CANCER

Two genome-wide interaction loci modify the association of nonsteroidal anti-inflammatory drugs with colorectal cancer

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Regular, long-term aspirin use may act synergistically with genetic variants, particularly those in mechanistically relevant pathways, to confer a protective effect on colorectal cancer (CRC) risk. We leveraged pooled data from 52 clinical trial, cohort, and case-control studies that included 30,806 CRC cases and 41,861 controls of European ancestry to conduct a genome-wide interaction scan between regular aspirin/nonsteroidal anti-inflammatory drug (NSAID) use and imputed genetic variants. After adjusting for multiple comparisons, we identified statistically significant interactions between regular aspirin/NSAID use and variants in 6q24.1 (top hit *rs72833769*), which has evidence of influencing expression of TBC1D7 (a subunit of the TSC1-TSC2 complex, a key regulator of MTOR activity), and variants in 5p13.1 (top hit *rs350047*), which is associated with expression of PTGER4 (codes a cell surface receptor directly involved in the mode of action of aspirin). Genetic variants with functional impact may modulate the chemopreventive effect of regular aspirin use, and our study identifies putative previously unidentified targets for additional mechanistic interrogation.

INTRODUCTION

Aspirin, a nonsteroidal anti-inflammatory drug (NSAID), is inversely associated with colorectal cancer (CRC) risk. In meta-analyses and systematic reviews of large observational studies, regular long-term use of aspirin is associated with CRC risk reduction of 20 to 30% (*1*–*3*). Reduction of CRC risk was also observed in well-designed clinical trials of colorectal neoplasia outcomes among individuals with Lynch syndrome or prior colorectal adenoma or CRC (*4*–*10*). However, the precise mechanism of action has not yet been fully elucidated, although several modes of action have been suggested for aspirin's anticancer effects (*3*, *11*). Despite a potential overlap in mechanism (i.e., inhibition of prostaglandin synthesis), the relationship of non-aspirin NSAIDs (henceforth simply termed "NSAIDs") and CRC risk is less consistent, potentially owing to more heterogeneous use in published studies, contamination of non-aspirin NSAID categories with aspirin use, or confounding by indication for use (i.e., individuals with higher inflammatory states). Because not all studies specifically differentiate between aspirin and other NSAID use, additional study of the impact of this broader drug class on CRC risk is warranted. Genetic variation is a key individual factor that likely interacts with aspirin and NSAIDs to ultimately determine CRC risk. In general, gene-drug interaction studies aim to clarify these relationships and implicate regions involved in the mode action (*12*), which may identify subpopulations of individuals that might most benefit from an aspirin preventive strategy, particularly in light of the potential harms.

In this analysis, we conducted a genome-wide interaction scan (GWIS) of regular aspirin/NSAID use and imputed genetic markers. A previous GWIS, conducted on a smaller subset of individuals, identified interaction loci in regions 12p12.3 and 15q25.2 (*13*). We expanded upon that analysis by greatly increasing the sample size and using additional statistical methods that improve power to detect interaction loci and infer functional impact.

RESULTS

Regular aspirin/NSAID use and CRC risk

Our combined study sample included 72,667 individuals (30,806 cases and 41,861 controls) with known aspirin/NSAID use status and CRC outcome status (table S1). We considered regular aspirin and/or NSAID use as a combined variable (aspirin/NSAID) because not all studies collected data on aspirin use separate from other NSAID use, and aspirin and NSAIDs likely have common anticancer mechanisms. Complete study inclusion and exclusion criteria and how regular aspirin/NSAID use is defined are described in Materials and Methods. Secondary analyses restricted to studies with information on aspirin use only are also presented (N = 72,137; 30,574 cases and 41,563 controls). Regular aspirin/NSAID use was less prevalent among CRC cases compared to controls (34% versus 40%, respectively) as was aspirin use alone (27% of cases versus 31% of controls). As expected, CRC cases tended to be older, had a higher body mass index (BMI) and energy intake, had a greater proportion of family history of CRC, were less educated, and were more likely to be exposed to other known risk factors including heavy alcohol intake and tobacco smoking compared to controls.

In meta-analyses of study-specific associations, regular aspirin/NSAID use [odds ratio (OR) = 0.76; 95% confidence interval (CI) 0.72 to 0.81] and aspirin use alone (OR = 0.80; 95% CI = 0.76 to 0.84) were associated with reduced CRC risk (Fig. 1). There was statistically significant cross-study heterogeneity in the aspirin/NSAIDS and aspirin-only associations, which appeared to be largely due to study design (fig. S1). The estimated reductions in CRC risk were more pronounced in case-control studies for aspirin/NSAID use ($OR_{aspirin/NSAID}$ = 0.67, 95% CI = 0.62 to 0.72) than in cohort studies ($OR_{aspirin/NSAID}$ = 0.85, 95% CI = 0.80 to 0.91) ($P_{het} < 0.001$). Similar trends in estimates were observed for regular use of aspirin-only (case-control studies: $OR_{aspirin}$ = 0.72, 95% CI = 0.67 to 0.77; cohort studies: $OR_{aspirin}$ = 0.88, 95% CI = 0.83 to 0.94) ($P_{het} < 0.001$). Further adjustment by established CRC risk factors—including BMI, alcohol intake, smoking, and red meat consumption—did not substantially change OR estimates of aspirin/NSAID use (table S2). Analyses stratified by sex show nominally stronger inverse associations with regular aspirin/NSAID use and CRC risk among women compared to men (multivariate model $P_{interaction}$ = 0.014), but there were no statistically significant sex differences for aspirin use

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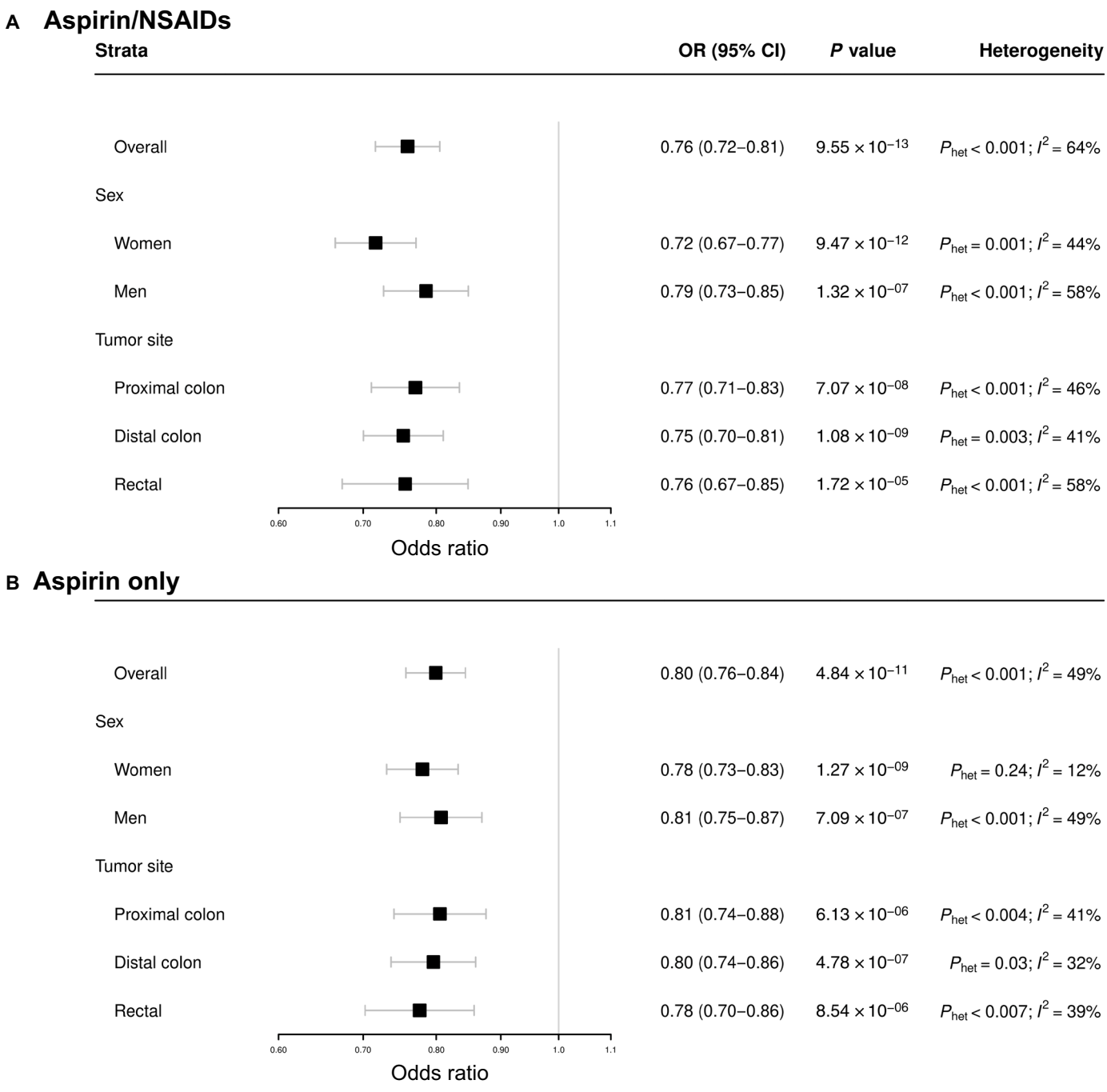

Fig. 1. Association of regular aspirin/NSAID use with CRC according to sex and tumor location. Results from meta-analysis of association between regular use of (**A**) aspirin/NSAID or (**B**) aspirin-only and colorectal cancer, overall and stratified by sex and tumor site. Models adjusted for age and sex. Heterogeneity measures include Cochran's Q statistic p-value (P_{het}) and Higgin's statistic (I^2), which describes the proportion of observed variance due to heterogeneity and not attributed to sampling error.

only (table S2). There were no notable differences in associations when stratifying models by tumor site (table S2).

GWIS results

We found no evidence of genomic inflation or residual population stratification in the genome-wide scan one degree of freedom (df) Gene x Environment (GxE) test *P* values for aspirin/NSAID or aspirin-only exposure variables (fig. S2). We identified a statistically significant interaction between regular aspirin/NSAID use and *rs72833769* (chr6:12577203 T/C, $P = 1.27 \times 10^{-8}$; Table 1), a marker in locus 6q26 upstream of gene *PHACTR1* (Fig. 2A). The overall minor allele frequency for this single-nucleotide polymorphism (SNP) was 0.067 (Table 1). This interaction for *rs72833769* was similarly significantly associated when restricted to aspirin use only ($P_{aspirin} = 4.02 \times 10^{-9}$; Fig. 2B). While *rs72833769* did not have a direct marginal association with CRC risk (OR = 1.00; 95% CI = 0.96 to 1.05),

Table 1. Significant results from genome-wide interaction scans of regular aspirin/NSAID use and CRC risk. SNP, single-nucleotide polymorphism; Chr, chromosome; BP Position, base pair position based on NCBI Build37. "Overall" is the *P* value for the 1-df GxE test (*rs72833769*) or two-step procedure (*rs350047*). Imputed SNPs were coded as expected gene dosage. Multiplicative interaction terms were modeled as the product of Aspirin/NSAIDs and each SNP of interest. All statistical tests were two-sided.

Method	SNP	Chr	BP Position	Locus	Gene	Ref	Alt	Alt allele freq (1000G)	Exposure	*P* value (overall)	EDGE *P* value (step 1)	EDGE *P* value (step 2)	*P* value (3 df)
1–degree-of-freedom (df) GxE													
	rs72833769	6	12577203	6p24.1	Upstream of *PHACTR1*	T	C	0.02	Aspirin/NSAID	1.27×10^{-8}	–	–	–
									Aspirin only	4.02×10^{-9}	–	–	–
Two-step (EDGE) and 3-df													
	rs350047	5	40252294	5p13.1	*LINC00603* (upstream of *PTGER4*)	C	T	0.41	Aspirin/NSAID	8.20×10^{-8}	5.22×10^{-6}	4.41×10^{-5}	6.50×10^{-9}
									Aspirin only	2.00×10^{-8}	9.40×10^{-6}	1.08×10^{-5}	3.12×10^{-9}

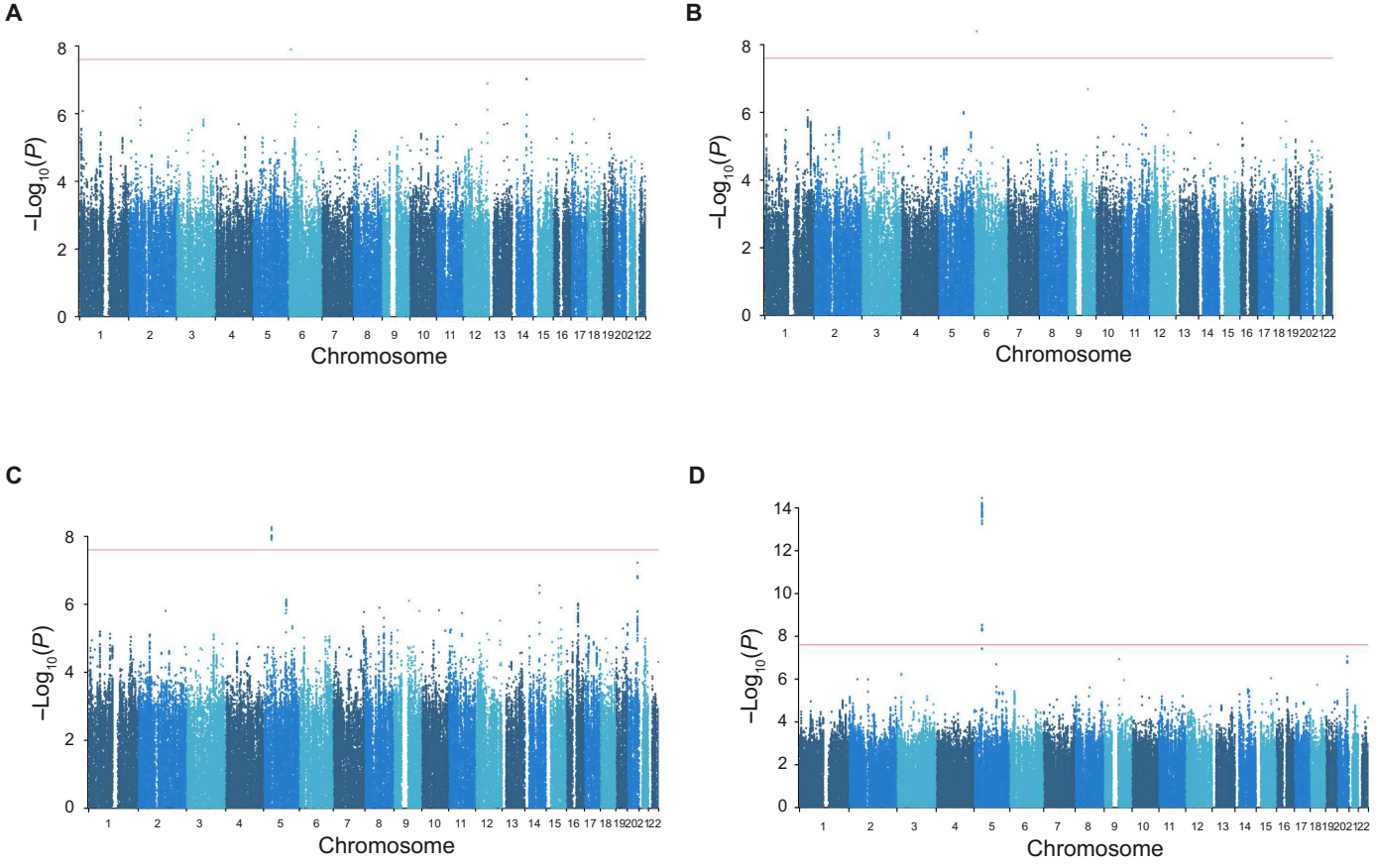

Fig. 2. Manhattan plots of genome-wide interaction scans. (**A**) traditional logistic regression interaction test (1-df) for aspirin/NSAID (**B**) traditional logistic regression interaction test (1-df) for aspirin-only, (**C**) 3-df joint test for aspirin/NSAID, and (**D**) 3-df joint test for aspirin-only. Red line represents a genome-wide significance threshold of $5 \times 10^{-8}/2.5$ after adjustment for multiple testing.

stratified analyses showed regular aspirin/NSAID use or aspirin-only use were significantly associated with lower CRC risk only among homozygous carriers of the common allele T (aspirin/NSAID: $OR_{TT} = 0.73$, 95% CI = 0.71 to 0.76; aspirin only: $OR_{TT} = 0.76$, 95% CI = 0.73 to 0.79) (Tables 2 and 3 and fig. S3, A and B). In contrast, regular aspirin/NSAID use was not significantly associated with risk of CRC among individuals carrying the CT or CC genotype (aspirin/NSAID: $OR_{CT} = 0.94$, 95% CI = 0.85 to 1.03; $OR_{CC} = 1.44$, 95% CI = 0.82 to 2.54; aspirin-only: $OR_{CT} = 1.00$, 95% CI = 0.90 to 1.11; $OR_{CC} = 1.40$, 95% CI = 0.75 to 2.59). Adjustment for additional CRC risk factors did not materially affect the estimate of interaction (Tables 2 and 3, model 2). In addition, interaction effects for this SNP did not differ substantially by sex or tumor subsite, although interaction estimates were modestly stronger for colon locations than rectal tumors (table S3A).

We identified a second locus *rs350047* (chr5:40252294 C/T, $MAF_{1000\ Genomes} = 0.48$) of interest using both our two-step EDGE method (fig. S3) and our 3-df joint test (Table 1). On the basis of the two-step testing, this SNP shows significant evidence of interaction with aspirin-only use; interactions were marginally significant for aspirin/NSAID use. For aspirin/NSAID use, *rs350047* achieved a step 1 "EDGE" *P* value of 5.22×10^{-6} and step 2 "GxE" *P* value of 4.41×10^{-5} (overall two-step *P* value = 8.2×10^{-8}). The corresponding *P* values for aspirin use only were 9.40×10^{-6} for step 1 and 1.08×10^{-5} for step 2, which in combination achieve genome-wide significance (overall two-step *P* value = 2.0×10^{-8}) (Table 1). This locus was statistically significant for both exposure variables based on 3-df joint test (aspirin/NSAID $P = 6.5 \times 10^{-9}$; aspirin only $P = 3.12 \times 10^{-9}$). Combined, the consistency across methods supports the identification of this hit. This genetic variant lies in locus 5p13.1 upstream of *PTGER4* and *LINC00603*, within a genomic region identified in a previously published GWAS of CRC risk and tagged by deletion-insertion marker *rs58791712* (*14*). Stratified analyses showed that regular aspirin/NSAID use and aspirin only use was inversely associated with CRC risk across all genotype groups, but the association was of greater magnitude among homozygous carriers of the T allele (aspirin/NSAID: $OR_{TT} = 0.67$, 95% CI = 0.63 to 0.72; aspirin-only: $OR_{TT} = 0.69$, 95% CI = 0.64 to 0.74) compared to heterozygous or homozygous carriers of the C allele (aspirin/NSAID: $OR_{CT} = 0.78$, 95% CI = 0.74 to 0.81; $OR_{CC} = 0.81$, 95% CI = 0.76 to 0.87; aspirin-only: $OR_{CT} = 0.81$, 95% CI = 0.77 to 0.85; $OR_{CC} = 0.85$, 95% CI = 0.79 to 0.91) (Tables 2 and 3 and fig. S3, C and D). Adjustment for additional CRC risk factors similarly did not affect the estimates of this interaction (Tables 2 and 3, model 2). The interaction is somewhat stronger among men than women (table S3B), although the three-way interaction *P* value was not statistically significant ($P_{GxExSex} = 0.07$). Similarly, interaction effects at this locus were similar in magnitude across tumor site (table S3B). In our genome-wide scan for rare variants, we did not find any significant interactions with aspirin/NSAID or aspirin-only use.

Interactions stratified by CRC molecular subtypes

Case counts with available tumor marker information on *BRAF* and *KRAS* mutation status and the presence of CpG island methylator phenotype (CIMP) or microsatellite instability (MSI) are summarized in table S4. Generally, when fitting traditional case-control logistic regression models, interactions between aspirin/NSAID use and *rs72833769* or *rs350047* are replicated within the subset of cases with available tumor-marker data, at a significance level $P < 0.05$ (Fig. 3; overall cases versus controls). In stratified analyses according to the presence or absence of molecular subtype markers, significant association for the interaction between *rs72833769* and aspirin/NSAIDs were limited to tumors absent of each of the available tumor markers (Fig. 3A; *BRAF* wild-type, CIMP-low/negative, non–MSI-high, and *KRAS* wild-type tumors versus controls: all $P < 0.05$); however, estimates for the interaction between cases absent for these molecular markers and those positive for the individual markers were not significantly heterogeneous (all $P_{het} > 0.05$). In contrast, significant association for the interaction between *rs350047* and aspirin/NSAID is observed only among cases positive for *BRAF* mutation, CIMP-high, or MSI-high compared to controls, but not among cases with *KRAS* mutation nor absent any individual marker (Fig. 3B). Similarly, no significant heterogeneity between cases with the molecular marker present versus absent was observed (all $P_{het} > 0.05$), although the estimates for *BRAF* mutant versus wild-type and CIMP-high versus CIMP-low/negative approached significance. Further restricting the analysis to aspirin use alone did not materially alter estimates, but the reduced sample size resulted in slight attenuation of observed statistical significance.

Functional follow-up

The regional plot for *rs72833769* (6p24.1) shows several genes in the vicinity of this locus, including *PHACTR1* and *END1* (fig. S5A). Several data sources provide evidence that the region tagged by rs72833769 plays a regulatory role in the transcription of neighboring genes. In CRC tumor and normal tissue, and CRC cell lines derived from work by Cohen *et al.* (*15*), the lead SNP *rs72833769* showed little evidence of functional activity. However, several SNPs in linkage disequilibrium (LD) with *rs72833769* coincided with accessible chromatin regions based on H3K27ac markers primarily in CRC tumors (table S5A and fig. S6A). This region also contains overlaps with regulatory regions based on H3K4me1, H3K4me3, H3K9ac, and H3K27 histone modification signals in connective, gastrointestinal, and immune cell types (*16*). ENSEMBL queries also show several regulatory features in this locus, in addition to a transcript region for lncRNA RP11-125 M16.1 (table S5A).

Evidence for an expression quantitative trait locus (eQTL) was less pronounced. None of the lead or LD SNPs were significant eQTLs for any gene/tissue in the GTEx v.8 database. In the Barcelona and University of Virginia genotyping and RNA sequencing (BarcUVa-Seq) dataset, a single SNP *rs12194512* [LD SNP, coefficient of determination (R^2) = 0.36] was a significant eQTL with *GFOD1*, a gene approximately 800 kb upstream of the main finding. While *rs72833769* was not specifically identified as an eQTL for *PHACTR1*, it was significantly associated with predicted *PHACTR1* expression ($P = 8.4 \times 10^{-6}$). Last, in the eQTLGEN database, we found significant eQTLs with LD SNPs *rs499627* ($R^2 = 0.32$) and *rs538788* ($R^2 = 0.24$) for the expression of *TBC1D7* (table S5A).

The regional plot for *rs350047* (5p13.1) shows that the SNP lies within a long noncoding RNA region, *LINC00604*, and is in LD with a known GWAS region identified in 2018 by Schmit *et al.* (*14*) (fig. S5B). This locus appears to reside in a region with little histone modifications or deoxyribonuclease (DNAse) accessible sites based on evidence from CRC normal and tumor tissues and CRC cell lines (table S5B and fig. S6B). However, SNPs in LD with *rs350047* overlap with functionally active sites in connective and immune cells (*17*). Several LD SNPs are significant eQTLs for *PTGER4* in GTEx v.8 suprapubic epithelial cells. This finding is

Table 2. Associations between aspirin/NSAID intake and CRC risk stratified by *rs72833769*. Odds ratios (ORs) and 95% confidence intervals (CIs) calculated from traditional interaction model with an interaction term. Model 1: Covariates include age (continuous), sex, study, and the first three principal components. Model 2: Includes all covariates in Model 1 + smoking (never/ever), alcohol consumption (nondrinkers; moderate, 1 to 28 g/day; heavy, >28 g/day), BMI (continuous), and red meat intake (study and sex specific quartiles of red meat intake based on controls only).

	TT			CT			CC		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
Aspirin/NSAID									
Model 1									
Nonregular use	17,861	21,554	1.0 (Ref)	2,410	3,349	1.0 (Ref)	89	120	1.0 (Ref)
Regular use	9,011	14,697	0.73 (0.71–0.76)	1,374	2,073	0.94 (0.85–1.03)	61	68	1.44 (0.82–2.54)
Model 2									
Nonregular use	15,957	19,917	1.0 (Ref)	2,138	3,113	1.0 (Ref)	81	114	1.0 (Ref)
Regular use	8,159	13,423	0.72 (0.70–0.75)	1,243	1,930	0.90 (0.81, 0.99)	54	65	1.33 (0.72, 2.46)
Aspirin only									
Model 1									
Nonregular use	19,535	24,775	1.0 (Ref)	2,689	3,874	1.0 (Ref)	108	142	1.0 (Ref)
Regular use	7,134	11,229	0.76 (0.73–0.79)	1,066	1,497	1.00 (0.90, 1.11)	42	46	1.40 (0.75, 2.59)
Model 2									
Nonregular use	17,517	22,969	1.0 (Ref)	2,399	3,615	1.0 (Ref)	98	136	1.0 (Ref)
Regular use	6,427	10,180	0.76 (0.73–0.79)	958	1,384	0.96 (0.86, 1.07)	37	43	1.28 (0.66, 2.49)

Table 3. Associations between aspirin/NSAID intake and CRC risk stratified by *rs350047*. Odds ratios (ORs) and 95% confidence intervals (CIs) calculated from traditional interaction model with an interaction term. Model 1: Covariates include age (continuous), sex, study, and the first three principal components. Model 2: Includes all covariates in Model 1 + smoking (never/ever), alcohol consumption (nondrinkers; moderate, 1 to 28 g/day; heavy, >28 g/day), BMI (continuous), and red meat intake (study and sex specific quartiles of red meat intake based on controls only).

	CC			CT			TT		
	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)	Cases	Controls	OR (95% CI)
Aspirin/NSAID									
Model 1									
Nonregular use	5,179	6,921	1.0 (Ref)	10,141	12,453	1.0 (Ref)	5040	5649	1.0 (Ref)
Regular use	2,765	4,496	0.81 (0.76–0.87)	5,301	8,425	0.78 (0.74–0.81)	2380	3917	0.67 (0.63–0.72)
Model 2									
Nonregular use	4,630	6,410	1.0 (Ref)	9,057	11,480	1.0 (Ref)	4489	5254	1.0 (Ref)
Regular use	2,504	4,104	0.80 (0.75–0.86)	4,811	7,725	0.76 (0.72–0.80)	2141	3589	0.66 (0.61–0.71)
Aspirin only									
Model 1									
Nonregular use	5,690	7,958	1.0 (Ref)	11,157	14,340	1.0 (Ref)	5485	6493	1.0 (Ref)
Regular use	2,197	3,374	0.85 (0.79–0.91)	4,173	6,389	0.81 (0.77–0.85)	1872	3009	0.69 (0.64–0.74)
Model 2									
Nonregular use	5,107	7,394	1.0 (Ref)	9,999	13,272	1.0 (Ref)	4908	6054	1.0 (Ref)
Regular use	1,978	3,055	0.85 (0.79–0.91)	3,774	5,815	0.81 (0.77–0.85)	1670	2737	0.69 (0.64–0.74)

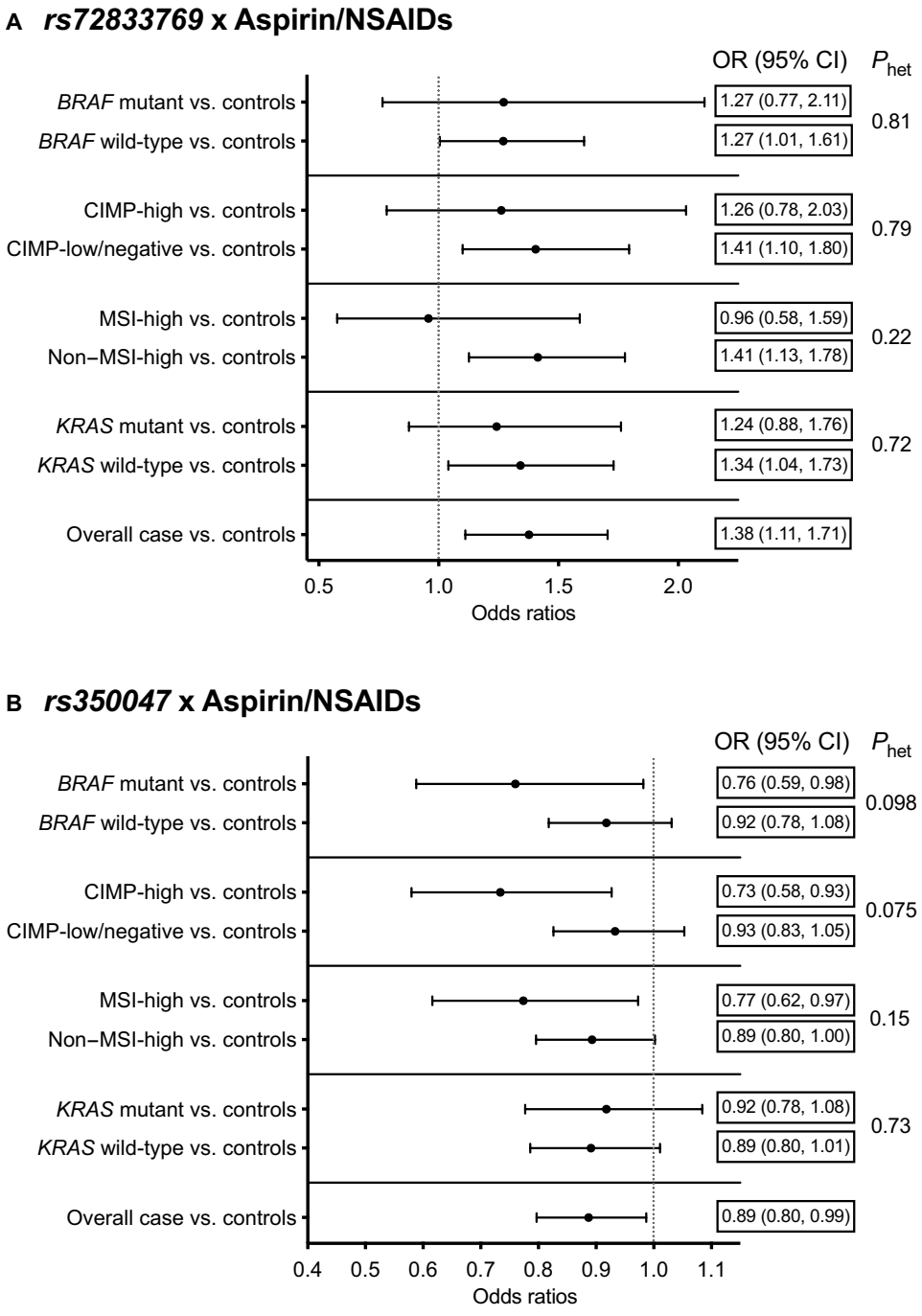

Fig. 3. Forest plots of GxE interactions for identified SNPs and aspirin/NSAID and risk of CRC stratified by tumor molecular subtypes. ORs (dots) and 95% CIs (error bars) are plotted for each case stratified by the presence (*BRAF* mutant; CIMP-high; MSI-high; *KRAS* mutant) or absence (*BRAF* wild type; CIMP-low/negative; Non-MSI-high; *KRAS* wild type) of the marker in cases versus controls (referent) for each identified SNP: (**A**) *rs72833769* and (**B**) *rs350047*. The *P* value for heterogeneity (P_{het}) between estimates for cases with the molecular marker present versus absent and the overall association of the interaction for cases with molecular marker data versus controls is also provided.

corroborated when querying eQTLGEN, where essentially the entire region contains significant eQTLs for *PTGER4* in blood cells (table S5B). Similarly, *rs350047* was not an identified eQTL for *PTGER4* expression but was significantly correlated with predicted expression in our data ($P = 2.2 \times 10^{-16}$). In addition to *PTGER4*, several LD SNPs were also significant eQTLs for *DAB2* in eQTLGEN, approximately 800 kb downstream from the lead SNP (table S5B). Last, we developed genetic models to fit interactions between regular aspirin use and predicted *PHACTR1* (upstream of *rs72833769*) and *DAB2/PTGER4* (near *rs350047*) expression based on the BarcUVa-Seq dataset but did not identify any significant interactions.

DISCUSSION

We report results of the largest genome-wide GxE interaction scan to date for CRC focused on aspirin and NSAIDs. Consistent with previous epidemiological studies, regular use of aspirin/NSAIDs was inversely associated with CRC risk in this analysis. The direct aspirin/NSAIDs associations did not differ substantially when stratified by sex or tumor site. Gene-environment interaction scans identified two previously undescribed interaction SNPs, *rs350047* (5p13.1) and *rs72833769* (6p24.1), and subgroup-specific analyses show different magnitudes of effects of aspirin/NSAIDs on CRC risk at each locus defined by genotype.

Our finding of a significant interaction for *rs350047* (5p13.1) and aspirin's preventive capacity is biologically plausible and potentially functionally relevant. *PTGER4* encodes PTGER4 (EP4), a major receptor for prostaglandin E2 (PGE_2). PGE_2 is one of the major proinflammatory factors that is produced constitutively in the intestinal epithelium and is elevated during periods of inflammation (*18*, *19*). Aberrant PGE_2 signaling via PTGER4 within the mucosal microenvironment promotes tumorigenesis and affects cellular differentiation processes central to mucosal injury repair (*20*–*23*). In the setting of cancer, PGE_2 has been found to induce proliferation of CRC stem cells and CRC liver metastasis in mouse models via EP4-dependent signaling pathways (*24*, *25*). Increased synthesis of PGE_2 [due to increased prostaglandin endoperoxide synthase 2 (PTGS2) expression] is observed in patients with CRC (*24*, *25*). Genetic deletion of *Ptgs2* or downstream PGE_2 receptors results in protection from tumorigenesis in CRC mouse models (*26*–*30*). In addition, 15-hydroxyprostaglandin dehydrogenase (*HPGD*; *15-PGDH*), the primary enzyme that catabolizes PGE_2, has been characterized as a tumor suppressor for several human cancers, including colorectal, and is ubiquitously down-regulated in CRC (*31*–*36*).

However, in the context of prevention, carefully orchestrated PGE_2-PTGER4 signaling mediated by PTGS2 in the mucosal microenvironment has been demonstrated to have a key role in healthy stem cell function and regenerative programming (*20*, *21*, *37*). The leading putative biological mechanism for the chemopreventive effects of aspirin and other nonselective NSAIDs centers on inhibition of PTGS1 and PTGS2, the enzymes that mediate conversion of arachidonic acid to PGE_2 (*3*). Direct inhibition of PGE_2 synthesis by aspirin has been confirmed in separate clinical trials, where aspirin intervention in patients at risk for CRC significantly reduces the major urinary metabolite of PGE_2 (*38*–*40*), 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranorprostane-1,20-dioic acid (PGE-M), an inflammatory biomarker that may predict individual risk for colorectal neoplasia and may have utility as an efficacy marker for aspirin prevention (*11*, *39*, *41*).

Thus, it is biologically plausible that the interaction SNP *rs350047* as an eQTL for *PTGER4* expression may substantially modify the PGE_2-PTGER4 signaling axis, aspirin's effect on this pathway and resultant tumorigenesis, and in turn an individual's risk for CRC. Moreover, the locus 5p13.1 has been previously implicated in investigations of cancer and inflammatory bowel disease. Genome-wide association analyses of CRC risk using data from a broader subset of the same Genetics and Epidemiology of Colorectal Cancer Consortium (GECCO)/Colon Cancer Family Registry (CCFR)/Colorectal Transdisciplinary Study (CORECT) consortia identified two independent risk loci in this region; Schmit *et al.* (*14*) reported a significant association between indel *rs58791712* (G/GT) and CRC risk, while Huyghe *et al.* (*42*) reported a separate hit upstream of this variant, (G/A). The association for *rs7708610* was genome-wide significant upon conditioning models for the previously identified hit at *rs58791712* (using a surrogate SNP), indicating the presence of an independent susceptibility locus (*42*). The correlation of the SNP *rs350047* showing interaction with *rs58791712* and *rs7708610* in 1000G European population is R^2 = 0.38 and 0.042, respectively. Furthermore, several analyses of inflammatory diseases, specifically for, but not limited to, Crohn's disease, also implicated the 5p13.1 region as a risk locus (*43*–*46*), although none of the reported markers are in LD with our main finding.

In contrast to *rs350047*, the functional evidence for rs72833769 (6p24.1) is less clear. However, a significant eQTL with *TBC1D7* might be of interest. This gene is a subunit of the TSC1-TSC2 complex, a key regulator of mammalian target of rapamycin (mTOR) activity, which is a proto-oncogene and downstream effector of the phosphatidylinositol 3-kinase/AKT pathway. As previously described in the context of pancreatic cancer (*47*), there is potential cross-talk between PGE_2 expression, PTGER4 activation, and the AKT/mTOR pathways. Moreover, in a large, recent study using human normal colon organoids, network analysis of RNA-seq data revealed that both *TBC1D7* and *GFOD1* were present within modules that were both significantly modulated by aspirin treatment in vitro and enriched for PGE_2-related pathways (*48*). Thus, it appears biologically plausible that aspirin/NSAID-mediated changes in PGE_2 expression can modulate the effectiveness of signal transduction to proliferative signals by these signaling axes.

These results also add to the existing evidence base for SNPs predicting aspirin's chemopreventive benefit for CRC. Nan *et al.* (*13*) previously reported significant interactions between aspirin use and variants in 12p12.3 (*rs2965667* and *rs10505806*) and 15q25.2 (*rs16973225*) for CRC risk in a subset of studies included in this investigation. Although these SNPs were not identified at genome-wide significance in our main analysis, we did confirm nominally significant interactions for these SNPs. While other prior studies have identified putative SNP-based biomarkers for an interaction of aspirin/NSAID use and CRC risk [summarized in prior reviews (*3*, *49*)], the functional relevance of the SNPs to their preventive mechanism for CRC has been limited, beyond putative indirect linkages with CRC-relevant or prostaglandin-signaling adjacent pathways or enzymes linked to aspirin pharmacokinetics. Similarly, prior studies that have performed targeted genotyping of specific pathways related to aspirin/NSAID chemoprevention (e.g., prostaglandin synthesis) include important a priori signals (*50*–*54*), such that the absence of previously studied functional genetic variants from the results here does not preclude their potential utility in precision prevention. Nonetheless, our study is not only the largest study to date to perform a GWIS and, thus, has the greatest power to eliminate potential false negatives, but also by extending our findings to our functional dataset, we have demonstrated that *rs350047* is within an eQTL for *PTGER4*, implicating a mechanistic link for this SNP and the effects of NSAIDs on PGE_2-PTGER4 signaling.

Beyond host genetics, other molecular epidemiology studies have demonstrated that aspirin/NSAID chemopreventive effects may be limited to tumors that would have otherwise developed via pathways sensitive to aspirin prevention. Specifically, these prior studies demonstrate that aspirin may have greater protective effects against *BRAF* wild-type (*55*) and *PIK3CA* mutant CRCs (*56*) or those tumors with higher PTGS2 expression (*57*) or against CRCs arising in individuals with intact *HPGD* expression (*58*). Our findings that interactions between aspirin/NSAID use and *rs350047* were statistically significant only when comparing *BRAF* mutant, CIMP-high,

or MSI-high cases to controls, whereas interactions between aspirin/NSAID use and *rs72833769* were limited to cases absent of these molecular markers compared to controls, suggests that host genetics may further influence aspirin's ability to differentially prevent tumors arising via separate tumorigenesis pathways (e.g., traditional versus serrated pathways). Combined, our findings support calls for a more nuanced, precision prevention approach to specifically identify subsets of individuals likely to benefit from aspirin/NSAIDs and improve broader, one-size-fits-all recommendations [i.e., only on the basis of age or conditioning on added risk for cardiovascular disease as has been the case for past U.S. Preventive Services Task Force recommendations (*59*, *60*)]. While some markers, such as *rs72833769*, may provide simpler, more qualitative guidance (i.e., go/no-go) for individual stratification, quantitative interaction markers, such as *rs350047*, will be critical to calibrating precision prevention recommendations to maximize net benefit among those more likely to benefit, particularly when they are linked to potential mechanisms of action. These findings can help identify interrelated modes of action that may help clarify differential anticancer effects associated with aspirin/NSAIDs, new and more effective therapeutic or prevention targets, or specific pathways individuals are on toward the development of cancer that may or may not be responsive to these agents. Moreover, quantitative measures may further help explain observed interindividual responses to preventive interventions, even among patients identified as likely to respond by qualitative measures (*11*). In all, a truly precise precision prevention approach likely requires the incorporation of both qualitative and quantitative genomic interaction markers of risk and response in context of the potential tumorigenesis pathways to which an individual is particularly susceptible to and additional individual risk factors.

Our study has several strengths, including being the largest GWIS of CRC and aspirin/NSAIDS to date, pairing this data with functional datasets that allow for the identification of eQTLs and using efficient and powerful statistical methods to improve power over standard GxE tests. A limitation of our study is that the resolution of data collected on aspirin use alone varied across studies and required us in some cases to group aspirin use, for which there is a clearer chemoprevention benefit established, with other non-aspirin NSAID use. While not ideal, we have performed careful data harmonization across the studies to ensure that we accurately have differentiated between aspirin/NSAID use, which may include aspirin only users along with users of both aspirin and/or other NSAIDs, and those who explicitly recorded aspirin use separately from other NSAID use. In addition, our study focuses on harmonized self-reported regular aspirin/NSAID use presumed to be representative of standard dose recommendations (≤325 mg/day) as this was most consistently collected across all studies. Although multiple lines of evidence support the notion that the chemopreventive association with aspirin is strongest 5 to 10 years after commencing continued regular use, we did not have sufficient information to consider the duration of exposure. Despite this measurement error, which is likely to attenuate risk estimates and lead to reduced power, we were able to show strong protective associations for aspirin/NSAIDs and identify two significant interactions. Nonetheless, future studies will need to examine whether the interactions observed for aspirin/NSAID use are specific to use of aspirin or extend to other non-aspirin NSAIDs and in context of other key factors including dose and duration of use. Our analysis is limited to individuals of European ancestry, thereby limiting the direct extension of these findings to different racial and ethnic populations. Last, we cannot rule out the effects of residual confounding, including from additional CRC risk factors (e.g., inflammatory bowel disease history) not otherwise accounted for in our analysis, or recall bias in our analysis.

In summary, we identified previously undescribed genetic loci that modify the protective effect of regular aspirin/NSAID use on CRC risk. Functional evidence presented in our investigation implicates genes directly involved in CRC-associated signaling pathways, such as PGE_2 synthesis/signaling in the case of *PTGER4*, and downstream pathways involved in tumorigenesis and proposed to be central to aspirin's protective mode of action, suggesting biological plausibility. Validation and additional functional work are necessary to confirm these findings. Furthermore, the likelihood of identifying additional interaction loci in the future can be improved via implementation of tissue-/cell-specific functional annotations, along with multi-ethnic GWIS.

MATERIALS AND METHODS

Study design

We pooled individual level genomic and epidemiological data from three consortia comprising individuals of European ancestry—the GECCO, the CORECT, and the CCFR consortia comprising a total of 52 studies. Study details have been previously published (*14*, *42*, *61*) and can be found in table S6. For cohort studies, nested case-control sets were assembled via risk-set sampling, while population-based controls were used for case-control studies. Clinical trials were treated as cohort studies and participants were matched according to trial arm. Cases were defined as CRC or advanced adenomas and were confirmed by medical records, pathological reports, or death certificate information. Controls were matched on age, sex, race, and enrollment date/trial group, when applicable. For the small subset of advanced adenoma cases, matched controls were found to be polyp-free on sigmoidoscopy or colonoscopy at the time of adenoma selection. All participants gave written informed consent, and studies were approved by their respective Institutional Review Boards.

Exposure and aspirin/NSAID use ascertainment

Analyses include individuals with complete exposure and covariate information. We excluded individuals based on discrepancies between reported and genotypic sex, cryptic relatedness, and duplicates. For any individual included in multiple studies, we selected a single record for them, prioritizing the study that genotyped on the more comprehensive platform. Collection of risk factor data and harmonization across contributing studies has been previously described (*62*). Combined use of any aspirin or non-aspirin NSAIDs at reference time is defined as "aspirin/NSAID use" (yes or no), comprising a final study sample size of 72,667 individuals (30,806 cases and 41,861 controls). When aspirin use was specifically queried separately from NSAID use, we defined regular use of aspirin at reference time as "aspirin only use" (yes or no) to conduct analyses limited to aspirin users ($N = 72{,}137$; 30,574 cases and 41,563 controls). Multivariate models include age (continuous), sex (male/female), study, smoking status (never/ever), alcohol consumption (nondrinkers; moderate, 1 to 28 g/day; heavy, >28 g/day), BMI (continuous), and red meat intake (study and sex-specific quartiles of red meat intake based on controls only) as specifically denoted. Individuals with missing covariate data were excluded from any model using the covariate.

Genotyping

The genotyping platforms used in each study are summarized in table S6. Briefly, genotyped SNPs were excluded on the basis of call rate (<95 to 98%), lack of Hardy-Weinberg equilibrium ($P < 1 \times 10^{-4}$), and discordant calls between duplicates. All autosomal SNPs of all studies were imputed to the Haplotype Reference Consortium r1.1 (2016) reference panel via the Michigan Imputation Server (*63*) and converted into a binary format for data management and analyses using R package BinaryDosage. Imputed common SNPs were filtered on the basis of a pooled minor allele frequency (MAF) ≥ 0.01 and imputation accuracy $R^2 > 0.8$. After imputation and quality control analyses, a total of more than 7.2 million SNPs were available for analysis. Principal components analysis for population stratification assessment was performed using PLINK 1.9 on 30,000 randomly sampled imputed SNPs with MAF > 0.05 and imputation accuracy $R^2 > 0.99$. Additional details on genotyping and quality control have been previously published (*42*).

Statistical analysis

We evaluated associations of CRC risk with regular aspirin/NSAID use and with aspirin only use and CRC risk using random-effects meta-analysis of study specific results to obtain summary ORs and 95% CIs across studies. Association tests were stratified by study design, sex, and tumor subsite. The latter was categorized into the following groups based on the following ICD-9 codes (otherwise excluded): proximal colon (153.0, 153.1,153.4, 153.6), distal colon (153.2,153.3, 153.7), and rectum (154.0, 154.1).

Common variant analysis

Genome-wide scans were conducted using GxEScanR, an R package that implements several methods for detecting GxE interactions (https://CRAN.R-project.org/package=GxEScanR). Imputed allelic dosages were modeled as continuous variables. Our analysis employs three primary methods: (i) traditional 1-df GxE logistic regression models, (ii) a two-step method using "D versus G" and "E versus G" joint information [the EDGE method (*64*)] as the step 1 filtering statistic and the 1-df GxE statistic for the step 2 test accounting for LD-based correlation among SNPs in step 2 (*65*), and (iii) a 3-df joint test that incorporates information from main effects, gene-exposure associations, and GxE interaction statistics in a single model. (*66*) For the 3-df test, we report only results with GxE P value $< 1 \times 10^{-4}$, since this test captures markers with significant results from any of the three sources of statistics. We adjusted the overall genome-wide significance threshold for each testing procedure to $5 \times 10^{-8}/2.5$ to account for multiple testing with methods that are statistically correlated. We adopt the following notation: E, regular aspirin/NSAID use; G, SNP; D, CRC outcome; and C, set of adjustment covariates. Traditional logistic regression models assessed interactions on a multiplicative scale by including an interaction term in the model $\text{logit}[\Pr(D = 1|G, E, \boldsymbol{C})] = \beta_0 + \beta_G G + \beta_E E + \beta_{\text{GxE}}\text{GxE} + \beta_C C$, testing $H0 : \beta_{\text{GxE}} = 0$. The typical focus of a GWIS is on the 1-df test of GxE interaction based on the null hypothesis $H_0 : \beta_{\text{GxE}} = 0$. This test is known to generally have low power, particularly in the context of discovering new interactions in a GWIS. To enhance our power to discover new GxE loci, we used the two-step EDGE method and 3-df joint tests summarized above. Additional details of these approaches are in the Supplementary Materials.

Functional follow-up

We leveraged the BarcUVa-Seq (https://barcuvaseq.org/) resource (*67*) to develop prediction models to test interactions between predicted gene expression and regular aspirin/NSAID use, in a method previously explored in GECCO consortium using GTEx data (*68*). Weights were generated using elastic net regularized regression models fit on BarcUVa-Seq gene expression (20,693 measured gene expressions) and HRC imputed genotypes. Imputed SNPs were filtered on the basis of MAF > 0.1 and imputation quality ($R^2 > 0.7$); models were adjusted for age, sex, RNA-seq batch, tissue location, principal components, and probabilistic estimation of expression residuals (PEER) factors (*69*). Weights were then used to predict gene expression in our study sample of 72,667 subjects. In total, 13,393 gene expressions were successfully predicted. Interaction tests were conducted using logistic regression with an interaction term between predicted genetic expression and aspirin/NSAID use and aspirin use alone.

We used LocusZoom v1.3 (*70*) to generate regional plots for significant findings to inspect and extend the association signal and LD, and position of findings relative to genes in the region. Measures of LD were estimated using European populations from the 1000 Genomes Project. The putative functional role of these SNPs and those in LD ($R^2 > 0.2$) at 500-kb flanking regions was investigated with relation to their potential contribution to gene expression regulation in two ways: first, by their physical location in regions of chromatin accessibility or histone modifications (variant enhancer loci) and, second, through their direct association with expression of nearby genes (eQTLs).

To assess the physical location of the SNPs in regions of chromatin accessibility or histone modifications, we queried overlaps between our findings and regions containing active enhancer elements in tissue from healthy and tumor colon samples in addition to CRC cell lines, obtained from previously analyzed assays for transposase-accessible chromatin with sequencing (ATAC-seq) data, DNase hypersensitivity sequencing, and H3K27ac histone chromatin immunoprecipitation sequencing datasets. (*15*) We extended this analysis to include additional tissue types by incorporating regulatory annotations of histone modifications from 10 groups of tissues, obtained from several resources (*71*, *72*) and compiled by Finucane *et al.* (*16*) Furthermore, we queried lead and LD SNPs against functional annotation databases from ENSEMBL using the Variant Effect Predictor tool. (*73*)

We checked for eQTLs using several resources: (i) GTEx v8, (ii) the colon transcriptome explorer (CoTrEx 2.0; https://barcuvaseq.org/cotrex/, accessed May 2021), a resource for transcriptomic data jointly developed by the University of BarcUVa-Seq, which includes eQTL from 445 epithelium-enriched healthy colon biopsies from ascending, transverse, and descending colon, and 3) eQTL results from eQTLGEN, a consortium of 37 cohorts pooling RNA sequencing data from whole blood samples.

Supplementary Materials

This PDF file includes:

Supplementary Materials and Methods
Sources of consortium funding
Figs. S1 to S6
Tables S1 to S6
References

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