcoxon signed rank test) compared to baseline. Thus, in this study, oral supplementation with EPA/DHA capsules did not have an effect on LNA concentrations.

4.1.3.7 Docosapentaenoic acid (DPA) concentration

As shown in section 1.2.3 – absorption and metabolism of omega-3 PUFAs, DPA is the direct elongation product of EPA. Mean IF DPA at baseline (0.03 ± 0.05 µg/ml) was lower than EPA at baseline (1.00 ± 0.52 µg/ml). Mirroring EPA concentration, first oral dosing of capsules resulted in a small 4-fold increase in mean IF DPA to 0.13±0.23 µg/ml, but this was not statistically significant (p=0.5, Wilcoxon signed rank test). The peak IF EPA concentrations observed in the subgroup of participants (#2,3,5,6 in Table 3) at V2, did not result in similar exponential increase in DPA concentrations at V2, indicating either potential saturation of enzymatic conversion of EPA to DPA, or insufficient time to convert EPA to DPA. After 4 weeks of oral supplementation, there was an increase in mean IF DPA to 0.07 ± 0.11 µg/ml, however this was not statistically significant (p=0.43). Again, this mirrored the EPA trend, albeit with much lower concentrations.

4.1.4 Concentration of omega-6 PUFAs in ileostomy fluid

Due to the reciprocal incorporation of omega-6 and omega-3 PUFAs into cell membranes – where an increase in omega-3 leads to reduction in omega-6 and increased membrane fluidity, together with competition in enzymatic pathways and inflammatory signalling, the effect of omega-3 PUFAs supplementation on omega-6 bioavailability in this cohort was explored.

4.1.4.1 Linoleic Acid (LA)

Mean IF LA concentration of 12.0 ±12 µg/ml was much higher than the EPA/DHA baseline concentration. After first dose, this dropped to mean IF LA of 5.2 ± 3.51 µg/ml, with participants #5,10 and 11 having the highest concentrations (Table 6). After 4 weeks, the mean IF LA was 9.41 ± 12.4 µg/ml accounting for the disproportionate rise in participant 11. There was no discernible trend in the participants bioavailability of LA with respect to EPA/DHA supplementation as #2,10 and 11 had the highest changes indicating possible intra-individual differences. Furthermore, the difference in final IF LA concentration from baseline was not statistically significant (p=0.7, Wilcoxon signed rank test)

Participant	Baseline (V1)	Post 1st oral dose (V2)	Post final oral dose (V3)
P1	1.3	3.1	1.9
P2	2.2	2.0	9.8
P3	28.1	3.1	
P4	2.9	2.0	0.8
P5	2.9	9.7	1.7
P6	15.8	4.2	12.9
P7	36.8	9.0	2.7
P8	6.0	2.4	3.9
P9	15.1	2.6	7.2
P10	3.5	8.4	10.7
P11	12.6	11.0	42.5

Table 6 Linoleic Acid concentrations after oral supplementation with omega-3 PUFAs. P3 – missing V3 value

4.1.4.2 Arachidonic Acid

Unlike LA, mean IF AA rose with first capsule dosing (5.62 ± 5.47 to 8.29±10.3 µg/ml). After 4 weeks, the AA concentration fell below baseline to 4.41 ± 3.76 µg/ml. Once again there was no statistical significance in the differences from baseline, indicating no consistent relationship between EPA/DHA supplementation and AA bioavailability in the small bowel lumen.

4.1.5 Concentrations of Oleic (OA), Stearic (SA) and Palmitic (PA) Acid.

At baseline, OA, PA, SA had a greater than ten-fold, 100-fold and 300-fold concentrations compared to EPA, respectively (see table 2). An initial fall in their concentration occurred after first oral EPA/DHA capsule supplementation perhaps indicating preferential enzymatic processing of EPA and DHA. With prolonged supplementation after 4 weeks, the OA, SA and PA concentrations

rebounded above baseline (see Table 3). This could be explained by the high prevalence of saturated fats in the Western diet consumed by our participants.

4.1.6 Concentration of omega-3 polyunsaturated fatty acids in red cell membranes

After 4 weeks of oral supplementation with 4g omega-3 PUFA (V3), red cell membrane EPA and DHA concentrations were consistently higher than at baseline (V1) as illustrated in Table 7 below. There was a statistically significant increase in both mean rbc EPA and DHA content. Mean rbc EPA rose from 1.6 ± 1.3% to 3.3 ± 2.0% (P=0.003; paired t-test) while rbc DHA rose from 5.3 ± 3.4% to 7.2 ± 2.7% (P=0.046; paired t-test). This data is consistent with previous studies on rbc FA incorporation after long term supplementation. (Neubronner et al., 2011; Offman et al., 2017)

This is further supported by a rise in the mean plasma EPA and DHA concentrations between visit 1 and visit 3 samples (Table 8 below). This supports the excellent participant compliance with capsule supplementation and indicates that the physiology of omega-3 PUFA absorption from a temporary loop ileostomy is relevant to individuals with an intact gastrointestinal tract.

FA	Baseline (V1)	Post 1st oral dose (V2)	Post final oral dose (V3)
LNA	0.31 (0.14)	0.21 (0.06)	0.23 (0.08)
EPA	1.59 (1.22)	1.53 (1.43)	3.31 (2.03)
DPA	3.36 (0.78)	3.06 (0.85)	3.39 (0.71)
DHA	5.45 (3.24)	4.57 (2.38)	7.2 (2.66)
LA	15.1 (4.74)	13.9 (5.03)	12.6 (3.47)
AA	14.8 (3.96)	13 (1.92)	13.3 (2.43)
OA	18.1 (2.65)	17.5 (2.37)	16.7 (1.73)
SA	23 (4.08)	23.8 (1.96)	24.6 (2.71)

Table 7 Mean (SD) FA concentrations (as a % total FA) in red cell membrane samples collected before (V1), after the first dose (V2) and after the final dose 28 days later (V3) of omega-3 PUFA capsules containing 4g of mixed 1:1 EPA and DHA.

FA	Baseline (V1)	Post 1st oral dose (V2)	Post final oral dose (V3)
LNA	21.2 (15.5)	20.2 (10.9)	12.3 (5.9)
EPA	75.6 (77.9)	97.4 (78.2)	153 (92)
DPA	25.6 (15.3)	24.9 (15.9)	20.9 (9)
DHA	70.1 (65.2)	83.5 (39.7)	116 (48.2)
LA	698 (599)	770 (466)	503 (233)
AA	245 (183)	249 (150)	173 (82.6)
OA	524 (432)	503 (299)	292 (122)
SA	121 (122)	89.3 (122)	41.8 (42.5)

Table 8 Mean (SD) FA concentrations (µg/ml) in plasma samples collected before (V1), after the first dose (V2) and after the final dose 28 days later (V3) of omega-3 PUFA capsules containing 4g of mixed 1:1 EPA and DHA

4.1.7 Ileal microbiota analysis

Fewer studies are available on ileal compared to faecal microbiota due to the complexities in obtaining samples, the temporal changes in the intestinal luminal environment, and variable bioinformatic analysis (Kastl et al., 2020). Thus the opportunity was taken to characterize this important microbiological ecosystem in relation to intestinal luminal omega-3 PUFA concentration.

4.1.8 Bacterial DNA extraction and purification

Of the 32 original IF samples, 9 samples did not yield enough DNA >10ng/ml or were deemed of high purity (260/230 ratio < 1) so the extraction was repeated. Two samples had no original IF volume left for a repeat of the extraction process while 7 samples underwent a repeat extraction. After a comparison between the original and the repeat sample, the sample with the greater DNA yield was taken for PCR. The measured DNA concentrations with ratios are illustrated in Table 9. Ninety-one percent of the bacterial DNA samples (n=29) had 260/280 ratios of >1.8 while 25% of these (n=8) had a 260/230 ratio of 1.8-2.2.

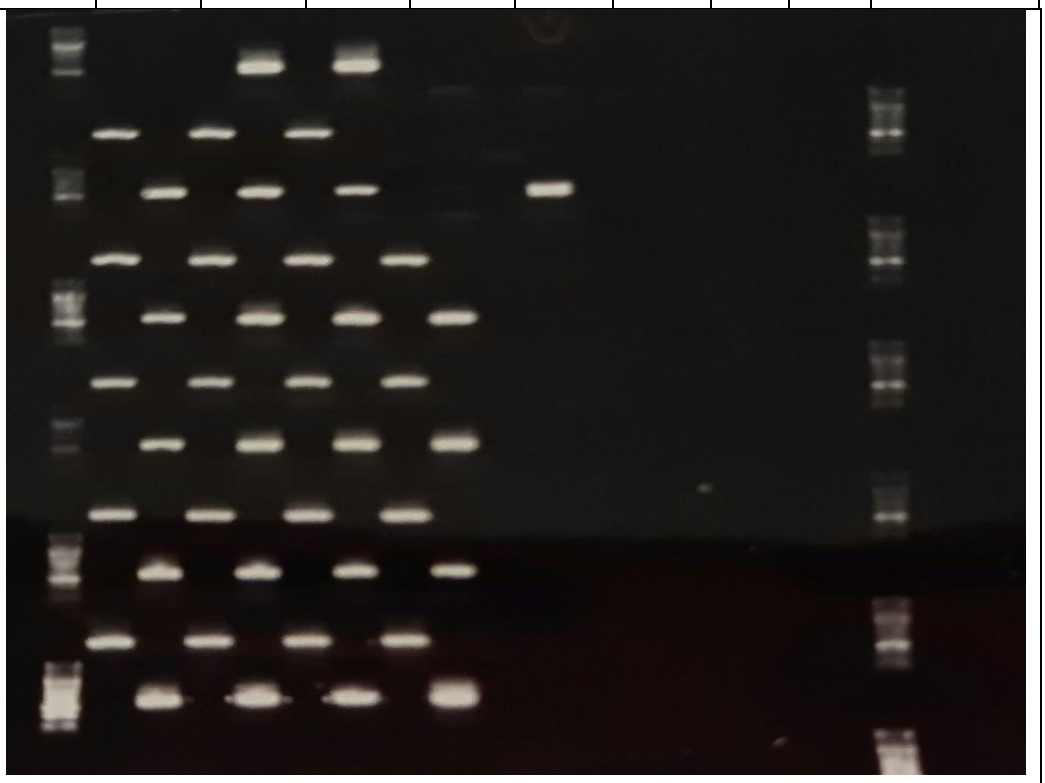
Ileostomy sample participant	DNA concentration(ng/ml)	260/280 ratio	260/230 ratio
P1-V1	32.26	1.91	1.26
P1-V2	75.95	2.1	2.1
P1-V3 [a,r]	110.62	2.12	1.46
P2-V1	32.03	1.99	1.2
P2-V2	26.22	2.17	1.46
P2-V3	106.22	2.02	1.27
P3 V1	71.32	2.03	1.61
P3-V2	143.5	2.1	2.14
P4-V1 [b,r]	25.41	2.16	0.71
P4-V2	54.39	2.15	1.44
P4-V3	69.1	2.21	1.21
P5-V1	39.22	2.2	1.86
P5-V2 [b,c]	1.14	-2.05	0.32
P5-V3	23.92	1.88	0.84
P6-V1	64.24	2.19	1.83
P6-V2	26.31	2.59	1.48
P6-V3	62	2.12	2.01
P7-V1 [a, r]	15.79	2.34	0.77
P7-V2	17.26	1.71	1.26
P7-V3 [a,b,r]	16.72	2.34	0.93
P8-V1 [b,c,r]	7.76	1.97	0.56
P8-V2	96.72	2.08	2.03
P8-V3	54.95	2.07	1.98
P9-V1 [b,d]	0.73	25.83	0.08
P9-V2	1.49	1.39	0.18
P9-V3	155.79	2.1	2.25
P10-V1 [b,r]	18.18	2.57	1.18
P10-V2	184.5	2.14	2.07
P10-V3	48.89	2.1	1.57
P11-V1	370.4	2.18	2.38
P11-V2	135.8	2.16	2.24
P11-V3	311.71	2.17	2.24

Table 9 Results of DNA extraction.

a=260/230 ratio < 1
b=low DNA concentration in sample led to repeat
c=repeat analysis yielded lower DNA concentration, so this sample was kept
d= not enough ileostomy fluid volume for repeat DNA analysis
r=repeat analysis

4.1.9 Gel electrophoresis of PCR products

Gel electrophoresis of the PCR products from amplification confirmed a PCR product of 300 base pair ladder. Successful bacterial DNA PCR product was detected in the E.coli positive control (lane E3) while the water (lane E1) and C2020 human cell lines (lane E2) were not detected. Lane A1 was kept blank while the D1- D3 lines corresponded with samples from another study that were included in this PCR amplification and sequencing run. The other samples in the D-column were an attempt to quantify bacterial DNA product in specific samples that had undergone bacterial DNA amplification. The gel electrophoresis read out can be seen below with the table laying out the participant's sample position in the wells (Table 10).

		A	B	C	D	E	F	G	H	
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										

		A	B	C	D	E	F	G	H
1			P4-V3	P8-V2	D1	WATER			
2		P1-V1	P5-V1	P8-V3	D2	c2020			
3		P1-V2	P5-V2 [b,c]	P9-V1 [b,d]	D3	E. coli			
4		P1-V3 [a,r]	P5-V3	P9-V2	P5-V2	BLANK			
5		P2-V1	P6-V1	P9-V3	P7-V1				
6		P2-V2	P6-V2	P10-V1 [b,r]	P7-V2				
7		P2-V3	P6-V3	P10-V2	P7-V3				
8		P3 V1	P7-V1 [a,r]	P10-V3	P8-V1				
9		P3-V2	P7-V2	P11-V1	P9-V1				
10		P4-V1 [b,r]	P7-V3 [a,b,r]	P11-V2	P9-V2				
11		P4-V2	P8-V1 [b,c,r]	P11-V3	P10-V1				
12	Blank								

Table 10 Gel electrophoresis and sample positioning on the 96-well plate.

a=260/230 ratio < 1
b=low DNA concentration in sample led to repeat
c=repeat analysis yielded lower DNA concentration, so this sample was kept
d= not enough ileostomy fluid volume for repeat DNA analysis
r=repeat analysis

4.1.10 Inter- and intra-individual variation in ileal microbiota

Consistent with reports on ileal microbiome (Booijink et al., 2010; Hayashi et al., 2005; X. Wang et al., 2003), the dominant genera in our participants' ileal effluent prior to supplementation consisted of *Bacteroides, Clostridium, Escherichia/Shigella, Turcibacter, Haemophilus* and *Streptococcus*. QIIME2 combined the 2 *Enterobacteriaceae* genera *Escherichia* and *Shigella* as a single genus category, which was then used in MEGAN for further analysis. Although more than 35 genera were identified, the top 10 genera made up >90% of the total operational taxonomic units (OTUs), illustrated below in Figure 7.

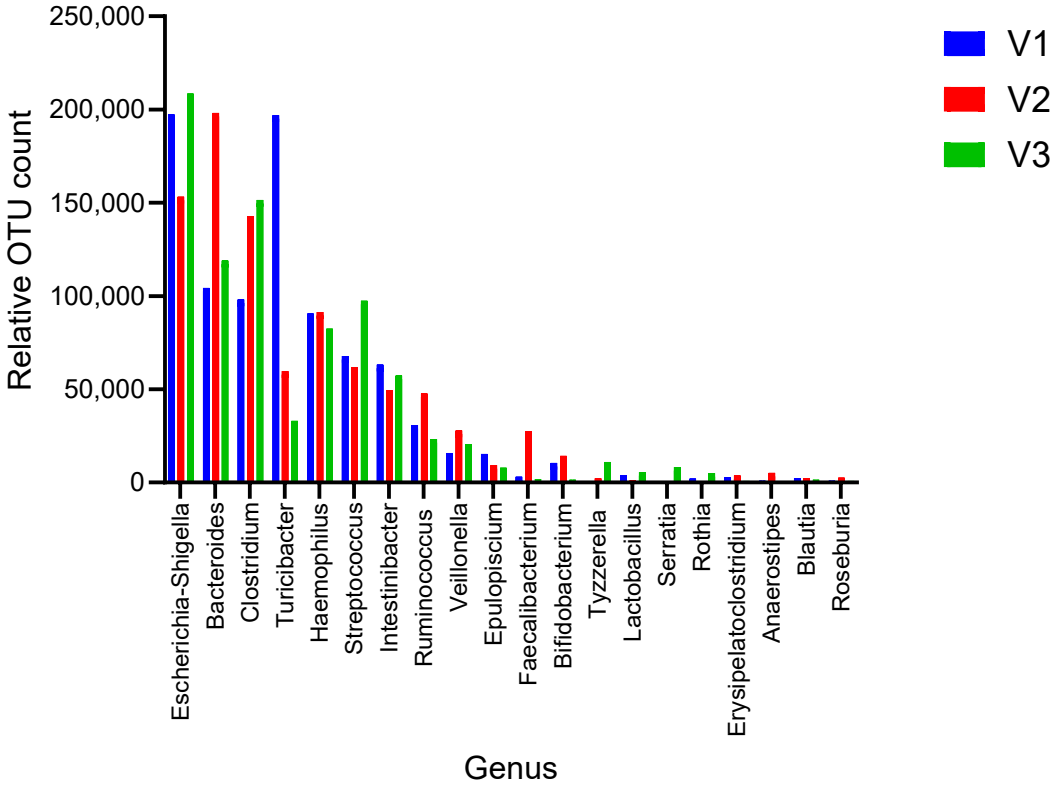


Figure 7 Distribution of top 20 bacterial genera in ileal fluid before (V1, blue), after the first (V2, red) and the final dose after 28 days (V3, green) of oral supplementation with omega-3 PUFA capsules. Columns represent the Operational Taxonomic Unit (OTU) counts for all participants who provided a sample.

As expected, there was wide variation in the ileal microbiome between individuals at baseline (visit 1) demonstrated by Bray-Curtis principal coordinate

analysis reflective of a highly individualistic microbiome. There were also large intra-participant differences between baseline - first (V1 - V2) and baseline - final (V1 - V3) oral dosing with omega-3 PUFA capsules. There was no significant difference across the visit profiles (PERMANOVA, p=0.93) with variability between the participants being the only significant variable (PERMANOVA, p=0.001). This data is consistent with the dynamic nature of small intestinal microbiome changes described in the literature (Figure 7).

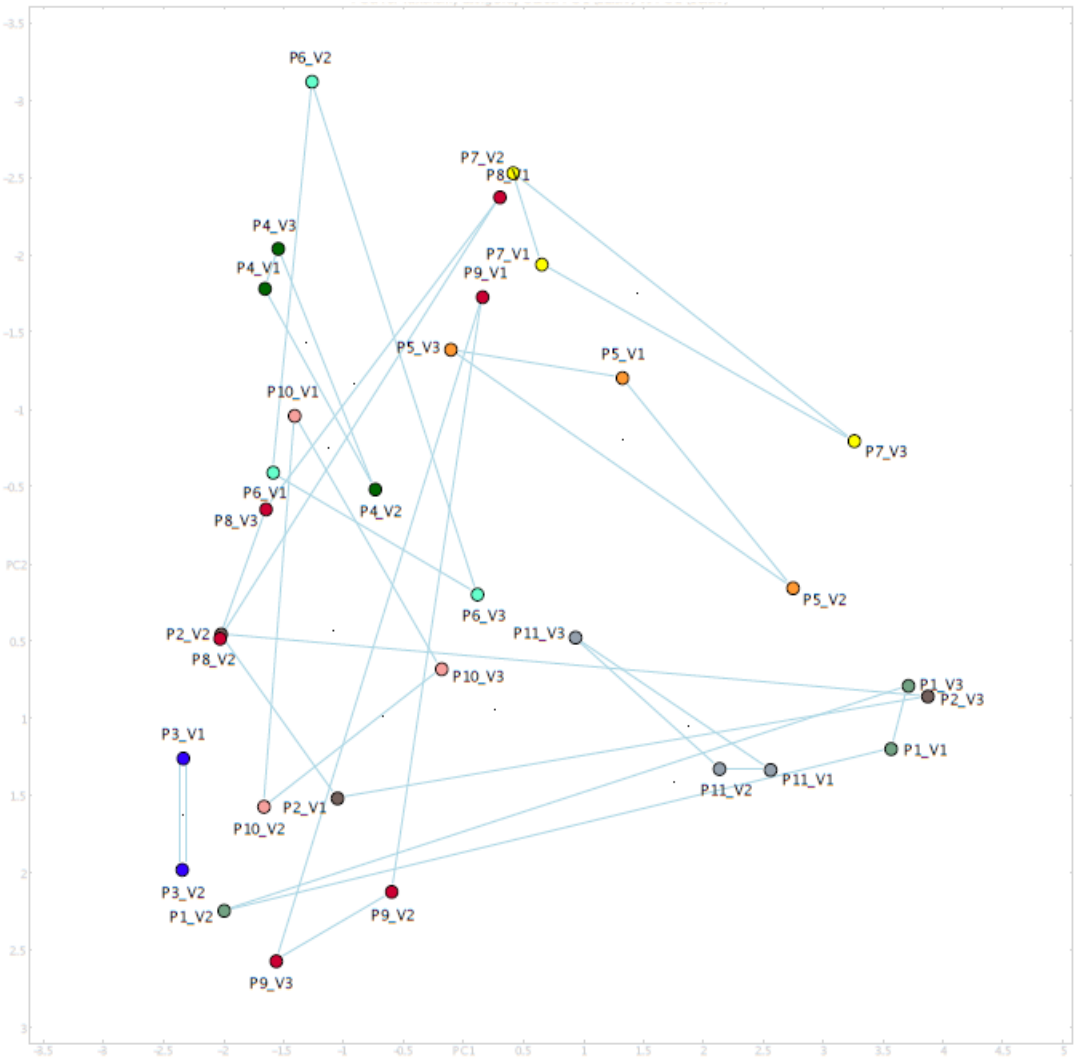


Figure 8 Bray-Curtis principal coordinate analysis of the ileal microbiome taxonomy, with blue lines joining the points before (V1), after the first (V2) and final dose after 28 days (V3) of oral supplementation with omega-3 PUFA capsules. Each participant is represented by a separate colour and number label with blue lines joining the time points. Participant 3 (P3, dark blue shade) did not have a V3 sample.

Examining the subgroup of participants that displayed a rise in EPA and DHA after first oral dosing (participants #2,3, 5 and 6), there was no discernible

difference or trend between V1 and V2 compared to the other subgroup that did not have an exponential change in EPA and DHA after first oral dosing (participants # 1, 4, 7 – 11). This suggests that a transient acute rise in luminal EPA/DHA concentration (over a few hours) does not produce a change in the ileal microbiome abundance or composition, above and beyond its natural variability. Subsequently, after final oral dosing (V3), the microbiome profile of this subgroup of participants (#2, 3, 5 and 6) did not consistently return to levels of abundance close to the V1 sample.

Even within the individual genera, major shifts in relative abundance (OTUs) between visits were observed in keeping with the inherent variability of the ileal microbiome. Examining the change in abundance from baseline after a few hours of oral dosing (difference between visit 2 and visit 1) and after 28 days of oral supplementation with omega-3 PUFA (difference between visit 3 and visit 1), the results are presented for the top 15 genera (Figure 9 below). No consistent trend in the relative abundance was observed across the individuals when comparing across time points. There was also no visible relation between the change in relative abundance between time points compared to baseline, although *Bacteroides* and *Veillonella* abundance was static or increased in 8 of the 11 participants across both time points.

Figure 9 Absolute difference in operational taxonomic units (OTUs, on y-axis) of the top 15 genera in ileostomy fluid, per participant (x-axis). Change in OTUs before, and either after the first visit (V2-1), or after the final dose visit (V3-1) following ≥28 days of oral supplementation with omega-3 PUFA capsules. Data are normalised to a 100,000 scale for each sample. Positive or negative figures denote an increase or decrease respectively, compared to baseline OTUs. There was no visit 3 data for P3.

4.1.11 Correlation between Ileostomy fluid and red blood cell omega-3 polyunsaturated fatty acids and ileal microbiota abundance

The relationship between genus abundance in IF and the IF fatty acid concentrations was then explored. Eight of the top 35 genera had zero OTUs in >5 samples, so these genera were excluded from our correlation analysis.

FA analysis demonstrated a significant rise in EPA and DHA (and DPA, a by-product of EPA elongation) concentration in IF after 28days of oral omega-3 PUFA supplementation. There was a strong correlation between this EPA/DHA rise and the change in abundance of several genera between the same time points (Fig 10 below). Higher concentrations of the supplemented omega-3 PUFA (EPA and DHA) in IF were associated with an increase in *Bacteroides*, that was not observed for the other FAs including the other omega-3 PUFA, LNA. Conversely, increase concentration of EPA and DHA in IF were associated with reduced abundance of 2 genera: *Granulicatella* and *Actinomyces*. This association was not shared by the other FAs measured. In contrast, a rise in the luminal concentration of LNA (the shorter-chain-omega-3 PUFA that was not supplemented) correlated with a rise in Bifidobacterium and fall in *Intestinibacter* and *Suterella*.

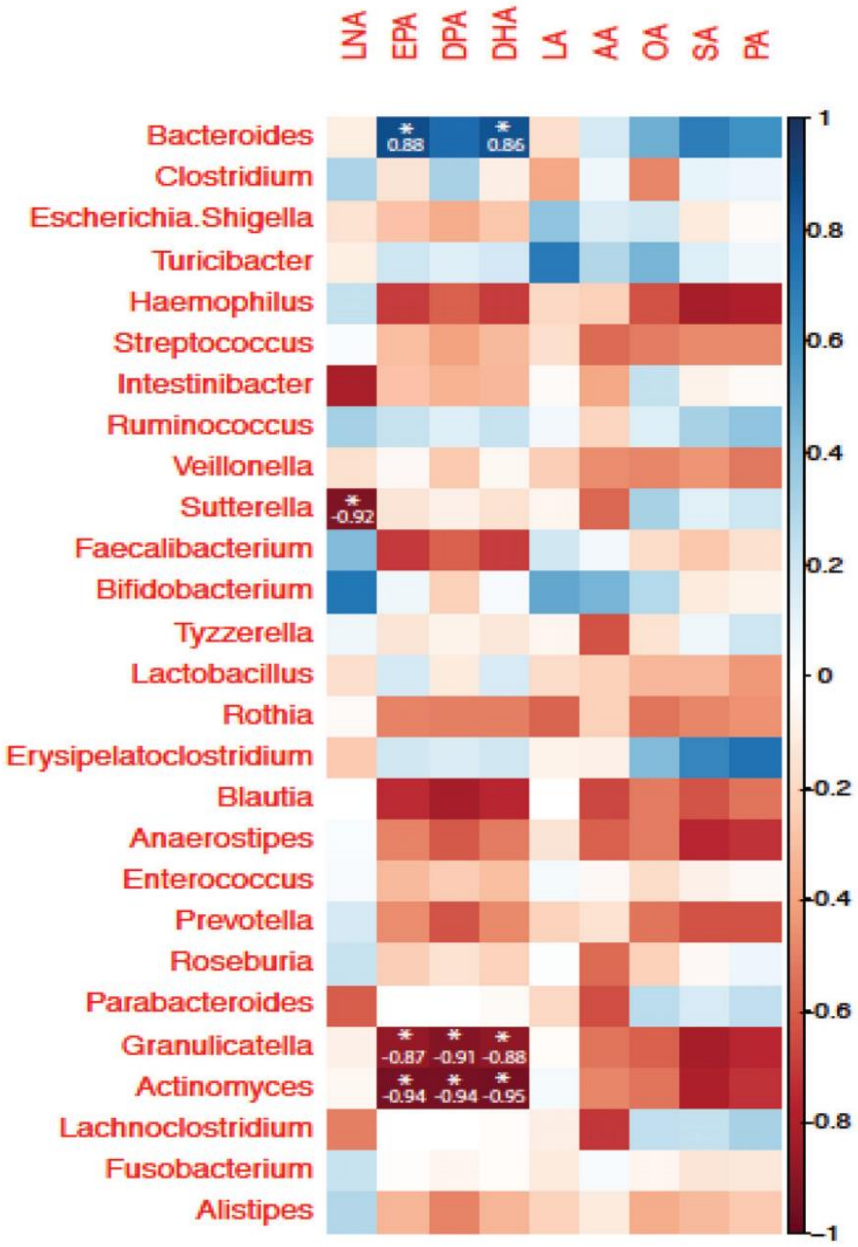


Figure 10 Correlation matrices for the difference in bacterial genus abundance and the difference in absolute ileal fluid fatty acid concentrations between baseline (V1) and after 28 days (V3) of treatment with omega-3 PUFA capsules in patients with a temporary ileostomy. Blue denotes a positive correlation. Red denotes a negative correlation. The strength of the Pearson correlation is denoted by the colour intensity (side-bar scale). Statistically significant ($P < 0.05$) relations are signified by an asterisk with the actual $r2$ value. EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; AA, arachidonic acid; DPA, ω-3 docosapentaenoic acid; LA, linoleic acid; LNA, alpha-linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.

By contrast, there was no strong correlation between the rise in EPA/DHA concentration in IF after the first capsules (V2) and relative abundance of genera (Figure 11). This confirms that acute supplementation with oral omega-3 PUFA does not alter the microbiome, unlike prolonged supplementation for several weeks.

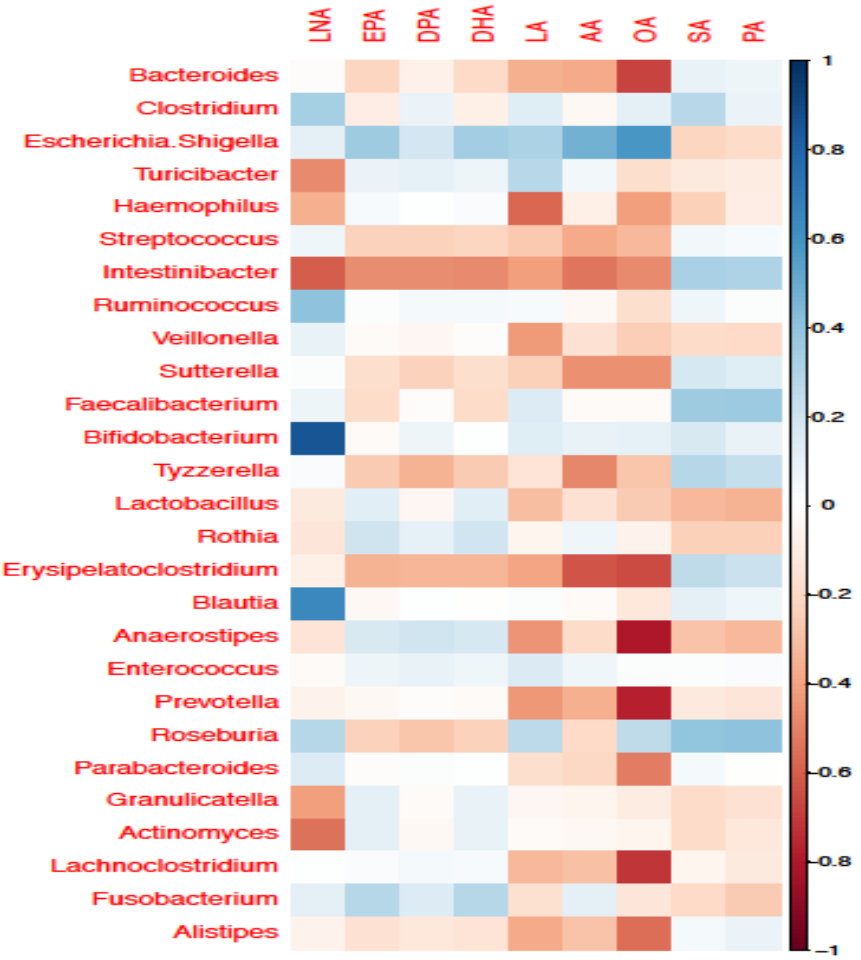


Figure 11 Correlation matrices for the difference in bacterial genus abundance and the difference in absolute ileal fluid fatty acid concentrations between baseline (V1) and first oral dose (V2) of omega-3 PUFA capsules in patients with a temporary ileostomy. Blue denotes a positive correlation. Red denotes a negative correlation. The strength of the Pearson correlation is denoted by the colour intensity (side-bar scale). Statistically significant ($P < 0.05$) relations are signified by an asterisk with the actual $r2$ value. EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; AA, arachidonic acid; DPA, ω-3 docosapentaenoic acid; LA, linoleic acid; LNA, alpha-linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid

Importantly, no significant correlations were observed between the change in bacterial abundance and the rise in rbc EPA/DHA at V3. This suggests that the relationship between the altered abundance of specific genera and concentrations of supplemented omega-3 PUFA are specific to the ileal luminal environment, rather than systemic concentrations of EPA and DHA. There were 4 isolated statistically significant negative correlations between the rbc content of FAs, including oleic acid, and 3 bacterial genera after first dosing, which lacks biological plausibility given the kinetics of FA incorporation into rbc membranes and could have resulted from multiple testing (See Figures 12 and 13)

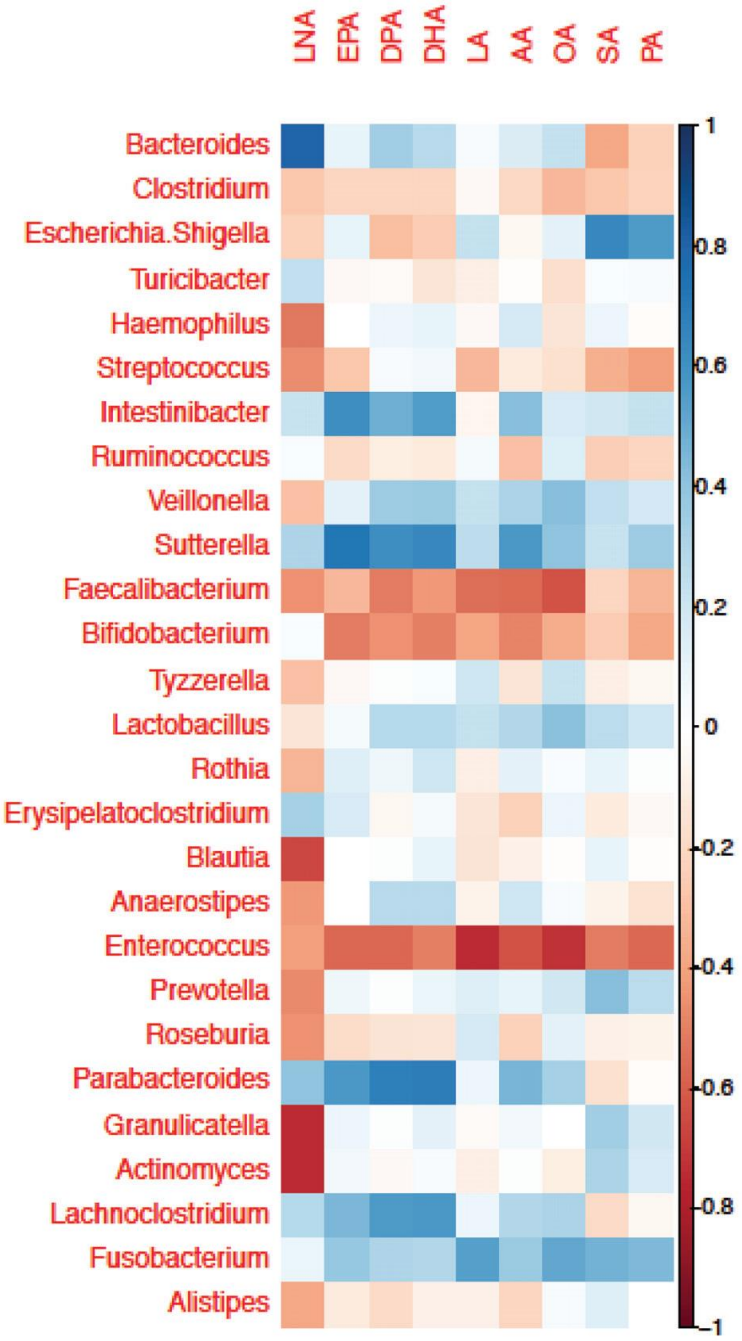


Figure 12 Correlation matrices for the difference in bacterial genus abundance and difference in red blood cell %FA content between baseline (V1) and final oral dose (V3) of omega-3 PUFA capsules in patients with a temporary ileostomy. Blue denotes a positive correlation. Red denotes a negative correlation. The strength of the Pearson correlation is denoted by the colour intensity (side-bar scale). Statistically significant ($P < 0.05$) relations are signified by an asterisk with the actual $r2$ value. EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; AA, arachidonic acid; DPA, ω-3 docosapentaenoic acid; LA, linoleic acid; LNA, alpha-linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid

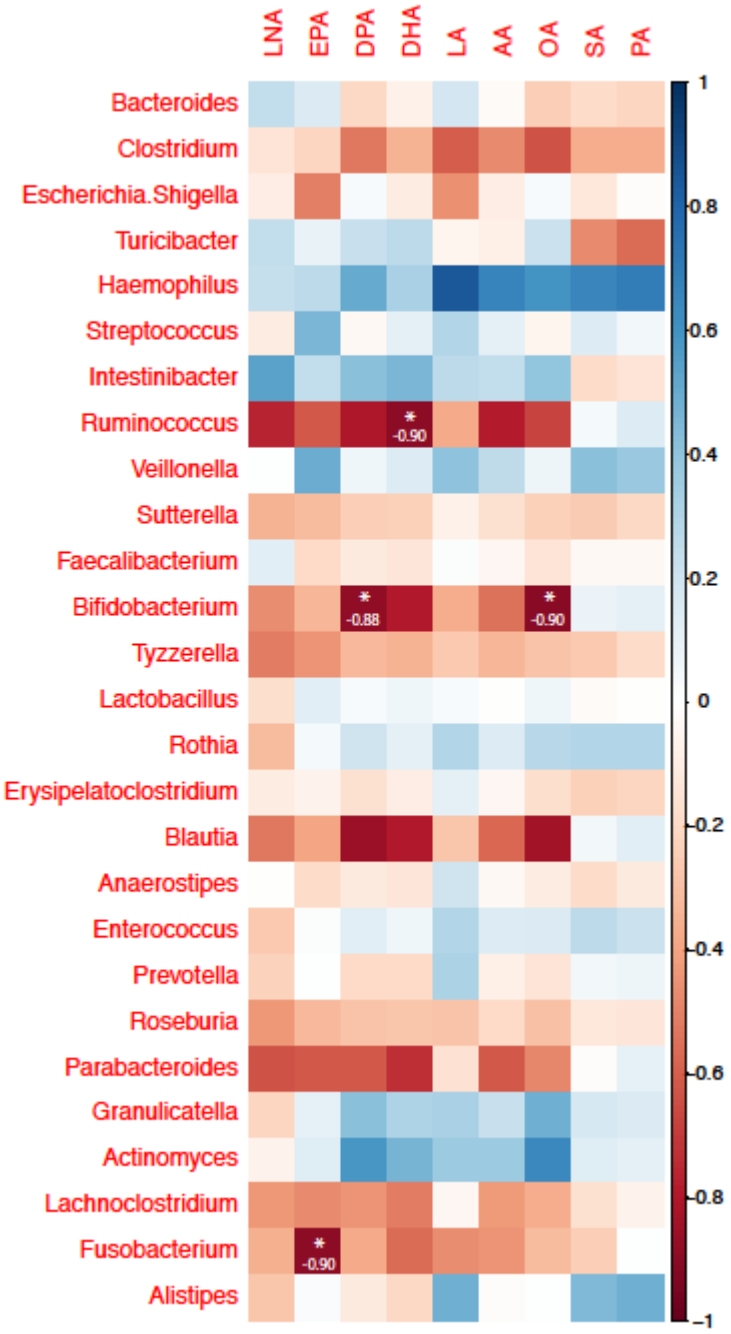


Figure 13 Correlation matrices for the difference in bacterial genus abundance and difference in red blood cell %FA content between baseline (V1) and first oral dose (V2) of omega-3 PUFA capsules in patients with a temporary ileostomy. Blue denotes a positive correlation. Red denotes a negative correlation. The strength of the Pearson correlation is denoted by the colour intensity (side-bar scale). Statistically significant ($P < 0.05$) relations are signified by an asterisk with the actual $r2$ value. EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; AA, arachidonic acid; DPA, ω-3 docosapentaenoic acid; LA, linoleic acid; LNA, alpha-linolenic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid

4.2 Discussion

4.2.1 Summary of key findings

This study demonstrated that twice-daily oral supplementation with 4g EPA/DHA capsules for a minimum of 28 days (delivering 1g each of EPA and DHA in FFA form, twice daily) resulted in an increase in IF EPA and DHA concentration. While there was an initial increase in luminal IF EPA and DHA concentrations in a subgroup of participants, this was not sustained with prolonged supplementation in the medium term (28 days). Consistent with equal dosing of EPA and DHA, similar changes in IF and rbc EPA, DHA and DPA but not LNA were measured across the study participants. This was associated with an increased relative abundance of *Bacteroides* and a reduction in other bacterial genera, despite the high variability of the distal small bowel microbiome between individuals and over time.

Consistent with current evidence (Dempsey et al., 2023; Schuchardt, Kräter, et al., 2024; Watson et al., 2016), prolonged oral supplementation with omega-3 PUFAs results in increased systemic EPA and DHA incorporation in the rbc membrane. However, findings from this study indicate that incorporation has limited correlation with changes in ileal microbiome abundance.

4.2.2 Implications of current findings

Assuming a 24 hour ileostomy output of 1000ml, one can estimate that an approximate EPA and DHA concentration of 7ug/ml in IF after 28d dosing could equate to as little as 1% of the orally administered dose appearing in the ileum (and hence the proximal colon) assuming that all luminal omega-3 PUFA derive directly from oral administration rather than indirectly from the small intestinal mucosa. This is a similar magnitude to the proportion of omega-3 PUFA (<1%) recovered in IF after oral supplementation with a smaller dose (<300mg) of EPA/DHA , irrespective of capsule or fortified food form, in a study by Sanguansri. One can conclude that proximal small bowel omega-3 PUFA absorption is highly efficient with a limited amount entering the colon lumen. (Sanguansri et al., 2013)

Consistent with equal dosing with EPA/DHA in this study, similar changes in their luminal concentration were measured in each participant. As expected, there were no other significant changes in the FA profile of IF after oral administration. This knowledge that micrograms per millilitre concentrations of EPA/DHA are generated in the distal small intestine after oral dosing, enough to cause shifts in the gut microbiome will inform *ex vivo* experiments using gut fermentation models

to investigate EPA- and DHA-derived bioactive lipid mediator production and provide further mechanistic insights into the chemopreventive actions of omega-3 PUFAs. (Isenring et al., 2023) One such experimental study from the Molecular Gastroenterology Group at LIMR is currently undergoing peer review.

This study was designed to compare IF samples a few hours after the first dose and after the last dose of omega-3 PUFA capsules (a minimum of 28 days later) preferably at an equivalent post-ingestion time points. Theoretically, this should allow firstly an estimation of direct oral delivery of omega-3 PUFA to the distal small intestine (visit 2 concentrations in previously omega-3 PUFA-"naïve" IF). Secondly, delivery of omega-3 PUFA to the distal small intestinal mucosa – hypothesised to occur only after medium-term (28-d) dosing (visit 3) that produces increased tissue (mucosal) and systemic omega-3 PUFA, with rbc EPA/DHA measured as a surrogate biomarker.

The acute rise in in IF EPA and DHA demonstrated in the subgroup of 4 individuals at V2, demonstrated rapid delivery to the distal ileum however, it confounded the comparison of V2 and V3 EPA and DHA in all participants. However, exclusion of this subgroup of individuals from this analysis confirmed that, although IF EPA/DHA concentrations were higher after medium term supplementation, there was no consistent or statistically significant increase in the IF bioavailability after oral supplementation for 28 days compared with that after the first dose. This suggests that sustained ileal exposure is more likely due to repeated luminal delivery from oral intake rather than omega-3 PUFA from the ileal mucosa release after tissue incorporation, perhaps through shed enterocytes. Thus ileal mucosal membrane contribution to distal ileal luminal (by extension proximal colonic) EPA/DHA content is minimal after prolonged supplementation.

The relatively high omega-3 PUFA concentrations in IF after first oral dosing, in 4 of the 11 participants may be explained by various confounding factors. This could be related to a lower oral or gastric absorption, size or composition of meals ingested by participants prior to sample collection, inter-individual variety in gastric emptying or bile salt secretion. Although dietary intake of the participants was recorded, it was not within the study protocol to standardise calorie intake or quantify fat composition of meals as this has been shown to affect systemic bioavailability of omega-3 PUFA (Davidson et al., 2012). Such a stipulation to this vulnerable participant group – cancer survivors – who were voluntarily agreeing to take part, while in some cases actively looking forward to the reversal of their ileostomy, would have added a significant hurdle to recruitment to the study. The reduction in IF omega-3 PUFA concentration after 28 days suggests that intestinal adaptation may have occurred in these individuals, as multiple studies

show that increased absorption leads to red cell systemic incorporation (Calder, 2020; Karageorgou et al., 2023; Schuchardt, Kräter, et al., 2024). A larger study with strict dietary monitoring would be required to quantify with greater precision, the size of the change in luminal IF EPA/DHA after a short period of oral dosing with omega-3 PUFA capsules. The only experimental protocol that could directly address the precise contribution of luminal delivery to intestinal bioavailability in humans would be measurement of EPA and DHA concentration in the intestinal lumen after parenteral administration of labelled omega-3 PUFA. Bypassing enteral digestion and absorption would permit quantification of the mucosal contribution to luminal concentrations. To our knowledge, there was no evidence of such a study in the literature so this represents an avenue for future research.

Historically, using 16S rRNA gene libraries, the jejunal and ileal microbiota were shown to consist mainly of simple families of facultative anaerobes and aerobes including but not limited to the *Enterococcus* group and *Bacteroides* group and L*actobacilli*, *Veillonella, Streptococcus, Gemella, Actinomyces,* consistent with our findings (Hayashi et al., 2005; Ruigrok et al., 2023; Villmones et al., 2022). Our data was consistent with the wide temporal variation between individuals and over time as previously reported (Booijink et al., 2010; Ruigrok et al., 2023). Interestingly, no consistent acute changes in the ileal microbiome were noted even when comparing the subgroup with elevated IF omega-3 PUFA concentrations after first oral dosing. However, with prolonged oral dosing, there was a strong correlation between the increase in EPA, DPA, DHA, but not LNA concentrations with increased concentrations and abundance of *Bacteroides* in ileal content.

The *Bacteroides* genus contains many SCFA-producing species (Fu et al., 2019), that are compatible with the effect of the same daily dose of omega-3 PUFA on SCFA-producing bacterial taxa in faecal sample from a healthy volunteer study (Watson et al., 2018) and more recent findings from a study of low-dose EPA (165mg daily) and DHA (110mg daily) administration on faecal *Bacteroides* abundance in healthy volunteers (Vijay et al., 2021). The latter study also reported that omega-3 PUFA supplementation was associated with increased plasma concentration of SCFAs (Vijay et al., 2021). Recent *in vitro* studies demonstrate increased abundance of *Akkermansia*, *Bifidobacterium* and *Roseburia*, boosting SCFA-production (propionate and butyrate) in response to EPA/DHA supplementation (Roussel et al., 2022; Salsinha et al., 2025). Together with our study, they confirm a mechanistic pathway of increased luminal availability resulting in microbiome compositional change and metabolite output.

Although this data demonstrates an association between a rising IF luminal omega-3 PUFA concentration and shifts in the abundance of specific genera

within the ileal microbiota, causality cannot be inferred. However, these findings signal a promising avenue for further research in this domain and a meaningful extension to the literature. Prior work in transgenic *Fat*-1 and *Fat*-2 mouse models has shown that alterations in the intestinal tissue content of omega-3 and omega-6 PUFAs can modulate the faecal microbiome independently of changes in dietary PUFA intake, potentially via a mechanism involving epithelial alkaline phosphatase expression (Kaliannan et al., 2015, 2019)

4.2.3 Strengths of the study

This human intervention study used a pure omega-3 PUFA formulation close to clinical trial formulations and dosage, rather than a complex mixture with various additives. As such, the effect of the 2 essential marine omega-3 PUFA, EPA and DHA, on intestinal bioavailability could be tested, without confounding with other fatty acids.

Assessing the small bowel microbiome is notoriously difficult due to obstacles in obtaining samples, processing, and analysis. Prior ileal content sampling studies relied on using invasive endoscopic sampling techniques (colonoscopy) or at autopsy (Hayashi et al., 2005) or bowel resections from participants with inflammatory bowel disease (Booijink et al., 2010). Other than the ileostomy formation, study participants did not have pre-existing inflammatory or functional bowel disorders. Although challenging to recruit, the use of participants with a reversible ileostomy allowed for non-invasive sampling. In addition, by combining ileal and blood sampling at a similar time after dosing during the intervention period, this study has provided significant insight into physiological and pharmacokinetics of omega-3 PUFA administration in this highly variable medium.

Surgical resection of the small bowel, results in alteration to gastrointestinal physiology, notably accelerated transit through the small bowel in the short to medium term. This may contribute to malabsorption, fluid losses until adaptive mechanisms take effect. (Boutté & Poylin, 2023) As such the protocol stipulated a 2 month duration from formation of loop ileostomy to recruitment into the study to minimise fluctuations due to intestinal adaptation that may confound results when comparing the differing abundance in ileal microbiota when comparing acute (first day) and prolonged (28days) dosing with omega-3 PUFA capsules. It should be noted that the participants had been living with a stoma for significantly longer – median 9 (range 2-18) months thus this reduces this theory as a potential explanation for the rapid increase in ileal EPA and DHA after oral dosing.

4.2.4 Limitations of the study

Although the study has demonstrated an association between ileal microbiome abundance and omega-3 PUFA concentrations in the small bowel, certain limitations to the study design need to be considered in the interpretation of the results.

The limited sample size rendered the study underpowered, increasing the odds of a type 1 statistical error. In designing the protocol, there was justifiable concern that recruitment would be challenging due to the low acceptability to volunteers, who were at a vulnerable time in their cancer treatment pathway, and were not getting any personal advantages by taking part in the study. Further constraints by standardising dietary fat intake, physical exertion or restricting antibiotic consumption, would have affected recruitment. Antibiotic use in particular, especially for the 6 months prior to enrolment onto the study was not assessed. I acknowledge that these factors may have acted as potential confounders in the interpretation of the results.

It is important to recognise that the terminal ileostomy content is only a proxy for luminal content in an intact gastrointestinal tract. A temporary ileostomy will alter gastrointestinal physiology, absorption dynamics and microbial ecology. Contamination from the peristomal skin may confound microbiome analysis (Kastl et al., 2020). Additionally, the 28-day supplementation period may not capture longer-term microbial adaptations in participants with an intact gastrointestinal tract, thus the study findings may not be generalisable to the general population with an intact GI tract.

Finally, this study provides novel data on mechanistic pathways and pharmacokinetics, however it does not establish causality, nor does it provide insight on downstream molecular antineoplastic biomarkers or clinical endpoints.

4.3 Conclusion

This study demonstrated that oral supplementation with omega-3 PUFA for a minimum of 28 days led to a rise in EPA and DHA in the ileal lumen, relevant to therapeutic use of omega-3 PUFA for ileocolonic disease. Despite the high variability in ileal microbiome between and within individuals, rising EPA and DHA concentrations in the ileal lumen were associated with an abundance of SCFA-producing *Bacteroides*. This provides insight on the contributory mechanism behind omega-3 PUFA use increasing luminal SCFA exposure, conferring a protective effect against colorectal cancer formation in the colon. Further studies are required to link this to improved health outcomes.

Chapter 5 The omega-3 polyunsaturated fatty acid content of human faecal samples: a secondary analysis of a randomised cross-over trial of oral omega-3 polyunsaturated fatty acid supplementation

5.1 Background

Omega-3 PUFA consumption is positively correlated with cancer prevention (Aldoori et al., 2022; Punia et al., 2019). Human studies on the impact of omega-3 PUFAs in curbing colonic tumorigenesis have focused on red cell membrane content of EPA/DHA (Watson et al., 2016) , and colonic mucosal biopsies (Sorensen et al., 2014; Tutino et al., 2019; West et al., 2010). Of the studies quoted in Aldoori's review, paired data directly correlating luminal colonic (or faecal) omega-3 PUFA concentrations with rbc EPA/DHA are sparse (Aldoori et al., 2022). Fresh from analysing the omega-3 PUFA concentrations in ileal fluid, this secondary analysis of a previously published trial in humans aims to explore and begin to fill this knowledge gap.

Watson and his team in Leeds, conducted a study in human volunteers to investigate the link between oral supplementation with EPA/DHA as a nutraceutical capsule or drink formulation and its effect on the faecal microbiome (Watson et al., 2018). They collected faecal samples from the participants and although they analysed the impact of PUFA supplementation on the microbiome, they linked this to tissue incorporation (rbc omega-3 PUFA levels) but did not quantify faecal PUFA levels. With the lead author's and institutional ethical approval, these samples were taken for further research. Using similar techniques of FA extraction from ileal fluid described in earlier chapters, a secondary analysis was performed to quantify the omega-3 PUFA levels in faecal samples obtained from this cross-over trial.

To the best of the author's knowledge, quantification of EPA/DHA content in faecal samples has not been described in the scientific literature. This information would be valuable for further in vivo and ex vivo studies of luminal EPA/DHA content, and its modulatory effects on the gut microbiome and biomarkers of inflammation.

5.1.1 Cross-over trial methodology

A recap of Watson's methodology is summarised in this section (Watson et al., 2018). Twenty-two healthy adult human volunteers that satisfied their inclusion/exclusion criteria, were randomised in a 1:1 manner. Half of the cohort took 4g of a mixed EPA/DHA 1:1 preparation, orally as soft-gel capsules (4x1g capsules in total with meals) daily. The other half took 4g mixed EPA/DHA as a drink (two 200ml Smartfish Remune drink cartons) daily. Each cohort took the supplements for 8 weeks then stopped. This was followed by a 12 week washout period. At the end of the washout period, the participants on capsules 'crossed over' to take the drink formulation and vice versa. Both groups took the supplements for another 8 weeks before stopping, again followed by a 12 week washout period.

Participants met with investigators at 5 intervals throughout the study, listed below:

- visit 1 (V1): baseline – prior to starting capsules or drinks first.
- visit 2 (V2): post-supplementation – after 8 weeks on capsules or drinks
- visit 3 (V3): washout – after 12 weeks off capsules or drinks. The new baseline prior to cross-over to drinks or capsules.
- visit 4 (V4): post-supplementation – after 8 weeks on drinks or capsules
- visit 5 (V5): washout – after 12 weeks off drinks or capsules

Those participants who took capsules first switched to drinks and vice versa after visit 3. Venous blood and urine samples were collected, and adverse event monitoring was conducted during each visit. Faecal samples were returned within 2 days of the visit.

Fatty acid extraction from red cell membrane was completed via LCMS using the method described earlier (Chapter 3.5.5). Bacterial DNA was extracted, purified, sequenced then analysed as described earlier (chapter 3.5.7). The study was completed in December 2015 after the final study 5 visit.

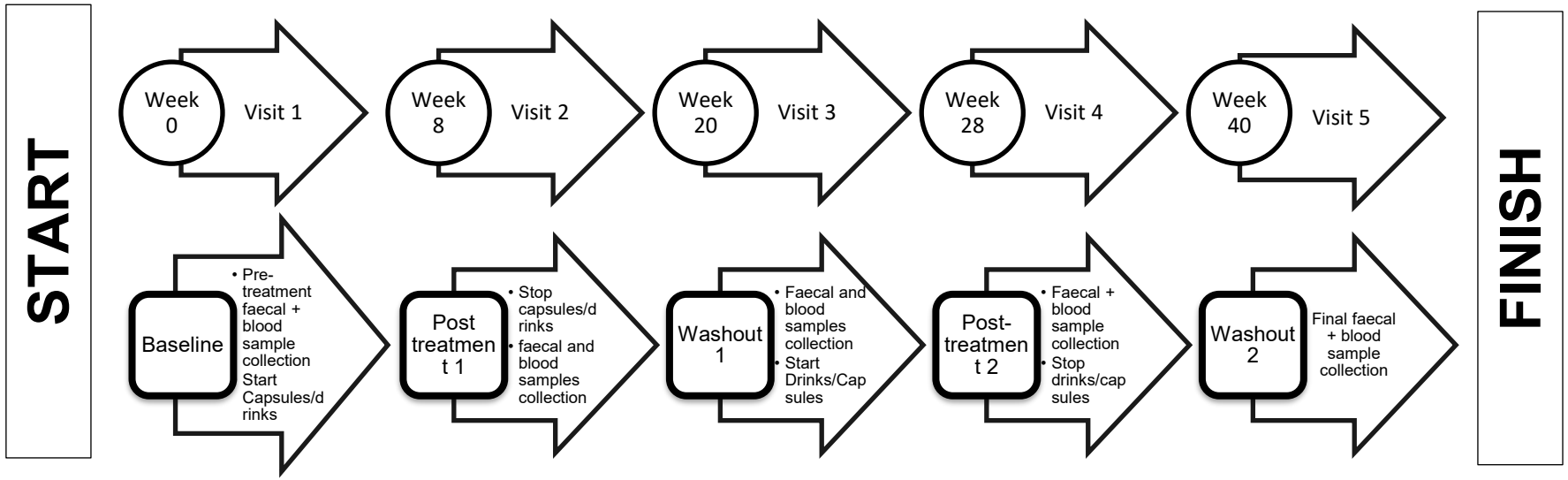


Figure 14 Timeline and Sampling schedule of cross-over trial

5.1.2 Fatty acid extraction, derivatisation and hydrolysis from faecal samples

This section describes in detail the methodology followed to extract and analyse FAs from Watson's faecal samples. A similar process, described earlier in chapter 3, was used for fatty acid extraction from faecal samples. Faecal samples were stored in a -80°C freezer until April 2018 when funding was available for further research. For each faecal sample, once thawed, 150mg was extracted to mix with 3ml of water and vortexing for 1 minute to form a slurry that was then stored at -80°C. These were transported on dry ice to the ICT in Bradford. With the assistance of collaborators at the institute, each sample was thawed and homogenised. A 50µl aliquot of faecal sample was transferred to an Eppendorf tube and mixed with 50 µl of distilled water (dH_2O), containing internal standard (2 µg/ml deuterated Arachidonic Acid – d8) then allowed to stand for 15 min. After 550µl Isopropanol was slowly added to the mixture, vortexing twice during a 1-hour incubation period at room temperature. Following incubation, 350 µl of chloroform was added. After a further 1h incubation, the aliquot was centrifuged at 10,000 × g for 5 min. The supernatant was transferred to a new Eppendorf tube and evaporated to dryness in a rotary evaporator (EZ-2 plus rotary evaporator, Genevac Ltd, Suffolk, UK). For hydrolysis, the faecal aliquot solution was reconstituted with acetonitrile. Further steps were conducted as described in chapter 3.5.5.2 and 3.5.5.3. Finally, the derivative mixture was injected into the LC-MS for an overall run time of 25 min. The flow rate was set at 0.5 ml/min and split post-column with 0.3 ml/min delivered to the MS. Samples were analysed in multiple reaction monitoring (MRM) mode for the following fatty acids: PA, LNA, LA, OA, SA, EPA, AA, DHA, DPA and deuterium-labelled internal standard (LNA-d8). Results were expressed as the percentage of each FA relative to the total FA peak area (%FA).

5.1.3 Statistical analysis

This was performed using Graph pad Prism 10 (Graph Pad Software, Inc). Individual omega-3 PUFA concentrations were expressed as a % of the total measured FA concentration. Absolute difference and fold changes in relative PUFA concentration were calculated between post-supplementation or washout and baseline values. Descriptive statistics were used to express changes across the course of the study. Group differences based on intervention, pre- and post-supplementation data were compared using the paired two-sided Student's t-tests. Non-parametric tests (Mann-Whitney U and Wilcoxon signed-rank where appropriate) were used for group analyses of faecal omega-3 PUFA profiles. Differences were expressed with their 95% confidence intervals, accepting a p value <0.05 as significant. Graphs and statistical analyses were performed using GraphPad Prism version 10 (GraphPad Software, Inc.). Correlations were assessed using Spearman's correlation coefficient.

5.2 Aims and objectives of secondary analysis

The aims of the analysis of PUFA levels in faecal samples were:

- To measure the faecal relative % FA content
- To measure the faecal EPA and DHA content of faecal samples at baseline, post supplementation and washout
- To assess any difference between the post supplementation and washout levels of faecal %EPA and DHA after capsules or drinks
- To assess the correlation between faecal and RBC EPA and DHA

5.3 Results

5.3.1 Primary results published in original study by Watson et al

Watson reported that 20 of the 22 participants completed at least one intervention and corresponding washout period while 16 of the 22 participants completed the study per protocol. The median duration on active oral supplementation was 57 days for capsules (n=20, range 55-63 days) and drinks (n=16, range 54-57). Compliance with both treatments was 100% and there were no severe adverse reactions. (Watson et al., 2018)

Ninety-seven out of a possible 100 venous blood samples from 20 participants underwent LCMS and were analysed. There were 3 missing samples from 2 participants. One participant did not undertake the drinks intervention after capsules so V4 or V5 samples were not available. Another participant failed to produce a post-supplementation (V2) sample after stopping the drinks intervention early. A post-washout (V3) sample was available for this participant. This is illustrated in figure 15:

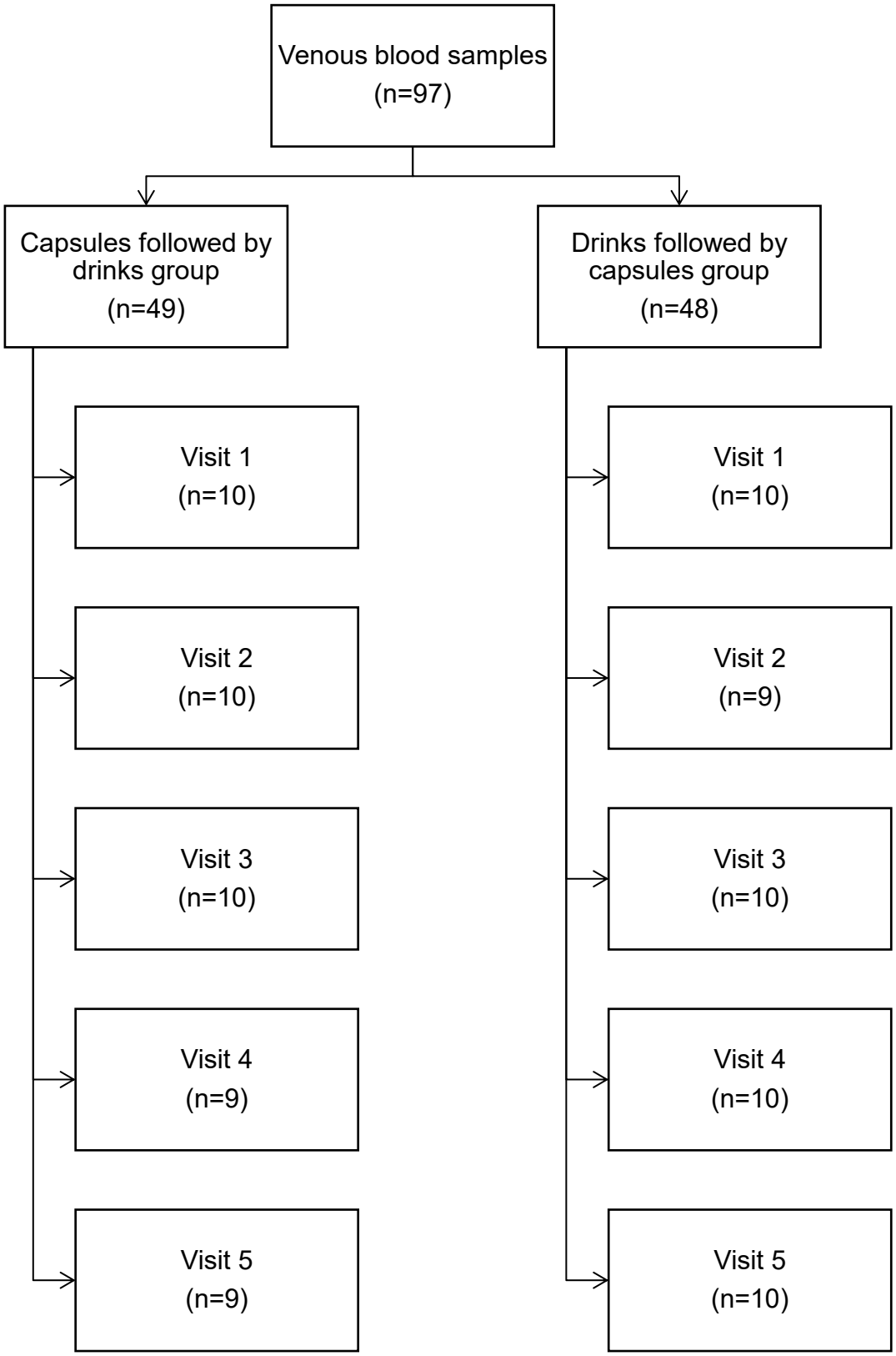


Figure 15 Flow diagram of venous blood samples from the Watson cross-over trial. (Watson et al., 2018)

5.3.2 Results of secondary analysis

This section describes the results of a secondary analysis of ninety-five out of a possible 100 faecal samples from 20 participants for LCMS and FA analysis. Five samples could not be analysed, because the volume left after microbiome analysis was insufficient for processing. These were:1 baseline (V1) sample pre-capsule supplementation, 3 post-treatment samples (1 with capsules (V4) and 2 with drink cartons (V2 and V4) and 1 washout (V5) sample post drinks as it wasn't supplied by the volunteer. See Figure 16 below:

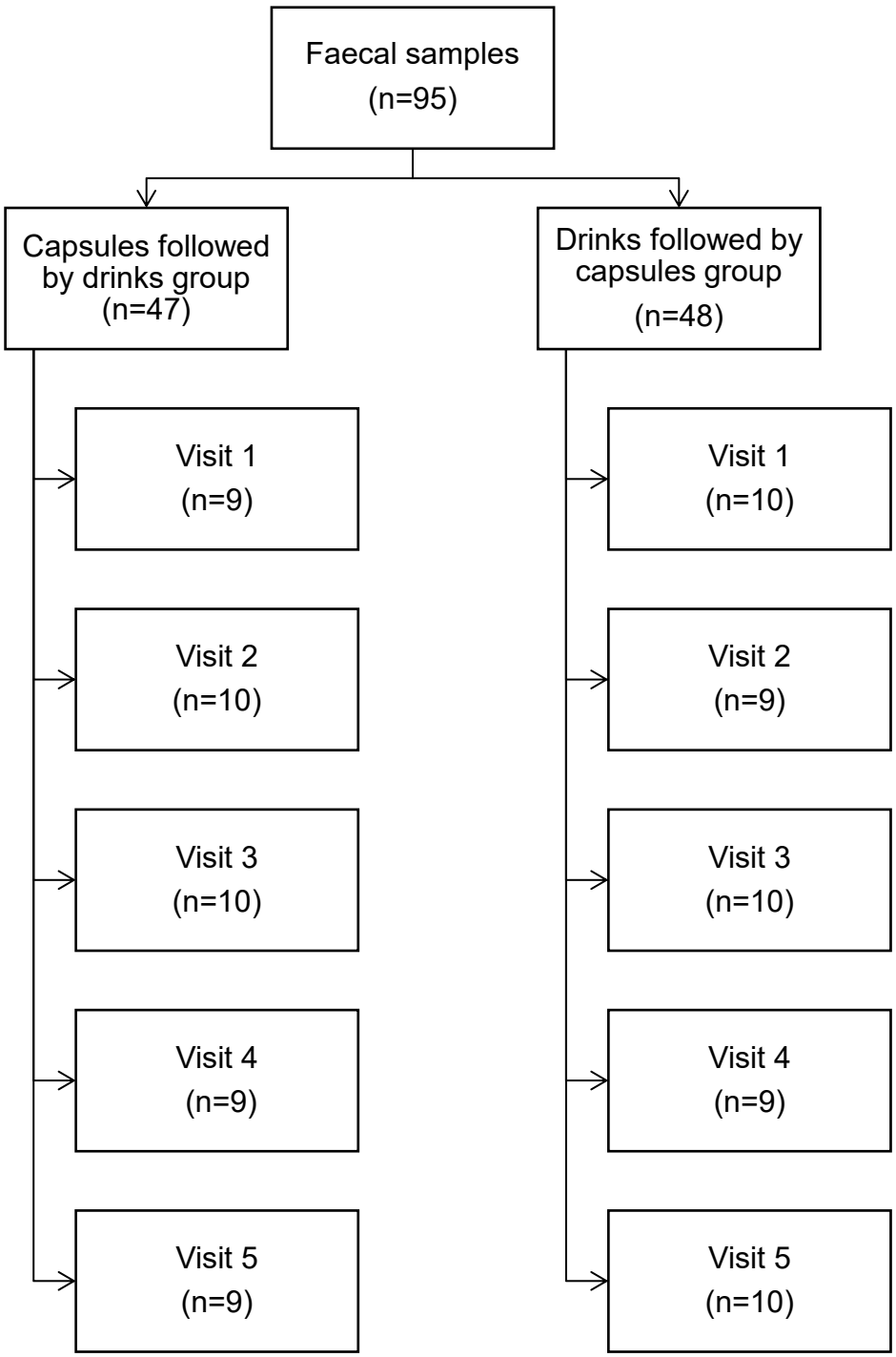


Figure 16 Flow diagram of faecal samples for secondary analysis

5.3.3 Faecal %EPA

The faecal %EPA measured across all phases of the study was <1% of the total %FA concentration, irrespective of the intervention (Figure 17).

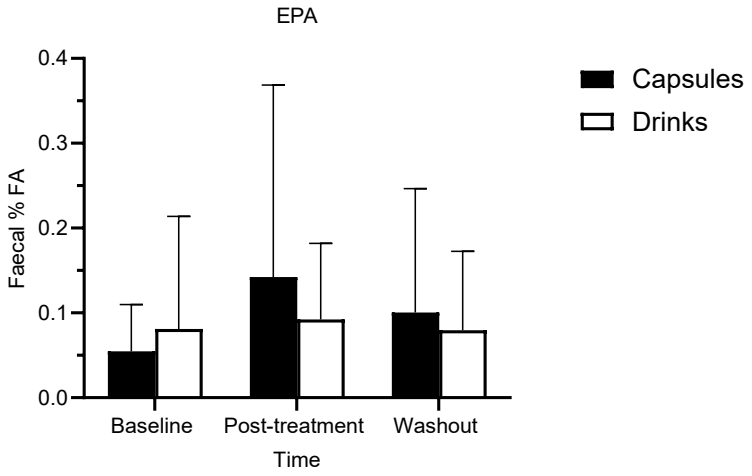


Figure 17 Mean change (+SD) of faecal EPA with each intervention.

The mean (Standard Deviation-SD) faecal %EPA was 0.05 (±0.05)% and 0.08% (±0.13)% before capsules and drinks respectively. There was mean two-fold increase in faecal % EPA to 0.14 (±0.23)% and 0.09 (±0.09)% post-supplementation with capsules and drinks respectively. Capsules resulted in a higher post-treatment rise in faecal EPA, compared to drinks (mean absolute difference from baseline of 0.09% vs 0.04% points respectively) however this was not statistically significant (p=0.632, Wilcoxon signed rank test).

Twelve weeks after stopping supplements (washout) there was an overall drop in mean faecal % EPA to 0.10 (±0.14)% and 0.08 ± (0.09)% for capsules and drinks respectively. The absolute difference between washout and baseline values was not statistically significant (p=0.98, Wilcoxon signed rank test) indicating an effective return to pre-treatment values (figure 18).

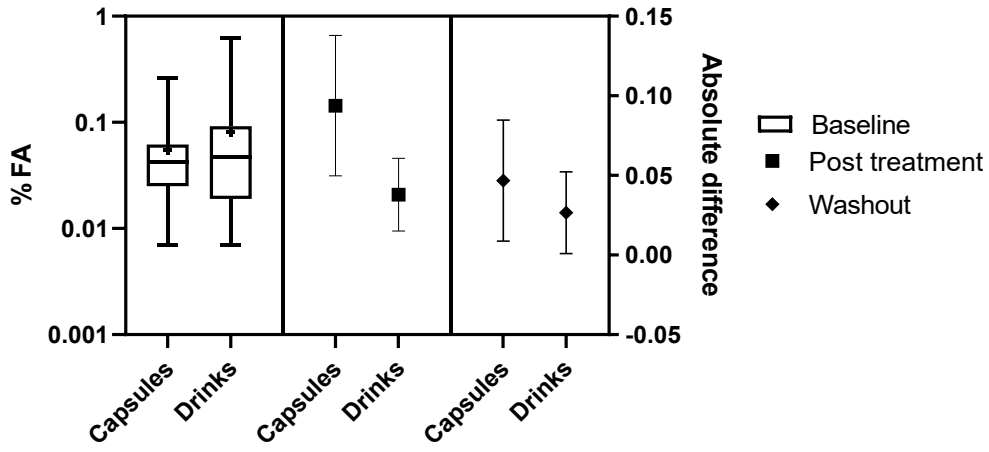


Figure 18: Faecal %EPA across all participants by intervention.

The box and whisker plot denotes median and range of baseline % EPA (left y-axis) with + to denote the mean. The symbols represent the mean and standard error (SEM) of the absolute differences (%,right y-axis): ■ (post-treatment - baseline %FA) and ♦ (washout - baseline %FA).

Individual response profiles were analysed (Figure 19). In the capsule arm, 13 individuals demonstrated an increase, while 5 had a decrease in faecal %EPA after 8 weeks. The mean absolute difference between the response groups was statistically significant (0.14±0.21% vs 0.02±0.01%, p=0.03, Mann-Whitney U). Upon stopping capsules, 12 patients promptly had a decrease in faecal %EPA (mean absolute difference 0.14 ± 0.25%) while 7 participants had higher washout than post-treatment faecal %EPA (mean absolute difference 0.10 ± 0.18%). There was no statistically significant difference in responses (p=0.23, Mann-Whitney U). Comparison of final washout values to the original baseline values showed high variability but no consistent trends nor differences between individuals (p=0.11, Mann-Whitney U test).

By contrast, supplementation with the drinks formulation showed modest changes across the various timepoints with fairly balanced split between participants. Twelve participants had an increase after baseline with 6 showing a decrease in faecal %EPA with no discernible trends or significant differences after supplementation (p=0.963, Mann-Whitney U). At washout, 11 participants also had an increase, while 8 had a decrease %EPA compared to baseline (p=0.741, Mann=Whitney U).

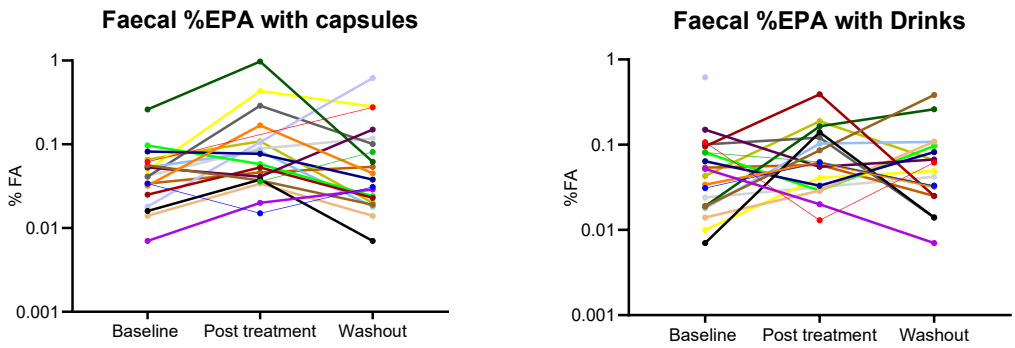


Figure 19 Individual participant response profiles for faecal %EPA with capsule and drinks supplementation. Each colour represents the same participant.

5.3.4 Faecal % DHA

The faecal %DHA measured across all phases of the study was also <1% of the total %FA concentration, irrespective of the intervention.

The mean baseline DHA was marginally lower than EPA across both intervention groups: 0.02 (±0.03)% DHA vs 0.05 (±0.05)% EPA in the capsule arm and 0.03 (±0.04)% DHA vs 0.08 (±0.13)% EPA in the drinks arm (see Figure 20).

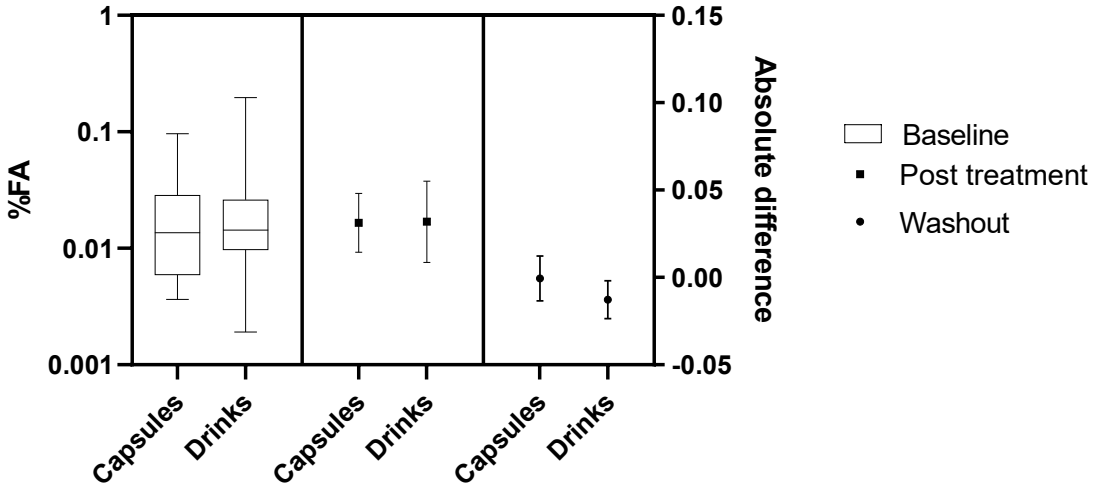


Figure 20 Faecal %DHA across all participants by intervention

The box and whisker plot denotes median and range of baseline % DHA (left y-axis) with + to denote the mean. The symbols represent the mean and standard error (SEM) of the absolute differences (%,right y-axis): ■ (post-treatment - baseline %FA) and ♦ (washout - baseline %FA).

Following oral supplementation, the mean faecal %DHA was 0.05 (±0.05)% and 0.06 (±0.07)% with capsules and drinks respectively. Although the absolute difference between post-treatment and baseline values for faecal DHA was 0.03% points; there was a 6-fold mean increase in DHA (compared to the 2-fold mean increase in EPA; post supplementation with either intervention (p=0.16, students' paired t-test).

Twelve weeks after stopping supplements (washout) there was an overall drop in the mean faecal % DHA to 0.02 (±0.04)% and 0.02 ± (0.02)% for capsules and drinks respectively. The absolute difference between washout and baseline values was not statistically significant (p=0.75, Mann-Whitney U test) indicating an effective return to pre-treatment values.

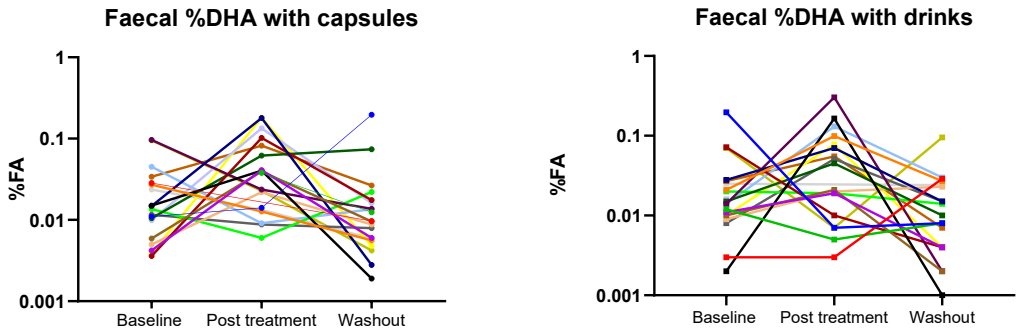


Figure 21 Individual participant response profiles for faecal %DHA with capsule and drinks supplementation. Each colour represents the same participant**.**

For faecal %DHA measurements (figure 21), 11 participants posted a post-supplementation accumulation with capsules while 13 did with drinks (mean = 0.08 ±0.06% compared with 0.08 ±0.08%; p=0.99, student's unpaired t-test). Of these, 8 individuals displayed a rise in faecal %DHA with both interventions. Conversely, only 2 showed a decline in faecal DHA. Only 6 of the 20 participants in the study showed a similar pattern irrespective of the intervention: 5 individuals (#6, #11, #12, #17, #18) displayed a rise while only 1 individual (#13) displayed a reduction in post-supplementation faecal EPA and DHA. (See Figure 22)

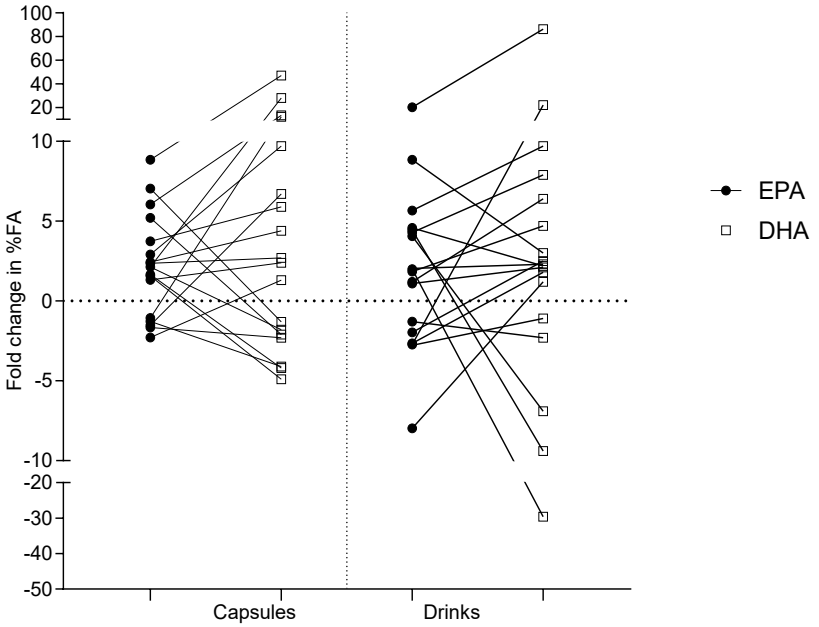


Figure 22 Fold changes in EPA and DHA after oral supplementation with capsules and drinks. Lines connect individual participants

5.3.5 Relationship between faecal and red cell membrane EPA

In their paper, Watson et al had concluded that 8 weeks of supplementation with EPA/DHA capsules and drinks led to a statistically significant increase in rbc relative abundance of EPA and DHA, with a resultant return to baseline 12 weeks after stopping supplements. (Watson et al., 2018)

Using the same cohort and biological samples from Watson's study, a secondary analysis was conducted extracting and quantifying faecal fatty acids. This revealed a rise in faecal % EPA and DHA during the supplementation period for both interventions. To further elucidate the relationship between colonic (luminal or mucosal) and systemic/tissue distribution of EPA and DHA – and provide insight on their bioavailability - the correlation between faecal and rbc omega-3 PUFA was explored.

Looking at the capsule arm first (figure 23), there was a weak positive correlation between rbc and faecal %EPA (Spearman r=0.14, p=0.56) at baseline but this was not significant. After 8 weeks, both faecal and rbc %EPA rose in keeping with

supplementation however there was a weak moderate negative correlation (r=-0.39, p=0.1) between both indicating that participants with faecal increases did not necessarily have the largest rbc EPA increases. This association was not statistically significant thus inference about tissue incorporation at the expense of luminal excretion or uptake into the colonic mucosal epithelium could not be firmly determined. In addition, the absolute difference between the faecal and rbc were negatively correlated (r=-0.25, p=0.31), thus rise in one did not equate to a similar increase in the other (figure 23D).

Finally, there was no correlation (r = -0.09, p = 0.70) between faecal and rbc EPA once supplements stopped, indicating that both dropped independently. Thus it can be concluded that there is no association between reduction in systemic EPA and increase in faecal EPA, implying that as levels falls, both behave independently of each other.

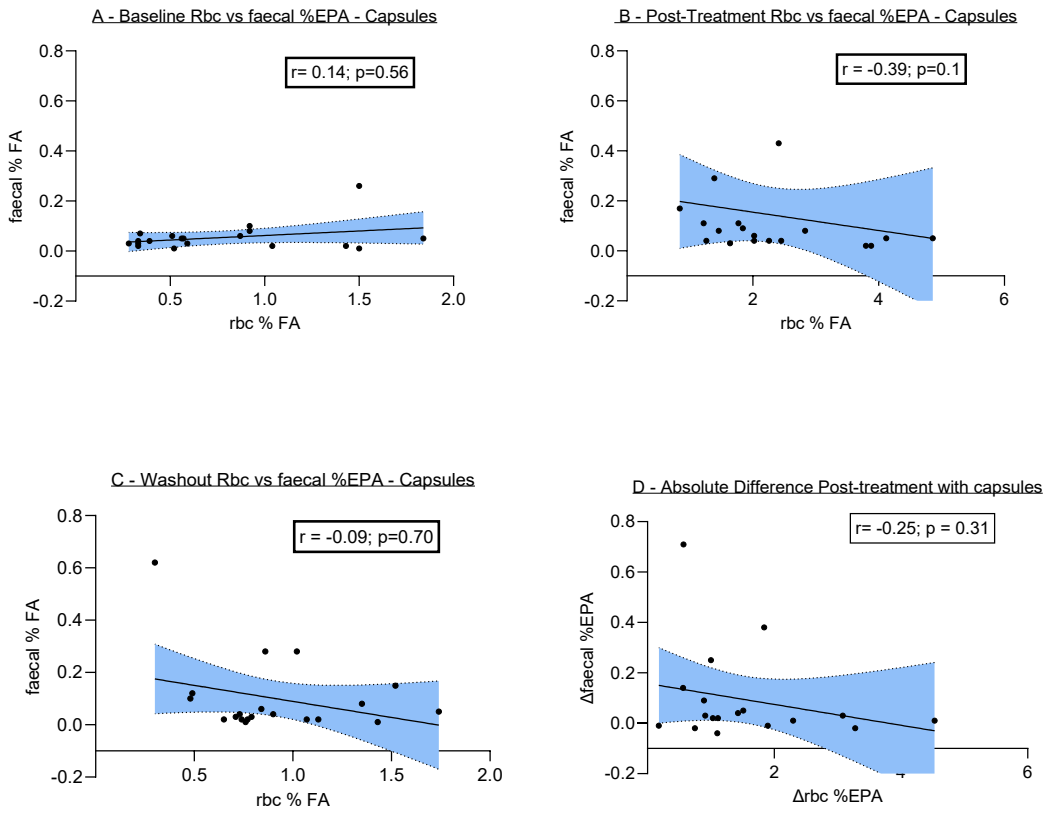


Figure 23 Correlations of Red cell membrane with faecal %EPA before (A), after 8 weeks of oral supplementation (B) and after 12 weeks of stopping supplementation with omega-3 PUFA capsules. Correlation of Change (absolute difference between post-supplementation and baseline %EPA) in faecal and red cell membrane %EPA (D). Spearman correlation coefficient quoted with p-values. Blue shaded area represents the 95% confidence interval of the p-values.

These changes were similar for EPA with drinks formulations, see Figure 24.

There was no correlation at baseline, a moderate negative correlation after supplementation that although stronger than with capsules, was still not statistically significant (r=-0.64, p=0.88). Washout resulted in a weak negative correlation that was not statistically significant.

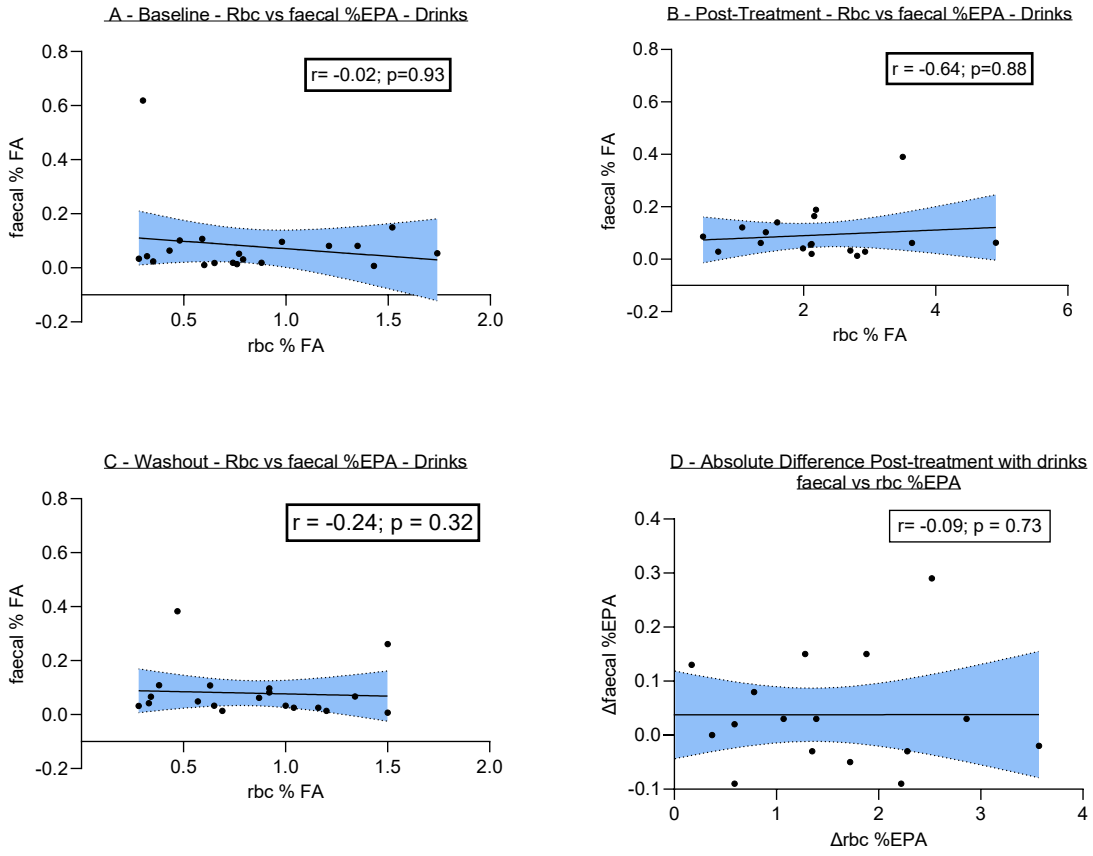


Figure 24 Correlations of Red cell membrane with faecal %EPA before (A), after 8 weeks of oral supplementation (B) and after 12 weeks of stopping supplementation with omega-3 PUFA Drinks. Correlation of Change (absolute difference between post-supplementation and baseline %EPA) in faecal and red cell membrane %EPA (D). Spearman correlation coefficient quoted with p-values. Blue shaded area represents the 95% confidence interval of the p-values.

5.3.6 Relationship between faecal and red cell membrane DHA

Prior to supplementation, there was no correlation between faecal and rbc DHA at baseline with either intervention(Figure 25 A and E). After 8 weeks of supplementation, there was a positive association between faecal and rbc DHA. This was statistically significant in the capsule arm (r=0.49, p=0.04). The absolute difference between post-treatment and baseline faecal and rbc DHA was also positively correlated (r=0.51; p=0.03) (Figure 25 A to D). Conversely this association with supplementation was negatively correlated in the drinks arm (Figure 25 E to H). As faecal FA rose, rbc DHA fell (r=-0.50;p=0.03). Similarly, this change reached statistical significance as demonstrated by the absolute difference in rbc and faecal DHA post-supplementation from baseline (r=-0.47; p=0.05).

After the 12 week washout period, there was no correlation between faecal and rbc DHA for capsules and drinks, mirroring EPA.

Supplementation resulted in an overall increase in DHA in faeces and rbc, similar to EPA. The total relative abundance of rbc DHA was higher than rbc EPA reflecting better systemic incorporation of DHA. The positive correlation with capsules was in contrast to the negative correlation with drinks, and both reached statistical significance indicating a real difference in the absorption and bioavailability between the colonic lumen and mucosa. (See Figure 25 below). Higher systemic incorporation of capsules was positively associated with higher faecal FA, could be explained by potential increased tissue uptake and metabolism or shedding of excess DHA from the enterocyte into stool. With drinks, lower tissue incorporation was associated with increased faecal DHA reflecting differences in the absorption pathway.

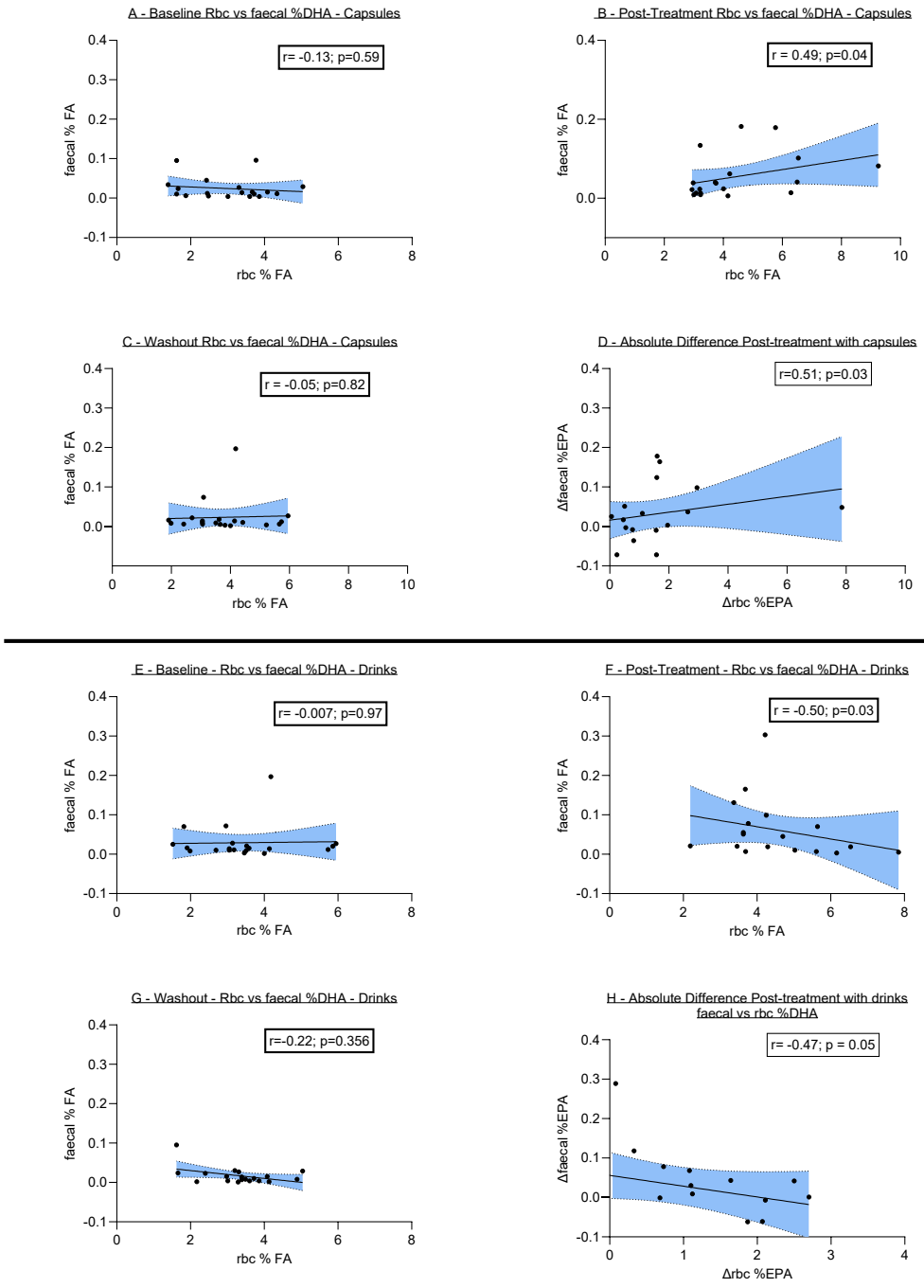


Figure 25 Correlation of red cell membrane with faecal %DHA before (A and E), after 8 weeks of oral supplementation (B and F) and after 12 weeks of stopping supplementation (C and G) with omega-3 PUFA capsules and drinks respectively. Correlation of Change (absolute difference between post-supplementation and baseline %DHA) in faecal and red cell membrane after capsules (D) and drinks (H). Spearman correlation coefficient quoted with p-values. Blue shaded area represents the 95% confidence interval of the p-values.

5.4 Discussion

5.4.1 Summary of key findings

This secondary analysis of a randomised cross-over trial investigated the effect of omega-3 PUFA supplementation - 4g of 1:1 EPA/DHA administered via capsule and drink formulations – on faecal EPA and DHA and explored their relationship with systemic (rbc) EPA/DHA.

At baseline, the relative abundance of EPA and DHA in the human faecal samples was <1% of the total FA abundance, as measured by LCMS/MS. This was similar to the baseline mean rbc %EPA in (0.77% and 0.88% in capsules and drinks arms respectively) in Watson's study. Mean rbc %DHA was higher than faecal DHA at baseline, in the same study (3.03% and 3.70% for capsules and drinks respectively) (Watson et al., 2018). DHA's longer carbon chain (22 vs 20 in EPA) and greater unsaturation (6 rather than EPA's 5 double bonds), improves membrane fluidity, and thus its uptake and tissue incorporation is higher than EPA's. (Jacobs et al., 2021; Schuchardt et al., 2022; Sherratt & Mason, 2018) This may explain its higher abundance in rbc, and reduced excretion in faeces. EPA on the other hand is more readily converted to other compounds thus lowering its tissue abundance compared to DHA.

Oral supplementation with both formulations resulted in a rise in faecal EPA/DHA. A greater number of participants in the capsule arm demonstrated a more significant increase in faecal EPA than DHA from baseline. There was high inter-individual variability and mixed responses to stopping capsules. While most participants exhibited a decline, others had paradoxical further increases in faecal EPA/DHA at washout, with levels not fully returning to baseline. Although the drink formulation also demonstrated a rise during supplementation, and a decline on stopping, there was no consistent difference between participants.

Comparing faecal and systemic relative FA abundance, there was no correlation between the two at baseline. With supplementation, the rise in rbc EPA did not have a strong positive correlation with the observed rise in faecal EPA, rather a weak negative association. After stopping supplements, rbc EPA also fell, however this was not correlated to the fall in faecal EPA. As both approached baseline, weak non-significant correlations persisted, indicating that systemic and faecal EPA concentrations were independent of each other.

5.4.2 Implications of current findings

The finding of <1% total EPA/DHA in faecal samples from this analysis is similar to the concentration in ileal effluent EPA/DHA detailed in Chapter 4, and is consistent with previous studies (Sanguansri et al., 2013) reflecting the highly efficient absorption of omega-3 PUFAs. Recent reviews on omega-3 PUFA bioavailability in human tissues advocate the of use plasma/red cell/platelet as biomarkers of tissue incorporation (Alijani et al., 2025). However, the influence of omega-3 PUFAs on the gut-blood axis remains poorly understood. This thesis contributes complimentary novel data, confirming ileal effluent EPA/DHA with associated microbiome shifts in the distal small bowel. Similar faecal EPA/DHA abundance suggests that it reaches the colonic lumen. Further work is required to determine whether the microbiome shifts observed in the ileal microbiome, translate to the colonic microbiome

Oral supplementation led to an increase in both faecal and Rbc EPA, indicating that participants were capable of digestion and delivery to the small intestine for absorption of the triglyceride-bound EPA/DHA in both formulations of the supplements. Notably, the capsule formulation resulted in a higher post-treatment rise in faecal EPA, compared to drink form. Given that lipid absorption is usually >95% efficient, this may reflect lower omega-3 PUFA absorption with capsules compared to drinks, resulting in increased faecal excretion. Results after washout hint at faecal EPA taking slightly longer to normalise after capsules. These differences in EPA absorption and excretion likely reflect confounding factors such as individual variability in enzymatic conversion, baseline dietary fat consumption, gut transit times, methodological issues in sample collection or formulation-related differences.

Systemic incorporation of EPA into red cell membrane occurred with supplementation followed by a predictable fall during washout. The absence of a strong correlation between faecal and rbc EPA after supplementation and the paradoxical increase in faecal EPA observed after washout in some participants, could be explained by either the aforementioned variation in gut digestion, or mucosal shedding of enterocytes containing mucosal EPA into the faeces. However, the failure to achieve statistical significance may indicate that these findings were unrelated and incidental.

The strengths of this study include the use of two formulations of supplements, with two separate biomarkers to concurrently explore systemic and luminal omega-3 PUFA bioavailability. The cross-over design meant that each participant acted as their control, reducing bias and minimising intra-individual variability. The washout period (≥12 weeks) was a suitable length to prevent carry-over

effects. (Alijani et al., 2025) The lipidomic assay method used was validated, robust and reproducible for investigation of omega-3 PUFAs in different cell and tissue pools (Volpato et al., 2017). The increase in rbc omega-3 PUFA abundance following supplementation is supported by the literature.

The results should be interpreted in light of the limitations of the study. Given the retrospective nature of this analysis and the small sample size, the original study was not powered to measure changes in faecal omega-3 PUFAs. There were inconsistencies in the timing, collection, transportation and storing of faecal samples. This may have resulted in oxidation of omega-3 PUFAs and inaccurate results. This can be minimised in future studies by using ethanol bottles for faecal specimens destined for metabolomics and microbiome analysis (Isokääntä et al., 2024). Future studies on omega-3 PUFA bioavailability should also incorporate suggested recommendations for standardised research protocols that reduce methodological inconsistencies between studies, as described in a recent review (Alijani et al., 2025).

Overall, this secondary analysis has shown a weak negative correlation between rising faecal and rbc omega-3 PUFAs after oral supplementation. This may reflect the shedding of colonic mucosal enterocytes into the lumen or excretion of the unabsorbed omega-3 PUFAs from the small intestine. Given the variable and dynamic nature of ileal effluent and colonic content, rbc omega-3 PUFAs remain the more reliable biomarkers of oral supplementation. Future work should explore whether colonic microbiome shifts or specific metabolite production (e.g. short-chain fatty acids) correlate with faecal omega-3 PUFA abundance.

Chapter 6 Summary and future work

Omega-3 PUFAs have demonstrated anti-neoplastic activity through multiple mechanisms including promotion of anti-inflammatory mediators and modulation of the gut microbiome in favour of SCFA producing bacterial taxa. (Watson et al., 2018; Wong & Yu, 2023) Mechanistic models still remain poorly understood, especially regarding how the bioavailability of ingested omega-3 PUFA supplements influences gastrointestinal and systemic incorporation. This body of work addresses this gap and contributes novel data to the evidence base.

In the first analysis, 28 days of supplementation with omega-3 PUFA was shown to significantly increase their luminal concentration in the distal small bowel. There was a concurrent increase in rbc EPA/DHA concentrations. This was associated with ileal microbiome shifts in favour of SCFA-producing taxa such as *Bacteroides* and *Firmicutes*. These findings serve as a proxy for colonic exposure. The secondary analysis, of a published cross-over trial of omega-3 PUFA supplementation with capsules and drinks formulations, determined faecal EPA/DHA concentrations, completing the journey on the gut-blood axis, elucidating pathways to systemic incorporation.

Baseline omega-3 PUFA concentration below 1% of total FA in ileal and colonic luminal samples were consistent with prior evidence of excellent GI absorption of omega-3 PUFAs. (Frydrych et al., 2025; Sanguansri et al., 2013) Increases in IF and faecal EPA/DHA support consistency in the trial protocol, participant compliance with supplementation and methodological rigor in capturing short-term changes. These findings provide clear evidence of GI accumulation with supplementation and contribute to understanding the pharmacokinetics of digestion and absorption.

Combining this with rbc EPA/DHA - a recognised biomarker of medium and long-term omega-3 PUFA status (Alijani et al., 2025) - allows assessment of tissue incorporation. The IF analysis showed that despite high temporal inter-individual variability; systemic incorporation increased with supplementation and dropped upon cessation. Notably, faecal EPA rise had a weak negative association with rbc EPA rise, suggesting variability in pharmacokinetics, GI absorption, or microbial interactions may modulate the response. While secondary analysis did not correlate faecal EPA with microbiome changes, as observed in the primary analysis - weak associations with rbc EPA – this would remain an inference and not establish causality.

Despite being limited by short-term interventions, small sample sizes, and high inter-individual variability; the study findings described in this thesis enhance the knowledge base on omega-3 PUFA luminal absorption, systemic incorporation and microbiome shifts. The observed association between supplementation and an increase in SCFA-producing *Bacteroides* in distal small bowel, supports similar shifts in SCFA-producing taxa in the colon in the literature, however the absence of a metabolomic endpoint is a limitation that would have provided mechanistic insight. A 6-week dietary intervention trial of daily supplementation of 69 healthy volunteers with 500mg omega-3 PUFA (165mg EPA, 110mg DHA) versus 20g of the prebiotic, inulin, combined metagenomic and metabolomics to provide a better understanding of the functional role of microbial species and communities in mediating immune benefits. The authors described significant increases in *Coprococcus* and *Bacteroides*. Additionally, they showed a positive association between the genus *Coprococcus* with the short-chain fatty acids iso-butyric and butyric acid production (Vijay et al., 2021).

As with the example above, the literature on gut microbiota modulation associated with omega-3 PUFA administration are not generalisable due to heterogeneity in formulation, dosing, duration of supplementation, study model, intra-individual variability, and disease phenotypes. (Vijay et al., 2021; L. Wang et al., 2021; Watson et al., 2018). However, the concentrations of omega-3 PUFAs in small bowel, determined from this study, could be used to refine such metabolomic endpoints through the use of *in vitro* human gut microbiota fermentation models. These range from deep-well plates to fully controlled bioreactors, that have been inoculated with different species to provide a controlled environment for studying the effects of prebiotics on the gut microbiota and exploring host-microbe interactions without host specific effects (Isenring et al., 2023; Rehman et al., 2024). One such study, using a Mucosal Simulator of the Human Intestinal Microbial Ecosystem (M-SHIME) recreated the ileum, ascending, transverse, and descending colon with luminal and mucus-associated microbiota, performing metabolomic and metagenomic assays after supplementation with omega-3 PUFAs for 7 days. (Roussel et al., 2022) Interestingly, their findings diverged from much of the in vivo literature, as they reported marked increase in *Akkermansia muciniphila*, a decrease in *Firmicutes* mucolytic bacteria in mucus luminal niches and increased proportions of propionate over butyrate – the latter SCFA being more commonly associated with omega-3 PUFA supplementation (Coker et al., 2022; M. Song, Chan, et al., 2020). The findings were limited by the inability to fully reproduce the lipid digestion and absorption due to the absence of intestinal epithelial cells, thus bioavailability could not be ascertained. The results of another study conducted

by Aldoori and colleagues in the UK (currently undergoing peer review), which uses the omega-3 PUFA concentrations derived from this study in an *in vitro* fermentation model, are eagerly anticipated. Provisional data suggest that omega-3 PUFAs increase SCFA production especially in the presence of inulin. This would provide the metabolomic endpoint that was lacking in this study currently. Furthermore, 16S RNA quantification of microbiome shifts has largely been replaced by shotgun metagenomics as the standard to obtain strain-level, functional and phylogenetic profiling. This metagenomic and metatranscriptomic measurements can now be used to study the epidemiology of the human microbiome for biomarkers and therapy with greater accuracy. (Beghini et al., 2021)

These human studies demonstrated excellent compliance and favourable safety profile of omega-3 PUFA supplements, with minimal dropout and no reports of serious adverse events. Supplement formulation was not shown to affect compliance but its potential influence on differential faecal EPA outcomes cannot be excluded. Furthermore, confounding factors such as BMI, dietary fat consumption, timing of sample collection likely impact systemic RBC incorporation.

Isotopic tracer studies would offer enhanced mechanistic detail into the interplay between luminal and systemic incorporation. In a crossover trial administering ^{13}C-labelled DHA as either a structured phospholipid or triacylglycerol, plasma kinetics revealed superior absorption and retention from the phospholipid form compared with triacylglycerol (Plourde et al., 2014). A secondary analysis later showed that gender differences, BMI and time affected omega-3 PUFA concentrations and distribution in tissues. (Loukil et al., 2025). These findings highlight the importance of delivery form and controlling confounders in studies of short-term bioavailability, though the long-term clinical implications remain uncertain.

While this work looked at blood, faecal biomarkers and microbiome shifts, it did not investigate molecular anti-inflammatory biomarkers implicated in the anti-neoplastic effects of omega-3 PUFAs. A recent study of 47 healthy adults using colonic mucosal and luminal stool brushings revealed contrasting findings: omega-3 PUFA supplementation did not significantly alter intestinal bacteria in healthy humans but increased diversity in luminal brushings predicted reduction in colonic PGE_2 – a potent anti-inflammatory mediator of omega-3 PUFA anticancer effect (Djuric et al., 2019). As with this studies, the sample size was small, thus a larger study would be beneficial.

In conclusion, larger well-controlled studies in both healthy and at risk populations with metagenomic and metabolomic endpoints are required to translate these experimental findings into clinically meaningful health outcomes.

List of references

Abdelhafez, A., Khabir, Z., Prestidge, C. A., Garcia-Bennett, A., & Joyce, P. (2025). The impact of formulation design on the oral bioavailability of omega-3 polyunsaturated fatty acids. *Food Research International*, *208*, 116171. https://doi.org/10.1016/j.foodres.2025.116171

Aglago, E. K., Huybrechts, I., Murphy, N., Casagrande, C., Nicolas, G., Pischon, T., Fedirko, V., Severi, G., Boutron-Ruault, M.-C., Fournier, A., Katzke, V., Kühn, T., Olsen, A., Tjønneland, A., Dahm, C. C., Overvad, K., Lasheras, C., Agudo, A., Sánchez, M.-J., … Gunter, M. J. (2020). Consumption of Fish and Long-chain n-3 Polyunsaturated Fatty Acids Is Associated With Reduced Risk of Colorectal Cancer in a Large European Cohort. *Clinical Gastroenterology and Hepatology*, *18*(3), 654-666.e6. https://doi.org/10.1016/j.cgh.2019.06.031

Alagiakrishnan, K., Morgadinho, J., & Halverson, T. (2024). Approach to the diagnosis and management of dysbiosis. *Frontiers in Nutrition*, *11*, 1330903. https://doi.org/10.3389/fnut.2024.1330903

Aldoori, J., Cockbain, A. J., Toogood, G. J., & Hull, M. A. (2022). Omega-3 polyunsaturated fatty acids: Moving towards precision use for prevention and treatment of colorectal cancer. *Gut*, *71*(4), 822–837. https://doi.org/10.1136/gutjnl-2021-326362

Aldoori, J., Zulyniak, M. A., Toogood, G. J., & Hull, M. A. (2025). Plasma n-3 Polyunsaturated Fatty Acid Levels and Colorectal Cancer Risk in the UK Biobank: Evidence of Nonlinearity, as Well as Tumor Site- and Sex-Specificity. *Cancer Epidemiology, Biomarkers & Prevention*, *34*(3), 394–404. https://doi.org/10.1158/1055-9965.EPI-24-1154

Alijani, S., Hahn, A., Harris, W. S., & Schuchardt, J. P. (2025). Bioavailability of EPA and DHA in humans – A comprehensive review. *Progress in Lipid Research*, *97*, 101318. https://doi.org/10.1016/j.plipres.2024.101318

Apprill, A., McNally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquatic Microbial Ecology*, *75*(2), 129–137. https://doi.org/10.3354/ame01753

Arnold, C., Markovic, M., Blossey, K., Wallukat, G., Fischer, R., Dechend, R., Konkel, A., von Schacky, C., Luft, F. C., Muller, D. N., Rothe, M., & Schunck, W.-H. (2010). Arachidonic acid-metabolizing cytochrome P450 enzymes are targets

of {omega}-3 fatty acids. *The Journal of Biological Chemistry*, *285*(43), 32720–32733. https://doi.org/10.1074/jbc.M110.118406

Astrazeneca. (2020, January 13). *Update on Phase III STRENGTH trial for Epanova in mixed dyslipidaemia*. https://www.astrazeneca.com/media-centre/press-releases/2020/update-on-phase-iii-strength-trial-for-epanova-in-mixed-dyslipidaemia-13012020.html#

Aung, T., Halsey, J., Kromhout, D., Gerstein, H. C., Marchioli, R., Tavazzi, L., Geleijnse, J. M., Rauch, B., Ness, A., Galan, P., Chew, E. Y., Bosch, J., Collins, R., Lewington, S., Armitage, J., Clarke, R., & Omega-3 Treatment Trialists' Collaboration. (2018). Associations of Omega-3 Fatty Acid Supplement Use With Cardiovascular Disease Risks: Meta-analysis of 10 Trials Involving 77 917 Individuals. *JAMA Cardiology*, *3*(3), 225–234. https://doi.org/10.1001/jamacardio.2017.5205

Bang, H. O., Dyerberg, J., & Nielsen, A. B. (1971). Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos. *Lancet (London, England)*, *1*(7710), 1143–1145. https://doi.org/10.1016/s0140-6736(71)91658-8

Banović Fuentes, J., Beara, I., & Torović, L. (2024). Regulatory Compliance of Health Claims on Omega-3 Fatty Acid Food Supplements. *Foods (Basel, Switzerland)*, *14*(1), 67. https://doi.org/10.3390/foods14010067

Baron, J. A., Barry, E. L., Mott, L. A., Rees, J. R., Sandler, R. S., Snover, D. C., Bostick, R. M., Ivanova, A., Cole, B. F., Ahnen, D. J., Beck, G. J., Bresalier, R. S., Burke, C. A., Church, T. R., Cruz-Correa, M., Figueiredo, J. C., Goodman, M., Kim, A. S., Robertson, D. J., … Summers, R. W. (2015). A Trial of Calcium and Vitamin D for the Prevention of Colorectal Adenomas. *New England Journal of Medicine*, *373*(16), 1519–1530. https://doi.org/10.1056/NEJMoa1500409

Beghini, F., McIver, L. J., Blanco-Míguez, A., Dubois, L., Asnicar, F., Maharjan, S., Mailyan, A., Manghi, P., Scholz, M., Thomas, A. M., Valles-Colomer, M., Weingart, G., Zhang, Y., Zolfo, M., Huttenhower, C., Franzosa, E. A., & Segata, N. (2021). Integrating taxonomic, functional, and strain-level profiling of diverse microbial communities with bioBakery 3. *eLife*, *10*, e65088. https://doi.org/10.7554/eLife.65088

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)*, *57*(1), 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Berg, J. M., Tymoczko, J. L., Stryer, L., & Stryer, L. (2002). *Biochemistry* (5th ed). W.H. Freeman.

Bertagnolli, M. M., Eagle, C. J., Zauber, A. G., Redston, M., Solomon, S. D., Kim, K., Tang, J., Rosenstein, R. B., Wittes, J., Corle, D., Hess, T. M., Woloj, G. M., Boisserie, F., Anderson, W. F., Viner, J. L., Bagheri, D., Burn, J., Chung, D. C., Dewar, T., … APC Study Investigators. (2006). Celecoxib for the prevention of sporadic colorectal adenomas. *The New England Journal of Medicine*, *355*(9), 873–884. https://doi.org/10.1056/NEJMoa061355

Blanksby, S. J., & Mitchell, T. W. (2010). Advances in Mass Spectrometry for Lipidomics. *Annual Review of Analytical Chemistry*, *3*(1), 433–465. https://doi.org/10.1146/annurev.anchem.111808.073705

Bodur, M., Yilmaz, B., Ağagündüz, D., & Ozogul, Y. (2025). Immunomodulatory Effects of Omega-3 Fatty Acids: Mechanistic Insights and Health Implications. *Molecular Nutrition & Food Research*, *69*(10), e202400752. https://doi.org/10.1002/mnfr.202400752

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Booijink, C. C. G. M., El-Aidy, S., Rajilić-Stojanović, M., Heilig, H. G. H. J., Troost, F. J., Smidt, H., Kleerebezem, M., De Vos, W. M., & Zoetendal, E. G. (2010). High temporal and inter-individual variation detected in the human ileal microbiota. *Environmental Microbiology*, *12*(12), 3213–3227. https://doi.org/10.1111/j.1462-2920.2010.02294.x

Boutté, H. J., & Poylin, V. (2023). High ileostomy output: A practical review of pathophysiology, causes, and management☆. *Seminars in Colon and Rectal Surgery*, *34*(2), 100955. https://doi.org/10.1016/j.scrs.2023.100955

Braeckman, R. A., Stirtan, W. G., & Soni, P. N. (2014). Pharmacokinetics of Eicosapentaenoic Acid in Plasma and Red Blood Cells After Multiple Oral Dosing With Icosapent Ethyl in Healthy Subjects. *Clinical Pharmacology in Drug Development*, *3*(2), 101–108. https://doi.org/10.1002/cpdd.84

Bray, F., Laversanne, M., Sung, H., Ferlay, J., Siegel, R. L., Soerjomataram, I., & Jemal, A. (2024). Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *74*(3), 229–263. https://doi.org/10.3322/caac.21834

Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanas, A., Konstam, M. A., Baron, J. A., & Adenomatous Polyp Prevention on Vioxx (APPROVe) Trial Investigators. (2005). Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *The New England Journal of Medicine*, *352*(11), 1092–1102. https://doi.org/10.1056/NEJMoa050493

Brown, T. J., Brainard, J., Song, F., Wang, X., Abdelhamid, A., & Hooper, L. (2019). Omega-3, omega-6, and total dietary polyunsaturated fat for prevention and treatment of type 2 diabetes mellitus: Systematic review and meta-analysis of randomised controlled trials. *BMJ*, l4697. https://doi.org/10.1136/bmj.l4697

Browning, L. M., Walker, C. G., Mander, A. P., West, A. L., Madden, J., Gambell, J. M., Young, S., Wang, L., Jebb, S. A., & Calder, P. C. (2012). Incorporation of eicosapentaenoic and docosahexaenoic acids into lipid pools when given as supplements providing doses equivalent to typical intakes of oily fish. *The American Journal of Clinical Nutrition*, *96*(4), 748–758. https://doi.org/10.3945/ajcn.112.041343

Bullman, S., Pedamallu, C. S., Sicinska, E., Clancy, T. E., Zhang, X., Cai, D., Neuberg, D., Huang, K., Guevara, F., Nelson, T., Chipashvili, O., Hagan, T., Walker, M., Ramachandran, A., Diosdado, B., Serna, G., Mulet, N., Landolfi, S., Ramon Y Cajal, S., … Meyerson, M. (2017). Analysis of Fusobacterium persistence and antibiotic response in colorectal cancer. *Science (New York, N.Y.)*, *358*(6369), 1443–1448. https://doi.org/10.1126/science.aal5240

Burdge, G. C., Jones, A. E., & Wootton, S. A. (2002). Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men*. *The British Journal of Nutrition*, *88*(4), 355–363. https://doi.org/10.1079/BJN2002662

Burkitt, D. P. (1971). Epidemiology of cancer of the colon and rectum. *Cancer*, *28*(1), 3–13. https://doi.org/10.1002/1097-0142(197107)28:1<3::aid-cncr2820280104>3.0.co;2-n

Calder, P. C. (2020). Eicosapentaenoic and docosahexaenoic acid derived specialised pro-resolving mediators: Concentrations in humans and the effects of age, sex, disease and increased omega-3 fatty acid intake. *Biochimie*, *178*, 105–123. https://doi.org/10.1016/j.biochi.2020.08.015

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Calviello, G., Serini, S., & Piccioni, E. (2007). n-3 polyunsaturated fatty acids and the prevention of colorectal cancer: Molecular mechanisms involved. *Current Medicinal Chemistry*, *14*(29), 3059–3069. https://doi.org/10.2174/092986707782793934

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics*, *10*(1), 421. https://doi.org/10.1186/1471-2105-10-421

Cancer Research UK. (2023). *Https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer#heading-One*. https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/bowel-cancer#heading-One

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S. M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J. A., Smith, G., & Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621–1624. https://doi.org/10.1038/ismej.2012.8

Center for Drug Evaluation and Research. (2020, September 4). *U.S. Food and Drug Administration. Guidance for Industry Bioavailability and Bioequivalence, Studies Submitted in NDAs or INDs—General Considerations. 2024.* https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioavailability-and-bioequivalence-studies-submitted-ndas-or-inds-general-considerations

Chang, J. P.-C., Tseng, P.-T., Zeng, B.-S., Chang, C.-H., Su, H., Chou, P.-H., & Su, K.-P. (2023). Safety of Supplementation of Omega-3 Polyunsaturated Fatty Acids: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Advances in Nutrition*, *14*(6), 1326–1336. https://doi.org/10.1016/j.advnut.2023.08.003

Chapelle, N., Martel, M., Toes-Zoutendijk, E., Barkun, A. N., & Bardou, M. (2020). Recent advances in clinical practice: Colorectal cancer chemoprevention in the average-risk population. *Gut*, *69*(12), 2244–2255. https://doi.org/10.1136/gutjnl-2020-320990

Chen, H.-M., Yu, Y.-N., Wang, J.-L., Lin, Y.-W., Kong, X., Yang, C.-Q., Yang, L., Liu, Z.-J., Yuan, Y.-Z., Liu, F., Wu, J.-X., Zhong, L., Fang, D.-C., Zou, W., & Fang, J.-Y. (2013). Decreased dietary fiber intake and structural alteration of gut microbiota in patients with advanced colorectal adenoma. *The American Journal of Clinical Nutrition*, *97*(5), 1044–1052. https://doi.org/10.3945/ajcn.112.046607

Chen, L., Tan, T., Wu, Q., Cui, F., Chen, Y., Chen, H., Zhao, Y., Xiang, X., Shan, Z., Tang, Y., & Deng, Q. (2025). Dietary polyunsaturated fatty acid and risk of gout: A cohort study integrating genetic predisposition and metabolomics. *European Journal of Epidemiology*, *40*(4), 427–439. https://doi.org/10.1007/s10654-025-01242-9

Chen, S.-H., & Chuang, Y.-J. (2002). Analysis of fatty acids by column liquid chromatography. *Analytica Chimica Acta*, *465*(1–2), 145–155. https://doi.org/10.1016/S0003-2670(02)00095-8

Chilton, F., Dutta, R., Reynolds, L., Sergeant, S., Mathias, R., & Seeds, M. (2017). Precision Nutrition and Omega-3 Polyunsaturated Fatty Acids: A Case for Personalized Supplementation Approaches for the Prevention and Management of Human Diseases. *Nutrients*, *9*(11), 1165. https://doi.org/10.3390/nu9111165

Cockbain, A. J., Volpato, M., Race, A. D., Munarini, A., Fazio, C., Belluzzi, A., Loadman, P. M., Toogood, G. J., & Hull, M. A. (2014). Anticolorectal cancer activity of the omega-3 polyunsaturated fatty acid eicosapentaenoic acid. *Gut*, *63*(11), 1760–1768. https://doi.org/10.1136/gutjnl-2013-306445

Coker, O. O., Liu, C., Wu, W. K. K., Wong, S. H., Jia, W., Sung, J. J. Y., & Yu, J. (2022). Altered gut metabolites and microbiota interactions are implicated in colorectal carcinogenesis and can be non-invasive diagnostic biomarkers. *Microbiome*, *10*(1), 35. https://doi.org/10.1186/s40168-021-01208-5

Das, U. N. (2006). Essential fatty acids: Biochemistry, physiology and pathology. *Biotechnology Journal*, *1*(4), 420–439. https://doi.org/10.1002/biot.200600012

Davidson, M. H., Johnson, J., Rooney, M. W., Kyle, M. L., & Kling, D. F. (2012). A novel omega-3 free fatty acid formulation has dramatically improved bioavailability during a low-fat diet compared with omega-3-acid ethyl esters: The ECLIPSE (Epanova(®) compared to Lovaza(®) in a pharmacokinetic single-dose evaluation) study. *Journal of Clinical Lipidology*, *6*(6), 573–584. https://doi.org/10.1016/j.jacl.2012.01.002

Dejea, C. M., Wick, E. C., Hechenbleikner, E. M., White, J. R., Mark Welch, J. L., Rossetti, B. J., Peterson, S. N., Snesrud, E. C., Borisy, G. G., Lazarev, M., Stein, E., Vadivelu, J., Roslani, A. C., Malik, A. A., Wanyiri, J. W., Goh, K. L., Thevambiga, I., Fu, K., Wan, F., … Sears, C. L. (2014). Microbiota organization is a distinct feature of proximal colorectal cancers. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(51), 18321–18326. https://doi.org/10.1073/pnas.1406199111

Dempsey, M., Rockwell, M. S., & Wentz, L. M. (2023). The influence of dietary and supplemental omega-3 fatty acids on the omega-3 index: A scoping review. *Frontiers in Nutrition*, *10*, 1072653. https://doi.org/10.3389/fnut.2023.1072653

Derbyshire, Birch, C. S., Bonwick, G. A., English, A., Metcalfe, P., & Li, W. (2024). Optimal omegas – barriers and novel methods to narrow omega-3 gaps. A narrative review. *Frontiers in Nutrition*, *11*, 1325099. https://doi.org/10.3389/fnut.2024.1325099

Derbyshire, E. (2019). Oily Fish and Omega-3s Across the Life Stages: A Focus on Intakes and Future Directions. *Frontiers in Nutrition*, *6*, 165. https://doi.org/10.3389/fnut.2019.00165

Djuric, Z., Bassis, C. M., Plegue, M. A., Sen, A., Turgeon, D. K., Herman, K., Young, V. B., Brenner, D. E., & Ruffin, M. T. (2019). Increases in Colonic Bacterial Diversity after ω-3 Fatty Acid Supplementation Predict Decreased Colonic Prostaglandin E2 Concentrations in Healthy Adults. *The Journal of Nutrition*, *149*(7), 1170–1179. https://doi.org/10.1093/jn/nxy255

Dong, R., Lin, H., Ding, Y., Chen, X., Shi, R., Yuan, S., Li, J., Zhu, B., Xu, X., Shen, W., Wang, K., Ding, D., & He, N. (2021). Effects of Docosahexanoic Acid on Gut Microbiota and Fecal Metabolites in HIV-Infected Patients With Neurocognitive Impairment: A 6-Month Randomized, Double-Blind, Placebo-Controlled Trial. *Frontiers in Nutrition*, *8*, 756720. https://doi.org/10.3389/fnut.2021.756720

Donohoe, D. R., Collins, L. B., Wali, A., Bigler, R., Sun, W., & Bultman, S. J. (2012). The Warburg Effect Dictates the Mechanism of Butyrate-Mediated Histone Acetylation and Cell Proliferation. *Molecular Cell*, *48*(4), 612–626. https://doi.org/10.1016/j.molcel.2012.08.033

Dyerberg, J., & Bang, H. O. (1979). Haemostatic function and platelet polyunsaturated fatty acids in Eskimos. *The Lancet*, *314*(8140), 433–435. https://doi.org/10.1016/S0140-6736(79)91490-9

Dziubańska-Kusibab, P. J., Berger, H., Battistini, F., Bouwman, B. A. M., Iftekhar, A., Katainen, R., Cajuso, T., Crosetto, N., Orozco, M., Aaltonen, L. A., & Meyer, T. F. (2020). Colibactin DNA-damage signature indicates mutational impact in colorectal cancer. *Nature Medicine*, *26*(7), 1063–1069. https://doi.org/10.1038/s41591-020-0908-2

Feeney, E. L., Daly, A., Dunne, S., Dible, V., Barron, R., Seratlic, S., Jacquier, J. C., O'Sullivan, M., Beresford, T., Jensen, S. K., & Gibney, E. R. (2023). Effect of reduced-calcium and high-calcium cheddar cheese consumption on the excretion

of faecal fat: A 2-week cross-over dietary intervention study. *European Journal of Nutrition*, *62*(4), 1755–1765. https://doi.org/10.1007/s00394-023-03118-8

Fenton, J. I., Gurzell, E. A., Davidson, E. A., & Harris, W. S. (2016). Red blood cell PUFAs reflect the phospholipid PUFA composition of major organs. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *112*, 12–23. https://doi.org/10.1016/j.plefa.2016.06.004

Fialkow, J. (2016). Omega-3 Fatty Acid Formulations in Cardiovascular Disease: Dietary Supplements are Not Substitutes for Prescription Products. *American Journal of Cardiovascular Drugs: Drugs, Devices, and Other Interventions*, *16*(4), 229–239. https://doi.org/10.1007/s40256-016-0170-7

Fini, L., Piazzi, G., Ceccarelli, C., Daoud, Y., Belluzzi, A., Munarini, A., Graziani, G., Fogliano, V., Selgrad, M., Garcia, M., Gasbarrini, A., Genta, R. M., Boland, C. R., & Ricciardiello, L. (2010). Highly purified eicosapentaenoic acid as free fatty acids strongly suppresses polyps in Apc(Min/+) mice. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *16*(23), 5703–5711. https://doi.org/10.1158/1078-0432.CCR-10-1990

Frydrych, A., Kulita, K., Jurowski, K., & Piekoszewski, W. (2025). Lipids in Clinical Nutrition and Health: Narrative Review and Dietary Recommendations. *Foods*, *14*(3), 473. https://doi.org/10.3390/foods14030473

Fu, X., Liu, Z., Zhu, C., Mou, H., & Kong, Q. (2019). Nondigestible carbohydrates, butyrate, and butyrate-producing bacteria. *Critical Reviews in Food Science and Nutrition*, *59*(sup1), S130–S152. https://doi.org/10.1080/10408398.2018.1542587

Gardner, F. H., & Cohen, P. (1966). Platelet Life Span. *Transfusion*, *6*(1), 23–31. https://doi.org/10.1111/j.1537-2995.1966.tb04688.x

Ghaddaf, A. A., Aziz, M., Alomari, M. S., Abdulhamid, A. S., Alharbi, F. A., Mullah, A. N., & Zaidi, S. F. (2021). Influence of aspirin on prevention of colorectal cancer: An updated systematic review and meta-analysis of randomized controlled trials. *International Journal of Colorectal Disease*, *36*(8), 1711–1722. https://doi.org/10.1007/s00384-021-03880-3

Gill, S. R., Pop, M., Deboy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., & Nelson, K. E. (2006). Metagenomic analysis of the human distal gut microbiome. *Science (New York, N.Y.)*, *312*(5778), 1355–1359. https://doi.org/10.1126/science.1124234

Giltay, E. J., Gooren, L. J., Toorians, A. W., Katan, M. B., & Zock, P. L. (2004). Docosahexaenoic acid concentrations are higher in women than in men because

of estrogenic effects. *The American Journal of Clinical Nutrition*, *80*(5), 1167–1174. https://doi.org/10.1093/ajcn/80.5.1167

Grady, W. M., & Markowitz, S. D. (2015). The Molecular Pathogenesis of Colorectal Cancer and Its Potential Application to Colorectal Cancer Screening. *Digestive Diseases and Sciences*, *60*(3), 762–772. https://doi.org/10.1007/s10620-014-3444-4

Hachem, M., Nacir, H., Picq, M., Belkouch, M., Bernoud-Hubac, N., Windust, A., Meiller, L., Sauvinet, V., Feugier, N., Lambert-Porcheron, S., Laville, M., & Lagarde, M. (2020). Docosahexaenoic Acid (DHA) Bioavailability in Humans after Oral Intake of DHA-Containing Triacylglycerol or the Structured Phospholipid AceDoPC®. *Nutrients*, *12*(1), 251. https://doi.org/10.3390/nu12010251

Han, Y.-M., Park, J.-M., Cha, J.-Y., Jeong, M., Go, E.-J., & Hahm, K. B. (2016). Endogenous conversion of ω-6 to ω-3 polyunsaturated fatty acids in *fat* -1 mice attenuated intestinal polyposis by either inhibiting COX-2/β-catenin signaling or activating 15-PGDH/IL-18: Inhibition of intestinal polyposis by ω-3 PUFAs in *fat* -1 mice. *International Journal of Cancer*, *138*(9), 2247–2256. https://doi.org/10.1002/ijc.29956

Harauma, A., Yasuda, H., Hatanaka, E., Nakamura, M. T., Salem, N., & Moriguchi, T. (2017). The essentiality of arachidonic acid in addition to docosahexaenoic acid for brain growth and function. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *116*, 9–18. https://doi.org/10.1016/j.plefa.2016.11.002

Harris, W. S., & Thomas, R. M. (2010). Biological variability of blood omega-3 biomarkers. *Clinical Biochemistry*, *43*(3), 338–340. https://doi.org/10.1016/j.clinbiochem.2009.08.016

Hawcroft, G., Volpato, M., Marston, G., Ingram, N., Perry, S., Cockbain, A., Race, A., Munarini, A., Belluzzi, A., Loadman, P., Coletta, P., & Hull, M. (2012). The omega-3 polyunsaturated fatty acid eicosapentaenoic acid inhibits mouse MC-26 colorectal cancer cell liver metastasis via inhibition of PGE2-dependent cell motility: EPA inhibits colorectal cancer liver metastasis. *British Journal of Pharmacology*, *166*(5), 1724–1737. https://doi.org/10.1111/j.1476-5381.2012.01882.x

Hayashi, H., Takahashi, R., Nishi, T., Sakamoto, M., & Benno, Y. (2005). Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *Journal of Medical Microbiology*, *54*(11), 1093–1101. https://doi.org/10.1099/jmm.0.45935-0

Hudert, C. A., Weylandt, K. H., Lu, Y., Wang, J., Hong, S., Dignass, A., Serhan, C. N., & Kang, J. X. (2006). Transgenic mice rich in endogenous omega-3 fatty acids are protected from colitis. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(30), 11276–11281. https://doi.org/10.1073/pnas.0601280103

Hull, M. A., Ow, P. L., Ruddock, S., Brend, T., Smith, A. F., Marshall, H., Song, M., Chan, A. T., Garrett, W. S., Yilmaz, O., Drew, D. A., Collinson, F., Cockbain, A. J., Jones, R., Loadman, P. M., Hall, P. S., Moriarty, C., Cairns, D. A., & Toogood, G. J. (2023). Randomised, placebo-controlled, phase 3 trial of the effect of the omega-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) on colorectal cancer recurrence and survival after surgery for resectable liver metastases: EPA for Metastasis Trial 2 (EMT2) study protocol. *BMJ Open*, *13*(11), e077427. https://doi.org/10.1136/bmjopen-2023-077427

Hull, M. A., Sprange, K., Hepburn, T., Tan, W., Shafayat, A., Rees, C. J., Clifford, G., Logan, R. F., Loadman, P. M., Williams, E. A., Whitham, D., & Montgomery, A. A. (2018). Eicosapentaenoic acid and aspirin, alone and in combination, for the prevention of colorectal adenomas (seAFOod Polyp Prevention trial): A multicentre, randomised, double-blind, placebo-controlled, 2 × 2 factorial trial. *The Lancet*, *392*(10164), 2583–2594. https://doi.org/10.1016/S0140-6736(18)31775-6

Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., Ruscheweyh, H.-J., & Tappu, R. (2016). MEGAN Community Edition—Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLOS Computational Biology*, *12*(6), e1004957. https://doi.org/10.1371/journal.pcbi.1004957

Isenring, J., Bircher, L., Geirnaert, A., & Lacroix, C. (2023). In vitro human gut microbiota fermentation models: Opportunities, challenges, and pitfalls. *Microbiome Research Reports*, *2*(1), 2. https://doi.org/10.20517/mrr.2022.15

Isokääntä, H., Pinto da Silva, L., Karu, N., Kallonen, T., Aatsinki, A.-K., Hankemeier, T., Schimmel, L., Diaz, E., Hyötyläinen, T., Dorrestein, P. C., Knight, R., Orešič, M., Kaddurah-Daouk, R., Dickens, A. M., & Lamichhane, S. (2024). Comparative Metabolomics and Microbiome Analysis of Ethanol versus OMNImet/gene•GUT Fecal Stabilization. *Analytical Chemistry*, *96*(22), 8893–8904. https://doi.org/10.1021/acs.analchem.3c04436

Jacobs, M. L., Faizi, H. A., Peruzzi, J. A., Vlahovska, P. M., & Kamat, N. P. (2021). EPA and DHA differentially modulate membrane elasticity in the presence of cholesterol. *Biophysical Journal*, *120*(11), 2317–2329. https://doi.org/10.1016/j.bpj.2021.04.009

Javaid, M., Kadhim, K., Bawamia, B., Cartlidge, T., Farag, M., & Alkhalil, M. (2024). Bleeding Risk in Patients Receiving Omega-3 Polyunsaturated Fatty Acids: A Systematic Review and Meta-Analysis of Randomized Clinical Trials. *Journal of the American Heart Association*, *13*(10), e032390. https://doi.org/10.1161/JAHA.123.032390

Kaliannan, K., Li, X.-Y., Wang, B., Pan, Q., Chen, C.-Y., Hao, L., Xie, S., & Kang, J. X. (2019). Multi-omic analysis in transgenic mice implicates omega-6/omega-3 fatty acid imbalance as a risk factor for chronic disease. *Communications Biology*, *2*, 276. https://doi.org/10.1038/s42003-019-0521-4

Kaliannan, K., Wang, B., Li, X.-Y., Kim, K.-J., & Kang, J. X. (2015). A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Scientific Reports*, *5*, 11276. https://doi.org/10.1038/srep11276

Karageorgou, D., Rova, U., Christakopoulos, P., Katapodis, P., Matsakas, L., & Patel, A. (2023). Benefits of supplementation with microbial omega-3 fatty acids on human health and the current market scenario for fish-free omega-3 fatty acid. *Trends in Food Science & Technology*, *136*, 169–180. https://doi.org/10.1016/j.tifs.2023.04.018

Kastl, A. J., Terry, N. A., Wu, G. D., & Albenberg, L. G. (2020). The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. *Cellular and Molecular Gastroenterology and Hepatology*, *9*(1), 33–45. https://doi.org/10.1016/j.jcmgh.2019.07.006

Khan, M., Rahman, M., Zaman, S., Jahangir, A., & Razu, M. (2015). *Omega-3 Polyunsaturated Fatty Acids from Algae.*

Kim, S. W., Rho, C. R., Kim, J., Xie, Y., Prince, R. C., Mustafa, K., Potma, E. O., Brown, D. J., & Jester, J. V. (2020). Eicosapentaenoic acid (EPA) activates PPARγ signaling leading to cell cycle exit, lipid accumulation, and autophagy in human meibomian gland epithelial cells (hMGEC). *The Ocular Surface*, *18*(3), 427–437. https://doi.org/10.1016/j.jtos.2020.04.012

Kiralan, M., Ketenoglu, O., & Kiralan, S. S. (2021). Trans fatty acids—Occurrence, technical aspects, and worldwide regulations. In *Studies in Natural Products Chemistry* (Vol. 70, pp. 313–343). Elsevier. https://doi.org/10.1016/B978-0-12-819489-8.00018-1

Koch, E., Wiebel, M., Hopmann, C., Kampschulte, N., & Schebb, N. H. (2021). Rapid quantification of fatty acids in plant oils and biological samples by LC-MS. *Analytical and Bioanalytical Chemistry*, *413*(21), 5439–5451. https://doi.org/10.1007/s00216-021-03525-y

Kraja, B., Muka, T., Ruiter, R., de Keyser, C. E., Hofman, A., Franco, O. H., Stricker, B. H., & Kiefte-de Jong, J. C. (2015). Dietary Fiber Intake Modifies the Positive Association between n-3 PUFA Intake and Colorectal Cancer Risk in a Caucasian Population. *The Journal of Nutrition*, *145*(8), 1709–1716. https://doi.org/10.3945/jn.114.208462

Kwao-Zigah, G., Bediako-Bowan, A., Boateng, P. A., Aryee, G. K., Abbang, S. M., Atampugbire, G., Quaye, O., & Tagoe, E. A. (2024). Microbiome Dysbiosis, Dietary Intake and Lifestyle-Associated Factors Involve in Epigenetic Modulations in Colorectal Cancer: A Narrative Review. *Cancer Control: Journal of the Moffitt Cancer Center*, *31*, 10732748241263650. https://doi.org/10.1177/10732748241263650

LaCourse, K. D., Johnston, C. D., & Bullman, S. (2021). The relationship between gastrointestinal cancers and the microbiota. *The Lancet. Gastroenterology & Hepatology*, *6*(6), 498–509. https://doi.org/10.1016/S2468-1253(20)30362-9

Lavelle, A., Nancey, S., Reimund, J.-M., Laharie, D., Marteau, P., Treton, X., Allez, M., Roblin, X., Malamut, G., Oeuvray, C., Rolhion, N., Dray, X., Rainteau, D., Lamaziere, A., Gauliard, E., Kirchgesner, J., Beaugerie, L., Seksik, P., Peyrin-Biroulet, L., & Sokol, H. (2022). Fecal microbiota and bile acids in IBD patients undergoing screening for colorectal cancer. *Gut Microbes*, *14*(1), 2078620. https://doi.org/10.1080/19490976.2022.2078620

Lee, J. Y., Sim, T.-B., Lee, J., & Na, H.-K. (2017). Chemopreventive and Chemotherapeutic Effects of Fish Oil derived Omega-3 Polyunsaturated Fatty Acids on Colon Carcinogenesis. *Clinical Nutrition Research*, *6*(3), 147. https://doi.org/10.7762/cnr.2017.6.3.147

Léveillé, P., Chouinard-Watkins, R., Windust, A., Lawrence, P., Cunnane, S. C., Brenna, J. T., & Plourde, M. (2017). Metabolism of uniformly labeled 13C-eicosapentaenoic acid and 13C-arachidonic acid in young and old men. *The American Journal of Clinical Nutrition*, *106*(2), 467–474. https://doi.org/10.3945/ajcn.117.154708

Linseisen, J., Grundmann, N., Zoller, D., Kühn, T., Jansen, E. H. J. M., Chajès, V., Fedirko, V., Weiderpass, E., Dahm, C. C., Overvad, K., Tjønneland, A., Boutron-Ruault, M.-C., Rothwell, J. A., Severi, G., Kaaks, R., Schulze, M. B., Aleksandrova, K., Sieri, S., Panico, S., … Nieters, A. (2021). Red Blood Cell Fatty Acids and Risk of Colorectal Cancer in The European Prospective Investigation into Cancer and Nutrition (EPIC). *Cancer Epidemiology, Biomarkers & Prevention*, *30*(5), 874–885. https://doi.org/10.1158/1055-9965.EPI-20-1426

Loukil, I., Ku, D., Vachon, A., Metherel, A., & Plourde, M. (2025). Omega-3 supplementation increases 13C-EPA but not 13C-DHA kinetics in healthy older adults after 13C-DHA single dose intake: A secondary analysis. *Nutrition (Burbank, Los Angeles County, Calif.)*, *136*, 112803. https://doi.org/10.1016/j.nut.2025.112803

Mallick, H., Ma, S., Franzosa, E. A., Vatanen, T., Morgan, X. C., & Huttenhower, C. (2017). Experimental design and quantitative analysis of microbial community multiomics. *Genome Biology*, *18*(1), 228. https://doi.org/10.1186/s13059-017-1359-z

Manson, J. E., Cook, N. R., Lee, I.-M., Christen, W., Bassuk, S. S., Mora, S., Gibson, H., Gordon, D., Copeland, T., D'Agostino, D., Friedenberg, G., Ridge, C., Bubes, V., Giovannucci, E. L., Willett, W. C., & Buring, J. E. (2019). Vitamin D Supplements and Prevention of Cancer and Cardiovascular Disease. *New England Journal of Medicine*, *380*(1), 33–44. https://doi.org/10.1056/NEJMoa1809944

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal*, *17*(1), 10. https://doi.org/10.14806/ej.17.1.200

Menni, C., Zierer, J., Pallister, T., Jackson, M. A., Long, T., Mohney, R. P., Steves, C. J., Spector, T. D., & Valdes, A. M. (2017). Omega-3 fatty acids correlate with gut microbiome diversity and production of N-carbamylglutamate in middle aged and elderly women. *Scientific Reports*, *7*(1), 11079. https://doi.org/10.1038/s41598-017-10382-2

Metherel, A. H., Irfan, M., Klingel, S. L., Mutch, D. M., & Bazinet, R. P. (2019). Compound-specific isotope analysis reveals no retroconversion of DHA to EPA but substantial conversion of EPA to DHA following supplementation: A randomized control trial. *The American Journal of Clinical Nutrition*, *110*(4), 823–831. https://doi.org/10.1093/ajcn/nqz097

Micha, R., Khatibzadeh, S., Shi, P., Fahimi, S., Lim, S., Andrews, K. G., Engell, R. E., Powles, J., Ezzati, M., Mozaffarian, D., & Global Burden of Diseases Nutrition and Chronic Diseases Expert Group NutriCoDE. (2014). Global, regional, and national consumption levels of dietary fats and oils in 1990 and 2010: A systematic analysis including 266 country-specific nutrition surveys. *BMJ (Clinical Research Ed.)*, *348*, g2272. https://doi.org/10.1136/bmj.g2272

Minoura, T., Takata, T., Sakaguchi, M., Takada, H., Yamamura, M., Hioki, K., & Yamamoto, M. (1988). Effect of dietary eicosapentaenoic acid on azoxymethane-induced colon carcinogenesis in rats. *Cancer Research*, *48*(17), 4790–4794.

Miyasaka, T., Yamada, T., Uehara, K., Sonoda, H., Matsuda, A., Shinji, S., Ohta, R., Kuriyama, S., Yokoyama, Y., Takahashi, G., Iwai, T., Takeda, K., Ueda, K., Kanaka, S., Ohashi, R., & Yoshida, H. (2024). Pks-positive Escherichia coli in tumor tissue and surrounding normal mucosal tissue of colorectal cancer patients. *Cancer Science*, *115*(4), 1184–1195. https://doi.org/10.1111/cas.16088

Moreira, A. P. B., Sabarense, C. M., Dias, C. M. G. C., Lunz, W., Natali, A. J., Glória, M. B. A., & Peluzio, M. C. G. (2009). Fish oil ingestion reduces the number of aberrant crypt foci and adenoma in 1,2-dimethylhydrazine-induced colon cancer in rats. *Brazilian Journal of Medical and Biological Research*, *42*(12), 1167–1172. https://doi.org/10.1590/S0100-879X2009001200008

Nabavi, S. F., Bilotto, S., Russo, G. L., Orhan, I. E., Habtemariam, S., Daglia, M., Devi, K. P., Loizzo, M. R., Tundis, R., & Nabavi, S. M. (2015). Omega-3 polyunsaturated fatty acids and cancer: Lessons learned from clinical trials. *Cancer and Metastasis Reviews*, *34*(3), 359–380. https://doi.org/10.1007/s10555-015-9572-2

Navarro, S. L., Neuhouser, M. L., Cheng, T.-Y. D., Tinker, L. F., Shikany, J. M., Snetselaar, L., Martinez, J. A., Kato, I., Beresford, S. A. A., Chapkin, R. S., & Lampe, J. W. (2016). The Interaction between Dietary Fiber and Fat and Risk of Colorectal Cancer in the Women's Health Initiative. *Nutrients*, *8*(12), 779. https://doi.org/10.3390/nu8120779

Neubronner, J., Schuchardt, J. P., Kressel, G., Merkel, M., von Schacky, C., & Hahn, A. (2011). Enhanced increase of omega-3 index in response to long-term n-3 fatty acid supplementation from triacylglycerides versus ethyl esters. *European Journal of Clinical Nutrition*, *65*(2), 247–254. https://doi.org/10.1038/ejcn.2010.239

Ng, C.-A. W., Jiang, A. A., Toh, E. M. S., Ng, C. H., Ong, Z. H., Peng, S., Tham, H. Y., Sundar, R., Chong, C. S., & Khoo, C. M. (2020). Metformin and colorectal cancer: A systematic review, meta-analysis and meta-regression. *International Journal of Colorectal Disease*, *35*(8), 1501–1512. https://doi.org/10.1007/s00384-020-03676-x

NHS, U. (2022, October 14). *Fish and shellfish. London: NHS (2022)*. https://www.nhs.uk/live-well/eat-well/food-types/fish-and-shellfish-nutrition/

Offman, E., Davidson, M., Abu-Rashid, M., Chai, P., & Nilsson, C. (2017). Systemic Bioavailability and Dose Proportionality of Omega-3 Administered in Free Fatty Acid Form Compared With Ethyl Ester Form: Results of a Phase 1 Study in Healthy Volunteers. *European Journal of Drug Metabolism and Pharmacokinetics*, *42*(5), 815–825. https://doi.org/10.1007/s13318-016-0398-2

O'Keefe, S. J. D., Li, J. V., Lahti, L., Ou, J., Carbonero, F., Mohammed, K., Posma, J. M., Kinross, J., Wahl, E., Ruder, E., Vipperla, K., Naidoo, V., Mtshali, L., Tims, S., Puylaert, P. G. B., DeLany, J., Krasinskas, A., Benefiel, A. C., Kaseb, H. O., … Zoetendal, E. G. (2015). Fat, fibre and cancer risk in African Americans and rural Africans. *Nature Communications*, *6*(1), 6342. https://doi.org/10.1038/ncomms7342

Pacetti, D., Mozzon, M., Lucci, P., & Frega, N. (2013). Bioactive Fish Fatty Acids: Health Effects and Their Use as Functional Food Ingredients. *Current Nutrition & Food Science*, *9*(4), 283–297. https://doi.org/10.2174/15734013113096660015

Pan, P., Huang, Y.-W., Oshima, K., Yearsley, M., Zhang, J., Yu, J., Arnold, M., & Wang, L.-S. (2018). Could Aspirin and Diets High in Fiber Act Synergistically to Reduce the Risk of Colon Cancer in Humans? *International Journal of Molecular Sciences*, *19*(1), 166. https://doi.org/10.3390/ijms19010166

Panda, C., Varadharaj, S., & Voruganti, V. S. (2022). PUFA, genotypes and risk for cardiovascular disease. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *176*, 102377. https://doi.org/10.1016/j.plefa.2021.102377

Parada, A. E., Needham, D. M., & Fuhrman, J. A. (2016). Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples: Primers for marine microbiome studies. *Environmental Microbiology*, *18*(5), 1403–1414. https://doi.org/10.1111/1462-2920.13023

Petersen, C., & Round, J. L. (2014). Defining dysbiosis and its influence on host immunity and disease. *Cellular Microbiology*, *16*(7), 1024–1033. https://doi.org/10.1111/cmi.12308

Piazzi, G., D'Argenio, G., Prossomariti, A., Lembo, V., Mazzone, G., Candela, M., Biagi, E., Brigidi, P., Vitaglione, P., Fogliano, V., D'Angelo, L., Fazio, C., Munarini, A., Belluzzi, A., Ceccarelli, C., Chieco, P., Balbi, T., Loadman, P. M., Hull, M. A., … Ricciardiello, L. (2014). Eicosapentaenoic acid free fatty acid prevents and suppresses colonic neoplasia in colitis-associated colorectal cancer acting on Notch signaling and gut microbiota. *International Journal of Cancer*, *135*(9), 2004–2013. https://doi.org/10.1002/ijc.28853

Plourde, M., Chouinard-Watkins, R., Rioux-Perreault, C., Fortier, M., Dang, M. T. M., Allard, M.-J., Tremblay-Mercier, J., Zhang, Y., Lawrence, P., Vohl, M.-C., Perron, P., Lorrain, D., Brenna, J. T., & Cunnane, S. C. (2014). Kinetics of 13C-DHA before and during fish-oil supplementation in healthy older individuals. *The American Journal of Clinical Nutrition*, *100*(1), 105–112. https://doi.org/10.3945/ajcn.113.074708

Punia, S., Sandhu, K. S., Siroha, A. K., & Dhull, S. B. (2019). Omega 3-metabolism, absorption, bioavailability and health benefits–A review. *PharmaNutrition*, *10*, 100162. https://doi.org/10.1016/j.phanu.2019.100162

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. *Nucleic Acids Research*, *41*(D1), D590–D596. https://doi.org/10.1093/nar/gks1219

R Core Team (2020). (n.d.). *R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.* http://www.r-project.org/index.html

Ragusa, A., Svelato, A., Santacroce, C., Catalano, P., Notarstefano, V., Carnevali, O., Papa, F., Rongioletti, M. C. A., Baiocco, F., Draghi, S., D'Amore, E., Rinaldo, D., Matta, M., & Giorgini, E. (2021). Plasticenta: First evidence of microplastics in human placenta. *Environment International*, *146*, 106274. https://doi.org/10.1016/j.envint.2020.106274

Ramírez, F., Shannon, L. J., Angelini, R., Steenbeek, J., & Coll, M. (2022). Overfishing species on the move may burden seafood provision in the low-latitude Atlantic Ocean. *Science of The Total Environment*, *836*, 155480. https://doi.org/10.1016/j.scitotenv.2022.155480

Rehman, A., Pham, V., Seifert, N., Richard, N., Sybesma, W., & Steinert, R. E. (2024). The Polyunsaturated Fatty Acids Eicosapentaenoic Acid and Docosahexaenoic Acid, and Vitamin K_1 Modulate the Gut Microbiome: A Study Using an In Vitro Shime Model. *Journal of Dietary Supplements*, *21*(2), 135–153. https://doi.org/10.1080/19390211.2023.2198007

Roussel, C., Anunciação Braga Guebara, S., Plante, P.-L., Desjardins, Y., Di Marzo, V., & Silvestri, C. (2022). Short-term supplementation with ω-3 polyunsaturated fatty acids modulates primarily mucolytic species from the gut luminal mucin niche in a human fermentation system. *Gut Microbes*, *14*(1), 2120344. https://doi.org/10.1080/19490976.2022.2120344

Ruigrok, R. A. A. A., Weersma, R. K., & Vich Vila, A. (2023). The emerging role of the small intestinal microbiota in human health and disease. *Gut Microbes*, *15*(1), 2201155. https://doi.org/10.1080/19490976.2023.2201155

Ruiz-Lopez, N., Usher, S., Sayanova, O. V., Napier, J. A., & Haslam, R. P. (2015). Modifying the lipid content and composition of plant seeds: Engineering the production of LC-PUFA. *Applied Microbiology and Biotechnology*, *99*(1), 143–154. https://doi.org/10.1007/s00253-014-6217-2

Russo, G. L. (2009). Dietary n−6 and n−3 polyunsaturated fatty acids: From biochemistry to clinical implications in cardiovascular prevention. *Biochemical Pharmacology*, *77*(6), 937–946. https://doi.org/10.1016/j.bcp.2008.10.020

Rzehak, P., Heinrich, J., Klopp, N., Schaeffer, L., Hoff, S., Wolfram, G., Illig, T., & Linseisen, J. (2009). Evidence for an association between genetic variants of the fatty acid desaturase 1 fatty acid desaturase 2 (FADS1 FADS2) gene cluster and the fatty acid composition of erythrocyte membranes. *The British Journal of Nutrition*, *101*(1), 20–26. https://doi.org/10.1017/S0007114508992564

Saini, R. K., & Keum, Y.-S. (2018). Omega-3 and omega-6 polyunsaturated fatty acids: Dietary sources, metabolism, and significance - A review. *Life Sciences*, *203*, 255–267. https://doi.org/10.1016/j.lfs.2018.04.049

Saito-Sasaki, N., Sawada, Y., & Nakamura, M. (2022). Maresin-1 and Inflammatory Disease. *International Journal of Molecular Sciences*, *23*(3), 1367. https://doi.org/10.3390/ijms23031367

Salsinha, A. S., Araújo-Rodrigues, H., Dias, C., Cima, A., Rodríguez-Alcalá, L. M., Relvas, J. B., & Pintado, M. (2025). Omega-3 and conjugated fatty acids impact on human microbiota modulation using an in vitro fecal fermentation model. *Clinical Nutrition (Edinburgh, Scotland)*, *49*, 102–117. https://doi.org/10.1016/j.clnu.2025.04.007

Sanguansri, L., Shen, Z., Weerakkody, R., Barnes, M., Lockett, T., & Augustin, M. A. (2013). Omega-3 fatty acids in ileal effluent after consuming different foods containing microencapsulated fish oil powder—An ileostomy study. *Food & Function*, *4*(1), 74–82. https://doi.org/10.1039/c2fo30133d

Sapieha, P., Stahl, A., Chen, J., Seaward, M. R., Willett, K. L., Krah, N. M., Dennison, R. J., Connor, K. M., Aderman, C. M., Liclican, E., Carughi, A., Perelman, D., Kanaoka, Y., SanGiovanni, J. P., Gronert, K., & Smith, L. E. H. (2011). 5-Lipoxygenase Metabolite 4-HDHA Is a Mediator of the Antiangiogenic Effect of ω-3 Polyunsaturated Fatty Acids. *Science Translational Medicine*, *3*(69). https://doi.org/10.1126/scitranslmed.3001571

Schaeffer, L., Gohlke, H., Müller, M., Heid, I. M., Palmer, L. J., Kompauer, I., Demmelmair, H., Illig, T., Koletzko, B., & Heinrich, J. (2006). Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. *Human Molecular Genetics*, *15*(11), 1745–1756. https://doi.org/10.1093/hmg/ddl117

Schuchardt, J. P., Beinhorn, P., Hu, X. F., Chan, H. M., Roke, K., Bernasconi, A., Hahn, A., Sala-Vila, A., Stark, K. D., & Harris, W. S. (2024). Omega-3 world map:

2024 update. *Progress in Lipid Research*, *95*, 101286. https://doi.org/10.1016/j.plipres.2024.101286

Schuchardt, J. P., Cerrato, M., Ceseri, M., DeFina, L. F., Delgado, G. E., Gellert, S., Hahn, A., Howard, B. V., Kadota, A., Kleber, M. E., Latini, R., Maerz, W., Manson, J. E., Mora, S., Park, Y., Sala-Vila, A., von Schacky, C., Sekikawa, A., Tintle, N., … Harris, W. S. (2022). Red blood cell fatty acid patterns from 7 countries: Focus on the Omega-3 index. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *179*, 102418. https://doi.org/10.1016/j.plefa.2022.102418

Schuchardt, J. P., & Hahn, A. (2013). Bioavailability of long-chain omega-3 fatty acids. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *89*(1), 1–8. https://doi.org/10.1016/j.plefa.2013.03.010

Schuchardt, J. P., Kräter, M., Schlögel, M., Guck, J., van Oirschot-Hermans, B. A., Bos, J., van Wijk, R., Tintle, N. L., Westra, J., Kerlikowsky, F., Hahn, A., & Harris, W. S. (2024). Omega-3 supplementation changes the physical properties of leukocytes but not erythrocytes in healthy individuals: An exploratory trial. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *202*, 102636. https://doi.org/10.1016/j.plefa.2024.102636

Scott, N., Whittle, E., Jeraldo, P., & Chia, N. (2022). A systemic review of the role of enterotoxic Bacteroides fragilis in colorectal cancer. *Neoplasia (New York, N.Y.)*, *29*, 100797. https://doi.org/10.1016/j.neo.2022.100797

Sender, R., Fuchs, S., & Milo, R. (2016). Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. *Cell*, *164*(3), 337–340. https://doi.org/10.1016/j.cell.2016.01.013

Serhan, C. N. (2014). Pro-resolving lipid mediators are leads for resolution physiology. *Nature*, *510*(7503), 92–101. https://doi.org/10.1038/nature13479

Serhan, C. N., & Levy, B. D. (2018). Resolvins in inflammation: Emergence of the pro-resolving superfamily of mediators. *Journal of Clinical Investigation*, *128*(7), 2657–2669. https://doi.org/10.1172/JCI97943

Serna, G., Ruiz-Pace, F., Hernando, J., Alonso, L., Fasani, R., Landolfi, S., Comas, R., Jimenez, J., Elez, E., Bullman, S., Tabernero, J., Capdevila, J., Dienstmann, R., & Nuciforo, P. (2020). Fusobacterium nucleatum persistence and risk of recurrence after preoperative treatment in locally advanced rectal cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*, *31*(10), 1366–1375. https://doi.org/10.1016/j.annonc.2020.06.003

Shalon, D., Culver, R. N., Grembi, J. A., Folz, J., Treit, P. V., Shi, H., Rosenberger, F. A., Dethlefsen, L., Meng, X., Yaffe, E., Aranda-Díaz, A., Geyer, P. E., Mueller-Reif, J. B., Spencer, S., Patterson, A. D., Triadafilopoulos, G.,

Holmes, S. P., Mann, M., Fiehn, O., … Huang, K. C. (2023). Profiling the human intestinal environment under physiological conditions. *Nature*, *617*(7961), 581–591. https://doi.org/10.1038/s41586-023-05989-7

Sherratt, S. C. R., & Mason, R. P. (2018). Eicosapentaenoic acid and docosahexaenoic acid have distinct membrane locations and lipid interactions as determined by X-ray diffraction. *Chemistry and Physics of Lipids*, *212*, 73–79. https://doi.org/10.1016/j.chemphyslip.2018.01.002

Shi, Y., & Burn, P. (2004). Lipid metabolic enzymes: Emerging drug targets for the treatment of obesity. *Nature Reviews Drug Discovery*, *3*(8), 695–710. https://doi.org/10.1038/nrd1469

Shrestha, R. P., Horowitz, J., Hollot, C. V., Germain, M. J., Widness, J. A., Mock, D. M., Veng-Pedersen, P., & Chait, Y. (2016). Models for the red blood cell lifespan. *Journal of Pharmacokinetics and Pharmacodynamics*, *43*(3), 259–274. https://doi.org/10.1007/s10928-016-9470-4

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis: An International Journal on Programmed Cell Death*, *5*(5), 415–418. https://doi.org/10.1023/a:1009616228304

Sinclair, H. M. (1956). Deficiency of essential fatty acids and atherosclerosis, etcetera. *Lancet (London, England)*, *270*(6919), 381–383.

Skulas-Ray, A. C., Wilson, P. W. F., Harris, W. S., Brinton, E. A., Kris-Etherton, P. M., Richter, C. K., Jacobson, T. A., Engler, M. B., Miller, M., Robinson, J. G., Blum, C. B., Rodriguez-Leyva, D., de Ferranti, S. D., Welty, F. K., & American Heart Association Council on Arteriosclerosis, Thrombosis and Vascular Biology; Council on Lifestyle and Cardiometabolic Health; Council on Cardiovascular Disease in the Young; Council on Cardiovascular and Stroke Nursing; and Council on Clinical Cardiology. (2019). Omega-3 Fatty Acids for the Management of Hypertriglyceridemia: A Science Advisory From the American Heart Association. *Circulation*, *140*(12), e673–e691. https://doi.org/10.1161/CIR.0000000000000709

Smith, P. M., Howitt, M. R., Panikov, N., Michaud, M., Gallini, C. A., Bohlooly-Y, M., Glickman, J. N., & Garrett, W. S. (2013). The Microbial Metabolites, Short-Chain Fatty Acids, Regulate Colonic T_{reg} Cell Homeostasis. *Science*, *341*(6145), 569–573. https://doi.org/10.1126/science.1241165

Song, C.-H., Kim, N., Nam, R. H., Choi, S. I., Jang, J. Y., Kim, E. H., Choi, J., Choi, Y., Yoon, H., Lee, S. M., & Seok, Y.-J. (2024). The Possible Preventative

Role of Lactate- and Butyrate-Producing Bacteria in Colorectal Carcinogenesis. *Gut and Liver*, *18*(4), 654–666. https://doi.org/10.5009/gnl230385

Song, M., & Chan, A. T. (2017). Diet, Gut Microbiota, and Colorectal Cancer Prevention: A Review of Potential Mechanisms and Promising Targets for Future Research. *Current Colorectal Cancer Reports*, *13*(6), 429–439. https://doi.org/10.1007/s11888-017-0389-y

Song, M., Chan, A. T., Fuchs, C. S., Ogino, S., Hu, F. B., Mozaffarian, D., Ma, J., Willett, W. C., Giovannucci, E. L., & Wu, K. (2014). Dietary intake of fish, ω-3 and ω-6 fatty acids and risk of colorectal cancer: A prospective study in U.S. men and women: Fish, ω-3 and ω-6 fatty acids and risk of CRC. *International Journal of Cancer*, *135*(10), 2413–2423. https://doi.org/10.1002/ijc.28878

Song, M., Chan, A. T., & Sun, J. (2020). Influence of the Gut Microbiome, Diet, and Environment on Risk of Colorectal Cancer. *Gastroenterology*, *158*(2), 322–340. https://doi.org/10.1053/j.gastro.2019.06.048

Song, M., Lee, I.-M., Manson, J. E., Buring, J. E., Dushkes, R., Gordon, D., Walter, J., Wu, K., Chan, A. T., Ogino, S., Fuchs, C. S., Meyerhardt, J. A., Giovannucci, E. L., & VITAL Research Group. (2020). Effect of Supplementation With Marine ω-3 Fatty Acid on Risk of Colorectal Adenomas and Serrated Polyps in the US General Population: A Prespecified Ancillary Study of a Randomized Clinical Trial. *JAMA Oncology*, *6*(1), 108–115. https://doi.org/10.1001/jamaoncol.2019.4587

Sorensen, L. S., Rasmussen, H. H., Aardestrup, I. V., Thorlacius-Ussing, O., Lindorff-Larsen, K., Schmidt, E. B., & Calder, P. C. (2014). Rapid incorporation of ω-3 fatty acids into colonic tissue after oral supplementation in patients with colorectal cancer: A randomized, placebo-controlled intervention trial. *JPEN. Journal of Parenteral and Enteral Nutrition*, *38*(5), 617–624. https://doi.org/10.1177/0148607113491782

Sprecher, H. (2000). Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica Et Biophysica Acta*, *1486*(2–3), 219–231. https://doi.org/10.1016/s1388-1981(00)00077-9

Stahl, A. (2001). Fatty acid transport proteins: A current view of a growing family. *Trends in Endocrinology and Metabolism*, *12*(6), 266–273. https://doi.org/10.1016/S1043-2760(01)00427-1

Stark, K. D., Van Elswyk, M. E., Higgins, M. R., Weatherford, C. A., & Salem, N. (2016). Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Progress in Lipid Research*, *63*, 132–152. https://doi.org/10.1016/j.plipres.2016.05.001

Sung, H., Siegel, R. L., Laversanne, M., Jiang, C., Morgan, E., Zahwe, M., Cao, Y., Bray, F., & Jemal, A. (2025). Colorectal cancer incidence trends in younger versus older adults: An analysis of population-based cancer registry data. *The Lancet Oncology*, *26*(1), 51–63. https://doi.org/10.1016/S1470-2045(24)00600-4

Surh, Y.-J. (2003). Cancer chemoprevention with dietary phytochemicals. *Nature Reviews. Cancer*, *3*(10), 768–780. https://doi.org/10.1038/nrc1189

Tang, Q., Jin, G., Wang, G., Liu, T., Liu, X., Wang, B., & Cao, H. (2020). Current Sampling Methods for Gut Microbiota: A Call for More Precise Devices. *Frontiers in Cellular and Infection Microbiology*, *10*, 151. https://doi.org/10.3389/fcimb.2020.00151

Taylor, M., Wood, H. M., Halloran, S. P., & Quirke, P. (2017). Examining the potential use and long-term stability of guaiac faecal occult blood test cards for microbial DNA 16S rRNA sequencing. *Journal of Clinical Pathology*, *70*(7), 600–606. https://doi.org/10.1136/jclinpath-2016-204165

Terrón-Camero, L. C., Gordillo-González, F., Salas-Espejo, E., & Andrés-León, E. (2022). Comparison of Metagenomics and Metatranscriptomics Tools: A Guide to Making the Right Choice. *Genes*, *13*(12), 2280. https://doi.org/10.3390/genes13122280

The ASCEND Study Collaborative Group. (2018). Effects of n−3 Fatty Acid Supplements in Diabetes Mellitus. *New England Journal of Medicine*, *379*(16), 1540–1550. https://doi.org/10.1056/NEJMoa1804989

The Earth Microbiome Project Consortium, Thompson, L. R., Sanders, J. G., McDonald, D., Amir, A., Ladau, J., Locey, K. J., Prill, R. J., Tripathi, A., Gibbons, S. M., Ackermann, G., Navas-Molina, J. A., Janssen, S., Kopylova, E., Vázquez-Baeza, Y., González, A., Morton, J. T., Mirarab, S., Zech Xu, Z., … Knight, R. (2017). A communal catalogue reveals Earth's multiscale microbial diversity. *Nature*, *551*(7681), 457–463. https://doi.org/10.1038/nature24621

Tu, M., Wang, W., Zhang, G., & Hammock, B. D. (2020). ω-3 Polyunsaturated Fatty Acids on Colonic Inflammation and Colon Cancer: Roles of Lipid-Metabolizing Enzymes Involved. *Nutrients*, *12*(11), 3301. https://doi.org/10.3390/nu12113301

Tutino, V., De Nunzio, V., Caruso, M. G., Veronese, N., Lorusso, D., Di Masi, M., Benedetto, M. L., & Notarnicola, M. (2019). Elevated AA/EPA Ratio Represents an Inflammatory Biomarker in Tumor Tissue of Metastatic Colorectal Cancer Patients. *International Journal of Molecular Sciences*, *20*(8), E2050. https://doi.org/10.3390/ijms20082050

van der Beek, C. M., Dejong, C. H. C., Troost, F. J., Masclee, A. A. M., & Lenaerts, K. (2017). Role of short-chain fatty acids in colonic inflammation, carcinogenesis, and mucosal protection and healing. *Nutrition Reviews*, *75*(4), 286–305. https://doi.org/10.1093/nutrit/nuw067

Vijay, A., Astbury, S., Le Roy, C., Spector, T. D., & Valdes, A. M. (2021). The prebiotic effects of omega-3 fatty acid supplementation: A six-week randomised intervention trial. *Gut Microbes*, *13*(1), 1863133. https://doi.org/10.1080/19490976.2020.1863133

Villmones, H. C., Svanevik, M., Ulvestad, E., Stenstad, T., Anthonisen, I. L., Nygaard, R. M., Dyrhovden, R., & Kommedal, Ø. (2022). Investigating the human jejunal microbiota. *Scientific Reports*, *12*(1), 1682. https://doi.org/10.1038/s41598-022-05723-9

Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., & Bos, J. L. (1988). Genetic alterations during colorectal-tumor development. *The New England Journal of Medicine*, *319*(9), 525–532. https://doi.org/10.1056/NEJM198809013190901

Volpato, M., Spencer, J. A., Race, A. D., Munarini, A., Belluzzi, A., Cockbain, A. J., Hull, M. A., & Loadman, P. M. (2017). A liquid chromatography-tandem mass spectrometry method to measure fatty acids in biological samples. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *1055–1056*, 125–134. https://doi.org/10.1016/j.jchromb.2017.04.030

Wang, G., Menon, S., Wilsack, L., Rehak, R., Lou, L., Turbide, C., Auger, J., Tremblay, A., Mathieu, O., Binda, S., Tompkins, T. A., Bruehlmann, S., & Andrews, C. N. (2024). Spatially and temporally precise microbiome profiling in the small intestine using the SIMBA capsule with X-ray tracking. *Frontiers in Microbiomes*, *3*, 1321624. https://doi.org/10.3389/frmbi.2024.1321624

Wang, L., Hang, D., He, X., Lo, C., Wu, K., Chan, A. T., Ogino, S., Giovannucci, E. L., & Song, M. (2021). A prospective study of erythrocyte polyunsaturated fatty acids and risk of colorectal serrated polyps and conventional adenomas. *International Journal of Cancer*, *148*(1), 57–66. https://doi.org/10.1002/ijc.33190

Wang, W., Yang, J., Nimiya, Y., Lee, K. S. S., Sanidad, K., Qi, W., Sukamtoh, E., Park, Y., Liu, Z., & Zhang, G. (2017). ω-3 Polyunsaturated fatty acids and their cytochrome P450-derived metabolites suppress colorectal tumor development in mice. *The Journal of Nutritional Biochemistry*, *48*, 29–35. https://doi.org/10.1016/j.jnutbio.2017.06.006

Wang, X., Heazlewood, S. P., Krause, D. O., & Florin, T. H. J. (2003). Molecular characterization of the microbial species that colonize human ileal and colonic

mucosa by using 16S rDNA sequence analysis. *Journal of Applied Microbiology*, *95*(3), 508–520. https://doi.org/10.1046/j.1365-2672.2003.02005.x

Watson, H., Cockbain, A. J., Spencer, J., Race, A., Volpato, M., Loadman, P. M., Toogood, G. J., & Hull, M. A. (2016). Measurement of red blood cell eicosapentaenoic acid (EPA) levels in a randomised trial of EPA in patients with colorectal cancer liver metastases. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *115*, 60–66. https://doi.org/10.1016/j.plefa.2016.10.003

Watson, H., Mitra, S., Croden, F. C., Taylor, M., Wood, H. M., Perry, S. L., Spencer, J. A., Quirke, P., Toogood, G. J., Lawton, C. L., Dye, L., Loadman, P. M., & Hull, M. A. (2017). A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. *Gut*, gutjnl-2017-314968. https://doi.org/10.1136/gutjnl-2017-314968

Watson, H., Mitra, S., Croden, F. C., Taylor, M., Wood, H. M., Perry, S. L., Spencer, J. A., Quirke, P., Toogood, G. J., Lawton, C. L., Dye, L., Loadman, P. M., & Hull, M. A. (2018). A randomised trial of the effect of omega-3 polyunsaturated fatty acid supplements on the human intestinal microbiota. *Gut*, *67*(11), 1974–1983. https://doi.org/10.1136/gutjnl-2017-314968

West, N. J., Clark, S. K., Phillips, R. K. S., Hutchinson, J. M., Leicester, R. J., Belluzzi, A., & Hull, M. A. (2010). Eicosapentaenoic acid reduces rectal polyp number and size in familial adenomatous polyposis. *Gut*, *59*(7), 918–925. https://doi.org/10.1136/gut.2009.200642

Wirbel, J., Pyl, P. T., Kartal, E., Zych, K., Kashani, A., Milanese, A., Fleck, J. S., Voigt, A. Y., Palleja, A., Ponnudurai, R., Sunagawa, S., Coelho, L. P., Schrotz-King, P., Vogtmann, E., Habermann, N., Niméus, E., Thomas, A. M., Manghi, P., Gandini, S., … Zeller, G. (2019). Meta-analysis of fecal metagenomes reveals global microbial signatures that are specific for colorectal cancer. *Nature Medicine*, *25*(4), 679–689. https://doi.org/10.1038/s41591-019-0406-6

Wong, C. C., & Yu, J. (2023). Gut microbiota in colorectal cancer development and therapy. *Nature Reviews. Clinical Oncology*, *20*(7), 429–452. https://doi.org/10.1038/s41571-023-00766-x

Yang, N., Sampathkumar, K., & Loo, S. C. J. (2017). Recent advances in complementary and replacement therapy with nutraceuticals in combating gastrointestinal illnesses. *Clinical Nutrition*, *36*(4), 968–979. https://doi.org/10.1016/j.clnu.2016.08.020

Yilmaz, B., Fuhrer, T., Morgenthaler, D., Krupka, N., Wang, D., Spari, D., Candinas, D., Misselwitz, B., Beldi, G., Sauer, U., & Macpherson, A. J. (2022).

Plasticity of the adult human small intestinal stoma microbiota. *Cell Host & Microbe*, *30*(12), 1773-1787.e6. https://doi.org/10.1016/j.chom.2022.10.002

Yin, X.-F., Ye, T., Chen, H.-L., Liu, J., Mu, X.-F., Li, H., Wang, J., Hu, Y.-J., Cao, H., & Kang, W.-Q. (2024). The microbiome compositional and functional differences between rectal mucosa and feces. *Microbiology Spectrum*, *12*(8), e0354923. https://doi.org/10.1128/spectrum.03549-23

Young, C., Wood, H. M., Fuentes Balaguer, A., Bottomley, D., Gallop, N., Wilkinson, L., Benton, S. C., Brealey, M., John, C., Burtonwood, C., Thompson, K. N., Yan, Y., Barrett, J. H., Morris, E. J. A., Huttenhower, C., & Quirke, P. (2021). Microbiome Analysis of More Than 2,000 NHS Bowel Cancer Screening Programme Samples Shows the Potential to Improve Screening Accuracy. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, *27*(8), 2246–2254. https://doi.org/10.1158/1078-0432.CCR-20-3807

Zarate, R., Jaber-Vazdekis, N., Tejera, N., Pérez, J. A., & Rodríguez, C. (2017). Significance of long chain polyunsaturated fatty acids in human health. *Clinical and Translational Medicine*, *6*(1). https://doi.org/10.1186/s40169-017-0153-6

Zepeda-Rivera, M., Minot, S. S., Bouzek, H., Wu, H., Blanco-Míguez, A., Manghi, P., Jones, D. S., LaCourse, K. D., Wu, Y., McMahon, E. F., Park, S.-N., Lim, Y. K., Kempchinsky, A. G., Willis, A. D., Cotton, S. L., Yost, S. C., Sicinska, E., Kook, J.-K., Dewhirst, F. E., … Johnston, C. D. (2024). A distinct Fusobacterium nucleatum clade dominates the colorectal cancer niche. *Nature*, *628*(8007), 424–432. https://doi.org/10.1038/s41586-024-07182-w

Zhang, H., Tian, Y., Xu, C., Chen, M., Xiang, Z., Gu, L., Xue, H., & Xu, Q. (2025). Crosstalk between gut microbiotas and fatty acid metabolism in colorectal cancer. *Cell Death Discovery*, *11*(1), 78. https://doi.org/10.1038/s41420-025-02364-5

Zuijdgeest-van Leeuwen, S. D., Dagnelie, P. C., Rietveld, T., Van Den Berg, J. W. O., & Paul Wilson, J. H. (1999). Incorporation and washout of orally administered *n* -3 fatty acid ethyl esters in different plasma lipid fractions. *British Journal of Nutrition*, *82*(6), 481–488. https://doi.org/10.1017/S0007114599001737