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Original research article

Plasma and rectal mucosal oxylipin levels during aspirin and eicosapentaenoic acid treatment in the seAFOod polyp prevention trial

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ABSTRACT

Background: Aspirin and eicosapentaenoic acid (EPA) have colorectal polyp prevention activity, alone and in combination. This study measured levels of plasma and rectal mucosal oxylipins in participants of the seAFOod 2 × 2 factorial, randomised, placebo-controlled trial, who received aspirin 300 mg daily and EPA 2000 mg free fatty acid, alone and in combination, for 12 months.

Methods: Resolvin (Rv) E1, 15-epi-lipoxin (LX) A_4 and respective precursors 18-HEPE and 15-HETE (with chiral separation) were measured by ultra-high performance liquid chromatography-tandem mass spectrometry in plasma taken at baseline, 6 months and 12 months, as well as rectal mucosa obtained at trial exit colonoscopy at 12 months, in 401 trial participants.

Results: Despite detection of *S*- and *R*- enantiomers of 18-HEPE and 15-HETE in ng/ml concentrations, RvE1 or 15-epi-LXA_4 were not detected above a limit of detection of 20 pg/ml in plasma or rectal mucosa, even in individuals randomised to both aspirin and EPA. We have confirmed in a large clinical trial cohort that prolonged (12 months) treatment with EPA is associated with increased plasma 18-HEPE concentrations (median [interquartile range] total 18-HEPE 0.51 [0.21–1.95] ng/ml at baseline *versus* 0.95 [0.46–4.06] ng/ml at 6 months [$P<0.0001$] in those randomised to EPA alone), which correlate strongly with respective rectal mucosal 18-HEPE levels ($r = 0.82$; $P<0.001$), but which do not predict polyp prevention efficacy by EPA or aspirin.

Conclusion: Analysis of seAFOod trial plasma and rectal mucosal samples has not provided evidence of synthesis of the EPA-derived specialised pro-resolving mediator RvE1 or aspirin-trigged lipoxin 15-epi-LXA_4. We cannot rule out degradation of individual oxylipins during sample collection and storage but readily measurable precursor oxylipins argues against widespread degradation.

1. Introduction

There is evidence that aspirin and the omega-3 polyunsaturated fatty acid (PUFA) C20:5*n*-3 eicosapentaenoic acid (EPA) have chemopreventative activity against colorectal cancer (CRC) [1,2]. However, the mechanism(s) of the anti-CRC activity of both agents remains unclear with several cyclooxygenase (COX)-dependant and -independent mechanisms being described, based largely on data from *in vitro* CRC cell studies and rodent CRC models [1,2].

A unifying hypothesis is that aspirin and EPA both inhibit COX-1- and COX-2-dependent synthesis of the pro-tumorigenic prostaglandin (PG) PGE_2 [1,2]. However, aspirin and EPA modify the activity of the COX isoforms in different ways [3]. Aspirin acetylates a serine residue in the active site of COX-1 and COX-2 leading to complete and irreversible inhibition of synthesis of the PG intermediate PGH_2 by COX-1, but alteration of COX-2 activity [4]. Acetylation of COX-2 results in inhibition of PGH_2 production, but also increased synthesis of an alternative hydroxylated 20-carbon oxylipin 15-hydroxyeicosatetraenoic acid

(HETE) (if omega-6 PUFA C20:4*n*-6 arachidonic acid [AA] is the COX substrate), with a chiral switch favouring the *R*-enantiomer over the usual *S*-configuration [4]. By contrast, EPA can displace AA from the plasma membrane substrate pool and acts as a reversible inhibitor of COX-1 activity. Eicosapentaenoic acid also acts as an inefficient alternative substrate for COX-2, leading to reduction in PGH_2, and hence PGE_2, synthesis, in favour of generation of a smaller amount of the equivalent 3-series PG, PGE_3 [3]. Furthermore, if EPA is the substrate for aspirin-acetylated COX-2, instead of AA, the equivalent omega-3 mono-hydroxylated oxylipin to 15-HETE, 18-hydroxyeicosapentaenoic acid (HEPE), is produced with a similar chiral switch favouring the *R*-enantiomer [5]. Both 15*R*-HETE and 18*R*-HEPE can act as substrates for other mono-oxygenases in a *cis*- or *trans*-cellular manner leading to formation of tri-hydroxylated derivatives 5*S*,6*R*,15*R*-trihydroxyeicosatetraenoic acid (also known as 15-epi-lipoxin [LX] A_4 or aspirin-triggered LX [ATL]) and 5*S*,12*R*,18*R*-trihydroxyeicosapentaenoic acid (also known as resolvin [Rv] E1) [4,5].

There is some evidence that 15-epi-LXA_4 and other ATLs have anti-tumorigenic properties *in vitro* [6], although the relevance of ATLs for the anti-CRC activity of aspirin *in vivo* is unclear [7]. Resolvin E1 and other so-called specialised pro-resolving mediators derived from EPA and the omega-3 PUFA C22:6*n*-3 docosahexaenoic acid (DHA) are believed to play a role in resolution of inflammation [8] and have anti-cancer properties in rodent models [6,9]. Resolvin E1 inhibits signalling by nuclear factor (NF) κB, a pivotal transcription factor controlling inflammation-associated carcinogenesis, and inhibits tumour formation [10,11]. In addition, there is some evidence that 18-HEPE may possess direct anti-cancer activity *per se*, although the downstream signalling pathway(s) has not been delineated and conversion to other bioactive oxylipins has not been ruled out [12].

The seAFOod polyp prevention trial tested the CRC chemopreventative activity of aspirin 300 mg daily and EPA 2000 mg free fatty acid equivalents daily *versus* placebo for 12 months in individuals aged 55–73 years with 'high risk' colorectal polyp findings (≥3 polyps, if one polyp was ≥10 mm; or ≥5 polyps of any size) at a screening colonoscopy using a 2 × 2 factorial design [13,14]. The primary 'at the margins' analysis compared aspirin and EPA separately against respective placebo groups, assuming no interaction between the two interventions [13,14]. Aspirin use was associated with a significant reduction in total colorectal polyp risk (measured as mean total polyp [including adenomatous and serrated polyps] number per participant) one year after clearance colonoscopy compared with placebo [13,14]. By contrast, EPA use was not associated with reduced total colorectal polyp risk but was associated with a statistically significant reduction in risk of left-sided (distal to the splenic flexure) adenomatous polyps [13,14]. However, it was also apparent from the analysis of the four individual treatment groups that individuals who were allocated both aspirin and EPA had fewer total and left-sided colorectal polyps compared with the other three treatment groups [13,14]. Subsequent *post-hoc* analysis confirmed that the incidence rate ratio for total colorectal polyps in the combined aspirin and EPA treatment arm was significantly lower than for the three other treatment arms [15].

Multiple clinical studies in a variety of health and disease settings have produced divergent observations regarding detection of RvE1 and 15-epi-LXA_4 in human blood and target tissues [7,9,16] and the role of these oxylipins in the anti-CRC activity of aspirin and EPA (and possible combination therapy) remains unclear [9]. Therefore, we measured levels of RvE1, 15-epi-LXA_4, as well as their respective precursors 18-HEPE and 15-HETE, in plasma and rectal mucosa in seAFOod trial participants before and during the trial intervention, in order to investigate whether increased levels of these oxylipins were associated with aspirin and EPA treatment and whether plasma levels of 18-HEPE were predictive of chemopreventative activity of EPA, alone and in combination with aspirin.

2. Methods

2.1. The seAFOod polyp prevention trial biobank

This study is part of a wider programme of investigations using the seAFOod trial biobank and post-trial English Bowel Cancer Screening Programme (BCSP) colonoscopy outcomes called STOP-ADENOMA (ISRCTN05926847). Ethical approval for this study was granted by London and Surrey Borders Research Ethics Committee (19/LO/1655). The seAFOod Trial biobank has been described elsewhere [14]. Biological samples were obtained, processed and stored as described in the Trial Laboratory Manual and detailed Trial report [14,17]. First participant, first visit was 11th November 2011 and last participant, last visit was 10th June 2017 [14].

2.2. Plasma and rectal mucosal sample collection and storage

In brief, a venous blood sample was obtained using a tourniquet at randomization (visit 1; at least 7 days after a BCSP screening colonoscopy), at trial visit 4 six months (± two weeks) later, and within two hours after the trial exit colonoscopy (first BCSP surveillance colonoscopy), which was 12 months after the screening procedure (visit 6). Participants were asked to stop Investigational Medicinal Product (IMP) the day before the visit 6 colonoscopy.

Blood was drawn directly into an EDTA tube, which was centrifuged (1000 RCF for 5 min) at 4°C after storage for a maximum of 30 min at 4°C. The plasma layer was transferred to separate cryovials for immediate storage locally at -20°C (for a maximum of 12 weeks) before transportation on dry ice to the central Trial Biobank (Institute of Cancer Therapeutics, University of Bradford) for long-term storage at -80°C. No sample underwent more than one freeze-thaw cycle before lipid extraction.

Four rectal biopsies were obtained from macroscopically normal mucosa at least 2 cm from a polypectomy site using 6 mm jaw forceps at the end of the trial exit colonoscopy (visit 6). Tissue was immediately placed in a -20°C freezer in two separate cryovials, before transportation to and storage at the Trial Biobank as above.

2.3. Laboratory methods

Plasma and rectal mucosal oxylipins were analysed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Authentic standards for 15*S*-HETE, 15*R*-HETE, 18*S*-HEPE, 18*R*-HEPE, 15*S*-lipoxin A_4 (LXA_4), 15*R*-lipoxin A_4 (15-epi-LXA_4), and (18*R*)-resolvin E1 were obtained from Cayman Chemical Co. (Ann Arbour, MI, USA). Deuterated 15*S*-HETE-d8 and RvE1-d4 were also obtained from Cayman Chemical Co. for use as an internal standard (IS).

Analytes were extracted from plasma and rectal mucosa using a solid-phase extraction (SPE) technique. Plasma samples were defrosted from -80 °C storage at room temperature before centrifugation at 5000 RCF. Plasma (500 µl) was added to 10 µl of each IS, and 250 µl 1% (v/v) acetic acid, prior to vortex mixing. Solid-phase extraction cartridges (Bond Elut C18, 500 mg, 3 ml; Agilent Technologies, UK) were pre-conditioned with 3 ml methanol followed by 3 ml acidified water (pH 3, acetic acid). The sample was applied to the cartridge, which was washed step-wise with 6 ml 15% methanol, 6 ml acidified water (pH 3) and 3 ml heptane. Analytes were eluted in 1 ml ethyl acetate and the eluate was evaporated to dryness using a Genevac EZ-2 evaporation system.

Rectal mucosa biopsy samples were kept on wet ice while a maximum two biopsies per participant were weighed (range 1.07–16.59 mg) before transfer to 2 ml reinforced tubes pre-filled with approximately six 1.4 mm ceramic beads, 10 µl each IS and 100 µl 1% (v/v) acetic acid. Biopsies were homogenised using an Omni Bead Ruptor 24 Bead Mill Homogeniser (Camlab, UK) and then extracted using a Bond Elut C18 (100 mg, 1 ml) SPE cartridge with the same, but smaller volume, washes. Analytes were similarly eluted in 1 ml ethyl acetate before

evaporation to dryness as above.

Plasma analyte samples were reconstituted in 50 μl 50% mobile phase (MP)A:50% MPB (composition of each MP described below). Biopsy analyte samples were reconstituted in 30 μl 50% MPA:50% MPB. The volume of all samples injected into the UPLC-MS/MS system was 5 μl.

UPLC-MS/MS analysis was performed on an Acquity H—Class UPLC system linked with a Xevo TQ-XS (Waters Corp, Milford, USA) tandem quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. To allow identification of chiral analytes, UPLC separation was achieved using an Acquity UPC2 Trefoil AMY1 column, 2.1 × 150 mm, 2.5 μm (Waters Corp) with a MPA of 0.2% (v/v) acetic acid, and MPB of 90:10 acetonitrile:isopropanol. Initial conditions of 45% MPA:55% MPB were held for 1 min, before the proportion of MPB was increased linearly to 65% at 5 min, then to 78.6% at 15 min, then back to initial conditions. Sample injection was every 30 min to allow for column re-equilibration to the start conditions. The flow rate was 300 μl/min. The Xevo mass spectrometer was operated in negative electrospray ion mode with a capillary voltage of 2.5 kV, cone voltage of 20 V, source temperature of 150 °C, and desolvation gas flow of 600 l/hr. Collision energies for all analyte MRM channels were established between 11 V and 18 V following method optimisation. MRM channels were set as 15S- and 15R-HETE m/z 319>175, 319>219; 18S- and 18R-HEPE m/z 317>215, 317>259; LXA$_4$ and 15-epi-LXA$_4$ m/z 351>115, 351>217; RvE1 m/z 349>161, 349>195; 15S-HETE-d8 m/z 327>182, 327>226; and RvE1-d4 m/z 353>162, 353>197. Distinct retention times for each R- and S- enantiomer were characterised during method optimisation.

15-HETE and 18-HEPE enantiomers were quantified against 15S-HETE-d8 with the calibration range 0.05–100 ng/ml. 15-LXA$_4$ epimers and RvE1 were quantified against RvE1-d4 with the calibration range 5–1000 pg/ml. Values were adjusted for biopsy wet weight to enable quantification against the IS. The limit of detection (LOD) for 15-HETE and 18-HEPE enantiomers was 10 pg/ml. The LOD for RvE1 and both 15-LXA$_4$ epimers was 20 pg/ml. The limit of quantification (LOQ) for all analytes was set at 50 pg/ml.

Red blood cell membrane fatty acid levels were previously measured by LC-MS/MS as described and were reported as% of total fatty acids [13,14].

2.4. Statistical analysis

Plasma oxylipin levels are reported as the concentration (ng/ml) and rectal mucosal oxylipin levels are reported as ng/mg wet weight of tissue.

Baseline (pre-treatment) plasma oxylipin levels were not normally distributed and were not normalized by log-transformation. Therefore, plasma oxylipin levels, which are reported as the median value and interquartile range (IQR), were compared according to clinical characteristics using non-parametric Mann-Whitney and Kruskal-Wallis tests for binary and multiclass variables, respectively.

Treatment effects on plasma and rectal mucosal oxylipin levels are described according to the trial treatment group allocation (placebos only, EPA alone, aspirin alone, and combination EPA and aspirin), to allow comparison of each individual intervention with combined EPA and aspirin therapy. For treatment effects, we report the levels of the S- and R-enantiomers of 15-HETE and 18-HEPE, as well as the total value (as the plasma ng/ml concentration or tissue ng/mg level). The ratio of the level of R- and S-enantiomers of each oxylipin is also reported.

Data are presented as unpaired plasma oxylipin values for each treatment group, according to trial visit, as paired data per participant in each treatment group, and as the V4/V1 level and V6/V1 level ratio, in order to display inter- and intra-individual variation in plasma oxylipin levels. In each case, box & whisker plots with logarithmic y axes were employed to display the distribution of plasma concentrations, including outlier values. Statistical comparisons of treatment effects were carried out using the Mann-Whitney test.

The relationship between the rectal mucosal level and the equivalent (visit 6) plasma level of individual oxylipins was investigated by the Pearson test.

The relationship between the plasma total 18-HEPE or 18R-HEPE level during treatment (visit 4) and total or left-sided colorectal polyp number at the seAFOod trial exit colonoscopy was investigated using a negative binomial generalized linear model (GLM), including the visit 4 18-HEPE level, sex, repeat colonoscopy at trial baseline (as per the primary seAFOod trial analysis), allocation to aspirin treatment (yes/no) and EPA treatment (either yes/no, or a continuous variable as intake per day [in grams] calculated using capsule compliance data from the seAFOod trial) as variables.

3. Results

Six hundred and seventy-six trial participants provided at least one plasma sample during seAFOod trial participation. One hundred and fifty-eight participants provided no visit 1 sample, or a visit 1 sample only, and were excluded. Only 30/138 plasma samples from the placebos-only group were analysed due to time and financial constraints (Table 1). Four visit 6 samples were excluded due to a temperature violation at a single research site. An additional five sets of V1-V4-V6 data, were excluded due to one or more analytical failures, resulting in a plasma oxylipin cohort of 401 trial participants (Table 1). This study population, from which one or more plasma oxylipin values were measured, had similar clinical characteristics to the overall seAFOod trial population, with the participants from the four randomised trial treatment arms, who contributed plasma oxylipin data, continuing to be well-matched for age, sex, body mass index (BMI) and tobacco smoking status (Supplementary Table 1).

3.1. Pre-treatment plasma oxylipin levels in seAFOod trial participants

Representative chromatograms for all analytes are available as Supplementary Data. No pre-treatment plasma samples had detectable RvE1, LXA$_4$ or 15-epi-LXA$_4$ above the LOD of 20 pg/ml. The median pre-treatment plasma total 15-HETE level was 1.02 (IQR 0.51, 3.15) ng/ml with a lower plasma total 18-HEPE level of 0.21 (IQR 0.10, 0.72) ng/ml (both $n = 400$). Plasma total 15-HETE and 18-HEPE levels according to sex, body mass index (BMI), diagnosis of diabetes, tobacco smoking history and alcohol use are described in Table 2. In this cohort of middle-aged individuals who had recently undergone clearance colonoscopy for multiple colorectal polyps, there was no statistically significant difference in plasma 15-HETE or 18-HEPE levels related to clinical characteristics, although a trend towards higher levels with increasing excess body weight was apparent (Table 2).

3.2. Plasma oxylipin levels during treatment with EPA and aspirin, alone and in combination, for 12 months

No on-treatment plasma sample from any of the treatment groups, including the combination EPA and aspirin group, had any detectable RvE1, LXA$_4$ or 15-epi-LXA$_4$ above the LOD at either 6 months (visit 4) or 12 months (visit 6). Based on current understanding of RvE1 synthesis,

Table 1

The number of plasma samples subjected to oxylipin analysis per seAFOod trial treatment allocation.

	placebos only	EPA	aspirin	EPA + aspirin	total
V1 (pre-treatment)	30[a]	114	126	130	400[b]
V4 (6 months)	30	114	126	131	401
V6 (12 months)	29	103	108	111	351

[a] Samples from only 30 of a possible 138 individuals who received placebos only were analysed.

[b] One visit 1 sample was lost during lipid extraction.

Table 2

Plasma total 15-HETE and 18-HEPE levels in seAFOod trial participants at baseline before intervention.

	n	15-HETE (ng/ml)[1]	P[2]	18-HEPE (ng/ml)[1]	P[2]
Sex					
Male	325	1.02 (0.52,3.14)	0.69	0.21 (0.10,0.76)	0.57
Female	75	1.06 (0.49,3.51)		0.20 (0.10,0.62)	
BMI					
Normal (<25 Kg/m^2)	65	0.90 (0.48,1.99)	0.10	0.17 (0.09, 0.65	0.25
Overweight (≥25 and <30 Kg/m^2)	134	0.98 (0.50,2.66)		0.20 (0.10,0.60)	
Obese (≥30 Kg/m^2)	120	1.24 (0.59,4.31)		0.28 (0.10,1.00)	
Diabetes					
No	363	1.02 (0.51,3.14)	0.78	0.21 (0.10,0.70)	0.91
Yes	37	1.03 (0.57,2.37)		0.20 (0.10,0.89)	
Tobacco smoking					
Never	144	1.00 (0.49,3.46)	0.60	0.22 (0.10,1.02)	0.43
Ever	197	0.98 (0.50,2.95)		0.19 (0.09,0.62)	
Current	59	1.19 (0.76,3.41)		0.28 (0.13,0.61)	
Alcohol intake					
None	62	1.22 (0.49,4.61)	0.21	0.23 (0.10,0.33)	0.15
1–7 units/week	136	1.02 (0.56,3.58)		0.10 (0.05,0.48)	
8–21 units/week	119	0.86 (0.45,2.77)		0.16 (0.08,0.61)	
≥22 units/week	82	1.27 (0.70,2.56)		0.28 (0.12,0.62)	

[1] Data are the median value and inter-quartile range.
[2] Wilcox test (two group comparison) or Kruskal-Wallis test (more than two group comparison).

we predicted that individuals who were randomised to combination EPA and aspirin treatment would be most likely to generate detectable levels of RvE1 [5,16]. In order to exclude potential confounding effects of poor compliance with EPA and/or aspirin, we identified a group of 10 participants from the combination treatment arm, who all had a visit 4 red blood cell (RBC) percentage EPA level of >3% (indicative of high EPA capsule compliance and cellular bioavailability) and had a low level (<50 pg/mg creatinine) of urinary 11-dehydro-thromboxane B$_2$ (confirming COX inhibition by aspirin) [18]. We confirmed that none of R- or S- RvE1, or LXA$_4$ or 15-epi-LXA$_4$ were detectable in either visit 4 or visit 6 samples, even in participants with objective biomarker evidence of combined EPA and aspirin use.

Although RvE1, or either 15-LXA$_4$ epimer were undetectable in plasma from the seAFOod trial biobank, the respective precursor oxylipins 18S- and 18R-HEPE, as well as 15S- and 15R-HETE, were detectable in ng/ml amounts in plasma (Supplementary Table 2) with wide inter-individual (Fig. 1A and 1B) and intra-individual (Fig. 2A and 2B) variability over time across all the placebo and active treatment groups. Overall, treatment with EPA was associated with an increase in plasma 18-HEPE concentration (Fig. 1A and 2A). The treatment effect of EPA at 12 months (visit 6) was less than that at 6 months (visit 4).

Participants were asked to stop IMP on the day before the trial exit colonoscopy. However, a few participants underwent blood sampling following the trial exit colonoscopy. Therefore, we performed a sensitivity analysis including only individuals who underwent blood sampling on the day of colonoscopy. This demonstrated a similar decrease in treatment effect at 12 months compared with 6 months (data not shown), ruling out prior cessation of treatment as an explanation for a drop-off in treatment effect. Capsule and tablet compliance was excellent during the seAFOod trial [14], but we cannot rule out a reduction in IMP compliance approaching the end of the trial intervention period before colonoscopy as an explanation for a lower treatment effect on 18-HEPE levels at visit 6 compared with visit 4. However, we believe that this is unlikely based on similar RBC % EPA levels at visit 6 compared with visit 4 [13,14].

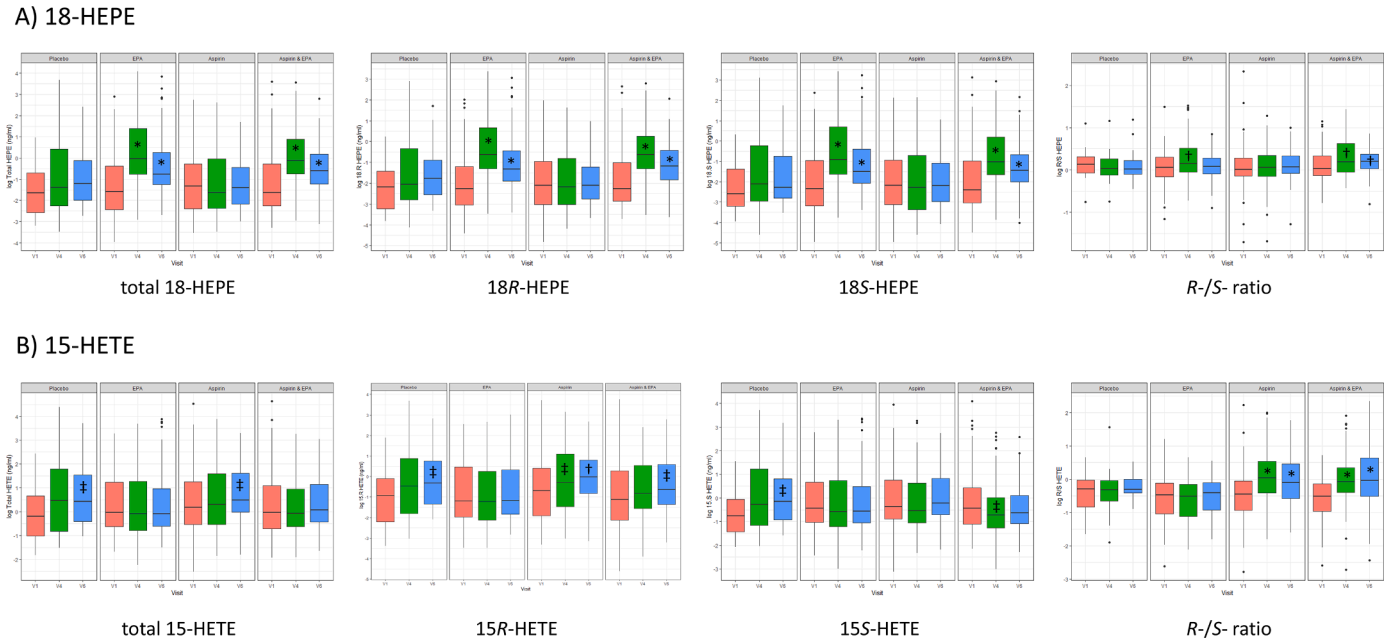Chiral analysis revealed that the increase in plasma 18-HEPE

A) 18-HEPE

B) 15-HETE

Fig. 1. Plasma 18-HEPE and 15-HETE concentrations at baseline (visit 1), during treatment (6 months; visit 4) and at the end of the intervention phase (12 months; visit 6) in seAFOod trial participants according to treatment group. Data are shown as box (median, 25th and 75th centile) & whisker (1.5 x IQR) plots with individual outlier values above/below 1.5 x IQR. For 18-HEPE and 15-HETE, the respective concentrations of the R- and S-enantiomers are shown in addition to the R-/S- ratio. Statistical comparisons with baseline (visit 1) values were performed using the Mann-Whitney test. *$P<0.0001$, †$P<0.01$, ‡$P<0.05$ for the comparison with visit 1 levels.
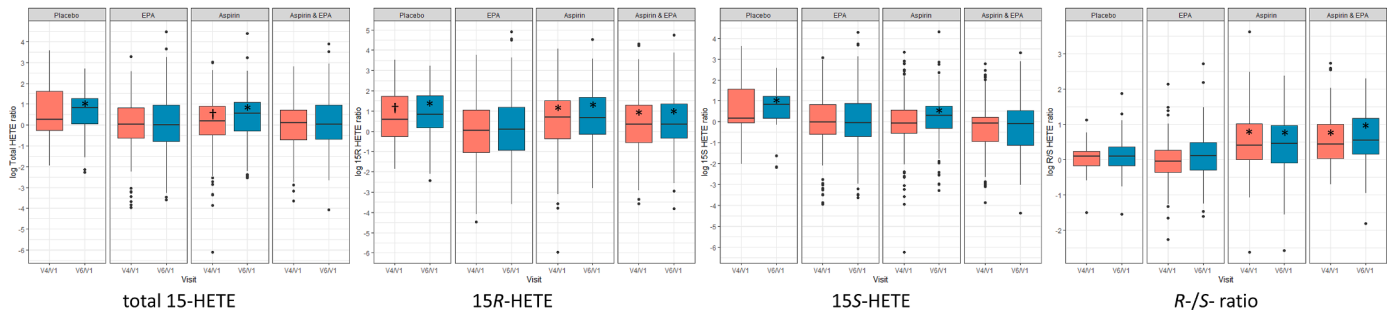
A) 18-HEPE
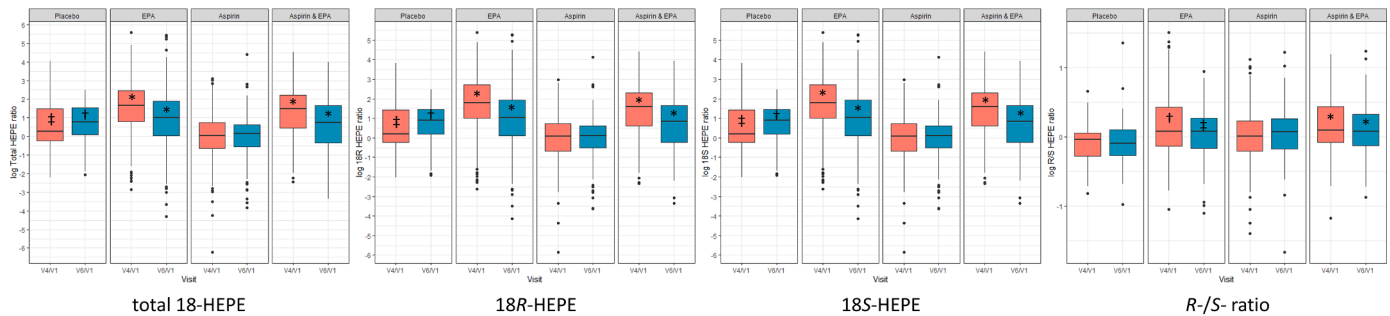
B) 15-HETE

Fig. 2. The ratio of plasma 18-HEPE and 15-HETE concentration at 6 months (visit 4) and at 12 months (visit 6) compared with the baseline, pre-treatment value (visit 1) in seAFOod trial participants according to treatment group. Data are shown as box (median, 25th and 75th centile) & whisker (1.5 x IQR) plots with individual outlier values above/below 1.5 x IQR. For 18-HEPE and 15-HETE, the respective concentrations of the *R*- and *S*-enantiomers are shown in addition to the *R*-/*S*- ratio. Statistical significance of the difference between the visit 4/visit 1 and visit 6/visit 1 ratios and unity (no change from baseline) was tested using the one-sample Wilcoxon rank sum test. *$P<0.0001$, †$P<0.01$, ‡$P<0.05$ for the difference from a ratio value = 1 (no change in plasma level compared with baseline).

concentration was explained by an increase in both *R*- and *S*-enantiomers of 18-HEPE (Figs. 1A and 2A), with a small, but statistically significant, increase in the relative amount of 18*R*-HEPE in the unpaired group (Figs. 1A and 2A).

We were also interested to determine the relationship between the plasma 18-HEPE concentration and the corresponding % EPA level in RBC membranes before (visit 1) and during the intervention phase of the trial at visit 4. Before trial intervention, there was a weak but statistically significant negative correlation between plasma total 18-HEPE concentration and the % EPA content of RBC membranes ($r = $ -0.27, $P<0.001$) in keeping with plasma membrane EPA mobilization leading to 18-HEPE synthesis (Fig. 3A). A similar relationship was observed during the intervention period with the relationship between plasma total 18-HEPE concentration and % RBC EPA content being maintained after EPA supplementation for 6 months ($r = $ -0.29; $P<0.001$; Fig. 3B).

Concurrent aspirin use was not associated with increased 18-HEPE levels compared with EPA treatment alone, nor with a higher *R*-/*S*-ratio than that observed with EPA treatment alone (Figs. 1A and 2A). Aspirin treatment did not increase plasma 18-HEPE levels in the absence of EPA supplementation (Figs. 1A and 2A).

However, aspirin treatment alone was associated with an increase in plasma 15-HETE concentration, which was abrogated by concurrent EPA supplement use (Figs. 1B and 2B). In contrast to treatment effects on the chiral ratio of 18-HEPE enantiomers, aspirin treatment was associated with a significant *S*- to *R*- switch in plasma 15-HETE (Figs. 1B and 2B). Interestingly, there was a significant increase in plasma 15-HETE concentration at visit 6 in individuals allocated placebos only, but no change in *R*-/*S*- ratio (Figs. 1B and 2B), perhaps associated with the colonoscopy preparation, or the procedure itself, driving non-acetylated-COX-dependant AA metabolism [19].

3.3. Rectal mucosal oxylipin levels during treatment with EPA and aspirin, alone and in combination, for 12 months

Rectal mucosa was available for oxylipin measurement from the colonoscopy at visit 6 after 12 months of trial intervention. A random sample of ten rectal mucosal samples was chosen from each treatment group for comparison of oxylipin levels with the corresponding visit 6 plasma sample.

In keeping with the plasma findings, neither RvE1, LXA₄, nor 15-epi-LXA₄, were detected in rectal mucosa in any of the treatment groups, including the combined EPA and aspirin trial treatment arm.

There was a statistically significant, strong correlation between the corresponding plasma and rectal mucosa level of 18-HEPE ($r = $ 0.82, $P<0.001$) and 15-HETE ($r = $ 0.60, $P<0.001$; Figs. 4A and 4B).

3.4. Does the plasma 18-HEPE concentration predict reduction in colorectal polyp risk associated with EPA treatment?

Although it is not clear whether 18-HEPE has direct anti-cancer activity [12], a recent report has described a negative correlation between the plasma and colonic tissue 18-HEPE level and colon tumour multiplicity in Pirc rats that had been treated with a stable EPA analogue (TP-252), with or without the non-steroidal anti-inflammatory drug naproxen [20]. Therefore, we explored the relationship between the plasma total 18-HEPE concentration at visit 4 (mid-treatment) and the colorectal polyp number recorded at the seAFOod trial exit colonoscopy. We generated separate models for total colorectal polyp number and left-sided (at or distal to the splenic flexure) colorectal polyp number, given that statistical significance for reduction in colorectal polyp number was only attained for left-sided colorectal polyps for EPA treatment in the seAFOod trial [1,2]. We also used the same multivariate model adding plasma 18R-HEPE level as a variable on the basis that the *R*-enantiomer is believed to be the precursor for RvE1 synthesis [5].

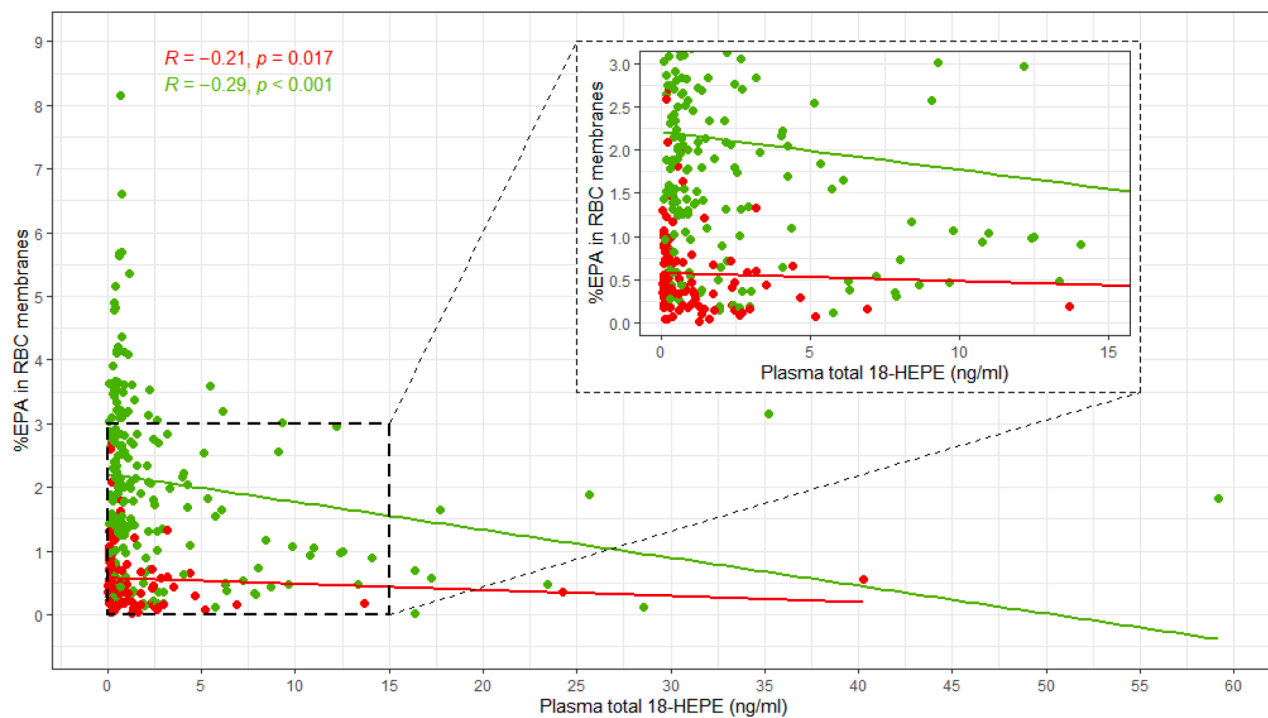In the negative binomial model for total colorectal polyp number,

A)
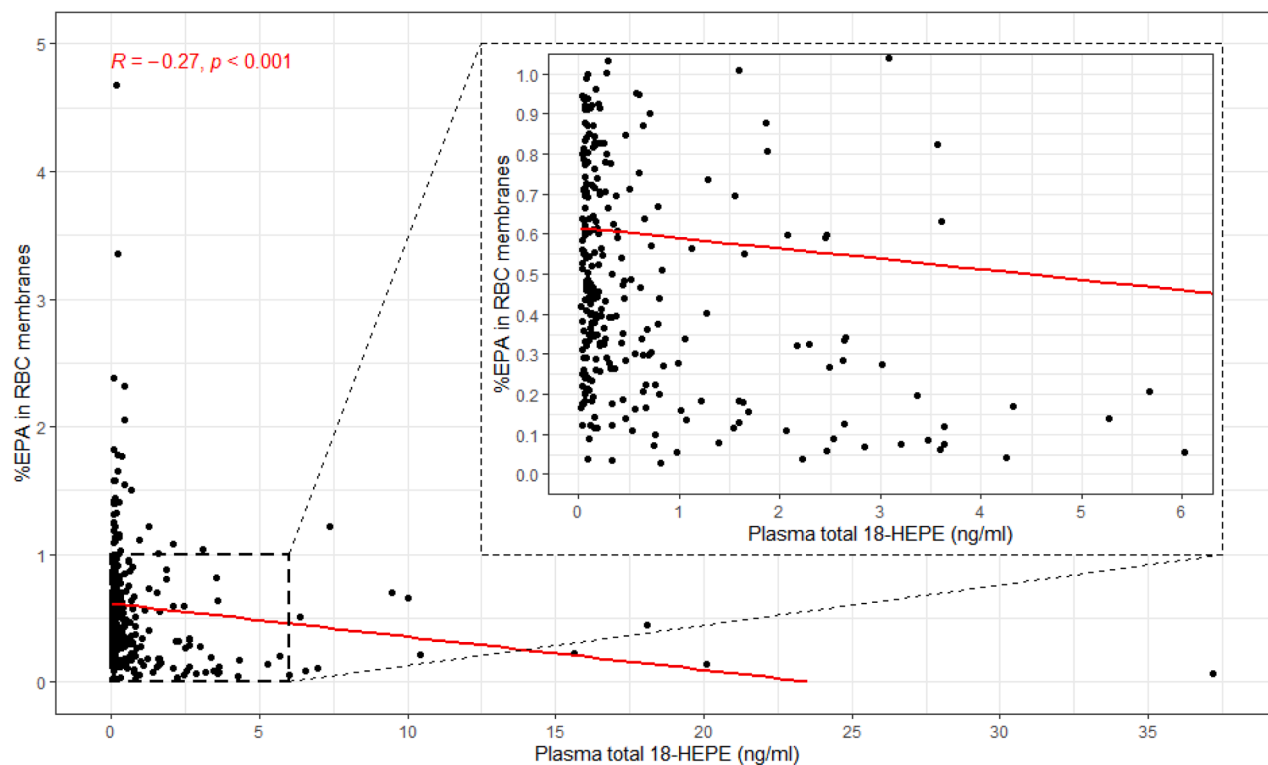
B)

Fig. 3. The relationship between the plasma total 18-HEPE concentration and % red blood cell EPA content in seAFOod trial participants. A) Baseline (visit 1) and B) after 6 months on treatment (visit 4) stratified by randomization to active EPA (green) or no EPA (red).
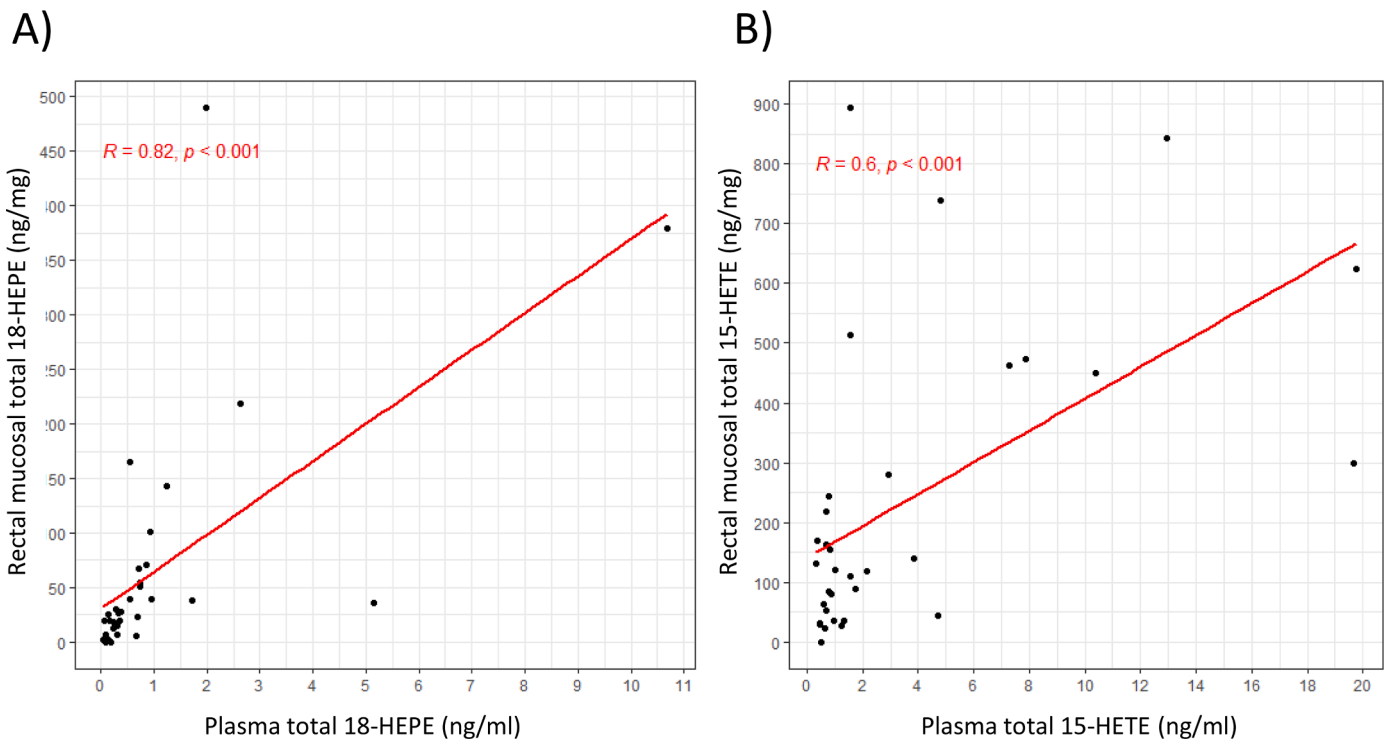
Fig. 4. The relationship between plasma oxylipin concentration and corresponding rectal mucosal oxylipin level at the end of the intervention period (12 months). A) Total 18-HEPE, and B) Total 15-HETE. Data points represent individual participants ($n = 30$) regardless of treatment allocation. R = Pearson's r value and P value.

neither plasma total 18-HEPE or 18*R*-HEPE levels predicted total colo-rectal polyp number (Table 3) or left-sided colorectal polyp number (data not shown) at the end of the treatment phase in the seAFOod trial. The only significant factor predicting reduced colorectal polyp number was randomization to active aspirin treatment in the seAFOod trial, in keeping with the primary finding of the trial (Table 3). Use of the EPA treatment variable as a binary treatment allocation, or as a continuous intake variable taking into account capsule compliance during the seAFOod trial (calculated as the mean EPA supplementation per day in grams), did not alter the GLM outcomes (data not shown).

4. Discussion

We did not detect RvE1 or either epimer of LXA$_4$ in plasma in any baseline or on-treatment sample from seAFOod trial participants above the LOD (20 pg/ml) for our LC-MS/MS method. Detection of pro-resolving oxylipin mediators in human plasma and serum, as well as a variety of other body fluids and tissues, is highly variable and is likely to be dependent on the sample collection protocol and assay methodology [16]. Despite careful quality assurance for blood sampling in our multi-site clinical trial [14], we cannot rule out that initial storage at -20°C and then prolonged storage of samples at -80°C (approximately 4–9 years) did not lead to degradation of these low abundance analytes. However, the precursor oxylipins 18-HEPE and 15-HETE were present in

plasma at concentrations observed in other studies [21–27], including those that also detected RvE1 [26,27], ruling out significant generalized oxylipin degradation over time. A study of oxylipin stability in human plasma EDTA samples concluded that a two-hour delay at room tem-perature before plasma processing and storage for up to one year at −80°C did not significantly affect plasma oxylipin measurements (including spiked, authentic RvE1) [28].

Several other clinical studies have also failed to detect RvE1 in plasma, including those with an omega-3 PUFA intervention [24,29,30]. Plasma total LXA$_4$ levels in the ng/ml range have been reported from a previous aspirin polyp prevention trial using an immunoassay that measured both LXA$_4$ epimers [7].

We were also unable to detect RvE1, LXA$_4$ or 15-epi-LXA$_4$ in rectal mucosa. This is the first study to investigate RvE1 levels in rectal mu-cosa, the target organ for CRC chemoprevention. A single study, which measured total LXA$_4$ levels by immunoassay, has reported pg/mg quantities of LXA$_4$ in human colorectal tissue [31].

By contrast, both *S*- and *R*-enantiomers of 18-HEPE and 15-HETE were readily detected in plasma and rectal mucosa. We did not detect any significant association between baseline, pre-treatment levels of these oxylipins and clinical characteristics in the seAFOod trial cohort, which is a middle-aged, male-predominant population with a high prevalence of excess body weight [13,14]. A previous study has reported that plasma 18-HEPE levels are higher in individuals with diabetes [25].

Table 3
Generalised linear model exploring the relationship between the plasma total 18-HEPE and 18*R*-HEPE level during treatment and the total colorectal polyp number at exit colonoscopy in the seAFOod trial. Significant co-variables ($P \leq 0.05$) are highlighted in bold text.

	Total 18-HEPE				18R-HEPE			
	Effect estimate	SE	Z value	P value	Effect estimate	SE	Z value	P value
Intercept	0.525	0.142	3.704	<0.001	0.528	0.142	3.725	<0.001
Plasma HEPE	0.012	0.012	1.059	0.290	0.024	0.025	0.954	0.340
aspirin	**-0.269**	**0.127**	**-2.123**	**0.034**	**-0.270**	**0.127**	**-2.131**	**0.033**
EPA	-0.216	0.129	-1.682	0.093	-0.217	0.129	-1.679	0.093
Sex (female)	-0.086	0.154	-0.556	0.579	-0.086	0.154	-0.557	0.577
Repeat colonoscopy	-0.008	0.165	-0.047	0.963	-0.009	0.165	-0.055	0.956

We have demonstrated that EPA treatment alone is associated with an increase in plasma 18-HEPE concentration. This has been reported in several previous, smaller studies up to 12 months in duration [21,23,24, 26,27,30,32,33]. Our report of oxylipin changes in the seAFOod trial describes a much larger cohort than prior mass spectrometric lipid analyses of other randomised trials with colorectal polyp and cancer outcomes [34,35] and highlights the significant intra- and inter-individual variability in plasma oxylipin levels over time, regardless of treatment allocation. The study by Norris and colleagues highlighted that a rapid and reversible increase in plasma 18-HEPE concentration is observed as early as two hours after low-dose endotoxin challenge [33], suggesting that intercurrent infections or other stressors may contribute to significant variability in plasma oxylipin levels over time during prolonged treatment. It should also be noted that we did not stipulate timing of blood sampling in the seAFOod trial in relation to eating. It is possible that post-prandial changes in oxylipin content of plasma lipoprotein fractions could contribute to intra-individual variability over time [36].

We did not observe an increase in plasma total 18-HEPE levels in EPA users who were also allocated to aspirin compared with those trial participants who were taking EPA alone. Similar findings have been reported after much shorter dosing (2–7 days) with the same amount of aspirin (300 mg daily) provided to healthy volunteers [27] and individuals with metabolic syndrome [26], who had already been taking a mixed omega-3 PUFA supplement (containing 1.4 g/day EPA-TG). The cellular and enzymatic source of 18-HEPE synthesis from EPA remains unknown. If acetylated-COX-2 (by aspirin) is not a dominant source of plasma 18-HEPE, an alternative pathway may be cytochrome P450 monooxygenase (CYP)-dependant synthesis [37]. Mammalian CYPs are believed to preferentially generate 17,18-epoxy- and n (20-)/n-1 (19-) hydroxyl derivatives of EPA [38]. However, at least one bacterial CYP can synthesise 18-HEPE [36] and it is possible that bacterial 18-HEPE synthesis contributes to the circulating plasma 18-HEPE pool [39]. However, the rapid (< 3 h) and reversible increase in plasma 18-HEPE observed in humans after intravenous lipopolysaccharide injection would be more consistent with immediate EPA release from host cell plasma membranes and/or rapid, inducible mammalian gene expression [33].

It remains unclear whether 18-HEPE has direct anti-CRC *per se* [12]. It is a weak ligand for peroxisome proliferator-activated receptors α and γ [39], which may mediate CRC chemopreventative activity [40].

We believe that the reduction in treatment effect of EPA on plasma 18-HEPE levels observed at 12 months is most likely to be due to an uncharacterized effect of bowel preparation (including a relative fast) for and/or the colonoscopy itself, which occurred 24 h before blood sampling. A previous study of EPA and DHA supplementation, which measured plasma 18-HEPE levels at 3 and 12 months after the start of the intervention, did not report blunting of the omega-3 PUFA effect on plasma oxylipin levels (including 18-HEPE) at 12 months compared with the earlier time-point [21]. It is also possible that the increase in plasma 15S-HETE at 12 months also relates to the colonoscopy intervention prior to sampling.

In contrast to the findings of Ostermann et al., who reported that an increasing daily intake of EPA was associated with a parallel linear increase in plasma EPA and 18-HEPE concentrations [21], we report a modest negative relationship between the RBC plasma membrane EPA content and the plasma 18-HEPE level measured at the same time-point, in keeping with release of membrane phospholipid-bound EPA for downstream metabolism.

Omega-3 PUFA-derived oxylipins have not been measured previously in human rectal mucosa. We show that rectal mucosal 18-HEPE content reflects that measured in plasma, which suggests that future clinical studies could use plasma levels as a surrogate for changes in the target organ (colorectal mucosa) for CRC prevention.

In contrast to the finding that the plasma 18-HEPE level is predictive of the chemopreventative activity of EPA (with or without naproxen) in a rat model of familial adenomatous polyposis [20], the plasma 18-HEPE level did not predict colorectal polyp multiplicity in seAFOod trial participants. As expected, the only significant factor in the multivariate model was aspirin use, in keeping with primary seAFOod trial analysis [13,14]. Further investigation of a role for 18-HEPE (or other oxylipins) as a predictive biomarker of therapeutic response to EPA and/or aspirin is needed in other large clinical studies.

In summary, in a large, randomised, placebo-controlled polyp prevention trial of aspirin and EPA, we did not detect circulating (plasma) or target tissue (rectal mucosal) oxylipins RvE1 or 15-epi-LXA$_4$, although proposed precursor mono-hydroxylated metabolites of EPA (18-HEPE) and AA (15-HETE) were measured readily. There was no evidence for a role for the 18-HEPE level as a predictive biomarker of chemoprevention response to EPA.

CRediT authorship contribution statement

H. Fuller: Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **A.D. Race:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **H. Fenton:** Formal analysis, Visualization, Writing – review & editing. **L. Burke:** Investigation, Methodology, Writing – review & editing. **A. Downing:** Formal analysis, Writing – review & editing. **E.A. Williams:** Funding acquisition, Writing – review & editing. **C.J. Rees:** Funding acquisition, Writing – review & editing. **L. C. Brown:** Formal analysis, Funding acquisition, Writing – review & editing. **P.M. Loadman:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Resources, Writing – review & editing. **M.A. Hull:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

CJR has received grant funding from Medtronic and was an expert witness for Olympus and ARC Medical. All the other Authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2023.102570.

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