Polymorphisms in *Cyclooxygenase*, *Lipoxygenase* and *TP53* genes predict colorectal polyp risk reduction by aspirin in the seAFOod polyp prevention trial

John R Davies[1]
Tracey Mell[1]
Harriett Fuller[1]
Mark Harland[1]
Rasha N M Saleh[2,3]
Amanda D Race[4]
Colin J Rees[5]
Louise C Brown[6]
Paul M Loadman[4]
Amy Downing[1]
Anne Marie Minihane[2, 7]
Elizabeth A Williams[8]
Mark A Hull[1]

[1] Leeds Institute of Medical Research, University of Leeds, Leeds, United Kingdom
[2] Nutrition and Preventive Medicine, Norwich Medical School, University of East Anglia, Norwich, United Kingdom
[3] Department of Clinical and Chemical Pathology, Faculty of Medicine, Alexandria University, Egypt
[4] Institute of Cancer Therapeutics, University of Bradford, United Kingdom
[5] Population Health Science Institute, Newcastle University, United Kingdom
[6] MRC Clinical Trials Unit at University College, London, United Kingdom
[7] Norwich Institute of Health Ageing, Norwich, United Kingdom
[8] Department of Oncology and Metabolism, University of Sheffield, United Kingdom

Running title: SNPs in COX, LOX and p53 modify chemoprevention by aspirin

Keywords: colorectal cancer; colorectal polyp; omega-3 polyunsaturated fatty acid; oxylipin; single nucleotide polymorphism

Corresponding author:

Professor Mark A Hull, Division of Gastrointestinal and Surgical Sciences, Leeds Institute of Medical Research, St James's University Hospital, University of Leeds, Leeds, LS9 7TF, United Kingdom

Telephone: +44 (0)113 343 8650

E-mail: M.A.Hull@leeds.ac.uk

Conflict of Interest disclosure: CJR has received grant funding from Medtronic and was an expert witness for Olympus and ARC Medical. All the other Authors declare that there are no conflicts of interest.

Contributorship Statement: JRD, RNMS, AMM, EAW and MAH participated in the conception and design of this study. MAH gained approvals for the study. JRD, TM, HF, MH, ADR, EAW and MAH were involved in the acquisition, analysis, or interpretation of data. Drafting of the manuscript was done by JRD and MAH. PML, EAW, CJR, LCB, and MAH obtained funding for the study. All the authors contributed to the critical review and final approval of the manuscript. JRD, TM, ADR and MAH have accessed and verified the underlying data. All authors were responsible for the decision to submit the manuscript.

Word Count: 4261

Number of Tables: 3

Number of Figures: 1

Abstract

Aspirin and eicosapentaenoic acid (EPA) reduce colorectal adenomatous polyp risk and affect synthesis of oxylipins including prostaglandin E_2. We investigated whether 35 single nucleotide polymorphisms (SNPs) in oxylipin metabolism genes such as *cyclooxygenase* [*PTGS*] and *lipoxygenase* [A*LOX*], as well as 7 SNPs already associated with colorectal cancer (CRC) risk reduction by aspirin (eg. *TP53*; rs104522), modified the effects of aspirin and EPA on colorectal polyp recurrence in the randomised 2x2 factorial seAFOod trial. Treatment effects were reported as the incidence rate ratio (IRR) and 95% confidence interval (CI) by stratifying negative binomial and Poisson regression analyses of colorectal polyp risk on SNP genotype. Statistical significance was reported with adjustment for the false discovery rate as the *P* and *q* value. Five hundred and forty-two (of 707) trial participants had both genotype and colonoscopy outcome data. Reduction in colorectal polyp risk in aspirin users compared with non-aspirin users was restricted to rs4837960 (*PTGS1*) common homozygotes (IRR 0.69 [95%CI 0.53,0.90]; *q*=0.06), rs2745557 (*PTGS2*) compound heterozygote-rare homozygotes (IRR 0.60 [0.41,0.88]; *q*=0.06), rs7090328 (*ALOX5*) rare homozygotes (IRR 0.27 [0.11,0.64]; q=0.05), rs2073438 (*ALOX12*) common homozygotes (IRR 0.57 [0.41,0.80]; *q*=0.05), and rs104522 (*TP53*) rare homozygotes (IRR 0.37 [0.17,0.79]; *q*=0.06). No modification of colorectal polyp risk in EPA users was observed. In conclusion, genetic variants relevant to the proposed mechanism of action on oxylipins are associated with differential colorectal polyp risk reduction by aspirin in individuals who develop multiple colorectal polyps. SNP genotypes should be considered during development of personalised, predictive models of CRC chemoprevention by aspirin.

Prevention Relevance Statement

Single nucleotide polymorphisms in genes controlling lipid mediator signalling may modify the colorectal polyp prevention activity of aspirin. Further investigation is required to determine whether testing for genetic variants can be used to target cancer chemoprevention by aspirin to those who will benefit most.

Introduction

A large body of evidence has accumulated from epidemiological observations, randomised colorectal polyp prevention trials, and long-term follow-up studies of colorectal cancer (CRC) outcomes after participation in vascular prevention trials, that regular aspirin use is associated with a 15-20% reduction in CRC risk [1-2]. However, aspirin use is not currently recommended for primary or secondary prevention of 'sporadic' CRC due primarily to concerns about the well-recognised gastro-intestinal and intra-cranial bleeding risk associated with aspirin treatment [1]. Therefore, a precision approach to primary CRC chemoprevention is needed in order to harness the preventative activity of aspirin in those individuals at highest risk of CRC, whilst avoiding aspirin use in those most at risk of harm. Several clinical factors are recognised to predict increased CRC risk (age, male sex, body fatness, family history, previous colorectal polyp history) and aspirin-related bleeding risk (age, hypertension, *Helicobacter pylori* infection) [3].

However, a multivariable risk prediction model utilising these clinical features has yet to emerge that would support precision chemoprevention largely because of the dearth of an accompanying biomarker(s) of personalised aspirin efficacy that could pinpoint individuals who are either sensitive to, or resistant to, the anti-CRC activity of aspirin [3]. There has been much interest in the stable urinary prostaglandin (PG) E_2 metabolite, PGE-M, as a predictive risk, or therapeutic response, biomarker of colorectal polyp prevention by aspirin [4], although it seems unlikely that a threshold pre-treatment or on-treatment urinary PGE-M level will have suitable test performance characteristics to enhance a clinically useful risk model [4].

An alternative approach has been to identify genetic variants that may be linked to differential risk reduction associated with aspirin or other non-steroidal anti-inflammatory drug (NSAID) use [5]. Investigation of single nucleotide polymorphisms (SNPs) in genes believed to be relevant to the mechanism of action of aspirin (*Prostaglandin G/H Synthase* [*PTGS*; also known as *Cyclooxygenase* {COX}]-1 and -2) in *post hoc* analyses of randomised colorectal polyp prevention trials of aspirin has demonstrated a possible interaction between aspirin and *PTGS2* SNP rs4648310 [6]. However, there were null associations for *PTGS2* SNPs rs20417, rs2745557, rs5277, rs5275, and rs20432, as well as *PTGS1* SNP rs3842787, in those randomised trial datasets [6-7].

COX-1 and COX-2 enzymes are direct acetylation targets of aspirin, leading to inhibition of downstream synthesis of the pro-tumorigenic PGE_2 from the omega-6 polyunsaturated fatty acid (PUFA) COX substrate C20:4*n*-6 arachidonic acid (AA) [8]. Alternatively, aspirin inhibition of COX activity leads to substrate diversion of AA towards lipoxygenase (LOX; encoded by *ALOX* genes)-mediated synthesis of other oxylipins with anti-tumorigenic activity such as leukotriene (LT) B_4 [9].

Moreover, expression of 15-PG dehydrogenase (encoded by *HPGD*), the enzyme responsible for inactivating PGE_2, has been shown to predict CRC risk reduction by aspirin [10].

Despite the relevance of other genes controlling oxylipin synthesis for the chemopreventative property of aspirin, the relationship between SNP genotypes in a wide range of genes controlling oxylipin synthesis and levels (including *PTGS1* and *PTGS2*, as well as *ALOX5*, *ALOX12* and *HPGD*) and the effect of aspirin on colorectal polyp risk has not been addressed previously in a randomised trial setting.

The omega-3 PUFA C20:5*n*-3 eicosapentaenoic acid (EPA) also has CRC chemoprevention activity [11]. It is an alternative substrate for multiple monooxygenases including the COX isoforms and the LOX family of enzymes [12]. EPA is an efficient COX-1 inhibitor and metabolism by COX (and LOX isoforms) leads to synthesis of 3- and 5-series oxylipins such as PGE_3 and LTB_5, which have reduced pro-inflammatory and pro-tumorigenic activity compared with their 2- and 4-series counterparts, perhaps contributing to the anti-CRC activity of EPA [12-14]. Therefore, the same genes controlling oxylipin synthesis and levels that could be relevant to aspirin chemoprevention could also modify anti-CRC activity of EPA.

Several other SNPs that are not directly related to oxylipin metabolism have also been demonstrated to modify CRC risk reduction associated with aspirin, for example rs6983267 [15] and rs2965667 [5], as well as being linked to increased CRC risk in other observational studies (rs1042522 [16]).

The seAFOod polyp prevention trial was a 2x2 factorial, randomised, placebo-controlled trial of aspirin 300 mg daily and EPA 2000 mg free fatty acid equivalents daily, alone or in combination, for 12 months in 707 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 screening colonoscopy in the English Bowel Cancer Screening Programme (BCSP) [17-18]. Trial

participants mirrored individuals undergoing screening colonoscopy after a positive faecal occult blood test (FOBt) in the BCSP with a male predominance and high prevalence of excess body weight [17-18]. The primary 'at the margins' analysis compared aspirin and EPA separately against their respective placebos, assuming no interaction between the two interventions [17-18]. While the seAFOod trial found no evidence for an effect of aspirin or EPA on the primary outcome of adenoma detection rate (the % of participants with one or more colorectal polyps), 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 treatment [17-18]. 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 [17-18].

We tested the relationship between SNPs in genes controlling oxylipin synthesis and levels, which are relevant for the putative mechanism(s) of the anti-cancer activity of aspirin and EPA, as well as SNPs in selected genes already linked to modification of CRC risk reduction by aspirin, and colorectal polyp outcomes in the seAFOod polyp prevention trial.

Materials and Methods

The seAFOod polyp prevention trial biobank

This study is part of a wider programme of investigations using the seAFOod trial biobank and post-trial BCSP colonoscopy outcomes called STOP-ADENOMA (ISRCTN05926847). The study was conducted in accordance with the Declaration of Helsinki. 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 [17]. Blood samples were obtained, and buffy coat leukocytes were collected and stored locally at -20^{o}C and in the central trial Biobank at -80^{o}C, as described in the Trial Laboratory Manual and detailed Trial report [18-19].

DNA extraction

DNA was extracted from a single buffy coat extract per seAFOod trial participant using an in-house phenol-chloroform method, followed by washing with absolute ethanol and storage at 4^{o}C in DNase/RNase-free water, prior to spectrophotometric assessment of the DNA quantity and quality. If any sample had a DNA yield <20 ng/µl, a second sample for that individual was obtained from the Biobank for DNA extraction.

SNP genotyping

Genotyping was carried out using the Fluidigm microfluidic SNP genotyping system (Fluidigm, San Francisco, CA), using custom-built SNP Type genotyping assays (Fluidigm, San Francisco, CA) for 92 SNPs (Supplementary Table 1).

A multiplexed pre-amplification PCR was carried out in order to increase the available template DNA and reduce allelic drop-out associated with low template concentration. DNA samples were run on 96.96 Dynamic Array™ IFCs (integrated fluidic circuits), which were primed and loaded using the IFC Controller HX (96.96 arrays), as per manufacturer's instructions. In total, 691 individual samples including 10 technical duplicates, 31 participant duplicate samples and 1 participant triplicate sample, were analysed, representing 648 individual participant DNA samples.

Genotyping PCR and measurement of endpoint fluorescent values were carried out using the Fluidigm BioMark™ HD system. Data were analysed and genotyping calls made using Fluidigm SNP Genotype Analysis software.

Fourteen SNPs failed quality control; one SNP was mono-allelic, one SNP was repeatedly called differently in duplicate sample runs, and twelve SNPs had an absence of genotype call >5% (Supplementary Table 1).

Therefore, genotype data for 78 SNPs were available for analysis inside the seAFOod trial database. Twelve SNPs did not satisfy Hardy-Weinberg equilibrium (P<0.05 with Benjamini-Hochberg correction for multiple testing). However, on inspection of scatter plots produced during Fluidigm genotyping, 'true calls' did not overlap with 'fails' suggesting that this was not caused by sampling error. Therefore, these SNPs were included in subsequent analyses in order to avoid missing a relationship with clinical outcomes given that these SNPs could be causally related to colorectal polyp development (and thus seAFOod trial recruitment) explaining absence of Hardy-Weinberg equilibrium.

This report is restricted to an analysis of the relationship between the 35 SNPs in genes controlling oxylipin synthesis and degradation (*PTGS1*; 7 SNPs: *PTGS2*; 5 SNPs: *ALOX5*; 8 SNPs: *ALOX12*; 10 SNPs: *ALOX15*; 3 SNPs: *HPGD***;** 2 SNPs), as well as 7 SNPs previously associated with differential CRC risk reduction in aspirin users [5,15-16], and colorectal polyp risk in the seAFOod polyp prevention trial. Genetic variants of interest in *PTGS* and *ALOX* genes were identified as part of the European Union-Biotechnology and Biological Sciences Research Council (UK)-funded Fatty Acid Metabolism (FAME) Consortium - Interlinking Diet with Cardio-metabolic Health. Briefly, all SNPs in *PTGS* and *ALOX* genes were grouped into linkage disequilibrium (LD) blocks using PLINK v1.9 software ($R^2 \geq 0.8$, minor allele frequency ≥ 5%) [20]. A tagging SNP for each LD block was selected based on prior disease association (from GWAS and pharmacogenetics databases), pathogenicity and position (within promotors, exons or splice sites).

Data on the relationship between the SNPs in genes controlling PUFA metabolism (Supplementary Table 1) with blood and rectal mucosal levels of PUFAs measured in the seAFOod polyp prevention trial, as well as colorectal polyp risk, are the subject of a separate report.

Statistical analysis

Linkage disequilibrium between SNPs in individual genes was analysed using the LDmatrix tool (National Institutes of Health, USA) to derive R^2 values for paired SNP relationships.

Only seAFOod trial participants, for whom there were trial colonoscopy outcome data, were included in this SNP analysis. Baseline characteristics (age, sex, body mass index [BMI], diabetes, tobacco smoking status, alcohol intake) of the seAFOod trial participants included in the SNP analysis were analysed using the Chi-squared test or Mann-Whitney U test, as appropriate, in order to confirm similarity with the original seAFOod trial cohort and ensure continuing balance across the trial treatment allocations.

Interactions of SNP genotypes with trial interventions were analysed 'at the margins' (ie active aspirin *versus* placebo aspirin, and active EPA *versus* placebo EPA, regardless of the other intervention in the 2 x 2 factorial trial), consistent with the primary seAFOod trial analysis (that assumed no treatment interaction) [17-18].

We analysed total colorectal (combined adenomatous and serrated-hyperplastic) polyp number per participant (previously termed adenoma per participant in the original seAFOod trial analysis [17-18]) in keeping with current colorectal polyp classification [21]. We also stipulated analysis of adenomatous polyp number per participant given that a secondary outcome of the seAFOod trial was that EPA (but also aspirin) specifically reduced risk of adenomatous polyps [17-18]. Descriptive data were tabulated for the possible genotypes for each SNP across the trial intervention comparisons (active aspirin *versus* placebo aspirin, and active EPA *versus* placebo EPA) with univariate statistical testing for each genotype by the Kruskal-Wallis rank test. Distribution dot-plots were also drawn to compare the colorectal polyp count distribution across genotypes for each SNP.

All SNPs were then used to stratify a negative binomial regression model of colorectal polyp number (justified by a positive-skewed distribution of individual colorectal polyp number values in the seAFOod trial [18]), which was adjusted for the hospital site where the colonoscopy occurred, and for whether a repeat colonoscopy (for example, for a polypectomy site check) had taken place at baseline, in order to aid comparison with the effect sizes for aspirin and EPA reported in the primary seAFOod trial analysis [17-18]. Data are reported as the incidence rate ratio (IRR) and 95% confidence interval (CI). For SNPs that had 40 or more rare homozygotes, the models were stratified at three levels (common homozygote, heterozygote, rare homozygote). In all other cases with fewer cases than the arbitrary threshold of 40 rare homozygotes, heterozygotes and rare homozygotes were collapsed to ensure a sufficient number of cases in the model [22]. For models that showed a statistically significant relationship ($P \leq 0.05$) between colorectal polyp number and

intervention for some strata, an interaction test between the SNP and intervention was conducted fitting the negative binomial regression model with additional terms for interaction between the treatment and SNP variables.

Each analysis was repeated using a Poisson regression model, with the same adjustments, in order to mirror the seAFOod trial analysis [17-18].

Statistical significance was specified as $P<0.05$. Given the relatively large number of individual analyses for SNP x colorectal polyp number interactions, as well as two interventions (aspirin and EPA) and two colorectal polyp outcomes, the positive false discovery rate (pFDR) was described by the *q* value for each of the models including individual SNPs [23].

All statistical analyses were conducted using Stata version 17.0.

Data availability

The data generated in this study are available upon request from the corresponding author and with approval from the study Sponsor (University of Leeds).

Results

seAFOod Trial participant samples

Six hundred and sixty-six trial participants had at least one buffy coat sample vial in the seAFOod trial biobank (Figure 1). There was no material in a single cryovial in four cases and the DNA yield was below 20 ng/ml in 14 cases despite DNA extraction from a second sample vial, leaving 648 individual participant DNA samples for SNP genotyping. Following characterisation by Fluidigm 96.96 IFC assay, one DNA sample yielded a SNP 'no call' rate of >20% and was excluded, leaving 647 individual participant SNP genotype profiles for analysis.

Five hundred and forty-two seAFOod trial participants, for whom SNP genotype data were available, also had colonoscopy outcome data from the seAFOod trial. Reasons for lack of primary (colorectal polyp) outcome data for some trial participants are detailed in the seAFOod trial CONSORT diagram [17]. The clinical characteristics of this study cohort are detailed in Table 1. The clinical features of the cohort used for the SNP analysis were not significantly different from the original randomized trial population (Table 1) [17]. There was also no significant difference in the clinical characteristics of the trial participants contributing to this genetic analysis according to treatment allocation to aspirin or EPA *versus* their respective placebos (Table 1).

Relationship between individual SNPs and colorectal polyp number according to seAFOod trial treatment allocation

PTGS1, *PTGS2* and *HPGD*

Comparison of total colorectal polyp number per participant between those allocated aspirin *versus* placebo according to genotype for each *PTGS1*, *PTGS2* and *HPGD* SNP revealed that *PTGS1* rs4837960 common homozygotes (G:G) and *PTGS2* rs2745557 compound heterozygotes (G:A)-rare homozygotes (A:A) were associated with a statistically significant reduction in total colorectal polyp number in aspirin users *versus* non-aspirin users in contrast to the other genotype (Supplementary Figure 1 and Supplementary Table 2). All the other *PTGS1*, *PTGS2* and *HPGD* SNPs did not reach statistical significance for a difference in total colorectal polyp number between aspirin and placebo users according to genotype (Supplementary Table 2). For *PTGS1* rs4837960, aspirin use was associated with a

reduction in total colorectal polyp risk in homozygotes for the major allele (IRR 0.69 [0.53, 0.90]; *P*=0.006; pFDR *q*=0.06), but not in individuals with one or more minor T alleles (IRR 0.91 [0.59, 1.40]; *P*=0.7) (Table 2). However, the P_{int} value for this SNP failed to reach the pre-specified threshold for statistical significance (P_{int}=0.3 for TT+GT vs GG; Table 2). A similar IRR value for rs4837960 was obtained in a Poisson model and using adenomatous polyp number per participant as the outcome. For the *PTGS2* SNP rs2745557, total colorectal polyp risk reduction was restricted to individuals with one or more minor (A) alleles (IRR 0.60 [0.41, 0.88]; *P*=0.009; pFDR *q*=0.06), but not common homozygotes (P_{int} = 0.2; Table 2). A similar IRR value for rs2745557 was also obtained for this *PTGS2* SNP in a Poisson model and using adenomatous polyp number per participant as the outcome.

None of the *PTGS1*, *PTGS2* or *HPGD* SNP genotypes, including rs4837960 and rs2745557, reached univariate statistical significance for a difference in total colorectal polyp number or adenomatous polyp number between individuals who received either active EPA or placebo EPA (Supplementary Figure 1).

ALOX5 and *ALOX12*

Univariate analysis of individual SNPs and total colorectal polyp number in aspirin and EPA users compared with individuals who were allocated to the respective placebo intervention demonstrated statistical significance for the *ALOX5* SNP rs7090328 and for three *ALOX12* SNPs (rs2073438, rs2920421 and rs2271316, which were in strong LD - R^2 between 0.671 and 0.871 for all pairwise comparisons), for aspirin users (Supplementary Figure 2 and Supplementary Table 2). In the negative binomial regression model, *ALOX5* rs7090328 was associated with modification of the effect of aspirin, with a reduction in total colorectal polyp risk in homozygotes for the minor allele (IRR 0.27 [0.11, 0.64]; *P*=0.003; pFDR *q*=0.05), but not in individuals with one or more major A alleles (IRR (AA) 0.81 [0.59, 1.11]; *P*=0.2), with a P_{int} value of 0.03 (Table 3). A similar IRR value for rs7090328 related to aspirin use was obtained in a Poisson model and using conventional adenomatous polyp number per participant as the outcome. For the *ALOX12* SNPs, total colorectal polyp risk reduction by aspirin was restricted to common homozygotes, but not those with one or more minor alleles (P_{int} = 0.02 for rs2073438 and rs2920421, and P_{int} = 0.06 for rs2271316; Table 3). Again, similar IRR values were also obtained for the *ALOX12* SNPs in a Poisson model and using adenomatous polyp number per participant as the outcome.

None of the *ALOX* SNP genotypes reached univariate statistical significance for a difference in total colorectal polyp number or adenomatous polyp number between individuals who received either active EPA or placebo EPA (Supplementary Figure 2).

SNPs associated with differential CRC risk reduction in aspirin users, as well as with overall CRC risk

The only SNP in the panel of polymorphisms previously linked to modification of CRC risk [4, 14-15], which demonstrated statistical significance for modification of the association of aspirin use and total colorectal polyp number, was rs104522, which is a SNP in the *TP53* tumour suppressor gene (Supplementary Figure 3 and Supplementary Table 2). In the negative binomial regression model, colorectal polyp risk reduction by aspirin was significant for rare homozygotes (IRR 0.37 [0.17, 0.79]; P = 0.01; pFDR q=0.06) with a P_{int} = 0.06, but not for individuals with one or more major alleles (Table 3). Similar results were obtained in the Poisson model and using adenomatous polyp number per participant as the outcome.

TP53 rs104522 genotype was not associated with any differential effect of EPA on total colorectal polyp number or adenomatous polyp number (Supplementary Figure 3).

Discussion

Using a panel of SNPs in genes controlling oxylipin signaling relevant to colorectal carcinogenesis and the pharmacology of aspirin and EPA, we report the association of several SNPs in genes encoding COX-1 (*PTGS1*), COX-2 (*PTGS2*), 5-LOX (*ALOX5*) and 12-LOX (*ALOX12*) with differential polyp prevention efficacy of aspirin, but not EPA, in the seAFOod trial.

The association of *PTGS1* SNP rs4837960 and *PTGS2* SNP rs2745557 with colorectal polyp risk reduction by aspirin has not been reported previously. We note that the P_{int} and pFDR values for these SNPs did not reach the pre-specified level for significance and the results require independent verification. The *PTGS1* SNP rs4837960 is in LD (R^2=0.93) with another *PTGS1* SNP rs3842787, which has been reported to interact with NSAID use for cancer risk [24], although null findings were reported for this SNP in the UKCAP colorectal polyp prevention trial [7]. Both rs4837960 and rs2745557 are intronic and the possible functional consequences of homozygosity for the major G allele at rs4837960, or presence of at least one minor allele (A) at rs2745557, are not known.

A previous analysis of the interaction between *PTGS2* SNPs and aspirin use related to colorectal polyp recurrence in the Aspirin/Folate Polyp Prevention Study (AFPPS) described a possible interaction between rs4648310 and aspirin use (81 mg daily) [6]. SNP rs4648310 is 8.7K base pairs downstream from rs2745557 and so is likely to have an association with aspirin efficacy independent of rs2745557 genotype.

We did not detect an interaction between the *PTGS2* promoter SNP rs20417 (-765G>C) and aspirin or EPA use in seAFOod trial participants. rs20417 has been reported to approach statistical significance (P=0.07) for an interaction with NSAID use for reduction in colorectal adenomatous polyp risk in one case-control study (494 cases and 584 controls), but not in another smaller study [25]. There was also no interaction between rs20417 and aspirin use in the UKCAP aspirin polyp prevention trial [26]. Our data support the contention that there is no interaction between rs20417 and aspirin use for colorectal polyp reduction. It should be noted that the AFPPS and UKCAP trial genotype studies used the 'adenoma detection rate' (the % number of individuals with one or more colorectal polyps at follow-up colonoscopy) as a measure of polyp recurrence risk [6-7], whereas we report the association of genotype with reduction in colorectal polyp number in the seAFOod trial (noting that the 'adenoma detection rate' was the null primary outcome of the seAFOod trial [17-18]). It will

be important, if possible, to perform meta-analysis of the aspirin polyp prevention trials that include genotype data, in order to harmonize colorectal outcome genotype-phenotype correlations (AFPPS and UKCAP studies collected colorectal polyp number as a secondary outcome).

One of the *ALOX12* SNPs that was associated with differential colorectal polyp prevention activity of aspirin in our study (the intronic SNP rs2920421) has been reported to interact with NSAID use in a case-control study of CRC risk [26]. In that study, NSAID use was associated with decreased CRC risk in heterozygous rs2920421 genotypes, but not major or minor homozygotes [26]. In a separate case-control study, rs2073438 (homozygotes for the minor A allele), which is in strong LD with rs2920421, was associated with reduced rectal cancer risk, but not colorectal polyp risk or an interaction with NSAID use [27]. Consistent with biological relevance of the *ALOX5* and *ALOX12* SNPs found to be associated with differential colorectal polyp risk in aspirin users in the seAFOod trial, these SNPs were all associated with expression quantitative trait loci for *ALOX5* and *ALOX12* in the sigmoid and transverse colon in the Genotype-Tissue Expression Project (GTEx) database.

We also demonstrated that homozygosity for the SNP (rs104522; G>C; Arg72Pro) in the coding region of the TP53 tumour suppressor gene was associated with colorectal polyp reduction in aspirin users in the seAFOod trial, in contrast with the group of individuals that had at least one major (G) allele, who did not demonstrate a reduction in colorectal polyp risk associated with aspirin use. At least one C allele at rs104522 has been reported to have an odds ratio of 1.16 (1.05, 1.30) for presence of one or more colorectal adenomas compared with homozygous G:G individuals in a meta-analysis of genetic association studies of colorectal adenomas [28]. The role of p53 in the pro-apoptotic activity of aspirin and other non-steroidal anti-inflammatory drugs remains unclear [29-30]. However, it is plausible that the alteration in p53 function associated with rs104522 could alter aspirin chemopreventative activity [31]. The relationship between rs104522 and colorectal polyp prevention activity of aspirin requires validation in an independent study, which would ideally have sufficient power to distinguish between the two colorectal polyp types (adenomatous and serrated) that have different molecular pathogenesis [32].

We did not observe any gene-supplement interaction for any SNP related to the modest effect of EPA use on colorectal polyp number in the seAFOod trial. The reduction in total colorectal polyp number associated with EPA use in the seAFOod trial was modest and just

failed to reach statistical significance [17-18]. However, we also tested the SNPs for modification of the effect of reduction in colorectal adenoma number by EPA that was a statistically significant finding from the seAFOod trial [17-18], which generated null findings. There has been no previous study of potential genetic modifiers of the effect of omega-3 PUFAs on CRC risk. However, COX-2 SNPs rs5275 and rs4648310 have been reported to modify the association between dietary omega-3 PUFA intake and prostate cancer risk [33-34].

Strengths of this study include the comprehensive coverage of the seAFOod trial population whereby 77% of 707 seAFOod trial participants had both colonoscopy and genotype data available. We used a relatively small, custom-built SNP array of relevant genes based on *a priori* knowledge of aspirin and EPA anti-cancer pharmacology [1,11]. We acknowledge the risk of type 1 statistical error given the number of SNPs that were evaluated, as well as two interventions and two colorectal polyp end-points. Ideally, these findings would be replicated in an independent prospective cohort or intervention trial of aspirin use. However, given the number of tests conducted, we saw more significant associations than would be expected by chance, which suggests that our results are unlikely to be explained by chance. Moreover, the respective *q* values for associations between SNPs and colorectal polyp prevention efficacy of aspirin suggest that we report true positive findings. In addition, the power of our study was limited for rare homozygote SNPs, for which the minor allele frequency was small (<0.3), if the effect size is only moderate (IRR>0.6). We also draw attention to the fact that the seAFOod trial population consisted of individuals who had undergone BCSP colonoscopy after a positive FOBt, thereby generating a predominantly male and white ethnicity cohort [17], which limits generalizability to other populations.

In summary, we report novel gene-chemoprevention (aspirin) interactions from the seAFOod polyp prevention trial. SNPs in COX-1, COX-2, LOX isoforms and TP53 should be further evaluated as biomarkers of aspirin chemoprevention efficacy, alone and in combination with other polymorphisms reported to predict colorectal polyp and/or CRC risk reduction by aspirin [3].

Acknowledgements

This project (NIHR128210) was funded by the Efficacy and Mechanism Evaluation (EME) Programme, an MRC and NIHR partnership (C.J. Rees, L.C. Brown, P.M. Loadman, E.A. Williams, M.A. Hull). The views expressed in this publication are those of the authors and not necessarily those of the MRC, NIHR or the Department of Health and Social Care. Microfluidic array design was supported by the European Union-Biotechnology and Biological Sciences Research Council (UK)-funded Fatty Acid Metabolism (FAME) Consortium - Interlinking Diet with Cardio-metabolic Health (BB/P028233/1; R.N.M. Saleh and A.M. Minihane). M.A. Hull and C.J. Rees are NIHR Senior Investigators. A. Downing and M.A. Hull are supported by Cancer Research UK grant C23434/A24939.

The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health, and by NCI, NHGRI, NHLBI, NIDA, NIMH, and NINDS. Data were accessed from the GTEx Portal on 08/17/2023.

The Authors wish to thank all the participants and research staff who contributed to the seAFOod polyp prevention trial. The Authors express their gratitude to Professors Tim Bishop and David Westhead for helpful review and advice. Professor Matthias Schulze provided useful advice about candidate SNPs. The Authors also acknowledge the significant contribution of Mr John Whelpton acting as the Patient & Public representative in the STOP-ADENOMA Study Group.

References

1. Drew DA, Chan AT. Aspirin in the prevention of colorectal neoplasia. *Ann Rev Med* 2021;**72**:415-430.
2. US Preventive Services Task Force. Aspirin use to prevent cardiovascular disease: US Preventive Services Task Force recommendation statement. *JAMA* 2022;**327**:1577-1584.
3. Drew DA, Cao Y, Chan AT. Aspirin and colorectal cancer: the promise of precision chemoprevention. *Nature Rev Cancer* 2016;**16**:173-186.
4. Drew DA, Schuck MM, Magicheva-Gupta MV, Stewart KO, Gilpin KK, Miller P *et al*. Effect of low-dose and standard-dose aspirin on PGE_2 biosynthesis among individuals with colorectal adenomas: A randomized clinical trial. *Cancer Prev Res* 2020;**13**:877-888.
5. Nan H, Hutter CM, Lin Y, Jacobs EJ, Ulrich CM, White E, *et al*. Association of aspirin and NSAID use with risk of colorectal cancer according to genetic variants. *JAMA* 2015;**313**:1133-1142.
6. Barry EL, Sansbury LB, Grau MV, Ali IU, Tsang S, Munroe DJ, *et al*. Cyclooxygenase-2 polymorphisms, aspirin treatment, and risk for colorectal adenoma recurrence – Data from a randomized clinical trial. *Cancer Epidemiol Biomarkers Prev* 2009;**18**:2726-2733.
7. Hubner RA, Muir KR, Liu J-F, Logan RFA, Grainge MJ, Houlston RS, *et al*. Polymorphisms in PTGS1, PTGS2 and IL-10 do not influence colorectal adenoma recurrence in the context of a randomized aspirin intervention trial. *Int J Cancer* 2007;**121**:2001-2004.
8. Wang D, Dubois RN. Prostaglandins and cancer. *Gut* 2006;**55**:115-122.
9. Velazquez JR, Teran LM. Aspirin-intolerant asthma: a comprehensive review of biomarkers and pathophysiology. *Clin Rev Allergy Immunol* 2013;**45**:75-86.
10. Fink SP, Yamauchi M, Nishihara R, Jung S, Kuchiba A, Wu K, *et al*. Aspirin and the risk of colorectal cancer in relation to the expression of 15-hydroxyprostaglandin dehydrogenase (HPGD). *Sci Transl Med* 2014;**6**:233re2.
11. Aldoori J, Cockbain AJ, Toogood GJ, Hull MA**.** Omega-3 polyunsaturated fatty acids: moving towards precision use for prevention and treatment of colorectal cancer. *Gut* 2022;**71**:822-837.
12. Smith WL. Cyclooxygenases, peroxide tone and the allure of fish oil. *Curr Opin Cell Biol* 2005;**17**:174-182.
13. Bortuzzo C, Hanif R, Kashfi K, Staino-Coico L, Shiff SJ, Rigas B. The effect of leukotrienes B and selected HETEs on the proliferation of colon cancer cells. *Biochim Biophys Acta* 1996;**1300**:240-246.
14. Hawcroft G, Loadman PM, Belluzzi A, Hull MA. The effect of the omega-3 polyunsaturated fatty acid eicosapentaenoic acid on E-type prostaglandin synthesis and EP4 receptor signalling in human colorectal cancer cells. *Neoplasia* 2010;**12**:618-627.

15. Nan H, Morikawa T, Suurinemi M, Imamura Y, Werner L, Kuchiba A, *et al*. Aspirin use, 8q24 single nucleotide polymorphism rs6983267, and colorectal cancer according to CTNNB1 alterations. *J Natl Cancer Inst* 2013;**105**:1852-1861.
16. Elshazli RM, Toraih EA, Elgaml A, Kandil E, Fawzy MS. Genetic polymorphisms of TP53 (rs1042522) and MDM2 (rs2279744) and colorectal cancer risk: An updated meta-analysis based on 59 case-control studies. *Gene* 2020;**734**:144391.
17. Hull MA, Sprange K, Hepburn T, Tan W, Shafayat A, Rees CJ, *et al*. Eicosapentaenoic acid and aspirin, alone and in combination, for the prevention of colorectal adenomas (seAFOod Polyp Prevention trial): a multicentre, randomised, double-blind, placebo-controlled, 2x2 factorial trial. *Lancet* 2018;**392**:2583-2594.
18. Hull MA, Sprange K, Hepburn T, Tan W, Shafayat A, Rees CJ, *et a*l. Eicosapentaenoic acid and/or aspirin for preventing colorectal adenomas during colonoscopic surveillance in the NHS Bowel Cancer Screening Programme: the seAFOod RCT. *Efficacy Mech Eval* 2019;**6**:4.
19. Hull MA, Sandell AC, Montgomery AA, Logan RF, Clifford GM, Rees CJ, *et al*. A randomized controlled trial of eicosapentaenoic acid and/or aspirin for colorectal adenoma prevention during colonoscopic surveillance in the NHS Bowel Cancer Screening Programme (The seAFOod Polyp Prevention trial): study protocol for a randomized controlled trial. *Trials* 2013;**14**:237.
20. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 2007;81: 559–575.
21. Rutter MD, East J, Rees CJ, Cripps N, Docherty J, Dolwani S, *et al*. British Society of Gastroenterology/Association of Coloproctology of Great Britain and Ireland/Public Health England post-polypectomy and post-colorectal cancer reseaction surveillance guidelines. *Gut* 2020;**69**:201-223.
22. Jenkins DG, Quintana-Ascencio PF. A solution to minimum sample size for regresssions. *PLoS One* 2020;**15**:e0229345.
23. Storey JD. The positive false discovery rate: a Bayesian interpretation and the *q*-value. *Ann Statist* 2003;**31**:2013-2035.
24. Nagao M, Sato Y, Yamauchi A. A meta-analysis of *PTGS1* and *PTGS2* polymorphisms and NSAID intake on the risk of developing cancer. *PLoS One* 2013;**8**:e71126.
25. Cross JT, Poole EM, Ulrich CM. A review of gene-drug interactions for nonsteroidal anti-inflammatory drug use in preventing colorectal neoplasia. *Pharmacogenomics J* 2008;**8**:237-247.
26. Resler AJ, Makar KW, Heath L, Whitton J, Potter JD, Poole EM, *et al*. Genetic variation in prostaglandin synthesis and related pathways, NSAID use and colorectal cancer risk in the Colon Cancer Family Registry. *Carcinogenesis* 2014;**35**:2121-2126.
27. Kleinstein SE, Heath L, Makar KW, Poole EM, Seufert BL, Slattery ML, *et al*. Genetic variation in the lipoxygenase pathway and risk of colorectal neoplasia. *Genes Chromosomes Cancer* 2013;**52**:437-449.

28. Naccarati A, Polakova V, Pardini B, Vodickova L, Hemminki K, Kumar R, *et al*. Mutations and polymorphisms in TP53 gene--an overview on the role in colorectal cancer. *Mutagenesis* 2012;**27**:211-218.
29. Montazeri Z, Theodoratou E, Nyiraneza C, Timofeeva M, Chen W, Svinti V, *et al*. Systematic meta-analyses and field synopsis of genetic association studies in colorectal adenomas. *Int J Epidemiol* 2016;**45**:186-205.
30. Dahabreh IJ, Linardou H, Bouzika P, Varvarigou V, Murray S. TP53 Arg72Pro polymorphism and colorectal cancer risk: a systematic review and meta-analysis. *Cancer Epidemiol Biomarkers Prev* 2010;**19**:1840-1847.
31. Watson AJ. An overview of apoptosis and the prevention of colorectal cancer. *Crit Rev Oncol Hematol* 2006;**57**:107-121.
32. Strum WB. Colorectal Adenomas. *N Engl J Med* 2016;**374**:1065-1075.
33. Hedelin M, Chang ET, Wiklund F, Belloco R, Klint A, Adolfsson J, *et al*. Association of frequent consumption of fatty fish with prostate cancer risk is modified by COX-2 polymorphism. *Int J Cancer* 2006;**120**:398-405.
34. Fradet V, Cheng I, Casey G, Witte JS. Dietary omega-3 fatty acids, *Cyclooxygenase-2* variation, and aggressive prostate cancer risk. *Clin Cancer Res* 2009;**15**:2559-2566.

Table 1: Clinical characteristics of the study cohort of 542 seAFOod trial participants who had a SNP genotype profile and trial colonoscopy outcome data. IQR, inter-quartile range. *missing Body mass index data for one case in the placebos only group and two cases in the EPA group.

	Placebos only	**Aspirin only**	**EPA only**	**Aspirin & EPA**	***P* for aspirin *v* no aspirin comparison**	***P* for EPA *v* no EPA comparison**	***P* for comparison of study cohort with n=165 excluded trial participants**
Number	142	138	126	136			
Age (median [IQR])	64.5 (7.9)	64.9 (6.2)	65.1 (6.2)	66.4 (7.7)	0.22	0.29	0.42
Sex (male:female [% female])	113:29 (20.4)	109:29 (21.0)	100:26 (20.6)	114:22 (16.2)	0.48	0.58	0.33
Body mass index (%)*					1.0	0.50	0.90
Normal (<24.9 Kg/m^2)	23 (16.2)	20 (14.5)	23 (18.3)	30 (22.1)			
Overweight (25.0-29.9 Kg/m^2)	64 (45.1)	60 (43.5)	51 (40.5)	60 (44.1)			
Obese (≥30.0 Kg/m^2)	54 (38.0)	58 (42.0)	50 (40.0)	46 (33.8)			
Diabetes (n [%])	20 (14.1)	14 (10.1)	13 (11.5)	10 (7.4)	0.18	0.20	0.16
Tobacco smoking					0.35	0.50	0.16
never	52 (36.6)	51 (37.0)	49 (38.9)	53 (39.0)			
ex-smoker	65 (45.8)	66 (47.8)	68 (54.0)	58 (42.6)			
current	25 (17.6)	21 (15.2)	9 (7.1)	25 (18.4)			
Alcohol units per week (median; IQR)	3 (1)	2 (1)	2 (1)	3 (1)	1.0	0.93	0.92

Table 2: SNP genotypes in *PTGS* genes that are associated with modification of the effect of aspirin on colorectal polyp number in the seAFOod polyp prevention trial. The incidence risk ratio (IRR) and 95% confidence interval (CI) for colorectal polyp number is for aspirin *versus* no aspirin in a negative binomial regression model.

SNP ID	Gene	Major allele	Minor allele	Common Homozygote				Heterozygote + rare Homozygote				*P* for interaction
				n	IRR (95% CI)	*P*	*q*	n	IRR (95% CI)	*P*	*q*	
rs4837960	*PTGS1*	G	T	397	0.69 (0.53, 0.90)	0.006	0.06	136 + 9	0.91 (0.59, 1.40)	0.7	0.8	0.3 (TT+ GT *v* GG)
rs2745557	*PTGS2*	G	A	347	0.83 (0.62, 1.09)	0.2	0.3	178 + 17	0.60 (0.41, 0.88)	0.009	0.06	0.2 (AA +GA *v* GG)

Table 3: SNP genotypes in *ALOX* genes and the *TP53* gene that are associated with modification of the effect of aspirin on colorectal polyp number in the seAFOod polyp prevention trial. The incidence risk ratio (IRR) and 95% confidence interval (CI) for colorectal polyp number is for aspirin *versus* no aspirin in a negative binomial regression model.

SNP ID	Gene	Major allele	Minor allele	Common Homozygote				Heterozygote				Rare Homozygote				*P* for interaction
				n	IRR (95% CI)	*P*	*q*	n	IRR (95% CI)	*P*	*q*	n	IRR (95% CI)	*P*	*q*	
rs7090328	*ALOX5*	G	A	284	0.81 (0.59, 1.11)	0.2	0.3	217	0.76 (0.54, 1.09)	0.1	0.3	39†	0.27 (0.11, 0.64)	0.003	0.05	0.8 (AG *v* GG) 0.03 (AA *v* GG)
rs2073438	*ALOX12*	G	A	236	0.57 (0.41, 0.80)	0.001	0.05	239	1.00 (0.71, 1.41)	1.0	1.0	56	0.90 (0.45, 1.83)	0.8	0.9	0.02 (AG *v* GG) 0.3 (AA *v* GG)
rs2920421	*ALOX12*	G	A	220	0.55 (0.39, 0.78)	0.001	0.05	254	0.96 (0.69, 1.34)	0.8	0.9	68	0.86 (0.45, 1.64)	0.7	0.8	0.02 (AG *v* GG) 0.3 (AA *v* GG)
rs2271316	*ALOX12*	G	C	175	0.56 (0.38, 0.82)	0.003	0.05	269	0.91 (0.66, 1.26)	0.6	0.8	96	0.86 (0.50, 1.49)	0.6	0.8	0.06 (CG *v* GG) 0.2 (CC *v* GG)
rs1042522	*TP53*	G	C	290	0.80 (0.59, 1.09)	0.2	0.3	205	0.82 (0.57, 1.18)	0.3	0.5	45	0.37 (0.17, 0.79)	0.01	0.06	0.9 (CG *v* GG) 0.06 (CC *v* GG)

†Rare homozygotes for rs7090328 were n=40 but missing data caused one case to drop out of the regression model.

Figure Legend

Figure 1. seAFOod trial participants and samples contributing to the gene x treatment interaction analysis. Six hundred and sixty-six of 707 seAFOod trial participants provided at least one buffy coat sample for DNA extraction. The number of samples lost during either DNA extraction or SNP genotyping is described at each stage. The final study population consisted of 542 participants for whom SNP genotype and trial exit colonoscopy data were available. EPA, eicosapentaenoic acid; SNP, single nucleotide polymorphism.

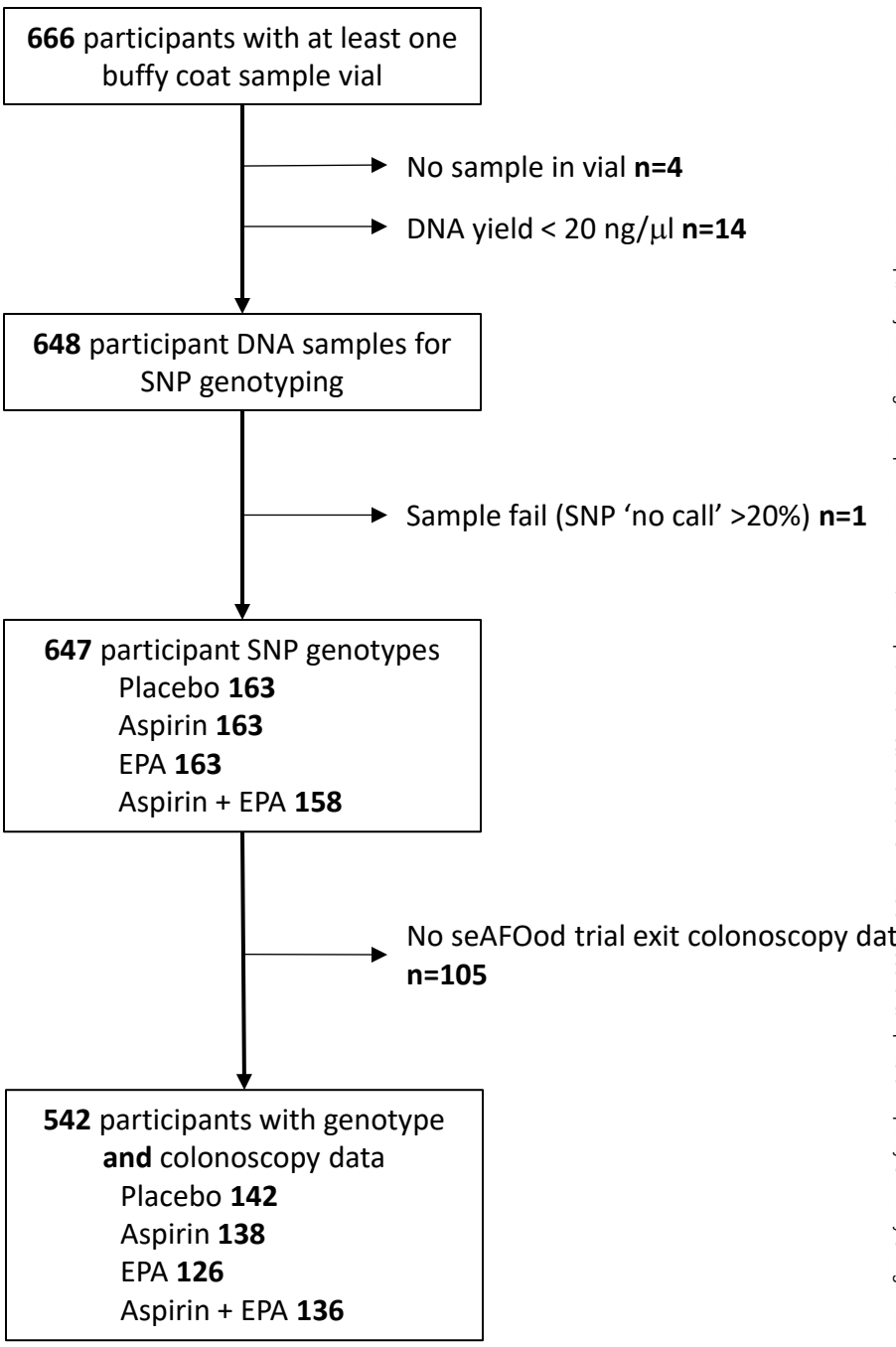

Figure 1