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(STOP-ADENOMA)

Understanding mechanisms of colorectal cancer chemoprevention using seAFOod Polyp Prevention Trial outcomes and its Biobank

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Abbreviations

AA	arachidonic acid
ADR	adenoma detection rate
ALA	alpha-linolenic acid
APC	*Adenomatous Polyposis Coli*
ATLs	aspirin-triggered lipoxins
BCSP	Bowel Cancer Screening Programme
BCSS	Bowel Cancer Screening System
BMI	body mass index
CIMP	CpG island methylator phenotype
COX	cyclooxygenase
CRC	colorectal cancer
CTPL	Clinical Trials Pharmacology Laboratory
CTU	Clinical Trials Unit
DHA	docosahexaenoic acid
DOB	date of birth
DPA	docosapentaenoic acid
DSPT	Data Security and Protection Toolkit
EDTA	ethylenediaminetetra-acetic acid
EPA	eicosapentaenoic acid
FAP	familial adenomatous polyposis
FFA	free fatty acid
FFAR	free fatty acid receptor
FFPE	formalin-fixed paraffin-embedded
FFQ	food frequency questionnaire
GCLP	Good Clinical Laboratory Practice
gDNA	genomic DNA
GI	gastrointestinal
HEPE	hydroxyeicosapentaenoic acid
HES	Hospital Episode Statistics
HETE	hydroxyeicosatetraenoic acid
HTA	Human Tissue Act
IHC	immunohistochemistry
IMP	Investigational Medicinal Product
IP	intellectual property
IRC	Integrated Research Campus
IRR	incidence rate ratio
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIDA	Leeds Institute of Data Analytics
LOD	limit of detection
LOX	lipoxygenase
MHRA	Medicines and Healthcare products Regulatory Agency
MoA	mechanism of action
mrm	multiple reaction monitoring
MSI	microsatellite instability
NCRAS	National Cancer Registration and Analysis Service
NF	nuclear factor
NIHR	National Institute for Health Research

ODR	Office for Data Release
ONS	Office for National Statistics
PCCRC	post-colonoscopy colorectal cancer
PCR	polymerase chain reaction
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
PGE-M	11α-hydroxy,9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid
PUFA	polyunsaturated fatty acid
qPCR	quantitative polymerase chain reaction
RBC	red blood cell
REC	Research Ethics Committee
RIS	Research and Innovation Service
RT-PCR	reverse transcription-polymerase chain reaction
Rv	resolvin
SAP	Statistical Analysis Plan
SCFA	short-chain fatty acid
SNP	single nucleotide polymorphism
TG	triglyceride
TTA	tissue transfer agreement
VRE	Virtual Research Environment

Summary of Research

The EME-funded seAFOod polyp prevention trial demonstrated that aspirin and the omega-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) both display colorectal cancer (CRC) chemopreventive activity, based on reduction in colorectal adenoma number, in a manner dependent on the type and location of colorectal neoplasia (*Lancet* 2018;392:2583-2594). It also suggested that the combination of aspirin plus EPA had greater efficacy than either agent alone, particularly for distal (left) colorectal lesions. Therefore, a precision medicine approach is needed in order to maximise efficacy of these chemoprevention agents. However, lack of understanding of mechanism(s) of action of both aspirin and EPA currently limits such an approach to CRC chemoprevention, which requires identification of biomarkers for risk stratification and therapeutic response prediction. Understanding the mechanistic basis of an interaction between aspirin and EPA also strengthens the justification for a subsequent clinical trial of combination aspirin and EPA treatment.

During the seAFOod polyp prevention trial, we amassed a comprehensive (greater than 95% coverage), high quality-assured Biobank of blood (plasma, leukocytes, and red blood cells), urine and rectal mucosa samples, aligned to detailed clinical (colonoscopy) trial outcomes. We have individual consent for laboratory analysis of samples and collection of post-trial clinical outcomes from all trial participants at randomisation. Therefore, we now have an unparalleled opportunity to utilise this resource in order to test hypotheses about mechanism(s) of action of aspirin and EPA, which are relevant to identification of biomarkers for risk stratification and response prediction, using a tissue collection unique in chemoprevention trials.

We will use a combination of laboratory techniques (mass spectrometry for lipid mediators, SNP genotype analysis, tissue gene expression [qPCR, immunohistochemistry]) and database interrogation (Bowel Cancer Screening [colonoscopy] outcomes, National Cancer Registration and Analysis Service [cancer] outcomes) in order to answer pre-specified questions regarding the molecular and clinical pharmacology of aspirin and EPA (particularly related to cyclooxygenase activity and lipid mediator signalling), as well as about endogenous factors controlling omega-3 PUFA levels and aspirin efficacy (genotype and diet).

Laboratory data will be linked to the existing seAFOod polyp prevention trial database in a secure virtual research environment (that is both ISO27001 and Data Security and Protection Toolkit [DSPT] compliant) within the Leeds Institute of Data Analytics. Proof-of-principle for this approach has been gained from the analysis of red blood cell fatty acid levels described in the primary seAFOod trial paper. Individual-level data from other sources (e.g. The NHS Bowel Cancer Screening System) will be obtained from Public Health England (PHE) and may be linked to other health outcomes data through the Cancer Research UK-funded CORECT-R CRC data repository that also sits within this environment.

Putative biomarkers identified by the mechanistic studies in STOP-ADENOMA can then be validated using existing trial datasets (eg. ASPIRED) and future polyp prevention trials.

Collectively, this study will drive forward a precision medicine approach to CRC chemoprevention highlighted by the seAFOod polyp prevention trial.

Plain English Summary

Despite advances in diagnosis and treatment of bowel (also known as colorectal) cancer, it remains a major cause of death. More attention needs to be focussed on ways to prevent bowel cancer, including the use of medicines and nutrients - an approach called chemoprevention. Colorectal cancer (CRC) develops over a number of years from a benign fleshy growth in the bowel called a polyp. Polyps are found and removed during a lower bowel camera test called a colonoscopy. The higher the number of polyps that anyone has, the higher that person's risk of CRC in the future.

The STOP-ADENOMA research team has recently completed a research study called the seAFOod Trial. That trial showed that the medicine aspirin (already used as an anti-inflammatory, as well as for prevention of heart attack and stroke), and a naturally-occurring oil found in fish called EPA, reduce the number of polyps detected in individuals at check-up who already had several polyps removed during a colonoscopy in the National Bowel Cancer Screening Programme. Aspirin and EPA reduced polyp number in different ways, depending on the type of polyp, and where in the bowel the polyp was situated. The trial also suggested that aspirin and EPA might work even better together.

Despite aspirin use for over a century, and widespread use of omega-3 fish oil as a health supplement, it is still not understood how they work against polyps and CRC. So, knowledge of how they work is needed in order to use them most effectively in the clinic.

A major legacy of the seAFOod Trial is a collection of blood, urine and tissue samples, which were collected from over 90% of the 709 people in the trial. We also have individual consent for research use of the samples and for access to future colonoscopy results after the trial. Laboratory results can be linked with the trial colonoscopy results for each participant in a secure, confidential database.

This means that the seAFOod Trial team are now in a unique position to ask questions about how aspirin and EPA work, each relevant to future best use of chemoprevention in people at increased risk of bowel cancer. We will;

1) See whether the reduction in bowel polyp recurrence persists at future check-up colonoscopies (or perhaps increases) after the trial has ended, which will tell us whether long-term treatment is needed, or not.
2) In the laboratory, measure the levels of several products of EPA and aspirin that are produced by the body, which will tell us whether combination aspirin plus EPA treatment is likely to give added benefit and which may identify blood test markers that tell us in advance whether aspirin and/or EPA is likely to work for prevention.
3) Address whether an EPA supplement, one's genetic profile and/or omega-3 intake in the diet is important on an individual level for whether aspirin prevents polyps and for whether EPA supplement use will be worthwhile. These results have the potential to lead to dietary guidance for individuals at higher risk of bowel cancer

Any marker that we identify as predicting benefit of aspirin and/or EPA on a personal level will need confirming 'in real life' in future clinical studies.

Background

The unmet clinical need for colorectal cancer chemoprevention

The comprehensive understanding of the natural history and molecular pathogenesis of colorectal cancer (CRC), combined with detailed knowledge of risk factors for colorectal carcinogenesis, that has been gained over the last four decades has, to date, not translated into effective prevention of this malignancy, which still remains the second most common cause of cancer-related death in the UK[1].

The preventability estimate for CRC is 54% based on known modifiable risk factors such as obesity and diet (for example, red and processed meat intake)[1]. Moreover, it is recognised that early diagnosis of CRC (the basis for occult blood test-based population screening in the UK) improves cancer outcomes[2] and that removal of pre-malignant precursor lesions (polyps) by endoscopic polypectomy reduces CRC incidence and mortality[3].

However, only 10% of CRCs are diagnosed through the UK Bowel Cancer Screening Programmes (BCSPs). Moreover, 'interval' CRCs occur despite careful endoscopic screening and surveillance, both within and outside the BCSP, with the post-colonoscopy CRC (PCCRC) rate (usually defined as a CRC diagnosis within three years) ranging between 2-8% of all colonoscopies at which a CRC is detected[4]. Lifestyle interventions (avoidance of obesity, physical exercise, and dietary change) have yet to have any significant prevention impact.

A complementary prevention strategy is chemoprevention (the use of drugs or nutritional agents) used in a primary (general population) prevention context, or alternatively in a secondary prevention setting targeted at individuals at high risk of subsequent CRC after endoscopic polypectomy or surgical resection of colorectal neoplasia[5].

Testing CRC chemopreventive efficacy

Colorectal polyp number is an established biomarker of subsequent CRC risk[6]. The colorectal polyp is also a clinically important lesion in its own right, leading to polypectomy (which is not without bleeding and/or perforation risk), as well as more frequent colonoscopic surveillance (thus increasing the overall burden on endoscopy resources)[6].

Therefore, the 'polyp prevention trial' has become the trial methodology of choice to test chemopreventive efficacy against 'sporadic' CRC using reduction in colorectal polyp recurrence (measured as the % of individuals with any polyp, or number of colorectal polyps per participant) during colonoscopic surveillance as a surrogate endpoint for CRC risk[6].

There is increasing recognition that the molecular pathogenesis of the early stages of colorectal carcinogenesis is not uniform[7]. There are two main endoscopic and histological subtypes of polyp that have malignant potential (the conventional dysplastic adenoma and serrated polyp [previously known as serrated adenoma, but changed to 'polyp' based on the absence of genuine dysplastic change in the majority of serrated lesions), which map to distinct molecular profiles (chromosomal instability driven by loss of function of the *Adenomatous Polyposis Coli* [*APC*] gene and microsatellite instability [MSI] usually

accompanied by a CpG island hypermethylation phenotype [CIMP], respectively)[7]. Conventional adenomas are distributed along the colo-rectum in a way that mirrors the relative frequency of CRCs throughout the colo-rectum (incidence in the left colon and rectum greater than the right colon [defined as proximal to the splenic flexure]). However, serrated polyps are over-represented in the right colon and are believed to contribute disproportionately to PCCRCs which are more likely to be right-sided and display MSI[7].

The seAFOod Polyp Prevention Trial

Proof-of-concept for CRC chemoprevention by the naturally occurring omega-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid (EPA) has been provided by a randomised trial in familial adenomatous polyposis (FAP) patients[8].

There are robust observational data from long-term follow-up of randomised trials with vascular endpoints[9], which back the assertion that aspirin prevents CRC, supported by a meta-analysis of four previous polyp prevention trials, which highlighted uncertainty about the optimal dose and target population for aspirin chemoprevention[10].

Therefore, we tested the hypotheses that EPA and aspirin prevent colorectal adenoma recurrence and are safe, alone and in combination, in individuals with 'high risk' colorectal neoplasia detected in the English BCSP. The EME-funded seAFOod polyp prevention trial was a multicentre, randomised, double-blind, placebo-controlled, 2 x 2 factorial trial of EPA free fatty acid 2 g daily and aspirin 300 mg daily, nested in the BCSP, that has now been published in *The Lancet*[11]. Seven hundred and nine individuals were randomised to receive a 12-month intervention before scheduled BCSP surveillance colonoscopy[11].

The seAFOod polyp prevention trial did not show any effect of EPA or aspirin on the primary endpoint of the percentage of individuals with any colorectal adenoma (the adenoma detection rate [ADR]) at surveillance colonoscopy[11]. However, the trial demonstrated that both EPA and aspirin have chemopreventive efficacy based on the reduction in colorectal adenoma number, which was a secondary outcome measure[11]. There was evidence of selectivity based on polyp type (conventional dysplastic adenoma or serrated polyp) and location within the colo-rectum (left *versus* right)[11]. We proposed that the historical primary endpoint in polyp prevention trials (the ADR) was less sensitive than changes in colorectal adenoma number in a BCSP, in which the ADR is exceptionally high (greater than 60%) in 'high risk' individuals and while it is used as an individual Colonoscopist Key Performance Indicator in the BCSP. Additionally, we highlighted that the difference in colorectal adenoma number has been used in multiple FAP chemoprevention trials, in which adenoma multiplicity is high, and that a change in colorectal adenoma number is arguably more biologically meaningful for cancer prevention than a patient-level colorectal adenoma incidence read-out (ADR)[11].

Aspirin use was associated with a reduction in mean total colorectal adenoma number per participant evidenced by an incidence rate ratio [IRR] of 0.78 [95%CI 0.68, 0.90], with preventive efficacy against conventional (IRR 0.82 [0.71, 0.94), serrated (IRR 0.46 [0.25,

0.87]) and right-sided (IRR 0.73 [0.61, 0.88]) lesions, but not left-sided (IRR 0.85 [0.69, 1.06]) colorectal adenomas.

There was evidence of more modest chemopreventive efficacy of EPA on conventional (IRR 0.86 [0.74, 0.99]) and left-sided (0.75 [0.60, 0.94]) colorectal adenomas, but not on total colorectal adenoma number (IRR 0.91 [0.79, 1.05]), serrated (IRR 1.44 [0.79, 2.60]) or right-sided (IRR 1.02 [0.85, 1.22]) colorectal adenomas.

On the basis of a similar magnitude risk reduction in the seAFOod polyp prevention trial compared with previous aspirin polyp prevention trials[10], aligned with the observational data on CRC incidence and mortality[9], we suggested that the risk reductions that we observed in the seAFOod polyp prevention trial were clinically meaningful, particularly given the excellent safety and tolerability profile of both agents[11].

Overall, colorectal adenoma number was reduced in the group who received EPA and aspirin together (166) compared with the other groups (238, 209 and 231)[11]. The 2x2 factorial trial was not powered to perform a formal 'inside the table' analysis of the four treatment arms. However, *post hoc* analysis confirmed that the reduced number of colorectal adenomas in the individuals who received combined treatment with aspirin and EPA was associated with an IRR of 0.75 (0.61, 0.93) compared with aspirin alone (unpublished data). Moreover, the excess of mild-to-moderate gastrointestinal (GI) adverse events observed in EPA users was not seen in those who used EPA in combination with aspirin suggesting better tolerability of combination treatment compared with omega-3 PUFA treatment alone[11].

The seAFOod Trial concluded that both EPA and aspirin have CRC chemoprevention efficacy, based on reduction of colorectal adenoma number, with the larger effect size of aspirin adding to the weight of evidence for its use in combination with endoscopic screening and surveillance, which currently provides sub-optimal protection, particularly against right-sided CRC.

The findings suggested that a precision medicine approach (addressing adenoma type and location) to CRC chemoprevention is necessary, which mirrors established best oncology practice in CRC treatment, which is now firmly based on molecular stratification of tumours.

The majority of participants in the seAFOod Polyp Prevention Trial were randomised to either active EPA free fatty acid (FFA) or placebo capsules (n=422). However, this formulation became unavailable during the intervention phase of the trial and the EPA Investigational Medicinal Product (IMP) was switched to an EPA triglyceride (TG) preparation (n=287), providing FFA dose-equivalence. Therefore, we have already measured EPA levels in all red blood cell (RBC) membranes and rectal mucosal samples in order to confirm that EPA incorporation was similar in users of the two EPA formulations. Individual PUFA profiles confirmed that baseline and post-treatment EPA levels in RBCs (an established surrogate for tissue PUFA incorporation) and the target tissue (rectal mucosa) were similar in users of the two active EPA formulations[11]. We also observed a moderate strength correlation between RBC and rectal mucosa levels (r=0.46), as well as wide variability in baseline and post-

treatment EPA levels despite excellent reported compliance and no change in dietary intake during the intervention[11]. A wide variation in omega-3 PUFA levels has been noted in previous intervention trials[12].

Factors controlling tissue omega-3 PUFA levels

Long-chain omega-3 PUFAs are naturally occurring substances with C20:5*n*3 (where Cx:y*n*z denotes the carbon chain length [x], the number of double-bonds [y], and the carbon position of the first double bond [z]) EPA and C22:6*n*3 docosahexaenoic acid (DHA) found in highest quantities in cold-water oily fish such as mackerel and sardines, and C18:3*n*3 alpha-linolenic acid (ALA) found predominantly in seed oils (such as chia, linseed, canola) oils. Conversion of ALA to EPA, and thenceforth DHA, is controlled by a series of enzymatic carbon-chain desaturation and elongation steps mediated by *FADS2/FADS1* and *ELOV2/ELOV5* genes, which each have functional genetic polymorphisms that likely underlie significant inter-individual variability in EPA and DHA synthesis from plant-derived ALA[13]. Omega-3 PUFA catabolism is dominated by beta-oxidation of PUFAs for energy harvesting through mitochondrial oxidative phosphorylation[14].

Therefore, in contrast to administration of a small molecule drug, the baseline EPA content in plasma membranes and the response to taking 'nutraceutical' purified EPA are dependent on dietary intake and endogenous omega-3 PUFA conversion under genetic control, as well as compliance and bioavailability factors relevant to oral administration of an EPA supplement.

Mechanisms of action of aspirin and EPA

Despite decades of research, the mechanism(s) of action (MoA) underlying anti-cancer activity of either aspirin or omega-3 PUFAs at the earliest stages of intestinal tumorigenesis remains unclear. Multiple putative MoA for aspirin (cyclooxygenase [COX] inhibition leading to reduced prostaglandin [PG] E_2 signalling, COX-independent activity [for example, nuclear factor {NF} κB signalling])[15] and EPA (COX inhibition leading to reduced PGE_2 signalling, reactive oxygen species generation, FFA receptor and peroxisome proliferator-activated receptor activation)[16] have been proposed, often solely on the basis of *in vitro* studies of high, supra-physiological drug concentrations on CRC cells with little, often no, attempt to confirm or refute the relevance of those findings in animal models and in humans. The seAFOod Trial is an excellent opportunity to understand MoA using human tissue aligned with clinical outcome data from a randomised trial. To this end, the EME-funded protocol originally included set-up and curation of a comprehensive Biobank along with a series of experiments, which the Investigators predicted would be relevant in advance of the clinical outcomes of the trial[17]. However, the time- and cost-extension to the trial granted by the EME Board, which was necessary after delays related to slow recruitment and the unexpected need to switch EPA and placebo capsule formulation, was predicated on cost-savings from the laboratory studies. Therefore, the only laboratory study that has been performed, to date, is measurement of PUFA levels in RBCs and rectal mucosa in order to confirm similar tissue EPA incorporation from the two EPA formulations[11].

The seAFOod polyp prevention trial Biobank

The seAFOod polyp prevention trial Biobank, which is situated in the Clinical Trials Pharmacology Laboratory (CTPL), Institute of Cancer Therapeutics at the University of Bradford is a unique, high quality-assured, comprehensive resource linked to trial outcomes. Details of the seAFOod Trial Biobank are available in the Trial protocol paper[17] and the completed NIHR Library Journals report[18]. At the end of the seAFOod trial (10/H0405/90), the biobank has been housed in the University of Bradford HTA-approved (Licensing no.12191) Research Tissue Bank.

During the trial, samples were stored at BSCP sites for between 1 and 696 days (median 115 days). 1378 (78%) sample sets were stored at BSCP sites for less than 6 months. Thirty (2%) sample sets were stored at BSCP sites for more than 12 months. The majority of sample sets (1021; 58%) were stored in BCSP sites at -20^{o}C (range -16 to -24^{o}C), with 230 (13%) sample sets stored at -40^{o}C (range -25 to -69^{o}C), and 524 (30%) sets stored at or below -70^{o}C.

One or more biological samples were received from 677 of 709 (95%) randomised seAFOod Trial participants. Seventy-three percent (519) of participants provided full sample sets of blood, urine and rectal mucosa from all three visits (baseline, 6 months, and 12 months). There were 76 participants who provided samples at two visits. Only 82 participants provided samples at a single visit. Overall, a total of 7323 biological samples were received (16,258 sample aliquots):

- 1715 plasma (6746 aliquots)
- 1714 leukocytes (1714 aliquots)
- 1707 RBCs (3421 aliquots)
- 1664 urine (3309 aliquots)
- 522 rectal biopsies (1068 aliquots)

Compliance with biological sample collection, defined as the proportion of sample sets expected (n=2127) that were received with at least one sample aliquot, was 80% (blood), 78% (urine), and 74% (rectal mucosa). Eighty percent of blood samples were obtained per protocol (refrigerated centrifugation within 30 minutes and transfer to the freezer within 60 minutes) with only a small number of protocol deviations including 1% of sample sets which suffered a temperature deviation (but no thaw) and 1% of sample sets which defrosted at some point.

Importantly, all samples were aliquoted so that multiple analyses would be possible without freeze-thaw damage. This is particularly important for the rectal mucosal samples, which will be subjected to different experimental approaches. To date, the only analysis that has been carried out on Biobank samples is measurement of RBC and rectal mucosal fatty acid profiles[11], which was prioritised after the unexpected capsule IMP switch.

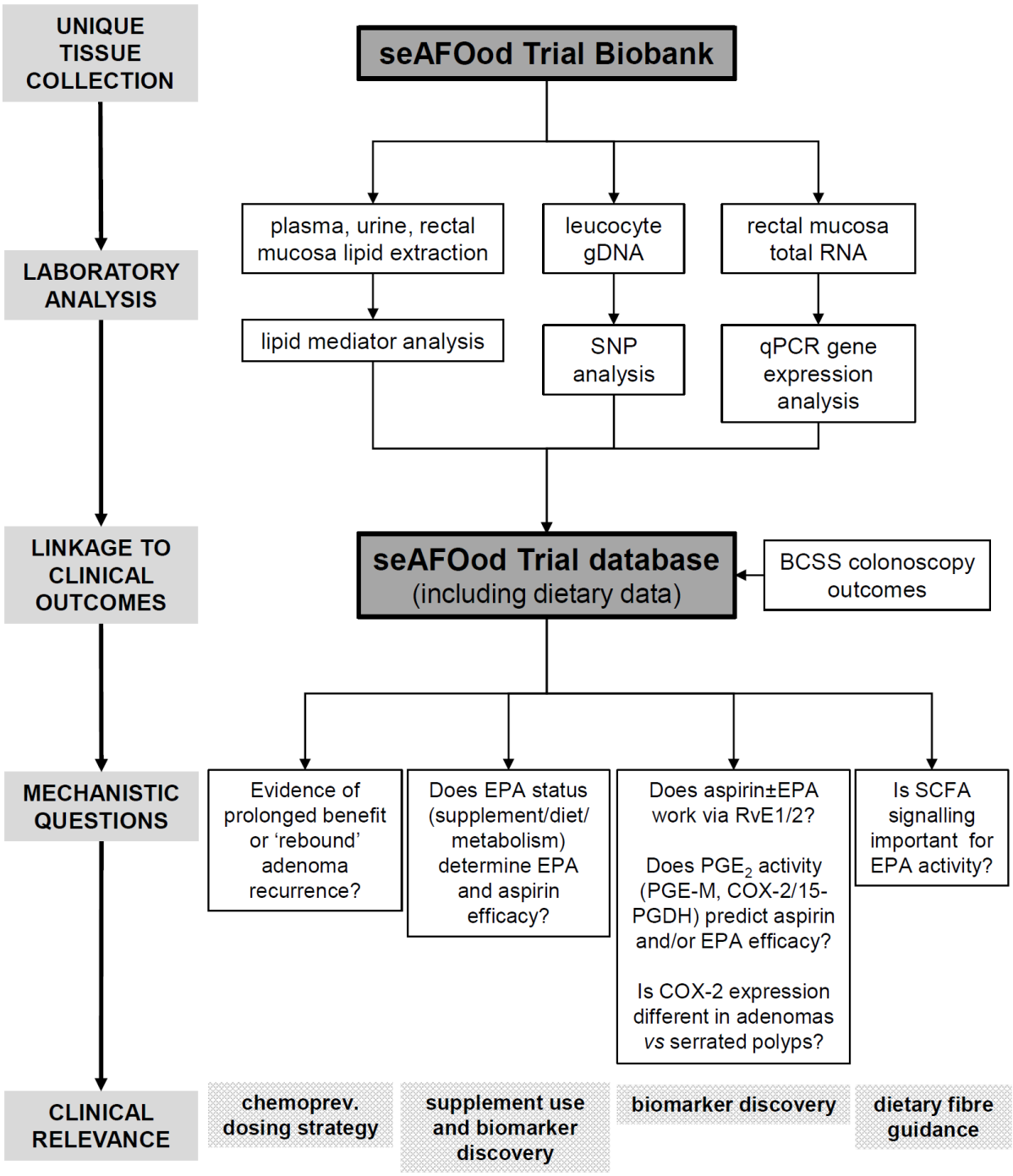
All samples remain stored in a dedicated -80°C freezer, which is connected to an emergency power supply in the CTPL and supported with a CO_2 back-up system and telephone alarm system. Freezer temperature is monitored daily.

The seAFOod Trial Biobank sample database is maintained by the CTPL. Samples are tracked via an internal CTPL ID, linking to the seAFOod Trial participant ID. The CTPL operates quality control procedures, as recommended by Medicines and Healthcare products Regulatory Agency (MHRA) Good Clinical Laboratory Practice (GCLP) guidelines. The database is stored on the University of Bradford server in the 'Secure' section, only accessible to nominated CTPL staff, as per the University of Bradford IT policy.

Mechanistic studies leading from the seAFOod Polyp Prevention Trial

The combination of the seAFOod Polyp Prevention Trial database and existing comprehensive biobank is a unique opportunity to address important mechanistic questions relevant to optimal use of both aspirin and EPA, under the terms of the individual consent provided by participants at the start of the trial (Figure 1).

Figure 1: STOP-ADENOMA – workstreams using the existing seAFOod Trial biobank and database



The clinical outcomes from the trial propose a paradigm shift in CRC chemoprevention strategy whereby the same precision medicine principles are applied to *prevention*, as are currently applied to CRC *treatment*, based on histological and molecular phenotyping. Addressing the mechanistic questions below that have been generated by the randomised trial will help to usher in a new era of precision prevention.

We have pre-specified five mechanistic questions that each address a hypothesis based on existing knowledge and outcomes from the seAFOod polyp prevention trial (Figure 1).

Hypothesis-driven questions about mechanism(s) of action of aspirin and EPA, resolution of which could lead to best use of these agents for CRC chemoprevention

1. Does chemoprevention by aspirin and EPA produce prolonged benefit (by inhibition of tumour initiation) or a 'rebound' increase in colorectal polyp incidence (by inhibition of tumour progression/growth)?

There are important consequences for the duration of any chemoprevention response and outcomes after cessation of therapy depending on whether chemoprevention agents inhibit formation of, or suppress growth of, benign precursor stages of CRC. Growth suppression (without inhibition of tumour initiation) is hypothesised to lead to a 'rebound' increase in colorectal polyp incidence whereby 'hidden' tumours are de-repressed and become detectable upon cessation of chemoprevention therapy. Knowledge of whether a 'rebound' increase in colorectal polyp number (indicative of increased CRC risk) occurs or whether there is prolonged benefit from taking a chemoprevention agent for a short time, will be key in order to define guidelines for duration of chemoprevention use and cessation of therapy, especially in the elderly, in whom increasing age is the main driver of aspirin-related bleeding risk.

2. Does individual EPA status at the start of chemoprevention (regardless of the source of EPA), and/or response to supplementation, predict response?

Knowledge of whether the absolute tissue level of EPA is important for subsequent colorectal neoplasia risk and/or efficacy of aspirin, regardless of the source of EPA ('nutraceutical' supplementation, dietary marine omega-3 PUFAs, conversion of plant-derived omega-3 PUFA) will be critical in order to best harness the modest anti-CRC activity of EPA observed in the seAFOod Trial and provide accurate guidance to the general public and at-risk groups about dietary omega-3 PUFA intake. An interaction between tissue EPA status and aspirin use would represent an exemplar nutrient-drug interaction and promote a precision medicine approach to aspirin chemoprevention in which individual baseline omega-3 PUFA status is considered alongside known (age) and potential (body mass index; BMI) biomarkers of benefit and risk from aspirin.

An alternative hypothesis is that the change in EPA level upon supplementation is important for EPA efficacy, with or without aspirin co-therapy. Proof that an increase in EPA incorporation during treatment is important for chemoprevention efficacy could lead to the use

of the tissue EPA response as a clinically useful predictive biomarker (increasingly realistic with the advent of whole blood spot omega-3 PUFA monitoring rather than venepuncture[19])

3. Does combined aspirin and EPA therapy lead to resolvin E synthesis?

The finding that combined aspirin and EPA treatment was associated with a significant reduction in colorectal adenoma risk compared with either agent alone has increased the spotlight on the mechanistic explanation for an interaction. One longstanding hypothesis (which was due to be tested in the original EME-funded seAFOod trial proposal) is that combined treatment leads to production of the lipid mediator resolvin (Rv) E1/E2 (Figure 2).

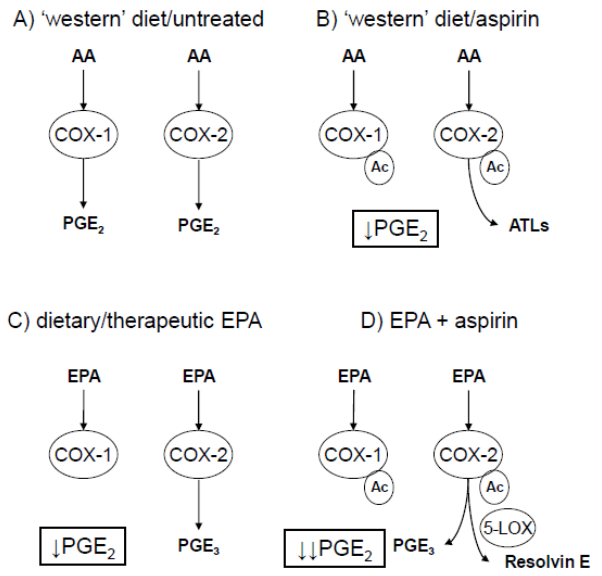


Figure 2. Mechanisms of action of aspirin and EPA targeting COX activity. A) In 'western' diets, omega-6 arachidonic acid C20:4*n*6 (AA) is the predominant substrate for the COX isoforms leading to production of PGE_2. **B)** Acetylation (Ac) of a serine residue in the COX active site by aspirin inhibits COX-1 entirely but alters activity of COX-2 leading to formation of aspirin-triggered lipoxins (ATLs). The overall result is reduced levels of pro-tumorigenic PGE_2. **C)** EPA competes with AA as substrate for both COX enzymes but enzymatic turnover (V_{max}) is 10-30 fold lower leading to reduced PGE_2 levels. In addition, EPA drives COX-2-dependent production of equivalent '3-series' PGs such as anti-tumorigenic PGE_3. **D)** The combination of aspirin and EPA is hypothesised to result in reduced PGE_2 levels, but also production of anti-inflammatory RvEs via downstream 5-lipoxygenase (5-LOX) metabolism, which may have anti-cancer activity and/or be a biomarker of therapeutic response to combination therapy.

Evidence that RvE synthesis occurs and is associated with reduced colorectal neoplastic risk may lead to the use of the E-type Rvs as a therapeutic response biomarker, as well as testing of stable RvE analogues as chemopreventive agents[20].

4. Does inhibition of PGE_2 production explain response to aspirin and EPA?

A strong candidate mechanism of chemopreventive activity of both aspirin and EPA is inhibition of COX activity (Figure 2). The ability to measure levels of the stable PGE_2 metabolite PGE-M in random urine samples now allows a simple read-out of COX activity at baseline and during treatment, in parallel with measurement of COX expression in colorectal mucosa. This mechanistic insight would lead to evaluation of this PGE_2 metabolite as a risk and/or therapeutic response biomarker.

Differential expression of the COX enzymes in conventional adenomas compared with serrated polyps could explain differential activity of EPA and aspirin against the two main polyp types. Surprisingly, COX expression in serrated lesions has not been well characterised compared with colorectal adenomas, in which both stromal (macrophage and fibroblast) cell and epithelial cell localisation has been reported[21].

5. Does mucosal expression of FFAR2 predict response to EPA?

The restriction of the anti-neoplastic activity of EPA to the left (distal) colon in the seAFOod polyp prevention trial combined with our recent finding that omega-3 PUFA supplementation alters the intestinal microbiome in favour of short-chain fatty acid (SCFA)-producing bacteria[22] supports the hypothesis that EPA has distal colonic activity through FFA receptor 2 (FFAR2)

signalling, expression of which is highest in the left colon. Clinical data supporting this hypothesis will lead to further investigation of the effect of omega-3 PUFA supplementation on colonic SCFA (propionate, butyrate and acetate) levels. Confirmation of the EPA-SCFA hypothesis could lead to evaluation of a dietary fibre (the natural source of SCFAs via bacterial metabolism)-omega-3 PUFA interaction, which would have wide applicability and appeal as a nutritional intervention for maintenance of GI health.

Aim and Objective of the STOP-ADENOMA project

The overall aim of the STOP-ADENOMA project is to perform mechanistic studies using the existing seAFOod Trial Biobank and clinical outcomes in order to develop further the biomarker-driven precision medicine approach to CRC chemoprevention by aspirin and EPA that was suggested by the results of the seAFOod polyp prevention trial.

The main objective is to provide mechanistic rationale for use of several putative risk and therapeutic prediction biomarkers that could find utility for clinical decision-making related to precision CRC chemoprevention.

Research Plan

The project has five distinct work-packages that are linked to the individual, pre-specified hypothesis-driven questions described above (Figure 1).

1. Does chemoprevention by aspirin and EPA produce prolonged benefit (by inhibition of tumour initiation) or a 'rebound' increase in colorectal polyp incidence (by inhibition of tumour progression/growth)?

A major advantage of nesting the seAFOod Polyp Prevention Trial in the English BCSP is that post-trial colonoscopy outcomes during ongoing surveillance are available through the BCSP Screening System (BCSS) database. Trial participants provided specific informed consent to such follow-up at trial entry.

By June 2020, all evaluable trial participants (n=707) will have undergone at least one further surveillance colonoscopy since the trial exit colonoscopy, at which the colorectal adenoma outcomes were assessed (88% of participants were re-classified after 'high-risk' surveillance colonoscopy at one year as 'intermediate risk' requiring a repeat colonoscopy at three years, the remaining individuals remained 'high risk' for further annual surveillance, all within the BCSP[2])[11]. Any necessary colonoscopic surveillance following the first surveillance procedure continues in the English BCSP as per current surveillance guidelines[23].

Colonoscopy (including histology) findings will be obtained directly from the BCSS, which sits inside PHE. In future, the data may be placed in the Cancer Research UK-funded COloRECTal cancer data Repository, known as CORECT-R, headed by Co-Investigator EM (REC 18/SW/0134). This is a resource in which numerous datasets relevant to colorectal cancer (including cancer registry, Hospital Episode Statistics and screening data) are linked and made available for research.

Colorectal adenoma and serrated polyp outcomes (number, size, histology, location) will be compared across the treatment arms with comparison at the trial factorial margins of aspirin users *versus* no aspirin users and EPA users *versus* no EPA users. Appropriate (including linear, logistic, Poisson or negative binomial) regression models will be adjusted for known important baseline characteristics eg. BMI and baseline colonoscopic findings, using regression models to produce IRRs for total colorectal adenomas and the subtypes specified in the original trial analysis[11], all adjusted for colonoscopy frequency.

We will not be able to access data on post-trial use of aspirin or EPA and it is possible that individuals may have begun to, and continued to, take either or both active agents after their trial participation. This could confound interpretation of post-trial colonoscopy outcomes. However, there is no evidence that this occurred to any significant extent following previous aspirin polyp prevention trials[24]. Many participants will have read the Plain English seAFOod polyp prevention trial results summary but only a handful of participants asked trial site staff for information on what treatment group they were randomised to when the trial completed. Therefore, there is no reason to believe that post-trial behaviour will be different after the seAFOod polyp prevention trial in any of the four treatment arms that participants were

allocated to in a double-blind manner. In order to strengthen our assertion that there has been no difference in post-trial aspirin use across the intervention groups, we will link patient-identifiable trial data to a composite of myocardial infarction and cerebrovascular events that have occurred in the trial population since the end of the trial in 2016, which will be obtained from Hospital Episode Statistics (HES; if we are able to use the CORECT-R resource for database linkage) and which will act as a proxy for aspirin use.

Given the number of individuals in the trial and modest post-trial follow-up duration (maximum 6 years), there are unlikely to be sufficient CRC events for interpretation of any possible treatment effect on cancer incidence. However, we will identify incident all-cancer cases through linkage to the National Cancer Registration and Analysis Service (NCRAS) and Civil Registration Data on mortality from the Office for National Statistics (ONS) (if the CORECT-R data resource is available for such linkage). We will specifically assess overall total cancer (excluding non-melanoma skin cancer) incidence during follow-up given the recent data from the ASPREE Trial that aspirin use in an elderly population (≥70 years) may be associated with increased overall cancer incidence and mortality in the short term[25].

2. Does individual EPA status at the start of chemoprevention (regardless of the source of EPA), and response to supplementation, predict response?

We already have a full dataset of fatty acid profiles (including EPA, but also ALA and DHA) at baseline, 6 months and 12 months for all evaluable participants[11]. There is also a comprehensive (80% of participants provided a pre- and post-treatment food frequency questionnaire [FFQ]) set of FFQs, which have already been used to confirm that there was no appreciable change in fish (marine omega-3 PUFA) intake between intervention groups and during the trial[11]. Separately, we have now estimated individual marine omega-3 PUFA (EPA, DHA) and plant omega-3 PUFA (ALA) intake using FETA software[26].

Genomic DNA will be isolated from 666 (94% of the trial population) individuals, from whom we have isolated leukocytes. We will perform SNP genotype analysis for known polymorphisms in the *FADS* gene cluster (eg. rs174546), for which there is evidence for association with baseline long-chain omega-3 PUFA levels, desaturase activity and response to supplementation, along with a minor allele frequency greater than 5%[12,27]. There is strong linkage disequilibrium between *FADS* SNPs allowing haplotype analysis and reconstruction[27]. We will collaborate closely with the ERA Healthy Diet for a Healthy Life-funded FAME team (see collaboration letter), who are already studying genetic polymorphisms in fatty acid-metabolising genes related to cardio-metabolic health, in order to co-ordinate testing of SNPs identified from publically accessible databases (NCBI HapMap, 1000genomes). We will also take the same approach with SNPs in genes that control lipid mediator synthesis including the lipoxygenase genes *ALOX5*, *ALOX12*, and *ALOX15*, as well as *PTGS2* (*COX-2*; rs4648261), *PTGS1* (*COX-1*), which may also be relevant for aspirin bioactivity[28]. Using this hypothesis-driven approach, we expect to study 30-40 variants and can remain reactive to latest developments in understanding of genetic determinants of omega-3 PUFA levels maintaining cardio-metabolic health.

We will combine omega-3 PUFA levels (EPA alone or total marine-derived omega-3 PUFAs [EPA + docosapentaenoic acid [DPA] + DHA]) with dietary intake and SNP haplotype in order to derive a predictive model for the relationship between dietary intake (marine- and plant-derived omega-3 PUFAs), combined with SNP haplotype, and 1) the baseline EPA level and 2) the response to EPA supplementation, separately. We will then explore the relationship between baseline EPA and total marine omega-3 PUFA level (as well as the corresponding post-treatment values), and trial colorectal adenoma outcomes, by analysing PUFA level tertiles/quartiles in both aspirin users and non-users in an EPA intervention-independent manner.

3. Does combined aspirin and EPA therapy lead to resolvin E synthesis?

Aspirin irreversibly acetylates a serine group in the active site of both COX enzymes[15]. This leads to complete inhibition of COX-1 activity (which underlies the anti-platelet activity of aspirin) but alters COX-2 function such that, if EPA is the substrate (rather than the usual omega-6 PUFA C20:4*n*6 arachidonic acid [AA]), the oxygenated product is 18*R*-hydroxyeicosapentaenoic acid (HEPE), which itself acts as a substrate for 5-lipoxygenase (5-LOX) leading to production of E-type Rvs (Figure 1) that have been proposed to have pro-resolving properties in acute inflammation models and may have anti-neoplastic activity by inhibition of NFκB signalling[29]. Resolvin E1 and 18*R*-HEPE have been detected in human plasma in septic patients[30]. However, there has been no supportive evidence from a large trial dataset that RvE1/2 is synthesised in humans taking aspirin. Although the two G protein-coupled receptors that transduce RvE1/2 signalling (ChemR23 and BLT1) are expressed by human colorectal epithelial cells (in-house unpublished data), it remains unknown whether RvE1/2 synthesis occurs in the human colorectum.

The preliminary finding that the combination of aspirin and EPA reduces colorectal adenoma number significantly in excess of either agent alone supports testing the 'resolvin hypothesis' by measuring 18*R*-HEPE and RvE1/2 levels in plasma from individuals in the combination treatment arm in comparison with plasma samples from the other treatment groups.

Plasma 18*R*-HEPE (*mrm* 317>133) and RvE1/2 (*mrm* 349>195/*mrm* 333>199/115) will be measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with chiral column analysis, which has a limit of detection (LOD) <100 pg. The proposed transcellular synthesis and paracrine activity of the Rvs suggests that quantification of Rvs in plasma may not be a good reflection of tissue bioactivity[31]. Therefore, we will prospectively measure RvE1/2 levels in our banked rectal mucosal samples (n=519) and use our established quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) assays to measure rectal mucosal expression of ChemR23 and BLT1 (Hutchinson J, Cancer Research UK Training Fellowship PhD thesis [unpublished]). The relationship between Rv level, expression of its receptors and colorectal adenoma recurrence in trial participants will be explored by multivariate analysis.

A related hypothesis is that individuals with high tissue levels of EPA, regardless of omega-3 PUFA supplement use or not, who take aspirin will synthesise RvEs that may contribute to the

anti-CRC activity of aspirin. Therefore, we will measure RvEs in samples from all four treatment arms (rectal mucosa; placebo 136; EPA 122; aspirin 134; EPA plus aspirin 127). The relationship between RvE levels and EPA content in RBCs and rectal mucosa will also be explored. Evidence that E-type Rvs are synthesised in individuals taking aspirin, regardless of omega-3 PUFA use, if endogenous EPA levels (driven by diet and genetic factors) are sufficiently high, will have profound implications for the field of nutrient (omega-3 PUFAs)-drug (aspirin) interactions if RvE1/2 levels are associated with reduced colorectal adenoma number. This would lead to prospective testing of the predictive value of individual EPA status for aspirin efficacy as a CRC chemopreventive agent.

In the absence of EPA as a substrate for aspirin acetylated COX-2, the usual COX substrate AA is converted via 15*R*-hydroxytetraenoic acid (HETE) and oxidation by 5-LOX to an alternative class of pro-resolving lipid mediators termed aspirin-triggered lipoxins (ATLs; Figure 1)[32]. Detection of EPA-derived RvEs in plasma and/or rectal mucosa will prompt measurement of ATLs (specifically 15*R*-epi-lipoxin A4), LC-MS/MS assay of which is established in Bradford following prior collaboration with Professor McAuley (Queen's University, Belfast)

4. <u>Does inhibition of prostaglandin E_2 production explain response to aspirin and EPA?</u>

Inhibition of the COX-PGE_2 pathway is established as a likely mechanism of the anti-cancer activity of both aspirin and EPA (Figure 1)[15-16]. This has led to evaluation of predictive biomarkers of aspirin chemoprevention in cohort studies including the urinary PGE-M level[33] and mucosal expression of 15-prostaglandin dehydrogenase (15-PGDH)[34].

Prostaglandin E_2 acts in a paracrine manner through cell-surface EP receptors but it is unstable and is converted to a stable metabolite 11α-hydroxy,9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (termed PGE-M), levels of which in the urine are a recognised measure of systemic PGE_2 exposure[33]. High baseline urinary PGE-M predicts lower colorectal adenoma risk in aspirin users[33]. Urinary PGE-M is the primary endpoint in the on-going ASPIRED trial of aspirin in patients with previous colorectal adenoma[35]. The LC-MS/MS assay for urinary PGE-M is established in Bradford and has been used by us in several studies including the Phase 2 EMT trial, in which EPA treatment was associated with reduced urinary PGE-M levels[12].

The seAFOod Trial is an ideal opportunity to test the effect of aspirin and EPA on urinary PGE-M, as well as determine the predictive value of the baseline urinary PGE-M value for chemopreventive activity of both aspirin and EPA. Unlike rectal mucosa, for which we only have samples from the exit trial colonoscopy because the baseline colonoscopy was performed informed trial consent was sought, we have three urine samples (baseline, 6 months, 12 months) from 422 participants (665 [94%] provided at least one urine sample) providing an excellent opportunity to perform longitudinal biomarker analysis.

15-PGDH catalyses the rate-limiting step in conversion of PGE_2 to the inactive 15-keto-PG metabolite[31]. 15-PGDH has been demonstrated to have tumour suppressor activity in pre-

clinical CRC models[34]. High mucosal 15-PGDH transcript levels predict lower colorectal adenoma risk in aspirin users[28]. Colorectal 15-PGDH mRNA levels are consistent, stable over time, and not affected by aspirin treatment[34]. Therefore, we will measure baseline rectal mucosal 15-PGDH transcript levels, as well as those for COX-2, by quantitative real-time PCR in all seAFOod trial participants (n=519)[36], thereby allowing us to validate 15-PGDH transcript levels as a biomarker of aspirin and/or EPA response, alone or in combination with the urinary PGE-M level at 12 months (end of treatment).

Combined baseline colorectal adenoma tissue expression of COX-2 and 15-PGDH predicts response to the selective COX-2 inhibitor celecoxib[37]. Therefore, we will obtain FFPE polypectomy specimens from the screening and surveillance colonoscopies of 100 participants from the top five recruiting sites under the terms of the tissue transfer agreements that were established during site R&I approval for the seAFOod Trial. Immunohistochemistry (IHC) for COX-2 and 15-PGDH will be performed using our established methods, which include published scoring protocols[21,36]. Twenty serrated lesions (47% of the total trial set) will be included in the experimental set with which to compare expression with conventional adenomas in order to test the hypothesis that COX-2 expression is lower in serrated polyps than conventional adenomas (thus generating the hypothesis that COX-2 expression explains the differential activity of EPA and aspirin on colorectal polyp subtypes, with aspirin having stronger effects on serrated lesions courtesy of its stronger COX-1 inhibitory activity).

5. Does mucosal expression of FFAR2 predict response to EPA?

FFAR2 (also known as GPR43) expression will be measured by RT-PCR on all rectal mucosal total RNA samples (n=519). Differential expression related to treatment arm will prompt analysis at the protein level by immunohistochemistry (rabbit polyclonal LS-A6598; LifeSpan BioSciences, Inc.). We will also analyse FFAR2 expression related to colorectal adenoma recurrence in EPA users.

Support for the hypothesis that FFAR2 expression in non-neoplastic rectal mucosa is required for EPA efficacy will prompt measurement of SCFA levels in faecal samples from our omega-3 PUFA intervention study in healthy volunteers[22], leading to further experimental medicine studies exploring the effect of an omega-3 PUFA-fibre interaction on colonic physiology and pathophysiology.

Laboratory methods

The following techniques will be performed in Leeds:

Genomic DNA extraction and SNP analysis

DNA will be extracted from leukocyte-rich EDTA-plasma using the Nucleon BACC2 genomic DNA extraction kit (GE Healthcare). SNP analysis will be performed using the Fluidigm 96.96 integrated fluidic circuit system (https://www.fluidigm.com/reagents/genotyping#overview) in order to provide the most time and cost-efficient SNP analysis using SNP Type allele-specific PCR probes for less than 50 variants.

Total RNA extraction and RT-PCR

Total RNA will be extracted from rectal mucosa using the RNeasy Mini kit (Qiagen) before DNase I treatment and reverse transcription with Superscript IV (Invitrogen). Real-time quantitative PCR will performed as described by us using an ABI7900 sequence detector and the SYBR™ Green system (ThermoFisher)[38].

Immunohistochemistry

Immunohistochemistry on FFPE tissue sections with visualisation using DAKO EnVision™+ (Dako UK Ltd) is routine in the Hull laboratory for multiple antigens including COX-2 and 15-PGDH[36]. Serial sections will be analysed wherever possible so that separate IHC scores can be combined and cellular immunoreactivity compared between sections.

Lipid mediator analysis will be performed in the Institute of Cancer Therapeutics in Bradford.

Lipid mediator measurement

Liquid chromatography- electrospray ionization triple quadrupole tandem mass spectrometry (LC-MS/MS) will be performed in Bradford under the terms of an existing, long-term over-arching agreement between Leeds and Bradford that covers the Trial biobank and multiple studies that have included LC-MS/MS measurement of fatty acids and other lipid mediators[11-12].

Lipid extraction with acid hydrolysis is established in-house including for rectal mucosa[11]. A Waters Acuity UPLC System module in combination with a Waters Quattro Premier XE triple quadrupole mass spectrometer will be used as described[35]. In addition to our published methodology for measurement of fatty acids[39], we will use established protocols for quantification of several lipid mediators including PGE_2, PGE_3 and PGE-M[40-41]. Chiral analysis of 18*R*-HEPE and RvE1/2 has already been established in-house based on published methods[42]. Extraction, following tissue homogenisation, is based on 96-well SPE plates and analysis via LC-MS/MS, as used for urinary PGE-M[41]. 18*R*-HEPE and RvE1/2 will be detected simultaneously from the same sample extract[42]. Our previous experience suggests that derivatisation will not be needed for any of the proposed analytes. Quantification of PGE-M, 18*R*-HEPE and RvE1 levels will be achieved using commercially available authentic

standards, with deuterated forms of PGE-M and RvE1 used as an internal control, as described[41].

Database management and governance

Although the seAFOod Trial was sponsored by the University of Leeds, the OpenClinica trial database was hosted by the Nottingham Clinical Trials Unit (CTU). For STOP-ADENOMA, the data has been exported as 'one dataset per form' .csv files, as well as annotated CRFs and other metadata, into a SQL, and then other, database file, as appropriate, which are housed in a secure environment (ISO27001 and DSPT compliant) at the University of Leeds.

The SQL database contains patient-identifiers (date of birth [DOB], initials, sex, and date/location of colonoscopy in the BCSP), which will allow linkage to other datasets. These identifiers will be held separately to the main trial outcome data in a linkage file that will also hold a unique study identifier for each participant that cannot be used to identify anyone but can be used to link across datasets (Figure 3). This linkage file will not be accessible to researchers involved in the study. Rather, this file will be held by a separate data linkage team working in the CORECT-R resource (REC no. 18/SW/0134) inside a Section 251 compliant area of the National Cancer Registration and Analysis Service in Public Health England. The linkage file will be used to identify relevant records detailing post-trial colonoscopy outcomes for trial participants in the BCSS reporting system termed OBIEE. If the CORECT-R resource eventually holds BCSS data, the relevant HES episodes and the details relating to causes of death from ONS mortality records will be linked inside CORECT-R. The research team will then have this pseudonymised information returned for linkage to the trial dataset (via the unique study identifier [the existing seAFOod Trial identifier codes based on trial site and participant number {XX/XXX}]) and subsequent analysis. In this way, the anonymity of trial participants will be completely protected (Figure 3). Only pseudonymised data will be used for external analysis by Co-Investigators in Sheffield (EW) and UCL (LB) and this will not include the BCSS data.

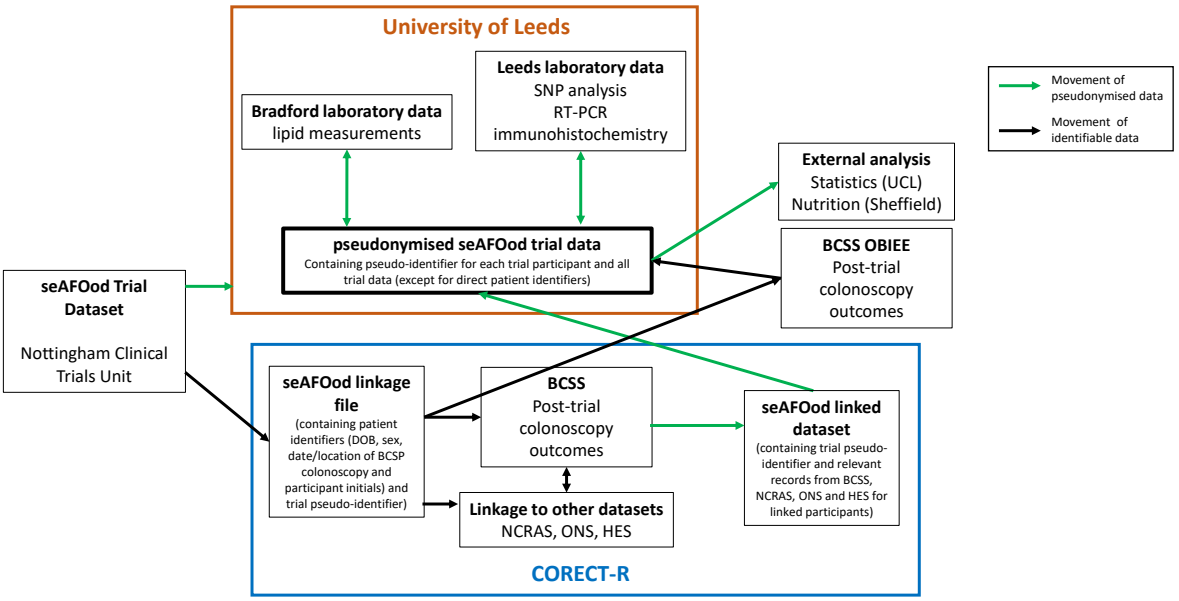


Figure 3: Data flow during STOP-ADENOMA. Identifiable data will be held in ISO27001 and DSPT compliant areas at both the University of Leeds and when in CORECT-R. In addition, CORECT-R has Section 251 approval to hold patient data without informed consent. All data-linkage using patient identifiers will be undertaken inside CORECT-R by approved individuals outwith the research team. In consequence, the trial dataset will always be fully anonymised to the research team. Until CORECT-R contains BCSS data, STOP-ADENOMA will access BCSS data directly from the BCSS reporting system OBIEE inside PHE.

Individual-level experimental data from Bradford and Leeds laboratories will be linked to treatment allocation and other clinical data eg. BMI, FFQs using the pseudonymised version of the trial database. Proof of principle for this approach has been obtained from the comprehensive PUFA analysis that has already been completed by the Bradford team, which was linked to the main trial database located in Nottingham, and which is now published[11].

Statistical analysis

Statistical analysis of the anonymised, linked clinical and laboratory database will be based on a pre-specified Statistical Analysis Plan (SAP) without prior inspection of linked baseline and outcome data. The SAP will be developed in LIDA with supervision from Investigator LB at University College, London. The SAP will be reviewed and updated at each study management group meeting in order to remain reactive to latest experimental findings and relevant literature, which may generate new testable hypotheses leading to new exploratory analyses. The SAP will be overseen by an independent Scientific Advisory Board (see *Project meetings and management*).

Plan for validation of the findings

It is important that new mechanistic insights leading to identification of a putative predictive or risk biomarker(s) are followed by validation studies. Although these would take place after the term of this proposal, the research team have important collaborations that will facilitate testing of prospective biomarkers using existing datasets and proposed randomised trials.

The COLO-PREVENT Polyp Prevention Trial (Cancer Research UK Clinical Research Committee full application – submitted June 2018) is a multicentre, open-label phase 2/3 trial

platform that, like the seAFOod polyp prevention trial, is embedded within the BCSP. In the main trial, the first proposed interventions to be tested against aspirin alone is metformin. A signal-seeking arm of the trial will simultaneously seek to gain proof of concept for resveratrol before this agent is considered for the main trial platform. There is a programme of blood and tissue sampling for pharmacokinetic/pharmacodynamic studies which will also be used to validate any findings from STOP-ADENOMA.

The Cancer Research UK-funded AsCaP Catalyst is focussed on understanding the MoA of the anti-cancer efficacy (prevention and treatment) as well as the bleeding risk, associated with aspirin. Collaboration with AsCaP will allow access to large trial sample collections for validation (eg. CAPP3 [NCT02497820], Add-aspirin [NCT02804815], ASPIRED [NCT02394769]).

Research expertise and roles of the team

There is a multi-disciplinary team of Investigators that includes the Chief Investigator of the seAFOod Trial (Professor Mark Hull, Leeds [MH]), an applied Biostatistician (Dr Louise Brown, UCL, London [LB]), an Epidemiologist with 'big data' expertise (Professor Eva Morris, Leeds [EM]) a Nutritionist (Dr Elizabeth Williams, Sheffield [EW]), an expert Endoscopist/Preventionist (Professor Colin Rees, Newcastle [CR]) and a Pharmacologist (Professor Paul Loadman, Bradford [PL]). The team are at the forefront of UK CRC screening and prevention research occupying key academic leadership roles.

There is no formal involvement from a CTU. Both Nottingham and Leeds CTUs have provided advice and both Units suggested that expertise in set-up and conduct of randomised trials was not required for STOP-ADENOMA, which will use a pre-formed biobank and trial database.

MH is the Lead Applicant and was CI of the seAFOod polyp prevention trial. He will take overall responsibility for the project along with LIDA, which will be responsible for the Information Governance surrounding the anonymised trial database and linkage to the laboratory data. MH is the Deputy Chair of the BCSP Research Advisory Committee.

LB will provide senior Biostatistician oversight aligned with her stratified medicine trial expertise, which includes a lead role in the FOCUS4, SCORT and Re-IMAGINE stratified oncology platforms.

EM is the Lead Investigator of the Cancer Research UK-funded CORECT-R programme, which aims to drive improvements in diagnosis and management of CRC through use of national-level outcomes data. She will provide academic oversight of data management for the project and will lead the work-stream focussed on post-trial colonoscopy and CRC outcomes. EM sits on the BCSP Research Advisory Committee.

EW provided nutritional input for the seAFOod polyp prevention trial, including initial analysis of the trial FFQ data in order to determine dietary omega-3 PUFA intake before and during the trial intervention. EW will provide expertise in assessment of omega-3 PUFA status linked to diet and genetic polymorphisms controlling PUFA fate.

CR is a Gastroenterologist and expert Endoscopist with an interest in multi-modal CRC prevention, particularly stratified approaches to endoscopic screening and surveillance. He was a member of the seAFOod Trial Management Group and remains invaluable for interpretation and prospective evaluation of any stratified chemoprevention approach in combination with colonoscopy that emerges from STOP-ADENOMA. He is the Chair of the NCRI CRC screening & prevention Clinical Studies Group. He is the Lead Investigator of the COLO-SPEED collaboration, which aims to use a network of endoscopy units and a consent-for-contact platform in order to accelerate clinical studies in CRC screening & prevention.

PL is a Pharmacologist with extensive experience of mass spectrophotometric measurement of lipids. He managed all aspects of sample collection, storage and QA testing at sites and for the central bio-repository in Bradford during the seAFOod polyp prevention trial. He will lead

on laboratory testing of bioactive lipid levels, as well as continuing oversight of the Biobank including sample transfer to Leeds for other laboratory analyses.

Although the seAFOod Polyp Prevention Trial benefited from patient and public involvement (PPI) throughout, the STOP-ADENOMA team includes a new Lead PPI representative in order to gain a fresh perspective on the trial results and potential impact of the findings from the proposed mechanistic research. The PPI Lead will also play an important role providing lay oversight of the information governance and data protection compliance within LIDA. STOP-ADENOMA will also benefit from oversight by the wider Patient and Public Group of Bowel Cancer Intelligence UK (https://bci.leeds.ac.uk/patient-public-group/), in which the CORECT-R repository is situated and of which the PPI Lead is already an established member.

Project meetings and management

There will be a monthly study management group teleconference, including the PPI representative, and junior staff based in Leeds and Bradford. There will be obligatory face-to-face meetings every 6 months in Leeds. The study will be overseen by a Scientific Advisory Board (SAB) of three members, consisting of expertise in CRC prevention, basic research into early stages of colorectal carcinogenesis, and applied bio-medical statistics. The SAB will meet at least six-monthly, providing oversight of the scientific strategy and any proposed changes to the exploratory analyses in the SAP.

Regulatory approvals

Although individual consent for use of trial data, long-term post-trial outcomes data and samples for all the proposed studies was provided by all trial participants as part of the informed consent provided at entry to the seAFOod Trial, this was dependent on future REC approval of the post-trial research. Therefore, this project is subject to new REC approval separate from the seAFOod Trial (Trent Research Ethics Committee 10/H0405/90).

The BCSP Research Advisory Committee approval for the work involving BCSP colonoscopy outcome data and its linkage to external datasets will be sought.

All the database linkages required will eventually be undertaken within the Cancer Research UK-funded Colorectal Cancer Data Repository (CORECT-R) led by EM, which exists inside PHE and exists to support the linkage, exploitation and enhancement of routine CRC data (see https://bci.leeds.ac.uk/). Within CORECT-R, many datasets including BCSS, NCRAS, and ONS are already linked and the seAFOod participants will be identified within the population-based data it contains using the approach described on page 27.

In the first instance, the post-trial colonoscopy outcome data will be identified directly in the BCSS reporting system termed OBIEE by PHE staff, for which PHE ODR approval will be sought. The de-identified data will be transferred to the master database inside University of Leeds. If CORECT-R contains BCSS data, separate REC and ODR approval may be obtained to perform linkage with other datasets including HES, ONS and NCRAS.

Tissue transfer agreements (TTAs) will be obtained from NHS Trusts in order to obtain FFPE polypectomy specimens from those Trusts who participated in the seAFOod Trial but who did not already complete a TTA.

Dissemination and Impact

The field of CRC chemoprevention has been held back by poor understanding of the MoA of candidate chemoprevention agents that are usually re-purposed drugs (aspirin [vascular prophylaxis], EPA [hypertriglyceridaemia]) and/or nutritional agents (omega-3 PUFAs, folate, vitamin D) with an existing excellent safety and tolerability profile, in which clinical efficacy assessment has outstripped understanding of MoA for the re-purposed indication. This has led to imprecision about potential clinical use of re-purposed agents including optimal dosing, the most appropriate target population and opportunities for combination treatment.

Recent publications have already highlighted the need for precision use of aspirin for vascular prophylaxis and cancer prevention based on age and body weight in order to maximise efficacy and minimise risk[43]. The seAFOod Trial has added the need to consider colorectal adenoma type and location when considering aspirin for CRC prevention.

The unique opportunity to utilise a rich and high quality-assured trial biobank to understand MoA of two agents which have clinical efficacy alone and in combination will have immediate impact in several domains; it will support aspirin-EPA as an exemplar drug-nutrient interaction, it will bolster the precision medicine approach to CRC chemoprevention, which was highlighted by the primary seAFOod trial report[11], and it will drive prospective validation and evaluation of therapeutic prediction biomarkers for aspirin use and EPA intake (supplement use or diet).

Lipid biomarker analyses will be performed to Good Clinical Laboratory Practice (GCLP) standards in Bradford, thus facilitating translation of any promising risk or therapeutic prediction biomarker(s) towards clinical use via validation in independent cohorts (see *Plan for validation of the findings*).

Although fatty acid levels were measured in RBC membranes in the seAFOod trial[11], there is increasing use of the whole blood spot for omega-3 PUFA monitoring, which has clear patient acceptability and cost advantages for fatty acid profiling in routine practice[44]. Future impact from STOP-ADENOMA about the predictive value of fatty acid levels will incorporate a switch to whole blood omega-3 PUFA measurements.

We aim to safeguard a legacy for the seAFOod Trial biobank as a unique sample set from a CRC chemoprevention trial, which will be discoverable for other scientists in the future. Access to the finite amount of residual rectal mucosa will be limited after we have completed our proposed studies but total RNA/cDNA from rectal mucosa will be made available for other researchers, as will gDNA for genomic studies, whenever possible. This is likely to be feasible through the NIHR National Biosample Centre when the study is adopted to the COLO-SPEED collaboration, which aims to use the NIHR Biosample centre to produce a sustainable CRC prevention bioresource.

Translation of chemoprevention efficacy results into effectiveness testing and the clinic is challenging. However, STOP-ADENOMA is timely given the momentum that a stratified approach to CRC screening, prevention and early diagnosis has achieved through COLO-

SPEED in an attempt to reduce the burden on endoscopic services in the UK. The COLO-SPEED collaboration has strong links to existing groups such as the UK Therapeutic Cancer Prevention Network and Independent Cancer Patient's Voice. The collaborative network-based approach linking to key decision-makers, in parallel with peer-reviewed publication, will maximise the ability of this research to change understanding and practice.

The use of chemoprevention as a CRC prevention strategy *in combination with* endoscopic surveillance of individuals at elevated risk of future colorectal neoplasia is highly topical at present given the likely adoption of increased colonoscopic surveillance intervals (from one to three years for 'high risk' individuals and three to five years for 'intermediate risk' individuals) in an effort to reduce strain on endoscopy services, and increased focus on PCCRCs, which are likely to increase, as a consequence.

We have already produced Plain English summaries of the seAFOod Trial results for participants and recruiting site staff. Similar summaries will be produced for those involved in the Trial and these can be used for wider out-reach.

We will engage with the University of Leeds Research and Innovation Service (RIS), which includes the technology transfer office. We will invite the RIS Commercialisation Team to study management group meetings at 6 monthly intervals in order to appraise RIS about potential intellectual property (IP) and commercialisation opportunities requiring due diligence leading to protection and management of the IP.

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