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TITLE: Mendelian randomization of circulating polyunsaturated fatty acids and colorectal cancer risk

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CONFLICTS OF INTEREST:

The authors declare no potential conflicts of interest.

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ABSTRACT (247/250 words)

Background: Results from epidemiologic studies examining polyunsaturated fatty acids (PUFAs) and colorectal cancer (CRC) risk are inconsistent. Mendelian randomization may strengthen causal inference from observational studies. Given their shared metabolic pathway, examining the combined effects of aspirin/NSAID use with PUFAs could help elucidate an association between PUFAs and CRC risk.

Methods: Information was leveraged from GWAS regarding PUFA-associated single nucleotide polymorphisms (SNPs) to create weighted genetic scores (wGSs) representing genetically-predicted circulating blood PUFAs for 11,016 non-Hispanic white CRC cases and 13,732 controls in the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO). Associations per standard deviation increase in the wGS were estimated using unconditional logistic regression. Interactions between PUFA wGSs and aspirin/NSAID use on CRC risk were also examined.

Results: Modest CRC risk reductions were observed per standard deviation increase in circulating linoleic acid ($OR_{LA}=0.96$; 95% CI=0.93-0.98; $p=5.2 \times 10^{-4}$), α -linolenic acid ($OR_{ALA}=0.95$; 95% CI=0.92-0.97; $p=5.4 \times 10^{-5}$); whereas modest increased risks were observed for arachidonic acid ($OR_{AA}=1.06$; 95% CI=1.03-1.08; $p=3.3 \times 10^{-5}$), eicosapentaenoic ($OR_{EPA}=1.04$; 95% CI=1.01-1.07; $p=2.5 \times 10^{-3}$), and docosapentaenoic acids ($OR_{DPA}=1.03$; 95% CI=1.01-1.06; $p=1.2 \times 10^{-2}$). Each of these effects were stronger among aspirin/NSAID non-users in the stratified analyses.

Conclusions: Our study suggests that higher circulating shorter-chain PUFAs (i.e., LA and ALA) were associated with reduced CRC risk, whereas longer-chain PUFAs (i.e., AA, EPA, and DPA) were associated with an increased CRC risk.

Impact: The interaction of PUFAs with aspirin/NSAID use indicates a shared CRC inflammatory pathway. Future research should continue to improve PUFA genetic instruments to elucidate the independent effects of PUFAs on CRC.

MANUSCRIPT: 4,334 words

Main Tables: 3

Supplementary Tables: 5

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide with an estimated 746,000 males and 614,000 females diagnosed in 2012.[1] Diet has been shown to play an important role in CRC development.[2,3] One nutrition-related inflammatory metabolite, prostaglandin E2 (PGE-2), is known to influence colorectal carcinogenesis[4] via promotion of tumor cell proliferation[5,6] and silencing of tumor suppressor and DNA repair genes.[7] PGE-2 is generated via metabolism of omega-6 polyunsaturated fatty acid (PUFA) arachidonic acid (AA) via the cyclooxygenase-2 (COX-2) enzyme[4] and is often overexpressed in CRC.[8,9] While omega-3 polyunsaturated fatty acids (PUFAs) are also metabolized by COX-2, they produce a different array of non-inflammatory eicosanoids which have not been implicated in carcinogenesis. Thus, PGE-2 levels may be competitively reduced by increasing levels of omega-3 PUFAs in the diet, which could be a potential strategy for CRC prevention.

Dietary intake of PUFAs have been studied in relation to CRC incidence; however, the results from epidemiologic investigations have been inconsistent.[10–12] One possible reason for these discrepancies in the epidemiologic literature may be related to error in accurately assessing dietary PUFA intake. For example, differential recall of dietary intake in case-control studies of CRC could lead to biased effect estimates. In cohort studies, repeated measurements would be ideal but are not feasible, and a pre-diagnostic measurement of PUFAs using an objective dietary biomarker may not accurately reflect dietary intake since the etiologically relevant period for CRC development is unclear. The observed inconsistencies could also be due to biases related to inappropriate confounding control, selection bias, or reverse causation. In addition to these methodologic considerations, it is important to consider aspirin and non-steroidal anti-inflammatory drug (NSAID) use in tandem with PUFAs given their shared

metabolic pathway via COX-2 and resulting PGE-2 production. A limited number of studies have examined the interaction between PUFAs and aspirin/NSAID use on CRC risk with inconsistent results.[13,14]

The goal of our study was to estimate potentially unbiased associations between genetically-predicted circulating PUFAs with CRC using the Mendelian randomization approach. The Mendelian randomization approach uses genetic variants as instrumental variables for an exposure, and given alleles are randomly assorted during conception (akin to a randomized trial), results from such analyses are less susceptible to confounding and other biases[15]. Our study was conducted among non-Hispanic whites using data from two large CRC consortia. Given the shared metabolism via COX-2, we further assessed the combined effects of genetically-predicted circulating PUFAs and aspirin/NSAID use on CRC risk.

METHODS

Study population

The current study leverages the Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO) consortium and the Colon Cancer Family Registry (CCFR), a pooled dataset of 14 studies of CRC with a total of 11,018 cases and 13,735 controls, all European ancestry. Details regarding the characteristics of individual studies included in the consortium have been published.[16–18] Briefly, medical records, pathologic reports, or death certificates were used to confirm colorectal cancer cases. Genotyped SNPs that did not meet the following criteria were excluded: (1) call rate <98%; (2) lack of Hardy-Weinberg equilibrium in the controls ($p < 1 \times 10^{-4}$); or (3) low minor allele frequencies.[16] All imputed SNPs had an $R^2 > 0.3$. Additional details regarding genotyping are published elsewhere.[19] Our study used individual-

level and summary statistics data from GECCO to conduct primary and sensitivity analyses. Additionally, summary statistics were available from the ColoRectal Transdisciplinary Study (CORECT) consortium, a pooled dataset comprised of 17 studies with a total of 18,682 cases and 11,225 controls are included. Study-specific sample sizes and genotyping platforms are provided in **Supplementary Table 1**. All study participants provided written informed consent, and all studies included in the consortia were approved by their respective institutional review boards.

Instrumental variable selection

Single nucleotide polymorphisms (SNPs) identified from published omega-6 and omega-3 PUFA GWAS conducted among individuals of European ancestry [20,21] were used as the genetic instruments for this Mendelian randomization analysis. The previous GWAS were conducted among the same individuals as part of the Cohorts for Heart and Aging Research in Genomic Epidemiology (i.e., CHARGE) Consortium. They reported associations between SNPs and plasma levels of omega-6 and omega-3 PUFAs (i.e., as a percentage of total fatty acids). The following nine SNPs were selected as they were all genome-wide significant (i.e., $p < 5 \times 10^{-8}$) and independent at $r^2 < 0.1$: rs10740118, rs174547, rs2727270, rs16966952, rs3798713, rs174538, rs780094, rs3734398, and rs2236212. The SNPs used in the six different genetic instruments (one instrument per PUFA) are summarized in **Table 1**, and further details are provided in **Supplementary Table 2**. Using the β estimates and effect allele frequencies (EAFs) specific to each SNP i , and the variance in PUFA levels from published GWAS [20,21], the percent variation explained by the n SNPs included in the six different genetic instruments were calculated as follows: $\sum_i^n [2\beta_i^2 (MAF)(1 - MAF) / \text{variance}(PUFA)] * 100$ [22]. In GECCO, the average imputation quality for imputed SNPs was $r^2 = 0.98$ (range: 0.97-0.99). In CORECT,

the average imputation quality was $r^2=0.99$ (range: 0.98-0.99).

Construction of weighted genetic scores

Weighted genetic scores (wGSs) were created using individual-level genotyped data in GECCO. For each PUFA, a wGS was constructed per individual as follows: $wGS = \sum_i^n \beta_i * dosage_i$; where n is the number of independent SNPs used for each PUFA instrument, β_i is the effect estimate (i.e., increase in percent of total plasma fatty acids) for SNP i (obtained from two GWAS examining omega-3 and omega-6 PUFAs within the same population [20,21]), and $dosage_i$ (range from 0-2) is the number of the effect alleles (i.e., alleles representing increased fatty acids levels) an individual possesses for SNP i . All GECCO participants had six different PUFA wGSs representing genetically-predicted circulating PUFA levels measured as a percentage of total plasma fatty acids. Excluding DHA's correlation with LA, AA, and ALA, the PUFA wGSs were highly correlated (**Supplementary Table 3**). No wGSs were simultaneously included in a single model.

Statistical analysis

Unconditional logistic regression adjusted for age, sex, study, and top three principal components for European ancestry was conducted to estimate associations between one standard deviation increase in genetically-predicted circulating PUFAs and CRC risk in GECCO. Matching factors including age, sex, and study were included in the models to avoid any bias due to control selection.[23] We also adjusted for principal components of European ancestry to account bias due to population stratification.[24,25] We also explored the association between each PUFA wGS with potential confounders including education (highest level completed),

family history (first-degree relative), regular aspirin/NSAID use (at any point during a participant's lifetime), body mass index (BMI; kg/m²), ever smoking (yes/no), alcohol use (g/day; compared to non-drinkers), folate intake (µg/day from diet), red meat consumption (serving/day), fruit and vegetable intake (servings/day), and sedentary behavior (hours/week; **Supplementary Table 4**). Only education, family history, aspirin/NSAID use, BMI, and fruit intake were found to be significantly associated ($p < 0.05$) with the six different PUFA wGSs. Results from the fully-adjusted model adjusting for these covariates were identical to those from the minimally-adjusted models.

Analyses were stratified by potential effect measure modifiers including sex, age [i.e., < 65 years (median age), ≥ 65 years], smoking use, regular aspirin/NSAID use, and BMI (i.e., ≤ 18.5 kg/m², 18.5-24.9, 25-30, and > 30). Statistically significant differences ($p < 0.05$) in strata were assessed via the likelihood ratio test using nested models for the multiplicative interaction term. Polytomous regression was conducted to estimate stratum-specific estimates by cancer site (i.e., rectal vs. colon, and separately for proximal and distal colon cancer).

Additive interactions were also conducted to assess the combined effects of genetically-predicted circulating PUFA levels and aspirin/NSAID use on CRC risk. All six PUFA-specific wGSs were dichotomized at the median representing “low” and “high” circulating levels. Using a common referent category, additive interactions were assessed statistically via calculation of the relative excess risk due to interaction (RERI) and its corresponding 95% confidence intervals.[26] All analyses were conducted using SAS Enterprise 7.13 (Cary, NC, USA) and “TwoSampleMR” package curated by MR-Base [27] in R 3.5.1 (R Foundation for Statistical Computing; <https://www.r-project.org/>).

Sensitivity analyses

Several sensitivity analyses were conducted in GECCO and CORECT. A fixed-effects inverse-variance weighted Mendelian randomization analysis[28] was conducted using summary statistics from PUFA GWAS and from the two consortia, GECCO and CORECT. The remaining analyses assessed the validity of the genetic instruments utilized in this study. Egger regression estimated a bias-reduced Mendelian randomization association in the presence of directional pleiotropy (i.e., when the average pleiotropic effects of all SNPs used in the instrument are either positive or negative), provided the effects of the instrument on the exposure is not correlated with any pleiotropic effects. Statistically significant intercepts from Egger regression indicate directional pleiotropy and was applied when three or more independent SNPs were included in the instrument (LA and DPA).[29] The weighted-median approach estimated the Mendelian randomization effect assuming at least 50% of SNPs used in the genetic instrument are invalid.[30] Corresponding 95% confidence intervals for the weighted-median estimate were calculated using bootstrapped standard errors. The weighted-median estimate was only conducted for the PUFAs with more than two SNPs in the instrument, and was not conducted for AA, ALA, DPA, or DHA. The multivariable Mendelian randomization was adjusted for the potential pleiotropic effects of the SNPs included in one PUFA instrument on circulating levels of other PUFAs and utilized all nine GWAS-identified SNPs and their PUFA-specific beta estimates.[31,32] Finally, for instruments with more than two SNPs, a “leave-one-out” analysis was conducted where the inverse-variance MR association was re-estimated after excluding the most influential SNP (determined via largest magnitude change in MR estimate after exclusion).[27] All sensitivity analyses using summary statistics were scaled to represent one standard deviation increase in genetically-predicted circulating PUFA levels.

RESULTS

The variants used in the six different PUFA genetic instruments are listed in **Table 1**. The instruments for α -linolenic acid (ALA) and docosahexaenoic acid (DHA) included one SNP each explaining 1.0% (i.e., rs174547) and 0.7% (i.e., rs2236212) percent of variation in PUFA levels, respectively. The instruments for eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) explained a higher proportion of variance in fatty acid levels with 2.1% and 11.6%, respectively. Comparatively, the SNPs associated with omega-6 PUFAs, linoleic acid (LA) and arachidonic acid (AA), explained a higher percent variation in fatty acid levels. Four SNPs were significantly associated with and explained anywhere between 8.8 to 23.6% of the variation in circulating LA levels (reported range from studies included in the omega-6 GWAS [20]). For AA, two SNPs (i.e., rs174547 and rs16966952) together explained more than 33% of variation in AA fatty acid levels, with rs174547 accounting for most of the variation explained.

Main effects and stratified analyses

In **Table 2**, a one standard deviation increase in wGSs for shorter-chain omega-6 and omega-3 fatty acids (i.e., LA and ALA) was associated with 4% to 5% reduced CRC risk ($OR_{LA}=0.96$, 95% CI=0.93-0.98, $p=5.2 \times 10^{-4}$; $OR_{ALA}=0.95$, 95% CI=0.92-0.97, $p=5.4 \times 10^{-5}$). An increased CRC risk was observed per standard deviation increase in circulating longer-chain omega-3 fatty acids, EPA ($OR_{EPA}=1.04$, 95% CI=1.01-1.07, $p=2.5 \times 10^{-3}$) and DPA ($OR_{DPA}=1.03$, 95% CI=1.01-1.06, $p=1.2 \times 10^{-2}$). No association was observed for DHA. The largest observed increased risk was for AA, the longer-chain omega-6 PUFA, where a 6% increased CRC risk was observed ($OR_{AA}=1.06$, 95% CI=1.03-1.08, $p=3.3 \times 10^{-5}$).

Stratified analyses are also presented in **Table 2**. Overall, most associations showed little evidence for varying by strata of different effect measure modifiers. Potential exceptions included a statistically significant multiplicative interaction for age (<65 years vs. ≥ 65 years; $p_{\text{interaction for LA}}=1.5 \times 10^{-2}$ and $p_{\text{interaction for ALA}}=0.04$) and regular aspirin/NSAID use ($p_{\text{interaction for AA}}=0.05$, $p_{\text{interaction for ALA}}=0.04$, and $p_{\text{interaction for EPA}}=1.4 \times 10^{-2}$). Among those ≥ 65 years, one standard deviation increase in genetically-predicted circulating ALA and LA reduced CRC risk by 7% and 8%, respectively ($OR_{LA, \geq 65 \text{ years}}=0.93$, 95% CI=0.89-0.96, $p=5.4 \times 10^{-5}$; $OR_{ALA, \geq 65 \text{ years}}=0.92$, 95% CI=0.89-0.96, $p=2.7 \times 10^{-5}$). Whereas among individuals <65 years, no statistically significant associations were observed. For longer-chain omega-3 PUFAs (i.e., EPA, DPA, and DHA), no differences across the age-stratified results were observed. For the longer-chain omega-6, one standard deviation increase in circulating AA levels was associated with an 8% increased CRC risk among those ≥ 65 years ($OR_{AA, \geq 65 \text{ years}}=1.08$, 95% CI=1.04-1.12, $p=2.7 \times 10^{-5}$), and no association was observed among those <65 years ($OR_{AA, < 65 \text{ years}}=1.03$, 95% CI=0.99-1.07, $p=0.08$). Among aspirin/NSAIDs non-users, a similar 8% increased risk was observed per standard deviation increase in circulating AA ($OR_{AA, \text{ aspirin/NSAID non-user}}=1.08$, 95% CI=1.04-1.11, $p=8.3 \times 10^{-6}$), whereas no association was observed ($OR_{AA, \text{ aspirin/NSAID user}}=1.02$, 95% CI=0.98-1.07, $p=0.34$) among users. For the short-chain omega-3 PUFA ALA, those individuals who were aspirin/NSAID non-users were observed to have a 7% reduced CRC risk per one standard deviation increase in circulating ALA levels ($OR_{ALA, \text{ aspirin/NSAID non-user}}=0.93$, 95% CI=0.90-0.96, $p=9.7 \times 10^{-6}$). Similar to longer-chain omega-6 AA, increased CRC risks were observed for higher levels of circulating longer-chain omega-3s EPA ($OR_{EPA, \text{ aspirin/NSAID non-user}}=1.07$, 95% CI=1.03-1.10, $p=1.7 \times 10^{-4}$) and DPA ($OR_{DPA, \text{ aspirin/NSAID non-user}}=1.05$, 95% CI=1.02-1.09, $p=2.4 \times 10^{-3}$) among aspirin/NSAID non-users; however this multiplicative

interaction was only statistically significant for EPA. Whereas among regular aspirin/NSAID users, null associations were observed for PUFAs in the stratified analysis.

Additive interaction with aspirin/NSAID use

In **Table 3**, additive interaction between PUFA-specific wGSs and regular use of aspirin/NSAID via a common referent category (i.e., “low” circulating PUFA levels and aspirin/NSAID non-users) are presented. Among those who were not regular aspirin/NSAID users (i.e., aspirin/NSAID non-users), high levels of circulating shorter-chain PUFAs (i.e., omega-6 LA and omega-3 ALA) was associated with an 11-13% reduction in CRC risk ($OR_{\text{high LA, aspirin/NSAID non-user}}=0.89$, 95% CI=0.84-0.95, $p=7.8 \times 10^{-4}$; $OR_{\text{high ALA, aspirin/NSAID non-user}}=0.87$, 95% CI=0.81-0.93, $p=4.1 \times 10^{-5}$). A 15% increased CRC risk was observed for higher levels of genetically-predicted circulating longer-chain omega-6 AA among aspirin/NSAID non-users ($OR_{\text{AA, aspirin/NSAID non-user}}=1.15$, 95% CI=1.07-1.23, $p=4.4 \times 10^{-5}$). Similar increased CRC risks were observed for higher circulating levels of longer-chain omega-3 PUFAs EPA ($OR_{\text{EPA, aspirin/NSAID non-user}}=1.12$, 95% CI=1.05-1.20, $p=7.6 \times 10^{-4}$) and DPA ($OR_{\text{DPA, aspirin/NSAID non-user}}=1.07$, 95% CI=1.00-1.15, $p=3.9 \times 10^{-2}$), among aspirin/NSAID non-users.

Among those with lower levels of genetically-predicted circulating PUFAs, use of aspirin/NSAIDs was associated with reduced CRC risk, with CRC risk reductions ranging from 24% ($OR_{\text{low AA, aspirin/NSAID user}}=0.76$, 95% CI=0.70-0.82, $p=8.4 \times 10^{-12}$) to 29% ($OR_{\text{low LA, aspirin/NSAID user}}=0.71$, 95% CI=0.65-0.77, $p=3.3 \times 10^{-17}$). Generally, among aspirin/NSAID users, higher levels of genetically-predicted PUFAs (namely LA and ALA) did not further reduce CRC risk compared to lower levels of PUFAs ($OR_{\text{high LA, aspirin/NSAID user}}=0.68$, 95% CI=0.63-0.73, $p=2.0 \times 10^{-20}$; $OR_{\text{high ALA, aspirin/NSAID user}}=0.65$, 95% CI=0.60-0.71, $p=3.2 \times 10^{-25}$). For

longer-chain PUFAs (i.e., omega-6: AA, and omega-3s: EPA, DPA, and DHA), among aspirin/NSAID users, the effect of higher circulating levels of these PUFAs modestly attenuated the CRC risk reductions observed compared to lower levels of AA, EPA, DPA, and DHA. However, the additive interactions presented did not significantly deviate from an additive model as measured via the RERI and corresponding 95% CIs. Overall, CRC risk reductions (likely driven by aspirin/NSAID use) were still observed in this subgroup ($OR_{\text{high AA, aspirin/NSAID user}}=0.82$, 95% CI=0.76-0.89, $p=1.9 \times 10^{-6}$; $OR_{\text{high EPA, aspirin/NSAID user}}=0.80$, 95% CI=0.74-0.87, $p=4.4 \times 10^{-8}$; $OR_{\text{high DPA, aspirin/NSAID user}}=0.77$, 95% CI=0.71-0.83, $p=8.1 \times 10^{-11}$; $OR_{\text{high DHA, aspirin/NSAID user}}=0.80$, 95% CI=0.73-0.87, $p=2.5 \times 10^{-7}$).

Summary statistics and sensitivity analyses results

The inverse-variance weighted fixed-effects Mendelian randomization results (**Supplementary Table 5**) using summary statistics were identical to those from the individual-level wGS results. For PUFAs with more than one SNP included in the instrument, statistically significant heterogeneity was observed for the inverse-variance weighted fixed-effects MR estimates for DPA ($p_{\text{heterogeneity}}=3.6 \times 10^{-4}$), indicating possibility for directional pleiotropy (i.e., when the effect on the outcome for each SNP included in the instrument is in the same direction).[15] The results in CORECT were identical to GECCO. Results from the weighted-median analyses were identical to the inverse-variance weighted fixed-effects MR, indicating that our estimates are robust when assuming half the variants included in the instrument are invalid.[30] No estimates from the multivariable MR approaches were statistically significant, which evaluated potential pleiotropy of SNPs included in one instrument on other PUFAs.[31,32] Results from the “leave-one-out” analysis (only possible for LA and DPA)

indicated that rs174547 was the most influential SNP in these two instruments, and removal of rs174547 from the PUFA instruments did not affect the overall results. The one exception being for DPA in the CORECT consortium where removal of rs174547 resulted in a 7% reduced CRC risk ($OR_{DPA}=0.93$, 95% CI=0.88-0.97, $p=2.1 \times 10^{-3}$).

DISCUSSION

In our study conducted among over 24,000 non-Hispanic white individuals from the GECCO consortium, we observed a 6% increased CRC risk among those with higher genetically-predicted circulating levels of omega-6 PUFA AA. Modest increased risks were observed for EPA and DPA. Modest risk reductions were observed for longer-chain omega-6 PUFA LA, and longer-chain omega-3 PUFAs ALA. These associations remained statistically significant among those ≥ 65 years and among aspirin/NSAID non-users. When stratified by aspirin/NSAID use, one standard deviation increase in circulating AA increased risk of CRC by 8% ($p_{interaction}=0.05$), and reduced risk by 7% for ALA ($p_{interaction}=0.04$). Regular users of aspirin/NSAIDs were observed to have 18-35% reduced risk of CRC regardless of their genetically-predicted levels of PUFAs. Our main effects results were confirmed using the summary statistics Mendelian randomization approach.

Not all the associations observed were consistent with our biologic hypothesis regarding omega-6 and omega-3 PUFAs. For example, a modest 4% reduction in CRC risk was observed for increases in genetically-predicted short-chain omega-6 LA levels, which is a pre-cursor to AA levels and subsequently PGE-2. One potential explanation for the risk reduction observed for the LA may be related to two variants included in the instrument that are part of the *FADS1* and *FADS2* genes (i.e., rs174547 and rs2727270, respectively) and are responsible for the conversion

of LA to AA. When incorporating these SNPs in the instrument, increased genetically-predicted levels of LA will result in lower downstream levels of AA and PGE-2, which could potentially reduce CRC risk. We also observed modest increased risks for higher genetically-predicted levels of potentially anti-inflammatory omega-3 PUFAs EPA and DPA. However, the risk reduction is consistent with a previous meta-analysis of LA intake on CRC risk[33], and with a previous Mendelian randomization study (also included data from the CCFR) conducted by May-Wilson et al. among 7 European cohorts ($OR_{LA}=0.95$, 95% CI= 0.93-0.98).[34] Furthermore, results for AA from May-Wilson et al. ($OR_{AA}=1.05$, 95% CI=1.02-1.07) are nearly identical to those presented in our study. Results for EPA, DPA, and DHA were in the same direction (except for EPA); however, the effect sizes reported in May-Wilson et al. have larger magnitudes but are less precise. We also observed slightly stronger associations among older (i.e., ≥ 65 years) compared to younger individuals for many of the PUFAs, which could be an indication of the cumulative effects of being genetically-predisposed to higher PUFA levels on CRC risk.

The benefits of taking aspirin/NSAID on CRC risk has been studied extensively.[35,36] GECCO has also reported risk reductions with aspirin/NSAID use ($OR=0.71$, 95% CI=0.66-0.77),[37] and the magnitude of the risk reduction was similar to the associations reported among the subgroup of aspirin/NSAID users when considering the interactions with circulating PUFAs. Notably, in the Nurses' Health Study, long-term aspirin use (i.e., >10 years) and NSAID use reduced CRC risk by 32%, and risk was reduced by over 50% ($OR=0.47$, 95% CI=0.31-0.71) among women taking more than 14 (325-mg) tablets per week.[35] The benefits of long-term aspirin use were corroborated in randomized and observational studies.[36] The recommendation to the United States Preventive Task Force for long-term aspirin use as a

preventive strategy for CRC was indicated for 10 years post-initiation.[38] In our study, aspirin/NSAID use was defined as regular use over an individual's lifetime and this definition varied according to study. Thus, it is possible that heterogeneity in assessment of aspirin intake may affect the association between long-term aspirin use and CRC risk in our study; however, the associations observed are consistent with previous investigations.

Hall and colleagues examined the interaction between PUFA levels and aspirin use on CRC risk among men in the Physicians' Health Study.[14] They reported reduced CRC risk with higher intake of long-chain omega-3 PUFAs (i.e., Quartile 4 vs. Quartile 1, $OR_{Q4vs.Q1}=0.34$, 95% $CI=0.15-0.82$) among non-aspirin users. Similar to our results, the potential added benefit of increasing long-chain omega-3 intake among aspirin users was minimal when compared to non-aspirin users with low omega-3 intake.[14] Among the Nurses' Health Study and Health Professionals Follow-up Study participants, the potential modification of marine omega-3 dietary intake by aspirin/NSAID use on CRC risk was evaluated but no significant heterogeneity was reported.[13] Another study examined pre-diagnostic levels of the urinary PGE-2 metabolite (PGE-M) on colorectal adenoma risk stratified by aspirin use (>2 tablets per week) in the Nurses' Health Study.[39] Aspirin use was only beneficial among individuals with high levels of PGE-M. Arachidonic acid uptake by COX-2 is reduced in the presence of NSAIDs in colon cancer cells.[40] Similarly, reduced binding of DHA to COX-2 was observed when combined with a selective COX-2 inhibitor celecoxib.[41] Inhibition of PUFA metabolism via the COX-2 enzyme in the presence of aspirin may help to explain the potential antagonism observed for the interaction between PUFAs and aspirin on CRC risk.

Our study has several strengths. First, we utilized data from two large consortia of approximately 25,000 and 30,000 subjects (for GECCO and CORECT, respectively) to estimate

potentially unbiased association between PUFAs and CRC risk using the Mendelian randomization approach. The availability of individual-level GECCO data and several covariates was helpful for assessing the association between the PUFA-specific wGSs with CRC risk factors. This is one way to assess the validity of the genetic instrument in a Mendelian randomization analysis (i.e., the instrument should not be associated with confounders of the exposure-disease association).[15] We adjusted for additional covariates that were found to be associated with the six different PUFA wGS; however, the results from the adjusted models were identical to the minimally-adjusted models. We also conducted stratified analyses to estimate the association between genetically-predicted PUFAs among different subgroups. Several Mendelian randomization sensitivity analyses were conducted to assess the robustness of the results in the presence of pleiotropy, but these analyses are likely underpowered due to the limited number of independent SNPs included. Finally, we are one of the few studies to assess the additive interaction between genetically-predicted circulating PUFAs along with aspirin/NSAID use on CRC risk.

While our study has many strengths, there are several opportunities for improvement in future investigations. There was indication of directional pleiotropy in the Mendelian randomization sensitivity analyses (for DPA), and for some of the PUFAs, we were unable to estimate an effect for sensitivity analyses using summary statistics (i.e., Egger regression, weighted-median approach, leave-one-out analysis) due to the limited number of SNPs used in the genetic instrument. Several of the wGSs were highly correlated with one another in the individual-level analysis, which would affect the estimation of independent PUFA effects. However, incorporating additional SNPs as part of the genetic instrument in the future will increase the percent variation explained and subsequently increase the strength of the genetic

instrument. Stronger genetic instruments will ultimately help to further elucidate independent PUFA effects and provide a better opportunity to assess influence of pleiotropy on the Mendelian randomization estimates. Furthermore, using new weights from future GWAS that examine associations with longer-term PUFA biomarkers (e.g., adipose tissue and red blood cell) will help to clarify the potential causal role of PUFAs on CRC risk. The power to detect an OR at least 1.05 at an $\alpha=0.05$ in our study ranged from approximately 5% (for DHA) to 62% (for AA), and is dependent upon the strength of the instrument.[42] Further, increasing the percent variation explained may allow for the detection of even smaller effects due to increased power. The associations derived from a Mendelian randomization analysis could help to identify the presence of a potential causal association between exposure and outcome. Many comparisons were made in this analysis and thus the potential for false-positive associations exists. However, most associations in our analysis remain statistically significant even after Bonferroni correction for multiple comparisons. Furthermore, our genetic instruments utilized SNPs previously reported to be associated with circulating PUFAs that have previously shown to have influence on carcinogenesis in experimental studies, and thus the analyses undertaken in this paper are based on an *a priori* biologic hypothesis. Finally, it would be worthwhile to conduct similar analyses in different populations to better understand the influence of PUFAs on CRC risk in populations where the ratio of omega-6 to omega-3 PUFAs may differ (e.g., Asians), and among populations where CRC risk is high (e.g., African Americans). Future investigations should consider identifying additional genetic variants associated with PUFA levels among different races which would facilitate conducting Mendelian randomization analyses in these populations.

Due to substantial amount of missing data for continuous measures of aspirin/NSAID use, we were unable examine the interaction between long-term aspirin/NSAID use and

circulating PUFAs on CRC risk. However, since selective COX-2 inhibitors may increase risk of cardiovascular disease with long-term use,[43] examining the potential added benefit of omega-3 PUFA intake with long-term use of selective COX-2 inhibitors may be futile realistically (unless among high-risk population subgroups). Finally, it is possible that the results from the additive interaction are subject to residual confounding given aspirin/NSAID use was self-reported.[44] Thus, future investigations with better long-term measures of aspirin/NSAID use should further examine the interaction with PUFAs, and also consider other potential biologic pathways.

In conclusion, we observed a 6% increased risk for CRC among those with higher genetically-predicted circulating levels of omega-6 PUFA AA, and similarly modest increased risks for longer-chain omega-3 PUFAs EPA and DPA. Risk reductions were observed among those with higher genetically-predicted circulating levels of short-chain omega-6 PUFA LA, and short-chain omega-3 PUFA ALA. Our study results indicate that among aspirin/NSAID users, the potential benefit of increasing long-chain omega-3 PUFAs may be minimal in terms of further reducing CRC risk. Results from the Mendelian randomization analysis using summary statistics corroborate our main effect findings. However, due to the limited number of variants used in some genetic instruments, an assessment of the influence of pleiotropy on our estimates could not be evaluated for all PUFAs. Given the small effects observed and the limited number of SNPs used in our genetic instruments, the clinical significance of our results is limited, and our results may only indicate a shared CRC inflammatory pathway for PUFAs and aspirin/NSAID use. Future Mendelian randomization studies should continue to improve the genetic instruments used which will help to further elucidate the effects of specific PUFAs on CRC risk.

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Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy or views of the International Agency for Research on Cancer/World Health Organization.

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